TECHNISCHE UNIVERSITÄT WIEN

DISSERTATION

Antimicrobial resistance and One Health: Occurrence of multiresistant human pathogenic bacteria in food and environment.

ausgeführt zum Zwecke der Erlangung des akademischen Grades einer Doktorin der Naturwissenschaften unter der Leitung von

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Declaration

"I confirm that this thesis is entirely my own work. All sources and quotations have been fully acknowledged in the appropriate places with adequate footnotes and citations. Quotations have been properly acknowledged and marked with appropriate punctuation. The works consulted are listed in the bibliography. This thesis has not been submitted to another examination panel in the same or a similar form, and has not been published."

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Acknowledgements

After an intensive period of three years, writing this note of thanks is the finishing touch on my dissertation. It has been a period of intense learning for me, not only in the scientific area, but also on a personal level. I would like to take this opportunity to thank those people who helped me throughout this period and made this thesis possible.

Firstly, I would like to express my sincere gratitude to my doctoral supervisor Prof. Dr. Robert Mach, who has always supported my work with great encouragement, with constructive suggestions and his guidance.

I would particularly like to single out my second supervisor, PD Dr. Werner Ruppitsch, his guidance helped me in all the time of research and writing of this thesis. His patience, motivation and understanding made him an outstanding advisor and mentor for my study.

A very special gratitude goes to Prof. Dr. Franz Allerberger, for his valuable comments, time and assistance. His strong support enabled to finalize this thesis.

To my colleagues and friends at the AGES institute, I thank them for their help and companionship.

Last but not least, I am grateful to my parents, Robert and Judith Lepuschitz, who provide me with everlasting moral and emotional support in my life. I would not have made it this far without them. I am also grateful to my other family members and friends who have supported me along the way.

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Zusammenfassung

Im Laufe der Evolution hat sich das Spektrum der Krankheitserreger, die eine Bedrohung für die Gesundheit und das Leben des Menschen darstellen, konstant verändert. Um deren Verbreitung einzudämmen, ist eine ständige Überwachung infektiöser Erreger als Maßnahme im Bereich der öffentlichen Gesundheit unerlässlich.

Das Auftreten beziehungsweise Wiederauftreten von Infektionskrankheiten kann durch unterschiedliche Faktoren verursacht werden. Der übermäßige Gebrauch und Missbrauch antimikrobieller Substanzen sind für eine zunehmende Antibiotika Resistenz der Krankheitserreger verantwortlich und erschweren notwendige medizinische Behandlungen damit erheblich. Sowohl Gram positive als auch Gram negative Bakterien entwickeln zunehmende Resistenzen gegen die jeweiligen Antibiotikatherapien. Reservoire von Krankheitserregern sind nicht nur auf Krankenhäuser, in denen der selektive Druck durch Antibiotika besonders hoch ist, beschränkt, sondern befinden sich auch in der Umwelt (z. B. Wasser, Lebensmittelproduktion, Landwirtschaft).

Insbesondere lebensmittelbedingte Krankheitserreger sind eine weitere treibende Kraft für die Ausbreitung von Infektionskrankheiten. Durch behördliche Kontrollen können Kontaminationsquellen zwar reduziert werden, jedoch können lebensmittelbedingte Infektionen in allen Altersgruppen auftreten und sind nach wie vor weit verbreitet. Daher sind regelmäßige Kontrollen und zugleich eine genaue Typisierung von Bakterien notwendig, um die Risiken einer Infektion und deren Ausbreitung zu minimieren.

Ziel dieses Projekts war es, natürliche Reservoirs und Quellen pathogener Mikroorganismen zu definieren. Dabei lag ein besonderer Schwerpunkt auf multiresistenten Mikroorganismen in Gewässern. Dazu wurden österreichische Flüsse auf die Präsenz humanpathogener, multiresistenter Erreger untersucht. Weiters wurde das Vorkommen eines Lebensmittel–assoziierten Erregers namens *Cronobacter sakazakii*, der besonders schwere Krankheitsausbrüche bei Säuglingen verursachen kann, im Zuge einer europäischen Multicenter-Studie im Jahr 2017 untersucht.

Mittels Ganzgenomsequenzierung wurde eine detaillierte Charakterisierung und Subtypisierung pathogener klinischer und lebensmittelbedingter Erreger durchgeführt und der jeweilige Antibiotikaresistenz- und Virulenz-Status bestimmt.

Die Ergebnisse des ersten Projekts zeigen, dass multiresistente Erreger, die in Krankenhäusern vorkommen auch im österreichischen Oberflächengewässer nachgewiesen werden können. Es gelang der Nachweis eines Methicillin-resistenten *Staphylococcus aureus* USA300 Isolats und der Nachweis von multiresistenten *Klebsiella pneumoniae* Isolaten, die mit den aus Krankenhäusern erhaltenen Isolaten eine genombasierte Übereinstimmung zeigten.

Der Nachweis humaner Krankheitserreger, wie dem *Staphylococcus aureus* USA300 Isolat und den *Klebsiella pneumoniae* Isolaten in österreichischen Flüssen hat gezeigt, dass Antibiotikaresistenzen nicht nur auf Krankenhäuser beschränkt sind, sondern von dort auch in die Umwelt gelangen können. Die anthropogene Verschmutzung durch Kläranlagen der jeweiligen Flüsse, wurde durch den Nachweis von multiresistenten *Klebsiella pneumoniae* Isolaten bestätigt, da sie von in Krankenhäusern gesammelten klinischen Isolaten nicht unterschieden werden konnten. Da Antibiotikagaben zur primären Behandlungsmethode gegen bakterielle Infektionen gehören, ist die zunehmende Ineffektivität alarmierend und erfordert Strategien, um eine Ausbreitung multiresistenter Stämme in der Umwelt zu minimieren.

Die europäische *Cronobacter sakazakii* Multicenter-Studie ermöglichte einen Überblick über die Situation der *Cronobacter* Infektionen in ganz Europa. Die Studie hat gezeigt, dass in Ländern, die 2017 *Cronobacter sakazakii* Isolate nachweisen konnten, keine europaweiten Ausbrüche vorlagen. Die korrekte Identifizierung von *Cronobacter* Spezies stellte sich als eine große Herausforderung für die teilnehmenden Laboratorien heraus und trug daher zu einer unterschätzten Verbreitung in ganz Europa bei. Die Etablierung eines Typisierungsschemas für Ausbruchsuntersuchungen ermöglichte die Entdeckung von vier bisher unveröffentlichten historischen Ausbrüchen (vor 2016).

Die Typisierung von Bakterien mittels Ganzgenomsequenzierung erlaubt eine Unterscheidung der Isolate in höchstmöglicher Auflösung und ist daher die Methode der Wahl, um Übertragungswege und Ausbrüche durch pathogene Erreger aufzudecken. Darüber hinaus ermöglichen genombasierten Sequenzdaten die Analyse und Charakterisierung von Genen, welche Antibiotikaresistenzen und Virulenzeigenschaften verursachen. Die Ergebnisse dieser Arbeit bestätigen, dass Ganzgenomsequenzierung besonders zur Überwachung, Erkennung von Ausbrüchen und zur Beobachtung der Entwicklung von Krankheitserregern geeignet ist.

Abstract

Throughout evolution, the spectrum of pathogens affecting human health and the thereby associated infectious diseases changed. To decrease dissemination, disease and death, control via constant public health surveillance is essential. Emergence respectively re-emergence of infectious diseases can be promoted by diverse factors. Amongst others, the use, overuse and misuse of antimicrobials lead to the growing emergence of infections, caused by increased antibiotic resistance of pathogens to medical treatment. Gram positive as well as Gram negative bacteria progressively resist antibiotic therapy. Reservoirs of such bacteria are not restricted to hospitals where the selective pressure is particularly high, but can also be found in the environment (e.g. water, food-production, husbandry). In addition, foodborne pathogens are a driving force for infectious diseases. Although regulatory controls can reduce sources of contamination, infections transmitted through food still remain common. Outbreaks caused by foodborne pathogens can appear in all age groups, therefore reliable regulatory control and accurate bacterial typing is important to reduce the risk of infection and dissemination.

The aim of this project was to define the natural reservoirs and sources of pathogenic bacterial organisms. One special focus was on multiresistant strains in the water environment. Main Austrian rivers were screened for the presence of multiresistant pathogens. Second, the occurrence of the foodborne pathogen *Cronobacter sakazakii*, which is associated with outbreaks of severe infections in infants, was investigated by a European multi-centre study in 2017. Clinical and foodborne pathogens were analysed in detail determining their antimicrobial resistance- and virulence status as well as characterization and subtyping of pathogenic isolates by whole genome sequencing (WGS).

Results of the first project revealed that human pathogens, which were simultaneously occurring in hospitals, have been detectable in Austrian surface water. This included the detection of a community acquired methicillin resistant *Staphylococcus aureus* USA300 isolate and the detection of multiresistant *Klebsiella pneumoniae* isolates, which resembled isolates from specimens obtained in hospitals.

Overall, the detection of human pathogens, such as the *Staphylococcus aureus* USA300 isolate in Austrian river water, shows that antimicrobial resistance is not restricted to hospitals, but that antibiotic resistant isolates spill over into the environment. Further, the occurrence of anthropogenic pollution by wastewater treatment plants has been affirmed by the detection of multiresistant *Klebsiella pneumoniae* isolates, which were indistinguishable with human isolates collected in hospitals. Since antibiotics belong to the treatment of choice against bacterial infections, the increasing ineffectiveness is alarming and demands attention and strategies to minimize the spread of multiresistant strains to the environment.

The European multi-centre study of *Cronobacter sakazakii* infections in humans allowed a comparison of the situation for *Cronobacter* infections across Europe. This study revealed the absence of European wide outbreaks in countries submitting *Cronobacter sakazakii* isolates in 2017. Species identification turned out to be a major challenge for participating laboratories, contributing to an underestimated prevalence across Europe. The establishment of a typing scheme for outbreak investigations enabled the detection of four previously unpublished historical outbreaks (before 2016).

Strain typing using WGS data discriminates isolates with the highest possible resolution and is therefore the method of choice for uncovering chains of transmission and outbreak investigations. In addition, WGS data allow the analysis and characterization of genes conferring antibiotic resistance and virulence. The findings corroborate, WGS as the recommended tool for surveillance, outbreak detection and for monitoring the evolution of pathogens.

Introduction

Emerging infectious diseases (EIDs) are defined as infections that have newly appeared in a population or either have existed previously but are rapidly increasing in incidence or geographic range [Morse 1995]. Throughout human history various EIDs, such as the Black Plague in the fourteenth century, the influenza pandemic from 1918 to 1920 or since the 1980s the acquired immune deficiency syndrome (AIDS/HIV), have caused incalculable misery and death [Morens *et al.* 2004]. The emergence or re-emergence of infectious diseases is caused by diverse factors, including: microbial genetic mutation (adaptation) [Neu 1992]; viral genetic recombination or re-assortment [Sharp *et al.* 2001]; changes in the population of hosts (e.g. human susceptibility to infection) [Espinal 2003]; switching from animal to human hosts [Beisel and Morens 2004]; human behavioural changes (especially movement and urbanization) [Holt 2018] and environmental factors (e.g. climate, changing ecosystems) [Hirk *et al.* 2016; Morens *et al.* 2004]. To minimize disease expansion, death and economic losses, the rapid detection of EIDs via constant public health surveillance by collection, analysis, interpretation and dissemination of health data is essential [Binder *et al.* 1999].

One factor, which significantly affects the emergence of infectious diseases, is the worldwide growing burden of antimicrobial resistance (AMR) in bacteria. The discovery of penicillin by Sir Alexander Fleming in 1928 started the modern era of antibiotics - thereby the use of antibiotics to cure human infections revolutionised the medical treatment and saved millions of lives - but in the past decades the rapid emergence of antibiotic resistant bacteria endangers the efficacy of antibiotics [Ventola 2015]. Some pathogens, such as methicillin resistant *Staphylococcus aureus* (MRSA), were particularly successful in acquiring resistance against most antibiotics and has a remarkable epidemiological history. MRSA are Gram positive bacteria and a most common cause of nosocomial infections, which have the ability to cause a wide variety of disease, ranging from skin and soft tissue infections to life-threatening septicaemia, endocarditis and toxic shock syndrome [Diep *et al.* 2006]. The history of MRSA shows the extraordinary power of these pathogens to adapt, since they are one of the most frequent pathogens in hospitals (hospital acquired (HA) MRSA), but they also increasingly appear in the community (community acquired (CA) MRSA) and they found a new ecological niche in animals (livestock associated (LA) MRSA) [Lakhundi and Zhang 2018]. Nevertheless, also Gram negative bacteria, such as multiresistant *Klebsiella pneumoniae* (*K. pneumoniae*) cause great public health concerns worldwide. The treatment of *K. pneumoniae* with broad-spectrum antibiotics began in the 1950s and since then they acquired a growing range of mechanisms to evade the treatment, including resistances to the treatment of choice: carbapenems. Nowadays, the threat of carbapenem resistant *K.*

pneumoniae is present throughout Europe and only very few therapeutic options are left to treat affected patients [Grundmann *et al.* 2010].

The constant change of epidemiology, host specificity, transmission routes as well as virulence- and antibiotic resistance attributes requires regularly monitoring [Lakhundi and Zhang 2018].

The use, overuse and misuse of antimicrobials in medicine, veterinary medicine and agriculture has led to dangerously high antibiotic resistance levels all over the world by adaption of bacteria to the treatment via new resistance mechanisms [Blaschitz *et al.* 2016; Hartl *et al.* 2017; Hartl *et al.* 2019; Jesumirhewe *et al.* 2017; Lepuschitz *et al.* 2017b; WHO 2015]. Bacteria acquire antibiotic resistance either spontaneously by mutation, via horizontal gene transfer (HGT) by transduction (exogenous DNA obtained via viruses), transformation (uptake of free DNA from the environment) or conjugation (direct cell-cell contact to other bacteria). This leads to antibiotic resistance through different mechanisms: exclusion of toxin by cell membrane; intracellular modification and/or deactivation of the antibiotic; extrusion from the cell; reduction in the sensitivity of the cellular target and intracellular sequestration [Taylor *et al.* 2011].

Especially hospitals are ecological niches for antimicrobial resistant bacteria and, due to the particularly high antimicrobial selective pressure, they play a major role in emergence and spread of resistant bacteria [Hocquet *et al.* 2016]. Resistant bacteria leave hospitals on colonized patients, but urban, hospital and pharmaceutical industry wastewater are among the main sources of antibiotics and antimicrobial resistant bacteria contamination in soil and water [Berendonk *et al.* 2015]. To date, efforts to prevent the emergence of antimicrobial resistant bacteria primarily focus on clinical relevant settings and no maximum contaminant levels for wastewater treatment plant (WWTP) effluents have been set by the European Union (EU) or other international institutions/organizations [Rizzo *et al.* 2013]. But particularly WWTP effluents have a high and diverse bacterial load and these environmental reservoirs are a suitable medium for the release, evolution, persistence and spread of antimicrobial resistant bacteria and AMR genes [Müller *et al.* 2018; Taylor *et al.* 2011; Voigt *et al.* 2019]. Further, human or animal associated bacteria intermingle with environmental bacteria, increasing the power of genetic variation and development of new resistance properties [Baquero *et al.* 2008]. There are diverse potential routes by which antimicrobial resistant bacteria and resistance genes can cycle among human population, terrestrial and aquatic systems, such as food, drinking water, bathing, fish keeping, irrigation, animals or husbandry among many others [Taylor *et al.* 2011].

An infection with antimicrobial resistant bacteria can occur either through direct contact to pathogens or via contact to environmental bacteria harbouring genes originating from pathogens, but at present it is not clear to which extent antimicrobial resistant bacteria

radiate back into clinical settings [Berendonk *et al.* 2015]. The occurrence of menacing antimicrobial resistant pathogens have been reported in recreational water numerous times and from a public health perspective, these findings need major attention [Lepuschitz *et al.* 2017a; Lepuschitz *et al.* 2018a; Lepuschitz *et al.* 2019b; Mahon *et al.* 2017; Zarfel *et al.* 2017; Zurfluh *et al.* 2013].

Another driving force of EIDs are emerging foodborne pathogens. Reservoirs for foodborne pathogens like *Campylobacter*, Shiga toxin-producing *Escherichia coli* (STEC), nontyphoidal *Salmonella* and *Listeria* are animals or the environment, and human are mostly associated hosts after foods are contaminated from those reservoirs. Others, like norovirus, hepatitis A or *Shigella* have human as primary reservoir and can be transmitted when infected humans contaminate foods. However, the source is not necessarily food, since some of these pathogens can also spread via water or animal contact [Braden and Tauxe 2013; Ruppitsch *et al.* 2017; Schlager *et al.* 2018]. Problems with food related diseases are caused by challenges facing food demand, production and broad distribution. The diffusion of foodborne pathogens sustains due to increased populations average age, urbanization, emigration, mass tourism and globalization of the food market [De Giusti *et al.* 2012]. Although some infections have been reduced by control efforts to prevent microbial contamination, other infections transmitted through food still remain common. The majority of these illnesses are not caused by known pathogens, therefore increased investigations are needed to discover and characterize new emerging pathogens [Tauxe 2002].

How important surveillance, detection and characterization of foodborne pathogens is, can be shown on the example of *Cronobacter sakazakii* (*C. sakazakii*). Outbreaks of severe infant infections (necrotizing enterocolitis, septicemia and meningitis) in neonatal intensive care units were caused by *Cronobacter* spp. [Ogrodzki and Forsythe 2017]. Already in 1950 the first *Cronobacter* was documented from a "dried milk" type product [Farmer 2015]. *Cronobacter* was first associated with powdered infant formula in 1988 [Muytjens *et al.* 1988] and since neonates are frequently fed with reconstituted powdered infant formula, it became the focus of attention to reduce the risk for neonates. Especially *C. sakazakii* clones belonging to multilocus sequence type (MLST) 4 were described to be highly stable clones with a high propensity for neonatal meningitis [Joseph and Forsythe 2011]. To reduce the neonatal and infant exposure to contaminated powdered infant formula, the FAO-WHO (Food and Agriculture Organization of the United Nations in collaboration with the World Health Organization) aimed to control *Cronobacter* as the first foodborne pathogen by improved criteria for powdered infant formula production and hygienic measurements for preparation [FAO/WHO 2004]. Additionally, for a reliable regulatory control, the correct identification and accurate bacterial taxonomy is essential. For example two out of five *Cronobacter* species (*C. sakazakii* and *C. malonaticus*) cannot be distinguished by 16S

rDNA sequencing because of the close relatedness [Forsythe 2018]. Issues with correct identification of *C. sakazakii* have been reported previously [Blažková *et al.* 2015; Jackson *et al.* 2015; Lepuschitz *et al.* 2018b] but with the advantages of whole genome sequencing (WGS) isolates can now be investigated in considerable detail [Lepuschitz *et al.* 2019a; Yan and Fanning 2015].

The surveillance of foodborne outbreaks with improved diagnostic testing in public health laboratories and the analysis of reported outbreaks to uncover vehicles, which account for outbreaks, are of utmost importance. Consequently, surveillance, e.g. by improved subtyping of pathogens via WGS routinely, and prevention, e.g. by showing an outbreak is caused by the same pathogen subtype and therefore may have the same source, can be improved [Braden and Tauxe 2013].

By now, international organizations have become aware of these emerging issues. To sustain human health, the so-called One Health concept supports that human, animal and environmental health are inextricably connected. The One Health movement seeks to promote, improve, and defend the health forces by uniting human medicine, veterinary medicine and environmentally related disciplines by worldwide collaborations [One Health Initiative 2019]. The WHO developed a global priority list of antimicrobial resistant bacteria [WHO 2017] to help prioritizing the research and development of new and effective antibiotics and a global action plan on antimicrobial resistance to improve awareness, strengthen knowledge and optimize the use of antibiotics [WHO 2015]. The Centers for Disease Control and Prevention (CDC) published a comprehensive analysis outlining the antibiotic-resistant threats in the United States [CDC 2014] and the European Centre for Disease Prevention and Control (ECDC) initiated the European Surveillance of Antimicrobial Consumption Network (ESAC-Net) and the European Antimicrobial Resistance Surveillance Network (EARS-Net). The European Commission released the European One Health Action Plan against Antimicrobial Resistance [European Comission 2017] to support the European Union and Member States in delivering innovative, effective and sustainable responses to fight against AMR. Further, the WHO has stated the emerging issues in water and infectious disease by classical and newly recognized pathogens for water and public health sectors [WHO 2002]. For improved detection and response to foodborne illnesses and outbreaks, public health institutes around the world from USA, Canada, Latin America and the Caribbean, Europe, Africa, the Middle East and Asia Pacific incorporated and established a public network called "PulseNet International" to link human cases with contaminated food sources [Nadon *et al.* 2017].

Beside different surveillance programs, typing methods for discriminating different bacterial isolates of the same species are essential tools for infection control and outbreak detection [Sabat *et al.* 2013]. For uncovering the evolutionary and phylogeographic spread of medical

significant microorganisms, molecular typing techniques have been of major importance for health authorities. Methods like pulsed field gel electrophoresis (PFGE), multi-locus sequence typing (MLST) or the use of DNA arrays have been proven to be useful to identify epidemiological relatedness among strains [Durand *et al.* 2018]. The implementation of standardised, validated protocols and analysis procedures enabled successful outbreak investigations within countries but also across borders [Nadon *et al.* 2017]. To date, the latest method for bacterial typing is WGS. Compared to previous typing methods, WGS is the most powerful tool for epidemiological investigations. It is a cost effective way to discover genome-wide variations, it has the highest discriminatory power to show transmission pathways, the ability to generate millions of reads in a single run and to construct the complete nucleotide sequence of a genome [Sabat *et al.* 2013]. In fact, the costs for WGS constantly decrease and in turn combines several classical methods to characterize isolates. However, the enormous data volume generated constitutes a major challenge and for the analysis of data bioinformatics support is needed [Durand *et al.* 2018]. By standardised procedures for WGS in public health laboratories, a public nomenclature (Bacterial Isolate Genome Sequence Database (BIGSdb), GenomeTrakr Network (US Food and Drug Administration)) and databases for specific questions (e.g. antimicrobial resistance, virulence genes, population structures), WGS can provide significant economic and public health benefits [Nadon *et al.* 2017].

Aims

The overarching goal of the project was to define the natural reservoirs and sources of pathogenic, multiresistant bacterial organisms, and their role in transmission to humans. The foundation was the concept of One Health, an approach facilitating the inseparableness between human health, animal health and ecosystem health. This included a detailed investigation of clinical and foodborne pathogens, their antimicrobial resistance- and virulence status as well as characterization and subtyping of pathogenic isolates by whole genome sequencing (WGS).

The main focus of our studies was the increasing public health burden of antimicrobial resistance. The aim was to proof the presence of human relevant, multiresistant pathogens in surface water. Isolates were derived from environmental samples, mainly surface water, serving as one of the main affected media of anthropogenic activity and pollution. Water samples from rivers were taken before and after passing through main Austrian cities to show the impact of wastewater effluents and anthropogenic pollution on the aquatic environment. Main Austrian rivers were screened for antimicrobial resistant microorganisms of Enterobacteriaceae (e.g. *E. coli*, *Klebsiella*, *Salmonella*), of enterococci, staphylococci and *Pseudomonas*. Further, the pathogens fulfilling the criteria of the WHO antibiotic-resistant priority pathogens list (WHO, 2017) were selected to determine the phenotypic antimicrobial resistance (AMR) and to evaluate the presence of AMR-genes, virulence-genes and plasmids by WGS. A detailed characterisation by WGS should assess the phylogenetic relatedness respectively diversity between collected environmental and clinical multiresistant strains. Clinical isolates were collected from hospitals located above the catchment basin of the sampled rivers to compare with environmental isolates. In order to monitor if wastewater treatment plant effluents are a reservoir for antimicrobial resistant pathogens, which contribute to their distribution and pose a risk factor for public health. Further reference sequences available from the National Center for Biotechnology Information (NCBI) were used to assess the relatedness to international described human pathogens (e.g. methicillin resistant *Staphylococcus aureus* USA300 clone, carbapenem resistant *Klebsiella pneumoniae*).

A second focus was on emerging foodborne pathogens, in particular on *Cronobacter sakazakii* (*C. sakazakii*). The public health burden of *C. sakazakii* is substantial, both in Europe and worldwide. However, there were no data available allowing a comparison of the situation for *C. sakazakii* infections in humans across Europe. To fill these data gaps, we organized a European, multi-centre, prospective prevalence pilot-study on *Cronobacter sakazakii* infections in humans (EUCRONI). Aim of this European multi-centre study was to determine the true prevalence of *C. sakazakii* as a causative agent in human infections

across Europe in 2017, to assess the extent of clonality for outbreak detection and to determine the antimicrobial in vitro susceptibility of human *C. sakazakii* isolates. National coordinators representing 24 European countries were requested to submit all *C. sakazakii* isolates obtained from clinical samples during 2017, but also current food isolates and historical isolates (with isolation-dates before 2017) to the Austrian Study Centre. Central testing comprised species identification by Matrix Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry (MALDI-TOF-MS), subtyping via whole genome sequencing (WGS) and determination of antimicrobial resistance.

Conclusion

The stated aims were accomplished by the publications included in this thesis-manuscript. One main result of this study was the detection and characterization of a community acquired methicillin resistant *Staphylococcus aureus* (CA-MRSA) USA300 isolate from a river sample in Austria in 2016. Further genetic analysis revealed a close relatedness to a clinical Austrian isolate as well as to a hypervirulent clinical strain from Canada, 2004. Reanalysis of the first documented clinical USA300 clones in Austria from 2007 demonstrated introduction of this clone from America to Austria on multiple occasions. Further, this paper describes the first genome based characterisation of an environmental USA300 isolate confirming the emerging global health threat caused by environmental AMR. While numerous environmental AMR studies have reported on Gram negative bacteria this example shows that also Gram positive bacteria might play their role in One Health. Considering the historical evolution of MRSA clones the emergence of new pathogenic clones is merely a question of time.

To show the impact of anthropogenic activities in urban areas on the aquatic environment, ten samples were collected from Austrian rivers, before and after flowing through cities. The screening for extended spectrum beta lactamase producing and carbapenem resistant *K. pneumoniae* isolates was negative in all five water samples collected upstream from cities, whereas all five samples taken downstream were positive. WGS based comparison of these water isolates to clinical isolates identified three cluster. Successfully connecting antimicrobial resistant isolates from hospitalized patients to contemporarily obtained river isolates provides further evidence of anthropogenic pollution by discharge of antimicrobial resistant bacteria via waste water treatment plants into the environment.

For appropriate actions further investigations are essential to determine the occurrence, risk, environmental impact of AMR in the environment as well as use of optimal wastewater treatment technologies. From a public health perspective these findings demand attention and strategies are required to minimize the spread of multi-resistant strains into the environment.

The European multi-centre study of *C. sakazakii* infections in humans (EUCRONI) in 2017 revealed a low prevalence in the participating countries submitting *C. sakazakii* isolates. Correct species identification within the *Cronobacter* group turned out to be a major challenge for participating laboratories, which might have played a role in underestimating the real prevalence. Whole genome sequencing performed on a clinical isolate, initially incorrectly specified as *Cronobacter*, led to the description of the first documented clinical *Siccibacter turicensis* isolate in Austria. Although clinical *C. sakazakii* isolates from 2017 showed a high genetic diversity and no outbreak was detected in 2017, characterization of the historical isolates gained during this study confirmed occurrence of four unpublished

historical outbreaks. This was achieved by the establishment of a core genome multi locus sequence type (cgMLST) scheme, which was therefore proven to be suitable for outbreak detection.

Overall, WGS is the method of choice for diverse molecular analysis purposes. It is suitable for characterization of microorganisms, identification of new genera or species, or by investigating for example the presence and composition of antimicrobial resistance genes, virulence genes and plasmids. Furthermore, the establishment of cgMLST schemes including all genes universally found in a particular genus or species, provides the highest possible resolution of strains and enables proper outbreak investigations. This can be especially useful for epidemiological studies in hospitals, to prevent or to find the source of outbreaks, but also in the surveillance of foodborne diseases. Therefore, WGS should be implemented in healthcare laboratories, to achieve correct species identification and to recognize the emergence respectively re-emergence of human pathogens. WGS is a tool that can provide huge benefits, not only for tracing chains of transmission and source identification, but for especially acting as surveillance tool, to monitor the evolution and dissemination of pathogens.

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Peer reviewed publications

Chapter 1

This chapter includes publications with focus on emerging pathogens in the water environment.

Peer-reviewed publications:

Lepuschitz S, Huhulescu S, Hyden P, Springer B, Rattei T, Allerberger F, Mach RL, Ruppitsch W. Characterization of a community-acquired-MRSA USA300 isolate from a river sample in Austria and whole genome sequence based comparison to a diverse collection of USA300 isolates. Sci Rep. 2018;8(1):9467.

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Hirk S, Huhulescu S, Allerberger F, **Lepuschitz S**, Rehak S, Weil S, Gschwandtner E, Hermann M, Neuhold S, Zoufaly A, Indra A. Necrotizing fasciitis due to *Vibrio cholerae* non-O1/non-O139 after exposure to Austrian bathing sites. Wien Klin Wochenschr. 2016;128(3-4):141-145.

Author's contribution: Sarah Lepuschitz performed the DNA isolation, preparation of WGS libraries and data analysis.

SCIENTIFIC REP**O**RTS

Characterization of a community-acquired-MRSA USA300 isolate from a river sample in Austria and whole genome sequence based comparison to a diverse collection of USA300 isolates

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The increasing emergence of multi-resistant bacteria in healthcare settings, in the community and in the environment represents a major health threat worldwide. In 2016, we started a pilot project to investigate antimicrobial resistance in surface water. Bacteria were enriched, cultivated on selective chromogenic media and species identification was carried out by MALDI-TOF analysis. From a river in southern Austria a methicillin resistant *Staphylococcus aureus* (MRSA) was isolated. Whole genome sequence analysis identified the isolate as ST8, *spa* type t008, SCCmecIV, PVL and ACME positive, which are main features of CA-MRSA USA300. Whole genome based cgMLST of the water isolate and comparison to 18 clinical MRSA USA300 isolates from the Austrian national reference laboratory for coagulase positive staphylococci originating from 2004, 2005 and 2016 and sequences of 146 USA300 isolates arbitrarily retrieved from the Sequence Read Archive revealed a close relatedness to a clinical isolate from Austria. The presence of a CA-MRSA USA300 isolate in an aquatic environment might pose a public health risk by serving as a potential source of infection or a source for emergence of new pathogenic MRSA clones.

Methicillin resistant strains of *Staphylococcus aureus* (MRSA) are the leading cause of nosocomial infections[1]. Manifestations vary from minor skin infection to fatal disease. MRSA have an outstanding ability to acquire antibiotic resistance genes leading to resistance to multiple antibiotic classes[2]. Since the late 1960s hospital acquired MRSA (HA-MRSA) became endemic in hospitals worldwide[3]. In the 1990s new clones affecting also healthy individuals in the community, featuring increased virulence as well as the power to spread easily, arose[4]. This so called community acquired MRSA (CA-MRSA) became prevalent worldwide with predominant clones in certain geographic areas. Clonal Complex (CC) 1 (USA400) and CC8 (USA300) are the major lineages in the United States, CC80 is the predominant lineage in Europe, CC59 and CC30 appear in Asia and the southwest Pacific[5,6]. CA-MRSA is now epidemic in the United States mainly due to dissemination of the USA300 clone which belongs to multi locus sequence type (MLST) 8/SCCmec IV and harbours the *lukS-lukF* genes, encoding the Panton-Valentine leukocidin (PVL) and the arginine catabolic mobile element (ACME) cluster[7]. While virulence of these strains enhances dissemination, the potential to acquire resistance to multiple antibiotic classes hinders treatment of MRSA infections[1]. CA-MRSA clones are not restricted to a geographical region. The ability

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of geographically predominant CA-MRSA clones to spread worldwide and to cause infections on other continents has been demonstrated[2]. In several European countries, where ST80 still represents the prevalent CA-MRSA clone, also infections due to the North American USA300 clone have been reported[8–11]. Consequently the identification of new potential infection sources is essential for infection control.

Antimicrobial resistance (AMR) is an increasing global problem and the fact that resistant pathogens are not restricted to clinical settings but can be increasingly found in the environment is alarming[12–14]. In aquatic systems, horizontal gene transfer (HGT) of resistance genes between bacteria leads to the evolution of AMR bacteria, which in turn find back into clinical settings. AMR dissemination of resistant strains occur through direct contact of humans to the AMR bacteria in the aquatic system or from contact to resistant environmental bacteria which pass resistance genes to human or animal pathogens[15]. So called high risk clones (HRCs), which are characterized by enhanced virulence and multiple antibiotic resistances, pose a serious public health risk[16]. Aanensen et al.[8] identified three key elements to tackle the public health threat caused by HRCs: genetic population structure and identification of HRCs, assessment of risks posed by virulence and resistance determinants and risk management by implementation of prevention and control strategies.

The Austrian Agency for Health and Food Safety, the major Austrian organization responsible for consumer protection and public health, started a pilot project analysing surface water samples from rivers and bathing sites with the aim to identify possible public health risks due to AMR HRCs in 2016[17]. Environmental screening for HRCs and high discriminatory typing identified a CA-USA300 isolate in a river water sample in Austria in 2016.

In this report we describe a detailed characterization of this USA300 MRSA isolate and its phylogenetic relatedness to clinical CA-MRSA USA300 isolates.

Results

Screening of the 12 water samples for MRSA revealed one sample from a river in southern Austria (46°38′14″N, 14°17′44″E) positive for MRSA (sample ID: W1). Whole genome sequencing (WGS) identified the river water isolate W1 as a USA300 strain. The isolate showed classical multi locus sequence type (MLST) sequence type (ST) 8, *spa* type t008, harboured SCCmecIV, the Panton-Valentine leucocidin (PVL) genes, the arginine catabolic mobile element (ACME) cluster and USA300 specific *cap5* mutations.

Genetic comparison of ST8 isolates. To assess relationship of the river water isolate W1 to clinical and food associated isolates, 18 clinical isolates from the Austrian national reference laboratory for coagulase positive staphylococci were characterized by WGS and sequences of 146 arbitrarily chosen USA300 isolates were retrieved from the Sequence Read Archive (SRA).

From the comparison of isolate W1 to in total 164 isolates, isolate W1 was indistinguishable from 99 USA300 isolates concerning ST8, *spa* type t008, SCCmecIV, PVL genes, the ACME cluster and *cap5* mutations. Eighteen isolates carried no ACME cluster, six isolates carried no PVL and four isolates carried no ACME cluster and no PVL. Thirty isolates had ST8, carried the ACME cluster and PVL but had different *spa* types: t024 (n = 5), t063 (n = 2), t068 (n = 1), t121 (n = 6), t16000 (n = 1), t2031 (n = 11), t622 (n = 3), t681 (n = 1). One isolate had ST8, *spa* type t622, PVL and no ACME cluster; one isolate had ST8, *spa* type t203, the ACME cluster and no PVL; four isolates had ST8, different *spa* types (t024 (n = 1), t190 (n = 2), t5271 (n = 1)) and carried no ACME cluster and no PVL. One isolate had ST3885, *spa* type t008, the PVL genes and the ACME cluster.

Whole genome based cgMLST phylogenetic analysis including *S. aureus* reference strain COL (accession no. NC_002951.2) (ST250), water isolate (W1), the reference strains FPR3757 (ATCC® BAA-1556™), TCH1516 (USA300-HOU-MR, ATCC® BAA-1717™), all clinical USA300 isolates (n = 18) from the Austrian national reference laboratory for coagulase positive staphylococci (from 2004, 2005 and 2016), and 143 genomes from isolates retrieved from SRA was performed and a minimum spanning tree was calculated (Fig. 1). Distance calculation between all 164 samples revealed a maximum allelic distance between samples of 351 and an average allelic distance of 93.5 across the MST. Clinical Austrian isolates differed among each other in minimum of two, a maximum of 327, and an average distance of 100 alleles. Based on the defined complex threshold (CT) of 24 allelic differences[18] 25 different complexes were obtained. Six out of 18 clinical Austrian isolates (H1/04-H3/04; H4/05-H6/05) showed close relatedness with a maximum allelic difference of 21 and were all located in complex 2 (Fig. 1) respectively in complex 1 (Fig. 2). Isolate H3/04, a human isolate from Austria in 2004, showed close relatedness to strains C2406 with an allelic difference of 14, USFL055 isolated in the United States in 2009 with an allelic difference of 22, N28973PS isolated in the United States in 2011 from turkey meat with an allelic difference of 24, and reference strain FPR3757 with an allelic difference of 23. Surface water isolate W1 differed by 54 alleles from C2406 and was closest related to the Austrian clinical isolate H5/16 differing by two alleles in cgMLST and by four alleles in the pan genome including the following targets from the reference genome *S. aureus* (SA)COL (accession no. NC_002951.2): SACOL1678 (luciferase, YP_186518.1, G996T), SACOL1708 (type III leader peptidase, YP_186547.1, C218T) in the core genome and the targets (n = 2) SACOL0289 (hypothetical protein, YP_185183.1, G56T, C57T, G84A, A105C) and SACOL0651 (hypothetical protein, YP_185536.1, C524A) in the accessory genome.

A total of 69 genes were identified referring virulence attributes in USA300 samples W1, H1/04-H3/04, H1/05-H6/05, H1/06, H1/16-H8/16, C2406 and FPR3757. Water isolate W1 harboured 61 virulence gene loci. Comparison of all these 21 isolates revealed 26 identical virulence genes and 60 identical virulence alleles between W1 and H5/16, 59 identical virulence alleles between W1, C2046 and reference strain FPR3757, and 64 identical virulence alleles between H3/04 and C2406 (Supplementary Table S1). All 21 isolates (W1, H1/04-H3/04, H1/05-H6/05, H1/06, H1/16-H8/16, C2406, FPR3757) carried the *agr* allele 1, all except H6/16 and H7/16 carried Panton-Valentine leucocidin (*lukS*-PV/*lukF*-PV), seventeen isolates carried the USA300 characteristic arginine catabolic mobile element (ACME) (W1, H1/04-H3/04, H3/05-H6/05, H1/06, H1/16, H3/16-H6/16, H8/16, C2406, FPR3757), and sixteen isolates carried *seq* (H1/04-H3/04, H2/05-H6/05, H1/06,H2/16-H5/16, H8/16, C2406, FPR3757).
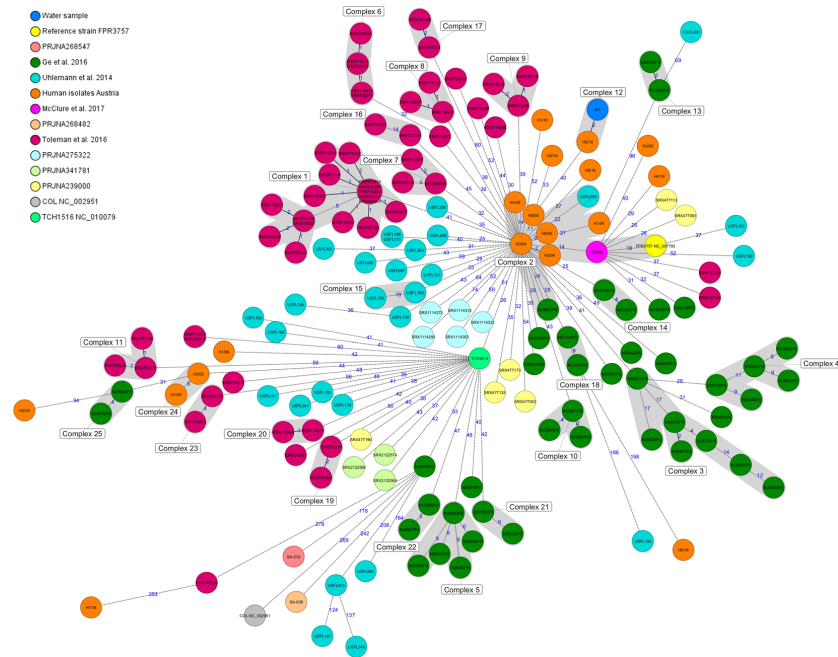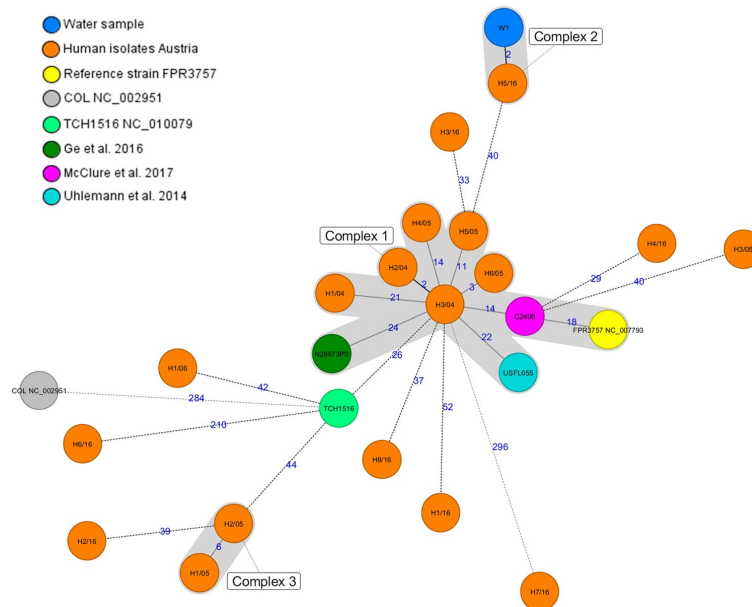
Figure 1. Minimum spanning tree for 165 MRSA isolates based on the cgMLST of *S. aureus*. Colours correspond to the origin of the samples. Each circle represents isolates with an allelic profile based on the sequence of 1,861 core genome targets. Blue numbers refer to the allelic differences between two isolates. Isolates with closely related genotypes were identified with a maximum of 24 allelic differences and are shaded in grey.

Phenotypic susceptibility testing revealed that the water isolate W1 was resistant to beta-lactams (benzylpenicillin, amoxicillin-clavulanic acid, cefoxitin), fluoroquinolones (ciprofloxacin, moxifloxacin) and erythromycin among the seventeen antibiotics tested (Table 1).

In total 25 genes conferring resistance to various antibiotics were identified in USA300 samples W1, H1/04-H3/04, H1/05-H6/05, H1/06, H1/16-H8/16 C2406 and FPR3757. The water isolate W1 harboured in total 14 resistance genes (Table 2) and showed complete concordance to H5/16, sharing nine identical resistance targets with FPR3757 and *fosB* with C2406. SNPs conferring resistance to ciprofloxacin were identified in 15 out of 21 isolates (except H1/04, H1/05, H2/05, H1/06, H2/16, H6/16), due to a L84S change in *gyrA*.

The presence of antimicrobial resistance against penicillin, methicillin, erythromycin and ciprofloxacin in isolate W1 was confirmed by in silico analysis using Mykrobe predictor.

Comparative analysis of isolate W1 to the closest relative isolate H5/16 via OrthoFinder revealed following differences in presence and absence of protein coding genes: additional presence of two domain-containing proteins (DUF443) and one hypothetical protein in isolate W1 and the additional presence of ten hypothetical proteins, four transposases, two family proteins (TIGR01741), one domain-containing protein (DUF5079), one immunoglobulin G-binding protein A and one transcriptional regulator in isolate H5/16.

Comparison of isolate W1 to isolate C2406 revealed nine additional hypothetical proteins, three domain-containing proteins (DUF443 (n = 2), DUF536), three transcriptional regulators, three transferases, two transporter, one penicillinase repressor BlaI, beta-lactam sensor/signal transducer BlaR1, penicillin-hydrolyzing class A beta-lactamase BlaZ, quinone oxidoreductase, one transposase and one ABC-F type ribosomal protection protein MsrA in isolate W1 and additional eight hypothetical proteins, two family proteins (TIGR01741), two transposases, one domain-containing protein (DUF5079) and one transcriptional regulator in isolate C2406 (Supplementary Table S2).

Additionally 15 genes in isolate W1 were identified for which no orthologue could be assigned in the other genomes in this comparison: two ATP-binding proteins, two family proteins (TIGR01741), two hypothetical proteins, one acyltransferase, one LysR family transcriptional regulator, one AI-2E family transporter, one homoserine dehydrogenase, one recombination protein RecJ and one DUF5079 domain-containing protein. Three genes revealed no significant result and could not be assigned to any known group of protein coding genes (Supplementary Table S3).

Antibiotic	MIC breakpoint (mg/L)		USA300 W1 (MIC)
	S≤	R>	
Benzylpenicillin	0.125	0.125	R (>32)
Amoxicillin-clavulanic acid	n.a	n.a	R (48)
Cefoxitin	4	4	R (>128)
Ciprofloxacin	1	1	R (>32)
Moxifloxacin	0.25	0.25	R (1.5)
Amikacin	8	16	S (1.5)
Gentamicin	1	1	S (0.125)
Teicoplanin	2	2	S (0.75)
Vancomycin	2	2	S (1)
Erythromycin	1	2	R (32)
Clindamycin	0.25	0.5	S (0.125)
Minocycline	0.5	1	S (0.064)
Linezolid	4	4	S (1.5)
Fosfomycin	32	32	S (0.5)
Fusidic acid	1	1	S (0.125)
Rifampicin	0.06	0.5	S (0.008)
Trimethoprim	2	4	S (0.38)

Table 1. Phenotypical resistance data for sample W1. MIC (minimum inhibitory concentration) breakpoints accord to EUCAST. S = sensitive, R = resistant, I = intermediate, n.a. = not applicable.

Figure 2. Minimum spanning tree for 25 MRSA isolates based on the cgMLST of *S. aureus*. Colours correspond to the origin of the samples. Each circle represents isolates with an allelic profile based on the sequence of 1,861 core genome targets. Blue numbers refer to the allelic differences between two isolates. Isolates with closely related genotypes were identified with a maximum of 24 allelic differences and are shaded in grey. Austrian isolates: W1, H1/04-H3/04, H1/05-H6/05, H1/06, H1/16-H8/16; reference strains: FPR3757, COL (NC_002951.2); closest related international isolates = C2406[26], N28973PS[39], TCH1516[38], USLF055[36].

The circular map (CGView Server V 1.0 (2007)) depicts sequence similarity between the reference strain FPR3757, isolates H5/16 and W1 and sequence similarity between COL (NC_002951.2), FPR3757 and isolate W1 in Fig. 3.

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Target	W1	H1/04	H2/04	H3/04	H1/05	H2/05	H3/05	H4/05	H5/05	H6/05	H1/06	H1/16	H2/16	H3/16	H4/16	H5/16	H6/16	H7/16	H8/16	C2406	FPR3757	Resistance against	Accession Nb.
aadD	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1	ND	ND	ND	aminoglycosides	BA000017.4
aphA3	ND	1	1	1	1	1	1	1	1	ND	1	ND	ND	ND	1	ND	ND	ND	1	ND	ND	aminoglycosides	CP009681.1
blaI	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	ND	1	7	ND	ND	beta-lactam	SRR016154
blaR	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	ND	15	15	6	ND	beta-lactam	SRR016154
blaZ	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	ND	1	10	6	ND	beta-lactam	SRR016154
ccrA2	6	NAT	6	6	6	6	6	6	6	NAT	6	6	6	6	6	6	ND	17	6	1	6	methicillin	NC010079
ccrB2	6	NAT	6	6	6	6	6	6	6	ND	6	6	15	6	6	6	ND	1	6	1	6	methicillin	NC010079
ccrC	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	NAT	ND	ND	3	ND	ND	methicillin	AP008934.1
dfrA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1	ND	1	trimethoprim	AE017171.1
ermA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1	ND	ND	ND	MLSBK	BA000017.4
fosB	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	fosfomycin	CP000046.1
lmrP	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	ND	1	various	CP000046.1
mecA	3	3	3	3	3	3	3	3	3	3	3	3	3	3	4	3	3	1	3	1	3	methicillin	BX571856.1
mecR-intact	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1	ND	ND	methicillin	BA000017.4
mecR-truncated	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	ND	FQC	1	ND	1	methicillin	CP000046.1
merA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1	ND	ND	SCCmec	NC013352.1
merB	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1	ND	ND	SCCmec	AB179623.1
mphC	2	2	2	2	2	2	2	2	2	2	2	2	FQC	FQC	2	2	ND	ND	2	ND	ND	MLSBK	NC017351
mprF	1	1	1	1	1	1	1	1	1	1	1	NAT	1	1	1	1	1	1	1	ND	1	methicillin-oxacillin	CP000046.1
msrA	2	2	2	2	2	2	2	2	2	2	2	2	2	FQC	2	2	ND	ND	NAT	ND	ND	MLSBK	NC017351
qacA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	NAT	ND	ND	ND	various	AF053771.1
sdrM	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	NAT	ND	1	various	CP000046.1
tetK	ND	ND	ND	ND	ND	ND	ND	4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	tetracycline	sampleLGO017
ugpQ	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	ND	1	SCCmec	CP000046.1
xylR	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2	ND	ND	SCCmec	BA000018.3

Table 2. Identified resistance genes and allele types in USA300 samples W1, H1/04–H3/04, H1/05–H6/05, H1/06, H1/16–H8/16, C2406 and FPR3757. Numbers accord to the respective allele type for each target; FQC = failed target QC procedure; NAT = new allele type; ND = not detected.

Discussion

The discharge, persistence and dissemination of AMR in nature are considered a major public health threat worldwide[19]. Therefore a strategy to better control the release of antibiotics into the environment and subsequently to prevent contact of bacteria from human and animal sources with environmental organisms[20] is of utmost importance.

Staphylococcus aureus was particularly successful in developing resistance to antibiotics. MRSA is listed as of high priority on WHOs antibiotic-resistant pathogens list (http://www.who.int/medicines/publications/global-priority-list-antibiotic-resistant-bacteria/en/). Since the first detection of MRSA with association to healthcare (HA-MRSA) settings in 1961[21], new epidemic MRSA clones have emerged affecting the community (CA-MRSA) or being associated with animals (LA-MRSA)[22–24]. Surveillance and characterisation of clinical, animal and environmental isolates is a public health imperative.

The MRSA isolate described was obtained from a water sample from a small river in southern Austria[24]. Characterisation of this isolate by WGS analysis revealed that the isolate has all characteristic features of CA-MRSA USA300[7]. CgMLST and wgMLST based gene-by-gene comparison[18] to eighteen clinical isolates (USA300) available from the National Reference Laboratory for Staphylococci including *Staphylococcus aureus* revealed a very close relatedness to one clinical USA300 isolate (H5/16) collected 2016 in Vienna, differing only by two alleles in their core- and by four alleles in their whole-genome targets. The water and patient isolates shared 60 identical virulence and 14 antibiotic resistance alleles. We were unable to find an epidemiological link between these two isolates. Four isolates collected in 2016 from a hospital located in one of the settlements next to this river, showed no close relatedness to the USA300 water isolate.

An interesting additional outcome of this WGS based typing study was that clinical USA300 isolates from Austria are a diverse population indicating that USA300 clones were introduced several times in Austria and are not all descendants from the first Austrian USA300 isolate from 2004. According to the WGS patterns all eight clinical Austrian USA300 isolates from 2016 are unrelated to each other. Thus in comparison to previous typing methods that did not allow a differentiation (same MLST, same *spa* type), WGS based typing is an important improvement representing an added information value for hospitals allowing a clear discrimination of outbreak and transmission events from unrelated isolates. In due consideration of staphylococcal mutation rates of one SNP per 15 weeks[25] we can assume that the water isolate W1 and several clinical isolates from Austria are descendants of the complex containing the hypervirulent USA300-C2406 strain from a patient with necrotizing pneumoniae isolated in Canada in 2004[26] and reference strain FPR3757[1]. In contrast to the aforementioned Austrian USA300 isolates from 2016, the first USA300 isolates detected in Austria in 2004 to 2005[10] belong to four different complex types. One complex contained isolates collected from two patients' stationary in the same hospital in 2005, whereof the initial one was derived from a postal worker processing post from the United States,
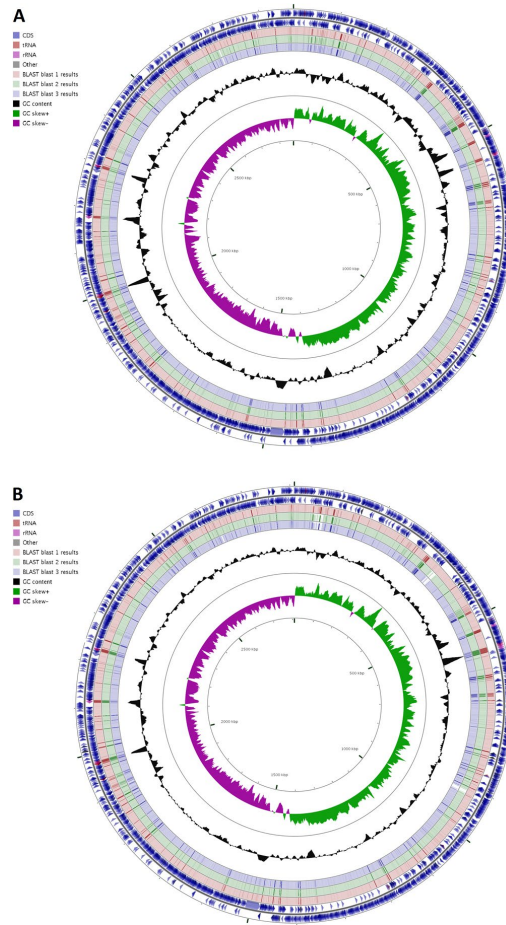
Figure 3. Comparative circular maps of *Staphylococcus aureus* genomes USA300_FPR3757 (NC_007793) and COL (NC_003951.2) to USA300 isolates. (**A**) The circles display the following information (from outside to inside): CDSs of USA300_FPR3757 on the + strand, CDSs of USA300_FPR3757 on the − strand, blastn result 1: USA300_FPR3757, blastn result 2: H5/16, blastn result 3: W1, GC content, GC skew + (green) and − (violet) value; (**B**) The circles display the following information (from outside to inside): CDSs of COL (NC002951.2) on the + strand, CDSs of COL (NC002951.2) on the − strand, blastn result 1: COL (NC002951.2), blastn result 2: USA300_FPR3757, blastn result 3: W1, GC content, GC skew + (green) and − (violet) value.

which had a clear transmission link. The second complex contained six isolates indicating a transmission or a common source of infection. Within this complex an epidemiological link to the United States could only be confirmed for one isolate (isolate ID H4/05) as well as the close relatedness between a couple (isolate ID H2/04 & H3/04) and an Austrian isolate from 2005 (isolate H6/05). CgMLST analysis shows that Austrian isolates of the second complex are closely related to several US isolates including C2046 and reference strain FPR3757 isolated from a HIV-positive patient in the United States in 2000[1] demonstrating a transfer. In contrast to the United States, where USA300 has become the most successful MRSA clone in the community as well as in hospitals, USA300 is still rare in most European countries and Austria. However, the fact that USA300 infections transmitted via contaminated environmental sources have been demonstrated[27] and the detection in a river water sample with close relatedness of the water isolate to a clinical isolate might indicate a possible risk for public health.

In conclusion, this is to our best knowledge the first genome based characterisation of an environmental USA300 isolate confirming the emerging global health threat caused by environmental AMR. While numerous environmental AMR studies have reported on Gram negative bacteria our example shows that also Gram positive

bacteria might play their role. Considering the historical evolution of MRSA clones the emergence of new pathogenic clones is merely a question of time.

For appropriate actions further investigations are essential to determine the occurrence, risk, environmental impact of AMR in the environment as well as use of optimal wastewater treatment technologies to alleviate this public health threat[28].

Methods

Microorganisms and Species Identification. In 2016 we collected 12 water samples from four rivers (Danube (n = 3, 48°14′53″N, 14°25′ 37″E; 48°14′45″N, 14°26′1″E; 48°14′32″N, 14°26′41″E), Glan (n = 3, 46°42′46″N, 14°6′23″E; 46°38′1″N, 14°18′56″E; 46°38′14″N, 14°17′44″E), Inn (n = 4, 47°16′35″N, 11°12′50″E; 47°15′55″N, 11°16′58″E; 47°16′14″N, 11°26′16″E; 47°16′6″N, 11°27′42″E), Traun (n = 1, 48°13′2″N, 14°15′26″E)) flowing through the Austrian provinces Carinthia, Salzburg, Tyrol, Lower Austria, Upper Austria, and Vienna. Samples were collected in sterile 500 ml sodium thiosulfate containing bottles (Corning® Gosselin™, NY, USA), 20 to 30 cm below the river surface and 50 to 100 cm apart from the river bank. For MRSA screening 100 ml aliquots of samples were filtrated and bacterial isolates were enriched by incubating the filter in tryptone soya broth (containing 3.5 mg/ml cefoxitin and 75 mg/ml aztreonam) at 37 °C overnight according to the standard protocol for isolating MRSA from dust samples (http://www.eurl-ar.eu/233-protocols.html, Isolation of MRSA from dust samples). To minimize the risk of contamination water sample filtration and cultivation were separated spatially, locally and temporally from cultivation of clinical samples. Laboratories were weekly screened for contaminations using air settlement plates and surface swabbing according to a standard operation protocol (SOP) following international guidelines[29]. For detection of methicillin resistant staphylococci, the overnight cultures were cultivated on BBL™ CHROMagar™ MRSA II according to the manufacturer's instructions (Becton Dickinson, Vienna, Austria). Mauve colonies from chromogenic media were sub-cultured and identified by matrix assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry.

The clinical MRSA USA300 isolates from the Austrian National Reference Laboratory for Coagulase Positive Staphylococci originating from 2016 (H1/16 to H4/16) came from a hospital located in a settlement beside the river (1,470 beds) in Carinthia and four isolates (H5/16 to H8/16) were from Vienna and from the province Lower Austria. Additionally isolates from the Austrian provinces Vienna, Lower Austria, Salzburg and Styria from 2004 (H1/04-H3/04), 2005 (H1/05-H6/05) and 2006 (H1/06) were analysed.

Antibiotic susceptibility testing. *In vitro* susceptibility was tested for 17 antibiotics (benzylpenicillin, amoxicillin-clavulanic acid, cefoxitin, ciprofloxacin, moxifloxacin, amikacin, gentamicin, teicoplanin, vancomycin, erythromycin, clindamycin, minocycline, linezolid, fosfomycin, fusidic acid, rifampicin, trimethoprim) by Etests (bioMérieux, Marcy-l'Ètoile, France) and interpreted according to EUCAST (European Committee on Antimicrobial Susceptibility Testing, EUCAST Clinical Breakpoint Tables v.7.1, valid from 2017-03-10).

Whole genome sequencing and data analysis. For whole genome sequencing (WGS) high quality genomic DNA (gDNA) was isolated using the MagAttract HMW DNA Kit (Qiagen, Hilden, Germany) and quantified with a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) using the dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Libraries of bacterial genomes were prepared using Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. A desired coverage of at least 50-fold was calculated with Sequencing Coverage Calculator (www.illumina.com/CoverageCalculator) and bacterial isolates were paired-end sequenced with a read length of 2 × 300 basepairs on a MiSeq instrument (Illumina, San Diego, CA, USA). SPAdes version 3.9.0[30] was used for read assembly and SeqSphere+ version 4.1.9 (Ridom, Münster, Germany) for strain characterisation using a recently published cgMLST scheme[18] and calculation of minimum spanning trees (MST). Phylogenetic relatedness between water and related clinical isolates was further visualized using CGView Server V 1.0(2007)[31].

Spa type[32], MLST (multilocus sequence type)[33], cgMLST (core genome MLST)[18], resistance genes and virulence genes[34] were extracted from WGS data using SeqSphere+ version 4.1.9. Mutations in *cap5* were identified according to the publication of Boyle-Vavra *et al.* (2015) and mutations in *gyrA* causing ciprofloxacin resistance were identified according to Sreedharan *et al.*[35,36]. Genotype-based antimicrobial-resistance was predicted for isolate W1 by using Mykrobe predictor for *Staphylococcus aureus*[37].

Genomes from Sequence Read Archive. One-hundred and forty-six isolates downloaded from the Sequence Read Archive (SRA) belonged to following Bioprojects: PRJEB2870 (n = 28)[38], PRJEB3174 (n = 55)[11], PRJNA224116 (n = 3)[1,39,40], PRJNA239000 (n = 6), PRJNA268482 (n = 1), PRJNA268547 (n = 1), PRJNA275322 (n = 5), PRJNA311554 (n = 43)[41], PRJNA341781 (n = 3), PRJNA345240 (n = 1)[26].

Analysis of gene presence and absence. Detailed analysis of additional genomic information was performed for 10 genomes: Reference strains C2406 (CP019590.1) and FPR3757 (CP000255.1) and isolates H1/04, H2/04, H3/04, H4/04, H5/04, H6/04, H05/16 and W1. The contigs of each assembly was filtered for a minimum length of 1,000 nucleotides. Genes were predicted using prodigal v. 2.6.3[42] with default parameters, orthologous groups where calculated with OrthoFinder v. 1.1.4[43]. Orthologous groups with differences in presence/absence were selected. Annotation of the orthologous groups and genes without group assignment was performed using NCBI-BLASTp v. 2.6.0+ with e-vaule cutoff of 0.01[44] and the RefSeq nr-protein database release 84[45]. The most frequent annotation in an orthologous groups was selected as description for the group.

Nucleotide accession number(s). This Whole Genome Shotgun project including ST8 isolates (H1/16-H8/16) from the Austrian national reference laboratory for coagulase positive staphylococci in 2016 and

the water isolate (W1) have been deposited at DDBJ/ENA/GenBank under the accession NKCP00000000 to NKCX00000000. The version described in this paper is version NKCP01000000 to NKCX01000000.

Data availability. All data generated or analysed during this study are included in this published article.

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Author Contributions

Designed the project: S.H., B.S., R.L.M., F.A., W.R.; Performed the experiments: S.L.; Analysed the data: S.L., S.H., P.H., B.S., T.R., W.R.; Wrote the manuscript: S.L., P.H., F.A., W.R. All authors reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-27781-8.

Competing Interests: The authors declare no competing interests.

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Draft Genome Sequence of a Community-Acquired Methicillin-Resistant *Staphylococcus aureus* USA300 Isolate from a River Sample

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ABSTRACT The increasing emergence of multiresistant bacteria in health care settings in the community and in the environment represents a major health threat worldwide. Here, we report the draft genome sequence of a community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) USA300 isolate (W1) from a small river in southern Austria.

In recent years, the rising evolution of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) has been prompted by the release of antibiotics as pollutants in aquatic environments (1). There, ARGs are able to persist and even spread through horizontal gene transfer, genetic mutation, and recombination of ARB (2). The dissemination of increasing antibiotic-resistant human pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA), is a growing public health threat; therefore, it is of major importance to understand the circumstances which enhance the evolution and distribution of ARGs (3).

In 2016, the Austrian Agency for Health and Food Safety started a pilot project to investigate antimicrobial resistance (AMR) in surface water. Among several water samples from diverse Austrian rivers, one sample from a small river in the province Carinthia contained a MRSA isolate. Whole-genome sequence analysis of this water isolate, W1, identified the main characteristics of community-acquired MRSA (CA-MRSA) USA300: sequence type 8 (ST8), *spa* type t008, staphylococcal cassette chromosome *mec* element type IV (SCC*mec* IV), Panton-Valentine leukocidin (PVL), and the arginine catabolic mobile element (ACME) cluster. This is the first report and draft genome sequence of a MRSA USA300 isolate derived from a water sample.

For the isolation of *S. aureus* from water samples, 100-ml aliquots were filtrated, and filters were incubated in thioglycolate at 37°C overnight. For the detection of MRSA, the overnight culture was plated on chromogenic medium (BBL CHROMagar MRSA II; Becton, Dickinson, Vienna, Austria). The water isolate was further identified as *Staphylococcus aureus* using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (Bruker, Billerica, MA). High-quality genomic DNA from an overnight culture was obtained using the MagAttract high-molecular-weight (HMW) DNA kit (Qiagen, Hilden, Germany). The quantification of input DNA was carried out with a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) using the double-stranded DNA (dsDNA) BR assay kit (Thermo Fisher Scientific). Library preparation for whole-genome sequencing was done with a Nextera XT kit (Illumina, Inc., San Diego, CA, USA), according to the manufacturer's protocol. Paired-end sequencing (2 × 300 bp) was performed on a MiSeq instrument (Illumina, Inc.) and generated 557,130

reads from 142,372,680 unassembled nucleotides. For assembly into a draft genome, raw reads were *de novo* assembled using SPAdes version 3.9.0 (4). Contigs were filtered for a minimum coverage of 5-fold and minimum length of 200 bp, which resulted in 380 contigs with a total of 3,016,290 nucleotides at a coverage of 50-fold. The NCBI Prokaryotic Genome Automatic Annotation Pipeline identified 3,348 genes, 3,267 coding sequences, 118 pseudogenes, 21 rRNA operons (9 complete, 12 partial), and 56 tRNA genes.

Accession number(s). This whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number NKCX00000000. The version described in this paper is version NKCX01000000.

ACKNOWLEDGMENT

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Whole genome sequencing reveals resemblance between ESBL-producing and carbapenem resistant *Klebsiella pneumoniae* isolates from Austrian rivers and clinical isolates from hospitals

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HIGHLIGHTS

- Detection of ESBL-, carbapenemase-producing *K. pneumoniae* in Austrian river water
- Relatedness of clinical and water isolates identified using WGS.
- Accordance of hospital wastewater effluent and water sampling location
- Evidence of anthropogenic pollution of river water in urban areas in Austria

GRAPHICAL ABSTRACT
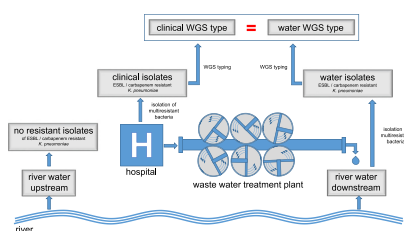
ARTICLE INFO

ABSTRACT

In 2016, the Austrian Agency for Health and Food Safety started a pilot project to investigate antimicrobial resistance in surface water. Here we report on the characterisation of carbapenem resistant and ESBL-producing *K. pneumoniae* isolates from Austrian river water samples compared to 95 clinical isolates recently obtained in Austrian hospitals.

Ten water samples were taken from four main rivers, collected upstream and downstream of major cities in 2016. For subtyping and comparison, public core genome multi locus sequence typing (cgMLST) schemes were used. The presence of AMR genes, virulence genes and plasmids was extracted from whole genome sequence (WGS) data.

In total three ESBL-producing strains and two carbapenem resistant strains were isolated. WGS based comparison of these five water isolates to 95 clinical isolates identified three clusters. Cluster 1 (ST11) and cluster 2 (ST985) consisted of doublets of carbapenem resistant strains (one water and one clinical isolate each). Cluster 3 (ST405) consisted of three ESBL-producing strains isolated from one water sample and two clinical specimens. The cities, in which patient isolates of cluster 2 and 3 were collected, were in concordance with the water sampling locations downstream from these cities. The genetic concordance

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between isolates from river water samples and patient isolates raises concerns regarding the release of wastewater treatment plant effluents into surface water. From a public health perspective these findings demand attention and strategies are required to minimize the spread of multiresistant strains to the environment.

1. Introduction

The Gram negative bacterium *Klebsiella pneumoniae* (*K. pneumoniae*) is a leading cause of human nosocomial infections, but can also be acquired in the community (Podschun and Ullmann, 1998; Shon and Russo, 2012). It can either be carried asymptomatically or can cause a wide spectrum of infections, for instance pneumonia; wound, soft tissue, urinary tract and bloodstream infections (Holt et al., 2015; Maatallah et al., 2014; Podschun and Ullmann, 1998).

The evolution, spread and emergence of bacterial antibiotic resistance represent one of the most important health care problems worldwide (Hawkey, 2008). Since the 1950s, infections caused by *Enterobacteriaceae* are treated with beta-lactam antibiotics. Following the introduction of broad-spectrum beta-lactam antibiotics, new extended spectrum beta-lactamases (ESBL) emerged (Grundmann et al., 2010). In 1996 the first carbapenemase, encoded by the bla_{KPC} gene, was detected in *K. pneumoniae* (Yigit et al., 2001). Subsequently, other carbapenemase-genes, such as bla_{NDM}, bla_{OXA-48}, bla_{VIM} and bla_{IMP-1} emerged (Fukigai et al., 2007; Kumarasamy et al., 2010; Miriagou et al., 2003; Wesselink et al., 2012). The global dissemination of carbapenemase-producing strains has been shown in rivers in different regions of the world (Khan et al., 2018; Mahon et al., 2017; Zarfel et al., 2017; Zurfluh et al., 2013).

Cumulating reports indicate that animals, food products and the environment may also constitute reservoirs for carbapenemase producing bacteria (Wyres and Holt, 2018; Zurfluh et al., 2013).

The discharge of hospital effluents into sewers are hotspots for antimicrobial resistant bacteria and antimicrobials (Duarte et al., 2018; Hocquet et al., 2016; Marti et al., 2014). The intermixture of bacteria from different anthropogenic sources (urban, industrial and agricultural waste) with environmental species may result in the transfer of antibiotic resistance genes. Subsequently this watery soup provides perfect conditions for the evolution of novel combinations of resistance genes (Amos et al., 2014) leading to the evolution and selection of new resistant species due to the presence of antibiotic residues in water (Chen et al., 2018; Gekenidis et al., 2018; Lupo et al., 2012; Rodriguez-Mozaz et al., 2015).

A mitigation of this critical situation can be achieved by consequent measurements leading to a reduction of antibiotic usage in hospitals and agriculture. This will reduce the risk of future emerging resistant pathogens, will also minimize the bacterial load in WWTPs subsequently increasing the efficiency of WWTPs in removing dangerous bacteria. In Austria with a total population of 8.75 million currently about 95% of the population are served by 1865 WWTPs (for a size >50 population equivalent (PE)) and 5% of the population are served by about 27,500 small WWTPs (<50 PE) (Langergraber et al., 2018). The most popular technologies for secondary treatment are conventional activated sludge (CAS), vertical flow (VF) wetlands as well as sequencing batch reactors (SBR), these processes can provide substantial but not complete removal of bacteria and this demands advanced treatment processes. However, 22.7% of small WWTPs use primary treatment only.

The aim of this study was to evaluate the diversity of ESBL and carbapenemase-harboring *K. pneumoniae* in water samples collected in four main Austrian rivers and to compare them with clinical isolates to identify possible sources of anthropogenic pollution.

2. Material and methods

2.1. Study design and strain isolation

Ten water samples were taken from main Austrian rivers, collected upstream (n = 5) and downstream (n = 5) of major cities in 2016, to screen for the presence of pathogenic, multiresistant bacterial organisms. Per sampling site, one 500 ml river water sample was collected upstream and one downstream (1 km to 3 km after waste water treatment plant effluent) from major Austrian cities: river Danube: cities Vienna and Linz; river Inn: city Innsbruck; river Glan: city Klagenfurt; river Traun: city Linz (Fig. 1).

A 100 ml sample aliquot was filtered and the filtrate incubated in BBL fluid thioglycollate medium (Becton Dickinson, NJ, USA) at 37 °C overnight. For extraction and detection of ESBL-producing and carbapenem resistant strains, 70 μl overnight cultures were plated on chromogenic media (chromID CARBA (bioMérieux, Marcy-l'Étoile, France), chromID ESBL (bioMérieux)). Per plate, morphologically different colonies were picked in triplicate and sub-cultured. Sub-cultivated single colonies were identified on species level by matrix assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry.

2.2. Whole genome sequencing and sequence data analysis

High-molecular-weight (100–200 kb) DNA was isolated from isolates using the MagAttract HMW DNA Kit (Qiagen, Hilden, Germany) and quantified fluorometrically with a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) using a target specific Qubit assay (dsDNA BR Assay Kit, Thermo Fisher Scientific).

To prepare ready-to-sequence libraries of bacterial genomes Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA) was used according to the manufacturer's protocol and paired end sequenced (2 × 300 bp) on an Illumina Miseq instrument. Sequencing coverage calculator (www.illumina.com/CoverageCalculator) was used for calculating a desired mean coverage of at least 50-fold. *De novo* assembly of raw reads was performed using SPAdes (version 3.9.0) (Bankevich et al., 2012) and NGS data interpretation was carried out with the analysis software SeqSphere⁺ (Ridom, Münster, Germany).

For phylogenetic analysis the MLST (multi-locus sequence type) (Diancourt et al., 2005) and the cgMLST (core genome multi-locus sequence type) were extracted from the whole genome sequence (WGS) data. Based on the defined *K. pneumoniae sensu lato* cgMLST in SeqSphere⁺, comprising of 2358 target genes, a gene-by-gene approach was used to compare genomes. Isolates were visualised as minimum spanning trees (MST) and genotypically related isolates were identified with a Complex Type (CT) Distance of 15 alleles (https://www.cgmlst.org/ncs/schema/2187931/). The definition "good core genome targets" was according to the criteria described in detail in Ruppitsch et al. (2015).

PlasmidFinder 1.3 (Carattoli et al., 2014) available from the Center for Genomic Epidemiology web server (http://www.genomicepidemiology.org/) and the Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2017) were used to search for the presence of plasmids and genes conferring antibiotic resistance. The bla_{SHV} alleles were refined using the Institut Pasteur BIGSdb database. The existence of virulence genes was investigated by using the virulence allele library from the
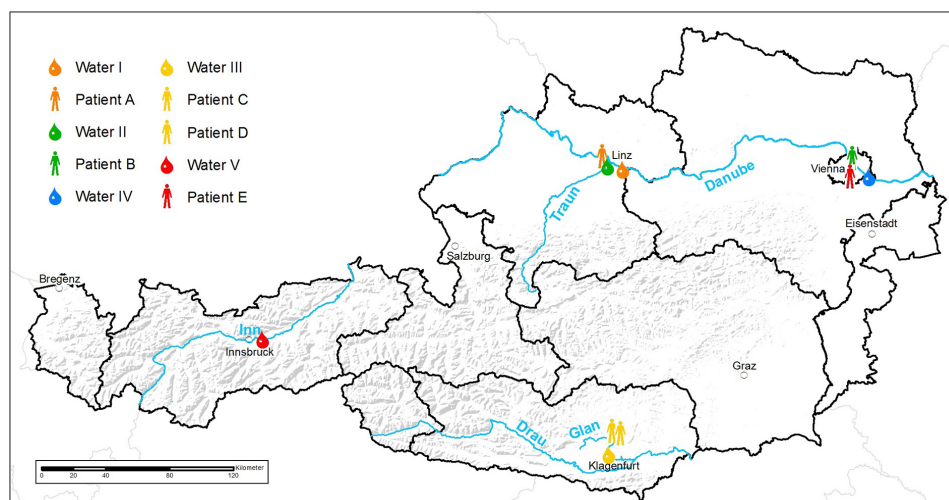
Fig. 1. Geographical map of Austria showing the sampling points for river-water samples yielding *K. pneumoniae* isolates and for the hospitals providing indistinguishable patient isolates. For water and clinical isolates belonging to the same MLST the same colour was assigned. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Institut Pasteur BIGSdb database for *K. pneumoniae* (http://bigsdb. pasteur.fr/klebsiella).

2.3. Collection of clinical isolates

To assess relationship of the river water isolates to clinical isolates, 95 isolates voluntarily provided by Austrian hospitals, available in the AGES "in-house" *K. pneumoniae* sequence database - comprising 95 isolates to date from 2011 (n = 4), 2012 (n = 1), 2015 (n = 8), 2016 (n = 46), 2017 (n = 24), 2018 (n = 12) were used for comparison.

2.4. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was determined for five water and four related clinical isolates with SensititreTM EUVSEC and EUVSEC2 plates (ThermoFisher Scientific, Waltham, USA) by microbroth dilution according to CLSI (Clinical and Laboratory Standards Institute) guidelines (CLSI standard M07). MIC values were interpreted according to EUCAST criteria (European Committee on Antimicrobial Susceptibility Testing, EUCAST Clinical Breakpoint Tables v.8.1, valid from 2018 to 05-15) for the following antibiotics: ampicillin, cefotaxime, ceftazidime, cefepime, ertapenem, imipenem, meropenem, gentamicin, ciprofloxacin and colistin.

Phenotypic detection of AmpC β-lactamase was carried out with Etest AmpC CN/CNI (bioMérieux).

2.5. Nucleotide sequence accession numbers

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank including the following accession numbers: QWWA00000000 (water I), QWVW00000000 (water II), QWVY00000000 (water III), QWVZ00000000 (water IV), QWVX00000000 (water V), QWWF00000000 (patient A), QWWE00000000 (patient B), QWWD00000000 (patient C), QWWC00000000 (patient D), QWWB00000000 (patient E). The version described in this paper is the first version.

3. Results

3.1. Whole genome sequencing analysis

The five water samples collected upstream from cities Vienna (river Danube), Linz (n = 2, river Danube and Traun), Klagenfurt (river Glan) and Innsbruck (river Inn) yielded neither ESBL-producing nor carbapenem resistant *K. pneumoniae* isolates. All five samples taken downstream from the cities contained multidrug-resistant *K. pneumoniae* isolates: two isolates were from the Danube (one obtained downstream from Linz (isolate ID: water I), one downstream from Vienna (water IV)), and one each from the remaining river samples of Traun (water II), Glan (water III) and Inn (water V).

For five *K. pneumoniae* isolates from water the MLST type was extracted from the assembly data and identified five different STs and five respective CTs: ST11/CT1302 (water I), ST323/CT266 (water V), ST405/CT1363 (water III), ST985/CT1362 (water II) and a previously not described ST3400/CT2200 (water IV). The five *K. pneumoniae* isolates were sequenced with an average coverage of 77-fold (53 to 100-fold) and comprised on average 99.4% (99.2 to 99.7%) called cgMLST alleles.

A cgMLST based comparison of these five water isolates to 95 clinical *K. pneumoniae* isolates from the AGES *K. pneumoniae* sequence database identified three clusters (Fig. A.1). Cluster 1 (ST11) consisted of isolates water I and patient A with three allelic differences in their cgMLST profiles (CT1302). Cluster 2 (ST985) consisted of isolates water II and patient B which shared the same cgMLST profile (CT1362). Cluster 3 (ST405) consisted of isolates water III, patient C and patient D. Isolates from patient C and patient D showed three respectively two allelic differences from the water III isolate in their cgMLST profiles (CT1363) (Figs. 1, 2). We further applied the previously published Pasteur cgMLST scheme (Bialek-Davenet et al., 2014) and found 0 (cluster 1 and 2) or 1 (cluster 3) intra-cluster allelic mismatches out of 634 loci. In contrast, there were 31 mismatches between the two ST323 isolates. All genotypically related clinical isolates in clusters 1–3 (Table 1) were collected in 2016 and were from hospitals in Linz (cluster 1), Vienna (cluster 2) and Klagenfurt (cluster 3).
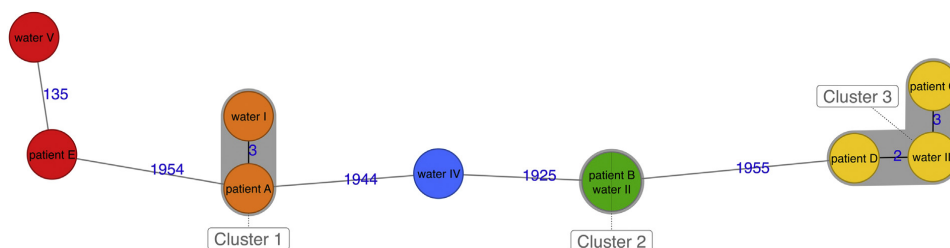
Sample ID	River	City	Good cgMLST targets	ST	Complex Type	Cluster
Water I	Danube	-	99.8 %	11	1302	Cluster 1
Patient A	-	Linz	99.8 %	11	1302	
Water II	Traun	-	99.2 %	985	1362	Cluster 2
Patient B	-	Vienna	99.2 %	985	1362	
Water III	Glan	-	99.7 %	405	1363	Cluster 3
Patient C	-	Klagenfurt	99.7 %	405	1363	
Patient D	-	Klagenfurt	99.7 %	405	1363	
Water IV	Danube	-	98.5 %	3400	2200	-
Water V	Inn	-	99.7 %	323	266	-
Patient E	-	Vienna	99.6 %	323	1577	-

Fig. 2. Minimum spanning tree including *K. pneumoniae* isolates collected from Austrian rivers and closely related clinical isolates collected from hospitals in Linz, Vienna and Klagenfurt in 2016. Each circle represents isolates with an allelic profile based on the cgMLST which consists out of 2358 alleles. Blue numbers correspond to the allelic differences between isolates; isolates with closely related genotypes are shaded in grey and marked as clusters. Isolates were coloured according to classical MLST. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Two river isolates, one from the Inn (water V) in Innsbruck and one from the Danube (water IV) in Vienna were singletons and showed no close relatedness to clinical isolates from Austria with 135 allelic differences (patient E collected in Vienna in 2016) and 1925 allelic differences to the closest related patient sample (patient B) (Fig. 2).

3.2. Phenotypic and genotypic antimicrobial resistance determination

In vitro susceptibility testing results of nine *K. pneumoniae* isolates are shown in Table A.1. All tested isolates were resistant to ampicillin, cefotaxime, ceftazidime, and cefepime; they were sensitive to colistin. Isolates from clusters 1 and 2, and from patients C and D were resistant to ertapenem too. Isolate water II was resistant to meropenem too. Isolates in cluster 3 and isolate water V were resistant to gentamicin too. All isolates except the isolates dubbed patient B and water IV were resistant to ciprofloxacin too. Out of all tested isolates only isolates of cluster 1 were confirmed as positive AmpC β-lactamase producer.

The analysis of antimicrobial resistance genes *via* the Comprehensive Antibiotic Resistance Database (CARD) identified 64 genes in total (Table 2) in the investigated *K. pneumoniae* isolates (n = 9), revealed resistances genes to beta-lactams, quinolones, aminoglycosides, phenicol,

sulphonamides, macrolide, rifampicin, trimethoprim, fosfomycin and fluoroquinolones. Both isolates of cluster 1 shared the same set of resistance genes (n = 35), isolates of cluster 2 shared 26 resistance genes in total, and isolates of cluster 3 shared a total of 28 resistance genes. Isolate water IV carried 20 antimicrobial resistance genes in total, including narrow-spectrum (native) bla_{SHV-1} and $bla_{CTX-M-15}$. Isolate water V carried a total of 30 antimicrobial resistance genes, including narrow-spectrum (native) bla_{SHV-1}, $bla_{CTX-M-15}$, bla_{TEM-1} and bla_{OXA-1} (Table 2).

3.3. Genotypic identification of virulence genes

Via the integrated *K. pneumoniae* virulence allele library (Bialek-Davenet et al., 2014; Lam et al., 2018) from Institut Pasteur database (http://bigsdb.pasteur.fr), 34 genes with attributes of virulence were detected (Table 3). Isolates belonging to the same ST shared the same set of virulence genes. Alleles of genes coding for yersiniabactin were identified and the observed combination was compared to the yersiniabactin sequence type (YbST) database from BIGSdb (Lam et al., 2018). Genes coding for yersiniabactin (*ybt*, n = 11) were identified in five isolates (cluster 1 and cluster 3) and the allelic profiles of YbST were assigned to sequence types YbST28 (ST11, n = 2) and YbST312

Table 1
Summarized data on patient isolates which were genotypically related to collected river samples.

Patient ID	Year of isolation	MLST	City of isolation	Age (years)	Sex	Specimen	Hospital ward
Patient A	2016	11	Linz	29	Male	Urine	Surgical ward
Patient B	2016	985	Vienna	2	Female	Urine	Paediatric ward
Patient C	2016	405	Klagenfurt	83	Female	Peritoneal fluid aspirate	Intensive care unit
Patient D	2016	405	Klagenfurt	81	Female	Urine	Urgent care center

MLST = multilocus sequence type.

(ST405, n = 3). Genes of type 3 fimbrial gene cluster (*mrk*), which is largely conserved within *K. pneumoniae*, and the ferric aerobactin receptor (*iutA*), which is part of the aerobactin gene cluster, were present in all nine isolates. Isolates from cluster 3 (water III, patient C, patient D; ST405), additionally carried genes which contribute to capsule formation (*kvgA, kvgS*), mediate uptake of ferric iron (*kfuA, kfuB, kfuC*) and genes belonging to the mammalian cell entry (*mce*)

cluster coding for microcin E492, a channel-forming bacteriocin with activity against mammalian cells (Hetz et al., 2002).

3.4. Plasmid identification

The strains contained a total of nine plasmids (IncFIB(K), IncFII(K), IncR, IncL/M, IncFIA(HI1), Col440I, Col440II, IncX5, IncFIB(Mar)) which

Table 2
Genotypic antimicrobial resistance determination for five isolates from river water and four genotypically related human isolates. The presence of a gene is represented by a "red box (+)".

Resistance Mechanism	Drug Class	target	water I ST11	patient A ST11	water II ST985	patient B ST985	water III ST405	patient C ST405	patient D ST405	water IV ST3400	water V ST323
antibiotic inactivation	aminoglycosides	AAC(3)-IIc					+	+	+		+
		AAC(6')-Ib			+	+					
		APH(3')-Ia	+	+							
		APH(3'')-Ib					+	+	+		+
		APH(6)-Id					+	+	+		+
		ANT(3'')-Ii-AAC(6')-IId fusion protein			+						
		aadA			+	+					
		aadA2	+	+							
		aadA15			+	+					
		aadA21			+						
		aadA24				+					
	aminoglycosides; fluoroquinolones	AAC(6')-Ib-cr	+	+			+	+	+		+
antibiotic efflux	aminoglycosides; aminocoumarins	baeR	+	+	+	+	+	+	+	+	+
antibiotic inactivation	cephalosporin	CTX-M-15	+	+			+	+	+	+	+
	cephalosporin; cephamycin	DHA-1	+	+							
	cephalosporin; penam	OXA-1	+	+			+	+	+		+
		OXA-10			+	+					
		OXA-48	+	+							
	cephalosporin; carbapenem; penam	SHV-1								+	+
		SHV-11	+	+							
		SHV-76					+	+	+		
		SHV-83			+	+					
	cephalosporin; penem; penam; monobactam	TEM-1					+	+	+		+
	cephalosporin; carbapenem; penem; penam; cephamycin	VIM-1			+	+					
antibiotic target replacement	diaminopyrimidine	dfrA12	+	+							
		dfrA14			+	+	+	+	+		+
antibiotic target alteration	elfamycin	EF-Tu mutation (R234F)				+	+	+	+	+	+
antibiotic efflux	fluoroquinolones	emrB	+	+	+	+	+	+	+	+	+
		emrR	+	+	+	+	+	+	+	+	+
antibiotic target alteration		qacH			+	+					
		parC mutation (S80I)	+	+							
antibiotic target protection		QnrB1					+	+	+		+
		QnrB4	+	+							
		QnrS1								+	
		QnrVC4			+	+					
antibiotic target alteration	fosfomycin	UhpT mutation (E350Q)	+	+	+	+	+	+	+	+	+
antibiotic inactivation		FosA5								+	
		FosA6	+	+	+	+	+	+	+		+
antibiotic inactivation	macrolides	mphA	+	+							
		Mrx	+	+							
antibiotic inactivation	phenicols	catB3	+	+			+	+	+		+
		catI	+	+							
antibiotic efflux		cmlA1				+					
		cmlA5			+	+					

(continued on next page)

Table 2 (*continued*)

antibiotic inactivation	rifamycin	**arr-3**	+	+								
antibiotic target replacement	sulfone; sulfonamides	**sul1**	+	+	+	+						
		sul2					+	+	+			+
antibiotic efflux	tetracyclines	**tet(C)**										+
antibiotic target alteration	triclosan	**gyrA mutation (S83F)**									+	
antibiotic efflux; reduced permeability to antibiotic	monobactam; cephalosporin; cephamycin; triclosan; glycylcycline; penem; carbapenem; penam; rifamycin; tetracycline; phenicol; fluoroquinolone	**marA**	+	+	+	+	+	+	+	+	+	+
antibiotic efflux	cephalosporin; tetracycline; triclosan; glycylcycline; phenicol; penam; rifamycin; fluoroquinolone	**marR**	+	+	+	+	+	+	+	+	+	+
		acrA	+	+	+	+	+	+	+	+	+	+
	cephalosporin; cephamycin; tetracycline; macrolide; penam; fluoroquinolone	**H-NS**	+	+	+	+	+	+	+	+	+	+
	penam; fluoroquinolone; macrolide	**CRP**	+	+	+	+	+	+	+	+	+	+
	tetracycline; fluoroquinolone	**adeF**	+	+	+	+	+	+	+	+	+	+
	tetracycline; triclosan; macrolide	**MexK**										+
	nitrofuran; tetracycline; glycylcycline; diaminopyrimidine; fluoroquinolone	**oqxA**	+	+	+	+	+	+	+	+	+	+
		oqxB	+	+								
	nitroimidazole	**msbA**	+	+	+	+	+	+	+	+	+	+
	pleuromutilin; streptogramin	**vgaC**	+	+							+	
antibiotic target alteration	cephamycin; monobactam; carbapenem; penam; cephalosporin	**PBP3 mutation (S357N, D350N)**	+	+	+	+	+	+	+	+	+	+
	nybomycin; fluoroquinolone	**gyrA mutation (S83I)**	+	+								
reduced permeability to antibiotic	monobactam; penem; cephalosporin; cephamycin; carbapenem; penam	**OmpK37**	+	+	+	+	+	+	+	+	+	+

showed between 95.95% and 100% identity to query sequences (Table 4). Isolates (n = 2) belonging to ST11 carried IncFIB(K), IncFII (K), IncR, IncL/M (pOXA-48). Whereas resistance genes *aadA2*, *dfrA12*, *mphA* are carried by IncFIB(K) plasmids, the OXA-48 carbapenemase gene is carried by IncL/M plasmids. Isolates (n = 2) belonging to ST985 carried IncFIB(K), IncFII(K), IncFIA(HI1), Col440I and Col440II. Isolates (n = 3) belonging to ST405 carried IncFIB(K) and IncFII(K). One isolate (ST3400) carried IncFIB(K), IncFII(K), IncR, IncX5, IncFIB (Mar) and one isolate (ST323) carried IncFIB(K) and IncFII(K). Water isolates had the same plasmid content as their related human isolates sharing the same MLST.

4. Discussion

The increasing number of antimicrobial resistant microorganisms poses a major problem for public health (WHO, 2015). Consequently, the World Health Organization (WHO) defined a list of global priority pathogens for antibiotic resistant bacteria to support the definition of priorities in research and development of new and effective drugs (http://www.who.int/medicines/publications/global-priority-list-antibiotic-resistant-bacteria/en/). Results of the European antimicrobial resistance surveillance report 2016 revealed that in Austria 9.6% of tested invasive *K. pneumoniae* isolates were resistant to 3rd generation cephalosporins and 0.7% were resistant to carbapenems (ECDC, 2017).

In 2016, the Austrian Agency for Health and Food Safety started a pilot project to survey the prevalence of clinically relevant, antibiotic resistant human pathogens in surface water. A common outcome of our study was that all water samples from rivers taken before a city were negative for ESBL and carbapenemase-producing *K. pneumoniae* whereas all samples taken one to three kilometers downstream of main Austrian cities wastewater plant release points were positive, which shows the impact of wastewater effluents and anthropogenic pollution on the aquatic environment (Amos et al., 2014).

A linkage of water and clinical isolates was demonstrated by WGS based comparison of isolates using two existing cgMLST schemes: https://www.cgmlst.org/ncs/schema/2187931/ and the Pasteur scheme (Bialek-Davenet et al., 2014). Three different clusters comprising water and clinical isolates were identified by both schemes. Isolates within the same cluster revealed similar antimicrobial resistance profiles, shared the same set of virulence genes and had the same plasmid content. Cluster 1 (ST11, CT1302) contained a water isolate collected downstream the waste water treatment plant (WWTP) of the city of Linz and a clinical isolate collected from a hospital in Linz in 2016, differing by three alleles in their cgMLST. ST11 is a common multidrug-resistant ST, mainly found in Asia and South America (Deleo et al., 2014; Dong et al., 2018; Munoz-Price et al., 2013). Although ST11 – OXA-48 outbreaks have been described in Europe previously (Jayol et al., 2016; Pérez-Blanco et al., 2018), the occurrence of this type in the clinic and in the environment in Austria may present an upcoming major public health threat. The capability of these strains to carry different classes of carbapenemases (OXA-48, VIM, NDM, KPC dramatically hampers medical treatment-options (Oteo et al., 2016; Pena et al., 2014; Voulgari et al., 2014). The ST11 isolates from our study were ESBL positive (*bla*_{CTX-M-15}, *bla*_{OXA-1}) and showed reduced susceptibility to carbapenems, attributable to the presence of *bla*_{OXA-48} located on a self-transferable IncL/M-type plasmid. Carbapenemase genes have been associated with multiple separate acquisition events mediated by plasmids of various sizes belonging to a huge range of incompatibility groups including broad and narrow host ranges, such as IncF, IncA/C, IncL/M, IncH, IncN and IncX3.6 (Voulgari et al., 2014). The occurrence of IncF-type, IncR and IncL/M plasmids in water as well as in patient isolates is highly alarming due to the possibility of acquiring further resistance genes and the occurrence of novel combinations of resistance genes (Amos et al., 2014). Additionally the detection of the yersiniabactin locus in these isolates reflects the virulence properties of these strains, since yersiniabactin is significantly associated with invasive infections in humans (Holt et al., 2015).

The second cluster (ST985, CT1362) contained a water isolate and a patient isolate (two-year-old baby girl with recurring urinary tract infection). These isolates were collected approximately 200 km away, which reveals for the first time the possible survival distance of multiresistant *K. pneumoniae* strains in river water. Both isolates had

Table 3
Identified virulence genes in five isolates from river water and four genotypically related patient isolates.

	water I ST11	patient A ST11	water II ST985	patient B ST985	water III ST405	patient C ST405	patient D ST405	water IV ST3400	water V ST323	
ybST	28	28			312	312	312			
ybtS	16	16			6	6	6			
ybtX	12	12			62	62	62			
ybtQ	4	4			60	60	60			
ybtP	3	3			4	4	4			
ybtA	3	3			1	1	1			yersiniabactin
irp2	35	35			145	145	145			
irp1	50	50			148	148	148			
ybtU	3	3			2	2	2			
ybtT	10	10			39	39	39			
ybtE	23	23			69	69	69			
fyuA	2	2			2	2	2			
mrkA	2	2	20	20	4	4	4		6	
mrkB	2	2	3	3	1	1	1	9	NAT	
mrkC	2	2	NAT	NAT	NAT	NAT	NAT		10	
mrkD	12	12	39	39	NAT	NAT	NAT		NAT	type 3 fimbrial gene cluster
mrkF			8	8	38	NAT	38		NAT	
mrkH	7	7	10	10	15	15	15	2	1	
mrkI	15	15	7	7	18	18	18	3	1	
mrkJ	12	12	6	6	1	1	1		6	
iutA	NAT	NAT	NAT	NAT	NAT	NAT	NAT	NAT	NAT	aerobactin transport
kvgA					2	2	2			contribute to capsule formation
kvgS					NAT	NAT	NAT			
kfuA					NAT	NAT	NAT			
kfuB					NAT	NAT	NAT			mediates uptake of ferric iron, intestinal colonization factor
kfuC					NAT	NAT	NAT			
mceA					1	1	1			
mceB					2	2	2			
mceC					1	1	1			
mceD					3	3	3			
mceE					2	2	2			mammalian cell entry (mce) gene cluster
mceG					NAT	NAT	NAT			
mceH					5	5	5			
mceI					NAT	NAT	NAT			
mceJ					NAT	NAT	NAT			

NAT = new allele type.

ESBL genes bla_{SHV-83} and bla_{OXA-10} and carbapenemase bla_{VIM-1}. Endemicity of VIM-producing *K. pneumoniae* isolates are mainly reported in Italy and Greece (Nordmann et al., 2011). First occurrence in Austria was reported the last decade and bla_{VIM} harboring *Enterobacter cloacae* isolates were recently found in Austrian surface water samples (Zarfel et al., 2017). To the best of our knowledge *K. pneumoniae* strains belonging to ST985 positive for bla_{VIM-1} have not been described before.

Table 4
Identified plasmids in five isolates from river water and four genotypically related patient isolates.

Accession no.	Plasmid	water I ST11	patient A ST11	water II ST985	patient B ST985	water III ST405	patient C ST405	patient D ST405	water IV ST3400	water V ST323
JN233704	IncFIB(K)	100.00 %	100.00 %	98.93 %	98.93 %	98.93 %	98.93 %	98.93 %	98.93 %	98.93 %
CP000648	IncFII(K)	100.00 %	100.00 %	98.65 %	98.65 %	95.95 %	95.95 %	95.95 %	97.97 %	95.95 %
DQ449578	IncR	100.00 %	100.00 %						99.20 %	
JN626286	IncL/M (pOXA-48)	100.00 %	100.00 %							
AF250878	IncFIA(HI1)			96.91 %	96.91 %					
CP023920.1	Col440I			100.00 %	100.00 %					
CP023921.1	Col440II			97.52 %	97.52 %					
MF062700.1	IncX5								99.65 %	
JN420336	IncFIB(Mar)								99.54 %	

The third cluster (ST405, CT1363) comprised one water isolate and two patient isolates differing in their cgMLST by two and three alleles. All isolates harbored ESBL genes bla_{SHV-76}, $bla_{CTX-M-15}$ and bla_{OXA-1}. ST405 is among the predominant clones in Spanish hospitals and carries the bla_{OXA-48} gene on IncL/M-type plasmids (Pérez-Vázquez et al., 2016). In contrast to the Spanish ST405 strains, bla_{OXA-48} was not present in our ST405 isolates. However, the Austrian ST405 isolates revealed by far the highest content of virulence genes. Genes belonging to yersiniabactin, genes of the type 3 fimbrial gene cluster, the ferric aerobactin receptor, which is part of the aerobactin gene cluster, genes which contribute to capsule formation, mediate uptake of ferric iron and genes belonging to the mammalian cell entry cluster (responsible for microcin E492 production) were present. The presence of these virulence genes enhances colonization and adherence to the host, invasive infections, and biofilm formation. The increased virulence potential of this clone subsequently might lead to an increase of community-acquired infections in young and healthy individuals (Clegg and Murphy, 2016).

Two ESBL positive water isolates, one with the new sequence type ST3400 and the other with ST323 were collected in the river Danube downstream from Vienna and in the river Inn downstream from Innsbruck; both lacked matching clinical *K. pneumoniae* isolates. Missing links from water to patient isolates were expected, since *K. pneumoniae* isolates are not routinely sent to the Austrian reference laboratory and comparison was therefore carried out on a limited number of sequenced clinical isolates available in the AGES *K. pneumoniae* sequence database. Both water-isolates carried IncF plasmids, among others, which have been termed "epidemic resistance plasmids" due to their ability to acquire resistance determinants and propensity to rapid dissemination (Mathers et al., 2015). They are specifically linked with certain beta-lactamase genes such as CTX-M-15, which was present in both water samples.

The screening of Austrian surface water revealed two carbapenem resistant and three ESBL-producing *K. pneumoniae* isolates in total, in five river samples taken downstream from WWTP effluents. The fact that we found these clinically important clones, of which three were indistinguishable from contemporarily collected patient isolates, indicates that pathogens find their way from hospitals into rivers, as described elsewhere recently (Amos et al., 2014; Hocquet et al., 2016; Khan et al., 2018; Mahon et al., 2017). Contaminated rivers provide a milieu for antibiotic-resistant bacteria to persist, disseminate, evolve and exchange antibiotic resistance determinants. This implicates the hazard of spreading to animals, humans and clinically relevant settings. However, the fact that patient isolates had corresponding genotypes to river isolates does not necessarily implicate them as direct contamination source, but rather, reflect the presence of such genotypes in the human population. We not only can confirm the previous finding, that WWTPs pose a worrying reservoir of highly resistant enteric bacteria in the environment (Amos et al., 2014), our results also show that hospital patients could be a source of multiresistant Gram negative organisms spilling into rivers when hospital effluents are not properly treated.

5. Conclusions

The results of this pilot study on the release of antimicrobial-resistant *K. pneumoniae* strains into the environment and the detection of clinically relevant strains in the environment is alarming and appears an emerging future public health problem, which demands increased attention. Based on our findings future projects should cover rivers from all over the country with repeated sampling to obtain a better picture of the situation. Immediate actions as a consequence of recent publications (Amos et al., 2014; Mahon et al., 2017; Zarfel et al., 2017; Zurfluh et al., 2013) and our study results include proper treatment of hospital effluents and operation of WWTPs with state-of-the-art techniques (Kistemann et al., 2008). We recommend the development of new strategies for treating WWTP effluents, and the establishment of a surveillance system - at least downstream the major cities - to monitor

for multiresistant clinically relevant bacterial species in surface water, especially such used for recreational activities.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.01.179.

CRediT authorship contribution statement

Sarah Lepuschitz: Investigation, Methodology, Data curation, Writing - original draft, Writing - review & editing. **Simone Schill:** Investigation, Methodology, Writing - review & editing. **Anna Stoeger:** Methodology, Writing - review & editing. **Shiva Pekard-Amenitsch:** Methodology, Data curation, Writing - review & editing. **Steliana Huhulescu:** Conceptualization, Data curation, Writing - review & editing. **Norbert Inreiter:** Investigation. **Rainer Hartl:** Investigation, Writing - review & editing. **Heidrun Kerschner:** Investigation, Writing - review & editing. **Sieglinde Sorschag:** Investigation, Writing - review & editing. **Burkhard Springer:** Conceptualization, Data curation, Writing - original draft, Writing - review & editing. **Sylvain Brisse:** Data curation, Writing - review & editing. **Franz Allerberger:** Conceptualization, Writing - original draft, Writing - review & editing. **Robert L. Mach:** Conceptualization, Writing - original draft, Writing - review & editing. **Werner Ruppitsch:** Conceptualization, Investigation, Data curation, Writing - original draft, Writing - review & editing.

Acknowledgements

Funding

Conflict of interest

None declared.

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Wiener klinische Wochenschrift
The Central European Journal of Medicine

Necrotizing fasciitis due to *Vibrio cholerae* non-O1/non-O139 after exposure to Austrian bathing sites

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Summary We report on two cases of necrotizing fasciitis of the lower leg due to nontoxigenic *Vibrio cholerae* (*V. cholerae*). A 73-year-old woman (case 1) and an 80-year-old man (case 2) were hospitalized with symptoms of necrotizing fasciitis on July 18 and August 15, 2015, respectively. In both cases, symptoms started the day after swimming in local ponds. Swabs gained intraoperatively and a blood culture from the male patient, yielded *V. cholerae* non-O1/non-O139, negative for cholera toxin gene *ctx* and positive for hemolysin genes *hly*A and *hly*B. Water samples taken from pond A on August 17, 2015 (32 days after exposure of case 1) and from pond B on August 20, 2015 (7 days after exposure of case 2) yielded non-O1/non-O139 *V. cholerae* in most-probable numbers of >11,000 per 100 ml each. The occurrence of two cases of necrotizing fasciitis within a 1 month period related to two Austrian non-saline bathing waters, previously not known to harbor *V. cholerae*, is probably linked to the prevailing extreme weather conditions (heat wave, drought) this summer in Austria. While case 1 was discharged in good clinical condition after 73 days, case 2 died after four months of hospitalization. Public health authorities are challenged to assess the effects of long-term climate change on pathogen growth and survival in continental bodies of fresh water.

Keywords *Vibrio cholerae* · Necrotizing fasciitis · Bathing sites · Climate change · Heat wave · Drought

Background

Vibrio (V.) cholerae is a gram-negative rod-shaped bacterium, which preferentially grows in warm (>15 °C) brackish and estuarine water [1, 2]. Only serogroups O1 and O139 are known to cause classic cholera [3–5]. The other approximately 200 serogroups of non-O1/non-O-139 *V. cholerae* rarely harbor cholera toxin (*ctx*), often carry hemolysin genes *hly*A and *hly*B and usually only cause self-limited gastroenteritis or mild extra intestinal symptoms [3, 6]. We report two cases of necrotizing fasciitis caused by non-O1/non-O139 *V. cholerae*, acquired by swimming at Austrian bathing sites, at the peak of a 2 month long heat period characterized by an extraordinarily low amount of rainfall, in 2015.

Case reports

Case 1

On July 18, 2015, a 73-year-old obese woman (body mass index 30.5) with hypertonia and diabetes mellitus presented herself to the emergency room of hospital A in Vienna, one day after swimming in pond A in the prov-

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ince of Lower Austria. She complained of severe pain in the left lower leg, livid-blue discoloration, local hyperthermia and of fever up to 38 °C. She recalled a minor excoriation on her left leg. Laboratory examinations demonstrated elevated C-reactive protein (26.0 mg/l, normal < 5 mg/l), a white blood cell count of 19.0 G/l (normal 4–9 G/l) and elevated serum-lactate (3.4 mmol/l, normal 0.5–1.6 mmol/l). A duplex sonography of the leg veins indicated a compartment syndrome. Initial surgical treatment consisted of bilateral fasciotomy (medial + lateral). Necrotizing fasciitis was diagnosed and swabs gained intraoperatively yielded non-O1/non-O139 *V. cholerae* (bacteriological results reported on day 7). Blood cultures remained sterile. Empiric antibiotic therapy initiated on day 1 consisted of ampicillin/sulbactam (3 g, tid, IV). On day 2 of hospitalization, C-reactive protein increased to 393.6 mg/l and white blood cell count to 26.4 G/l; serum procalcitonin was 33.48 ng/ml (normal < 0.5 ng/ml), antithrombin III activity was 66 % (normal 83–128 %) and the patient required intensive care. Antibiotic treatment was switched to a combination of piperacillin/tazobactam (4.5 g, tid, IV) and fosfomycin (8 g, tid, IV); the first of four soft tissue debridements was performed that day. On day 3, clindamycin (900 mg, tid, IV) was added; antithrombin III activity was 34 % and C-reactive protein 463.2 mg/l. On day 5, the patient became afebrile. On day 7, with arrival of bacteriological results showing *V. cholerae* susceptible to piperacillin/tazobactam, antibiotic therapy was deescalated to the latter antimicrobial for another 2 weeks. The isolates were also susceptible to ampicillin, trimethoprim-sulfamethoxazole, ciprofloxacin and tigecycline, tested according to the European committee on antimicrobial susceptibility testing (EUCAST) recommendations for enterobacteriaceae [7]. Wound swabs taken on day 3, 4, and 6 again yielded *V. cholerae*. The first culture-negative wound swab was on day 9. A stool sample on day 7 was negative for *V. cholerae*. On day 9, negative pressure wound therapy system (VAC, vacuum-assisted closure; KCI Corp, Vienna, Austria) was applied. On day 14, the patient was transferred to a surgical ward. Figure 1 depicts the wound status at that point in time. On day 22, a split skin-graft transplantation was done. The patient was transferred to the plasticsurgery ward on day 27, where she stayed till discharge on September 30, after 73 days of hospitalization. Figure 2 depicts the clinical status as of day 68.

Case 2

On August 15, 2015, an 80-year-old man presented himself to the emergency room of hospital B in the province Lower Austria, one day after swimming in local pond B. During the summer, the patient had repeatedly used this pond for swimming. He complained of increasing swelling and pain in his left lower leg, fever (38 °C), dyspnea, and malaise since last night. His medical history was unremarkable except for ichthyosis cutis and several episodes of cellulitis. He recalled a minor trauma after hit-

Fig. 1 Left lower leg of case 1 on day 14 of hospitalization

Fig. 2 Left lower leg of case 1 on day 68 of hospitalization

ting his left ankle on a table the day before swimming. The patient was admitted under the presumptive diagnosis of deep vein thrombosis and pulmonary embolism. His vital signs (blood pressure 78/36 mmHg; heart rate 130/min) deteriorated rapidly, and on day 2, by then the severely septic patient was admitted to intensive care unit. Blood cultures taken on day 2 yielded *V. cholerae* (bacteriological results reported on day 5). On day 3, bilateral fasciotomy and debridement were performed on his left lower leg and necrotizing fasciitis was diagnosed; swabs taken intraoperatively yielded growth of non-O1/non-O139 *V. cholerae*. Antibiotic therapy was initiated with piperacillin/tazobactam (4.5 g, tid, IV), tigecycline (100 mg, bid, IV), and metronidazole (500 mg, tid, IV). On day 5, the patient was transferred to an ICU specialized in infectious diseases (Hospital C, Vienna). Laboratory examinations demonstrated elevated C-reactive protein (265 mg/l, normal < 5 mg/l) and a white blood cell count of 35.0 G/l (normal 4–9 G/l). *V. cholerae* (isolated from wound swabs until day 7) was susceptible to ampicillin, trimethoprim-sulfamethoxazole, ciprofloxacin and tigecycline, tested according to the European committee on antimicrobial susceptibility testing (EUCAST) recom-

mendations for enterobacteriaceae [7]. On day 5, blood cultures were positive for *V. cholerae* and antibiotic treatment was switched to piperacillin/tazobactam (4.5 g, tid, IV) in combination with clindamycin (600 mg, tid, IV) and doxycycline (100 mg, bid, IV). Due to suspected ventilator-associated pneumonia, piperacillin/tazobactam was switched to meropenem (1 g, tid, IV); clindamycin was stopped on day 13. A total of five surgical revisions and application of negative pressure wound therapy system (VAC, vacuum-assisted closure, KCI Corp, Vienna, Austria) followed. After 5 weeks of hospitalization, a split skin-graft transplantation was performed. Case 2 died after four months of hospitalization.

Environmental investigations and subtyping

A total of seven *V. cholerae* isolates (two human isolates, two from pond A and three from pond B) tested by NGS lacked cholera toxin genes *ctx*A, *ctx*B, toxin-coregulated pilus (TCP), heat-stable enterotoxin gene *sto* and NAG-ST gene *stn*. All seven *V. cholerae* isolates were positive for El Tor like hemolysin genes *hly*A and *hly*B.

A water sample taken from pond A on August 17, 2015 (32 days after exposure of case 1) yielded non-O1/non-O139 *V. cholerae* in most-probable numbers (MPN) of >11,000 per 100 ml tested by MPN method [8] (enterococci: <15 MPN/100 ml; *Escherichia coli* (*E. coli*): <15 MPN/100 ml according to ÖNORM EN ISO 7899-1 and ÖNORM EN ISO 9308-3 respectively [9, 10]). Pond A (water-surface area: 20,000 m², maximum depth: 17 m) is a brick pond, located in a village of approximately 5000 inhabitants south of the city of Vienna. It is not registered as an EU bathing site but widely used for swimming.

A water sample taken from pond B on August 20, 2015 (7 days after exposure of case 2) yielded non-O1/non-O139 *V. cholerae* in numbers of >11,000 MPN per 100 ml (enterococci: 127 MPN/100 ml; *E. coli*: 161 MPN/100 ml). Pond B (water-surface area: 600 m², maximum depth: 3 m) is a former gravel quarry situated 20 km south of pond A. It is not registered as an EU bathing site, but used for swimming by locals.

Both ponds were resampled on September 1, 2015: pond A showed non-O1/non-O139 *V. cholerae* in numbers of 2,400 MPN/100 ml (enterococci: <15 MPN/100 ml; *E. coli*: <15 MPN/100 ml), pond B, 11,000 MPN/100 ml (enterococci: 15 MPN/100 ml; *E. coli*: <15 MPN/100 ml). Salient features of the two swimming sites are summarized in Table 1.

On August 17 and 18, a total of 90 of 175 Austrian bodies of water registered as EU bathing sites were tested for *V. cholerae*. Lake Neusiedl (located in the Austrian province Burgenland), three further bodies of water in Burgenland and three in the province Lower Austria tested positive for non-O1/non-O139 *V. cholerae*. Data on positive water samples are summarized in Table 2. With the exception of Asangteich, which showed 534 MPN/100 ml enterococci and 127 MPN/100 ml *E. coli*, all 90 bodies of water showed less than 400 MPN/100 ml enterococci and less than 1,000 MPN/100 ml *E. coli*.

Discussion

In Austria, occurrence of non-O1/non-O139 *V. cholerae* has so far only been known for Lake Neusiedl, a saline steppe lake in eastern Austria, bordering Hungary [3, 4, 11]. Huhulescu *et al.* [3] previously reported the occurrence of human cases of otitis externa, otitis media, mild diarrhea and one fatal case of septicemia in an immunocompromised patient after swimming in this lake; lake Neusiedl has a saline concentration approximately one-tenth of the Mediterranean Sea. In 2015, two cases of non-O1/non-O139 *V. cholerae* infections were documented in relation to Lake Neusiedl: A 21–year-old woman saw an otolaryngologist for otitis externa on August 17, 2015 and a 28-year-old male patient presented himself to an outpatient-clinic for infection of the urogenital tract ("bloody seminal fluid") on September 3, 2015 (unpublished data).

To our knowledge, the two cases of necrotizing fasciitis due to non-O1/non-O139 *V. cholerae* described here are the first cases documented in Austria. Necrotizing fasciitis due to non-O1/non-O139 *V. cholerae* has previously been reported in the scientific literature. In Europe, one isolated case of necrotizing fasciitis caused by non-O1/non O-139 *V. cholerae* (and associated with water exposure) was reported Italy (Mediterranean sea) [12]. In contrast to our Austrian cases, all those infections were associated with saline waters, as were singular reports of *V. cholerae* non-O1/non O-139 necrotizing fasciitis from the United States [13] and Taiwan [14, 15].

The occurrence of two cases of necrotizing fasciitis within a 1 month period related to two Austrian non-saline bathing sites, previously not known to harbor *V. cholerae*, is probably related to the prevailing extreme weather conditions (heat wave, drought) in Austria during this summer. Austria-wide, temperatures in July 2015

Table 1 Summarized data on bathing sites related to non-O1/non-O139 *Vibrio cholerae* necrotizing fasciitis

Sampling date	Bathing site	*V. cholera* non-O1/non-O139 in MPN/100 ml	Patient	Water temperature in °C	pH	Conductivity in µS/cm
Aug 18, 2015	Pond A	>11,000	Case 1	28.6	8.9	2,320
Aug 20, 2015	Pond B	>11,000	Case 2	20.5	8.9	1,242
Sept 01, 2015	Pond A	2,400	Case 1	25.2	9.0	2,260
Sept 01, 2015	Pond B	11,000	Case 2	25.0	8.4	1,220
MPN most-probable number						

Table 2 Summarized data of EU bathing waters harboring *Vibrio cholerae* non-O1/non-O139 in Austria, August 2015

Body of water	Bathing site	Province	*V. cholerae* non-O1/non-O139 in MPN/100 ml	Water temperature in °C	pH
Lake Neusiedl	Weiden	Burgenland	>11,000	24.9	8.8
	Neusiedl		360	24.6	8.7
	Breitenbrunn		4,600	24.4	8.8
	Rust		4,600	25.5	8.6
	Podersdorf		11,000	23.8	8.9
	Illmitz		>11,000	24.9	8.9
	Mörbisch		11,000	25.4	8.8
Lake Andau	Lake Andau	Burgenland	930	25.5	8.4
Lake Apetlon	Lake Apetlon	Burgenland	2,400	25.0	8.6
Zicksee	St. Andrä	Burgenland	2,100	21.3	8.9
Ausee	Blindenmarkt	Lower Austria	150	23.1	8.4
Lake Hohenau	Lake Hohenau	Lower Austria	1,500	24.2	9.0
Lake Seeschlacht	Langenzersdorf	Lower Austria	>11,000	24.6	8.4
MPN most-probable number					

were 3.1 °C higher than the average (mean) measured from 1981 to 2010. It was the warmest July on record since 1767. The rate of precipitation in July 2015 was 20 % below average (as measured from 1981 to 2010) Austria-wide. In Lower Austria, the deviation in precipitation was 41 % below average [16] and temperatures were +3.2 °C above average in July 2015. August 2015 deviated from the accepted average taken 1981–2010 by +2.7 °C, the fourth warmest month since records began in 1767; there was 35 % less rainfall than average in all of Austria. In Lower Austria, deviations in precipitation were −36 %, in temperatures +3.4 °C [17]. These climatic conditions most likely supported the growth of non-O1/non O-139 *V. cholerae* in these two ponds and in 7 % of the 90 EU bathing waters tested. This may also explain the high amount of *V. cholerae* (>11,000 MPN/100 ml) present in the two swimming ponds at the time of first testing, high numbers which did not correlate with an increase in enterococci and *E. coli*, the classical indicator organisms used for bathing water surveillance.

Public health authorities have already expressed increasing concern regarding the role of climate change in driving bacterial waterborne infectious diseases [18]. Associations between environmental changes observed in the Baltic area and the recent emergence of non-O1/non O-139 *V. cholerae* infections have prompted ECDC to implement a real-time model that uses daily updated remote sensing data to map environmental suitability for *Vibrio* growth in the Baltic Sea [19]. The overall occurrence of non-O1/non O-139 *V. cholerae* infections is still low. However, the two cases of necrotizing fasciitis described here and related to bathing sites in Austria raise important questions about environmental reservoirs of non-O1/non O-139 *V. cholerae* in view of increasing extreme weather conditions. Global warming of water bodies will inevitably lead to an increased occurrence of non-O1/non O-139 *V. cholerae* and resulting problems will not be restricted to the Baltic Sea. There is a need for centralized and systematic case reporting methods. Baker-Austin et al. [18] have asked for efforts to inform at-risk groups to prevent them from recreational contact with unsafe water during periods of sustained surface water temperature warming. However, our cases reveal that presently, at-risk groups are neither properly defined, nor are the presently applied EU-parameters for testing bathing waters able to allow proper risk assessment concerning non-O1/non O-139 *V. cholerae*. Public health authorities are challenged to assess the effects of long-term climate change on pathogen growth and survival in continental fresh water bodies and to determine routes of exposure as well as the role of host susceptibility in disease emergence.

Compliance with ethical standards

Conflict of interest
On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Chapter 2

This chapter includes publications with focus on novel antibiotic resistance mechanisms and multiresistant clinically relevant pathogens, which pose an increasing public health problem.

Peer-reviewed publications:

Lepuschitz S, Sorschag S, Springer B, Allerberger F, Ruppitsch W. Draft Genome Sequence of Carbapenemase-Producing *Serratia marcescens* Isolated from a Patient with Chronic Obstructive Pulmonary Disease. Genome Announc. 2017;5(46). pii: e01288-17. doi: 10.1128/genomeA.01288-17.

Blaschitz M, **Lepuschitz S**, Wagner L, Allerberger F, Indra A, Ruppitsch W, Huhulescu S. Draft Genome Sequence of a Vancomycin-Resistant and Vancomycin-Dependent *Enterococcus faecium* Isolate. Genome Announc. 2016;4(2). pii: e00059-16. doi: 10.1128/genomeA.00059-16.
Author's contribution: Marion Blaschitz and Sarah Lepuschitz contributed equally to this work. Sarah Lepuschitz performed the DNA isolation, library preparation for whole genome sequencing and data analysis.

Hartl R, Kerschner H, **Lepuschitz S**, Ruppitsch W, Allerberger F, Apfalter P. Detection of the *mcr-1* Gene in a Multidrug-Resistant *Escherichia coli* Isolate from an Austrian Patient. Antimicrob Agents Chemother. 2017;61(4).
Author's contribution: Sarah Lepuschitz performed whole genome sequence data analysis.

Jesumirhewe C, Springer B, **Lepuschitz S**, Allerberger F, Ruppitsch W. Carbapenemase-Producing Enterobacteriaceae Isolates from Edo State, Nigeria. Antimicrob Agents Chemother. 2017;61(8).
Author's contribution: Sarah Lepuschitz performed whole genome sequence data analysis.

Hirk S, **Lepuschitz S**, Cabal Rosel A, Huhulescu S, Blaschitz M, Stöger A, Stadlbauer S, Hasenberger P, Indra A, Schmid D, Ruppitsch W, Allerberger F. Draft Genome Sequences of Interpatient and Intrapatient Epidemiologically Linked *Neisseria gonorrhoeae* Isolates. Genome Announc. 2018;6(16). pii: e00319-18. doi: 10.1128/genomeA.00319-18.
Author's contribution: Sarah Lepuschitz performed whole genome sequence data analysis.

Hartl R, Kerschner H, Gattringer R, **Lepuschitz S**, Allerberger F, Sorschag S, Ruppitsch W, Apfalter P. Whole-Genome Analysis of a Human *Enterobacter mori* Isolate Carrying a *bla*IMI-2 Carbapenemase in Austria. Microb Drug Resist. 2019;25(1):94-96.

Author's contribution: Sarah Lepuschitz performed whole genome sequence data analysis.

Wisgrill L, **Lepuschitz S**, Blaschitz M, Rittenschober-Böhm J, Diab-El Schahawi M, Schubert S, Indra A, Berger A. Outbreak of Yersiniabactin-Producing *Klebsiella pneumoniae* in a Neonatal Intensive Care Unit. Pediatr Infect Dis J. 2018. doi: 10.1097/INF.0000000000002258. [Epub ahead of print]

Author's contribution: Sarah Lepuschitz performed whole genome sequence data analysis.

Cabal A, Schmid D, **Lepuschitz S**, Stöger A, Blaschitz M, Allerberger F, Ruppitsch W, Hell M. Nosocomial outbreak of *Streptococcus pyogenes* puerperal sepsis. Clin Microbiol Infect. 2018. doi: 10.1016/j.cmi.2018.11.028. [Epub ahead of print]

Author's contribution: Sarah Lepuschitz performed whole genome sequence data analysis.

Draft Genome Sequence of Carbapenemase-Producing *Serratia marcescens* Isolated from a Patient with Chronic Obstructive Pulmonary Disease

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ABSTRACT The occurrence of multidrug-resistant *Serratia marcescens* strains producing metallo-β-lactamases or extended-spectrum β-lactamases represents a serious public health threat. Here, we report the draft genome sequence of a multidrug-resistant carbapenemase-producing *Serratia marcescens* isolate recovered from the bronchoalveolar lavage specimen of a patient suffering from chronic obstructive pulmonary disease (COPD).

*S*erratia marcescens, first described in 1819, belongs to the family of *Enterobacteriaceae* and is a motile rod-shaped Gram-negative bacterium (1). *Serratia* species are omnipresent in the environment, and *S. marcescens* is classified as an important nosocomial pathogen causing a wide range of infections, including, most notably, urinary tract infection and bloodstream infection (2). Besides several potential virulence factors, one important feature of clinical *S. marcescens* is its ability to acquire antimicrobial resistance. VIM-metallo-β-lactamase (MBL)-producing isolates have the ability to hydrolyze almost all β-lactams and have been described in association with outbreaks worldwide (3).

In 2017, in Austria, *S. marcescens* strain at10508 was cultured from a 68-year-old male patient with clinical signs of chronic obstructive pulmonary disease (COPD), pneumonia, brain abscess due to a *Nocardia* sp., diabetes mellitus type 2, liver cirrhosis, coronary heart disease, ascites, and pleural effusion. Antimicrobial resistance was determined using BD Phoenix (Becton Dickinson, Franklin Lakes, NJ, USA), yielding the following results: ampicillin (resistant [R]), ampicillin-sulbactam (R), amoxicillin-clavulanic acid (R), piperacillin (R), piperacillin-tazobactam (R), cefazolin (R), cefepime (R), cefotaxime (R), ceftazidime (R), cefuroxime (R), ertapenem (R), imipenem (R), meropenem (R), ciprofloxacin (R), levofloxacin (R), amikacin (sensitive [S]), gentamicin (S), tobramycin (R), tetracycline (R), tigecycline (intermediate [I]), colistin (R), fosfomycin (R), and trimethoprim-sulfamethoxazole (R).

For whole-genome sequencing, high-molecular-weight DNA was isolated from an overnight culture on Mueller Hinton agar plates (BioMérieux, Marcy-l'Étoile, France) using the MagAttract HMW DNA kit (Qiagen, Hilden, Germany). Using the double-stranded DNA (dsDNA) BR assay kit (Thermo Fisher Scientific, Waltham, MA, USA), 1 ng of input DNA was quantified with a Qubit 2.0 fluorometer (Thermo Fisher Scientific). Library preparation to obtain ready-to-sequence libraries was done with a NexteraXT kit (Illumina, Inc., San Diego, CA, USA). Paired-end sequencing (2 × 300 bp) was performed using a MiSeq system (Illumina, Inc.) and generated 3,174,214 reads from 687,587,445

unassembled nucleotides. Raw reads were *de novo* assembled into a draft genome using SPAdes version 3.9.0 (4). Contigs were filtered for a minimum coverage of 5 and minimum length of 200 bp, which resulted in 272 contigs with a total of 5,687,772 nucleotides at a coverage of 133-fold.

Antimicrobial resistance genes were identified using the ResFinder tool (5) from the Center of Genomic Epidemiology (CGE) (http://www.genomicepidemiology.org) and included bla_{VIM-1}, bla_{ACC-1}, bla_{SRT-2}, *aadA1*, *aadA16*, *aac(6')-Ic*, *aac(6')Ib-cr*, *qnrB6*, *tet*(41), *dfrA27*, *arr-3*, *catA1*, and *sul1*, conferring resistance to β-lactam antibiotics, aminoglycosides, quinolones, tetracyclines, trimethoprim, rifampin, phenicol, and sulfonamides. The PlasmidFinder tool from CGE (6) identified two plasmids (IncHI2, IncHI2A). The NCBI Prokaryotic Genome Automatic Annotation Pipeline identified 5,674 genes, 5,552 coding sequences, 194 pseudo-genes, 19 rRNA operons (9 complete, 10 partial), and 82 tRNA genes.

Accession number(s). This whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number NPIX00000000. The version described in this paper is version NPIX01000000.

ACKNOWLEDGMENT

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Draft Genome Sequence of a Vancomycin-Resistant and Vancomycin-Dependent *Enterococcus faecium* Isolate

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Vancomycin-resistant enterococci have emerged as major nosocomial pathogens worldwide. While antimicrobial pressure promotes nosocomial colonization with these enterococci, prolonged exposure to vancomycin may foster the transition from vancomycin resistance to vancomycin dependence. Here, we report the draft genome sequence of a vancomycin-dependent *Enterococcus faecium* isolate showing partial teicoplanin dependence.

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Vancomycin-resistant enterococci (VRE) are major nosocomial pathogens worldwide. While antimicrobial pressure promotes nosocomial colonization with VRE, prolonged exposure to vancomycin may foster the transition from vancomycin resistance to dependence (1). Enterococci showing growth on medium containing 6 μg/ml vancomycin and an MIC of >8 μg/ml are considered vancomycin resistant. Strains unable to grow in the absence of 6 μg/ml vancomycin, despite multiple subcultures, are considered vancomycin dependent (1). Vancomycin-dependent enterococcus (VDE) was first described in 1993 (2, 3). To our knowledge, only twenty-five cases of VDE have been described worldwide so far (4).

We report here the draft genome of a VRE/VDE isolate obtained in Austria in 2007 from an oncology patient 1 month after cessation of teicoplanin therapy (5). The described strain of VDE showed resistance to penicillin (MIC, 64 μg/ml), ampicillin (MIC, 128 μg/ml), amoxicillin-clavulanate (amoxicillin MIC, >256 μg/ml), erythromycin (MIC, 8 μg/ml), clindamycin (MIC, 8 μg/ml), ciprofloxacin (MIC, >2 μg/ml), moxifloxacin (MIC, >2 μg/ml), fusidic acid (MIC, 4 μg/ml), and low-level resistance to gentamicin (MIC, 32 μg/ml) when tested according to Clinical and Laboratory Standards Institute (CLSI) standards (6). The strain was susceptible to quinupristin-dalfopristin (MIC, <0.5 μg/ml), oxytetracycline (MIC, <0.5 μg/ml), linezolid (MIC, 2 μg/ml), chloramphenicol (MIC, 8 μg/ml), and mupirocin (MIC, 2 μg/ml). The strain showed intermediate resistance to teicoplanin when tested on Mueller-Hinton agar (bioMérieux, Marcy l'Etoile, France) using a 30-μg teicoplanin disc (Oxoid, Basingstoke, United Kingdom) or a teicoplanin Epsilon test strip (AB Biodisk, Solna, Sweden) (MIC, 8 μg/ml). It also showed partial teicoplanin dependence, i.e., it grew in the area of low concentration of teicoplanin (0.016 to 6 μg/ml) but was inhibited in the area with high teicoplanin concentrations (8 to 256 μg/ml). The strain showed high-level resistance to vancomycin (MIC, >256 μg/ml) and complete vancomycin dependence when

tested on Mueller-Hinton agar (bioMérieux) with a 30-μg vancomycin disk (Oxoid) or a vancomycin Epsilon test strip (AB Biodisk). The strain also showed *in vitro* reversion to vancomycin-nondependent vancomycin-resistant *Enterococcus faecium* mutants.

The MagAttract high-molecular-weight (HMW) DNA kit (Qiagen, Hilden, Germany) was used to isolate genomic DNA from overnight cultures grown on Mueller-Hinton agar plates (bioMérieux) with a 30-μg vancomycin disk (Oxoid) and a 30-μg teicoplanin disk (Oxoid). The fragment library was prepared using the Nextera XT kit (Illumina, Inc., San Diego, CA) and 1 ng of genomic DNA. Paired-end sequencing (2 × 300 bp) was performed on a MiSeq (Illumina, Inc.), generating 1,365,744 reads from 333,173,006 unassembled nucleotides. Raw reads were *de novo* assembled into a draft genome using Velvet version 1.1.04 (7). Contigs were filtered for a minimum coverage of 5 and minimum length of 200 bp, which resulted in 232 contigs with a total of 2,949,766 nucleotides at a coverage of 95-fold. A total of 2,953 genes, 2,878 coding sequences, 95 pseudogenes, 7 rRNA genes, and 64 tRNA genes were identified using the NCBI Prokaryotic Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/).

Nucleotide sequence accession numbers. This whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession no. LQRS00000000. The version described in this paper is the first version, LQRS01000000.

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Detection of the *mcr-1* Gene in a Multidrug-Resistant *Escherichia coli* Isolate from an Austrian Patient

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ABSTRACT Since colistin resistance based on the plasmid-encoded *mcr-1* gene was first described, this resistance gene in *Enterobacteriaceae* has been found worldwide. These organisms are typically of heterogeneous genetic background and show exceptional clonal diversity. We describe the first confirmation of *mcr-1* in a human *Escherichia coli* strain cultured from a surveillance stool sample of an Austrian oncology patient.

Since the first description of colistin resistance based on the plasmid-encoded *mcr-1* gene, the occurrence of this resistance gene in *Enterobacteriaceae* has been described in Europe and many other areas worldwide (1–3). These organisms are typically of heterogeneous genetic background and show exceptional clonal diversity (4). We describe the first confirmation of *mcr-1* in a human *Escherichia coli* strain cultured from a surveillance stool sample of an oncology patient in Austria.

The *E. coli* strain (isolate 204965) was isolated in June 2016 from a surveillance stool sample of a 60-year-old female patient with acute myeloid leukemia secondary to a myelodysplastic syndrome in Linz, Upper Austria. The patient did not present any symptoms of infection and had no recent travel history. Consecutively collected stool samples revealed the persistence of the strain for at least 3 weeks; no further screening results were available after that period. Screening cultures from other body sites, including urine and throat swab samples, revealed no further colonization. The multidrug-resistant phenotype of the isolate presented in Table 1 indicated the presence of extended-spectrum β-lactamase (ESBL) in combination with resistance to aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole (5). ESBL was confirmed phenotypically by a positive double-disk synergy test between ceftazidime and clavulanic acid.

According to our routine two-step approach for multidrug-resistant enterobacteria, extended susceptibility testing, including colistin, fosfomycin, and tigecycline, was applied to the isolate. The initial colistin MIC of 2 μg/ml (Etest, bioMérieux, France) was categorized susceptible, according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints. Due to the recent EUCAST warning concerning the use of colistin gradient tests, subsequent broth microdilution was done (UMIC, biocentric, France), which resulted in an MIC of 4 μg/ml, indicating resistance to colistin (6).

The presence of *mcr-1* was then confirmed by PCR, as previously described, and whole-genome sequencing (WGS) (1, 7). For WGS, high-quality genomic DNA (gDNA) was isolated from an overnight culture using the MagAttract HMW DNA kit (Qiagen,

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TABLE 1 Susceptibility pattern of isolate 204965

Antibiotic	Result[a]
Ampicillin	R (>256)
Amoxicillin-clavulanic acid	R (128)
Cefuroxime	R (16)
Cefotaxime	I (2)
Ceftazidime	R (16)
Cefepime	R (16)
Ceftolozane-tazobactam	R (8)
Meropenem	S (0.032)
Gentamicin	R (32)
Tobramycin	R (8)
Amikacin	S (2)
Ciprofloxacin	R (>32)
Tigecycline	S (0.5)
Trimethoprim-sulfamethoxazole	R (>32)
Fosfomycin	S (8)

[a]S, susceptible; I, intermediate; R, resistant. Results are based on EUCAST disk diffusion method. Etest results (μg/ml) are given in parentheses.

Hilden, Germany). One nanogram of gDNA was used to prepare the fragment library with the Nextera XT kit, and paired-end sequencing (2 × 300 bp) was performed on a MiSeq (both Illumina Inc., San Diego, CA, USA). There were 1,860,146 raw reads generated from 480,250,526 unassembled nucleotides. Raw reads were *de novo* assembled into a draft genome using Velvet version 1.1.07 (8). Contigs were filtered for a minimum coverage of 5 and minimum length of 200 bp, which resulted in 327 contigs with a total of 5,259,094 nucleotides at a coverage of 91-fold. There were 5,788 genes, 5,427 coding sequences, 239 pseudogenes, 122 RNA genes, and 2 CRISPR (clustered regularly interspaced short palindromic repeat) arrays identified by the NCBI prokaryotic genome automatic annotation pipeline. The PlasmidFinder, ResFinder, FimTyper, and SerotypeFinder tools from the Center for Genomic Epidemiology were used for WGS data analysis, which revealed the presence of plasmids IncHI2, p0111, IncX4, IncHI2A, IncFII (pRSB107), IncQ1, and IncFIB (AP001918); *fimH* type f-54; serotype O9:H9; and the resistance genes listed in Table 2 (7, 9, 10). The $bla_{TEM-154}$-containing contig (8,459 bp) matched *E. coli* plasmid R1 transposon Tn*4* (GenBank accession number HM749966.1) to 99% (3 mismatches) (11). The presence of this complex mutant TEM-type ESBL has not yet been associated with *mcr-1* carriage (2, 12). To assess the classic multilocus sequence type (MLST), ST10 was extracted *in silico* from WGS data using the Warwick MLST scheme. Finally, the contig containing *mcr-1* (15,163 bp) was submitted to GenBank using the basic local alignment search tool (BLAST), and IncHI2 was identified as the *mcr-1*-carrying plasmid showing 99% identity (one mismatch) to plasmid pS38, an IncHI2 plasmid already described as carrying *mcr-1* (13, 14).

After finding the isolate described above and following an ECDC (European Centre for Disease Prevention and Control) rapid risk assessment, 221 suspected carbapenemase-producing *Enterobacteriaceae* (CPE) isolates from the nationwide surveillance system (CARBA-Net) for CPE, archived at the Austrian National Reference Centre for Nosocomial Infections and Antimicrobial Resistance, underwent colistin MIC deter-

TABLE 2 Resistance gene profile of isolate 204965

Antibiotic substance class	Gene (mutation) detected by WGS
Polymyxins	*mcr-1*
Oxyiminocephalosporins	$bla_{TEM-154}$
Carbapenems	None found
Aminoglycosides	*aadA1, aadA2, strA, strB, aac(3)-IIa*
Fluoroquinolones	*gyrA* (Leu83, Asn87), *parC* (Ile80)
Dihydrofolate reductase inhibitors	*sul1, sul2, sul3, dfrA1*
Phenicols	*cmlA1*
Tetracyclines	*tet(A)*

mination by broth microdilution (15). Seven such isolates (5 *K. pneumoniae*, 2 *Enterobacter* spp.) were resistant to colistin and were screened for the presence of *mcr-1*. None of these isolates showed a positive PCR result, which is in line with the observation that colistin resistance may be determined by multiple chromosomal and plasmid-encoded resistance mechanisms (4). To the best of our knowledge, this *mcr1*-mediated colistin resistance is the first instance described and reported in a human *E. coli* strain in Austria.

Accession number(s). This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession MSEK00000000. The version described in this paper is version MSEK01000000.

ACKNOWLEDGMENTS

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Carbapenemase-Producing *Enterobacteriaceae* Isolates from Edo State, Nigeria

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The emergence and spread of carbapenem-resistant *Enterobacteriaceae* (CRE) are a global health problem that is of great concern to public health services (1, 2). The purpose of this study was to determine the frequency of CRE in three Nigerian hospitals and to characterize the resistance mechanisms of such isolates.

A total of 218 consecutive clinical isolates of *Enterobacteriaceae* based on inclusion criteria were collected from March to May 2015 at three medical microbiology laboratories of hospitals in Edo State, Nigeria (see Table S1 in the supplemental material). Screening for carbapenem resistance was performed using meropenem and ertapenem discs (10 μg; Oxoid, United Kingdom) according to EUCAST guidelines (3). The Kirby-Bauer susceptibility testing technique (4) and Etest method were carried out, and results were interpreted using EUCAST criteria (5). Identification of the involved resistance mechanisms was determined by whole-genome sequencing (WGS).

Out of 218 consecutive clinical *Enterobacteriaceae* isolates, 9 (4.1%) were further investigated due to cutoff values above the EUCAST screening recommendations (Table 1). All isolates showed resistance to piperacillin-tazobactam and amoxicillin-clavulanic acid, all but isolate Ec4349 showed resistance to fluoroquinolones and cefotaxime, and all but two isolates each showed resistance to ceftazidime (Ec4349 and Ecl2840_1) and ertapenem (Ec4349 and Ecl10_14_15). Only Ec4349, Ecl2845, Ecl2840_1, and EclQ9 were sensitive to cefepime and aztreonam. The carbapenemase inactivation method (6) revealed positive results for all nine CRE isolates. By application of WGS, one *Klebsiella pneumoniae* isolate harbored the $bla_{OXA-181}$ gene; two *K. pneumoniae* isolates harbored the bla_{NDM-1} gene. The bla_{OXA-48} gene was detected in one *Escherichia coli* isolate, one *K. pneumoniae* isolate, and four *Enterobacter cloacae* isolates, respectively. All isolates had a single carbapenemase resistance gene on their draft genome fragment. Thirteen plasmid incompatibility groups were identified among the nine CRE isolates. Multilocus sequence typing (MLST) grouped the nine isolates into five sequence types.

Previous reports from Nigeria on molecular characterization of carbapenem resistance genes have identified genes such as bla_{VIM}, bla_{GES}, bla_{NDM}, $bla_{OXA-181}$, and bla_{KPC} (7–10). bla_{OXA-48}, obtained from our study, has only been determined phenotypically. To the best of our knowledge, our findings, where six out of nine carbapenemase-producing isolates harbored the bla_{OXA-48} gene, represent the first molecular determination of the bla_{OXA-48} gene for Nigeria. The presence of different plasmid replicon types in carbapenemase-producing *Enterobacteriaceae* (CPE) underlines their importance in the dissemination of resistance genes. The IncL/M plasmid type was found in all of our OXA-48-producing isolates, correlating with previous reports indicating that

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TABLE 1 Characteristics of carbapenem-resistant *Enterobacteriaceae* isolates

Isolate[a]	Hospital[b]	Clinical specimen	MIC values (μg/ml)[c]	Resistance genes	ST[d]	GenBank accession no.[e]	Plasmid replicon(s)[f]
Kp1337LF	UBTH	Urine	MEM, 0.75; ETP, 3; COL, 1	aac(6')Ib-cr, aac(3)-IIa, str A, aadA1, str B, bla$_{TEM-1B}$, bla$_{CTX-M-15}$, bla$_{SHV-11}$, bla$_{OXA-18-1}$, bla$_{OXA-1}$, mph(A), catA1, catB3, qnrS1, qnrB2, oqxA, oqxB, sul1, tet(A), dfrA15	11	SAMN06704546	IncFIB(Mar), ColKP3, IncX3,* IncFII(K), IncFIB(K), IncR
Kp852	UBTH	Urine	MEM, 24; ETP, 32; COL, 1	aadA1, aac(3)-IIa, aacA4, aph(3')-VIa, arm A, aadA2, aac(6')Ib-cr, bla$_{SHV-28}$, bla$_{CTX-M-15}$, bla$_{NDM-1}$, bla$_{OXA-1}$, msr(E), mphE, catB3, oqxB, oqxA, aac(6')-Ib-cr, qnrB1, sul1, tet(A), dfrA12	15	SAMN06704523	Col(BS512), IncFIB(pKPHS1), IncFII, IncFIA(HI1), IncR, IncFIB(K), IncFIB(Mar), IncHI1B*
Kp852K	UBTH	Urine	MEM, 32; ETP, 24; COL, 0.75	aadA1, aac(3)-IIa, aacA4, aph(3')-VIa, arm A, aadA2, aac(6')Ib-cr, bla$_{SHV-28}$, bla$_{CTX-M-15}$, bla$_{NDM-1}$, bla$_{OXA-1}$, msr(E), mphE, catB3, oqxB, oqxA, aac(6')-Ib-cr, qnrB1, sul1, tet(A), dfrA12	15	SAMN06704522	Col(BS512), IncFIB(pKPHS1), IncFII, IncFIA(HI1), IncR, IncFIB(K), IncFIB(Mar), IncHI1B*
Kp872	UBTH	Endocervical swab	MEM, 4; ETP, 1.5; COL, 0.38	str A, str B, aadA2, aac(3)-IIa, bla$_{CTX-M-15}$, bla$_{OXA-48}$, bla$_{TEM-1B}$, bla$_{SHV-11}$, oqxB, oqxA, sul1, sul2, tet(D), dfrA14	340	SAMN06704513	IncFIA(HI1), IncR, IncL/M(pOXA-48)*
Ecl10_14_15	CH	Peritoneal fluid	MEM, 1.5; ETP, 0.75; COL, 0.75	bla$_{ACT-5}$, bla$_{OXA-48}$, qnrB1, dfrA14	78	SAMN06704512	IncL/M(pOXA-48)*
Ecl2845	UBTH	Urine	MEM, 0.75; ETP, 2; COL, 0.75	bla$_{ACT-5}$, bla$_{OXA-48}$	78	SAMN06704511	IncL/M(pOXA-48)*
EclQ9	UBTH	Unidentified source	MEM, 0.75; ETP, 1.5; COL, 0.75	bla$_{ACT-5}$, bla$_{OXA-48}$	78	SAMN06703827	IncL/M(pOXA-48)*
Ecl2840_1	UBTH	Urine	MEM, 4; ETP, 4; COL, 1	bla$_{ACT-5}$, bla$_{OXA-48}$	78	SAMN06703828	IncL/M(pOXA-48)*
Ec4349	UBTH	Urine	MEM, 0.25; ETP, 0.75; COL, 0.38	str A, str B, bla$_{TEM-1B}$, bla$_{OXA-48}$, sul2, tet(A), dfrA14	1408	SAMN06703826	Col3M, IncL/M(pOXA-48),* IncR

[a]Ec, *Escherichia coli*; Kp, *Klebsiella pneumoniae*; Ecl, *Enterobacter cloacae*.
[b]UBTH, University of Benin Teaching Hospital; CH, Central Hospital, Benin.
[c]MEM, meropenem; ETP, ertapenem; COL, colistin.
[d]ST, sequence type.
[e]Accession numbers are shown for the carbapenem resistance gene.
[f]*, plasmid replicon type harboring the carbapenem resistance gene.

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the current spread of OXA-48 β-lactamase producers is mainly related to the diffusion of this plasmid (11).

Occurrence of CPE has been reported globally (12–14). In Nigeria, most previous reports characterized CRE phenotypically (15–17). Only a few studies used molecular methods, which are considered the "gold standard" for identification of carbapenemase-producing bacteria (12, 18). Carbapenem resistance is of particular concern as carbapenems are often the last available treatment option for infections due to multidrug-resistant *Enterobacteriaceae* (19). To the best of our knowledge, we are reporting the first genomic characterization of CRE from Nigeria. Detailed characterization of CRE is required to combat this worldwide emerging threat and improve patient outcomes.

Accession number(s). WGS results from our isolates have been deposited in GenBank under the accession numbers listed in Table 1.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC .00255-17.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.

ACKNOWLEDGMENTS

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Draft Genome Sequences of Interpatient and Intrapatient Epidemiologically Linked *Neisseria gonorrhoeae* Isolates

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ABSTRACT *Neisseria gonorrhoeae* is the causative agent of gonorrhea and was identified by the World Health Organization as an urgent public health threat due to emerging antibiotic resistance. Here, we report 13 draft genome sequences of *N. gonorrhoeae* isolates derived from two epidemiologically linked cases from Austria.

*N*eisseria gonorrhoeae is the etiological agent of the sexually transmitted disease gonorrhea, and it poses a public health threat due to the emergence of multidrug-resistant strains (1–4). Whole-genome sequencing is considered a powerful strategy to elucidate chains of transmission (5). Here, we announce the draft genome sequences of 13 epidemiologically linked *N. gonorrhoeae* isolates.

Two vaginal swabs, taken from a 3-year-old girl on 10 January 2018, and her rectal swab, gained on 13 January 2018, yielded *N. gonorrhoeae* colonies on Chocolat PolyViteX VCAT3 agar plates (bioMérieux, Marcy-l'Étoile, France). A 46-year-old male household member was sampled on 13 January 2018, and *N. gonorrhoeae* colonies grew from a rectal swab. Eight single colonies from the child and five from the adult were further analyzed.

For each isolate, antimicrobial susceptibility was determined according to the EUCAST recommendations for gonococci (6). All 13 isolates showed resistance to penicillin G (median MIC, 6 μg/ml; range, 1.5 to 32 μg/ml), tetracycline (median MIC, 24 μg/ml; range, 24 to 64 μg/ml), and ciprofloxacin (median MIC, 0.75 μg/ml; range, 0.5 to 1.5 μg/ml), but were susceptible to ceftriaxone, cefixime, and azithromycin.

Genomic DNA isolation, whole-genome sequencing, assembly, and contig filtering were performed as described previously (7). Paired-end sequencing (2 × 300 bp) generated 348,172 to 847,328 reads, with a mean coverage of 41- to 89-fold. The NCBI Prokaryotic Genome Automatic Annotation Pipeline identified 2,654 to 2,720 genes, 2,604 to 2,664 coding sequences, 273 to 305 pseudogenes, 3 to 6 rRNA genes, and 47 to 51 tRNA genes.

Antimicrobial resistance genes were identified using the Comprehensive Antibiotic Resistance Database (CARD) (8). All 13 isolates had *gyrA*, *N. meningitidis* PBP2 and *rpsJ*, and the efflux genes *farA*, *farB*, *macA*, *macB*, *mtrC*, *mtrD*, and *mtrR*. In addition, bla_{TEM-1} was detected in three child and three household member isolates. Three isolates from the child and one from the household member carried bla_{TEM-90}. One child isolate had $bla_{TEM-150}$, and another one carried $bla_{TEM-150}$ plus the efflux gene *patA*.

All 13 isolates belonged to multilocus sequence type (MLST) 1588 (ST1588). An *ad hoc* core genome MLST (cgMLST) scheme comprising 1,524 targets was established using strain MS11 (ATCC BAA-1833) as a reference. Child isolates differed by zero to three alleles and household member isolates by zero to one alleles; the maximum interindividual variability of the isolates was five allelic differences. In the course of comparison with the Austrian Agency for Health and Food Safety (AGES) *N. gonor-*

TABLE 1 Fourteen *N. gonorrhoeae* isolates included in BioProject PRJNA433931

Strain	GenBank accession no.	No. of contigs	Total length (bp)
980035-18	PTPT00000000	173	2,234,140
980036-18	PTPS00000000	155	2,227,585
980037-18	PTPR00000000	203	2,214,439
980038-18	PTPQ00000000	193	2,239,226
980039-18	PTPP00000000	164	2,214,439
980040-18	PTPO00000000	191	2,232,201
980041-18	PTPN00000000	156	2,226,960
980042-18	PTPM00000000	168	2,223,307
980043-18	PTPL00000000	160	2,217,312
980044-18	PTPK00000000	137	2,214,035
980045-18	PTPJ00000000	141	2,210,712
980046-18	PTPI00000000	140	2,217,914
980047-18	PTPH00000000	199	2,209,742
980016-16	PTPG00000000	178	2,233,553

rhoeae whole-genome database (currently covering 452 isolates from the years 2014 to 2018), all but one isolate differed by at least 303 alleles. An isolate gained in 2016 (strain 980016-16) from a urethral swab of an epidemiologically unrelated 32-year-old male patient, registered in the same Austrian province as the two described case patients, showed a six-allele difference. From these results, we propose a complex-type threshold of a maximum of five allelic differences for direct transmission events of *N. gonorrhoeae*. Our findings underline the considerable potential of whole-genome sequencing (WGS) to document chains of transmission.

Accession number(s). This whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession numbers shown in Table 1. The versions described in this paper are the first versions.

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Whole-Genome Analysis of a Human *Enterobacter mori* Isolate Carrying a *bla*_{IMI-2} Carbapenemase in Austria

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Enterobacterales belonging to the genus Enterobacter are well-known human pathogens and *bla*_{IMI} carrying strains have been described in several countries. From Austria, we report on the first human *Enterobacter mori* isolate, typically a plant pathogen, which showed an undescribed multilocus sequence type and carried a *bla*_{IMI-2} carbapenemase.

Keywords: *Enterobacter mori*, *bla*_{IMI-2}, Austria, carbapenemase

Introduction

*E*NTEROBACTERALES BELONGING TO the genus *Enterobacter* are well-known pathogens for the kingdoms of *plantae* and *animalia*. *Enterobacter aerogenes*, *Enterobacter cloacae*, *Enterobacter agglomerans*, *Enterobacter gergoviae*, *Enterobacter amnigenus*, and *Enterobacter taylorae* are important human pathogens with a wide spectrum of disease.[1] *Enterobacter mori* is commonly known as a plant pathogen causing bacterial wilt of mulberry, and has not yet been linked to human disease.[2]

We report on a clinical *E. mori* isolate (laboratory ID no. 308485), which was recovered from the ear swab of a 59-year-old outpatient suffering from acute otitis externa. Symptoms had reportedly developed after visiting a thermal bath in the federal state of Carinthia. An empiric therapy using topical ciprofloxacin was initiated; the patient was subsequently lost to follow-up. Neither data on earlier antibiotic treatment nor data on recent travel history were available. As the isolate showed *in vitro* resistance toward carbapenems (doripenem, ertapenem, imipenem, and meropenem), the strain was referred to the National Reference Center for Nosocomial Infections and Antimicrobial Resistance (NRZ) for further characterization.

Materials and Methods

Identification of the isolate

Initial species identification in the local clinical laboratory was performed by using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) and confirmed by the same method in the NRZ (Biotyper IVD, Bruker Daltonics, Bremen, Germany). API 20E (bioMérieux, Marcy l'Etoile, France) was applied for the analysis of biochemical characteristics.

Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MICs) were determined using gradient diffusion (Etest; bioMérieux) on Mueller–Hinton agar plates with the exception of colistin, where broth microdilution was used. MICs were interpreted according to EUCAST 2016 clinical breakpoints.

Phenotypic confirmation of underlying resistance mechanisms

Production of an AmpC enzyme was confirmed phenotypically by combination disk test (CDT) (AmpC detection set, Mast Diagnostica, Reinfeld, Germany). Phenotypic carbapenemase confirmation included the modified Hodge test,[3] and the CDT (KPC, MBL, and OXA-48 confirm kit, Rosco, Denmark).

Molecular biology

PCR targeting *bla*_{IMI} was performed as previously described.[4] In addition, whole-genome sequencing (WGS) was applied. For WGS, high-quality genomic DNA (gDNA) was isolated using the MagAttract HMW DNA Kit (Qiagen, Hilden, Germany). For preparing the fragment library with the Nextera XT kit, 1 ng gDNA was used, then paired end sequencing was performed on a MiSeq (both Illumina, Inc., San Diego, CA).

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Results

Identification of the isolate

MALDI-TOF MS indicated the presence of *E. cloacae* complex in both laboratories. Database query using Speciesfinder 1.2 (https://cge.cbs.dtu.dk/services/SpeciesFinder/) did not result in reliable identification (<98% identity with *E. cloacae* FP929040). However, the whole-genome-based identification using a tetra correlation search in JSpeciesWS yielded a highest score of 0.99972 to *E. mori* type strain LMG 25706 genome and only 0.99723 to *E. cloacae* WCHECI-1060.[5] To substantiate the species identification by WGS, ribosomal multilocus sequence type (rMLST)[6] was performed and the complete 16S rDNA (1,554 bp) and *groEL* (1,634 bp) were extracted and assembled from WGS reads and blasted against the NCBI *Enterobacter* spp. database.

The rMLST analysis resulted in a 100% match to *E. mori*. The 16S rDNA had 99.5% identity (7 mismatches) to *E. mori* type strain LMG 25706, 99.0% (16 mismatches) to *E. aerogenes* KCTC2190, and 98.4% (25 mismatches) to *E. cloacae* ATCC13047. The *groEL* sequence of the isolate matched the *E. mori* type strain LMG 25706 by 99.5% (8 mismatches) and the *E. cloacae* ATCC13047 by 96.6% (55 mismatches).[7] Biochemical analysis revealed no lysine decarboxylase activity but the ability of the isolate to utilize D-arabitol, which was in line with the phenotypic characteristics of *E. mori* type strain LMG 2570 as previously described.[2]

Antimicrobial susceptibility testing

At the NRZ, carbapenem resistance (all MICs >32 mg/L) was confirmed. Furthermore, the susceptibility pattern for other antibiotic substances was in line with an *Enterobacterales* isolate harboring a chromosomal AmpC enzyme, which is summarized in Table 1.

TABLE 1. SUSCEPTIBILITY PATTERN OF ISOLATE 308485

Antibiotic	
Ampicillin	R (>256)
Amoxicillin–clavulanic acid	R (64)
Cefuroxime	R (16)
Cefotaxime	S (0.5)
Ceftazidime	S (1)
Cefepime	S (0.5)
Ceftolozane–tazobactam	S (0.5)
Meropenem	R (>32)
Imipenem	R (>32)
Doripenem	R (>32)
Ertapenem	R (>32)
Gentamicin	S (0.25)
Tobramycin	S (1)
Amikacin	S (1)
Ciprofloxacin	S (0.064)
Tigecycline	S (0.5)
Trimethoprim–sulfamethoxazole	S (0.064)
Colistin	S (0.5)
Fosfomycin	S (16)

Minimum inhibitory concentrations in mg/L are given in parentheses. S, susceptible; I, intermediate; R, resistant.

Phenotypic confirmation of underlying resistance mechanisms

The CDTs indicated the presence of an AmpC enzyme as well as an Ambler class A carbapenemase. In addition, the modified Hodge test yielded a positive result for meropenem, ertapenem, and imipenem, which was also indicative of a carbapenemase.

WGS analysis of resistance genes and genetical background

WGS-generated raw reads were *de novo* assembled into a draft genome using Velvet version 1.1.04.[8] CARD and ResFinder were used to detect resistance genes (Table 2) and PlasmidFinder was used for plasmid detection.[9–11] Phenotypic resistance to carbapenem could be linked to bla_{IMI-2}. The bla_{IMI-2} carbapenemase containing contig (16,065 bp) matched *E. cloacae* plasmid pDSMZ16690 GenBank accession number NZ_CP017185.1 to 94%, which has already been reported as bla_{IMI-2} carrying in a clinical *E. coli* isolate from China (https://www.ncbi.nlm.nih.gov/nuccore/10956 04776).

In silico determination of the classical multilocus sequence type (MLST) from WGS data using the *E. cloacae* MLST scheme resulted in a previously undescribed MLST comprising new alleles for *gyrB* (308), *leuS* (354), and *pyrG* (283). Allele sequences were submitted for curation and assigned to the new ST 1009 by the National Center for Global Health and Medicine in Japan.

Discussion

To the best of our knowledge, this is the first report of a bla_{IMI-2} expressing isolate from Austria. Since the first description of the $bla_{NMC-A/IMI}$ enzyme in 1984, bla_{IMI} carrying *Enterobacter* spp. isolates has infrequently been reported from various countries across the globe, including the United States, Canada, China, and Europe.[12–14] These hitherto rare organisms are typically of heterogeneous genetic background.[4] bla_{IMI} can also be found in *Enterobacterales* species other than *Enterobacter*, such as *Escherichia coli* and *Klebsiella variicola*.[15] Since 2010, 565 *Enterobacterales* isolates with decreased carbapenem susceptibility from Austria have been investigated for carbapenemase production at the NRZ without such organisms being detected. Nevertheless, the characteristic resistance pattern, that is, resistance to carbapenems with phenotypic indication of an Ambler class A enzyme and concomitant susceptibility to third-generation cephalosporins, should prompt an evaluation for bla_{IMI}. As most commercial assays do not detect the genes encoding this

TABLE 2. RESISTANCE GENE PROFILE OF ISOLATE 308485

Antibiotic substance class	Genes detected by whole-genome sequencing
Cephamycins	bla_{ACT-29}
Carbapenems	bla_{IMI-2}
Fluoroquinolones	*qnr*E
Fosfomycin	*fos*A-2
Phenicols	*cat*

enzyme, such isolates should best be referred to a reference laboratory with extended diagnostic capabilities.

In addition, this is the first description of a clinically relevant *E. mori* isolate. WGS, rMLST, 16S rDNA, as well as *groEL* analysis revealed highest consensus to *E. mori* type strain LMG 25706 and resulted in an unequivocal identification as *E. mori*. The hypothesized role of *E. mori* as a human pathogen, however, warrants further proof since we are reporting on a single case only and the isolate originated from an external ear canal swab, prone to possible contamination. However, commercially available MALDI-TOF MS-based identification systems presently lack reference spectra for this rare species and automated biochemical identification platforms cannot distinguish *E. mori* from *E. cloacae* complex because the crucial discriminative reactions, lysine decarboxylase activity and D-arabitol fermentation, are not part of their panels. Thus, the role of *E. mori* as a causative agent of human disease may have been missed so far and manufacturers should be encouraged to include this species in their databases.

Nucleotide accession number(s)

This whole genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession NFZM00000000. The version described in this paper is version NFZM01000000.

Disclosure Statement

No competing financial interests exist.

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Outbreak of Yersiniabactin-Producing *Klebsiella pneumoniae* in a Neonatal Intensive Care Unit

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Conflict of interest: The authors declare no potential conflict of interest.

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Abstract

Background: The Gram-negative bacterium *Klebsiella pneumoniae* is a frequent pathogen causing outbreaks in neonatal intensive care units (NICUs). Some *Enterobacteriaceae* can acquire the ability to sequester iron from infected tissue by secretion of iron-chelating compounds such as yersiniabactin. Here we describe an outbreak and clinical management of infections due to a highly virulent yersiniabactin-producing, non-multiresistant *Klebsiella pneumoniae* strain in a NICU. Outbreak investigation and effectiveness assessment of multidisciplinary infection control measurements to prevent patient-to-patient transmission of highly-pathogenic *Klebsiella pneumoniae* were undertaken.

Methods: Outbreak cases were identified by isolation of *Klebsiella pneumoniae* from blood or stool of infants. Clinical data were abstracted from medical charts. *Klebsiella pneumoniae* isolates were genotyped using whole genome sequencing and yersiniabactin production was evaluated by luciferase assay.

Results: Fourteen cases were confirmed with eight symptomatic and six colonized patients. Symptomatic patients were infants of extremely low gestational and chronological age with fulminant clinical courses including necrotizing enterocolitis and sepsis. Whole genome sequencing for bacterial isolates confirmed the presence of an outbreak. All outbreak-isolates produced yersiniabactin.

Conclusion: Yersiniabactin-producing *Klebsiella pneumoniae* can display a high pathogenicity in extremely premature infants with low chronological age. This outbreak also underlines the considerable potential of today's infection control systems for recognizing and controlling nosocomial infections in highly vulnerable populations.

Keywords: preterm neonate, yersiniabactin-producing *Klebsiella pneumoniae*, outbreak, necrotizing enterocolitis, neonatal sepsis

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Introduction

The Gram-negative bacterium *Klebsiella pneumoniae* is a well-recognized pathogen of nosocomial infections in intensive care units and has been reported to be responsible for at least 15% of Gram-negative nosocomial infections (1, 2). One characteristic of *K. pneumoniae* is the capacity to silently colonize the gastrointestinal tract of patients and hospital staff without causing symptoms (3). Such carriers often remain unrecognized, resulting in impaired infection and outbreak control (4).

In recent years, outbreaks with multiresistant *K. pneumoniae* have been reported worldwide with different sources and reservoirs such as bath soap (5), breast milk (6) and ultrasonography gel (7). In the majority of outbreaks an epidemiologically proven environmental source was missing, suggesting the possibility of patient-to-patient and staff-to-patient transmission. Since *K. pneumoniae* can survive for hours on human skin, the likelihood of transmission through skin contact is high (8).

In recent years, different *Klebsiella* strains have shown the potential to acquire pathogenic properties as well as multidrug-resistance (9, 10). One pathogenic factor is the production of siderophores. Iron is an important bacterial micronutrient needed in numerous biological processes including metabolic cycles, gene regulation and DNA synthesis (11). Iron-deprivation mechanisms are an important metabolic adjustment during the host immune response against invading pathogens. Thus, by production of siderophores, bacteria possess the ability to sequester and uptake iron from infected tissues. Members of the *Enterobacteriaceae* family, including *K. pneumoniae*, have been found to produce different siderophores (12). The expression of the siderophore yersiniabactin is associated with virulence in *Yersinia* species and was found to spread horizontally with high frequency among *Enterobacteriaceae* species (13). However, the potential contribution of the yersiniabactin production to pathogenicity in *K. pneumoniae* and other *Enterobacteriaceae* causing extraintestinal infections remains undefined. Premature infants, especially extremely

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premature infants below 1000 grams birth weight, are highly susceptible to severe bacterial infections resulting in high mortality and morbidity (14).

In May 2016, an outbreak of yersiniabactin-producing *K. pneumonia*e occurred in the neonatal intensive care unit (NICU) at the Medical University of Vienna. Here we report the clinical course, genomic profiling of the *K. pneumoniae* strain as well as the multidisciplinary management leading to immediate control of the outbreak

Methods

Study center and data collection

The Department of Neonatology at the Medical University Vienna/General Hospital Vienna is a tertiary care academic center consisting of two neonatal intensive care units (NICUs, 22 beds, level IV) and two intermediate care wards (24 beds). The outbreak primarily occurred in the NICU connected to the prenatal ward and obstetric department. This NICU consists of 12 beds separated into 3 bays with 4 beds each and is geographically separated from the other neonatal wards. In general, the average nurse:patient ratio is 1:1.7 during day-time and 1:2.4 during night shifts. The probiotic preparation Infloran® (*Lactobacillus acidophilus & Bifidobacterium bifidum*) is routinely used in our institution for prevention of necrotizing enterocolitis (NEC). We do not use enteral antibiotics for NEC prevention. Demographic and clinical data of affected patients were retrieved from the patient *data* management system (PDMS) ICCA (Phillips Healthcare). *K. pneumoniae* culture results and antibiotic resistance profiles were analyzed using the monitoring of microorganism (MOMO) database of Medexter Healthcare as described previously (15). NEC was classified according to modified Bell´s staging criteria (16).

Luciferase-Assay

Production of the siderophore yersiniabactin was quantified indirectly using a luciferase reporter assay as described elsewhere (17, 18). Briefly, bacterial strains were cultivated in NBD medium for 24 hours at 37°C. Next, bacteria were pelleted by centrifugation and the

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supernatant was added to the indicator strain WR 1542 harbouring plasmid pACYC5.3L. All the genes necessary for yersiniabactin uptake, i.e. *irp6, irp7, irp8, fyuA, ybtA*, are located on this plasmid. Furthermore, the reporter plasmid is equipped with a fusion of the *fyuA* promoter region with the luciferase reporter gene. The amount of yersiniabactin can be quantified semi-quantitatively, as yersiniabactin-dependant upregulation of *fyuA* expression is determined by luciferase activity of a *fyuA-luc* reporter fusion.

Infection Control – Outbreak Investigation

After the rapid clinical deterioration of two extremely premature infants in the same night, the infection control team was consulted and microbiological cultures of blood and stool samples of index patients were sent for microbiological evaluation. A case was defined as the occurrence of *K. pneumoniae* isolated from culture of any specimen collected during the outbreak period. Active surveillance of all patients at the NICU was initiated to prospectively identify additional cases. Infants with rectal colonization with *K. pneumoniae* were immediately isolated and cohorted in one room. Working on patient beds was only allowed using barrier precautions with single-use gowns and gloves. During the epidemiologic investigation, all staff members were re-educated on proper hand hygiene by the infection control (IC) team. Infection control practices in the NICU were reviewed including an reassessment of all invasive procedures. Direct observations of routine work flow were conducted by the IC team. The reprocessing of medical equipment was reevaluated and all special equipment identified as potential outbreak sources used in the NICU was examined. Environmental cultures were collected from all surfaces in patient-care areas, from sinks, ultrasound probe gels and medical equipment. Environmental cleaning as well as reprocessing of infant beds was reassessed. From day 1 to 7, the NICU was closed for new admissions. Thereafter, newly admitted infants were cohorted with surveillance culture-negative infants in a separated ward room. Additionally, all patients were cared for under barrier precautions using single-use gowns and gloves until the last patient with *Klebsiella* spp. colonization was

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discharged home. After the implementation of the immediate outbreak management, we did neither observe novel *K. pneumoniae* related infections nor colonizations of the pathogen. Affected infants were isolated during the entire hospital stay and the last colonized patient was discharged after four months of hospitalization.

Microbiological analysis

All isolates were identified to species level using standard microbiological methods for culture and identification in our clinical microbiology laboratory. Antimicrobial resistance testing was performed according to EUCAST recommendations (www.eucast.org).

Whole genome sequencing and data analysis

High quality genomic DNA was extracted from 14 *K. pneumoniae* isolates from overnight cultures by using the MagAttract HMW DNA Kit (Qiagen, Hilden, Germany) and quantified with a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, USA) using the dsDNA BR Assay Kit (Thermo Fisher Scientific). Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) was carried out according to the manufacturer´s protocol to obtain ready to sequence bacterial genomic libraries. The libraries were paired end sequenced (2 x 300 bp) on an Illumina Miseq instrument. Genome assemblies were performed *de novo* using Velvet (version 1.1.07) (19), and whole genome sequence (WGS) data interpretation was carried out with the SeqSphere[+] analysis software (20).

The multi-locus sequence type (MLST) (21) and the core genome multi-locus sequence type (cgMLST) were extracted from the WGS data. The *K. pneumoniae* sensu lato cgMLST scheme (defined by John Rossen and Dag Harmsen) comprising 2358 core and 1946 accessory genes was used for outbreak analysis with the defined cluster threshold of 15 allelic differences and for the calculation of minimum spanning trees (MST). For detection of virulence genes, the allelic library of genes associated with virulence in *K. pneumoniae* from BIGSdb (http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html) was integrated into SeqSphere[+]. This Whole Genome Shotgun project has been deposited at

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DDBJ/ENA/GenBank under the accessions PHGE00000000 to PHGR00000000. The versions described in this paper are versions PHGE01000000 to PHGR01000000.

Statistical Analysis

Patient characteristics are presented as mean ± standard deviation or as frequency within the study group if not stated otherwise. Continuous variables were analyzed using 2 sample t-test and categorical variables were analyzed using Fisher-Exact test. A p-value <0.05 was considered statistically significant. Statistical analysis was performed using SPSS 24.0 (IBM, Armonk, NY).

Results

Demographic data of infants

A total of 14 patients were affected by the *K. pneumoniae* outbreak in May 2016. Eight of those infants had a symptomatic course: the two index patients developed a fulminant NEC and died within 24 hours; three infants developed NEC with conservative treatment (2 cases of NEC stage Ib, 1 case of NEC stage IIa) and three further infants suffered from sepsis. Asymptomatic patients were colonized with the *K. pneumoniae* strain but did not develop signs of infections during the hospital stay. Symptomatic patients displayed lower gestational age (25.11 ± 0.90 vs. 26.90 ± 2.44 weeks of gestational age (WOG), p=0.07), lower birth weight (709 ± 157 vs. 966 ± 241 gram; p=0.03) and were significantly younger at time of diagnosis of colonization with *K. pneumoniae* (17.75 ± 12.57 vs. 43.00 ± 20.56 days postpartum; p=0.01). Demographic data are summarized in table 1.

Time-course of the outbreak

Data of involved patients are summarized in figure 1. Within one night, two extremely premature infants with an uncomplicated previous course acutely deteriorated from spontaneous breathing on CPAP to the full-blown picture of fulminant NEC within hours. Index patient 1 (25+4 WOG, 5 days old) had a blood culture taken and intraabdominal swabs (obtained during emergency laparotomy); all were positive for *K. pneumoniae*. Within an

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hour, Patient 2 (24+4 WOG, 8 days old, triplet III, also uncomplicated on CPAP until that night), located in another room, developed fulminant pan-NEC and died. On the following day, Patient 3 (24+4 WOG, 9 days old, triplet II) and 4 (24+4 WOG, 9 days old, triplet I), located in the same room as Patient 2, exhibited signs of sepsis. Blood cultures were positive for *K. pneumoniae*. Bacterial cultures from vaginal swabs and the amniotic membrane-tissue taken from the triplet's mother showed negative results. On day 4 of the outbreak, Patient 5 (24+6 WOG, 13 days old) displayed clinical signs of sepsis and showed a positive blood culture with *K. pneumoniae*. Patient 6 (25+6 WOG, 22 days old) developed NEC Stage II with a positive rectal swab for *K. pneumoniae*. Both infants were located in the bay next to Patients 2 to 4. Patient 7 (24+1 WOG, 31 days old) and Patient 8 (26+6 WOG, 41 days old) showed subtle signs of NEC stage 1 with occult and gross blood in stool but stable vital signs on day 1 of the outbreak. Both patients were assigned later to the outbreak as rectal surveillance cultures were positive for *K. pneumoniae*. Immediately after the fulminant death of the index cases, all admitted infants at our four neonatal wards (two NICUs, two NIMCs) were screened for rectal colonization with *K. pneumoniae*. We identified six colonized asymptomatic infants at one of our intermediate care wards showing no clinical symptoms of *K. pneumoniae* infection during their entire hospital stays.

Infection Control

Lack of space between and around beds as well as a very high turn-over rate of patients in the NICU were identified during the infection control assessment as contributing factors. No breaches in reprocessing procedures were recognized. All environmental cultures were negative for *K. pneumoniae*, therefore no environmental source as cause of the outbreak could be identified. Nevertheless, multi-use gel containers for ultrasound probes were replaced against single-use containers. Inconsistencies in the environmental cleaning procedures of patient-care areas including bed reprocessing could be observed and led to retraining sessions

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for cleaning personel. Hand hygiene teaching sessions for all health care workers were reinforced during the outbreak and regular hand hygiene audits implemented.

WGS outbreak analysis

All 14 *K. pneumoniae* isolates were sequenced with a minimum of 98% of good cgMLST targets and an average coverage of 84-fold. All isolates were assigned to classical MLST 664 and cgMLST 1306. Based on the cgMLST the group including symptomatic patients (n=8) had a maximum allelic difference of 3 (average distance 0.96), the group including asymptomatic patients had a maximum allelic difference of 5 (average distance 3.53), and between these two groups the maximum allelic difference was 5 (average distance 2.29). Based on the cgMLST and the accessory genome the group of symptomatic patients had a maximum allelic difference of 3 (average distance 1.68), the group including asymptomatic patients had a maximum allelic difference of 8 (average distance 5.67) and between these two groups the maximum allelic difference was 7 (average distance 3.67) (Figure 2).

Via the integrated virulence database from BIGSdb all alleles (n=11) belonging to yersiniabactin were detected (Table 2). The locus *irp*1 was identified as new allele, submitted to the BIGSdb database and a new allele number was assigned (irp1_new). The new combination of yersiniabactin loci revealed a new yersinibactin sequence type (YbST= new).

Discussion

To the best of our knowledge, this is the first description of an outbreak of a yersiniabactin-producing *K. pneumoniae* strain in a neonatal intensive care unit. Using WGS for bacterial isolates we were able to confirm the presence of an outbreak among the respective *K. pneumoniae* isolates. Inclusion of the pan genome (cgMLST plus accessory genome analysis) did not reveal major differences in comparison to the cgMLST analysis and thus confirmed the outbreak cluster.

Invasive infection with the described bacterial strain led to fulminant NEC and sepsis courses in extremely premature infants. The clinical course of infected infants appeared to be

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influenced by gestational age as well as chronological age. Only extremely premature infants with low chronological age developed fulminant disease upon infection with yersiniabactin-producing *K. pneumoniae*. The premature gut, especially of infants born prior to 28 weeks of gestation, exhibits various immaturities rendering the intestine vulnerable to exogenous factors leading to dysbalance in microbial colonization. This can result in exaggerated inflammatory response of the immature intestinal epithelium to luminal bacteria and poor intestinal microperfusion (16, 22). Thus, the "leaky gut" of extremely premature infants and the higher virulence of siderophore producing bacteria might predispose those infants to higher epithelial damage with increased bacterial translocation leading to an excessive systemic inflammation. This hypothesis is underlined by the asymptomatic clinical courses of affected patients with higher gestational and chronological age where a more mature gut was not affected at all by yersiniabactin-producing *K. pneumoniae*. In our setting, a protective isolation strategy prevented the transmission of *K. pneumoniae* to newly admitted premature infants.

Over the last decade, numerous publications delivered novel insights into the relationship between colonization of the gut and health/disease status (23). In premature infants, a lower diversity of the premature gut colonization seems to be a risk factor for NEC (24). These findings correlate with clinical observations that infants who receive antibiotics for a longer duration display a higher risk for developing NEC (25). On the other side, oral decontamination with antibiotics in the first weeks of life resulted in a lower NEC incidence in low birth weight infants (26). Thus, the combination of diversity and dysbiosis seems to be important to develop a stable gut homeostasis in preterm infants. The lower diversity can be tackled by probiotics, which show promising results in NEC prevention (27). Several randomized-controlled trials as well as consequently conducted Meta-Analyses showed a clear benefit for probiotics in premature infants (28, 29). Interestingly, a large multicenter randomized placebo-controlled trial using *Bifidobacterium breve* alone showed no effect on

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the NEC incidence in very low birth weight infants (30). Thus, the optimal probiotic strain and dosage in premature infants needs to be elucidated.

Whether routine screening for rectal colonization can prevent outbreaks in NICUs is controversially discussed in the literature. Whereas some authors concluded that routine mucosal cultures are inefficient for the prediction of late-onset sepsis in neonatal intensive care units (31) or might even be harmful by enticing to overuse of antibiotics (32), others have found beneficial effects of a once-weekly screening strategy for MDRO (33, 34). Likewise, whereas in the UK guidelines claim that "*There is currently insufficient evidence on clinical effectiveness to recommend weekly screening in NICUs*" (35), the RKI in Germany stipulates at least once weekly rectal screening cultures in VLBWI in German NICUs (36). All guidelines and authors arguing in favour of a routine screening strategy, however, recommend screening for particular pathogens only, such as MDRO or pathogens particularly relevant in NICUs such as *Serratia marcescens*, but not screening for all gut pathogens. Although we had performed routine rectal MDRO screening at our NICU, the yersiniabactin-producing K. pneumoniae strain was not recognized as an harmful strain due to an normal antibiogram. Hence, we only realized that transmission inside the ward had been going on when the first two patients simultaneously developed fulminant NEC and subsequent routine screening and genotyping unveiled colonization of 6 additional patients with the same - microbiologically "harmless" – pathogen. As a result of the outbreak experience, we switched to a routine once-weekly screening strategy for all pathogens, irrespective of antibiogram, although there are no data in the literature supporting this approach.

In conclusion, we describe the first outbreak with a yersiniabactin producing Klebsiella strain in a NICU. Extremely low gestational age and birthweight as well as colonization in the first weeks of life were significant risk factors for fulminant and invasive infections with this pathogen. Although not suspicious by conventional microbiology, this pathogen has to be added to the list of pathogens with particular virulence in chronologically young extremely

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premature infants. Whether routine screening for all pathogens (including microbiologically "harmless" strains) and/or protective isolation of extremely premature infants in the first weeks of life to prevent "unphysiologic" colonization can prevent fulminant infections as described in this report will have to be tested in future trials.

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Figure 1 Timeline of the outbreak in the neonatal ward. The first week of the outbreak is shown on a daily basis (D = day). After the first week, culture results from the conducted rectal surveillance cultures are shown in weekly frequencies (W = weeks; dotted line). Pat. = Patient; Black box = case of death; red box = first detection in blood culture; yellow box = onset of disease; green box = positive surveillance culture

Figure 2 MST (minimum spanning tree) based on the core genome and the accessory genome of *K. pneumoniae*. Fourteen outbreak isolates are shown as circles; allelic differences between isolates are represented by blue numbers between isolates. The outbreak cluster was identified with a maximum allelic difference of 15, all fourteen isolates lie within this definition. Symptomatic patients are colored in orange; asymptomatic patients in light blue. MST based on cgMLST (core genome Multilocus Sequence Typing) comprising 2358 core target genes (left) and MST based on cgMLST plus 1946 accessory target genes (right).

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Table 1 Demographic data of symptomatic (n=8) and asymptomatic (n=6) patients colonized with *K. pneumoniae*.

	Symptomatic Patients n = 8	Asymptomatic Patients n = 6	p- value
Gestational age, wks (mean ± SD; min-max)	25.11 ± 0.90 (24.14 – 26.86)	26.90 ± 2.44 (24.14 – 31.28)	0.07
Birth weight, g (mean ± SD; min-max)	709 ± 157 (440 - 915)	966 ± 241 (625 - 1370)	0.03*
Age at disease onset, d (median; min-max)	12.5 (5 - 41)	n.r.	n.r.
Age at first detected colonization, d (median; min-max)	12.5 (5 - 41)	43 (19 - 74)	0.01*
Male, n (%)	4 (50%)	3 (50%)	1.00
Multiples, n (%)	4 (50%)	1 (16.7%)	0.30
Necrotizing enterocolitis, n (%)	5 (62.5%)	0 (0%)	0.03*
Sepsis, n (%)	3 (37.5%)	0 (0%)	0.20
Blood culture positive, n (%)	4 (50%)	0 (0%)	0.08
Death, n (%)	2 (25%)	0 (0%)	0.47

Continuous variables were analyzed using 2 sample t-test and categorical variables were analyzed using Fisher-Exact test. *p < 0.05; n.r. = non-relevant;

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Table 2 Typing scheme for yersiniabactin virulence operon.

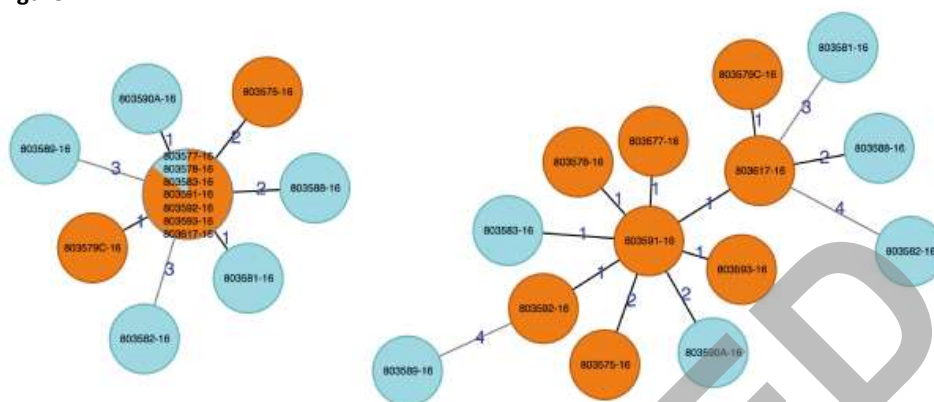| YbST	loci	*ybtS*	*ybtX*	*ybtQ*	*ybtP*	*ybtA*	*irp2*	*irp1*	*ybtU*	*ybtT*	*ybtE*	*fyuA*
new	allele	6	4	20	61	1	253	new	2	76	4	2

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Figure 1

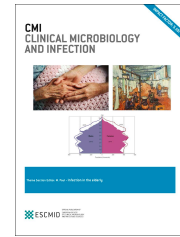|	D0	D1	D2	D3	D4	D5	D6	D7	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12	W13	W14	W15	W16
Pat. 1	†																						
Pat. 2	🟨	†																					
Pat. 3			B				🟩		🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩		
Pat. 4	🟩			B	🟩					🟩		🟩											
Pat. 5	🟩				B				🟩	🟩			🟩										
Pat. 6					🟨	🟩			🟩		🟩	🟩		🟩								🟩	
Pat. 7		🟨							🟩	🟩	🟩	🟩		🟩	🟩								
Pat. 8		🟨			🟩				🟩	🟩	🟩		🟩			🟩	🟩	🟩					

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Figure 2

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Accepted Manuscript

Nosocomial outbreak of *Streptococcus pyogenes* puerperal sepsis

Adriana Cabal, Daniela Schmid, Sarah Lepuschitz, Anna Stöger, Marion Blaschitz, Franz Allerberger, Werner Ruppitsch, Markus Hell

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1   To the Editor

2   **Nosocomial outbreak of *Streptococcus pyogenes* puerperal sepsis**

3

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24   To the Editor,

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26   Infections with *Streptococcus pyogenes* in females after delivery cause significant morbidity

27   worldwide [1] and are mostly due to *S. pyogenes emm* Type 28 [2]. Although the rate of

28   nosocomial postpartum *S. pyogenes* infections tremendously decreased within the past

29   century due to improved hygienic conditions during delivery, health care workers remain a

30   potential source of infection [3].

31   In July 2018, an outbreak of postpartum *S. pyogenes* infections occurred among patients in the

32   same obstetric ward of an Austrian hospital. The aim of the investigation was to identify the

33   source and mode of transmission by descriptive epidemiology and Whole Genome

34   Sequencing (WGS)-based typing.

35   In July 2018, four pregnant women with a median age of 33 years (range 33 – 35 years)

36   admitted for delivery to an obstetric ward of an Austrian hospital, developed signs and

37   symptoms of sepsis within four days after delivery. Vaginal swabs were available from all

38   patients and blood cultures were collected from two of these. Specimens were cultured on

39   Columbia CNA agar and presumed *S. pyogenes* colonies were confirmed by MALDI TOF

40   MS (Bruker Daltonik GmbH, Hamburg, Germany). DNA isolation and WGS including

41   assembly and *contig* filtering were performed as described previously [4]. SeqSphere+

42   software was used to extract the classical multilocus sequence type (MLST) from the WGS

43   data and to define a new core genome multilocus sequence typing (cgMLST) scheme. For this

44   purpose, we compared all *S. pyogenes* available at NCBI with complete (n=59) or

45   chromosome (n=3) status to the reference genome of *S. pyogenes* GAS M1 reference strain.

46   The final typing scheme comprised 1127 core genome targets and 528 accessory genome

47   targets. A Minimum Spanning Tree (MST) was created to visualize allelic differences

48   between the isolates. In addition, we determined the *emm* subtype, assessed the presence of

49 virulence genes and the region of difference 2 (RD2) and characterized the CovR/S regulatory

50 system using the online tools BLAST (http://www.ncbi.nlm.nih.gov/blast) and VFanalyzer

51 (http://www.mgc.ac.cn/VFs/). We used the *emm*28.4 reference genome of MGAS 6180 for

52 comparison.

53 Within active case finding, vaginal, pharyngeal swabs and blood cultures were obtained from

54 two additional patients of the obstetric ward, who had developed increased body temperature

55 after delivery. Pharyngeal swabs were collected on a voluntary basis from one medical doctor

56 involved in one of the deliveries out of 24 staff members working at the ward. Already before

57 assisting in the four deliveries, one of the 12 ward midwives, started to suffer from a

58 panaritium at the middle finger of her right hand, which was then swabbed for the purpose of

59 source identification (fig 1a). Microbiological workup and when appropriate, molecular

60 typing, were performed as described above. The study used anonymized patient data only;

61 ethic approval was not required as puerperal sepsis is a mandatorily notifiable disease by the

62 Austrian Epidemic Act. Specimens from the two patients with increased temperature and the

63 medical doctor were negative for *S. pyogenes*. The panaritium swab from the midwife tested

64 positive for *S. pyogenes*.

65 The six isolates obtained from the four septic patients and the involved midwife were

66 MLST52, *emm*28.0 and carried the RD2. In addition, among all virulence genes found (data

67 not shown), *eno* and *sda* genes, encoding for streptococcal enolase and streptodornase-alpha

68 respectively, were not shared by the reference strain MGAS 6180. We found no mutations in

69 the CovR/S regulatory system.

70 CgMLST analysis revealed that the six isolates differed by 0-2 alleles (fig 1b). Including the

71 accessory genome, isolates differed by three alleles (tree not shown). In order to establish a

72 cluster threshold, we included five *S. pyogenes* strains from a previous puerperal sepsis

73 outbreak in Australia, which shared the *emm* type and MLST (*emm*28, MLST52) with the

74 Austrian outbreak isolates. Three out of five Australian strains belonged to a cluster, as

75   identified using SNP analysis [5]. CgMLST confirmed the cluster found by SNP analysis

76   since the three isolates differed only by 0-1 alleles. From these findings, we propose a

77   preliminary cluster threshold of ≤5 alleles for *S. pyogenes*.

78   In-depth interview of the midwife revealed that inappropriate hand disinfection likely

79   occurred before donning gloves and after removing gloves during childbirth assistance due to

80   her finger bandage.

81   In view of the findings of the descriptive epidemiology and cgMLST analysis, the source of

82   this point outbreak is likely to be the midwife having been suffering from a panaritium when

83   assisting in all deliveries. Intensified training in hand hygiene for health care workers, in

84   particular for hospital midwives, was immediately launched. No further cases were detected

85   within the following two months.

86   In conclusion, this is the first time having elucidated a nosocomial outbreak of *S. pyogenes* -

87   caused puerperal sepsis by use of WGS in Austria. The pre-existent recommendation of

88   reliable inspection of midwives' hand for skin infection prior to delivery or of glove-use

89   during delivery assistance plus hand disinfection with and alcohol-based handrub before and

90   after glove use should be constantly reinforced to prevent postpartum infections with *S.*

91   *pyogenes*.

92

93   **Accession number(s):** This Whole Genome Shotgun project has been deposited at

94   DDBJ/ENA/GenBank under the accessions QYUD00000000, QYUE00000000,

95   QYUF00000000, QYUG00000000, QYUH00000000 and QZEU00000000). The versions

96   described in this paper are versions QYUD01000000, QYUE01000000, QYUF01000000,

97   QYUG01000000, QYUH01000000 and QZEU01000000.

98

99 **Acknowledgements**

106 **Conflict of interests**

107 All co-authors in this manuscript declare that they did to receive payment or services from a

108 third party. Also, they do not have other relationships or activities that readers could perceive

109 to have influence the manuscript writing.
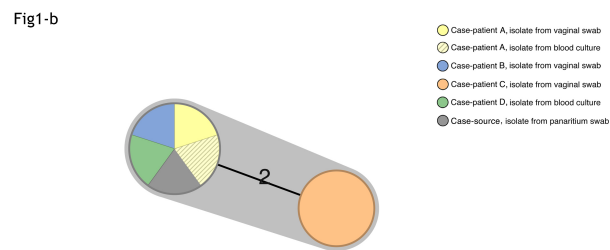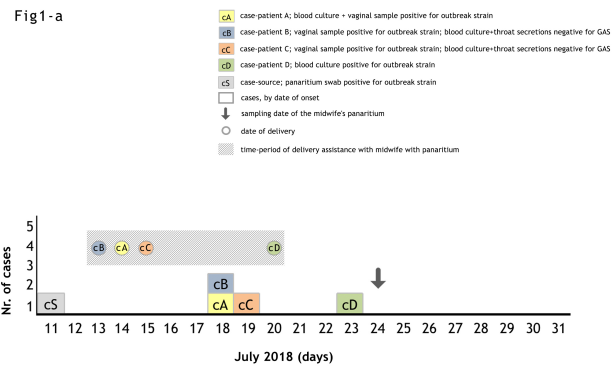
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124

125   **Figures**

126   **Figure 1.** (A) Timeline of the case-patients (cA-cD) by date of onset and date of delivery and

127   the case-midwife cS (source) by date of onset and date of sampling. (B) Minimum spanning

128   tree showing the number of allelic differences among the six *S. pyogenes* isolates in their core

129   genome.

Fig1-a

cA case-patient A; blood culture + vaginal sample positive for outbreak strain
cB case-patient B; vaginal sample positive for outbreak strain; blood culture+throat secretions negative for GAS
cC case-patient C; vaginal sample positive for outbreak strain; blood culture+throat secretions negative for GAS
cD case-patient D; blood culture positive for outbreak strain
cS case-source; panaritium swab positive for outbreak strain
☐ cases, by date of onset
↓ sampling date of the midwife's panaritium
○ date of delivery
▨ time-period of delivery assistance with midwife with panaritium

Fig1-b

○ Case-patient A, isolate from vaginal swab
◐ Case-patient A, isolate from blood culture
● Case-patient B, isolate from vaginal swab
● Case-patient C, isolate from vaginal swab
● Case-patient D, isolate from blood culture
● Case-source, isolate from panaritium swab

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Chapter 3

This chapter includes publications with focus on prevalence and emergence of foodborne pathogens.

Peer-reviewed publications:

Lepuschitz S, Ruppitsch W, Pekard-Amenitsch S, Forsythe SJ, Cormican M, Mach RL, Piérard D, Allerberger F, and the EUCRONI Study Group. European multi-centre study on occurrence of *Cronobacter sakazakii* in clinical samples. Emerg Infect Dis. 2019. doi: 10.3201/eid2503.181652. Accepted for publication

Lepuschitz S, Pekard-Amenitsch S, Haunold R, Schill S, Schriebl A, Mach R, Allerberger F, Ruppitsch W, Forsythe SJ. Draft Genome Sequence of the First Documented Clinical *Siccibacter turicensis* Isolate in Austria. Genome Announc. 2018;6(18). pii: e00380-18. doi: 10.1128/genomeA.00380-18.

Ruppitsch W, Monschein S, **Lepuschitz S**, Allerberger F, Springer B. Letter to the editor: Livestock-associated meticillin-resistant *Staphylococcus aureus* (LA-MRSA), Austria, 2013. Euro Surveill. 2017;22(46).
Author's contribution: Sarah Lepuschitz performed whole genome sequence data analysis.

Schlager S, **Lepuschitz S**, Ruppitsch W, Ableitner O, Pietzka A, Neubauer S, Stöger A, Lassnig H, Mikula C, Springer B, Allerberger F. Petting zoos as sources of Shiga toxin-producing *Escherichia coli* (STEC) infections. Int J Med Microbiol. 2018;308(7):927-932.
Author's contribution: Sarah Lepuschitz performed whole genome sequence data analysis.

Multicenter Study of *Cronobacter sakazakii* Infections in Humans, Europe, 2017

Sarah Lepuschitz, Werner Ruppitsch, Shiva Pekard-Amenitsch, Stephen J. Forsythe,
Martin Cormican, Robert L. Mach, Denis Piérard, Franz Allerberger, the EUCRONI Study Group[1]

Cronobacter sakazakii has been documented as a cause of life-threating infections, predominantly in neonates. We conducted a multicenter study to assess the occurrence of *C. sakazakii* across Europe and the extent of clonality for outbreak detection. National coordinators representing 24 countries in Europe were requested to submit all human *C. sakazakii* isolates collected during 2017 to a study center in Austria. Testing at the center included species identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, subtyping by whole-genome sequencing (WGS), and determination of antimicrobial resistance. Eleven countries sent 77 isolates, including 36 isolates from 2017 and 41 historical isolates. Fifty-nine isolates were confirmed as *C. sakazakii* by WGS, highlighting the challenge of correctly identifying *Cronobacter* spp. WGS-based typing revealed high strain diversity, indicating absence of multinational outbreaks in 2017, but identified 4 previously unpublished historical outbreaks. WGS is the recommended method for accurate identification, typing, and detection of this pathogen.

*C*ronobacter sakazakii is a motile, gram-negative, rod-shaped opportunistic pathogen of the family *Enterbacteriaceae* (*1*). In 2007, organisms previously classified as *Enterobacter sakazakii* were reassigned to the new genus *Cronobacter*, which now consists of 7 species: *C. sakazakii*, *C. condimenti*, *C. dublinensis*, *C. malonaticus*, *C. muytjensii*, *C. turicensis*, and *C. universalis* (*2,3*). *C. sakazakii* has been isolated from various environments (e.g., domestic environments and manufacturing plants), clinical sources (e.g., cerebrospinal fluid, blood and sputum), food (e.g., cheese, meat, and vegetables), and animals (e.g., rats and flies) (*4,5*).

Author affiliations: Austrian Agency for Health and Food Safety, Vienna, Austria (S. Lepuschitz, W. Ruppitsch, S. Pekard-Amenitsch, F. Allerberger); Vienna University of Technology, Vienna (S. Lepuschitz, R.L. Mach); foodmicrobe.com, Keyworth, United Kingdom (S.J. Forsythe); National University of Ireland, Galway, Ireland (M. Cormican); Universitair Ziekenhuis Brussel, Brussels, Belgium (D. Piérard)

Most reported cases of illness caused by *C. sakazakii* are in infants <2 months old (*6,7*). Premature infants and infants with underlying medical conditions are at the greatest risk for illness. Numerous outbreaks caused by *C. sakazakii* have been traced to contaminated powdered infant formula (*8*). Powdered infant formula is not a sterile product, and the ability of *C. sakazakii* to tolerate dry conditions enables it to survive for long periods in the final powdered product (*9*).

The screening of food (particularly powdered formula) was proposed to reduce the risk to neonatal and infant health (*10,11*). The most common syndromes of foodborne infection in infants include necrotizing enterocolitis (NEC), bacteremia, and meningitis (*12,13*). Examples of outbreaks of illness in hospital neonatal units caused by *C. sakazakii* associated with powdered infant formula have been compiled by Iversen and Forsythe (*6*) and by Lund (*8*).

A few cases of illness (usually nongastrointestinal) in adults caused by *C. sakazakii* have been reported. In most of these cases the adults had underlying diseases, and no evidence of foodborne transmission was reported (*14,15*).

We performed a multicenter study of *C. sakazakii* infections in humans (EUCRONI) to determine the occurrence of *C. sakazakii* in clinical microbiology laboratories across Europe. We also assessed the extent of clonality for human *C. sakazakii* isolates.

Material and Methods

Study Design

EUCRONI consisted of national coordinators (EUCRONI study group members) from 24 countries in Europe. Coordinators had to actively approach all medical microbiology laboratories to collect human *C. sakazakii* isolates (1 per patient) in their respective countries during 2017. Human historical isolates (with isolation dates before 2017) were also accepted. The 24 participating countries were arbitrarily chosen to reflect a wide geographic and

[1]Members of the EUCRONI study group are listed at the end of this article.

socioeconomic range (Figure 1). Isolates were transferred to the study center (Austrian Agency for Health and Food Safety, Vienna, Austria) for whole-genome sequencing (WGS), matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) analysis, and antimicrobial drug susceptibility testing. We submitted data capture forms to national coordinators to collect the following demographic data: patient age and sex, patient status (colonized or infected), specimen source, type of healthcare facility requesting the microbiologic culture, and date of specimen collection.

Species Identification and DNA Extraction

We cultured isolates on Columbia blood agar plates (bio-Mérieux, http://www.biomerieux.com/) overnight at 37°C. We performed species identification by using MALDI-TOF Biotyper (Bruker, https://www.bruker.com) and MBT Compass IVD 4.1.60 (Bruker) according to the manufacturer's instructions. We conducted isolation, quantification, and WGS of genomic DNA according to methods described by Lepuschitz et al. (16). We used Sequencing Coverage Calculator (http://www.illumina.com) for calculation of a desired mean coverage of ≥80-fold.

WGS Data Analysis

We de novo assembled raw reads by using SPAdes version 3.9.0 (17) and processed them in SeqSphere+ (Ridom GmbH, https://www.ridom.de) for bacterial typing. We deposited the genome sequences in the PubMLST *Cronobacter* database (https://pubmlst.org/Cronobacter) under accession nos. 2403 and 2495–2552. To determine the core genome multilocus sequence type (cgMLST) gene set, we performed a genome-wide gene-by-gene comparison by using the MLST+ target definer function of SeqSphere+
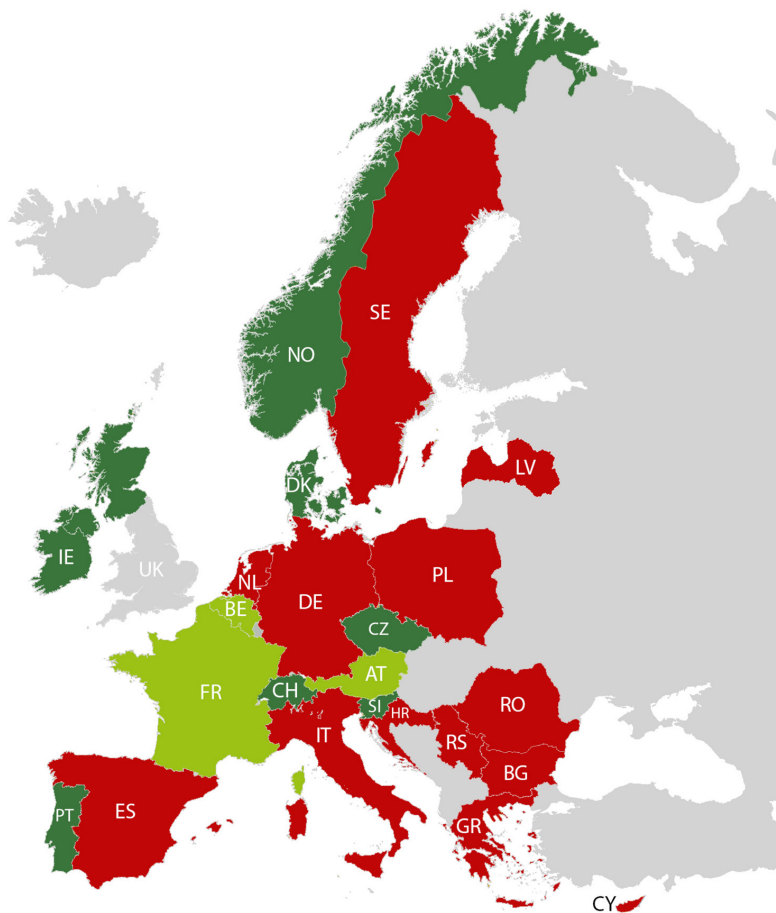

Figure 1. Countries participating in a multicenter study of *Cronobacter sakazakii* infections in humans, Europe, 2017. Dark green indicates the 8 countries that sent *C. sakazakii* isolates to the study center in Austria; light green indicates the 3 countries where historical outbreaks were detected; and red indicates the 13 countries that participated but did not provide isolates. AT, Austria; BE, Belgium; BG, Bulgaria; CH, Switzerland; CY, Cyprus; CZ, Czech Republic; DE, Germany; DK, Denmark; ES, Spain; FR, France; GR, Greece; HR, Croatia; IE, Ireland; IT, Italy; LV, Latvia; NL, Netherlands; NO, Norway; PL, Poland; PT, Portugal; RO, Romania; RS, Serbia; SE, Sweden; SI, Slovenia; UK, United Kingdom.

as described previously (*18*) with default parameters and the complete genome of *C. sakazakii* strain ATCC BAA-894 (*19*) as reference genome, all complete *C. sakazakii* genomes available at GenBank, 8 isolates retrieved from whole-genome shotgun sequencing projects, and 4 *C. sakazakii* isolates sequenced at the Austria study center as query genomes. We extracted sequences of the 7 genes comprising the allelic profile of the classical MLST scheme and queried them against the *C. sakazakii* MLST database (*1*), assigning classical sequence types (STs) in silico. We obtained additional species confirmation by using JSpeciesWS (*20*) and ribosomal MLST (*21*). We included 23 *C. sakazakii* historical isolates from 4 different outbreaks (F. Allerberger, 2016; F. Barbut, 2010–2016; G. Feierl, 2009; D. Piérard, 1997–1998, all unpub. data; Appendix Table 1, https://wwwnc.cdc.gov/EID/article/25/3/18-1652-App1.xlsx) and 3 reference strains, ATCC BAA-894 (*19*), ATCC29544 (PRJNA224116), and NCTC 8155 (PRJNA224116), to determine the level of microevolution.

Antimicrobial Resistance Testing

We performed in vitro susceptibility testing with the VITEK 2 Compact System (bioMérieux) and interpreted the VITEK 2 AST196 card according to European Committee on Antimicrobial Susceptibility Testing criteria for *Enterobacteriaceae* (Clinical Breakpoint Tables version 8.0, http://www.eucast.org/ast_of_bacteria/previous_versions_of_documents). For detection of antibiotic resistance genes, we used the Comprehensive Antibiotic Resistance Database (*22*) with default settings "perfect" and "strict" for sequence analysis. We tested isolates in SeqSphere+ for *Cronobacter*-specific variant *ampC* (e.g., CSA-1, CSA-2, CMA-1, and CMA-2) (*23*).

Results

Strain Collection and Primary Species Identification

During the study period, 11 of 24 national coordinators (Figure 1) provided 77 presumptive *C. sakazakii* isolates previously identified by conventional biochemical testing, local MALDI-TOF MS analysis (Bruker Biotyper and VITEK MS), locally performed *Cronobacter* genus- and species-specific PCRs, or 16S rRNA gene sequence analysis. These 77 isolates consisted of 36 human isolates from 2017 and 41 historical human isolates obtained during 1964–2016. The participating laboratories, using local conventional phenotypical methods or local MALDI-TOF MS analysis, incorrectly identified 18 (23.4%) of 77 human isolates as *C. sakazakii*.

MALDI-TOF MS analysis in the study center identified 69 of 77 isolates as *C. sakazakii;* 1 isolate from 2017 yielded low-confidence identification (log[score] value 1.70–1.99). We assigned 7 clinical isolates from 2017 and 1 historical clinical isolate from 2005 to other species (Table 1). The WGS-based species identification using JSpeciesWS and rMLST confirmed MALDI-TOF MS identification results in all but 10 of the 69 isolates. WGS indicated that 5 isolates were *C. malonaticus*, 2 were *C. turicensis*, 1 was *C. dublinensis*, 1 was *C. universalis*, and 1 was *Siccibacter turicensis* (Table 1; Appendix Table 1).

Human *C. sakazakii* Isolates Collected in 2017

In total, 21 *C. sakazakii* isolates from 21 patients were collected in 2017 in 9 participating countries in Europe. Case-fatality ratio (within 30 days after specimen collection) was 2 of 21 case-patients (Table 2).

Molecular Typing of Bacterial Isolates

The defined cgMLST gene set consisted of a total of 2,831 core and 1,017 accessory targets. Of 77 sequenced isolates, 59 isolates were confirmed as *C. sakazakii*. These isolates had on average 99.4% of good core genome targets (97.7% to 99.9%) (*18*) and revealed in total 17 different sequence types (STs) (Table 3).

Core genome comparison of 59 *C. sakazakii* isolates and the 3 reference strains revealed an average allelic difference of 2,402 and a maximum allelic difference of 2,724 (Figure 2). Isolates clustered in the minimum-

Table 1. Comparison of MALDI-TOF mass spectrometry and whole-genome sequencing results for 77 isolates submitted as *Cronobacter sakazakii* in a multicenter study of *C. sakazakii* infections in humans, Europe, 2017*

MALDI-TOF	Whole-genome sequencing	Total no. isolates	Human isolates detected in 2017	Historical human isolates
C. sakazakii	*C. sakazakii*	59	21	38
C. sakazakii	*C. dublinensis*	1	1	–
C. sakazakii	*C. malonaticus*	5	4	1
C. sakazakii	*C. turicensis*	2	1	1
C. sakazakii	*C. universalis*	1	1	–
C. sakazakii	*Siccibacter turicensis*	1	1	–
Enterobacter aerogenes	*Kluyvera intermedia*	1	1	–
E. asburiae	*E. cloacae*	2	2	–
E. asburiae	*E. asburiae*	1	–	1
Klebsiella oxytoca	*Klebsiella oxytoca*	1	1	–
Kosakonia cowanii	*Kosakonia cowanii*	2	2	–
Paenibacillus pasadenensis	*Paenibacillus pasadenensis*	1	1	–

*MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; –, no isolates detected.

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Table 2. Characteristics of patients enrolled and *Cronobacter sakazakii* isolates collected in a multicenter study of *C. sakazakii* infections in humans, Europe, 2017*

Sample ID	Country of origin	Patient age, y/sex	Specimen source	Death within 30 d	MLST
802520	Austria	73/F	Stool	No	630
7750-17	Austria	<1/M	Blood	Yes	4
16862-17	Austria	77/F	Blood	No	37
808921	Austria	69/F	Stool	No	21
56487-17	Austria	78/M	Urine	No	17
101807-17	Austria	77/M	Blood	No	1
9929-17	Austria	5/M	Stool	No	17
EUCRONI016	Belgium	61/M	Urine	No	13
EUCRONI012	Belgium	78/M	Wound	No	31
1481-17	Czech Republic	80/F	Rectal swab	No	8
436-17	Czech Republic	31/M	Rectal swab	No	4
10965-17	Czech Republic	74/M	Rectal swab	No	4
D97986	Denmark	85/F	Sputum	No	1
17007483	Denmark	69/M	Urine	No	58
423410	Ireland	65/M	Blood	No	12
170215-0130	Norway	87/M	Blood	Yes	17
M732000	Portugal	60/M	Urine	No	40
80357408-17	Scotland	73/F	Stool	No	33
80363028-17	Scotland	71/M	Urine	No	4
07_2005	Slovenia	54/M	Tracheal aspirate	No	184
2017C1	Switzerland	55/F	Cervix uteri	No	40

*MLST, multilocus sequence type.

spanning tree to their respective MLST. Eight isolates belonging to ST1 included 2 stool isolates from neonates with a common epidemiologic link in Austria in 2009; these 8 isolates showed 1 allelic difference and were most closely related (203 alleles difference) to reference ATCC BAA-894, an isolate collected from powdered formula in the United States in 2001. That outbreak affected 2 neonates with necrotizing enterocolitis (both male, age 10 days and 12 days) hospitalized in the same neonatal intensive care unit.

Twelve isolates belonged to ST4, of which 3 were confirmed isolates from infants. Two infant isolates belonged to an outbreak cluster with a common epidemiologic link detected in Austria in 2016; these isolates shared the same cgMLST profile and showed a maximum of 47 allelic differences to the historical reference strain NCTC 8155 (from milk, United Kingdom, 1950). This outbreak again affected 2 neonates (neonate A: female, age 22 days, positive blood culture, fatal outcome; neonate B: male, age 16 days, positive respiratory tract specimen) hospitalized in the neonatal intensive care unit of another hospital in Austria. The third infant isolate was a 2017 ST4 isolate from a case in Austria with a fatal outcome and was most closely related (302 allelic differences) to a historical strain from Denmark isolated in 2003.

Six clinical isolates assigned to ST8 consisted of 2 historical human isolates from Canada (date of isolation unknown). These 6 isolates shared the identical core genome profile and had 1 allelic difference to reference strain ATCC29544 (from an infant, United States, 1970).

Nine human isolates assigned to ST21 comprised an historical outbreak cluster from France collected during 2010–2016. The outbreak included 3 female patients (mean

age 62 years) and 5 male patients (mean age 68 years); initial specimens were abscess material from the digestive tract (n = 1), ascites fluid (n = 1), respiratory tract specimens (n = 2), and rectal swab specimens (n = 4). Eight of these 9 isolates showed the same core genome genes, and 1 yielded 1 allelic difference.

All 10 isolates assigned to ST155 belonged to a historical outbreak among infants in Belgium during 1997–1998; the isolates originated from blood cultures (n = 2), stool specimens (n = 2), rectal swab specimens (n = 4), and respiratory tract specimens (n = 2). The first positive sample was collected in November 1997; the remaining 9 specimens were obtained during August–September 1998. Eight

Table 3. In silico evaluation of MLSTs for *Cronobacter sakazakii* strains in a multicenter study of *C. sakazakii* infections in humans, Europe, 2017*

MLST	Total no. isolates	Human isolates detected in 2017	Historical human isolates
1	7	2	5
12	1	1	–
13	1	1	–
148	1	–	1
155	10	–	10
17	3	3	–
184	1	1	–
21	10	1	9
31	2	1	1
33	1	1	–
37	1	1	–
4	11	4	7
40	2	2	–
50	1	–	1
58	1	1	–
630	1	1	–
8	5	1	4

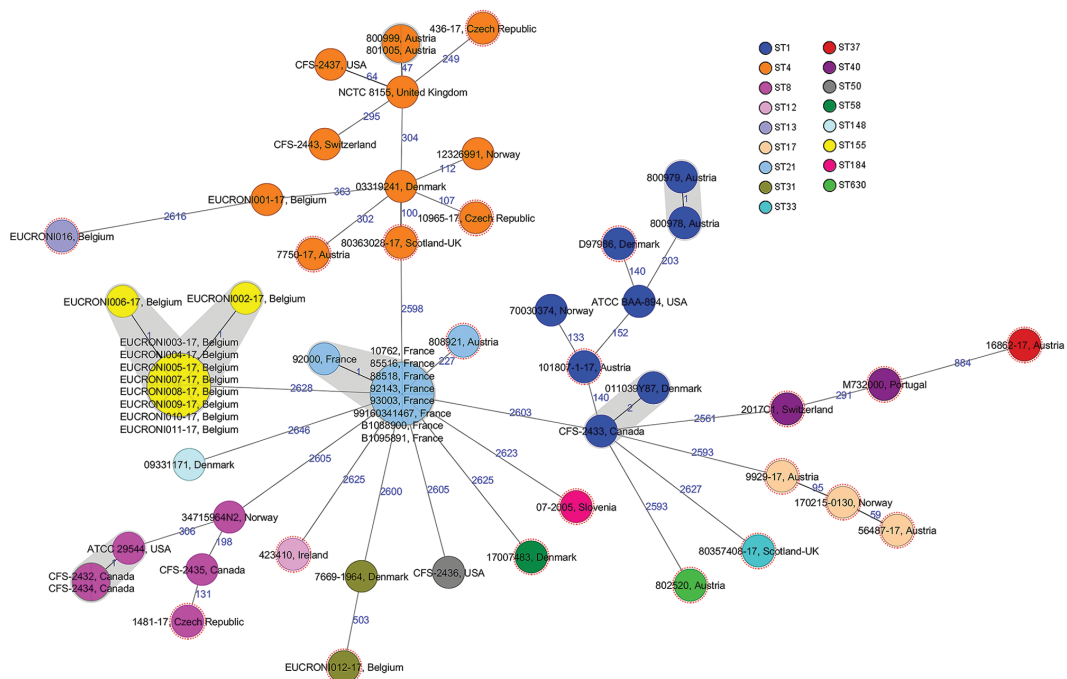*MLST, multilocus sequence type; –, no isolates detected.

Figure 2. Minimum-spanning tree of 59 *Cronobacter sakazakii* isolates, including 21 human isolates from 2017 and 38 historical human isolates, from 11 countries in Europe. Each circle represents isolates with an allelic profile based on the core genome multilocus sequence type, which consists of 2,831 alleles. Blue numbers indicate the allelic differences between isolates; isolates with closely related genotypes are shaded in gray. Isolates were colored according to classical multilocus sequence type, labeled with the country of isolation and the respective sample identification. Nodes encircled with a dotted red line were collected in 2017. Ireland additionally provided 7 historical isolates originating from Canada (n = 4), United States (n = 2), and Switzerland (n = 1). For comparison, sequence data of reference strains ATCC BAA-894 (United States, ST1); ATCC29544 (United States, ST8); NCTC 8155 (United Kingdom, ST4) were included. ST, sequence type.

of the 10 isolates shared the same cgMLST profile, and 2 had 1 allelic difference.

In total, 27 of 38 historical isolates were most closely related (≤1 allelic difference) to other historical isolates; 11 were singletons. All 21 isolates collected in 2017 were singletons, and no close relatedness was evident (≥100 allelic differences) between historical isolates and isolates from 2017.

In Vitro and In Silico Antimicrobial Resistance Analysis
In vitro susceptibility testing of 21 human *C. sakazakii* isolates from 2017 revealed 20 *C. sakazakii* isolates that were susceptible to all 14 tested antibiotics (Appendix Table 2). One isolate was resistant to ampicillin, cefotaxime, gentamicin (intermediate), moxifloxacin, and trimethoprim/sulfamethoxazole.

Of 21 *C. sakazakii* isolates, 12 isolates carried the efflux genes *emrB*, *msbA*, *patA*, regulatory systems modulating antibiotic efflux *CRP*, *marA*, *emrR*, *marR*, H-NS, antibiotic

target protection gene *msrB*, and the determinant of fosfomycin resistance *glpT*. Seven isolates had in addition the antibiotic protection gene *vgaC*. One isolate had additionally the efflux gene *norB*, the antibiotic inactivation gene *fosX*, and the antibiotic target alteration gene *mprF*. One isolate had the additional antibiotic inactivation genes *aac(6')-Ib-cr*, *aadA16*, *aadA2*, *ant(2'')-Ia*, *arr-3*, *catB3*, *CTX-M-9*, *OXA-1*, the antibiotic target protection gene *qnrA1*, and the antibiotic target replacement gene *sul1*.

The presence of variant *ampC* was confirmed for all 21 isolates. Seventeen isolates harbored CSA-2, and 4 isolates harbored CSA-1 (Appendix Table 2).

Discussion
The aim of our 2017 *C. sakazakii* study was to assess the occurrence of this opportunistic pathogen in countries of Europe, characterize the isolates, and recognize possible multinational outbreaks. Our finding that only 59 of 77 presumptive *C. sakazakii* isolates had the species-identification

C. sakazakii confirmed at the central study center shows that correct identification of *Cronobacter* spp. is still a challenge for many routine laboratories.

The prevalence of reported *C. sakazakii* cases was low, with only 11 (45.8%) of 24 participating countries submitting *C. sakazakii* isolates. Clinical isolates from 2017 showed high genetic diversity, indicating that neither multinational nor national outbreaks occurred in 2017 in the 24 countries studied. However, characterization of the historical isolates obtained during this study confirmed occurrence of 4 previously unpublished historical outbreaks: 2 outbreaks from 2009 and 2016 in Austria, 1 from Belgium during 1997–1998, and 1 from France during 2010–2016. Hospitals affected by nosocomial *C. sakazakii* outbreaks might still be reluctant to publish possibly food-related outbreaks or nosocomial infections, especially in the case of affected infants and particularly in the case of related fatalities.

Strain typing using classical MLST identified a total of 17 STs among 59 sequenced *C. sakazakii* isolates. Our addition of a new ad hoc cgMLST scheme consisting of 2.831 core target genes provides more discriminative power for outbreak investigation and source tracking than the standard 7-loci MLST scheme.

The dominant STs found among our clinical *C. sakazakii* isolates from 2017 were ST4, ST17, ST1, and ST40, a distribution consistent with results from other studies (*1*). The medical literature often links *C. sakazakii* ST4 with powdered infant formula–associated outbreaks in infants (*3*). In our study, the sole strain (7750-17) affecting an infant (a 3-month-old baby boy who died) was ST4, isolated from a blood culture.

Antibiotic treatment is essential in the care of a patient with a confirmed *Cronobacter* infection. The traditional antibiotic regime for *Cronobacter* spp. was ampicillin in combination with either gentamicin or chloramphenicol. In view of claimed resistance to ampicillin and most first- and second-generation cephalosporins, it has been suggested that carbapenems or third-generation cephalosporins be used with an aminoglycoside or trimethoprim/sulfamethoxazole (*24*). In our study, antimicrobial resistance testing showed susceptibility to all tested antibiotics for 20 of 21 human isolates from 2017. In comparison to other members of the family *Enterobacteriaceae*, *Cronobacter* strains seem to be more susceptible against so-called "key access antibiotics" of the World Health Organization's Model List of Essential Medicines (*25*), such as ampicillin, aminoglycosides, chloramphenicol and third-generation cephalosporins (the last is included in the List of Essential Medicines only for specific, limited indications) (*26*). For all isolates, we confirmed the presence of 1 of 4 tested *ampC* β-lactamase variants, which confer phenotypic resistance exclusively to first-generation cephalosporins (e.g.,

cephalothin) but not to ampicillin (*23*). A few studies have reported *Cronobacter* isolates conferring multidrug resistance (*26*), a phenomenon observed in our study only for 1 strain from Slovenia.

Correct species identification within the *Cronobacter* group was a major challenge for 7 of 11 participating laboratories. This identification problem is consistent with numerous misidentifications reported in the literature (*27,28*). The discrepancies in correct *Cronobacter* spp. identification on genus and species level between the study center in Austria and the primary testing laboratories using MALDI-TOF MS is probably attributable to outdated databases used by primary testing laboratories. Nevertheless, our study showed that the overall MALDI-TOF MS performance for *Cronobacter* spp. identification on species level is insufficient and misleading. The databases contained data for *C. sakazakii* only, and therefore all 7 species of the genus *Cronobacter* were identified as *C. sakazakii*. In addition, although a database comment indicated that *Cronobacter* could only be identified on genus level, the MALDI-TOF MS result simulated the highest identification score for *C. sakazakii*. This shortfall should be corrected by an update of the MALDI-TOF MS databases to enable accurate *Cronobacter* identification at the species level. In comparison, WGS-based species identification represents a major improvement to conventional identification methods and MALDI-TOF MS (*29*). Therefore, we recommend the use of WGS-based identification tools and databases for identification of species within the *Cronobacter* group.

Adults were the main affected age group in our study. All but 2 of the isolates from 2017 originated from adults. This finding confirms the results from previous recent studies (*14,30*) and contradicts statements in numerous medical textbooks, postulating that infants are more often affected than adults (*8,31–33*).

Our study has some limitations. Lack of information (e.g., detailed epidemiologic and clinical patient data) and misidentification on genus and species level might have played a role in underestimating the real prevalence rate; 13 of the 24 participating countries did not find or did not submit *C. sakazakii* isolates.

In conclusion, this *C. sakazakii* study in Europe revealed a high strain diversity, which points to highly diverse infection sources and an absence of national or multinational outbreaks in 2017. Correct identification of *C. sakazakii* still poses a diagnostic challenge to many laboratories, and the use of such imperfect detection systems might explain the low prevalence of reported clinical *C. sakazakii* isolates found in this study. WGS data must be used for accurate species identification and high-resolution strain typing. We recommend the inclusion of *C. sakazakii* as a notifiable organism by public health authorities.

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Draft Genome Sequence of the First Documented Clinical *Siccibacter turicensis* Isolate in Austria

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ABSTRACT The nonpathogenic species *Siccibacter turicensis* is closely related to members of the food-associated pathogenic genus *Cronobacter* and has been detected in fruit powders, formula, spices, and herbs. Here, we report on the first clinical isolate of *S. turicensis*, recovered from the labial angle of a patient with angular cheilitis.

Members of the genus *Siccibacter* and family *Enterobacteriaceae* are characterized to be Gram negative, coccoid to rod shaped, peritrichously flagellated, weakly oxidase positive, catalase positive, and facultatively anaerobic (1). As close relatives of the foodborne pathogenic members of the genus *Cronobacter*, *Siccibacter* species can cause severe clinical infections in infants and immunocompromised adults; thus, their correct identification is of utmost importance. To date, *S. turicensis* has been isolated from various foods but has not been described in clinical samples (2).

In April 2017, a 40-year-old patient with a 6-month history of perleche saw a dermatologist. A swab taken from her mouth angle grew two types of Gram-negative rods, which were initially diagnosed as *Cronobacter sakazakii* and *Escherichia vulneris*. Topical treatment with gentamicin resulted in the healing of this angular cheilitis. Whole-genome sequencing performed on the assumed *Cronobacter* isolate revealed it to be *Siccibacter turicensis*.

The isolation of high-molecular-weight DNA from a bacterial overnight culture was carried out with a MagAttract HMW DNA kit (Qiagen, Hilden, Germany) and quantified with a Qubit version 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) using the double-stranded DNA broad-range (dsDNA BR) assay kit (Thermo Fisher Scientific). A NexteraXT kit (Illumina, Inc., San Diego, CA, USA) was used for library preparation. Whole-genome sequencing was done with 300-bp paired-end reads on an Illumina MiSeq instrument using the MiSeq reagent kit with V3 chemistry (Illumina). The *de novo* genome assembly was completed using SPAdes version 3.9.0 (3) and resulted in 171 contigs with a total of 4,224,698 nucleotides and a 58.4% GC content. Species confirmation was done via ribosomal multilocus sequence typing (4), and following submission to the *Cronobacter* PubMLST database (5), we assigned a new sequence type (ST), ST635, to the isolate (*Cronobacter* PubMLST ID 2411). The NCBI Prokaryotic Genome Annotation Pipeline (PGAP) identified 4,169 genes, 4,059 coding sequences, 128 pseudogenes, 24 rRNA operons (9 complete, 15 partially), and 76 tRNAs. Antimicrobial resistance genes were identified via the Comprehensive Antibiotic Resistance

Database (CARD) (6) and included the antibiotic efflux genes CRP, *emrB*, *emrR*, H-NS, *marA*, *marR*, *msbA*, and *patA*, as well as the antibiotic inactivation gene *fosA2* and the antibiotic target alteration gene *glpT*. *In vitro* susceptibility testing with the Vitek 2 compact system (bioMérieux, Marcy-l'Étoile, France)—interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical break-point tables version 8.0 (valid from 1 January 2018)—revealed the isolate to be resistant to fosfomycin and sensitive to ampicillin, amoxicillin-clavulanic acid, piperacillin-tazobactam, cefuroxime-axetil, cefoxitin, cefotaxime, ceftazidime, cefepime, aztreonam, imipenem, meropenem, amikacin, gentamicin, ciprofloxacin, moxifloxacin, tigecycline, and trimethoprim-sulfamethoxazole.

Accession number(s). This whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number PYEP00000000. The version described in this paper is the first version, PYEP01000000.

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Letter to the editor: Livestock-associated meticillin-resistant *Staphylococcus aureus* (LA-MRSA), Austria, 2013

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To the editor: In their article titled 'Livestock-associated meticillin-resistant *Staphylococcus aureus* (LA-MRSA) among human MRSA isolates, European Union/European Economic Area countries, 2013', Kinross et al. recently reported on the occurrence of LA-MRSA in humans [1]. The results were obtained by an ECDC initiated study documenting the identification of LA-MRSA (i.e. CC398 and 'other' LA-MRSA) in European Union/European Economic Area countries (EU/EEA) countries and the MRSA subtyping capacity and availability in EU/EEA national or regional reference laboratories. ECDC National Focal Points for Antimicrobial Resistance (AMR) were invited to designate a primary and alternate contact person with expertise in molecular surveillance of MRSA for public health purposes and with access to data for the survey in their respective countries; 27 of 30 EU/EEA countries responded to this request for data.

Data for Austria was missing in the report. We, the National Reference Laboratory (NRL) for coagulase-positive staphylococci, including *Staphylococcus aureus*, hereby report the missing data. In 2013, 250 human *S. aureus* isolates were obtained for typing: 18 isolates (7.2%) were of sequence type (ST)398 and belonged to five different spa types and six different cluster types (Table) [2]. Except for nine further human isolates of ST1 (spa type 127), no 'other LA-MRSA' was documented in 2013. All isolates were Panton–Valentine leukocidin (PVL)-negative.

Within Decision 2012/506/EU on case definitions for reporting communicable diseases, reporting of MRSA in the EU/EEA is included as a 'Special health issue' of 'Antimicrobial resistance' [3]. The Institute for Medical Microbiology and Hygiene, Austrian Agency for Health and Food Safety (AGES) in Graz was entrusted by the Austrian Ministry of Health with the tasks of a NRL for Staphylococcus aureus in 2007, and since then operates a sentinella system based on five hospitals. Our Austrian data with ST398 and ST1 (t127) accounting for 10.8% of 250 clinical MRSA-isolates tested, fit well with those 9.7% reported from the other nine NRLs that reported data from clinical samples only [1].

The increasing proportion of ST398 clonal complex strains, termed 'LA-MRSA in isolates from human samples requires special attention. Much uncertainty remains about the origin and public health implications of LA-MRSA. AGES has started to survey the proportion of MRSA isolates from humans that were ST 398 in 2007 [4-6]. In the light of the increasing spread of LA-MRSA in Europe, Kinross et al. advocate that EU/EEA countries should consider periodically repeating this survey to monitor changes. They furthermore suggest that isolates from veterinary sources be included in such monitoring to systematically document potential reservoirs and transmission pathways to inform measures for prevention and control. We support such initiative and are pleased to contribute to this endeavour.

Spa-Types, geographical origin and type of sample of LA-MRSA isolates (n = 18) and human isolates of ST1 (spa type 127) (n = 9), Austrian National Reference Laboratory for coagulase-positive staphylococci, including Staphylococcus aureus, 2013

Sequence Type	Spa-Type	No. of isolates	Primary laboratory/province	Sample	CT (cgMLST)
398	t011	14	Hospital A Carinthia (n = 8) Hospital B Lower Austria (n = 4) Hospital C Vienna (n = 2)	Nose swab (n = 4) Urine (n = 1) Throat swab (n = 2) Perianal swab (n = 1) Wound swab (n = 5) Unknown (n = 1)	46, 1103, 395, 604, 98
	t034	1	Hospital A	Wound swab	1716
	t108	1	Hospital B	Unknown	Not done
	t571	1	Hospital D Vienna	Wound swab	Not done
	t3423	1	Hospital A	Urine	Not done
1	t127	9	Hospital A (n = 4) Hospital B (n = 4) Hospital C (n = 1)	Blood culture (n = 1) Wound swab (n = 4) Pleurocentesis fluid (n = 1) Bronchoalveolar lavage (n = 1) Unknown (n = 2)	Not done

cgMLST = core genome multilocus sequence typing; CT = cluster type; LA-MRSA: Livestock-associated meticillin-resistant *Staphylococcus aureus*.

Conflict of interest

None declared.

Authors' contributions

WR, FA and BS wrote the draft manuscript. All authors corrected and approved the final version.

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Petting zoos as sources of Shiga toxin-producing *Escherichia coli* (STEC) infections

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A B S T R A C T

Despite their general low incidence, Shiga toxin-producing *Escherichia (E.) coli* (STEC) infections are considered an important public health issue due to the severity of illness that can develop, particularly in young children. We report on two Austrian petting zoos, one in Tyrol (2015) and one in Vorarlberg (2016), which were identified as highly likely infection sources of STEC infections. The petting zoo related cases involved a case of hemolytic uremic syndrome (HUS) due to STEC O157:HNM in 2015 and an outbreak of STEC O157:H7 infections affecting five young children and two adults in 2016. The HUS case accounted for 2.8% of the 36 STEC O157:HNM/H7 infections notified in Austria in 2015 (5,9% of 17 HUS cases). The seven cases described for 2016 accounted for 4.0% of the 177 human STEC infections documented for Austria in 2016, and for 19.4% of the 36 STEC O157:HNM/H7 infections notified that year. The evaluation of the STEC infections described here clearly underlines the potential of sequence-based typing methods to offer suitable resolutions for public health applications. Furthermore, we give a state-of-the-art mini-review on the risks of petting zoos concerning exposure to the zoonotic hazard STEC and on proper measures of risk-prevention.

1. Introduction

Microbiological and epidemiological investigations of transmission chains play a central role in prevention and control of infections (Krause, 2009). Even investigations that identify the source of an outbreak after its natural ending can contribute to preventing re-emergence and avoiding similar future occurrences. Whereas the incidence of sporadic notifiable illness can be seen as unavoidable, the occurrence of an outbreak almost always indicates inadequate application of hygiene standards. The core function of local public health authorities is to identify and verify such poor standards (Krause, 2009). Investigations of transmission chains are thus an instrument for evaluating and improving existing preventive measures (Reingold, 1998).

Although the majority of Shiga toxin-producing *Escherichia (E.) coli* (STEC) outbreaks occur as a result of contaminated food or water, numerous outbreaks have been traced back to direct contact with animals or indirect contact via fair, farm, and petting zoo environments (Conrad et al., 2017). Research suggests that individuals with repeated exposure to enteric pathogens, such as those living or working on farms, may become less susceptible to infection (Belongia et al., 2003; Hale et al.,

2012). However, today most members of the public do not have direct interaction with farms in their daily lives and therefore are more susceptible.

Over the last decades, there has been a significant rise in the popularity of open farms, farm holidays and petting zoos (Stirling et al., 2007). Visitors have access to animals such as goats, sheep, lambs, rabbits, kittens, donkeys, guinea pigs and puppies, which makes these attractions particularly popular among children. This close association, promoted through activities such as feeding and handling the animals, has led to reports of zoonotic transmission of several mainly gastrointestinal infections, including STEC (Conrad et al., 2017; Stirling et al., 2007; McMillian et al., 2007; Weese et al., 2007; Erdozain et al., 2013; Heuvelink et al., 2002; Warshawsky et al., 2002; Goode et al., 2009).

Besides the ability of a typing method to clearly identify isolates that are involved in an outbreak, the typing method must accurately differentiate outbreak strains from non-outbreak isolates. Pulsed-field gel electrophoresis (PFGE) was initially described in 1983 (Schwartz et al., 1983) and still represents the *gold standard* in molecular typing of most bacterial species. In contrast to the situation in forensic human genetics, where—with the exception of monozygotic twins –

Table 1
Animals sampled in an epidemiologically linked petting zoo in Tyrol in 2015.

	Number of individual animals sampled	STEC-positive	STEC O157:HNM-positive	Analyzed in PFGE and NGS	By PFGE, indistinguishable from HUS case isolate
Goat	20	15/20	1	0	–
Sheep	26	26/26	7	6	1 (sheep no. 4)
Llama	2	2/2	1	1	0
TOTAL	48	43/48	9/48	7/48	1/48

indistinguishable DNA fingerprinting patterns prove epidemiological relatedness, in bacteriology even epidemiologically unrelated STEC isolates can yield PFGE patterns indistinguishable from each other, without any causal relation (Schmid et al., 2014). Explanations for this might be a monomorphic population structure, which is known from *E. coli* O157:H7, or the limitation of the PFGE typing method used. On the other hand, the maximum resolution of next generation sequencing (NGS) poses a major challenge in determining meaningful similarity thresholds for grouping related isolates, to provide an appropriate level of discrimination for source attribution. The definition of such thresholds will be a major task for public health authorities, using well-defined outbreak scenarios of the pathogen species of interest. The applied method has to allow for some genetic diversity between isolates from human and animal/environmental sources, but only to the degree that it can still be assumed that they originate from the same source (Schmid et al., 2014).

Here we report on two events of STEC transmission related to Austrian petting zoos. A German case of HUS due to STEC O157:HNM in 2015 and an outbreak of STEC O157:H7 infection affecting five young children and two adults in 2016 were investigated using PFGE-typing. Using these strains from successfully investigated STEC infections, we compared newly generated NGS-results with the retrospective historic PFGE analyses results. The evaluation of the STEC infections described here clearly underlines the potential of sequence-based typing methods to offer suitable resolutions for public health applications. Furthermore, we give a state-of-the-art mini-review on the risk of petting zoos concerning exposure to the zoonotic hazard STEC and on proper measures of risk-prevention.

2. Two episodes of illness related to Austrian petting zoo visits

2.1. Patient populations

A German one year old girl suffered from HUS due to STEC O157:HNM (isolate titled: case 2015). Onset of illness was 19th of May 2015. A petting zoo in Tyrol was identified epidemiologically (and microbiologically; see below) as the highly likely source of infection.

An outbreak of STEC O157:H7 infection affecting five young children and two adults occurred in Austria in 2016 (for age and gender see Table 2, isolates titled: cases 2016). A petting zoo in Vorarlberg was identified epidemiologically as the highly likely source of infection. All dates of STEC O157:HNM/H7-isolation are mentioned in Table 4.

2.2. Material and methods

Organisms were cultured in EHEC-Direkt-Medium (Axon Lab, Baden, Switzerland) for 18–24 h and PCR was used to test for *stx1* plus *stx2* (Reischl et al., 2002) and for a O157-serotype specific gene according to EU-Reference Laboratory for *E. coli*, VTEC_Method_02 (EU Reference Laboratory for E. coli, 2013). In case of positivity isolation of STEC was conducted on SMAC, CT-SMAC (Oxoid, Basingstoke, UK) and CHROMagar O157 (CHROMagar, Paris, France). All gained STEC O157 isolates were O-and H-typed according to the protocol published by (Ørskov et al. (1977)). Pulsed-field gel electrophoresis (PFGE) profiles

were generated according to the standardized laboratory protocol developed by the Centers for Disease Control and Prevention, USA (Standard Operating Procedure for PulseNet PFGE of *Escherichia coli* O157:H7, 2018). Genomic DNA isolation, whole genome sequencing using an Illumina MiSeq instrument (Illumina Inc., San Diego, CA, USA), assembly and contig filtering were performed as described previously (Lepuschitz et al., 2017; Hartl et al., 2017). Assembled genomes were compared using the Enterobase core genome (cg)MLST scheme (Enterobase.warwick.ac.uk. Available at: http://enterobase.warwick.ac.uk) using SeqSphere + with a cluster type threshold of ten allelic differences. Minimum spanning tree was visualized in SeqSphere +.

2.3. Initial analysis using pulsed-field gel electrophoresis

Concerning the German HUS case in 2015, we received the patient's STEC O157:HNM-isolate in June 2015 from the Bavarian health authorities. Fecal samples from 48 animals of an epidemiologically linked Tyrolean petting zoo (20 goats, 26 sheep, 2 llamas) were gained four days later. Fifteen of the 20 goats, all of the 26 sheep and both llamas were found STEC positive (Table 1). Results of PFGE analysis of STEC isolates from the HUS case and from 7 of 9 STEC O157:HNM positive animals are depicted in Fig. 1. Sheep no. 4 yielded an STEC O157:HNM isolate indistinguishable from the patient's strain by PFGE using *Xba*I as restriction enzyme. The remaining six animal isolates clustered with the patient's isolate with two bands difference. For the 2015 German HUS case we were able to provide epidemiological and PFGE microbiological proof of a causative connection to an Austrian petting zoo.

For the outbreak investigation of the 2016 cases we analyzed stool samples from the 7 persons involved, 5 children between 14 months and 5 years of age and 2 adults (Table 2). Dates for onset of illness ranged from 10. July till 23. September. From an epidemiologically linked petting zoo in Vorarlberg individual fecal samples from 5 goats, 1 pooled fecal sample from goats, individual fecal samples from 2 donkeys and pooled fecal samples from the donkeys and from alpine ibexes were collected on 01.09.2016 (Table 3). Results of PFGE analysis of isolates from 5 cases and from 2 goats are depicted in Fig. 2. In this outbreak of gastroenteritis a petting zoo in Vorarlberg was epidemiologically identified as common source: all six goat samples and an ibex sample were STEC positive. The donkey samples remained STEC negative. Fourteen individual STEC O157:H7 strains were cultured from the two goats. Using *Xba*I as restriction enzyme, they were clearly distinguishable from the patients' isolates. Epidemiological investigation showed that all seven cases had direct contact to goats. In this 2016 outbreak, a causative connection could be proven only epidemiologically. The lack of microbiological prove does not surprise, as it is well known that ruminants shed the pathogen intermittently.

2.4. Retrospective analysis using next generation sequencing

From the 2015 German HUS case investigation, the patient's STEC O157:HNM isolate and seven animal isolates (the llama isolate and 6 sheep isolates) (entitled Complex 1 in Fig. 3) and from the 2016 outbreak, five patients' strains (entitled Complex 2 in Fig. 3) and two goat isolates (entitled Complex 3 in Fig. 3) were available for NGS analysis.

Table 2

Epidemiological data on cases epidemiologically linked to a petting zoo in Vorarlberg in 2016 (m = male; f = female).

	Sex	Age (years)	Date of contact with goats	Hand washing after animal contact?	Analyzed in PFGE and NGS	Symptoms and date of onset
Case 1	M	4	7.7.2016	Yes	Yes	bloody diarrhea, 10.7.2016
Case 2	F	1	8.8.2016	No	Yes	diarrhea, 13.8.2016
Case 3	F	37	8.8.2016	No	No, no strain isolated	diarrhea, 20.8.2016
Case 4	M	1	7.8.2016	No	Yes	diarrhea, 23.8.2016
Case 5	M	5	7.8.2016	Yes, without soap	Yes	vomiting, diarrhea, 21.8.2016
Case 6	M	58	7.8.2016	Yes, without soap	No, no strain isolated	No symptoms
Case 7	F	2	16.9.2016	No	Yes	fever, vomiting, urinary tract infection, 23.9.2016
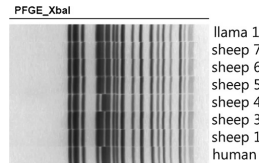
Fig. 1. PFGE: STEC O157:HNM isolates from a German HUS case and from seven animal samples gained from a petting zoo in Tyrol in 2015 using *Xba*I as restriction enzyme.

In concordance to PFGE analysis, NGS analysis also revealed that all seven animal isolates clustered with the patient's strain. The isolate of sheep no. 4, which was considered indistinguishable by PFGE analysis, differed by five alleles, three more differences than found for the isolate of sheep no. 5. NGS underlined the causative role of the Tyrolean petting zoo as source of the epidemiologically linked HUS case.

In accordance to the results of PFGE analysis, NGS confirmed that the two goat isolates clearly differed from the five human isolates epidemiologically linked to the petting zoo in Vorarlberg.

2.5. Public health interventions

The responsible public health authority enforced the installation of facilities for proper hand hygiene after contact to animals and ordered attachment of information-signs fostering obedience to proper hygiene (hand-washing after animal contact; no food-consumption in contact area). The owner of the petting zoo in Vorarlberg also removed his two STEC O157 positive goats from the areas, where direct contact to visitors could be possible.

3. Conclusions

With the efficiency and the decreasing costs of next generation sequencing, the technology is being introduced rapidly into clinical and public health laboratory practice (Besser et al., 2018). One of the basic assumptions in molecular epidemiology is that phylogeny approximates epidemiology, i.e. patients are more likely to be epidemiologically associated if the pathogens that made them ill are closely related phylogenetically than if they are not. Similarly, if pathogens from food or the environment are phylogenetically related to clinical isolates a causal relationship between the two is likely (Besser et al., 2018). Thus, strain

Table 4

Isolates connected to the outbreaks in 2015 and 2016 undergoing next generation sequencing: sample-ID, date of isolation, outbreak-ID, accession number.

Sample-ID	Date of isolation	Outbreak-ID	Accession number
MRV-15/00236	3.6.2015	case-2015	PYPP00000000
MRV-15/00250	3.7.2015	sheep 1-2015	PYPR00000000
MRV-15/00252	3.7.2015	sheep 3-2015	PYPO00000000
MRV-15/00253	3.7.2015	sheep 4-2015	PYPN00000000
MRV-15/00254	3.7.2015	sheep 5-2015	PYPM00000000
MRV-15/00255	3.7.2015	sheep 6-2015	PYPL00000000
MRV-15/00256	3.7.2015	sheep 7-2015	PYPK00000000
MRV-15/00258	7.7.2015	llama 1-2015	PYPJ00000000
MRV-16/00378	1.8.2016	case 1-2016	PYPI00000000
MRV-16/00457	25.8.2016	case 2-2016	PYPH00000000
MRV-16/00466	31.8.2016	case 4-2016	PYPQ00000000
MRV-16/00472	1.9.2016	case 5-2016	PYPG00000000
MRV-16/00492	7.9.2016	goat 1-2016	PYPF00000000
MRV-16/00493	7.9.2016	goat 2-2016	PYPE00000000
MRV-16/00605	21.10.2016	case 7-2016	PYPD00000000
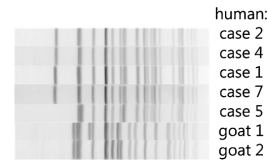
Fig. 2. PFGE: STEC O157:H7 isolates from five outbreak cases and two animal samples gained from a petting zoo in Vorarlberg in 2016 using *Xba*I as restriction enzyme.

typing can be used to confirm (or refute) a causal association between a human infection and an animal source and thereby is an important tool to convince the public of this law of nature. However, like any other subtyping or strain characterization method, this correlation between epidemiology and phylogeny is incomplete and sequencing data cannot stand on their own but should always be interpreted taking all available epidemiological information into consideration. Furthermore, absence (at the often delayed time of testing) of the microbial hazard in epidemiologically linked animals must never be misinterpreted as evidence of absence of a causative link. Nevertheless, the excellent resolution provided by NGS in the two petting zoo-related events

Table 3

Animals sampled in an epidemiologically linked petting zoo in Vorarlberg during the outbreak-investigation in 2016.

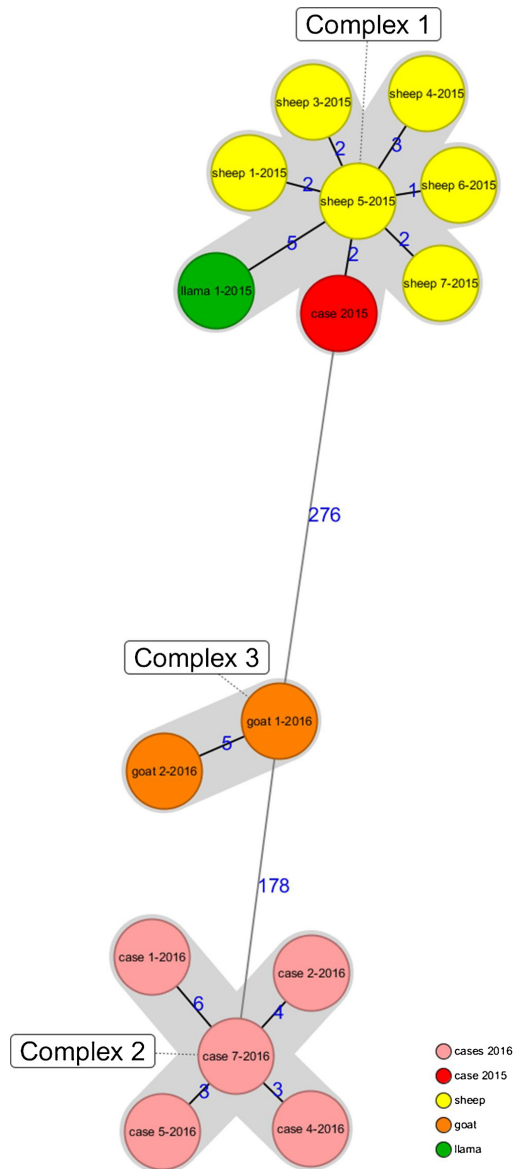|	Number of individual animals sampled	Number of pooled fecal samples	STEC-positive	STEC O157:H7-positive	Analyzed in PFGE and NGS	By PFGE, indistinguishable from isolates of human cases
Goats	5		5/5	2	2	0
		1	1/1	1	0	–
Donkeys	2		0/2	0	0	–
		1	0/1	0	0	–
Alpine ibex	0	1	1/1	0	0	–
TOTAL	7	3	7/10	3/10	2/10	0/10

Fig. 3. Minimum spanning tree from all clinical and animal STEC O157 isolates. A cluster type threshold of 10 allelic differences was applied. Complex 1: German HUS case 2015 and associated animals; complex 2: cases 2016; complex 3: epidemiologically associated goats to complex 2 cases. Allelic differences given in blue numbers (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

contracted from direct or indirect animal contact. Although the transmission of STEC infections is low compared with other zoonotic pathogens, it is a significant issue because of its very small infective dose (as few as 10–100 cells), and the severity of illness that can develop, particularly in young children. Serotype O157:H7 is a particularly dangerous STEC strain; the top 4 non-O157 STEC serogroups causing human disease include O26, O103, O111, and O145. An estimated 8% and 6% of respective non-O157 and O157 STEC are due to direct animal contact in the United States annually (Hale et al., 2012). In Austria, the seven cases described for 2016 accounted for 4.0% of the 177 human STEC infections of 2016, and for 19.4% of the 36 STEC infections caused by STEC O157:HNM/H7 notified that year. The HUS case accounted for 2.8% of the 36 STEC O157:HNM/H7 infections notified in Austria in 2015 (5.9% of 17 HUS cases).

Open farms and similar premises often provide the only significant opportunity for the general public to have close contact with animals, and they fulfill an important educational role and provide major contributions to the tourism and leisure industries. The potential risk is the exposure of people to zoonotic hazards such as STEC (Daly and Hill, 2015). The first recorded outbreaks of STEC O157 associated with an animal exhibit occurred in England (1994), affecting 7 individuals, with 4 developing HUS, followed by Wales (1995) with another 3 individuals affected with 1 developing HUS (LeJeune and Davis, 2004). A notable outbreak linked to STEC O157:H7 occurred in Ontario, Canada (1999), where a petting zoo was associated with 155 probable cases and seven culture-confirmed infections (Warshawsky et al., 2002). Another noteworthy outbreak occurred at the North Carolina State Fair in 2004, where 108 STEC O157:H7 cases were reported (41 laboratory-confirmed), with 20 patients hospitalized and 15 developing HUS (Goode et al., 2009). A laboratory investigation concluded that STEC O157:H7 in sheep and goat feces was responsible for contamination of the petting zoo environment (Stirling et al., 2007; Goode et al., 2009).

The prevalence of STEC O157 among the wide range of species found on animal amenity premises has been extensively researched. Between 1997 and 2017, Pritchard et al. investigated 31 public health amenity premises in England and Wales (Pritchard et al., 2009). STEC O157 was confirmed in one or more species on 19 (61.3%) of the premises. On positive premises, the highest mean proportion of positive samples (29.0%) was in cattle, followed by sheep (24.4%), donkeys (14.6%), pigs (14.3%), horses (12.3%) and goats (9.9%) (Pritchard et al., 2009). In 2006, Keen et al. (2006) isolated STEC O157:H7 from livestock at 31 (96.9%) of 32 fairs in two U.S. states. Prevalence of STEC O157:H7 was the highest from cattle (11.4%), followed by sheep and goats (3.6%), and swine (1.2%).

There are three primary tools to reduce risk of zoonotic disease transmission: awareness of risks involved with human-animal interactions, sanitation and awareness of risky behaviors (Pritchard et al., 2009). Proper behavior and handwashing can significantly reduce the risks of physical injury and zoonotic disease infection (Erdozain et al., 2015; Centers for Disease Control and Prevention, 2011; Health Protection Agency (HPA), 2011). High-risk behavior and poor hygienic practices by visitors at animal amenity premises are well known, as confirmed by the findings of our 2016 outbreak. McMillian et al. (2007) observed 991 attendees at six petting zoos in Tennessee (USA). Of these, 74% had direct contact with animals, 87% had contact with potentially contaminated surfaces in animal contact areas, 49% had hand-to-face contact, and 22% ate or drank in animal contact areas. Studying petting zoos in Kansas and Missouri, Erdozain et al. found that of 574 visitors observed for hand hygiene compliance, only 37% attempted any type of hand hygiene (Erdozain et al., 2013). Weese et al. observed hand hygiene compliance of 0–77% (mean value 30.9%) at 36 petting zoos in Canada (Weese et al., 2007).

We claim that the risk of people acquiring an infection from animals depends more on the degree of contact and the precautions adopted than the prevalence of colonization in a particular species. Carriage of STEC by cattle and sheep can range from low to very high ($\geq 10^4$

described, clearly supports the rapid shift of the 'state of the art' technology and the practice of typing for public health to NGS data analysis for outbreak studies (European Centre for Disease Prevention and Control, 2016).

Regulations cannot provide complete protection from illnesses

colony forming units [CFU]/g of feces) (Baker et al., 2016). Individual cattle or small ruminants that shed more than 10^4 CFU/g feces are termed super-shedders (Baker et al., 2016). Though only a few individuals within a herd or flock may be super-shedders, they can be responsible for widespread animal-to-animal transmission and contamination of the environment. One study estimated that 96% of all *E. coli* O157:H7 shed by a group of cattle came from only 9% of the animals in the herd (Omisakin et al., 2003). However, identification of super-shedders can be difficult, as shedding of STEC levels $\geq 10^4$ CFU/g appears to be highly intermittent (McPherson et al., 2015; Munns et al., 2015).

Daly and Hill studied the role of animal exposure in Shiga toxin-producing *E. coli* infections in South Dakota in 2012; 10.3% of total STEC cases reporting animal contact had documented exposure to a petting zoo (Daly and Hill, 2016). The most frequent animal exposure reported by their patients was that of touching or petting a goat, which was reported by all four patients. Three patients reported touching or petting a sheep. All four had multiple contacts with animals during their visits. Two of four patients reported washing their hands upon leaving the exhibit. One patient reported eating or drinking in the exhibit area. Allerberger et al. even reported a case of gastroenteritis in a boy who – supervised by his elementary school teacher - drank raw goat milk at a show farm. Human sorbitol non-fermenting O157:HNM isolates and animal isolates from the goat were indistinguishable by PFGE (Allerberger et al., 2001).

Whether vaccination could be an effective public health control measure for animal exhibits and petting zoos is unclear (Conrad et al., 2017). Research indicates that vaccination does not consistently reduce prevalence of STEC O157:H7 in cattle feces (Stanford et al., 2014). Vaccination may reduce but not eliminate the pathogen, making it necessary to employ a suite of interventions to reduce zoonotic risk (Snedeker et al., 2012). Currently, no vaccines for *E. coli* O157:H7 in small ruminants are commercially available.

Treatment of animals with antimicrobials is also not a practical option, because it has been shown to prolong shedding and could contribute to antimicrobial resistance (Conrad et al., 2017; Béraud et al., 2008). Antimicrobial treatment cannot reliably eliminate colonization, prevent shedding, or protect against recolonization and may often fail to target the pathogen of interest (National Association of State Public Health Veterinarians, 2013).

Routine testing of animals is not recommended as a reliable means of preventing infection, as most pathogens are shed intermittently. In addition, the inherent limitations of laboratory tests make it difficult to identify and remove infected animals from the herd or flock rapidly (Conrad et al., 2017; McMillian et al., 2007). As the true STEC prevalence in livestock animals may be close to 100% and there are no sound countermeasures against STEC carriage and shedding by ruminants yet, knowledge of the (most likely) presence of STEC in a premise would not result in any other recommendations as given below. Cost-extensive routine testing of animals in animal exhibits and petting zoos would not help to prevent human infections.

Those who provide public access to animals should inform visitors about the risk of transmission from animals to humans and possible prevention strategies. The most effective protective measures against enteric illnesses include educating the public, increasing overall awareness of the risks and the emphasizing the importance of hand hygiene, as well as providing access to hand-washing facilities (Conrad et al., 2017). Food and beverages should be prepared, served, and consumed in separate areas, where animals cannot enter. Activities such as eating and drinking, smoking, or anything that involves the hand interacting with the mouth should be avoided in interaction areas. Children younger than 5 years old, the elderly, pregnant women, and immunocompromised individuals should take extra care, as they are at a higher risk of being infected. Washing facilities (running water, soap, and disposable towels) should be available in both the interacting and animal-free areas and in sufficient quantities to cope with the numbers of visitors. Signs should be displayed to highlight the need to wash hands before eating or leaving the interaction area (Stirling et al., 2007). However, despite the high prevalence of STEC in animals and the environment, the risk of acquiring the infection in petting zoos, open farms, and other animal premises is very small in relation to the large numbers of visitors each year. Therefore, it is necessary to balance this small risk against the undoubted benefits of allowing the public to interact with farm animals.

3.1. Nucleotide sequence accession numbers

This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank as described in Table 4. The version described in this paper is the first version.

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Curriculum Vitae

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Education

02/2016 – 03/2019	**Doctor of Philosophy (PhD)** Vienna University of Technology, Vienna (Austria)
09/2013 – 02/2016	**Master of Science (MSc)** in Molecular Biology with focus on Molecular Medicine University of Vienna, Vienna (Austria)
09/2009 – 07/2013	**Bachelor of Science (BSc)** in Molecular Biology Graz University of Technology, Graz (Austria)
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Work Experience

02/2016 – 03/2019	**Doctoral thesis** Vienna University of Technology, Research Area of Biochemical Technology, Institute of Chemical, Biological and Environmental Engineering & AGES (Austrian Agency for Health and Food Safety) - Institute for medical Microbiology and Hygiene, Vienna (Austria) Title "Antimicrobial resistance and one health: Occurrence of multiresistant human pathogenic bacteria in food and environment." Supervisors: Prof. Dr. Robert Mach (Vienna University of Technology) & PD Mag. Dr. Werner Ruppitsch (AGES)

03/2015 – 02/2016	**Master thesis**
	AGES (Austrian Agency for Health and Food Safety) - Institute for medical Microbiology and Hygiene, Vienna (Austria)
	Title: "Subtyping of livestock-associated methicillin-resistant *Staphylococcus aureus* CC398 isolates by next generation sequencing"
	Duties: different DNA isolation methods, next generation sequencing, data analysis with SeqSphere$^+$ (Ridom)
	Supervisor: PD Mag. Dr. Werner Ruppitsch
08/2014 – 09/2014	**Internship**
	Medical University Vienna - Skin & Endothelium Research Division (SERD), Vienna (Austria)
	Duties: Histochemical staining of human and mouse tissues, antibody testing, Immunofluorescence and Laser Scanning Microscopy, human melanoma cell culture, western blot, endothelial sprouting assays, migration, invasion and proliferation assays, retroviral infection
11/2013 – 03/2014	**Internship**
	AGES (Austrian Agency for Health and Food Safety) - Institute for medical Microbiology and Hygiene, Vienna (Austria)
	Duties: Several molecular methods for subtyping of livestock-associated methicillin-resistant *Staphylococcus aureus*, PCR, antibiotic resistance profiles
07/2012 – 08/2012	**Bachelor thesis**
	National Institution for Veterinary Examinations, Klagenfurt (Austria)
	Title: "Comparison of analytical sensitivity of two qPCR methods"
	Duties: Conventional and real time PCR, DNA extraction from biological samples, gelelectrophoresis
	Supervisor: Mag. Dr. med. vet. Vogl Gunther
08/2011	**Internship**
	Klinikum Klagenfurt am Wörthersee - Institute for Pathology and Institute for Laboratory Diagnosis and Microbiology, Klagenfurt (Austria)
	Duties: Molecular diagnostics in a histological and cytological laboratory, basics of histochemistry, DNA extraction, PCR, screening of gynaecological cytological smear
08/2010	**Internship**
	Carinthian Institute for Food Analysis and Quality Control, Klagenfurt (Austria)
	Duties: Quality control for microbiological diagnosis, general laboratory work in molecular diagnostics, Salmonella and EHEC diagnostics by PCR, DNA hybridisation for detection of EHEC, conventional and real time PCR, work with plasmids, cultivation of Salmonella, DNA extraction from biological samples, gelelectrophoresis

Additional Information

Publications

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Awards

Research funding: "Use of whole genome sequencing technologies for in depth investigation of antimicrobial resistant bacterial isolates from surface water", Austrian Society of Antimicrobial Chemotherapy (ÖGACH), November 2017, Vienna, Austria (5.000 Euro)

Research funding: "European, multi-centre, prospective prevalence pilot-study of *Cronobacter sakazakii* infections in humans (EUCRONI 2017)", ESCMID Food- and Water-borne Infections Study Group (EFWISG), April 2017, Vienna, Austria (3.000 Euro)

Travel grant: "The superiority of NGS in tracing chains of transmission: a retrospective analysis of the first documented nosocomial transmission of LA-MRSA *spa*-type t011 in an Austrian hospital, 2010-2011", 35th Annual conference Austrian Society of Hygiene, Microbiology and Preventive Medicine (ÖGHMP), June 2015, Zell am See, Austria (400 Euro)

Merit Scholarship: Granted annually to students with an outstanding academic record and are intended to recognize and reward exceptional performance. University of Vienna, January 2015 (750 Euro)