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Transmission of ESBL-producing Enterobacteriaceae and their mobile genetic elements – identification of sources by whole genome sequencing: study protocol for an observational study in Switzerland

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Abstract

Introduction

Extended-spectrum beta-lactamases (ESBL)-producing Enterobacteriaceae were first described in relation with hospital-acquired infections. In the 2000s, the epidemiology of ESBL-producing organisms changed as especially ESBL-*E. coli* was increasingly described as an important cause of community-acquired infections, supporting the hypothesis that in more recent years ESBL-producing Enterobacteriaceae have probably been imported into hospitals rather than vice versa. Transmission of ESBL-producing Enterobacteriaceae is complicated by ESBL genes being encoded on self-transmissible plasmids, which can be exchanged among the same and different bacterial species. The aim of this research project is to quantify hospital-wide transmission of ESBL-producing Enterobacteriaceae on both the level of bacterial species and the mobile genetic elements, and to determine if hospital-acquired infections caused by ESBL-producers are related to strains and mobile genetic elements predominantly circulating in the community or in the healthcare setting. This distinction is critical in prevention since the former emphasizes the urgent need to establish or reinforce antibiotic stewardship programs, and the latter would call for more rigorous infection control.

Methods and analysis

This protocol presents an observational study, which will be performed at the University Hospital Basel and in the city of Basel, Switzerland. ESBL-producing Enterobacteriaceae will be collected from any specimens obtained by routine clinical practice or by active screening in both in-and outpatient settings, as well as from wastewater samples and foodstuffs, both collected monthly over a 12-month period for analyses by whole genome sequencing.

Bacterial chromosomal, plasmid and ESBL-gene sequences will be compared within the cohort to determine genetic relatedness and migration between humans and their environment.

Ethics and dissemination

This study has been approved by the local ethics committee (Ethikkommission Nordwest-und Zentralschweiz) as a quality control project (Project-ID 2017-00100). The results of this study will be published in peer-reviewed medical journals, communicated to participants, the general public and all relevant stakeholders.

Strengths and limitations of this study

- This study applies an interdisciplinary, "one-health" approach, using state of the art technologies to investigate diverse sources entertaining and promoting the current epidemic of extended-spectrum beta-lactamases (ESBL)-producing Enterobacteriaceae.

- The single center study design may limit generalizability to other settings.

- Lack of systematic surveillance of all patients entering and exiting the healthcare institution may result in missed transmission events.

- The obtained knowledge on transmission of ESBL-producing Enterobacteriaceae on both the level of bacterial species and mobile genetic elements will provide a sound foundation for tailoring specific measures to prevent further spread.

Introduction

Transmission of extended-spectrum beta-lactamases (ESBL)-producing Enterobacteriaceae challenges healthcare facilities worldwide regarding the implementation of effective infection control measures to limit further nosocomial spread. ESBL-producing Enterobacteriaceae were first described in 1983[1] in relation to hospital-acquired infections and have rapidly increased globally ever since. In the 2000s, the epidemiology of ESBL-producing organisms changed as especially *E. coli* producing the CTX-M ESBL-type was increasingly described as an important cause of community-acquired urinary tract infections worldwide,[2] supporting the hypothesis that in more recent years ESBL-producing Enterobacteriaceae have probably been imported into hospitals rather than vice versa. Possible community-sources may include foodstuffs[3] and colonization resulting from global travel, especially to the Indian subcontinent.[4] Furthermore, ESBL-producing Enterobacteriaceae have been recovered in water samples from Swiss rivers and lakes,[5] possibly constituting an underappreciated exposure route for dissemination of antibiotic resistance. Transmission of ESBL-producing Enterobacteriaceae is further complicated by ESBL genes being encoded on self-transmissible plasmids, which can be exchanged among the same and different species of Enterobacteriaceae. The contribution of this horizontal transfer of plasmids carrying ESBL genes between Enterobacteriaceae to the epidemiology of ESBL-producers, however, remains elusive, as analyses of plasmids is challenging due to their flexible architecture and the non-availability of suitable reference sequences, as they are far less conserved than bacterial chromosomes.

Overall, multiple levels of transmission of strains, plasmids and possibly genes, as well as different reservoirs, such as healthcare settings, foodstuffs and water, require investigation using a "one-health"-approach to elucidate the most important drivers of the current ESBL-epidemic. Detailed knowledge on the specific contribution of these different sources is currently lacking, despite being a key requirement for tailoring effective infection control

interventions in healthcare settings to limit further spread. Typing of bacterial strains is the prerequisite for studying transmission. Until recently, techniques such as pulsed-field gel electrophoresis (PFGE) or multilocus sequence typing (MLST) were considered the reference standards,[6] however, these lack the resolution to differentiate closely related strains, which is needed for a deeper understanding of the current ESBL-epidemic. Furthermore, these techniques are not able to distinguish mobile genetic elements.[7] Whole genome sequencing (WGS) allows the identification of single-nucleotide polymorphisms (SNPs) that differentiate chromosomes and mobile genetic elements at the highest possible resolution, therefore enabling investigation of strain and plasmid relatedness by phylogenetic analyses, and ultimately leading to detailed exploration of transmission pathways.[8] This technique has to date not been applied to determine transmission of both strains and plasmids in a large epidemiological study including clinically relevant ESBL-Enterobacteriaceae-strains and isolates recovered from community settings. A key resource at our institution is a strain collection of all ESBL-producing Enterobacteriaceae recovered from any specimens obtained by routine clinical practice in both in and outpatient settings since 2003, representing a unique collection of clinically relevant strains over time.

Aim and objectives

The aim of this research project is to investigate the transmission of ESBL-producing Enterobacteriaceae on both the level of bacterial chromosomes and mobile genetic elements, and to determine the source of hospital-acquired infections. This data is critical for prevention since community-acquisition emphasizes the urgent need to establish or reinforce antibiotic stewardship programs, and nosocomial acquisition would call for more rigorous infection control. The specific aims include:

1. To quantify the extent of hospital-wide transmission of ESBL-producing Enterobacteriaceae by assessing the genetic relatedness of ESBL-producing Enterobacteriaceae among patients and over time.

2. To estimate the contribution of horizontal gene transfer to the spread of ESBL-producing Enterobacteriaceae in both hospital- and community settings.

3. To determine migration of ESBL-producing Enterobacteriaceae between humans and their environment (i.e. foodstuffs and wastewater samples) by comparing genetic relatedness of strains and plasmids recovered from patients and the environment, as a "one-health"-approach.

Methods and analysis

Setting

The University Hospital Basel is a tertiary academic care center admitting more than 35,000 adult patients annually and comprising 813 beds. It provides acute care and hospital services in the city of Basel (approximate population, 200,000) and serves as a referral center for patients requiring specialized medical care for the north-western part of Switzerland. Its outpatient clinics provide both general and specialized medical care.

In keeping with current guidelines, the following infection control practices are in effect at our institution:

Active Surveillance for ESBL-carriage

Since 2003, all patients with recovery of ESBL-producing Enterobacteriaceae from any specimens obtained by routine clinical practice in both in-and outpatient settings are routinely screened to determine further colonization sites. In addition, all patients with known ESBL-carriage are screened, whenever they are readmitted to the hospital. Furthermore, the following patient populations are routinely screened for ESBL carriage at our institution: 1) all patients hospitalized for at least 24 hours in the same room as a patient colonized or infected with an ESBL-producing Enterobacteriaceae, 2) since 2013, patients admitted to the intensive care units and requiring mechanical ventilation, and 3) since 2015, all patients transferred directly from a hospital abroad and all admitted asylum seekers. Screening for ESBL-carriage is carried out by selective plating of rectal swabs, swabs from any open wounds or drainages, as well as urine samples from patients with foley catheters.[9]

Standard precautions

Standard precautions have been implemented at our institution since 1999. They include the proper use of hand hygiene (as indicated in the WHO and CDC guidelines)[10 11] for any patient contact and the use of personal protective equipment (i.e. gloves, gowns, masks, eye protection) for procedures involving contact with body fluids as outlined by the CDC guidelines.[10]

Contact precautions

Contact precautions involve assignment to a single room, as well as use of gloves and gowns by both healthcare workers and visitors on entrance.[10]

As of 1999, contact precaution measures were implemented for all patients infected or colonized with ESBL-producing Enterobacteriaceae. From January 2012 contact precaution measures were discontinued for patients infected or colonized with ESBL-*E. coli,* based on the finding of low transmission rates at our institution.[12]

Strain collection at the microbiology laboratory of the University Hospital Basel

Since January 2003, all ESBL-producing Enterobacteriaceae isolates recovered from any specimens obtained by routine clinical practice in both in and outpatient settings of the University Hospital Basel, Switzerland have been collected and stored at the Division of Clinical Microbiology, representing a unique collection of clinically relevant strains. Serial isolates from the same patient are collected and stored if the date of isolation is over two months from detection of the first strain.

Study design

We propose a two-stage approach:

a) **Retrospective study** (addresses study aims 1 and 2): Whole genome sequencing will be performed on representative ESBL-strains collected from January 2003 to December 2016 at the University Hospital Basel. The epidemiological relationships (as defined below) between patients with genetically related strains of ESBL-producing Enterobacteriaceae and cases with genetically related plasmids carrying the respective ESBL genes will be assessed.

b) **Prospective study** (addresses study aims 1 and 2 and additionally study aim 3): Whole genome sequencing will be performed on representative ESBL-strains collected over a two-year study period at the University Hospital Basel, from samples from the wastewater system of both the hospital and the city of Basel, and from foodstuff samples collected from both the hospital and the city of Basel. Foodstuffs will be collected monthly from two stores and wastewater samples from representative sites of the wastewater network system in each of the ten postal code districts of the city of Basel over one to two years. The epidemiological relationships (as defined below) between patients, as well as environmental samples with genetically related strains of ESBL-producing Enterobacteriaceae and cases with genetically related plasmids carrying the respective ESBL genes will be assessed.

Epidemiologic data and classification

The following data is systematically assessed and available for all patients admitted to our institution or treated in one of the outpatient clinics: demographics, movement throughout the hospital (including detailed information regarding rooms and wards occupied during the hospital stay), home postal code districts and assigned general medical practices.

Epidemiological relationships between genetically related cases will be classified as follows:

- **Hospital room contact:** patients hospitalized in the same room during the same time period or up to one week after discharge

- **Hospital ward contact:** patients hospitalized on the same hospital ward during the same time period or up to one week after discharge
- **Hospital-wide contact:** patients hospitalized during the same time period or up to one week after discharge
- **Outpatient contact:** patients receiving outpatient care within the same outpatient clinic or at the same general medicine practice.
- **Community contact (between patients):** patients living in the same postal code district.
- **Community contact (between patients and the environment)** patients, foodstuff samples and/or wastewater samples deriving from the same postal code district.

Sample collection

Patient samples

All ESBL-producing Enterobacteriaceae recovered from any specimens obtained by routine clinical practice or by active screening (as detailed above) in both in- and out-patient settings of the University Hospital Basel, Switzerland, have been collected and stored at the Division of Clinical Microbiology since January 2003. This collection will be continued throughout the study period.

Wastewater samples

Wastewater samples will be collected monthly during the prospective part of the study. In collaboration with the civil engineering department of the city of Basel (Tiefbauamt Basel Stadt), 20 specific sampling sites reflecting the different postal code areas of the city (two per postal code area) have been chosen. In addition, wastewater will be collected from the main wastewater outlet of the University Hospital Basel.

Samples from foodstuff

Samples from foodstuff will be collected monthly during the prospective part of this study. Foodstuffs will be bought from two stores from each different postal code area. In addition, foodstuffs will be collected from the hospital kitchen.[13 14] Based on the results of prior studies confirming the presence of ESBL-producing Enterobacteriaceae in foodstuffs in Switzerland, we plan to investigate the following foodstuffs: poultry[13] (detection of ESBL-producers in 50% of samples, personal communication Kantonales Laboratorium Basel-Stadt), fresh herbs, sprouts[15] (detection of ESBL-producers in 17% of samples, personal communication Kantonales Laboratorium Basel-Stadt) and salads. Both local and imported produce will be sampled.[16]

Sample processing

The patient specimens will be analysed for ESBL-producers according to the routine diagnostic procedures in the Clinical Microbiology laboratory of the University Hospital Basel. Food and environmental samples will be processed in close collaboration with the local health authority (Kantonales Laboratorium Basel-Stadt). Isolates identified as producing ESBL will be subjected to WGS for further epidemiological investigations.

Molecular epidemiology based on WGS

Investigation of the spread and transmission of ESBL-producers will be carried out using WGS on two important levels. First, we will analyse the bacterial chromosomal sequences to follow transmission of the bacteria. This will allow for the detection of classical spread of ESBL-producing bacteria. Second, we will analyse the ESBL-encoding plasmids to see whether transmission is occurring between bacterial strains. Transmission of a plasmid or even the uptake of free plasmids could result in ESBL spread between different lineages of bacteria or even between different Enterobacteriaceae species.

To identify the transmission events of bacteria and their plasmids, we will combine different WGS approaches, like Illumina® and long read sequencing technologies. For analysis of bacterial chromosomes, various methodologies will be applied and compared in order to get the most accurate phylogenetic relationships among all the strains. Core-genome MultiLocus Sequence Typing (cgMLST) and SNP-based methods will be evaluated in this respect.

In the second part we will compare the above chromosomal phylogenies with those generated by similar analysis of the ESBL-plasmids, such that we can observe horizontal transfer. Using WGS we generate information about the plasmids of each isolate in parallel to the chromosomal sequences. We are currently setting up an allele based analysis pipeline, interrogating the assemblies of all isolate genomes for the presence of plasmid replicons [17], genes encoding ESBL enzymes and other antibiotic resistances.

Quantitative data analyses and modelling

Based on the sequencing data, we will reveal to what extent patients with and without an epidemiological link cluster together, giving a proxy for the transmission of bacterial strains. Significant clustering in plasmid phylogenies where strains have more divergent chromosomal phylogenies will indicate horizontal gene transfer. Furthermore, assessing the transmission rates before and after omitting contact isolation precautions at the University Hospital Basel in 2012 for both bacteria and mobile elements will enhance our knowledge on the spread of multi-resistance in a hospital setting, thus directly impacting contact isolation policies in healthcare settings.

All phylogenies based on WGS will be inferred using BEAST v2.0[18] employing our previously developed tool for quantifying transmission rates.[19] BEAST is a Bayesian inference tool, which takes into account the phylogenetic uncertainty observed in such closely related pathogen data. Furthermore, BEAST infers trees with branch lengths denoting calendar time allowing us to link transmission times in the tree with epidemiological links.

In the final "one-health" approach, we will use phylogenies based on ESBL strains from patients, foodstuffs and wastewater samples to determine the amount of transmission of bacteria and plasmids between these different sources. Again the phylogeny is interpreted as a proxy for transmission. If the strains or mobile genetic elements from different sources (i.e. hospital, foodstuffs, wastewater) cluster with each other in the phylogeny, but strains from different sources do not mix in the phylogeny, we have an indication for very limited interaction between sources. On the other hand, mixing of strains in the phylogeny from different sources indicates ongoing transmission between sources. Quantification of transmission events will allow us to analyse the contribution of each source to human ESBL-infections.

The methodologies for data analyses and modelling could be subject to certain modifications during the execution of the project

Limitations

This study has some important limitations. The single center study design may limit generalizability to other settings and the lack of systematic surveillance of all patients entering and exciting the healthcare institution may result in missed transmission events. To extend the scope of our study, in a next step our strains could be also compared with genomes from other studies in order to contextualize our isolates against a global collection More detailed analysis of isolates which share plasmid markers (*e.g.* both isolates share an identical ESBL encoding gene and at least one identical replicon sequence) will require optimised assemblies of the genomic data. ESBL plasmids are very large (up to 200kb) and present technical challenges with regards to assembly. We are currently evaluating several strategies. First, improvement of *de novo* sequence assembly by optimizing the bioinformatics data analysis pipeline by exclusion of chromosomal sequences. Second, sequencing of purified plasmids to yield higher and more even coverage than when sequencing the complete genome, enabling better assembly. Therefore, we are currently testing various commercially available kits to efficiently recover such large plasmids. Third, application of long read sequencing strategies. Pacbio® and MinION® sequencing yield read lengths of several kilobases. The combination of multiple technplogies will improve the quality of the genome assemblies obtained by Illumina, either by scaffolding the previously obtained contigs, or by performing de novo assembly with the long reads and further quality control with the short Illumina® reads. Fourth, we will use already established tools such as Placnet[20] to reconstruct the plasmids based on additional information such as scaffolding, coverage and database comparisons. Finally, the obtained full plasmid sequences from key reference isolates will be incorporated into a local database together with already published sequences for resistance plasmids, which can be used as reference to calculate relatedness and transmission of plasmids in more detail. Mapping against a comprehensive database of plasmids will allow us

to thoroughly analyse the relatedness of all plasmids. The database can also be published and made available to the public to facilitate downstream projects on plasmid transmission.

Ethics and dissemination

This study has been approved by the local ethics committee (Ethikkommission Nordwest-und Zentralschweiz) as a quality control project (Project-ID 2017-00100).

We plan to present the results of this research project at national and international scientific meetings. We aim to publish our results in open access journals so they are widely available to interested international audiences. We aim to make our sequencing data available to the research community so that distribution of strains and plasmids can be assessed on both a national and international level. Such a database could provide a sound basis for tracking outbreaks and transmission between institutions and countries.

We expect our results to inform infection control strategies in healthcare settings. The division of Infectious Diseases and Hospital Epidemiology of the University Hospital Basel will tailor their infection control interventions accordingly. On a national level, our implementation partner will be SwissNoso, which publishes national guidelines for infection control measures in healthcare settings, as well as the Swiss Society for Infectious Diseases and the Federal Office of Public Health, which are currently developing strategies for national Antibiotic Stewardship interventions.

Our results may reveal foodstuffs as a relevant source of transmission of ESBL-producers and may suggest changes to wastewater management. The responsible authorities on the cantonal level (the cantonal laboratory, which belongs to the cantonal health authorities) are directly involved in this research project.

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Author's contributions

TS: Co-applicant, contributed to designing the protocol, reviewed intellectual content of this paper.

DM: Co-applicant, contributed to designing the protocol, reviewed intellectual content of this paper.

LAB: Advises on the sequencing methodology, reviewed this paper for intellectual content.

JH: Advises on the phylogenetic analysis, reviewed this paper for intellectual content.

RS: Advises on environmental sampling strategies, reviewed this paper for intellectual content.

AE: Advises on the sequencing methodology, reviewed this paper for intellectual content.

HSS: Advises on the sequencing methodology, reviewed this paper for intellectual content.

LE: Collection and analysis of environmental samples, reviewed this paper for intellectual content.

PB: Advises on collection and analysis of environmental samples, reviewed this paper for intellectual content.

PH: Advises on collection and analysis of environmental samples, reviewed this paper for intellectual content.

CB: Advises on collection and analysis of environmental samples, reviewed this paper for intellectual content.

STS: Principal investigator and corresponding author, initiated, designed and drafted the protocol, coordinated all collaborations.

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Competing interests statement

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Abstract

Introduction

Extended-spectrum beta-lactamases (ESBL)-producing Enterobacteriaceae were first described in relation with hospital-acquired infections. In the 2000s, the epidemiology of ESBL-producing organisms changed as especially ESBL-*E. coli* was increasingly described as an important cause of community-acquired infections, supporting the hypothesis that in more recent years ESBL-producing Enterobacteriaceae have probably been imported into hospitals rather than vice versa. Transmission of ESBL-producing Enterobacteriaceae is complicated by ESBL genes being encoded on self-transmissible plasmids, which can be exchanged among the same and different bacterial species. The aim of this research project is to quantify hospital-wide transmission of ESBL-producing Enterobacteriaceae on both the level of bacterial species and the mobile genetic elements, and to determine if hospital-acquired infections caused by ESBL-producers are related to strains and mobile genetic elements predominantly circulating in the community or in the healthcare setting. This distinction is critical in prevention since the former emphasizes the urgent need to establish or reinforce antibiotic stewardship programs, and the latter would call for more rigorous infection control.

Methods and analysis

This protocol presents an observational study, which will be performed at the University Hospital Basel and in the city of Basel, Switzerland. ESBL-producing Enterobacteriaceae will be collected from any specimens obtained by routine clinical practice or by active screening in both in-and outpatient settings, as well as from wastewater samples and foodstuffs, both collected monthly over a 12-month period for analyses by whole genome sequencing.

Bacterial chromosomal, plasmid and ESBL-gene sequences will be compared within the cohort to determine genetic relatedness and migration between humans and their environment.

Ethics and dissemination

This study has been approved by the local ethics committee (Ethikkommission Nordwest-und Zentralschweiz) as a quality control project (Project-ID 2017-00100). The results of this study will be published in peer-reviewed medical journals, communicated to participants, the general public and all relevant stakeholders.

Strengths and limitations of this study

- This study applies an interdisciplinary, "one-health" approach, using state of the art technologies to investigate diverse sources entertaining and promoting the current epidemic of extended-spectrum beta-lactamases (ESBL)-producing Enterobacteriaceae.

- The single center study design may limit generalizability to other settings.

- Lack of systematic surveillance of all patients entering and exiting the healthcare institution may result in missed transmission events.

- The obtained knowledge on transmission of ESBL-producing Enterobacteriaceae on both the level of bacterial species and mobile genetic elements will provide a sound foundation for tailoring specific measures to prevent further spread.

Introduction

Transmission of extended-spectrum beta-lactamases (ESBL)-producing Enterobacteriaceae challenges healthcare facilities worldwide regarding the implementation of effective infection control measures to limit further nosocomial spread. ESBL-producing Enterobacteriaceae were first described in 1983[1] in relation to hospital-acquired infections and have rapidly increased globally ever since. In the 2000s, the epidemiology of ESBL-producing organisms changed as especially *E. coli* producing the CTX-M ESBL-type was increasingly described as an important cause of community-acquired urinary tract infections worldwide,[2] supporting the hypothesis that in more recent years ESBL-producing Enterobacteriaceae have probably been imported into hospitals rather than vice versa. Possible community-sources may include foodstuffs[3] and colonization resulting from global travel, especially to the Indian subcontinent.[4] Furthermore, ESBL-producing Enterobacteriaceae have been recovered in water samples from Swiss rivers and lakes,[5] possibly constituting an underappreciated exposure route for dissemination of antibiotic resistance. Transmission of ESBL-producing Enterobacteriaceae is further complicated by ESBL genes being encoded on self-transmissible plasmids, which can be exchanged among the same and different species of Enterobacteriaceae. The contribution of this horizontal transfer of plasmids carrying ESBL genes between Enterobacteriaceae to the epidemiology of ESBL-producers, however, remains elusive, as analyses of plasmids is challenging due to their flexible architecture and the non-availability of suitable reference sequences, as they are far less conserved than bacterial chromosomes.

Overall, multiple levels of transmission of strains, plasmids and possibly genes, as well as different reservoirs, such as healthcare settings, foodstuffs and water, require investigation using a "one-health"-approach to elucidate the most important drivers of the current ESBL-epidemic. Detailed knowledge on the specific contribution of these different sources is currently lacking, despite being a key requirement for tailoring effective infection control

interventions in healthcare settings to limit further spread. Typing of bacterial strains is the prerequisite for studying transmission. Until recently, techniques such as pulsed-field gel electrophoresis (PFGE) or multilocus sequence typing (MLST) were considered the reference standards,[6] however, these lack the resolution to differentiate closely related strains, which is needed for a deeper understanding of the current ESBL-epidemic. Furthermore, these techniques are not able to distinguish mobile genetic elements.[7] Whole genome sequencing (WGS) allows the identification of single-nucleotide polymorphisms (SNPs) that differentiate chromosomes and mobile genetic elements at the highest possible resolution, therefore enabling investigation of strain and plasmid relatedness by phylogenetic analyses, and ultimately leading to detailed exploration of transmission pathways.[8] This technique has to date not been applied to determine transmission of both strains and plasmids in a large epidemiological study including clinically relevant ESBL-Enterobacteriaceae-strains and isolates recovered from community settings. A key resource at our institution is a strain collection of all ESBL-producing Enterobacteriaceae recovered from any specimens obtained by routine clinical practice in both in and outpatient settings since 2003, representing a unique collection of clinically relevant strains over time.

Aim and objectives

The aim of this research project is to investigate the transmission of ESBL-producing Enterobacteriaceae on both the level of bacterial chromosomes and mobile genetic elements, and to determine the source of hospital-acquired infections. This data is critical for prevention since community-acquisition emphasizes the urgent need to establish or reinforce antibiotic stewardship programs, and nosocomial acquisition would call for more rigorous infection control. The specific aims include:

1. To quantify the extent of hospital-wide transmission of ESBL-producing Enterobacteriaceae by assessing the genetic relatedness of ESBL-producing Enterobacteriaceae among patients and over time.

2. To estimate the contribution of horizontal gene transfer to the spread of ESBL-producing Enterobacteriaceae in both hospital- and community settings.

3. To determine migration of ESBL-producing Enterobacteriaceae between humans and their environment (i.e. foodstuffs and wastewater samples) by comparing genetic relatedness of strains and plasmids recovered from patients and the environment, as a "one-health"-approach.

Methods and analysis

Setting

The University Hospital Basel is a tertiary academic care center admitting more than 35,000 adult patients annually and comprising 813 beds. It provides acute care and hospital services in the city of Basel (approximate population, 200,000) and serves as a referral center for patients requiring specialized medical care for the north-western part of Switzerland. Its outpatient clinics provide both general and specialized medical care.

In keeping with current guidelines, the following infection control practices are in effect at our institution:

Active Surveillance for ESBL-carriage

Since 2003, all patients with recovery of ESBL-producing Enterobacteriaceae from any specimens obtained by routine clinical practice in both in-and outpatient settings are routinely screened to determine further colonization sites. In addition, all patients with known ESBL-carriage are screened, whenever they are readmitted to the hospital. Furthermore, the following patient populations are routinely screened for ESBL carriage at our institution: 1) all patients hospitalized for at least 24 hours in the same room as a patient colonized or infected with an ESBL-producing Enterobacteriaceae, 2) since 2013, patients admitted to the intensive care units and requiring mechanical ventilation, and 3) since 2015, all patients transferred directly from a hospital abroad and all admitted asylum seekers. Screening for ESBL-carriage is carried out by selective plating of rectal swabs, swabs from any open wounds or drainages, as well as urine samples from patients with foley catheters.[9]

Standard precautions

Standard precautions have been implemented at our institution since 1999. They include the proper use of hand hygiene (as indicated in the WHO and CDC guidelines)[10 11] for any patient contact and the use of personal protective equipment (i.e. gloves, gowns, masks, eye protection) for procedures involving contact with body fluids as outlined by the CDC guidelines.[10]

Contact precautions

Contact precautions involve assignment to a single room, as well as use of gloves and gowns by both healthcare workers and visitors on entrance.[10]

As of 1999, contact precaution measures were implemented for all patients infected or colonized with ESBL-producing Enterobacteriaceae. From January 2012 contact precaution measures were discontinued for patients infected or colonized with ESBL-*E. coli,* based on the finding of low transmission rates at our institution.[12]

Strain collection at the microbiology laboratory of the University Hospital Basel

Since January 2003, all ESBL-producing Enterobacteriaceae isolates recovered from any specimens obtained by routine clinical practice in both in and outpatient settings of the University Hospital Basel, Switzerland have been collected and stored at the Division of Clinical Microbiology, representing a unique collection of clinically relevant strains. Serial isolates from the same patient are collected and stored if the date of isolation is over two months from detection of the first strain.

Study design

We propose a two-stage approach:

a) **Retrospective study** (addresses study aims 1 and 2): Whole genome sequencing will be performed on representative ESBL-strains collected from January 2003 to December 2016 at the University Hospital Basel. The epidemiological relationships (as defined below) between patients with genetically related strains of ESBL-producing Enterobacteriaceae and cases with genetically related plasmids carrying the respective ESBL genes will be assessed.

b) **Prospective study** (addresses study aims 1 and 2 and additionally study aim 3): Whole genome sequencing will be performed on representative ESBL-strains collected over a two-year study period at the University Hospital Basel, from samples from the wastewater system of both the hospital and the city of Basel, and from foodstuff samples collected from both the hospital and the city of Basel. Foodstuffs will be collected monthly from two stores and wastewater samples from representative sites of the wastewater network system in each of the ten postal code districts of the city of Basel over one to two years. The epidemiological relationships (as defined below) between patients, as well as environmental samples with genetically related strains of ESBL-producing Enterobacteriaceae and cases with genetically related plasmids carrying the respective ESBL genes will be assessed.

Epidemiologic data and classification

The following data is systematically assessed and available for all patients admitted to our institution or treated in one of the outpatient clinics: demographics, movement throughout the hospital (including detailed information regarding rooms and wards occupied during the hospital stay), home postal code districts and assigned general medical practices.

Epidemiological relationships between genetically related cases will be classified as follows:

- **Hospital room contact:** patients hospitalized in the same room during the same time period or up to one week after discharge

- **Hospital ward contact:** patients hospitalized on the same hospital ward during the same time period or up to one week after discharge
- **Hospital-wide contact:** patients hospitalized during the same time period or up to one week after discharge
- **Outpatient contact:** patients receiving outpatient care within the same outpatient clinic or at the same general medicine practice.
- **Community contact (between patients):** patients living in the same postal code district.
- **Community contact (between patients and the environment)** patients, foodstuff samples and/or wastewater samples deriving from the same postal code district.

Sample collection

Patient samples

All ESBL-producing Enterobacteriaceae recovered from any specimens obtained by routine clinical practice or by active screening (as detailed above) in both in- and out-patient settings of the University Hospital Basel, Switzerland, have been collected and stored at the Division of Clinical Microbiology since January 2003. This collection will be continued throughout the study period.

Wastewater samples

Wastewater samples will be collected monthly during the prospective part of the study. In collaboration with the civil engineering department of the city of Basel (Tiefbauamt Basel Stadt), 20 specific sampling sites reflecting the different postal code areas of the city (two per postal code area) have been chosen. In addition, wastewater will be collected from the main wastewater outlet of the University Hospital Basel.

Samples from foodstuff

Samples from foodstuff will be collected monthly during the prospective part of this study. Foodstuffs will be bought from two stores from each different postal code area. In addition, foodstuffs will be collected from the hospital kitchen.[13 14] Based on the results of prior studies confirming the presence of ESBL-producing Enterobacteriaceae in foodstuffs in Switzerland, we plan to investigate the following foodstuffs: poultry[13] (detection of ESBL-producers in 50% of samples, personal communication Kantonales Laboratorium Basel-Stadt), fresh herbs, sprouts[15] (detection of ESBL-producers in 17% of samples, personal communication Kantonales Laboratorium Basel-Stadt) and salads. Both local and imported produce will be sampled.[16]

Sample processing

The patient specimens will be analysed for ESBL-producers according to the routine diagnostic procedures in the Clinical Microbiology laboratory of the University Hospital Basel. Food and environmental samples will be processed in close collaboration with the local health authority (Kantonales Laboratorium Basel-Stadt). Isolates identified as producing ESBL will be subjected to WGS for further epidemiological investigations.

Molecular epidemiology based on WGS

Investigation of the spread and transmission of ESBL-producers will be carried out using WGS on two important levels. First, we will analyse the bacterial chromosomal sequences to follow transmission of the bacteria. This will allow for the detection of classical spread of ESBL-producing bacteria. Second, we will analyse the ESBL-encoding plasmids to see whether transmission is occurring between bacterial strains. Transmission of a plasmid or even the uptake of free plasmids could result in ESBL spread between different lineages of bacteria or even between different Enterobacteriaceae species.

To identify the transmission events of bacteria and their plasmids, we will combine different WGS approaches, like Illumina® and long read sequencing technologies. For analysis of bacterial chromosomes, various methodologies will be applied and compared in order to get the most accurate phylogenetic relationships among all the strains. Core-genome MultiLocus Sequence Typing (cgMLST) and SNP-based methods will be evaluated in this respect.

In the second part we will compare the above chromosomal phylogenies with those generated by similar analysis of the ESBL-plasmids, such that we can observe horizontal transfer. Using WGS we generate information about the plasmids of each isolate in parallel to the chromosomal sequences. We are currently setting up an allele based analysis pipeline, interrogating the assemblies of all isolate genomes for the presence of plasmid replicons [17], genes encoding ESBL enzymes and other antibiotic resistances.

Quantitative data analyses and modelling

Based on the sequencing data, we will reveal to what extent patients with and without an epidemiological link cluster together, giving a proxy for the transmission of bacterial strains. Significant clustering in plasmid phylogenies where strains have more divergent chromosomal phylogenies will indicate horizontal gene transfer. Furthermore, assessing the transmission rates before and after omitting contact isolation precautions at the University Hospital Basel in 2012 for both bacteria and mobile elements will enhance our knowledge on the spread of multi-resistance in a hospital setting, thus directly impacting contact isolation policies in healthcare settings.

All phylogenies based on WGS will be inferred using BEAST v2.0[18] employing our previously developed tool for quantifying transmission rates.[19] BEAST is a Bayesian inference tool, which takes into account the phylogenetic uncertainty observed in such closely related pathogen data. Furthermore, BEAST infers trees with branch lengths denoting calendar time allowing us to link transmission times in the tree with epidemiological links.

In the final "one-health" approach, we will use phylogenies based on ESBL strains from patients, foodstuffs and wastewater samples to determine the amount of transmission of bacteria and plasmids between these different sources. Again the phylogeny is interpreted as a proxy for transmission. If the strains or mobile genetic elements from different sources (i.e. hospital, foodstuffs, wastewater) cluster with each other in the phylogeny, but strains from different sources do not mix in the phylogeny, we have an indication for very limited interaction between sources. On the other hand, mixing of strains in the phylogeny from different sources indicates ongoing transmission between sources. Quantification of transmission events will allow us to analyse the contribution of each source to human ESBL-infections.

The methodologies for data analyses and modelling could be subject to certain modifications during the execution of the project

Limitations

This study has some important limitations. The single center study design may limit generalizability to other settings and the lack of systematic surveillance of all patients entering and exciting the healthcare institution may result in missed transmission events. To extend the scope of our study, in a next step our strains could be also compared with genomes from other studies in order to contextualize our isolates against a global collection More detailed analysis of isolates which share plasmid markers (*e.g.* both isolates share an identical ESBL encoding gene and at least one identical replicon sequence) will require optimised assemblies of the genomic data. ESBL plasmids are very large (up to 200kb) and present technical challenges with regards to assembly. We are currently evaluating several strategies. First, improvement of *de novo* sequence assembly by optimizing the bioinformatics data analysis pipeline by exclusion of chromosomal sequences. Second, sequencing of purified plasmids to yield higher and more even coverage than when sequencing the complete genome, enabling better assembly. Therefore, we are currently testing various commercially available kits to efficiently recover such large plasmids. Third, application of long read sequencing strategies. Pacbio® and MinION® sequencing yield read lengths of several kilobases. The combination of multiple technplogies will improve the quality of the genome assemblies obtained by Illumina, either by scaffolding the previously obtained contigs, or by performing de novo assembly with the long reads and further quality control with the short Illumina® reads. Fourth, we will use already established tools such as Placnet[20] to reconstruct the plasmids based on additional information such as scaffolding, coverage and database comparisons. Finally, the obtained full plasmid sequences from key reference isolates will be incorporated into a local database together with already published sequences for resistance plasmids, which can be used as reference to calculate relatedness and transmission of plasmids in more detail. Mapping against a comprehensive database of plasmids will allow us

to thoroughly analyse the relatedness of all plasmids. The database can also be published and made available to the public to facilitate downstream projects on plasmid transmission.

Ethics and dissemination

This study has been approved by the local ethics committee (Ethikkommission Nordwest-und Zentralschweiz) as a quality control project (Project-ID 2017-00100).

We plan to present the results of this research project at national and international scientific meetings. We aim to publish our results in open access journals so they are widely available to interested international audiences. We aim to make our sequencing data available to the research community so that distribution of strains and plasmids can be assessed on both a national and international level. Such a database could provide a sound basis for tracking outbreaks and transmission between institutions and countries.

We expect our results to inform infection control strategies in healthcare settings. The division of Infectious Diseases and Hospital Epidemiology of the University Hospital Basel will tailor their infection control interventions accordingly. On a national level, our implementation partner will be SwissNoso, which publishes national guidelines for infection control measures in healthcare settings, as well as the Swiss Society for Infectious Diseases and the Federal Office of Public Health, which are currently developing strategies for national Antibiotic Stewardship interventions.

Our results may reveal foodstuffs as a relevant source of transmission of ESBL-producers and may suggest changes to wastewater management. The responsible authorities on the cantonal level (the cantonal laboratory, which belongs to the cantonal health authorities) are directly involved in this research project.

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Author's contributions

TS: Co-applicant, contributed to designing the protocol, reviewed intellectual content of this paper.

DM: Co-applicant, contributed to designing the protocol, reviewed intellectual content of this paper.

LAB: Advises on the sequencing methodology, reviewed this paper for intellectual content.

JH: Advises on the phylogenetic analysis, reviewed this paper for intellectual content.

RS: Advises on environmental sampling strategies, reviewed this paper for intellectual content.

AE: Advises on the sequencing methodology, reviewed this paper for intellectual content.

HSS: Advises on the sequencing methodology, reviewed this paper for intellectual content.

LE: Collection and analysis of environmental samples, reviewed this paper for intellectual content.

PB: Advises on collection and analysis of environmental samples, reviewed this paper for intellectual content.

PH: Advises on collection and analysis of environmental samples, reviewed this paper for intellectual content.

CB: Advises on collection and analysis of environmental samples, reviewed this paper for intellectual content.

STS: Principal investigator and corresponding author, initiated, designed and drafted the protocol, coordinated all collaborations.

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Competing interests statement

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