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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-producing Escherichia 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 emphasises the urgent need to establish or reinforce antibiotic stewardship programmes, and the latter would call for more rigorous infection control.

Methods and analysis This protocol presents an observational study that 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 inpatient 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 in relation to hospital-acquired infections and have rapidly increased globally since the former emphasises the urgent need to establish or reinforce antibiotic stewardship programmes, and the latter would call for more rigorous infection control.

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-centre study design may limit generalisability 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 19831 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 Escherichia coli producing the CTX-M...
ESBL type was increasingly described as an important cause of community-acquired urinary tract infections worldwide, 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 food-stuffs and colonisation resulting from global travel, especially to the Indian subcontinent. Furthermore, ESBL-producing Enterobacteriaceae have been recovered in water samples from Swiss rivers and lakes, 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 are 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 or multilocus sequence typing (MLST) were considered the reference standards; 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. 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. 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-producing 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 inpatient 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. These data are critical for prevention since community acquisition emphasises the urgent need to establish or reinforce antibiotic stewardship programmes, 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 (ie, 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 centre 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 centre for patients requiring specialised medical care for the northwestern part of Switzerland. Its outpatient clinics provide both general and specialised 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 inpatient and outpatient settings are routinely screened to determine further colonisation 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 hospitalised for at least 24 hours in the same room as a patient colonised or infected with 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.
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 Centers for Disease Control and Prevention (CDC) guidelines)\textsuperscript{10,11} for any patient contact and the use of personal protective equipment (ie, gloves, gowns, masks and eye protection) for procedures involving contact with body fluids as outlined by the CDC guidelines.\textsuperscript{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.\textsuperscript{10}

As of 1999, contact precaution measures were implemented for all patients infected or colonised with ESBL-producing Enterobacteriaceae. From January 2012, contact precaution measures were discontinued for patients infected or colonised with ESBL-producing \textit{E. coli}, based on the finding of low transmission rates at our institution.\textsuperscript{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 inpatient 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 2 months from detection of the first strain.

Study design
We propose a two-stage approach:

a. Retrospective study (addresses study aims 1 and 2): WGS 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): WGS will be performed on representative ESBL strains collected over a 2-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 1–2 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.

Epidemiological data and classification
The following data are 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 hospitalised in the same room during the same time period or up to 1 week after discharge
- hospital ward contact: patients hospitalised on the same hospital ward during the same time period or up to 1 week after discharge
- hospital-wide contact: patients hospitalised during the same time period or up to 1 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 inpatient and outpatient 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 (Tiefbaumat 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.\textsuperscript{13,14} Based on the results of prior studies...
confirming the presence of ESBL-producing Enterobact-
ериaceae in foodstuffs in Switzerland, we plan to inves-
tigate the following foodstuffs: poultry\textsuperscript{15} (detection of
ESBL producers in 50\% of samples, personal commu-
nication, Kantonales Laboratorium Basel-Stadt), fresh
herbs, sprouts\textsuperscript{15} (detection of ESBL producers in 17\%
of samples, personal communication, Kantonales Labora-
torium Basel-Stadt) and salads. Both local and imported
produce will be sampled.\textsuperscript{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-pro-
ducers 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-pro-
ducing bacteria. Second, we will analyse the ESBL-encod-
ing 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 method-
ologies will be applied and compared in order to get the
most accurate phylogenetic relationships among all the
strains. Core-genome MLST and SNP-based methods will
be evaluated in this respect.

In the second part, we will compare the above chro-
mosomal 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 chro-
mosomal sequences. We are currently setting up an allele-
based analysis pipeline, interrogating the assemblies of all
isolate genomes for the presence of plasmid replicons,\textsuperscript{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 phylog-
enies 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 multiresistance
in a hospital setting, thus directly impacting contact iso-
lar policies in healthcare settings.

All phylogenies based on WGS will be inferred using
BEAST V.2.0\textsuperscript{18} employing our previously developed tool
for quantifying transmission rates.\textsuperscript{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 trans-
mission times in the tree with epidemiological links.

In the final ‘one-health’ approach, we will use phyloge-
ny based on ESBL strains from patients, foodstuffs and
wastewater samples to determine the amount of trans-
mission 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 (ie, hospital, foodstuffs that waste-
water) 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. However, 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-
centre study design may limit generalisability 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
contextualise our isolates against a global collection. More
detailed analysis of isolates that share plasmid markers
(eg, 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 chal-
enges with regards to assembly. We are currently evalu-
ating several strategies. First, improvement of de novo
sequence assembly by optimising 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 technologies 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\textsuperscript{29} 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**

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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