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Molecular characterisation of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* isolates from hospital and ambulatory patients in Germany

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ABSTRACT

The increase of *Escherichia coli* producing extended-spectrum β-lactamases (ESBL) in hospitals and their emergence as intestinal colonisers of healthy humans is of concern. Transmission ways and the extent of spread of distinct *E. coli* clones or ESBL genes among humans and animals via the food chain or the environment is a matter of debate.

In this study we determined ESBL genotypes in *E. coli* isolates ($n = 233$) resistant to 3rd generation cephalosporins from hospitals and medical practices using PCR and sequencing. Bacterial strain typing was performed by PCR-based phylogrouping, multilocus sequence typing (MLST) and a ST131-specific PCR. Results showed that CTX-M-15 (50.4%), CTX-M-1 (28.4%) and CTX-M-14 (5.6%) were the most common ESBL types. Especially, CTX-M-15 was associated with *E. coli* ST131 of phylogenetic group B2, which was the dominant sequence type among our isolates (35.8%). MLST typing revealed 40 different sequence types (STs), with ST131, ST410, ST10 and ST38 as the most prevalent ones.

Our findings give an overview of the current distribution of ESBL-producing *E. coli* isolates from humans in Germany. *E. coli* O25b:H4-ST131 was confirmed to be the most common clone, which is known for its successful dissemination worldwide. Although heterogeneity among the isolates was found, several successful clones previously described in animals (ST410, ST10) also occurred in our isolate collection. Further detailed investigations of ESBL-producing isolates from different habitats are needed to evaluate possible transfer ways.

1. Introduction

The dramatic increase of *Escherichia coli* with resistance to 3rd generation cephalosporins (e.g. cefotaxime and ceftazidime) in the last ten years is an international challenge for public health systems (ECDC, 2013). According to data of the nationwide surveillance system ARS the rate of cefotaxime-resistant *E. coli* isolated in German hospitals increased from 6.5% in 2008 to 12.6% in 2014 (https://ars.rki.de/; 08/18/2015). The majority of these *E. coli* is multidrug-resistant due to a combination of resistance to β-lactams (penicillins and 3rd generation cephalosporins) and other antimicrobial substances (fluoroquinolones, aminoglycosides, sulfonamides) (ECDC, 2013).

Resistance to 3rd generation cephalosporins in *E. coli* is mainly caused by acquisition of genes encoding extended-spectrum β-lactamases (ESBLs) or AmpC β-lactamases (ECDC, 2013; Pfeifer et al., 2010). ESBL genes are mostly located on conjugative plasmids

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and transferable into other enterobacterial species facilitating their further spread (Carattoli, 2013). Nowadays, the most common ESBLs in *E. coli* are CTX-M-type enzymes (Naseer and Sundsfjord, 2011). Apart from plasmid transfer the success of CTX-M in *E. coli* from human patients is caused by spread of distinct clonal lineages e.g. *E. coli* O25b:H4-ST131 (Rogers et al., 2011).

Data on ESBL-producing *E. coli* from German hospitals in 2004 and 2008 showed that CTX-M-15 (53%) and CTX-M-1 (34%) were the most common ESBL-types (Pfeifer et al., 2010). In 2011 the interdisciplinary research project "RESET" (www.reset-verbund.de) was initiated to investigate ESBL-producing *E. coli* from humans, livestock animals and food. Here we present the results of a RESET-study on identification and characterisation of a representative number of ESBL-producing *E. coli* isolates from human patients in German hospitals and medical practices.

2. Materials and methods

2.1. Bacterial isolates

In 2011, diagnostic laboratories throughout Germany including laboratories of the Limbach-Group were asked to collect *E. coli* with ESBL-phenotype. Since these local laboratories perform microbiological diagnostics for different medical practices and hospitals in their commuting area, every laboratory was asked to provide five ambulant *E. coli* isolates (isolates from outpatients with urinary tract infections in medical practices) and five nosocomial *E. coli* isolates (isolates from hospitalised patients, identification 48–72 h after admission). Study criteria were resistance to cefotaxime and/or ceftazidime and/or a confirmed ESBL phenotype (inhibition by clavulanic acid). Identification of the bacterial species and determination of the ESBL phenotype was performed in these laboratories using matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS; Bruker, Billerica, MA, USA) and VITEK 2 (bioMérieux, Marcy-l'Étoile, France), respectively. Between January 2011 and December 2012 a total of 24 laboratories located in eleven of the 16 German federal states sent 233 *E. coli* isolates (128 nosocomial *E. coli* and 105 ambulant *E. coli* from medical practices) for further analyses to the Robert Koch Institute.

2.2. Antimicrobial susceptibility testing and resistance gene screening

At the Robert Koch Institute the antimicrobial susceptibilities to nine antibiotics (ampicillin, cefotaxime, ceftazidime, cefoxitin, nalidixic acid, ciprofloxacin, gentamicin, amikacin, and trimethoprim–sulfamethoxazole) were determined for all 233 *E. coli* isolates by microbroth dilution (according to EUCAST and CLSI, respectively). Susceptibilities to imipenem, meropenem and ertapenem were tested by disk diffusion (Oxoid Ltd., Basingstoke, UK). All results were interpreted according to the current recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints version v 5.0 (http://www.eucast.org/clinical_breakpoints) and the results of nalidixic acid were interpreted according to recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2012). Additionally, ESBL- or AmpC-production was confirmed by a disk test set (AmpC and ESBL Detection Set D68C, MAST Group, UK).

Presence of β-lactamase genes was tested in all 233 isolates by PCR amplification and sequencing of different β-lactamase genes ($bla_{\text{TEM-like}}$, $bla_{\text{SHV-like}}$, $bla_{\text{CTX-M-like}}$, $bla_{\text{OXA-1-group}}$, $bla_{\text{CMY-like}}$, $bla_{\text{DHA-like}}$, $bla_{\text{ACC-like}}$, $bla_{\text{KPC-like}}$, $bla_{\text{OXA-48-like}}$, $bla_{\text{VIM-like}}$, $bla_{\text{NDM-like}}$) (Eller et al., 2013). Furthermore, screening for plasmid-mediated quinolone resistance (PMQR) genes was performed by PCR amplification and sequencing of *qnrA*-like, *qnrB-like* and *qnrS-like* genes and methyltransferase gene *aac(6′)Ib-cr* (Eller et al., 2013).

2.3. Bacterial strain typing

All 233 *E. coli* isolates were assigned to one of the four main phylogenetic groups using a PCR-based assay (Clermont et al., 2000). To identify isolates of the clonal lineage O25b:H4-ST131, a rfbO25b-ST131-specific PCR was performed (Blanco et al., 2009). Another ST131 specific PCR-based assay, identifying isolates of the clonal linages O25b:H4-ST131 and O16:H5-ST131, was applied for isolates with discrepant results (Johnson et al., 2014). To compare the results, 83 ESBL-positive *E. coli* isolates from community-acquired rectal colonisations of a case-control study performed at the Charité, Berlin (Leistner et al., 2013) and 211 ESBL-positive *E. coli* isolates from healthy humans in Bavaria, Germany (Valenza et al., 2014) were additionally screened using the allele specific PCR for O25b:H4-ST131 (Blanco et al., 2009). Both of these studies were conducted within the RESET research consortium.

Independent from the ST131-PCR results thirty nosocomial *E. coli* and 19 ambulant *E. coli* were randomly selected for multilocus sequence typing (MLST) using the MLST scheme described by (Wirth et al., 2006). In addition, MLST was performed for 27 isolates from the case-control study (Leistner et al., 2013) and 22 isolates from healthy German persons (Valenza et al., 2014; Supplementary Table 1). To determine the sequence types (STs) PCR amplification of the seven housekeeping gene fragments was performed using DreamTaq PCR Master Mix 2x (Thermo Fisher Scientific Inc., Waltham MA, USA); MLST primer sequences and PCR conditions can be found at http://mlst.ucc.ie/mlst/dbs/Ecoli/documents/primersColi_html. The sequence of the PCR products was analysed either by "lightrun96" sequencing (GATC Biotech AG, Konstanz, Germany) or by using the 3130xl Genetic Analyzer (Applied Biosystems Inc.) using the BigDye Terminator v.3.1Cycle Sequencing Kit (Applied Biosystems Inc.) according to manufacturer's instructions. The resulting gene sequences were aligned, trimmed, quality controlled, and allele types were determined via Megablast in Geneious® 7.1.4 (Biomatters Ltd, Auckland, New Zealand) using allele references from http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/Downloads_HTML. Finally, the STs were determined in the database at http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/.

Additionally, the STs of 54 randomly selected ESBL-positive *E. coli* isolates from all three studies (ambulant $n = 12$; nosocomial $n = 16$; case control study, $n = 10$; healthy humans $n = 16$) were obtained from whole genome sequences (Supplementary table S1). For this, genomic DNA was isolated using Purelink Genome DNA Mini Kit (Invitrogen, Germany) according to the manufacturer's instruction. Whole genome sequencing was carried out on a MiSeq instrument (Illumina, Netherlands) using a Nextera XT library (Illumina, Netherlands) with 2×300 bp paired-end reads. The data were assembled to contigs using Spades (version 3.0) (Bankevich et al., 2012). The STs following the scheme of Wirth et al., 2006 were identified using the web-based tool MLST 1.7 (Larsen et al., 2012).

Isolates with ambiguous results in the ST131 typing ($n = 15$) were selected for serotyping using a micro-agglutination assay with specific rabbit antisera (Sifin, Berlin, Germany, and Statens Serum Institute, Copenhagen, Denmark). When whole genome sequences of the isolates were available the SerotypeFinder Tool (https://cge.cbs.dtu.dk/services/SerotypeFinder/) was used for serotype identification.

3. Results

Antimicrobial susceptibility testing revealed that all 233 isolates were resistant to ampicillin but remained susceptible to carbapenems. A total of 98.6% of the nosocomial *E. coli* and 100% of the ambulant isolates were resistant to cefotaxime. The rates of resistance to ceftazidime were 78.1% and 79% for nosocomial and

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