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High diversity of beta-lactamases in the General Hospital Vienna verified by whole genome sequencing and statistical analysis

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ABSTRACT

The detailed analysis of antibiotic resistance mechanisms is essential for understanding the underlying evolutionary processes, the implementation of appropriate intervention strategies and to guarantee efficient treatment options. In the present study, 110 β-lactam-resistant, clinical isolates of *Enterobacteriaceae* sampled in 2011 in one of Europe's largest hospitals, the General Hospital Vienna, were screened for the presence of 31 β-lactamase genes. Twenty of those isolates were selected for whole genome sequencing (WGS). In addition, the number of β-lactamase genes was estimated using biostatistical models.

The carbapenemase genes bla_{KPC-2}, bla_{KPC-3}, and bla_{VIM-4} were identified in carbapenem-resistant and intermediate susceptible isolates, bla_{OXA-72} in an extended-spectrum β-lactamase (ESBL)-positive one. Furthermore, the observed high prevalence of the acquired bla_{DHA-1} and bla_{CMY} AmpC β-lactamase genes (70%) in phenotypically AmpC-positive isolates is alarming due to their capability to become carbapenem-resistant upon changes in membrane permeability. The statistical analyses revealed that approximately 55% of all β-lactamase genes present in the General Hospital Vienna were detected by this study. In summary, this work gives a very detailed picture on the disseminated β-lactamases and other resistance genes in one of Europe's largest hospitals.

1. Introduction

Already in 1940, Abraham et al. reported the inactivation of penicillin by an isolated bacterial enzyme that belongs to the very diverse group of β-lactamases comprising four different classes (Abraham and Chain, 1940). This classification system is based on the amino acid sequence similarity of the various enzymes (Ambler, 1980). Additionally, a functional classification system is used based on the different hydrolytic activity of the β-lactamases (Bush and Jacoby, 2010). Class A, C and D β-lactamases have a serine-based active site, and although they share very little DNA sequence similarity, 3D-structure analyses suggest a common protein precursor (Hall and Barlow, 2003, 2004). The catalytic mechanism involves the formation of an acyl-enzyme intermediate. Class B enzymes have a zinc-dependent reaction site and are also referred to as metallo-β-lactamases. The metal-ion is involved in the processing of the substrate and the intermediates (Page and Badarau, 2008). What all β-lactamases have in common is that they hydrolyse the core structure of the β-lactam antibiotics, the β-lactam ring.

Today, more than 950 unique, naturally occurring β-lactamases have been described and their number is steadily increasing due to the careless use of antibiotics (Bush, 2010). Studies addressing this huge genetic diversity in clinical environments are rare and focus mainly on the detection of a handful of clinically highly abundant genes. One of the largest studies was conducted by Lascols et al., screening more than 1000 extended spectrum beta-lactamase (ESBL)-positive isolates worldwide for the presence of bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{KPC} (Lascols et al., 2012). Although the high number of samples is impressive, such studies do not provide information on the statistical significance of the obtained data. Statistical models such as the species richness estimators ACE and Chao1 and the rarefaction curves are used in microbial ecology to estimate the total number of certain genes detectable within an experimental setup (Hartmann and Widmer, 2006; Pitta et al., 2010; Stevens and Ulloa, 2008). A main motivation behind these calculations is to avoid over- or under-sampling when addressing the genetic diversity of a habitat and to facilitate the comparison

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of the genetic diversity of different habitats despite varying sample numbers.

Although the dissemination of β-lactamase genes in Europe follows global trends, regional differences can be observed with a higher incidence of β-lactamase-positive strains in southern parts of the continent. The question remains if this gradient exists due to varying healthcare systems, climate conditions, travel behaviour or other factors. Central Europe is located in between the borders of this north-south antibiotic resistance gradient and thus may be an indicator for future trends. The presence of the β-lactamase genes bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{OXA-1}, bla_{KPC}, bla_{VIM}, and bla_{NDM} was already confirmed in Austria, but the investigations focused only on the detection of these genes and information on the presence of other β-lactamases such as plasmid-mediated AmpCs, OXA-48, etc. is missing (Duljasz et al., 2009; Eisner et al., 2006; Heller et al., 2012; Hoenigl et al., 2012; Huemer et al., 2011; Prelog et al., 2008; Zarfel et al., 2011a,b).

In the present study, we selected 110 β-lactam-resistant clinical isolates of *Enterobacteriaceae* from the General Hospital Vienna and screened them for a wide range of β-lactamase genes. These phenotypically ESBL-, AmpC- or carbapenemase-positive isolates were analysed by PCR and the detected genes were Sanger-sequenced, in parallel, the isolates were also characterised with a new padlock probe-based detection assay identifying 31 β-lactamase genes (Barisic et al., 2013). After these characterisations, 20 isolates were selected for whole genome sequencing (WGS) using the IonTorrent platform, and in the subsequent sequence data analyses, the isolates were not only screened for β-lactamases but also other antibiotic resistance genes. WGS of an isolate was performed if Sanger-sequencing identified a new gene not listed in Genbank or if the observed antibiotic resistance phenotype could not be explained using PCR and the padlock probe assays. Subsequently, the obtained DNA sequence data was used for statistical calculations estimating the total number of the β-lactamase genes in the General Hospital Vienna.

2. Materials and methods

2.1. Susceptibility testing and phenotypical detection of resistance mechanisms

The clinical isolates were tested for antibiotic susceptibility on Mueller-Hinton agar plates, using the disc diffusion test according to the EUCAST (European Committee on Antimicrobial Susceptibility Testing) recommendations (versions 1.2 and 1.3, The European Committee on Antimicrobial Susceptibility Testing – EUCAST 2011). In the case of resistance to third generation cephalosporins and/or cefoxitin in disc diffusion testing, *Enterobacteriaceae* known to possibly harbour plasmid-encoded AmpC enzymes based on phenotypical testing were screened for the presence of AmpC β-lactamases by a combined-disc test (AmpC ID Confirm Kit; Rosco Diagnostica A/S, Taastrup, DK) according to the manufacturer's instructions. In organisms that produce an inducible chromosomal AmpC β-lactamase, identification alone was indicative of AmpC production (Thomson, 2010). As in primary disc diffusion testing, extended-spectrum cephalosporins (cefuroxime 30 μg, cefpodoxime 10 μg, cefotaxime 5 μg, cefepime 30 μg,) were put around a central amoxicillin-clavulanate (20 μg-10 μg) disc in a way to enable inhibitor-based synergisms. ESBL-producing *Enterobacteriaceae* could be recognized based upon observation of this defining criterion (Moland and Thomson, 1994). In the case of cefoxitin resistance and lacking synergism phenomena, a 20 mm-double-disc synergy test between an amoxicillin-clavulanate and a cefepime disc was performed to avoid missing ESBL-positive phenotypes masked by concomitant AmpC production (Garrec et al., 2011). According to EUCAST antimicrobial wild-type distributions of microorganisms (http://mic.eucast.org/Eucast2/SearchController/search.jsp?action=performSearch&BeginIndex=0&Micdif=dif&NumberIndex=50&Antib=177&Specium=-1&Discstrength=10), meropenem inhibition zone diameter $\leqslant$ 0.25 mm in primary disc diffusion testing was chosen to screen isolates with elevated carbapenem minimal inhibitory concentrations (MIC), which had to undergo confirmatory phenotypic testing in cases where meropenem Etest (bioMérieux, Marcy-l'Étoile, France) revealed MICs $\geqslant$ 0.5 mg/l (http://mic.eucast.org/Eucast2/SearchController/search.jsp?action=performSearch&BeginIndex=0&Micdif=mic&NumberIndex=50&Antib=177&Specium=-1). Ambler class A β-lactamases, metallo-β-lactamases, or AmpC β-lactamases in combination with efflux pumps or reduced permeability were recognized phenotypically by KPC + MBL Confirm ID Kit (Rosco Diagnostica A/S, Taastrup, DK), while Ambler class D β-lactamases where detected by the modified Hodge test (Lee et al., 2010). The KPC + MBL Confirm ID Kit identifies a reduces permeability with AmpC β-lactamases only and not with class A and B β-lactamases. The minimum inhibitory concentration (MIC) values were only recorded for meropenem in carbapenemase-positive isolates.

2.2. Bacterial isolates and DNA extraction

For this study, 110 phenotypically ESBL-, AmpC- and carbapenemase-positive clinical isolates collected at the Division of Clinical Microbiology of the Medical University and General Hospital Vienna between January and September 2011 were genetically characterised (Supplementary Table 1). These consisted of 56 consecutively selected ESBL-positive bacterial strains from an equal number of different patients between January and April 2011 and 54 isolates comprising β-lactamases other than ESBLs consecutively selected between June and September 2011. The ethical clearance to carry out this study was obtained from the ethics committee of the Medical University of Vienna (approval number: 2137_001).

The isolates were grown on BD Columbia blood agar plates with 5% sheep blood (BD Diagnostic Systems, Sparks, USA) at 37 °C overnight. Cells were transferred with an inoculation loop into 1.5 ml microcentrifuge tubes with 500 μl ddH_2O, incubated at 100 °C for ten minutes and plunged into liquid nitrogen for one minute. The thermal cell lysis was repeated two more times. Upon centrifugation at 13,000 rpm for 1 min, the supernatant was used for the subsequent PCR and the padlock probe assay as DNA template. For the WGS experiments, genomic DNA was extracted using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

2.3. DNA amplification, Sanger-sequencing and padlock probes assay

Amplification reactions were conducted using both new primers listed in Supplementary Table 2 and published primers (Supplementary Table 3) to double-check the PCR results. The new primers were designed using the software package ARB (Ludwig et al., 2004) and quality checked using the NetPrimer software (Premier Biosoft, 2012). The final primer concentration was 1 μM in the PCR mix. The reagents of the Mastermix 16S Basic PCR kit (Molzym, Bremen, Germany) were used at a total volume of 50 μl. One microlitre of the cell lysate was added per reaction. The thermal cycling was carried out by an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing for 30 s (annealing temperatures listed in Supplementary Tables 2 and 3), elongation at 72 °C for 80 s, and a final elongation cycle at 72 °C for 10 min. Negative controls without DNA template were used in all experiments. The PCR products were subsequently purified according to the manufacturer's protocols using the MinElute PCR Purification kit (Qiagen, Hilden,

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