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Detection of VIM, NDM and OXA-48 producing carbapenem resistant Enterobacterales among clinical isolates in Southern Hungary

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ABSTRACT

Infections caused by carbapenem-resistant Enterobacterales (CRE) present an important therapeutic problem, as there are limited number of effective therapeutic alternatives available. In this study, phenotypic and genotypic methods were used to characterize carbapenemase-production and other resistance-determinants (AmpC and ESBL-production, efflux pump-overexpression) in 50 isolates (*Klebsiella* spp. $n = 35$, *Escherichia coli* $n = 12$ and *Enterobacter cloacae* complex $n = 3$) collected at the Albert Szent-Györgyi Clinical Center (University of Szeged) between 2014 and 2017. Minimum inhibitory concentrations of meropenem, sulfamethoxazole/trimethoprim, tigecycline, amikacin, moxifloxacin, colistin and fosfomycin were also determined. 24% of isolates were AmpC-producers, while 30% carried $bla_{\text{CTX-M}}$ ESBL-genes. Carbapenemase-genes were detected in 18 (36%) of the tested isolates: in 2 isolates bla_{NDM}, in 6 isolates $bla_{OXA\text{-}48\text{-}like}$ and in 12 isolates, bla_{VIM} was detected by PCR. The species-distribution for isolates positive for carbapenemase-genes was the following: *Klebsiella pneumoniae* $n = 11$, *Klebsiella oxytoca* $n = 1$, *E. coli* $n = 5$, *E. cloacae* complex $n = 1$. Efflux pump-overexpression based on the PAβN-screening agar was shown in $n = 3$ of the tested strains. In nine isolates (18%), carbapenemase and ESBL-genes were detected simultaneously. Highest levels of resistance were noted for fosfomycin (74%) and moxifloxacin (70%), while all isolates were susceptible to colistin. Among applied phenotypic tests in this study the modified carbapenem inactivation method (mCIM) proved to be the most accurate one compared to that of PCR results.

KEYWORDS

carbapenem-resistant Enterobacterales, colistin, fosfomycin, carbapenemase, carbapenem inactivation method

INTRODUCTION

Carbapenems, namely imipenem (introduced in 1985), meropenem (1996), ertapenem (2003) and doripenem (2007) are β-lactam-antibiotics with some of the broadest spectrum and bactericidal activity; they are effective in the therapy of infections caused by aerobic and anaerobic Gram-positive and Gram-negative pathogens (including non-fermenters, with the exception of ertapenem) [1, 2]. Initially, carbapenems were exclusively used as monotherapy for life-threatening infections, however, after the rise and global spread of extended-spectrum

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β-lactamase (ESBL)-producing Enterobacterales, it has become an established clinical practice to use these agents in first-line empirical therapy [2, 3]. Nevertheless, the extensive use of these agents resulted in the emergence of carbapenem-resistant Gram-negative strains [4]. From a clinical standpoint, carbapenem-resistant Gram-negative bacilli (CRGNB) present an important therapeutic problem, as there are limited number of safe and effective therapeutic alternatives available [5, 6]. Carbapenem resistance may develop through a variety of cellular mechanisms: alteration in membrane permeability and porin loss (e.g., ΔOmpK in *Klebsiella pneumoniae*), overexpression of efflux pumps, changes in penicillin-binding proteins; however, the most prevalent mechanism of resistance is through the production of specific β-lactamases called carbapenemases, capable of hydrolyzing these antibacterial drugs [4, 7–10]. The differentiation of carbapenemase-producing carbapenem resistant Enterobacterales (CP-CRE; with *Klebsiella* spp. represented in highest numbers) from non-carbapenemase producing carbapenem resistant Enterobacterales (CRE) is of utmost importance, as the resistance-determinants of CP-CREs are readily transferable on plasmids or integrons, having a role in nosocomial outbreaks and global dissemination [11, 12]. Based on sequence similarity, carbapenemases are classified into Ambler Class A e.g. KPC, SME, NMC-A, IMI, PER, GES, SFO, SFC and IBC), Class D (e.g. OXA-23 group, OXA-48-group) and Class B (e.g. VIM, GIM, SIM, NDM, IMP, IND, AIM, DIM and SPM) enzymes; while the first two groups consist of serine-β-lactamases, the members of the latter group are exclusively metallo-β-lactamases [13, 14]. Different carbapenemases have varying epidemiological significance around the world and in Europe, based on their origins and dissemination levels: ST258 bla_{KPC}s most likely have spread to Europe from the United States, while ST512 bla_{KPC}s are suggested to have spread to Europe from Israel [15]; bla_{VIM-1} emerged in Greece and bla_{OXA-48} emerged in Turkey and spread to Europe through the Mediterranean [16]. In Hungary, the first published case of carbapenemase-production in an Enterobacterales clinical isolate was reported in a KPC-2 and SHV-12-producing *K. pneumoniae*; the isolate originated from a patient, who has been previously hospitalized in Greece and caused a local outbreak in the northeastern region of the country [16, 17].

Various laboratory methods are available for the detection of CP-CRE strains, although the methods-of-choice are highly dependent on the epidemiological situation and the economic possibilities of the given healthcare settings [18]. Molecular methods, such as polymerase chain reaction (PCR) and whole-genome sequencing (WGS) are considered as the gold standard for detection of carbapenemase genes, nevertheless, their high laboratory costs make them unsuitable for many smaller, low-resource laboratories [19]. In addition, molecular methods are only capable of detecting already known resistance-determinants. Thus, carbapenemase-detection is usually carried out as a step-wise process: if carbapenemase-production is suspected based on the results of antimicrobial susceptibility testing, phenotypic screening (e.g., chromogenic media) or confirmatory tests (detection of diffusible carbapenemases, carbapenemase-inhibitor assays, assessment of carbapenem hydrolysis activity by measuring pH-change or spectrophotometry), followed by molecular methods, if available [18–21]. Additionally, the use of lateral flow assays, microarray technology and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for carbapenemase-detection has also been described [22, 23].

In the suspicion of CP-CRE arises, rapid screening and verification is essential, both to ensure appropriate therapy to the affected patients and to allow for infection control measures to take place [16, 18]. Knowledge on the local epidemiology of each healthcare institution regarding carbapenemase-producing strains is of utmost importance, as this may aid the selection of the ideal methodology for their screening [16, 18]. The aim of our study was the characterization of Enterobacterales strains suspected of carbapenemase-production in a low-prevalence setting in Hungary, through the use of various phenotypic and genotypic methods.

MATERIALS AND METHODS

Bacterial strains, identification, inclusion criteria

Fifty (n = 50) Enterobacterales strains, isolated from clinical samples between 2014 and 2017 at the Institute of Clinical Microbiology (Albert Szent-Györgyi Clinical Center, Szeged, Hungary) were included in this study. The identification of these isolates was carried out using MALDI-TOF MS. Mass spectrometry was performed by Microflex LT MALDI Biotyper (Bruker Daltonics, Bremen, Germany) instrument, using the MALDI Biotyper RTC 3.1 software and the MALDI Biotyper Library 3.1 for the spectrum analysis (Bruker Daltonics, Germany). The sample preparation, methodology, and technical specification of the MALDI-TOF MS measurements were described elsewhere [24]. Inclusion of these strains was based on the screening criteria recommended by the ESCMID/EUCAST guidelines (meropenem disk diameter <28 mm) [25].

Minimum inhibitory concentrations (MICs) of meropenem and ancillary antibiotics

MICs of meropenem (MER), sulfamethoxazole/trimethoprim (SXT), tigecycline (TIG), amikacin (AMK) and moxifloxacin (MOX) were determined by E-tests (Liofilchem, Roseto degli Abruzzi, Italy) on Mueller-Hinton agar plates (Oxoid, Basingstoke, UK). MIC determination for colistin (COL) was carried out using the broth microdilution method in cation-adjusted Mueller-Hinton broth (MERLIN Diagnostika, Berlin, Germany) [24, 26]. MIC determination for fosfomycin (FOS) was carried out using the agar dilution method, on Mueller-Hinton agar plates supplemented with 25 mg/L glucose-6-phosphate and varying concentrations of FOS. The interpretation of the results was based on EUCAST Clinical Breakpoints v.9 (http://www.eucast.org). In case of isolates other than *E. coli*, epidemiological cut-off values were used for tigecycline (MIC≤1 mg/L as susceptible, MIC>1 mg/L as resistant) [26].

Phenotypic detection of AmpC-β-lactamases and ESBL-enzymes

The strains included in the study were screened for AmpC-β-lactamase-production by cefoxitin disks (30 μg; Oxoid, Basingstoke, UK) [27]. Phenotypic verification of AmpC-β-lactamase and ESBL-production was carried out using AmpC-ESBL Detection Set (MAST Diagnostica, Reinfeld, Germany) and VITEK 2 Compact ID/AST (bioMérieux, Marcy-l'Étoile, France), according to the manufacturer's instructions.

Phenotypic screening and verification of carbapenemase-production

For phenotypic screening of carbapenemase-production, the chromID CARBA SMART agar (CARB/OXA; bioMérieux, Marcy-l'Étoile, France) was used; this medium consists of a nutrient base, combining different peptones, three chromogenic substrates enabling the detection of specific metabolic enzymes for *E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Serratia* spp. and *Citrobacter* spp., in addition to a proprietary mixture of antibiotics, favoring the selective growth of carbapenemase-producers [28]. The phenotypic verification of carbapenemase-production was carried out using the modified cloverleaf (or Hodge) test and the modified carbapenem inactivation method (mCIM), as previously described [29, 30]. In both assays, meropenem disks (10 μg; Oxoid, Basingstoke, UK) were utilized and *E. coli* ATCC 25922 was used as an indicator organism.

Phenotypic detection of efflux pump-overexpression

The effects of phenylalanine-arginine β-naphthylamide (PAβN), on the MICs of meropenem were detected using the agar dilution method described previously [27]. During the experiments, the concentration of PAβN was 40 μg/mL in the agar base. Two-fold decrease in the MICs of meropenem in the presence of PAβN (a compound with well-known efflux-pump inhibitory activity [30]), compared to the MIC values without the inhibitor was considered as positivity for efflux pump overexpression.

Molecular detection of ESBL and carbapenem-resistance genes

Molecular detection of resistance genes encoding for ESBLs and carbapenemases was carried by multiplex PCR with previously described protocols [31–33].

Statistical analyses

Based on the data provided during our experiments, sensitivity, specificity, positive and negative predictive values (SN, SP, PPV and NPV, respectively) were calculated for each test, as described previously [34].

RESULTS

Sample types and species composition of the isolates

During the 4-year study period, 50 Enterobacterales isolates with meropenem disk diameters under 28 mm were detected: the majority (n = 35) of these isolates were *Klebsiella* spp. (*K. pneumoniae*: n = 34, *Klebsiella oxytoca*: n = 1), while *E. coli* (n = 12) and *E. cloacae* complex (n = 3) were also suspected for carbapenemase-production in lower numbers. These isolates originated from the following samples: urine: n = 19 (midstream urine: n = 14, catheter-specimen urine: n = 5), feces: n = 14, tracheal aspirate: n = 7, abscess: n = 4, aerobic wound culture and bile: n = 3, respectively.

MICs of tested antibiotics

The MICs of tested antibiotics, including MIC_{50}, MIC_{90} values, MIC ranges and the percentage of resistant isolates are presented in Table 1. The highest levels of resistance were observed for FOS (n = 37, 74%), followed by MOX (n = 35, 70%), while non-susceptibility of the isolates for SXT and AMK were around 50%. All tested isolates were susceptible to colistin, with MIC values ranging between 0.25 and 1 mg/L. Based on EUCAST breakpoints, n = 14 (28%) of isolates showed MICs above the resistance breakpoint (8 mg/L) for meropenem, with MICs ranging between 0.25 and 32 mg/L. Highest MICs were observed for the bla_{NDM} and several bla_{VIM}-expressing strains, while in n = 4 (8%) of cases, strains carrying $bla_{OXA48\text{-like}}$ enzymes showed MICs in low-to-moderate range (0.5–1 mg/L) (Table 1.).

Phenotypic and genotypic detection of resistance-determinants

Based on the cefoxitin screening disk, 14 out of 50 isolates (28%) were suspected of AmpC β-lactamase-production, while based on the AmpC-ESBL Detection Set, n = 12 (24%) of these isolates were phenotypically verified as AmpC-producers. The AmpC-ESBL Detection Set showed ESBL-production in n = 15 (30%) isolates; in line with phenotypic results, $bla_{CTX\text{-}M}$-type ESBL-genes were detected in all of these isolates. In n = 6 (12%) of isolates, AmpC and ESBL co-production was verified, both by phenotypic and genotypic methods.

Using the modified cloverleaf test, 20 isolates showed positive results for carbapenemase-production, while this number was n = 19 for the chromID CARBA SMART agar and n = 18 for the mCIM assay. Carbapenemase-genes were

Table 1. MIC values of meropenem and ancillary antibiotics on the tested bacterial strains

	Resistant strains (n, %)	MIC range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)
Meropenem	14 (30%)	0.25–32	8	16
Amikacin	24 (48%)	1–16	4	16
Colistin	0 (0%)	0.25–1	0.5	1
Fosfomycin	37 (74%)	1–64	16	64
Moxifloxacin	35 (70%)	0.064–1	0.25	1
Sulfamethoxazole/trimethoprim	24 (48%)	0.064–16	2	8
Tigecycline	16 (32%)	0.064–1	0.25	1

detected by PCR in $n = 18$ (36%) of the tested isolates: in 2 strains $bla_{NDM,}$ in 6 strains were $bla_{OXA\text{-}48\text{-}like}$ and in 12 bla_{VIM} was detected. In nine isolates (18%), carbapenemase and ESBL-genes were detected simultaneously (bla_{VIM} and $bla_{\text{CTX-M}}$ co-detection in $n = 7$ cases, $bla_{\text{OXA-48-like}}$ and $bla_{\text{CTX-M}}$ co-detection in $n = 2$ cases). The species-distribution for isolates positive for carbapenemase-genes was the following: *K. pneumoniae* $n = 11$, *K. oxytoca* $n = 1$, *E. coli* $n = 5$, *E. cloacae* complex $n = 1$. No ESBL or carbapenemase-encoding genes or phenotypic AmpC-production were detected in $n = 5$ (10%) of isolates. Efflux pump-overexpression based on the PAβN-screening agar was shown in 3 (6%) of the tested strains only: one isolate was ESBL-positive, one was an AmpC-producer, while in one isolate, no other resistance determinant could be detected.

Based on the comparative analysis of phenotypic and genotypic carbapenemase detection methods (considering molecular methods as the gold standard), three isolates presented false-positive (17/20 were true positives) and one presented as false-negative (29/30 were true negatives), while the chromogenic screening agar showed once false-positive (18/19 were true positives). False positivity was noted in case of an AmpC-producer (*E. coli*), an AmpC-ESBL co-producer (*E. cloacae* complex) and an AmpC-efflux pump over-expresser (*K. pneumoniae*); in contrast, the strain presenting as false-negative in both methods was a $bla_{\text{OXA-48-like}}$-producer. The mCIM method was completely in line with the results of genotypic testing (zero false positives or false negatives). Determination of the predictive power of the individual screening and verification tests is presented in Table 2.

DISCUSSION

Members of the Enterobacterales order are common causes of both community-acquired and nosocomial infections, including bloodstream-infections, lower respiratory tract infections and urinary tract infections [35]. Carbapenems have become essential components of therapy in infections caused by extended-spectrum cephalosporin resistant strains, especially for vulnerable patient groups, such as children, pregnant women and the elderly [1–4, 36]. Although intrinsic resistance to carbapenems has been previously described in *Stenotrophomonas maltophilia* (conferred by the metallo-β-lactamase L1), acquired carbapenem-resistance (through mutational events or via horizontal gene transfer) has emerged as a significant clinical problem in the last 15–20 years [37, 38]. The spread of carbapenem-resistant strains in nosocomial settings, where MDR Gram-negative bacteria are already endemic, is especially daunting [39]. These pathogens may acquire resistance determinants, conferring resistance for multiple antibiotic classes, leading to virtually untreatable infections [40]. CP-CRE species have now been reported worldwide; carbapenemases have mainly been found in *K. pneumoniae*, and to a much lesser extent in *E. coli* and other members of the Enterobacterales order [41]. Based on estimation of the Centers for Disease Control and Prevention (CDC), carbapenem-resistant Enterobacterales species may be causative agents in up to 9,000 infection and 600 excess deaths per year in the US [42]. Similarly, the Burden of Antimicrobial Resistance Collaborative Group estimated that in Europe, around 16,000 infections and 2000 attributable deaths occurred in 2015 due to carbapenem-resistant *K. pneumoniae* [43]. The distribution of carbapenemase-producing isolates worldwide has been recently summarized by the Center for Disease Dynamics, Economics and Policy database: according to this database, $bla_{\text{OXA-48-like}}$ is endemic in Africa, $bla_{\text{OXA-48-like}}$, bla_{NDM}, bla_{KPC} and bla_{IMP} in Asia, bla_{KPC} in the American Continent and $bla_{\text{OXA-48-like}}$, bla_{VIM}, bla_{KPC} and bla_{IMP} in Europe [44]. Based on the data of the ECDC Surveillance on Antimicrobial Resistance in 2017 and 2018, Greece had the highest levels of carbapenem-resistant isolates (64.7 and 63.9% for *K. pneumoniae* and 1.6 and 2.0% for *E. coli*), followed by Italy (26.8 and 29.7% for *K. pneumoniae*) and Romania (22.5 and 29.5% for *K. pneumoniae*) [45]; this is not surprising, as Greece has been considered the epicenter of CP-CRE strains on the continent, where the spread of these strains usually showed a south-to-north gradient [37, 38]. In Hungary, the ratio of carbapenem-resistant *E. coli* strains was <0.1% for both years, while for *K. pneumoniae*, the percentage of relevant isolates was still very low (0.2 and 0.3%, respectively) [23, 42].

The costs, turnaround-time, accuracy, the information provided by these screening and phenotypic tests and the expected output (clinical, local or regional epidemiology or national/international surveillance) all need to be taken into consideration when opting for a carbapenemase-detection strategy [46]. While some test may be suitable for local microbiology laboratories for rapid therapeutic decisions, others will only be available to reference laboratories. The phenotypic detection of carbapenemases is hindered by their diversity, the pronounced differences in hydrolytic ability and their frequent co-occurrence with other resistance mechanisms influencing β-lactam resistance [47, 48]. For example, in strains with ESBLs or AmpC-hyperproduction, coupled with porin loss and/or efflux pump-overexpression, a similar phenotype may be observed to carbapenemase-production in phenotypic tests [47, 48]. On the other hand, some carbapenemase-enzymes do not confer high MICs to the isolates, therefore there is a risk that they will be missed by disk diffusion/gradient tests [49]. In addition, the local epidemiology of carbapenemases also influences the phenotypic tests employed, based on the sensitivity of these tests for the specific enzymes [16, 18]. The advantage of inhibitor tests is that they may be used to distinguish between different types (i.e. serine or metallo-β-

Table 2. Calculated predictive power of the individual screening and verification tests employed the study

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Modified cloverleaf (Hogde) test	94.7	91.4	85.7	96.9
chromID CARBA SMART	100	96.9	94.7	100
Modified carbapenem inactivation method (mCIM)	100	100	100	100

lactamases) or enzymes [47, 48]. The poor sensitivity and specificity of the modified cloverleaf (Hodge) test has been documented previously: false-positive results are common in other β-lactamase (ESBL or AmpC) producers, with or without porin mutations or efflux pumps, while some types of carbapenemases ($bla_{\text{OXA-48-like}}$ and bla_{NDM}-types, although the introduction of Zn^{2+} to the culture media results in increased sensitivity for the latter type) have shown false-negative results [49]. Several modifications and adjustments have been published with the aim of improving the detection parameters of this assay [50]. The advantage of screening media (e.g., chromID CARBA SMART utilized in this study) is that they are ready-to-use, assistant-friendly and ease of interpretation and sensitivity/specificity of this method has been demonstrated to be around 96–100/97–100% in several studies [26, 51]; nevertheless, the verification of culture results with additional phenotypic tests is recommended. The modified carbapenem-inactivation method (mCIM) is easy-to-perform and the panels can be prepared in-house; this assay has also demonstrated sensitivities/specificities ≥98% in many publications [27, 52]. The use of the CarbaNP confirmatory test (and the numerous iterations of this method) is becoming more and more widespread: the method is based on the direct detection of carbapenem hydrolysis, and similarly to the mCIM test, both commercial and in-house panels are available to use [53]. Nonetheless, molecular methods (predominantly single or multiplex PCR-based, with results in 2–6 hours) remain the reference for identification and differentiation of carbapenemases and this may be followed by sequencing, if available [18, 54]. Their cost and the need for trained technicians however, are significant drawbacks.

While there has been some developments in the introduction of new β-lactam/non-boronic or boron-based β-lactamase-inhibitor combinations (e.g., ceftolozane/tazobactam, ceftazidime/avibactam, meropenem/vaborbactam) in therapy for Class A carbapenemases, we currently do not possess adequate β-lactam-therapy for metallo-β-lactamase-producing (Class B) strains [55]; these Zn^{2+}-dependent enzymes are inhibited by metal chelators, such as EDTA or dipicolinic acid, but no clinically-relevant metallo-β-lactamase-inhibitor has been identified thus far [56]. An additional therapeutic concern is the possibility of strains possessing more than one type of carbapenemase, eliminating β-lactams as potential therapeutic options [56]. Carbapenemases in Enterobacterales are mostly plasmid-encoded and are often associated with resistance-determinants to other antibiotics, especially for fluoroquinolones and aminoglycosides [57]. Colistin is nephrotoxic and neurotoxic polycationic peptide, which is usually considered as a last-resort agent in MDR Gram-negative infections [58]; in our study, all of the tested isolates were susceptible. Nonetheless, the increasing frequency of reports on colistin-resistance in Enterobacterales and non-fermenters is a serious concern; in the EuSCAPE Survey (European survey of carbapenemase-producing *Enterobacteriaceae*) 28.3% of CRE isolates were also colistin-resistant [59]. TIG resistance was observed in 32% of isolates; this agent has been introduced with great promise in the therapy of MDR Gram-negative infections, however, the black-box warning issued by the FDA (due to increased overall mortality in TIG-treated patients) and low tissue penetration and serum-levels, it did not live up to the expectations. The use of intravenous FOS in the therapy of MDR Gram-negative bacteria has been extensively reported as a promising alternative [60]; nevertheless, 74% of our isolates tested were resistant to this antibiotic. In a Turkish study, the susceptibilities of CP-CRE isolates were assessed and the authors reported 76.2% of isolates as COL-resistant and 67.4% as FOS-resistant [26]. On the other hand, the literature is scarce on the relevance of SXT-therapy for CP-CRE infections: in our study, 48% of isolates were still susceptible to SXT; in the CRACKLE-1 cohort study in the US, 29% of pathogens in CP-CRE infections were susceptible to SXT and one-third of these affected patients were successfully treated with the drug [61]. In contrast, in a survey involving $n = 181$ CP-CRE isolates, Baran et al. showed that SXT-resistance in *E. coli* and *K. pneumoniae* was 61 and 55%, respectively [62]. CP-CRE isolates have very few or no therapeutic options (depending on resistance rates to ancillary antibiotics) available. Thus, their optimal monitoring and rapid detection for therapeutic, infection control and epidemiological purposes is needed. The findings of our study indicate that out of the 50 suspected isolates, presence of carbapenemase-genes verified in 18 strains, and the phenotypic methods utilized (especially the chromogenic media for screening and mCIM for verification) were appropriate to use in our settings. Nevertheless, every laboratory and/or healthcare institution needs to pre-test various testing methods and consult the national reference laboratory to select for adequate detection methods for carbapenemase-producers.

Conflict of interest: The authors declare no conflict of interest, monetary or otherwise.

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