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Emergence of Carbapenemase-producing *Enterobacteriaceae*, a Public Health Threat: a Romanian Infectious Disease Hospital Based Study

Emergența *Enterobacteriaceaelor* producătoare de carbapenemaze, o amenințare pentru sănătatea publică: un studiu realizat într-un spital românesc de boli infecțioase

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Abstract

Introduction: Hospital-acquired infections caused by *Enterobacteriaceae* producing different types of carbapenem-hydrolyzing enzymes are now commonly observed and represent a great limitation for antimicrobial therapy. The purpose of the study was to evaluate the emergence of carbapenem-resistant *Enterobacteriaceae* among the strains isolated from hospitalized patients to the National Institute of Infectious Diseases, Bucharest (NIID) and the identification of different types of carbapenemases, using phenotypic methods.

Materials and methods: Between January - June 2014, 587 strains of *Klebsiella pneumoniae*, *Enterobacter* species and *E.coli* were isolated from various clinical specimens. We were included all non-susceptible strains to carbapenems, according to EUCAST 2014 clinical breakpoints, as determined by using microdilution MicroScan Panels (Siemens Healthcare Diagnostics). The modified Hodge test (MHT) was performed as phenotypic confirmatory test for carbapenemase production according to CLSI guidelines and the combination disk test (KPC, MBL, OXA-48 Confirm kit, Rosco Diagnostica) according to EUCAST guidelines.

Results: A total of 45 non-repeat *Enterobacteriaceae* (32 strains *Klebsiella pneumoniae*, 5 strains *E.coli*, 8 strains *Enterobacter* spp) were identified as non-susceptible to one or more carbapenems (93,33% ertapenem, 53,33% meropenem, 48,88% imipenem). Most strains were isolated from urine (75,55%). MHT was positive in 55,6% (25/45) of carbapenem-resistant strains; in 24 cases the carbapenem-hydrolyzing enzyme was identified as: OXA-48-like (n=16), KPC (n=4), MBL (n=1), KPC + MBL (n=2) and MBL + OXA-48-like (n=1). All carbapenemase-positive strains were 100% resistant to 3rd and 4th generation cephalosporins, showing less resistance to tigecycline (12.5% resistant and 25% intermediate), colistin (37.5%) and fosfomycin (41.6%).

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Conclusion: During 6 months period, there were isolated 7,66% (45/587) carbapenem-resistant Enterobacteriaceae (*K. pneumoniae* 21,47%, *E. coli* 1,23%). Twenty four strains were carbapenemase-producers. The most frequent carbapenemase isolated in our study was OXA-48-like.

Keywords: Enterobacteriaceae, resistance, carbapenemase

Rezumat

Introducere: Infecțiile produse de Enterobacteriaceae producătoare de carbapenemaze sunt tot mai frecvent întâlnite în practica medicală și limitează serios antibioticoterapia. Am evaluat emergența enterobacteriilor rezistente la carbapeneme din tulpinile izolate de la pacienții internați în Institutul Național de Boli Infecțioase (INBI) din București și identificarea diferitelor tipuri de carbapenemaze, utilizând metode fenotipice.

Materiale si metode: În perioada ianuarie-iunie 2014 au fost izolate 587 tulpini de *Klebsiella pneumoniae*, *Enterobacter species* și *E.coli* din diferite prelevate clinice. În studiu au fost incluse toate tulpinile rezistente la unul sau mai multe carbapeneme conform indicațiilor din EUCAST 2014.

Testul Hodge modificat (MHT) a fost efectuat pentru confirmarea fenotipică a producerii de carbapenemază conform ghidului CLSI, iar combinația de discuri Rosco (KPC, MBL, OXA-48 Confirm kit, Rosco Diagnostica) s-a utilizat conform ghidului EUCAST în același scop.

Rezultate: Un total de 45 tulpini non-duplicate de Enterobacteriaceae (32 tulpini *Klebsiella pneumoniae*, 5 tulpini *E.coli*, 8 tulpini *Enterobacter spp.*) au fost identificate ca rezistente sau intermediare la unul sau mai multe carbapeneme (93,33% ertapenem, 53,33% meropenem, 48,88% imipenem). Majoritatea tulpinilor au fost izolate din urină (75,55%). MHT a fost pozitiv în 55,6% (25/45) dintre tulpinile rezistente la carbapeneme; la 24 dintre ele a fost identificată enzima care hidrolizează carbapenemele: tip OXA-48 (n=16), KPC (n=4), MBL (n=1), KPC + MBL (n=2) și MBL + OXA-48-like (n=1). Toate tulpinile producătoare de carbapenemază au fost 100% rezistente la cefalosporinele de generația a 3-a și a 4-a, cea mai mică rezistență fiind la tigeciclină (12,5% rezistent, 25% intermediar), colistin (37,5%), fosfomicină (41,6%).

Concluzii: Într-o perioadă de 6 luni s-au izolat 7,66% (45/587) Enterobacteriaceae rezistente la carbapeneme (*K. pneumoniae* 21,47%, *E. coli* 1,23%). Dintre acestea aproximativ jumătate au fost producătoare de carbapenemază. Cel mai frecvent tip de carbapenemază detectat a fost OXA-48.

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Introduction

First carbapenems were introduced in Romania in 2001 to treat patients having infections with ESBL-producing Enterobacteriaceae, hospitalized in intensive-care units and infectious diseases wards. From 2009 these antibiotics were largely used in others medical wards, since the prevalence of ESBL-producing strains increased and they became important in the treatment of health-care associated and severe community-acquired infections. Western countries were using earlier carbapenems and specific carbapenem resistance was first reported more than 20 years ago (1).

Carbapenem resistance in Enterobacteriaceae may be caused by one of the following mechanisms: (a) extended spectrum beta-lactamases (ESBL) or cephalosporinases (AmpC) combined with a decreased permeability due to loss or alteration in outer membrane porins (2); (b) carbapenemase production (3). The need to distinguish between mechanisms of carbapenem resistance is determined by the evidence that the carbapenemase-producing strains are likely resistant to all carbapenems and other beta-lactam agents and they are carried on self-conjugative plasmids, leading to rapid spread, especially in hospital settings. (4) The plasmids carrying carbapenemase genes could be carriers for oth-

er resistance determinants and identification of carbapenemase-producing strains is important for epidemiological reasons and public health potential threats. Detection of carbapenem resistance mechanisms seems to have limited clinical value; the standard of care is to report susceptibility and to use carbapenems with results “as tested”, at least with newer clinical breakpoints. (5)

The aim of our study was to determine to which extent the Enterobacteriaceae (*Klebsiella pneumoniae*, *Escherichia coli* and *Enterobacter spp.*) isolated at the National Institute of Infectious Diseases “Prof. Dr. Matei Bals”, Bucharest, are carbapenem resistant and which type of resistance mechanism has been involved (including characterization of main types of carbapenemases).

Material and methods

Between January 1st and June 30 2014, 587 non-duplicate isolates of *E. coli* (n=404), *K. pneumoniae* (n=149) and *Enterobacter spp.* (n=34) were isolated from various clinical specimens from patients admitted to the National Institute of Infectious Diseases “Prof. Dr. Matei Bals”. Ready to use culture media (BioMerieux, France) such as blood agar (COS), chocolate agar (Polivitex) and CLED agar were used for isolation of strains. All plates were incubated overnight at 37°C and identification of etiological agents was performed using an automated MicroScan Walk Away 96 Plus (Siemens Healthcare Diagnostics, USA) system.

Initial antibiotic susceptibility testing was performed using microdilution MicroScan Panels and interpretation as susceptible, intermediate and resistant was done according to the EUCAST 2014 guidelines. Further studies were performed on all non-susceptible strains to one or more carbapenems according to the EUCAST 2014 clinical breakpoints (MIC >0.5 µg/ml for

ertapenem and >2 µg/ml for meropenem and imipenem).

The modified Hodge test (MHT) was performed as a phenotypic confirmatory test for carbapenemase production according to CLSI guidelines, on Mueller Hinton agar (BioMerieux, France) using 10 µg ertapenem disks (Oxoid, USA). For all carbapenem resistant strains the combination disk test (KPC, MBL and OXA-48 Confirm kit, Rosco Diagnostica, Denmark) was used as a phenotypic confirmatory test for carbapenem hydrolyzing enzyme (following the manufacturer’s instructions), according to EUCAST guidelines (Version 1.0 December 2013). The Rosco kit has in composition the following cartridges of tablets: meropenem (10 µg), meropenem (10 µg) + phenylboronic acid (KPC and AmpC inhibitor), meropenem (10 µg) + cloxacillin (AmpC inhibitor), meropenem (10 µg) + dipicolinic acid (MBL inhibitor) and temocillin (30 µg). Identification of specific carbapenemase was performed according to the manufacturer’s instructions.

All carbapenem-resistant strains were tested for ESBL and AmpC production. The ESBL production was signaled by the automated system (MicroScan) and confirmed by plating the strains on ESBL Brilliance Agar (Oxoid, UK) and/or by disk diffusion test according to EUCAST guidelines. AmpC production was determined by using the *Etest* (BioMerieux, France) with cefotetan and cefotetan + cloxacillin.

For all carbapenemase-producing strains the susceptibility to colistin was verified using the *Etest*.

Statistical analysis: “Z” test was used to compare two independent proportions and was considered statistically significant at $p \leq 0.05$.

Results

Forty-five non-duplicate isolates of *Enterobacteriaceae* (45/587=7.66%) were non-sus-

ceptible to one or more carbapenems as follows: 93.33% ertapenem, 53.33% meropenem, 46.66% imipenem (Table I). These were mostly isolated from urine (34/45=75.55%), followed by those from sputum and bronchial lavage (4/45=8.88%), wounds (3/45=6.66%), blood (2/45=4.44%) and central venous catheters (2/45=4.44%). The carbapenem-resistant strains isolated were: *K. pneumoniae* (32/149=21.48%), *Enterobacter spp.* (8/34=23.52%) and *E. coli* (5/404=1.24%) (Table II). The risk of being carbapenem-resistant was significantly higher for *K pneumoniae* when compared with *E coli*: $z=8.451$, $p<0.0002$ and for *Enterobacter spp* versus *E. coli*: $z=7.356$, $p<0.0002$.

The modified Hodge Test was positive in 55.6% (25/45) of the tested strains. Production of carbapenemase (24/45=53.33%) was detected by the combination disk test (Rosco Diagnostica) as follows: 16 strains OXA-48-like, 4 strains KPC, 1 strain MBL and 3 strains were detected as double carbapenemase producers: 2 KPC+MBL and 1 MBL+OXA-48-like.

K. pneumoniae and *Enterobacter spp* were more frequently carbapenemase producers than *Escherichia coli*, with $z=0.00021$ and $p<0.0001$. As previously known, the production of carbapenemase is associated with other resistance determinants, so these strains are resistant to almost all beta-lactams and non-beta-lactams, which leads to multidrug- and pan-drug resistant isolates (Figure 1). Lower antimicrobial resistance for carbapenemase-producing isolates was shown for amikacin (29.16%), followed by colistin and tigecyclin (37.50% each) and fosfomycin (41.66%).

All carbapenem-resistant strains (45/45) were ESBL-producers and 33.33% (15/45) produced both ESBL and AmpC. Among the carbapenemase-producing strains 54.16% (13/24) produced both ESBL and AmpC. The co-production of AmpC and ESBL was found in only 19.04% (4/21) of the non-carbapenemase-producing strains.

Almost all AmpC negative carbapenemase-producing strains were OXA-48-like

Table I. Results of the antibiotic susceptibility testing for carbapenems in carbapenem-resistant strains (n=45)

Interpretation Antibiotic	Susceptible (No. of strains)	Intermediate (No. of strains)	Resistant (No. of strains)
Ertapenem	3	6	36
Meropenem	21	6	18
Imipenem	23	7	15

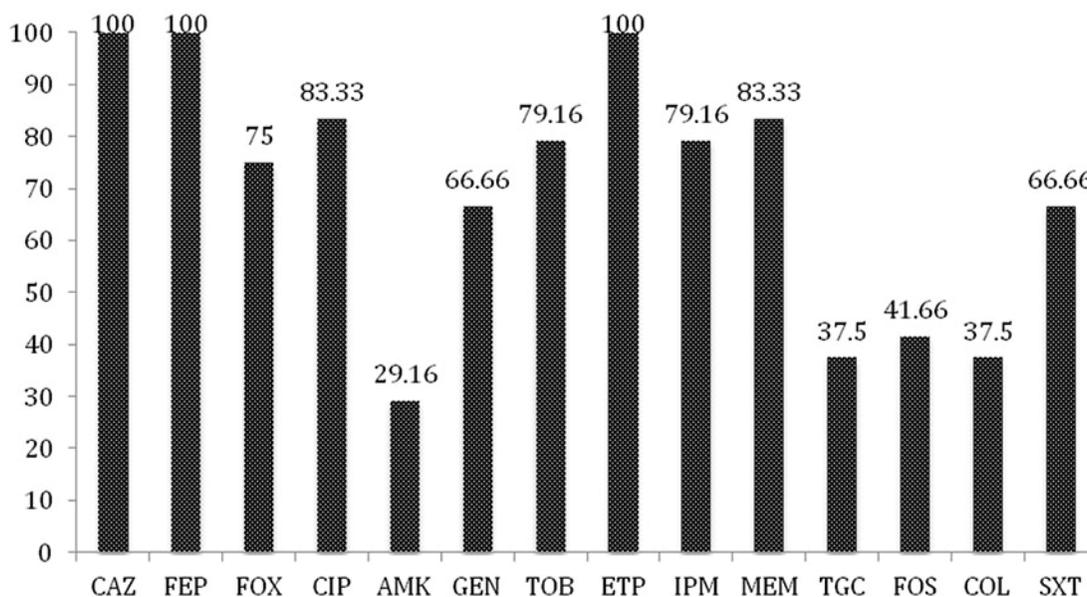
Interpretation criteria (EUCAST guideline 2014):

- ertapenem: susceptible when MIC ≤ 0.5 $\mu\text{g/ml}$ and resistant when MIC > 1 $\mu\text{g/ml}$;

- meropenem and imipenem: susceptible when MIC ≤ 2 $\mu\text{g/ml}$, resistant when the MIC > 1 $\mu\text{g/ml}$;

Table II. *Klebsiella pneumoniae*, *Escherichia coli* and *Enterobacter spp.* isolated in 6 months

Microorganism	Carbapenem-susceptible	Carbapenem-resistant N (%)	Carbapenemase-positive strains	Total
<i>E. coli</i>	399	5 (1.23%)	4	404
<i>K. pneumoniae</i>	117	32 (21.47%)	15	149
<i>Enterobacter spp.</i>	26	8 (23.52%)	5	34
Total	542	45 (7.66%)	24	587



CAZ = ceftazidime, FEP = cefepime, CIP = ciprofloxacin, AMK = amikacin, GEN = gentamicin, TOB = tobramycin, ETP = ertapenem, IPM = imipenem, MEM = meropenem, TGC = tigecycline, FOS = fosfomicin, COL = colistin, SXT = trimethoprim-sulfamethoxazole

Fig. 1. Antibiotic resistance (%) of carbapenemase-producing Enterobacteriaceae (n=24)

(n=10), except one which was positive for both MBL and OXA-48-like carbapenemases.

Discussions

The classification and detailed properties of carbapenemases in *Enterobacteriaceae* have been extensively reported (4); they belong to three classes of beta-lactamases according to the Ambler classification: A, B (metallo- β -lactamases) and D (enzymes of the OXA-48 type). The most frequent carbapenemases produced by *Enterobacteriaceae* are KPC, VIM, IMP, NDM and OXA-48-like, and the prevalence varies among countries: KPC is frequently reported in USA, Greece, Israel but a wide dissemination was also shown recently in Italy (6); OXA-48 has a high prevalence in Turkey (7) and in Mediterranean countries (8, 9).

In a recent study on carbapenemase-producing *Enterobacteriaceae* in the Baltic countries and western Russia it was shown that only 77 out

of 9757 *K. pneumoniae* and *E. coli* strains were phenotypically carbapenem non-susceptible. (10) Compared to them, in our study the carbapenem-resistance is a concerning problem (37 out of 553). In the EARS-Net report for 2013, the population weighted-mean for *K. pneumoniae* resistant to carbapenems was 8.3%; the highest level of resistance being in Greece (59.4%), followed by Italy (34.3%) and Romania (20.5%). (11)

In a survey performed in European Union member states, EuSCAPE, most participants (national experts in this area) declared sporadic cases of carbapenemase-producing *Enterobacteriaceae*. There are scarce data for Romania, only sporadic cases per year being reported before 2012, but a constant increase since then is mentioned in international medical journals and reports. (8, 11)

The National Institute of Infectious Diseases "Prof. Dr. Matei Bals" is the main infectious disease hospital in Romania, having around 30000

admissions every year, from Bucharest and the southern region of Romania. Data obtained from this study describe a worrying situation. More than half of carbapenem-resistant strains produced carbapenemase and this result is not significantly different from that of a study from a different region of Romania where 9 out of 13 *Enterobacteriaceae* resistant to carbapenems were producing carbapenem-hydrolyzing enzymes (12); emergence of carbapenemase-producing strains seems to be present in various Romanian regions.

The combination disk test from Rosco identified single (n=21) and also double carbapenemase-producing strains (n=3), as previously published Miriagou et al. (13)

In case of the 21 carbapenem-resistant strains without evidence of carbapenemase production, porin deficiency in combination with ESBL production could be assumed as a cause of decreased susceptibility to carbapenems. Additionally, 4 strains were co-producing AmpC lactamases and ESBL.

The carbapenemase-producing strains were less resistant to colistin, tigecycline, fosfomicin besides amikacin and, as other studies revealed, these represent potential drugs of choice in treating infections caused by these microorganisms, but larger clinical studies are needed. (14, 15)

To conclude, identification of the increasing number of carbapenemase-producing *Enterobacteriaceae* is of great importance in order to evaluate the treatment with carbapenems in critically ill patients and for the epidemiology-control of health-care associated infections.

This study has some limitations: as described in literature, we might have lost some *Enterobacteriaceae* (less than 10%) that are resistant or have reduced susceptibility to carbapenems due to the commercial system used (MicroScan panels for antibiotic susceptibility testing) designed to identify isolates with MIC over clinical break-points and not over the epidemiological cut-off.

(16) Also, further molecular studies are needed in order to determine the carbapenemase types for all carbapenemase-producers and to compare the results with those from other countries since there are few data at this time for Romania.

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Potential conflicts of interest

All authors: no conflict.

All authors had equal contribution in preparing this article

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