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Carbapenemase Producing Enterobacterales in Romania: Investigating the Origins

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Abstract

Carbapenemase-producing Enterobacterales (CPE) have emerged and spread in Romania since 2010. According to the reports of the EuSPACE (European survey of carbapenemase-producing Enterobacteriaceae) the epidemiological stage of the CPE expansion in Romania has shifted from sporadic occurrence in 2013 directly to inter-regional spread in 2014-2015. In this study we aimed to provide data from the timeframe when the dissemination of the carbapenemase genes in Romania began, by retrospectively analyzing CPE strains in a tertiary care university hospital. During the period of November 2012 – October 2013 we found 107 CPE (8.78%) out of 1219 non-duplicate Enterobacterales strains. 26 isolates of various Enterobacterales species carried bla_{NDM-1}, 83 Klebsiella pneumoniae strains were positive for $bla_{OXA-48-like}$ and 2 of these co-harboured bla_{NDM-1}. The increased incidence of OXA-48 producing K. pneumoniae was linked to a two-peaked hospital outbreak during February and May 2013. The percentage of 24.3% of NDM-1 producers was alarming due to the diversity of involved species and the higher resistance levels to carbapenems compared with $bla_{OXA-48-like}$ gene carriers. Plasmid replicon typing revealed a great diversity of plasmids in NDM-1-positive strains, belonging to incompatibility groups A/C, FII, FIIk, HI2, L and M. The strong connection between certain plasmid groups and host species suggests that the transfer of broad host-range plasmids through conjugation does not play the main role in the successful spread of bla_{NDM-1} among Enterobacterales species.

Keywords: *carbapenem resistance, carbapenemase genes, plasmid, NDM-1, minimum inhibitory concentration*

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Introduction

Over the last decade, the spread of carbapenemase-producing *Enterobacterales* (CPE) has been recognized as a major threat to healthcare worldwide (1, 2, 3). Romania participated in the "European survey of carbapenemase-producing *Enterobacteriaceae* (EuSCAPE)" project launched by the European Centre for Disease Prevention and Control (ECDC) in 2012 and also several studies have been published concerning the CPE (4, 5, 6), nevertheless, there are still insufficient data available for a comprehensive description of the molecular epidemiology and characterization of these strains in our country.

The emergence of CPE in Tirgu-Mures Emergency Clinical County Hospital dates back to 2010. The first nine isolates recovered during the period between January 2010 and September 2012 harboured $bla_{\text{NDM-1}}$ or $bla_{\text{OXA-48-like}}$ genes or both (7). This research aims to follow on from that previous study by testing for CPE isolates and the presence of $bla_{\text{NDM-1}}$, $bla_{\text{OXA-48-like}}$, bla_{KPC}, as well as bla_{VIM}, bla_{IMP}, bla_{GIM} and bla_{SPM} genes for the following one year period between November 2012 and October 2013. It investigated whether or not there was a significant change in the prevalence of NDM-1 or OXA-48 producing *Enterobacterales* or an emergence of other types of carbapenemases. It also evaluated the resistance level to carbapenems of each CPE strain, correlating the minimal inhibitory concentration (MIC) values with the types of the carbapenemase genes identified. Moreover, it analyzed the plasmid profile of the $bla_{\text{NDM-1}}$-positive strains in order to identify possible pathways through which this resistance gene managed to spread between different bacterial species.

We consider that the CPE strains isolated in this specific period of time are of a great importance since the occurrence of CPE was only sporadic and the dissemination of the carbapenemase genes in Romania, especially of $bla_{\text{NDM-1}}$, had just begun.

Materials and methods

Between November 2012 and October 2013, all *Enterobacterales* strains isolated from any type of clinical or screening sample referred to the Microbiology Laboratory of the Tirgu-Mures Emergency Clinical County Hospital and found non-susceptible to any of the three tested carbapenems: ertapenem, meropenem and imipenem by routine antibiotic susceptibility testing were screened for carbapenemase production. For this purpose the modified Hodge test (8) was used. All tested strains were conserved by freezing at -70°C in 20% glycerol. Molecular analysis of the strains was performed according to protocols adapted after others (9) via two multiplex PCR (polymerase chain reaction) assays targeting $bla_{\text{NDM-1}}$, $bla_{\text{OXA-48-like}}$, bla_{KPC}, respectively bla_{VIM}, bla_{IMP}, bla_{GIM} and bla_{SPM} genes. All strains were further investigated to determine the MIC of imipenem, meropenem and ertapenem by the broth microdilution method. The results were interpreted according to the EUCAST Clinical Breakpoint Table v. 7.0. The statistical analysis was performed with GraphPad software.

Plasmid detection was performed on NDM-1 positive strains. Molecular identification of plasmids was carried out with a PCR based replicon typing method (PBRT 2.0 kit, Diatheva, Italy) according to the manufacturer's instructions.

Results

A total of 1219 non-duplicate *Enterobacterales* strains were isolated and tested during the routine workup of all clinical and screening samples. Of these strains, 119 (9.8%) were resistant to at least one carbapenem. The species distribution was as follows: 96 *Klebsiella pneumoniae*, 9 *Serratia marcescens*, 9 *Enterobacter cloacae*, 1 *Escherichia coli*, 1 *Serratia liquefaciens*, 1 *Pro-*

teus mirabilis, 1 *Providencia stuartii* and 1 *Serratia plymuthica*. 118 tested as Hodge positive, of which 107 harboured carbapenemase genes while in 12 strains none of the tested genes were detected.

The distribution of the 107 samples according to their type: 54 respiratory tract specimens, 15 specimens from skin and soft tissue infections, 14 urine, 9 blood, 7 stool, and 8 other specimens (indwelling catheter tips, bile, cerebrospinal fluid, pleural fluid, peritoneal fluid). Of these 8 were screening samples: 7 stool specimens and 1 pharyngeal swab.

Twenty-six isolates carried bla_{NDM-1}: 9 *E. cloacae*, 7 *K. pneumoniae*, 8 *S. marcescens*, 1 *P. mirabilis* and 1 *S. liquefaciens*. Eighty-three *Klebsiella pneumoniae* strains were positive for $bla_{OXA-48-like}$ and 2 of these co-harboured bla_{NDM-1}. No other *Enterobacterales* species were harbouring $bla_{OXA-48-like}$ and none of the CPE had bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{GIM} or bla_{SPM}.

The yearly distribution of the CPEs recovered from clinically significant samples is presented in Fig. 1.

In 11 strains with positive Hodge test none of the tested carbapenemase genes were detected and from all the carbapenem-resistant strains only one was Hodge-negative. This latter strain did not harbour any carbapenemase genes either.

All 119 strains were highly resistant to ertapenem, the MIC_{50} value being 256µg/ml. One non-CPE strain was susceptible to both meropenem and imipenem, 2 non-CPE strains were susceptible to imipenem and intermediate susceptible to meropenem. Further 48 strains were interpreted as being susceptible or intermediate susceptible to imipenem only, 46 of them harboured $bla_{OXA-48-like}$ and 2 were non-CPE strains.

The MIC values of the $bla_{OXA-48-like}$-positive, respectively the bla_{NDM-1}-positive CPE isolates were statistically compared. The resistance levels of the strains harbouring the bla_{NDM-1} gene were significantly higher ($p<0.0001$). The MIC distributions of the $bla_{OXA-48-like}$-positive and the

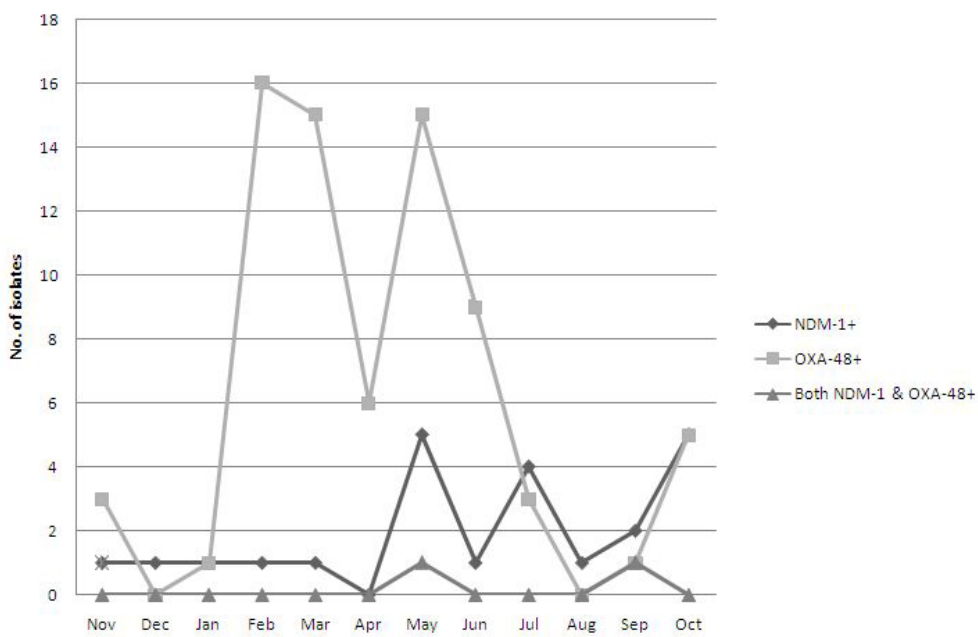

Fig. 1. Yearly distribution of CPE strains during one year period between November 2012 and October 2013 isolated from clinically significant samples

bla$_{NDM-1}$-positive strains are presented in Fig. 2, the MIC_{50} and MIC_{90} values are presented in Table 1.

The two strains harbouring both resistance genes showed high-level resistance to imipenem (128 µg/ml) and meropenem (256 µg/ml) and a relatively lower resistance level to ertapenem (64 µg/ml), MIC values of the two strains being identical.

Plasmid replicon typing was performed on 23 out of the 26 NDM-1 producing strains (3 strains were lost during storage: one *P. mirabilis* and two *E. cloacae* strains). One out of 8 *E. cloacae* strains was positive for the replicon FII denoting the presence of a plasmid belonging to IncF family, the other 7 strains harboured replicon HI2 belonging to IncHI family and 6 of them were positive for IncM as well. Besides HI2 and M, one strain was positive also for A/C and R replicons. In one *K. pneumoniae* strain no plasmid replicon was detected, the other five strains harboured an R replicon, unassigned to any known incompatibility group. Four strains of these co-harboured one or two replicons associated specifically to *Klebsiella spp*., belonging to plasmids from the IncF complex: FII, FIIk - a divergent FII replicon, and FIB KN – the replicon identifying the most common plasmid found in *Klebsiella spp*., the pKpN3. One strain was positive for the L replicon as well. All 9 strains of *Serratia spp.* were positive for the L replicon and one stain of *S. marcescens* co-harboured the replicons HI2 and A/C.

Discussion

In 2013 we characterized the first emerging CPE strains in Romania isolated from clinical samples collected between January 2010 and September 2012 (7). The incidence of CPE in that time period was less than 1% of the total *Enterobacterales* isolates. Similar incidence was reported by others (10) for the same time interval and the same geographical area, namely the central part of Romania. A more recent study conducted in the eastern region of the country revealed a still relatively low incidence of CPE about 2% (11), however, Rafila et al. (5) reported an incidence of more than 7.5% from the southern region of Romania. In our present study we found an incidence of carbapenem resistant *Enterobacterales* of 9.8%, of which 8.78% carried carbapenemase genes. The increased overall incidence was linked to a two-peaked hospital outbreak of OXA-48 producing *K. pneumoniae* during February and May 2013. After infection control measures being applied, the incidence dropped in the following months.

No clustering was observed in the incidence of NDM producing *Enterobacterales* during this period. The predominance of the OXA-48-positive strains was consistent with data reported by others (4,5), this type of carbapenemase being the most rapidly spreading carbapenemase throughout Europe (12). Still, the percentage of 24.3 of NDM-1 producers from all the CPE was alarming due to the diversity of the spe-

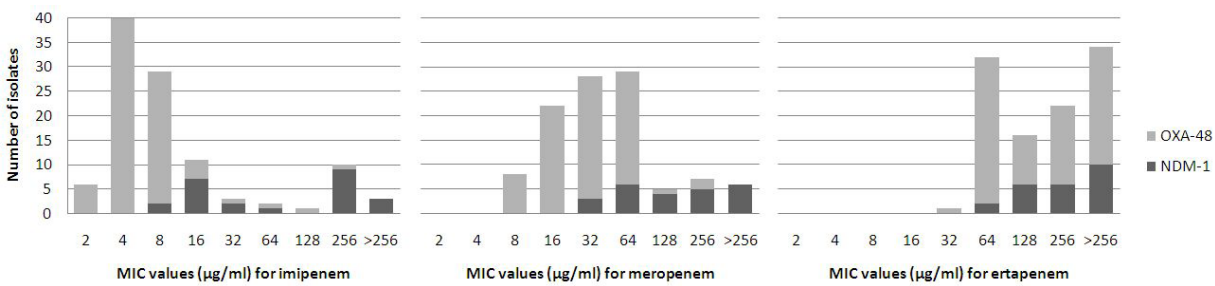

Fig. 2. MIC distributions of the *bl*$_{aOXA-48-like}$-positive (N=81) and the *bla*$_{NDM-1}$-positive strains (N=24)

Table 1. The MIC_{50} and MIC_{90} values for imipenem, meropenem and ertapenem in case of $bla_{OXA-48-like}$-positive (N=81) and the bla_{NDM-1}-positive strains (N=24)

Antibiotic	Carbapenemase genes	Range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
Imipenem	$bla_{OXA-48-like}$	2 - 256	4	8
	bla_{NDM-1}	8 - >256	64	>256
Meropenem	$bla_{OXA-48-like}$	8 - >256	32	64
	bla_{NDM-1}	32 - >256	128	>256
Ertapenem	$bla_{OXA-48-like}$	32 - >256	128	>256
	bla_{NDM-1}	64- >256	256	>256

cies harbouring the bla_{NDM-1} gene, which underlines the high interspecies transferability of this gene (13). Moreover, the high resistance level to all carbapenems limits the efficacy of carbapenem-based combination therapy, which is MIC-dependent (14). Due to the small number of isolates of only 26 NDM-positive strains the MIC_{50} and MIC_{90} values may not be fully representative, but they were clearly higher than the MIC of the OXA-48-positive strains. KPC-positive strains were not detected among our strains. In Romania, the first *K. pneumoniae* strains harbouring bla_{KPC} were described by Lixandru et al. (4) during a survey conducted in a later period, between November 2013 and April 2014. Since then, only a few studies have reported KPC-positive CPE in Romania (4,15).

In 2016 Timofte et al. (6) analyzed a collection of strains isolated from our hospital and described the probable transfer of the bla_{NDM-1} gene via IncFIIs plasmid between *S. marcescens* and *K. pneumoniae* strains, while bla_{OXA-48} was detected only in *K. pneumoniae* strains associated with an IncL type plasmid. The countrywide dissemination of *P. stuartii* strains harbouring bla_{NDM-1} genes on IncA/C plasmids, described recently (16), raised the question whether this broad host-range conjugative plasmid played a role in the spread of carbapenemase genes already in the first years of occurrence of CPE in Romania or it emerged later. Surprisingly, we detected the A/C replicon in just one *E. cloacae* and one *S. marcescens* strain and in contrast to the findings of Timofte et al. (6) one of the 2 double-positive (NDM-1 and OXA-48) *K. pneumoniae* strains

Table 2. Plasmid replicons identified in NDM-1-positive CPE strains

Isolate	Plasmid replicon	Genotype
K. pneumoniae	Negative	NDM
K. pneumoniae	R	NDM
K. pneumoniae	R, FIIK, FII	NDM
K. pneumoniae	R, FIIK, FII	NDM+OXA
K. pneumoniae	R, FIB KN	NDM
K. pneumoniae	R, L, FIIK, FIB KN	NDM+OXA
E. cloacae	FII	NDM
E. cloacae	HI2	NDM
E. cloacae	HI2, M	NDM
E. cloacae	HI2, M	NDM
E. cloacae	HI2, M	NDM
E. cloacae	HI2, M	NDM
E. cloacae	HI2, M	NDM
E. cloacae	HI2, M, A/C, R	NDM
S. liguefaciens	L	NDM
S. marcescens	L	NDM
S. marcescens	L	NDM
S. marcescens	L	NDM
S. marcescens	L	NDM
S. marcescens	L	NDM
S. marcescens	L	NDM
S. marcescens	L	NDM
S. marcescens	L	NDM
S. marcescens	L, HI2, A/C	NDM

did not contain the IncL plasmid. A close relationship between the plasmid groups and the host species can be observed, the host-specificity of plasmids being well known (17), however, there is a discrete overlapping of the replicon types detected in different species: HI2 and A/C in *E. cloacae* and *Serratia spp*., L replicon in *K. pneumonia* and *Serratia spp*. and the R replicon detected both in *K. pneumoniae* and *E. cloacae*. The detection of broad host-range plasmids (IncL and IncA/C) and intermediate host-range plasmid (IncH) might suggest the possibility of interspecies transfer of the bla_{NDM-1} gene but it does not explain the success rate of its dissemination. The IncA/C plasmid can reside in virtually any *Enterobacterales* species and it is the most common plasmid associated with the bla_{NDM-1} gene (18), but our findings suggest that bacteria rather show a preference toward certain plasmid types. It seems that the transfer of plasmids through conjugation does not play the main role in the dissemination of this resistance gene, but rather the transposition of the gene from one plasmid type to another, as proposed by Wailan et al. (18)

The interim results of the EuSPACE project (19), based on the self-assessment by national experts in February - March 2013, showed that, in Romania, the occurrence of the CPE was only sporadic (corresponding to epidemiological stage 1). This stage remained unchanged between 2010-2013, however, according to the final report of the EuSPACE project (20), the epidemiological stage of CPE spread in Romania shifted directly to stage 4 (corresponding to inter-regional spread) in 2014-2015. The increase of carbapenem non-susceptibility rate in our hospital from under 1% to almost 10% within one year, along with the increased incidence of CPE-s up to almost 90% of the total carbapenem non-susceptible isolates, the large hospital outbreak of OXA-48 producing *K. pneumoniae* and the diversity of the species harbouring bla_{NDM-1} strongly suggests that there was an underestimation of the epidemiological stage in Romania in 2013. Similar conclusion was reached by Lixandru et al. (4) based on the results of their survey conducted between November 2013 – April 2014 in the southern, south-eastern, and north-eastern part of Romania allowing the updating of the epidemiological stages of several types of carbapenemases from 1 to 4. Both studies highlighted the shortcomings of the surveillance programme based on voluntary participation of a few laboratories only and drew attention to the necessity of more controlled and mandatory surveys. The findings of an active surveillance scheme conducted in three hospitals from different regions of Romania revealed great differences between the circulating carbapenemase-producer Gram-negative bacteria showing diversity both in the detected carbapenemase genes and the bacterial species which harboured them. Based on the published data about the CPE in Romania it becomes clear that isolated studies performed in different geographical areas of the country in different timeframes cannot provide a comprehensive overview of the situation, hence, the measures to be taken against the further spread of the CPE at national level cannot be established.

Our study has some limitations: being a retrospective study, some strains were lost during storage and data from a single hospital were analyzed. We described the molecular background of the resistance at the level of plasmids, but further tests, like genotyping, would be needed to fully characterize the genetic environment of CPE strains. Transconjugation and gene mobilization studies could provide useful information regarding the probable routes of dissemination of carbapenemase genes between different bacterial species and between different plasmid types.

Conclusions

Our study provided an insight into the diversity of plasmidic profiles of the emerging CPE

strains. The interspecies transfer of *bla*$_{NDM}$ could not be linked to one specific plasmid. Sharing of several plasmids by different species has been documented. However, it is obvious that the interactions between hosts-plasmids-genes need to be analyzed more in detail in order to fully understand the mechanisms behind the successful spread of these feared bacteria, the CPE.

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Author contributions

SM: methodology, investigation, resources, data curation, draft preparation, review and editing, visualization, project administration. KEV: methodology, investigation, review and editing. ES: conceptualization, methodology, review and editing, supervision.

Conflict of interest

None declared.

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