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CARBAPENEM RESISTANCE IN NON-FERMENTATIVE BACTERIAL SPECIES AND IN *ENTEROBACTERIACEAE* ISOLATES FROM HOSPITALIZED PATIENTS IN DIFFERENT HEALTH-CARE SETTINGS

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Abstract

Aim. *Carbapenem-resistant strains have been increasingly reported over the last few years. In this study we used laboratory records to determine the occurrence of carbapenem-resistant strains from hospitalized patients with emphasis on the comparative analysis of the incidence in various health-care settings.*

Materials and methods. *From January 2012 to November 2012 and from May 2013 to November 2013, we evaluated 566 strains (Acinetobacter spp., Pseudomonas aeruginosa, Escherichia coli, and Klebsiella spp.). All isolates were tested and analyzed according to their antibiotic resistance phenotypic pattern. Laboratory results were correlated with data regarding admission in different clinical wards.*

Results. *Among 566 isolates, 191 carbapenem-resistant or carbapenem-intermediate strains (33.74%) were detected. Non-fermentative species were the most prevalent carbapenem-resistant organisms, 80.62% of 191 carbapenem-resistant or carbapenem-intermediate strains isolated were Acinetobacter spp., and 17.27% of 191 were Pseudomonas aeruginosa. Apart from that, only 4 (2.09%) carbapenem-resistant Enterobacteriaceae (CRE) strains were identified. We identified 59.30% of 172 strains isolated from patients hospitalized in anesthesia and intensive care units non-susceptible to carbapenems. The main mechanism associated with carbapenem resistance could be the production of carbapenemase in combination with impermeability.*

Conclusions. *Our study demonstrates that infections with carbapenem-resistant strains are correlated with hospitalization in intensive care units. Our data showed a predominant carbapenem-resistant Acinetobacter spp. strain in intensive care units.*

Keywords: β-lactamase, carbapenems, antibiotic resistance, Gram-negative

Introduction

Nosocomial and community-acquired Gram-negative bacilli infections due to acquired carbapenemases are increasingly reported worldwide and the spread of carbapenem-resistant strains among hospitalized patients has become an increasing cause of concern [1,2]. This phenomenon has an important impact in the duration of hospitalization, mortality rate, and health care costs [3]. Carbapenems (imipenem, meropenem, doripenem, ertapenem) are frequently used as a last therapeutic option for the treatment of infections caused by multidrug resistant non-fermenters and extended spectrum β-lactamase (ESBL) *Enterobacteriaceae* [4,5]. Mechanisms associated with carbapenem resistance are complex and involve various genes. Impermeability and production of carbapenemases (enzymes that inactivates carbapenems) are the most important molecular mechanisms that mediate carbapenem resistance [6,7]. Impermeability due to the loss of OprD porin is the most common mechanism described in *Pseudomonas aeruginosa* [8,9]. A recent study rigorously demonstrated the involvement of transferable elements in the selection of carbapenem-resistant *Pseudomonas*

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aeruginosa. A novel insertion sequence IS*Pa*8 discovered in the genome of *Pseudomonas aeruginosa* has a critical role in the insertional disruption in porin gene *opr*D [10]. In *Klebsiella pneumonia*e there are two major outer membrane porins, OmpK35/36, involved both in carbapenem resistance but also in virulence. In this particular case, porins deficiency is associated with virulence decrease associated only with ertapemen resistance [11,12]. Another important mechanism involved in carbepenem resistance is the acquisition of gene-encoded carbapenemases. Furthermore, apart from transferable carbapenemases, it is well known that most important non-fermenters isolated from nosocomial infections (*Acinetobacter* spp. and *Pseudomonas aeruginosa*) have also chromosomal geneencoded AmpC-type cephalosporinase [13]. Functional and molecular characterization of carbapenemases, the most powerful beta-lactamases, is continuously reviewed due to the huge diversity of these enzymes [14,15,16,17]. Carbapenemases are β-lactamases belonging to molecular Ambler class A (penicillinase), class B (metalloenzymes involved in natural resistance), and class D (oxacillinases) [18,19,20]. Even though class D carbapenemses are almost exclusively found in *Acinetobacter* spp., there are some reports of *Pseudomonas aeruginosa* clinical isolates that produce these type of beta-lactamases [21,22]. Even though the amino acid sequences for some carbapenemeses are available, the source of the acquired carbapenemases remain unknown [23,24,25]. A recent study has shown a synergistic effect between the aztreonam and polyamines (spermine and spermidine) when carbapenem-resistant *Acinetobacter* spp. were tested [26]. A new challenge in the treatment of nosocomial infections arises from the emerging carbapenem-resistant strains. Antibiotic use is the main cause of evolution of antibiotic resistance and there is a real concern regarding the evolution of a resistance gene [27,28].

This study was undertaken to evaluate the extent of hospital-acquired infection with carbapenem-resistant strains in different clinical wards. The analysis of the data was limited to the comparison of the phenotypic patterns of four Gram-negative species: *Acinetobacter* spp., *Pseudomonas aeruginosa*, *Escherichia coli,* and *Klebsiella* spp. isolated from various clinical specimens.

Materials and methods

Bacterial strains and culture conditions

In this observational retrospective study we reviewed the microbiological data collected from the records of 540 individual patients hospitalized from January 2012 to November 2012 and from May 2013 to November 2013 in the County Emergency Clinical Hospital Cluj-Napoca. The patients were admitted in different hospital wards. Five hundred and sixty six strains (*Acinetobacter* spp., *Pseudomonas aeruginosa*, *Escherichia coli,* and *Klebsiella* spp.) were isolated. The collection included unique bacterial isolates and excluded duplicate isolates. Clinical strains were isolated from broncho-alveolar lavage, urine, peritoneal drainage, blood, wounds, catheter specimens, and other specimens. All specimens were inoculated on culture media: blood agar, MacConkey agar, Sabouraud media, and Chapman media. For urine samples CLED media was used as a selective culture media. All strains were identified according to their cultural appearance and their regular biochemical reactions.

Antimicrobial susceptibility

Non-fermentative isolates were tested for the following antibiotics: imipenem (10 mcg), meropenem (10 mcg), amikacin (30 mcg), gentamicin (10 mcg), ceftazidime (30 mcg), ciprofloxacin (5 mcg), cefoperazone/sulbactam (75/30 mcg), piperacillin/tazobactam (100/10 mcg), and colistin (10 mcg). *Enterobacteriaceae* isolates were tested for: imipenem (10 mcg), meropenem (10 mcg), amikacin (30 mcg), gentamicin (10 mcg), ceftazidime (30 mcg), ciprofloxacin (5 mcg), and cefoperazone/sulbactam (75/30 mcg). The new CLSI standards do not have criteria for cefoperazone/sulbactam. To interpret cefoperazone/sulbactam susceptibility, the zone of inhibition was compared with the breakpoints approved for cefoperazone [29,30,31]. Quality control strains *E.coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were tested. Only 43 strains (11 strains of *Acinetobacter* spp., three strains of *Pseudomonas aeruginosa*, 11 strains of *Escherichia coli,* and 18 strains *Klebsiella* spp.) were also tested using VITEK-2 automatic system (BioMerieux) and the European Committee on Antimicrobial Susceptibility Testing. EUCAST served as a reference procedure (http://www.eucast.org) [32]. According to CLSI recommendation, carbapenem-resistant or carbapenem-intermediate strains were considered as non-susceptible to carbapenems.

Ethical statement

All data collected in the present study were originated from our laboratory, therefore, the verbal or written consent of the patients was not obtained. However, before introducing and analyzing the data in our study, all identifiable data regarding the patients were removed.

Results

The 540 patients included in our study were hospitalized in 11 clinical wards: general surgery unit (213; 39.44%), anesthesia and intensive care unit (172; 25.92%), neurosurgery unit (29; 5.37%), neurosurgery intensive care unit (50; 9.25%), nephrology unit (57; 10.55%), internal medicine unit (9; 1.66%), gynecology unit (2; 0.37%), dermatology unit (3; 0.55%), orthopedic unit (3; 0.55%), oto-rhino-laryngology unit (1; 0.18%), and ophthalmology unit (1; 0.18%).

The clinical specimens analyzed for further identification were: wound exudate (drainage) (233; 43.14%), urine (139; 25.74%), broncho-alveolar lavage (108; 20%), blood (25; 4.62%), cerebrospinal fluid (17;

3.14%), peritoneal fluid (7; 1.29%), central venous catheter (6; 1.11%), bile (2; 0.37%), ascites aspirate (1; 0.18%), pleural aspirate (1; 0.18%), and vaginal discharge (1; 0.18%).

In this retrospective study we included 566 non-repetitive strains: *Acinetobacter* spp. (161; 28.44%), *Pseudomonas aeruginosa* (62; 10.95%), *Escherichia coli* (213; 37.63%), and *Klebsiella* spp. (130; 22.96%).

In the section below, we summarize the antibiotic resistance pattern of bacterial strains as well as carbapenem resistance occurrence in different clinical wards. In table I we underlined the importance of carbapenem resistance in the non-fermentative species, particularly in *Acinetobacter* spp., which had a higher resistance rate to carbapenems compared to *Pseudomonas aeruginosa.* Even though we identified only four strains of carbapenem-resistant *Enterobacteriaceae* (CRE) (table II), the results cannot be generalized to the other clinical units and to other populations of patients because the actual study is limited to existing recorded data.

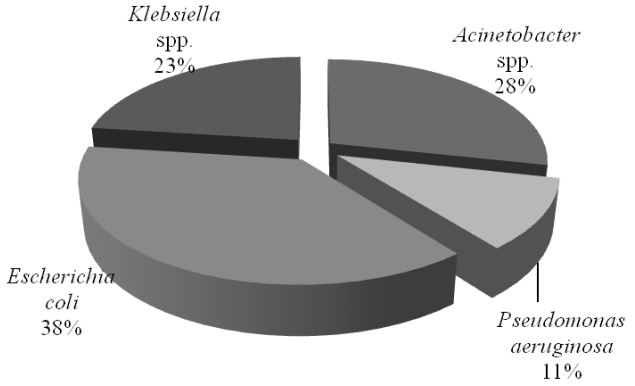

Figure 1. Gram-negative bacilli species isolated for further comparative analyses.

Among the 566 strains included in our study, 191 (33.74%) carbapenem-resistant or carbapenem-intermediate strains were detected. We noticed that non-fermenters were the predominant carbapenem-resistant species: 93.16% of 161 *Acinetobacter* spp. and 48.38% of 62 *P.aeruginosa* strains tested were non-susceptible to carbapenems. Only four CRE strains were identified: two (0.93% of 213 tested) CRE *Escherichia coli* and two (1.5% of 130 tested) CRE *Klebsiella* spp. isolates. The value of our results is closely connected with CLSI recommendation for disk diffusion method. CLSI and EUCAST standards are up-dated regularly. Adequate detection of carbapenem-resistant micro-organisms has a major impact in the management of nosocomial infections and in the appropriate choice of antimicrobial therapy, therefore a reliable detection in clinical laboratory is imperative.

It should be assumed that resistance to carbapenem is due to the impermeability of the membrane in addition to carbapemase production (oxa- or metallo-beta-lactamase). According to the Bush classification [16] class B metallo-beta-lactamases do not hydrolyze aztreonam. In our

Table I. Susceptibility of the non-fermenters to various antibiotics; S (susceptible), I (intermediate), and R (resistant).

Antibiotic	*Acinetobacter* spp (N=161)			*Pseudomonas aeruginosa* (N=62)		
	S	I	R	S	I	R
Imipenem	7 (4.35%)	4 (2.48%)	**150 (93.17%)**	29 (46.77%)	3 (4.84%)	**30 (48.39%)**
Meropenem	8 (4.97%)	3 (1.86%)	**150 (93.17%)**	29 (46.77%)	3 (4.84%)	**30 (48.39%)**
Amikacin	28 (17.39%)	8 (4.97%)	125 (77.64%)	29 (46.77%)	5 (8.07%)	28 (45.16%)
Gentamicin	17 (10.56%)	3 (1.86%)	141 (87.58%)	17 (27.42%)		45 (72.58%)
Ceftazidime	2 (1.24%)	3 (1.86%)	156 (96.90%)	30 (48.39%)		32 (51.61%)
Ciprofloxacin	7 (4.35%)	7 (4.35%)	147 (91.30%)	24 (38.71%)	1 (1.61%)	37 (59.68%)
Cefoperazone/sulbactam	20 (12.42%)	6 (3.73%)	135 (83.85%)	26 (41.94%)	8 (12.9%)	28 (45.16%)
Colistin	161 (100%)			62 (100%)		
Piperacillin/tazobactam	8 (4.97%)		153 (95.03%)	27 (43.55%)	1 (1.61%)	34 (54.84%)

Table II. Susceptibility of the *Enterobacteriaceae* species to various antibiotics.

Antibiotic	*Escherichia coli* (N=213)			*Klebsiella* spp (N=130)		
	S	I	R	S	I	R
Imipenem	211 (99.06%)		**2 (0.94%)**	128 (98.46%)		2 (1.54%)
Meropenem	211 (99.06%)		**2 (0.94%)**	128 (98.46%)		2 (1.54%)
Amikacin	197 (92.49%)	5 (2.35%)	11 (5.16%)	106 (81.54%)	12 (9.23%)	12 (9.23%)
Gentamicin	158 (74.18%)	4 (1.88%)	51 (23.94%)	69 (53.08%)	1 (0.77%)	60 (46.15%)
Ceftazidime	96 (45.07%)		117 (54.93%)	38 (29.23%)		92 (70.77%)
Ciprofloxacin	157 (73.72%)	9 (4.22%)	47 (22.06%)	60 (46.15%)	16 (12.31%)	54 (41.54%)
Cefoperazone/sulbactam	201 (94.37%)	4 (1.88%)	8 (3.75%)	96 (73.85%)	10 (7.69%)	24 (18.46%)

study we identified only 31 isolates tested for aztreonam. Among them, 70.96% were susceptible to aztreonam. In table III one carbapenem-resistant *Pseudomonas aeruginosa* isolate is shown to demonstrate resistance to aztreonam. It is assumed that it could elaborate a class D, OXA ("oxacillin-hydrolyzing") β-lactamase [21,33]. Two carbapenem-resistant *Pseudomonas aeruginosa* isolates do not hydrolyze aztreonam, that particular phenotypic pattern being suggestive for class B (metallo-beta-lactamase) [34]. CRE strains included in this study were not tested for aztreonam.

The analysis of our data has revealed 43 cases of co-infections with carbapenem-resistant micro-organisms and other bacterial species. In figure 2 there are 33 cases of co-infection with carbapenem-resistant non-fermentative species and other bacterial or *Candida* species. We noticed the importance of infection due to *Enterobacteriaceae* species susceptible to carbapenems in association with carbapenem-resistant micro-organisms, therefore we took into consideration all the *Enterobacteriaceae* species isolated: *E.coli, Proteus* spp., *Morganella* spp. and *Providencia* spp. (data not shown).

A particular cause of concern is co-infection with two carbapenem-resistant species. Our data revealed seven (1.29%) cases of co-infection with carbapenem-resistant *Acinetobacter* spp. and carbapenem-resistant *P.aeruginosa.* One patient (0.18%) was infected with CRE *Klebsiella* spp., carbapenem-resistant *Acinetobacter* spp., and methicillin resistant *Staphylococcus aureus* (MRSA). It is well known that *Enterobacteriaceae* play an important role in interspecies spreading of plasmids containing gene-encoding carbapenemases. We noticed 11 (2.03%) patients with co-infection with carbapenem-resistant strains and *Enterobacteriaceae* susceptible to carbapenems.

Among the 191 isolates, *Acinetobacter* spp. was the most prevalent carbapenem-resistant organism; 154 (80.62%), 87 (45.54%) strains were isolated from anesthesia and intensive care units.

Figure 3 shows the comparative distribution of carbapenem non-suceptible and carbapenem susceptible strains isolated from five health-care settings. Among 172 strains isolated from the patients hospitalized in anesthesia and intensive care unit, 102 (59.30%) strains were carbapenem-resistant or carbapenem-intermediate. From the neurosurgery intensive care unit only 50 strains were analyzed, therefore we can only have a general perspective of the prevalence of resistance to carbapenems in that clinical ward, 35 (70%) strains being non-susceptible to carbapenems.

Our findings highlighted a strong correlation of carbapenem-resistant species infections with admission to intensive care units. Comparison of the data obtained from different clinical wards revealed that intensive care units were more likely to be contaminated with carbapenem-resistant strains. Fewer carbapenem-resistant strains were isolated from the general surgery unit - 40 (18.7%) - than from the anesthesia and intensive care unit 102 (59.30%). Even though the strains isolated from other clinical wards were fewer, we can still obtain some interesting results. While 35 (70%) carbapenem-resistant or carbapenem-intermediate strains were isolated in the neurosurgery intensive care unit, only 11 (37.93%) such strains were

Table III. Comparative analysis of the susceptibility to aztreonam (ATM) of 31 isolates. S (susceptible), I (intermediate), R (resistant).

	P. aeruginosa		*Escherichia coli*	*Klebsiella* spp.	
	carbapenem resistant	carbapenem susceptible	carbapenem susceptible	carbapenem susceptible	
ATM - S	2 (6.45%)	5 (16.13%)	12 (38.71%)	3 (9.68%)	**22 (70.97%)**
ATM - I	1 (3.23%)	6 (19.35%)			7 (22.58%)
ATM - R	1 (3.23%)	1 (3.23%)			2 (6.45%)
Total	4 (12.90%)	12 (38.71%)	12 (38.71%)	3 (9.68%)	31 (100%)

Table IV. Prevalence of carbapenem-resistant or carbapenem-intermediate isolates by clinical wards.

	carbapenem-resistant or carbapenem-intermediate isolates				
Clinical wards	*Acinetobacter* spp	*Pseudomonas aeruginosa*	*Escherichia coli*	*Klebsiella* spp	Total
anesthesia and intensive care unit	87 (45.54%)	14 (7.32%)		1 (0.52%)	**102 (53.40%)**
general surgery unit	30 (15.71%)	8 (4.18%)	2 (1.047%)		40 (20.94%)
neurosurgery-intensive care unit	28 (14.65%)	7 (3.66%)			35 (18.32%)
neurosurgery unit	7 (3.66%)	3 (1.57%)		1 (0.52%)	11 (5.75%)
gynecology unit	1 (0.52%)				1 (0.52%)
nephrology unit	1 (0.52%)	1 (0.52%)			2 (1.07%)
Total	**154 (80.62%)**	33 (17.27%)	2 (1.047%)	2 (1.047%)	191 (100%)

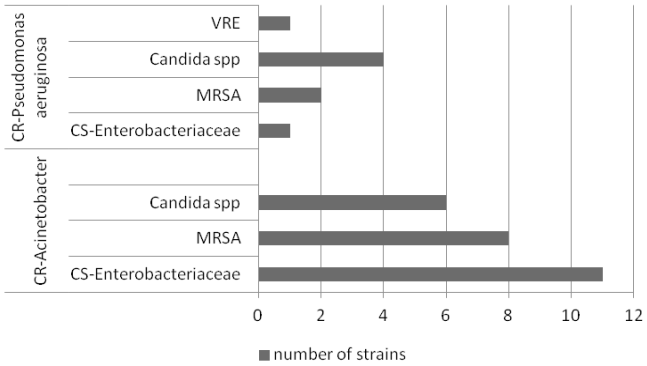

Figure 2. The number of cases with co-infection with carbapenem-resistant (CR)-non-fermentative species with carbapenem-susceptible (CS)-*Enterobacteriaceae*, methicillin resistant *Staphylococcus aureus* **(**MRSA), vancomycin resistant *Enterococcus* spp. (VRE) and *Candida* spp.

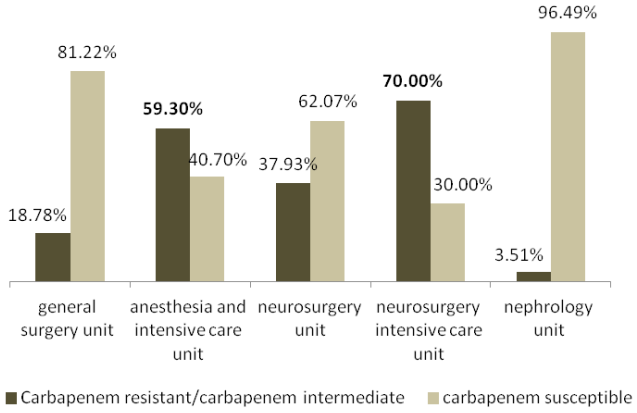

Figure 3. Comparative analysis of carbapenem-resistant or carbapenem-intermediate isolates and carbapenem-susceptible isolates incidence in various clinical wards.

noticed in the neurosurgery unit. Early detection of carbapenem-resistant strains is crucial in limiting the spread of infection and in monitoring the treatment of patients with severe illnesses.

Discussion

Early recognition and treatment of carbapenem-resistant species must become a clinical priority for all hospitalized patients because resistance to carbapenems is often associated with resistance to other classes of antibiotics. Carbapenems as last-resort antibiotics recommended in eliminating ESBL-producing Enterobacteriaceae could no longer be a viable choice as monotherapy.

The number of appropriate antibiotics for the carbapenem-resistant micro-organisms infections is limited. Amikacin remains a viable option of treatment in some cases. Other authors recommend gentamycin instead [35]. In our study we did not identify any non-fermentative strains that exhibited resistance to colistin. In spite of its side effects [36,37], colistin had a remarkable antibiotic activity against carbapenem-resistant *P.aeruginosa*, its efficiency being even higher compared with a three-drug combination of aztreonam, ceftazidim and amikacin [38]. Colistin in combination with cefoperazone/sulbactam or tigecycline could have a synergistic effect against carbapenem-resistant *Acinetobacter* spp. [39].

There are certain recommendations regarding the treatment of patients colonized or infected with carbapenem-resistant micro-organisms: single room ward, enhanced environmental cleaning during hospitalization, hospital-stay length, active surveillance of the patients transferred from countries or institutions with epidemic or endemic carbapenem-resistant Gram-negative strains occurrence [40,41]. However, making a clear distinction between colonization and infection with carbapenem-resistant strains could be a difficult task [42].

There are several limitations to our study, including the lack of clinical information regarding the risk factors, treatment provided, days of hospitalization, and data regarding previous hospitalizations. In the clinical laboratory the accurate identification of carbapenem- resistant strains can be difficult to achieve due to methodological limitations, such as the availability of selective CRE agar [43] or Hodge test, although this test has a variable efficiency in detecting carbapenemase producing isolates [44]. However, the study has some strength such as the analysis of a sizeable quantity of strains, which allows to draw important conclusions regarding the dissemination and subsequent epidemics of carbapenem-resistant micro-organisms mainly in intensive care units. In addition, the phenotypic pattern of the strains isolated suggests that a combination of antimicrobial resistance mechanism contributes to the resistance to carbapenems used in therapy. Although some antibiotics are not constantly tested, aztreonam for example, we can still draw the same general conclusions about the genetic mechanisms involved in the resistance to carbapenems, based on the phenotypic patterns of the clinical isolates included in our study.

We assumed that among carbapenem-resistant strains, carbapenemase-producing strains are most commonly involved in infections following hospitalization of patients with severe illness. Molecular methods for accurate identification of carbapenem-resistant strains are not included in our clinical laboratory procedures. As a result, a reliable detection of the carbapenem-resistance mechanisms was not performed.

Conclusions

Surveillance of antimicrobial resistance in hospitals isolates provides useful guidelines for microbiologists and clinical practitioners, therefore accurate susceptibility testing is essential. All Gram-negative bacilli with a reduced susceptibility to meropenem or imipenem by disk diffusion method should be further tested for the production of carbapenemases. Patients hospitalized in intensive care units showed a trend toward higher risk of infections with carbapenem-resistant strains. Unlike *Enterobacteriaceae*,

non-fermentative bacilli were much more frequently involved in maintaining and spreading of carbapenem-resistant genes in hospital environment. *Acinetobacter* spp. as a major cause of hospital-acquired infection is a versatile bacteria which could develop very efficient resistance mechanisms under the selective pressure generated by antibiotic prescription.

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