Resistance and Virulence Features in Carbapenem-resistant *Acinetobacter baumannii* Community Acquired and Nosocomial Isolates in Romania

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We aimed to identify the virulence and antimicrobial resistance features in Carbapenem Resistant Acinetobacter baumannii (CRAB) strains isolated from hospital settings and compare them with those isolated in the same period of time from community acquired (CA) infections in Bucharest, south of Romania. A total number of 93 A. baumannii strains were isolated in majority from hospitalized patients and from CA infections. The resistance and virulence mechanisms of the strains were characterized by phenotypic and genotypic methods. The antibiotic resistance profiles in H and CA A. baumannii isolates revealed high percentages of carbapenem-resistance in both H and CA isolates. The ciprofloxacin resistance was found very closed in both types of isolates (84%/83.33%). CRAB H and CA isolates revealed the intrinsec carbapenemase OXA-51and the acquired carbapenemases OXA-23, OXA-24, IMP,and VIM-2. The bla$_{OXA-23}$ gene was identified in different plasmid types (GR2-Aci1, GR6-pACICU2). rep135040, p3S18 and Aci6 in H A. baumannii isolates. The most frequently expressed virulence factor was lipase and DN-ase. OXA-51-like alleles corresponding to the two main sequence groups were identified as bla$_{OXA66}$ (63.63% of the isolates) and respectively, bla$_{OXA-69}$ (38.39%) and revealed the corresponding type of ompAand csuE sequence grouping. AphA6 (24%/16.6%), AphA1 (16%/16.6%) and aadB (9.3%/5.5%) genes were responsible for aminoglycosides resistance. Our survey revealed a high drug resistance in A. baumannii isolates. Different plasmid groups containing CRAB isolates may facilitate the bla$_{OXA23}$ dissemination.

Keywords: carbapenem resistance, virulence, community acquired, nosocomial infections

Acinetobacter baumannii is recognized as an opportunistic nosocomial pathogen, mainly in immunocompromised patients being frequently associated with therapeutic failures, due to its multi-drug (MDR), extended-drug (XDR) or even pan-drug resistance (PDR) phenotypes. Carbapenems were the antibiotics of choice for infections treatment caused by this organism, but resistance to carbapenems is becoming common, and very few therapeutic options remain. Mortality rates associated with Carbapenem Resistant *Acinetobacter baumannii* (CRAB) isolates are steadily growing at present [1, 2]. In *A. baumannii* clinical isolates five groups of acquired, chromosomal or plasmid located CHDLs (class D β-lactamases) with variable geographic distribution have been identiûed, i.e.: OXA-23, OXA-24/-40, OXA-58, OXA-143 and OXA-235 [3]. OXA-23 is the most worldwide distributed enzyme in *A. baumannii*, having been implicated in outbreaks in multiple European (including Romania and other Eastern European countries), Asian and American countries and Oceania [4-6]. There have been revealed that overexpression of the *bla*$_{OXA-51-like}$ gene intrinsic in *A. baumannii* was responsible for carbapenem resistance. The overexpression is due to the acquisition of a promoter provided by an insertion sequence (IS) element, IS*Aba1*, inserted upstream of the carbapenemase gene [7].

The acquisition of genes encoding aminoglycoside-modifying enzymes (AMEs) has been a main cause of resistance to aminoglycosides in *A. baumannii* [8]. Different AME-encoding genes, such as *aphA1*, *aphA6*, *aphA15*, *aacC1*, *aacC2*, *aacA4*, *aadB*, *aadA1*, and *aadA4*, have been detected in clinical isolates of *A. baumannii* [8,9]. Many of these genes (for example, *aac C1*, *aacA4*, and *aadA1*) are located on class 1 integrons [10]. In contrast, the *aphA1* and *aphA6* genes have always been surrounded by IS elements, forming different composite transposon structures [7].

Numerous potential virulence factors have been revealed in *A. baumannii* strains, including biofilm formation [several factors contribute to biofilm formation such as the Csu pili [11] encoded by csuE gene, the autoinducer synthase *AbaI*, part of the *quorum sensing* (QS) system [12], the outer membrane protein A (encoded by OmpA gene) which facilitates the adhesion to host epithelial cells], complement resistance [13], iron acquisition characteristics, capsule, outer membrane protein phospholipases, alteration in penicillin-binding proteins [14]. Phospholipases C and D are responsible for epithelial cell invasion [15, 16]; the siderophore acinetobactin [17]; the polysaccharidic capsule [18]; and a penicillin-binding protein 7/8 [19] are important for

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survival and dissemination in human serum. Growth in serum has been demonstrated to upregulate iron acquisition systems, genes associated with epithelial cell adherence and DNA uptake, as well as numerous putative antibiotic efflux pumps, leading to increased antibiotic tolerance [20]. In addition, lipopolysaccharide (LPS) is an important cell envelope component, which influence the pathogenic potential of *A. baumannii* by the O-polysaccharide chain (O-antigen) [21].

Regarding *A. baumannii* infections the most predisposed are patients from ICU in which this pathogen may cause serious infection and, thus, contributes substantially to the considerable mortality of this population [22]. Although the attention of *A. baumannii* infections has been focused on hospitalized patients, there is another patient population that may be affected by this important pathogen, namely, patients in the community setting that have some form of morbidity, especially in the tropical and sub-tropical area [23].

Originally, we are interested in identifying the relationship between virulence and antimicrobial resistance in CRAB strains from both hospital settings and the community, the ARGs, their transfer and dissemination into the community.

Experimental part

Material and methods

The study included 93 recently isolated (Aug-Nov 2017) *A. baumannii* strains, which were isolated in majority from hospitalized patients (H) (n=75) and from CA infections (n=18). The hospital strains were identified by BD Phoenix and the CA ones by mass spectrometry using MALDI Biotyper and MicroScan Walk Away 96. Carbapenemases and virulence genes were searched by PCR.

Antibiotic resistance

The antibiotic susceptibility was determined by Kirby-Bauer standard disk diffusion method [using the antibiotics recommended by CLSI, 2017: meropenem (MEM), imipenem (IMP), ertapenem (ETP), cephalotin (CEF) ceftriaxon (CTX), cefuroxime (CXM), cefoxitin (FOX), ceftazidim (CAZ), aztreonam (ATM), cefepime (FEP), amoxicillin-clavulanic acid (AMC), piperacilin-tazobactam (PIP-TZP), ciprofloxacin (CIP), levofloxacin (LEV) gentamycin (GEN), amikacin (AMK), nitrofurantoin (NIT), trimethoprim-sulfamethoxazole (SXT), tetracycline (TET), tigecycline (TIG) and colistin] and quality control was performed with *Pseudomonas aeruginosa* ATCC 27853 and automated methods (BD Phoenix and Vitek II).

Evaluation of the soluble enzymatic factors

The virulence phenotypes were investigated by performing enzymatic tests for the expression of the following soluble virulence factors: haemolysins, pore forming toxins (lecithinase, lipase), proteases (caseinase, gelatinase), amylase and aesculin hydrolysis.

Genetic support of AR and virulence in CRAB

The genetic support of the resistance (carbapenemases and aminoglycosides table 1), Plasmid analysis included identification of replicase genes:19 PCR amplifications were devised to detect 27 replicase genes, which were grouped into 19 homology groups (GRs) on the basis of their nucleotide sequence similarities (table 2), BIOPFILM biofilm producing virulence factors (table 3) and global lineage in CRAB (table 4) was investigated by simplex and multiplex PCR, using a reaction mix of 20µL (PCR Master Mix 2X, Thermo Scientific) containing 1 µL of bacterial DNA extracted using the alkaline extraction method. In this purpose, 1-5 colonies of bacterial cultures were suspended in 1.5 ml tubes containing 20 µL solution of NaOH (sodium hydroxide) and SDS (sodium dodecyl sulphate). The following step was the addition of 180 µL of TE buffer (TRIS+EDTA) 1X and centrifugation at 13000 rpm for 3 min. All PCR reactions were performed using the Thermal Cycler machine Bio-Rad.

Table 1
THE TARGET GENES SCREENED FOR CARBAPENEMASES AND AMINOGLYCOSIDES

Target gene	Primer name	Sequence	Amplicon size (bp)	Reference
bla_{OXA-23}	OXA-23-F OXA-23-R	5'-ATGAGTTATCTATTTTTGTC-3' 5'-TGTCAAGCTCTTAAATAATA-3'	501	[24]
bla_{OXA-24}	OXA24/40-F OXA24/40-R	5' GCAGAAAGAAGTAAARCGGGT3' 5' CCAACCWGTCAACCAACCTA3'	270	[25]
bla_{OXA-51}	OXA-51-F OXA-51-R	5'-TAATGCTTTGATCGGCCTTG-3' 5'-TGGATTGCACTTCATCTTGG-3'	353	[24]
bla_{OXA-58}	OXA-58-F OXA-58-R	5'-AAGTATTGGGGCTTGTGCTG-3' 5'-CCCCTCTGCGCTCTACATAC-3'	599	[24
$bla_{OXA-143}$	OXA-143-F OXA-143-R	5'-TGGATTGCACTTCATCTTGG-3' 5'-TGGCACTTTCAGCAGTTCCT-3'	180	[26]
$bla_{OXA-235}$	OXA-235-F OXA-235-R	5'-TTGTTGCCTTTACTTAGTTGC-3' 5'-CAAAATTTTAAGACGGATCG-3'	700	[26]
bla_{IMP}	blaIMP-F blaIMP-R	GGAATAGAGTGGCTTAAYTCTC GGTTTAAYAAAACAACCACC	232	[25]
bla_{VIM-2}	blaVIM -2-F blaVIM-2-R	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	800	[27]
AME's	aphA6-F aphA6-R	ATGGAATTGCCCAATATTATTC TCAATTCAATTCATCAAGTTTTA	797	[26]
	aphA1-F aphA1-R	CAACGGGAAACGTCTTGCTC ATTCGTGATTGCGCCTGAG	455	
	aacA4-F aacA4-R	ATGACTGAGCATGACCTTGCG TTAGGCATCACTGCGTGTTCG	518	
	aadB-F aadB-R	ATGGACACAACGCAGGTCGC TTAGGCCGCATATCGCGACC	524	
	aadA1-F aadA1-R	ATGAGGGAAGCGGTGATCG TTATTTGCCGACTACCTTGGTG	254	
	aacC1-F aacC1-R	ATGGGCATCATTCGCACATGTAGG TTAGGTGGCGGTACTTGGGTC	456	

Target gene	Primer name	Sequence	Amplicon size (bp)	Reference
Gr1 (p1ABSDF001)	gr1-F gr1-R	5′-CATAGAAATACAGCCTATAAAG-3′ 5′-TTCTTCTAGCTCTACCAAAAT-3′	330	[28]
GR2 (Aci1/Aci2)	gr2-F gr2-R	5′-AGTAGAACAACGTTTAATTTTATTGGC-3′ 5′-CCACTTTTTTTAGGTATGGGTATAG-3	851	
GR3 (Aci3/Aci7)	gr3-F gr3-R	5′-TAATTAATGCCAGTTATAACCTTG-3 5′-GTATCGAGTACACCTATTTTTTGT-3′	505	
GR4 (Aci4)	gr4-F gr4-R	5′-GTCCATGCTGAGAGCTATGT-3′ 5′-TACGTCCCTTTTTATGTTGC-3′	508	
GR5 (Aci5)	gr5-F gr5-R	5′-AGAATGGGGAACTTTAAAGA-3′ 5′-GACGCTGGGCATCTGTTAAC-3′	220	
GR6 (Aci6)	gr6-F gr6-R	5′-AGCAAGTACGTGGGACTAAT-3′ 5′- AAGCAATGAAACAGGCTAAT-3′	662	
GR7 (p3ABSDF002)	gr7-F gr7-R	5′-GAACAGTTTAGTTGTGAAAG-3′ 5′-TCTCTAAATTTTTCAGGCTC-3′	885	
GR8 [Aci8, repM (Aci9)]	gr8-F gr8-R	5′-AATTAATCGTAAAGGATAATGC-3′ 5′-GACATAGCGATCAAATAAGC-3′	233	
GR9 (p3ABSDF0009)	gr9-F gr9-R	5′-GCAAGTTATACATTAAGCCT-3′ 5′-AAAAATAAACGCTCTGATGC-3′	191	
GR10 (AciX)	gr10-F gr10-R	5′-TTTCACTAGCTACCAACTAA-3′ 5′-ACACGTTGGTTTGGAGTC-3′	371	
GR11 (p1ABAYE0001)	gr11-F gr11-R	5′-GGCTATTCAAAACAAAGTTAC-3′ 5′-GTTTCCTCTCTTACACTTTT-3′	852	
GR12 (p2ABSDF0001)	gr12-F gr12-R	5′-TCATTGGTATTCGTTTTTCAAAACC-3′ 5′-ATTTCACGCTTACCTATTTGTC-3′	165	
GR13 (p3ABAYE0002)	gr13-F gr13-R	5′-CAAGATCGTGAAATTACAGA-3′ 5′-CTGTTTATAATTTGGGTCGT-3′	780	
GR14 (p4ABAYE0001)	gr14-F gr14-R	5′-TTAAATGGGTGCGGTAATTT-3′ 5′-GCTTACCTTTCAAAACTTTG-3′	622	
GR15 (p3ABSDF0018)	gr15-F gr15-R	5′-GGAAATAAAAATGATGAGTCC-3′ 5′-ATAAGTTGTTTTTGTTGTATTCG-3′	876	
GR16 (repApAB49)	gr16-F gr16-R	5′-CTCGAGTTCAGGCTATTTTT-3′ 5′-GCCATTTCGAAGATCTAAAC-3′	233	
GR17 (A1s_3471)	gr17-F gr17-R	5′-AATAACACTTATAATCCTTGTA-3′ 5′-GCAAATGTGACCTCTAATATA-3′	380	
GR18 (p2ABSDF00025)	gr18-F gr18-R	5′-TCGGGTTATCACAATAACAA-3′ 5′-TAGAACATTGGCAATCCATA-3′	676	
GR19 (rep135040)	gr19-F gr19-R	5′- ACGAGATACAAACATGCTCA-3′ 5′- AGCTAGACATTTCAGGCATT-3′	815	

Table 2
PRIMERS USED TO DETECT THE REPLICASE GENE GROUPS IN THE *A. Baumannii* PCR-BASED REPLICON TYPING SCHEME

Target gene	Primer name	Sequence	Amplicon size (bp)	Reference
Biofilm producing virulence factors	ompA-F ompA-R	5'- CGCTTCTGCTGGTGCTGAAT -3' 5'- CGTGCAGTAGCGTTAGGGTA -3'	531	[26]
	epsA-R epsA-R	5'- AGCAAGTGGTTATCCAATCG-3' 5' - ACCAGACTCACCCATTACAT-3'	451	

Table 3
INVESTIGATED BIOFILM PRODUCING VIRULENCE FACTORS

Multiplex 1			
Primer	**Sequence**	**Amplicon Size (bp)**	**Reference**
Group1ompAF306	5'-GATGGCGTAAATCGTGGTA-3'	355	[29]
Group1and2ompAR660	5'-CAACTTTAGCGATTTCTGG-3'		
Group1csuEF	5'-CTTTAGCAAACATGACCTACC-3'	702	
Group1csuER	5'-TACACCCGGGTTAATCGT-3'		
Gp1OXA66F89	5'-GCGCTTCAAAATCTGATGTA-3'	559	
Gp1OXA66R647	5'-GCGTATATTTTGTTTCCATTC-3'		
Multiplex 2			
Group2ompAF378	5'-GACCTTTCTTATCACAACGA-3'	343	
Group1and2ompAR660	5'-CAACTTTAGCGATTTCTGG-3'		
Group2csuEF	5'-GGCGAACATGACCTATTT-3'	580	
Group2csuER	5'-CTTCATGGCTCGTTGGTT-3'		
Gp2OXA69F169	5'-CATCAAGGTCAAACTCAA-3'	162	
Gp2OXA69R330	5'-TAGCCTTTTTTCCCCATC-3'		

Tabel 4
PRIMERS USED IN THE IDENTIFICATION OF THE GLOBAL LINEAGES OF THE STRAINS -*A. baumannii*

Results and discussions

The antibiotic resistance profiles in H and CA *A. baumannii* isolates revealed high percentages of carbapenem-resistance in both H and CA isolates, i.e. imipenem (82.66%/77.77%), meropenem (84%/83.33%) as well as for SXT (85.33%/77.77%) and aminoglycosides (85.33%/66.66%). The ciprofloxacin resistance was closed in both types of isolates (84%/83.33%) (fig. 1). A. *baumannii* has acquired a huge genetic repertoire *via* horizontal gene transfer that makes it virulent and resistant to any environmental pressures [30-33]. Antibiotic susceptibility testing in this study showed that all *A. baumannii* isolates were resistant to the commercially available antibiotics with the exception of colistin. Previously in Romania, Vaduva et al., revealed the presence of beta-lactamase producers nosocomial *A. baumannii* strains from Timisoara hospital and a very closer aminoglycosides resistance profile [34].

CRAB H and CA isolates revealed the intrinsec carbapenemase OXA-51(58.6%/55.5%) and the acquired

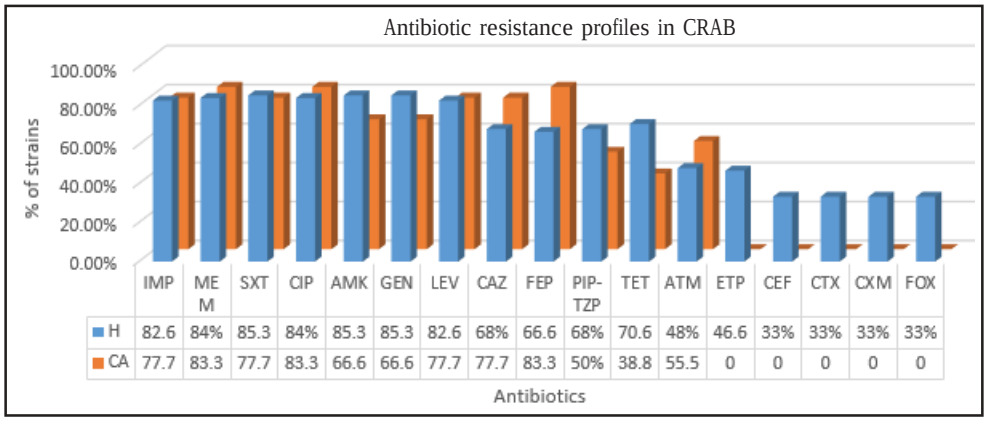

Fig. 1. Graphic representation of antibiotic resistance profiles (%) in analysed CRAB strains

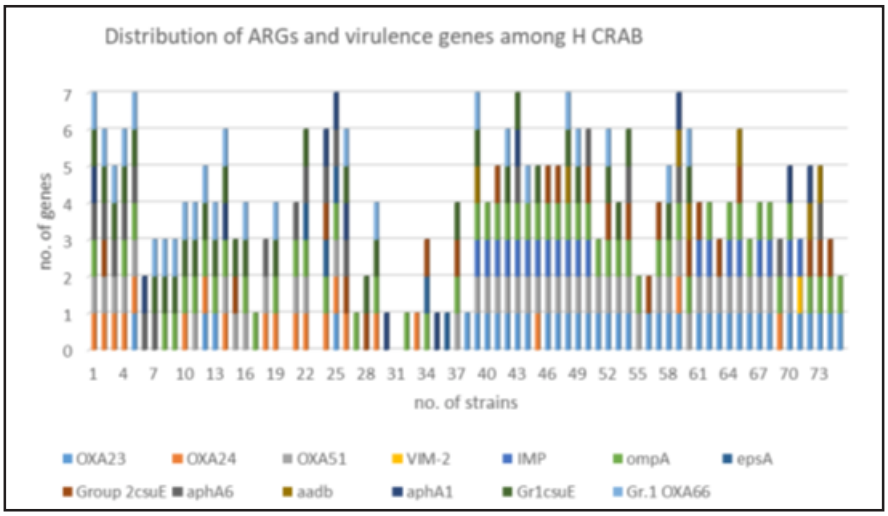

Fig. 2. Graphic representation of antibiotic resistance and virulence genes in analysed H strains

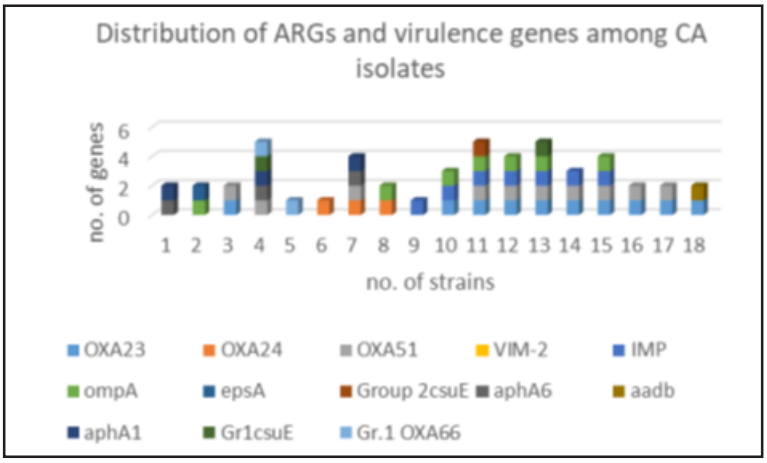

Fig. 3. Graphic representation of antibiotic resistance and virulence genes in analysed CA strains

carbapenemases OXA-23 (50%/55.5%), OXA-24 (26.6%/16.6%) IMP (26.66%/22.22%) and VIM-2 (1.33%/0%) (fig. 2, 3).

In Bucharest, one study performed on *A. baumannii* nosocomial strains recovered from clinical infections in patients hospitalized in ICU between 2001-2003 demonstrated the association of class 1 integrons with bla_{IPM-1}, bla_{VIM-2}, bla_{OXA-24} and bla_{OXA-25} genes [35]. In Iasi hospitals the presence of bla_{IMP-13} in nosocomial *A. baumannii* strains was revealed [36]. Data revealed by our research team (2012-2013) demonstrated that *A. baumannii* strains that were investigated harboured the class D carbapenemase OXA-23 [37]. Previous studies in Timisoara, Arad and Resita indicated that CHLD in *A. baumannii* is encoded by chromosomally - located bla_{OXA-23}, with the insertion sequence IS*Aba1* detected upstream and the strains belonged to the ST2 and ST1 clones [6]. More recently one study from our department highlights a remarkable mobility for bla_{OXA-23}-Tn*2008* and surrounding structures (identified in plasmid or chromosome of different clones) and also describes for the first time the spread of Tn*aph6*-carrying pACICU2-like plasmids in *A. baumannii* in Europe [27]. A pilot study from three Romanian hospitals – Iasi and Targu-Mures (2014-2015) demonstrated the presence of carbapenemases OXA-23, OXA-24/72 in *A. baumannii* nosocomial isolates [38].

Mammina et al., in 2012 revealed the presence in a high percentage of bla_{OXA-23} gene in nosocomial CRAB isolated ICU patients in Palermo Italy and belonging to ST2 [39].

Very recently Petrova et al., demonstrated a higher prevalence of OXA-23 *A. baumannii* producers isolated from different Bulgarian hospitals between 2010-2014 but opposite with our results they didn't observe the presence of OXA-24 and overexpression of OXA-51 in any of the analysed isolates [40].

In our study bla_{OXA-23} gene was identified in different plasmid types (GR2-Aci1, GR6-pACICU2). rep135040

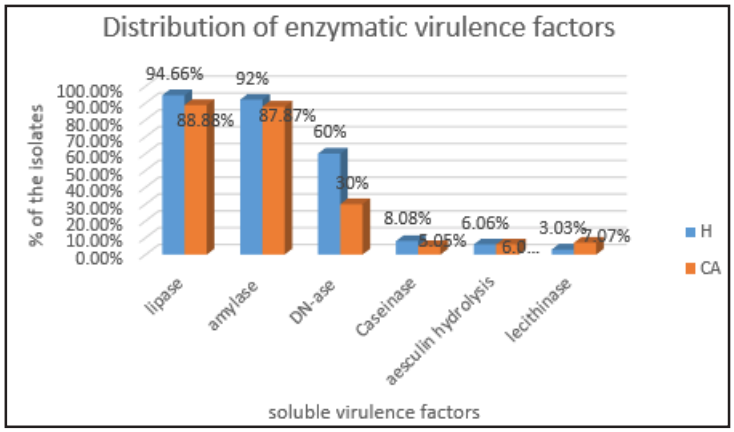

Fig. 4. Graphic representation of enzymatic virulence factors production in CRAB isolates.

(100%) and p3S18 (100%), followed by Aci6 (67%) in H *A. baumannii* isolates.

The multidrug resistant profile of the isolates was also heightened by the presence of AME's namely the phosphotransferase AphA6 (24%/16.6% of the strains), AphA1 (16%/16.6%) and acetyltransferases aadB (9.3%/ 5.5%). Nowak et al., in 2014 reported in MDR *A. baumannii* isolated in Poland the following aminoglycoside resistance genes: aphA1, aphA6,and aacC1 [41]. The genes encoding AMEs can be disseminated *via* integrons, and expression of AMEs enable bacteria to catalyze the modification of amino and hydroxyl groups on sugar moieties, such as aminoglycosides [42].

Carbapenem and aminoglycosides resistance has been associated to nonenzymatic mechanisms revealed by changes on the outer membrane proteins [ompA biofilm-producing virulence factor (66.66% of the analysed strains)], mechanism [43] demonstrated also by different authors. The outer membrane protein A of *A. baumannii* represent one of the most abundant surface protein associated with the apoptosis of epithelial cells through mitochondrial targeting [44]. OmpA is also the major nonspecific channel in *A. baumannii* and appears to be essential for this organism's high levels of intrinsic resistance to different antibiotics [45]. Several reports have been demonstrated that *A. baumannii* possesses Omp's which interfere with carbapenem resistance, for e.g. in 2002, Limansky *et al.* demonstrated that imipenem resistance was associated with the loss of a 29-kDa Omp in clinical isolates of *A. baumannii* in which no carbapenemase activity had been detected [46].

Regarding the soluble virulence factors, the decreasing frequently expressed were lipase (94.66%/88.88%), amylase (92%/87.87%), DN-ase (60%/30%), caseinase (8.08%/5.05%); aesculin hydrolysis (6.06%/6.06%) and lecithinase (3.03%/7.07%) (Fig. 4).

OXA-51-like alleles corresponding to the two main sequence groups were identified as bla_{OXA66} (63.63% of the isolates) and respectively, bla_{OXA-69} (38.39%) and revealed the corresponding type of ompA and csuE sequence grouping (fig. 2, 3). There have been revealed a link between the production of some of the naturally occurring OXA-51/69-like oxacillinases and carbapenem resistance in *A. baumannii*. Despite the relatively weak ability of these enzymes to hydrolyse carbapenems, it has been shown that these oxacillinases may sometimes be overexpressed, resulting in a decreased level of susceptibility to carbapenems and the presence of IS*Aba1*element upstream of the $bla_{OXA-51/69}$-like gene (J. Turton, N. Woodford and T. Pitt, personal communication).

Conclusions

The significant levels of antibiotic resistance in CRAB strains highlights the need for continuous surveillance and epidemiological studies, of not only hospital, but also CA isolates. The elucidation of the genetic context of resistance in CRAB isolates with different origins could reveal further clinically important associations, and help to better understand the interaction between antimicrobial resistance and virulence in *A.baumannii*.

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Abbreviation

CRAB - Carbapenem Resistant *Acinetobacter baumannii*
AMEs - aminoglycoside-modifying enzymes
H - hospitalized patients
CA - Community acquired infections

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