Biochemical, Virulence and Resistance Features in Bacterial Strains Recovered from Hospital Surfaces after Decontamination with Quaternary Ammonium Compounds, Triclosan and Iodine Desinfectatnts

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The purpose of this study was to investigate the resistance and virulence markers in microbial strains isolated from the hospital environment, recovered after surfaces decontamination with quaternary ammonium compounds, triclosan, iodine disinfectants, in order to predict their role in healthcare-associated infections. The resistance phenotypes were established using disk diffusion and double-disk diffusion test. Simplex and multiplex PCR assays were used to identify the genetic support of cell-associated and soluble virulence factors The Gram-negative bacterial strains isolated from hospital surfaces after decontamination were resistant in high proportions to beta-lactam antibiotics, including penicillins and associations with beta-lactamase inhibitors, third and fourth generation cephalosporins and carbapenems (encoded by bla_{TEM}, $bla_{NDMlike}$, $bla_{CTX\text{-}Mlike}$, $bla_{OXA\text{-}48like}$ genes), quinolones (QnrA, gyrB, parE genes), aminoglycosides (aac3la gene), and tetracyclines. Regarding the virulence profiles, P. aeruginosa strains revealed the plcH gene (86.6%) followed by plcH, ExoS, algD and ExoU, while the Enterobacteriaceae strains, fimH (24%), followed by papC, sfaDE, hlyA, cnf1, eaea and VT2 genes. S. aureus isolates revealed the SCCmec cassette of type II (40%), followed by type V, IIIA and type IV (2B) and the clfA, clfB, and can adhesion genes. These findings demonstrate that incorrect practices in biocides use, regarding contact time and concentrations or instruments and surfaces insufficient cleaning before disinfection can decrease their effect and favor the persistence of resistant and virulent microbial strains in the hospital environment. The multiple drug resistance and virulence determinants, encoded by diverse genetic elements suggest the potential of these strains to persist and initiate hospital-associated infections.

Keywords: resistance, virulence, biocides

The continuously and increasingly emergence and spread of microbial resistance worldwide has led to the introduction of disinfection procedures based on widespread used biocides in hospital (instruments, various devices, surfaces and air) and food industry. However, despite their broad action, with multiple unspecific target sites at the level of microbial cell (e.g., cytoplasmic membrane, nucleic acids, fatty acids, proteins, enzymes), the efficacy of biocides can be affected by intrinsic factors, like: enzymatic degradation, permeability barrier, biocide concentration, contact time but also by acquired resistance mechanisms [1-3]. The bacterial cells grown in biofilms were found to be 150 to 3000 times more resistant to disinfectants, mainly due to poor penetration of the biofilm polysaccharide matrix [4, 5]. Although it has been demonstrated that the mutation is a rare mechanism for resistance acquisition to biocides, this was documented for triclosan (FAB I) [6-8] and quaternary ammonium compounds (qacG) resistance [9-11].

Experimental part

Bacterial strains and virulence analysis

A total number of 60 bacterial strains belonging to *Escherichia coli* (n=10), *Klebsiella pneumoniae* (n=15), *Pseudomonas aeruginosa* (n=15) and *Staphylococcus aureus* (20) were recovered in 2016 from hospital surfaces after decontamination with quaternary ammonium compounds, triclosan and iodine disinfectants in Public Health Diagnostic and Research Laboratory.

The strains identification was performed with traditional biochemical tests and the automated miniApi system [12, 13]. The antibiotic susceptibility testing was performed using the disk diffusion method (Kirby-Bauer), following the CLSI recommendations (2015 and 2016 editions).

Molecular analysis

The genetic support of the resistance (carbapenemases, ESBLs, aminoglycosides and quinolones) and virulence in Gram-negative rods (table 1) was investigated by simplex and multiplex PCR, using a reaction mix of 20μL (PCR Master Mix 2x, Thermo Scientific) containing 1μL of bacterial DNA extracted using the alkaline extraction method (table 2). 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 amplification program was runned on a *PCR thermal Corbett* machine. The genotypic

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Table 1

PRIMERS SEQUENCES USED IN SIMPLEX AND MULTIPLEX PCR ASSAYS FOR CARBAPENEM, QUINOLONE AND AMINOGLYCOSIDES RESISTANCE GENES AND VIRULENCE GENES IN ANALYZED *ENTEROBACTERIACEAE* AND *P. AERUGINOSA* STRAINS

The gene	Primer	Nucleotide sequence	Amplicon size	References
bla$_{OXA-48}$	OXA-F OXA-R	5´GCGTGGTTAAGGATGAACAC 3´ 5´CATCAAGTTCAACCCAACCG 3´	438	[17]
bla$_{IMP}$	IMP-F IMP-R	5´GGAATAGAGTGGCTTAA(C/T)TCT C 3´ 5´GGTTTAA(C/T)AAAACAACCACC 3´	232	
bla$_{VIM}$	VIM-F VIM-R	5´GATGGTGTTTGGTCGCATA 3´ 5´CGAATGCGCAGCACCAG 3´	390	
bla$_{NDM}$	NDM-F NDM-R	5´GGTTTGGCGATCTGGTTTTC 3´ 5´CGGAATGGCTCATCACGATC 3´	621	
bla$_{PER}$	PER-F PER-R	5'-AATTTGGGCTTAGGGCAGAA-3' 5'-ATGAATGTCATTATAAAAGC-3'	925	[18]
bla$_{TEM}$	TEM-F TEM-R	5'-ATGAGTTTTCAACATTTTCG-3' 5'-TTACCAATGCTTAATCAG TG-3'	861	[19]
bla$_{SHV}$	SHV-F SHV-R	5'-GCCCTCACTCAAGGATGTAT-3' 5'-TTAGCGTTGCCAGTGCTCGA-3'	888	[20]
bla$_{CTX-M}$	CTX-M-F CTX-M-R	5'-CGCTGTTGTTAGGAAGTGTG-3' 5'-GGCTGGGTGAAGTAAGTGAC-3'	730	[21]
QnrA	QnrAm-F QnrAm-R	5'-AGA GGA TTT CTC ACG CCA GG-3' 5'-TGC CAG GCA CAG ATC TTG AC-3'	580	[22]
QnrB	QnrBm-F QnrBm-R	5'-GGM ATH GAA ATT CGC CAC TG-3' [M = A or C, H = A or C or T] 5'-TTT GCY GYY CGC CAG TCG AA-3' [Y = C or T]	264	
QnrS	QnrSm-F QnrS-R	5'-TCT AAA CCG TCG AGT TCG GCG-3' 5'-GCGCGAGATGACCCGCCGCA-3'	820	
GyrB	GyrB-F GyrB-R	5'-GCGCGAGATGACCCGCCGCA-3' 5'-CTGGCGGAAGAAGAAGGTCAACA-3'	465	[18]
ParE	ParE-F ParE-R	5'CGGCGTTCGTCTCGGGCGTGGTGAAGG3' 5'TCGAGGGCGTAGTAGATGTCCTTGCCGA3'	670	
HlyA	HlyA-F HlyA-R	5'-AACAAGGATAAGCACTGT TCTGGC T-3' 5'-ACCATATAAGCGGTCATT CCC GTC A-3'	1,177 bp	[25]
sfaD/E	sfaD/E-F sfaD/E-R	5'-CGGAGGAGTAATTACAAACCTGGCA -3' 5'- CTCCGGAGAACTGGGTG ATCTTA C-3'	408 bp	
PapC	PapC-F PapC-R	5'-GACGGCTGTACTGCAGGGTGTGGC-3' 5'-ATATCCTTTCTGCAGGGATGCAATA-3'	328 bp	
FimH	FimH-F FimH-R	5'-TGC AGA ACG GAT AAG CCG TGG -3' 5'- GCA GTC ACC TGC CCT CCG GTA -3'	508 bp	[26]
cnf1	cnf1-F cnf1-R	5'- GAA CTT ATT AAG GAT AGT-3' 5'-CAT TAT TTA TAA CGC TG-3'	543kb	[27]
eaea	Eaea-F Eaea-R	5'- GGCTCAATTTGCTGAGACCACGGTT-3' 5'- GCAAATTTAGGTGCGGGTCAGCGTT-3'	494 bp	[28]
bfpA	BfpA-F BfpA-R	5'- CAATGGTGCTTGCGCTTGCT-3' 5'- GCCGCTTTATCCAACCTGGT-3'	324bp	
Eaf	Eaf-F Eaf-R	5'- CAGGGTAAAAGAAAGATGATAA-3' 5'- TATGGGGACCATGTAATTATCA-3'	397bp	
AggR	AggR-F AggR-R	5'- CGATGTATACACAAAAGAAGGA -3' 5'- GCCTAATGAAATATGATGGTACT-3'	640bp	
EAggE	EAggE-F EAggE-R	5'- CTGGCGAAAGACTGTATCAT-3' 5'-CAATGTATAGAAATCCGCTGTT-3'	630bp	
VT1	VT1-F VT1-R	5'-GAAGAGTCCGTGGGATTACG-3' 5'- AGCGATGCAGCAGCTATTAATAA-3'	130bp	
VT2	VT2-F VT2-F	5'- AAGAAGATGTTTATGGCGGT-3' 5'- CACGAATCAGGTTATGCCTC-3'	346bp	

Protease IV	TCF TCR	5′-TATTTCGCCGACTCCCTGTA-3′ 5′-GAATAGACGCCGCTGAAATC-3	134	[16]
algD	algD-F algD-R	5′-ATGCGAATCAGCATCTTTGGT-3′ 5′-CTACCAGCAGATGCCCTCGGC-3′	1310	
plcH	plcH-F plcH-R	5′-GAAGCCATGGGCTACTTCAA-3′ 5′-AGAGTGACGAGGAGCGGTAG-3′	307	
plcN	plcN-F plcN-R	5′-GTTATCGCAACCAGCCCTAC-3′ 5′-AGGTCGAACACCTGGAACAC-3′	466	
exoU	exoU-F exoU-R	5′- CCGTTGTGGTGCCGTTGAAG-3′ 5′- CCAGATGTTCACCGACTC G-3′	134	
ExoT	exoT-F exoT-R	5′-AATCGCCGTCCAACTGCATGCG-3′ 5′- TGTTCGCCGAGGTACTGCTC-3′	152	
ExoA	Eta1B Eta2	5′-AACCAGCTCAGCCACATGTC -3′ 5′-CGCTGGCCCATTCGCTCCAGCGCT-3′	207	
ExoS	ExoSf2 ExoSr2	5′-ATC GCTTCAGCAGAGTCCGTC-3′ 5′-CAGGCCAGATCAAGGCCGCGC-3′	1352	
CIF2	CIF2 F2 CIF2 R2	5’-TTC GAG TTG CTG ATG AAG AAG G-3’ 5’-ATT TAC CAC AAG GAC TAC CAG C-3’	495	[14]
RIF5	RIF5 F10 RIF5 R13	5’-TTC TTA AGT ACA CGC TGA ATC G-3’ 5’-GTC ACA GTA ATT CCA TCA ATG C-3’	414	
ccrB2	ccrB2 F2 ccrB2 R2	5’-AGT TTC TCA GAA TTC GAA CG-3’ 5’-CCG ATA TAG AAW GGG TTA GC-3’	311	
mecI	mecI P2 mecI P3	5’-ATC AAG ACT TGC ATT CAG GC-3’ 5’-GCG GTT TCA ATT CAC TTG TC-3’	209	
mecA	mecA P4 mecA P7	5’-TCC AGA TTA CAA CTT CAC CAG G-3’ 5’-CCA CTT CAT ATC TTG TAA CG-3’	162	
SCCmecVJ1	SCCmecV J1 F SCCmecV J1 R	5’-TTC TCC ATT CTT GTT CAT CC-3’ 5’-AGA GAC TAC TGA CTT AAG TGG-3’	377	
Dcs	dcs F2 dcs R1	5’-CAT CCT ATG ATA GCT TGG TC-3’ 5’-CTA AAT CAT AGC CAT GAC CG-3’	342	
Kdp	kdp F1 kdp R1	5’-AAT CAT CTG CCA TTG GTG ATG C-3’ 5’-CGA ATG AAG TGA AAG AAA GTG G-3’	284	
SCCmecIIIJ1	SCC mec III J1 F SCCmec III J1 R	5’-CAT TTG TGA AAC ACA GTA CG-3’ 5’-GTT ATT GAG ACT CCT AAA GC-3’	243	
TypeI	Type I-F Type I-R	5’-GCT TTA AAG AGT GTC GTT ACA GG-3’ 5’-GTT CTC TCA TAG TAT GAC GTC C-3’	613	[15]
TypeII	Type II-F Type II-R	5’-CGT TGA AGA TGA TGA AGC G-3’ 5’-CGA AAT CAA TGG TTA ATG GAC C-3’	398	
TypeIII	Type III-F Type III-R	5’-CCA TAT TGT GTA CGA TGC G-3’ 5’-CCT TAG TTG TCG TAA CAG ATC G-3’	280	
TypeIVa	Type IVa-F Type IVa-R	5’-GCC TTA TTC GAA GAA ACC G-3’ 5’-CTA CTC TTC TGA AAA GCG TCG-3’	776	
TypeIVb	Type IVb-F Type IVb-R	5’-TCT GGA ATT ACT TCA GCT GC-3’ 5’-AAA CAA TAT TGC TCT CCC TC-3’	493	
TypeIVc	Type IVc1-F Type IVc1-R	5’-TCT ATT CAA TCG TTC TCG TAT T-3’ 5’-TCG TTG TCA TTT AAT TCT GAA CT-3’	200	
TypeIVd	Type IVd1-F Type IVd1-R	5’-AAT TCA CCC GTA CCT GAG AA-3’ 5’-AGA ATG TGG TTA TAA GAT AGC TA-3’	881	
TypeV	Type V-F Type V-R	5’-GAA CAT TGT TAC TTA AAT GAG CG-3’ 5’-TGA AAG TTG TAC CCT TGA CAC C-3’	325	
ccrC	ccrC-F ccrC-R	5’-CGT CTA TTA CAA GAT GTT AAG GAT AAT-3’ 5’-CCT TTA TAG ACT GGA TTA TTC AAA ATA T-3’	495	

Table 2

THE COMPOSITION OF THE REACTION MIX

The gene	Concentration						Final volume
	primer	$MgCl_2$	Dntp	DNA Taq-pol	Reaction buffer	DNA	
bla_{VIM}, bla_{IMP}, bla_{TEM} bla_{CTX-M} bla_{NDM}	0.5µM	1.2mM	2µM	0.2Uµl	1x	10x	20µL

characterization of the SCCmec cassette types in *S.aureus* strains was performed using simplex and multiplex PCR. Two reactions were performed using the multiplex PCR with five and four pairs of specific primers respectively for the various sequences of the SCCmec cassette. Their classification and parameters used to conduct the reactions followed the protocol developed by Miheirico et al. [14] and Zhang et al. [15]. The detection of the specific virulence genes was performed by three simplex PCR and three multiplex PCR assays according with previous published protocols [16].

Results and discussions

P. aeruginosa strains isolated from hospital surfaces after decontamination were resistant in high proportions to ticarcillin (53.33%), third and fourth generation cephalosporins (ceftazidime=53.33% and cefepime= 46.66%), ciprofloxacin (33.33%), gentamicin (26.66%), carbapenems (imipenem and meropenem) (20%), piperacillin (20%), piperacillin+tazobactam (13.33%).

Regarding the antibiotic resistance genes our study revealed that 20% of the analyzed strains resistant to meropenem and imipenem produced imipenemase and 26.6% of the analyzed strains revealed the presence of *bla*TEM gene. No quinolone resistance genes were found in the investigated strains and only one isolate revealed the aac3Ia gene which confers aminoglycoside resistance. Concerning the virulence profiles of *P. aeruginosa* strains, the molecular analysis through PCR arrays showed that all the analyzed strains revealed the *plcH* gene, in 86.6% of the strains were found *plcH, ExoS* and *AlgD* genes and only 33.3% of *P. aeruginosa* harbored the *ExoU* gene. In *P. aeruginosa* strains, the plcH gene encodes for a phospholipase that promotes the degradation of the erythrocyte membrane, exposing the inner layer to PlcN that could then hydrolyze the phospatidylserine residues present on this level.

In 2016 Porumbel et al., revealed the presence of the both PlcH and PlcN genes in 27 MDR nosocomial strains isolated from hospitalized patients with primary cardiovascular diseases, The presence of algD gene encoding for alginate production in the majority of tested strains demonstrates their possible involvement in infections with biofilm formation [29]; 18% of the analyzed strains expressed the ExoS gene - an anti-phagocytic factor which could affect eukaryotic cell cytoskeletal structure [30] and less frequently, the ExoU gene a highly virulence marker in *P. aeruginosa* isolates from hospital-acquired pneumonia [31].

The *E. coli* and *Klebsiella pneumoniae* strains exhibited â-lactam [60% of the isolates were resistant to cefepime; 56% to piperacillin-tazobactam; 52% to ceftazidime; 40% to carbapenems (imipenem and meropenem) and 32% to amoxicillin-clavulanic acid and piperacillin]; amino-glycosides (52% of the strains were resistant to amikacin and 48% to gentamicin and tobramycin); quinolones (48% were resistant to ciprofloxacin); tetracyclines (56%) resistance. The results of PCR analysis for carbapenemases and ESBLs genes revealed the presence of $bla_{NDMlike}$ gene (28% of *K. pneumoniae* isolates and 20% of *E.coli* strains); $bla_{CTX\text{-}Mlike}$ gene in 28% of *E.coli* and 24% of *K. pneumoniae* isolates and $bla_{OXA\text{-}48like}$ gene only in 4% of the *K. pneumoniae* strains. The genetic findings suggest the possible nosocomial origin of the respective strains. The carbapenemases and ESBLs genes may reside within the same plasmids and, therefore, be spread together. Quinolone resistance was revealed by QnrA gene (16% of *E.coli* and 4% of *K. pneumoniae);* gyrB gene (25% of *E.coli* and 16% of *K. pneumoniae);* and parE gene (25% of *E.coli* isolates).

Regarding the virulence genes of the *Enterobacteriaceae* strains, our results have revealed the presence of fimH gene in the case of 24% of the investigated strains; papC and sfaDE (20%); hlyA and cnf1 genes (12%); eaea and VT2 genes (4%). The FimH gene encodes for type I fimbriae which are involved in the colonization of urinary tract, which may suggest a crucial role of this virulence gene in infections caused by *E. coli* [32]. Similar results were obtained. The study performed by Chelariu et al., in 2016 on 133 strains isolated from stool samples in patients with metabolic syndrome admitted in Cardiology and Internal Medicine Units of Hospital Prof. C-tin Angelescu also revealed the fimH as the most frequent virulence marker. The sfaDE gene represents an important adhesin of uropathogenic *E. coli* strains causing urinary tract infections in humans [33] and The HlyA is a pore-forming toxin, which belongs to the family of RTX (repeats in toxin) toxins that are widespread among the Gram-negative pathogens [34]. In 2015, Grosu et al., also revealed in a high percent the expression of fimH virulence factor in *E.coli* strains isolated from urinary tract infections from the ambulatory sector of Central Laboratory Regina Maria Hospital in Bucharest, Romania from January to December 2014 [35-37].

In *S. aureus* isolates the molecular analysis through PCR arrays showed that 40% of the isolates revealed the SCCmec cassette of type II, followed by type V and IIIA (30%) and type IV (2B) (20%). Regarding the virulence genes, the molecular analysis using PCR methods showed that 40% of the analyzed strains expressed both the clumping factor (clf) A and B genes and 50% of the isolates presented the collagen adhesin (cna) gene. The finding that, among the investigated *S. aureus* strains, the majority present adhesions, demonstrates that these strains have the potential to initiate an infectious process.

Conclusions

Incorrect practices in biocides use, regarding contact time and concentrations or instruments and surfaces insufficient cleaning before disinfection can decrease their effect and favor the persistence of resistant and virulent microbial strains in the hospital environment. Moreover, microorganisms can be incorporated in different organic substrata, like pus, blood, serum, food waste or may grow in biofilms, that may also impair the effect of disinfectants. Therefore, monitoring of biocides efficacy and resistance should be implemented as a key component of the hospital infections control strategies.

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