Antibioresistance and Virulence Factors Spectrum in Several Bacterial Strains Isolated from Polluted Environments

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Abstract

Most studies concerning antibiotic resistance are performed on strains isolated from the clinical environment. This phenomenon should not be neglected in bacterial strains isolated from natural or polluted environments, because the phenomenon of virulence and antibiotic resistance occurred in these environments and can contribute to maintaining a reservoir of virulence and antibiotic resistance. This study aimed to assess the resistance factors as well as the antibiotic resistance pattern in bacterial strains isolated from environments polluted with oil products or heavy metals. The strains under study exhibited virulence factors, 85% of the strains had the ability to synthesize amylases, 65% to hydrolyze esculin and gelatin, 50% to produce lecithinases, and hemolysins, and 40% to metabolize casein. To some bacterial strains, the *bla*NDM and *bla*IMP resistance genes were identified. These microorganisms showed intermediate resistance to Carbapenem, Monobactam and Aminoglycoside antibiotic classes. Pollutants exert selective pressure on native microorganisms contributing to the development of defense mechanisms against pollution stress, these mechanisms can be used in virulence and antibiotic resistance.

Introduction

The phenomenon of antibiotic resistance is a topic of real interest, being studied extensively especially in the last decades, in connection with the increasing use of antibiotics [1].

Uncontrolled use of antibiotics in human and veterinary medicine has a major impact on human health, as well as implications on the ecosystems, leading to the selection of antibiotic-resistant bacterial strains, which represent today a real problem for the effective treatment of bacterial infections [2].

Martinez, 2009, mentioned the adverse effects of antibiotics upon aquatic ecosystems, causing imbalances in the specific microbiota and increasing the incidence of antibiotic-resistant bacteria.

Internationally, most studies concerning antibiotic resistance are performed on strains isolated from the clinical environment. The first references to the phenomenon of antibiotic resistance in strains isolated from the external environment were made by EUCAST (European Committee for Antimicrobial Susceptibility Testing), the environmental microorganisms representing true reservoirs of resistance genes. This is why the expanding of researches on antibiotic resistance and virulence of microorganisms from polluted environments becomes a must.

Materials And Methods

Biological material

The study was carried out on 20 bacterial strains isolated from environments polluted with oil products or heavy metals, belonging to the Department of Microbiology of the Bucharest Institute of Biology (IBB) collection; 17 strains are classified in the *Pseudomonas* genus, and the other three in *Shewanella*, *Alcaligenis* and *Serratia* genera, respectively. The taxonomic classification of the strains was made in advance of the present study.

The antibiotic susceptibility/resistance spectrum of the strains under study was assessed by the Kirby-Bauer disc diffusion method, following EUCAST provisions.

Inoculation was performed by the pour plate technique on the Müller-Hinton medium with a bacterial suspension corresponding to 0.5 McFarland turbidity standard. Antibiotic-impregnated discs (bioMérieux), standardized (according to CLSI 2018 Clinical Laboratory Standards Institute) were applied to the inoculated plates. The plates were incubated for 18–24 hours at 37°C, and the results read by measuring the diameters of the growth inhibition zones generated by different antibiotics, according to CLSI 2018 tables, with standardized critical points for the diffusimetric method.

Antibiotic resistance was also being considered when bacterial colonies visible to the naked eye appeared in the area of inhibition.

The microbial strains were tested against Imipenem (IMP), Piperacillin + tazobactam (TZP), Aztreonam (ATM), Cefepim (FEP), Piperacycline (PRL), Meropenem (MEN), Amikacin (AK), Colistin (CT), Tobramycin (TOB), Gentamicin (CN), Doripenem (DOR) and Ciprofloxacin (CIP).

Highlighting the expression of **soluble virulence factors** was performed by biochemical tests, using specific culture media, which were incubated at 37°C for 24-72h according to the methodology described by [4].

Hemolysin production was assessed by inoculating the strains in agar with 5% sheep blood. The bacterial strains that can produce hemolysins show a transparent halo.

The presence of **lecithinases** was estimated by the interest bacterium onto an solid medium containing egg yolk. Lecithinase synthesis results in a white opaque zone of precipitation that spreads beyond the edge of the colony.

Lipase detection was carried out by the spot method on agar supplemented with 1% Tween 80 (sorbitol monooleate). The presence of an opaque precipitation zone around the growth area, given by the formation of insoluble calcium oleate crystals (crystals formed between the released fatty acids and Ca^{2+}) was considered a positive reaction.

Assessment of **amylases** was performed on 1% starch supplemented agar. After the spot-seeding of the bacterial strains and incubation, the Petri dishes were flooded with Lugolol solution. The formation of a yellow ring around the inoculation sites is considered a positive reaction.

To highlight the proteases (caseinase and gelatinase), the strains were seeded on agar added with 15% casein and 3% gelatin, respectively. The strains that secrete caseinase have a precipitation zone around the culture spots due to the formation of calcium para-caseinate. Strains that can synthesize gelatinase liquefy entirely or partially the medium.

DNase presence was assessed by growing the microorganisms on agar medium with DNA and toluidine blue. After incubation, a pink halo is observed around the culture spot of positive strains, while the rest of the medium remains blue [4, 5].

Hydrolysis of esculin was estimated by cultivation on esculin and iron citrate supplemented medium. The strains that have the ability to hydrolyze esculin cause blackening of the medium, due to the esculetol resulted from the hydrolysis of esculin and its combination with the iron salts present in the medium [4, 5].

Highlighting of antibiotic resistance genes by PCR method

In order to perform PCR reactions, the following mixture was used: 12.5 µl Maxter Mix Promega, 20 µM forward primer – 1 µl, 20 µM reverse primer – 1 µl, DNA – 1 µl, and sterile Mili-Q water up to 25 µl / tube. The PCR reaction was performed with a Mastercycler pro S equipment (Eppendorf, Hamburg, Germany). The amplification conditions for the three pairs of primers were: initial denaturation at 93°C – 5 min, followed by 35 cycles of 92°C for 1min, 50°C for 50s, 72°C for 1.30 min. and a final extension of 72°C for 10 min.

PCR was used for signaling the amplicons for the blaVIM, blaNDM, blaIMP genes [6, 7], the following primers were used: blaIMP (5'GGTTTGGCGATCTGGTTTTC3') (5'CGGAATGGCTCATCACGATC3'); blaNDM (5'GATGGTGTGTTGGTCGCATA3') (5'CGAATGCGCAGCACCAG3'); blaVIM (5'GGAATAGAGTGGCTTAAYTCTC3') (5'GGTTTAAAYAAAACAACCACC3'). After amplification the PCR product was analysed on 1% agarose gels-1X TAE and run at 3V/cm for 2 h [7, 8] with ethidium bromide and visualized under ultraviolet light (SynGene System), products were determined by comparison with a molecular-sized standard (1kb DNA ladder Promrga) [9].

Results And Discussion

The strains under study were Gram-negative, oxidase-positive, and catalase-negative. Most microbial strains were sensitive to the tested antibiotics (Table 1), and only six were resistant to one or more antibiotics, representing 30% of the tested strains. *Pseudomonas spp.* (M27) and *Pseudomonas spp.* (Psfl4) were resistant to one antibiotic and the strains *Alcaligenisspp.* and *Pseudomonasspp* (CMM2) were resistant to two antibiotics: the first to IMP and ATM and the latter to ATM and MEM. T *Pseudomonas spp.* (Ps3c) and *Pseudomonas aeruginosa* (C16) had multiple antibiotic resistance, being resistant to seven and eight antibiotics, respectively, with a similar phenotypic pattern of resistance.

Table 1
Evaluation of antibiotic resistance of some bacterial strains isolated from polluted environments

Antibiotic Strain	IMP	ATM	TZP	FEP	PRL	MEM	AK	CIP	DOR	TOB	CT	CN
<i>Pseudomonas spp.</i> (M23)	s	s	s	s	s	s	s	s	s	s	s	s
<i>Pseudomonas spp.</i> (4-Pa)	s	s	s	s	s	s	s	s	s	s	s	s
<i>Pseudomonas spp.</i> (M28)	s	s	s	s	s	s	s	s	s	s	s	s
<i>Pseudomonas spp.</i> (M26)	s	s	s	S	S	s	s	s	s	s	s	s
<i>Pseudomonas spp.</i> (M24)	s	s	s	s	s	s	s	s	s	s	s	s
<i>Pseudomonas spp.</i> (M27)	s	s	s	s	s	s	s	s	s	R	s	s
<i>Alcaligenisspp.</i>	R	R	s	s	s	s	s	s	s	s	s	s
<i>Pseudomonas putida</i> (P1)	s	s	s	s	s	s	s	s	s	s	S	s
<i>Pseudomonas putida</i> (P2)	s	s	s	s	s	s	s	s	s	s	S	s
<i>Shewanella spp.</i> (SH)	s	s	s	S	S	s	s	s	s	s	S	s
<i>Pseudomonas spp.</i> CMM1	s	s	s	s	s	s	s	s	s	s	S	s
<i>Pseudomonas spp.</i> CMM2	S	R	s	s	s	R	s	s	s	s	s	s
<i>Pseudomonas spp.</i> (Psfl4)	s	s	s	s	s	s	s	s	s	s	s	R
<i>Pseudomonas spp.</i> (Ps3c)	R	R	R	s	s	R	s	R	R	R	s	S
<i>Pseudomonas aeruginosa</i> (C16)	R	R	R	s	s	R	s	R	R	R	s	R
<i>Pseudomonas spp.</i> Ps3d	s	s	s	S	S	s	s	s	s	s	S	s
<i>Pseudomonas spp.</i> (D8.1)	s	s	s	s	s	s	s	s	s	s	s	s
<i>Pseudomonas spp.</i> (S3)	s	s	s	s	s	s	s	s	s	s	s	s
<i>Pseudomonas spp.</i> (D7.2)	s	s	s	s	s	s	s	s	s	s	s	s
<i>Serratia spp.</i> (VF)	s	s	s	s	s	s	s	s	s	s	s	s
R-resistance, S- sensitive												

The similarity can be attributed to the pressures exerted by pollutants. Originating from oil-polluted environments, these strains have developed similar resistance mechanisms under the selective action of contaminants.

The resistance level of the strains isolated from polluted environments (Fig. 1) was evaluated according to the resistance intervals proposed by the European Center for Infectious Disease Control (ECDC/European Resistance Surveillance Network EARS-Net) for bacterial strains isolated from the human patients. Thus: <1% = insignificant, 1 to < 5% = very low, 5 to < 10% = low; 10 to < 25% = intermediate; 25 to < 50% = high; >50% = very high (http://ecdc.europa.eu/en/activities/surveillance/EARS-Net/database/Pages/maps_report.aspx).

Analyzing the resistance level of the studied strains, an insignificant resistance was found to EFF (4th generation Cephalosporins), PRL (Penicillin class), and CT (Polymyxine class), while for TZP, CIP (Fluoroquinolone class), DOR (Carbapeneme class), and CN (Aminoglycosides class) the exhibited resistance was low. The tested strains showed intermediate resistance to antibiotics belonging to Carbapenems (IMP, MEN) 15%, Monobactams (ATM) 20%, and Aminoglycosides (TOB) 15%.

The spectrum of soluble virulence factors in bacterial strains isolated from different polluted environments

The presence of soluble virulence factors in the in the tested microorganisms was assessed by analyzing the phenotypic results of solubilization tests, which demonstrated the ability of these strains to produce enzymes involved in both pathogenesis and bacterial survival in the external environment.

In the present study, we analyzed eight virulence factors, these being more or less present with different levels of expression.

Hydrolysis of esculin

In both natural and interstitial environments, the amounts of Fe^{2+} required for bacterial metabolism were low, and the microorganisms have developed specific mechanisms for the acquisition of iron ions. Thus, the biochemical property of esculin hydrolysis (a complex heteroside) to esculetin may be a nonspecific mechanism of iron acquisition, representing a virulence factor as well.

65% of the studied strains, could hydrolyze esculin (Table 2). Because they are strains isolated from polluted environments, this property can favor the development of bacteria in the external environment poor in iron ions, but it can also be a virulence factor when invading the human body.

Amylase was produced by 85% of the studied strains isolated from various polluted environments. The bacteria that have the ability to synthesize this enzyme have the advantage that they can survive in environments with limited resources (Table 2).

Table 2
Presence of virulence factors in the tested microorganisms

Enzyme Strain	Esculinase	Amylase	Cazeinase	Gelatinase	Hemolysin	Lecitinase	Lipase	DNase
<i>Pseudomonas</i> spp. (M23)	-	+	-	+	+	+	-	-
<i>Pseudomonas</i> spp. (4-Pa)	-	-	-	+	-	-	-	-
<i>Pseudomonas</i> spp. (M28)	+	+	-	-	+	+	-	-
<i>Pseudomonas</i> spp. (M26)	+	+	+	+	+	-	-	-
<i>Pseudomonas</i> spp. (M24)	+	-	+	-	-	+	-	-
<i>Pseudomonas</i> spp. (M27)	+	-	+	+	+	+	-	-
<i>Alcaligenis</i> spp. (Alc)	+	+	-	+	+	+	-	-
<i>Pseudomonas putida</i> (P1)	-	+	-	-	-	-	-	-
<i>Pseudomonas putida</i> (P2)	-	+	-	-	-	-	-	-
<i>Shewanella</i> spp. (SH)	-	+	-	+	-	-	-	-
<i>Pseudomonas</i> spp. (CMM1)	-	+	+	+	-	+	+	-
<i>Pseudomonas</i> spp.(CMM2)	-	+	+	+	-	+	+	-
<i>Pseudomonas</i> spp. (Psfl4)	-	+	+	+	+	-	+	-
<i>Pseudomonas</i> spp. (Ps3c)	-	+	+	-	+	-	-	-
<i>Pseudomonas aeruginosa</i> (C16)	-	+	-	+	-	-	-	-
<i>Pseudomonas</i> spp.(Ps3d)	-	+	-	-	+	-	-	-
<i>Pseudomonas</i> spp. (D8.1)	+	+	-	+	+	+	-	-
<i>Pseudomonas</i> spp. (S3)	+	+	-	-	-	+	-	-
<i>Pseudomonas</i> spp. (D7.2)	+	+	+	+	+	+	-	-
<i>Serratiaspp.</i> (VF)	-	+	-	+	-	-	-	-
+ positive - negative								

Instead, **caseinase** was synthesized by 8 bacterial strains, representing 40% of the tested strains. This extracellular protease hydrolyzes proteins to peptides and amino acids, giving a competitive advantage to the producing strains for colonization of a particular niche and survival in different environments.

Gelatinase gives the strains the ability to invade and disseminate in the host organism. Also, this enzyme is involved in the survival and maintenance of the reservoir of microorganisms in the external environment.

Out of the 20 strains under test, 13 produced gelatinase, which means 65% of the strains can degrade gelatin.

Four bacterial strains among the tested ones synthesized a low amount of enzyme because they did not completely liquefy the whole amount of culture medium on which they grew.

Hemolysins(extracellular enzymes) were present in 50% of the strains isolated from polluted environments. These enzymes cause tissue damage, as an expression of the pathogenicity of various bacterial species.

On egg yolk containing medium, 10 strains exhibited the capacity to synthesize **lecithinases**, representing 50% of the tested strains.

Only three strains, *Pseudomonas spp* (Psfl4), *Pseudomonas fluorescens* (CMM2), and *Pseudomonas spp*(CMM1), produced **lipases** in the medium with Tween-80.

Neither strain under test possessed the ability to synthesize **DNase**, this being a competitive disadvantage in the ecological niche of origin.

All tested strains showed one or more virulence factors, the strain *Pseudomonas spp.* (D7.2) exhibiting the most, six out of the eight tested.

Investigation of molecular markers of antibiotic resistance

In the present study, we aimed to investigate the *blaVIM*, *blaIMP*, *blaNDM* genes; these genes are encoders of metallo- β -lactamases (carbapenemases), which contain Zn^{2+} at the active site [10]. Bacterial strains that synthesize these enzymes are resistant to penicillins, cephalosporins, carbapenems, and β -lactamase inhibitors. They do not hydrolyze aztreonam and are sensitive to bivalent metal chelating agents, such as EDTA (ethylenediaminetetraacetic acid).

For the *blaNDM* gene, in addition to the amplicon of interest indicated in the literature with a size of 475 bp [11], 2014) secondary amplicons were obtained in most of the tested strains (Fig. 2).

The strains *Pseudomonas spp* CMM1, *Pseudomonas spp*(M28), *Pseudomonas spp.* (M23), *Pseudomonasputida* (P2), *Alcaligenis spp.*, *Pseudomonas spp.* (S3), *Pseudomonas spp.* (4-Pa), *Pseudomonas spp.*(8.1)no amplification of the sequence of interest was found. Furthermore, we found that the genotypic expression is different from the phenotypic one. Although some bacterial strains possessed genetic support, they did not exhibit the phenomenon of resistance to the tested antibiotics.

Following the amplification of the *blaIMP* gene, a positive PCR reaction was found in *Pseudomonas spp* (Psfl4), *Pseudomonas spp.* (Ps3c), *Pseudomonas aeruginosa* C16, *Pseudomonas spp.* 8.1. The amplicons obtained in the four strains had different dimensions from those indicated in the literature, of 587 bp [12, 13], in three of the four positive strains, the amplicon was much larger, over 1000 bp, while the amplicon obtained in the case of *Pseudomonas spp* (Psfl4) was about 250 bp (Fig. 3).

In the case of the *blaVIM* gene amplification, the results were negative in all 20 bacterial strains isolated from polluted environments. The *blaVIM* gene was rarely reported in bacterial strains isolated from the external environment, being

frequently found in the clinical environment [14, 15] and wastewaters from hospitals [16].

Conclusions

The tested strains showed intermediate resistance to antibiotics from the Carbapenem, Monobactam, and Aminoglycoside classes;

The tested bacterial strains exhibited at least one virulence factor;

Similar to strains isolated from the clinical environment, the strains isolated from contaminated environments possess the *bla**NDM* and *bla**IMP* resistance genes;

Bacterial strains from polluted environments can be a reservoir of virulence and antibiotic resistance and can contribute to the vertical and horizontal transmission of these phenomena;

Declarations

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Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

"Not applicable" in this section, because, this manuscript does not report on or involve any animals, humans, human data, human tissue or plants.

Consent for publication statement

Not applicable" in this section.

Availability of data and material

All data generated or analyzed during this study are included in this published article. Data are available from the authors upon reasonable request, are also found at ICUB (Research Institute of the University of Bucharest).

Code availability

Not applicable for that section

Authors' contributions

The authors contributed equally to the preparation of this article

Ethics approval

Not applicable for that section

Consent to participate

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Not applicable for that section

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Figures

Figure 1

Distribution of antibiotic resistance markers (%) in strains isolated from different polluted environments

Figure 2

BlaNDM amplicon electrophoresis

Figure 3

BlaIMP amplicons electrophoregram (587 bp)