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Pseudomonas aeruginosa: Multi-Drug-Resistance Development and Treatment Options

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1. Introduction

Antibiotic resistance is a worldwide problem of major importance. Isolations in some countries of multi-drug-resistant (resistant to three or more classes of antimicrobials), extensively-drug-resistant (resistant to all but one or two classes) or even pan-drug-resistant (resistant to all available classes) Gram-negative pathogens are causing therapeutic problems and- in the same time- are posing infection control issues in many hospitals. In fact, numerous studies highlight the link between multi-drug-resistance and increased morbidity and mortality, increased length of hospital stay and higher hospital costs [1-4].

Pseudomonas aeruginosa is a Gram-negative opportunistic nosocomial pathogen responsible for a wide range of infections that may present high rates of antimicrobial resistance. The genome of this microorganism is among the largest in the bacterial world allowing for great genetic capacity and high adaptability to environmental changes. In fact, *P. aeruginosa* has 5567 genes encoded in 6.26 Mbp of DNA while *Escherichia coli* K12 for example has 4279 genes encoded in 4.46 Mbp and *Haemophilus influenzae* Rd has 1.83 Mbp encoding 1714 genes [5]. This large genetic armamentarium- that can be further enriched with the addition of genes acquired by transferable genetic elements via horizontal gene transfer- is a major contributing factor to its formidable ability to develop resistance against all known antibiotics.

Generally, antibiotic resistance mechanisms of *P. aeruginosa* can be divided in intrinsic and acquired. Intrinsic refers to resistance that is a consequence of a large selection of genetically-encoded mechanisms and acquired refers to resistance that is achieved via the acquisi-

tion of additional mechanisms or is a consequence of mutational events under selective pressure.

2. Intrinsic resistance of *Pseudomonas aeruginosa*

P. aeruginosa shows inherent resistance to antimicrobial agents through a variety of mechanisms: (1) decreased permeability of the outer membrane, (2) efflux systems which actively pump antibiotics out of the cell, and (3) production of antibiotic-inactivating enzymes [6].

2.1. Outer membrane permeability

The outer membrane of Gram-negative bacteria is a barrier which prevents large hydrophilic molecules to pass through it. Aminoglycosides and colistin interact with lipopolysaccharides changing the permeability of the membrane in order to pass whereas beta-lactams and quinolones need to diffuse through certain porin channels.

Bacteria produce two major classes of porins: general; which allow almost any hydrophilic molecule to pass [7] and specific; which have binding sites for certain molecules, allowing them to be oriented and pass in the most energy-efficient way [8].

Most bacteria posses lots of general porins and relatively few specific ones. However, the exact opposite occurs for *P. aeruginosa* that expresses mainly specific porins [7].

2.2. Efflux systems

P. aeruginosa expresses several efflux pumps that expel drugs together with other substances out of the bacterial cell. These pumps consist of three proteins: (1) a protein transporter of the cytoplasmatic membrane that uses energy in the form of proton motive force, (2) a periplasmic connective protein, and (3) an outer membrane porin [5].

Most antibiotics- except polymyxins- are pumped out [9,10] by these efflux systems (Table 1) therefore their first two components are named multidrug efflux (Mex) along with a letter (e.g. MexA and MexB). The outer membrane porin is called Opr along with a letter (e.g. OprM) [11].

2.3. Antibiotic-inactivating enzymes

P. aeruginosa belongs to the SPICE group of bacteria (*Serratia* spp., *P. aeruginosa*, Indole positive *Proteus*, *Citrobacter* spp., *Enterobacter* spp.). These microorganisms share a common characteristic: the ability to produce chromosomal-encoded and inducible AmpC beta-lactamases. These are cephalosporinases that hydrolyze most beta-lactams and are not inhibited by the beta lactamase inhibitors.

Another endogenous beta-lactamase produced by *P. aeruginosa* is the class D oxacillinase PoxB [12,13]. This enzyme however has only been found in laboratory mutants and is not clinically significant.

Efflux system	Efflux pump family	Substrates	References
MexAB-OprM	Resistance Nodulation Division (RND)	Fluoroquinolones Aminoglycosides β-Lactams (preferably Meropenem, Ticarcillin) Tetracycline Tigecycline Chloramphenicol	[17]
MexCD-OprJ	Resistance Nodulation Division (RND)	Fluoroquinolones β-Lactams (preferably Meropenem, Ticarcillin) Tetracycline Tigecycline Chloramphenicol Erythromycin Roxythromycin	[17]
MexEF-OprN	Resistance Nodulation Division (RND)	Fluoroquinolones β-Lactams (preferably Meropenem, Ticarcillin) Tetracycline Tigecycline Chloramphenicol	[17] [18]
MexXY-OprM	Resistance Nodulation Division (RND)	Fluoroquinolones Aminoglycosides β-Lactams (preferably Meropenem, Ticarcillin, Cefepime) Tetracycline Tigecycline Chloramphenicol	[17]
AmrAB-OprA	Resistance Nodulation Division (RND)	Aminoglycosides	[19]
PmpM	Multidrug And Toxic compound Extrusion (MATE)	Fluoroquinolones	[17]
Mef(A)	Major Facilitator Superfamily (MFS)	Macrolides	[20]
$ErmE_{PAF}$	Small Multidrug Resistance (SMR)	Aminoglycosides	[21]

Table 1. Efflux systems of *P. aeruginosa*.

3. Antipseudomonal treatment

Despite the intrinsic resistance of *P. aeruginosa* to many antimicrobials, some antibiotics are active against this microorganism [14]. Those used more frequently belong to three antibiotic classes: (1) Beta-lactams, (2) Quinolones and (3) Aminoglycosides (Table 2).

3.1. Beta-lactams

Beta-lactams bind to and inactivate penicillin-binding proteins (PBPs) that are transpeptidases involved in bacterial cell wall synthesis [15]. The group of beta-lactam antibiotics includes penicillins, cepholosporins, monobactams and carbapenems. The beta-lactams that are most active against *P. aeruginosa* are: Piperacillin and ticarcillin (penicillins), ceftazidime (3rd generation cephalosporin), cefepime (4th generation cephalosporin), aztreonam (monobactam), imipenem, meropenem and doripenem (carbapenems).

3.2. Quinolones

Quinolones are synthetic antimicrobials that block DNA replication by inhibiting the activity of DNA gyrase and topoisomerase IV [16]. The fluorquinolones with anti-pseudomonal activity are ciprofloxacin, levofloxacin and ofloxacin.

Antibiotic Class	Mechanism of action	Drug
Penicillins	Bacterial cell wall synthesis inhibition	Ticarcillin
Penicillin / Beta-lactamase inhibitor	Bacterial cell wall synthesis inhibition	Ticarcillin/Clavulanic acid
		Piperacillin/Tazobactam
Cefalosporins	Bacterial cell wall synthesis inhibition	Ceftazidime
		Cefepime
Monobactams	Bacterial cell wall synthesis inhibition	Aztreonam
Carbapenems	Bacterial cell wall synthesis inhibition	Imipenem
		Meropenem
		Doripenem
Fluoroquinolones	Block of DNA synthesis	Ciprofloxacin
		Levofloxacin
		Ofloxacin
Aminoglycosides	Protein synthesis inhibition	Gentamycin
		Tobramycin
		Amikacin

Table 2. Commonly used anti-pseudomonal drugs.

3.3. Aminoglycosides

Aminoglycosides inhibit protein synthesis by binding to the 30S or 50S ribosomal subunit [22]. Drugs of this antibiotic class that can be used against *P. aeruginosa* are tobramycin, amikacin and gentamicin. Aminoglycosides are associated with ototoxicity and nefrotoxicity [23]. Because of these adverse effects and because of their narrow therapeutic range, aminoglycosides are used in combination with agents belonging to other antibiotic classes. The only treatment in which aminoglycosides are recommended as monotherapy is that of urinary tract infections due to *P. aeruginosa* [14].

4. Acquired resistance of *Pseudomonas aeruginosa*

Apart from being resistant to a variety of antimicrobial agents, *P. aeruginosa* develops resistance to anti-pseudomonal drugs as well. This acquired resistance is a consequence of mutational changes or the acquisition of resistance mechanisms via horizontal gene transfer and can occur during chemotherapy [24]. Mutational events may lead to over-expression of endogenous beta-lactamases or efflux pumps, diminished expression of specific porins and target site modifications while acquisition of resistance genes mainly refers to transferable beta-lactamases and aminoglycoside-modifying enzymes (Table 3).

Resistance to	Resistance mechanism
Beta-lactams	Endogenous beta-lactamases
	Acquired beta-lactamases
	Efflux
	Diminished permeability
Fluoroquinolones	Target site mutations
	Efflux
Aminoglycosides	Aminoglycoside-modifying enzymes
	Efflux
	16S rRNA methylases
Polymyxins	LPS modification

Table 3. Resistance mechanisms of *P. aeruginosa* to anti-pseudomonal drugs.

4.1. Resistance to beta-lactams

Resistance to beta-lactam antibiotics is multi-factorial but is mediated mainly by inactivating enzymes called beta-lactamases. These enzymes cleave the amide bond of the beta-lactam ring causing antibiotic inactivation and are classified according to a structural [25] and a functional [26] classification.

Among the beta-lactams, carbapenems are the most efficient against *P. aeruginosa*. These agents are stable to the hydrolytic effect of the majority of the beta-lactamases including the Extended Spectrum Beta-Lactamases (ESBLs) [27]. For this reason, the enzymes that possess carbapenemase activity, namely the carbapenemases [28], will be discussed separately in this section.

4.1.1. Expression of endogenous beta-lactamases

Resistance to beta-lactams in clinical isolates is commonly due to the presence of AmpC beta-lactamases [29-36]. Furthermore, the production of AmpC beta-lactamases in *P. aeruginosa* can be induced by a number of beta-lactam antibiotics such as benzyl penicillines, narrow spectrum cephalosporins and imipenem [37]. In fact, this mutational derepression is one of the most common mechanisms of resistance to beta-lactams in *P. aeruginosa* [29,32,33,36].

AmpC enzymes are not carbapenemases, they posses however a low potential of carbapenem hydrolysis and their overproduction combined with efflux pumps over-expression and/or diminished outer membrane permeability has been proven to lead also to carbapenem resistance in *P. aeruginosa* [38].

4.1.2. Acquired beta-lactamases

Acquired beta-lactamases are typically encoded by genes which are located in transferable genetic elements such as plasmids or transposons [39] often on integrons [40-49]. Integrons are genetic elements that capture and mobilize genes [50]. Other genetic elements associated with transferable resistance in *P. aeruginosa* are the mobile insertion sequences called ISCR elements [49,51-53].

Different types of transferable beta-lactamases have been found in clinical *P. aeruginosa* isolates around the world (Table 4).

Among them, carbapenemases are of major clinical importance because they inactivate carbapenems together with other beta-lactams. Ambler class A ESBLs hydrolyze penicillins, narrow- and broad-spectrum cephalosporins and aztreonam [54]. Some TEM and SHV enzymes do not possess broad-spectrum cephalosporinase activity and are called restricted-spectrum beta-lactamases. Class D OXA beta-lactamases are a heterogenous group of enzymes and not all share the same properties. Generally, most of them show a preference for cloxacillin over benzylpenicillin. They confer resistance to amino- and carboxypenicillins and narrow – spectrum cephalosporins even though some of them are ESBLs and a few members of the class present carbapenemase activity [24].

4.1.3. Carbapenemases

P. aeruginosa is the species in which all types of transferable carbapenemases, except SIM-1 [55], have been detected. The class B carbapenemases that bear Zn^{2+} in their active center [56] are the most frequent around the world in *P. aeruginosa* isolates and are called metallo-beta-lactamases (MBLs). They hydrolyse *in vitro* all beta-lactams except aztreonam and are the major cause of high-level carbapenem resistance. Genes that encode MBLs are commonly found as

Ambler molecular class	Bush-Jacoby-Madeiros group	Enzymes	References
A	2b	TEM-1, -2, -90, -110, SHV-1	[57,58]
	2be	PER-1, -2	[10]
		VEB-1, -2, -3	[53]
		TEM-4, -21, -24, -42, -116	[59-62]
		SHV-2a, -5, -12	
		GES/IBC-1, -2, -5, -8, -9	
		BEL	
		LBT 802	
		CTX-M-1, -2, -43	
	2c	PSE-1 (CARB-2), PSE-4 (CARB-1), CARB-3, CARB-4, CARB-like, AER-1	[10] [63]
	2f	KPC-2, -5	[64,65]
B	3	IMP-1, -4, -6, -7, -9, -10, -12, -13, -15, -16, -18, -22	[10] [47] [66-76]
		VIM-1, -2, -3, -4, -5, -7, -8, -11, -13, -15, -16,-17, -18	
		SPM-1	
		GIM-1	
		AIM-1	
		NDM-1	
C	1	AmpC	[77]
D	2d	OXA	[10]
		LCR-1	[12]
		NPS-1	[54] [57] [78-80]

Table 4. Beta-lactamases found in *P. aeruginosa* isolates.

gene cassettes in integrons and are transferable [42]. Interestingly, more resistance genes for other antibiotic classes can be present in the same integrons contributing thus in the development of a multi-drug resistant phenotype.

IMP and VIM type MBLs were first identified in Japan [81] and Italy [82] respectively and have spread though all continents since then. Other metallo-enzymes are more geographically restricted. SPM-1, after causing outbreaks in Brazil [28], has been found in Basel [83] in a single isolate recovered from a patient previously hospitalized in Brazil. GIM-1 and AIM-1 were

reported from Germany [41] and Australia [84] and did not spread elsewhere. Finally, the only report for NDM-1 in *P. aeruginosa* was made from Serbia [76].

Ambler class A carbapenemase KPC was first reported in *P. aeruginosa* isolates in Colombia [64] but KPC-producing *P. aeruginosa* isolates have not been reported from other continents except Latin America. KPCs present high rates of carbapenem hydrolysis and inactivate all other beta-lactams including aztreonam.

Enzymes GES/IBC belong to the same enzymatic class but their carbapenemase activity is not as high as that of the KPCs. It may become important however if combined with diminished outer membrane permeability or efflux over-expression. For *P. aeruginosa*, GES-2 has been reported in South Africa [85] and IBC-2 in Greece [86].

Class D carbapenemases like OXA-198 have been found in *P. aeruinosa* isolates although such findings are rather rare for this species [87]. The most clinically important carbapenemases are summarized in Table 5.

Ambler molecular class	Bush-Jacoby-Madeiros group	Carbapenemases
A	2f	KPC
B	3	IMP enzymes
		VIM enzymes
		SPM-1
		GIM-1
		AIM-1
		NDM-1

Table 5. Clinically important carbapenemases found in *P. aeruginosa* isolates.

4.1.4. Efflux systems over-expression

Among the various efflux systems of *P. aeruginosa*, MexAB-OprM, MexXY-OprM and MexCD-OprJ play an important role in developing beta-lactam resistance [88]. Between these three, MexAB-OprM accommodates the broadest range of beta-lactams [24], is by far the better exporter of meropenem [24] and is most frequently related to beta-lactam resistance in clinical *P. aeruginosa* isolates [33,89]. The efflux pumps may be over-expressed in some isolates [90] contributing thus, together with other mechanisms in the development of multi-drug resistance [24].

4.1.5. Diminished permeability

OprD is a specific porin of the outer membrane of *P. aeruginosa* through which carbapenems (mainly imipenem) enter into the periplasmic space [91]. Diminished expression [92] or mutational loss [93] of this porin is the most common mechanism of resistance to carbapenems [24,94] and is frequently associated with efflux pumps and/or AmpC over-expression [36,38].

Diminished expression or loss of the OprD porin is a frequent phenomenon during imipenem treatment [95].

4.2. Resistance to fluoroquinolones

High-level resistance to fluoroquinolones is mediated by target site modifications. Efflux plays a contributing role as well [96,97] and the two mechanisms often coexist [32,98-100].

4.2.1. DNA gyrase and topoisomerase IV mutations

Gyrase and topoisomerase are comprised by two subunits each. DNA gyrase (GyrA and GyrB) is the main target of fluoroquinolones in *P. aeruginosa*. Consequently, mutations are most common for this enzyme rather than for topoisomerase IV (ParC and ParE) [98-102]. Highly resistant isolates have multiple mutations in *gyrA* and/or *parC* [98,101-103] while mutations regarding the other subunits are less frequently encountered [100-102,104].

4.2.2. Efflux pumps contribution

Four efflux pumps contribute to fluoroquinolone resistance: MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM [105] as a consequence of mutational events in their repressor genes [24]. Among these, MexAB-OprM, MexCD-OprJ, and MexEF-OprN have been associated to fluoroquinolone resistance in clinical isolates [31,105-107] whereas MexXY-OprM has only been linked rarely to such type of resistance [106].

4.3. Resistance to aminoglycosides

Acquired resistance to aminoglycosides is mediated by transferable aminoglycoside-modifying enzymes (AMEs), rRNA methylases and derepression of endogenous efflux systems [24,108,109].

4.3.1. Aminoglycoside-modifying enzymes

Modification and subsequent inactivation of aminoglycosides is achieved by three deferent mechanisms: (1) acetylation, by aminoglycoside acetyltransferases (AACs), (2) adenylation, by aminoglycoside nucleotidyltransferases (ANTs), and (3) phosphorylation, by aminoglycoside posphoryltransferases (APHs) [108].

Genes encoding AMEs are typically found on integrons together with other genes responsible for transferable resistance for other antibiotic classes. This way AMEs become important determinants for the development of multi-drug resistance in *P. aeruginosa* and other species [24,108,109].

Enzymatic families that acetylate the 3 and 6' position of the antibiotic are the most common. Five subfamilies of AAC(3) and two of AAC(6') have been described for *P. aeruginosa*, each one presenting different preferences for aminoglycoside substrates (Table 6).

Among the nucleotidyltransferases, ANT(2')-I is the most frequently encountered in *P. aerugiosa.* This enzyme is present in isolates showing resistance to gentamicin and tobramycin but not to amikacin [109].

Almost all phosphoryltransferases of *P. aeruginosa* act in the 3' position of the aminoglycoside molecule [24]. However, they have less clinical importance because of the fact that they inactivate aminoglycosides that are not routinely used for the treatment of *P. aeruginosa* infections such as kanamycin and neomycin [109]. The enzymes of this family that inactivate anti-pseudomonal aminoglycosides are APH(3')-VI [110-112], APH(3')-IIb-like [113] and APH(2'') [110]. Despite being reported in some cases, these enzymes remain rare for clinical *P. aeruginosa* isolates [24].

4.3.2. Efflux systems

Resistance to aminoglycosides in *P. aeruginosa* can occur independently of aminoglycoside-modifying enzymes in cystic fibrosis patients. This type of resistance has been reported in several studies [99,118-120] and is attributable to over-expression of the MexXY-OprM efflux pump.

4.3.3. 16S rRNA methylases

Methylation of the 16S rRNA of the A site of the 30S ribosomal subunit interferes with aminoglycoside binding and consequently promotes high-level resistance to all aminoglycosides [24]. Different 16S rRNA methylases have been described for *P. aeruginosa*: RmtA [112,121], RmtB [122], ArmA [122,123] and RmtD which is commonly found together with the MBL SPM-1 in Brazil [124,125].

5. Treatment options for MDR *Pseudomonas aeruginosa*

Different combinations of the aforementioned mechanisms may be present in a single *P. aeruginosa* isolate leading to simultaneous resistance to various anti-pseudomonal compounds. The most potent combination is obviously that of a carbapenemase producing isolate usually enriched by resistance to quinolones and aminoglycosides leaving very limited options for antimicrobial treatment.

As far as newer carbapenem compounds are concerned, data suggest that doripenem does not offer advantages over other carbapenems against carbapenemase producing strains [126].

Tigecycline is an option for Gram-negative MDR pathogens but it cannot be used against *P. aeruginosa*, *Morganella morganii*, *Proteus* spp. and *Providencia* spp. because it is intrinsically vulnerable to their chromosomal-encoded efflux pumps [127].

Furthermore, time-kill studies on 12 MBL-producing *P. aeruginosa* isolates performed with aztreonam alone and in combination with ceftazidime and amikacin, showed bactericidal activity against one and eight isolates respectively. In the same study, colistin was bactericidal against all 12 isolates [128].

Category	Enzymatic family	Subfamily	Substrates	References
Acetyltransferases (AAC)	AAC(3)	I	Gentamicin	[11]
		II	Gentamicin	[48]
			Tobramycin	[108,109]
		III	Gentamicin	
			Tobramycin	
		IV	Gentamicin	
		VI	Gentamicin	
			Tobramycin	
	AAC(6′)	I	Tobramycin	[108,109]
			Amikacin	
		II	Tobramycin	
			Gentamicin	
Nucleotidyltransferases (ANT)	ANT(2′)	I	Gentamicin	[109]
			Tobramycin	
	ANT(4′)	IIa	Tobramycin	[114,115]
			Amikacin	
		IIb	Tobramycin	
			Amikacin	
	ANT(3′)		Streptomycin	[108]
Phosphoryltransferases (APH)	APH(3′)	II	Kanamycin	[109]
			Neomycin	[116]
		IIb	Kanamycin	[117]
		IIb-like	Amikacin (weakly)	[113]
		VI	Amikacin	[110-112]
			Isepamicin	
	APH(2′′)		Gentamicin	[110]
			Tobramycin	

Table 6. Aminoglycoside-modifying enzymes found in *P. aeruginosa* isolates.

In fact, polymyxins and colistin in particular, are quite effective in the treatment of MDR *P. aeruginosa* infections [129,130]. The target of colistin is the bacterial cell membrane. More precisely, colistin interacts with the lipid A of lipopolysaccharides, allowing penetration through the outer membrane by displacing Ca^{2+} and Mg^{2+}. The insertion between the phospholipids leads to loss of membrane integrity and consequent bacterial cell death [131]. There are reports of resistance to polymyxin B [132-134] and colistin [135-137] in clinical isolates but they remain to date relatively rare for *P. aeruginosa* [24]. While in many cases the mechanism of clinical polymyxin resistance is unknown, substitution of the lipopolysaccharide lipid A with aminoarabinose has been shown to contribute to polymyxin resistance *in vitro* [138] and

cystic fibrosis isolates [139]. Colistin is frequently associated with nephro- and neurotoxicity but both these adverse effects seem to be dose-dependent and reversible [140].

Another interesting option for the treatment of MDR *P. aeruginosa* is fosfomycin, an old antibacteial that has regained attention because of its *in vitro* activity against such isolates [140]. Fosfomycin inactivates the enzyme pyruvil-transferase, which is required for the synthesis of the cell wall peptidoglycan. In a review of the existing fosfomycin studies, 81.1% of 1529 patients were successfully treated for infections caused by *P. aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis, Enterobacter* spp. and *Klebsiella* spp. Fosfomycin was administered together with aminoglycosides, cephalosporins and penicillines [141]. More studies are needed however to determine the future role of fosfomycin against MDR *P. aeruginosa* isolates.

6. Combination therapy

The application of combination therapy instead of monotherapy in cases of non-MDR *P. aeruginosa* remains to date a controversial issue [14]. Combination treatment against MDR strains instead seems to be some times necessary (for example in cases of pan-resistance or resistance to all except a single agent). In such cases better results are expected by the additive or subadditive activity of a combination or by the enhancement of a single active agent by an otherwise inactive drug [142].

Several old and newer studies have showed the increased activity *in vitro* of various antibiotic combinations against MDR *P. aeruginosa* (Table 7) even though, the mechanisms of positive interaction between the various agents are rarely known [142].

Antibiotic combination	References
Ticarcillin, Tobramycin, Rifampin	[143]
Cephalosporins, Quinolones	[144]
Ceftazidime, Colistin	[145]
Macrolides, Tobramycin, Trimethoprim, Rifampin	[146]
Polymyxin B, Rifampin	[147]
Polymyxin B, Imipenem	[148]
Colistin, Meropenem	[149]

Table 7. Enhanced activity of antibiotic combinations against MDR *P. aeruginosa*.

7. Conclusion

P. aeruginosa is a nosocomial pathogen of particular clinical concern not only because of its extraordinary resistance mechanisms armamentarium but also for its formidable ability to

adapt very well to the hospital environment. There are important challenges in the treatment of MDR *P. aeruginosa* strains and their isolation in healthcare settings poses serious infection control issues. For these reasons, the prudent use of antibiotics, mainly those used as last resort treatment like carbapenems is of outmost importance in order to prevent evolutionary pressure that may lead to the emergence of highly resistant clones.

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