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Antibiotic resistance genes from the environment: a perspective through newly identified antibiotic resistance mechanisms in the clinical setting

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Abstract

Soil bacteria may contain antibiotic resistance genes responsible for different mechanisms that permit them to overcome the natural antibiotics present in the environment. This gene pool has been recently named the 'resistome', and its components can be mobilized into the microbial community affecting humans because of the participation of genetic platforms that efficiently facilitate the mobilization and maintenance of these resistance genes. Evidence for this transference has been suggested or demonstrated with newly identified widespread genes in multidrug-resistant bacteria. These resistance genes include those responsible for ribosomal methylases affecting aminoglycosides (*armA, rtmB*), methyltransferases affecting linezolid (*cfr*) or plasmid-mediated efflux pumps conferring low-level fluoroquinolone resistance (*qepA*), all of which are associated with antibiotic-producing bacteria. In addition, resistance genes whose ancestors have been identified in environmental isolates that are not recognized as antibiotic producers have also been recently detected. These include the *qnr* and the bla_{CTX} genes compromising the activity of fluoroquinolones and extended-spectrum cephalosporins, respectively. The application of metagenomic tools and phylogenetic analysis will facilitate future identification of other new resistance genes and their corresponding ancestors in environmental bacteria, and will enable further exploration of the concept of the resistome as being a unique reservoir of antibiotic resistance genes and genetic elements participating in resistance gene transfer.

Keywords: Clinical setting, environment, metagonomic, resistance genes, resistome

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Introduction

During the last decade, an increase in rates of antimicrobial resistance has been recognized worldwide, and an increased frequency of multidrug-resistant (MDR) isolates in the clinical setting has been demonstrated. Moreover, the term extreme drug resistance has been applied to those isolates for which there are no available therapeutic options [1]. The accelerated emergence and description of new mechanisms of resistance and the recognition of efficient surrounding genes and genetic platforms facilitating capture, transfer and expression of resistance determinants are also subjects of intense investigation. These studies also include the origin of the resistance determinants and the corresponding surrounding genetic platforms.

Pathways for resistance

To become resistant to antimicrobial agents, bacterial communities use different strategies, including both natural and engineering responses [2]. Natural responses include: (i) the use of pre-existing bacterial machinery, i.e. the use of resistance genes from other bacteria; and (ii) gene variation (mutation) in pre-existing genes (pro-active response) or in acquired genes (post-active response). Some of these strategies require engineering processes involving different genetic platforms that are used for mobilization, acquisition and assembly of foreign resistance genes. In addition to these mechanisms, bacterial growth in biofilms might also enhance resistance to antimicrobial agents (physiological response).

Resistance response using pre-existing machinery from natural antibiotic-producing bacteria is widely recognized in resistance pathogens. Table 1 illustrates different examples involving

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Table 1. Natural reservoirs of resistance determinants affecting different antimicrobial agents

Antimicrobial group	Mechanisms	Related natural protein	Natural reservoirs
New resistance genes associated with antibiotic-producing organisms			
Aminoglycosides	Acetylation	Histone acetylases	*Streptomyces*
	Phosphorylation	Protein kinases	
	16S ribosomal RNA methylation	Methylases	*Streptomyces*, *Micromonospora*
Tetracyclines	Efflux (mar)	Major facilitator superfamily EF-Tu, EF-G	*Streptomyces*
Chloramphenicol	Acetylation	Acetylases	*Streptomyces*
	Efflux (mar)	Major facilitator superfamily EF-Tu, EF-G	
Macrolides	Target mutation	50S ribosomal subunit	*Streptomyces*
New resistance genes associated with non-antibiotic-producing organisms			
Fluoroquinolones	Topoisomerase protection	QnrA-like protein	*Shewanella algae*
		QnrS-like protein	*Vibrio splendidus*
β-Lactams	Hydrolases	PBP (transpeptidases)	*Kluyvera* spp.

PBP, penicillin-binding protein.

classic and new antibiotic resistance mechanisms. Most of these have been associated with *Streptomyces* spp. recovered from soil, and involve inactivating resistance mechanisms, efflux processes or antibiotic target modifications [2]. In the last few years, it has been demonstrated that antibiotic resistance mechanisms can also originate in environmental bacteria that apparently do not produce antibiotics. Most mechanisms are cryptic resistance determinants in the natural bacteria that are efficiently expressed in the new host. This fact is exemplified by the CTX-M extended-spectrum β-lactamases (ESBLs), which constitute one of the most successfully spread resistance mechanisms affecting expanded-spectrum cephalosporins, or by the Qnr-related topoisomerase protective proteins, which compromise fluoroquinolone activity [3,4].

The $bla_{\text{CTX-M}}$ genes have been associated with *Kluyvera* spp., whereas the *qnr* genes have been associated with *Shewanella algae*. Both comprise environmental isolates that have not been recognized as antibiotic producers, but carry a $bla_{\text{CTX-M}}$ gene and a *qnrA* resistance gene, respectively. In addition, these organisms do not show intrinsic resistance to either expanded-spectrum cephalosporins or fluoroquinolones. On the other hand, isolates in the clinical setting harbouring these genes are of clinical significance and are rising to prominence within the MDR isolates [3,5].

Environmental antibiotic resistome and antibiotic extended resistome

In recent years, metagenomic tools have identified resistance genes in isolates recovered from the environment [6–8]. These genes form part of pre-existing machineries in bacterial isolates capable of producing substances with antimicrobial activity or, as recently recognized, as natural intermicrobial signalling molecules [9]. The term 'antibiotic resistome' was proposed for the collection of all antibiotic resistance genes in microorganisms, including those from pathogenic and non-pathogenic bacteria. This term exploits the concept of a unique reservoir of antibiotic resistance genes. It includes resistance genes in antibiotic producers and precursor genes that, under appropriate selective pressure, evolve to act as resistance elements. Most are cryptic resistance genes that are not naturally expressed in environmental isolates [8].

D'Acosta *et al.* [6] were the first to use the antibiotic resistome concept when constructing a library of up to 480 *Streptomyces* sp. strains recovered from different environmental sources and subsequently screened against 21 antibiotics. Within this library, they identified resistance genes in all strains and resistance mechanisms affecting not only natural but also synthetic antimicrobials. Some of these resistance mechanisms, e.g. inactivation mechanisms affecting daptomycin or telithromycin, two of the newest antimicrobials introduced in therapeutics, have not yet been characterized in clinical isolates.

This metagenomic approach has been expanded and is also now applied to surrounding resistance genes and genetic elements participating in resistance gene transfer, and is termed the 'antibiotic extended resistome' [7]. This concept is useful for the exploration of environmental diversity in different ecosystems, including broad settings (e.g. healthcare settings of nosocomial transmission) or specific compartments, even in a single patient (e.g. the intestinal compartment), and could be useful for prediction of the future evolution of antibiotic resistance [10]. The analysis of some of the recent antibiotic resistance mechanisms described in the clinical setting reinforces these approaches.

New resistance genes associated with antibiotic-producing organisms

Aminoglycoside-modifying enzymes, efflux-based mechanisms affecting tetracylines, acetylases modifying chloramphenicol and RNA methylases conferring resistance to macrolides (Table 1), among others, have been traditionally considered

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to be resistance mechanisms, the corresponding resistance determinants for which can be found in natural antibiotic-producing bacteria. This list has now been enlarged with the identification of new resistance genes occurring in these bacteria.

Ribosomal methylases affecting aminoglycosides

Methylation of the 16S rRNA is the most recently described resistance mechanism affecting aminoglycosides [11]. It was first identified in a *Citrobacter freundii* isolate recovered in Poland in 2002, and then in *Pseudomonas aeruginosa* isolates in Japan and in *Klebsiella pneumoniae* isolates in France in 2003. Although few recent reports have been published, it has been also described in other members of the *Enterobacteriaceae*, including *Proteus mirabilis*, *Serratia marcescens*, *Salmonella* spp., *Enterobacter aerogenes*, *Escherichia coli*, *Shigella flexneri*, *Klebsiella oxytoca*, and *Acinetobacter* spp. Most of these isolates were recovered in clinical settings. In the future, we will surely encounter an increasing number of publications depicting the epidemiological situations in which this resistance mechanism has clinical relevance.

The production of the aminoglycoside methylases has been associated with different gene families, including the *armA*, *rmtA*, *rmtB*, *rmtC* and *rmtD* genes. The amino acid and nucleotide alignment demonstrates that these genes have originated from the *Actinomicetales*, including *Streptomyces* and *Micromonospora* spp. [12]. These genes are linked with insertion sequences (ISs) that have shown integration, mobilization and expression functions, such as IS*CR1* (formerly ORF 513) or IS*Ecp1*, similar to those encountered with other emerging resistance genes. All these sequences are carried in transposable platforms within conjugative plasmids, allowing potential spread within bacterial populations. Moreover, it is important to note that 16S rRNA methylase genes have been commonly associated with other resistance genes that are currently well dispersed in human clinical and veterinary isolates, such as bla_{BLEE} (CTX-M, SHV), plasmid bla_{AmpC}, $bla_{\text{SPM-1}}$, different aminoglycoside-modifying enzyme genes, and plasmid-mediated quinolone resistance genes (*qepA*). All of these resistance genes allow potential co-selection and maintenance processes in compartments with high antibiotic use [11]. Recent studies in isolates of human and animal origin have shown that some of these genes (e.g. *armA*) are associated with a class 1 integron platform structure harbouring IS*26*, whereas other genes (e.g. *rmtC*) are associated with complex potential transposons linked to IS*Ecp1* [13–15].

Methyltransferases affecting linezolid

Linezolid is an oxazolidinone compound that is active against Gram-positive isolates, including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococcal isolates. It is a synthetic bacteriostatic compound that inhibits protein synthesis by binding at the P-site in the ribosomal 50S subunit. Linezolid-resistant isolates have rarely been described. Resistance is mostly due to mutations in domain V of 23S RNA, mainly described in clinical methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococcal isolates and laboratory mutants of *Streptococcus pneumoniae* [16]. Deletion in genes encoding the L4 riboprotein has also been described in *S. pneumoniae*, and these deletions confer cross-resistance to macrolides, oxazolidinones and chloramphenicol [17]. Moreover, efflux pumps affecting linezolid have been identified in *Mycobacterium tuberculosis* [18].

Recently, a methyltransferase affecting the linezolid ribosome target and caused by the *cfr* gene was recognized in a clinical *Staphylococcus aureus* isolate recovered in Colombia [19]. The Cfr methyltransferase modifies adenosine at position 2503 in 23S rRNA in the large ribosomal subunit. Although linezolid is a synthetic antibiotic, the *cfr* gene is thought to be of natural origin and could be responsible for resistance mechanisms involving protection against natural antibiotics whose site of action overlaps that of linezolid. The Cfr methyltransferase also affects chloramphenicol, lincosamides, pleuromutilins and streptogramin A antibiotics. In this *Staphylococcus aureus* isolate, the *cfr* gene was located in the chromosome linked to the *ermB* gene, which confers resistance to erythromycin through dimethylation of A2058 in 23S rRNA. A chromosomal location of the *ermB*/*cfr* operon was previously identified in transposable elements within a conjugative plasmid in coagulase-negative staphylococci of animal origin [20]. This demonstrates dissemination of a resistance mechanism of potential environmental origin affecting a synthetic antibiotic, in this case, linezolid.

Plasmid-mediated quinolone resistance associated with efflux pumps

Traditionally, quinolone resistance mechanisms have been mainly due to target modifications and efflux pumps. Resistance due to target modifications is determined by topoisomerase mutations (*gyrA* mutations in Gram-negative bacteria, and *parC* in Gram-positive bacteria), whereas resistance due to efflux pumps involves different families of

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efflux pumps. Both mechanisms are chromosomally mediated and are increasingly recognized in clinical isolates. During the last few years, plasmid-mediated quinolone resistance mechanisms have been unexpectedly found, including: (i) Qnr topoisomerase-protective proteins; (ii) the AAC(6′)-Ib-cr quinolone-modifying enzyme; and (iii) the QepA efflux pump [4,5,21,22]. The last of these, due to the *qepA* gene, was first characterized in multiresistant *E. coli* isolates recovered in Japan in 2002, which also harboured other resistance genes such as $bla_{CTX\text{-}M\text{-}12}$, $bla_{TEM\text{-}1}$, *rmtB*, and *mphA*. The *qepA* gene has also been characterized in Europe (Belgium) in bacterial collections recovered from 2000 to 2005. These isolates, like those recovered in Japan, harboured other resistance genes, such $bla_{CTX\text{-}M\text{-}14}$, $bla_{TEM\text{-}1}$, *dfrA17*, *sul1*, *tet(A)*, *catI*, *ant39*, and *rmtB*, giving a multidrug resistance phenotype.

Sequence analysis of the *qepA* gene and alignment with other genes have revealed a potential origin in transport systems (MFS efflux pumps) from environmental isolates, such as *Actinomycetales* (*Nocardia farcinica*, *Streptomyces globisporus*, and *Streptomyces clavuligerus*). Recent studies demonstrated that the *qepA* gene is linked to transposable elements and IncF1 plasmids, allowing mobilization among bacterial isolates [21,22]. These results suggest that there will be future appearances of this resistance mechanism.

But, from a phenotypic view, wild-type isolates expressing the *qepA* gene have higher MICs than those of transconjugants, because of the coexistence of other resistance genes affecting quinolones in the former. Although the QepA efflux pumps confer only a slight incremental increase in MIC values, their presence might favour the development of mechanisms expressing even greater resistance [5]. Coexistence of the *qepA* gene with mechanisms conferring higher fluoroquinolone resistance might also favour the fixation of this newly described resistance gene in the bacterial resistome.

New resistance genes associated with environmental isolates not recognized as antibiotic producing isolates

As noted, two new types of resistance genes, the *qnr* and the bla_{CTX} determinants affecting fluoroquinolones and extended-spectrum cephalosporins, respectively, exemplify this possibility of new means of antimicrobial resistance appearing among environmental isolates [3,4]. Both of these recently described genes and mechanisms are expanding rapidly within clinical isolates.

Qnr Topoisomerase protective proteins

The Qnr plasmid-mediated resistance mechanism was identified in 1998 in *K. pneumoniae* isolates producing β-lactamase (FOX-5), which affects extended-spectrum cephalosporins, including metoxi-cephalosporins [23]. The new mechanism confers protection of topoisomerase from fluoroquinolones [4]. Like the *qepA* gene, despite conferring only low-level resistance to fluoroquinolones, it facilitates the selection of isolates with high-level resistance, including those with topoisomerase mutations [24]. There are different families of Qnr proteins, QnrA, QnrB and QnrS, that share variable amino acid similarities. QnrA belongs to the pentapeptide-repeat family, which is defined by a tandem of five specific amino acid repeats within the protein. Some of these proteins, including McbG, a pentapeptide-repeat protein with 19.6% amino acid identity with QnrA, protects against other naturally occurring peptides affecting DNA gyrase, such as microcin B17. Similar proteins have been identified in *Mycobacterium smegmatis* and *M. tuberculosis* [4].

The presence of *qnr* genes has been mainly identified in clinical isolates of *Enterobacteriaceae* with other resistance mechanisms, such as CTX-M ESBL and other newly described β-lactamases (VEB-1 and plasmid AmpC-derived enzymes), aminoglycoside-modifying enzymes, or plasmid-mediated efflux resistance mechanisms [4]. The origin of *qnrA*-like genes was determined using a PCR-based strategy on a series of Gram-negative organisms. The reservoir was identified as *Shewanella algae*, an environmental species from marine and fresh water. Four variants of *qnrA* (*qnrA2–qnrA5*) were found to be chromosomally mediated in strains of *Shewanella algae* [25]. Moreover, the progenitor of *qnrS* genes was also found in *Vibrio splendidus*, and genes similar to *qnr* with 40–70% amino acid identity were found in the chromosomes of other water-borne species, including *Vibrio vulnificus*, *Vibrio parahaemolyticus* and *Photobacterium profundum* [26]. These findings indicate that gene exchange has occurred between these environmental organisms and *Enterobacteriaceae*, and also support speculation that, under current intensive fluoroquinolone selective pressure, such genes have entered circulation on mobile genetic elements [4].

A recent report has shown an unusual occurrence of the *qnrS2* fluroquinolone resistance determinant in environmental *Aeromonas* spp. recovered from a river in an urban area in France. The *qnrS2* determinant was harboured in a newly described genetic platform defined as a mobile insertion cassette (‘mic’), resembling a transposon within IncU2 plasmids,

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which are ubiquitous in a wide range of environments. Both observations increase the possibility of dissemination outside of natural environmental compartments, and also underscore that these bacteria may play a role as a reservoir of the *qnrS* genes in an aquatic environment, as already shown for other resistance genes such as *tet* genes affecting tetracyclines [27].

Despite these findings, *qnr* gene mobilization could have also been performed by the same genetic elements that have mobilized ribosomal methylase genes affecting aminoglycosides or bla_{CTXM} genes hydrolysing expanded-spectrum cephalosporins (see below). In fact, the search for the surrounding platform of *qnr* genes facilitated the identification of their genetic linkage with IS*CR1*, which has been associated with all previously mentioned new resistance genes [3,4,11].

CTX-M ESBLs

The CTX-M ESBLs exemplify one of the most successful resistance mechanisms that have emerged and spread during recent years. This family of β-lactamases was first described 20 years ago, almost simultaneously in Germany and Argentina. It encompasses five different groups of enzymes (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25), all of them displaying potent hydrolytic activity against cefotaxime, although new variants may also effectively hydrolyse ceftazidime [3].

In recent years, a dramatic increase in these β-lactamases has been found worldwide in different compartments, particularly in *E. coli* in the community setting and also in veterinary isolates. Reasons for this increase include its association with ISs participating in gene mobilization, such as IS*Ecp1* or IS*CR1* and phage-related sequences, all of them within conjugative plasmids. Although these plasmids are widely disseminated among clinical isolates and are easily transferred, it is important that bla_{CTX-M} genes and surrounding genes may also be linked to class 1 integrons allocated within transposable platforms resembling Tn*21* subfamilies. These structures can integrate resistance gene cassettes and are associated with resistance to biocides and mercurial compounds that can participate in co-selection processes. Moreover, it is not unusual to find associations of bla_{CTX-M} genes with those coding for new emerging resistance mechanisms, e.g. 16S RNA methylases (*armA* and *rmtB* genes), Qnr proteins (*qnrA*, *qnrB* or *qnrS*), acetylases affecting fluroquinolones (*aac(6′)-Ib-cr*) and systems pumping out fluoroquinolones (*qepA*); these genes are found in the same genetic platforms.

From an evolutionary point of view, different *Kluyvera* species have been recognized as progenitors of bla_{CTX-M} genes: *Kluyvera ascorbata* for the CTX-M-1 and CTX-M-2 groups, and *Kluyvera georgiana* for the CTX-M-8 group. In addition, phylogenetic analysis showed that bla_{CTX-M} genes have been mobilized from the chromosome of *Kluyvera* spp. approximately ten times more frequently than genes coding for other class A β-lactamases, which has facilitated the spread of the bla_{CTX-M} genes. Moreover, the bla_{CTX-M} genes are descended from a common ancestor that was incorporated in ancient times into the chromosome of the ancestor of *Kluyvera* spp. through horizontal transfer [28]. The expression of bla_{CTX-M} in *Kluyvera* is modest, as isolates belonging to this genus are normally susceptible to expanded-spectrum cephalosporins. This is not the case in *Enterobacteriaceae*.

The importance of *Kluyvera* spp. in nature in recruiting resistance genes has not been sufficiently investigated. It can also act as a donor of resistance genes to other bacteria. In fact, the ability of IS*Ecp1* to mobilize the chromosomal bla_{CTX-M} gene from one of these *Kluyvera* progenitors was shown in an *in vitro* experiment, and its potential role for ensemble in conjugative plasmids was also demonstrated [29].

Conclusions

The ancestors of most of the new resistance genes found in MDR clinical isolates have been identified both in antibiotic-producing bacteria and in environmental isolates not recognized as antibiotic producers. Mobilization of these genes from soil bacteria might have occurred by the use of similar genetic strategies, with final insertion in genetic platforms enabling efficient transfer to other organisms. These platforms might recruit multiple resistance genes. The wide use of antimicrobials might determine co-selection processes that ensure persistence of these genes in bacterial communities. The application of metagenomic tools and phylogenetic analysis will not only facilitate future identification of other new resistance genes and their corresponding ancestors in environmental bacteria, but will also exploit the concept of the resistome as a unique reservoir of antibiotic resistance genes and genetic elements participating in resistance gene transfer.

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