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Natural antibiotic resistance and contamination by antibiotic resistance determinants: the two ages in the evolution of resistance to antimicrobials

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The study of antibiotic resistance has been historically concentrated on the analysis of bacterial pathogens and on the consequences of acquiring resistance for human health. The development of antibiotic resistance is of course extremely relevant from the clinical point of view, because it can compromise the treatment of infectious diseases as well as other advanced therapeutic procedures as transplantation or anticancer therapy that involve immunosuppression and thus require robust anti-infective preventive therapies. Nevertheless, the studies on antibiotic resistance should not be confined to clinical-associated ecosystems. It was evident soon after introducing antibiotics for human therapy, that bacteria were able to develop resistance, not just as the consequence of mutations in the targets of antibiotics, but by acquiring genes conferring resistance to antimicrobials (Abraham and Chain, 1940). Since those genes were not present before in the human bacterial pathogens, the only suitable source for them was the environmental microbiota, and indeed the presence of *R*-factors (resistance plasmids) in pristine environments without any record of contact with antibiotics was described in the first studies of antibiotic resistance in the field (Gardner et al., 1969).

Given that the origin of antibiotic resistance is the environmental microbiota, it would be necessary to study resistance in natural, non-clinical habitats in order to fully understand the cycle of acquisition of resistance by human pathogens. However, until recently the studies on antibiotic resistance in natural ecosystems have been fragmentary. The availability of metagenomic tools as well as high-throughput sequencing techniques is allowing describing in depth the presence of resistance genes in different ecosystems. Indeed, the use of functional genomic and metagenomic techniques has served to show that natural ecosystems, including not just soils but human gut as well, contain a large number of elements that, upon transfer to a new host, can confer resistance to any type of antimicrobial (D'Costa et al., 2006; Sommer et al., 2009). These include natural antibiotics, which are produced by the environmental microbiota, and synthetic antimicrobials, as quinolones.

One important question from an evolutionary point of view is the function of these resistance genes in their natural environmental hosts (Davies and Davies, 2010). Whereas for naturally produced antibiotics a protective role for resistance genes in the producers organisms (or those coexisting with producers Laskaris et al., 2010) might be foreseen (Benveniste and Davies, 1973), this explanation is not suitable for synthetic antibiotics as quinolones. Indeed, it has been described that the origin of the quinolone resistance gene QnrA, which is now widespread in plasmids present in human pathogens is the environmental non-antibiotic producer *Shewanella algae* (Poirel et al., 2005). This means that a gene that confers resistance in a human pathogen does not necessary play the same role in its original host (Martinez et al., 2009a). The finding that several proteins, involved in basic processes of the bacterial physiology, contribute to intrinsic resistance to antibiotics (Fajardo et al., 2008; Laskaris et al., 2010; Linares et al., 2010), further supports the concept that resistance genes, acquired through horizontal gene transfer by human pathogens, might have evolved in their original host to play a different role than resisting the activity of antimicrobials in natural ecosystems.

We can thus distinguish two ages in the evolution of antibiotic resistance genes. For billions of years (until the use of antibiotics by humans), these genes have been usually chromosomally encoded and had evolved for different purposes. Some of them, as those found in antibiotic producers, likely evolved for detoxifying the original host from the antibiotic it produces, although a role in the biosynthesis of the antibiotic itself has been proposed as well for some of them (Benveniste and Davies, 1973; Doyle et al., 1991). Others, as beta-lactamases might be involved in the biosynthesis of the cell wall (Jacobs et al., 1994; Massova and Mobashery, 1998), whereas others as multidrug efflux pumps might serve for different purposes including the trafficking of signaling molecules, detoxification of metabolic intermediates, or extrusion of plant-produced compounds among others (Martinez et al., 2009b). Like in the case of antibiotics, which do not necessarily have an inhibitory function at the concentrations in which they are present in natural ecosystems (Linares et al., 2006; Yim et al., 2007; Fajardo and Martinez, 2008), the fact that a plasmid-encoded gene produces resistance to antibiotics upon its expression in a new host, is not an unequivocal prove that it confers resistance as well in its original host. This reflection serves to show the relevance of the second age in the evolution of antibiotic resistance determinants. Once a gene is introduced in a new host in which it lacks its original biochemical and genetic context, its function is limited to antibiotic resistance (Baquero et al., 2009). This change of function without changing the sequence of the gene itself, has been named as exaptation (Gould and Vrba, 1982), and is the consequence of the strong selective pressure exerted by antibiotics in the last decades from the time they were introduced for therapy.

Two important aspects are emerging from the studies of natural resistome. First, the environmental microbiota contains a much larger number of resistance genes than those seen to be acquired by bacterial pathogens (Wright, 2007; Davies and Davies, 2010). Furthermore, different ecosystems contain different resistance genes,

which means that we are still far away to have a consistent estimation on the number of potential resistance genes present in natural ecosystems. Finally, the origin of most resistance genes currently found in transferrable elements is still ignored, despite genes (and genetic structures) belonging to the same families are regularly found in different ecosystems, including deep terrestrial subsurface (Brown and Balkwill, 2009), ice (Miteva et al., 2004), and even the permafrost (D'Costa et al., 2011), which have not been in contact with human contaminants. Second, those genes present in mobile elements in human bacterial pathogens can be found nearly everywhere, including pristine ecosystems or wild animals not supposed to be in contact with antibiotics (Martinez, 2009). This indicates that pollution with antibiotic resistance genes is widely spread and that resistance genes can persist even in the absence of a positive selection pressure. The analysis of historical soil archives has shown a consistent increase on the presence of antibiotic resistance genes since 1940 (Knapp et al., 2010), which is a clear prove of the contamination by antibiotic resistance elements of natural ecosystems and the resilience of those elements for their elimination.

In this situation, which type of studies are needed to analyze in depth the role that natural ecosystems may have on the development of resistance in human bacterial pathogens? In my opinion, these studies have two faces (Martinez, 2008). One consists on the analysis of the genes already present in bacterial pathogens. In other words, we will study mainly contamination by antibiotic resistance determinants and how this contamination might increase the risks for the dissemination of those elements (Martinez, 2009). These studies might serve to define reservoirs, elements for enrichment and dissemination of resistance (as wild birds Simoes et al., 2010) or hotspots for the transfer of resistance as waste-water treatment plants (Baquero et al., 2008). For instance, a recent study has shown that soil composition and in particular the presence of heavy metals might enrich for the presence of antibiotic resistance genes in natural ecosystems (Knapp et al., 2011). The other type of studies consists on the analysis, using functional assays, of novel resistance genes in different ecosystems (D'Costa et al., 2006, 2011; Sommer et al., 2009). These studies are useful for defining novel mechanisms of resistance, but making risks assessments on whether those novel antibiotic resistance genes will be transferred to new hosts is likely unsuitable (Martinez et al., 2007). On the other hand tracking the source of currently known resistance gene has demonstrated to be a very difficult task. We have to be extremely careful for assigning the origin of resistance determinants. Only when the genes are nearly identical (as QnrA) and the gene is present in several strains of the original host, with the same synteny and without any sign of a recent acquisition event, we can firmly establish this host being the origin. The report of genes that are highly similar (even above 90%) to antibiotic resistance genes demonstrate their belonging to the same phylogenetic group, not that one is the origin of the other. Does it mean that we will be unable of tracking the source of resistance genes and to propose from this information valuable strategies for reducing antibiotic resistance? I do not believe that. It has been already determined that QnrA was originated in *S. algae* (Poirel et al., 2005) and that chromosomally encoded *qnr* genes are mainly present in water-dwelling bacteria (Sanchez et al., 2008). This suggests that the source of transferrable quinolone resistance is the water microbiota and puts a focus on the effect that the use of quinolones in aquaculture might have had for the emergence and dissemination of these resistance elements (Cabello, 2006).

The study on antibiotic resistance in natural ecosystems and its role on the maintenance and spread of clinically relevant resistance determinants is still in its infancy. It is surprising that large efforts have been used to study the risks for the dissemination of resistance that may have the release of genetic modified organisms containing resistance genes in their chromosomes, whereas the study of the effect of the discharge of human wastes, which contain bacterial pathogens harboring the resistance genes that have demonstrated to be really relevant, in the elements that are important for their dissemination has received few attention if any. Studies in this new field are needed in order to understand the mechanisms involved in the emergence, spread, maintenance, and evolution of antibiotic resistance.

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