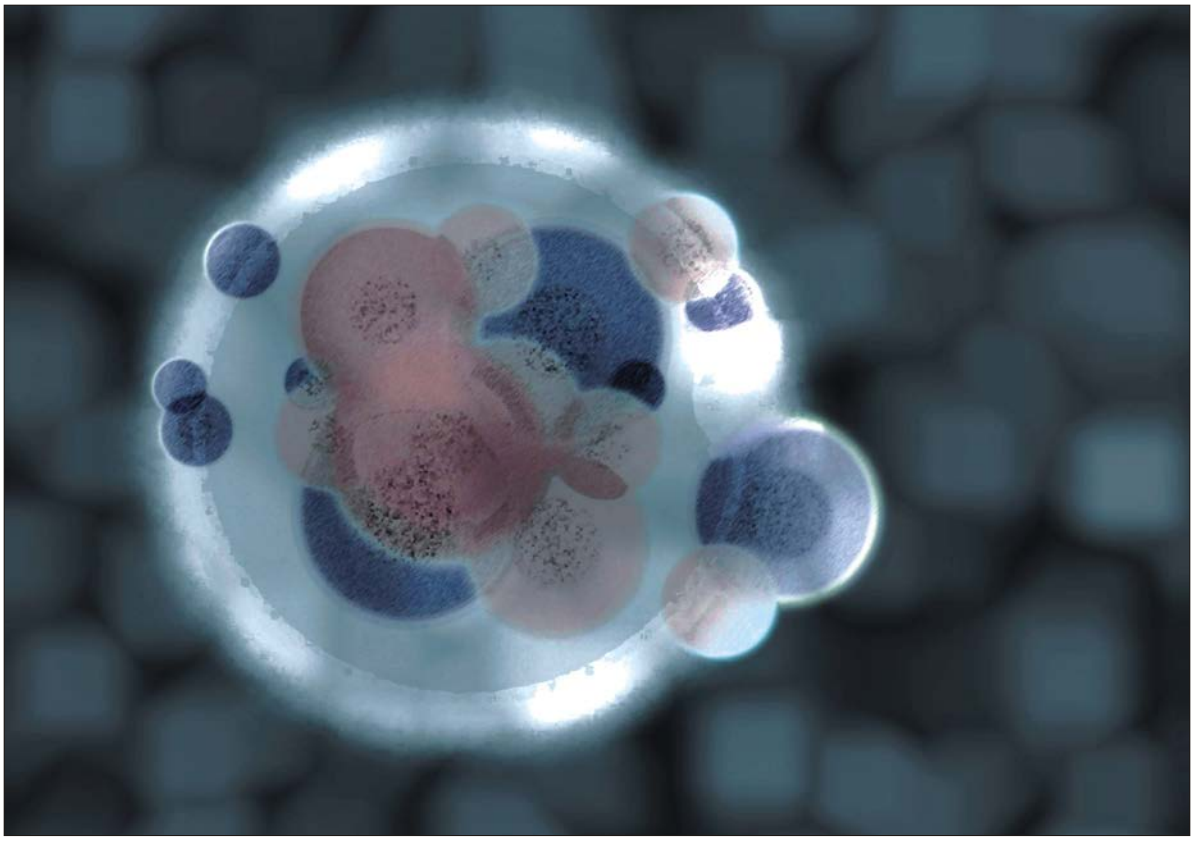

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Role of Non-clinical Environments in the Selection of Virulence and Antibiotic Resistance Determinants in Pathogenic Bacteria

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Abstract: The acquisition of genomic islands and plasmids from a common gene pool, has shaped the evolution of bacterial pathogens. Interestingly, determinants used for infection can be also used (at least in some cases) for surviving in natural environments and the same antibiotic resistance determinants can be found in infective and environmental bacteria. This suggests that natural environments might have a relevant role in the evolution of human pathogens. It is review about the potential role of natural (non-clinical) environments in the evolution of bacterial pathogens.

Key words: Antibiotic resistance, opportunistic pahogens, evolution, genomic islands, pathogenicity islands

INTRODUCTION

The World Health Organization has stated that infectious diseases account for around 20 million deaths a year (one in two deaths in developing countries) and are the main cause of mortality and morbidity in the world[1]. Antibiotics have been historically very successful in the treatment of these diseases. However, several bacterial species have evolved to antibiotic resistance[2]. Antibiotic resistance can be developed either through mutation[3] either through the acquisition of antibiotic resistance genes by horizontal gene transfer[4]. It was earlier stated that antibiotic resistance genes could be originated in the antibiotic-producer organisms and afterwards transferred to pathogenic bacteria[5]. Besides that, some genes involved in the resistance to other environmental toxic compounds produced either by plants or by other bacteria might also have a role in antibiotic resistance[6]. In any case, it seems clear that antibiotic resistance genes might have been originated in environmental bacteria and transferred to the pathogenic ones after introduction of antibiotics for the therapy of infectious diseases.

It has been found that in several occasions, bacterial virulence determinants are present in pathogenicity islands acquired by horizontal transfer from unknown bacteria[7]. Since the genomes or environmental bacteria contain fitness islands also acquired by horizontal gene transfer, it seems that the spread of genomic island is an efficient method for the evolution of bacteria independently of their habitat. It is then possible that pathogenicity islands might have originated in the environment, where they increase the fitness (fitness islands) of bacteria containing them[7]. Gene flow between environmental bacteria and human-colonizing microorganism may then contribute to the evolution of bacterial pathogens. It is important to notice here that several bacterial pathogens have an environmental, non-clinical, reservoir[8]. These include highly resistant opportunistic pathogens and other organisms like *Vibrio cholerae* with an important role in the infectious diseases and with a free-living style[9].

Since virulent bacteria might have a non-clinical habitat, the selective forces acting in environmental, non-clinical ecosystems may have shaped their evolution as a whole and the horizontal flow of antibiotic resistance and virulence genes from environmental bacteria to human pathogens.

Antibiotic resistance genes in non-clinical environments: Mutations in target genes are involved in the development of antibiotic resistance[3]. Besides that, it has been shown that acquisition of antibiotic resistance genes by horizontal gene transfer[4] has a highly relevant role in the emergence and further spread of antibiotic resistance genes among pathogenic bacteria.

The analysis of Gram-negative pathogens from the pre-antibiotic era demonstrated that they contain the same amount of plasmids belonging to the same incompatibility groups that can be found today in these bacteria[10]. This indicates that pathogenic bacteria have acquired (and are acquiring) antibiotic resistance genes from non-clinical environments after introduction of antibiotics for treating infections. In some occasions, these genes have been originated in the antibiotic producers. Examples are the tetracycline resistance genes *otrA* and *otrB* that have been found in the tetracycline-producing organism

Streptomyces rimosus and in pathogenic micobacteria[11]. However, for most antibiotic resistance genes found in pathogenic bacteria, an identical counterpart has not been yet described in antibiotic producers. It is clear that the amount of producers so far characterized (and eventually sequenced) is extremely low, thus it is possible that those antibiotic resistance genes might be present in so far unknown antibiotic producers.

Increased evidence supports the idea that some antibiotic resistance genes might have a functional role different to antibiotic resistance. Conspicuous examples of these genes are chromosomal beta-lactamases, Multidrug (MDR) efflux pumps and some aminoglycoside-modifying enzymes. Whereas its has been suggested that some β-lactamases[12] and aminoglycosides acetyl transferases[13,14] might have a role in the metabolism of peptidoglycan, MDR pumps have been involved in intercellular signalling[15-17] and resistance to compounds such as solvents[18,19], detergents[20], heavy metals[21], biocides[22] or toxic compounds[23] present in plants exudates (Table 1). Those compounds can be found in natural, non-clinical environments and contribute to the selection of MDR determinants. In fact, opportunistic pathogens with an environmental origin are usually more resistant than classical pathogens[24]. This clearly states that non-clinical environments constitute a reservoir that contributes to the selection and further spread of antibiotic resistance genes and intrinsic antibiotic resistant bacteria, which can then have a role in the development of infections.

One example of this is the presence of quinolone-extruding MDR pumps in *P. aeruginosa*. Quinolones are synthetic antibiotics introduced for therapy in late 1960s. The analysis of environmental *P. aeruginosa* strains isolated before the invention of quinolones demonstrated that all of them were able to efflux those antibiotics through MDR efflux pumps[25]. It is clear that these antibiotic resistance elements have not been selected in Nature some hundred thousands of years ago to avoid the activity of a family of synthetic antibiotics that has just forty years lifetime. Noteworthy, quinolones are good substrates for several MDR pumps present in the chromosomes of different bacterial species[26]. This strongly suggests that, at least in some cases, determinants with a relevant role in the development of antibiotic resistance in clinical settings have a different function in natural environments. This function is largely unknown in most cases, so that the reasons for the emergence, further evolution and spread in Nature of these determinants remain to be clearly established.

Table 1: Substrates of multidrug efflux pumps with relevance for the bacterial fitness during infections of in non-clinical envirouments

Infections	Non-clinical environments
Antibiotics for treatment	Antibiotics from producers
Quorum sensing signals	Quorum sensing signals
Antimicrobial peptides produced by humans	Antimicrobial peptides produced by plants or animals
Bile salts	Plant-produced antibacterials
Fatty acids	Heavy metals (natural or from pollution)
Detergents	Detergents (pollution)
Biocides and disinfectants	Solvents (pollution)

On the origins of pathogenicity islands: The analysis of an increasing number of bacterial genomes has demonstrated that horizontal gene transfer has an important role in the evolution of prokaryotes[27]. One of the elements that contribute to the rapid evolution of pathogens is the presence in their genome of pathogenicity islands[28]. These genetic elements have a different G+C content than the rest of the bacterial chromosome, indicating that they have been acquired from different bacterial species. It has been found that, for several pathogens, virulence genes are located on mobile (or formerly mobile) elements derived from lysogenic bacteriophages or plasmids.

Although pathogenicity islands were firstly described in bacterial pathogens, more recent works have shown that similar elements are present as well in the chromosomes of environmental-non-pathogenic prokaryotes. This feature has highlighted the concept that horizontal transfer of large DNA fragments (now named genomic islands) contributes to the evolution of pathogenic but also of non-pathogenic and environmental bacterial species[7]. One evolutionary advantage of the acquisition of genetic islands is that large number of genes that confer new traits to the recipient cell can be transferred as a whole. This allows the bacteria receiving these genetic elements to colonize novel ecosystems (like human body in the case of human pathogens).

Since genetic islands contribute to the evolution of pathogens, an important question would be the origin of these genetic elements. The catalogue of bacterial infectious diseases is fairly exhaustive so that, it is difficult to think that the great number of pathogenicity islands present in human pathogens have been originated from other still unknown human pathogens. The possibility of extinct pathogens as the source of these islands is possible but also unsuitable. If pathogenicity islands have not been originated in human pathogens, two possibilities remain: their origin is in bacteria causing infections in animals, or they have been originated in environmental non-pathogenic bacteria.

Pathogenicity islands frequently carry genes coding elements (Table 2) like systems for iron uptake[29,30],

Table 2: Functions encoded by genes present in pathogenicity islands

Function	Advantage conferred
Degradation of aromatic compounds	Growth in contaminated environments[57]
Nitrogen fixation/nodulation	Beneficial plant/host interactions[58]
Resistance to multiple antibiotics	Survival to antibiotic treatment[59]
Production of toxins	Colonization (and eventually killing) of the host[60]
Iron uptake	Better growth during infections and in environments with low concentrations of available iron[29,30]
Expression of type III secretion systems	Interference with host-cell functions[31]
Expression of adhesions	Colonization of host surfaces and inert materials[32]
Production of bacteriocins	Increases competitiveness[61]

secretion systems[31], determinants involved in bacterial attachment to surfaces[32] or proteases[33,34] that can be useful for colonizing different environments[35] from human body to plants rhizhosphere including the degradation of dead bodies or the life inside eukaryotic cells (amoeba, protozoa, invertebrates, plants and animals) in natural non-clinical environments. As we will see below, bacteria can use the same elements for infecting plants, mammals, nematodes, protozoa[36]... so that an environmental origin for pathogenicity islands is a suitable hypothesis. In fact, it has been recently proposed that invertebrates might be a source of emerging human pathogens[35]. This does not mean that horizontal transfer between different pathogens is not possible. In fact, enterohemorrhagic *E. coli* isolates contain pathogenicity islands and virulence plasmids similar to those found in *Shigella* sp.[37-39], indicating either a common origin of these elements, either their transfer from one organism to the other.

A clear example of evolution of bacterial pathogens and the role of the environment on such evolution is *Yersinia pestis*[40]. This bacterial species can be divided in three biovars and these biovars are thought to be responsible of the three major pandemic plagues: the Justinian plague, the Black Death and the modern plague[41]. It is thought that *Y. pestis* evolved from *Yersinia pseudotuberculosis* 1500 to 20000 years ago[41]. *Y. pseudotuberculosis* is found mainly in natural environments and is a frequent cause of animal infections. This bacterial species shares the same ecosystems (for instance rodents and fleas) with other microorganisms and it is thought that the step-wise acquisition of different pathogenicity islands and virulence plasmids in these non-human habitats leads the emergence of *Y. pestis*. It is important to remember here that the acquisition of novel traits by horizontal gene transfer allows bacteria to colonize novel environments. Polymicrobial infections are not common, thus it is unsuitable that horizontal gene transfer (either pathogenicity islands or antibiotic resistance genes) may happen during infection. This transfer requires the existence of a common genetic pool shared by the members of complex microbial communities, like those present in Natural environments, from which pathogenic bacteria may eventually be transferred to humans. In the case of *Y. pestis*, it is thought that, after acquiring pathogenicity islands from other members of the community, increases in the population size of certain rodents associated to changes in human behaviour might have trigger the selection and further expansion of this dangerous human pathogen[41,42].

Opportunistic pathogens with an environmental (non-clinical) origin: Opportunistic infections occur in people with basal diseases, intubated, immunodepressed or in general with their natural defenses against infection dramatically impaired. Whereas before the antibiotic era, these persons were mainly infected by their own commensal organisms, the huge utilization of antibiotics has changed the landscape of opportunistic infections so that several opportunistic bacterial pathogens today are environmental bacteria highly resistant to antibiotics used at hospitals[24,43].

Opportunistic pathogens do not infect regularly the community, but their relevance as infective agents has increased recently with the aging of the population, diseases like AIDS or cystic fibrosis and medical techniques such as immunosuppression for transplantation, intubation or catheterization among others. Since these pathogens are not classical virulent bacteria that might have evolved in close contact with humans to acquire a virulent phenotype, the most suitable possibility should be that the virulence determinants of opportunistic pathogens have been selected to cover a function in their environmental (non-clinical) habitat[8].

One interesting point would the be to analyze whether the infective isolates of a given opportunistic pathogen present any characteristic feature to justify their virulence or by contrast all the isolates (clinical and environmental) present the same characteristics and infection is just the consequence of the predisposing conditions of the patient. The analysis of several different isolates of *P. aeruginosa* has demonstrated that, at least for this important opportunistic pathogen, there are no clear differences between clinical and environmental populations of this bacterial species. Using a combination of pulse field gel electrophoresis and analysis of single nucleotide polymorphism, it was found that the same clones could be found in natural and clinical environments[44]. It has been also described that

environmental *P. aeruginosa* contain virulence genes, are cytotoxic and have active mechanisms of antibiotic resistance, features important for bacterial virulence. On the other hand, clinical isolates can degrade oil-derived hydrocarbons, a feature characteristic of environmental bacteria[25]. It then seems that clinical and environmental isolates of *P. aeruginosa* are both genetically and functionally equivalent, suggesting that all of them may produce an infection. It is important to notice here that, although the genome of *P. aeruginosa* contains genomic islands[45,46], there is not any evidence of a role on virulence of the determinants coded in these islands. Furthermore, some elements such as the operons coding the proteins of the Type III secretion system, that are usually inside pathogenicity islands in the genome of virulent bacteria, are not forming a part of any genomic island in *P. aeruginosa*, although they are present in its genome. All of this clearly states that the determinants that *P. aeruginosa* uses for infecting humans also play a role in the environment.

Besides mammals, *P. aeruginosa* can colonize and infect plants[47] and it has been found that the virulence factors used for colonizing humans are the same as those used for the colonization of plants. Further work has demonstrated that *P. aeuginosa* can infect mammals, insects, plants, worms and molds using a similar repertoire of virulence determinants[36,48]. It is then clear that in the case of opportunistic pathogens with an environmental origin, the ecological interactions in non-clinical environments have shaped their genome, without the need of a constant contact with human being.

Environmental changes and selection of clinically relevant traits in bacterial pathogens: All along the review, it is stated that the environment may have a relevant role on the selection and spread of antibiotic resistance and virulence determinants in bacterial populations. If this hypothesis is true, environmental changes either natural either due to anthropogenic forces, may produce changes in bacterial populations and the genetic elements shared by different genomes, which at the end might influence the evolution of bacterial pathogens.

One example of natural changes with a clear influence in human infectious diseases is the seasonal incidence of cholera epidemics in the Ganges delta region of Bangladesh and India. Besides being an important human pathogen, this bacterial species is a part of the normal flora in estuarine environments, being the water a vehicle for its transmission[49]. A correlation between abundance of this bacteria in water and cholera epidemics (twice a year) has been found and it was suggested that

Table 3: Selectable markers present in replicons containing antibiotic resistance genes

Selectable marker	Selection
Adhesin	Adhesion to surfaces[62]
Microcin	Competition with other bacteria[63]
Colicin	Competition with other bacteria[62]
Lantibiotics	Competition with other bacteria[64]
Cytolysin	Infection of metazoa and lysis of unicellular eukaryotes[65]
Resistance to disinfectants	Decontamination procedures[66]
Iron uptake	Infection and environments with low concentrations of available iron[29,67]
Resistance to heavy metals	Survival in heavy-metal contaminated environments[54]

environmental concentration of *Vibrio* species might increase as the consequence of plankton blooms driven by seasonal changes in aquatic conditions[9,50]. A role for changes in the temperature due to El Niño-Southern Oscillation has been suggested[51]. More recently, it has been found that there is an inverse correlation between the presence of either cholera bacteriophages or phage-susceptible *V. cholerae* strains. It was then proposed that phages play a predominant role in ending cholerae epidemics[52]. All these data support the notion that the behaviour of *V. cholerae* in aquatic environments is extremely important for understanding the epidemicity of this bacterial species. The fact that bacteriophages might end cholera epidemics may account for the explosive cholera epidemics that occurred upon the introduction of these orgamisms in previously cholera-free regions such as Africa or South America[49].

Enrichment in pathogenic bacterial populations may happen as the consequence of natural variations in the environment. The same might occur as the consequence of anthropogenic forces. *P. aeruginosa* is bacterial species that can grow and multiply in a wide range of water sources including river water, seawater and wastewater. A study of *P. aeruginosa* diversity in a Belgian river has demonstrated that the same clonal complexes could be found at any of the sites used for sampling. However, the abundance of total number of *P. aeruginosa* was higher in the most polluted part of the river[53]. It is then clear than human activities challenging the environment may also affect the populations of pathogenic bacteria.

Environmental changes might also select antibiotic resistant bacteria or antibiotic resistance genes without the need of antibiotic selective pressure[6]. MDR pumps can efflux, not only antibiotics, but other toxic compounds like solvents[18,19], detergents[20] or biocides[22]. Although it has not been demonstrated, enrichment in bacteria capable to resist these agents and thus antibiotic resistant, might be possible in contaminated environments. Antibiotic resistant genes may also be

selected if they are present in replicons containing another type of selectable marker (Table 3). One example of this is the association of heavy-metal resistance and antibiotic resistance in the same genetic element[54]. Heavy-metal contamination is the most important contamination problem in industrial societies[55]. In the presence of heavy metals, the genetic elements containing antibiotic resistance and heavy-metal resistance genes will be selected, producing a burst of antibiotic resistance genes in these types of environments. Heavy-metal contaminated environments contain a higher percentage of antibiotic-resistant bacteria than non-contaminated ones and bacteria isolated from contaminated soils have more plasmids than bacteria isolated from non-contaminated environments[56]. Horizontal transfer of bacteria is higher in heavy-metal contaminated soils, so that the risk of spread of plasmid-encoded antibiotic resistance genes may increase with contamination.

CONCLUSIONS

Two of the areas of highest impact in microbiology are the studies on infectious diseases and the environmental microbiology. Research in these areas usually goes in parallel, without crossing overs. However, several bacterial pathogens have a non-clinical natural environment. The analysis of the influence of the environment and its changes on the evolution of bacterial pathogens is required to understand in deep the physiology and the behaviour of human pathogens. This understanding is needed in order to develop novel strategies for fighting infections.

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