Persistence and dissemination of antimicrobial resistances in aquatic systems

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1. Introduction

With the advent of the antibiotic age, more and more drugs were developed to treat serious infections, and their indiscriminate and irrational use has led to the development of resistance determinants to prevent their own demise and the selection of drug-resistant microbes [1-3]. Thus, misdiagnosis, unnecessary prescriptions, improper use of antibiotics by patients and/or the use of antibiotics as additives in animal feed greatly affect the increase of resistant organisms [4].

The emission of bacteria into the aquatic environment, for example through wastewater, also favors the genetic exchange with previously non-resistant populations, thereby increasing the dispersion of this resistant capacity in environmental bacteria [5]. So, natural environments are reservoirs of resistant bacteria and resistance genes where anthropogenic-driven selective pressures may be contributing to the persistence and dissemination of genes usually relevant in clinical environments [6]. In this context, Baquero *et al*. [7] indicated that an important part of the dispersal and evolution of antibiotic-resistant bacteria depends on water environments.

The persistence of antibiotic resistant bacteria and the ability to disseminate their genetic information in aquatic environments is largely determined by their capacity to survive under adverse conditions.

Biotic and abiotic factors affect the survival patterns of allochthonous bacteria. Although abiotic factors (adverse temperatures, depletion of nutrients, light, salinity and others) may not remove resistant bacteria, they can induce viable but nonculturable state in some non-differentiating bacteria [8-11]. In this state, cells are nonculturable but retain activity and are able to exchange genetic information and, in some cases, to recover culturability. On the other hand, biotic factors, mainly predation by protozoa [12-14], effectively eliminate prey bacteria and may be a crucial process to restrict bacterial dissemination and so, gene resistance spread.

The exchange of genetic information is conditioned by the density and the physiological state of the cells implied in the process, as well as by the physicochemical conditions of the system where it is performed [15]. While the transfer of genetic material by conjugation among free-cells could be very restricted in aquatic environments, it could be important in wastewater treatment plants where bacterial density is high, antibiotics are present and the solid fractions (flocs and sludge) promote the permanence and contact among large numbers of bacteria [16, 17].

The main aim of this review is to give a brief account of the environmental factors that may affect the exchange of antimicrobial resistance information, basically by conjugation, in aquatic systems.

2. Permanence of plasmid carrier bacteria in aquatic ecosystems

Antibiotic-resistance is present in bacteria from different aquatic systems [6, 18-20], even in remote locations not appreciably impacted by anthropogenic activities [21, 22]. This fact could be attributed to the ability of antibiotic resistant bacteria to persist in these environments and/or to transfer their mobile genetic information.

Several studies and reviews have described the behavior of allochthonous bacteria, including plasmid-carrier bacteria, which are released directly or through wastewaters in rivers and coastal seawaters. In these systems their persistence, and the permanence of their genetic information, depends on both biotic and abiotic environmental factors [23-25].

Slater *et al.* [26] have indicated that plasmid carriage is only beneficial to the host under certain environmental conditions if they confer a fitness advantage, but this beneficial effect is countered by the permanent fitness cost associated with plasmid carriage. In this sense, Barcina *et al*. [27] found differences in the persistence between *Escherichia coli* strains carrying plasmids that confer resistance to the negative effect of visible light and their isogenic plasmid free strains. However, studies on the influence of plasmids in bacterial survival are contradictory. Some authors have reported that in aquatic environments, in absence of selection, plasmid-bearing strains can survive as well as their counterparts plasmid-free, or even better [28-33]. Thus, Flint [34] indicated that possession of antibiotic resistance plasmids does not enhance survival or cause a faster rate of decay, and concluded that the metabolic burden imposed by a plasmid is not a factor influencing survival under starvation conditions. In contrast, Smith and Bidochka [35] have

demonstrated that the maintenance of plasmids is significantly improved by reductions in plasmid size, and that plasmids with high number of copies reduce the fitness of the bacterial host.

In any case, environmental factors affect survival of allochthonous bacteria in aquatic systems. Abiotic parameters such as suboptimal temperature, depletion of nutrients, light and others induce the adoption of the viable but nonculturable (VBNC) phenotype in some non-differentiating bacteria [8-11]. In this state, bacteria lose their ability to grow on culture media but remain metabolically active [36]. This fact raises serious questions about the routine use of quality testing methods based on the growth of bacteria on culture media [8]. Some studies have demonstrated that plasmid-carrier strains also enter into the VBNC state [28, 33] and, moreover, plasmids are maintained in these VBNC cells [37-39]. Nevertheless, Muela *et al*. [33] indicated that plasmids are negatively affected by environmental stress and described that both chromosomal and plasmid DNA content decreases during the starvation-survival process of *E. coli* in river water.

Anyway, several studies [39, 40] have demonstrated that cells in VBNC state retain virulence (plasmid codified) and should be considered by researchers and government regulators involved in public health.

Protozoan predation is one of the most important factors for the elimination of bacteria, including those harboring resistance plasmids, in aquatic environments [12, 13, 41, 42] and may be a crucial process to restrict bacterial dissemination and so, gene resistance spread. Arana *et al.* [43] demonstrated, by using a GFP (green fluorescent protein) tagged *E. coli* strain, that in presence of the river microbial community, the *E. coli* cells appeared to be ingested before cellular deterioration could occur. Thus, predation reduces the quantitative importance of the VBNC population of *E. coli* in aquatic systems. However, the result of the bacteria-protozoa interactions may not be so negative for resistant bacteria and plasmid transfer in the environment. In this sense, Ahmetagic *et al.* [44] demonstrated that in environments containing large numbers of protozoa, bacteria using efflux pumps (codified in plasmids) to remove toxins, may have an evolutionary advantage over other bacteria, and indicated that bacterial plasmids and phages encode the synthesis of toxic molecules which inhibit protozoan predation. Moreover, Borella *et al.* [45] indicated that for *Legionella*, the bacterial-protozoan interaction contributes to the amplification of the population in water systems and acts as a reservoir of infection since *Legionella* survives within its protozoa host, converting its predator into a vector [46].

3. Transfer of plasmids by conjugation in aquatic systems

In aquatic systems, antimicrobial resistance genes can be horizontally transferred to native bacteria [20, 21, 24]. Antibiotic resistance transfer is mainly attributed to conjugation since many antibiotic resistance genes are situated on mobile elements, such as plasmids and conjugative transposons, whereas transformation and transduction are usually more limited. Moreover, conjugation of broad-host-range plasmids enables DNA to be transferred over genus and species borders [47-49].

Although the high percentage of resistant bacteria in diverse environments seems to indicate that gene transfer processes take place naturally [20, 24, 50] in aquatic systems, Davison [5] and Kümmerer [51] have observed that the transfer of resistant genes is particularly favored by the presence of antibiotics. Anyway, Van Elsas and Bailey [24] conjecture that the ecologically highly relevant factors determining survival of plasmid carrying strains (adverse temperature, limitation of nutrients, salinity, etc.) could act as determinants of natural gene transfer.

Information about horizontal gene transfer in aquatic ecosystems has been usually obtained from studies with microcosms [47]. Obviously, these studies have limitations in reproducing the natural aquatic systems and discount the role of factors potentially affecting the process, but they provide information hardly obtainable in changing natural systems.

Early studies, carried out in laboratory microcosms, demonstrated the influence of abiotic factors such as temperature, availability of nutrients and/or coincubation (mating) time [15, 52-54] in the conjugative process.

Low temperatures and scarcity of nutrients affect negatively this process among *E. coli* strains [34, 55]. However, Fry and Day [56] established that the maximum transfer for pQM85 plasmid in *Pseudomonas* occurred at 10ºC. These different results are not surprising if we consider that, at least for bacteria, the optimum survival temperature is below the optimum growth temperature [57]. So, psychrotrophic and psychrophilic bacteria show three separate survival domains, cold (below approximately 15ºC), optimal and up from the optimal temperature [58-60], while in mesophiles the range is continuous, being low temperatures less stressful than high ones [57]. In any case, these factors affect bacterial survival and activity and consequently, the plasmid transfer as it is an active process [61].

Adhesion to surfaces also influences plasmid transfer, since depending on the shape and characteristics of the plasmid-encoded pili, plasmids might be easier transferred on surfaces [62, 63]. Additionally, bacterial hosts are likely to adhere to surfaces where nutrients are concentrated, cells are protected and cell-to-cell contacts are promoted [24, 47, 64]. Therefore, the plasmid transfer is higher among cells attached to surfaces than among cells in the aquatic phase [65].

Moreover, biotic factors, such as densities of donor (D) and recipient (R) populations (and their proportion, D/R) as well as their physiological status, have been demonstrated to affect the conjugative gene exchange [28, 54, 61, 66]. Fernández-Astorga *et al*. [55] reported a direct relationship between the density of parent cells (D+R) and the number of

transconjugants detected, obtaining the maximum transfer frequency with 10^8 CFU/ml and a D/R proportion comprised between 1 and 10.

Some studies have also revealed that, in general, plasmid-transfer frequencies depend on the physiological status of donor rather than of recipient cells [61, 67]. Therefore, the entry of donor cells into the VBNC state influences negatively conjugative processes [28, 67]. However, Chandrasekaran *et al.* [66] indicated that the nonculturable state and nutrient deprived condition may not limit plasmid transfer in *P. fluorescens*. Their results suggested that terrestrial antibiotic resistant bacteria that reach seawater may be responsible for the prevalence of resistance genes in the marine environment.

Another factor to be considered is the interaction with the native microbial population [5]. The presence of microbial communities leads to a reduced conjugal plasmid transfer frequency due to predation, bacteriophages, inhibition and competition with natural microbial communities [43, 68]. Conversely, Ueki *et al*. [69] described the enhancement of conjugal plasmid pBHR1 transfer among bacteria in the presence of extracellular metabolic products excreted by *Microcystis aeruginosa.*

As it has been previously indicated, most of the information about the transfer process has been obtained under laboratory conditions and raises doubts about whether they reflect the real processes in the environment.

4. Wastewater treatment plants: a hot spot for antibiotic resistance transference

Liquid wastes are produced by everyday human operations and by agricultural and industrial operations. Untreated wastewater potentially contains a variety of chemicals and biological constituents hazardous to human health and to the environment, so that they are treated to prevent the transmission of diseases, contamination of land and the direct or indirect pollution of aquatic systems. Wastewater treatment plants (WWTP) have been designed to reduce the concentration of dissolved organic carbon, nitrogen and phosphorous and eliminate pathogens from the influent. Treatment includes physical, chemical, and biological processes with the aim to produce an environmentally safe effluent and suitable sludge for disposal or reuse. Several studies have confirmed a significant reduction not only of the usually monitored physicochemical parameters (biological oxygen demand, chemical oxygen demand, total and suspended solids, etc.), but also of the microbiological parameters (heterotrophic plate count, fecal indicator bacteria, total bacteria, etc.), as consequence of the treatment [70-73].

Many researchers have recognized wastewater treatment plants as reservoirs for antibiotic resistant bacteria and as an important environment for horizontal gene transfer [4, 7, 72, 74, 75]. Biological treatment processes create an environment potentially suitable for selection and increase of resistant bacteria. Investigators argue different reasons not mutually exclusive. In WWTP, bacteria are continuously exposed to antibiotics at sub-inhibitory concentrations and to high nutrient contents. Moreover, the high bacterial density and the surfaces could promote the cell-to-cell contact and so, genetic exchange.

The occurrence of antibiotics in sewage waters may facilitate the selection of antibiotic resistant genes and antibiotic resistant bacteria [4, 7, 74, 76]. In fact, correlations between high concentration of antibiotics in sewage and elevated levels of resistant bacteria have been reported [7, 20, 74].

On the other hand, enhanced bacterial survival in wastewater with respect to river water [77, 78] has been attributed to more abundant nutrients that offset cellular injuries. The same reason could explain how, although plasmids transfer decreases, this ability is maintained for longer periods of time than in oligotrophic environments [77]. Recently, horizontal transfer of antibiotic resistant genes was studied in sewage and lake water by Shakibaie *et al*. [79], who observed that the rate of conjugation was twofold higher in sewage than in lake water.

These facts, the enhanced survival of plasmid carrier strains and the high frequency of plasmid transfer, could effectively contribute to the proliferation of plasmid carrier strains in wastewater, but only if we consider the possibility that transconjugant bacteria will become potential donors.

Another fact to consider is the bacterial adhesion to solid surfaces (flocs, filters) that takes places in WWTP. The activated sludge process is based on the adhesion of microorganisms into flocs and their subsequent concentration in sludges. Flocs contain high concentration of nutrients and cells, and floc structure may protect against predation by bacteriovorous protozoa in addition to provide greater opportunities for cell-to-cell contact (see above factors affecting plasmid transfer by conjugation). Besides, sludge and biosolid samples contain a high concentration of antibiotic-resistant bacteria [80-82].

In the last years, the application of new and easier methods to detect active cells, coupled with the use of molecular biology methods in WWTP studies, has demonstrated the presence of an important fraction of active cells, non-detectable by traditional methods based on culturability. Muela *et al*. [71] estimated this fraction ranging from 5 to 86% of total bacteria. This variation has been also reported by other authors [83-84]. This fraction includes VBNC bacteria (see above) and other active bacteria as chemolithotrophic bacteria or uncultured bacteria only detectable by molecular methods [11, 71, 85] which may contribute to the antibiotic resistance transference in wastewater [74, 76]. In addition, these molecular methods have provided data which suppose a direct demonstration of gene transfer in the wastewater environment [81].

5. Concluding remarks

The development of new antibiotics as well as their increasing and, occasionally, indiscriminate and irrational use provokes the release of antibiotic and antibiotic-resistant bacteria via sewage or treated wastewater to the environment. Wastewater plants constitute an appropriate environment for survival of plasmid-carrier bacteria and for dissemination of their resistances by conjugation processes, so both treated effluents and sludges are a way of dissemination in natural aquatic systems. However, stressful conditions, common in natural aquatic systems, as adverse temperature, scarcity of nutrients, predation by protists and others, could interfere with some effectiveness in the subsequent spread of resistance in natural environments.

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