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Marta Colomer Lluch

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Thesis presented by **Marta Colomer Lluch** to obtain the degree of Doctor by the University of Barcelona

Memòria presentada per **Marta Colomer Lluch** *per optar al grau de Doctora per la Universitat de Barcelona*

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Thesis developed under the supervision of Dr. Joan Jofre Torroella and Dr. Mª Teresa Muniesa Pérez in the Department of Microbiology, Faculty of Biology, University of Barcelona

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Universitat de Barcelona

Professors Joan Jofre Torroella and Mª Teresa Muniesa Pérez from the University of Barcelona

DECLARE THAT,

Marta Colomer Lluch has performed the work entitled ***Antibiotic resistance genes in the viral DNA fraction of environmental samples / Gens de resistència a antibiòtics en el DNA de la fracció vírica de mostres ambientals*** under our supervision in order to obtain the degree of Doctor by the University of Barcelona and that this thesis fulfils the requirements to obtain the International Doctor Mention, and that this work is ready to be presented from the present day.

Signature,

Joan Jofre Torroella

Mª Teresa Muniesa Pérez

Barcelona, April 2014

A la meva família.

“Learn from yesterday, live for today, hope for tomorrow.

The important thing is to not stop questioning.”

Albert Einstein.

"Le rôle de l'infiniment petit dans la nature est infiniment grand".

Louis Pasteur.

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PRESENTATION OF THE THESIS

The PhD thesis presented here has as a main objective the study of antibiotic resistance genes clinically relevant in the DNA fraction of bacteriophage particles isolated from environmental samples of different origin in order to determine the importance of bacteriophages as vehicles for the mobilization of antibiotic resistance genes between bacteria.

Specifically, a broad range of antibiotic resistance genes were studied as representative of the main groups recently described in our geographical area belonging to three β-lactamases (*bla*$_{TEM}$, *bla*$_{CTXM-1}$ and *bla*$_{CTX-M-9}$), the *mecA* gene conferring resistance to methicillin in staphylococci, and the quinolones resistance genes *qnrA* and *qnrS*.

To achieve these goals samples of urban wastewater, river water and animal faecal wastes were analysed quantifying the antibiotic resistance genes of interest in bacteriophages DNA.

During the development of this Thesis, it was attempted to optimize the available methodology for bacteriophage DNA extraction, as well as the necessary controls to guarantee the amplification of encapsidated DNA and to remove any free DNA in the samples and any possible vesicles containing DNA.

In addition, the ability of phage-encoded genes to confer antibiotic resistance in bacterial strains was assessed by performing transfection experiments.

It was also studied the influence of various compounds involved in the induction of the lytic cycle of temperate bacteriophages, on the abundance of antibiotic resistance genes in DNA from the phage fraction in wastewater samples.

Finally, due to the importance of horizontal gene transfer as a mechanism for antibiotic resistance dissemination in clinical and environmental settings, transduction experiments were attempted, although unsuccessfully, to reproduce *in vitro* the process that would take place in nature.

The research developed in this Thesis is divided into 5 studies included in 4 chapters: (1) Antibiotic resistance genes in the bacteriophage DNA fraction of water samples (urban wastewater, river water and wastewater with animal faecal wastes); (2) Quinolone resistance genes (*qnrA* and *qnrS*) in bacteriophage particles from wastewater samples and the effect of inducing agents on packaged antibiotic resistance genes; (3) Evaluation of ARGs in the DNA of bacterial and bacteriophage fraction in wastewater samples from Tunisia and comparison with results obtained in Barcelona area; (4) Detection of quinolone-resistant *Escherichia coli* isolates belonging to clonal groups O25b:H4-B2-ST131 and O25b:H4-D-ST69 in water samples from Barcelona area.

Each of the studies has given rise to a scientific article already published or submitted for scientific publication.

In accordance with the requirements for the international mention, English and Catalan were the chosen languages for the development of this memory. This thesis is based on the published and submitted articles that have been included in the publications section. All articles were written in English and are accompanied by a summary in Catalan at the beginning of each publication. It has also been included an introduction, the main objectives and the conclusions in both languages. The discussion has been written entirely in English.

PRESENTACIÓ DE LA TESI

La tesi doctoral que es presenta a continuació té com a objectiu principal l'estudi de gens de resistència a antibiòtics de rellevància clínica en la fracció de DNA de partícules de bacteriòfags aïllades de diferents tipus de mostres ambientals per tal de determinar la importància dels bacteriòfags com a vehicles de mobilització de gens de resistència a antibiòtics entre bacteris.

Concretament, s'ha estudiat un ampli espectre de gens de resistència a antibiòtics com a representants dels grups principals descrits actualment en la nostra àrea geogràfica corresponent a tres β-lactamases (bla_{TEM}, bla_{CTXM-1} i $bla_{CTX-M-9}$), el gen *mec*A de resistència a meticil·lina en estafilococs, i els gens de resistència a quinolones *qnrA* i *qnrS*.

Per això s'han analitzat diversos tipus de mostres procedents d'aigua residual municipal, d'aigua de riu i d'aigua residual amb contaminació fecal animal per tal de quantificar els gens de resistència a antibiòtics d'interès en DNA aïllat de bacteriòfags.

Durant els diferents estudis s'ha intentat optimitzar la metodologia d'extracció de DNA de bacteriòfags així com els controls corresponents per garantir l'amplificació de DNA encapsidat i l'eliminació de qualsevol DNA lliure present a les mostres i de qualsevol possible vesícula amb DNA al seu interior.

Per altra banda, també s'ha determinat la capacitat funcional dels gens de resistència detectats en DNA de fags i per això s'han realitzat experiments de transfecció a partir de soques bacterianes sensibles a un determinat antibiòtic amb l'objectiu d'incorporar la resistència i per tant, esdevenir resistents a l'antibiòtic en qüestió.

També, s'ha estudiat la influència de determinats compostos implicats en la inducció del cicle lític de bacteriòfags temperats, en l'augment en el nombre de còpies de gens de resistència a antibiòtics en DNA present en la fracció de fags de l'aigua residual.

Finalment, degut a la importància de la transferència horitzontal de gens com a mecanisme de dispersió de la resistència a antibiòtics en el medi ambient i en clínica s'han dut a terme experiments de transducció, malauradament sense èxit, per tal d'intentar reproduir *in vitro* el procés que tindria lloc de manera natural.

El treball desenvolupat al llarg d'aquesta tesi s'ha dividit en 5 estudis agrupats en 4 capítols: (1) Gens de resistència a antibiòtics en la fracció d'DNA de bacteriòfags en mostres d'aigua (aigua residual municipal, aigua de riu i aigua residual amb contaminació fecal animal); (2) Gens de resistència a quinolones (*qnrA* i *qnrS*) en partícules de bacteriòfags en mostres d'aigua residual i avaluació de l'efecte d'agents inductors en els gens de resistència a antibiòtics encapsidats; (3) Avaluació de gens de resistència a antibiòtics en DNA de la fracció bacteriana i de bacteriòfags en mostres d'aigua residual amb contaminació fecal humana i animal de Tunísia i comparació amb els resultats obtinguts a l'àrea de Barcelona; (4) Detecció d'aïllaments d'*Escherichia coli* resistents a quinolones dels grups clonals O25b:H4-B2-ST131 i O25b:H4-D-ST69 en mostres d'aigua de l'àrea de Barcelona.

Cadascun dels capítols ha donat lloc a un o més articles científics publicats o sotmesos a revistes científiques.

D'acord amb els requisits exigits per a l'obtenció de la menció internacional, les llengües escollides per a realitzar aquesta memòria han estat el català i l'anglès. La base d'aquesta tesi són els articles publicats i sotmesos que s'inclouen a l'apartat de publicacions de la tesi. Tots els articles han estat escrits en anglès i s'acompanyen del corresponent resum en català a l'inici de cada publicació. També s'ha incorporat una introducció, uns objectius i conclusions finals en ambdues llengües. La discussió s'ha realitzat íntegrament en anglès.

LIST OF ABBREVIATIONS/LLISTAT D'ABREVIATURES

ºC	Degree Celsius
ARGs	Antibiotic resistance genes
bp	Base pairs
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
OD	Optical density
ECDC	European Center for Disease Control and Prevention
EDTA	Ethylenediaminetetraacetic acid
ESBLs	Extended-spectrum ß-lactamases
g	Centrifugal force
GC	Gene copy
HGT	Horizontal gene transfer
kb	Kilobases
kDa	KiloDaltons
MDR	Multidrug-resistant bacteria
MGEs	Mobile genetic elements
MRSA	Methicillin-resistant *Staphylococcus aureus*
NCBI	National Center for Biotechnology Information
n.d.	Not determinated
P	Probability, variable of statistical significance
PCR	Polymerase chain reaction
PFU	Plaque forming units
qPCR	Quantitative PCR
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate

SSCmec	Staphylococcal cassette chromosome mec
SOMCPH	Somatic coliphages
spp	Specie
TBE	Tris/Borate/EDTA
TSA	Tryptone soya agar
UV	Ultraviolet light
V	Volts
WHO	World Health Organization
WWTP	Wastewater treatment plant

CONTENT OF THE THESIS/*CONTINGUT DE LA TESI*

1. GENERAL INTRODUCTION / *INTRODUCCIÓ GENERAL*

1. GENERAL INTRODUCTION

1.1. Bacteriophages

Mobile genetic elements (MGEs) are typically identified as fragments of DNA that encode a variety of virulence and resistance determinants as well as the enzymes that mediate their own transfer and integration in the new host (Frost *et al*., 2005). MGEs may consist of insertion sequences (IS), transposons, intragenic chromosomal elements (ICEs), plasmids, pathogenicity islands, chromosome cassettes and some bacteriophages (Lupo *et al.*, 2012).

In this thesis, we will focus on bacteriophages and especially in their potential role as reservoris and vehicles in the dissemination of antibiotic resistance genes (ARGs).

Bacteriophages (or phages) are viruses that infect bacteria and were discovered independently in 1915 by Twort and in 1917 by d'Herelle (Adams, 1959; Duckworth, 1976). Bacteriophages are ubiquitous in nature and are probably the most abundant entities on Earth with an estimated total population of 10^{30}-10^{32} (Suttle, 1994; Brüssow and Hendrix, 2002; Ashelford *et al*., 2003; Jofre, 2003; Chibani-Chennoufi *et al*., 2004).

Bacteriophages are extremely diverse showing different morphologies, lifestyles and genomic composition, with dsDNA tailed phages accounting for 95% of all the phages reported in the literature (Mc Grath and van Sinderen, 2007). Phages also vary in structure, ranging from the most simple to the most elaborated and complex, with different sizes and shapes but essentially each phage particle (virion) contains its nucleic acid genome (DNA or RNA) enclosed in a protein or lipoprotein coat, or capsid; the combined nucleic acid and capsid form the nucleocapsid. Many phages also contain additional structures such as tails or spikes (Kutter and Sulakvekidze, 2005).

Although phages carry all the information to direct their own reproduction in an appropriate host, they lack of machinery for generating energy and have no ribosomes for making proteins. Thus, bacteriophages can only replicate in a susceptible host bacterial cell by using the enzymatic cell system in order to duplicate their structures, mainly proteins and nucleic acids (Adams, 1959; Waldor and Friedman, 2005).

The spectrum of bacteria that can be infected by a given phage, termed *host range*, depends on the presence of bacterial receptors recognizable by the phage. The target host for each phage is a specific group of bacteria, often a subset of one species, but several related species can sometimes be infected by the same phage (Kutter and Sulakvekidze, 2005). Many phages have a narrow host range infecting a limited number of strains of a given bacteria species. However, others known as *polyvalent bacteriophages* have been reported to have a wide host range that crosses the boundaries of different bacterial species in a genus, as in *Enterococcus* (Mazaheri *et al*., 2011); of different genera in a family, for example in *Enterobacteriaceae* (Souza *et al*., 1972; Evans *et al*., 2010); or different taxa, for example between *Gammaproteobacteria* and *Betaproteobacteria* (Jensen *et al*., 1998); and between Gram-positive and Gram-negative bacteria (Khan *et al.*, 2002). As an example, similar prophages have been detected in bacteria of different species of *Clostridium* and *Bacillus* (Shan *et al*., 2012).

1.1.1. Bacteriophages life cycles

Based on the outcome of phage infection of the host cell, phages can follow two different life cycles (Adams, 1959) (Figure 1):

- **Lytic cycle:** Following adsorption of the phage to the specific receptor of the susceptible bacterial host cell, the phage genome is injected into the bacterial cytoplasm. In the lytic pathway, the genome is replicated independently of the bacterial chromosome and several copies of the phage genome are produced. Meanwhile, the phage late genes are transcribed and translated to yield the protein components of the heads and/or tails, which assemble independently in the bacterial cytoplasm. The phage capsid protein assembles and the genome is then spooled into the new phage heads. The DNA-containing heads are then joined to the performed tails (if tailed phages) leading to the production of new phages. Further phage-encoded proteins (very late proteins) burst the host cell and release the progeny of phages into the medium finally causing bacterial cell death.

The phages that can only follow the lytic pathway are called **virulent phages.** T4 phage and ΦX174 are examples of virulent phages.

- **Lysogenic cycle:** Certain phages, once they infect the host cell, can either enter lytic or lysogenic cycle and are known as **temperate phages**.

  In the lysogenic pathway, the genome of the temperate phage becomes part of the bacterial genome, replicating along with the host, either integrated in the host chromosome (this is the case for most prophages), or by making the phage genome circular and remaining as an independent replicon (*e.g.* P1 phage).

  More in detail, after phage attachment to the bacterial receptor, the phage genome is injected within the host cell and usually circularizes. By means of the product of the phage gene encoding for an integration enzyme (integrase), the phage genome becomes integrated in the bacterial chromosome usually in a specific locus yielding the quiescent phage, called **prophage**. By means of the integrase, there is a recognition of a specific site in the bacterial chromosome, called *att*B (attachment site of the bacteria). Phage and chromosomal DNAs become joined by a sequential series of DNA breaking and joining reactions. As a result of integrative recombination, the phage chromosome becomes attached to the bacterial chromosome, afterward resembling a normal cellular gene. It remains in this condition indefinitely, being replicated as its host cell replicates to make a clone of cells all containing prophages. Cells containing prophages are said to be **lysogenic** (or **lysogens**). Therefore, the bacterial cell incorporates phage genes and is able to express them; the process is called **lysogenic conversion**.

  Temperate prophages may occasionally come out of its quiescent condition and switch to the lytic cycle, in a process called **induction.** Once in the lytic cycle, phages propagate causing at the end the lysis of the host cell and being released as new phage particles (virions).

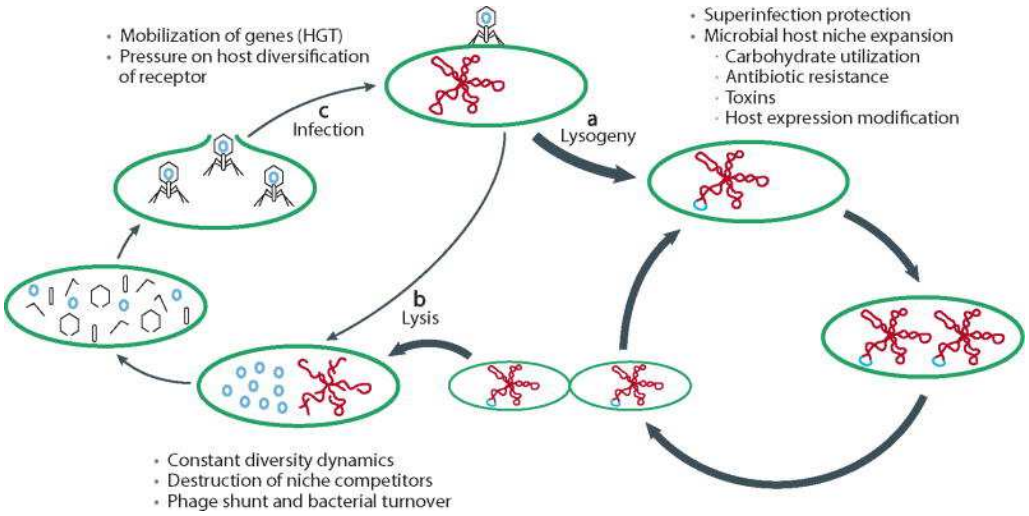

Figure 1. Lytic and lysogenic cycles of bacteriophages (Reyes *et al*., 2012).

It is important to highlight that usually these virions cannot infect other cells from the lysogenic culture since all of them are carrying the same prophage in their genome giving *immunity* to the attack of a virion of the same phage, although cells still vulnerable to the infection by other phages. Because of their immunity condition, temperate phages only make plaques when there are plated in a non-lysogenic strain. Some exceptions have been however found, as temperate Shiga toxin encoding bacteriophages (Serra-Moreno *et al*., 2008).

Summarizing, **temperate phages** have then a choice of reproductive modes when they infect a new host cell. Sometimes the infecting phage initiates a lytic cycle, resulting in the lysis of the cell and the release of new phage particles as previously mentioned. Alternatively, the infecting phage may initiate a quiescent state called a *prophage*, often integrated into the host genome, but sometimes maintained as a plasmid. It remains in this condition indefinitely, being replicated as its host cell reproduces to make a clone of cells all containing prophages (lysogenic cells). Occasionally, one of these prophages comes out of its quiescent condition and enters the lytic cycle. The factors affecting the choice to lysogenize or to reenter into a lytic cycle (induction) are called inductors or **inducing agents** and are described in the next section.

The plaques of lysis generated by temperate phages could be slightly different compared to the ones formed by virulent phages. Instead of being clear plaques as virulent phages do, they are usually slightly turbid. Plaques are visible for the lysis of the cell following the lytic cycle and the turbidity observed is caused because many of the phages are conducting lysogenic cycles instead of strictly lytic cycle and the lysogenic cells are growing within the plaque.

Lambda (λ), P1, P22, and Mu bacteriophages infecting *Enterobacteriaceae* and various phages infecting *Lactobacillaceae* are among the best-known temperate phages.

Temperate phages can help protect their hosts from infection by other similar phages (*superinfection*) and by means of lysogenic conversion can lead to significant changes in the properties of their hosts. Some authors have emphasized the importance of temperate phages as "replicons endowed with horizontal transfer capabilities" (Briani *et al.,* 2001) and they have probably been major factors in bacterial evolution by moving segments of genomes into different bacteria.

1.1.2. Inducers of lytic cycle in temperate bacteriophages

Prophage induction can take place either spontaneously or stimulated by inductors. Some compounds, either natural or introduced by human activity in the environment, may act as inducing agents leading to phage replication and increase in the number of phage particles that will be released from the host cell. The most relevant inducers are described below:

- *Mutagenic agents:* Mutagenic agents or any agent that can cause DNA damage, such as radiations as UV, can induce the phage lytic cycle in many lysogens.

- *Different classes of antibiotics*: Certain antibiotics, used in human therapy or animal husbandry as growth promoters, are able to induce phages from their lysogens. Among others, those antibiotics affecting DNA or those activating the bacterial SOS response will cause phage induction. Quinolones, such as ciprofloxacin or norfloxacin have been widely used for induction of phages (Goerke *et al*., 2006; Rolain *et al*., 2009; Looft, 2012; Meessen-Pinard *et al*., 2012) and Qnr-prophage induction in the

presence of quinolones has been demonstrated with intestinal populations (Modi *et al*., 2013).

Other antibiotics like trimethoprim, furazolidone and ciprofloxacine are potent SOS inducers and they are reported to induce *stx* gene expression in EHEC O157:H7 (Kimmitt *et al*., 2000). Ampicillin has also been shown to induce prophages (Maiques *et al*., 2006).

- *Mitomycin C*, which damages DNA by cross-linking complementary strands, is considered an antitumoral agent. This compound is commonly used in laboratory practice as an agent for temperate phage induction through activation of the SOS response (Fuchs *et al*., 1999; Livny and Friedman, 2004; Muniesa *et al*., 2004).

- *Chelating agents:* EDTA and sodium citrate are chelating agents. EDTA was reported to increase the number of copies of Shiga toxin gene (*stx*) in temperate Stx phages when a culture of a lysogenic strain for a Stx phage is treated with 20mM EDTA, even in the absence of RecA (Imamovic and Muniesa, 2012).

- *Oxidizing agents:* Reactive oxygen species (ROS), such as hidrogen peroxide, are strong oxidizing agents that can decompose into free radicals and cause DNA damage to bacteria, which leads to prophage induction (Kutter and Sulakvekidze, 2005).

Such factors may play important roles in enhancing the frequency of gene exchange in environments such as farms, hospitals, and sewage systems, which provide ideal conditions for ARGs acquisition.

1.1.3. Bacteriophage transduction

Bacteriophages mediate horizontal gene transfer (HGT) through transduction. **Transduction** refers to the process by which a DNA fragment is transferred from one bacterial cell to another using a bacteriophage particle as a vector. The size of DNA fragments that can be

packaged into a phage particle is limited by the size of the phage capsid, but can reach upwards of 100 kb.

Transduction by bacteriophages includes any sort of bacterial DNA, from linear chromosome fragments to all sorts of MGEs such as plasmids, genomic islands, transposons and insertion elements (Mann and Slauch, 1997).

Transduction was traditionally considered to occur at very low frequencies around once every 10^7-10^9 phage infections, but recent studies show that transduction takes place in the environment at a remarkably high rate with frequencies greater than previously thought (Chiura, 1997; Evans *et al.,* 2010; Kenzaka *et al*., 2010). For example, transduction of genes for the global marine phage population between marine bacteria has been calculated to take place in the oceans at the rate of about 20 million billion times per second, although the real numbers will probably be lower due to smaller transduction efficiency and more rapid phage decay in the ocean than in the laboratory. (Bushman, 2002).

There are two types of transduction, **generalized transduction** and **specialized transduction**:

- **Generalized transduction**: Generalized transduction is so named because essentially any fragment of bacterial DNA from any location in its genome can be packaged into a phage head, instead of phage DNA, and then be transferred between cells by this mechanism. Phage particles that have encapsidated bacterial DNA are called *transducing particles*. Generalized transducing particles can be produced during lytic growth of either virulent or temperate phages (Thierauf *et al*., 2009). Phages that package DNA by a headful mechanism can occasionally misfire and package a headful of host cell DNA instead. Upon infection of a new host cell, the DNA can sometimes become incorporated into the genome of the new host.

  Generalized transduction is a prominent means of gene transfer between bacteria. The generalized transducing particles filled with host DNA rather than phage DNA cannot propagate, but may infect a new cell, hence still contribute substantially to bacterial exchange in nature.

Examples of phages known to be capable of generalized transduction are P1, Mu, P22, T1, T4, KB1 and ES18 phages.

Generalized transduction process can be exemplified by bacteriophage P1, a temperate phage that infects a variety of Gram-negative bacteria, which was isolated from a lysogenic strain of *Escherichia coli*. Upon translocation into the host cytoplasm, the linear double-stranded P1 DNA circularizes by recombination. During lytic growth, the circular genome of P1 initiates several rounds of bidirectional replication before switching to rolling circle replication that produces long concatamers of double-stranded P1 DNA. P1 encodes a phage endonuclease that recognizes a specific ***pac*** sequence on the phage DNA and cuts the DNA at this site to initiate headful packaging into an empty phage head. When packaging has been completed, the excess DNA is cleaved by a sequence independent mechanism. Subsequent rounds of packaging are then initiated from the cleaved DNA and these packaging reactions continue processively until several heads are filled with DNA. When a cell lyses, it releases the new phage particles. (Sternberg and Coulby, 1990; Thierauf *et al*., 2009). The generalized transduction occurs when the endonuclease cuts by mistake sequences on the host bacterial chromosome that are homologous to the P1 *pac* site. When P1 infects a cell, occasionally the P1 endonuclease cuts one or more of these chromosomal sites DNA and package it into P1 phage heads. These phage heads contain only bacterial DNA and no phage DNA. The P1 particles carrying bacterial DNA (transducing particles) can inject this DNA into a new hostand the DNA once inside the new cell can then recombine into the chromosome by homologous recombination. These transducing particles will not be able, however, to conduct lysis or lysogeny in the new host.

About 30% of the phage particles in a P1 lysate contain host DNA rather than phage DNA. Given the relative sizes of the *E. coli* and P1 genomes, approximately 1 in 1,500 phage particles in a lysate will carry a given gene from the donor. All genes of the donor (including plasmid genes) present the same probabilities of being mobilized by this mechanism.

- **Specialized transduction:** In contrast to generalized transduction, it results from the aberrant excision of a prophage from a specific integration site in the bacterial chromosome, packaging both phage DNA and a fragment of adjacent DNA from the bacterial genome into a single phage particle. By means of specialized transduction the phage can package only its DNA and the specific bacterial DNA flanking the attachment site of an integrated prophage (Miller *et al*., 2004; Thierauf *et al*., 2009).

  Because the types of sequences that can be transferred are so restricted, it seems likely that specialized transduction is not a major contributor to gene transfer in the environment compared to generalized transduction. Nevertheless, the frequency of transduction of this specific DNA fragment is high since all the phage particles produced after induction of the prophage carry this particular fragment.

  A few examples of phages capable of specialized transduction have been reported being lambda phage the most well-known.

Usually, temperate phages that cause specialized transduction immediately circularise their DNA after infection of the bacterial host, by using the *cos* (*co*hesive end) sites. When these phages package their DNA after induction of lytic cycle they use the *cos* sites to cleave their DNA concatemers and these DNA segments cut at the *cos* sites are packaged in the phage heads. Therefore, theoretically, they are not able to produce generalized transduction, which needs to package DNA by a *pac*-mechanism. Only few examples of *cos*-packaging phages causing generalized transduction are found in the literature (Sternberg, 1986; Campoy *et al*., 2006).

1.1.4. Ubiquity and abundance of bacteriophages

Bacteriophages exist in large quantities and are widely distributed in different natural environments, wherever their bacterial hosts live, such as gastrointestinal tracts of humans and animals where gut bacteria are associated with their specific phages communities (Breitbart *et al*., 2003, 2008; Minot *et al*., 2011), sewage water (Cantalupo *et al*., 2011), human and animal faeces (Letarov and Kulikov, 2009; Victoria *et al*., 2009; Reyes *et al*.,

2010), soil (Weinbauer, 2004), plants (Gill and Abedon, 2003), marine systems (Angly *et al*., 2006), lakes (Ogunseitan *et al*., 1990), river water, etc. and even in extreme environments (Le Romancer *et al*., 2007), with variable numbers that seem to depend on bacterial abundance and activity (Table 1).

Origin	Concentration	Reference
Deep sea environments	10^4-10^5 VLP[a]/mL	Paul and Kellog, 2000
Coastal environments	10^6-10^7 VLP/mL	Paul and Kellog, 2000
Productive lakes or estuarine waters	10^8 -10^9 VLP	Hennes and Suttle, 1995
Limnetic and marine sediments	>10^8 -10^9 VLP	Danovaro *et al.*, 2002
Solar saltern	10^8-10^{10} VLP/mL	Boujelben *et al*., 2012
Soil or rizosphere	10^7-10^8 VLP/g	Ashelford *et al*., 2003
Intestinal content	5x10^7-10^{10} VLP/g	Lepage *et al*., 2008
Intestinal mucosa	>10^8 VLP/mm^2	Letarov and Kulivov, 2009
Sputum of patient with broncho-pulmonary infections	10^3-10^7 [b]PFU on *Pseudomonas aeruginosa*	Tejedor *et al*., 1982
Plant's microbial communities	>10^6 PFU/g of leave tissue of bacteriophages infecting *Erwinia*	Ritchie and Kloss, 1977
Activated sewage sludge	>10^9 VLP/mL	Otawa *et al*., 2006
Raw municipal wastewater	10^8 VLP/mL	Rosario *et al*., 2009
Potable and well water	10^5-10^6 VLP/mL	Rosario *et al*., 2009

Table 1. Ubiquity and abundance of bacteriophages (Muniesa *et al*., 2013). *[a]VLP: Virus-like particles ; [b]PFU: Plaque forming units.*

Transmission electron microscopy and epifluorescence microscopy, together with the development of molecular techniques, such as genome sequencing and metagenomic analysis, have allowed the detection of bacteriophages in environments in a way that was not possible previously, independently of their infectivity, revealing that phages are much more abundant than previously thought (Breitbart *et al*., 2004; Weinbauer, 2004; Srinivasiah *et al*., 2008), and indicating that natural phage communities are reservoirs of the greatest uncharacterized diversity on Earth, with an enormous variety of environmental niches and survival strategies (Weinbauer, 2004). Many of these studies are based in analysis of the viral fraction of a given sample but since in most environments studied phages are the main part of the viral fraction (Dinsdale *et al*., 2008), it can be assumed that the viral DNA evaluated in these studies will belong mostly to bacteriophages.

The idea that bacteriophages play an important role in microbial ecology is widely accepted nowadays. On the one hand, by infecting and lysing infected bacteria, they contribute remarkably to bacteria mortality, thus they regulate the numbers of certain bacterial populations in a given environament; and by releasing organic compounds through cell lysis they have an important impact on the cycling of organic matter in the biosphere (Suttle, 1994). On the other hand, they control microbial diversity by selecting for some types of bacteria that are resistant to their attacker (Scanlan and Buckling, 2012), thus changing the proportions of bacterial species or strains in a community, and also influencing the evolution of bacterial genomes through HGT by transduction.

Considering the wide occurrence of phages in the environment, and the high concentration of phages in some water bodies (Weinbauer, 2004; Srinivasiah *et al*., 2008), and since they can transduce genes among their bacterial hosts, they are expected to play a crucial role of genetic transfer in water habitats mediated by phages (Ripp and Miller, 1995; Brabban *et al.,* 2005; Parsley *et al*., 2010b). Transduction probably constitutes one of the main gene transfer mechanisms and of genome evolution for bacteria in water habitats.

1.1.5. Persistence of bacteriophages in the environment

Phages, either virulent or temperate, show a higher resistance to natural and anthropogenic stress factors than bacteria usually persisting better in aquatic environments than their bacterial hosts do (IAWPRC, 1991; Muniesa *et al*., 1999; Durán *et al*., 2002; Jofre, 2007). This higher survival and the abundance of phages carrying ARGs in animal and human wastewater (Muniesa *et al*., 2004; Minot *et al*., 2011; Looft *et al*., 2012) makes them suitable candidates as vehicles for the mobilization of the environmental pool of ARGs between bacteria in different biomes that contribute to the maintenance and emergence of new resistances (Sano *et al*., 2004). In addition, due to the structural characteristics of phages, with phage-encapsidated DNA protected from degradation, their persistence in the environment is higher than free DNA (either linear fragments or plasmids), which is more sensitive to nucleases, temperature, predation and radiation (Lorenz and Wackernagel, 1994; Dupray *et al*., 1997; Zhu, 2006).

Phages may survive in special environments without the loss of their infectious capabilities and are able to transfer genes by transduction. This observation supports the notion that the contribution of phages to gene transfer in natural extra-intestinal environments and in human-generated environments could be greater than that of plasmids or transposons, with a lower persistence in the environment (Lorenz and Wackernagel, 1994; Dupray *et al*., 1997; Zhu, 2006). In clinical settings, however, plasmids and transposons are probably most relevant MGEs for horizontal antibiotic resistance transfer because their lower environmental persistence is not a limitation in a human body.

Indeed, their high level of specificity, long-term survivability, and ability to reproduce rapidly in appropriate hosts displayed by phages contribute to the maintenance of a dynamic balance among the wide variety of bacterial species in any natural ecosystem. When no appropriate hosts are present, many phages can maintain their ability to infect for a long period of time unless damaged by external agents.

Although phages vary greatly in their sensitivity to various chemical and physical agents, certain general principles have been described. For example, DNA phages are very susceptible to UV light in the range of 260 nm as well as in the far UV. Other factors potentially affecting phages include:

- *pH*: Phages generally are stable at pH 5 to 8, and many are stable down to pH 3 or 4, but it depends on each phage.

- *Temperature:* Temperature is a crucial factor for bacteriophage replication and survavility (Hurst *et al.,* 1980; Yates *et al.,* 1985; Nasser and Oman, 1999; Olson *et al*., 2004). It plays a fundamental role in attachment, penetration, multiplication, and the length of the latent period (in the case of lysogenic phages). At lower than optimal temperatures, fewer phage genetic material penetrate into bacterial host cells; therefore, fewer of them can be involved in the multiplication phase. Higher temperatures can prolong the length of the latent stage. Moreover, temperature determines the occurrence, viability, and storage of bacteriophages.

- *Urea and urethane*: Phages are often quite sensitive to protein-denaturing agents such as urea and urethane, but the level of inactivation depends on both concentration and temperature and differs for different phages.

- *Detergents:* Not surprisingly, detergents generally have far less effect on phages than they do on bacteria, although the few phages that are enveloped in membranes are quite susceptible.

- *Chelating agents:* Chelating agents, in contrast, have strong effects on some phages depending mainly on cations requirements for adsorption or capsid assembly.

- *Chloroform*: Chloroform has little or no effect on nonenveloped phages.

- *Mutagenic agents*: such as mustard gas, nitric oxide, and UV light can inactivate phages and, as mentioned before, can also induce the lytic cycle in many lysogens.

1.2. Antibiotics

1.2.1. Antibiotics: definition and history

The term antibiotic (Greek. anti, "against"; bios, "life") was originally referred to a natural compound produced mainly by moulds or other microorganisms that kills bacteria which cause disease in humans or animals. Nowadays, we refer to an **antibiotic** as a chemical substance produced by a microorganism or a synthetic derivative that inhibits the growth of (bacteriostatic) or kills (bactericidal) other sensitive microorganisms.

Before the early 20th century, treatments for infections were based on traditional medicine. Many ancient cultures, including the ancient Egyptians, the ancient Greeks, the Chinese, and Indians of central America used specially selected mould and plant materials and extracts to treat infections, but without understanding the connection of their antibacterial properties and the treatment of diseases.

In the 19th century, prior to the introduction of antibiotics and antiseptic treatments, more than half of all surgical patients developed infections so scientists began to devote time to searching for drugs that would kill these disease-causing bacteria. In Germany, Paul Ehrlich discovered the synthetic antibacterial Salvarsan (arsphenamine). But it was not until in 1928 that Alexander Fleming observed that colonies of the bacterium *Staphylococcus aureus* could be destroyed by a product secreted by the fungus of the genus *Penicillium*, demonstrating antibacterial properties, although he was not able to purify the molecule (penicillin).

In 1932, at the Bayer Laboratories in Germany, a research team developed the first sulfonamide and first commercially available antibacterial, Prontosil, which had a relatively broad effect against Gram-positive cocci, but not against enterobacteria.

A key breakthrough came in 1945 with the development, mass production and distribution of penicillin. Purified penicillin displayed potent antibacterial activity against a wide range of bacteria and had low toxicity in humans. Furthermore, its activity was not inhibited by biological constituents unlike the synthetic sulfonamides. The discovery of such a powerful antibiotic was unprecedented and the development of penicillin led to renewed interest in

the search for antibiotic compounds with similar efficacy and safety. These findings, together with the discovery of streptomycin by Selman Waksman, opened the era of antibiotics.

Initially, after being introduced into clinical practice in the 1940s, antibiotics were extremely efficient and transformed medical care by dramatically reducing illnesses and deaths from bacterial infectious diseases that were leading causes of human morbidity and mortality. Importantly, it was recognized that certain types of bacteria, particularly actinomycetes and streptomycetes, often produced compounds with antibiotic properties leading to systematic efforts to isolate environmental antibiotic producing bacteria.

The world discovered and produced more than 20 novel classes of antibiotics between 1930 and 1962 with a peak during the 1960s (Coates *et al*., 2002; Powers, 2004) (Figure 2). Since then, new antibiotic development has dramatically fallen off and only five new classes of antibiotics have been marketed (Butler and Buss, 2006; Hair and Kean, 2007) mainly due to the problem of bacteria becoming resistant very fast every time a new antibiotic is introduced. The mainstrean approach to combat emerging and re-emerging resistent pathogens has been the modification of existint antibiotics.

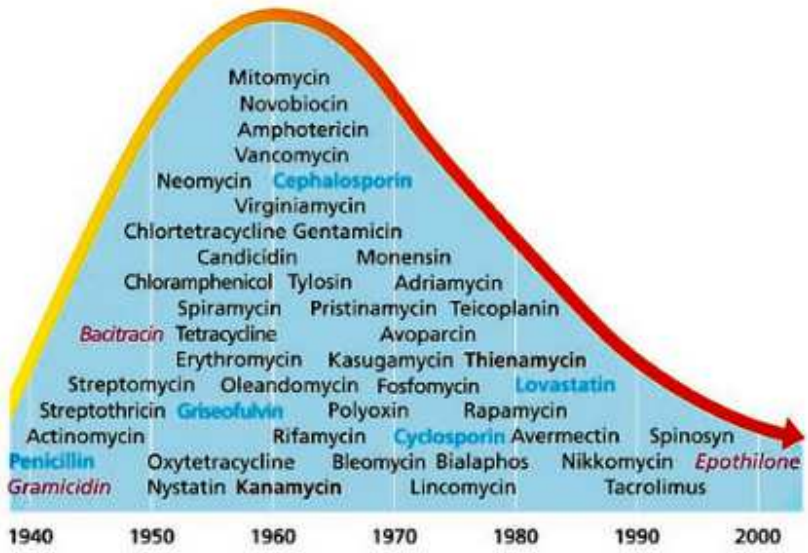

Figure 2. Development of new antibiotics during the past 70 years (scivdet.net).

1.2.2. Use of antibiotics

As mentioned before, the discovery and industrial production of antibiotics in the middle of the previous century has been one of medicine's greatest achievements. The use of antibiotics has revolutionised the treatment of bacterial infectious diseases both in humans and in animals by reducing their morbidity and mortality and has also contributed substantially to human's increased life expectancy. They are also an essential tool for modern medicine and common procedures such as surgeries or to to prevent infections in chemotherapy for cancer, which could not be performed without the availability of potent antibiotics.

Since then, ever-increasing amounts of antibiotics have been extensively produced and used in different applications, some of them mentioned below:

- Therapeutic/prophylactic use in humans
- Therapeutic/prophylactic use in animals (*e.g.* livestock, poultry, pigs) including aquaculture
- Production purposes: to enhance growth food-producing animals (growth promoters) and to improve feed efficiency in animals
- Therapeutic/prophylactic use in agriculture
- Therapeutic/prophylactic use in household pets
- Use as biocides in toiletries and in hand care and household cleaning products
- Culture sterility, cloning, and selection in research and industry

It should be noted that therapeutic use in humans accounts for less than half of all applications of antibiotics produced commercially. Actually, antibiotics are used in greater quantities in healthy food-producing animals than in the treatment of disease in human patients. (Davies and Davies, 2010). Although the use of antibiotics for promoting growth is not necessary and was abandoned in the EU in 2006, there are still many other countries using them for this purpose.

1.2.3. Antibiotics classification

Nowadays different classes of antibiotics are known and they are commonly classified based on their mechanisms of action, chemical structure, or spectrum of activity (Neu, 1992). Antibiotics can for instance inhibit protein synthesis, like aminoglycosides, chloramphenicol, macrolides, and tetracyclines or interact with the synthesis of DNA and RNA, such as quinolones and rifamycins. Other groups inhibit the synthesis of, or damage the bacterial cell wall as β-lactams and glycopeptides do, or modify the energy metabolism of the bacterial cell like sulfonamides and trimethoprim. In Table 2 are presented the main classes of antibiotics currently used and their mechanism of action.

Antibiotic class	Antibiotic	Species range	Primary target	Pathways affected
Fluoroquinolones				
DNA synthesis inhibitor	Nalidixic acid, ciprofloxacin, norfloxacin, levofloxacin and gemifloxacin	Aerobic Gram-positive and Gram-negative species, some anaerobic Gram-negative species (*C. perfringes*) and *M. tuberculosis*	Topoisomerase II (DNA gyrase), topoisomerase IV	DNA replication, SOS response, cell division, ATP generation, TCA cycle, Fe–S cluster synthesis, ROS formation, and envelope and redox-responsive two-component systems
Trimethoprim–sulfamethoxazole				
DNA synthesis inhibitor	Co-trimoxazole (a combination of trimethoprim and sulfamethoxazole in a 1:5 ratio)	Aerobic Gram-positive and Gram-negative species	Tetrahydrofolic acid synthesis inhibitors	Nucleotide biosynthesis and DNA replication
Rifamycins				
RNA synthesis inhibitor	Rifamycins, rifampin and rifapentine	Gram-positive and Gram-negative species, and *M. tuberculosis*	DNA-dependent RNA polymerase	RNA transcription, DNA replication and SOS response
β-lactams				
Cell wall synthesis inhibitors	Penicillins (penicillin, ampicillin, oxacillin), cephalosporins (cefazolin, cefoxitin ceftriaxone, cefepime) and carbapenems (imipenem)	Aerobic and anaerobic Gram-positive and Gram-negative species	Penicillin-binding proteins	Cell wall synthesis, cell division, autolysin activity (regulated by LytSR–VncRS two-component system), SOS response, TCA cycle, Fe–S cluster synthesis, ROS formation, and envelope and redox-responsive two-component systems
Glycopeptides and glycolipopeptides				
Cell wall synthesis inhibitor	Vancomycin; teicoplanin	Gram-positive species	Peptidoglycan units (terminal d-Ala-d-Ala dipeptide)	Cell wall synthesis, transglycosylation, transpeptidation and autolysin activation (VncRS two-component system)
Lipopeptides				
Cell wall synthesis inhibitors	Daptomycin and polymixin B	Gram-positive species (daptomycin), Gram-negative species (polymixins)	Cell membrane	Cell wall synthesis and envelope two-component systems

Antibiotic class (cont.)	Antibiotic	Species range	Primary target	Pathways affected
Aminoglycosides				
Protein synthesis inhibitors	Gentamicin, tobramycin, streptomycin and kanamycin	Aerobic Gram-positive and Gram-negative species, and *M. tuberculosis*	30S ribosome	Protein translation (mistranslation by tRNA mismatching), ETC, SOS response, TCA cycle, Fe–S cluster synthesis, ROS formation, and envelope and redox-responsive two-component systems
Tetracyclines				
Protein synthesis inhibitors	Tetracycline and doxycycline	Aerobic Gram-positive and Gram-negative species	30S ribosome	Protein translation (through inhibition of aminoacyl tRNA binding to ribosome)
Macrolides				
Protein synthesis inhibitors	Erythromycin and azythromycin	Aerobic and anaerobic Gram-positive and Gram-negative species	50S ribosome	Protein translation (through inhibition of elongation and translocation steps) and free tRNA depletion
Streptogramins				
Protein synthesis inhibitors	Pristinamycin, dalfopristin and quinupristin	Aerobic and anaerobic Gram-positive and Gram-negative species	50S ribosome	Protein translation (through inhibition of initiation, elongation and translocation steps) and free tRNA depletion
Phenicols				
Protein synthesis inhibitors	Chloramphenicol	Gram-positive and Gram-negative species including *B. fragilis*, *N. meningitidis*, *H. influenzae* and *S. pneumoniae*	50S ribosome	Protein translation (through inhibition of elongation step)

Table 2. Antibiotics classification. Antibiotic class, examples of antibiotics, species range, main target and pathways affected are listed for the most used antibiotics (modified from Kohanski *et al*., 2010).

1.3. Antibiotic resistance

Antibiotic resistance can be defined as the ability of some microorganism populations to survive and multiply in the presence of an antibiotic to which it was originally sensitive.

Upon the introduction of antibiotics, it was assumed that the evolution of antibiotic resistance was unlikely based on the fact that the frequency of mutations generating antibiotic resistance was negligible (Davies, 1994). Unfortunately, time has proven the opposite. Nobody anticipated that bacteria would react so fast by adapting themselves by developing resistance to antibiotics using a wide variety of mechanisms. Moreover, HGT was specially unexpected. Later on, it was discovered that the existence of natural resistances actually began before the first antibiotic was characterized (Abraham and Chain, 1988; van Hoek *et al*., 2011).

Genes conferring resistance to antibiotics are widespread in nature and these genes are frequently swapped among pathogens. The wide use of antibiotics provides a powerful selection pressure causing the fraction of bacteria containing resistance genes to increase overtime. As a consequence of this, resistance to a new antibiotic typically arises 2 to 5 years after its introduction. (Bushman, 2002).

Several years before the introduction of penicillin as a therapeutic a bacterial penicillinase was already identified. Once the antibiotic was used widely, resistant strains became prevalent. The identification of a bacterial penicillinase before the use of the antibiotic can now be appreciated in the light of recent findings that a large number of ARGs are components of natural microbial populations (D'Costa *et al*., 2006). Another example can be found in streptomycin, which was introduced in 1944 for the treatment of tuberculosis. Mutant resistant strains of *Mycobacterium tuberculosis* were found to arise during the patients' treatment. Similarly, other antibiotics that were discovered, large-scale produced and introduced into clinical practice showed a similar course of events in the appearance of mechanisms that allowed bacteria to become resistant (Davies and Davies, 2010).

Figure 3 shows the sequence of discovery and concomitant development of antibiotic resistant for the major classes of antibiotics.

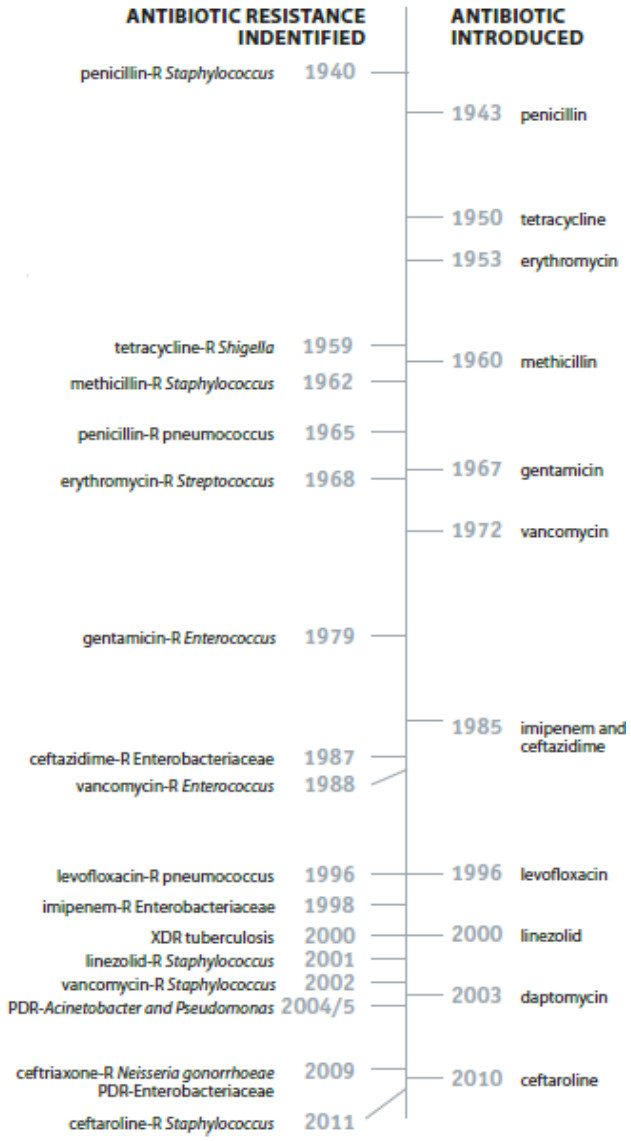

Figure 3. Timeline of antibiotic deployment and the evolution of antibiotic resistance. The year each antibiotic was introduced is depicted on the right side of the timeline, and the year resistance to each antibiotic was observed is depicted on the left side of the timeline (CDC, 2013).

Thus, antibiotic resistance has become a serious and growing phenomenon in contemporary medicine and has emerged as one of the pre-eminent public health concerns of the 21st century (WHO, 1996).

An estimated 25,000 people die every year in Europe from antibiotic-resistant bacteria and at least 23,000 as a direct result of antibiotic-resistant infections and many more die from other conditions that are complicated by an antibiotic-resistant infection. Regarding Spain, up to 2,000 people die every year as a direct result of inefficient antibiotic response due to antibiotic resistance (ECDC, 2009).

The emergence and rapid dissemination of antibiotic-resistant pathogens are a growing menace to all people regardless of age, gender, or socioeconomic background and they endanger people in developed and in less-developed countries (Aminov, 2009). Examples of clinically important microbes that are rapidly developing resistance to available antimicrobials are, on the one hand, Gram-negative pathogens, which are particularly worrisome because they are becoming resistant to nearly all drugs that would be considered for treatment. The most serious Gram-negative infections are health-care associated, and the most common pathogens are *Enterobacteriaceae* (*E. coli* and *Klebsiella pneumoniae*), *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. On the other hand, there are resistant bacteria among Gram-positive bacteria, such as *S. aureus*, *Streptococcus* and *Enterococcus* causing important infections (Table 3).

Examples of clinically important multidrug-resistant bacteria	Type of infections
Methicillin-resistant *Staphylococcus aureus* (MRSA)	Skin, bone, lung and bloodstream infections
Vancomycin-resistant enterococci (VRE)	Skin, bone, lung and bloodstream infections
ESBL-producing *Enterobacteriaceae*: *Escherichia coli* *Klebsiella pneumoniae*	Pneumonia, urinary tract infections, bloodstream infections, foodborne infections, infections transmitted in health care settings
Carbapenemase-producing *Enterobacteriaceae* (*e.g. Klebsiella pneumoniae*)	Infections transmitted in health care settings
Multidrug-resistant *Pseudomonas aeruginosa*	Infections transmitted in health care settings
Clostridium difficile	Infections transmitted in health care settings
Drug-resistant *Salomonella* spp.	Foodborne infections
Multidrug-resistant *Acinetobacter* spp.	Infections transmitted in health care settings

Table 3. Clinically important MDR bacteria and the most important type of infections caused (WHO, 2012).

Prudent antibiotic use and comprehensive infection control strategies targeting all healthcare sectors (acute care hospitals, long-term care facilities and ambulatory care) are the cornerstones of effective interventions that aim to prevent selection and transmission of antibiotic-resistant bacteria. (ECDC, 2013b). However, several are the causes of the occurrence of antibiotic resistance.

1.3.1. Causes of antibiotic resistance

The causes of antibiotic resistance are complex but resistance can occur as a natural phenomenon or be enhanced due to anthropogenic activities.

- **Natural occurrence:** There is evidence that naturally occurring antibiotic resistance is common and is a consequence of evolution via natural selection. The ARGs in nature (non-clinical settings) may be transferred from non-disease-causing bacteria to those that do cause disease, leading to clinically significant antibiotic resistant bacteria. Recent studies have uncovered the presence of ARGs in the gut flora of people who live in isolated areas apparently untouched by modern civilization and not exposed to antibiotic therapies (Pallecchi *et al*., 2007, 2008; Bartoloni *et al.*, 2008), and in ecosystems with no human-produced antibiotic presence as varied as varied as soil (D'Costa *et al*., 2011), pristine waters (Lima-Bittencourt *et al*., 2007) or a microcave isolated for over 4 million years (Bhullar *et al*., 2012). These examples highlight the potential importance of environmental bacteria as a source of resistance genes.

- **Human activities:** The predominant role of anthropogenic activities in the generation of environmental reservoirs of antibiotic resistance cannot be disputed. Since the industrialisation of antibiotic production, ever-increasing amounts of antibiotics designated for human applications have been manufactured, used clinically, released to the environment, and widely disseminated, thus providing constant selection and maintenance pressure for populations of resistant strains in all environments and changing the distribution and increasing the abundance of resistance genes.

In medicine: Although there is no data about the levels of antibiotic-resistant bacteria before the widespread use of antibiotics, selective pressure from their use has undoubtedly played a role in the development of multidrug resistance varieties and in the spread of resistance between bacterial species.

In medicine, the major problem of the emergence of resistant bacteria is due to **misuse and overuse of antibiotics**. Antibiotics are misused and over-used in all regions, in both developing and developed countries, being a particularly serious global problem. In Europe, some countries are using three times the amount of antibiotics per head of population compared to other countries with similar disease profiles (Levy and Marshall, 2004; WHO, 2011) (Figure 4). Several studies have demonstrated that patterns of antibiotic usage greatly affect the number of resistant bacteria that develop (Figure 5).

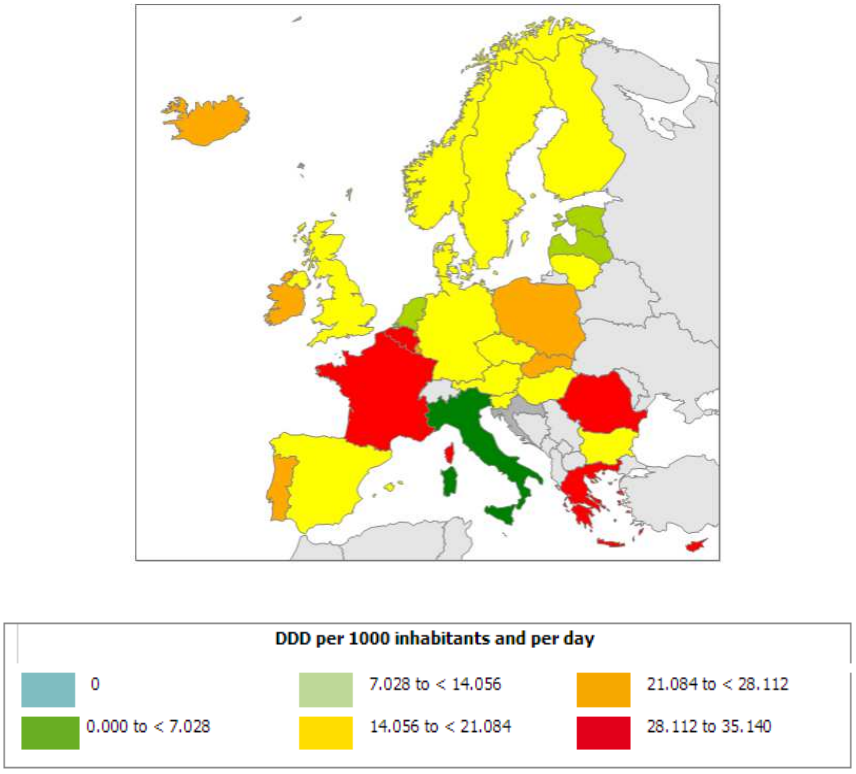

Figure 4. Antimicrobial consumption in ATC group J01 (antibacterials for systemic use) in the community (primary care sector) in Europe, reporting year 2011. *DDD: Defined daily dose,* ESAC-Net (ECDC, 2013a).

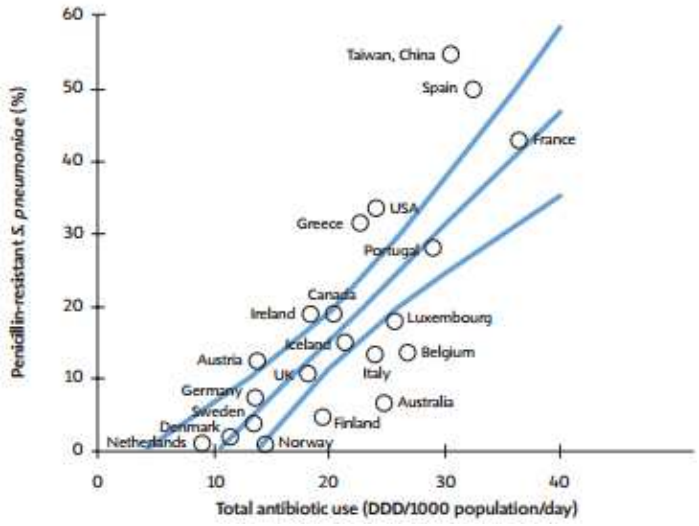

Figure 5. Correlation between antibiotic consumption and penicillin-resistant *S. pneumoniae* in different European countries. (Albrich *et al*., 2004).

Other factors contributing towards increased resistance include **incorrect diagnosis, inappropriate prescription of antibiotics** and **improper use of antibiotics by patients**. Doctors prescribe antibiotics to patients who do not need them, while patients do not adhere to their treatment causing the risk of antibiotic resistance. **Poor hand hygiene** by hospital staff has been associated with the spread of resistant microorganisms, and an increase in hygiene compliance results in decreased rates of resistant bacteria (Swoboda *et al*., 2004; Girou *et al*., 2006).

In industry: Some practices in the pharmaceutical manufacturing industry (wastewater spills from pharmaceutical industry) and household use of antibacterials in soaps and other products can contribute towards the likelihood of creating antibiotic-resistant strains.

In animals: The emergence of antibiotic resistant microorganisms in human medicine is primarily the result of the use of antibiotics in humans, although the use of antibiotics in animals is also partly responsible. Traditionally, there has been extensive use of antibiotics in animal husbandry.

Antibiotics are used in animals that are intended for human consumption, such as cattle, pigs, chickens, fish, etc. as mentioned in a previous section. The resistant bacteria in animals due to antibiotic exposure can be transmitted to humans via three pathways: through the consumption of animal products (milk, meat, eggs, etc.), from close or direct contact with animals or other humans, or through the environment.

The WHO concluded that inappropriate use of antibiotics in animal husbandry is an underlying factor that contributes to the emergence and spread of antibiotic resistant bacteria and the use of antibiotics as growth promoters in animal feeds should be prohibited, in the absence of risk assessments.

1.3.2. Consequences of antibiotic resistance

As mentioned before, antibiotic resistance is a serious threat to public health worldwide with important consequences. Although poorly quantified, it has been reported that individuals with antibiotic-resistant infections have longer duration of illness and poorer prognoses, require prolonged and/or costlier treatments, extend hospital stays, necessitate additional doctor visits and healthcare use and result in greater disability and higher rates of mortality compared with infections that are easily treatable with antibiotics (Laxminarayan *et al.,* 2013).

The costs of treating antibiotic resistant infections place a significant burden on society by adding considerable and avoidable costs to the health care system. This burden is likely to grow larger as the number of cases of drug-resistant illness increases. (WHO, 2011).

1.3.3. Mechanisms of antibiotic resistance

The **resistome** is the collection of all the antibiotic resistance genes and their precursors, including those circulating in pathogenic bacteria, antibiotic producers, and benign non-pathogenic microorganisms found either free living in the environment or as commensals of other organisms. It also includes resistance genes in antibiotic producers and

Bacteria have become resistant to antimicrobials through a number of mechanisms (Figure 6 and Table 4) (Finley *et al*., 2013):

- Permeability changes in the bacterial cell wall which restricts antimicrobial access to target sites
- Active efflux of the antibiotic from the microbial cell
- Enzymatic modification of the antibiotic
- Degradation of the antimicrobial agent
- Acquisition of alternative metabolic pathways to those inhibited by the drug
- Modification of antibiotic targets
- Overproduction of the target enzyme

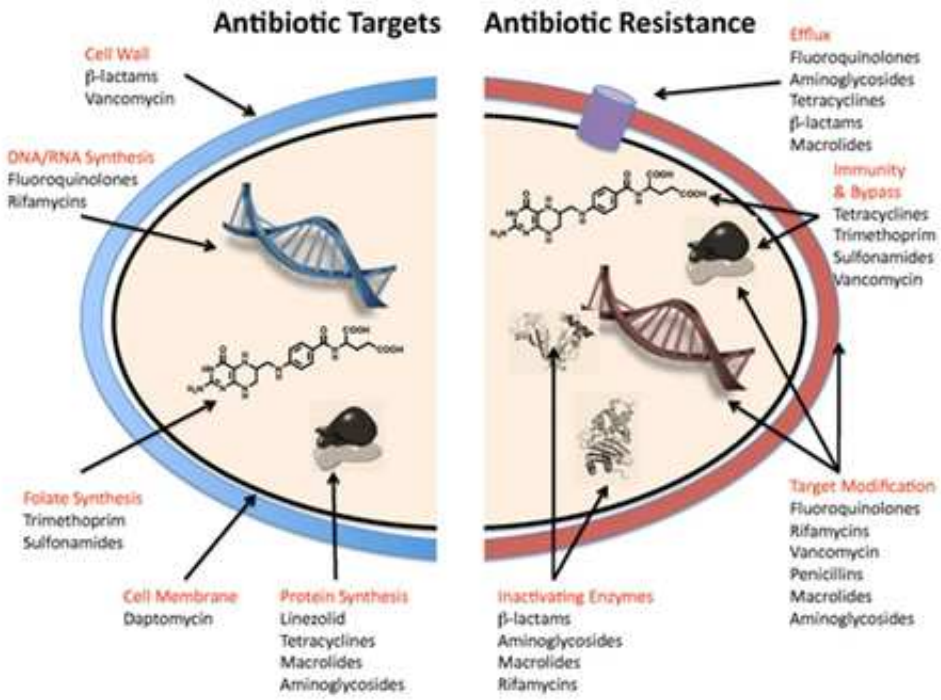

Figure 6. Antibiotic targets and mechanisms of resistance (Wright, 2010).

Antibiotic class	Example(s)	Target	Mode(s) of resistance
β-lactams	Penicillins (ampicillin), cephalosporins (cephamycin), penems (meropenem), monobactams (aztreonam)	Peptidoglycan biosynthesis	Hydrolisis, efflux, altered target
Aminoglycosides	Gentamicin, streptomycin, spectinomycin	Translation	Phosphorylation, acetylation, nucleotydation, efflux, altered target
Glycopeptides	Vancomycin, teicoplanin	Peptidoglycan biosynthesis	Reprogramming, peptidoglycan bioynthesis
Tetracyclines	Minocycline, tigecycline	Translation	Monooxigenation, efflux, altered target
Macrolides	Erythromycin, azithrommicin	Translation	Hydrolisis, glycosylation, phosphorylation, efflux, altered target
Lincosamides	Clindamycin	Translation	Nucleotidylation, efflux, altered target
Streptogramins	Synercid	Translation	C-O lyase (type B streptogramins), acetylation (type A streptogramins), efflux, altered target
Oxazolidinones	Linezolid	Translation	Efflux, altered target
Phenicols	Chloramphenicol	Translation	Acetylation, efflux, altered target
Quinolones	Ciprofloxacin	DNA replication	Acetylation, efflux, altered target
Pyrimidines	Trimethoprim	C_1 metabolism	Efflux, altered target
Sulfonamides	Sulfamethohxazole	C_1 metabolism	Efflux, altered target
Rifamycins	Rifampin	Transcription	ADP-ribosylation, efflux, altered target
Lipopeptides	Daptomycin	Cell membrane	Altered target
Cationic peptides	Colistin	Cell membrane	Altered target, efflux

Table 4. Modes of action and resistance mechanisms of commonly used antibiotics (Davies and Davies, 2010).

These resistance mechanisms can be intrinsic or achieved by mutations or by acquisition of genetic material through HGT (Figure 7):

- **Intrinsic resistance:** Occurs naturally in all or most strains of species and are chromosomally encoded (*e.g.* Gram-negative bacteria are resistant to vancomycin). The intrinsic resistome is a naturally occurring phenomenon that predated antibiotic chemotherapy and is present within the genome of a bacterial species (pathogens and non-pathogens). It is independent of antibiotic selective pressure. (D'Costa *et al*., 2006; Dantas *et al*., 2008; Sommer *et al*., 2009; Cox and Wright, 2013).

- **Acquired resistance:** Results either by mutations of the existing DNA or by acquisition of foreign DNA through HGT.

  Mutations: Mutations would lead to a vertical transmission of the resistance gene (VGT). For example, mutations on chromosomal genes codifying for the antibiotic target, or in the genes that regulate different cellular functions that could lead to the activation or inhibition of genes implicated in the mechanism of resistance (*e.g.* hyper expression of inactivating enzymes, hyper expression of pump efflux bombs or inhibition of porines).

  HGT: The transfer of MGEs between cells is known as **horizontal gene transfer** (HGT) in contrast to **vertical gene transfer** (VGT), which is the transmission of genetic information from parent to progeny cell. (Malachowa and DeLeo, 2010). HGT can occur as prokaryote-to-prokaryote, prokaryote-to-eukaryote, and eukaryote to eukaryote transfer of DNA (Jain *et al*., 1999; Keeling and Palmer, 2008). HGT is a key contributor to evolutionary change playing a crucial role in the plasticity of the genome, allowing bacteria to adjust readily to new environments. Selective pressure from the environment drives enrichment for specific genes that promote fitness and survival in a given environment.

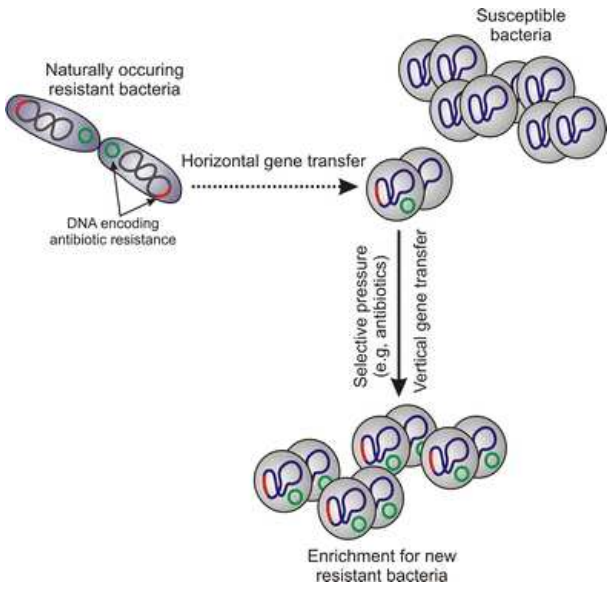

Figure 7. Horizontal and vertical gene transfer (Malachowa and DeLeo, 2010).

Three broad mechanisms mediate efficient movement of DNA between bacterial cells through HGT: transformation, conjugation and transduction (Figure 8):

- **Transformation**: Involves the simple acquisition by a cell of free DNA from the surrounding environment and the expression of that DNA in the recipient cell. For some bacteria, transformation is induced by specific environmental conditions, whereas others are always "competent" for transformation. Transformation is carried out by dedicated cellular machinery that binds the extracellular DNA and transfers it across the cellular membrane. (*e.g.* resistance acquisition in Gram-positive bacteria, *Streptococcus pneumoniae*, *Streptococcus* from the viridans group).

- **Conjugation**: Direct transfer of DNA from one cell to another, basically, between bacterial cells. Conjugation involves direct cell-to-cell contact and generally a special apparatus, *pilus*, for binding the donor and the recipient cells. A DNA strand from the donor cell is extruded into the recipient cell, so the recipient can acquire new characters encoded in the transferred DNA.

The genes for the conjugation apparatus are commonly found on the DNA that becomes transferred, usually as extrachromosomal DNA circles such as plasmids. Conjugation has been considered the most relevant mechanism of antibiotic resistance transfer between bacteria and their diffusion especially in clinical settings.

- **Transduction**: Transfer of a DNA sequence from one bacterial cell to another by a bacteriophage. Transduction was discussed in detail in section 1.1.3.

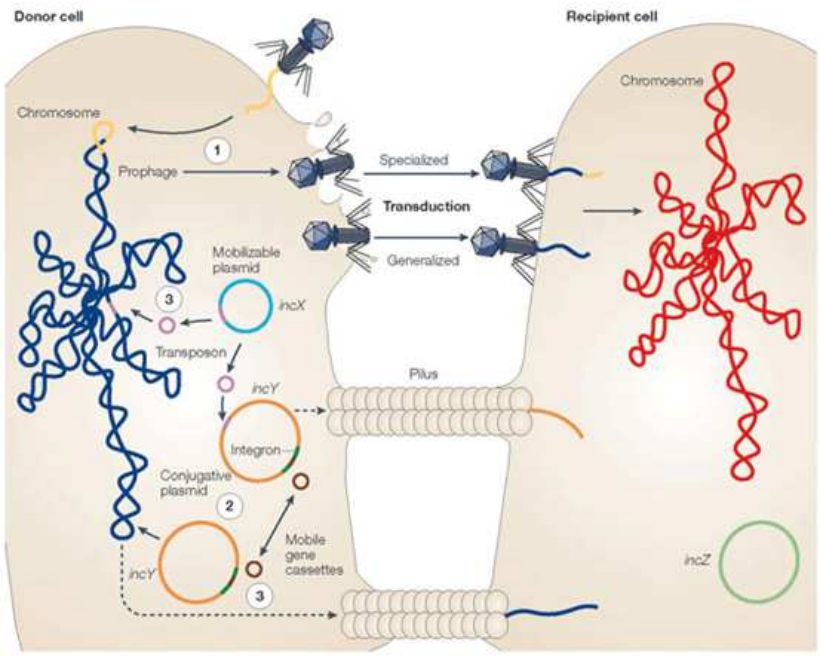

Figure 8. Acquisition of MGEs by HGT between bacterial cells. (1) *Transduction*; (2) *Conjugation*; (3) T*ransposition. Transformation* not shown (Frost *et al*., 2005).

Especially relevant is the transference of MGEs by transposition. In **transposition**, collinear DNA segments move from one location in the genome to another in the same cell. Transposons typically encode the enzymes that redirect braking and joining reactions involved in transposition, and the sites of action for these enzymes. Transposition is not an HGT mechanism *per se,* however, in a few cases, transposition reactions are directly associated with HGT linking cellular sequences to vehicles for HGT between cells, such as conjugative plasmids or viruses, resulting in their mobilization.

1.4. Antibiotic resistance genes

Enterobacteriaceae have become one of the most important causes of nosocomial and community acquired infections. β-lactams and fluoroquinolones constitute the main therapeutic choices to treat infections caused by these microorganisms but, unfortunately, resistances by different mechanisms have been reported worldwide over the years. Regarding Gram-positives, methicillin-resistant *S. aureus* (MRSA) is the most notorious multi-drug resistant hospital pathogen.

In this section the description of acquired resistance against several classes of antibiotics, β-lactams and fluoroquinolones, is detailed summarized along with the mechanisms of action. These resistances are reviewed in detail here because will be evaluated in the present thesis.

1.4.1. β-lactams and β-lactams resistance genes

History and mechanism of action of β-lactams:

As mentioned before, the first antibiotic discovered was a β-lactam (penicillin) and, since then, many β-lactam antibiotics have been developed. By definition, all β-lactam antibiotics have a β-lactam nucleus in their molecular structure, which confers them their main characteristics: their mechanism of action and their low toxocity. They work by inhibiting the cell wall synthesis by binding to penicillin-binding proteins (PBPs) in bacteria and interfering with the structural cross linking of peptidoglycans and as such preventing terminal transpeptidation in the bacterial cell wall. As a consequence, they weaken the cell wall of the bacterium and finally results in cytolysis or death due to osmotic pressure (Andes and Craig, 2005).

The β-lactam antibiotic family includes penicillins and derivatives, cephalosporins, carbapenems, monobactams, and β-lactamase inhibitors. β-lactamase inhibitors, like clavulanic acid, do contain the β-lactam ring, but they exhibit negligible antimicrobial activity and are used in combination with β-lactam antibiotics to overcome resistance in bacteria secreting β-lactamases, for which they compete, which otherwise inactivate most penicillins.

It is important to highlight that β-lactams account for approximately two-thirds, by weight, of all antibiotics administered to humans and are also widely used in veterinary medicine. (Lachmayr *et al*., 2009).

Resistance mechanisms and β-lactams ARGs:

Resistance to β-lactam antibiotics is an increasing problem and β-lactamase production (extended spectrum β-lactamases, plasmid-mediated AmpC enzymes and carbapenemases) is the most common and important mechanism of drug resistance in Gram-negative bacilli (Dallenne *et al*., 2010). Regarding Gram-positives, MRSA is the most notorious multi-drug resistant hospital pathogen, and became resistant to β-lactam antibiotics by acquisition of *mecA* gene in its chromosomal DNA.

- *β-lactamases:*

The first β-lactamase was identified in *E. coli* prior to the release of penicillin for use in medical practice (Abraham and Chain, 1988) and to date, over 1,150 chromosomal, plasmid and transposon β-lactamases have been described (Bush and Jacoby, 2010; Drawz and Bonomo, 2010).

β-lactamases are enzymes which hydrolyse the β-lactam ring of the antibiotic molecule, and thus inactivate the antibiotic (Jacoby, 2005). Among them, extended spectrum β-lactamases (ESBLs) are of great concern.

β-lactamases are mainly classified using the Ambler scheme based on protein sequence, which grades to molecular classes (A through D), and using the Bush-Jacoby and Madeiros scheme based on functional groups, which use the characteristic of the enzymes, including their hydrolytic substrate profile and response to inhibitors. ESBLs are included in the 2be Bush-Jacoby and Madeiros functional group belonging to the molecular class A β-lactamases from the Ambler's classification (Ambler, 1980; Madeiros, 1984; Bush and Jacoby, 2010).

In general, **ESBLs** described in pathogens and human commensals are plasmid-mediated bacterial enzymes and are able to hydrolyse a wide variety of β-lactam antibiotics including

penicillins, fisrt-, second-, third- and fourth-generation cephalosporins (*e.g.* cefotaxime and ceftazidime) and monobactams (*e.g.* aztreonam), thus conferring resistance to a broad range of β-lactams. In addition, ESBLs producers generally remain susceptible to cephamycins and carbapenems, but are inhibited by other β-lactamase inhibitors (*e.g.* clavulanic acid, sulbactam, tazobactam).

Since their first description in 1983, (Knothe *et al*., 1983) ESBLs have been increasingly reported worldwide most frequently in *Enterobacteriaceae,* mainly in *E. coli* and *Klebsiella* species*,* and in a minor extent in *Salmonella enterica and Serratia marcescens,* but they have also been found in other bacterial species such as *P. aeruginosa* and *A. baumannii*.

Because ESBL-producing strains are resistant to a wide variety of commonly used antimicrobials, their proliferation poses a serious global health concern that has complicated treatment strategies for patients. According to a recent review (Davies and Davies, 2010), there has been a dramatic increase in the number of β-lactamases since the 1980s due almost exclusively to class A and D β-lactamases. ESBLs belonging to class A mainly include TEM, SHV, CTX-M, VEB, and GES enzymes. For example, in Spain the prevalence of ESBL-producing *E. coli* increased 8-fold from 2000 to 2006 (Díaz *et al*., 2010).

a) bla_{TEM}:

Of the β-lactamases, the TEM family represents one of the most clinically significant, widely distributed and with a high diversity and prevalence. The first in this group to be discovered, TEM-1, was described in the early 1960s in an *E. coli* strain isolated in Greece from a patient named Temoneira (TEM as acronym for Temoneira) and is considered broad spectrum and hydrolyses the early cephalosporins, in addition to many penicillins (Datta and Kontomichalou, 1965). TEM-1 has become one of the most commonly encountered β-lactamases and is ubiquitous among *Enterobacteriaceae*.

Most ESBLs have evolved by amino acid substitutions (point mutations) from native β-lactamases TEM-1, TEM-2, and SHV-1 found in *Enterobacteriaceae* (Bradford, 2001), especially *E. coli* and *Klebsiella* species, leading to a broad substrate specificity of the

enzymes to include third-generation cephalosporins (*e.g.* cefotaxime and ceftazidime) and monobactams (*e.g.* aztreonam). TEM-3 was then characterized, discovered in *K. pneumoniae* isolates recovered from intensive care unit patients in France, which has an increased substrate spectrum, including third generation cephalosporins, but is susceptible to β-lactamase inhibitors such as clavulanic acid (Sirot *et al*., 1987). The majority of TEM types discovered subsequently are ESBLs resulting in over 165 variants described to date (www.lahey.org/studies).

b) $bla_{CTX\text{-}M}$:

Recently, a shift in the distribution of different ESBLs has occurred, with a dramatic increase of CTX-M enzymes over TEM and SHV variants and nowadays are the most prevalent ESBLs in *Enterobacteriaceae* in Europe and other areas in the world (Coque *et al*., 2008a). Over the 1980s and the 1990s, the TEM- and SHV-ESBLs were predominant in the ESBL landscape, mainly associated with nosocomial outbreaks involving *K. pneumoniae* and to a lesser extent in *E. coli* and other *Enterobacteriaceae,* whereas CTX-M was less prevalent. On the first decade of the 2000s, there was an accelerated evolution and extraordinary dispersion of CTX-M enzymes and nowadays they are confined not only to the hospital setting but also to the community with *E. coli* being the most important pathogen producing these enzymes (Cantón and Coque, 2006; Coque *et al*., 2008a).

CTX-M enzymes were first discovered in 1989 nearly simultaneously in Europe and South America. A cefotaxime-resistant but ceftazidime susceptible *E. coli* isolate was recovered from the ear of a 4-month-old child suffering from otitis media in Munich and the enzyme responsible for that phenotype was named as CTX-M-1 (CTX as acronym for cefotaxime and -M from Munich). In South America, a *Salmonella typhimurium* isolate showing resistance to cefotaxime was recovered in patients suffering from meningitis, septicaemia or enteritis and the enzyme, with a different isoelectric point than the one described in Germany, was named CTX-M-2 (Bauernfeind *et al.*, 1990, 1992). Although it was not since 2004 when it was related to CTX-M-3, in 1986 a cefotaxime-resistant *E. coli* isolate was recovered from the faecal flora of a laboratory dog in Japan.

CTX-M enzymes, initially confined in *E. coli*, *K. pneumoniae* and *Salmonella* spp., have rapidly emerged in other *Enterobacteriaceae* (*Enterobacter* spp., *S. marcescens*, *Citrobacter* spp., *P. aeruginosa*) and have also been described in non-*Enterobacteriaceae* species.

Different variants of CTX-M ESBLs are grouped in 5 clusters (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25), all displaying potent hydrolytic activity against cefotaxime, although new variants may also effectively hydrolyse ceftazidime, with their distribution varying greatly depending on the geographic area. The CTX-M family, with upwards more than 120 variants being identified so far (www.lahey.org/studies), are highly successful at transmission and are currently recognized as the most widespread and threatening mechanism of antibiotic resistance, both in clinical and community settings (Valverde *et al*., 2004; Cantón and Coque, 2006; Pitout and Laupland, 2008).

Particularly, CTX-M-15 and CTX-M-14 enzymes are the most important being reported in human, animal and environment compartments all over the world (Cantón *et al*., 2008; Hawkey and Jones, 2009; Dolejska *et al*., 2011; Hiroi *et al*., 2012) (Figure 9). In Spain, there has been reported high local prevalence of CTX-M-9, CTX-M-10, CTX-M-14 and CTX-M-15 enzymes among inpatients, outpatients and healthy individuals (Figure 10).

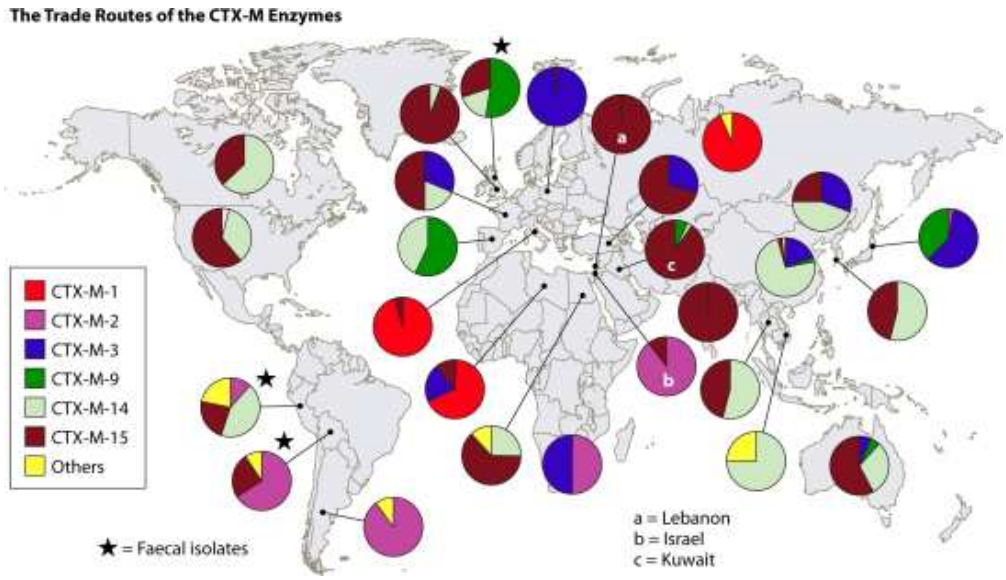

Figure 9. Worldwide distribution of different classes of CTX-M β-lactamases (Davies and Davies, 2010).

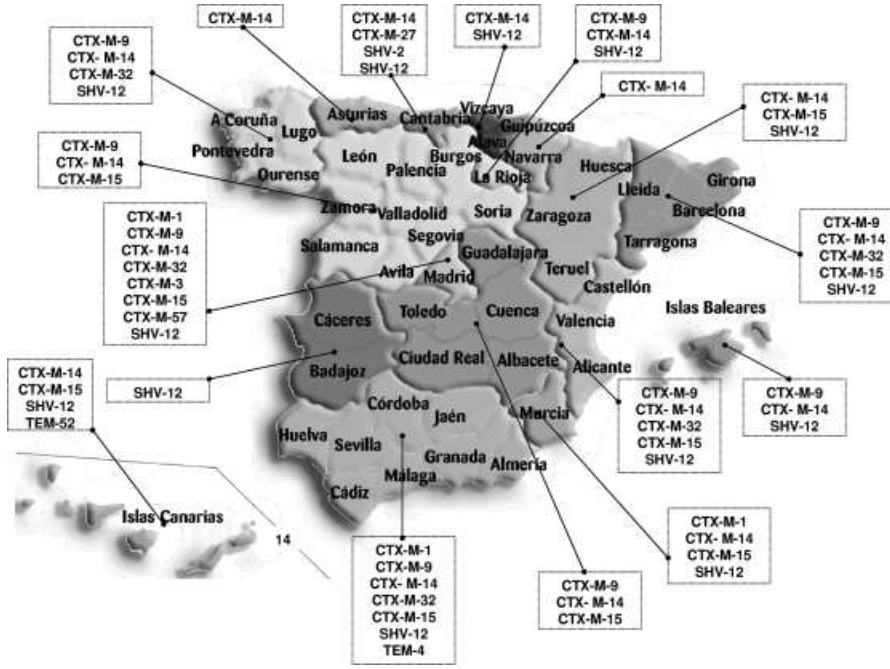

Figure 10. Distribution of ESBL *E. coli* isolates in Spain (Díaz *et al*., 2010).

E. coli is the most frequently involved species and recently, the international spread of the *E. coli* O25b:H4-ST131 clone producing CTX-M-15 and other β-lactamases have been described which is thought to be responsible for the pandemic dissemination of the CTX-M-15 enzyme (Coque *et al*, 2008b; Nicolas-Chanoine *et al*., 2008). Despite that CTX-M-15 is the most widely ESBLs linked to this clonal group (ST131), other different variants of CTX-M have been reported lately such as CTX-M-9, CTX-M-14 and CTX-M-32. Noteworthy was the detection for the first time in poultry farms of this clonal group producing CTX-M-9 with very similar macrorestriction profile and virulence genes to those observed in clinical human isolates (Mora *et al*., 2010).

Interestingly, phylogenetic analyses suggest that CTX-Ms were not originated by mutations form previous plasmid mediated enzymes, but through mobilization of chromosomal *bla* genes from different *Kluyvera* species, which have subsequently spread among pathogenic and non-pathogenic relevant bacteria. This successful distribution seems to be related to the interplay of different selective forces. Firstly, those derived from the association of $bla_{\text{CTX-M}}$ genes with particular genetic platforms (IS, integrons, transposons, plasmids and to a lesser

extent by bacteriophages) and/or specific bacterial clonal complexes. (Coque *et al*., 2008b; Nicolas-Chanoine *et al*., 2008). Secondly, the strong selective pressures exerted by the widespread and concomitant use of different classes of antibiotics on clinical bacteria, driving the emergence and dispersal of novel co-resistant CTX-M producing strains, particularly to aminoglycosides and fluoroquinolones, and more intensely active variants (Cavaco *et al*., 2008; Pitout and Laupland, 2008; Cantón and Ruiz-Garbajosa, 2011; Tacao *et al*., 2012).

c) Other β-lactamases

Other non-TEM, non-SHV enzymes have also been described in some European countries (PSE, PER, GES, IBC, OXA types) and "new β-lactamases" conferring resistance to carbapenems, such as metallo- β-lactamases and KPC carbapenemases, or to cephamycins, such as CMY enzymes, have more recently emerged and are often associated with ESBLs.

In addition to the production of β-lactamases, resistance can also be due to possession of altered PBPs making the antibiotic less effective at disrupting cell wall synthesis. One of these proteins is the product of gene *mecA* in the *Staphylococcus* genera.

- *mecA*:

Over the years, it has been shown by numerous ecological studies that (increased) antibiotic consumption contributes to the emergence of antibiotic resistance in various bacterial genera (MARAN, 2007; NethMap, 2008). A clear example of the link between antibiotic dosage and resistance development is the rise of MRSA which appeared for the first time in 1960 (Jevons *et al*., 1963).

The genus *Staphylococcus* consists of Gram-positive bacteria that colonize human or animal skin and mucosal membranes being part of the normal flora and thus commensal microorganisms. However, they are also opportunistic pathogens and, among staphylococci, *S. aureus* is the most invasive species and an etiological agent of diverse human and animal diseases, including skin infections, abscesses, food poisoning, toxic shock syndrome,

septicaemia, endocarditis and pneumonia (Weems, 2001; van Belkum, 2006; DeLeo and Chambers, 2009).

Penicillin was the first antibiotic used for the treatment of *S. aureus* infections. Although it was initially highly effective, today over 90% of human *S. aureus* strains are resistant to this antibiotic (Olsen *et al*., 2006). In 1960, methicillin, a penicillinase-insensitive penicillin, was developed for the treatment of multi-drug resistant *S. aureus.* However, approximately a year after its introduction, the first methicillin-resistant *S. aureus* (MRSA) was isolated (Jevons *et al*., 1963), which by 1970s became spread all over the wold. Since then, MRSA has become one of the most prominent causes of both health care settings and community acquired bacterial infections worldwide (DeLeo and Chambers, 2009; Motoshima *et al*., 2010).

S. aureus is known to adapt rapidly to selective pressures imparted by the human host, promptly responding to antibiotics, making it resistant and increasingly difficult to treat. Actually, MRSA is considered a multidrug-resistant pathogen because is resistant to practically all the β-lactam antibiotics that have been developed to date and to most of macrolides, aminoglycosides, chloramphenicol, fluoroquinolones, and tetracyclines (Lee, 2006). Alternatively, MRSA infections are treated with vancomycin, but unfortunately more recently, *S. aureus* acquired vancomycin resistance elements from enterococci, resulting in the emergence of vancomycin-resistant *S. aureus* (VRSA) (Chang *et al*., 2003; Weigel *et al*., 2003; Zhu *et al*., 2008). Linezolid, quinupristin/dalfopristin, daptomycin, ceftaroline and tigecycline are used to treat more severe infections that do not respond to glycopeptides such as vancomycin.

MRSA infections are estimated to affect more than 150,000 patients anually in the European Union and are associated with significant morbidity, mortality, and hospitalization costs (Köck *et al*., 2010). MRSA cause infections in health care facilities, being endemic in many hospitals worldwide (UK, Japan, USA) and in the last decade MRSA has also emerged in community settings causing skin and soft tissue infections, blood-borne infections, and pneumonia (Deresinski, 2005; Gemmell *et al*., 2006; Otter and French, 2010). Besides, MRSA

have recently been identified in food of animal origin, and some outbreaks were linked to the consumption of contaminated food products or colonized food handlers (Jones *et al*., 2002; Lee, 2006). More recently, MRSA have been found to colonise or infect livestock and humans exposed to those animals in several countries (van Cleef *et al*., 2011; Köck *et al*., 2010).

New MRSA clones emerge because of the exogenously acquisition of methicillin resistance by previously methicillin-sensitive *S. aureus* (MSSA) strains. Clinically significant methicillin resistance is conferred by expression of the *mecA* gene, which encodes a modified penicillin-binding protein (PBP2a or PBP2') and has low affinity for β-lactam antibiotics. PBP2a catalyses the formation of cross-bridges in bacterial cell peptidoglycan and facilitates cell-wall synthesis in the presence of methicillin/oxacillin and other β-lactams leading to the resistance phenotype (Hartman and Tomasz, 1984; Rizek *et al*., 2011).

The *mecA* gene complex resides on a mobile genetic element, the **staphylococcal cassette chromosome** (SCC*mec*). Although SSCmec elements are highly divers in their estructural organization and genètic content, basically SCC*mec* is comprised of the *mecA* gene complex, containing the *mecA* gene, together with its regulator genes (*mecI* and *mecR)*, the insertion sequence IS431*mec*, site-specific cassette chromosome recombinases (*ccr*) necessary for site-specific integration and excision of the element, terminal and direct inverted repeats, and the specific chromosomal insertion site within a conserved open reading frame (*orf*X). (Hiramatsu *et al*., 2002; Deurenberg *et al*., 2007). By the time this thesis was prepared, eleven major SSC*mec* types have been classified to date according to size and composition (I to XI) (Ma *et al*., 2002; Zhang *et al.* 2009; http://www.sscmec.org).

This element is also found in several other staphylococcal species, coagulase-negative staphylococci (CoNS), and it is believed that SCC*mec* probably originates in coagulase-negative staphylococci and integrates site-specifically into de *S. aureus* genome. Thus, MR-CoNS may constitute a reservoir for SSC*mec* acquisition by *S. aureus*. Due to the species-independent conservation of the gene complex, it is believed that the transfer of the SCCmec occurs frequently (Okuma *et al*., 2002).

As a consequence of the limited ability of *S. aureus* to acquire DNA from the environment (low natural competence) compared to bacteria such as *Streptococcus* spp*., Neisseria* spp*.* or *Bacillus subtilis*, most of the intercellular transfer of staphylococcal plasmids occurs by transduction or conjugation. Particularly, temperate bacteriophages can integrate into the staphylococcal genome as a prophage (Mann, 2008; Goerke *et al*., 2009).

1.4.2. Quinolones and quinolone resistance genes

History and mechanism of action of quinolones:

Quinolones are fully synthetic and bactericidal antibacterial agents used widely both in human and veterinary medicine. The targets of quinolone molecules are the type II topoisomerases DNA gyrase (topoisomerase II) and DNA topoisomerase IV (Drlica and Zhao, 1997), which are essential for bacterial growth by controlling the DNA supercoiling to facilitate replication, transcription, recombination and DNA repair (Hawkey, 2003). Quinolones act by inhibiting the action of DNA gyrase and topoisomerase IV and as a result DNA synthesis is blocked and the bacteria are killed.

The clinically available quinolones have been classified into several generations based on their spectrum of activity (Ball, 2000). The first generation quinolone (Q1G), nalidixic acid, was discovered in 1962 (Lesher *et al*., 1962) and others such as oxolinic acid and pipemidic acid were latter developed being used in veterinary. During the 1980s, the second generation quinolones (Q2G) were developed by addition of a fluorine atom at position C-6 to the quinolone nucleus yielding to the fluoroquinolones (*e.g.* norfloxacin, ciprofloxacin or ofloxacin) with potent activity against Gram-negative, some Gram-positive bacteria (such as *S. aureus*) and intracellular bacteria positives (Hooper, 2001; King *et al*., 2000). In the 1990s, third generation of (fluoro) quinolones (Q3G) were developed showing increased activity against Gram-positive bacteria (*S. pneumoniae*) (*e.g.* sparfloxacin, levofloxacin) and anaerobic bacteria (Hooper, 2001; King *et al*., 2000).

Even if the main factors leading to resistance to quinolones and fluoroquinolones are related to chromosomal mutations in the drug targeted genes, the discovery during the last decades

of a series of plasmid-encoded resistance mechanisms has contributed to speculate about the origin and enhancing factors of that transferable resistance. Interplay between an environmental and animal source on one side, and the human clinical pathogens on the other side seem to have an important role in the emergence of the quinolone resistance.

Resistance mechanisms and quinolone ARGs:

Resistance to quinolones and fluoroquinolones is being increasingly reported among human but also veterinary isolates during the last two or three decades (Figure 11), very likely as a consequence of the large clinical use of these antibiotics.

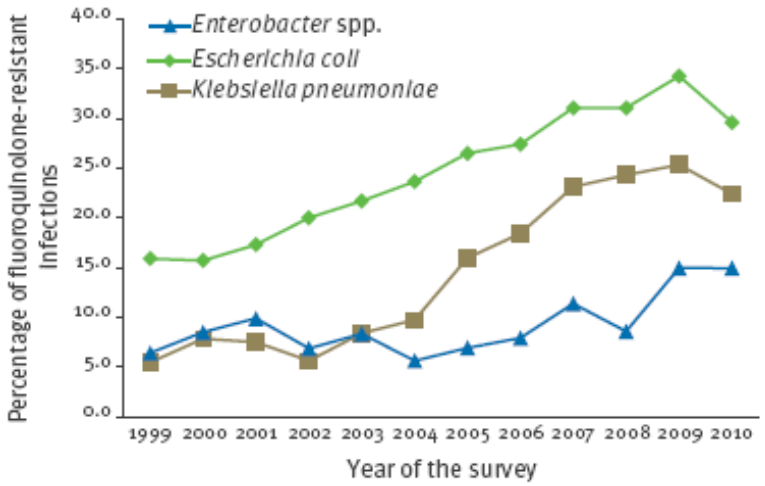

Figure 11. Annual rates of *Enterobacteriaceae* resistant to fluoroquinolones in Spain, 1999-2010 (Asensio *et al*., 2011).

For decades, the mechanisms of resistance to quinolones were believed to be only chromosome-encoded, due to the modifications of molecular targets (DNA gyrase and topoisomerase IV), decreased outer-membrane permeability (related to porine loss), and overexpression of naturally occurring efflux pumps (Hooper, 2001; Ruiz, 2003; Jacoby, 2005). However, the emergence of plasmid-mediated quinolone resistance (PMQR) has been reported since 1998 (Robicsek *et al.*, 2006; Courvalin, 2008; Martínez-Martinez *et al*., 2008).

Although these PMQR determinants confer low level resistance to quinolones and/or fluoroquinolones, they are a favourable background for selection of additional chromosome-encoded quinolone resistant mechanisms. They supply a degree of reduced quinolone susceptibility, enough for microorganisms to survive in the presence of quinolones, while resistance mutations occur sequentially rather than simultaneously.

The first PMQR determinant was reported in 1998 corresponding to the Qnr protein (QnrA1). Acquired Qnr proteins belong to a pentapeptide repeat family and to date, six families of Qnr proteins have been described: QnrA, QnrB, QnrC, QnrD, QnrS, QnrVC. *Qnr* genes are highly diverse with 7 *qnrA*, 73 *qnr*B, 1 *qnr*C, 2 *qnr*D, 9 *qnrS* and 5 *qnrVC* genes identified (http://www.lahey.org/qnrStudies). *Qnr* genes have been reported worldwide from unrelated enterobacterial species and are usually associated with mobile elements (Cambau *et al*., 2006).

1.4.3. Antibiotic resistance determinants in viral communities

There are only a few examples of antibiotic resistance genes described as elements of phage genomes. However, there have been examples of ARGs, and even entire mobile genetic elements, being mobilized by phage transduction, as reported in several bacterial genera (Blahová *et al*., 1993; Willi *et al*., 1997; Schmieger and Schicklmaier, 1999; Del Grosso *et al*., 2011; van Hoek, 2011).

In vitro, phages transduce resistance to imipenem aztreonam and ceftazidime in *P. aeruginosa* by generalized transduction (D'Costa *et al*., 2006). The epidemic strain *Samonella enterica* serovar Typhimurium DT104, characterized by various multirestistance patterns, has been reported phage-mediated transfer of ampicillin, chloramphenicol and tetracycline resistances (Blahová *et al*, 1993; Schmieger and Schicklmaier, 1999). In addition, 95% of the strains contained complete inducible prophage genomes, and 99% of these phages were capable of generalized transduction of chromosomal host markers and plasmids (Schicklmaier *et al*., 1998; Cloeckaert *et al*., 2001). *Bacillus anthracis* temperate phage Wβ encodes demonstrable fosfomycin resistance (Schuch and Fischetti, 2006).

Early descriptions of *Streptococcus pyogenes* resistant to antibiotics revealed that when treated with mitomycin C to induce phages lead to transduction of tetracycline resistance or multiresistance acquisition to chloramphenicol, macrolides, lincomycin and clindamycin (Ubukata *et al*., 1975). Evidence has also been presented that prophages participate in the dissemination of erythromycin-resistance phenotype in *S*. *pyogenes* clinical isolates (Hyder *et al*., 1978; McShan, 2000).

Varga *et al*. recently reported a high frequency of transduction of penicillinase and tetracycline resistance plasmids within MRSA clone US300, one of the *S. aureus* clones with the greatest spread worldwide (Varga *et al*., 2012).

Other indirect evidence for beta-lactam antibiotics mobilized by phages has been reported. CTX-M-10 was linked to a phage-related element, which disseminates among *Enterobacteriaceae* in a hospital (Oliver *et al*., 2005).

Metagenomic studies of viral communities indicate that sequences corresponding to ARGs were detected in the viral communities of the human gut (Minot *et al*., 2011) and in an activated sludge WWTP (Parsley *et al*., 2010a). As indicated before, in most environments studied, phages are the main part of the viral fraction (Dinsdale *et al*., 2008); therefore it can be assumed that the viral DNA evaluated in these studies will belong mostly to bacteriophage. Moreover, taking into account that in the viral fraction phage particles performing generalized transduction are present many host genes can be found in the viral fraction. Other studies revealed many short sequences putatively encoding ARGs in cystic fibrosis sputum and only three in the non-cystic fibrosis sputum (Willner *et al*., 2009). Several years before that, it was reported the abundance of viral particles carrying sequences of $bla_{\text{OXA-2}}$, $bla_{\text{PSE-1}}$, $bla_{\text{PSE-4}}$ and $bla_{\text{PSE-type}}$ genes in the viral fraction of raw municipal wastewater (Muniesa *et al*., 2004) (Table 5).

Resistance gene or protein	Antibiotic	Source (natural reservoir of bacteria)	Reference
bcrA	Bacitracin	Swine fecal microbiomes, human gut viromes, cystic fibrosis sputum microbiota	Minot *et al*. 2011; Fancello *et al*., 2012; Looft *et al*., 2012
bla_{OXA-2}, bla_{PSE-1}, bla_{PSE-4}, $bla_{PSE\text{-}type}$	β-lactam antibiotics	Sewage	Muniesa *et al*., 2004
$bla_{CTX-M-10}$	β-lactam antibiotics	*Enterobacteriaceae*	Oliver *et al*., 2005
bla_{CMY-2}	Ampicillin	*Salmonella enterica*	Schmieger *et al*., 1999
dfrAa	Trimethoprim	Swine fecal microbiomes	Looft *et al*., 2012
Fluoroquinolone resistance genes	Fluoroquinolones	Cystic fibrosis sputum microbiota	Fancello *et al*., 2012
macB	Macrolides	Swine fecal microbiomes	Looft *et al*., 2012
mefA	Macrolides	*Streptococcus pyogenes*	Looft *et al*., 2012
tetA, *tetB*	Tetracycline	*S. enterica*	Schmieger *et al*., 1999
tetW	Tetracycline	Swine fecal microbiomes, human gut viromes	Minot *et al*., 2011; Looft *et al*., 2012
tet37	Tetracycline	Swine fecal microbiome	Looft *et al*., 2012
Genes not annotated	b-lactam antibiotics	Human gut viromes, cystic fibrosis sputum microbiota	Willner *et al*., 2009; Minot *et al*. 2011; Fancello *et al*., 2012
Vancomycin resistance genes	Vancomycin	Swine fecal microbiomes, human gut viromes	Minot *et al*., 2011; Looft *et al*., 2012
Genes not annotated	Fosfomycin resistance	Prophage Wb *Bacillus anthracis*	Schuch *et al*., 2006
Genes not annotated	Tetracycline, gentamicin	*Enterococcus*	Mazaheri *et al*., 2011
Not determined	Erythromycin	*S. pyogenes*	Hyder *et al*., 1978
Not determined	Tetracycline, chloramphenicol, macrolides, lincomycin, clindamycin	*S. pyogenes*	Ubukata *et al*., 1975
Not determined	Imipenem, cefotaxime, ceftazidime, aztreonam, kanamycin, streptomycin	*Pseudomonas aeruginosa*	Blahová *et al*., 1993

Predicted proteins (cont.)	Antibiotic	Source (natural reservoir of bacteria)	Reference
Acriflavin resistance protein	Acriflavin	Viral metagenomes from an activated sludge microbial assemblage	Parsley *et al*., 2010a
Class A b-lactamase	β-lactam antibiotics	Viral metagenomes from an activated sludge microbial assemblage	Parsley *et al*., 2010a
Drug resistance transporter Bcr/CflA	ND	Viral metagenomes from an activated sludge microbial assemblage	Parsley *et al*., 2010a
Glyoxalase/bleomycin resistance	ND	Viral metagenomes from an activated sludge microbial assemblage	Parsley *et al*., 2010a
TetC	Tetracycline	Viral metagenomes from an activated sludge microbial assemblage	Parsley *et al*., 2010a
Tetracycline-resistant transposon Tn916	Tetracycline	Phage Aa phi ST1 in *Actinobacillus actinomycetemcomitans*	Willi *et al*., 1997
Chloramphenicol resistance of plasmid pKT210	Chloramphenicol	Phages Aa phi ST1 and Aa phi 23 in *A. actinomycetemcomitans*	Willi *et al*., 1997
Streptogramin acetyl transferase	Streptogramin	Human gut viromes	Minot *et al*., 2011

Table 5. ARGs and predicted proteins in the genome of bacteriophages, phage-related elements or in the viral DNA fraction of diverse biomes (Muniesa *et al*., 2013).

1.4.4. Antibiotic resistance dissemination and the role of the environment

Numerous types of anthropogenic activity, including antibiotic use in hospitals and veterinary settings, human community, farms, agriculture, aquaculture, other non-human applications of antibiotics, and waste disposal, among others, are reactors where the usage of antibiotics selects for resistant bacteria and promotes gene exchange through HGT. In addition, the environment has an important role in facilitating the development and dissemination of ARGs with water bodies such as rivers, streams, wastewater effluents, and lakes being of great relevance (Figure 12).

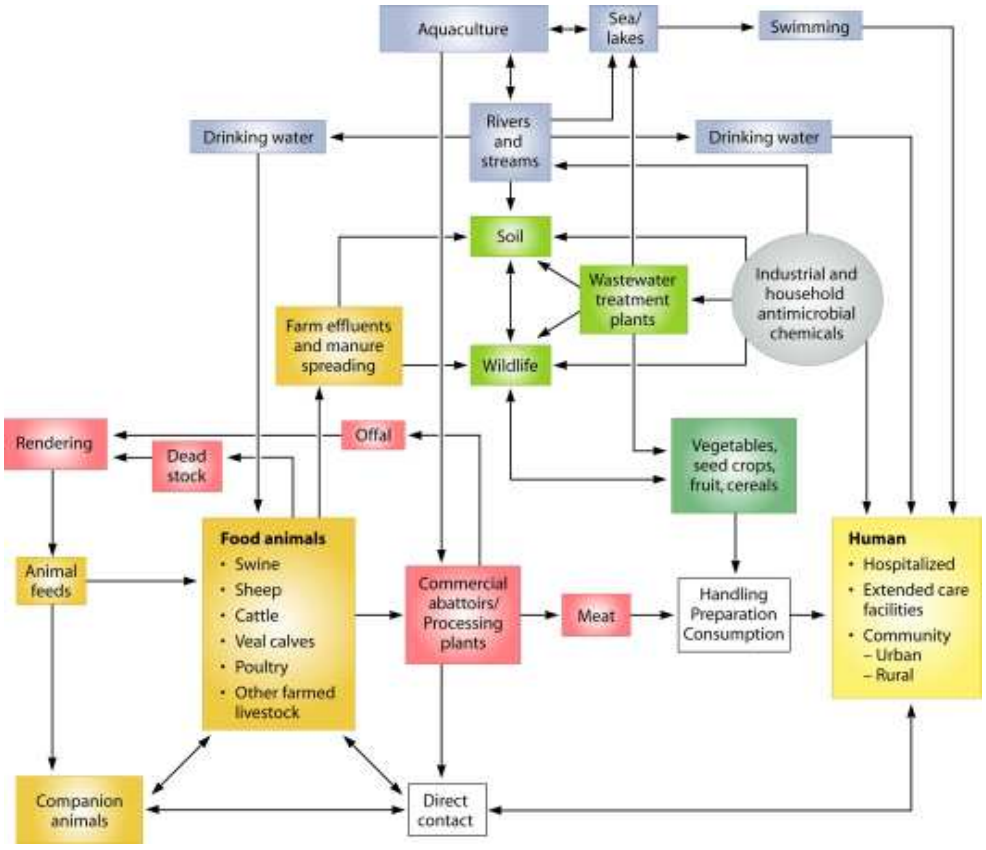

Figure 12. Dissemination of antibiotics and antibiotic resistance within agriculture, community, hospital, wastewater treatment, and associated environments (Davies and Davies, 2010).

Considering that bacteria do not respect the boundaries of ecological compartments, there is always a continuous flow of genetic information by HGT between different ecological compartments. HGT is highly important in the evolution and transmission of resistance genes between species in the environment and includes the movement of resistance genes from pathogenic bacteria to environmental bacteria, as well as the reverse; that is, emergence of novel mechanisms of acquired resistance in pathogens, genes that originally were present in non-pathogen bacteria. Once the potential antibiotic resistance genes enter, even in small numbers or low frequencies, into the commensal/pathogenic human/animal microbiota, the antibiotic selection immediately leads to the amplification and dissemination of these genes.

Therefore, the main scope of this thesis is to study the abundance of ARGs with clinical relevance in the DNA packaged in bacteriophage particles from environmental samples. These samples have fecal contamination of diferent human and animal origin and belong to different geographical areas. We attempt to study the potential role of phages as vectors for the HGT in the mobilization and spread of these bacteriophage-mediated antibiotic resistances. In addition, this study intends to evaluate the influence of various compounds involved in the induction of the lytic cycle of temperate bacteriophages on the abundance of antibiotic resistance genes in DNA from the bacteriophage fraction in wastewater samples.

With these goals, we have defined the following objectives described in the next section and detailed in each chapter.

1. INTRODUCCIÓ GENERAL

1.1. Bacteriòfags

Els **elements genètics mòbils** (*MGEs*) són considerats típicament com fragments de DNA que codifiquen una varietat de determinants de virulència i de resistència així com també els enzims per a la seva transferència i integració en el nou hoste (Frost *et al*., 2005). Els *MGEs* poden ser seqüències d'inserció, transposons, elements cromosòmics intragènics, plasmidis, illes de patogenicitat, cassets cromosòmics i també alguns bacteriòfags.

Aquesta tesi s'ha centrat en els bacteriòfags i, concretament, en el seu paper potencial com a reservoris i vehicles en la disseminació de gens de resistència a antibiòtics.

Els **bacteriòfags** (o fags) són virus que infecten bacteris i van ser descoberts de manera independent el 1915 per Twort i el 1917 per d'Herelle (Adams, 1959; Duckworth, 1976). Els bacteriòfags són ubics a la natura i probablement les entitats més abundants de la Terra, amb una població estimada de 10^{30}-10^{32} (Brüssow i Hendrix, 2002; Ashelford *et al*., 2003; Jofre, 2003; Chibani-Chennoufi *et al*., 2004; Suttle, 1994).

Els bacteriòfags són extremadament diversos, amb diferents morfologies, estils de vida i composició genòmica, essent els fags amb cua de doble cadena de DNA el 95% de tots els fags descrits a la literatura (Mc Grath i van Sinderen, 2007). Els fags també varien en la seva estructura, de manera que podem trobar des de fags simples fins a fags amb estructures molt elaborades i complexes amb mides i formes diferents. Però essencialment cada partícula fàgica (virió) conté el seu genoma d'àcid nucleic (DNA o RNA) envoltat d'una càpsida proteica o lipoproteica; el conjunt d'àcid nucleic i càpsida formen la nucleocàpsida. Molts fags, a més, poden contenir estructures addicionals com cues o espícules (Kutter i Sulakvekidze, 2005).

Tot i que els fags porten la informació necessària per a la seva pròpia replicació en un hoste adequat, els manca la maquinària necessària per a generar energia i no tenen ribosomes per a la síntesi de proteïnes. Així, els bacteriòfags només es poden replicar en un bacteri hoste sensible mitjançant l'ús del sistema enzimàtic cel·lular per tal de duplicar les seves

estructures, principalment proteïnes i àcids nucleics (Adams, 1959; Waldor i Friedman, 2005).

L'espectre de bacteris que poden ser infectats per un determinat fag, l'anomenat *espectre d'hoste*, depèn de la presència de receptors identificables pel fag. L'hoste diana per cada fag és un grup específic de bacteris, sovint un subgrup d'una espècie, però diverses espècies relacionades poden, a vegades, ser infectades pel mateix fag (Kutter i Sulakvekidze, 2005). Molts fags tenen un espectre d'hoste reduït i infecten un nombre limitat de soques d'una espècie bacteriana. En canvi, d'altres coneguts com *bacteriòfags polivalents* es caracteritzen per tenir un ampli espectre d'hoste que creua els límits d'espècies bacterianes diferents en un gènere, com en el cas d' *Enterococcus* (Mazaheri *et al*., 2011); o de gèneres diferents en una família, per exemple en *Enterobacteriaceae* (Souza *et al*., 1972; Evans *et al*., 2010); o fins i tot taxes diferents, per exemple entre *Gammaproteobacteria* i *Betaproteobacteria* (Jensen *et al*., 1998), i entre bacteris Gram-positius i Gram-negatius (Khan *et al.*, 2002). A més, profags similars han estat detectats en bacteris d'espècies diferents de *Clostridium* i *Bacillus* (Shan *et al*., 2012).

1.1.1. Cicles de vida dels bacteriòfags

Segons al resultat de la infecció fàgica de la cèl·lula hoste, els fags poden seguir dos cicles de vida diferents (Adams, 1959). (NOTA: les figures es poden trobar a la versió anglesa de la introducció) (Figura 1):

- **Cicle lític:** Una vegada el fag s'adsorbeix al receptor específic del bacteri hoste sensible, el genoma del fag és injectat al citoplasma bacterià. En la via lítica, el genoma del fag es replica independentment del cromosoma bacterià i es produeixen diverses còpies del genoma del fag. Mentrestant, els gens tardans del fag són transcrits i traduïts per donar lloc als components proteics dels caps i/o les cues, els quals s'uneixen per formar les càpsides dins el citoplasma bacterià. Un cop formada la càpsida proteica, el genoma és introduït als nous caps fàgics. Els caps que contenen DNA són units a les cues (en el cas de fags amb cua) donant lloc a la producció de nous fags.

Proteïnes molt tardanes codificades pel fag (*very late proteins*) lisen la cèl·lula hoste i alliberen la progènie de fags al medi causant finalment la mort cel·lular de l'hoste bacterià.

Els fags que només poden seguir el cicle lític s'anomenen **fags virulents**. Els fags T4 i ΦX174 són exemples de fags virulents.

- **Cicle lisogènic:** Alguns fags, una vegada infecten el bacteri hoste, poden o bé entrar al cicle lític o bé al cicle lisogènic, són els anomenats **fags temperats**.

  En el cicle lisogènic, el genoma del fag temperat esdevé part del genoma bacterià, replicant junt amb l'hoste, ja sigui integrat en el cromosoma hoste (és el cas de la majoria de profags), o bé en forma de genoma circular mantingut com a replicó independent (per exemple, el fag P1).

  Més detalladament, després de la unió al receptor bacterià, el genoma del fag és injectat a l'interior del citoplasma bacterià i generalment circularitzat. Mitjançant l'enzim d'integració codificat pel fag (integrasa), reconeix un lloc específic en el cromosoma bacterià *attB* (*attachment site of the bacteria*) i el genoma del fag és integrat en un locus específic donant lloc a la forma quiescent del fag anomenada **profag**.

  El DNA fàgic i cromosòmic s'uneixen mitjançant un seguit de reaccions seqüencials. Com a resultat d'aquesta recombinació el cromosoma del fag esdevé part del cromosoma bacterià comportant-se com un gen bacterià més. Es manté en aquesta condició de manera indefinida, replicant-se a la mateixa vegada que ho fa l'hoste per produir un clon de cèl·lules bacterianes totes elles portadores de profags. Les cèl·lules que contenen profags s'anomenen **lisogèniques** (o **lisògens**). Per tant, en aquest cas, el bacteri incorpora gens fàgics i és capaç d'expressar-los, és el procés conegut com a **conversió lisogènica**.

Ocasionalment, els fags temperats poden sortir del seu estat de quiescènscia i canviar al cicle lític en el procés d'**inducció**. Una vegada en el cicle lític, els fags es propaguen causant finalment la lisis del bacteri hoste i són alliberats com a noves partícules fàgiques (virions).

És important destacar que, generalment, aquests virions no poden infectar altres cèl·lules del cultiu lisogènic ja que totes elles són portadores del mateix profag en el seu genoma i per tant confereixen *immunitat* a l'atac per un virió del mateix fag, tot i que continua essent vulnerable a la infecció per altres fags. Degut a la seva condició d'immunitat, els fags temperats només generen clapes quan són sembrats en una soca no-lisogènica. Tanmateix, s'han descrit excepcions com en el cas dels bacteriòfags temperats codificadors de la toxina shiga (Serra-Moreno *et al*., 2008).

En resum, els **fags temperats** doncs, poden escollir el mode de reproducció quan infecten un nou bacteri hoste. A vegades el fag infectiu inicia el cicle lític donant lloc a la lisi de la cèl·lula i alliberament de noves partícules fàgiques tal i com s'ha descrit anteriorment. Alternativament, el fag infectiu pot iniciar l'estat quiescent de *profag*, sovint integrat en el genoma hoste, però a vegades mantingut en forma de plasmidi. El fag pot romandre en aquesta condició indefinidament replicant al mateix temps que ho fa l'hoste donant lloc a cèl·lules lisogèniques. Ocasionalment, un d'aquests profags pot sortir del seu estat de quiescència i entrar al cicle lític. Els factors que afecten en la tria de lisogenitzar o de reentrar al cicle lític (inducció) s'anomenen **agents inductors** i es descriuen al següent apartat.

Les clapes de lisi generades per fags temperats poden ser lleugerament diferents a les formades per fags virulents. En lloc de ser clapes clares com les produïdes pels fags virulents, són més tèrboles. Les clapes són visibles per la lisis de la cèl·lula que segueix el cicle lític i la terbolesa observada és deguda a què molts dels fags estan duent a terme cicles lisogènics, i no de l'estricte cicle lític, i les cèl·lules lisogèniques creixen dins la placa.

Els fags Lambda (λ), P1, P22, i Mu, diversos fags que infecten *Enterobacteriaceae*, i fags que infecten *Lactobacillaceae* es troben entre els fags temperats més coneguts.

Els fags temperats poden ajudar a protegir els seus hostes bacterians d'altres fags similars (*superinfecció*) i mitjançant el procés de conversió lisogènica poden conduir a canvis significatius en les propietats dels seus hostes.

Alguns autors han fet èmfasi en la importància dels fags temperats com a "replicons dotats de capacitat per la transferència horitzontal" i que probablement han estat factors importants en l'evolució bacteriana mitjançant la mobilització de segments de genomes en diversos bacteris (Briani *et al.,* 2001).

1.1.2. Inductors del cicle lític en bacteriòfags temperats

La inducció dels **profags** pot tenir lloc espontàniament o bé estimulada per inductors. Alguns compostos, tant naturals com introduïts per l'activitat antropogènica al medi ambient, poden actuar com a agents inductors donant lloc a la replicació dels fags i a un augment en el nombre de partícules fàgiques que seran alliberades de la cèl·lula hoste. Els inductors més rellevants es descriuen a continuació:

- *Agents mutagènics:* Agents mutagènics o qualsevol agent que causi danys en el DNA, com la llum UV, poden induir el cicle lític dels fags en molts lisògens.

- *Antibiòtics*: Alguns antibiòtics utilitzats en teràpia humana o en ramaderia són capaços d'estimular la producció de fags a partir de lisògens. Entre d'altres, els antibiòtics que afecten el DNA o els que activen la resposta SOS bacteriana donaran lloc a la inducció fàgica. Quinolones com la ciprofloxacina o la norfloxacina s'han utilitzat àmpliament per a la inducció de fags (Goerke *et al*., 2006; Rolain *et al*., 2009; Looft, 2012; Meessen-Pinard *et al*., 2012), i en poblacions intestinals s'ha demostrat la inducció de profags-Qnr en presència de quinolones (Modi *et al*., 2013).

  Altres antibiòtics com la trimetoprima, la furazolidona i la ciprofloxacina són potents inductors de la resposta SOS i també s'ha descrit que indueixen l'expressió del gen *stx* en EHEC O157:H7 (Kimmitt *et al*., 2000). L'ampicil·lina també s'ha demostrat que indueix profags (Maiques *et al*., 2006).

- *Mitomicina C*: és considerada un agent antitumoral i actua intercalant-se en les cadenes complementàries del DNA i causant dany en aquest. Aquest compost s'utilitza generalment en laboratoris com a agent inductor de la resposta SOS en fags temperats (Fuchs *et al*., 1999; Livny i Friedman, 2004; Muniesa *et al*., 2004).

- *Agents quelants:* L'EDTA i el citrat sòdic són agents quelants. S'ha descrit que l'EDTA incrementa el nombre de còpies del gen *stx* en fags temperats Stx quan un cultiu lisogènic de Stx es tractava amb EDTA 20mM, fins i tot en absència de RecA (Imamovic i Muniesa, 2012).

- *Agents oxidants:* Les espècies reactives d'oxigen (ROS), com el peròxid d'hidrogen, són agents oxidants molt forts que es poden dissociar en radicals lliures i causar danys en el DNA bacterià, donant lloc a la inducció de pròfags (Kutter i Sulakvekidze, 2005).

Tots aquests factors tindrien un paper important en l'augment de la freqüència d'intercanvi gènic en ambients com granges, hospitals, sistemes de tractament d'aigües residuals, etc. els quals proporcionarien les condicions ideals per a l'adquisició de gens de resistència.

1.1.3. Transducció

La transferència horitzontal de gens en bacteriòfags té lloc per mitjà del fenomen de transducció. La **transducció** fa referència al procés pel qual un fragment de DNA és transferit d'una cèl·lula bacteriana a una altra mitjançant una partícula fàgica com a vector. La mida dels fragments de DNA que poden ser encapsidats en una partícula fàgica es troba limitada per la mida de la càpsida fàgica, però pot arribar fins a 100 kb. La transducció per bacteriòfags inclou qualsevol tipus de DNA bacterià, des de fragments lineals fins a tot tipus de *MGEs* com plasmidis, illes genòmiques, transposons i elements d'inserció (Mann i Slauch, 1997).

Tradicionalment es considerava que la transducció tenia lloc a freqüències molt baixes de l'ordre d'una cada 10^7-10^9 infeccions fàgiques, però estudis recents mostren que la transducció de lloc al medi ambient a taxes considerablement altes amb freqüències més

elevades de les que s'havien considerat anteriorment (Chiura, 1997; Evans *et al.,* 2010; Kenzaka *et al*., 2010). Per exemple, la transducció de gens pel global de la població de fags marins entre bacteris marins es calcula que té lloc als oceans 20 milions de milions de vegades per segon, encara que els valors segurament són inferiors degut a la menor eficiència de transducció i major deteriorament dels fags en els oceans que al laboratori (Bushman, 2002). A més a més, en els darrers anys, la transducció fàgica ha anat adquirint certa rellevància en clínica, essent un exemple clar l'adquisició de penicil·linases per *Staphylococcus.* (Bushman, 2002; Kenzaka *et al*., 2010).

Podem distingir dos tipus de transducció, la transducció generalitzada i la transducció especialitzada:

- **Transducció generalitzada:** Rep aquest nom perquè bàsicament qualsevol fragment de DNA bacterià de qualsevol localització en el genoma pot ser encapsidat en un fag, en lloc de DNA fàgic, i després ser transferit entre bacteris mitjançant aquest mecanisme. Les partícules fàgiques que contenen DNA bacterià encapsidat s'anomenen *partícules transductants*. Les partícules de transducció generalitzada poden ser produïdes durant el cicle lític tant de fags virulents com de fags temperats (Thierauf *et al*., 2009). Els fags que encapsiden DNA poden, de tant en tant, per error encapsidar DNA del bacteri hoste en lloc de DNA fàgic. En el moment de la infecció d'una nova cèl·lula hoste, a vegades el DNA pot ser incorporat al genoma del nou hoste.

  La transducció generalitzada és un mecanisme important de transferència de gens entre bacteris. Les partícules de transducció generalitzada amb DNA hoste (en lloc de DNA fàgic) no es poden porpagar però tot i així poden contribuir de manera substancial a l'intercanvi genètic entre bacteris a la natura.

  Els fags P1, Mu, P22, T1, T4, KB1 or ES18 són exemples de fags capaços de dur a terme transducció generalitzada.

El procés de transducció generalitzada es pot exemplificar en el fag P1, un fag temperat que infecta diversos bacteris Gram-negatius i que es va aïllar d'una soca lisogènica d'*E.coli*. Després de la translocació al citoplasma de la cèl·lula hoste, el DNA bicatenari lineal del fag P1 circularitza per recombinació. Durant el creixement lític, el genoma circular de P1 inicia diverses rondes de replicació bidireccional abans de canviar a la replicació en cercle rodant que produeix concatèmers llargs de cadena bicatenària de DNA. El fag P1 codifica una endonucleasa que reconeix una seqüència específica *pac* en el DNA fàgic i talla el DNA en aquest punt per iniciar l'encapsidació. Quan l'encapsidació s'ha completat, l'excés de DNA és tallat per un mecanisme independent de seqüència. Aleshores tenen lloc rondes subseqüents d'encapsidació a partir del DNA tallat fins que diversos caps són plens amb DNA. Quan té lloc la lisi cel·lular s'alliberen noves partícules fàgiques. (Sternberg i Coulby, 1990; Thierauf *et al*., 2009). La transducció generalitzada té lloc quan l'endonucleasa talla per error seqüències en el cromosoma hoste bacterià que són homòlogues a les del lloc *pac* de P1. Quan el fag P1 infecta una cèl·lula, de tant en tant, l'endonucleasa de P1 talla un o més d'aquests llocs cromosòmics i encapsida aquest fragment de DNA en càpsides de P1 de manera que aquestes contenen només DNA bacterià i no DNA fàgic.

Les partícules de P1 portadores de DNA bacterià (partícules transductants) poden injectar aquest DNA en un nou hoste, i una vegada el DNA es troba al citoplasma pot recombinar en el cromosoma bacterià per recombinació homòloga. Cal tenir present que aquestes partícules transductants no podran produir la lisi en el nou hoste ja que els manquen els gens responsables per a la replicació fàgica i lisi, ni tampoc lisogènia (Figura 2).

Un 30% de les partícules fàgiques en un lisat de P1 contenen DNA hoste en lloc de DNA fàgic. Si es consideren les longituds dels genomes d'*E. coli* i P1, aproximadament 1 de cada 1.500 partícules fàgiques en un lisat portarà un determinat gen de l'hoste donador.

- **Transducció especialitzada:** A diferència de la transducció generalitzada, la transducció especialitzada té lloc com a resultat de l'excisió aberrant d'un profag d'un lloc específic d'integració del cromosoma bacterià, que resulta en l'encapsidació de DNA fàgic i un fragment de DNA adjacent del genoma bacterià en una única partícula fàgica. Per mitjà de la transducció especialitzada els fags només poden encapsidar el seu DNA i el DNA bacterià flanquejant a l'*attachment site* d'un profag integrat (Miller *et al*., 2004; Thierauf *et al*., 2009). Tots els gens de l'hoste donador (inclosos gens de plasmidis) tenen les mateixes probabilitats de ser mobilitzats per aquest mecanisme.

  Degut a què els tipus de seqüències que poden ser transferides són tan limitades, és probable que la contribució de la transducció especialitzada en la transferència de gens al medi ambient no sigui tan important en comparació amb la transducció generalitzada. No obstant, la freqüència de transducció d'aquest fragment de DNA específic és més alta ja que totes les partícules fàgiques produïdes després de la inducció del profag portaran aquest fragment en concret.

  S'han descrit diversos exemples de fags capaços de dur a terme transducció especialitzada, essent el fag Lambda el més ben estudiat.

  Generalment, els fags temperats que fan transducció especialitzada circularitzen immediatament el seu DNA després de la infecció al bacteri hoste a partir dels llocs *cos* (*cohesive end*). Quan aquests fags encapsiden el seu DNA després de la inducció del cicle lític, utilitzen els llocs *cos* per tallar els seus concatèmers de DNA i aquests segments són empaquetats en les càpsides fàgiques. Per tant, en teoria, no serien capaços de produir transducció generalitzada, la qual requereix el mecanimse *pac*. A la literatura hi ha pocs exemples descrits de fags *cos*-packaging causants de transducció generalitzada (Sternberg, 1986; Campoy *et al*., 2006).

1.1.4. Ubiqüitat i abundància dels bacteriòfags

Els bacteriòfags es troben en grans quantitats i àmpliament distribuïts en diversos entorns naturals, allà on habiten els seus hostes bacterians, com per exemple en el tracte intestinal d'humans i animals on els bacteris intestinals es troben associats amb les seves poblacions de fags específiques (Breitbart *et al*., 2003, 2008; Minot *et al*., 2011), en aigua residual (Cantalupo *et al*., 2011) en femtes humanes i d'animals, (Letarov and Kulikov, 2009; Victoria *et al*., 2009; Reyes *et al*., 2010), sòl (Weinbauer, 2004), plantes (Gill and Abedon, 2003), sistemes marins (Angly *et al*., 2006), llacs (Ogunseitan *et al*., 1990), aigua de riu, etc. i, fins i tot, en ambients extrems (Le Romancer *et al*., 2007), amb nombres variables que semblen dependre de l'abundància i activitat bacteriana (Taula 1).

Les tècniques de microscopia electrònica de transmissió i de microscopia d'epifluorescència, juntament amb el desenvolupament de tècniques moleculars com la seqüenciació genòmica i les anàlisis metagenòmiques, han permès la detecció de bacteriòfags en ambients en els quals no era possible abans, independentment de la seva infectivitat, i també han revelat que els fags són més abundants del què es considerava anteriorment (Breitbart *et al*., 2003; Weinbauer, 2004; Srinivasiah *et al*., 2008). Aquests estudis indiquen que les poblacions naturals de fags són reservoris de la diversitat més gran encara no caracteritzada, amb una enorme varietat de nínxols ambientals i d'estratègies de supervivència (Weinbauer, 2004).

Molts d'aquests estudis es basen en l'anàlisi de la fracció vírica de la mostra, però com en la gran part d'ambients estudiats, els fags són la part majoritària de la fracció vírica (Dinsdale *et al*., 2008), es pot assumir que el DNA víric avaluat en aquests estudis pertany majoritàriament a bacteriòfags.

La idea que els bacteriòfags tenen un paper important en l'ecologia microbiana està àmpliament acceptada actualment. Per una banda, a través de la infecció i lisi dels bacteris infectats, contribueixen a la mortalitat bacteriana, i per tant, regulen els nombres de determinades poblacions bacterianes en un ambient donat; i amb l'alliberament de compostos orgànics conseqüència de la lisi cel·lular tenen un impacte important en el cicle de la matèria orgànica de la biosfera (Suttle, 1994). Per altra banda, controlen la diversitat

microbiana a través de la selecció de determinats bacteris resistents als seus atacants (Scanlan and Buckling, 2012), canviant així les proporcions d'espècies o soques bacterianes en una població i, conseqüentment, influenciant en l'evolució de genomes bacterians mitjançant la transferència horitzontal de gens en el procés de transducció.

Tenint en compte l'abundància de fags en el medi ambient i les seves elevades concentracions en ambients aquàtics (Weinbauer, 2004; Srinvivasiah *et al*., 2008), juntament amb la seva capacitat per transduir gens entre els seus hostes bacterians, és d'esperar la seva contribució clau en la transferència de gens en ambients aquàtics (Ripp i Miller, 1995; Brabban *et al.,* 2005; Parsley *et al*., 2010b), essent la transducció probablement un dels principals mecanismes implicats en l'evolució genòmica de bacteris en hàbitats aquàtics.

1.1.5. Persistència dels bacteriòfags en el medi ambient

Els fags, ja siguin virulents o temperats, presenten una persistència més elevada a factors d'estrès externs naturals i antropogènics que els bacteris, i generalment persisteixen millor en ambients aquàtics que ho fan els seus bacteris hoste (IAWPRC, 1991; Muniesa *et al*., 1999; Durán *et al*., 2002). Aquesta supervivència superior i l'abundància de fags portadors de gens de resistència en aigües residuals humanes i animals (Muniesa *et al*., 2004; Minot *et al*., 2011; Looft *et al*., 2012) fa dels fags candidats adients com a vehicles per a la mobilització del *pool* ambiental de gens de resistència a antibiòtics entre bacteris en biomes diferents contribuint així al manteniment i aparició de noves resistències (Sano *et al*., 2004). A més a més, degut a les característiques estructurals dels fags, amb el DNA encapsidat protegit de la degradació, la seva persistència a l'ambient és superior a la de DNA lliure (fragments lineals o plasmidis), el qual és més sensible a nucleases, temperatura, predació i radiació (Lorenz i Wackernagel, 1994; Dupray *et al*., 1997; Zhu, 2006).

Els fags poden sobreviure en ambients especials sense perdre la seva capacitat infectiva i poden transferir gens per transducció. Aquesta observació recolzaria la idea que la contribució dels fags en la transferència horitzontal de gens en ambients naturals extra-intestinals i en ambients humanitzats seria major que la de plasmidis o transposons, els quals tenen una persistència ambiental inferior (Lorenz i Wackernagel, 1994; Dupray *et al*.,

1997; Zhu, 2006). En canvi, en entorns clínics, plasmidis i transposons són segurament els *MGEs* més rellevants per la transferència horitzontal de resistència a antibiòtics ja que la seva menor persistència ambiental no suposa una limitació en el cos humà.

En efecte, l'elevat nivell d'especificitat, supervivència a llarg termini i capacitat per multiplicar-se ràpidament en un hoste adequat que presenten els fags, contribueix al manteniment d'un equilibri dinàmic entre espècies bacterianes en qualsevol ecosistema. Quan no hi ha hostes adequats, molts fags poden mantenir la seva capacitat infectiva intacta durant un llarg període de temps, sempre i quan no siguin danyats per agents externs.

Tot i que els fags tenen sensibilitats diverses a agents químics i físics, es poden establir algunes generalitats. Per exemple, tots els fags són molt sensibles a la llum UV en el rang de 260 nm així com a l'UV llunyà. Altres factors que potencialment afectarien els fags serien:

- *pH*: En general, els fags són estables de pH 5 a 8, i molts són estables a pHs inferiors a 3 o 4, però depèn de cada fag.

- *Temperatura:* La temperatura és un factor crucial en la replicació i supervivència dels bacteriòfags (Hurst *et al.,* 1980; Yates *et al.,* 1985; Nasser i Oman, 1999; Olson *et al.,* 2004). Té especial importància en els processos d'adsorció, penetració, multiplicació i durada del període de latència (en el cas dels fags lisogènics). Per una banda, a temperatures inferiors a l'òptima, hi ha una menor penetració del material genètic del fag a l'interior del bacteri hoste; per tant, un menor nombre de fags es veuran implicats en la fase de multiplicació. Per altra banda, temperatures elevades poden prolongar la durada de l'estat de latència. A més, la temperatura determina l'abundància, viabilitat i conservació dels bacteriòfags.

- *Urea i uretà*: Els fags sovint són bastant sensibles a agents desnaturalitzants de proteïnes com l'urea i l'uretà, però el nivell d'inactivació depèn tant de la concetració com de la temperatura, i l'efecte varia segons el tipus de fags.

- *Detergents:* Els detergents, com és d'esperar, generalment tenen molt menys efecte en fags que en bacteris; només alguns fags embolcallats en membranes són sensibles a detergents.

- *Agents quelants:* En canvi, els agents quelants, tenen efectes molt potents en fags que requereixen de cations per a l'adsorció o per a la formació de la càpsida.

- *Cloroform*: El cloroform té poc o cap efecte en fags sense embolcall.

- *Agents mutagènics*: agents com el gas mostassa, l'òxid nítric, i la llum UV inactiven els fags i, com s'ha esmentat anteriorment, poden induir el lític cicle en molts lisògens.

1.2. Antibiòtics

1.2.1. Antibiòtics: definició i història

El terme antibiòtic (del grec. anti, "contra"; bios, "vida") es referia originalment a un compost natural produït principalment per fongs o un altres microorganismes i el qual mata els bacteris causant de malalties en humans i animals. Actualment, ens referim a **antibiòtic** com a una substància química produïda per un microorganimse o bé un derivat sintètic que és capaç d'inhibir el creixement (bacteriostàtic) o matar (bactericida) altres microorganismes sensibles.

Abans del segle XX els tractaments de les infeccions es basaven en medicina tradicional. Moltes de les civilitzacions antigues com egipcis, grecs, xinesos i indis d'Amèrica central utilitzaven floridures específiques i extractes de plantes pel tractament d'infeccions però sense comprendre la connexió entre les seves propietats antibacterianes i el tractament de malalties.

Durant el segle XIX i prèviament a la introducció dels antibiòtics i tractaments antisèptics, més de la meitat dels pacients quirúrgics desenvolupaven infeccions i com a conseqüència els científics es van començar a interessar en la recerca de medicaments capaços de combatre els bacteris causants d'aquestes malalties. A Alemanya, Paul Ehrlich va descobrir el primer antimicrobià sintètic, Salvarsan (arsfenamine). Però no va ser fins el 1928 que Alexander Fleming va observar que el creixement de colònies del bacteri *Staphylococcus aureus* podia ser inhibit per acció d'una substància secretada pel fong del gènere *Penicillium,* demostrant així les seves propietats antibacterianes, tot i que no va ser capaç de purificar-ne la molècula, la penicil·lina.

El 1932, als Laboratoris Bayer d'Alemanya, un equip d'investigació va desenvolupar la primera sulfonamida i el primer antibacterià disponible al mercat, el Prontosil, el qual tenia un efecte relativament ampli contra cocs Gram-positius però no contra enterobacteris.

Un avenç clau va tenir lloc el 1945 amb el desenvolupament i producció a gran escala de la penicil·lina. La penicil·lina purificada presentava una potent activitat antibacteriana contra

un ampli espectre de bacteris i amb una baixa toxicitat pels humans. A més, la seva activitat no es veia inhibida per components biològics a diferència del què succeïa amb les sulfonamides sintètiques. El descobriment d'un antibiòtic tant potent era un fet inaudit i el desenvolupament de la penicil·lina va donar lloc a un interès renovat en la recerca de compostos antibiòtics d'eficàcia i seguretat similars.

Aquestes troballes, junt amb el descobriment de l'estreptomicina per Selman Waksman, van obrir l'era dels antibiòtics.

A partir de la seva introducció als anys 40, els antibiòtics van transformar la medicina i van reduir dràsticament les malalties i les morts per infeccions bacterianes, les quals eren causes principals de mortalitat i morbiditat en humans. Encara més, es va descobrir que determinats tipus de bacteris, sobretot actinomicets i estreptomicets, sovint produïen compostos amb propietats antibiòtiques, fet que va donar lloc a esforços per aïllar bacteris ambientals productors d'antibiòtics.

Actualment, es considera que es van descobrir i produir més de 20 classes noves d'antibiòtics entre 1930 i 1962 amb un gran auge en els anys 60 (Coates *et al*., 2002; Powers, 2004) (Figura 2). Des d'aleshores però, la troballa i desenvolupament de nous antibiòtics s'ha reduït de manera molt considerable amb només unes cinc classes noves d'antibiòtics comercialitzades (Butler i Buss, 2006; Hair i Kean, 2007), principalment degut al problema de l'aparició ràpida de bacteris resistents cada vegada que un nou antibiòtic és introduït al mercat. Per tal de combatre els patògens resistents en molts casos s'ha optat per una aproximació basada en la modificació dels antibiòtics ja existents en lloc del descobriment de nous.

1.2.2. Ús dels antibiòtics

Tal i com s'ha esmentat anteriorment, el descobriment i producció d'antibiòtics en la primera meitat del segle passat ha estat un dels grans èxits de la medicina. L'ús d'antibiòtics ha revolucionat el tractament de malalties infeccioses bacterianes, tant en humans com en animals, i ha permès reduir la morbiditat i mortalitat associades, així com augmentar

l'esperança de vida de la població. Els antibiòtics són també essencials en medicina moderna i en procediments com cirurgies, o per evitar infeccions en tractaments de quimioteràpia per càncer, els qual no es podrien realitzar sense disposar d'antibiòtics potents.

Des d'aleshores, quantitats cada vegada més importants d'antibiòtics s'han anat produint i utilitzant en aplicacions diverses com les que es detallen a continuació:

- Ús terapèutic/profilàctic en humans
- Ús terapèutic/profilàctic en animals (bestiar boví, porcí, aviram, etc.) incloent-hi l'aqüicultura
- Producció: per augmentar el creixement d'animals destinats a la producció d'aliments (factors de creixement) i millorar l'eficàcia nutritiva en animals
- Ús terapèutic/profilàctic en agricultura
- Ús terapèutic/profilàctic en animals domèstics
- Ús com a biocides en productes de neteja
- Ús en esterilitat de cultius, clonació, i selecció en investigació i indústria

Cal destacar que l'ús terapèutic en humans representa menys de la meitat de totes les aplicacions dels antibiòtics comercialment disponibles. De fet, els antibiòtics són utilitzats en major quantitat en animals destinats a la producció d'aliments que en el tractament de malalties en pacients (Davies i Davies, 2010). Encara que l'ús d'antibiòtics per a l'augment del creixement animal en ramaderia va ser prohibit a la Unió Europea el 2006, encara hi ha molts altres països que els utilitzen amb aquesta finalitat.

1.2.3. Classificació dels antibiòtics

Avui dia, les diferents classes d'antibiòtics que es coneixen es classifiquen basant-se en el seu mecanisme d'acció, estructura química, o espectre d'activitat (Neu, 1992). Els antibiòtics, per exemple, poden actuar inhibint la síntesi proteica, com és el cas d'aminoglicòsids, cloramfenicol, macròlids i tetraciclines; o interactuar en la síntesi de DNA i RNA com fan

quinolones i rifamicines. Altres grups inhibeixen la síntesi o danyen la paret cel·lular bacteriana com per exemple els β-lactàmics i glicopèptids, o d'altres modifiquen el metabolisme energètic de la cèl·lula bacteriana com sulfonamides i trimetoprim.

A la Taula 2 es presenten les classes principals d'antibiòtics utilitzats actualment i el seu mecanisme d'acció.

1.3. Resistència a antibiòtics

La **resistència als antibiòtics** es pot definir com la capacitat d'algunes poblacions de microorganismes de sobreviure i multiplicar-se en presència d'un antibiòtic al qual originàriament eren sensibles.

Amb la introducció dels antibiòtics, inicialment es va assumir que l'evolució de resistències era poc probable basant-se en el fet que la freqüència de mutació per generar resistència als antibiòtics era insignificant (Davies, 1994). Desgraciadament, el temps ens ha demostrat el contrari. Ningú no podia anticipar que els bacteris serien capaços de reaccionar tan ràpid adaptant-se mitjançant el desenvolupament de resistències i utilitzant una àmplia varietat de mecanismes. A més, la transferència horitzontal de gens va ser especialment inesperada. Més endavant, es va descobrir que l'existència de resistències naturals va tenir lloc, de fet, abans que es caracteritzés el primer antibiòtic (Abraham i Chain, 1988; van Hoek, 2011).

Els gens que confereixen resistència als antibiòtics es troben disseminats a la natura i freqüentment distribuïts entre patògens. L'ampli ús d'antibiòtics proporciona una potent pressió de selecció afavorint el creixement de la fracció bacteriana portadora de les resistències. Com a conseqüència, la resistència a nous antibiòtics sorgeix típicament de 2 a 5 anys després de la introducció d'aquests (Bushman, 2002).

Anys abans de la introducció de la penicil·lina com a agent terapèutic es va identificar una penicil·linasa bacteriana. Una vegada l'antibiòtic es va utilitzar àmpliament, les soques resistents van esdevenir prevalents. La identificació d'una penicil·linasa bacteriana prèvia a l'ús d'antibiòtic es pot apreciar ara a la llum de descobriments recents que revelen nombrosos gens de resistència a antibiòtics com a components naturals de poblacions microbianes (D'Costa *et al*., 2006). Un altre exemple el podem trobar en l'estreptomicina, la qual va ser introduïda el 1944 pel tractament de la tuberculosi i durant el tractament de pacients amb l'antibiòtic van aparèixer soques mutants resistents de *Mycobacterium tuberculosis*. De la mateixa manera, altres antibiòtics que es van descobrir, produir a gran escala i introduir en clínica, van donar lloc al desenvolupament de mecanismes per a l'aparició de bacteris resistents (Davies i Davies, 2010).

La Figura 3 mostra la seqüència del descobriment i desenvolupament corresponent de resistència a antibiòtics per a la majoria de classes d'antibiòtics.

Així, la resistència a antibiòtics ha esdevingut un fenomen en augment en la medicina contemporània i ha aparegut com una de les preocupacions de salut pública més importants del segle XXI (WHO, 1996).

Es calcula que unes 25.000 persones moren cada any a Europa degut a bacteris resistents a antibiòtics i com a mínim unes 23.000 com a resultat directe d'infeccions resistents i moltes d'altres per complicacions derivades. A nivell d'Espanya, unes 2.000 persones moren cada any com a resultat de la resposta ineficient a antibiòtics degut a l'aparició de resistències (ECDC, 2009).

L'aparició i ràpida disseminació de patògens resistents a antibiòtics representen una amenaça cada vegada més gran per a la població independentment de l'edat, sexe, o origen sòcio-econòmic, i posen en perill a persones tant de països desenvolupats com subdesenvolupats (Aminov, 2009). Exemples de bacteris clínicament rellevants que han desenvolupat resistència als antibiòtics disponibles actualment són, per una banda, patògens Gram-negatius, els quals són especialment preocupants perquè esdevenen resistents a quasi tots els antibiòtics d'elecció per al tractament de malalties. Les infeccions més greus per Gram-negatius estan relacionades amb l'assistència sanitària, essent els patògens més comuns enterobacteriàcies (*E. coli* i *K. pneumoniae*), *P. aeruginosa*, *A. baumannii*. Per altra banda, hi ha bacteris resistents entre els bacteris Gram-positius com *S. aureus*, *Streptococcus* i *Enterococcus* els quals són causants d'infeccions importants. A la Taula 3 es detallen els bacteris multi-resistents clínicament més rellevants i les infeccions més importants que causen.

L'ús prudent d'antibiòtics i estratègies de control d'infecció dirigides a tots els sectors d'atenció sanitària (unitats de cures intensives, ambulatoris,...) són els pilars de les intervencions destinades a la prevenció de la selecció i transmissió de bacteris resistents a antibiòtics (ECDC, 2013). Però són diverses les causes de l'aparició de resistències.

1.3.1. Causes de la resistència a antibiòtics

La resistència a antibiòtics pot tenir lloc com un fenomen natural o bé augmentar degut a les activitats antropogèniques.

- **Aparició natural:** Hi ha evidències que la resistència a antibiòtics té lloc de manera natural i és conseqüència de l'evolució via selecció natural. Els gens de resistència a antibiòtics a la natura (no en ambients clínics) poden ser transferits des de bacteris no patògens a aquells que causen malalties, donant lloc a l'aparició de bacteris resistents a nivell clínic.

  Estudis recents han descobert la presència de gens de resistència a antibiòtics a la flora intestinal de poblacions allunyades de la civilització moderna i no exposades a antibiòtics (Pallecchi *et al*., 2007, 2008; Bartoloni *et al.*, 2008), i en ecosistemes amb absència d'antibiòtics produïts per humans tan diversos com el sòl (D'Costa *et al*., 2011), aigües prístines (Lima-Bittencourt *et al*., 2007) o una microcova aïllada durant més de 4 milions d'anys (Bhullar *et al*., 2012). Aquests exemples destaquen el paper potencial dels bacteris ambientals com a font de gens de resistència a antibiòtics.

- **Activitats antropogèniques:** El paper predominant de les activitats humanes en la generació de reservoris ambientals de resistències a antibiòtics és en l'actualitat àmpliament acceptat.

  Des de la industrialització de la producció d'antibiòtics, aquests s'han produït en grans quantitats, destinat a múltiples aplicacions en humans, alliberat al medi ambient i disseminat àmpliament, proporcionant una pressió de selecció constant de poblacions de soques resistents en el medi ambient i canviant la distribució dels gens de resistència i augmentant-ne també la seva abundància.

  En medicina: Tot i que no hi ha dades sobre els nivells de bacteris resistents a antibiòtics abans de l'ús generalitzat dels antibiòtics, la pressió selectiva exercida derivada del seu ús ha jugat, sens dubte, un paper en el desenvolupament i disseminació de resistències entre espècies bacterianes.

En medicina, el problema més important de l'aparició de bacteris resistents és degut al **mal ús i abús d'antibiòtics**, el qual té lloc tant en països desenvolupats com en desenvolupament, essent un problema global molt greu. Dades d'Europa revelen que alguns països utilitzen fins a tres vegades la quantitat d'antibiòtics per persona en comparació amb d'altres països amb perfils similars de malalties (Levy i Marshall, 2004; WHO, 2011) (Figura 4). Diversos estudis també han demostrat que determinats patrons en la utilització d'antibiòtics afecten de manera considerable el nombre de resistents que es desenvolupen (Figura 5).

Altres factors que contribuirien a la resistència inclourien **diagnòstics incorrectes, la prescripció inadequada d'antibiòtics** i **l'ús inadequat d'antibiòtics per part dels pacients.** Sovint, els metges recepten antibiòtics a pacients que no els necessitarien i alguns pacients no respecten el seu tractament causant risc d'aparició de resistències. Una **pobra higiene de mans** del personal d'hospital s'ha associat amb la disseminació de microorganismes resistents, i una millora d'aquesta resulta en una disminució de les taxes de bacteris resistents (Swoboda *et al*., 2004; Girou *et al*., 2006).

En la indústria: Algunes pràctiques en la indústria farmacèutica com els abocaments de les seves aigües residuals o l'ús domèstic de sabons o altres productes que contenen antimicrobians poden contribuir en la probabilitat de generar bacteris resistents.

En animals: L'ús d'antibiòtics en animals és també responsable, en part, de l'aparició de resistències en humans.

Tradicionalment, s'ha fet un ús intensiu dels antibiòtics en ramaderia animal i com s'ha esmentat prèviament, els antibiòtics també s'han utilitzat en animals destinats al consum humà com bestiar boví, oví, porcí, aviram, peix, etc. Degut a l'exposició a antibiòtics, els bacteris resistents que s'originen en animals poden ser transmesos als humans principalment per aquestes tres vies: a través del consum de productes

animals (llet, carn, ous, etc.), per contacte directe amb animals o altres persones, i per mitjà del medi ambient.

De fet, l'OMS va concloure que l'ús indegut d'antibiòtics en ramaderia és un factor subjacent que contribueix a l'aparició i disseminació de bacteris resistents a antibiòtics i que l'ús d'antibiòtics com a promotors de creixement en animals hauria d'estar prohibit.

1.3.2. Conseqüències de la resistència a antibiòtics

Tal i com s'ha esmentat anteriorment, la resistència a antibiòtics és una greu amenaça per a la salut pública mundial amb conseqüències importants. Tot i que no s'ha quantificat, s'ha descrit que individus amb infeccions resistents presenten una duració més llarga de la seva malaltia i pitjor prognosi, requereixen tractaments més llargs i/o cars, estades més llargues en hospitals, visites addicionals de metges, i tot això donaria lloc a taxes més elevades de mortalitat en comparació amb les infeccions que són fàcilment tractables amb antibiòtics (Laxminarayan *et al.,* 2013).

A més a més, els costos per al tractament d'infeccions resistents suposa una enorme càrrega per la societat ja que afegeix costos considerables al sistema sanitari i es preveu que incrementi a mesura que augmentin el nombre de casos de malalties resistents. (WHO, 2011).

1.3.3. Mecanismes de resistència a antibiòtics

El **resistoma** comprèn tota la col·lecció de gens de resistència a antibiòtics i els seus precursors, incloent aquells circulant en bacteris patògens, productors d'antibiòtics, i bacteris no patògens, tant de vida lliure al medi ambient com comensals d'altres microorganismes. El resistoma també inclouria els gens de resistència a antibiòtics en productors d'antibiòtics i gens precursors els quals, sota una determinada pressió selectiva, es desenvoluparien per actuar com a elements de resistència.

Els bacteris han esdevingut resistents als antibiòtics mitjançant diversos mecanismes (Figura 6 i Taula 4) (Finley *et al*., 2013):

- Canvis de permeabilitat en la paret cel·lular que restringeixen l'accés de l'antibiòtic a la seva diana
- Bombeig actiu de l'antibiòtic a l'exterior de la cèl·lula bacteriana
- Modificació enzimàtica de l'antibiòtic
- Degradació de l'antibiòtic
- Adquisició de rutes metabòliques alternatives d'aquelles inhibides per l'antibiòtic.
- Modificació de la diana de l'antibiòtic
- Sobreproducció de l'enzim diana

Aquests mecanismes de resistència poden ser intrínsecs o bé adquirits per mutacions o per adquisició de material genètic mitjançant la transferència horitzontal de gens (*HGT*) (Figura 7):

- **Resistència intrínseca:** Té lloc de manera natural en totes o la majoria de soques d'una espècie i estan codificades al cromosoma bacterià (per exemple, els bacteris Gram-negatius són resistents a la vancomicina). El resistoma intrínsec és un fenomen natural que precedeix la teràpia amb antibiòtics i que es troba en el genoma de les espècies bacterianes (patògenes i no-patògenes) i és independent de pressió selectiva (D'Costa *et al*., 2006; Dantas *et al*., 2008; Sommer *et al*., 2009; Cox i Wright, 2013).

- **Resistència adquirida:** És el resultat o bé de mutacions en el DNA existent o bé de l'adquisició de DNA exogen mitjançant *HGT.*

  Mutacions: Les mutacions donen lloc a la transferència vertical de gens (*VGT*) de resistència. Per exemple, mutacions en gens cromosòmics que codifiquen per la diana de l'antibiòtic, o en gens que regulen diferents funcions cel·lulars poden

conduir a l'activació o inhibició de gens implicats en el mecanisme de resistència (ex: hiperexpressió d'enzims inactivants, hiperexpressió de bombes de flux o inhibició de porines).

HGT: La transferència de *MGEs* entre cèl·lules es coneix com a **transferència horitzontal** de gens (*HGT*), en contraposició a la transferència vertical de gens la qual té lloc entre una cèl·lula parental i una cèl·lula de la seva progènie (Malachowa i DeLeo., 2010). L'*HGT* pot tenir lloc com a transferència de DNA de procariota a procariota, de procariota a eucariota, i d'eucariota a eucariota (Jain *et al*., 1999; Keeling i Palmer, 2008).

L'*HGT* contribueix de manera clau al canvi evolutiu jugant un paper important en la plasticitat del genoma de manera que permet l'adaptació ràpida dels bacteris a nous ambients. La pressió de selecció del medi ambient condueix al manteniment de gens determinats que promouen *fitness* i supervivència en un entorn determinat.

La transferència eficient de DNA entre bacteris mitjançant l'*HGT* té lloc per tres mecanismes principals: transformació, conjugació i transducció (Figura 8):

- **Transformació**: Implica la captació de DNA lliure de l'entorn circumdant per la cèl·lula i l'expressió d'aquest DNA a la soca receptora. Per alguns bacteris, la transformació és induïda per condicions ambiental específiques, mentre que d'altres són sempre "competents" per a la transformació. En la transformació hi intervé maquinària cel·lular específica que s'uneix al DNA extracel·lular i el transfereix a través de la membrana cel·lular (ex: adquisició de la resistència en bacteris Gram-positius com *S. pneumoniae* i *Streptococcus* del grup viridans).
- **Conjugació**: És el procés de transferència de DNA d'una cèl·lula a una altra, sobretot, entre bacteris, en el qual es requereix contacte directe cèl·lula-cèl·lula. En la conjugació generalment hi intervé una estructura específica, *pilus*, que permet la unió de la cèl·lula donadora amb la

receptora. Un fragment de DNA de la cèl·lula donadora és introduït a la receptora de manera que aquesta última adquireix les noves característiques codificades en el DNA transferit. Els gens necessaris per a l'aparell de conjugació es troben generalment en el DNA que és transferit, generalment, com a DNA extracromosòmics circular com plasmidis.

La conjugació és el mecanisme més rellevant en la transferència de gens de resistència a antibiòtics entre bacteris i en la seva disseminació, sobretot en ambients clínics.

- **Transducció**: És la transferència d'una seqüència de DNA d'un bacteri a un altre mitjançant un bacteriòfag. El procés de transducció s'ha tractat en detall a l'apartat 1.1.3.

Un altre mecanisme rellevant en l'*HGT* de *MGEs* és la transposició. En la **transposició** segments de DNA col·lineals són moguts d'una localització del genoma a una altra dins la mateixa cèl·lula. Típicament, els transposons codifiquen els enzims encarregats de les reaccions de trencament i unió implicats en la transposició així com els llocs d'acció d'aquests enzims. Cal tenir present que la transposició no és un mecanisme d'*HGT per se,* tot i així, en alguns casos, les reaccions de transposició es troben directament associades amb *HGT*, ja que enllacen seqüències cel·lulars a vehicles per a l'*HGT* entre cèl·lules, com plasmidis conjugatius o virus, donant lloc a la seva mobilització.

1.4. Gens de resistència a antibiòtics

Les enterobacteriàcies han esdevingut una de les causes més importants d'infeccions nosocomials i comunitàries. Els β-lactàmics i les fluoroquinolones són els antibiòtics de primera elecció pel tractament d'aquest tipus d'infeccions però, malauradament, al llarg dels anys s'han anat descrivint resistències adquirides per diferents mecanismes desenvolupades per aquests bacteris arreu del món. Pel què fa a Gram-positius, els *S. aureus* resistents a meticil·lina (*MRSA*) són els patògens multi-resistents més importants en hospitals.

En aquest apartat es detalla la descripció de diverses resistències adquirides a diferents antibiòtics, centrant-se en β-lactàmics i (fluoro)quinolones, juntament amb el seu mecanisme d'acció. S'han inclòs aquestes resistències perquè són les que s'han estudiat en aquesta tesi.

1.4.1. β-lactàmics i gens de resistència a β-lactàmics

Història i mecanisme d'acció

Com s'ha esmentat anteriorment, el primer antibiòtic en ser descobert va ser un β-lactàmic, la penicil·lina.

Els β-lactàmics són un ampli grup d'antibacterians bactericides que tenen en comú un nucli amb un anell β-lactàmic a la seva estructura molecular el qual els hi confereix les seves característiques més importants: el mecanisme d'acció i la baixa toxicitat. Funcionen inhibint la síntesi de la paret cel·lular bacteriana per unió a proteïnes d'unió a penicil·lines (*PBPs*) interferint en l'estructura del peptidoglicà i com a conseqüència impedeixen la transpeptidació terminal a la paret cel·lular. Com a resultat es produeix una debilitació de la paret cel·lular bacteriana que condueix a la citòlisi i mort per pressió osmòtica (Andes i Craig, 2005).

La família dels β-lactàmics inclou penicil·lines i derivats, cefalosporines, carbapenems, monobactams i inhibidors de β-lactamases. Els inhibidors de β-lactamases, com l'àcid clavulànic, no tene l'anell β-lactàmic i tenen una activitat antimicrobiana intrínseca

insignificant, però degut a la seva similitud en l'estructura química s'utilitzen en combinació amb els antibiòtics β-lactàmics per vèncer els bacteris resistents que secreten β-lactamases, les quals sinó inactivarien la majoria de β-lactàmics.

És important destacar que els β-lactàmics representen aproximadament dos terços, per pes, de tots els antibiòtics administrats en humans i també són àmpliament utilitzats en veterinària (Lachmayr *et al*., 2009).

Mecanismes de resistència i gens de resistència a β-lactàmics:

La resistència als β-lactàmics és un problema en augment i la producció de β-lactamases (β-lactamases d'espectre ampliat, enzims AmpC plasmídics i carbapenemases) és el mecanisme més freqüent i important de resistència en bacils Gram-negatius (Dallenne *et al*., 2010). En Gram-positius, els *MRSA* són els patògens multi-resistents més prevalents en hospitals, els quals han esdevingut resistents als β-lactàmics per adquisició del gen *mecA* en el seu cromosoma.

- *β-lactamases:*

La primera β-lactamasa va ser identificada en *E. coli* prèviament a l'ús de la penicil·lina en clínica (Abraham and Chain, 1988) i fins avui, s'han descrit més de 1150 β-lactamases cromosòmiques, plasmídiques i en transposons (Bush and Jacoby, 2010; Drawz and Bonomo, 2010). Les β-lactamases són enzims que hidrolitzen l'anell β-lactàmic de la molècula de l'antibiòtic inactivant-la (Jacoby, 2005). Entre les β-lactamases, les anomenades β-lactamases d'espectre ampliat (*ESBLs*) són de gran preocupació.

Bàsicament, les β-lactamases s'agrupen segons la classificació d'Ambler, basada en la seqüenciació a nivell proteic, en quatre classes (A-D) i en la classificació de Bush-Jacoby i Madeiros, basada en les característiques funcionals d'aquests enzims com el perfil de substrat hidrolític o la resposta a inhibidors. Les *ESBLs* es troben en el grup fincional 2be de Bush-Jacoby i Madeiros i pertanyen a la classe A d'Ambler (Ambler, 1980; Madeiros, 1984; Bush i Jacoby, 2010).

En general, les ***ESBLs*** descrites en comensals i patògens humans, són enzims bacterians mediats per plasmidis capaços d'hidrolitzar una gran varietat d'antibiòtics β-lactàmics incloent penicil·lines, cefalosporines de primera a quarta generació (ex: cefotaxima i ceftazidima) i monobactams (ex: aztreonam) i, per tant, confereixen resistència a un ampli espectre de β-lactàmics. Generalment, els priductros d'*ESBLs* es mantenen sensibles a cefamicines i carbapenems, els quals són inhibits per altres inhibidors de β-lactamases (ex: àcid clavulànic, sulbactam o tazobactam).

Des de la seva primera identificació el 1983 (Knothe *et al*., 1983), cada vegada s'han descrit més *ESBLs* arreu del món, de manera més freqüent en entereobacteriàcies, sobretot en *E. coli* i *Klebsiella*, i en menor grau en *S. enterica* i *S. marcescens*. Però també s'han trobat en altres espècies bacterianes com *P. aeruginosa* i *A. baumannii*.

Degut al fet que les soques productores d'*ESBLs* són resistents a molts dels antibiòtics utilitzats habitualment, la seva proliferació suposa un greu problema de salut global i complica les estratègies de tractament. Segons un estudi recent (Davies and Davies, 2010), s'ha produït un augment espectacular en el nombre de β-lactamases des dels anys 80, sobretot de les classes A i D. La classe A inclou principalment els enzims TEM, SHV, CTX-M, VEB, i GES. Per exemple, a Espanya la prevalença d'*E. coli* productores d'*ESBLs* va augmentar 8 vegades de l'any 2000 al 2006 (Díaz *et al*., 2010).

a) bla_{TEM}:

D'entre les β-lactamases, la família TEM representa una de les clínicament més significatives, àmpliament distribuïda, i amb una elevada diversitat i prevalença. El primer enzim del grup que es va descobrir va ser TEM-1, el qual va ser descrit per primera vegada als anys 60 en una soca d' *E. coli* aïllada a Grècia d'un pacient anomenat Temoneira (TEM com a acrònim de Temoneira) i hidrolitza les cefalosporines primerenques i moltes penicil·lines (Datta i Kontomichalou, 1965). TEM-1 ha esdevingut una de les β-lactamases més comuns en enterobacteriàcies.

La majoria d'*ESBLs* han evolucionat a partir de substitucions aminoacídiques (mutacions puntuals) de les β-lactamases natives TEM-1, TEM-2, i SHV-1 d'enterobacteriàcies (Bradford, 2001), sobretot d'*E. coli* i *Klebsiella*, amb una especificitat de substrat més àmplia dels enzims per incloure cefalosporines de tercera generació (ex: cefotaxima i ceftazidima) i monobactams (ex: aztreonam). Més endavant es va caracteritzar TEM-3, descoberta en soques de *K. pneumoniae* aïllades de pacients en unitats de cures intensives a França, les quals presentaven un espectre de substrat ampliat, incloent cefalosporines de tercera generació, però eren sensibles a inhibidors β-lactamases com l'àcid clavulànic (Sirot *et al*., 1987). Des d'aleshores s'han anat descobrint noves variants de TEM i actualment n'hi ha fins a més de 165 descrites (www.lahey.org/studies).

b) $bla_{\text{CTX-M}}$:

Recentment però, ha tingut lloc un canvi en la distribució de les diferents *ESBLs* amb un augment considerable dels enzims CTX-M, per davant de TEM i SHV, i s'han convertit actualment en les *ESBLs* més prevalents en enterobacteriàcies a Europa i a moltes altres zones del món (Coque *et al*., 2008). Durant els anys 80 i 90, les variants de TEM i SHV eren les *ESBLs* predominants, associades sobretot a brots nosocomials de *K. pneumoniae* i en menor grau d'*E. coli* i d'altres enterobacteriàcies, mentres que CTX-M era menys prevalent. A la primera dècada del 2000 es va produir una evolució accelerada i dispersió extraordinària dels enzims CTX-M i actualment es troben no només en l'entorn hospitalari sinó també fora, entre la població sana, essent *E. coli* el patogen més important productor d'aquests enzims (Cantón i Coque, 2006; Coque *et al*., 2008a).

Els enzims CTX-M es van descobrir per primera vegada el 1989 quasi simultàniament a Europa i a Amèrica del Sud. Per una banda, es va aïllar a Munic una soca d'*E. coli* resistent a cefotaxima però sensible a ceftazidima de l'oïda d'un nen amb otitis mitjana i l'enzim responsable del fenotip es va anomenar CTX-M-1 (CTX com acrònim per cefotaxima i -M de Munic). Per altra banda, a Amèrica del Sud es va aïllar una soca de *Salmonella typhimurium* resistent a cefotaxima de pacients amb meningitis, septicèmia o enteritis i l'enzim, el qual presentava un punt isoelèctric diferent al descrit a Alemanya, es va anomenar CTX-M-2

(Bauernfeind *et al.*, 1990, 1992). Tot i que no va ser fins el 2004 quan es va associar a CTX-M-3, ja al 1986 s'havia aïllat una soca d'*E. coli* resistent a cefotaxima de la flora fecal d'un gos a Japó.

Els enzims CTX-M, estaven confinats inicialment a *E. coli*, *K. pneumoniae* i *Salmonella* spp. però ràpidament van aparèixer en d'altres enterobacteriàcies (*Enterobacter* spp., *S. marcescens*, *Citrobacter* spp., *P. aeruginosa*) i han estat descrits també en espècies que no pertanyen a les enterobacteriàcies.

Les diverses variants de CTX-M s'agrupen en 5 clústers (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25), tots ells caracteritzats per una potent activitat hidrolítica davant la cefotaxima, tot i que hi ha noves variants que també poden hidrolitzar ceftazidima, amb una distribució que varia molt segons l'àrea geogràfica. La família de CTX-M, amb més de 120 variants identificades fins al moment (www.lahey.org/studies), és extremadament exitosa en la seva transmissió i actualment està reconeguda com el mecanisme de resistència a antibiòtic més disseminat, tant en entorns clínics com fora d'ells (Valverde *et al*., 2004; Cantón i Coque, 2006; Pitout i Laupland, 2008).

Concretament, els enzims CTX-M-15 i CTX-M-14 són els més importants i s'han descrit tant en humans, com en animals i al medi ambient a tot el món (Cantón *et al*., 2008; Hawkey i Jones, 2009; Dolejska *et al*., 2011; Hiroi *et al*., 2012) (Figura 9). A Espanya, les variants CTX-M-9, CTX-M-10, CTX-M-14 i CTX-M-15 són les més prevalents tant en pacients de consulta externa com hospitalitzats i en individus sans (Figura 10).

E. coli és l'espècie bacteriana més freqüentment implicada, i en els darrers anys, s'ha descrit la disseminació internacional del clon d'*E. coli* O25b:H4-ST131 productor de CTX-M-15 i altres β-lactamases el qual es considera responsable de la difussió de l'enzim CTX-M-15 (Coque *et al*., 2008b; Nicolas-Chanoine *et al*., 2008).

Tot i que CTX-M-15 és l'*ESBL* majoritàriament associada a aquest grup clonal (ST131), també s'han descrit altres variants com CTX-M-9, CTX-M-14 i CTX-M-32. Cal destacar la detecció per primera vegada d'aquest grup clonal productor de CTX-M-9 en granges d'aviram amb perfils

de macroresitricció i gens de virulència molt similars a aquells observats en aïllaments clínics en humans (Mora *et al*., 2010).

Diverses anàlisis filogenètiques suggereixen que els enzims CTX-M no s'haurien originat a partir de mutacions en enzims anteriors mediats per plasmidis, sinó mitjançant la mobilització de gens *bla* cromosòmics de diferents espècies de *Kluyvera*, les quals s'haurien disseminat posteriorment entre bacteris patògens i no patògens. L'èxit d'aquesta distribució sembla estar relacionada amb la interacció de diferents forces selectives. En primer lloc, aquelles derivades de l'associació de gens $bla_{\text{CTX-M}}$ amb plataformes genètiques específiques (*IS*, integrons, transposons, plasmidis i en menor grau per bacteriophages) i/o complexes clonals bacterians específics (Coque *et al*., 2008b; Nicolas-Chanoine *et al*., 2008). En segon lloc, les fortes pressions de selecció exercides per l'ús generalitzat i concomitant de diverses classes d'antibiòtics en clínica, donarien lloc a l'aparició i dispersió de noves soques productores de CTX-M co-resistents, sobretot a aminoglucòsids i fluoroquinolones (Cavaco *et al*., 2008; Pitout i Laupland, 2008; Cantón i Ruiz-Garbajosa, 2011; Tacao *et al*., 2012).

c) Altres β-lactamases

Altres enzims no-TEM, no-SHV també s'han descrit en països europeus (PSE, PER, GES, IBC, OXA *types*) i les denominades "noves β-lactamases" que confereixen resistència a carbapenems, com metal·lo-β-lactamases i carbapenemases KPC, o a cefamicines, com els enzims CMY, han sorgit més recentment i sovint han estat associades amb *ESBLs*.

A més de la producció de β-lactamases, la resistència a β-lactàmics, pot ser deguda també a a la presència de proteïnes PBPs modificades de manera que l'antibiòtic esdevé menys efectiu a l'hora d'interrompre la síntesi de la paret cel·lular. Una d'aquestes proteïnes és el producte del gen *mecA* en el gènere *Staphylococcus.*

- *mecA*:

Amb el pas del temps, nombrosos estudis han demostrat que un augment en el consum d'antibiòtics contribueix a l'aparició de resistències en diversos gèneres bacterians (MARAN, 2007; NethMap, 2008). Un exemple clar de la relació entre la dosi d'antibiòtics i el desenvolupament de resistències és l'augment de *MRSA*, el qual va aparèixer per primera vegada el 1960 (Jevons *et al*., 1963).

El gènere *Staphylococcus* està format per bacteris Gram-positius que colonitzen la pell d'humans i animals i membranes mucoses essent part de la flora normal, i per tant, bacteris comensals. No obstant, també són patògens oportunistes, i d'entre els estafilococs, *S. aureus* és l'espècie més invasiva i agent etiològic de diverses patologies en humans i animals com per exemple infeccions de pell, abscessos, intoxicacions alimentàries, síndrome del xoc tòxic, septicèmia, endocarditis i pneumònia (Weems, 2001; van Belkum, 2006; DeLeo i Chambers, 2009).

La penicil·lina va ser el primer antibiòtic utilitzat per al tractament d'infeccions d'*S. aureus*. Encara que inicialment era molt efectiva, actualment un 90% de les soques humanes d'*S. aureus* són resistents a aquest antibiòtic (Olsen *et al*., 2006).

El 1960, es va desenvolupar la meticil·lina, una penicil·linasa insensible a penicil·lina, pel tractament d'*S. aureus* multi-resistent. Però, aproximadament un any després de la seva introducció, es va aïllar la primera soca d'*S. aureus* resistent a meticil·lina (*MRSA*) (Jevons *et al*., 1963), i als anys 70 es trobava disseminada per tot el món. Des d'aleshores, *MRSA* ha esdevingut una de les causes més importants d'infeccions bacterianes adquirides tant en àmbits d'atenció sanitària com a la població en general arreu del món (DeLeo i Chambers, 2009; Motoshima *et al*., 2010).

S. aureus destaca per la seva capacitat d'adaptar-se ràpidament a pressions selectives generades per l'hoste humà, responent ràpidament als antibiòtics i esdevenint resistent i essent cada vegada més difícil de tractar. De fet, *MRSA* és considerat un patogen multi-resistent ja que és resisitent a pràcticament tots els antibiòtics β-lactàmics

desenvolupats fins ara i a la majoria de macròlids, aminoglucòsids, cloramfenicol, fluoroquinolones i tetraciclines (Lee, 2006).

Alternativament, les infeccions per *MRSA* es tracten amb vancomicina, la qual ha resultat ser eficaç fins que malauradament, fa uns anys han aparegut soques d'*S. aureus* que han adquirit elements de resistència a vancomicina d'enterococs, donant lloc a l'aparició d'*S. aureus* resistents a vancomicina (*VRSA*) (Chang *et al*., 2003; Weigel *et al*., 2003; Zhu *et al*., 2008). Antibiòtics com linezolid, quinupristina/dalfopristina, daptomicina, ceftarolina i tigeciclina s'utilitzen per tractar infeccions més severes que no responen a glicopèptids com la vancomicina.

Les infeccions per *MRSA* s'ha estimat que afecten més de 150.000 pacients anualment a la Unió Europea i s'associen amb morbiditat, mortalitat elevada i generen elevats costos per a la sanitat (Köck *et al*., 2010). *MRSA* causa infeccions nosocomials, essent endèmic en molts hospitals (ex: Regne Unit, Japó, Estats Units) i en l'última dècada també ha sorgit en altres entorns comunitaris causant infeccions de pell i teixits, infeccions sanguínies i pneumònia entre d'altres. A més, *MRSA* també s'ha identificat en aliments d'origen animal, i alguns brots han estat relacionats amb el consum de productes contaminats o amb manipuladors d'aliments colonitzats per aquest bacteri (Jones *et al*., 2002; Lee, 2006). Més recentment s'ha descrit en diversos països la colonització o infecció per *MRSA* de bestiar i humans exposats a aquests animals (van Cleef *et al*., 2011; Köck *et al*., 2010).

Els clons de *MRSA* apareixen per l'adquisició exògena de la resistència a meticil·lina per una soca d'*S. aureus* inicialment sensible a meticil·lina (*MSSA*). La resistència a meticil·lina la confereix l'expressió del gen *mecA*, el qual codifica una proteïna d'unió a penicil·lina modificada (PBP2a o PBP2') amb baixa afinitat pels antibiòtics β-lactàmics. PBP2a catalitza la formació de ponts d'entrecreuament en el peptidoglicà de la paret bacteriana i facilita la síntesi de la paret en presència de meticil·lina/oxacil·lina i d'altres β-lactàmics donant lloc al fenotip resistent (Hartman i Tomasz, 1984; Rizek *et al*., 2011).

El complex del gen *mecA* resideix en un element genètic mòbil, el **casset cromosòmic estafilocòcic** (SCC*mec*). Tot i que els elemens són molt diversos en la seva organització

estructural i en el seu contingut genètic, l'SCC*mec* consta del complex del gen *mecA*, que conté el gen *mecA*, juntament amb els seus gens reguladors (*mecI* and *mecR)*, la seqüència d'inserció IS431*mec*, les *recombinases site-specific cassette chromosome recombinases* (*ccr*) necessàries per a la integració i excisió específica de lloc de l'element, repeticions invertides directes i terminals, i el lloc cromosòmic específic d'inserció amb una pauta de lectura oberta conservada (*orf*X) (Hiramatsu *et al*., 2002; Deurenberg *et al*., 2007). Fins el moment de la preparació d'aquesta tesi i segons la seva longitud i composició, es poden distingir fins a onze tipus principals de SSC*mec* (I a XI) (Ma *et al*., 2002; Zhang *et al.* 2009; http://www.sscmec.org).

L'element SSC*mec* també es pot trobar en altres espècies d'estafilococs com els estafilococs coagulasa negatius (CoNS), i es creu que probablement té l'origen en CoNS i s'hauria integrat en el genoma d'*S. aureus* de manera que els CoNS multi-resistents constituirien un reservori per a l'adquisició de SSC*mec* per part d'*S. aureus*. Degut a la conservació independent d'espècie d'aquest complex gènic, es considera que la transferència de SSC*mec* té lloc freqüentment (Okuma *et al*., 2002). Cal tenir present però que la capacitat d'*S. aureus* per adquirir DNA del medi ambient és baixa (competència natural baixa) si es compara amb bacteris com *Streptococcus* spp., *Neisseria* spp. o *Bacillus subtilis*, i per tant, gran part de la transferència de gens intercel·lular tindria lloc a través de plasmidis pels processos de conjugació o transducció. Concretament, s'ha demostrat que els bacteriòfags temperats poden integrar-se en el genoma estafilocòcic com a profags (Mann, 2008; Goerke *et al*., 2009).

1.4.2. Quinolones i gens de resistència a quinolones

Història i mecanisme d'acció

Les quinolones són antibiòtics bactericides totalment sintètics que s'utilitzen tant en medicina humana com animal. Les dianes que les quinolones són els enzims DNA girasa (topoisomerasa II) i DNA topoisomerasa IV (Drlica i Zhao, 1997), enzims essencials pel creixement bacterià ja que controlen el superenrotllament del DNA per facilitar la replicació, transcripció, recombinació i reparació del DNA (Hawkey, 2003). Les quinolones actuen

inhibint l'acció d'aquests enzims i com a resultat es produeix el bloqueig de la síntesi de DNA i la mort bacteriana.

Les classificacions més recents divideixen les quinolones en diverses generacions basant-se en el seu espectre d'activitat (Ball, 2000). Les primeres quinolones van ser les quinolones de primera (*Q1G*), amb l'àcid nalidíxic descobert el 1962 (Lesher *et al*., 1962) i d'altres com l'àcid oxolínic i l'àcid pipemídic desenvolupats posteriorment i utilitzats en veterinària. En els anys vuitanta es va desenvolupar la segona generació de quinolones (*Q2G*) per addició d'un àtom de fluor a la posició C-6 del nucli de la quinolona donant lloc a les fluoroquinolones (ex: norfloxacina, ciprofloxacina o ofloxacina) que presentaven activitat potent contra Gram-negatius alguns Gram-positius com *S. aureus* i bacteris intracel·lulars (Hooper, 2001; King *et al*., 2000). Els anys noranta van donar pas a les (fluoro)quinolones de tercera generació (*Q3G*) amb major activitat davant Gram-positius (*S. pneumoniae*) (ex: esparfloxacina i levofloxacina) i bacteris anaeròbics.

Tot i que els principals factors que condueixen a la resistència a quinolones i fluoroquinolones estan relacionats amb mutacions cromosòmiques en els gens diana de l'antibiòtic, el descobriment en les darreres dècades de diversos mecanismes de resistència codificats en plasmidis ha contribuït a l'especulació sobre l'origen i els factors desencadenants d'aquestes resistències transferibles. La interacció entre una font ambiental i animal per una banda, i els patògens humans en clínica per una altra, semblen tenir un paper important en l'aparició de la resistència a quinolones.

Mecanismes de resistència i gens de resistència:

L'augment de la resistència a quinolones i fluoroquinolones és un fenomen cada vegada més descrit en aïllaments d'humans però també en veterinària en les darreres dues o tres dècades molt probablement com a conseqüència de l'ús generalitzat d'aquests antibiòtics (Figura 11). Durant dècades, es creia que els mecanismes de resistència a quinolones només estaven codificats a nivell cromosòmic, degut a modificacions als enzims DNA girasa i topoisomerasa IV, a la disminució de la permeabilitat de la membrana externa (associada a la pèrdua de porines), o a la sobreexpressió de bombes de flux (Hooper, 2001; Ruiz, 2003;

Jacoby, 2005). Tanmateix, des de 1998 s'ha descrit l'aparició de resistència a quinolones mediada per plasmidis (*PMQR*) (Robicsek *et al.*, 2006; Courvalin, 2008; Martínez-Martinez *et al*., 2008).

Encara que aquests determinants *PMQR* determinants confereixen un nivell baix de resistència quinolones i/o fluoroquinolones, és suficient per a la supervivència dels bacteris en presència de quinolones, mentre tenen lloc les mutacions de resistència de seqüencialment i no simultàniament.

El primer determinant *PMQR* es va identificar el 1998 i corresponeia a la proteïna Qnr (QnrA1). Les proteïnes Qnr adquirides pertanyen a una família de repeticions de pentapèptids. A dia d'avui, s'han descrit fins a sis famílies de proteïnes Qnr: QnrA, QnrB, QnrC, QnrD, QnrS i QnrVC. Els gens *qnr* són molt diversos amb 7 *qnrA*, 73 *qnr*B, 1 *qnr*C, 2 *qnr*D, 9 *qnrS* and 5 *qnrVC* identificats (http://www.lahey.org/qnrStudies). Els gens *qnr* s'han descrit a tot el món en espècies diverses d'enterobacteris i es troben associats generalment a elements genètics mòbils (Cambau *et al*., 2006).

1.4.3. Determinants de resistència a antibiòtics en poblacions víriques

Hi ha només alguns exemples de gens de resistència a antibiòtics identificats com a elements de genoma de fags. Tot i així, hi ha diversos exemples de gens de resistència a antibiòtics, i fins i tot d'elements genètics mòbils sencers, mobilitzats per transducció fàgica, com s'ha descrit en diversos gèneres bacterians (Blahová *et al*., 1993; Willi *et al*., 1997; Schmieger i Schicklmaier, 1999; Del Grosso *et al*., 2011; van Hoek, 2011).

In vitro, s'ha demostrat que els fags transdueixen resistència a imipenem, aztreonam i ceftazidima en *P. aeruginosa* per transducció generalitzada (D'Costa *et al*., 2006). La soca de *Samonella enterica* serovar Typhimurium DT104, característica per diversos patrons de multi-resistència, és capaç de transduir gens de resistència a ampicil·lina, cloramfenicol i tetraciclina (Blahová *et al.*, 1993; Schmieger i Schicklmaier, 1999). A més, el 95% de les soques tenien genomes complets de profags induïbles, i el 99% d'aquests fags eren capaços de realitzar transducció generalitzada de marcadors cromosòmics de l'hoste i de plasmidis

(Schicklmaier *et al*., 1998; Cloeckaert *et al*., 2001). Un altre exemple és el cas del fag temperat Wβ de *Bacillus anthracis* codifica resistència a fosfomicina (Schuch i Fischetti, 2006).

S'ha descrit també que *Streptococcus pyogenes* resistent a diversos antibiòtics, en ser tractat amb mitomicina C per a la inducció de fags donava lloc a la transducció de resistència a tetraciclina o a l'adquisició de multi-resistència a cloramfenicol, macròlids, lincomicina i clindamicina (Ubukata *et al*., 1975). Altres estudis demostren que profags participen en la disseminació de la resistència a eritromicina en el fenotip d'*Streptococcus* en aïllaments clínics (Hyder *et al*., 1978; McShan, 2000).

Varga *et al*. Han descrit recentment una elevada freqüència de transducció de penicil·linases i plasmidis de resistència a tetraciclina en el clon US300 de *MRSA,* un dels clons de *MRSA* més estesos (Varga *et al*., 2012).

També s'han descrit evidències indirectes de resistència a β-lactàmics mobilitzada per fags amb CTX-M-10 associat a un element fàgic i disseminat entre enterobacteriàcies en un hospital (Oliver *et al*., 2005).

Estudis metagenòmics de poblacions víriques van permetre la detecció de seqüències corresponents a gens de resistència a antibiòtics a l'intestí humà (Minot *et al*., 2011) i en llots activats d'una EDAR (Parsley *et al*., 2010a). Com s'ha comentat anteriorment els fags són la part majoritària de la fracció vírica (Dinsdale *et al*., 2008), i per tant, es pot assumir que el DNA víric analitzat en aquests estudis correspon majoritàriament a bacteriòfags. A més, tenint present que a la fracció vírica s'hi troben partícules fàgiques que realitzen transducció generalitzada, es poden trobar molts gens d'hoste bacterià a la fracció vírica.

Altres estudis revelen nombroses seqüències curtes potencialment codificadores per gens de resistència a antibiòtics en mostres d'esput de fibrosi quística i només tres en l'esput no associat a fibrosi quística (Willner *et al*., 2009). Anys enrere també es va descriure l'abundància de partícules víriques portadores de les seqüències dels gens bla_{OXA-2}, bla_{PSE-1}, bla_{PSE-4} i $bla_{PSE-type}$ en la fracció vírica d'aigua residual urbana (Muniesa *et al*., 2004) (Taula 5).

1.4.4. Disseminació de la resistència a antibiòtics i el paper del medi ambient

Diverses activitats antropogèniques com l'ús d'antibiòtics en hospitals, veterinària, granges, agricultura, aqüicultura i d'altres aplicacions juntament amb les aigües residuals de rebuig entre d'altres, poden actuar com a reactors on l'ús d'antibiòtics selecciona bacteris resistents i promou l'intercanvi de gens per *HGT*. A més, el medi ambient juga un paper important facilitant el desenvolupament i la disseminació dels gens de resistència en ambients aquàtics com rius, rierols, llacs, aigua residual, etc (Figura 12).

Tenint present que els bacteris no respecten els límits dels compartiments ecològics, hi ha sempre un flux continu d'informació genètica entre diferents compartiments ecològics. L'*HGT* ha esdevingut molt important en l'evolució i transmissió de gens de resistència entre espècies al medi ambient i inclou el moviment de resistències de bacteris patògens a bacteris ambientals, i viceversa; és a dir, l'aparició de nous mecanismes de resistència adquirida en patògens a partir de gens que originalment es trobaven en bacteris no patògens. Una vegada els potencials gens de resistència a antibiòtic entren a la microbiota comensal/patogènica d'humans o animals,, encara que sigui en baixes freqüències, en presència d'antibiòtic la selecció actua immediatament conduint a l'amplificació i disseminació d'aquests gens.

El plantejament principal d'aquesta tesi és l'estudi de l'abundància de gens de resistència a antibiòtics amb rellevància clínica en la fracció de DNA de partícules de bacteriòfags presents en mostres ambientals d'orígens diversos de la nostra àrea geogràfica, i l'estudi del paper potencial dels bacteriòfags com a reservoris i vectors per a la mobilització i disseminació d'aquestes resistències mitjançant l'*HGT*. També es pretén avaular la influència de diversos compostos implicats en la inducció del cicle lític de bacteriòfags temperats, en l'abundància de gens de resistència a antibiòtics en el DNA de la fracció bacteriòfags en mostres d'aigua residual.

Els objectius definits en detall es descriuen al següent apartat i es troben detallats en cadascun dels capítols.

2. OBJECTIVES / *OBJECTIUS*

2. OBJECTIVES

2.1. Chapter 1: Antibiotic resistance genes in the bacteriophage DNA fraction of water samples (urban wastewater, river water and wastewater with animal faecal wastes)

Study 1: Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples.

The main objectives of this study are described below:

- Development of a methodology to evaluate ARGs in the bacteriophage DNA fraction of environmental water samples.
- Design of a qPCR set for the $bla_{\text{CTX-M-1}}$ gene for the amplification of cluster 1, which is composed of 31 variants, including CTX-M-1, 3, 10, 11 and 15.
- Quantification by qPCR of three genes conferring resistance to β-lactam antibiotics, two β-lactamase genes (bla_{TEM} and $bla_{\text{CTX-M-1}}$) and the *mec*A gene in the phage and bacterial fraction DNA from environmental water samples from urban sewage and river water.
- Evaluation of the ability of phage-encoded genes detected to confer antibiotic resistance in bacterial sensitive host strains.

Study 2: Bacteriophages carrying antibiotic resistance genes in fecal waste from cattle, pigs and poultry.

The main objectives of this study are described below:

- Design of a qPCR set for the $bla_{\text{CTX-M-9}}$ gene for the amplification of cluster 9, which detects the most abundant variants of the cluster, including CTX-M-9, 13, 14, 16 a 19, 21 and 27.
- Quantification by qPCR of antibiotic resistance genes bla_{TEM}, $bla_{\text{CTX-M}}$ clonal groups 1 and 9, and *mecA* gene in the bacterial and phage DNA fraction from animal faecal wastes to determine the potential role of bacteriophages in the dissemination of antibiotic resistance genes in animal settings.

2.2. Chapter 2: Quinolone resistance genes (*qnrA* and *qnrS*) in bacteriophage particles from wastewater samples and the effect of inducing agents on packaged antibiotic resistance genes.

Study 3: Quinolone resistance genes (*qnrA* and *qnrS*) in bacteriophage DNA from wastewater samples and the effect of inducing agents on bacteriophage-encoded antibiotic resistance genes.

The main objectives of this study are described below:

- Design of a qPCR set for the quantification by qPCR of quinolone antibiotic resistance genes *qnA,* which detects variants *qnrA1-A7,* and *qnrS,* which detects variants *qnrS1-S6*.
- Quantification by qPCR of antibiotic resistance genes *qnrA* and *qnrS* in the phage DNA fraction of faecally polluted environmental samples.
- Evaluation of the influence of phage inducers (mitomycin C, ciprofloxacin, EDTA and sodium citrate) and physical parameters on the abundance of antibiotic resistances in the phage DNA fraction of bacterial populations from wastewater samples.
- Evaluation of the influence of phage inducers (mitomycin C, ciprofloxacin, EDTA and sodium citrate) and physical parameters on infectious somatic coliphages of bacterial populations from wastewater samples.

2.3. Chapter 3: Evaluation of ARGs in the DNA of bacterial and bacteriophage fraction in wastewater samples from Tunisia and comparison with results obtained in Barcelona area.

Study 4: Antibiotic resistance genes in Tunisian and Spanish wastewaters and their use as markers of antibiotic resistance patterns within a population.

The main objectives of this study are described below:

- Quantification by qPCR of three β-lactamase genes (bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}9}$), two quinolone resistance genes (*qnrA* and *qnrS*) and the *mec*A gene in phage and bacterial fraction DNA from environmental water samples from urban sewage samples from two WWTPs and from a slaughterhouse in Tunisia.
- Comparison of the ARGs content in both bacteria and phage DNA from the samples from Tunisia with the results previously obtained in Barcelona area.
- Evaluation of the ARGs detection as a marker to study the antibiotic resistance patterns within a population.

2.4. Chapter 4: Detection of quinolone-resistant *Escherichia coli* isolates belonging to clonal groups O25b:H4-B2-ST131 and O25b:H4-D-ST69 in water samples from Barcelona area.

Study 5: Detection of quinolone-resistant *Escherichia coli* isolates belonging to clonal groups O25b:H4-B2-ST131 and O25b:H4-D-ST69 in raw sewage and river water in Barcelona, Spain.

The main objectives of this study are described below:

- Evaluation of the prevalence of the emerging clonal group O25b:H4-B2-ST131 in water environments with faecal pollution (urban sewage and river water) in Barcelona.
- Determination of the antibiotic sensitivity pattern and *ESBL*s production of the *E. coli* O25b isolates.
- Study of 30 virulence genes of ST131 environmental isolates.

- Comparison of the macrorestriction profiles, virulence gene content and antibiotic resistance patterns of ST131 environmental isolates from this study with clinical isolates of the same clone causing human extraintestinal infections in Spain.

Summarizing, the main scope of this Thesis was the evaluation of the abundance of antibiotic resistance genes clinically relevant in the DNA fraction of bacteriophage particles isolated from environmental samples of different origin, including wastewater samples, river water and animal wastewater samples, in order to study the role of bacteriophages as vehicles for the mobilization of antibiotic resistance genes between bacteria.

2. OBJECTIUS

2.1. Capítol 1: Gens de resistència a antibiòtics en la fracció de DNA de bacteriòfags de mostres d'aigua (aigua residual municipal, aigua de riu i aigua residual animal).

Estudi 1: *Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples*

En aquest estudi es van plantejar els objectius detallats a continuació:

- Desenvolupar una metodologia per avaluar gens de resistència a antibiòtics en la fracció de DNA de bacteriòfags de mostres d'aigua ambientals.
- Disseny de la sonda i encebadors per a la qPCR del gen $bla_{\text{CTX-M-1}}$ per a l'amplificació del clúster 1, el qual consta de 31 variants, incloent CTX-M-1, 3, 10, 11 i 15.
- Detecció i quantificació per qPCR de tres gens que confereixen resistència a antibiòtics β-lactàmics, dos gens codificadors de β-lactamases (bla_{TEM} i $bla_{\text{CTX-M-1}}$) i el gen *mec*A, en la fracció de DNA fàgic i DNA bacterià de mostres d'aigua residual municipal i de riu.
- Avaluació de la capacitat dels gens de resistència a antibiòtics detectats de conferir resistència a soques hoste bacterianes sensibles.

Estudi 2: *Bacteriophages carrying antibiotic resistance genes in fecal waste from cattle, pigs and poultry.*

En aquest estudi es van plantejar els objectius detallats a continuació:

- Disseny de la sonda i encebadors per a la qPCR del gen $bla_{\text{CTX-M-9}}$ per a la quantificació del clúster 9, el qual detecta les variants més abundants del clúster (CTX-M-9, 13, 14, 16 a 19, 21 i 27).
- Detecció i quantificació per qPCR dels gens de resistència a antibiòtics (bla_{TEM}, $bla_{\text{CTX-M}}$ grups clonals 1 i 9 i *mecA*) en la fracció de DNA fàgic de mostres amb residus fecals de diferents animals per tal de determinar el paper potencial dels bacteriòfags en la disseminació de gens de resistència a antibiòtics en entorns animals.

2.2. Capítol 2: Gens de resistència a quinolones (*qnrA* and *qnrS*) en partícules de bacteriòfags de mostres d'aigua residual i efecte d'agents inductors en els gens de resistència a antibiòtics encapsidats.

Estudi 3: *Quinolone resistance genes (qnrA and qnrS) in bacteriophage particles from wastewater samples and the effect of inducing agents on packaged antibiotic resistance genes.*

En aquest estudi es van plantejar els objectius detallats a continuació:

- Disseny de la sonda i encebadors per a la qPCR dels gens de resistència a quinolones *qnrA* i *qnrS*.
- Detecció i quantificació per qPCR de dos gens de resistència a quinolones, *qnrA* i *qnrS*, en el DNA de fags de mostres ambientals amb contaminació fecal.
- Avaluació de la influència de factors inductors de fags (mitomicina C, ciprofloxacina, EDTA i citrat sòdic) i de paràmetres físics en l'abundància de gens resistència a quinolones en DNA de fags de poblacions bacterianes de mostres d'aigua residual.
- Avaluació de la influència de factors inductors de fags (mitomicina C, ciprofloxacina, EDTA i citrat sòdic) i de paràmetres físics en la capacitat infectiva dels colifags somàtics de poblacions bacterianes de mostres d'aigua residual.

2.3. Capítol 3: Avaluació dels gens de resistència a antibiòtics en la fracció de DNA bacterià i de bacteriòfags en mostres d'aigua residual de Tunísia i comparació amb els resultats obtinguts a l'àrea de Barcelona.

Estudi 4: *Antibiotic resistance genes in Tunisian and Spanish wastewaters and their use as markers of antibiotic resistance patterns within a population.*

En aquest estudi es van plantejar els objectius detallats a continuació:

- Detecció i quantificació per qPCR de tres gens codificadors de β-lactamases (bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$ i $bla_{CTX\text{-}M\text{-}9}$), dos gens de resistència a quinolones (*qnrA* and *qnrS)* i el gen *mec*A en la fracció de DNA fàgic i DNA bacterià de mostres de dues EDARs i d'un escorxador de Tunísia.
- Comparació del contingut de gens de resistència a antibiòtics en DNA bacterià i DNA fàgic de les mostres de Tunísia amb els resultats obtinguts prèviament a Barcelona.
- Avaluació de la detecció de gens de resistència a antibiòtics com a marcadors per a l'estudi dels patrons de resistència a antibiòtics en una determinada població.

2.4. Capítol 4: Detecció d'aïllaments d'*Escherichia coli* resistents a quinolones dels grups clonals O25b:H4-B2-ST131 i O25b:H4-D-ST69 en mostres ambientals de l'àrea de Barcelona.

Estudi 5: *Detection of quinolone-resistant Escherichia coli isolates belonging to clonal groups O25b:H4-B2-ST131 and O25b:H4-D-ST69 in raw sewage and river water in Barcelona, Spain.*

En aquest estudi es van plantejar els objectius detallats a continuació:

- Avaluació de la prevalença del grup clonal O25b:H4-B2-ST131 en ambients aquàtics amb contaminació fecal (aigua residual i aigua de riu) de l'àrea de Barcelona.
- Determinació del patró de sensibilitat a antibiòtics i producció d'*ESBL*s dels aïllaments d'*E. coli* O25b.
- Estudi de 30 gens de virulència dels aïllaments ambientals d'ST131.

- Comparació dels perfils de macrorestricció, gens de virulència i patrons de resistència a antibiòtics dels aïllaments ambientals de ST131 d'aquest estudi, amb aïllaments clínics humans del mateix clon causants d'infeccions extraintestinals a Espanya.

En resum, l'objectiu general d'aquesta tesi és l'avaluació de l'abundància de gens de resistència a antibiòtics de rellevància clínica en la fracció de DNA de partícules de bacteriòfags aïllades a partir de mostres ambientals de diferent origen, incloent aigua residual, aigua de riu i aigua residual animal, per tal d'estudiar el paper dels bacteriòfags com a vehicles en la mobilització de gens de resistència a antibiòtics entre bacteris.

3. PUBLICATIONS

3. PUBLICATIONS

3.1. Chapter 1: Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples (wastewater, river water and animal wastewater).

❖ ARTICLE 1

Títol: Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples

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RESUM

Introducció

La resistència a antibiòtics és un problema global que augmenta la morbiditat i mortalitat causades per les infeccions bacterianes (WHO, 1996). Molts gens de resistència a antibiòtics tenen el seu origen en microorganismes del medi ambient i poden ser transferits a bacteris que es troben en ambients clínics. La transmissió dels determinants de resistència pot tenir lloc mitjançant la transferència horitzontal de gens a través d'elements genètics mòbils com plasmidis, transposons, o com plantegem en aquest estudi, bacteriòfags (Muniesa *et al*., 2004; Witte, 2004; Brabban *et al*., 2005).

Tot i que es disposa de molta informació sobre la contribució de plasmidis i transposons en la mobilització de gens de resistència, es desconeix el paper dels bacteriòfags en aquest aspecte. Es creu que, de la mateixa manera que poden mobilitzar gens de virulència en el medi ambient (per exemple toxines), també poden participar de manera important en la transferència horitzontal de gens de resistència a antibiòtics.

Els antibiòtics β-lactàmics presenten una elevada eficàcia clínica, baixa toxicitat i són àmpliament utilitzats pel tractament d'infeccions bacterianes. Un dels mecanismes de resistència a aquest tipus d'antibiòtic en bacteris Gram-negatius és la producció de β-lactamases (Hawkey *et al*., 2009). En els darrers anys, les β-lactamases d'espectre ampliat (*ESBL*s) s'han estès àmpliament i poden ser adquirides mitjançant la transferència horitzontal de gens i mobilitzades a partir de bacteris ambientals. Concretament, bla_{TEM} i $bla_{CTX\text{-}M}$ són les famílies d'*ESBL*s més prevalents arreu.

La resistència en Gram-positius també està molt distribuïda, com és el cas de les infeccions per *Staphylococcus aureu*s resistent a meticil·lina (*MRSA*), sobretot associades a infeccions nosocomials (Ito *et al*., 2001). En els *MRSA*, el gen *mec*A, localitzat en una illa genètica mòbil (SSCmec), codifica per a una proteïna d'unió a penicil·lina que confereix resistència a meticil·lina (Ito *et al*., 2003; Lindsay i Holden, 2006).

Així doncs, tenint present que els bacteriòfags són la major part de la fracció vírica en gran part d'ambients (Dinsdale *et al*., 2008) pretenem estudiar el paper dels fags com a vehicles en la disseminació de gens de resistència a antibiòtics en el medi ambient.

Objectius

En aquest estudi es van plantejar els objectius detallats a continuació:

- Desenvolupar una metodologia per avaluar gens de resistència a antibiòtics en la fracció de DNA de bacteriòfags de mostres d'aigua ambientals.
- Disseny de la sonda i encebadors per a la qPCR del gen $bla_{CTX\text{-}M\text{-}1}$ per a l'amplificació del clúster 1, el qual consta de 31 variants, incloent CTX-M-1, 3, 10, 11 i 15.
- Detecció i quantificació per qPCR de tres gens que confereixen resistència a antibiòtics β-lactàmics, dos gens codificadors de β-lactamases (bla_{TEM} i $bla_{CTX\text{-}M\text{-}1}$) i el gen *mec*A en la fracció de DNA fàgic i DNA bacterià de mostres ambientals d'aigua residual municipal i d'aigua de riu.

- Avaluació de la capacitat dels gens de resistència a antibiòtics detectats de conferir resistència a soques hoste bacterianes sensibles.

Resultats i discussió

Es van seleccionar els gens bla_{TEM} i $bla_{CTX\text{-}M\text{-}1}$ ja que són *ESBL*s molt prevalents i àmpliament distribuïdes en la nostra zona d'estudi. El clúster 1 de CTX-M es troba molt distribuït a Espanya i a Europa, en particular CTX-M-15 és una variant que ha emergit en els darrers anys i s'ha estès molt. Es va incloure el gen *mec*A per l'augment en la incidència de les infeccions causades per *MRSA*.

Els tres grups de gens que es van quantificar per qPCR en la fracció d'DNA fàgic i DNA bacterià de 30 mostres: 15 mostres procedents d'aigua residual de l'entrada de l'estació depuradora d'aigües residuals (EDAR) de Gavà (Barcelona) i 15 d'aigua del riu Llobregat a l'entrada de Sant Joan Despí (Barcelona).

Cal remarcar que en tot moment es van realitzar els controls necessaris per garantir que el DNA bacterià no encapsidat no es va amplificar (processos de filtració, tractaments amb cloroform i DNAasa).

Els tres gens de resistència a antibiòtics van ser detectats per qPCR en ambdós tipus de mostra.

Es van trobar entre 10^2 i 10^4 còpies de gen/mL (CG/mL) de bla_{TEM} en la fracció de DNA fàgic d'aigua residual mentre que en aigua de riu els valors per aquest gen es trobaven de mitjana un ordre de magnitud per sota (Figura 2). Comparativament, com era d'esperar, els valors obtinguts en DNA de fags van ser inferiors als de DNA bacterià, essent estadísticament significatives les diferències detectades. Tot i així, les mostres 3 i 9 en aigua residual i la mostra 2 en aigua de riu van presentar una concentració de bla_{TEM} més elevada en DNA de fags que en DNA bacterià.

En aigua residual es van detectar de mitjana entre 1.5-3 unitats logarítmiques de còpies de $bla_{CTX\text{-}M\text{-}1}$ en DNA fàgic per mL d'aigua residual i menys d'1 unitat logarítmica per mL d'aigua

de riu. En aquest cas, les diferències de nombre de còpies de *bla*$_{CTX-M-1}$ detectades en aigua residual i de riu van ser significatives. En DNA bacterià, el nombre de còpies de *bla*$_{CTX-M-1}$ va ser de fins a 4 log_{10} per mL d'aigua residual i de quasi 3 log_{10} per mL d'aigua de riu (Figura 4).

Per *mec*A els valors en DNA bacterià en mostres d'aigua residual van ser superiors que en aigua de riu, però en DNA fàgic algunes mostres tenien valors inferiors en aigua residual. Per altra banda, la variabilitat del contingut de *mec*A en DNA fàgic i bacterià en mostres d'aigua residual va ser major que en les d'aigua de riu. De mitjana, els valors de nombre de còpies del gen *mec*A detectats en DNA fàgic en aigua residual i aigua de riu van ser bastant similars, fet que suggeriria que el gen *mec*A detectat provindria de fags d'origen diferent als que trobem en ambients amb contaminació fecal humana però les dades obtingudes no permeten discernir-ne l'origen (Figura 5).

Posteriorment a la detecció i quantificació dels tres gens de resistència en DNA fàgic es va voler avaluar la capacitat d'aquests gens de resistència a antibiòtics de conferir resistència a soques hoste bacterianes sensibles. Per això, es va procedir a la transfecció de DNA fàgic portador dels gens de resistència quantificats a les soques hoste *E. coli* C600 i *E. coli* WG5, ambdues sensibles a ampicil·lina i resistents a àcid nalidíxic. Després es van seleccionar els tranfectants ampicil·lina i àcid nalidíxic resistents i es va fer una avaluació de les colònies resistents que presentaven els gens de resistència mitjançant PCR convencional.

Es van detectar més clons resistents quan es van utilitzar com a soca hoste *E. coli* WG5 que *E. coli* C600. *bla*$_{TEM}$ es va trobar en major percentatge que *bla*$_{CTX-M-1}$ i *bla*$_{CTX-M-1}$ no es va detectar en *E. coli* C600. En cap cas es van presentar ambdós gens en un mateix clon resistent. En tot cas, aquests experiments demostraven que els gens presents al DNA fàgic eren funcionals i capaços de generar resistència.

Per últim, es van intentar diversos experiments de transducció de les partícules fàgiques portadores de gens de resistència a antibiòtics detectats utilitzant *E. coli* com a soca hoste però malauradament no es van poder obtenir transductants. Cal tenir present que en el procés de transducció intervenen diversos factors limitants com són la presència d'una soca hoste adequada i sensible i d'unes condicions òptimes per a la transducció. A més a més, el

fet que els fags amb resistència a antibiòtics no hagin de ser necessàriament partícules infeccioses fan que l'obtenció de transductants sigui una tasca encara més dificultosa. També, la presència a les mostres de molts fags lítics per les soques receptores compliquen enormement la selecció de transductants, si la transducció té lloc a baixa freqüència.

Conclusions

Els tres gens de resistència a antibiòtics quantificats per qPCR van ser detectats en DNA fàgic tant en les mostres d'aigua residual com en aigua de riu. El gen bla_{TEM} va ser el que va presentar les densitats més elevades, seguit de $bla_{CTX\text{-}M\text{-}1}$ i *mec*A.

Els gens de resistència a antibiòtics detectats en DNA fàgic són capaços de conferir resistència a una soca hoste bacteriana sensible.

Aquest estudi demostra que els gens de resistència a antibiòtics es poden detectar en DNA fàgic, i per tant, que els fags poden actuar com a reservori de gens de resistència a antibiòtics al medi ambient.

Informe sobre el factor d'impacte de l'article 1

L'article ***Antibiotic resistance genes in the bacteriophage fraction of environmental samples*** va ser publicat online el 3 de març de 2011 a la revista *PloS ONE* que es troba inclosa en el primer quartil (Q1) de l'àrea temàtica de *Biology* (12/85). L'any 2011 la revista *PloS ONE* va presentar un factor d'impacte de 4.092.

En el moment de la presentació d'aquest informe aquest article ha estat citat 36 vegades segons Web of Science.

Informe de participació de l'article 1

La doctoranda Marta Colomer Lluch ha realitzat el disseny de la sonda i encebadors per a la qPCR del gen $bla_{CTX\text{-}M\text{-}1}$, així com el disseny dels encebadors per a l'amplificació per PCR convencional dels gens bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$ i *mec*A. És també responsable de la recollida i transport de les mostres, de l'anàlisi dels paràmetres microbiològics i del processament de les mostres. La doctoranda ha dut a terme les extraccions de DNA bacterià i DNA fàgic de les mostres, la seva quantificació i la posterior anàlisi i processat de les dades obtingudes. També ha contribuït en la realització dels experiments de microscòpia electrònica. Finalment, ha participat en la redacció de l'article i en l'elaboració de taules i figures així com en la seva difusió en diverses ocasions.

Dr. J. Jofre Dra. M. Muniesa

PLoS one

Antibiotic Resistance Genes in the Bacteriophage DNA Fraction of Environmental Samples

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Abstract

Antibiotic resistance is an increasing global problem resulting from the pressure of antibiotic usage, greater mobility of the population, and industrialization. Many antibiotic resistance genes are believed to have originated in microorganisms in the environment, and to have been transferred to other bacteria through mobile genetic elements. Among others, β-lactam antibiotics show clinical efficacy and low toxicity, and they are thus widely used as antimicrobials. Resistance to β-lactam antibiotics is conferred by β-lactamase genes and penicillin-binding proteins, which are chromosomal- or plasmid-encoded, although there is little information available on the contribution of other mobile genetic elements, such as phages. This study is focused on three genes that confer resistance to β-lactam antibiotics, namely two β-lactamase genes (blaTEM and blaCTX-M9) and one encoding a penicillin-binding protein (mecA) in bacteriophage DNA isolated from environmental water samples. The three genes were quantified in the DNA isolated from bacteriophages collected from 30 urban sewage and river water samples, using quantitative PCR amplification. All three genes were detected in the DNA of phages from all the samples tested, in some cases reaching 104 gene copies (GC) of blaTEM or 102 GC of blaCTX-M and mecA. These values are consistent with the amount of fecal pollution in the sample, except for mecA, which showed a higher number of copies in river water samples than in urban sewage. The bla genes from phage DNA were transferred by electroporation to sensitive host bacteria, which became resistant to ampicillin. blaTEM and blaCTX were detected in the DNA of the resistant clones after transfection. This study indicates that phages are reservoirs of resistance genes in the environment.

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Introduction

Recognized as a global problem [1], antibiotic resistance increases the morbidity and mortality caused by bacterial infections, as well as the cost of treating infectious diseases. The threat from resistance (particularly multiple resistance in bacterial strains that are widely disseminated) is serious. The key factors contributing to this threat are the pressure of increased antibiotic usage (in both human and animal medicine), greater mobility of the population and industrialization [2,3]. Many potentially life-threatening infections, generally regarded as diseases from the past due to the success of antibiotics and vaccines, have returned as resistance increasingly hampers successful therapy and prophylaxis [4].

Microorganisms produce many antimicrobials in nature [5,6]. These antibiotic-producing organisms have also become resistant to the antibiotics they produce, and the genes that confer such resistance can be transferred to other non-resistant bacteria. The presence of antibiotics in the environment may provide long-term selective pressure for the emergence and transmission of these resistance-conferring genes in non-producing organisms [5,7]. Given that many genera found in diverse environments carry resistance determinants [6], it is feasible that antibiotic-resistance genes have originated in the environment and that they could have been transferred from the environment to pathogenic bacteria, which are currently found in clinical settings. [8]. The transfer from the environment to clinical settings might have occurred through horizontal gene transfer, which is the most effective mechanism to accelerate the dispersal of antibiotic-resistance genes. The mobile genetic elements (MGEs) for the horizontal transfer of such genes most commonly studied are plasmids, transposons or, as a few reports suggest, bacteriophages [9–11].

Several studies have focused on antibiotic resistance codification in plasmids or transposons, and there is also interesting information about the extent of antibiotic resistance genes in a given environment (the so-called "resistome") [8,12]. However, there is less information on the potential contribution of phages to antibiotic resistance-gene transfer, despite calls for research in this field. Recent reports [2,11] conclude that the horizontal transfer of genetic information by phages is much more prevalent than previously thought, and that the environment plays a crucial role in the phage-mediated transfer of antibiotic-resistance genes [2,13]. Since many antibiotic resistance genes are plasmid-encoded, much effort has been devoted to the study of plasmids and less to the study of phages carrying genes for antibiotic resistance. However, many reports available suggest that phages can mobilize resistance genes and confer resistance, and some authors suggest that mobilization can occur through generalized transduction [14–18]. Only a few reports have analyzed antibiotic resistance genes in phage DNA isolated from wastewater environments [9,19].

β-lactam antibiotics are characterized by clinical efficacy and low toxicity and they are thus widely used as antimicrobials. One mechanism of resistance to β-lactam antibiotics in Gram-negative bacilli involves the production of β-lactamases [3]. Among other Gram-negative bacteria, members of the family *Enterobacteriaceae* commonly express plasmid-encoded β-lactamases (e.g. TEM/SHV), which confer resistance to penicillins. More recently, extended-spectrum β-lactamases (ESBLs) evolved, conferring resistance to penicillins and oxymino-cephalosporins. EBSLs are sometimes mutant derivatives of TEM/SHV, but they are also mobilized from environmental bacteria (e.g. CTX-M) [20]. Most β-lactamases are acquired by horizontal gene transfer and the novel β-lactamase genes that emerge dramatically spread worldwide, causing both nosocomial and community-onset infections [3].

Resistance in Gram-positive bacteria is also widely distributed and increasing. This is the case for the emergence of community-associated methicillin-resistant *Staphylococcus aureus* (MRSA), a development that has blurred the distinction between hospital and community strains [21]. In *S. aureus*, *mec*A, a gene encoding for a penicillin-binding protein that confers resistance to methicillin, is located on a mobile genomic island, the Staphylococcal Cassette Chromosome mec (SCCmec) [22,23]. In addition to the resistance genes carried on SCC*mec*, *S. aureus* can also harbor resistance genes on other sites of the genome, such as Tn*554*, as well as on plasmids [23]. Antibiotic use and environmental factors contribute to the emergence and spread of resistance in *S. aureus*, which is a common cause of serious and life-threatening infections.

Here we focused on two β-lactamases (bla_{TEM} and $bla_{\mathrm{CTX\text{-}M}}$) and a penicillin-binding protein (*mec*A). bla_{TEM} belongs to class A serine β-lactamases, which have been described in epidemiological studies; $bla_{\mathrm{CTX\text{-}M}}$ and bla_{TEM} are the most prevalent broad-spectrum β-lactamases and the most widely distributed enzymes worldwide [24–26]. *mec*A was included in this study because of the increasing incidence of infections caused by MRSA. The three genes were quantified by real-time PCR in the viral DNA fraction of water samples contaminated with fecal pollution. Since in most environments studied, phages are the main part of the viral fraction [27], it can be assumed that the DNA isolated from the viral fraction will belong mostly to bacteriophages. We sought to highlight the potential role of phages in the spread of these genes in the aquatic environment.

Results

Microbiological parameters

The numbers of aerobic bacteria and *Escherichia coli* were relatively homogeneous in all the urban sewage and river water samples tested (Table 1). These values were in accordance with previous water analyses from the same source [28–30]. River water samples showed significantly lower numbers ($P<0.05$) than urban sewage and these differences are attributed to the lower fecal input received by river water. The numbers of resistant bacteria were slightly lower than the total bacteria, as expected. Since bacteria are difficult to recover from the environment because of the stressed conditions of bacterial cells, the method and the low concentration of ampicillin (35 mg/ml) used were intended to prevent the inhibition of growth. Similar concentrations of ampicillin were reported before for the isolation of ampicillin-resistant bacteria [31]. We further tested 10% of all the colonies isolated in LB agar plates (35 mg/l) for sensitivity at higher concentrations of ampicillin (100 mg/l). At this concentration all the isolates were resistant to the antibiotic.

Table 1. Samples analyzed and microbiological parameters.

Sample	Urban sewage		River	
	Average $\log_{10}$ CFU/ml	SD	Average $\log_{10}$ CFU/ml	SD
N	15		15	
Aerobic bacteria	6.47	0.32	3.71	0.37
E. coli	4.75	0.64	1.22	0.56
S. aureus	2.29	0.36	1.88	0.11
Aerobic bacteria ap^R	6.22	0.24	3.12	0.45
E. coli ap^R	4.14	0.34	0.80	0.56
S. aureus met^R	1.51	0.20	0.00	-
Somatic coliphages[a]	4.43	0.30	2.42	0.39

[a]PFU/ml.

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To determine the number of *S. aureus* strains in the samples, 25% of the yellow-pigmented colonies obtained in each plate of agar 110 medium were further confirmed by catalase and with the Slidex Staph Plus kit. Depending on the plates, from 80% to 90% of the colonies were confirmed as *S. aureus*. The numbers of these bacteria presented (Table 1) are a correction of the percentage of positive colonies among the total number of yellow colonies detected in the agar plate. We detected S. *aureus* MRSA in sewage but not in river water.

Somatic coliphages, proposed as viral fecal indicators of pollution [29], were analyzed to determine the presence of bacteriophages infecting *E. coli* in the samples studied. As for bacterial indicators, the numbers of somatic coliphages were relatively homogeneous in all the samples tested (Table 1) and also in accordance with previous analyses of samples from the same source [28–30].

Direct observation of bacteriophages in sewage and river water

In addition to the evaluation of infectious somatic coliphages in the samples, direct observation of bacteriophages present in the water samples was conducted by electron microscopy. Tailed bacteriophages (Figure 1) belonging to different morphological types were observed, with a greater abundance of phages with contractile tail with *Myoviridae* morphology and non-contractile tail with *Siphoviridae* morphology. Variations in capsid and tail size were observed, as expected for bacteriophages that can infect different bacterial genera. Non-tailed virus particles were also observed, although in this case it could not be determined by morphology whether they were bacterial viruses or viruses infecting other hosts.

Antibiotic resistance genes in the phage and bacterial fraction of sewage and river water bla_{TEM} genes

The set of primers and probe used [25], which included amplification of more than 145 TEM variants, allowed efficient screening of bla_{TEM} genes in the environmental samples. From 10^2 to10^4 bla_{TEM} gene copies (GC) were detected in the phage DNA fraction of one ml of urban sewage (Figure 2), while in river water the average was one order of magnitude lower. In both types of sample, these values indicate that phage DNA contains a large number of bla_{TEM} gene copies. As explained in the methods

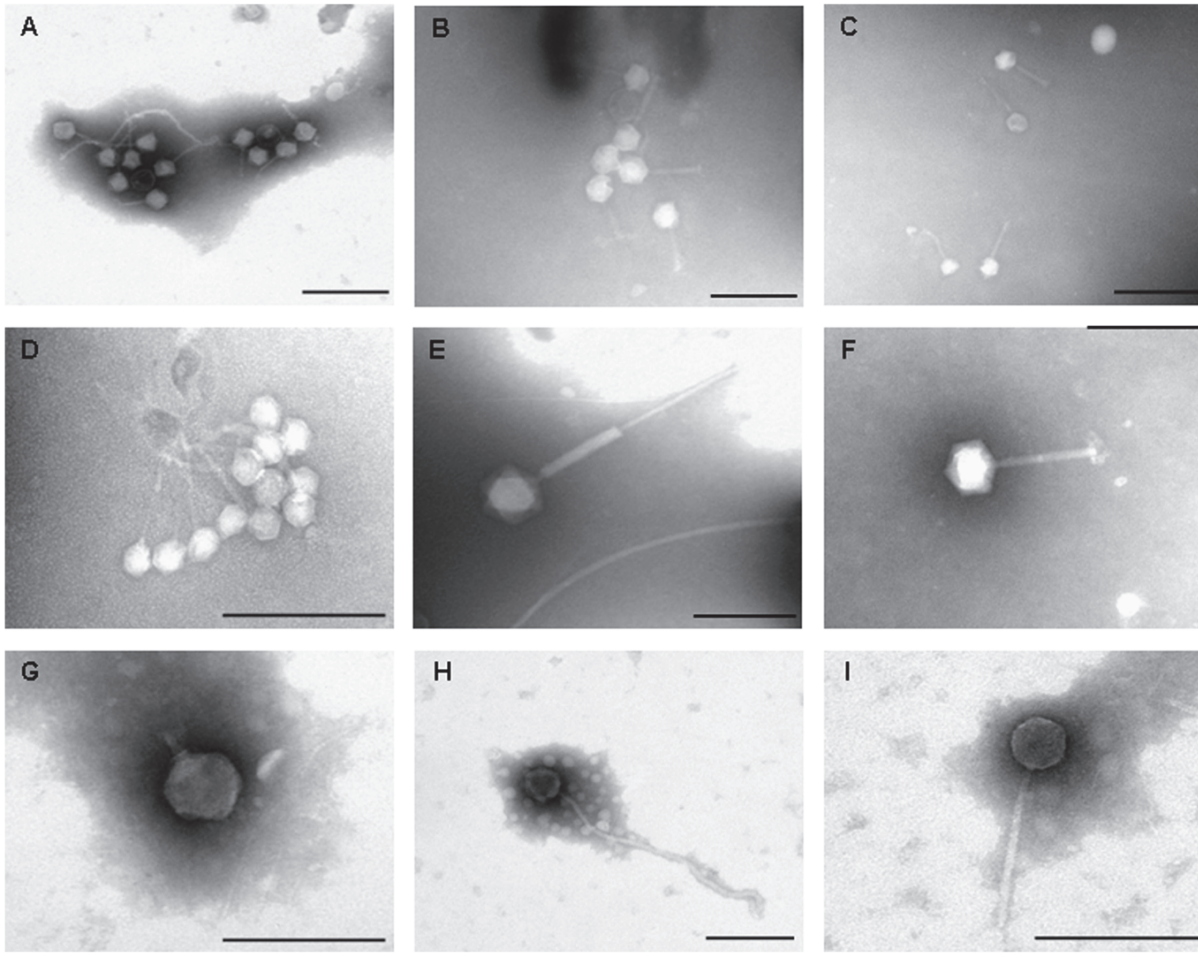

Figure 1. Electron micrographs of bacteriophages present in sewage and river water. A–B. Group of phages with *Myoviridae* and *Siphoviridae* morphology from sewage. C. *Myoviridae* phages from river water. D: group of *Siphoviridae* phages from sewage. E–F. *Myoviridae* phages from sewage. G: *Podoviridae* phage from sewage. H–I. *Siphoviridae* phages from sewage and river water respectively. Bar 200 nm.
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section, a careful approach was performed to rule out that DNA from a non-viral origin was amplified in the qPCR, and controls were performed during phage DNA extraction. To this end, controls of the samples, taken after DNase treatment, but before the phage DNA was extracted from the capsid, were used as template for conventional PCR for eubacterial 16S rDNA and for qPCR for the three antibiotic resistance genes. These controls showed negative values for eubacterial 16SrDNA as well as for the three antibiotic resistance genes, which confirmed that the samples were free of bacterial DNA or non-encapsidated DNA, and that our results were due to amplification of DNA located within the viral particles. These controls were performed in all the samples tested.

The number of copies of bla_{TEM} genes detected in the phage DNA fraction of the samples were, as expected, lower than in bacterial DNA; however, this difference was less than one order of magnitude (Figure 2). Differences in GC/ml found between bacterial and phage DNA were significant ($P<0.05$). Moreover, a few samples (Samples 3 and 9 in sewage and Sample 2 in river water, Figure 2) showed a higher concentration of bla_{TEM} in phage DNA than in bacterial DNA.

$bla_{\mathrm{CTX\text{-}M}}$ genes

To our knowledge, quantitative real-time PCR probes that are universal for the most common variations of $bla_{\mathrm{CTX\text{-}M}}$ genes have not previously been reported, and so a primer set for these genes was developed in this study. The nucleotide sequence for diverse $bla_{\mathrm{CTX\text{-}M}}$ genes was aligned in a search for common sequences. As expected, the five clusters described for the CTX-M family did not share conserved regions (see references [32,33] for review and presentation of a CTX-M cluster), so it was impossible to design a common qPCR for all the CTX-M variants. We selected Cluster 1 (composed of 31 variants described so far, including CTX-M-1, 3, 10, 11 and 15) [34], which is widespread in Europe and Spain [25,35]. Alignment of some CTX-M Cluster 1 sequences (Figure 3) showed several regions from which primers and probe can be selected according to the requirements for the design of primers and probes for qPCR, established in the Primer Express Software version 3.0 (Applied Biosystems). The Taqman PCR assay developed was valid for quantitative measurements of all Cluster 1 CTX-M variants assayed, except CTX-M-12, 30 and 60, which did not match the sequence of the lower primer (Figure 3). Standard curves were repeatable and the amplification

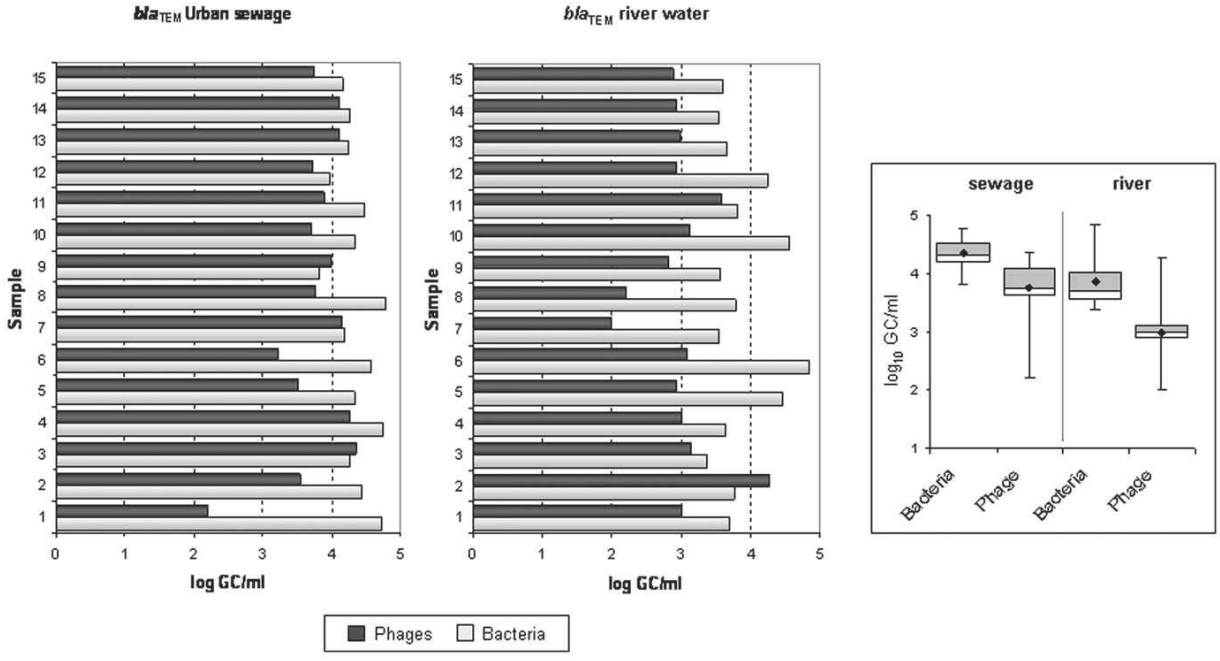

Figure 2. Number of copies of bla_{TEM} genes (GC/ml) in urban sewage and river water samples in phage and bacterial DNA. On the left side of the figure, bar chart of the gene copies detected for each sample, dark grey for phage DNA and light grey for bacterial DNA. On the right side of the figure, the box plot chart shows the averaged values obtained from all samples from the same origin. Within the box plot chart, the cross-pieces of each box plot represent (from top to bottom) maximum, upper-quartile, median (black bar), lower-quartile, and minimum values. Black diamond shows the mean value. The grey boxes in the box plot chart include samples showing values within the 75th percentile and white boxes samples showing values within the within the 25th percentile.
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efficiency (*E*) of our reactions ranged from 95%-100%. Controls performed with several *E. coli* strains harboring different CTX-M genes from Cluster 1 confirmed the validity of the qPCR set designed.

The number of copies of $bla_{CTX\text{-}M}$ detected in phage DNA in sewage ranged from 1.5 to 3 $\log_{10}$ units, while fewer than one $\log_{10}$ units were still detected in one ml of river water (Figure 4). Differences between the number of copies of the $bla_{CTX\text{-}M}$ genes in

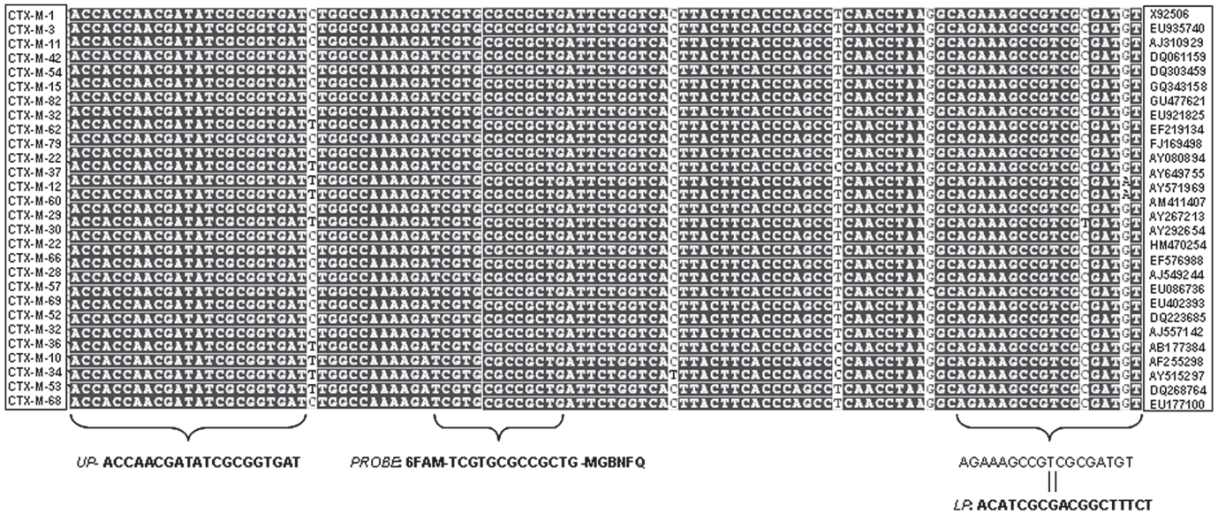

Figure 3. Primers and TaqMan assay probe resulting from the alignment of $bla_{CTX\text{-}M}$ genes from Cluster 1. Conserved nucleotides are marked in bold, non-conserved nucleotides in white. Sequence reverse and complementary is shown for lower primer. Right column indicate the GenBank accession number of each gene.
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phage and bacterial DNA were significant ($P<0.05$) in both sewage and river water. The number of copies of the gene detected in phage DNA were from <1 to 2.5 $\log_{10}$ units lower than in bacterial DNA, with few exceptions (sewage sample 6). The number of copies of the $bla_{\text{CTX-M}}$ genes in bacterial DNA was as high as 4 $\log_{10}$ units in 1 ml of sewage and almost 3 $\log_{10}$ units in 1 ml of river water.

mecA

All samples showed the presence of *mec*A in either bacterial or phage DNA. While values in bacterial DNA were higher in sewage, in phage DNA the average and also inter-sample comparison showed that some samples of sewage presented lower values than river water samples. The variability of the *mec*A content in phage and bacterial DNA in urban sewage samples was greater than in river samples (Figure 5).

Ability of phage-encoded genes to confer antibiotic resistance in bacterial strains

To evaluate whether the antibiotic resistance sequences in phage DNA correspond to potential active genes able to confer resistance in a bacterial background, phage DNA from sewage samples 6, 7 and 15, was transfected in two *E. coli* recipient hosts (C600nal^{R} and WG5), both of which nalidixic acid-resistant and ampicillin-sensitive. After transfection, *E. coli* colonies were selectively grown in Chromocult ap/nal plates (Table 2). 25% of the ap/nal resistant *E. coli* colonies in each plate were randomly selected and analyzed for *bla* genes using conventional PCR with the respective primers (Table 2) and confirmed by sequencing. More ap-resistant clones were detected using WG5 as recipient than C600nal^{R}. Analysis of the *bla* genes located in each clone showed from 0–10% of the clones harbouring bla_{TEM} or $bla_{\text{CTX-M}}$. Among these, more clones harboring bla_{TEM} and $bla_{\text{CTX-M}}$ were also found with WG5. bla_{TEM} was detected in a greater percentage of colonies than $bla_{\text{CTX-M}}$ in both host strains and no clones were detected for $bla_{\text{CTX-M}}$ in C600nal^{R} on two of the three samples assayed (Table 2). Accordingly, the densities of bla_{TEM} genes in the sewage samples used were greater than densities of $bla_{\text{CTX-M}}$ (Figs. 2 and 4 respectively). Both genes were never detected simultaneously in a single clone. Other clones showing nal/ap resistance were not harboring the two *bla* genes analyzed, suggesting that other gene conferring ampicillin resistance could have been transferred.

Discussion

Genes of antibiotic resistance are present in bacterial chromosomes and they are detected in plasmids when analyzed in clinical settings, but there is controversy as to how these genes originate and how they reach the pathogenic strains found in hospitals. Several authors indicate a plausible environmental origin of these genes, and we suggest here that phages could be suitable candidates as intermediates between the original bacteria and the clinical isolate.

The genes examined in the present study are the most widely distributed. TEM has been reported worldwide [36] and CTX-M is currently the most widespread and threatening mechanism of antibiotic resistance, particularly in community-acquired infections [25]. The qPCR set designed for CTX-M detected one of the five main clusters described for $bla_{\text{CTX-M}}$ genes [20]. Cluster 1 is one of the most diversified groups, which is of particular interest because of the recently described international spread and changing epidemiology of clones carrying the CTX-M-15 variant [3,5,34,37]. Although the qPCR set detected other types in addition to type 15, the prevalence of $bla_{\text{CTX-M-1}}$ in phage DNA (Figure 4) indicates this cluster is abundant in environmental phage DNA. Our results may be applicable to other CTX-M clusters, and it is feasible that the other clusters would also be detectable in phage DNA. Recent studies suggest that the CTX-

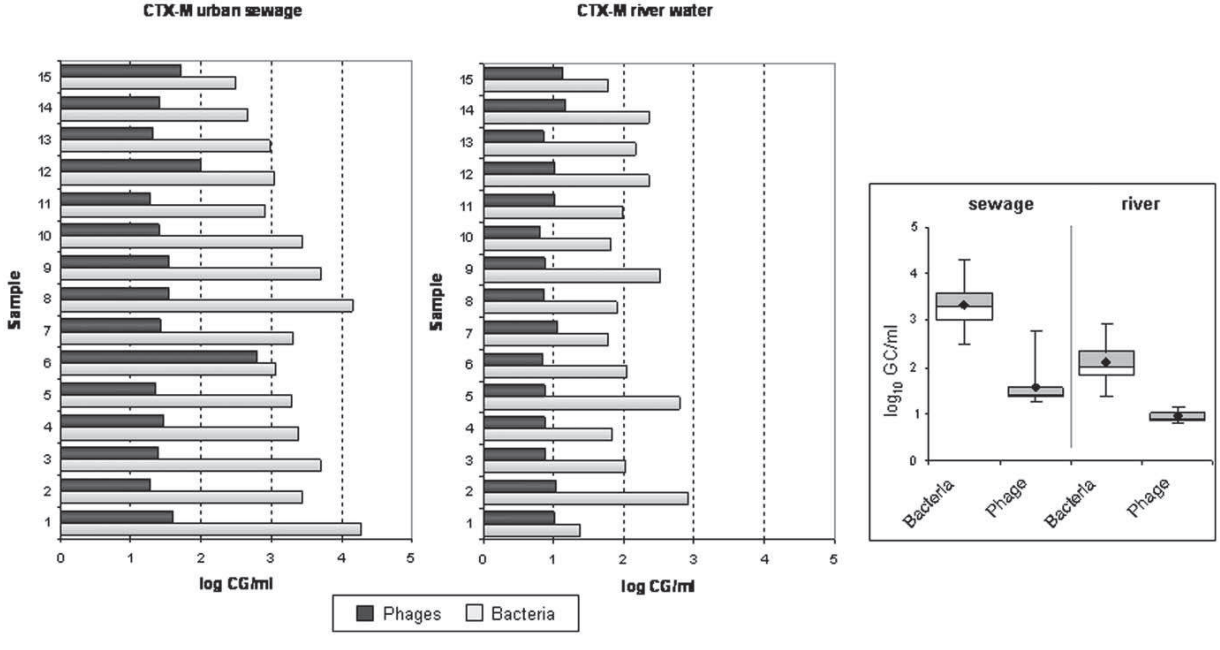

Figure 4. Number of copies of $bla_{\text{CTX-M}}$ genes (GC/ml) in urban sewage and river water samples in phage and bacterial DNA and box plot of averaged values.
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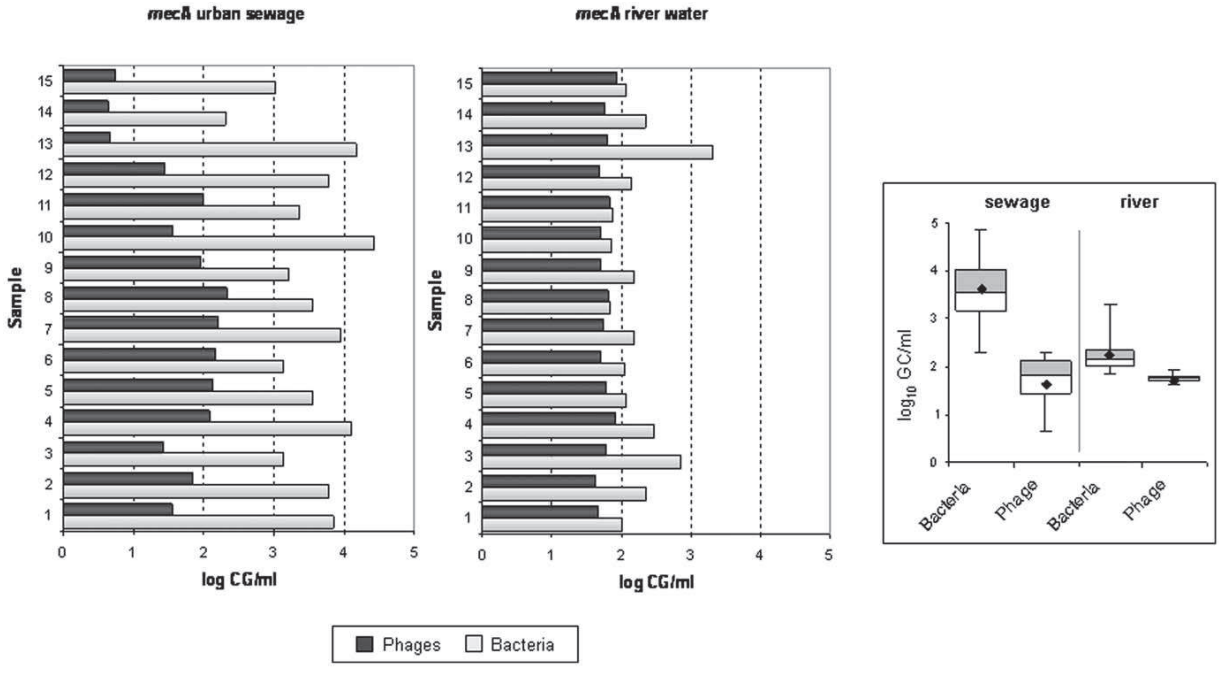

Figure 5. Number of copies of *mec*A (GC/ml) in urban sewage and river water samples in phage and bacterial DNA and box plot of averaged values.
doi:10.1371/journal.pone.0017549.g005

M-type derives from chromosomal genes from several *Kluyvera* species and that it is rapidly mobilized from these species to a number of genetic platforms [20], such as insertion sequences, integrons, transposons and plasmids.

We detected MRSA in sewage. although in other studies *Staphylococcus* was not detected in municipal wastewater [38], or it was detected but not quantified [39]. The results of *mec*A in phage DNA showed a lack of correlation with fecal pollution in the samples, since averaged values of sewage and river water were similar. This suggests that the *mec*A detected came from phages other than those found in human fecal pollution. Although the sewage samples analyzed contain exclusively human fecal pollution river samples in this study carried mostly human fecal pollution but also some animal fecal pollution [29], as well as autochthonous freshwater bacteria. Since previous experiments with these urban sewage samples indicated that the values of fecal pollutants are highly consistent over time [30], the variability in the number of copies of the *mec*A detected in phages supports the hypothesis of an origin other than the human fecal load. Our results do not allow us to discern whether the gene derives from animals or autochthonous microorganisms.

S. aureus can mobilize fragments of its chromosome, the pathogenicity islands, or with helper phages [40]. The transfer of *S. aureus* phages into and out of isolates may occur in nature or during the course of colonization or infection of patients [23]. The number of copies of *mecA* detected in phage DNA supports our hypothesis that, regardless of its origin, *mec*A is located in phages in aquatic environments. This wide spread of *mec*A could have

Table 2. Transfection of phage DNA isolated from sewage in *E. coli* WG5 and C600 strains.

		Sample number		
		Sewage 6	**Sewage 7**	**Sewage 15**
	μg of phage DNA transfected	2.60	1.14	1.74
Ampicillin/Nal WG5	N° of ap/nal resistant clones[a]	552	422	310
	% bla_{TEM}[b]	10.0	13.6	16.6
	%bla_{CTX-M}[b]	6.8	1.7	13.3
Ampicillin/Nal C600nal^R	N° of ap/nal resistant clones[a]	89	101	42
	% bla_{TEM}	3.6	7.7	6.2
	%bla_{CTX-M}	0	1.2	0

[a]Averaged number of colonies per plate after transduction.
[b]Percentage of colonies where these genes have been detected by PCR and confirmed by sequencing.
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influenced the emergence of community-acquired strains, which are responsible for serious diseases in healthy individuals [41].

The occurrence of antibiotic resistance genes in the viral DNA fraction of water samples provides new insights into the extent to which ecosystems serve as pools of resistance genes and suggests that phage DNA can act as reservoirs of these genes. However, our results do not indicate whether these genes confer resistance in a given bacterial host. To elucidate this point, a set of experiments aiming to transduce the genes from phage particles isolated from the samples in *E. coli* was attempted. Unfortunately, as shown in other studies [42], this approach might need to identify a suitable and sensitive host strain (*E. coli* or others) that would support infection with these phages and subsequent transduction. The search for the suitable host and the right conditions for transduction to occur is likely a complicated task. Moreover, the phages in which antibiotic resistance genes were detected are not necessarily infectious particles. We were therefore unable to achieve transduction of the antibiotic resistance to a bacterial host strain (data not shown), although more efforts will be made to pursue this objective.

However, we were able to demonstrate that the sequences corresponding to resistance genes detected in phage DNA can confer resistance to a recipient bacteria. Using *E. coli* as a Gram-negative host we generated resistant clones after transfection of phage DNA. This approach avoids the requirement of a suitable host strain and the need for phage infectivity, and only requires a suitable genetic background in which the genes can be expressed. The bla_{TEM}, $bla_{\text{CTX-M}}$ genes were transferred into the host strains, which then became resistant to the respective antibiotics after transfection of environmental phage DNA. This demonstrates that these genes can be expressed in a bacterial genetic background.

A similar experimental approach was attempted with environmental phage DNA carrying *mec*A in an *S. aureus mec*A^{-} strain, although no methicillin-resistant colonies were obtained (data not shown). This is not surprising since methicillin resistance is conferred by acquisition of the SCC*mec* element, which includes a type-specific *ccr* complex, and the *mec* complex, which includes *mec*A and its regulatory genes [22,43]. Although a complete SCC*mec* element may not be needed, at least a complete *mec* complex seems to be necessary for the expression of methicillin-resistance. *mec*A is always localized within *mec* complexes in all reported MRSA isolates and it is never transferred alone. The various SCCmec elements are between 21 and 67 kb, so it is unlikely that a phage would carry such a long, active SCC*mec* element, which could then be transferred and confer resistance.

Several reports relate wastewater and antibiotic resistance [39,44,45]. Many characteristics of wastewater make it a highly suspect medium for the spread of antibiotic resistance genes, i.e., the presence of antibacterials from household products (soaps, detergents, etc.), the presence of antibiotics that have been excreted by humans or disposed of down the drain, and a high bacterial load. The evolution of MGEs, which allow horizontal gene transfer, depends on the selective forces operating on them, independently of the host strain. However, these elements often encode products with a selective value for the host, and bacteria increase their fitness and diversity when they acquire these elements. In this case, the incorporation of antibiotic resistance in environments with high antibacterial concentrations would guarantee the survival of the bacterial host.

There are only a few examples of antibiotic resistance genes identified as elements of phage chromosomes. However, phages mobilize antibiotic resistance genes through generalized transduction, as reported in several bacterial genera [14–16]. Other "phage-like particles" may also be responsible for the spread of antibiotic resistance genes [46]. *In vitro*, phages transduce resistance to imipenem, aztreonam and ceftazidime in *Pseudomonas aeruginosa* by generalized transduction [12]. The epidemic strain *Salmonella enterica* serovar Typhimurium DT104, characterized by various multiresistance patterns, transduces some of the resistance genes [14]. *Bacillus anthracis* temperate phage encodes demonstrable fosfomycin resistance [47]. Since 1970s evidence has been presented that prophages participate in the dissemination of erythromycin-resistance phenotype *Streptococcus* infections [17,48]. The *mefA* gene, encoding macrolide resistance, is associated with a 58.8-kb chimeric genetic element composed of a transposon inserted into a prophage in *S. pyogenes* [18]. The *ermA* gene, a erythromycin resistance determinant, is located on an integrated conjugative element present in Streptococcus strain GAS [49]. These mobile elements identified for macrolide transfer can contribute to mobilization of the genes studied here. However experimental identification of the resistance determinant within a phage is needed.

Other indirect evidence for beta-lactam antibiotics mobilized by phages has been reported. CTX-M-10 was linked to a phage-related element which disseminates among *Enterobacteriacea* in a hospital [46]. We agree with these authors that the transfer of $bla_{\text{CTX-M-10}}$ from the chromosome of *Kluyvera* spp. to a transferable plasmid may have been mediated by transduction by a phage. Genetic analyses of *Kluyvera* phages revealed high homology with phages infecting *E. coli* [50]. This observation indicates that recombination between the two phages facilitated gene exchange between these bacterial genera. In 1972, Smith [51] reported ampicillin resistance conferred by phage infection, but these studies were not pursued. We previously described the presence of phages encoding sequences of $bla_{\text{OXA-2}}$, $bla_{\text{PSE-1}}$ or $bla_{\text{PSE-4}}$ *and* bla_{PSE}-type genes in sewage. This was the first report of the contribution of phages to the spread of β-lactamase genes in the environment [9], although the genes detected were not quantified.

Phages, either lytic or temperate, usually persist better in water environments than their bacterial hosts do [28,29]. This higher survival makes them suitable candidates for transferring genes among bacteria. Due to the structural characteristics of phages, their persistence in the environment is higher than free DNA (either linear fragments or plasmids), which is more sensitive to nucleases, temperature, predation and radiation [52–54]. This observation supports the notion that the contribution of phages to gene transfer in natural extra-intestinal environments and in human-generated environments is greater than that of plasmids or transposons. Plasmids and transposons may be the main routes for antibiotic resistance transfer In clinical settings. However, the fact that they are degraded faster than phages limits their role as MGEs in the environment.

The presents study shows that phages carry antibiotic resistance genes able to confer resistance to a bacterial strain. The possibility of transfer of these genes that lead to the emergence of new clones will depend on the susceptibility of infection of the recipient strains as well as the environmental conditions, but it could be assumed that it is likely to occur, although probably at a low frequency. In-depth analysis of the environmental dissemination of phages carrying antibiotic resistance genes outside the clinical setting could increase information about the antibiotic resistance genes circulating among the healthy human population, and their influence on the generation of resistance in the environment. Antibiotic resistance will continue to develop more rapidly than the new antimicrobial agents generated to treat infections, and mobilization through MGEs ensures dissemination of these genes. In many examples, the presence of antibiotics will increase SOS responses, which allows the mobilization of MGEs carrying

antibiotic resistance genes, thereby ensuring their own dissemination [55]. It is, therefore, crucial to determine the mechanisms behind the spread of antibiotic resistance genes and to identify the new genes before they become a public health problem.

Materials and Methods

Bacterial strains, bacteriophages and media

E. coli strain C600 containing pGEM vector was used as a control for bla_{TEM}. *E. coli* strains isolated from sewage during this study were used as controls for $bla_{CTX\text{-}M}$ genes carrying types CTX-M 1, 3, 10, 11, 15 and 34. *S. aureus* MRSA isolated from a human patient was used as a positive control for *mec*A. *E. coli* strain WG5 (a nalidixic acid-resistant mutant) (ATCC 700078) (anonymous) and strain C600nal^R [56] were used as host for transfection experiments.

Luria-Bertani (LB) agar or broth was used for routine bacterial propagation. Chromocult® Coliform Agar (Merck, Darmstadt, Germany) and Staphylococcus Medium 110 (Difco Laboratories, France) were used to evaluate background flora. When necessary, media were supplemented with ampicillin (35 mg/l or 100 mg/l), 10 mg/l methicillin, or nalidixic acid (25 mg/l) (Sigma-Aldrich, Steinheim, Germany).

Samples

Urban sewage. We used 15 sewage samples collected from the influent of an urban sewage plant that serves the urban area of Barcelona, including a number of cities and towns, of approximately 500 000 inhabitants. Samples were collected regularly approximately every 15 days over six months.

River sample. Fifteen samples were collected from the Llobregat river, near Barcelona, a watercourse that receives mixed human and animal contamination. Samples were collected regularly approximately every 15 days over six months.

Microbiological parameters

Aerobic bacteria present in the samples and grown in TSA were evaluated by performing decimal dilutions of the sample in PBS, plating 0.1 ml of each dilution in TSA and incubating plates in aerobic conditions at 37°C for 18 h. *E. coli* was detected using Chromocult as an indicator of bacterial fecal pollution by the membrane filtration method, as described elsewhere [57]. Somatic coliphages, proposed as indicators of viral fecal pollution [58], were enumerated using the ISO method [59]. The estimation of total bacteria and *E. coli* resistant to β-lactam antibiotics was performed as described above but using TSA and Chromocult respectively supplemented with 35 mg/l of ampicillin.

Estimation of *S. aureus* in the same samples was done with Staphylococcus Medium 110 (Difco Laboratories, France), which was incubated at 37°C for 48 h for the isolation of staphylococci. For the estimation of methicillin-resistant *S. aureus*, agar plates supplemented with 10 mg/l methicillin (Sigma-Aldrich. Spain) were used. Colonies grown in this medium that showed yellow-orange pigment were suspected of being *S. aureus*. This was confirmed with the Slidex Staph Plus (Biomerieux España, Madrid. Spain).

Standard PCR procedures

PCRs were performed with a GeneAmp PCR system 2700 (Applied Biosystems, Barcelona, Spain). The DNA template was prepared directly from two colonies of each strain suspended in 50 µl of double-distilled water and heated to 96°C for 10 min prior to the addition of the reaction mixture. Purified bacterial or phage DNA was diluted 1:20 in double-distilled water. The oligonucleotides used to amplify *mec*A, bla_{TEM} or $bla_{CTX\text{-}M}$ are described in Table 3. Five µl of each PCR product was analyzed by agarose (1.5%) gel electrophoresis and bands were visualized by ethidium bromide staining. When necessary, PCR products were purified using a PCR Purification Kit (Qiagen Inc., Valencia, USA).

qPCR procedures

Preparation of standard curves. For the generation of standards for the qPCR assays, a plasmid construct was used. The 569-bp fragment of TEM, the 356-bp fragment of CTX-M, and the 434-bp fragment of *mec*A, all obtained by conventional PCR with the primers described in Table 3, and purified as described above, were cloned with a pGEM-T Easy vector for insertion of PCR products, following the manufacturer's instructions (Promega, Barcelona, Spain). The construct was transformed by electroporation into *E. coli* DH5α electrocompetent cells. Cells were electroporated at 2.5 kV, 25 F capacitance and 200 Ω resistance.

Colonies containing the vector were screened by conventional PCR to evaluate the presence of the vector containing each insert. The presence of the insert in the vector and its orientation was assessed by conventional PCR and sequencing, as described above, using the primers in Table 3. The vector containing the insert was purified from the positive colonies using the Qiagen Plasmid Midi purification kit (Qiagen Inc., Valencia, CA, USA) and the concentration of the vector was quantified by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies. Thermoscientifics. Wilmington. USA). The reaction product was linearized by digestion with *Xmn*I restriction endonuclease (Promega Co., Madison, USA). The restricted product was purified and quantified again.

To calculate the number of construct gene copies (GC), the following formula was used: [concentration of the pGEM-T-Easy::*insert* (ng/µl)/molecular weight (ng/mol)] $\times 6.022 \times 10^{23}$ molecules/mol = n° molecules pGEM-T-Easy::*insert*/µl. The number of GC/µl of the stock prepared for each gene was calculated. Serial decimal dilutions of this stock were made in double-distilled water to prepare the standard curve for qPCR. The standard dilutions were then aliquoted and stored at −80°C until use. Three replicates of each dilution were added to each qPCR reaction.

blaCTX-M primers and probe set. Using the software tool Primer Express 3.0 (Applied Biosystems), primers and probes were selected for use in a standardized TaqMan amplification protocol. All primers and FAM-labeled fluorogenic probes were commercially synthesized by Applied Biosystems (Spain). CTX-M probe was a Minor groove binding probe with a FAM reporter (FAM: 6-carboxyfluorescein) and a non-fluorescent quencher (NFQ). Primers and probes were used under standard conditions in a Step One Real Time PCR System (Applied Biosystems, Spain). Primer and probe specificity was determined with sequence alignments using BLAST and NCBI data entries. The primers and probe set was tested for cross-reactions with the respective sensitive strains. Amplification was performed in a 20 µl reaction mixture with the TaqMan Environmental Real Time PCR Master Mix 2.0 (Applied Biosystems, Spain). The mixture contained 2 µl of the DNA sample or quantified plasmid DNA. Thermal cycler conditions were as follows: an initial setup of 10 min at 95°C, and forty cycles of 15 s of denaturation at 95°C, and 1 min of annealing/extension at 60°C. All samples were run in triplicate, as well as the standards, and positive and negative controls. The number of GC was defined as the average of the triplicate data obtained.

Table 3. Oligonucleotides used in this study.

Target gene	PCR	Sequence	Conditions	Amplimer (bp)	Reference
16SrDNA	UP	AAGAGTTTGATCCTGGCTCAG	95°C 5 min (1 cycle); 95°C 1 min, 42°C 0.5 min, and 72°C 2 min (35 cycles), 72°C 2 min (1 cycle).	1503	[61]
	LP	TACGGCTACCTTGTTACGACTT			
TEM PCR	UP	CTCACCCAGAAACGCTGGTG	95°C 5 min (1 cycle). 94°C, 15 s, 63°C 1 min, 72°C, 1.3 min (30 cycles). 72°C, 4 min (1 cycle).	569	This study
	LP	ATCCGCCTCCATCCAGTCTA			
TEM qPCR	UP	CACTATTCTCAGAATGACTTGGT	50°C 2 min (1 cycle). 95°C 15 min (1 cycle) 94°C for 15 s and 60°C 1 min (45 cycles).	85	[36]
	LP	TGCATAATTCTCTTACTGTCATG			
	Probe	6FAM-CCAGTCACAGAAAAGCAT-CTTACGG-MGBNFQ			
CTX-M-1 PCR	UP	ACGTTAAACACCGCCATTCC	95°C 5 min (1 cycle). 94°C, 15 s, 60°C 1 min, 72°C, 1.3 min (30 cycles) 72°C, 4 min (1 cycle).	356	This study
	LP	TCGGTGACGATTTTAGCCGC			
CTX-M-1 qPCR	UP CTX-M	ACCAACGATATCGCGGTGAT	50°C 2 min (1 cycle). 95°C 15 min (1 cycle) 94°C for 15 s and 60°C 1 min (45 cycles).	101	This study
	LP CTX-M	ACATCGCGACGGCTTTCT			
	Probe	6FAM – TCGTGCGCCGCTG- MGBNFQ			
MecA PCR	UP	ATACTTAGTTCTTTAGCGAT	95°C 5 min (1 cycle). 94°C, 15 s; 48°C 1 min, 72°C, 1.3 min (30 cycles). 72°C, 4 min (1 cycle).	434	This study
	LP	GATAGCAGTTATATTTCTA			
MecA qPCR	UP	CGCAACGTTCAATTTAATTTTGTTAA	50°C 2 min (1 cycle). 95°C 10 min (1 cycle). 95°C for 15 s and 60°C 1 min (40 cycles)	92	[38]
	LP	TGGTCTTTCTGCATTCCTGGA			
	Probe	FAM-AATGACGCTATGATCCCAATCT-AACTTCCACA-TAMRA			

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To screen for PCR inhibition, dilutions of the standard were spiked with environmental DNA and the experimental difference was compared to the true copies of the target genes in the standards. Inhibition of the PCR by environmental DNA was not detected.

Purification of phage DNA

Fifty ml of sewage and 100 ml of river water samples were passed through low protein-binding 0.22-μm-pore-size membrane filters (Millex-GP, Millipore, Bedford, MA). When necessary, several filter units were used to filter the whole volume. This allowed us to partially purify viral particles from the samples- The viruses were then 100-fold concentrated by means of protein concentrators (100 kDa Amicon Ultra centrifugal filter units, Millipore, Bedford, MA), following the manufacturer's instructions. The total volume was reduced to 0.5 ml. The centrifugation time varied depending on the sample and ranged from 10–90 min. The viral concentrate was recovered from the tube and the volume was adjusted to 2 ml with double distilled sterile water. Samples were treated with DNase (100 units/ml of the viral concentrate) to eliminate free DNA outside the phage particles.

Control of non-phage DNA. An aliquot of the sample at this stage was evaluated to rule out the presence of bacterial or non-encapsidated DNA. After DNase treatment, but before desencapsidation, the samples were used as template for conventional PCR of eubacterial 16SrDNA (Table 3) and for qPCR of the three antibiotic resistance genes (Table 3). This control was to ensure that the DNase treatment had removed all the non-encapsidated DNA from the samples.

DNA from the viral particles was isolated by proteinase K digestion and phenol/chloroform (1:1) (v:v) treatment [59]. The mixture phenol/chloroform/phage lysate was added to Phase Lock Gel tubes (5- Prime, VWR International, Madrid, Spain) and centrifuged following the manufacturer's instructions. The DNA from the supernatant was precipitated using 100% ethanol and 3M sodium acetate, and the volume was adjusted to 250 μl. DNA was further purified by using Microcon YM-100 centrifugal filter units (Millipore, Bedford, MA), following the manufacturer's instructions. Purified DNA was eluted in a final volume of 50 μl and evaluated by agarose (0.8%) gel electrophoresis. The bands were then viewed by ethidium bromide staining. The concentration and purity of the phage DNA extracted was determined by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies. Thermoscientifics. Wilmington. USA).

Purification of bacterial DNA

Fifty ml of sewage and 100 ml of river water samples were passed through 0.45 μm polyvinylidene fluoride (PVDF) DURAPORE® membrane filters (Millipore, Bedford, Massachusetts), described by the manufacturer as low protein-binding membranes. These allowed the phages to pass through whilst bacteria were retained on the surface of the filter. To remove phages retained on the filters, 10 ml of PBS was added to the surface of the filter, gently agitated and removed by filtration. Two washing steps allowed high (99%) phage reduction without significant loss of bacteria [60]. The membrane containing retained bacteria was recovered in 4 ml of LB. The suspension was centrifuged at

3000 g for 10 min. To recover DNA from both Gram-positive and Gram-negative bacteria, the pellet was suspended in 180 µl of enzymatic solution (20 mg/ml lysozyme; 25 mg/ml lisostaphine, 20 mM Tris-HCl, pH = 8.0; 2 mM EDTA; 1,2% Triton) and incubated for 30 min at 37°C. DNA was then extracted using a QIAamp DNA Stool Mini Kit (Qiagen Inc., Valencia, USA), following the manufacturer's instructions.

Transfection with antibiotic resistance genes

Twenty µl of phage DNA prepared as described above from three sewage samples (samples 6, 7 and 15) was transfected by electroporation into ap-sensitive, nal-resistant *E. coli* WG5 and C600nal^R strains (each culture containing 5×10^8 CFU/ml). Electrocompetent cells were prepared and phage DNA was electroporated as described above and incubated for 2 h in LB at 37°C. The clones were selected on Chromocult plates supplemented with ap/nal. A 25% of the ap/nal-resistant colonies were randomly selected and screened for the presence of bla_{TEM} and $bla_{\text{CTX-M}}$ genes with the corresponding primers for conventional PCR (Table 3). Positive amplification of the genes was confirmed by sequencing.

Electron microscopy

The sewage and river samples were used as a source of bacteriophages. Viruses from the samples were partially purified by filtration and 100-fold concentrated (sewage) or 1000-fold concentrated (river water), by means of protein concentrators (100 kDa Amicon Ultra centrifugal filter units, Millipore, Bedford, MA), following the manufacturer's instructions. Ten-µl of each virus suspension was deposited on copper grids with carbon-coated Formvar films and stained with 2% KOH phosphotungstic acid (pH 7.2) for 2.0 min. Samples were examined in a JEOL JEM-1010 electron microscope operating at 80 kV.

Sequencing and sequence analyses

The amplified DNA of each resistance gene cloned into the pGEM-T-Easy vector used to generate the standard was confirmed by sequencing. Amplicons of bla_{TEM}, $bla_{\text{CTX-M}}$ and *mec*A, generated by conventional PCR with primers described in Table 3, were electrophoretically analyzed in a 1% agarose gel, and bands were viewed by ethidium bromide staining. The bands were excised from the agarose gel and purified using a QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA, USA), following the manufacturer's instructions. The purified amplicons were used as a template for sequencing. Sequencing was performed with an ABI PRISM Big Dye 3.1 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Spain) in an ABI PRISM 3730 DNA Analyzer (Applied Biosystems, Spain), following the manufacturer's instructions. All sequences were performed at least in duplicate.

Nucleotide sequence analysis searches for homologous DNA sequences in the EMBL and GenBank database libraries were carried out using Wisconsin Package Version 10.2, Genetics Computer Group (GCG), (Madison, WI). BLAST analyses were performed with the tools available on the National Institutes of Health (NIH) webpage: http://www.ncbi.nlm.nih.gov. Sequences were assembled with the MultAlin program available on the web page: http://bioinfo.genotoul.fr/multalin/multalin.html.

Statistical analyses

Computation of data and statistical tests were performed using the Statistical Package for Social Science software (SPSS). One-way analysis of variance (ANOVA) tests were used to evaluate the differences between microbiological parameters in sewage and river samples and the differences between the resistance genes detected in bacterial and phage DNA. Evaluations were based on a 5% significance level in both cases (P 0.05). The box-plot graph used to compare the number of detected copies of the genes was done using EXCEL software (Microsoft® EXCEL 2000). The calculations performed to generate the box-plot graph included mean, standard deviation, media, quartiles and minimum and maximum values for each group of samples.

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Author Contributions

Conceived and designed the experiments: MM JJ. Performed the experiments: MM MCLL. Analyzed the data: MCLL MM JJ. Contributed reagents/materials/analysis tools: MM JJ. Wrote the paper: MCLL MM JJ.

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❖ **ARTICLE 2**

Títol: Bacteriophages carrying antibiotic resistance genes in fecal waste from cattle, pigs and poultry

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RESUM

Introducció

Els antibiòtics, com s'ha esmentat anteriorment, són àmpliament utilitzats en el tractament d'infeccions en humans i en animals així com també com a factors promotors del creixement animal en ramaderia.

L'ús i abús d'antibiòtics en humans i animals ha exercit una important pressió selectiva en les comunitats bacterianes i és considerada una de les principals causes de l'emergència de resistències a antibiòtics (American Academy of Microbiology, 2009; Hawkey *et al*., 2009).

Junt amb la producció d'antibiòtics sintètics, cal considerar que molts bacteris ambientals produeixen antibiòtics per tal de competir amb altres bacteris. Conseqüentment, molts d'ells contenen gens de resistència a antibiòtics com a mecanisme de protecció. Així, quan aquests bacteris arriben a ambients clínics els gens de resistència a antibiòtics dels microorganismes d'origen ambiental es seleccionen positivament a l'enfrontar-se a elevades concentracions d'antibiòtics, donant lloc a l'emergència i difusió de la resistència a antibiòtics com a conseqüència de la forta pressió selectiva (Martínez, 2008).

Estudis recents consideren que els fags podrien tenir més rellevància del que es considerava anteriorment en la transferència horitzontal de gens de resistència a antibiòtics i que el medi ambient hi jugaria un paper essencial.

En aquest cas ens plantegem l'estudi del paper potencial que juguen els fags en la disseminació de gens de resistència a antibiòtics en entorns d'origen animal.

Objectius

En aquest estudi es van plantejar els objectius detallats a continuació:

- Disseny de la sonda i encebadors per a la qPCR del gen $bla_{CTX\text{-}M\text{-}9}$ per a la quantificació del clúster 9, el qual detecta les variants més abundants del clúster (CTX-M-9, 13, 14, 16 a 19, 21 i 27).

- Detecció i quantificació per qPCR dels gens de resistència a antibiòtics (bla_{TEM}, $bla_{CTX\text{-}M}$ grups clonals 1 i 9 i *mecA*) en la fracció de DNA fàgic de mostres amb residus fecals de diferents animals per tal de determinar el paper potencial dels bacteriòfags en la disseminació de gens de resistència a antibiòtics en entorns animals.

Resultats i discussió

En aquest estudi es van analitzar 8 mostres de purins de granges de vaques, 9 mostres d'aigua residual d'escorxador de porcs, 16 mostres d'aigua residual d'escorxador d'aus, 10 mostres amb barreja de contingut fecal procedent d'aviram, ànecs, conills, gossos i gats domèstics, i 28 mostres fecals de vaques recollides a partir d'excrements de pastures del Pirineu.

El grau de contaminació fecal de les mostres es va establir mitjançant l'enumeració de colifags somàtics (indicadors vírics de contaminació fecal) i d'*E. coli*.

Els clústers 1 i 9 de $bla_{CTX\text{-}M}$ van ser detectats en DNA fàgic de totes les mostres sense diferències significatives ($P>0.05$) entre clústers amb valors mitjans entre 10^2-10^3 CG/mL. Les densitats de $bla_{CTX\text{-}M\text{-}1}$ van ser lleugerament superiors en les mostres d'aigües residuals d'escorxadors de porcs. Les mostres d'escorxador d'aus presentaven una prevalença superior ($P<0.05$) del clúster 9 en comparació amb la resta de mostres. De fet, es va incorporar en aquest estudi el nou assaig de qPCR per a la quantificació de $bla_{CTX\text{-}M\text{-}9}$, donada la prevalença d'alguns dels gens d'aquest grup en animals. Els nostres resultats concorden

amb el fet que el grup *bla*$_{\text{CTX-M-9}}$ és molt prevalent en aviram a Espanya, i que les variants CTX-M-9 i CTX-M-14 són les més freqüentment aïllades en animals (Patterson, 2003; Coque *et al*., 2008a; Rodríguez-Baño *et al*., 2008) (Figura 1).

Totes les mostres van ser positives pel gen *bla*$_{\text{TEM}}$, essent el que es va trobar en densitats més elevades respecte els altres gens de resistència a antibiòtics analitzats (valors màxims de fins a 10^5-10^6 CG/mL). De mitjana, les mostres d'escorxador d'aus contenien el major nombre de còpies de *bla*$_{\text{TEM}}$ i les d'escorxador de porc les que menys (Figura 1).

En el cas de *mecA*, va ser el gen que va presentar major nombre de mostres negatives i densitats inferiors en comparació a la resta de gens quantificats. Aquest fet era d'esperar ja que la qPCR del gen *mec*A detecta un únic gen i la resta una família de gens. *mecA* es va trobar principalment en mostres d'aigua residual dels escorxadors de porcs i d'aviram (Figura 1).

Posteriorment, es van seleccionar 24 amplicons dels gens de resistència a antibiòtics quantificats que presentaven major nombre de còpies, es van tornar a amplificar per PCR convencional i es va dur a terme la seva seqüenciació. Es va poder confirmar la seva identitat per homologia amb les seqüències dipositades tot i que no es va poder discriminar entre variants al·lèliques degut a què presentaven els mateixos percentatges d'homologia.

D'aquest estudi es desprèn que els gens de resistència a antibiòtics analitzats es troben en DNA fàgic de mostres amb contaminació fecal d'origen animal de la mateixa manera que també s'ha comprovat prèviament en altres tipus de mostra com aigua residual humana i aigua de riu. Cal considerar que els bacteriòfags, degut a les seves característiques estructurals resisteixen millor en ambients aquàtics que els seus hostes bacterians i que el DNA lliure. La seva elevada supervivència i l'abundància de fags portadors de gens de resistència a antibiòtics en aigua residual animal i humana recolzaria la idea dels fags com a vectors per a la mobilització de gens de resistència a antibiòtics entre biomes.

Per altra banda, els resultats obtinguts en mostres procedents de femtes de vaques, les quals no han estat en granges durant tota l'estació d'estiu i que només han consumit pastures,

sustentaria la hipòtesi que les resistències a antibiòtics tindrien origen a la natura i l'ús d'antibiòtics causaria pressió de selecció per a l'emergència de les resistències, ja que aquests animals no han estat exposats a antibiòtics, i per tant, les resistències es trobarien de manera natural en el medi ambient.

Conclusions

Els gens de resistència a antibiòtics *bla*$_{CTX-M}$ clústers 1 i 9, *bla*$_{TEM}$ i *mecA* es troben en DNA fàgic de mostres amb contaminació fecal animal, essent *mecA* el gen menys prevalent detectat a les mostres amb contaminació fecal animal.

Tenint en compte els resultats obtinguts, es conclou que els animals poden actuar com a reservoris de gens de resistència a antibiòtics i els fags poden actuar com a vehicles per a la disseminació d'aquestes resistències.

Informe sobre el factor d'impacte de l'article 2

L'article ***Bacteriophages carrying antibiotic resistance genes in fecal waste from cattle, pigs and poultry*** va ser publicat online l'1 d'agost de 2011 a la revista *Antimicrobial Agents and Chemotherapy* que es troba inclosa en el primer quartil (Q1) de l'àrea temàtica de *Microbiology* (21/114) i *Pharmacology&Pharmacy* (24/261). L'any 2011 la revista *Antimicrobial Agents and Chemotherapy* va presentar un factor d'impacte de 4.841.

En el moment de la presentació d'aquest informe aquest article ha estat citat 17 vegades segons Web of Science.

Informe de participació de l'article 2

La doctoranda Marta Colomer Lluch ha realitzat el disseny de la sonda i encebadors per a la qPCR del gen $bla_{CTX\text{-}M\text{-}9}$. També ha realitzat el processament de les mostres i les anàlisis dels paràmetres microbiològics. La doctoranda ha dut a terme la quantificació de les mostres de DNA fàgic, les diverses seqüenciacions i la posterior anàlisi i processat de les dades obtingudes. Finalment ha participat en la redacció de l'article i en l'elaboració de taules i figures així com en la difusió dels resultats en congressos nacionals i internacionals.

Dr. J. Jofre Dra. M. Muniesa

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Bacteriophages Carrying Antibiotic Resistance Genes in Fecal Waste from Cattle, Pigs, and Poultry[▽]

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This study evaluates the occurrence of bacteriophages carrying antibiotic resistance genes in animal environments. bla_{TEM}, bla_{CTX-M} (clusters 1 and 9), and *mecA* were quantified by quantitative PCR in 71 phage DNA samples from pigs, poultry, and cattle fecal wastes. Densities of 3 to 4 $\log_{10}$ gene copies (GC) of bla_{TEM}, 2 to 3 $\log_{10}$ GC of bla_{CTX-M}, and 1 to 3 $\log_{10}$ GC of *mecA* per milliliter or gram of sample were detected, suggesting that bacteriophages can be environmental vectors for the horizontal transfer of antibiotic resistance genes.

Antibiotics are widely used to protect human health and to increase the growth rate of animals in livestock husbandry. The use and abuse of antibiotics in humans and animals have exerted selective pressure on bacterial communities, resulting in the emergence of resistances (1, 22). There are concerns about the potential impact of antibiotic residues in the aquatic environment, where many antibiotics are discharged (26, 36).

In addition to the antibiotics synthesized for therapy, many antibiotics are produced by environmental microorganisms (15, 34). These organisms host antibiotic resistance genes (ARGs) that protect them from the antibiotics they produce (16). Environmental bacteria that do not produce antibiotics themselves also carry ARGs conserved as a consequence of the selective pressure of antibiotics in certain environments (26, 36) or used for different purposes (14). Therefore, when bacteria reach clinical settings, these ARGs from environmental origins are challenged with high concentrations of antibiotics and antibacterial resistance evolves and emerges under the strong selective pressure that occurs during the treatment of infections (26).

Many ARGs are acquired by bacteria through conjugative transfer by mobile elements (plasmids or integrative and conjugative elements), by transformation by naked DNA, or by transduction by bacteriophages (36). Compared with other genetic vectors, less is known about the contribution of phages to antibiotic resistance transfer. Recent reports (1, 7, 10) suggest that the horizontal transfer of ARGs by phages is much more widespread than previously believed and that the environment plays a crucial role in it (7).

This study was focused on bla_{TEM} and bla_{CTX-M}, which encode β-lactamases that are widespread among Gram-negative pathogens (11), and *mecA*, which encodes penicillin-binding protein 2a (PBP2a), associated with methicillin resistance in staphylococci (32). Quantification of these genes by quantitative PCR (qPCR) was done in the viral DNA fraction of animal fecal wastes. Assuming that phages are the major part of the viral fraction in most environments (17), we sought to highlight the potential role of phages in the spread of ARGs in animal settings.

This study was conducted with archived fecal wastes collected from several slaughterhouses and farms in Spain. We analyzed 8 cattle slurries, 9 wastewater samples from abattoirs slaughtering pigs and 16 from poultry slaughter, 10 wastewater samples containing mixed fecal wastes of poultry, ducks, rabbits, and domestic dogs and cats, and 28 fecal samples aseptically collected from cowpats in summer pastures in the Pyrenees mountains. The fecal contamination in the samples was established by enumerating fecal coliforms and *Escherichia coli* (9). Somatic coliphages, proposed as fecal viral indicators, allowed evaluation of the levels of bacteriophages (2, 5) The samples showed levels of bacterial and viral indicators that were relatively homogeneous (Table 1) and similar to those previously reported (5, 23).

Phage DNA was purified from the samples as described previously (10, 23). Samples were treated with DNase (100 units/ml) to rule out the possibility of nonphage DNA contamination. For this, an aliquot taken after DNase treatment and before disencapsidation was evaluated using conventional PCR of eubacterial 16S ribosomal DNA (rDNA) (Table 2) and using qPCR.

Conventional PCRs for ARGs were performed as described previously (10) (Table 2), using environmental *E. coli* strains and a clinical isolate of methicillin-resistant *Staphylococcus aureus* (MRSA) as controls for bla_{TEM}, bla_{CTX-M}, and *mecA*, respectively. TaqMan qPCR assays (Table 2) were used for quantification of ARGs using standards as previously described (10).

A real-time qPCR oligonucleotide set for bla_{CTX-M} cluster 1 (10) and a new set for bla_{CTX-M} cluster 9, developed in this study, were used to detect CTX-M in phage DNA isolated from animal wastes. The new qPCR oligonucleotides for bla_{CTX-M} cluster 9 detect the most abundant variants of the cluster (CTX-M-9, 13, 14, 16 to 19, 21, and 27) (6) and showed a detection limit of 13 gene copies (GC) (threshold cycle of 31).

bla_{CTX-M} clusters 1 and 9 were detected in phage DNA (Fig. 1A and B) without significant differences ($P > 0.05$, analysis of variance [ANOVA]) between the occurrence of the clusters. The densities of cluster 1 were slightly higher in swine samples,

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TABLE 1. Fecal coliforms and *E. coli* as bacterial indicators and somatic coliphages as viral indicators detected in animal waste and fecal samples

Sample type	No. of samples	No. [CFU/ml or CFU/g (SD)] of:		No. [PFU/ml or PFU/g (SD)] of somatic coliphages
		Fecal coliforms	*E. coli*	
Cattle slurry	8	1.7×10^5 (1.5×10^5)	3.3×10^4 (8.2×10^3)	1.1×10^4 (1.4×10^4)
Pig wastewater	9	1.4×10^6 (1.4×10^6)	4.6×10^5 (1.1×10^5)	7.6×10^5 (1.1×10^6)
Poultry wastewater	16	9.4×10^5 (3.6×10^5)	7.8×10^4 (2.2×10^4)	1.8×10^4 (1.8×10^4)
Mixed slurry[a]	10	3.2×10^5 (1.0×10^6)	2.2×10^4 (6.5×10^4)	8.5×10^3 (1.2×10^4)
Cowpats	28	7.9×10^3 (3.2×10^3)	3.9×10^3 (7.2×10^3)	4.0×10^3 (4.2×10^3)

[a] Slurries were from a farm with fecal loads from diverse animal origins.

while poultry samples showed a significantly ($P < 0.05$) higher prevalence of cluster 9. CTX-M-1 was previously detected in phage DNA from municipal sewage of the same area (10), although at lower densities than in animal wastes. CTX-M is currently the most prevalent β-lactamase family in many countries (11). CTX-M cluster 1 is the most prevalent in pig isolates in Spain (13), and within this cluster, CTX-M-15 is the most widely distributed (12, 27). CTX-M cluster 9 is the most prevalent in poultry in Spain (13, 27), and within it, CTX-M-9 and CTX-M-14 are the most frequent in animal isolates (11). Both CTX-M-15 and CTX-M-9 have been linked to *E. coli* O25b:H4, a serious human pathogen worldwide (12).

The qPCR oligonucleotide set for bla_{TEM} (Table 2) (24) showed positive results in all samples and higher densities ($P < 0.05$) than were found for the other ARGs (Fig. 1C). Poultry waste carried the highest number of copies, and pig waste the lowest. bla_{TEM} is the most prevalent β-lactamase in *E. coli* isolates from food of animal origin and from healthy livestock (8). This prevalence is consistent with the high densities of bla_{TEM} detected in phage DNA in this study and in municipal sewage (10).

The qPCR for *mecA* (33) showed lower densities than qPCR for the other ARGs (Fig. 1D) and registered more negative samples. This was expected because this qPCR oligonucleotide set detects a specific gene instead of a family (TEM or CTX-M) spread among numerous bacterial genera. Swine and poultry showed a significantly ($P < 0.05$) higher prevalence than the other sources (Fig. 1D). *mecA* has been detected in isolates from domestic animals (20, 21, 25, 31) and in phage DNA from municipal sewage (10). The presence of *mecA* in animals has been associated with antimicrobial usage, contact with humans, and farm hygiene. Transmission is from humans to domestic animals (31) or from animals to farmers (20).

Twenty-four amplicons of the ARGs, selected according to the highest GC densities, were generated by conventional PCR and sequenced (23). All amplicons were confirmed as to their identity, although for some β-lactamase genes, discrimination between allelic variants was not possible, since the sequences were partial.

Indirect evidence suggests that selective pressures have mobilized ARGs from their initial chromosomal location in bacteria (4, 32). Phages persist better in aquatic environments than their bacterial hosts (3, 18) and, due to their structural characteristics, better than free DNA (37). This higher survival and the abundance of phages carrying ARGs in animal and human wastewater (10, 28) support the notion that phages are

TABLE 2. Oligonucleotides used in this study

Target gene	Reaction	Oligonucleotide	Sequence	Amplimer size (bp)	Reference
bla_{TEM}	TEM PCR	UP	CTCACCCAGAAACGCTGGTG	569	10
		LP	ATCCGCCTCCATCCAGTCTA		
	TEM qPCR	UP	CACTATTCTCAGAATGACTTGGT	85	24
		LP	TGCATAATTCTCTTACTGTCATG		
		Probe	6FAM-CCAGTCACAGAAAAGCATCTTACGG-MGBNFQ		
$bla_{\mathrm{CTX\text{-}M}}$ cluster 1	CTX-M-1 PCR	UP	ACGTTAAACACCGCCATTCC	356	10
		LP	TCGGTGACGATTTTAGCCGC		
	CTX-M-1 qPCR	UP CTX-M	ACCAACGATATCGCGGTGAT	101	10
		LP CTX-M	ACATCGCGACGGCTTTCT		
		Probe	6FAM-TCGTGCGCCGCTG-MGBNFQ		
$bla_{\mathrm{CTX\text{-}M}}$ cluster 9	CTX-M-9 PCR	UP	ACGCTGAATACCGCCATT	346	This study
		LP	CGATGATTCTCGCCGCTG		
	CTX-M-9 qPCR	UP CTX-M	ACCAATGATATTGCGGTGAT	85	This study
		LP CTX-M	CTGCGTTCTGTTGCGGCT		
		Probe	6FAM-TCGTGCGCCGCTG-MGBNFQ		
mecA	MecA PCR	UP	ATACTTAGTTCTTTAGCGAT	434	10
		LP	GATAGCAGTTATATTTCTA		
	MecA qPCR	UP	CGCAACGTTCAATTTAATTTTGTTAA	92	33
		LP	TGGTCTTTCTGCATTCCTGGA		
		Probe	FAM-AATGACGCTATGATCCCAATCTAACTTCCACA-TAMRA		
	16S rDNA	UP	AAGAGTTTGATCCTGGCTCAG	1,503	29
		LP	TACGGCTACCTTGTTACGACTT		

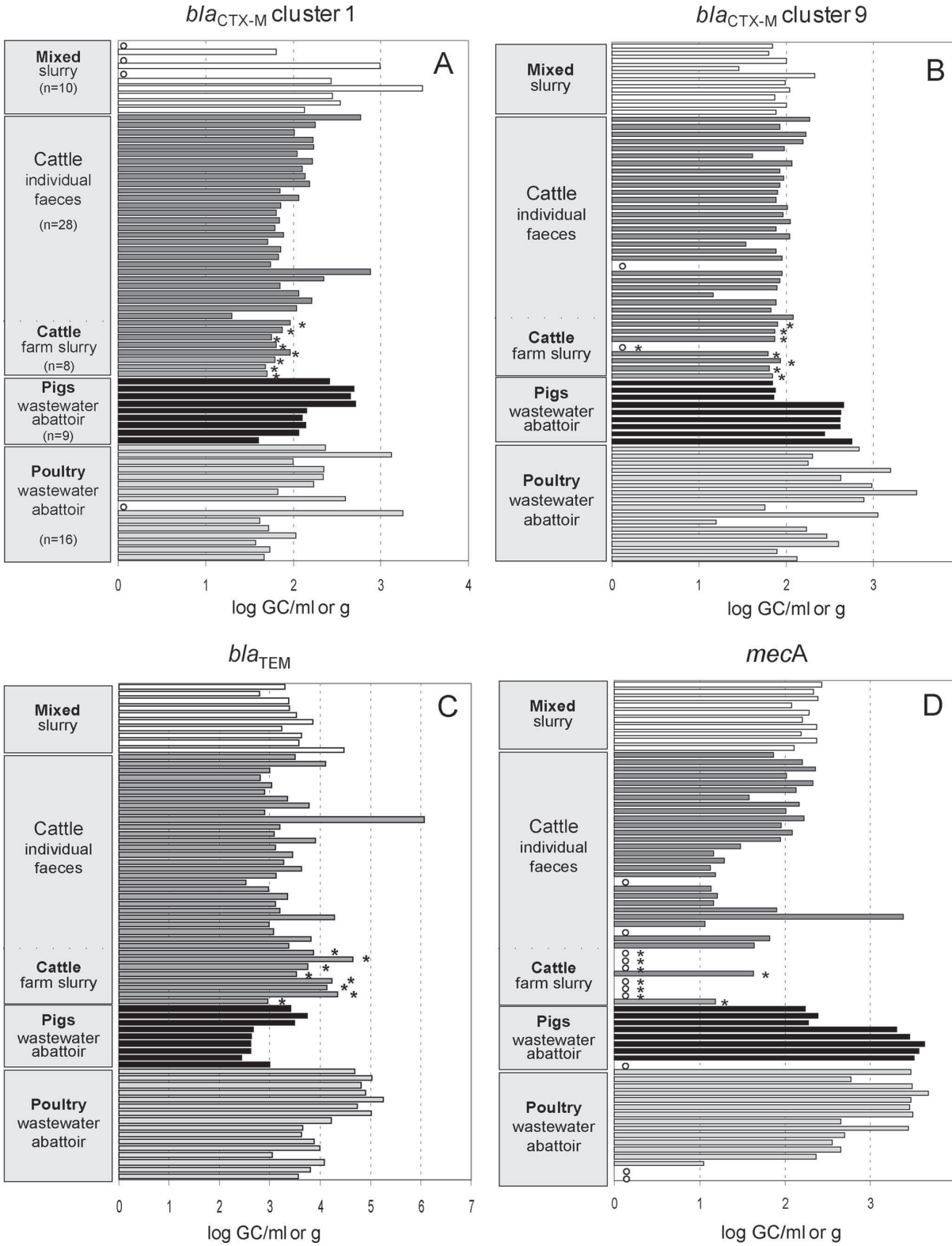

FIG. 1. Number of detected copies (log GC/ml or log GC/g) of each ARG in DNA isolated from farm fecal wastes (mixed samples), cattle, pigs, and poultry. Shown are the number of detected copies of bla_{CTX-M} genes of cluster 1 (A), bla_{CTX-M} genes of cluster 9 (B), bla_{TEM} genes (C), and *mecA* genes (D). White circles indicate those samples showing results that were negative or below the detection limit. Asterisks indicate cattle slurries from a farm, while the rest of the cattle samples were fecal samples from summer pastures.

vehicles for mobilization of the environmental pool of ARGs that contribute to the maintenance and emergence of new resistances.

Despite the recent efforts of many international health organizations (19, 20, 30, 35) that recommend a controlled use of antibiotics and to withdraw their use in animal husbandry, new resistances continue to emerge. This could suggest that the origin of resistances is not the antibiotic pressure but the ARGs present in the environmental pool. The results for cattle feces presented here support this hypothesis, since these animals graze on pasture outside the farms and, thus, are not exposed to antibiotics. The study of this environmental pool and of the mechanisms of ARG mobilization, such as bacteriophages, could provide an early warning system for future clinically relevant resistance mechanisms.

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3.2. Chapter 2: Quinolone resistance genes (*qnrA* and *qnrS*) in bacteriophage particles from wastewater samples and the effect of inducing agents on packaged antibiotic resistance genes.

❖ **ARTICLE 3**

Títol: Quinolone resistance genes (*qnrA* and *qnrS*) in bacteriophage particles from wastewater samples and the effect of inducing agents on packaged antibiotic resistance genes

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RESUM

Introducció

Recentment la resistència a quinolones i fluoroquinolones en enterobacteris ha experimentat un increment molt important a nivell mundial. El principal mecanisme de resistència a quinolones implica mutacions en els gens dels enzims DNA girasa i DNA topoisomerasa IV (Rodríguez-Martínez *et al*., 2011). Tot i així, també s'han descrit plasmidis portadors de gens de resistència a quinolones (*PMQR*) (Cano *et al*., 2009). Els gens de *PMQR* s'han trobat en ambients aquàtics i d'animals de granja suggerint el medi aquàtic com a ambient per a la disseminació de *PMQR* entre animals i humans mitjançant elements genètics mòbils.

Els bacteriòfags són elements genètics mòbils molt abundants i tal com s'esmenta en els capítols anteriors, s'han detectat gens de resistència a antibiòtics en la fracció de DNA de fags de mostres ambientals amb contaminació fecal.

És conegut que diversos factors poden promoure la transferència de gens entre bacteris per mobilització dels elements genètics mòbils. Concretament, els bacteriòfags temperats poden

ser induïts dels seus hostes bacterians per acció de diversos compostos ambientals i químics que produeixen l'activació del seu cicle lític. Els bacteriòfags, un cop activat el seu cicle lític, es multipliquen i surten del bacteri hoste, causant-li la lisi i propagant-se fora de la cèl·lula, o bé com a partícules fàgiques amb DNA de l'hoste (transducció generalitzada). Factors com la radiació ultraviolada, agents quelants, i els antibiòtics, en particular les fluoroquinolones, poden causar la inducció del cicle lític dels bacteriòfags (Allen *et al*., 2011; Taylor *et al*., 2011; Looft *et al*., 2012).

Objectius

En aquest estudi es van plantejar els objectius detallats a continuació:

- Disseny de la sonda i encebadors per a la qPCR dels gens de resistència a quinolones *qnrA* i *qnrS*.
- Detecció i quantificació per qPCR de dos gens de resistència a quinolones, *qnrA* i *qnrS*, en el DNA de fags de mostres ambientals amb contaminació fecal.
- Avaluació de la influència de factors inductors de fags (mitomicina C, ciprofloxacina, EDTA i citrat sòdic) i de paràmetres físics en l'abundància de gens resistència a quinolones en DNA de fags de poblacions bacterianes de mostres d'aigua residual.
- Avaluació de la influència de factors inductors de fags (mitomicina C, ciprofloxacina, EDTA i citrat sòdic) i de paràmetres físics en la capacitat infectiva dels colifags somàtics de poblacions bacterianes de mostres d'aigua residual.

Resultats i discussió

En aquest estudi es va fer la detecció i quantificació dels gens *qnr* en DNA de fags a partir de 18 mostres d'aigua residual, 18 mostres d'aigua de riu i 28 mostres d'aigua residual animal d'origen boví. Es van seleccionar els gens *qnrA* i *qnrS* per la seva prevalença i rellevància clínica en el nostre entorn. Per això es van dissenyar les qPCRs específiques per a *qnrA*, la qual permet la detecció de 7 variants (*qnrA1-A7*), i de *qnrS* per a la detecció de 6 variants (*qnrS1-S6*).

El 100% de les mostres d'aigua residual i d'aigua de riu van ser positives pel gen *qnrA,* així com un 71.4% de les mostres d'aigua amb contaminació d'origen animal. Les densitats mitjanes van ser de $2.3x10^2$ CG/mL en aigua residual i de $7.4x10^1$ CG/mL en aigua residual animal. (Figura 1).

qnrS va ser menys prevalent, amb un 38.9% de les mostres d'aigua residuals positives per aquest gen, un 22.2% de les d'aigua de riu i només una mostra d'aigua residual animal. Tot i tenir una prevalença més baixa, les densitats de *qnrS* van arribar a assolir valors de 10^3CG/mL. (Taula 1).

Per altra banda, amb la finalitat d'avaluar la influència de factors inductors de lisogènia en l'abundància de gens de resistència a quinolones en DNA de fags, aquests factors es van usar per induir fags a partir de les poblacions bacterianes presents en mostres d'aigua residual. Es van tractar 50mL d'aigua residual amb mitomicina C, ciprofloxacina, EDTA i citrat sòdic en presència o absència de glucosa, en condicions d'aerobiosi i anaerobiosi, i a diverses temperatures. L'abundància de gens de resistència en DNA de fags de les mostres induïdes es va comparar amb les mostres en absència d'inductors.

Tant els dos gens de resistència a quinolones, *qnrA* i *qnrS*, com les β-lactamases bla_{TEM} i $bla_{CTX\text{-}M\text{-}1}$ van incrementar de manera molt significativa el seu nombre de còpies en DNA fàgic quan es va tractar l'aigua residual amb dos agents quelants, EDTA i citrat sòdic. En canvi, altres agents inductors, com la mitomicina C i la ciprofloxacina, no van causar cap augment del nombre de còpies dels gens de resistència estudiats en DNA fàgic. Per tal d'estimular el creixement dels bacteris presents a les mostres, es va analitzar en paral·lel mostres induïdes amb o sense glucosa, però no es van observar diferències significatives en presència o absència de glucosa. Per altra banda, per tal de potenciar el creixement de bacteris autòctons a les mostres, els experiments es van repetir en paral·lel a 37ºC i a 22ºC, i ambdues temperatures tampoc van demostrar diferències entre sí, tot i que el gen *qnrS* va presentar el major increment de fins a 4.46 $\log_{10}$ en presència d'EDTA i glucosa a 22ºC. (Taula 2 i 3).

De manera addicional, es va analitzar l'efecte dels agents inductors en els colifags somàtics infecciosos presents a les mostres d'aigua residual incubades en les diferents condicions. Aquests experiments estaven dirigits a avaluar si els inductors mostraven efecte sobre fags virulents que poden formar clapes de lisi o si es podia detectar un augment de clapes de lisi deguda a la inducció de fags temperats presents en els bacteris de les mostres. La infectivitat dels fags es va confirmar per la seva capacitat de generar clapes de lisi en una monocapa de la soca hoste *E. coli*. WG5. Els agents inductors no van tenir efecte en les densitats de colifags somàtics detectats en comparació amb els controls (Figura 3).

La mobilització de gens de resistència a antibiòtics per fags s'ha descrit que podria estar causada per transducció generalitzada (Schmieger, 1999) i els resultats obtinguts en aquest estudi recolzarien aquesta idea. Les partícules fàgiques de transducció generalitzada contenen fragments de DNA bacterià enlloc de DNA fàgic a l'interior de la seva càpsida i no són capaces de causar lisis en una soca hoste, i per tant, no són detectables en placa. Tot i així, poden introduir el seu DNA en una soca hoste susceptible i transduir els gens que mobilitzen.

Així, els fags portadors de gens *qnr* i d'altres gens de resistència a antibiòtics serien majoritàriament partícules de transducció generalitzada. Per una banda, els agents inductors han demostrat no tenir un efecte en les densitats de fags virulents d'aigua residual que infecten la soca d'*E. coli* WG5 i aquestes densitats no es veuen influenciades per la quantitat de fags temperats que podrien ser induïts a partir dels bacteris presents a la mostra. Per altra banda, el nombre de còpies de gens *qnr* en DNA fàgic no augmenta en presència d'agents inductors del cicle lític de fags temperats (mitomicina C i ciprofloxacina) però sí ho fa després d'afegir EDTA o citrat sòdic.

Tot i que es desconeixen els mecanismes pels quals l'EDTA causaria aquest efecte, aquest podria ser degut a les seves propietats quelants que afectarien la membrana donant lloc a una resposta d'estrès de membrana (Vaara, 1992; Bury-Moné *et al*., 2009).

Malgrat tot, es desconeix si les partícules fàgiques induïdes per EDTA o citrat sòdic són fags completament funcionals. De fet, s'ha descrit que els fags de transducció generalitzada

poden presentar alteracions en l'especificitat d'empaquetament del DNA donant lloc a la formació de partícules transductants en major freqüència. L'efecte dels agents quelants podria causar aquesta alteració en l'empaquetament de les partícules i augmentar el seu nombre, fet que concordaria amb l'augment de gens de resistència a antibiòtics en DNA empaquetat en presència d'agents quelants.

Conclusions

En aquest estudi es mostra que els gens de resistència a quinolones *qnrA* i *qnrS* es troben en la fracció de DNA fàgic de mostres ambientals d'aigua residual, aigua de riu i aigua residual amb contaminació fecal animal.

No es va observar un increment en el nombre de còpies dels gens de resistència a antibiòtics en DNA de la fracció fàgica quan les mostres d'aigua residual es van tractar amb inductors de fags temperats (mitomicina C i ciprofloxacina). En canvi, quan aquestes es van tractar amb EDTA i citrat sòdic s'hi va detectar un increment significatiu, probablement produït per les propietats quelants d'aquests agents.

Cap dels agents inductors utilitzats va resultar tenir efecte en l'augment del nombre de colifags somàtics de l'aigua residual tractada. Això, juntament amb el fet que els inductors de fags temperats no van causar un increment de còpies de gens *qnr* en DNA fàgic, suggeriria que els fags codificadors per *qnr* podrien ser partícules de transducció generalitzada i no pròpiament fags temperats.

Aquest estudi, per tant, confirmaria la contribució dels fags en la mobilització de gens de resistència a antibiòtics i el paper del medi ambient i de determinats inductors en la disseminació d'aquestes resistències mitjançant fags.

Informe sobre el factor d'impacte de l'article 3

L'article ***Quinolone resistance genes (qnrA and qnrS) in bacteriophage particles from wastewater samples and the effect of inducing agents on packaged antibiotic resistance genes*** va ser publicat online el 23 de gener de 2014 a la revista *Journal of Antimicrobial Chemotherapy* que es troba inclosa en el primer quartil (Q1) de l'àrea temàtica de *Infectious diseases* (7/70), *Microbiology* (16/116) i *Pharmacology&Pharmacy* (18/261). L'any 2012 la revista *Journal of Antimicrobial Chemotherapy* va presentar un factor d'impacte de 5.338.

Informe de participació de l'article 3

La doctoranda Marta Colomer Lluch ha realitzat el disseny de la sonda i encebadors per a la qPCR dels gens *qnrA* i *qnrS*. És també responsable de la recollida i transport de les mostres així com de les anàlisis dels paràmetres microbiològics i processament de les mostres. La doctoranda ha dut a terme la totalitat dels experiments incloent-hi les extraccions de DNA bacterià i DNA fàgic de les mostres, la seva quantificació i la posterior anàlisi i processat de les dades obtingudes. Finalment ha participat en la redacció de l'article i en l'elaboració de taules i figures així com en la difusió dels resultats en congressos nacionals i internacionals.

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Quinolone resistance genes (*qnrA* and *qnrS*) in bacteriophage particles from wastewater samples and the effect of inducing agents on packaged antibiotic resistance genes

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Objectives: This study quantifies quinolone antibiotic resistance genes (*qnrA* and *qnrS*) in DNA of phage particles isolated from faecally polluted waters and evaluates the influence of phage inducers on the abundance of antibiotic resistance genes in packaged DNA.

Methods: *qnrA* and *qnrS* were quantified by qPCR in DNA of phage particles isolated from 18 raw urban wastewater samples, 18 river samples and 28 archived samples of animal wastewater. The bacterial fraction of the samples was treated with mitomycin C, ciprofloxacin, EDTA or sodium citrate under different conditions, and the number of resistance genes in DNA of phage particles was compared with the non-induced samples.

Results: *qnrA* was more prevalent than *qnrS*, with 100% of positive samples in urban wastewater and river and 71.4% of positive samples in animal wastewater. Densities of *qnrA* ranged from 2.3×10^2 gene copies (GC)/mL in urban wastewater to 7.4×10^1 GC/mL in animal wastewater. *qnrS* was detected in 38.9% of urban wastewater samples, in 22.2% of river samples and only in one animal wastewater sample (3.6%). Despite the lower prevalence, *qnrS* densities reached values of 10^3 GC/mL. Both *qnr* genes and other resistance genes assayed (bla_{TEM} and $bla_{CTX\text{-}M}$) showed a significant increase in DNA of phage particles when treated with EDTA or sodium citrate, while mitomycin C and ciprofloxacin showed no effect under the different conditions assayed.

Conclusions: This study confirms the contribution of phages to the mobilization of resistance genes and the role of the environment and certain inducers in the spread of antibiotic resistance genes by means of phages.

Keywords: phages, EDTA, sodium citrate

Introduction

Quinolones and fluoroquinolones are antimicrobials commonly used in clinical and veterinary medicine.[1] The first quinolone, nalidixic acid, was introduced into clinical use in 1962 and in the mid-1980s ciprofloxacin, a fluoroquinolone with a wide spectrum of *in vitro* antibacterial activity, first became available clinically.[2] Resistance to quinolones in enterobacteria has become common and is dramatically increasing worldwide.[3,4]

The main mechanism of quinolone resistance involves the accumulation of mutations in the genes coding for the target bacterial enzymes of the fluoroquinolones, DNA gyrase and DNA topoisomerase IV,[5] which protect against the inhibitory activity of quinolones. However, the traditional understanding of quinolone resistance as a mutational phenomenon has not provided a completely satisfactory explanation for the frequency with which it has arisen. Such a phenomenon might be better accounted for by horizontally transferable elements. These supply a degree of reduced quinolone susceptibility, enough for microorganisms to survive in the presence of quinolones, while resistance mutations occur sequentially rather than simultaneously. Since 1998, when *qnrA1* was discovered,[6] several plasmid-mediated quinolone resistance (PMQR) genes have been described.[7] More recent findings suggest that the *qnr* genes in circulation could have originated in the chromosomes of water-dwelling or other environmental organisms.[8] In the face of intense quinolone pressure, such genes have entered circulation on mobile genetic elements.[9]

Acquired Qnr proteins belong to a pentapeptide repeat family. To date, six families of Qnr proteins have been described: QnrA, QnrB, QnrC, QnrD, QnrS and QnrVC. *qnr* genes are highly diverse, with 7 *qnrA*, 73 *qnrB*, 1 *qnrC*, 2 *qnrD*, 9 *qnrS* and 5 *qnrVC* genes

identified (http://www.lahey.org/qnrStudies/). Qnr genes have been reported worldwide from unrelated enterobacterial species and are usually associated with mobile elements.[10,11]

A series of studies have identified the environment as a reservoir of PMQR genes, with farm animals and aquatic habitats being significantly involved. Recent observations suggest that the aquatic environment might constitute the original source of PMQR genes, which then spread among animal or human isolates by means of several mobile genetic elements.[12,13]

Besides plasmids, other studies highlight the abundance of phage particles as mobile genetic elements carrying antibiotic resistance genes (ARGs) in the environment.[14–16] Metagenomic analysis confirms the abundance of ARGs in viral DNA in diverse biomes.[17] However, there is still little information on phages carrying ARGs in environmental settings.

Several factors could promote gene transfer among bacteria in an aquatic environment.[18] The lytic cycle of temperate bacteriophages could be activated by various environmental factors and chemicals. Activation of the phage lytic cycle that leads to prophage excision from the bacterial chromosome is called phage induction, and continues with the generation of new phage particles until the bacterial host lyses or bursts, releasing the phages outside the cell. Among others, some antibiotics, and specifically fluoroquinolones, cause phage induction and subsequent gene transduction.[19,20]

In this study we focused first on the qPCR quantification of two quinolone resistance genes (*qnrA* and *qnrS*) in DNA in phage particles isolated from environmental samples. These genes were selected because they are widely distributed in our region and clinically relevant.[21] We also wanted to evaluate the influence of phage-inducing factors on the number of quinolone-resistance genes in DNA in phage particles after induction of the bacterial populations in the water samples. For this purpose, we quantified by qPCR four ARGs (bla_{TEM}, $bla_{CTX\text{-}M}$, *qnrA* and *qnrS*) in the phage DNA fraction of wastewater samples treated with phage inducers: mitomycin C as an inducer of the SOS response, ciprofloxacin as a quinolone antibiotic and EDTA and sodium citrate as chelating agents. Mitomycin C is a compound commonly used for induction of temperate phages.[22,23] In addition, some quinolones, such as ciprofloxacin, have also been used for induction of phages.[24–26] Both compounds activate the SOS response through RecA activation. EDTA and sodium citrate were also applied because they have been reported to increase the number of copies of Shiga toxin gene (*stx*) in temperate Stx phages when a culture of a strain lysogenic for a Stx phage is treated with 20 mM EDTA, even in the absence of RecA.[27]

Materials and methods

Samples

Eighteen raw urban wastewater samples were collected between autumn 2009 and spring 2013 from the influent of a wastewater treatment plant in the Barcelona area, including a number of cities and towns, with ~500 000 inhabitants. Eighteen river samples were collected monthly between winter 2010 and summer 2012 from the Llobregat river near the Barcelona area, which is subject to anthropogenic pressure.[15] Twenty-eight archived wastewater samples from animals (27 from cattle and 1 from poultry) that were collected from several slaughterhouses and farms in Spain were included in this study.[16]

All samples were collected in sterile containers, transported to the laboratory at 5 ± 2°C within 2 h of collection and processed immediately for bacterial counts and further experiments. Archived samples had been stored at −70°C for >1 year.

Bacterial strains and media

The clinical *Escherichia coli* strain 266 was used as a control for *qnrA* and the environmental *Enterobacter cloacae* strain 565 was used as a control for *qnrS*. *E. coli* WG5 (ATCC 700078) was used as a host for evaluation of somatic coliphages.[28] Luria–Bertani (LB) agar or broth was used for routine bacterial propagation. Tryptic soy agar (TSA) and Chromocult® Coliform Agar (Merck, Darmstadt, Germany) were used for the detection of heterotrophic bacteria and *E. coli* respectively. For the detection of resistant bacteria, media were supplemented with 32 mg/L ampicillin (Sigma-Aldrich, Steinheim, Germany), 25 mg/L nalidixic acid (Sigma-Aldrich) or ciprofloxacin at 4, 1, 0.4 and 0.1 mg/L (Sigma-Aldrich). A self-inducible Cdt bacteriophage was used as negative control for phage induction.[29]

Microbiological parameters

The extent of faecal contamination in the samples was established by counting the total heterotrophic bacteria grown at 37°C and *E. coli* as bacterial indicators. Heterotrophic bacteria and *E. coli* were analysed by the membrane filtration method, in line with previously standardized methods.[30] Briefly, serial decimal dilutions of urban wastewater and river water were filtered through 0.45 mm pore membrane filters (0.45 μm and 47 mm white-gridded EZ-Pak® Membrane Filters, Millipore). Membranes were placed upside up on the respective agar media and incubated at 37°C for 18 h. To evaluate the presence of bacteria resistant to antibiotics, samples were processed as described above and incubated in TSA or Chromocult® coliform agar for 2 h at 37°C. Then, membranes were transferred to TSA or Chromocult® coliform agar containing the respective antibiotics and further incubated at 37°C for 18 h.

Somatic coliphages, proposed as faecal viral indicators, were included to get an indication of the levels of virulent bacteriophages in the samples,[28] and to evaluate the effect of the inducers on the presence of virulent phages that could have been induced from the bacterial population. This method allows detection of many virulent infectious phages from a faecal sample with a single host strain (*E. coli* WG5) and through the double agar layer plaque assay.[28]

Standard PCR procedures

Conventional PCRs for ARGs bla_{TEM} and $bla_{CTX\text{-}M}$ were performed as described in previous studies.[15] For *qnr* amplification, *qnr*-positive strains were used as positive controls and PCR was performed using the oligonucleotides described in Table S1 (available as Supplementary data at *JAC* Online) with a GeneAmp PCR system 2700 (Applied Biosystems, Barcelona, Spain). An aliquot of 5 μL of each PCR product was analysed by agarose (0.8%) gel electrophoresis and bands were viewed by ethidium bromide staining. When necessary, PCR products were purified with a PCR Purification Kit (Qiagen Inc., Valencia, USA).

qPCR procedures

For quantification of each ARG in DNA in phage particles (phage DNA), TaqMan qPCR assays using standards prepared as previously described for bla_{TEM} and $bla_{CTX\text{-}M}$ genes were used.[15]

A real-time qPCR assay for *qnrA* and *qnrS* was developed in this study to detect both the quinolone resistance genes in phage DNA isolated from urban wastewater samples. The qPCR oligonucleotides for *qnrA* detected seven variants (*qnrA*1–7) of the gene and showed a detection limit of 3.1 gene copies (GC)/μL (threshold cycle of 33.5). The new qPCR developed for *qnrS* detected six variants (*qnrS*1–6) and showed a detection limit of 8.3 GC/μL (threshold cycle of 30) (Figure 1).

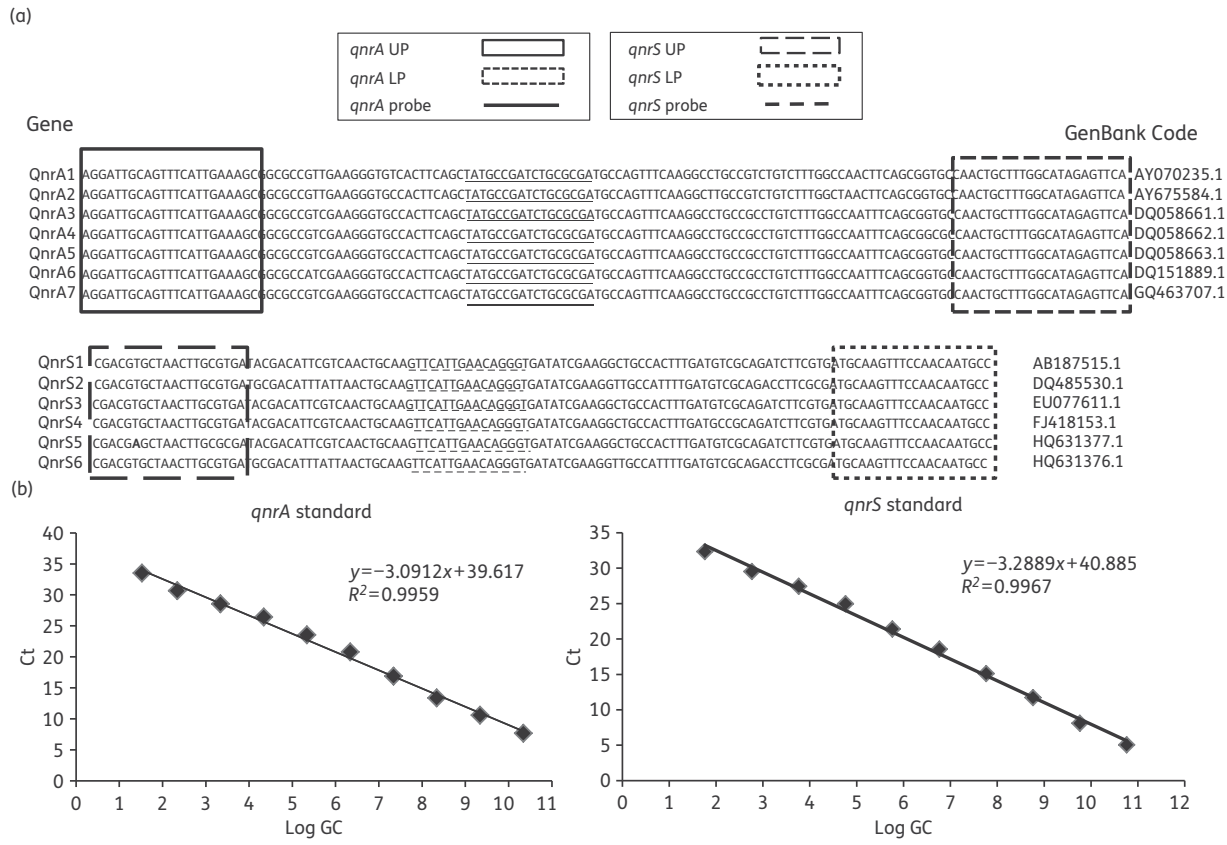

Figure 1. (a) Primers and TaqMan probe resulting from the alignment of *qnrA* genes and *qnrS* genes. Sequences of the probe, upper primer (UP) and lower primer (LP) are shown for each gene. The column on the right indicates the GenBank accession numbers of some of the genes used in the alignment. (b) Standard curves for *qnrA* and *qnrS* qPCR.

For the generation of standards for the qPCR assays, a plasmid construct was used. The 565 bp fragment of *qnrA* and the 425 bp fragment of *qnrS*, both obtained by conventional PCR as described above and purified using a gel extraction kit (PureLink Quick Gel Extraction and PCR Purification Combo Kit, Invitrogen Carlsbad, USA), were cloned with a pGEM-T Easy Vector (Promega, Barcelona, Spain) for insertion of PCR products, following the manufacturer's instructions. The construct was transformed by electroporation into *E. coli* DH5α electrocompetent cells. Cells were electroporated at 2.5 kV, 25 F capacitance and 200 Ω resistance in a BTX ECM 600 Electroporation System (Harvard Apparatus, Inc., MA, USA). Colonies containing the vector were screened by conventional PCR to evaluate the presence of the vector containing each insert. The presence of the insert in the vector and its orientation were assessed by PCR and sequencing. The vector containing the insert was purified from the positive colonies using the Qiagen Plasmid Midi purification kit (Qiagen Inc., Valencia, USA) and the concentration of the vector was quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Thermoscientifics, Wilmington, USA). The reaction product was linearized by digestion with XmnI restriction endonuclease (Promega Corp., Madison, USA). The restricted product was purified and quantified for use in the qPCR assays.

The GC/μL value of the stock prepared was calculated for each gene and the standard curve for qPCR was prepared as previously described.[15] Three replicates of each dilution were added to each qPCR.

Using the software tool Primer Express 3.0 (Applied Biosystems), primers and probes were selected for use in a standardized TaqMan amplification protocol. Primers and 6-carboxyfluorescein (FAM)-labelled fluorogenic probes were commercially synthesized by Applied Biosystems (Spain). *qnrS* and *qnrA* probes were minor-groove binding (MGB) probes with an FAM reporter and a non-fluorescent quencher (NFQ). Primers and probes were used under standard conditions in a StepOne Real-Time PCR System (Applied Biosystems, Spain). Primer and probe specificities were determined with sequence alignments using BLAST and NCBI data entries. They were tested for cross-reactions with the respective susceptible strains. They were amplified in a 20 μL reaction mixture with TaqMan Environmental Real-Time PCR Master Mix 2.0 (Applied Biosystems, Spain). The reaction contained 7 μL of the DNA sample or quantified plasmid DNA. Thermal cycling was performed under standard conditions as indicated by the manufacturer of the StepOne Real-Time PCR system. Briefly, an initial setup of 10 min at 95°C was followed by 40 cycles of 15 s of denaturation at 95°C and 1 min of annealing/extension at 60°C. All samples were run in duplicate, as well as the standards and positive

and negative controls. The GC value was defined as the average of the duplicate data obtained.

To screen for PCR inhibition, dilutions of the standard were spiked with environmental DNA and the experimental difference was compared with the known number of copies of the target genes in the standards. Inhibition of PCR by environmental DNA was not detected.

Sequencing

The amplimers cloned to prepare the qPCR standards were sequenced with an ABI PRISM BigDye 3.1 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Spain), following the manufacturer's instructions and with the primers shown in Table S1. All sequencing was performed at least in duplicate.

Purification of DNA in phage particles

To purify DNA in phage particles from the samples (phage DNA), 50 mL of each sample was centrifuged at 3000 **g** for 10 min, passed through low protein-binding 0.22 μm pore membrane filters (Millex-GP, Millipore, Bedford, MA) and then 100-fold concentrated at 3000 **g** by means of protein concentrators (100 kDa Amicon Ultra centrifugal filter units, Millipore, Bedford, MA), following the manufacturer's instructions. The concentrate was recovered from the tube and treated with chloroform to rule out the presence of possible vesicles containing DNA, and then treated with DNase (100 U/mL of the phage lysate) to eliminate any free DNA that might be present in the samples outside the phage particles. After heat inactivation of the DNase, and to confirm that bacterial or free DNA containing the target genes had been removed from the sample, an aliquot of the phage lysate at this stage was amplified for eubacterial 16S rDNA by conventional PCR and the different ARGs by qPCR (Table S1).

DNA from the phage fraction was isolated from phage lysates as previously described.[15,16] The concentration and purity of the DNA extracted were determined with a NanoDrop ND-1000 spectrophotometer.

Assays with inducing agents

To evaluate the effects of different compounds on phage induction from the bacterial populations in the samples, 50 mL of raw urban wastewater was incubated with the addition of mitomycin C (0.5 mg/L), ciprofloxacin (4, 1, 0.4 and 0.1 mg/L), EDTA (20 mM, pH = 7.2) or 0.2 M sodium citrate, in aerobic conditions under agitation (180 rpm). When indicated, the samples were supplemented with glucose (0.1 mg/mL) and analysed at 37°C for 18 h or at 22°C for 48 h. Phage DNA was purified from the samples with or without inducing agent and used as a template for the quantification of the ARGs by qPCR. For anaerobic conditions, samples were incubated in anaerobic jars.

Statistical analyses

Data and statistical tests were performed using the Statistical Package for Social Science (SPSS) software. One-way analysis of variance (ANOVA) was used to evaluate the differences between the resistance genes detected in phage DNA and the difference in the densities of virulent phages in the induction experiments. Evaluations were based on a 5% significance level in both cases, where a P value of <0.05 was considered to denote a significant difference.

Results

Prevalence of heterotrophic bacteria and E. coli in urban wastewater and river water samples

The samples showed levels of bacterial and viral indicators that were relatively homogeneous in all the urban and river water samples tested (Table 1) and similar to those previously reported from the same source.[15,31] River water samples showed significantly lower numbers ($P<0.05$) than urban wastewater ones; the differences were attributed to the lower faecal input received by river water. The numbers of resistant bacteria were lower than the numbers of bacteria cultured without antibiotics, as expected, though the numbers of resistant bacteria were still quite high, suggesting that bacteria resistant to some antibiotics that were common in the water samples of our study.

Total heterotrophic bacteria showed average values of 7 $\log_{10}$ cfu/mL and 5 $\log_{10}$ cfu/mL of *E. coli* in urban wastewater (Table 1). Most heterotrophic bacterial isolates were resistant to ampicillin and nalidixic acid and showed densities of <1 logarithm below those of the colonies obtained without antibiotic selection. River water samples showed values at least 3 $\log_{10}$ units lower than urban wastewater for all microbiological parameters.

Table 1. Samples analysed and microbiological parameters

	Urban wastewater (n=18)		River water (n=18)	
	average $\log_{10}$ cfu/mL (SD)	differences between absence and presence of antibiotics	average $\log_{10}$ cfu/mL (SD)	differences between absence and presence of antibiotics
Heterotrophic bacteria	7.33 (6.81)	—	4.16 (3.72)	—
E. coli	5.58 (5.21)	—	1.76 (1.63)	—
Heterotrophic bacteria AMP^R	7.11 (6.96)	0.22	4.04 (4.08)	0.12
Heterotrophic bacteria CIP^R	5.30 (5.09)	2.03	1.83 (2.00)	2.33
Heterotrophic bacteria NAL^R	6.99 (6.83)	0.34	3.59 (3.08)	0.57
E. coli AMP^R	5.10 (4.77)	0.48	1.63 (1.41)	0.13
E. coli CIP^R	4.61 (4.19)	0.97	0.81 (0.62)	0.95
E. coli NAL^R	4.98 (4.45)	0.60	1.04 (1.06)	0.72
Somatic coliphages	4.41 (0.51)	—	2.39 (0.44)	—

AMP^R, ampicillin resistant; CIP^R, ciprofloxacin resistant; NAL^R, nalidixic acid resistant.

Resistant *E. coli* also showed values close to those of the bacteria grown without antibiotic selection, particularly when using ampicillin. Ciprofloxacin was the antibiotic showing the largest difference (>2 $\log_{10}$ cfu/mL), in both heterotrophic bacteria and *E. coli*, compared with the wastewater and river samples without antibiotics.

It should be noted that the antibiotic concentrations used in these studies were in the low range, in accordance with other reports analysing resistance in the environment,[32] in order to avoid inhibition of the growth of stressed or damaged bacterial cells.

Prevalence of qnrA and qnrS in the DNA of the phage fraction of the samples

The *qnrA* gene was more prevalent than *qnrS* in the phage DNA of the samples analysed. All urban wastewater and river samples were positive for *qnrA* in the phage DNA as were 71.4% of the archived animal wastewater samples. On average, river water and animal wastewater showed *qnrA* densities less than one order of magnitude below those of urban wastewater (Figure 2).

*qnr*S was less prevalent, detected in 38.9% of urban wastewater, 22.2% of river water and 3.6% of animal wastewater samples (Figure 2). The densities of the genes detected in the few positive samples were, however, higher than the values observed for *qnr*A and were higher in urban and animal wastewater than in river water.

For *qnrA*, reference values obtained from bacterial DNA in urban wastewater samples were on average 1.8×10^3 versus 1.3×10^2 GC/mL in river water samples. On average, *qnrS* showed densities of 2.0×10^5 GC/mL in urban wastewater and 6.9×10^3 GC/mL in river water samples.

Controls described in the Materials and methods section, designed to rule out the presence of non-encapsidated DNA and DNA in vesicles, showed negative values, which indicated that the results obtained were due to amplification of the DNA contained within viral particles.

Comparing the differences between urban wastewater and river water for the prevalence of *qnr* genes in phage DNA with the differences observed for quinolone-resistant bacteria (Table 1), wider differences between resistant bacteria were found. This could be attributable to several facts. First, resistance in bacteria is caused by genes other than *qnr*; second, we were detecting a fraction of quinolone-susceptible autochthonous bacteria in river water, while a larger fraction of faecal bacteria from humans is present in urban wastewater; third, there is stronger persistence of *qnr* in phages, protected within the phage capsid in the river environment, while bacterial DNA could have been degraded or bacteria could have been grazed.

Phage inducers: mitomycin C, ciprofloxacin and EDTA

Mitomycin C, ciprofloxacin and EDTA were assayed in wastewater samples to study whether these compounds could induce phages carrying ARGs from the bacterial population in the sample and hence increase the number of ARGs in the phage DNA fraction of the induced samples. Since these experiments were intended to induce phages from the bacterial population occurring in the samples, glucose was added to the samples to stimulate bacterial metabolism. Assuming that part of the bacterial population in the samples consisted of allochthonous (faecal) bacteria but also autochthonous (environmental) bacteria, the experiments were performed at 37°C for 18 h, but also at 22°C for 48 h for optimal growth of both types of population.

The GC/mL values of each gene in the phage DNA of the samples induced were compared with those of the non-induced samples assayed under the same conditions. The most striking result was that samples incubated with EDTA (and, as shown later, with sodium citrate), with or without glucose showed a significant (ANOVA, $P<0.05$) increase in *qnr* GC/mL in phage DNA when compared with the non-induced samples (Table 2). *qnrS* showed the highest number of GC/mL in phage DNA, reaching differences of up to 4.46 $\log_{10}$ when EDTA was present and the samples were incubated at 22°C without glucose. Under the other conditions, the increase in the density of *qnr*S with EDTA varied from 2.6 to 4 $\log_{10}$ units (Table 2).

Regarding the presence or absence of glucose and the influence of temperature, there were no significant differences (ANOVA $P>0.05$) between the number of GC/mL in phage DNA when samples were incubated at 37 or 22°C or when glucose was added.

In this study, we decided to seek ARGs other than *qnr* to verify whether the inducing agents would cause a similar effect. bla_{TEM}, $bla_{\text{CTX-M}}$ and *mecA* were analysed (Table 2). bla_{TEM} and, to a lesser extent, $bla_{\text{CTX-M}}$ showed a significant ($P<0.05$) increase in the GC/mL value in phage DNA after addition of EDTA, with differences of 2.34–3.31 $\log_{10}$ for bla_{TEM} and 0.82–1.60 $\log_{10}$ for $bla_{\text{CTX-M}}$. However, there was no significant ($P>0.05$) increase in GC/mL for the *mecA* gene when the inducers were present. Whether this was due to the gene itself or because this gene was located in Gram-positive bacteria could not be decided from our data.

Remarkably, the differences (increase or reduction in GC numbers) were not significant ($P>0.05$) (Table 2) when samples were incubated with mitomycin C or ciprofloxacin, known to be inducers of temperate phages by means of activation of RecA and therefore the SOS pathway. In some cases densities after induction were lower than those of the non-induced samples, probably due to the inherent uncertainty of the method. Given the results obtained with ciprofloxacin, we assayed different concentrations of the antibiotic (0.4, 1 or 10 mg/L) but, as observed at 4 mg/L, there were no significant ($P>0.05$) differences in the increase in GC/mL of any of the ARGs in phage DNA. Results given are for 4 mg/L only.

To rule out a possible effect of EDTA on the qPCR assay or on the extraction method that might cause the increase in GC number, we also evaluated as a control another temperate phage available within our research group. This phage harbours the cytolethal distending toxin (Cdt-V).[29] The phage has been reported as self-inducible, since it does not increase the number of phages induced after treatment with any of the inducing agents assayed here. No significant ($P>0.05$) increase in the GC number of Cdt phages in phage DNA after EDTA treatment was observed in this study (data not shown), and was reported previously.[29] These results confirm that the effect of the chelating agents was not due to any interference in the extraction or amplification process.

Sodium citrate

Since the effect observed by EDTA was attributable to its chelating properties,[27] we attempted to assay whether the chelating effect

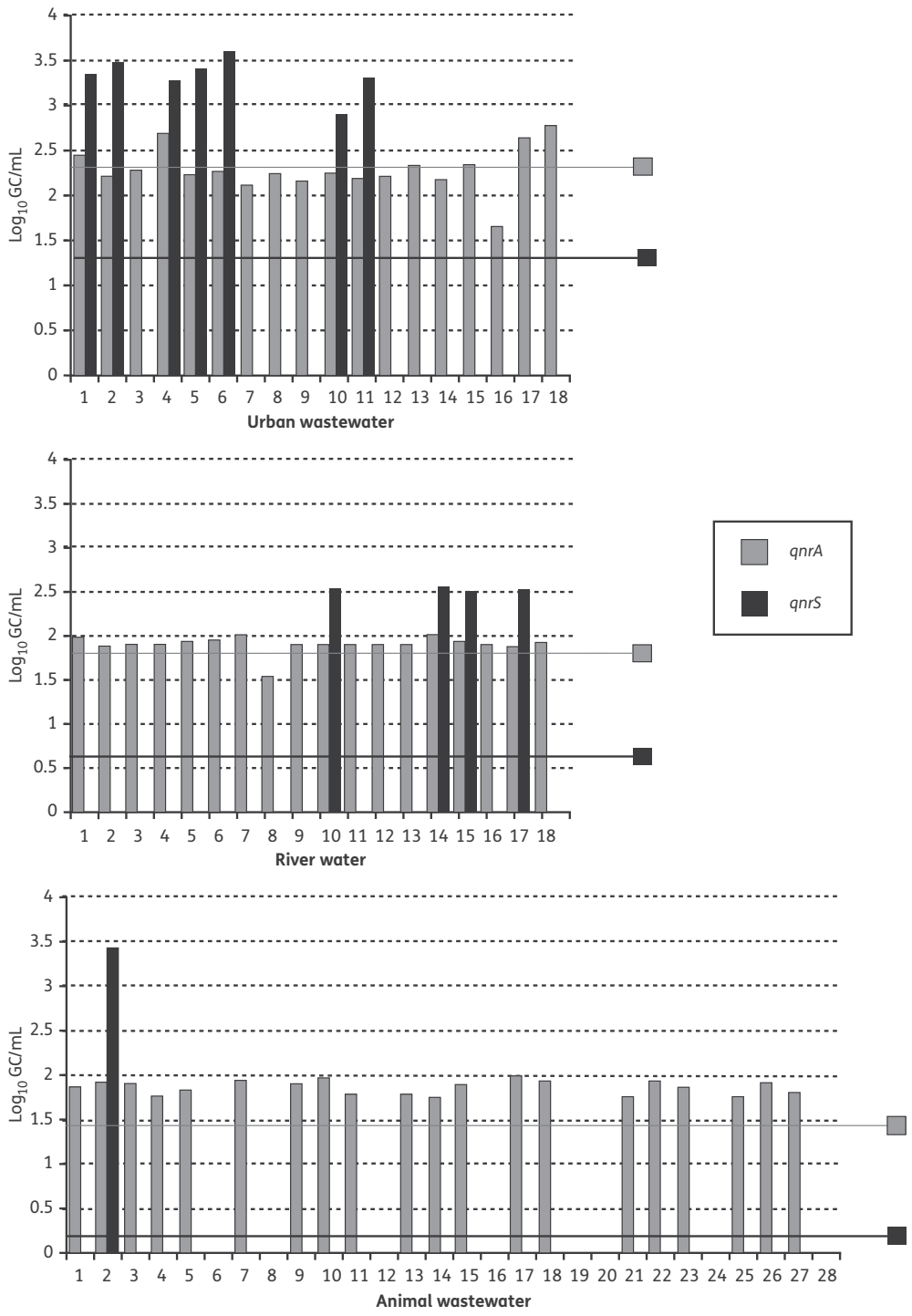

Figure 2. Densities of *qnrA* and *qnrS* genes (GC/mL) in phage DNA of raw urban wastewater, river and animal wastewater samples. Horizontal lines show the averaged values for *qnrA* and *qnrS*.

Table 2. Differences expressed as $\log_{10}$ GC/mL of bla_{TEM}, $bla_{CTX\text{-}M}$, *qnrA*, *qnrS* and *mecA* in phage DNA obtained from induced versus non-induced samples with and without glucose and incubated at 37°C and 22°C under aerobic conditions

		Differences between induced and non-induced samples ($\Delta\log_{10}$ GC/mL)											
		mitomycin C−control				ciprofloxacin (4 mg/L)−control				EDTA−control			
		−glucose		+glucose		−glucose		+glucose		−glucose		+glucose	
Gene		37°C	22°C	37°C	22°C	37°C	22°C	37°C	22°C	37°C	22°C	37°C	22°C
bla_{TEM}	inducer−control	−0.04	0.06	0.01	−0.53	−0.12	0.21	0.23	−0.46	**2.34**	**3.31**	**2.58**	**2.60**
	standard error	0.22	0.03	0.21	0.11	0.18	0.21	0.04	0.02	0.30	0.28	0.37	0.55
$bla_{CTX\text{-}M}$	inducer−control	0.12	−0.38	−0.12	0.10	0.16	0.44	0.49	−0.62	**0.82**	**1.01**	**1.60**	**1.27**
	standard error	0.06	0.55	0.01	0.12	0.28	0.30	0.34	0.17	0.20	0.35	0.15	0.69
qnrA	inducer−control	−0.24	−0.54	0.21	0.18	−0.52	−0.88	−0.76	−0.48	**0.88**	**1.01**	**1.52**	**1.50**
	standard error	0.52	0.10	0.56	0.05	0.53	0.24	0.07	0.07	0.41	0.52	0.43	0.53
qnrS	inducer−control	−0.14	0.31	−0.28	0.40	−0.47	0.57	−0.22	0.09	**2.85**	**4.46**	**2.60**	**4.00**
	standard error	0.25	0.10	0.06	0.11	0.17	0.23	0.18	0.07	0.27	0.13	0.38	0.24
mecA	inducer−control	0.13	0.08	0.45	0.08	0.05	0.49	−0.27	0.02	0.17	0.19	0.06	0.22
	standard error	0.07	0.04	0.25	0.04	0.11	0.30	0.22	0.07	0.12	0.01	0.09	0.03

		sodium citrate−control			
		−glucose		+glucose	
Gene		37°C	22°C	37°C	22°C
bla_{TEM}	inducer−control	**2.74**	**2.73**	**2.14**	**0.95**
	standard error	0.21	0.27	0.45	0.51
$bla_{CTX\text{-}M}$	inducer−control	**1.06**	**0.93**	**0.86**	**0.67**
	standard error	0.10	0.08	0.04	0.01
qnrA	inducer−control	**0.85**	**1.77**	**0.65**	**1.95**
	standard error	0.29	0.20	0.03	0.01
qnrS	inducer−control	**1.90**	**2.00**	**0.61**	**0.88**
	standard error	0.44	0.45	0.22	0.30
mecA	inducer−control	ND	ND	ND	ND
	standard error	—	—	—	—

ND, not detected.
Results are expressed as the mean of five independent experiments and the standard errors are indicated. Bold font indicates a significant increase (ANOVA $P<0.05$).

was the cause of the increase in ARGs in phage DNA. For this purpose samples were treated with another chelator, sodium citrate, which, like EDTA, is a chelator of Ca^{2+} and Mg^{2+}. Results observed with sodium citrate in *qnrA*, *qnrS*, bla_{TEM} and $bla_{CTX\text{-}M}$ showed the same effect as EDTA. Sodium citrate significantly ($P>0.05$) increased the number of ARGs in phage DNA of induced samples compared with non-induced samples (Table 2). The highest increase in GC number was observed for bla_{TEM}. As observed previously when using EDTA, no increase in the GC number of *mecA* in phage DNA was observed, and the use of Cdt phage as control did not result in any increase in the number of induced phages.

Anaerobic conditions

Samples incubated under anaerobic conditions did not show a significant ($P>0.05$) increase when using mitomycin C or ciprofloxacin (Table 3). In contrast, when using chelating agents there was a clear effect, shown by a significant ($P<0.05$) increase in the GC number of bla_{TEM} and *qnr* with EDTA. $bla_{CTX\text{-}M}$ genes showed an increase with EDTA, but it was not significant. Only *qnrS* with sodium citrate showed a significant increase. Results showed the same trends as assays under aerobic conditions.

Effect of inducing agents on virulent phages

We assayed the influence of the inducing agents on infectious somatic coliphages. The infectivity of the phages was confirmed by their ability to generate plaques of lysis on a monolayer of *E. coli* WG5 used as a host strain. Five urban wastewater samples were treated with mitomycin C, ciprofloxacin, EDTA and sodium citrate, and somatic coliphages present in the samples were evaluated before and after treatment with the four inducing agents (Figure 3). The differences between induced samples and controls were mostly not significant ($P>0.05$). On the few occasions when the difference was significant (e.g. with EDTA or sodium citrate) the induced sample showed lower density than the control. Only samples treated with mitomycin C and glucose showed a non-significant ($P<0.05$) increase, of <0.5 $\log_{10}$ unit.

Discussion

The mobilization of ARGs described in phages could be caused by generalized transduction.[33] The phage particles causing generalized transduction do not contain the phage genome, but fragments of bacterial DNA. As these particles are unable to cause lysis in a host strain, they might not be detectable by plaque assay or leave traces of phage DNA in the recipient cell. Nevertheless, they can infect a susceptible host and transduce the genes that they are mobilizing. A plausible interpretation of our results would be that the phages carrying *qnr* (as well as other ARGs) could be mainly generalized transducing particles. One argument to support this hypothesis is that the inducing agents have no effect on the densities of virulent phages and that these densities are not influenced by the amount of temperate phages that could be induced from the bacteria present in the sample, if any. This is either because the phages induced by chelating agents were not producing visible lytic plaques or because the increase in the number of phages did not overcome the number of virulent phages already present in the samples.

Table 3. Differences expressed as $\log_{10}$ GC/mL of bla_{TEM}, $bla_{CTX\text{-}M}$, *qnrA*, *qnrS* and *mecA* in phage DNA obtained from induced versus non-induced samples at 37°C and 22°C under anaerobic conditions

		Differences between induced and non-induced sample ($\Delta\log_{10}$ GC/mL)							
		mitomycin C−control		ciprofloxacin (4 mg/L)−control		EDTA−control		sodium citrate−control	
Gene		37°C	22°C	37°C	22°C	37°C	22°C	37°C	22°C
bla_{TEM}	inducer−control	−0.05	−0.18	0.01	−0.23	**1.36**	**1.31**	0.34	0.22
	standard error	0.14	0.29	0.26	0.01	0.39	0.01	0.25	0.19
$bla_{CTX\text{-}M}$	inducer−control	0.02	−0.18	−0.29	−0.21	0.34	0.19	0.23	0.22
	standard error	0.10	0.17	0.58	0.59	0.09	0.10	0.16	0.29
qnrA	inducer−control	−1.31	−0.59	−1.62	−1.10	**1.50**	**2.67**	0.22	0.34
	standard error	0.14	0.02	0.16	0.04	0.17	0.02	0.04	0.06
qnrS	inducer−control	0.07	−0.24	−0.26	−0.28	**3.31**	**3.27**	**1.55**	**1.58**
	standard error	0.01	0.12	0.15	0.06	0.08	0.26	0.54	0.32
mecA	inducer−control	0.03	0.25	−0.30	0.01	−0.08	−0.33	0.11	0.17
	standard error	0.12	0.00	0.01	0.10	0.10	0.15	0.07	0.07

Results are expressed as the mean of five independent experiments and the standard errors are indicated. Bold font indicates a significant increase (ANOVA $P<0.05$).

A second argument to support the hypothesis of generalized transducing particles is that the numbers of phages carrying *qnr* did not increase after addition of agents inducing the lytic cycle of temperate phages, which work well on many specialized transducing phages, such as mitomycin C or ciprofloxacin, though they did increase after addition of EDTA or sodium citrate. It is not clear whether the mechanisms of EDTA-mediated phage induction are similar to those of other inducing agents and whether the differences in induction observed with EDTA and mitomycin C may be attributed to activation of different pathways that lead to activation of the phage lytic cycle.[27] Considering its chemical structure, it is not to be expected that EDTA enters the bacterial cell,[34] and the uptake of EDTA is limited to a few strains able to use it as the sole source of carbon, nitrogen and energy.[34] Thus, its effect must be due to external influence, very likely on the envelope of bacteria, since EDTA is known to induce envelope stress responses.[35,36] Chelation of membrane cations from outside the cell should cause stress in the bacterial envelope.[36] This was suspected since a similar effect was observed when using another chelating agent, sodium citrate. In contrast, mitomycin C is known to activate the SOS response through RecA activation, leading to suppression of lysogeny control and induction of lytic cycles in temperate phages.[22,23,27] The inducing properties of EDTA are attributed to its chelating effects and have been reported to be independent of RecA.[27] However, there is no evidence that the particles induced by EDTA or citrate are completely functional temperate phages in all cases. It is known that phages performing generalized transduction, such as P22,[37] can give altered packaging specificity, leading to the formation of transducing particles at increased frequencies. This could be a possible cause of the increase in ARGs in DNA from phage particles observed when chelating agents were used.

Recently, Modi *et al*.[38] demonstrated that treatment with antibiotics increases the number of ARGs in the intestinal phageome of mice. As in our study, these authors found that phage encapsulation was stimulated by stress, plausibly as a mechanism to increase the robustness of the populations in the gut under these circumstances. It is reasonable to assume that other stressing factors, such as chelators, could lead to a similar effect. The higher number of gene copies in the bacterial cell after treatment with chelators would result in better chances for these genes to be later mobilized by transduction. If our assumption is correct and chelating agents stimulate generalized transducing particles, then this is consistent with no increase in the densities of virulent infectious phages observed, since phage-derived generalized transducing particles will not be able to cause plaques of lysis, although they will be able to transduce the ARGs to susceptible bacterial recipients.

We ran the induction experiments in this study because we believed that the use of quinolones, used as inducers of temperate phages, could stimulate the induction of Qnr-encoding phages and thus contribute to the transference of their own resistance. However, this was not confirmed by our experiments. A non-significant increase in the densities of *qnr* copies in phage DNA was observed only under a few conditions. In theory, Qnr prophage induction may occur in the presence of quinolones, and this has been demonstrated with intestinal populations.[38] However, it was not observed using the natural bacterial populations in our samples, even when using different ciprofloxacin concentrations, which may be because the density or physiological state of the bacteria was different.

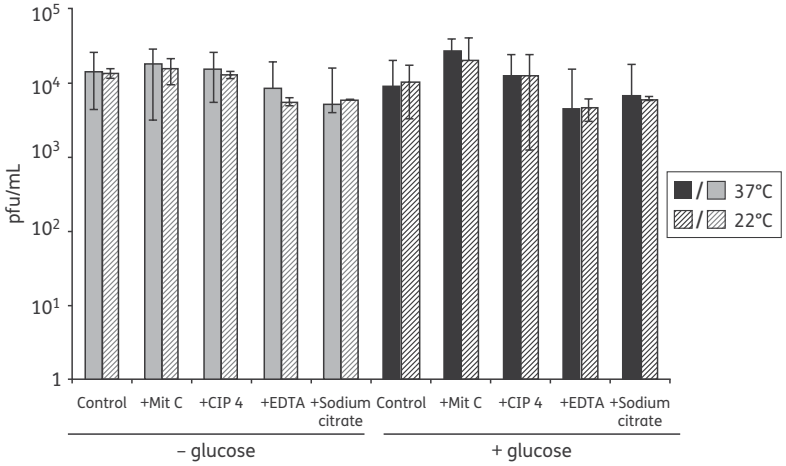

Figure 3. Effect of inducers on the densities of virulent somatic coliphages in raw urban wastewater, evaluated with and without inducing agents (mitomycin C, ciprofloxacin, EDTA and sodium citrate) at 22°C and 37°C. The pfu/mL values were determined following the ISO 10705-2 method for determination of somatic coliphages. Data presented are the means of five independent experiments. Error bars indicate SD. Mit C, mitomycin C; CIP 4, ciprofloxacin (4 mg/L).

EDTA is a common chelating agent (Mg^{2+} and Ca^{2+}) and antioxidant that is used as a food additive and has applications in medicine, cosmetics and pharmaceutical products.[39] It is also used to treat acute hypercalcaemia and lead poisoning.[39,40] As an antimicrobial agent, EDTA acts by disrupting the structure of the outer membrane of bacteria, so making this more permeable and therefore accessible to other antimicrobial agents.[35] Similarly, sodium citrate has several applications in food as a preservative (in Europe as food additive E331; https://webgate.ec.europa.eu/sanco_foods/main/index.cfm) and in medical usage as an anticoagulant. The effect of EDTA, also observable with sodium citrate, on the densities of ARGs in general and of quinolones in particular in the bacteriophage DNA fraction must be considered when applying them, since these agents could increase the number of particles harbouring these genes, increasing the possibilities of gene transfer and the generation of new resistant clones.

As indicated previously for other ARGs,[15,16,41] mobilization of quinolone resistance genes from their chromosomal or plasmid location and transduction to new clones could contribute to the spread of resistance and to a quick emergence of new resistant clones, already observed in practice,[9] which will represent the greatest challenge and the most important concern for the treatment of microbial infections in the future.

Conclusions

In summary, we showed that quinolone-resistance genes *qnrA* and *qnrS* are found in the bacteriophage DNA fraction isolated from urban wastewater, river water and animal faecal samples. Due to the unknown nature of these phage particles, we evaluated the potential induction of these phages by known inducing agents. There was no increase in GC number in DNA of phage particles when treated with typical inducers of temperate phages (mitomycin C or ciprofloxacin), but a significant increase when treated with EDTA or sodium citrate, probably due to their chelation properties. No increase in infectious lytic coliphages was found with any treatment. This, together with the fact that inducers of temperate phages did not cause an increase in Qnr phages, suggests that the *qnr*-encoding phages could be in fact generalized transducing particles. The presence of *qnr*-encoding phage particles and the effect caused by some chelating agents strongly increase the spread of some ARGs and also create the possibility of new transduction events that might cause the emergence of new resistant strains.

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Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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3.3. Chapter 3: Evaluation of ARGs in the DNA of bacterial and bacteriophage fraction in wastewater samples from Tunisia and comparison with results obtained in Barcelona area.

❖ ARTICLE 4

Títol: Antibiotic resistance genes in Tunisian and Spanish wastewaters and their use as markers of antibiotic resistance patterns within a population

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RESUM

Introducció

La resistència a antibiòtics, tant en patògens humans com en animals, és un fenomen d'especial preocupació a nivell global, sobretot per l'increment de gens de resistència a antibiòtics com a conseqüència de la introducció dels antibiòtics en ambients antropogènics. Tot i així, s'han observat diferències marcades en els perfils de resistència de bacteris patògens entre països i àrees geogràfiques diferents i la disseminació de les resistències no hauria tingut lloc de manera simultània a tot arreu (Klugman, 2002; Robicsek *et al.*, 2006; Nübel *et al*., 2008; Kumarasamy *et al.*, 2010; Rodriguez-Martinez *et al*., 2011).

Per tant, es considera que hi ha una progressió temporal respecte l'abundància d'uns determinats gens de resistència a antibiòtics i la seva distribució geogràfica que faria possible la utilització d'aquests gens de resistència com a eina per tal d'analitzar els patrons de resistència a antibiòtics en una determinada àrea (Hawkey i Jones, 2009; Paniagua *et al*., 2010). Fins ara, la majoria d'estudis sobre la distribució i epidemiologia de les resistències s'han basat en l'aïllament de patògens procedents de l'ambient clínic però aquesta podria ser una aproximació esbiaixada, ja que és ben conegut que bacteris comensals tant en

humans com en animals contenen també gens de resistència a antibiòtics i han demostrat jugar un paper important en l'aparició i disseminació d'aquests (Salyers *et al*., 2004; Sommer *et al*., 2009; Rolain, 2013; Wasyl *et al*., 2013). Així doncs, les poblacions microbianes associades als entorns humans i animals serien adequades per a l'obtenció d'informació sobre l'epidemiologia de les resistències.

Concretament, l'aigua residual, tant d'origen humà com animal, conté gens de resistència a antibiòtics els quals representen els determinants de resistència predominants en la microbiota intestinal associada a humans i animals (Schwartz *et al*., 2003; Tennstedt *et al*., 2003; Volkman *et al*., 2004; Szczepanowski *et al*., 2009; Novo *et al*., 2013; Rizzo *et al.*, 2013) i per tant, poden aportar informació rellevant sobre les resistències que es troben circulant en la població sana en un determinat ambient o àrea geogràfica. Generalment, els estudis de detecció de gens de resistència a antibiòtics en aigües residuals mitjançant tècniques genòmiques s'han centrat en DNA bacterià. Tot i així, cal tenir present que el viroma, el qual està majoritàriament constituït per bacteriòfags, conté també nombrosos gens de resistència a antibiòtics tal i com han descrit diversos estudis (Muniesa *et al*., 2004; Parsley *et al*., 2010a) i s'ha esmentat en capítols anteriors.

Aquest estudi es va realitzar a partir de mostres procedents de dues EDARs de Tunísia, situades una al sud de Kebili i l'altra a Tunis capital, així com també d'un escorxador de Tunis capital per tal d'avaluar l'abundància de diversos gens de resistència a antibiòtics en DNA bacterià i DNA de la fracció fàgica per establir-ne la comparació amb els resultats obtinguts prèviament a l'àrea de Barcelona.

Objectius

En aquest estudi es van plantejar els objectius detallats a continuació:

- Detecció i quantificació per qPCR de tres gens codificadors de β-lactamases (bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$ i $bla_{CTX\text{-}M\text{-}9}$), dos gens de resistència a quinolones (*qnrA* and *qnrS)* i el gen *mec*A en la fracció de DNA fàgic i DNA bacterià de mostres de dues EDARs i d'un escorxador de Tunísia.

- Comparació del contingut de gens de resistència a antibiòtics en DNA bacterià i DNA fàgic de les mostres de Tunísia amb els resultats obtinguts prèviament a Barcelona.
- Avaluació de la detecció de gens de resistència a antibiòtics com a marcadors per a l'estudi dels patrons de resistència a antibiòtics en una determinada població.

Resultats i discussió

En aquest estudi es van analitzar 6 gens de resistència a antibiòtics. Per una banda, tres β-lactamases: bla_{TEM}, que conté més de 145 variants, el clúster 1 de bla_{CTX-M}, el qual inclou les variants CTX-M-1, 3, 10, 11 i 15, i el clúster 9 de bla_{CTX-M}, el qual inclou les variants CTX-M-9, 13, 14, 16, to 19, 21 a 27. bla_{TEM} i bla_{CTX-M} són les *ESBL*s més àmpliament distribuïdes mundialment entre bacteris Gram-negatius, i concretament, CTX-M-9 i CTX-M-14 són els grups de bla_{CTX-M} més freqüents en aïllaments animals (Patterson, 2003; Coque *et al*., 2008a; Rodrıíguez-Baño *et al*. 2008). Per altra banda, també es van analitzar dos gens de resistència a quinolones: *qnrA* (variants *qnrA1* a *qnrA7*) i *qnrS* (variants *qnrS1* a *qnrS6*) ja que les quinolones i fluoroquinolones són antibiòtics d'ús habitual en clínica i veterinària i la resistència a aquests ha augmentat de manera molt significativa en els darrers anys i s'ha trobat associada a elements genètics mòbils com plasmidis i DNA fàgic en aigua residual (Hooper, 2001; Paterson, 2006). Finalment, el gen *mecA* el qual confereix resistència a meticil·lina en *S. aureus* (*MRSA*).

La quantificació dels diversos gens de resistència a antibiòtics es va realitzar utilitzant la mateixa metodologia dels estudis anteriors de manera que ens va permetre comparar la distribució dels gens de resistència a antibiòtics entre dues zones, Tunísia i Barcelona, amb característiques sòcio-econòmiques, culturals, geogràfiques i climàtiques ben diferents.

En aquest estudi es van analitzar mostres d'aigua residual de l'EDAR 1 al sud de Tunísia (7 mostres d'DNA bacterià i 7 d'DNA fàgic), i de l'EDAR 2 al nord de Tunísia (14 mostres d'DNA bacterià i 16 d'DNA fàgic), i d'un escorxador (5 mostres de DNA bacterià i 5 d'DNA fàgic).

bla_{TEM} vas ser el gen més prevalent en tots tres punts de mostreig. De mitjana, ambdues EDARs, 1 i 2, van presentar valors similars en el nombre de còpies de gen de bla_{TEM} en DNA

bacterià, amb 4.1 i 4.3 unitats logarítmiques de CG/mL, respectivament. En canvi, les densitats de les mostres d'escorxador van ser lleugerament inferiors i més diverses (3.8 unitats logarítmiques de CG/mL de mitjana) (Figura 1). De fet, bla_{TEM} va ser la primera *ESBL* descrita a Tunísia el 1994 i actualment continua sent-ho (Chouchani *et al*., 2011).

Respecte $bla_{CTX\text{-}M\text{-}1}$, la seva prevalença va ser inferior en totes les mostres, amb un 28.6% de les mostres de l'EDAR 1 positives en DNA bacterià, un 14.3% de l' EDAR 2 i un 40% de les mostres d'escorxador amb densitats molt similars d'entre 2.8 a 3 unitats logarítmiques de CG/mL. Si considerem el DNA fàgic, només una mostra procedent de l'EDAR 2 va resultar positiva per aquest gen. Comparativament, $bla_{CTX\text{-}M\text{-}9}$ va ser més prevalent que $bla_{CTX\text{-}M\text{-}1}$ (Figura 3), tant en DNA bacterià com en DNA de partícules fàgiques. Cal tenir present que la primera $bla_{CTX\text{-}M}$ va ser descrita a Tunísia el 2005, pertanyent al grup 9, mentre que les del grup 1 es van descriure inicialment el 2005 i en els darrers anys han estat les més freqüentment descrites (Chouchani *et al*., 2011). Així, es podria considerar que a Tunísia aquests gens es troben probablement encara en fase de dispersió i menys estesos que bla_{TEM}.

Només una mostra va ser positiva per *mecA* mentre que totes van resultar negatives per aquest gen en DNA fàgic. Aquests resultats concordarien amb la baixa prevalença de *mecA* descrita a Tunísia (Kesah *et al*., 2003).

Si ens fixem en *qnrA*, la seva prevalença en DNA bacterià va ser superior que la de *qnrS* tant en ambdues EDARs com en les mostres d'escorxador (Figura 4). Un 71.4% de les mostres de les EDAR 1 i 2 van ser positives per aquest gen i totes les d'escorxador amb densitats mitjanes de 4.5 $\log_{10}$ de CG/mL. En canvi, només 4 de 16 mostres van ser positives per aquest gen en DNA fàgic corresponents a les EDAR 1 i 2, mentre que aquest gen no es va detectar en DNA fàgic de mostres d'escorxador. L'altre gen de resistència a quinolones, *qnrS*, es va trobar en un 14.3% de les mostres de l'EDAR 1, en un 28.6% de les de l'EDAR 2 i en un 20% de les mostres d'escorxador en DNA bacterià amb valors de 4 a 4.3 unitats logarítmiques. En canvi, en el DNA de partícules fàgiques només es va trobar en mostres de l'EDAR 2 (20% de positius) amb valors mitjans de 3.5 $\log_{10}$ unitats logarítmiques de CG/mL.

La disseminació d'aquests gens està en continua evolució (Strahilevitz *et al*., 2009; Rodriguez-Martinez *et al.,* 2011), essent *qnrA* el primer descrit i *qnrS* el més freqüent actualment arreu (Rodriguez-Martinez *et al*., 2011). Els estudis sobre gens *qnr* a Tunísia i països veïns no presenten diferències rellevants entre *qnrA* i *qnrS* (Rodriguez-Martinez *et al*., 2011), tot i que *qnrA* és lleugerament més prevalent (Dahmen *et al*., 2010).

En realitzar la comparació de l'abundància dels gens de resistència analitzats a Tunísia i Barcelona s'observen tendències similars però també diferències destacables.

$bla_{TEM,}$ es troba present en ambdues àrees en totes o quasi totes les mostres i en concentracions similars. La prevalença dels gens *qnrA,* $bla_{\text{CTX-M-9,}}$ $bla_{\text{CTX-M-1}}$ i *qnrS* és relativament semblant respecte a ordre d'abundància tot i que a Tunísia les concentracions detectades van ser inferiors. Aquest fenomen pot estar relacionat amb les diferències existents entre les dues àrees d'estudi. Barcelona, degut a la seva situació geogràfica, rep un elevat nombre de visitants i és un important focus de ramaderia intensiva. A més a més, Espanya és una regió d'elevat consum d'antibiòtics, considerat moderat-alt tant en clínica (ECDC, 2013a) com en veterinària (European Medicines Agency, 2013). Aquests fets, la convertirien en un bon lloc per a l'aparició, selecció i disseminació de bacteris resistents.

Les clares diferències observades pel què fa al gen *mecA*, el qual és abundant en les mostres de Barcelona però és quasi absent a Tunísia, tindrien com a possible explicació el tipus de pràctiques en ramaderia, majoritàriament oví a Tunísia, i amb una predominança de la indústria porcina a l'àrea de Barcelona. La ramaderia, principalment porcina, és considerada com una font important de *MRSA* (De Neeling *et al*., 2007; Khanna *et al*., 2008; Feingold *et al*., 2012).

Conclusions

Com a conclusió d'aquest estudi es desprèn que els gens de resistència a antibiòtics analitzats es troben tant en la fracció de DNA bacterià com en la fracció de DNA fàgic d'aigua residual amb contaminació fecal humana i animal en l'àrea de Tunísia, essent la seqüència per ordre d'abundància: bla_{TEM} > *qnrA* > $bla_{\text{CTX-M-9}}$ >$bla_{\text{CTX-M-1}}$ > *qnrS* > *mecA.*

Les densitats dels gens de resistència a antibiòtics detectats varien segons la seva disseminació i les característiques de l'àrea estudiada.

L'avaluació de gens de resistència a antibiòtics en aigües residuals seria una eina adequada com a marcadors per l'estudi de patrons de resistències en una determinada població.

Informe sobre el factor d'impacte de l'article 4

L'article ***Antibiotic resistance genes in Tunisian and Spanish wastewaters and their use as markers of antibiotic resistance patterns within a population*** ha estat sotmès per publicació el febrer del 2014 a la revista *Environment International* que es troba inclosa en el primer quartil (Q1) de l'àrea temàtica de *Environmental Sciences* (6/210). L'any 2012 la revista va presentar un factor d'impacte 6.248.

Els canvis suggerits pels revisors per a l'acceptació de l'article s'estan duent a terme en el moment de la presentació d'aquesta memòria.

Informe de participació de l'article 4

La doctoranda Marta Colomer Lluch ha realitzta les extraccions d'DNA bacterià i DNA fàgic de les mostres, la seva quantificació, la posterior anàlisi i processat de les dades obtingudes i la seva comparació amb els resultats obtinguts anteriorment. Finalment ha participat en la redacció de l'article i en l'elaboració de taules i figures.

Dr. J. Jofre Dra. M. Muniesa

Antibiotic resistance genes in Tunisian and Spanish wastewaters and their use as markers of antibiotic resistance patterns within a population

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Abstract

The emergence and increased prevalence of antibiotic resistance genes in the environment may pose a serious global health concern. The aim of the study was to evaluate the abundance of several antibiotic resistance genes (ARGs) in bacterial and bacteriophage DNA via real-time qPCR in samples from three different sampling points in Tunisia: two wastewater treatment plants (WWTP1 and 2), and one wastewater from abattoirs slaughtering different animals, and to compare them with results previously obtained in the Barcelona area, in northeast Spain.

The study was conducted using archived samples collected between 2011 and 2012. Eight ARGs were quantified by qPCR from total and phage DNA fraction obtained from all samples. Three β-lactamases (*bla*$_{TEM}$, *bla*$_{CTX-M}$ cluster 1 and *bla*$_{CTX-M}$ cluster 9), two quinolone resistance genes (*qnrA* and *qnrS*), the *mecA* gene that confers resistance to methicillin in *Staphylococcus aureus* were evaluated.

bla$_{TEM}$ was the most prevalent ARG detected at all three sampling points with densities ranging from 3.8 to 4.3 $\log_{10}$units. *bla*$_{CTX-M-9}$ was more prevalent than *bla*$_{CTX-M-1}$ both in bacterial and DNA within phage particles in all samples analysed. Of all the samples analysed, only one from WWTP 1 contained the *mecA* gene in bacterial DNA. None of the phage DNA contained the *mecA* gene. *qnrA* was more prevalent than *qnrS* in bacterial and phage DNA from all sampling points.

In conclusion, our study shows that ARGs are found in the bacterial and phage DNA fraction of human and animal wastewater. The densities of the ARGs vary depending on the shedding of each ARG by each population and the characteristics of each area. Thus, the evaluation of ARGs in wastewaters seems to be suitable as marker reflecting the antibiotic resistance patterns of the population.

1. Introduction

Antibiotic resistance, both in human and animal pathogens, has emerged since the introduction of antibiotics in anthropogenic environments, compromising public and animal health worldwide. Antimicrobial resistance is a major global health problem, but marked variations in the resistance profiles of bacterial pathogens are found between countries and different geographic areas.

The appearance of antibiotic resistances are modulated by events of co-option, mutation, recombination and/or horizontal gene transfer between strains and, once have occurred are subjected to natural selection that allows the widespread dispersal of the strains due to current globalization. The spread of resistance varies both temporally and geographically (Klugman, 2002; Kumarasamy et al., 2010; Nübel et al., 2008; Robicsek et al., 2006; Rodriguez-Martinez et al., 2011). Consequently, there is a temporal progression in the abundance of the corresponding antibiotic resistance genes (ARGs) and their geographical distribution that allows the occurrence of ARGs to be used to analyse the patterns of antibiotic resistance of a given population (Hawkey and Jones, 2009; Paniagua et al., 2010), which could be useful to detect changes on these patterns. To date, studies of the distribution and epidemiology of resistance have mostly been based on, and hence biased towards, the study of pathogens isolated in the clinical environment.

However, it is well known that it is not only pathogens that evolve these ARGs or gene arrangements, but that human and animal commensals also play an important role in the appearance and spread of these genes (Rolain, 2013; Salyers et al., 2004; Sommer et al., 2009; Wasyl et al., 2013). Therefore, microbial populations associated with humans and animals should be a good setting for searching for information about the epidemiology of resistance.

In addition, the microbiological composition of some wastewaters, such as municipal sewage or abattoir wastewaters, is mostly due to human and animal gut microbiomes. It is thus likely that the ARGs found in bacterial populations in wastewaters represent the ARGs dominating in environments in contact with man-made antibiotics. The presence of ARGs and resistant bacteria in wastewaters has been widely reported (Novo et al., 2013; Rizzo et al., 2004; Schwartz et al., 2003; Szczepanowski et al., 2009; Tennstedt et al., 2003; Volkman et al., 2004).

The potential for detecting these ARGs by genomic techniques makes the characterization of the resistome, total or partial, of the microbial populations present in wastewaters feasible. These sorts of studies are typically focused on bacterial DNA. However, the virome, mostly constituted by bacteriophages, has also recently been reported to contain abundant and diverse ARGs in the human gut (Minot et al., 2011; Quirós et al., 2014) and in human and animal wastewater (Colomer et al., 2011a, 2011b; Muniesa et al., 2004; Parsley et al., 2010). Moreover, the fact that genomic studies can be performed independently of culture allows the use of archival sampling. So, although the finding of resistance determinants in a bacterium might not correspond to its phenotypic resistance, the presence of ARGs detected and quantified by molecular techniques will certainly provide relevant information regarding the ARGs circulating in a given environment or geographical area.

This study was conducted using archived samples from raw sewage entering two municipal and one abattoir wastewater treatment plants (WWTPs) in Tunisia, and aimed to evaluate the abundance of several ARGs in bacterial and bacteriophage DNA via real-time qPCR and to compare this with results previously obtained in the Barcelona area in Spain.

The following ARGs were studied: three β-lactamases: bla_{TEM}, which includes more than 145 variants, $bla_{CTX\text{-}M}$ cluster 1, which includes variants CTX-M-1, 3, 10, 11 and 15, and $bla_{CTX\text{-}M}$ cluster 9, which includes variants CTX-M-9, 13, 14, 16 to 19, 21 and 27; two quinolone resistance genes: *qnrA* (including variants *qnrA1* to *qnrA7*) and *qnrS* (including variants *qnrS1* to *qnrS6*); and the *mecA* gene, which confers resistance to methicillin in *Staphylococcus aureus* (MRSA). Quantification was performed using previously described methodology (Colomer Lluch et al., 2011a, b) allowing us to compare the distribution of the ARGs in two different areas that differ in socio-economic and cultural characteristics, climate (especially in the southern location sampled in Tunisia), and geographic background.

These ARGs were selected because bla_{TEM} and $bla_{CTX\text{-}M}$ have been reported to be the most widely distributed extended spectrum β-lactamases (ESBLs) worldwide among Gram-negative pathogenic bacteria, while CTX-M-9 and CTX-M-14 are the most frequent $bla_{CTX\text{-}M}$ groups in animal isolates (Coque et al., 2008; Patterson, 2003; Rodrıíguez-Baño et al., 2008). *mecA* was selected because it confers resistance in Gram positive bacteria that showed a high prevalence in our geographical area (Colomer-Lluch et al., 2011a, b). With regard the quinolones and fluoroquinolones, these are antimicrobials that are commonly used in clinical and veterinary medicine. Resistance to quinolones is dramatically increasing and has been described in both chromosome and acquired mobile genetic elements (MGEs) such as plasmids and in phage DNA in wastewater (Colomer-Lluch et al., 2013; Hooper, 2001; Paterson, 2006).

2. Materials and Methods

2.1. Sampling settings

Samples from Tunisia comprise incoming raw sewage to WWTPs sampled in two locations between 2011 and 2014. The first location was a WWTP treating water equivalent to 20,000

inhabitants corresponding to three towns in a pre-desert area (very warm in summer and low yearly rainfall) in South Tunisia (WWTP 1) with contribution of waste from tourist facilities. The second location was a municipal medium-load WWTP in a metropolitan area in North Tunisia (WWTP 2), the origin of its treated influents being domestic. The third raw wastewater was collected from an abattoir slaughtering mostly sheep and to a lesser extent cattle (Fig. 1). Samples from Spain comprise incoming raw sewage to a WWTPs treating water equivalent to 500,000 inhabitants in the area of Barcelona between 2011 and 2014.

The faecal load of the samples studied as measured by the concentration of somatic coliphages (Jebri et al., 2012) was similar for samples from Tunisia and Spain showing faecal loads as reported in the Barcelona area for these types of sample (Muniesa et al., 2012). The samples were collected in sterile conditions, frozen at -80ºC and stored in dry ice until laboratory analysis. In total, 26 samples were used for total DNA extraction and 28 for DNA extraction from the viral (phage) fraction, followed by quantification by real-time qPCR.

Fig. 1. Geographic location of the three Tunisian sampling points: WWTP 1, WWTP 2 and the slaughterhouse.

2.2. Purification of bacterial DNA

Five mL of each sample was passed through a 0.45 µm EZ-Pak® membrane filter (Millipore, Bedford, Massachusetts), described by the manufacturer as a low protein-binding membrane. This filter allowed the phages to pass through whilst bacteria were retained on the surface of the filter. The membrane containing retained bacteria was recovered in 5 mL of LB. The suspension was then centrifuged at 3000x*g* for 10 min. To recover bacterial DNA, the pellet was resuspended in 200 µL of fresh LB. DNA was then extracted using a QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, USA), following the manufacturer's instructions. Bacterial DNA was resuspended in a final volume of 200 µL and DNA concentration was evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Thermoscientifics, Wilmington, USA).

2.3. Purification of DNA in the phage particles

Recovery of phage particles was performed according to the USEPA protocol (USEPA, 2006). Briefly, 0.05 M $AlCl_3$ was added to 100 mL of well-mixed samples to a final concentration of 0.0005 M and pH was adjusted to 3.5. After centrifugation, the pellet was resuspended in 100 mL of buffered 10% beef extract (pH 8) (LP029B; Oxoid). Polyethylene glycol (PEG) precipitation was performed by adding 50% (w/v phosphate solution pH 7.2) PEG 2000 (Sigma) to the resuspended pellet. After rigorous agitation, the mixture was kept at 4ºC overnight and then centrifuged at 8000x*g* for 90 min at 4ºC. The pellet obtained was then suspended in 5 mL phosphate buffer (pH 7.2) and finally filtered through 0.22 µm nitrocellulose filters. After that, extraction of DNA inside phage particles was performed as previously described (Colomer-Lluch et al., 2011a), with a few modifications, from 1 mL of virus suspension. The viral suspension was treated with chloroform (1:10) in order to avoid the presence of possible vesicles containing DNA, followed by DNase treatment (100

units/mL) for 1 hour at 37ºC to remove any possible free DNA that might be present outside the phage particles. After that, DNase was heat inactivated at 80ºC for 10 minutes. DNA extraction was performed via proteinase K treatment and phenol:chloroform treatment (Colomer-Lluch et al., 2011a; Sambrook and Russell, 2001).

2.4. qPCR procedures

2.4.1. Standard curves

Each ARG under study was amplified by conventional PCR, purified and then cloned into a pGEM-T Easy vector for insertion of PCR products, following the manufacturer's instructions (Promega, Barcelona, Spain), as previously described (Colomer-Lluch et al., 2011). After obtaining and purifying the plasmid construct, the DNA concentration was quantified using a NanoDrop ND-1000 spectrophotomoter (NanoDrop Technologies, Thermoscientifics, Wilmington, USA). Standard curves were generated using serial decimal dilutions of the standard carrying the targeted genes, and the C_T values obtained by qPCR were plotted against log gene copy number (Colomer-Lluch et al., 20011a, b).

2.4.2. Primers and probe design

In brief, the primers and probes for each ARG (Table 1) were designed using the software tool Primer Express 3.0 and commercially synthesized (Applied Biosystems, Spain) and were used in a standardized TaqMan amplification protocol in a Step One Real Time PCR System as previously described (Colomer-Lluch et al., 2011). All probes were Minor groove binding probes with a FAM reporter (FAM: 6-carboxyfluorescein) and a non-fluorescent quencher (NFQ). All samples were run in triplicate, along with the standards, and positive and negative controls. The efficiency (*E*) of our reactions ranged from 95% to 100%.

Table 1.

qPCR primers and probes for qPCR assays used in this study

Target gene	PCR	Sequence	Amplimer (bp)	Ref.
bla_{TEM}	UP	CACTATTCTCAGAATGACTTGGT	85	Lachmayr *et al*., 2009
	LP	TGCATAATTCTCTTACTGTCATG		
	Probe	6FAM-CCAGTCACAGAAAAGCATCTTACGG-MGBNFQ		
$bla_{CTX-M-1}$	UP	ACCAACGATATCGCGGTGAT	101	Colomer-Lluch *et al*., 2011a
	LP	ACATCGCGACGGCTTTCT		
	Probe	6FAM – TCGTGCGCCGCTG-MGBNFQ		
$bla_{CTX-M-9}$	UP	ACCAATGATATTGCGGTGAT	85	Colomer-Lluch *et al*., 2011b
	LP	CTGCGTTCTGTTGCGGCT		
	Probe	6FAM – TCGTGCGCCGCTG- MGBNFQ		
mecA	UP	CGCAACGTTCAATTTAATTTTGTTAA	92	Volkman *et al*., 2004
	LP	TGGTCTTTCTGCATTCCTGGA		
	Probe	6FAM-AATGACGCTATGATCCCAATCTAACTTCCACA-TAMRA		
qnrA	UP	AGGATTGCAGTTTCATTGAAAGC	138	Colomer-Lluch *et al*., 2014
	LP	TGAACTCTATGCCAAAGCAGTTG		
	Probe	6FAM-TATGCCGATCTGCGCGA-MGBNFQ		
qnrS	UP	CGACGTGCTAACTTGCGTGA	118	Colomer-Lluch *et al*., 2014
	LP	GGCATTGTTGGAAACTTGCA		
	Probe	6FAM –AGTTCATTGAACAGGGTGA-MGBNFQ		

3. Results

3.1. Controls

3.1.1. qPCR controls to exclude non-encapsidated DNA

The phage particle DNA extraction protocol used was always accompanied by several controls in order to ensure that the results obtained were only due to the amplification of the DNA contained within the capsid of bacteriophage particles. Additionally to the chloroform and DNase treatments mentioned above, the following steps were performed:

3.1.2. Absence of non-packaged DNA contamination

To rule out the possibility of contamination with free DNA outside the phage particles, an aliquot of the sample taken after DNase treatment and before desencapsidation was evaluated. At this stage, the samples were used as a template for conventional PCR of eubacterial 16S rDNA and as a template for the qPCR assay of each antibiotic resistance gene.

Both amplifications should be negative, confirming that DNase has removed all non-encapsidated DNA from the samples.

Confirmation of DNase activity

To verify the success of the DNase treatment, serial decimal dilutions of the construct used for the standards with vector pGEM*::antibiotic resistance gene*, were treated with DNase followed by the heat inactivation of the enzyme. The reactions containing the DNA corresponding to the dilutions of the standard, theoretically degraded by DNase activity, were amplified by the qPCR set of the respective antibiotic resistance gene. The results showed negative amplification, indicating that the DNase was able to remove the DNA added, even at high DNA concentrations.

3.1.3. Inactivation of DNase by heat treatment

The success of DNase inactivation by heat treatment was evaluated. Without such inactivation, any remaining DNase would degrade the qPCR primers and probe, leading to negative results for the controls caused by DNase activity rather than the absence of non-encapsidated DNA.

To test inactivation, serial decimal dilutions of the construct used for the standards pGEM*::antibiotic resistance gene* were added to the DNase controls (an aliquot of the sample taken after DNase treatment and before desencapsidation). After DNase heat inactivation, reactions were amplified by the corresponding qPCR assay and the values were compared with the direct evaluation of the same dilution of the standard containing each gene of study with the respective qPCR assay. If the DNase had been inactivated, we would expect the same amplification results in the controls with DNase as in the original dilutions of the standard containing pGEM*::antibiotic resistance gene*. The results showed almost the same Ct values

in the DNase controls compared with the original standards (only one Ct below the Ct of the corresponding dilution of the standard). This confirmed that the DNase was well inactivated by heat treatment and did not interfere in the subsequent qPCR reaction. The slight differences observed in the Ct values obtained could be attributed to a small amount of DNA degradation caused by the heat treatment.

3.2. Densities of ARGs in bacterial DNA and in DNA in phage particles

Data for all ARGs was obtained from Tunisian waters. Data for *bla*$_{TEM}$, *bla*$_{CTX-M-1}$ and *bla*$_{CTX-M-9}$, *mecA* and *qnrA* and *qnrS* genes in Barcelona samples was previously reported and comparison with Tunisian waters is done on the basis of the previous results.

3.2.1. bla$_{TEM}$

bla$_{TEM}$ was the most prevalent ARG detected in Tunisia at all three sources and almost all samples were positive in both bacterial DNA and in DNA in phage particles. The densities of *bla*$_{TEM}$ detected in DNA within the phage capsids of the samples were, as expected, lower than in bacterial DNA, with 1–1.5 $\log_{10}$ units of difference on average. These values were similar (same order of magnitude) to previous results obtained at a WWTP in Barcelona, Spain.

The prevalence of *bla*$_{TEM}$, either in bacterial DNA or in DNA in phage particles, showed low variability between samples and thus the results are presented as averaged values (Fig. 2). On average, samples from WWTP 1 and 2 contained a similar number of gene copies of *bla*$_{TEM}$ in bacterial DNA, at 4.3 $\log_{10}$ units and 4.1 $\log_{10}$ units respectively, while the densities detected in the slaughterhouse samples were slightly lower (3.8 $\log_{10}$ units) and more variable (Fig. 2).

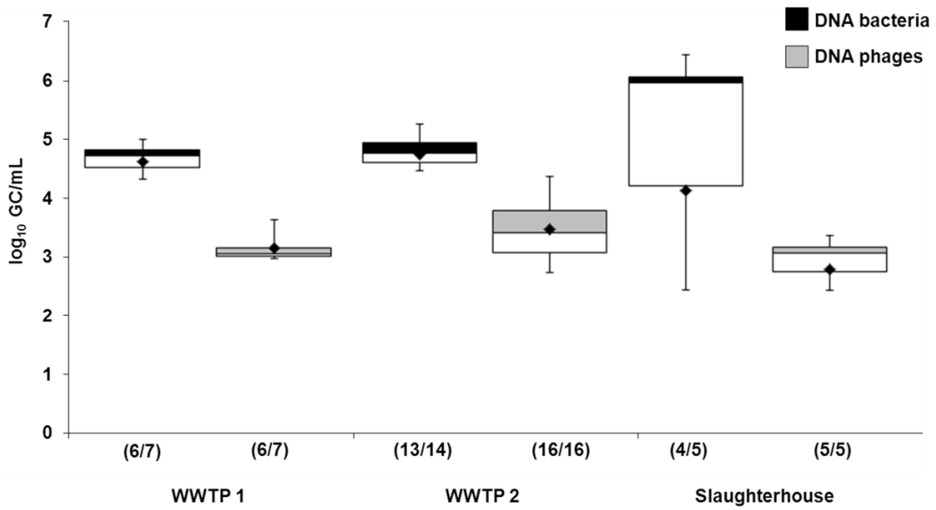

Fig. 2. Box plot of averaged values of *bla*$_{TEM}$ genes (log GC/mL) from WWTPs 1 and 2 and the slaughterhouse. Each box plot indicates the counts obtained from samples from the same source. The cross-pieces in each box plot represent (from top to bottom) the maximum, upper-quartile, median (black bar), lower-quartile, and minimum values. The coloured boxes include samples showing values within the 75th percentile and white boxes samples showing values within the 25th percentile. Black diamond shows the mean value.

3.2.2. bla$_{CTX\text{-}M\text{-}1}$ *and bla*$_{CTX\text{-}M\text{-}9}$

bla$_{CTX\text{-}M\text{-}1}$ was less prevalent than *bla*$_{CTX\text{-}M\text{-}9}$ in all Tunisian samples (Fig. 3), with 28.6% of the samples from WWTP 1 being positive for *bla*$_{CTX\text{-}M\text{-}1}$ in bacterial DNA, along with 14.3% from WWTP 2 and 40% from the slaughterhouse. The densities in samples from all three sources were similar, ranging from 2.8 to 3 $\log_{10}$ units. DNA in phage particles displayed a very low prevalence and only one sample from WWTP 2 was positive for *bla*$_{CTX\text{-}M\text{-}1}$.

In comparison, *bla*$_{CTX\text{-}M\text{-}9}$ was more prevalent than *bla*$_{CTX\text{-}M\text{-}1}$ (Fig. 3), both in bacterial DNA and in DNA within phage particles in all samples analysed. On average, 2.7 $\log_{10}$ units of GC were detected in bacterial DNA from WWTP 1, while 3.2 $\log_{10}$ units were detected on average in the samples from WWTP 2 and the slaughterhouse. In terms of DNA in phage particles, 26.7% of the samples from WWTP 2 were positive for this gene, along with 12.5% from

WTTP 1, whereas none of the slaughterhouse samples contained *bla*$_{CTX\text{-}M\text{-}9}$ in encapsidated DNA.

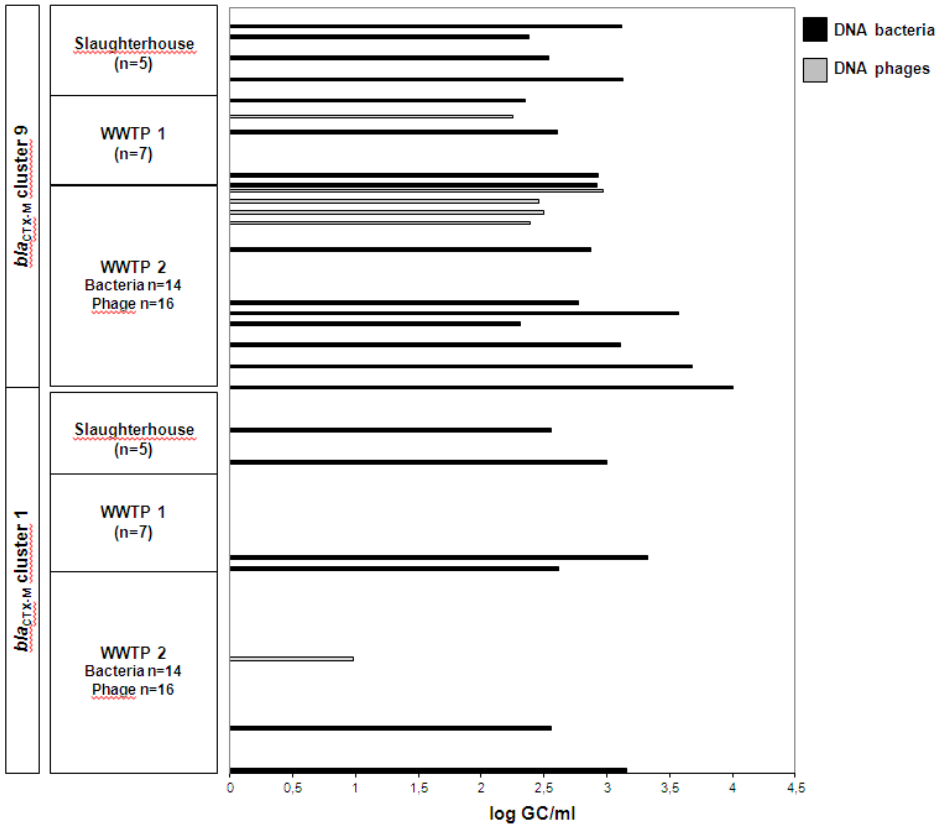

Fig. 3. Densities of *bla*$_{CTX\text{-}M}$ cluster 1 and *bla*$_{CTX\text{-}M}$ cluster 9 (log GC/mL) in bacterial DNA and in DNA of the phage fraction (phage DNA) from individual samples from WWTPs 1 and 2 and the slaughterhouse. The number of samples processed for each DNA is indicated.

3.2.3. mecA

Of all the Tunisian samples analysed, only one from WWTP 1 contained the *mecA* gene in bacterial DNA, with 2.78 $\log_{10}$ units of GC detected. None of the DNA packaged in phage particles isolated from the samples contained the *mecA* gene.

3.2.4. qnrA and *qnrS*

qnrA was more prevalent in bacterial DNA than *qnrS* in samples from both WWTPs and the slaughterhouse (Fig. 4). In bacterial DNA, *qnrA* was detected in 71.4% of the samples from WWTP 2 and WWTP 1, and all the samples of animal origin were positive for this gene. On average, *qnrA* densities in bacterial DNA were similar regardless of its origin (average of 4.5 $\log_{10}$ units), although densities varied slightly between samples. Regarding DNA in phage particles, only 4 out of 16 samples were positive for *qnrA*. These samples were from WWTP 2 (26.7%) and WWTP 1 (12.5%), while no *qnrA* was detected in phage DNA from slaughterhouse samples.

qnrS in bacterial DNA was found in 28.6% of the samples from WWTP 2, 14.3% from WWTP 1 and 20% from the slaughterhouse. No remarkable differences were detected in the number of gene copies of *qnrS* between sources, with densities ranging from 4 to 4.3 $\log_{10}$ units. However, in phage DNA *qnrS* was only present in samples from WWTP 2 (20.0% of samples), with 3.5 $\log_{10}$ units of gene copies on average.

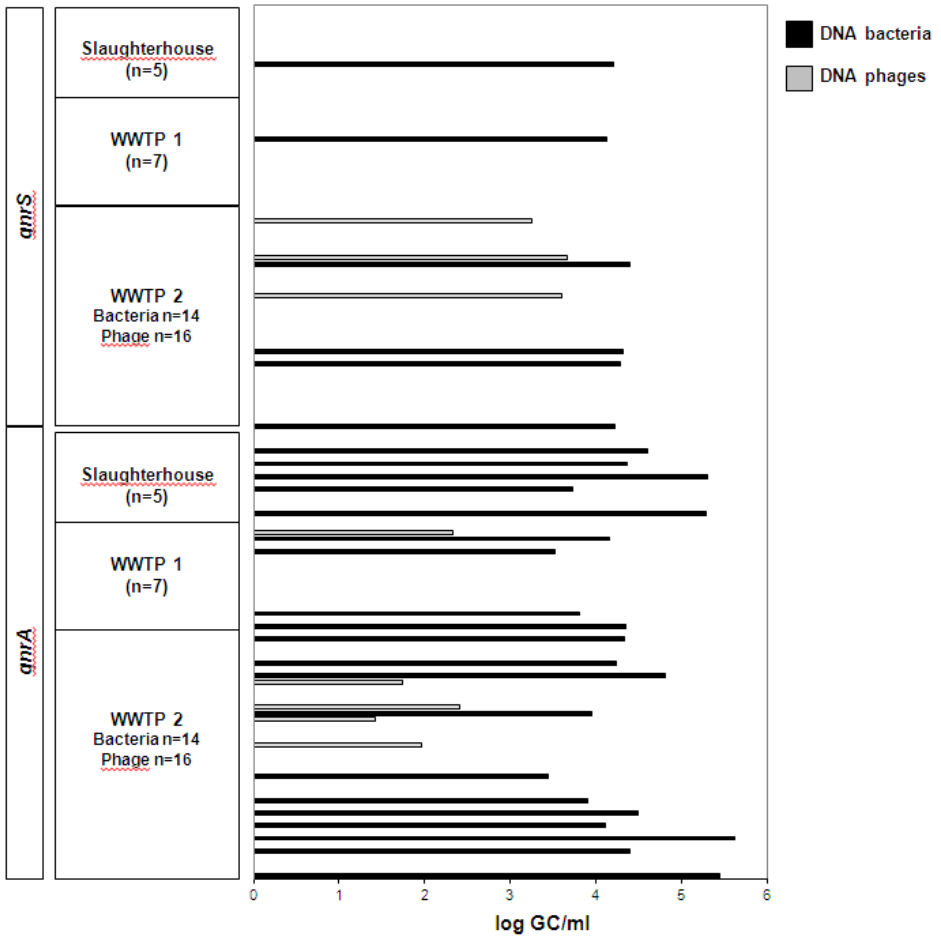

Fig. 4. Densities of *qnrA* and *qnrS* (log GC/mL) in bacterial DNA and in DNA of the phage fraction (phage DNA) from individual samples from WWTPs 1 and 2 and the slaughterhouse. The number of samples processed for each DNA is indicated.

4. Discussion

There was a clear difference in the occurrence and concentrations of the ARGs studied in the Tunisian samples between bacterial DNA and DNA from the viral fraction of the samples. As described in other studies, the density of ARGs detected in bacteriophages was lower than that in bacterial DNA (Colomer et al., 2011a, 2011b) but followed the same trend. Likewise, despite minor differences the ranking in terms of occurrence and concentration of each ARG was similar in the three types of sample studied.

bla$_{TEM}$ and *bla*$_{CTX-M}$ β-lactamases are the most prevalent ESBLs and the most widely distributed worldwide (Díaz, 2010; Patterson, 2003; Rodriguez-Baño et al., 2008). *bla*$_{CTX-M}$, which continues to have a rapidly changing epidemiology (Hawkey and Jones, 2009), has been mobilized from its chromosomal location in *Kluyvera* species, via highly efficient mobile genetic elements, into high-risk multiresistant clones that have spread on a global scale (D'Andrea et al., 2013).

bla$_{TEM}$ was the most prevalent ESBL in the studied samples. In fact, it was the first ESBL to be described in Tunisia in 1994 and it remains the most commonly reported (Chouchani et al., 2011). *bla*$_{CTX-M}$, were less abundant than *bla*$_{TEM}$ both in terms of occurrence and concentration in the study samples, with cluster 9 being more prevalent than group 1 but with similar concentrations between the two clusters in positive samples. These genes are probably less widespread in strains from Tunisia than *bla*$_{TEM}$. The first *bla*$_{CTX-M}$ reported in Tunisia in 2005 belonged to group 9, whereas group 1 was first described in 2005; in the last few years the most frequently reported ESBL has been *bla*$_{CTX-M-15}$, belonging to *bla*$_{CTX-M}$ cluster 1 (Chouchani et al., 2011).

Regarding the *qnr* genes, the occurrence of *qnrA* was significantly higher than that of *qnrS*, although samples containing *qnrS* showed higher concentrations of the latter. This is not due to the detection limit of the method, and thus should be interpreted as rare shedding but of high levels. Again, as in the case of *bla*$_{CTX-M}$, the frequency of these ARGs is increasing very actively (Rodriguez-Martinez et al., 2011; Strahilevitz et al., 2009), with *qnrA* being the first gene described, but *qnrS* now being the most frequently reported worldwide (Rodriguez-Martinez et al., 2011). Reports of *qnr* genes in Tunisia and neighbouring countries do not show much difference between *qnrA* and *qnrS* (Rodriguez-Martinez et al., 2011), with *qnrA* being only slightly more prevalent than *qnrS* (Dahmen et al., 2010).

mecA sequences were absent or quasi-absent in the samples analysed in this study. This is consistent with the low prevalence of methicillin-resistant *Staphylococcus aureus* in Tunisian isolates (Kesah et al., 2003).

Therefore, the ranking of abundance, as evaluated by occurrence and concentration, of ARGs in bacterial DNA and in DNA packaged in bacteriophages in Tunisia was bla_{TEM} > *qnrA* > $bla_{CTX\text{-}M\text{-}9}$ >$bla_{CTX\text{-}M\text{-}1}$ > *qnrS* > *mecA.*

Comparisons with data on the same ARGs in Barcelona revealed both similarities and some remarkable differences. First, the faecal load of the samples tested was similar, so any differences in concentrations of ARGs cannot be attributed to differences in faecal load. There was a clear similarity in occurrence of $bla_{TEM,}$ which was present at both locations in all or almost all samples at similar concentrations. This is consistent with the fact that bla_{TEM} is the most prevalent β-lactamase worldwide. The similarity in the values of bla_{TEM}, together with the concentrations of other ARGs such as *qnrA*, $bla_{CTX\text{-}M}$ group 9, $bla_{CTX\text{-}M}$ group 1 and *qnrS* in the positives samples and the low detection limits of all the genes studied, clearly indicates that their prevalence in the Tunisian samples was not significantly influenced by transport, and consequently that the differences found reflect the actual abundance of the studied genes in Tunisia and Barcelona.

The ranking of abundance of *qnrA*, $bla_{\text{CTX-M-9,}}$ $bla_{\text{CTX-M-1}}$ and *qnrS* genes was similar in the two locations, although with lower occurrence and concentrations in Tunisia. This may be attributed to several differences between the two areas of study. Barcelona, because of its geographical situation, receives a high number of foreign visitors, and intensive animal husbandry is important. This, together with the high consumption of antibiotics in Spain

(moderate to high in human medicine (European Centre for Diseases Prevention, 2013) and veterinary medicine (European Medicines Agency, 2013), make it a good location for the appearance and selection of antibiotic resistant bacteria.

There was also a clear difference regarding *mecA*, which was abundant in samples from Barcelona, but rare in samples from Tunisia. In this case the difference could be attributed to differences in livestock production, mainly involving sheep and goats in Tunisia, and pigs in the Barcelona area. Livestock, and particularly pigs, are viewed as an important source of methicillin-resistant *Staphyloccocus aureus* (De Neeling et al., 2007; Feingold et al., 2012; Khanna et al., 2008), which may further explain this observation. It also reinforces the notion that geographical spread of MRSA over long distances and across cultural borders is a rare event (Nübel et al., 2008).

The study of a qPCR fragment in the DNA of the bacteriophage fraction of the samples allows quantification of the ARGs in phage particles, but it does not provide enough information to elucidate the nature of these particles. Few ARGs have been described within the genome of temperate bacteriophages (see Muniesa et al., 2013 for review). Without excluding this possibility, previous studies within our group (Colomer-Lluch et al., 2014) suggest that mostly generalized transducing phage particles are the ones detected in the studies analysing the phage fraction of different samples (Schmieger and Schicklmaier 1999; Parsley et al., 2010; Minot et al., 2011; Modi et al., 2013). The phage particles causing generalized transduction do not contain the phage genome, but fragments of bacterial DNA. As these particles are unable to cause lysis in a host strain, they might not be detectable by plaque assay or leave traces of phage DNA in the recipient cell. Nevertheless, they can infect a susceptible host and transduce the genes that they are mobilizing.

Many bacteriophages have a narrow host range infecting a limited number of strains of a given species, but others (polyvalent bacteriophages) can infect a wide host range that would allow transduction among different taxa. Phages might therefore play a crucial role in the early stages of transfer of the chromosomally located resistomes (and also plasmids) by randomly mobilizing ARGs from environmental bacteria to commensal bacteria or pathogens, probably through generalized transduction.

The environment may provide an ideal setting for the acquisition and dissemination of antibiotic resistance. Moreover, environmental ARGs constitute a reservoir nourished either from environmental bacteria or from pathogens reaching environmental settings. From this pool, ARGs can be responsible of the generation of new pathogens, including uncultured microorganisms, or to provide old ones with an improved resistome. For all these reasons, environmental monitoring of ARGs is essential to increase our understanding of how ARGs spread within a community, particularly assuming that ARGs are “easy to get, but very hard to lose” pollutants.

5. Conclusions

In conclusion, the bacterial and the phage fractions of human and animal wastewater contain variable amounts of ARGs, which differ according to the level of expansion of the different resistance genes, and vary between geographical areas that differ in socio-economic and cultural characteristics, climate and geographic background. Consequently, the study of ARGs in wastewater is an interesting option for studying antibiotic resistance epidemiology and may even be a good instrument for detecting resistance before it appears in clinical settings.

Acknowledgments

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3.4. Chapter 4: Detection of quinolone-resistant *Escherichia coli* isolates belonging to clonal groups O25b:H4-B2-ST131 and O25b:H4-D-ST69 in water samples from Barcelona area

❖ **ARTICLE 5**

Títol: Detection of quinolone-resistant *Escherichia coli* isolates belonging to clonal groups O25b:H4-B2-ST131 and O25b:H4-D-ST69 in raw sewage and river water in Barcelona, Spain

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RESUM

Introducció

El grup clonal intercontinental d'*E. coli* productor de la β-lactamasa CTX-M-15 representa una gran preocupació en salut pública degut al seu potencial de virulència (Woodford *et al*., 2004; Coque *et al*., 2008b; Nicolas-Chanoine e*t al*., 2008). Aquest grup clonal pertany al grup filogenètic B2, al serotip O25b:H4 i a la seqüència tipus multilocus ST131, i es caracteritza per la resistència a diverses classes d'antibiòtics i per la seva capacitat d'adquirir diferents mecanismes de resistència.

El clon O25b:H4-B2-ST131 s'ha descrit que circula entre humans però també entre animals (Ewers *et al*., 2010; Mora *et al*., 2010; Platell *et al*., 2011). Tot i que es desconeix la raó de l'èxit de la seva disseminació al medi ambient, és clar que la contaminació fecal pot contribuir-hi de manera important.

La presència de gens de resistència a antibiòtics en bacteris en ambients aquàtics és una preocupació cada vegada més important ja que s'han descrit microorganismes portadors de gens de resistència a un ampli ventall d'antibiòtics en aigües residuals procedents

d'hospitals, aigües residuals amb contaminació fecal animal, aigües residuals d'EDARs, aigua de riu, entre d'altres (Baquero *et al*., 2008; Zhang *et al*., 2009) Però es disposa de poca informació i de dades sobre el clon O25b:H4-B2-ST131 en la nostra zona geogràfica.

Objectius

En aquest estudi es van plantejar els objectius detallats a continuació:

- Avaluació de la prevalença del grup clonal O25b:H4-B2-ST131 en ambients aquàtics amb contaminació fecal (aigua residual i aigua de riu) de l'àrea de Barcelona.
- Determinació del patró de sensibilitat a antibiòtics i producció d'*ESBL*s dels aïllaments d'*E. coli* O25b.
- Estudi de 30 gens de virulència dels aïllaments ambientals d'ST131.
- Comparació dels perfils de macrorestricció, gens de virulència i patrons de resistència a antibiòtics dels aïllaments ambientals de ST131 d'aquest estudi, amb aïllaments clínics humans del mateix clon causants d'infeccions extraintestinals a Espanya.

Resultats i discussió

Per aquest estudi es van utilitzar 10 mostres d'aigua residuals procedents de l'Estació depuradora d'aigües residuals (EDAR) de Gavà i Prat (Barcelona) la qual abasteix una àrea urbana d'entre 500.000 a un milió d'habitants, recollides durant el període de la tardor de 2009. Per altra banda, es van escollir 6 mostres d'aigua del riu Llobregat (sotmès a una pressió antropogènica important) recollides mensualment la primavera-estiu de 2010. Els valors de coliforms fecals en les mostres de riu es troben 3-4 ordres de magnitud per sota respecte els de l'aigua residual, essent l'aigua de riu un bon exemple de mostra amb contaminació recent però amb menys contaminació que l'aigua residual.

Es va realitzar l'aïllament de soques d'*E. coli* resistents a ampicil·lina mitjançant el mètode de filtració per membrana en plaques que contenien ampicil·lina (32 μg/mL). Posteriorment, de les soques d'*E. coli* resistents aïllades es va fer l'amplificació del gen *rfbO25b* associat al grup

clonal O25b:H4-B2-ST131. En total, es va obtenir un 12.3% d'aïllaments d'*E. coli* O25b positius en aigua residual i un 9.9% en aigua de riu; en conjunt, 75 aïllaments *E. coli* O25b (Taula 1).

Dels 75 aïllaments d'*E. coli* positius per O25b obtinguts es va determinar el seu grup clonal mitjançant la tècnica de *Multilocus sequence typing* (*MLST)*. 51 van pertànyer al grup clonal O25b:H4-B2-ST131, mentre que els altres 24 corresponien a una seqüència tipus multilocus diferent, O25b:H4-D-ST69. Dels aïllaments d'ST131, 25 procedien d'aigua residual i 26 d'aigua de riu. En canvi, 23 dels 24 aïllaments d'ST69 es van obtenir a partir de mostres d'aigua residual i només una era d'aigua de riu.

Per altra banda, es va establir el patró de sensibilitat a antibiòtics de cada aïllament i es va avaluar la producció d'*ESBL*s. Un 75% dels aïllaments d'ST131 i el 100% dels aïllaments d'ST69 van ser resistents a àcid nalidíxic i un 61% dels ST131 van ser també resistents a fluoroquinolones, mentre que cap dels ST69 ho era. Només un 12% dels ST131 eren productors d'*ESBL*s (CTX-M-15 i CTX-M-1). De cada aïllament, a més, es va avaluar la presència de gens de virulència i la relació de perfils de restricció per *PFGE.* Dels 30 gens de virulència analitzats, 21 es van detectar com a mínim en un aïllament. Tant els aïllaments d'ST131 com d'ST69 contenien de 4 a 13 gens de virulència però en comparar els dos grups clonals es van observar diferències significatives ja que els aïllaments del grup clonal ST131 contenien un nombre molt superior de gens de virulència en comparació amb els aïllaments d'ST69 (Taula 2).

Cal destacar que, com s'ha descrit en estudis previs (Mora *et al*., 2010; Blanco *et al*., 2011; Coelho *et al*., 2011), els aïllaments d'O25b:H4-B2-ST131 positius pel gen *ibeA* presentaven major virulència que els *ibeA* negatius. El 100% dels *ibeA* positius contenien 8 o més gens de virulència mentre que només un 6% dels *ibeA* negatius els contenien. Contràriament, cap dels clons d'O25b:H4-D-ST69 va ser positiu per *ibeA* i cap contenia més de 4 gens de virulència.

Addicionalment, 63 aïllaments es van analitzar per la tècnica d'electroforesi en camp pulsant (*PFGE*): 18 O25b:H4-B2-ST131 *ibeA*+, 33 O25b:H4-B2-ST131 *ibeA*- i 12 dels 24

O25b:H4-D-ST69 (Figura 1) els resultats del qual mostraven que els aïllaments d'ST131 presentaven una elevada diversitat genètica mentre que aquesta era molt menor en els aïllaments d'ST69.

La comparació dels perfils de macrorestricció, gens de virulència i patrons de resistència dels 51 aïllaments ambientals d'O25b:H4-B2-ST131 d'aquest estudi amb 59 aïllaments clínics humans d'aquest mateix grup clonal d'un sondeig de vigilància Espanyol, confirma que els aïllaments d'O25b:H4-B2-ST131 de mostres d'aigua residual presenten un contingut de gens de virulència i perfils de macrorestricció similars als d'origen humà. En canvi, els 12 aïllaments ambientals del grup clonal O25b:H4-D-ST69 van presentar un contingut de gens de virulència, perfils de macrorestricció i perfils de PFGE molt diferents dels dels 22 aïllaments de CGA-D-ST69 causants d'infeccions en humans (Figura 1 i Figures S1 i S2).

Conclusions

En aquest estudi es demostra la circulació d' *E. coli* resistents a quinolones dels grups clonals O25b:H4-B2-ST131 i O25b:H4-D-ST69 en aigua residual i aigua de riu a Barcelona.

Dels 51 aïllaments d'ST131, un 12% van ser productors d'*ESBL*s (5 aïllaments productors de CTX-M-15 de 3 mostres d'aigua residual i només 1 aïllament de CTX-M-1 d'aigua de riu).

Els aïllaments d'O25b:H4-B2-ST131 de mostres no patològiques obtingudes d'aïllaments d'aigua de residual urbana i de riu presentaven perfils de restricció i virulència similars als aïllaments trobats en humans.

Aquest estudi és la primera descripció del grup clonal O25b:H4-D-ST69 (en el moment de la seva publicació).

Informe sobre el factor d'impacte de l'article 5

L'article ***Detection of quinolone-resistant Escherichia coli isolats belonging to clonal groups O25b:H4-B2-ST131 and O25b:H4-D-ST69 in raw sewage and river water in Barcelona, Spain*** va ser publicat online l'abril de 2013 a la revista *Journal of Antimicrobial Chemotherapy* que es troba inclosa en el primer quartil (Q1) de l'àrea temàtica de *Infectious diseases* (7/70), *Microbiology* (16/116) i *Pharmacology&Pharmacy* (18/261). L'any 2012 la revista *Journal of Antimicrobial Chemotherapy* va presentar un factor d'impacte de 5.338.

En el moment de la presentació d'aquest informe aquest article ha estat citat 1 vegada segons Web of Science.

Informe de participació de l'article 5

Aquest estudi s'ha realitzat en col·laboració amb el grup de recerca del Laboratori de Referència d'*E. coli* (LREC) de la Universitat de Santiago de Compostel·la, Lugo.

La doctoranda Marta Colomer Lluch és la responsable de la recollida i transport de les mostres. També ha realitzat el processament de les mostres, les anàlisis dels paràmetres microbiològics així com els aïllaments de les soques bacterianes d'*E .coli* i l'avaluació de la prevalença del grup clonal O25b i de la presència de CTX-M-15 i CTX-M-1 per PCR. Finalment, ha participat en la redacció de l'article i en l'elaboració de taules així com en la difusió dels resultats en congressos nacionals i internacionals

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Detection of quinolone-resistant *Escherichia coli* isolates belonging to clonal groups O25b:H4-B2-ST131 and O25b:H4-D-ST69 in raw sewage and river water in Barcelona, Spain

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Objectives: The present study was carried out to evaluate the prevalence of clonal group O25b:H4-B2-ST131 in water environments with faecal pollution (urban sewage and river water) in the north-east of Spain and to study the virulence gene content of environmental isolates and to compare them with isolates causing human extraintestinal infections in Spain.

Methods: This study was performed with 10 sewage samples (collected in Catalonia, north-eastern Spain, in autumn 2009 from the influent raw urban sewage of a wastewater treatment plant that serves a large urban area) and 6 river water samples (collected monthly from February to April 2010 in the Llobregat river catchment area, near Barcelona, a watercourse subjected to heavy anthropogenic pressure). *Escherichia coli* colonies were screened by PCR for the *rfbO25b* gene associated with the clonal group O25b:H4-B2-ST131. Sequence types (STs), serotypes, virulence genes, PFGE profiles, antimicrobial resistance and extended-spectrum β-lactamase (ESBL) enzymes were determined in 75 *E. coli* isolates positive for the O25b molecular subtype.

Results: Of the 75 O25b-positive isolates, 51 belonged to the O25b:H4-B2-ST131 clonal group and the remaining 24 belonged to clonal group O25b:H4-D-ST69. The majority of ST69 isolates (23 of 24) were isolated from urban sewage, whereas ST131 isolates were isolated from urban sewage (25 isolates) as well as from river water (26 isolates). ST131 and ST69 isolates carried 4–13 virulence genes, the majority (82%) being quinolone resistant.

Conclusions: We showed the presence of *E. coli* isolates belonging to clonal groups O25b:H4-B2-ST131 and O25b:H4-D-ST69 in raw sewage and river water in Barcelona. Furthermore, we observed that the environmental O25b:H4-B2-ST131 isolates showed similar virulence and macrorestriction profiles to clinical human isolates. To our knowledge, this is the first study describing the O25b:H4-D-ST69 clonal group.

Keywords: CTX-M-15, *E. coli*, ESBLs, virulence genes

Introduction

Recently, an intercontinental *Escherichia coli* clonal group producing CTX-M-15 with a high virulence potential has been reported and represents a major public health concern.[1–3] This clonal group belongs to the B2 phylogenetic group, to the serotype O25b:H4 and to the multilocus sequence type ST131 (where ST stands for sequence type). The O25b:H4-B2-ST131 clonal group is characterized by co-resistance to several classes of antibiotics and is able to acquire different mechanisms of resistance. This clonal group is commonly associated with the dissemination of fluoroquinolone resistance and only some (<10%) isolates produce extended-spectrum β-lactamases (ESBLs).[4–9] The O25b:H4-B2-ST131 isolates possess numerous virulence determinants, being the causative agents of 12% of extraintestinal infections in humans and accounting for 23% of multidrug-resistant extraintestinal pathogenic *E. coli* (ExPEC) isolates in Spain.[10,11]

Currently, it is assumed that O25b:H4-B2-ST131 isolates circulate not only among humans but also among animal hosts. Thus, this clonal group has been recently identified as a cause of clinical infections in companion animals and poultry.[12–14]

The reasons underlying the successful dissemination of O25b:H4-B2-ST131 are unknown, as is the extent to which it has contaminated the wider environment. Nevertheless, it is clear that faecal pollution can contribute to the spread of this clone in the environment and could play a role in its dissemination. The global occurrence of antibiotic resistance genes in bacteria in water environments is an increasing concern, and there are many reports of microorganisms that carry genes encoding resistance to a broad range of antibiotics found in hospital wastewater and animal production wastewater as well as in urban sewage, wastewater treatment plant (WWTP) effluents, surface water, river water, groundwater and drinking water.[15,16] There is, however, scarce information about clone O25b:H4-B2-ST131. Recent reports from the UK[17] and the Czech Republic[18] indicate that isolates from the clonal group O25b:H4-B2-ST131 can be isolated from urban sewage and river water, but the virulence genotypes were not reported and the environmental isolates were not compared with human ST131 ExPEC isolates. Furthermore, there are no reports about the environmental prevalence of this clone in Spain. For these reasons, the present study was carried out to evaluate the prevalence of clonal group O25b:H4-B2-ST131 in environmental waters with faecal pollution (urban sewage and river water) in the north-east of Spain. Another aim was to characterize and compare the virulence gene content of environmental isolates belonging to ST131 and to other clonal groups detected in this study with isolates causing human extraintestinal infections in Spain.

Materials and methods

Samples

This study was performed with 10 sewage samples collected in Catalonia, north-eastern Spain, in autumn 2009, from the influent raw urban sewage of a WWTP that serves a large urban area consisting of a number of cities and towns of ∼1000000 inhabitants. In addition, six samples were collected monthly from February to April 2010 in the Llobregat river catchment area, near Barcelona, a watercourse subjected to heavy anthropogenic pressure. The scarce and irregular contribution of animal faecal contaminants has its origin in husbandry activities in the upper course and occasional wild birds in the lower course.[19] The river samples were selected because the water there consistently contains numbers of faecal coliform bacteria ∼3–4 orders of magnitude lower than the numbers in sewage, and will allow evaluation of the prevalence of the clone in samples containing recent pollution but are not so heavily polluted as raw sewage.[19] All samples were collected in sterile containers, transported to the laboratory at 5±2°C within 2 h of collection and processed immediately for enumeration of bacteria.

The *E. coli* isolates of this study were compared with 59 O25b:H4-B2-ST131 and 22 CGA-D-ST69 previously characterized *E. coli* isolates causing human extraintestinal infections in Spain.[11]

Microbiological parameters and bacterial isolation

Aerobic bacteria and *E. coli* present in the samples were evaluated by a membrane filtration method as standardized previously.[20] Briefly, decimal serial dilutions of urban sewage and river water in PBS were filtered through 0.45 μm pore-size membranes (Millipore, Mosheim, France), and the membranes were placed on the corresponding agar media. Aerobic bacteria were grown in Trypticase soy agar (TSA), *E. coli* enumeration was performed on Chromocult® coliform agar (Merck, Darmstadt, Germany), and plates were incubated in aerobic conditions at 37°C for 18 h.

To evaluate the presence of aerobic bacteria and *E. coli* isolates showing resistance to ampicillin, samples were processed as described above and incubated in TSA or in Chromocult® coliform agar for 2 h at 37°C. Then, membranes were transferred to TSA or Chromocult® coliform agar containing 32 mg/L ampicillin (Sigma-Aldrich, Steinheim, Germany) and further incubated at 37°C for 18 h. The ampicillin concentration used reflected the breakpoint concentration for testing with *E. coli*, and was previously reported for isolation of bacteria from the environment.[20,21]

For *E. coli* isolation, 20–50 blue colonies of each sample suspected to be *E. coli* were isolated from Chromocult® coliform agar plates containing ampicillin. Indole-positive *E. coli* colonies were screened by PCR for the *rfbO25b* gene associated with the clonal group O25b:H4-B2-ST131.[22] In total, 662 colonies were screened (390 colonies from 10 samples of raw sewage and 272 colonies from 6 samples of Llobregat river). The 75 *E. coli* isolates positive for the O25b molecular subtype were identified as *E. coli* using the MicroScan WalkAway automated system (Siemens Healthcare Diagnostics Ltd).

Bacterial DNA isolation and standard PCR procedures

Each colony was resuspended in 25 μL of double-distilled sterile water, heat treated in a bath at 95°C for 10 min and immediately transferred to ice-cold absolute ethanol. Samples were then centrifuged at 16000 **g** for 5 min and 2 μL of the supernatant was used as a template for PCR amplification. PCR amplifications were performed with a GeneAmp PCR System 2400 (Perkin-Elmer, PE Applied Biosystems, Barcelona, Spain). Molecular subtype O25b was identified based on a recently described molecular approach, which amplifies a 300 bp fragment with primers rfb.1bis and rfbO25b.r.[5,22] Afterwards, the isolates were confirmed to be O25b:H4 by serotyping.

O and H typing

Determination of O and H antigens was carried out using the method previously described by Guinée *et al.*[23] with all available O (O1–O181) and H (H1–H56) antisera. Additionally, the specific O25a and O25b molecular subtypes and the flagellar H4 gene (*fliC*-H4) were determined by PCR using oligonucleotide primers described elsewhere.[22,24]

Phylogenetic grouping and multilocus sequence typing (MLST)

Isolates were assigned to one of the four main phylogenetic groups of *E. coli* (A, B1, B2, D) by using the multiplex PCR-based method of Clermont *et al.*[25] MLST was achieved as previously described by gene amplification and sequencing of the seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) according to the protocol and primers specified at the *E. coli* MLST web site (http://mlst.ucc.ie/mlst/dbs/Ecoli). The allelic profile of the seven gene sequences and the STs were obtained via the electronic database at the *E. coli* MLST web site.[26]

Antimicrobial susceptibility and ESBL typing

Susceptibility to antibiotics was analysed by broth microdilution. Minimal inhibitory concentrations were determined using a MicroScan WalkAway automated system according to the manufacturer's instructions. Intermediate susceptibility was not considered as resistant. Resistance was interpreted based on the recommended breakpoints of the CLSI.[27]

Suggestive evidence of ESBL production was defined as synergy between amoxicillin/clavulanate and cefotaxime or ceftazidime. To determine the genotype of ESBLs, PCR and sequencing were performed using the TEM-, SHV-, CTX-M-1 and CTX-M-9 group-specific primers as reported previously.[28]

Virulence genotyping

The presence of 30 virulence genes was analysed as documented previously,[10,11,29–31] using primers specific for genes and operons that encode extraintestinal virulence factors (VFs) characteristic of ExPEC: *fimH*, $fimAv_{MT78}$, *papEF* (positive results were tested for *papG I*, *papG II*, *papG III* and *papG IV* alleles), *sfa/focDE*, *afa/draBC* (positive results were tested for *afa* operon FM955459), *bmaE*, *gafD*, *cnf1*, *cdtB*, *sat*, *hlyA*, *iucD*, *iroN*, *kpsM II* (establishing *neuC*-K1, K2 and K5 variants), *kpsM III*, *cvaC*, *iss*, *traT*, *ibeA*, *malX*, *usp* and *tsh*.

PFGE

PFGE analysis with XbaI digestion was performed as described previously.[29] The PFGE profiles were analysed with the BioNumerics fingerprinting software (Applied Maths, St-Martens-Latem, Belgium). Cluster analysis of Dice similarity indices based on the unweighted pair group method using arithmetic linkages (UPGMA) was done to generate a dendrogram describing the relationship among the PFGE profiles. Isolates were considered related if their Dice similarity index was >85%, according to the criterion of Tenover *et al.*[32] (a difference of six bands or less).

Statistical analysis

Comparisons of proportions were tested using Fisher's exact test. For each comparison, a P value of <0.05 was considered to denote a significant difference.

Results and discussion

Prevalence of aerobic bacteria and E. coli in water samples

Results showed values of total aerobic bacteria averaging 6 $\log_{10}$ units of aerobic bacteria cfu and 4 $\log_{10}$ units of *E. coli* cfu per mL of sewage (Table 1). Among these, most isolates showed resistance to ampicillin (32 mg/L), and the densities of ampicillin-resistant colonies detected were <1 $\log_{10}$ units cfu/mL below the densities obtained without ampicillin. River water samples showed densities of both parameters 3 $\log_{10}$ units less than urban sewage water samples. These values were in accordance with recent reports of samples from the same origin taken on different dates.[33]

Human and animal pathogenic and potentially pathogenic bacteria are constantly released with wastewater into the water environment. Many characteristics of faecal polluted water make it a highly suspect medium for the spread of antibiotic resistance genes, i.e. the presence of antibacterials from household products (soaps, detergents, etc.), the presence of antibiotics that have been excreted by humans or disposed of down the drain, and a high bacterial load. An important part of the dispersal and evolution of antibiotic-resistant bacterial organisms depends on water environments. In water, bacteria of different origins (human, animal and environmental) are able to mix, and resistance evolves as a consequence of promiscuous exchange and shuffling of genes, genetic platforms and genetic vectors.[15]

Prevalence of clonal groups O25b:H4-B2-ST131 and O25b:H4-D-ST69 in water samples

Characteristic β-glucuronidase-positive *E. coli* colonies (presenting a blue colour) grown on the Chromocult® coliform agar plates were randomly selected and tested by PCR for the *rfbO25b* gene, and 20–50 colonies per sample were subcultured. All colonies were further confirmed as *E. coli* by the indole test, as recommended by the manufacturer (Merck). In 15 of the 16 samples analysed, at least one positive isolate of molecular subtype O25b was obtained. In total, 75 positive O25b isolates were identified (Table 1). The averaged prevalence of O25b isolates was calculated for each sample [(number of O25b isolates/total number of *E. coli* isolates in the sample) ×100]. The average for all samples was 12.3% in raw sewage and 9.9% in river water.

Of the 75 O25b-positive isolates, 51 belonged to the O25b:H4-B2-ST131 clonal group while the remaining 24 belonged to O25b:H4-D-ST69 (Table 2). The majority of ST69 isolates were isolated from urban sewage (23 of 24 isolates from 4 of 10 samples), whereas 25 ST131 isolates were isolated from urban sewage (from 9 of 10 samples) and 26 from river water (from 6 of 6 samples) (Table 1). It should be remembered that, as for urban sewage, isolates from Llobregat river were most likely of human origin, since there is a very low possibility of animal faecal contamination in the lower course of this river.

In the present study, we showed that ST69 isolates belonged to serotype O25b:H4 by using serotyping and PCR typing (O25b rfb variant and *fliC*-H4). Thus, the serotype O25b:H4 usually associated with B2-ST131 isolates can also be found in isolates belonging to other clonal groups. Previously, other examples have been reported in this line, e.g. isolates of serotype O1:K1:H7 belonging to clonal groups B2-ST95 and D-ST59[29] and isolates of serotype O2:H6 belonging to clonal groups B2-ST998 and D-ST115.[34]

Quinolone resistance and ESBL production

The majority (75%) of 51 ST131 and all 24 ST69 isolates were resistant to nalidixic acid and a significant proportion (61%) of ST131 isolates were also fluoroquinolone resistant, while no ST69 isolates were fluoroquinolone resistant. However, ESBL production was detected in only 6 (12%) of the 51 O25b:H4-B2-ST131 isolates (5 CTX-M-15-producing isolates from three raw sewage samples and 1 CTX-M-1-producing isolate from river water). All 24 isolates belonging to the clonal group O25b:H4-D-ST69 were negative for ESBL production. This prevalence is similar to that observed in a Spanish national survey of *E. coli* causing extraintestinal infections in humans performed in 2009.[11] In that study, 6 (10%) of the 59 human ST131 isolates were positive for CTX-M-15, whereas none of the 22 human ST69 isolates produced ESBL enzymes. Thus, the Spanish environmental and human O25b:H4-B2-ST131 clonal group occurs frequently as a quinolone-resistant but cephalosporin-susceptible pathogen.

Dhanji *et al.*[17] sampled River Thames water from three urban sites (City of London) on two occasions (1 week apart, 23 and 30 March 2010) and recovered O25b:H4-B2-ST131 *E. coli* isolates

Table 1. Microbiological analysis of the water samples and prevalence of clonal groups O25b:H4-B2-ST131 and O25b:H4-D-ST69

	Aerobic bacteria (cfu/mL)	Aerobic bacteria + 32 mg/L AMP (cfu/mL)	*E. coli* (cfu/mL)	*E. coli* + 32 mg/L AMP (cfu/mL)	O25b isolates[a]	O25b:H4-B2-ST131[b]	O25b:H4-D-ST69[b]
Urban sewage ($n=10$)							
average	3.7×10^6	1.9×10^6	5.7×10^4	1.9×10^4	48	25	23
max	9.4×10^6	3.9×10^6	2.8×10^5	7.9×10^4		-	-
min	1.8×10^6	5.5×10^5	1.6×10^4	5.0×10^3		-	-
River water ($n=6$)							
average	7.0×10^3	2.4×10^3	4.0×10^1	1.5×10^1	27	26	1
max	9.9×10^3	1.4×10^4	1.4×10^2	8.5×10^1		-	-
min	8.7×10^2	2.0×10^2	2.5×10^0	1.4×10^0		-	-

AMP, ampicillin.
[a]Number of O25b-positive isolates among colonies selected randomly from the ampicillin-resistant *E. coli* colonies.
[b]Number of isolates of ST131 and ST69 among the 75 O25b isolates.

only on 23 March. This study showed that, among a total of 30 ciprofloxacin-resistant isolates, 20 belonged to the O25b:H4-B2-ST131 clonal group. Ten of 20 ST131 isolates harboured CTX-M-14 enzyme but none produced CTX-M-15, which is the most common ESBL among human clinical ST131 isolates.

Similarly, Dolejska *et al.*[18] detected O25b:H4-B2-ST131 isolates in 12 (27%) of 45 wastewater samples taken daily between November 2008 and February 2009 from the outflow of a municipal WWTP in Brno city (Czech Republic). Water samples were cultivated on MacConkey agar with cefotaxime and 17 of 19 O25b:H4-B2-ST131 isolates were positive for CTX-M-15 enzyme.

To our knowledge, this is the first study to detect the O25b:H4-D-ST69 clonal group. In previous studies, the ST69 was observed in isolates of clonal group A (CGA) belonging to phylogenetic group D and showing different O serogroups with the H18 flagellar antigen. CGA-D-ST69 accounted for up to 50% of trimethoprim/sulfamethoxazole-resistant urinary tract infections due to *E. coli* in the USA and 4% of total human extraintestinal infections caused by *E. coli* in Spain.[11,35] CGA isolates belonging to serotypes O11:H18, O15:H18, O17:H18, O44:H18, O17,O77:H18, O73:H18, O77:H18 and O86:H18 were present in four of seven sewage effluents collected from geographically dispersed areas of the USA.[36]

Virulence factors

ExPEC isolates typically possess diverse specialized VFs, including adhesins, toxins, iron acquisition siderophores, polysaccharide capsules and other miscellaneous factors that enable them to colonize host surfaces, injure host tissues and avoid host defence systems. The 75 isolates belonging to the clonal groups O25b:H4-B2-ST131 and O25b:H4-D-ST69 in the present study were analysed by PCR for the presence of 30 genes encoding VFs typical of ExPEC that cause urinary tract infections, sepsis and meningitis. Of the 30 virulence genes tested, 21 were detected in at least 1 isolate each. Isolates carried 4–13 virulence genes. Comparison of the virulence gene content of the two clonal groups showed significant differences (Table 2 and Figure 1). ST131 isolates carried a significantly higher number of virulence genes (mean 8.2, range 4–13) than ST69 isolates (all showed the same four virulence genes).

A total of 62 (83%) of 75 O25b:H4 isolates from water samples in this study satisfied criteria for ExPEC status according to a modification of the criteria of Johnson *et al.*,[37] as they carried two or more of the *papEF*, *sfa/focDE*, *afa/draBC*, *iucD* and *kpsM II* genes.

As in previous studies,[10–12] O25b:H4-B2-ST131 isolates carrying the *ibeA* gene (invasion of brain endothelium) in the present study exhibited a higher virulence gene content than *ibeA*− isolates. Thus, 100% (18 of 18) of the *ibeA*+ isolates carried eight or more virulence genes versus 6% (2 of 33) of the O25b:H4-B2-ST131 *ibeA*− isolates ($P<0.001$). On the contrary, none of the O25b:H4-D-ST69 isolates was positive for the *ibeA* gene ($P<0.001$) and none carried more than four virulence genes (Table 2).

Thirteen (72%) of 18 O25b:H4-B2-ST131 *ibeA*+ isolates were recovered from river water versus 5 (28%) from urban sewage ($P=0.02$).

Interestingly, 31 (94%) of 33 O25b:H4-B2-ST131 *ibeA*− isolates exhibited resistance to ciprofloxacin and 18 (54%) resistance to tobramycin. In contrast, none of the 18 ST131 *ibeA*+ isolates was resistant to fluoroquinolones ($P<0.001$) and only 1 (6%) isolate was resistant to tobramycin ($P<0.001$).

Previously, Nicolas-Chanoine *et al.*,[1] studying the virulence genotypes of 36 CTX-M-15-producing O25b:H4-B2-ST131 human isolates from eight countries and three continents, found similar pathotypes to those detected in the present study among O25b:H4-B2-ST131 *ibeA*− isolates, with the difference that only 5 of 51 ST131 isolates in the present study were CTX-M-15 producing.

Remarkably, the 12 O25b:H4-D-ST69 environmental isolates showed the presence of *papG* allele I and the absence of group 2 capsule genes. In contrast, none of the 22 CGA-D-ST69 isolates causing infections in humans characterized in a previous study[11] showed the *papG* allele I, and 11 of those 22 carried group 2 capsule genes (*kpsM II-K5*).

Macrorestriction profiles by PFGE: comparison with clinical human isolates

Sixty-three isolates were analysed by PFGE: 18 O25b:H4-B2-ST131 *ibeA*+ isolates, 33 O25b:H4-B2-ST131 *ibeA*− isolates and 12 of the

Table 2. Prevalence of virulence genes in environmental isolates of clonal groups O25b:H4-B2-ST131 and O25b:H4-D-ST69

	Clonal groups			P^a		
Virulence genes	O25b:H4-B2-ST131 *ibeA*+ (n=18)	O25b:H4-B2-ST131 *ibeA*− (n=33)	O25b:H4-D-ST69 (n=24)	ST131 *ibeA*+ versus ST131 *ibeA*−	ST131 *ibeA*+ versus ST69	ST131 *ibeA*− versus ST69
Adhesins						
fimH	18 (100%)	31 (94%)	24 (100%)			
$fimAv_{MT78}$	0	0	0			
papEF	15 (83%)	2 (6%)	24 (100%)	<0.001		<0.001
papG I	0	0	24 (100%)		<0.001	<0.001
papG II	0	2 (6%)	0			
papG III	15 (83%)	0	0	<0.001	<0.001	
papG IV	0	0	0			
sfa/focDE	0	0	0			
afa/draBC	1 (6%)	1 (3%)	0			
afaFM955459	0	0	0			
bmaE	0	0	0			
gafD	0	0	0			
Toxins						
cnf1	6 (33%)	1 (3%)	0	0.006	0.004	
cdtB	9 (50%)	0	0	<0.001	<0.001	
sat	2 (11%)	31 (94%)	0	<0.001		<0.001
hlyA	6 (33%)	1 (3%)	0	0.006	0.004	
Siderophores						
iucD	16 (89%)	31 (94%)	24 (100%)			
iroN	14 (78%)	0	0	<0.001	<0.001	
Capsules						
kpsM II	18 (100%)	20 (61%)	0	0.001	<0.001	<0.001
kpsM II-K2	0	0	0			
kpsM II-K5	17 (94%)	20 (61%)	0	0.009	<0.001	<0.001
neuC-K1	1 (6%)	0	0			
kpsM III	0	0	0			
Miscellaneous						
cvaC	14 (78%)	0	0	<0.001	<0.001	
iss	14 (78%)	0	0	<0.001	<0.001	
traT	18 (100%)	27 (82%)	24 (100%)			
ibeA	18 (100%)	0	0	<0.001	<0.001	
malX (PAI)	18 (100%)	33 (100%)	0		<0.001	<0.001
usp	18 (100%)	33 (100%)	0		<0.001	<0.001
tsh	1 (6%)	0	0			
ExPEC status	18 (100%)	20 (61%)	24 (100%)	0.001		<0.001
Isolates with at least eight virulence genes	18 (100%)	2 (6%)	0	<0.001	<0.001	

Continued

24 O25b:H4-D-ST69 isolates. Only 4 of 16 isolates obtained from sample US-8 were selected for PFGE typing since the 16 ST69 isolates showed the same virulence gene content and antibiotic resistance profile. The dendrogram derived from analysis of the macrorestriction profiles generated three different groups corresponding to isolates O25b:H4-B2-ST131 *ibeA*+ (I), O25b:H4-B2-ST131 *ibeA*− (II) and O25b:H4-D-ST69 (III), characteristically defined by their virulence profiles and resistance patterns

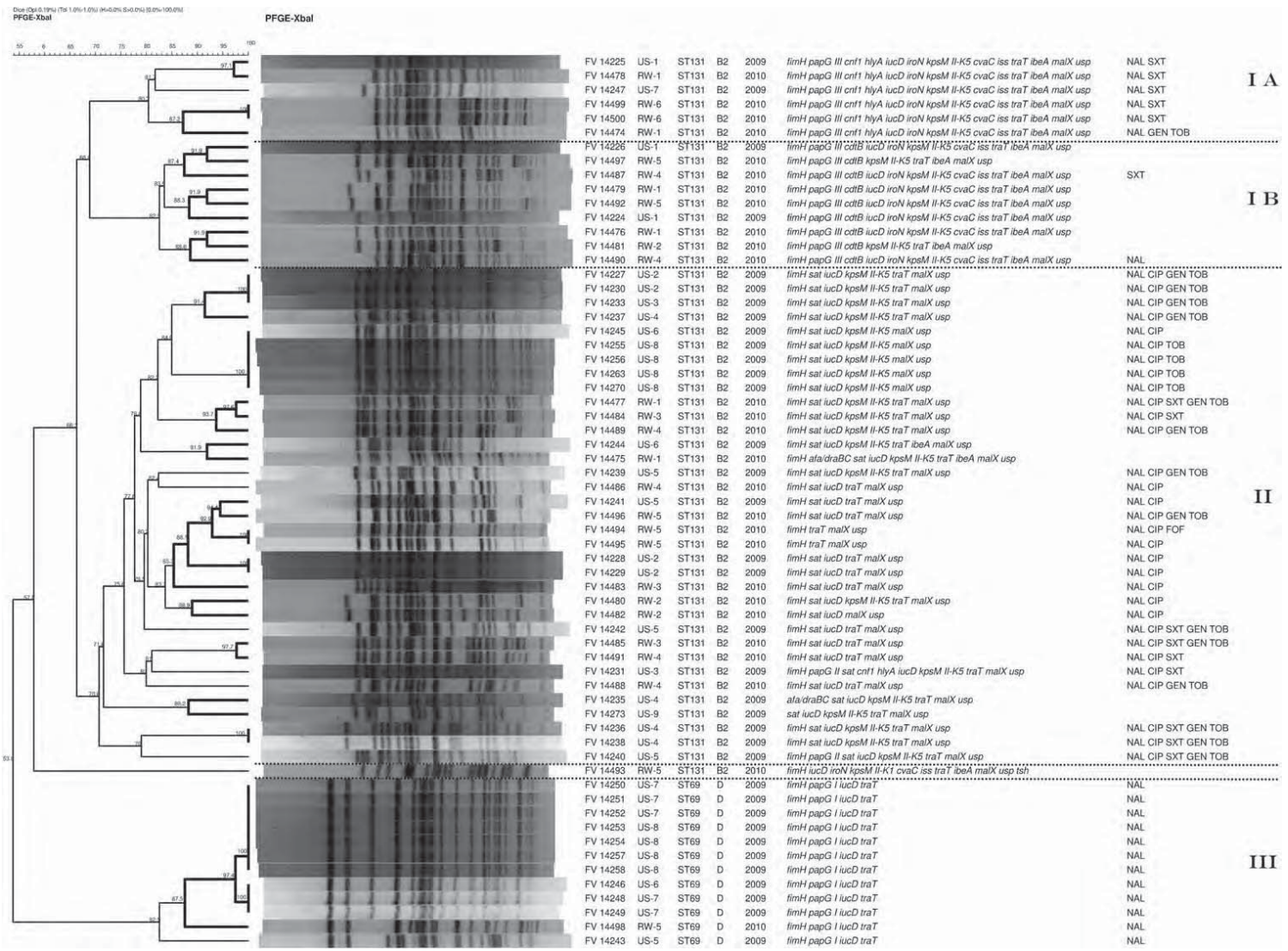

Figure 1. PFGE of XbaI-digested DNA from the 63 *E. coli* O25b:H4 isolates from urban sewage (US) and river water (RW). The dendrogram was obtained with the UPGMA algorithm based on the Dice similarity coefficient and applying 1% tolerance in the band position. Isolate designation, origin, ST, phylogenetic group, year of isolation, virulence gene profile and antibiotic resistance pattern are shown on the right. Isolates FV14227, FV14230, FV14231, FV14233 and FV14240 were positive for CTX-M-15 and isolate FV14493 was positive for CTX-M-1. NAL, nalidixic acid; CIP, ciprofloxacin; GEN, gentamicin; TOB, tobramycin; SXT, co-trimoxazole; FOF, fosfomycin.

(Figure 1). ST131 isolates showed a higher genetic diversity (41 pulsotypes with 14 clusters of ≥85% similarity), while the ST69 isolates showed only 4 pulsotypes with 1 cluster of ≥85% similarity grouping 11 of 12 isolates. Interestingly, ST131 isolates with different PFGE profiles and virulence gene contents were isolated from the same water sample in 11 cases (samples RW-1, RW-2, RW-3, RW-4, RW-5, US-1, US-2, US-3, US-4, US-5 and US-6).

Comparing the macrorestriction profiles, virulence genes and resistance patterns of the 51 environmental O25b:H4-B2-ST131 isolates of the present study with 59 clinical human isolates of this clonal group from a Spanish national survey,[11] we can confirm that the O25b:H4-B2-ST131 isolates from the water samples of this study showed similar virulence gene contents and macrorestriction profiles to those of human origin (Figure S1, available as Supplementary data at *JAC* Online). Among the 110 isolates, a total of 20 clusters with similarity ≥85% were observed. Interestingly, eight of these clusters included clinical human isolates and environmental isolates from both urban sewage and water samples.

In contrast, the 12 environmental isolates of clonal group O25b:H4-D-ST69 showed very different virulence gene contents, resistance patterns and PFGE profiles from the 22 CGA-D-ST69 isolates causing infections in humans[11] (Figure S2, available as Supplementary data at *JAC* Online).

Conclusions

In summary, we showed the presence of quinolone-resistant *E. coli* isolates belonging to clonal groups O25b:H4-B2-ST131 and O25b:H4-D-ST69 in raw sewage and river water in Barcelona. Furthermore, environmental O25b:H4-B2-ST131 isolates showed similar virulence and macrorestriction profiles to clinical human isolates. To our knowledge, this is the first study to detect the O25b:H4-D-ST69 clonal group.

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Transparency declarations

None to declare.

Supplementary data

Figures S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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4. GENERAL DISCUSSION

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The emergence of and increased antibiotic resistance among bacteria is a major problem for public health worldwide (WHO, 1996; American Academy of Microbiology, 2009; Hawkey and Jones, 2009).

Although mutations in antibiotic target genes (Martínez and Baquero, 2000) were supposed to be the primary cause of antibiotic resistance in the early antibiotic era, it soon became evident that acquisition of antibiotic resistance determinants by mobile genetic elements (MGEs) through horizontal gene transfer (HGT) has a major role on the development and spread of antibiotic resistance among bacteria (Davies, 1994). For example, the analysis of bacterial isolates from the "preantibiotic" era demonstrated that the plasmids carried by pathogenic bacteria where essentially the same that can be found today. Although plasmids were common, they did not carry ARGs, so it has been assumed that the acquisition and further dissemination among pathogenic bacterial populations of antibiotic resistance is the consequence of strong selective pressure as a result of antibiotic therapy (Hughes and Datta, 1983). If these genes are not present in the pathogenic bacteria, they must have originated in the environmental bacteria, sometimes as an antibiotic protective mechanism but sometimes with a different function. Interestingly, in the last years several studies revealed a great abundance of ARGs in many environmental ecosystems barely in contact with human produced and released antibiotics (Lima-Bittencourt *et al*., 2007; Martínez, 2008; D'Costa *et al*., 2011; Bhullar *et al*., 2012) indicating the existence of intrinsically resistance bacteria in the environment independent of the selective pressure imposed by the anthropogenic use of antibiotics.

Therefore, pathogenic bacteria can be resistant to antibiotics either because they contain the determinants for resistance in their genome or because they acquire ARGs from an exogenous source. The most straightforward selection is antibiotic treatment although non-antibiotic compounds (e.g. biocides, detergents, etc.) can also select antibiotic resistant bacteria. Antibiotics will select intrinsically resistant bacterial species, bacteria that have acquired ARGs by HGT or antibiotic resistant mutants.

As ARGs are present in chromosomes of environmental bacteria and they are often detected in MGEs when analysed in clinical settings, usually as plasmids and transposons (genomic islands or integrons), some authors suggest an environmental origin of these genes, which then may be transferred to pathogenic microorganisms through HGT. Many studies have focused on HGT of ARGs encoded in plasmids, transposons and other MGEs because of their relevance in clinics. However, little is known about the potential contribution of phages in this matter. We hypothesise that phages could be suitable candidates as intermediates mobilizing ARGs between the original environmental bacteria and the pathogenic clinical isolates. The hypothesis is based in the fact that bacteriophages can mobilize ARGs and transfer them to a suitable bacterial host strain by means of transduction.

Metagenomic studies of viral communities from different biomes, which are mostly composed by bacteriophages, confirm that substantial percentages of the viral particles present in most environments contain bacterial genes, including ARGs (Breitbart *et al*., 2004; Angly *et al*., 2006; Breitbart *et al*., 2008; Rosario *et al*., 2009; Victoria *et al*., 2009; Willner *et al*., 2009; Parsley *et al*., 2010; Cantalupo *et al*., 2011; Minot *et al*., 2011; Looft *et al*., 2012) providing indirect evidences that the mobilization of bacterial genes, and within it, the ARGs, mediated by phages is more relevant than previously thought.

Samples used in our study were municipal sewage samples containing mostly human faecal pollution and river water samples having a low faecal input from both human and animal origin and the corresponding autochthonous bacteria.

Within the present thesis, it was evaluated the prevalence of ampicillin-resistant and fluoroquinolone-resistant heterotrophic bacteria and *E. coli* in urban wastewater and river water samples. The numbers of resistant heterotrophic bacteria and *E. coli* were lower than the values of bacteria cultured without antibiotics, although levels of resistant bacteria were still high. There are several reports about the occurrence of resistant bacteria in the environment, but there is not so many data available about the kind of samples used in our study. These analyses about resistant bacteria were performed mostly to confirm the presence of resistant bacteria that we already suspected.

In addition to the evaluation of ampicillin and fluoroquinolone-resistant bacteria, we analysed more specifically the occurrence of a given *E. coli* clonal group causing extraintestinal infections, the widespread O25b:H4-B2-ST131 *E. coli* producing CTX-M-15, which has been recently reported as an emerging pathogen of great concern. This correspond to the latest study of this Thesis, **Study 5**, which was performed through collaboration between our research group and the *E. coli* Reference Laboratory group (LREC), located in Lugo, Spain.

This clonal group is characterized by co-resistance to several antibiotics, able to acquire different mechanisms of resistance and with a high virulent potential. It is also commonly associated with the dissemination of fluoroquinolone resistance and some isolates produce ESBLs. Before this study, there was little information about this clone and no reports about its environmental prevalence in Spain.

We identified 75 O25b-positives isolates, 51 belonged to the O25b:H4-B2-ST131 clonal group, equally obtained from urban and river water, and 24 belonged to O25b:H4-D-ST69 mostly from urban sewage. In this study we described ST69 isolates belonging to serotype O25b:H4. Therefore, we showed that the serotype O25b:H4 usually associated with B2-ST131 can also be found in isolates belonging to other clonal groups as reported in other examples, *e.g.* isolates of serotype O1:K1:H7 belonging to B2-ST95 and D-ST59 (Mora *et al*., 2009), and isolates of serotype O2:H6 belonging to B2-ST998 and D-ST115 clonal groups (Cortés *et al*., 2010).

The results about the ESBL production showed similar prevalence to that reported in a Spanish national survey of *E. coli* causing extraintestinal infections in humans in 2009. We can conclude that the Spanish environmental and human O25b:H4-B2-ST131 clonal group occurs frequently as a quinolone-resistant but cephalosporin-susceptible pathogen.

ExPEC (extraintestinal pathogenic *E. coli*) isolates typically harbours diverse specialized virulence factors and this was observed for ST131 isolates of our study, while ST69 presented lower prevalence of virulence genes. In accordance with previous reports, ST131 isolates carrying the *ibeA* gene, which encodes a virulence factor found in some ExPEC strains from

the B2 phylogenetic group, exhibited higher virulence gene content than *ibe*A- isolates (Mora *et al*., 2010; Blanco *et al*, 2011; Coelho *et al*., 2011).

The analysis of antibiotic resistant bacterial isolates in the samples of our study showed high numbers of resistant bacteria and a wide spread of clinically relevant clones. The environment has been reported as a reservoir of bacteria harbouring ARGs. Studies of distribution and epidemiology of resistance performed so far are mostly based, and hence biased, in the study of pathogens isolated in the clinical environment (Bradford, 2001; Blanco *et al*., 2009; Díaz *et al*., 2010; Otter and French, 2010; Paniagua *et al*., 2010; Ortega *et al*., 2012). The samples of this study could be in contrast considered as a mirror of the human or animal population and taken as a model of what is happening outside clinical settings.

Once established the occurrence of antibiotic resistant bacteria in the samples of our study, this thesis has been focused on the detection of clinically relevant ARGs in the DNA from the bacteriophage fraction of environmental samples to evaluate the role of bacteriophages as reservoirs and vehicles for the dissemination of antibiotic resistance determinants. In addition, trying to get a clue on the nature of the phage particles detected, it was studied the effect of some phage inducing agents on the abundance of ARGs in DNA packaged in phage particles. Finally, a comparison study was carried out between two very different geographic areas to evaluate the distribution of the ARGs in phage particles DNA compared to the bacterial DNA.

To determine the occurrence of ARGs in the viral fraction of the samples, we developed a qPCR assay for each of the ARGs of study which enables their detection in phage DNA. Real-Time qPCR is a highly sensitive technique which can provide quick quantitative data on the presence of the targeted gene in the DNA content of phage particles. Comparison with a standard permits the absolute quantification of the number of copies of the targeted ARG. In all reactions included in this study, the efficiency ranged from 95 to 100%. Although results obtained from Real-Time PCR cannot provide the information about the potential infectivity of the particles packaging ARGs, they provide valuable information about phages or phage-derived particles acting as reservoirs of ARGs. The limit of detection of the qPCR

assays was considered at the point that the standard curves constructed with serial decimal dilutions used for quantification did not provide a suitable consistency when replicates are amplified. Although few samples showed positive amplification, in those cases that these results were below our defined limit, the samples were accounted as negatives. In this sort of studies we would rather prefer to record a false negative than a false positive amplification.

In our study, we have been particularly accurate with the methodology used to ensure that we were only extracting packaged DNA (DNA inside phage particles) and that neither free nor bacterial DNA was amplified. It is therefore important to emphasise that all samples were treated with high DNase concentrations as reported in other viral metagenomic studies (Willner *et al*., 2009). All phage DNA extractions included a chloroform step to remove possible vesicles containing DNA (Ciofu *et al*., 2000; Yaron *et al*., 2000; Kulp and Kuehn, 2010) and a strong DNase treatment step to eliminate any free DNA outside the phage particles whether chromosomal or plasmidic. In addition, controls were performed to evaluate that the DNase was properly removed after the treatment and that it could not influence subsequent amplifications. Moreover, in all qPCR reactions, controls to exclude non-encapsidated DNA were included (see appendix 4 for detailed protocols). Our controls confirmed the absence of non-packaged DNA in the phage DNA fraction of our samples.

Our studies were designed to determine the prevalence and abundance first of the bla_{TEM}, $bla_{\text{CTX-M}}$ and *mecA* ARGs in the bacteriophage DNA fraction of environmental samples, and later of *qnrA* and *qnrS*. Our results revealed a great abundance of these ARGs in phage particles, although densities detected in the phage DNA fraction of the samples were always lower than in the bacterial DNA.

bla_{TEM} and $bla_{\text{CTX-M}}$ are the most widely distributed β-lactamases worldwide (Rodríguez-Baño *et al*., 2008). The set of primers and probe for the amplification of bla_{TEM} allowed efficient screening of more than 145 variants (Lachmayr *et al*., 2009) and $bla_{\text{CTX-M}}$ cluster 1 included 31 variants described so far (including CTX-M-1, 3, 10, 11 and 15) which was of particular interest because of the recently described geographic spread of bacterial clones carrying

CTX-M-15 variant (Pitout and Laupland, 2008; Cantón, 2009; Hawkey and Jones, 2009; Novais *et al*., 2010).

Results from **Study 1** indicated that both bla_{TEM} and $bla_{CTX\text{-}M}$ are abundant in phage DNA both in municipal sewage and river water samples with higher densities of bla_{TEM} genes being in accordance with the wide distribution of both genes worldwide.

We were able to detect *mec*A gene in phage and bacterial DNA in sewage, although in other studies MRSA was not detected (Volkman *et al.*, 2004) or it was detected but not quantified (Börjesson *et al.*, 2009). Nevertheless, the occurrence of MRSA has been demonstrated in US municipal wastewater with secondary-treated wastewater being a potential source of exposure to these bacteria in occupational settings and reuse applications (Rosenberg *et al*., 2012) and in hospital wastewater released into the sewerage system (Thompson *et al.*, 2013).

Averaged values of *mecA* in bacterial DNA for both sewage and river water samples were similar, indicating an origin other than the human faecal load although the results obtain do not allow discerning the origin, which could be other than human faecal wastes, as for example animal faecal wastes or autochthonous bacteria. The densities of *mec*A detected in phage DNA supports the notion that, regardless of its origin, *mec*A can be located on phages in aquatic environments.

Genetic and genome studies of wastewater treatment plants have shown that they are rich reservoirs of ARGs and resistant microorganisms (Szczepanowski *et al*., 2009). Many characteristics of wastewater such as the presence of antibiotics excreted by humans or disposed down the drain, the presence of antibiotics from household products like soaps or detergents, together with a high bacterial load make it a suitable medium for the spread of ARGs. Thus, the ARGs found in bacterial populations in wastewaters represent the ARGs dominating in environments in contact with man-made antibiotics.

Previous work from our research group, using urban sewage and river water samples from the same location and other authors have also highlighted the occurrence of ARGs and

virulence-related genes in the phage DNA fraction from wastewater samples (Muniesa *et al*., 2004, Tanji *et al*., 2003; Dumke *et al*., 2006).

Following the detection and quantification of bla_{TEM}, $bla_{CTX\text{-}M}$ and *mecA* in phage DNA fraction of samples with human faecal contamination, it was decided to deepen about these ARGs in another setting containing preferably faecal pollution from animal origin to assess the prevalence of ARGs in the viral DNA fraction of animal faecal wastes.

In **Study 2**, a new set for $bla_{CTX\text{-}M}$ cluster 9 was developed to detect the most abundant variants of cluster 9 (CTX-M-9, 13, 14, 16, 17, 18, 19, 21, and 27), described as widely spread among animal isolates (Bonnet, 2004; Mora *et al*., 2010).

This study was conducted with faecal wastes collected from several slaughterhouses and farms in our geographical area as well as individual cowpats. In total, 8 cattle slurries, 9 wastewater samples from abattoirs slaughtering pigs and 16 from poultry slaughter, 10 wastewater samples containing mixed faecal wastes of poultry, ducks, rabbits, and domestic dogs and cats, and 28 faecal samples aseptically collected from cowpats in summer pastures in the Pyrenees (Catalonia) were analysed.

Data from study 2 showed that all samples were positive for bla_{TEM} with significant higher densities than for the other ARGs. Poultry waste carried the highest number of bla_{TEM} copies while pig wastes the lowest. These results would be in accordance to the fact that bla_{TEM} is the most prevalent β-lactamase in *E. coli* isolates from healthy livestock and from food of animal origin by the time this study was performed (Briñas *et al*., 2002).

CTX-M-1 and CTX-M-9 were detected in phage DNA with no significant differences between the clusters, and only a few samples were negative or below the detection limit. Densities of cluster 1 were slightly higher in swine samples, while poultry samples showed a higher occurrence of cluster 9. These results support previous reports where CTX-M cluster 1 is the most prevalent in pig isolates in Spain (Cortés *et al*., 2010), and within this cluster, CTX-M-15 is the most widely distributed (Coque *et al*., 2008b; Mora *et al*., 2010). CTX-M cluster 9 is the most prevalent in poultry in Spain (Cortés *et al*., 2010, Mora *et al*., 2010), and, within it,

CTX-M-9 and CTX-M-14 are the most frequent in animal isolates (Coque *et al*., 2008a). Noteworthy, CTX-M-15 and CTX-M-9 have been linked to *E. coli* serotype O25b:H4, a serious human pathogen worldwide (Coque *et al*., 2008b) analysed in Study 5.

Comparing the results from Study 2 with Study 1, the prevalence of bla_{TEM} is consistent with the high densities of this ARG detected in phage DNA in sewage samples. Higher densities of CTX-M-1 were detected in samples from animal origin than in human raw sewage.

Some samples were negative for *mec*A in phage DNA in animal samples, particularly from cattle origin. *mec*A has been detected in farm and domestic animals (Lee, 2006; Strommenger *et al*,. 2006; Goldburg *et al*., 2008; Graveland *et al*., 2010; Köck *et al*., 2010) and the presence of *mecA* in animals has been associated in some cases with antimicrobial usage, contact with humans, and farm hygiene. Transmission is not clear, some reports indicate it can be from humans to domestic animals (Strommenger *et al*,. 2006), but others also reported transmission from animals to farmers, as described in pig farms in Europe (Goldburg *et al*., 2008). Later in this discussion we will report the evaluation of Tunisian wastewaters where *mec*A is not present plausibly due to the absence of *mec*A related animals.

Interestingly, phage DNA from cowpats samples showed abundance of ARGs. These animals were unlikely to have had any recent contact with anthropogenically-introduced β-lactam antibiotics, since they graze on pasture in the mountains outside the farms the whole spring and summer season (samples were collected in the early autumn), and, therefore are not exposed to antibiotics. These data support previous studies where bacteria in environments that are not contaminated with antibiotics from anthropogenic practices share ARGs, with human and animal pathogens (Forsberg *et al*., 2012; Tacao *et al*., 2012). For example, high levels of antibiotic resistance were found in *E. coli* from an isolated human population in Bolivia (Bartoloni *et al*., 2004) with little access to modern health care and minimal contact with people outside the community. The resistance genes in the remote community (such as bla_{TEM}-like genes) closely matched genes from antibiotic-exposed environments (Pallecchi *et al*., 2007). Another study from Tacao *et al*. focused on $bla_{CTX\text{-}M}$ and compared resistomes in

polluted and unpolluted rivers and found that the level of diversity among CTX-M-like genes from unpolluted river was much greater than in polluted ones; the majority of CTX-M-like genes found in polluted waters were similar to chromosomal ESBL and with lower diversity (Tacao *et al*., 2012).

The next step was to focus on *qnr* genes (**Study 3**) because their emergence has been described recently as an important resistant mechanism in enterobacteria and is dramatically increasing worldwide and also because they can be found in MGEs such as plasmids. We focused on *qnrA* and *qnrS* because of their importance in clinics and wide distribution in our area of study (Lavilla *et al*., 2008).

As in for the other ARGs, *qnrA* and *qnrS* were both detected in the phage DNA fraction of the samples, being *qnrA* more prevalent than *qnrS* in all samples analysed. *qnrS*, however, showed higher densities in the samples where it was detected. Comparing the differences between the prevalence of *qnr* genes in phage DNA with the differences observed for quinolone-resistant bacteria in urban sewage and river water, wider differences between resistant bacteria were found. These could be attributable to several reasons. First, the resistance to quinolones in bacteria is caused by other means than *qnr* genes; second, in river, it was detected a fraction of quinolone-sensitive autochthonous bacteria, while a large fraction of faecal bacteria from humans is present in urban wastewater; or third, that there is a stronger persistence of *qnr* genes in phage particles, which have been described to persist better than their hosts in these environments (Durán *et al*., 2002; Jofre, 2007).

The analyses of Tunisian waters (**Study 4**) were done in collaboration with the *Unité de Microbiologie et de Biologie Moléculaire*, CNSTN from Tunisia, and attempted to evaluate the abundance of all the ARGs analysed up to now in bacterial and bacteriophage DNA and to compare the results with the data previously obtained in Barcelona area. It was performed to use the information generated as an epidemiological tool, to study the distribution of these ARGs between two different areas varying in socio-economic and cultural characteristics and in climate and geographic background.

As described in studies in Barcelona, the densities detected in the ARGs in bacteriophages in the three types of samples analysed were lower both in occurrence and concentration than in the bacterial DNA, but follow the same trends.

bla$_{TEM}$ was the most prevalent ARG detected in accordance with the fact that *bla*$_{TEM}$ was the first ESBL to be described in Tunisia in 1994 and it still remains the most reported (Chouchani *et al*., 2011), and matching with the high prevalence observed in Barcelona.

bla$_{CTX\text{-}M\text{-}9}$ was more prevalent than *bla*$_{CTX\text{-}M\text{-}1}$ both in bacterial and DNA within phage particles in all samples evaluated from Tunisia. Averaged densities of cluster 9 in phage DNA were very similar to those obtained in Barcelona study. Regarding cluster 1, more positive samples were detected in bacterial DNA in Barcelona wastewaters but densities were very similar in both areas. Both *bla*$_{CTX\text{-}M\text{-}1}$ and *bla*$_{CTX\text{-}M\text{-}9}$ were more prevalent in the phage DNA fraction of the wastewaters analysed from Barcelona

By contrast, clear differences were observed regarding *mec*A comparing the two areas. *mecA* was abundant in the samples from Barcelona and was almost absent in the samples analysed in Tunisia, matching well with the low prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in isolates of Tunisian (Kesah *et al*., 2003). In this case the differences in livestock raising in Tunisia, mostly ovine, and the Barcelona area, which hostess a very important rising of porcine and transforming industry may explain further the differences observed. Livestock, mostly porcine is viewed as an important source of MRSA (De Neeling *et al*., 2007; Khanna *et al*., 2008; Feingold *et al*., 2012). This observation reinforces as well the idea that geographical spread of MRSA over long distances and across cultural borders is a rare event (Nübel *et al*., 2008). As discussed above, is not clear whether the origin of *mec*A goes from human to animal or vice versa. In view of the present results and the absence of *mec*A in an area without *mec*A-related animals, the transmission from animals to humans seems likely.

qnrA showed a significantly higher occurrence than *qnrS* in both WWTPs and in the slaughterhouse samples of study in bacterial and phage DNA. Reports of *qnr* genes in Tunisia and neighbouring countries do not show great differences between *qnrA* and *qnrS*

(Rodriguez-Martinez et al., 2011), with slightly higher prevalence of *qnrA* comparing with *qnrS* (Dahmen *et al*., 2010). The same pattern was observed in urban wastewater, river and animal wastewater samples analysed in Barcelona area.

The results obtained in Tunisia are in accordance with other descriptions pointing out that geographical distribution of ARGs makes possible to use the occurrence of ARGs to study the patterns of antibiotic resistance within a population (Hawkey and Jones, 2009; Paniagua *et al*., 2010).

At this point of the Thesis we have shown the prevalence of ARGs in the phage DNA fraction of municipal sewage, river and animal faecal polluted waters. Some of the aspects regarding the biology of this phenomenon will be discussed below and can be found in the different studies in the publications section.

The occurrence of ARGs in the viral DNA fraction of environmental samples supports the idea of ecosystems serving as pools of ARGs and suggests that encapsidated DNA can act as reservoir of these genes. However, the results were based on the detection of a fragment of each gene by qPCR. Our qPCR data do not indicate essential information such as the nature of the bacteriophage particles implicated or whether the genes detected were able to confer resistance to a given bacterial host.

To elucidate these points, a set of experiments were performed in order to transduce ARGs from phage particles isolated from the samples using *E. coli* as a bacterial host. Unfortunately, we faced some experimental difficulties in the process of generation and detection of the potential transductants with an environmental pool of bacteriophages. The main obstacles that makes very difficult and did not allow us detecting successful transduction could be that i) phage particles detected could not be infectious. An infectious phage should encounter its suitable bacterial host strain (*E. coli* or others) and overcome defence host systems and subsequent transduction by integrating its genome within the host genome. Moreover, the frequency of ARGs transduction might be expected to be very low and the infectivity of the phages carrying ARGs could not be evaluated by qPCR; ii) phages could be infectious but the host strain used could be not susceptible to these particular

phages or; iii) lytic phages present in the same phage pool cause the lysis of the host strain before transduction could have taken place or before the transductants could have been selected on liquid medium. Transduction is most likely when the ratio of input phage to recipient bacteria is low so that cells are infected with only a single phage particle; with multiple phage infection, bacteria are likely to be killed by the lytic phages in the lysate.

In order to circumvent the limitations of the susceptibility of the host strain to be infected by the phages carrying ARGs, another approach was attempted. By means of transfection experiments, we evaluated whether the antibiotic resistance sequences on phage DNA particles were functional genes able to confer resistance to a recipient bacterium. We were able to demonstrate this point by using *E. coli* laboratory strains in which bla_{TEM} and $bla_{CTX\text{-}M}$ from phage DNA were transfected successfully by electroporation, showing that these genes from environmental phage DNA can be expressed in a bacterial genetic background (**Study 1**). Regarding *mec*A, a similar experiment was attempted with environmental phage DNA carrying *mec*A in an *S. aureus mec*A$^-$ host strain, although in this case, no methicillin resistant transfectants were obtained. This could be due to the fact that a complete *mec* complex might be needed for the expression of the resistance (Berger-Bachi and Rohrer, 2002; Ito *et al*., 2003). The size of such a fragment would make unlikely for a phage to carry it.

To obtain additional information about the nature of the bacteriophages carrying ARGs, it was decided to further analyse the sequences of the genes and their flanking regions. For the first approach, we used conventional PCR, which allowed generation of longer fragments to amplify some of the ARGs from phage DNA of our samples, selected from those with the highest gene copy densities. The ARGs amplified from phage DNA showed 100% homology with sequences available in databases, although for some β-lactamase genes, it was not possible to discriminate between variants since sequences were partial. For the second approach, an enzymatic digestion of bla_{TEM} and $bla_{CTX\text{-}M\text{-}1}$ from transformed strains previously obtained was performed followed by a ligation step. Then, inverted primers specifically designed for the amplification of the flanking regions of bla_{TEM} and $bla_{CTX\text{-}M\text{-}1}$ were used in a conventional PCR. The bands obtained were extracted, purified and sequenced. However, the information from sequencing the flanking regions of the ARGs showed bacterial

sequences (data not shown) and did not provide relevant information about the nature of the phage particles carrying ARGs, although it reinforces the notion of being generalized transducing particles. Therefore, the fact that the sequences analysed showed only bacterial DNA, although does not hamper the presence of temperate phages carrying ARGs suggest that we are mostly detecting generalized transducing particles.

To get further insights about the biology of the phages carrying ARGs, we attempted to analyse their induction processes. Some quinolones, such as ciprofloxacin, are known as inducers of temperate phages by means of activation of RecA, this is, the SOS pathway (Fuchs *et al*., 1999; Livny and Friedman, 2004; Goerke *et al*., 2006; Rolain *et al*., 2009; Messen-Pinard *et al.*, 2012). Within this study, we wanted to know if the presence of quinolones in a sample could stimulate the induction of Qnr-encoding phages and, thus, the presence of the antibiotic itself could contribute to the transference of their own resistance. However, this was not confirmed by our experiments using the natural bacterial populations in our samples, even when using different ciprofloxacin concentrations, which may be because of the densities or the physiological state of the bacteria or because of the nature of the population. We added mitomycin C treatment, since it is another well-known inducer of the lytic cycle of temperate phages (Fuchs *et al*., 1999; Livny and Friedman, 2004; Muniesa *et al*., 2004). But it did also not affect the densities of *qnr* genes in the phage DNA fraction. Therefore, ciprofloxacin and mitomycin C failed to induce phages and showed no effect in increasing *qnr* genes in the phage DNA fraction from the bacterial population from urban sewage samples.

Recently, Modi *et al*. (Modi *et al*., 2013) demonstrated that prolonged antibiotic treatment, particularly ciprofloxacin and ampicillin, leads to the enrichment of phage-encoded genes that confer resistance to the administered antibiotic in intestinal populations in mice, and confirmed that the phageome becomes broadly enriched for functionally beneficial genes under stress-related conditions However, the findings of our studies do not confirm these observations; although this could be attributable to the different environmental conditions suffered by bacteria within an intestinal gut compared with the environmental bacteria used in our studies. In addition, some results reported by Modi *et al*. (Modi *et al*., 2013) could be

discussed since the use of antibiotics in their model for a certain period of time would have selected antibiotic resistant bacteria within the animals. It would be, therefore an expectable increase in the number of ARGs in their bacterial population, increasing the chances that these could be mobilized by phages, hence generating the enrichment of phage-encoded genes observed.

In contrast, both *qnr* genes, as well as bla_{TEM} and $bla_{CTX\text{-}M}$, showed a significant increase in GC in phage DNA when treated with EDTA. *mec*A gene was not affected by any of the inducers at any of the conditions analysed. Investigating whether the chelation properties of EDTA could contribute to phage induction and leading to an increase in the abundance of ARGs, we introduced other chelating agent, sodium citrate. Sodium citrate had similar effect on increasing the amount of ARGs to the one obtained with EDTA, and this led us to an assumption that the increase of ARGs in phage DNA might be due to the chelating properties of EDTA and citrate.

We wanted firstly to rule out the possible artefact effect of EDTA and sodium citrate on the extraction method or in the qPCR assay. With this purpose, we evaluated as a control the Cdt (cytolethal distending toxin) temperate phage available in our research group, which has been reported as self-inducible (Allué-Guardia *et al*., 2013). No significant increase in the densities of GC of Cdt phages in phage DNA after EDTA or sodium citrate treatment was observed, confirming that the chelating effect was not due to any interference in the extraction or amplification processes. The lack of increase of *mecA* in phage DNA also confirms this fact, although the reason of this lack of effect is not known. Since this gene is found in staphylococci, it could be attributable to a different effect of chelating agents on phage induction in gram positive bacteria.

EDTA is a common chelating agent (Mg^{2+} and Ca^{2+}) and has been described to act by disrupting the structure of the outer membrane of bacteria, so making this more permeable and, therefore, accessible to other antimicrobial agents and causing stress in the bacterial envelope. Both EDTA and sodium citrate have several applications such as in food industry and in medicine. The mechanism that causes an increase of ARGs in phage particles after

treatment with chelating agents is not known. However, since it has been described that phages performing generalized transduction, such as P22, can give altered packaging specificity leading to the formation of generalized transducing particles at increased frequencies (Jackson *et al*., 1982), it could be speculated that this could be a possible cause of the increase in ARGs in DNA from phage particles observed when chelating agents were used.

The effect of chelating agents on the densities of ARGs in general and of quinolone resistance genes in particular, in the bacteriophage DNA fraction must be considered when applying them, since these agents could increase the number of particles harbouring these genes, increasing the possibilities of gene transference and the generation of new resistant clones.

We also analysed infectious somatic coliphages in the samples after treatment with the inducing agents to determine if there was an induction of temperate phages that can be observed by analysis of infectious phage particles. The inducing agents had no effect on the densities of infectious coliphages and therefore they had no influence on the induction of temperate phages from the bacteria present in the sample that can be detected with the host strain used in these experiments (*E. coli* WG5). These results might be explained either because they are not producing visible lytic plaques in the host strain used or because the increase in the number of phages did not overtake the number of virulent phages in the samples.

The lack of increase in infectious lytic coliphages together with the fact that inducers of temperate phages did not cause an increase of ARGs-phages and the nature of the flanking sequences would suggests that the phages detected in our study could be in fact generalized transducing particles.

As explained in the introduction of this Thesis, phage particles causing generalized transduction are phage particles that have packaged fragments of bacterial DNA from any location in its genome into a phage head, instead of phage DNA. These particles would not be affected by compounds inducing temperate phages conducting specialized transduction,

since they are not necessarily present as prophages in a genome and are not mobilized by RecA induction as many temperate phages do. However, they could increase their number if any compound could cause stress that would lead to an erroneous encapsidation. Modi *et al*. describe that phage encapsulation could be stimulated by stress (Modi *et al*. 2013); plausibly as a mechanism to increase the robustness of the populations in the gut under these circumstances. Chelating agents could be responsible of this stress since at least EDTA has been described as responsible of membrane stress responses (Imamovic and Muniesa, 2012). Moreover, as generalized transducing particles do not carry phage DNA, they should be unable to cause lysis in a host strain and they might not be detectable by plaque assay or leave traces of phage DNA in the recipient cell (*defective particles*). Nevertheless, they still introduce the bacterial DNA in a susceptible host and transduce the genes that they are mobilizing.

Taken together, the information obtained in the five studies and the previous data from other authors, suggest that phages, or more specifically, generalized transducing phage particles, may play an important role in mobilizing determinants of resistance to antibiotics, especially in nature. Due to their structure and composition, phages persist quite successfully in the environment and are relatively resistant to anthropogenic stressors (IAWPRC, 1991; Muniesa *et al*., 1999; Durán *et al*., 2002). Bacteriophages persist much better than the bacteria in habitats where the host bacteria are aliens suggesting that phages may have good chances of mobilizing genes between different environments. Owing to the structural characteristics of phages, with phage-encapsulated DNA protected from degradation, their persistence in the environment is also much higher than of free DNA, which is more sensitive to nucleases, temperature and radiation (Lorenz and Wackernagel, 1994; Dupray *et al*., 1997; Zhu, 2006).

Therefore, their resistance to environmental stressors, together with their ubiquity and great abundance (Weinbauer, 2004), and since they can transfer ARGs by both generalized (probably very common according with our results) and specialized transduction (probably less frequent, but still supported by some descriptions (Coetze, 1975; Pereira *et al*., 1997), make phages suitable vectors for the mobilization and spread of ARGs between and within

biomes. Additionally, the existence of the so called polyvalent phages, which are broad host range generalized transducing phages, make them also a possible vector for the transfer of genes between strains, species, and even genera.

ARGs present in naturally resistant environmental bacteria are typically mediated by a resistance genes belonging to the cell's core genes. Examples of the latter are the chromosomally encoded β-lactamases found in *Enterobacteriaceae*, as for example in species of *Kluyvera*, *Rahnella* and *Ewingella* (Poirel *et al*., 2010; Lupo *et al*., 2012), which are considered environmental species. CTX-M-type genes detected in clinical isolates are located mostly within plasmids, although some studies suggest that they derive from chromosomal genes, particularly from *bla* genes present in different *Kluyvera* species, and that they are mobilized from these species at an unusually high rate through diverse genetic platforms (Barlow *et al*., 2008). The origin of *mec*A is unknown, but in *Staphylococcus fleurettii,* a commensal bacterium in animals, *mec*A is chromosomally located (Tsubakishita *et al*., 2010), and could be a highly probable origin of *mec*A.

The same genes have been found in pathogens and commensal bacteria both in human and animals microbiomes after the introduction in clinics of a given antibiotic. The presence of the antibiotic act by selecting those genes either alone or located in the genetic mobile platforms and induce their mobilization from its initial location by various mechanisms. Many of the antibiotic resistance determinants found in clinical isolates are typically acquired through and located in MGE, allowing their horizontal transfer to other bacterial strains (pathogens, commensal or environmental). Usually, conjugation mediated by plasmids has been considered the most important mode of ARG transmission by HGT. Our study shows that phages could be suited vehicles of transmission in the environment.

Thus, bacteriophage mediated transfer might be crucial in mobilization and transfer of chromosomally located ARG of environmental bacteria to human and animal pathogens. Most likely, a plausible explanation would be the incorporation of the environmental ARGs in animal and human microbiomes through commensal bacteria, due to their major abundance (van den Bogaard and Stobberingh, 2000). From commensal ARG will move to pathogens

belonging to the same genetic exchange community. Once in a body-associated microbiome where well-established genetic communities exist, the maintenance and entrance of an ARG in a proficient MGE will need of recombination, point mutation and mobilization and transfer events. The pressure exerted by antibiotics will favour the process of incorporation of genes in MGEs and their permanence in the body-associated microbiomes. Then, the spread of resistant bacteria from animals to humans and vice versa will play an important role on the spread and maintenance in anthropogenic environments of these genes from environmental bacteria.

Phages and other transfer agents contribute to the mobilization of ARGs from environmental microbes to pathogens and then back again from pathogens to new environmental microorganisms. The study of this environmental pool and the mechanisms of ARGs mobilization, like bacteriophages, could provide an early warning system for future clinically relevant antibiotic resistance mechanisms.

5. CONCLUSIONS / *CONCLUSIONS*

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The studies developed in this Thesis have led to obtain results that allow the following main conclusions:

- Targeted Real-Time PCR produces quick quantitative data on the presence of ARGs in the phage DNA fraction from environmental samples.

- β-lactamases bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$, the *mecA* gene, and the quinolone-resistance genes *qnrA* and *qnrS,* are detected in the bacteriophage DNA fraction of sewage water, river water and animal faecal samples from Barcelona area, being bla_{TEM} and $bla_{CTX\text{-}M\text{-}1}$ the most prevalent.

- The high prevalence of ARGs in phage DNA from cattle faeces obtained from animals that have not previously been exposed to antibiotics indicates that these ARGs should be naturally occurring in the environment.

- bla_{TEM} and $bla_{CTX\text{-}M\text{-}1}$ ARGs from phage particles are functional and able to confer resistance to a suitable bacterial host strain by transfection.

- Sequencing of the flanking regions of the genes encoded in phage particles did not revealed phage sequences, but only plasmid or bacterial sequences.

- The lack of increase in the number of copies of the ARGs studied in packaged DNA when treated with typical inducers of temperate phages (mitomycin C or ciprofloxacin) suggests that these might not be specialized transducing temperate bacteriophages.

- However, a significant increase is observed when treated with EDTA and sodium citrate, probably due to their chelation properties.

- The presence of ARGs-encoding phage particles and the effect caused by some chelating agents strongly increase the spread of some antibiotic resistance genes and also create the possibility of new transduction events that might cause the emergence of new resistant strains.

- No increase in infectious lytic coliphages was found after induction with any inducing agent.

- Considering the results observed, phage particles containing ARGs could be, in fact, generalized transducing particles derived from lytic bacteriophages. These particles would be still infectious and able to transfer the ARG to a receptor bacteria, but not able to propagate, induce or generate plaques of lysis. The genes inside phage transducing particles would correspond to bacterial genes.

- The quinolone-resistant *E. coli* strains belonging to clonal groups O25b:H4-B2-ST131 and O25b:H4-D-ST69 with high virulence gene content has been shown in raw sewage and river water in Barcelona, showing an environmental spread of these clones.

- Environmental O25b:H4-B2-ST131 isolates showed similar virulence and macrorestriction profiles to clinical human strains.

- To our knowledge this is the first time the O25b:H4-D-ST69 clonal group has been detected in municipal and river water samples in the Barcelona area.

- In Tunisian waters, distribution of some of the genes showed similarities with Barcelona waters, being bla_{TEM} was the most prevalent ARG, followed by $bla_{CTX\text{-}M\text{-}9}$, which was more prevalent than $bla_{CTX\text{-}M\text{-}1}$ and *qnr* genes, both in bacterial and DNA within phage particles in all samples analysed.

- The low prevalence of the *mec*A gene in bacterial DNA and its absence in phage DNA could be attributed to differences in livestock production, mainly involving sheep and goats in Tunisia, and not involving pigs, which are considered as an important source of methicillin-resistant *S. aureus* (MRSA).

- The study of ARGs in wastewater seems to be an interesting option for studying antibiotic resistance patterns within a population in a given area.

5. CONCLUSIONS

Els estudis desenvolupats en aquesta tesi han donat lloc a l'obtenció d'una sèrie de resultats, les conclusions principals dels quals es detallen a continuació:

- La tècnica de *Real-Time PCR* permet l'obtenció ràpida de dades quantitatives sobre la presència de gens de resistència a antibiòtics en la fracció de DNA fàgic de mostres ambientals.

- Les β-lactamases bla_{TEM}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$, el gen *mecA,* i els gens de resistència a quinolones *qnrA* i *qnrS,* van ser detectats en la fracció de DNA de bacteriòfags de mostres d'aigua residual, d'aigua de riu i mostres amb contaminació fecal animal de l'àrea de Barcelona, essent bla_{TEM} i $bla_{CTX\text{-}M\text{-}1}$ els més prevalents.

- L'elevada prevalença de gens de resistència a antibiòtics en DNA fàgic de mostres de vaques les quals no han estat exposades prèviament a antibiòtics indica que aquests gens de resistència es trobarien de manera natural en el medi ambient.

- Els gens de resistència a antibiòtics bla_{TEM} i $bla_{CTX\text{-}M\text{-}1}$ detectats en DNA fàgic són capaços de conferir resistència a una soca hoste bacteriana sensible i adequada mitjançant transfecció.

- Les regions flanquejants dels gens de resistència a antibiòtics codificats en partícules fàgiques no revelaven seqüències fàgiques, sinó seqüències bacterianes i plasmídiques.

- La manca d'augment en el nombre de còpies de gens de resistència a antibiòtics estudiats en el DNA de partícules fàgiques en aigua residual quan es va tractar amb inductors de fags temperats (mitomicina C i ciprofloxacina) suggereix que no es tractaria de bacteriòfags temperats de transducció especialitzada.

- En canvi, quan es va tractar l'aigua residual amb EDTA i citrat sòdic es va detectar un increment significatiu en el nombre de còpies de gens de resistència a antibiòtics en el DNA de partícules fàgiques, probablement produït per les propietats quelants d'aquests agents.

- La presència de partícules fàgiques codificadores de gens de resistència a antibiòtics i l'efecte causat per alguns agents quelants augmenta considerablement la disseminació d'alguns gens de resistència i també crea la possibilitat de nous esdeveniments de transducció que podrien causar l'emergència de noves soques resistents.

- Cap dels agents inductors utilitzats va resultar tenir efecte en l'augment del nombre de colifags somàtics en aigua residual.

- Tenint en compte els resultats observats, les partícules fàgiques portadores de gens de resistència a antibiòtics serien partícules de transducció generalitzada derivades de bacteriòfags lítics. Aquestes continuarien essent infeccioses i capaces de transferir la resistència a una soca receptora, però incapaces de propagar, induir o generar clapes de lisi. Els gens continguts al seu interior correspondrien a gens bacterians.

- S'han aïllat soques d'*E. coli* resistents a quinolones dels grups clonals O25b:H4-B2-ST131 i O25b:H4-D-ST69 amb elevat contingut de gens de virulència en aigua residual i aigua de riu a Barcelona, demostrant la disseminació ambiental d'aquests clons.

- Els aïllaments d'O25b:H4-B2-ST131 de mostres no patològiques obtingudes d'aigua residual urbana i de riu presentaven perfils de restricció i virulència similars als d'aïllaments clínics del mateix clon trobats en humans.

- Aquest estudi és la primera descripció de la detecció del grup clonal O25b:H4-D-ST69 en aigua residual municipal i aigua de riu a l'àrea de Barcelona.

- A les aigües de Tunísia analitzades, la distribució d'alguns gens de resistència presentava similituds amb les aigües de Barcelona, essent bla_{TEM} el més prevalent, seguit de $bla_{CTX\text{-}M\text{-}9}$, el qual va ser més prevalent que $bla_{CTX\text{-}M\text{-}1}$ i que els gens *qnr*, tant en DNA bacterià com en DNA de partícules fàgiques en totes les mostres analitzades.

- La baixa prevalença del gen *mec*A en DNA bacterià i la seva absència en DNA fàgic es consideraria un reflex de les diferències en les pràctiques de ramaderia de Tunísia, principalment ovina i caprina, la qual no implicaria bestiar porcí, font important d'*S. aureus* resistent a meticil·lina (*MRSA*).

- L'estudi de gens de resistència a antibiòtics en aigua residual seria una opció interessant per a l'estudi de patrons de resistència d'una població en una àrea determinada.

6. REFERENCES

6. REFERENCES

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7. APPENDICES

APPENDIX 1: Other publications

Other publications of the same author not included in this study (chronological order):

1. **Muniesa M, Colomer-Lluch M, Jofre J.** 2013. Potential impact of environmental bacteriophages in spreading antibiotic resistance genes. *Future Microbiol.* 8(6):739-751.

2. **Muniesa M, Colomer-Lluch M, Jofre J.** 2013. Could bacteriophages transfer antibiotic resistance genes from environmental bacteria to human-body associated bacterial populations? *Mob. Genet. Elements.* 3(4):e25847.

3. **Quirós P, Colomer-Lluch M, Martínez-Castillo A, Miró E, Argente M, Jofre J, Navarro F, Muniesa M.** 2014. Antibiotic-resistance genes in the bacteriophage DNA fraction of human fecal samples. *Antimicrob. Agents. Chemother*. 58(1):606-609.

Review
Future Microbiology

Potential impact of environmental bacteriophages in spreading antibiotic resistance genes

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The idea that bacteriophage transduction plays a role in the horizontal transfer of antibiotic resistance genes is gaining momentum. Such transduction might be vital in horizontal transfer from environmental to human body–associated biomes and here we review many lines of evidence supporting this notion. It is well accepted that bacteriophages are the most abundant entities in most environments, where they have been shown to be quite persistent. This fact, together with the ability of many phages to infect bacteria belonging to different taxa, makes them suitable vehicles for gene transfer. Metagenomic studies confirm that substantial percentages of the bacteriophage particles present in most environments contain bacterial genes, including mobile genetic elements and antibiotic resistance genes. When specific genes of resistance to antibiotics are detected by real-time PCR in the bacteriophage populations of different environments, only tenfold lower numbers of these genes are observed, compared with those found in the corresponding bacterial populations. In addition, the antibiotic resistance genes from these bacteriophages are functional and generate resistance to the bacteria when these genes are transfected. Finally, reports about the transduction of antibiotic resistance genes are on the increase.

The WHO has identified that increasing antibiotic resistance among bacteria is a major problem for public health on a global scale. The causes of this increase in resistance are frequently attributed to overuse and incoherent application of antibiotics in humans, together with the use of antibiotics in animal husbandry [1]. However, concurrently, a growing body of evidence points to the potentially important role of environmental microorganisms from ecosystems in which the presence of antibiotics produced by humans is expected to be very low or completely absent. Such ecosystems are as varied as soil [2], a microcave isolated for over 4 million years [3] and pristine waters [4].

Environmental bacteria seem to be an unrestricted source of resistance genes, probably because these genes have emerged in bacteria that produce antibiotics, which are mainly found in environments with limited nutritional resources. There are also resistance genes in bacteria that share habitats with antibiotic producers. Finally, many antibiotic resistance genes are not primarily resistance genes, but can easily be converted to antibiotic resistance genes and are thus known as the hidden resistome [2]. Bearing in mind that the production of antibiotics is considered a competitive advantage for microorganisms living in environments with scarce nutritional resources, it seems likely that antibiotic resistance genes are more abundant in the microbiomes of noncontaminated ecosystems than in the microbial communities of humans and animals not suffering the pressure of antibiotics. It seems clear nowadays that environmental bacteria are an unlimited source of genes that may act as resistance genes when transferred to pathogenic microorganisms through horizontal gene transfer.

Moreover, bacteria in environments that are not contaminated with antibiotics from anthropogenic practices share antibiotic resistance genes, or resistomes, with human and animal pathogens [5,6]. A study by Tacao *et al.* focused on extended-spectrum β-lactamase and cefotaxime-hydrolyzing β-lactamase (CTX-M) and compared resistomes in polluted and unpolluted rivers [6]. They found that: the level of diversity among CTX-M-like genes from unpolluted rivers was much greater than in polluted ones; the majority of CTX-M-like genes found in polluted waters were similar to chromosomal extended-spectrum β-lactamase such as $\beta\text{-lactamase}_{\text{RAHN-1}}$; and diversity was much lower in the polluted river, revealing the presence of different genetic mobile platforms previously described for clinical strains. A good example is found when looking at β-lactamases and Enterobacteriaceae. Available information reveals that nowadays, many β-lactamases present in genetically mobile platforms

Keywords
■ antibiotic resistance ■ bacteriophages ■ horizontal gene transfer ■ lysogeny ■ transduction

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Could bacteriophages transfer antibiotic resistance genes from environmental bacteria to human-body associated bacterial populations?

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Environments without any contact with anthropogenic antibiotics show a great abundance of antibiotic resistance genes that use to be chromosomal and are part of the core genes of the species that harbor them. Some of these genes are shared with human pathogens where they appear in mobile genetic elements. Diversity of antibiotic resistance genes in non-contaminated environments is much greater than in human and animal pathogens, and in environments contaminated with antibiotic from anthropogenic activities. This suggests the existence of some bottleneck effect for the mobilization of antibiotic resistance genes among different biomes. Bacteriophages have characteristics that make them suitable vectors between different biomes, and as well for transferring genes from biome to biome. Recent metagenomic studies and detection of bacterial genes by genomic techniques in the bacteriophage fraction of different microbiota provide indirect evidences that the mobilization of genes mediated by phages, including antibiotic resistance genes, is far more relevant than previously thought. Our hypothesis is that bacteriophages might be of critical importance for evading one of the bottlenecks, the lack of ecological connectivity that modulates the pass of antibiotic resistance genes from natural environments such as waters and soils, to animal and human microbiomes. This commentary concentrates on the potential importance of bacteriophages in transferring resistance genes from the environment to human and animal body microbiomes, but there is no doubt that transduction occurs also in body microbiomes.

Keywords: bacteriophages, transduction, lysogeny, horizontal gene transfer, antibiotic resistance

Abbreviations: ARG, antibiotic resistance genes; MGE, mobile genetic elements

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Antibiotic Resistance in Pristine Environments

Emergence and spread of resistance to antibiotics is hampering one of the major achievements of the history of medicine, which is the minimization of the effects of infectious diseases, mostly of those caused by bacteria. Traditionally, it was thought that the selective pressure caused by the overuse and misuse of antibiotics in human medicine and animal husbandry was the major, if not the unique, cause of this occurrence.[1] In the last years, the number of evidences about the ubiquity and abundance of antibiotic resistance genes in diverse environments has increased. These studies have shown that there is a great abundance of antibiotic resistance genes (ARG) in many environmental ecosystems barely in contact with human produced and released antibiotics, that suggests an important role of environmental microorganisms as source and reservoirs of resistance genes.[2] Thus, a controversial question about microbial resistance origin is whether it is the result of human activity or rather a result of the joint evolution of antibiotic production and antibiotic resistance pathways that evolved during millions of years in the environment, or both.[3–5] Indeed, antibiotic resistance seems to be very antique.[6] The diversity of resistance determinants in different bacteria is larger in natural environments not contaminated with antibiotic from anthropogenic

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Antibiotic Resistance Genes in the Bacteriophage DNA Fraction of Human Fecal Samples

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A group of antibiotic resistance genes (ARGs) (*bla*$_{TEM}$, *bla*$_{CTX\text{-}M\text{-}1}$, *mecA*, *armA*, *qnrA*, and *qnrS*) were analyzed by real-time quantitative PCR (qPCR) in bacteriophage DNA isolated from feces from 80 healthy humans. Seventy-seven percent of the samples were positive in phage DNA for one or more ARGs. *bla*$_{TEM}$, *qnrA*, and, *bla*$_{CTX\text{-}M\text{-}1}$ were the most abundant, and *armA*, *qnrS*, and *mecA* were less prevalent. Free bacteriophages carrying ARGs may contribute to the mobilization of ARGs in intra- and extraintestinal environments.

Antibiotic resistance may be obtained by spontaneous mutations or acquired by the incorporation of antibiotic resistance genes (ARGs) (1). ARGs spread between cells by using genetic platforms known as mobile genetic elements (MGEs). The most commonly studied MGEs are plasmids, transposons, integrons, and, more recently, bacteriophages (2).

Bacteriophages or phage-related elements carry ARGs in Gram-positive (3–6) and Gram-negative (7–10) bacteria. Recently, some studies have suggested that the role of phages carrying ARGs in the environment is much more important than previously thought (2, 11–13). Abundant ARGs have been reported in the bacteriophage DNA fraction of fecally contaminated water (14–16), and metagenomic analyses indicate that there are abundant ARGs in viral DNA (17). As a result of their higher incidence in clinical settings, much effort has been devoted to the study of plasmids, integrons, and transposons. However, there is little information on phages carrying ARGs in clinical settings.

This study analyzes a group of ARGs in phage DNA isolated from stool samples. The ARGs studied include two groups of beta-lactamase genes from Gram-negative bacteria (*bla*$_{TEM}$ and *bla*$_{CTX\text{-}M\text{-}1\ group}$); *mecA*, responsible for resistance to methicillin in *Staphylococcus* spp.; *armA*, a gene which confers high-level resistance to aminoglycosides in Gram-negative bacteria; and *qnrA* and *qnrS*, plasmid-mediated genes that provide some degree of reduced quinolone susceptibility.

The study was performed using 80 human fecal samples from 46 females and 34 males from 6 months to 102 years of age who visited the Sant Pau Hospital (Barcelona, Spain) during a 6-month period. Stool samples were processed according to conventional protocols for the isolation of enteropathogenic bacteria, rotavirus, and adenovirus and were microscopically examined for protozoa. Only samples that were negative for these pathogens were included in the study. None of the patients selected was involved in a food-borne outbreak or showed any severe gastrointestinal pathology. To our knowledge, none of the patients were receiving antibiotic treatment during the time of the study, although previous antibiotic treatments could not be excluded.

Fecal samples were homogenized to a 1:5 (wt/vol) dilution in phosphate-buffered saline (PBS) by magnetic stirring for 15 min. Fifty milliliters of the homogenate was centrifuged at 3,000 ×g, and the phage lysate was purified and concentrated as described previously (15, 16). Phage suspensions were treated with DNase (100 U/ml) to eliminate free DNA outside the phage particles. To confirm total removal of nonencapsidated DNA, eubacterial 16S rRNA genes and the different ARGs (see Table S1 in the supplemental material) were evaluated in the sample after DNase treatment and before its disencapsidation.

Phage DNA was extracted from the suspension as previously described (16, 18). Total DNA (including Gram-positive and Gram-negative bacterial and viral DNA) was extracted from 200 μl of the homogenate by use of a QIAamp DNA stool minikit (Qiagen Inc., Valencia, CA) in accordance with the manufacturer's instructions.

Standard and quantitative PCR (qPCR) procedures for *bla*$_{TEM}$, *bla*$_{CTX\text{-}M\text{-}1\ group}$, and *mecA* were performed as previously described (16). The *armA* qPCR assay was designed using the sequence of *armA* in plasmid pMUR050 (NC_007682.3) from an *Escherichia coli* pig isolate (19). pMUR050 was also used to generate standard curves (16). The *armA* qPCR assay has an average efficiency of 98.4% and a detection limit of 2.74 gene copies (GC). The *qnrA* qPCR assay detects seven variants (*qnrA* 1 to 7), and the *qnrS* qPCR assay detects six variants (*qnrS* 1 to 6) (20). The 565-bp fragment of *qnrA* was obtained from *E. coli* strain 266, and the 425-bp fragment of *qnrS* was obtained from the environmental strain *Enterobacter cloacae* 565 isolated from sewage. Both fragments were cloned in pGEM-T-Easy vector (Promega, Barcelona, Spain) to generate the standard curves (16). The *qnrA* qPCR assay showed 98.2% efficiency and a detection limit of 3.1 GC/μl, and the *qnrS* assay showed 99.4% efficiency and a detection limit of 8.3 GC/μl. All qPCR assays (see Table S1 in the supplemental material) were performed under standard conditions (15, 16). To

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APPENDIX 2: Composition of growth media and buffers

1. Culture media composition

Chromocult® Coliform Agar (Merck®):

26.5 g Chromocult agar

1 L H_2O distilled H_2O

Add 2 mL of *E. coli*/coliform selective supplement for each 500 mL. Heat to boiling with frequent agitation until completely dissolved. Do not sterilize by autoclaving and do not overheat. Pour it into plates and store plates at 4ºC.

LB agar:

1 L LB broth

15 g bacteriologic agar

Sterilise by autoclaving at 121°C for 15 minutes. Pour into plates and store plates at room temperature or 4ºC.

LB (Luria-Bertani) broth:

10 g pancreatic digest of casein (tryptone)

5 g yeast extract

10 g NaCl

1 L distilled H_2O

pH= 7.0 – 7.2

Sterilise by autoclaving at 121°C for 15 minutes and store it at room temperature and/or 4ºC.

LBss (semi-solid):

1 L LB broth

7 g bacteriologic agar

Sterilise by autoclaving at 121°C for 15 minutes and store it at room temperature and/or 4ºC.

TSA (Trypticasein Soya Agar):

40 g TSA

1 L double distilled H_2O

Sterilise by autoclaving at 121°C for 15 minutes. Pour into plates and store plates at room temperature or 4ºC.

TSB broth (Trypticasein Soya Broth):

17 g tryptone

3 g soytone (peptic digest of soybean meal)

5 g NaCl

2.5 g KH_2PO_4

2.5 g dextrose

1 L distilled H_2O

pH= 7.0 – 7.2

Sterilise by autoclaving at 121°C for 15 minutes and store it at 4ºC.

2. Buffers composition

PBS (Phosphate Buffered Saline):

8 g NaCl

0.2 g KCl

0.2 g KH_2PO_4

1.15 g Na_2HPO_4

1 L double distilled H_2O

Adjust pH= 7.1 – 7.2 and sterilise by autoclaving at 121°C for 15 minutes and store it at room temperature.

SM buffer:

5.8 g NaCl

2 g Mg_2SO_4 x 7 H_2O

50 mL Tris HCl 1M, pH=7.5

0.1 g gelatine

950 mL double distilled H_2O

Sterilise by autoclaving at 121°C for 15 minutes and store it at room temperature.

Proteinase K buffer:

2 mL Tris HCl 1M, pH=8

2 mL EDTA 0.5M, pH=8

10 mL SDS (10%)

Add double distilled H_2O to a final volume of 100 mL. Prepare it with sterile solutions. Proteinase K buffer is used in combination with Proteinase K, Roche® (0.2 mg/mL final concentration)

TBE 10X (Tris Boric EDTA buffer):

109 g Tris base

55.6 g boric acid

9.2 g EDTA

1 L double distilled H_2O

Sterilise by autoclaving at 121°C for 15 minutes and store it at room temperature.

3. Other reagents

Sodium citrate 0.2 M:

Prepare from 2M and then dilute to obtain 0.2M:

58.82 g sodium citrate

100 mL double distilled H_2O

Filter by 0.22 µm sterilization of aqueous solution filters.

Mitomycin C (0.5 mg/mL) (Sigma):

0.5 mg Mitomycin C

1 mL double distilled H_2O

Store it in the dark at 4ºC.

EDTA 20 mM:

0.74 g EDTA

100 mL double distilled H_2O

Adjust pH=7.2

Sterilise by autoclaving at 121°C for 15 minutes and store it at room temperature.

Agarose gel for electrophoresis:

150 mL TBE 1X

Agarose adjusted to different concentrations: 0.8 - 2% w/v (weight percentage per volume).

Ethidium bromide for staining of DNA agarose gel:

Add 200 µL of the solution stock (1%; w/v) of ethidium bromide (Merck®) to 1 L of double distilled H_2O.

4. Antibiotics:

Antibiotic	Final concentrations used (µg/mL)
Ampicillin	32,50,100
Ciprofloxacin	0.1, 0.4, 1, 4
Nalidixic acid	25
Oxacillin	1, 2, 4

APPENDIX 3: Detailed protocols used in this study

The absence of standardized methods to concentrate and obtain DNA from viral particles easy to use in laboratories, with the lowest cost and the best efficiency is one of the main obstacles to analyse, detect and quantify phages and their genetic material from environmental samples. The main steps to analyse ARGs in phage DNA from environmental samples are: sampling, concentration, removal of inhibitors, non-encapsidated DNA removal, and specific detection of the ARGs of study.

It is necessary to properly separate and concentrate the phage particles from samples in small volumes before proceeding to the detection analysis. The main steps to concentrate phages from environmental samples are based on: centrifugation, filtration of the sample to separate bacteria from viral particles by size using a 0.22 µm filter, concentration of the phage particles, phage DNA extraction, for example by using chloroform and phage DNA precipitation (the detailed protocol for phage DNA extraction is detailed below).

An important point to consider is the presence of certain components that may interfere in the detection process of the ARG, which can be together with the phage particles. Components such as polysaccharides, phenols and cati ons are well-known inhibitors, which may be present in the sample.

Non-phage DNA removal is a critical point to guarantee the absence of bacterial DNA, free DNA and DNA contained in vesicles. For this matter, the optimization of the bacteriophage DNA extraction protocol was performed.

1. DNA extraction methods

Phage and bacterial DNA were extracted from several samples: bacterial cultures, wastewater samples, river samples, animal wastewater samples and faecal stools. Initial preparation and volume of sample varied:

- Wastewater: 50 mL of sample were processed as described below.
- River water: 100 mL of sample was processed as described below.

- Animal wastewater samples: 50 mL of sample were processed as described below.
- Animal faecal samples: 2.5 g of each faecal sample were homogenized in 50 mL of PBS.

The studies developed in this thesis always used the same method for phage and bacterial DNA extraction in order to generate consistent and comparable results.

a) <u>Phage DNA extraction protocol (modified from Sambrook and Russell, 2001)</u>

1. To separate the phage fraction from the bacterial cells and other particles, the sample was centrifuged at 3000 *xg* 10 minutes at 4ºC. The supernatant was filtered through low protein binding 0.22 µm pore size filter membrane (Millex-GP, Millipore, Bedford, MA).
2. Concentration of the volume: In order to reduce the volume of the samples were concentrated by using Amicon Ultra 15 mL tubes (100 kDa Amicon Ultra centrifugal filter units, Millipore, Bedford, MA) by centrifuging at 3000 *xg* 10 minutes. The process was repeated until the volume was 0.5 mL when possible.
3. Chloroform treatment: chloroform (1:10 v/v) was added to the concentrate sample
4. DNase treatment: 20 µL of DNase (100 mg/mL) was added to the concentrate sample and incubated 1h at 37ºC. DNase was then inactivated at 80ºC for 10 minutes. At this point, an aliquot of the sample was taken and kept as a control of efficiency of the removal of any DNA not encapsidated (absence of bacterial or free DNA). Proteinase K treatment: 6 µL of Proteinase K (0.2 mg/mL) and 250 µL of Buffer Proteinase K were added per 0.5 mL of sample and incubated 1h at 55ºC.
5. Sample was mixed with phenol-chloroform (1:1 v/v) in a 2 mL Phase Lock Gel tubes (5- Prime, VWR International, Madrid, Spain) and centrifuged. Then, chloroform (1:1 v/v) was added to the sample and the mixture was centrifuged again at 16,000 *xg* 5 minutes at room temperature.

6. DNA precipitation: DNA was precipitated with 0.1 volume of 3M sodium acetate and 2 volumes of absolute ethanol and kept overnight at 4ºC.
7. DNA recovery: Precipitated DNA was collected by centrifugation at 16,000 *xg* 30 minutes. The supernatant was removed carefully and the DNA was washed in 300 µL ethanol (70%) and centrifuged at 16000 *xg* 30 min.
8. DNA pellet was air dried 2-3 h and eluted in sterile double-distilled water.

b) Bacterial DNA extraction protocol

The respective volumes of samples of study were passed through 0.45 µm polyvinylidene fluoride (PVDF) DURAPORE® membrane filters (Millipore, Bedford, Massachusetts), described by the manufacturer as low protein-binding membranes. These allowed the phages to pass through whilst bacteria were retained on the surface of the filter. To further removing phages retained on the filters, 10 mL of PBS was added to the surface of the filter, gently agitated and removed by filtration. Two washing steps allowed a high (99%) phage reduction without significant loss of bacteria (Muniesa *et al*., 2005). The membrane containing retained bacteria was recovered in 5 mL of LB. The suspension was centrifuged at 4000 *xg* for 10 minutes. Given that the bacterial fraction included Gram-positive and Gram-negative bacteria, and in order to recover DNA from both, the pellet containing bacteria was suspended in 180µl of enzymatic solution (20mg/mL lysozyme; 20mM Tris-HCl, pH=8.0; 2mM EDTA; 1,2% Triton) and incubated for 30 minutes at 37ºC. DNA was then extracted using a QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, USA), following the manufacturer's instructions.

2. Membrane filtration method for bacterial enumeration

Aerobic bacteria and *E. coli* present in the samples were evaluated by a membrane filtration method as standardized previously (Anonymous, 1998).

Briefly, decimal serial dilutions of urban and sewage water in PBS were filtered through 0.45 µm polyvinylidene fluoride (PVDF) DURAPORE® membrane filters (Millipore, Bedford, Massachusetts) and the membranes were placed on the corresponding agar media. Aerobic

bacteria were grown in TSA and *E. coli* in Chromocult® coliform agar (Merck, Darmstadt, Germany), and plates were incubated in aerobic conditions for 18h.

To evaluate aerobic bacteria and *E. coli* resistant to antibiotics (ampicillin, nalidixic acid, ciprofloxacin), samples were processed as described above but incubated in TSA and Chromocult® coliform agar for 2 hours at 37ºC and then the membranes were transferred to TSA or Chromocult® coliform agar containing the corresponding antibiotic and further incubated at 37ºC for 18h.

Colonies were enumerated and suspected resistance colonies were screened for the presence of the resistant gene of study by conventional PCR.

3. Transformation of constructs containing ARGs into competent cells by electroporation

1. From an overnight culture of the strain (*E. coli* DH5α) 2 mL of the culture were inoculated in 20 mL of LB and incubated at 37ºC under agitation. When the OD_{600} reached 0.3-0.5 the bacterial growth was stopped by keeping it on ice for 10 minutes.
2. The culture was then centrifuged 10 minutes at 3000 *xg* at 4ºC.
3. The pellet was resuspended with 500 µL of ice-cold double-distilled water.
4. Cells were centrifuged for 10 seconds at 10,000 *xg* and the pellet was washed with 500µL of ice-cold water. The washing step was repeated for 6-7 times in order to remove salts present in the culture.
5. Finally, it was resuspended with 100 µL of double-distilled water.
6. The ligation mixture[a] was previously prepared as indicated in the table below and incubated overnight at 4°C to reach the maximal ligation efficiency. Alternatively, the reactions were incubated for 1 hour at room temperature.

Reagent	Volume/reaction
2X Rapid Ligation Buffer	5 µL
pGEM® -T vector	1 µL
PCR product	X* µL
T4 DNA ligase	1 µL
Nuclease free water to a final volume of 20 µL	10 µL

*Molar ratio PCR *product::vector* (3:1)

7. The ligation mixture was added to the electrocompetent cells, gently mixed and introduced in an electroporation cuvette. Electroporation of the electrocompetent cells was performed at 2.5 kV, 25 F capacitance and 200 Ω resistance.
8. 3mL of LB was added to the electroporated mixture and incubated 2-3 hours at 37ºC without shaking.
9. The culture was plated in LB agar plates with ampicillin (100µg/mL) for pGEM-T-easy vector constructs (pGEM-T Easy vector has an ampicillin resistance gene as a marker) and incubated overnight at 37ºC.
10. Colonies grown on the plates, potentially containing the vector with insert were screened by conventional PCR to evaluate the presence of the construct and the orientation of the insert by using the primers listed in Table 6. The insert amplification was confirmed by sequencing.
11. The colonies containing the vector with the insert was subcultured and from it, the construct was purified by using a standard kit (e.g. Invitrogen, Qiagen) following the manufacturer instructions.
12. The construct concentration was quantified by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technilogies. Thermoscientifics. Wilmington, USA).

APPENDIX 4: PCR and qPCR reactions

1. Conventional PCR assay conditions

PCR (Dreem Taq Green PCR Master Mix, Fermentas®)

Reagent	Volume/reaction
Dreem Taq	12.5 µL
Upper primer (30 µM)	0.5 µL
Lower primer (30 µM)	0.5 µL
dd H_2O	9 µL
DNA sample	2.5 µL
Final volume	25 µL

PCR (Roche®)

Reagent	Volume/reaction
Taq Roche®	0.4 µL
dNTPs	0.4 µL
Buffer	5 µL
Upper primer (30 µM)	1 µL
Lower primer (30 µM)	1 µL
dd H_2O	38.2 µL
DNA sample	4 µL
Final volume	50 µL

Sequencing (Applied Biosystems®)

Reagent	Volume/reaction
Big Dye M. Mix 3.1	1 µL
Buffer Big Dye 3.1	3 µL
Primer 5 µM	1 µL
Sample DNA	µL
dd H_2O	11 µL
DNA sample	X µL
Final volume	10µL

X=100ng DNA/100pb

DIG labelling of a DNA Probe dUTP method (Roche®)[a]

Reagent	Volume/reaction
H_2O bd	35.6 µL
Buffer 10X	5 µL
$MgCl_2$	1 µL
dNTPs (10 mM)	1 µL
Upper primer (30 µM)	1 µL
Lower primer (30 µM)	1 µL
Taq 2units	0.4 µL
DNA sample	5 µL
Final volume	50 µL

[a] Mix the following reagents and perform the PCR using the conditions for the corresponding amplimer.

2. Real time qPCR

The development and optimization of molecular techniques has allowed overcoming some disadvantages of traditional techniques. Nowadays, quantitative PCR (qPCR) is one of the techniques most used in microbiology laboratories and it allows to quantify the number of gene copies (GC) present in a sample easily and a non-very expensive way. This is a very sensitive and specific technique when primers and probe are properly designed, it is also easy to standardize and makes it possible to analyse an important number of samples at the same time. However, it has certain limitations, mainly due to the presence of inhibitors that may interfere in the amplification process.

General conditions

DNA obtained from different samples (wastewater, river water, animal wastewater) was used for quantification of antibiotic resistance genes by Real-Time qPCR (Applied Biosystems). The extraction and purification of DNA from phage and bacterial fraction was described above. Each 20 µL Real-Time PCR reaction contained:

Reagent	Volume/reaction
Primers and TaqMan probe (Applied Biosystems)	3 µL
Taqman® Environmental Master Mix 2.0 (Applied Biosystems)	10 µL
Sample or dilution of plasmid	1 µL
Final volume	20 µL

Real-Time PCR conditions:

- Initial step: 95ºC for 10 min
- Denaturation: 95ºC for 15 s
- Extension/Annealing: 60ºC for 40 cycles

Creating Standard Curves with Plasmid DNA Templates for use in Real-Time PCR

Plasmids containing each cloned targeted antibiotic resistance gene sequences were used as standards in Real-Time qPCR.

The amplified fragment obtained by conventional PCR with the primers described in Table 6, and purified as described before, was cloned into a pGEM-T Easy vector for insertion of PCR products, following the manufacturer's instructions (Promega, Barcelona, Spain). The construct was transformed by electroporation into *E.coli* DH5α electrocompetent cells following the protocol described above.

Once plasmid construct was obtained and purified the DNA concentration was quantified using a NanoDrop ND-1000 spectrophotomoteer (NanoDrop Technologies. Thermoscientifics. Wilmington, USA).

Serial decimal dilutions of the stock were made in double-distilled water to prepare the standard curve for qPCR. Then, standard dilutions where aliquoted and stored at -80ºC until use. Three replicates of each dilution were added to each qPCR reaction.

The number of construct gene copies (GC) per µL for every pGEM-T-Easy vector::*antibiotic resistance gene* was calculated by assuming average molecular mass of 660Da for 1 bp of dsDNA. The calculation was achieved using the following equation:

$$\textbf{Gene copy (GC) / µL sample} = [X \text{ ng/µL DNA} \times P_{pGEM:ARG} \times 660 \text{ g/mol}] \times 6.022 \times 10^{23}$$

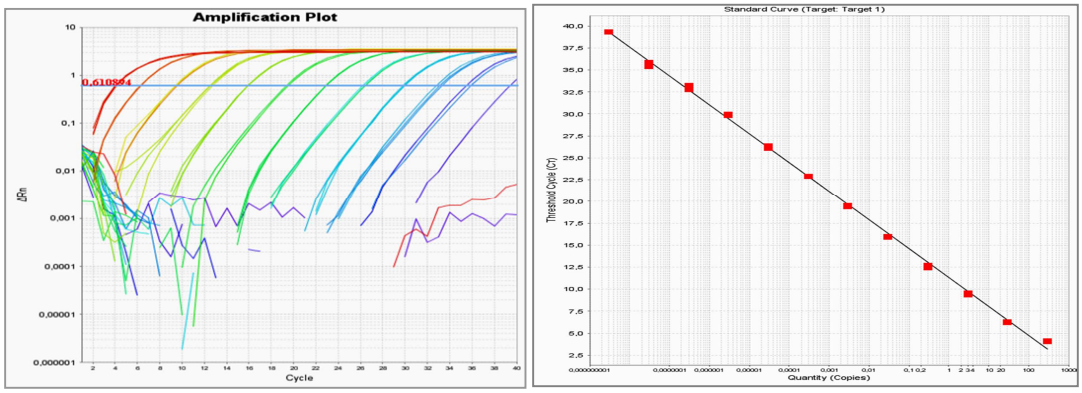
X: DNA concentration of the pGEM-T-Easy vector::ARG (ng/µL)

$\mathbf{P_{pGEM::ARG}}$: plasmid length with ARG insert (bp)

Average molecular weight of a double-stranded DNA molecule= 660 g/mol

Avogadro's number= $6.023e^{23}$ molecules/1 mol

Standard curves were generated using serial decimal dilutions of pGEM::*ARG* stock per X and the C_T values obtained were plotted against log gene copy number. C_T slope method was used to determine the amplification efficiency, R^2 and y-intercept of the standards.



The efficiency (*E*) was calculated using the formula:

$$E = 10^{(-1/slope)} - 1$$

Detection limit was considered in when stock dilution was no longer consistent with the previous dilution pattern.

qPCR controls to exclude non-encapsidated DNA

The protocol used for DNA extraction from phage fraction in all samples analysed in the different studies was always accompanied with several controls performed in order to ensure that the results obtained were only due to the amplification of the DNA contained within the capsid of bacteriophage particles. Two additional steps in the protocol of encapsidated DNA extraction were used:

1. Chloroform treatment: Once samples were concentrated, a chloroform treatment was performed in order to avoid possible vesicles containing DNA with protein encapsidated particles not affected by this treatment. Although not very likely, DNA has been described associated with membrane vesicles.

2. DNase treatment: Samples were treated with DNAse (100 units/mL) of the phage lysate) to eliminate free DNA outside the phage particles. After the treatment, DNase was heat inactivated for 10 minutes at 80ºC in order to stop the enzyme activity.

Controls to exclude non-encapsidated DNA

To rule out the possibility of non-phage DNA contamination, an aliquot of the sample taken after DNase treatment and before desencapsidation was evaluated. At this stage, the samples were used as template for conventional PCR of eubacterial 16S rDNA and as template for the qPCR assay of each antibiotic resistance gene. Both amplifications should be negative confirming that DNase has removed all non-encapsidated DNA from the samples.

Confirmation of the DNase activity

To verify the good performance of the DNase treatment, additional controls were also performed in preliminary experiments. Serial decimal dilutions of the construction stock pGEM*::antibiotic resistance gene* were treated with DNase followed by the heat inactivation of the enzyme. Then, the reactions containing the DNA corresponding to the dilutions of the standard, theoretically degraded by DNase activity, were amplified by the qPCR set of the respective antibiotic resistance gene. Results showed negative amplification, indicating that the DNase was able to remove the DNA added, even at higher concentrations.

Inactivation of the DNase by heat treatment

We wondered whether the DNase was inactivated by the heat treatment. Otherwise, remaining DNase would degrade the qPCR primers and probe, leading to negative results of the controls caused by the DNase activity instead of the absence of non-encapsidated DNA.

To test this, serial decimal dilutions of the construction stock *pGEM:antibiotic resistance gene* were added to the DNase controls (an aliquot of the sample taken after DNase treatment and before desencapsidation). After DNase heat inactivation, reactions were amplified by qPCR in parallel to the standards of the gene of study. If the DNase was well

inactivated, we would expect the same amplification results in the controls with DNase than in the original dilutions of the standard containing *pGEM::antibiotic resistance gene*. Results showed nearly the same Ct values in the DNase controls compared with the original standards (only one Ct below the Ct of the corresponding dilution of the standard). This confirmed that the DNase was well inactivated by heat treatment and did not interfere in the subsequent qPCR reaction. The slightly differences observed in the Ct values obtained could be attributable to small degradation of the DNA caused by the heat treatment.

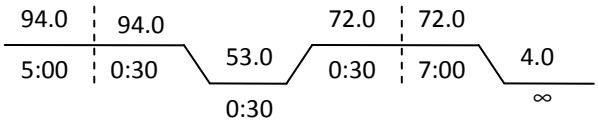
APPENDIX 5: Conventional PCR and qPCR conditions

1. Conventional PCR

Conventional PCR reactions were performed in 25 cycles:

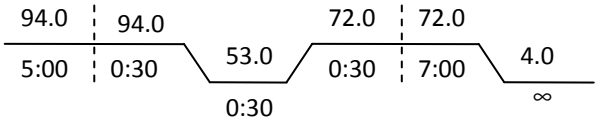
PCR *bla*$_{CTX-M}$:

ctxC1+ ctxC2



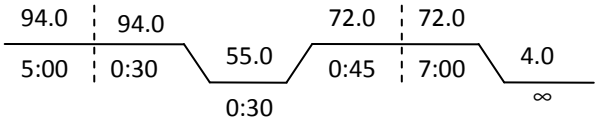
PCR *bla*$_{CTX-M-9}$:

ctx-m-9up + ctx-m-9lp



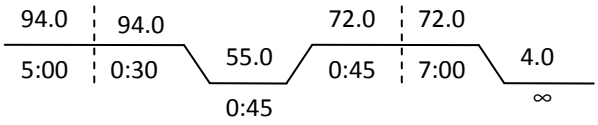
PCR *bla*$_{CTX-M-15}$:

ctx-m-15up + ctx-m-15lp



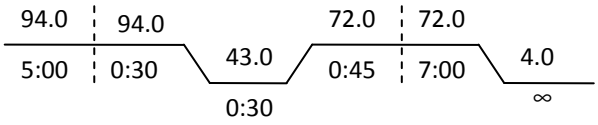
PCR *bla*$_{TEM}$:

temup + temlp



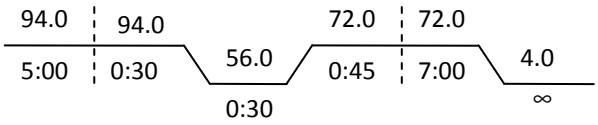
PCR *mecA*:

mecAup + mecAlp



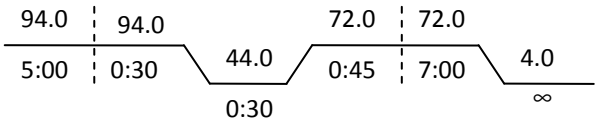
PCR *nuc*:

nuc1 + nuc2



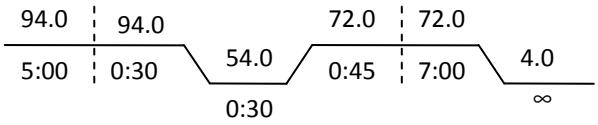
PCR pGEM:

pGEMT7



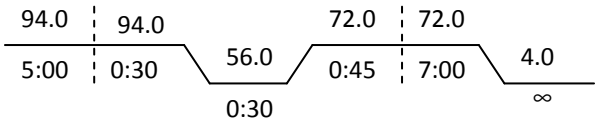
PCR *qnrA*:

qnrAup + qnrAlp



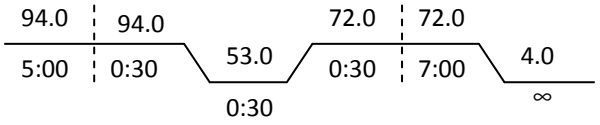
PCR *qnrS*:

qnrSup + qnrSlp



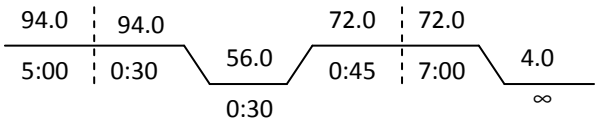
PCR *rfb.O25*:

rfb.1bis + rfb025b.r



PCR 16S rDNA (35 cycles):

27f + 1492r



2. Real-time qPCR

Real-time qPCR reactions were performed as followed:

50ºC 2 min (1 cycle), 95ºC 15 min (1 cycle), 94ºC for 15 s and 60ºC 1 min (40 cycles)

Gene	Primers	Sequence	Amplimer (bp)	Reference
***bla*$_{TEM}$ PCR**	UP	CTCACCCAGAAACGCTGGTG	569	This study
	LP	ATCCGCCTCCATCCAGTCTA		
***bla*$_{TEM}$ qPCR**	UP	CACTATTCTCAGAATGACTTGGT	85	Lachmayr *et al*., 2009
	LP	TGCATAATTCTCTTACTGTCATG		
	Probe	6FAM-CCAGTCACAGAAAAGCATCTTACGG-MGBNFQ		
***bla*$_{TEM}$ PCR inv**	UP	CACCAGCGTTTCTGGGTGAG		This study
	LP	TAGACTGGATGGAGGCGGAT		
***bla*$_{CTX-M}$ PCR**	UP	ATGTGCAGCACCAGTAAAGT	545	This study
	LP	ACCGCGATATCGTTGGTGG		
***bla*$_{CTX-M-1}$ PCR**	UP	ACGTTAAACACCGCCATTCC	356	This study
	LP	TCGGTGACGATTTTAGCCGC		
***bla*$_{CTX-M-1}$ qPCR**	UP	ACCAACGATATCGCGGTGAT	101	This study
	LP	ACATCGCGACGGCTTTCT		
	Probe	6FAM – TCGTGCGCCGCTG-MGBNFQ		
***bla*$_{CTX-M-15}$ PCR inv**	UPinv	GGAATGGCGGTGTTTAACGT		This study
	LPinv	GCGGCTAAAATCGTCACCGA		
***bla*$_{CTX-M-9}$ PCR**	UP	ACGCTGAATACCGCCATT	346	This study
	LP	CGATGATTCTCGCCGCTG		
***bla*$_{CTX-M-9}$ qPCR**	UP	ACCAATGATATTGCGGTGAT	85	This study
	LP	CTGCGTTCTGTTGCGGCT		
		6FAM – TCGTGCGCCGCTG-MGBNFQ		
***mec*A PCR**	UP	ATACTTAGTTCTTTAGCGAT	434	This study
	LP	GATAGCAGTTATATTTCTA		
***mec*A qPCR**	UP	CGCAACGTTCAATTTAATTTTGTTAA	92	Volkman *et al*., 2004
	LP	TGGTCTTTCTGCATTCCTGGA		
	Probe	6FAM-AATGACGCTATGATCCCAATCTAACTTCCACA-TAMRA		
***qnrA* PCR**	UP	ACGCCAGGATTTGAGTGAC	565	Lavilla *et al*., 2004
	LP	CCAGGCACAGATCTTGAC		
***qnrA* qPCR**	UP	AGGATTGCAGTTTCATTGAAAGC	138	This study
	LP	TGAACTCTATGCCAAAGCAGTTG		
	Probe	6FAM-TATGCCGATCTGCGCGA-MGBNFQ		

Gene (cont.)	Primers	Sequence	Amplimer (bp)	Reference
***qnrS* PCR**	UP	AAGTGATCTCACCTTCACCGCTTG	425	This study
	LP	TTAAGTCTGACTCTTTCAGTGATG		
***qnrS* qPCR**	UP	CGACGTGCTAACTTGCGTGA	118	This study
	LP	GGCATTGTTGGAAACTTGCA		
	Probe	6FAM –AGTTCATTGAACAGGGTGA-MGBNFQ		
***nuc* PCR**	UP	GCGATTGATGGTGATACGGTT		This study
	LP	AGCCAAGCCTTGACGAACTAAAGC		
***rfbO25b* PCR**	rfb.1bis	ATACCGACGACGCCGATCTG	300	Blanco *et al*., 2009
	rfbO25b.r	TGCTATTCATTATGCGCAGC		
16S rDNA	27f	AAGAGTTTGATCCTGGCTCAG	1503	Sander and Schmieger, 2001
	1492r	TACGGCTACCTTGTTACGACTT		
pGEM	UP	TGTAATACGACTCACTAT		Serra-Moreno *et al*., 2008

Table 6. Oligonucleotides used in this study.