ABSTRACT

The epidemiology of resistance plasmids is a major issue for the description of antimicrobial resistance diffusion. The identification of related plasmids associated to specific resistance genes may help to follow the spread of epidemic plasmids, opening new epidemiological scenarios on the mechanism of diffusion of antimicrobial resistance. This aspect is particularly interesting when applied to collections of plasmids that are playing a major role in the diffusions of Extended Spectrum Beta Lactamases (ESBLs) such as CTX-M, SHV, TEM.

The aim of this thesis is the molecular characterization of plasmids conferring drug resistances in different collections of *Enterobacteriaceae* of human and animal origin. Several example of epidemic plasmids will be discussed: the IncHI2 plasmids carrying $bla_{\text{CTX-M-9}}$ or $bla_{\text{CTX-M-2}}$ genes identified from human and animal isolates of *Escherichia coli* and *Salmonella* from Spain, Belgium and UK; the IncI1 family of plasmids characterized by specific virulence factors, carrying the $bla_{\text{CMY-2}}$, $bla_{\text{TEM-52}}$ and $bla_{\text{CTX-M-1}}$ genes from *Salmonella* and *E. coli* of human and animal origin; the IncN plasmids carrying the gene codifying the metallo-β-lacatamase VIM-1 from human isolates of *Klebsiella pneumoniae* and *E. coli* in Greece; the IncA/C_2 plasmids carrying specific resistance genes such as $bla_{\text{CMY-2}}$, $bla_{\text{CMY-4}}$, $bla_{\text{VIM-4}}$ and $bla_{\text{VEB-1}}$ from different *Enterobacteriaceae* isolated worldwide; different plasmid replicons (IncFII, IncA/C_1, IncI1) carrying the ESBL SHV-12 from human and animal origin.

The comparative analysis of plasmid backbones allowed to ascertain the diffusion of common, emerging plasmids and helped to determine the evolution of these plasmids by acquisition of relevant resistance genes by a panoply of mobile genetic elements and illegitimate recombination events.

ABSTRACT

L'epidemiologia dei plasmidi di resistenza è il principale mezzo per descrivere la diffusione delle resistenze agli antimicrobici. L'identificazione di plasmidi correlati associati a specifici geni di resistenza può aiutare a tracciare la disseminazione di plasmidi epidemici, aprendo nuovi scenari epidemiologici sui meccanismi di diffusione delle resistenze agli antimicrobici. Questo aspetto è particolarmente evidente quando si considerano plasmidi che sono associati alla diffusione delle Beta Lattamasi a Spettro Esteso (ESBL) come gli enzimi CTX-M, SHV, TEM.

Lo scopo di questa tesi è stato quello di caratterizzare plasmidi che conferiscono resistenza a farmaci rilevanti per la terapia umana in differenti collezioni di *Enterobacteriaceae* di origine umana e animale. Verranno discussi diversi esempi di plasmidi epidemici quali: i plasmidi IncHI2 che trasportano i geni $bla_{\text{CTX-M-9}}$ o $bla_{\text{CTX-M-2}}$ provenienti da isolati di origine umana e animale di *Escherichia coli* e *Salmonella* dalla Spagna, Belgio e Inghilterra; i plasmidi IncI1 caratterizzati da specifici fattori di virulenza, che trasportano i geni $bla_{\text{TEM-52}}$ e $bla_{\text{CTX-M-1}}$ derivanti da isolati di *Salmonella* e *E. coli* di origine umana e animale; i plasmidi IncN che trasportano i geni codificanti la metallo-β-lattamasi VIM-1 da isolati umani di *Klebsiella pneumoniae* and *E. coli* dalla Grecia; plasmidi IncA/C_2 associati a specifici geni di resistenza come $bla_{\text{CMY-2}}$, $bla_{\text{CMY-4}}$, $bla_{\text{VIM-4}}$ e $bla_{\text{VEB-1}}$ provenienti da diverse *Enterobacteriaceae* isolate in differenti parti del mondo, ed infine, i plasmidi associati all'enzima SHV-12 isolati in diverse *Enterobacteriaceae* di origine umana e animale.

L'analisi mediante comparazione della struttura plasmidica ha permesso di rilevare la diffusione di plasmidi emergenti e consente di tracciare i percorsi evolutivi e di diffusione attraverso l'acquisizione di una vastità di elementi genetici mobili associati ai geni di resistenza.

KEY WORDS:

plasmid, replicon, antibiotic-resistance, *Enterobacteriaceae*, incompatibility group, extended-spectrum-β-lactamase, quinolone.

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Dottorato di Ricerca in Microbiologia Medica e Immunologia

XX CICLO

PLASMID-MEDIATED ANTIMICROBIAL RESISTANCE IN GRAM-NEGATIVE BACTERIA

Tutor: Dr.ssa ALESSANDRA CARATTOLI

Dottoranda: Dr.ssa ALESSIA BERTINI

Coordinatore: Prof. CARLO FEDERICO PERNO

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1. INTRODUCTION

1.1. Gram-Negative Bacteria

A bacterium is a unicellular microorganism, typically a few micrometers in length. Individual bacteria have a wide-range of shapes, ranging from spheres to rods to spirals. Bacteria are ubiquitous in every habitat on Earth, growing in soil, acidic hot springs, radioactive waste, seawater, and deep in the Earth's crust[1]. These microorganisms are vital in recycling nutrients, and many important steps in nutrient cycles depend on bacteria, such as the fixation of nitrogen from the atmosphere. However, most of bacteria have not been characterized, and only about half of the phyla of bacteria have species that can be cultured in the laboratory[2].

Bacteria were first observed by Anton van Leeuwenhoek in 1676, using a single-lens microscope of his own design. He called them "animalcules" and published his observations in a series of letters to the Royal Society[3]. The name *bacterium* was introduced much later, by Christian Gottfried Ehrenberg in 1828, and is derived from the Greek word βακτήριον -α , *bacterion -a* , meaning "small staff". Identification of bacteria in the laboratory is particularly relevant in medicine, where the correct treatment is determined by the bacterial species causing an infection. Consequently, the need to identify human pathogens was a major impetus for the development of techniques to identify bacteria.

A technique to identify bacteria is the Gram stain, developed in 1884 by Hans Christian Gram; the Gram reaction is based on the structure of the bacterial cell wall.

In Gram-positive bacteria, the purple crystal violet stain is trapped by the layer of peptidoglycan which forms the outer layer of the cell. In Gram-negative bacteria, the outer membrane prevents the stain from reaching the peptidoglycan layer in the periplasm. The outer membrane is then per-

meabilized by acetone treatment, and the pink safranin counterstain is trapped by the peptidoglycan layer[4]. This outer membrane is responsible for protecting the bacteria from several antibiotics, dyes, and detergents which would normally damage the inner membrane or cell wall (peptidoglycan).

1.1.1. *Enterobacteriaceae*

Enterobacteriaceae are Gram-negative, oxidase-negative, rod-shaped bacteria. They are distributed worldwide: in water, in soil and as normal intestinal flora in humans and many animals. They live as saprophytically, as symbionts, epiphytes, and parasites. Their host range includes animals ranging from insects to humans, as well as fruits, vegetables, grains, flowering plants, and trees. One of the reasons that the *Enterobacteriaceae* have been so widely studied is due to their obvious impact on human and animal health and on agricultural practice.

Gram-negative bacteria of the *Enterobacteriaceae* family are important causes of urinary tract infections (UTIs), bloodstream infections, hospital-and healthcare-associated pneumonias, and various intra-abdominal infections. Within this family, *Escherichia coli* is a frequent cause of UTIs, *Klebsiella* spp and *Enterobacter* spp are important causes of pneumonia, and all of the *Enterobacteriaceae* have been implicated in bloodstream infections and in peritonitis, cholangitis, and other intra-abdominal infections. Additionally, organisms such as *Salmonella* produce gastroenteritis and subsequently, in some patients, invasive infection. Emerging resistance in *Enterobacteriaceae* is a significant problem that requires immediate attention. Resistance related to production of extended-spectrum β-lactamases (ESBLs) is a particular problem in the handling of *Enterobacteriaceae* infections, but other mechanisms of resistance are also emerging, leading to multidrug resistance and threatening to create panresistant species.

In recent years there has been a steady increase in frequency of ESBLs producting *Klebsiella pneumoniae* and *E. coli*[5] and fluoroquinolone resistance is emerging in Gram-negative pathogens worldwide[6]. Although antibiotic resistance is a natural expression of evolution and bacterial genetics, certain factors are thought to contribute immensely to enhance the expression and spreading of this bacterial inherent potentiality. Overuse of antibiotics and consequent antibiotic selective pressure is thought to be the most important factor contributing to the appearance of different kinds of resistant bacteria. The more often a drug is used, the more likely bacteria are to develop resistance to it[5].

1.2. Mechanisms of resistance

Throughout history, there has been a continual battle between humans and the multitude of microorganisms that cause infection and disease. Bubonic plague, tuberculosis, malaria, and more recently, the human immunodeficiency virus/acquired immunodeficiency syndrome pandemic, have affected substantial portions of the human population, causing significant morbidity and mortality. Beginning around the middle of the 20th century, major advances in antibacterial drug development and other means of infection control helped turn the tide in favour of humans. The situation dramatically improved when penicillin became available for use in the early 1940s. However, the euphoria over the potential conquest of infectious diseases was short lived[7]. Almost as soon as antibacterial drugs were deployed, bacteria responded by manifesting various forms of resistance. As antimicrobial usage increased, so did the level and complexity of the resistance mechanisms exhibited by bacterial pathogens. Mechanisms of antibiotic resistance and the over coming methods are thus an intense area of research.

Bacteria may manifest resistance to antibacterial drugs through a variety of mechanisms. Some species of bacteria are **innately resistant** to more or 1 class of antimicrobial agents. In such cases,

all strains of that bacterial species are likewise resistant to all the members of those antibacterial classes. Of greater concern are cases of **acquired resistance**, where initially susceptible populations of bacteria become resistant to an antibacterial agent and proliferate and spread under the selective pressure of use of that agent. Several mechanisms of antimicrobial resistance are readily spread to a variety of bacterial genera. First, the organism may acquire genes encoding enzymes, such as β-lactamases, that destroy the antibacterial agent before it can have an effect. Second, bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell before it can reach its target site and exert its effect. Third, bacteria may acquire several genes for a metabolic pathway which ultimately produces altered bacterial cell walls that no longer contain the binding site of the antimicrobial agent. Fourth, bacteria may acquire mutations that limit access of antimicrobial agents to the intracellular target site via down regulation of porin genes[8].

Thus, normally susceptible populations of bacteria may become resistant to antimicrobial agents through mutation and selection, or by acquiring from other bacteria the genetic information that encodes resistance. Spontaneous mutations may cause resistance by (a) altering the target protein to which the antibacterial agent binds by modifying or eliminating the binding site, (b) up regulating the production of enzymes that inactivate the antimicrobial agent, (c) down regulating or altering an outer membrane protein channel that the drug requires for cell entry, or (d) up regulating pumps that expel the drug from the cell. In all of these cases, strains of bacteria carrying resistance conferring mutations are selected by antimicrobial use, which kills the susceptible strains but allows the newly resistant strains to survive and grow. Acquired resistance that develops due to chromosomal mutation and selection is termed *vertical evolution*.

Bacteria also develop resistance through the acquisition of new genetic material from other resistant organisms. This is termed *horizontal evolution*, and may occur between strains of the same spe-

cies or between different bacterial species or genera. Mechanisms of genetic exchange include conjugation, transduction, and transformation[9].

Transformation (Fig. 1) involves the passage of DNA to a recipient through a specific medium. This process of transferring genetic material is mostly observed *in vitro* in the molecular biology laboratory and almost never occurs in nature.

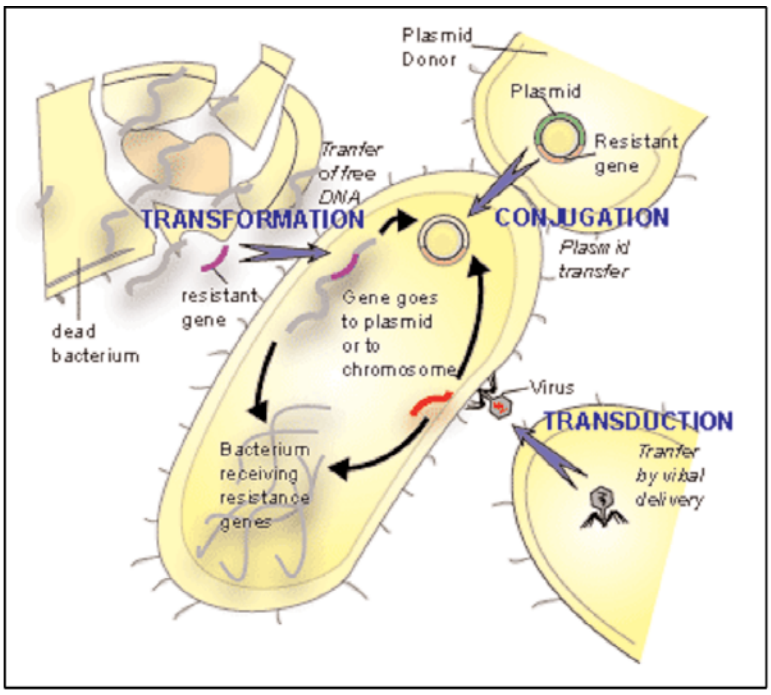

Fig 1: Mechanisms of genetic exchange: Horizontal Gene Transfer.

Transduction (Fig. 1) is a process whereby DNA is transferred from a donor to a recipient by way of a host, a phage. It is still unknown whether this process causes clinically observed resistance to antibiotics. Because this process is highly dependent on specific phages, it may occur only within certain bacterial species. Only a limitated amount of DNA that can be packed into the head of a phage can normally be transferred. Transduction therefore cannot be responsible for multiple drug resistance.

Conjugation (Fig. 1) is the process when DNA transfers from a donor to a recipient by simple, direct cell-to-cell contact. The process allows for the passage of more than one functional gene at a

time, so that multiple resistance could occur within a single step. Furthermore, many different organisms can act as recipients, allowing DNA (resistant genes) to be donated freely from different sources. Resistance can be passed from commensals in the gut to a pathogens existing in the same environment. Conjugation is thus an important and highly efficient process for transferring genes, and the acquisition of resistance by most pathogens is probably a result of this process. Resistance is believed to be mediated either by resistance plasmids (R plasmids) or transposable elements (transposons).

1.3. Plasmids

A plasmid is defined as a double stranded, circular or linear DNA molecule capable of autonomous replication. They are mostly a phenomenon of the prokaryotic world, although they exist in some groups of primitive eukaryotes as yeast, fungi and cellular slime moulds[10]. Plasmids are not organisms. They belong to a special biological category of extrachromosomal, accessory DNA elements[11, 12].

By definition, plasmids do not carry genes essential for growth of their host under no stressed conditions. Plasmids are a fashionable field of scientific research, since they are still studied as excellent models of DNA replication as well as in biotechnology.

Natural plasmids have systems guarantying their autonomous replication but also mechanisms controlling the copy-number and ensuring stable inheritance during cell division. Many plasmids can promote their horizontal transfer among bacteria of different genera and kingdoms, through the conjugation process. However, the presence of plasmids must have some biological cost for the bacterial host. It is therefore plausible that a selfish element that does not benefit its host can be eliminated from the bacterial population. The plasmid-encoded functions have been extensively investigated and there is ample evidence that natural plasmids have evolved as an integral part of the bacterial genome, providing additional functions to their host[13]. In many cases the selective advantage of a plasmid appears obvious, because it carries genes

that confer a selectable phenotypic character under specific niche conditions[14]. The best example of phenotypic advantage associated to a plasmid is given by the antimicrobial resistance. Resistance genes located on plasmids offer immediate advantage to their host under antimicrobial pressure. However, the real advantage of the plasmid location of resistance genes is not evident: plasmid-located resistance genes can be successfully transferred to the bacterial chromosome, therefore disfavoring the plasmid maintenance. An advantage of plasmid location for selectable genes has been hypothesized in the relative higher copy number of plasmids, influencing the expression levels by increasing the gene dosage. However, there are numerous other factors influencing the chromosome/plasmid harmony and evolution. Plasmids must be thought like autonomous selfish molecules, encoding extra genetic information, establishing complex relationships with the recipient host and playing multiple roles in the bacterial population.

Bacterial plasmid genome sequence comparisons provided the historical events through which plasmids are assembled. Their evolution seems to proceed by the assemblage of modular components by transposition, homologous recombination, and illegitimate recombinational events[15].

This is particularly evident when the phenomenon of antimicrobial resistance is analysed. Antimicrobial resistance arises from a complex multifactorial process supported by a panoply of mobile genetic elements that contain and transfer resistance determinants. Resistance genes located on plasmids move from one bacterium to another, conferring phenotypic characteristics. Several resistance plasmids have been described to carry virulence factors, such as bacteriocins, siderophores, cytotoxins, or adhesion factors[16] and virulence plasmids have been described to carry resistance genes[17-19]. For plasmids carrying virulence and resistance linked determinants, an infective population will be selected for antimicrobial resistance, and antimicrobial resistance pressure will select the virulence traits. However, once those determinants have been selected in the bacterial host, they can evolve further and eventually be transferred to other bacterial population. The acquisition of antimicrobial resistance genes on virulence plasmids, could represent a novel tool in bacterial evolution, implementing adaptive strategies to explore and colonise novel hosts and environments[16].

Classification and taxonomy of plasmids is needed mainly for practical medical purposes of surveying the spread of multiple-resistance plasmids. Plasmids are usually classified in incompatibility (Inc) groups, defined as the inability of two plasmids to be propagated stably in the same cell line[20]. Because incompatibility is a property universally inherited by plasmids and was discovered to be a manifestation of their biological relatedness, plasmid classification is based on this property. About 30 incompatibility groups are recognized among plasmids in enteric bacteria. Since incompatibility is the expression of a replicon maintenance function, there is a correlation between DNA homology of essential parts of replicons (*rep* genes) and incompatibility[21]. A method of identification has been based on a replicon typing using a bank of rep probes to hybridize with plasmid DNAs. This work has supported and refined the previous plasmid taxonomy and modernized the classical approach based on plasmid segregation[22]. But this methodology cannot be easily applied to a large number of strains and its application has been limited by the laborious and time-consuming work required. For these reasons in the 2005, we developed a new method for the identification of the major replicons of plasmids circulating among the *Enterobacteriaceae*[23].

In this method eighteen specific primer pairs were designed, on the basis of the multiple comparative analysis of nucleotide sequence on the EMBL Gene Databank, for HI1, HI2, I1-Iγ, X, L/M, N, FIA, FIB, W, Y, P, FIC, A/C, T, $FIIA_S$, F, K and B/O replicons, representative of the major plasmid incompatibility groups circulating among the[22]. The specificity of the PCR-based replicon typing (PBRT) was then tested on 61 reference enteric plasmids (58 positives and 3 negatives) originally isolated from 16 different bacterial species. On this reference plasmids the PBRT was more specific than the hybridization-based method. This was particularly evident when applied to FII, FIC, I1-Iγ, K and B/O replicons that exhibit considerable cross-reactions by hybridization-based *inc/rep* typing, thus limiting the use of this method in the classification of plasmids[22].

To ascertain the specificity and sensibility of the method, it was applied to a collection of 20 epidemiologically-unrelated multidrug-resistant *Salmonella enterica* strains of 10 different seroty-

pes isolated in Italy[24]. These isolates, previously analyzed for antimicrobial resistance genes, show a wide diffusion of the *strA-strB* and *tet(*A) resistance genes and 7 of 20 strains also carried integrons encoding resistance to trimethoprim, kanamycin, sulfonamides and streptomycin (Table 1).

Table 1: PBRT applaied to a collection of multidrug resistant *S. enterica* isolates

Isolate	Source	Serotype	Resistance [a]	*inc/rep*	*strA* [b]	*tetA* [b]	*int* [b]
17/24	Animal	Agona	SmSxTTe	HI2	+	+	+
17/20	bovin meat	Anatum	GmNaSmSxTTe	HI2	+	+	+
17/17	Turkey	Blockley	CfKmNaNmSmTe	N	+	+	-
17/34	meat food	Blockley	KmNaNmSmTe	N, A/C	+	+	-
17/41	swine meat	Blockley	KmNmSmTe	N	+	+	-
17/8	Turkey	Bredeney	ApAmcCfCmEnNaSmSxTTe	HI2, I1	+	+	+
17/3	dog stool	Bredeney	KmNmSmSuTe	neg	+	-	-
27/30	pork sausage	Give	ApSmSxTTe	I1	+	+	+
17/16	Duck	Hadar	ApAmcCfKmNmSmTe	X	+	+	-
17/9	Duck	Hadar	ApNmSmSxTTe	X	+	+	-
17/37	Chicken	Hadar	ApCfEnNaSmTe	N	+	+	-
17/35	Chicken	Hadar	ApAmcCfEnNaSmTe	neg	+	+	-
17/4	Turkey	Heidelberg	ApAmcNaSmTe	N	+	+	-
17/29	swine meet	Heidelberg	ApCfSmSxTTe	N	-	+	-
17/6	Swine	Heidelberg	CmGmKmNmSmSxTTe	A/C	-	+	+
17/40	chicken	London	ApAmcCfEnNaSuSmTe	A/C	+	+	-
17/31	Swine	London	SmSuTe	neg	+	+	-
17/27	Turkey	Saintpaul	ApAmcCfGmKmNaNmSmSuTe	HI2	+	-	+
17/21	Turkey	Saintpaul	ApAmcCfEnGmKmNaNmSmSxTTe	HI2	+	-	+
17/13	Chicken	Senftenberg	ApAmcCfClCtxSmSxT	neg	+	-	-

[a]: ampicillin (Ap), amoxicillin-clavulanic acid (Amc), cephalothin (Cf), colistin (Cl), chloramphenicol (Cm), ciprofloxacin (Cp), enrofloxacin (En), streptomycin (Sm), sulfonamides (Su), tetracycline (Te), trimethoprim-sulfamethoxazole (SxT), kanamycin (Km), gentamicin (Gm), nalidixic acid (Na), neomycin (Nm), cefotaxime (Ctx). Strains showing both Su and SxT resistance phenotypes are reported as SxT. [b]: *strA-strB, tet(A)* and the integrons (*int*) were previously described [24].

The PBRT detected the presence of N, I1, HI2, A/C and X replicons in several isolates. Integrons were associated with I1-, HI2- and A/C-positive strains, but not with the N-positive strains. These results suggest the presence of recurrent and common plasmids in epidemiological unrelated *Salmonella* isolates, indicating the successful spread of these genetic determinants in zoonotic pathogens.

1.4. β -LACTAM ANTIBIOTICS

Penicillin G (benzylpenicillin) was the first β-lactam antibiotic introduced into clinical practice. Building on unprecedented clinical success, β-lactam antibiotics now include: penicillinase-resistant, amino-, carboxy-, indanyl-, and ureido-penicillins; first- to fifth-generation cephalosporins; monobactams; and carbapenems (Fig. 2). The distinctive structural feature of a β-lactam is the highly reactive four-member ring[25, 26]. All β-lactam antibiotics are bactericidal agents that inhibit cell wall synthesis.

The glycan component of this rigid structure consists of alternating units of *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG), the former having short peptide stems attached to it. Peptides in adjacent glycan strands are cross-linked, producing the characteristic net structure of the peptidoglycan. Bacterial transpeptidases (detected as penicillin-binding proteins, PBPs) are essential enzymes that catalyze this crosslinking step. β-lactams are sterically similar to the penultimate D-Ala-D-Ala of the pentapeptide that is attached to NAM; hence PBPs mistakenly use penicillin as a substrate for cell wall synthesis and the transpeptidase (or carboxypeptidase) is acylated. The acylated PBP cannot hydrolyze the β-lactam and subsequent steps in cell wall synthesis are hindered while autolysis by cell wall degrading (autolytic) enzymes continues. Bacterial cells become permeable to water, rapidly take up fluid, and eventually lyse[27, 28].

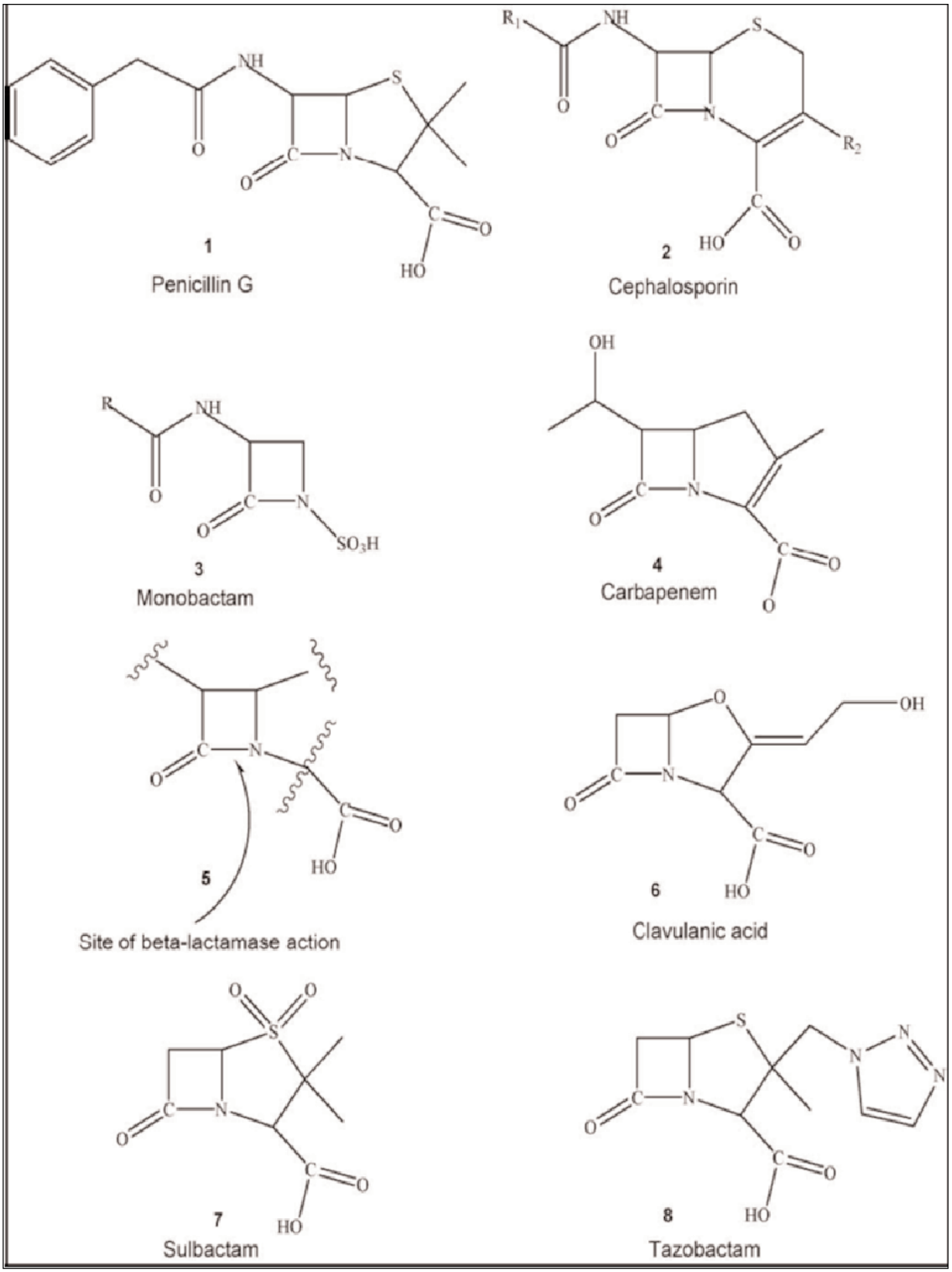

Fig. 2: Chemical structures of beta-lactams (1–4), site of action of beta-lactamases (5), and chemical structures of beta-lactamase inhibitors used in clinical practice (6–8).

1.4.1. β-LACTAM RESISTANCE

There are three major ways bacteria avoid the bactericidal effect of β-lactams:

(a) *Production of β-lactamases*. β-lactamases are bacterial enzymes that hydrolyze the β-lactam ring and render the antibiotic inactive before it reaches the PBP target. The underlying structural kinship that β-lactamases share with PBPs allows these enzymes to bind, acylate, and use a strategically located water molecule to hydrolyze and thereby inactivate the β-lactam[29].

(b) *Altered PBPs that exhibit low affinity for β-lactam antibiotics*. Examples are PBP 2x of *Streptococcus pneumoniae* and PBP 2' (PBP2a) of *Staphylococcus aureus*[30]. These PBPs are relatively resistant to inactivation by penicillins and are able to assume the functions of other PBPs when the latter are inactivated.

(c) *Lack or diminished expression of outer membrane proteins (OMPs) in gram-negative bacteria*. The loss of OMPs restricts the entry of certain β-lactams into the periplasmic space of gram-negative bacteria and hence access to PBPs on the inner membrane. Imipenem resistance in *Pseudomonas aeruginosa* and *K. pneumoniae* can arise from the loss of OMP D2 and of OmpK36, respectively[31-33].

The destruction of β-lactams by β-lactamases is the most important resistance mechanism in gram-negative bacteria.

1.4.2. β-LACTAMASE CLASSIFICATION AND PROPERTIES

β-lactamases are globular proteins that characteristically have an α-helices, β-pleated sheets, and share similar structural features. β-lactamases are categorized based on similarity in amino acid sequence (Ambler classes A through D) or on substrate and inhibitor profile (Bush-Jacoby-Medeiros Groups 1 through 4) (Table 2)[34-38].

Table 2: ß-lactamase classification system

Ambler classification system		
Class A	**penicillinases**	**TEMs, SHVs, PC1, CTX-Ms, SME-1, EPC,1**
Class B	**metallo-ß-lactamases**	**IMP-1, VIM-1, Cer A**
Class C	**cephalosporinases**	**AmpCs, CMY-2, ACT-1**
Class D	**oxacillinases**	**OXA-1**
Bush-Jacoby-Medeiros classification		
Group 1	**cephalosporinases**	**AmpCs, CMY-2, ACT-1, MIR-1**
Group 2	**hydrolyze estende-spectrum cephalosporinases; clavulanate resistant all clavulanic acid susceptible**	
2a	**penicillinase**	**PC1 from *S. aureus***
2b	**broad-spectrum penicillinase**	**TEM-1, SHV-1, TEM-2**
2be	**ESBLs**	**SHV-2, TEM-10, CTX-Ms**
2br	**inhibitor resistant**	**TEMs, IRTs, TEM-30, TEM-31**
2c	**carbenecillin hydrolyzing**	**PSE-1**
2d	**oxacillin hydrolyzing**	**OXA-10, OXA-1**
2e	**cephalosporinases inhibited by clavulanate**	**FEC-1**
2f	**carbapenemases**	**KPC-1, SME-1**
Group 3	**metallo-ß-lactamases**	**IMP-1, VIM-1, Cer A**
Group 4	**hydrolyze imipenem, inhibited by EDTA, resistant to clavulanate miscellaneous**	

Ambler Class A enzymes (Bush Group 2) are penicillinases that are susceptible to β-lactamase inhibitors. The TEM-1 and SHV-1 β-lactamases, Group 2b, are the β-lactamases usually found in *E. coli* and *K. pneumoniae* that confer resistance to penicillins (ampicillin and piperacillin)[35].

Ambler Class B enzymes (Bush Group 3) are MBLs that use one of two zinc (Zn^{2+}) atoms for inactivating penicillins and cephalosporins. In bacteria, MBLs confer resistance to carbapenems, cephalosporins and penicillins. Bacteria possessing MBLs are among the most resistant phenotypes encountered by clinicians. MBLs are inhibited by chelating agents (EDTA), but not by clavulanic acid or sulfones. The *bla* genes encoding MBLs are found on a variety of genetic elements (chromosome, plasmid, integrons, etc.)[39]. Clinically important MBLs that are widespre-

ad are the IMP-type and VIM-type of *P. aeruginosa*. Nearly 20% of imipenem-resistant *P. aeruginosa* strains possess a MBL.

Ambler class C enzymes (Bush Group 1) include the chromosomally encoded AmpC type β-lactamses found in *Citrobacter freundii*, *Enterobacter aerogenes* and *Enterobacter cloacae*, *Morganella morganii*, *P. aeruginosa* and *Serratia marcescens*. Bacteria possessing AmpC β-lactamases are resistant to penicillins, β-lactamase inhibitors, cefoxitin, cefotetan, ceftazidime, ceftriaxone, and cefotaxime. Aztreonam and cefepime are usually more active against bacteria possessing class C β-lactamases. Historically, it was thought that *ampC* genes encoding class C β-lactamases were located exclusively on the chromosome but, within the last 17 years, an increasing number of *ampC* genes have been found on plasmids[40]. These have mostly been acquired by AmpC-deficient pathogenic bacteria, which consequently are supplied with new and additional resistance phenotypes.

Ambler classD enzymes (Bush group 2f) are serine β-lactamases that are able to hydrolyze oxacillin (hence oxacillinases or OXA β-lactamases). The OXA enzymes of *Acinetobacter baumannii* and *P. aeruginosa* represent the most structurally diverse and rapidly growing group of β-lactamases[41, 42]. Depending on the OXA enzyme, these β-lactamases confer resistance to penicillins, cephalosporins, extended spectrum cephalosporins (OXA-type ESBLs) or carbapenems (OXA-type carbapenemases). OXA enzymes are relatively resistant to clavulanic acid inactivation, but are inhibited by sodium chloride[43, 44].

1.5. Quinolones

Quinolones and fluoroquinolones are among the most important antibacterial drugs, used extensively for treatment of bacterial infections both in human and veterinary medicine. It has been over four decades since the first of the quinolones, nalidixic acid, was introduced for cli-

nical use in 1962[45]. Fluoridation of the quinolone molecule at the C-6 position in the 1970s yielded norfloxacin, the first fluoroquinolone, which entered the clinic in 1986[46]. Ciprofloxacin, perhaps the most important as well as the most used fluoroquinolone, was introduced into the clinical market in 1987. Since then, structural revisions of the quinolone molecules (with fluoroquinolones or naphthyridones as the core structures) have provided numerous new agents suited to the treatment of a variety of bacterial infections. Older quinolones are mostly active against Gram-negative bacteria and newer ones have a broad spectrum of activity, with enhanced activity against Gram-positive pathogens[45-47,48]. However, the future utility of these drugs is threatened by the increasing rate of emergence of quinolone-resistant bacteria. Indeed, quinolone resistance is seen widely in a number of clinical pathogens and thus poses a major public health concern[49-52]. Ironically, this reality (i.e. currently high fluoroquinolone resistance) is largely contrary to what was predicated in the early stage of fluoroquinolone development in the 1980s[53]. Because quinolone drugs are xenobiotics and have not been found to occur naturally, it was expected that quinolone resistance would occur rarely.

Several mechanisms have been known to account for the emergence of quinolone resistance.

1.5.1. Mode of action

The quinolone agents are direct inhibitors of DNA synthesis by targeting two related but functionally distinct and essential type II topoisomerases, DNA gyrase (a protein complex of GyrA2B2 encoded chromosomally by genes *gyrA* and *gyrB*) and topoisomerase IV (ParC2E2 encoded by *parC* and *parE*), although the two targets have differential affinity for different quinolone drugs[54-57]. By binding to the enzyme–DNA complex, the quinolones stabilise DNA strand breaks created by the two topoisomerases. Ternary complexes of drug, enzyme and DNA block

progress of the replication fork. Cytotoxicity of fluoroquinolones likely occurs as a two-step process involving conversion of the topoisomerase–quinolone–DNA complex to an irreversible form and generation of a double-strand break by denaturation of the topoisomerase[55].

1.5.2. Resistance mechanisms

Resistance to quinolones can be either intrinsic or acquired.

Intrinsic resistance: Naturally occurring or wild-type bacterial species display innate susceptibility or resistance to antimicrobials. Levels of such intrinsic resistance to individual quinolones in bacterial species are determined by the susceptibility of the drug targets to the drugs[56-58] as well as the expression status of endogenous multidrug efflux pumps[59-62]. Studies with purified topoisomerases indicate that quinolones have different activities against their two targets. Whilst in Gram-negative bacteria the DNA gyrase is often the primary target for quinolones, topoisomerase IV plays a major role as the quinolone target in Gram-positive bacteria. Some newer fluoroquinolones apparently have stronger affinity for topoisomerase IV and thus display enhanced antibacterial activity against Gram-positive bacteria[56-63, 64]. Bacteria such as *E. coli*, *P. aeruginosa* and *S. aureus* produce basal expression levels of multidrug efflux pumps[54]. These energy-dependent efflux pumps are composed either of three components in Gram-negative bacteria or of a single component in Gram-positive bacteria, and they accommodate a variety of structurally unrelated antimicrobials that includes quinolones/fluoroquinolones, β-lactams, aminoglycosides, macrolides, amphenicols, tetracyclines, dyes, detergents and/or organic solvents[65]. The well-studied representatives of multidrug efflux pumps responsible for intrinsic resistance to quinolones include MexAB-OprM of *P. aeruginosa*[59, 66], AcrAB-TolC of *E. coli*[67] and NorA of *S. aureus*[68, 69]. Inactivation of these efflux pump systems confers on the bacteria enhanced susceptibility to quinolones and other antibacterials.

Acquired resistance: Acquired resistance to quinolones is mainly mediated by chromosomal mutations that either alter the targets of the quinolone drugs (i.e. DNA gyrase and topoisomerase IV)[54, 70, 71] or activate expression of the multidrug efflux pumps for which quinolones are substrates[65, 72, 73]. Decreased uptake as a result of porin reduction also contributes to quinolone resistance and this often interplays synergistically with other resistance mechanisms such as efflux pumps to increase resistance levels[65, 74]. The target alterations most frequently occur to the *gyrA* gene, particularly within a small, limited region of the gene called the "quinolone resistance-determining region" (QRDR)[75] . The most common mutations include Ser83Phe, Ser83Leu and Asp87Asn of the *E. coli* GyrA[76]. The drug resistance level varies with the mutations and the bacterial species. In species such as *P.* and *S. aureus*, single mutations are sufficient to cause clinically important levels of resistance. Multiple mutations are often required to generate clinically important resistance in *E. coli*, *Campylobacter jejuni*, *Salmonella* spp. and *Neisseria gonorrhoeae*[77]. The significant contribution of drug efflux pumps to resistance to quinolones and other antimicrobials was recognised (particularly in *P. aeruginosa*) a decade ago[67, 78]. Newer fluoroquinolones and other antimicrobials are also subject to efflux[79, 80]. The majority of clinically important multidrug efflux pumps in Gram-negative bacteria fall into the resistance-nodulation-division (RND) superfamily, and the expression of these efflux pumps is well regulated in wild-type cells under the control of the regulators, either repressors (in most cases) or activators. Inducible mutations in the regulatory genes increase the expression of the drug efflux pumps and hence contribute significantly to acquired quinolone resistance such as seen in the *nalB*, *nfxB*, *nfxC* and *nalC* mutations of *P. aeruginosa*. Many of these overexpressed efflux pumps also export other structurally unrelated classes of antimicrobials and hence confer multidrug resistance phenotypes on bacteria. Furthermore, plasmids are now recognised as an important contributor to quinolone resistance.

1.5.3. Plasmid-mediated quinolone resistance

Recently, three plasmid-mediated quinolone resistance (PMQR) mechanisms have been described.

The first comprises *qnr* genes that encode target protection proteins of the pentapeptide repeat family[81, 82]. Three *qnr* genes, *qnrA*, *qnrB*, and *qnrS*, and their allelic variants have been described so far. Although the protein products of *qnrA1* through *qnrA5*, *qnrB1*, *qnrB2*, *qnrS1*, and *qnrS2* all provide low-level quinolone resistance, considerable variability can be observed in their amino acid sequences[83-86].

The second mechanism involves the *aac(6')-Ib-cr* gene, which encodes a new variant of the common aminoglycoside acetyltransferase. Two single-nucleotide substitutions at codons 102 and 179 in the wild-type allele *aac(6')-Ib* enable the gene product to be capable of acetylating and thus reducing the activity of some fluoroquinolones, including norfloxacin and ciprofloxacin[87].

The third mechanism, involving the fluoroquinolone efflux pump protein QepA, has been described very recently[88]. The *qepA* gene encoding a putative protein of 511 amino acids, was identified on plasmid pHPA of *E. coli* C316. It resides on a putative transposable element, flanked by two copies of IS*26*.

2. OBJECTIVE

The aim of this thesis is the molecular characterization of plasmids conferring drug resistances in different collections of *Enterobacteriaceae* of human and animal origin.

The epidemiology of resistance plasmids is a major issue for the description of antimicrobial resistance diffusion. The identification of related plasmids associated to specific resistance genes may help to follow the spread of epidemic plasmids, opening new epidemiological scenarios on the mechanism of diffusion of antimicrobial resistance. This aspect is particularly interesting when applied to collections of plasmids that are playing a major role in the diffusions of Extended Spectrum Beta Lactamases (ESBLs) such as CTX-M, SHV, TEM. Several example of epidemic plasmids will be discussed:

1) plasmids encoding resistance to newer β-lactams such as the IncN plasmids carrying the gene codifying the metallo-β-lacatamase VIM-1 from human isolates of *K. pneumoniae* and *E. coli* in Greece; the IncA/C_2 plasmids carrying specific resistance genes such as bla_{CMY-2}, bla_{CMY-4}, bla_{VIM-4} and bla_{VEB-1} from different *Enterobacteriaceae* isolated worldwide;

2) the IncI1 family of plasmids characterized by specific virulence factors, carrying the bla_{CMY-2}, bla_{TEM-52} and $bla_{CTX-M-1}$ genes from *Salmonella* and *E. coli* of human and animal origin;

3) the IncHI2 plasmids carrying $bla_{CTX-M-9}$ or $bla_{CTX-M-2}$ genes identified from human and animal isolates of *E. coli* and *Salmonella* from Spain, Belgium and UK.

The comparative analysis of plasmid backbones allows to ascertain the diffusion of common, emerging plasmids and helps to determine the evolution of these plasmids by acquisition of relevant resistance genes by a panoply of mobile genetic elements and illegitimate recombination events.

3. MATERIALS AND METHODS

3.1. Total genomic DNA purification

Total DNAs from strains containing studied plasmids were extracted by Wizard Genomc DNA Purification Kit (Promega), by the following procedure: one colony from each strain was inoculated in 3 ml of LB (Luria-Bertani broth) containing a suitable concentration of antibiotic for selection, for 18 hours at 37 °C. 1 ml of overnight cultures were transferred in a 1.5 ml microcentrifuge tube and centrifuged at 13000 rpm for 2 minutes to pellet the cells. To lyse the cells, the pellets were resuspended in 600 µl of Nuclei Lysis Solution, incubated at 80 °C for 5 minutes and cooled to room temperature. 3 µl of RNase Solution (4 mg/ml) were added to cell lysates and incubated at 37 °C for 15-60 minutes. 200 µl of Protein Precipitation Solution were added to the RNase-treated cell lysates and vortexed vigorously for 20 seconds. The samples were incubated on ice for 5 minutes and then centrifuged at 13000 rpm for 3 minutes. The supernatants containing the DNA were transferred in a clean 1.5 microcentrifuge tube containing 600 µl of room temperature isopropanol. The samples were gently mixed by inversion until the thread-like strands of DNA formed a visible mass. Then, they were centrifuged at 13000 rpm for 2 minutes. The supernatants were carefully removed and 600 µl of room temperature 70% ethanol were added. The samples were centrifuged at 13000 rpm for 2 minutes, the supernatants were carefully removed and the DNAs were dried at 37 °C for 10 minutes. The extracted DNAs were rehydrated by 100 µl of distilled water. These solutions were used for PCR.

3.2. Plasmid DNA purification

The studied plasmids were extracted by Qiagen Plasmid Midi Kit. In brief, one colony from each strain was inoculated in 25 ml of LB (Luria-Bertani broth) containing a suitable concentra-

tion of antibiotic for selection, for 18 hours at 37 °C. The overnight cultures were centrifuged at 6000 g for 15 minutes at 4 °C to pellet the cells. The pellets were resuspended in 4 ml of Buffer P1 (50 mM TRIS-HCl, 10 mM EDTA, pH 8.0 containing 100 µg/ml RNasiA). 4 ml of Buffer P2 [200 mM NaOH, 1% SDS (w/v)] were added to the samples, mixed thoroughly by vigorously inverting 4-6 times and incubated at room temperature for 5 minutes. Then 4 ml of chilled Buffer P3 (3.0 M potassium acetate, pH 5.5) were added to the cell lysates, mixed thoroughly by vigorously inverting 4-6 times and incubated on ice for 15 minutes. The samples were centrifuged for 30 minutes at 4 °C. The supernatants containing the plasmids were applied to anion-exchange based columns previously equilibrated by applying 4 ml of Buffer QBT [750 mM NaCl, 50mM MOPS pH 7.0, 15% isopropanol (v/v), 0.15% Triton X-100 (v/v)]. The columns were washed twice with 10 ml of Buffer QC [1.0 M NaCl, 50mM MOPS pH 7.0, 15% isopropanol (v/v)]. The plasmidic DNAs were eluted with 5 ml of Buffer QF [1.25 M NaCl, 50mM MOPS pH 8.5, 15% isopropanol (v/v)]. The plasmidic DNAs were precipitated by adding 3.5 ml of room temperature isopropanol and centrifuged at 15000 g for 30 minutes at 4 °C. The pellets were washed with 2 ml of room temperature 70% ethanol and centrifuged at 15000 g for 10 minutes. The pellets were air-dried and resuspended in 60 ìl of distilled water.

3.3. Polymerase Chain Reaction Assays

The amplification of gene targets of the PBRT was done preparing a 25 µl of mixture containing: 400 ng of genomic DNA, 200 µM of dNTP (Roche Diagnostic), 1 µM of each couple of primers, 1X PCR Buffer (Roche Diagnostic), 1.25U of Taq DNA-polymerase (Roche Diagnostic). The used primers, the target genes, the sizes of the amplicons and the running conditions are previously described[89].

The amplification of antibiotic resistance genes carried by plasmids were done using plasmidic DNA. The used primers, the target genes, the sizes of the amplicons and the running conditions are previously described[90-94].

3.4. DNA SEQUENCING

The amplified PCR products of interest and the clones in this study were sequenced by an automatic sequencing Pharmacia Biothec (Bio-Fab Research, Pomezia, Italy).

The sequencing primers were synthesized by Primm s.r.l. (Milan, Italy).

The electhropherograms were analyzed by the "Chromas" program (www.tecnelysium.com).

The nucleotide sequence analysis were obtained by NCBI "BLAST" program (www.ncbi.nlm.nih.gov).

3.5. PLASMID ANALYSIS BY RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

To analyze plasmid polymorphisms, restriction were performed on aliquots of 10 µg of plasmidic DNA incubated for 1 hour at 37 °C in a mixture containing 20U of a suitable restriction enzyme and appropriate buffer at 1X concentration, according to manufacturer's instruction (Roche Diagnostics), in a 30 µl of final volume. The restriction enzyme used in this study was PstI. The restriction fragments were separated by agarose gel electrophoresis in TBE buffer 1X, containing ethidium bromide 0.5 µg/ml; the run condition was at constant voltage (15V/cm) for 3 hour. After the running the gel was visualized under UV light by the digital camera Kodak (version 3.5) and transferred on a nylon membrane positively charged (Roche Diagnostics) to further analysis.

3.6. Southern blot hybridization experiments

To transfer the digested restriction fragments on a nylon membrane, the agarose gel was treated for 15 minutes with HCl 0.25M, denaturized in NaOH 0.5M+NaCl 1.5M for 30 minutes, neutralized in Tris-HCl 0.5M pH 7.6 for 40 minutes and finally transferred on a nylon membrane positively charged in 20X SSC, for 1 night, according the method described by Southern[95]. The DNA was covalently fixed by UV light for 4 minutes. The nylon membranes were used for hybridization experiments by digoxigenin labelled probes with antibiotic resistance genes (bla_{CMY}, bla_{VIM-1}, bla_{SHV-12}, bla_{TEM-52}, QnrA1) and *rep* genes (A/C_1, A/C_2, I1, N, F).

The membranes were pre-hybridized at 68 °C for 2 hours in a solution containing SSC 5X, N-laurosylsarcosine 0.1% (p/v), blocking solution 1%, SDS 0.02% (p/v) and salmon sperm DNA. Then, the probe (previously boiled for 10 minutes at 100 °C and cooled in ice) was added. Its final concentration was 5-25 ng/ml. The hybridization was at 68 °C for 1 night. Successively, the membrane was washed twice at 68 °C for 5 minutes with SSC 2X + SDS 0.1% and twice at 68 °C for 15 minutes with SSC 0.1X + SDS 0.1%. The DNA fragments recognised by the probe were detected by chemiluminescence, incubating the membrane for 30 minutes with the anti-digoxigenin-alkaline phosphatase diluted 1:10000 in a maleic acid buffer containing blocking solution able to block the not specific sites and detecting the alkaline phosphatase by a specific substrate, the CSPD. The signal was detected by a X-ray film (X-OMAT-AR, Kodak).

4. RESULTS

4.1. Replicon typing of plasmids encoding resistance to newer β-Lactams

The PCR based replicon typing (PBRT) was applied to a collection of resistance plasmids carried by 26 *E. coli* transconjugants or transformants obtained from epidemiologically unrelated clinical isolates of *Enterobacteriaceae* associated with community- or hospital-acquired infections in the United States, Italy and Greece. The resistance plasmids carried genes encoding β-lactamases of Ambler class A (SHV-12), B (VIM-1 or VIM-4), and C (CMY-2, CMY-4, or CMY-13) (Table 3), which represent key emerging resistance determinants to extended-spectrum cephalosporin and carbapenems[90].

The plasmid donors from the United States consisted of 4 previously characterized extended-spectrum cephalosporin (ESC) resistant *Salmonella* isolates submitted to the National Antimicrobial Resistance Monitoring System (NARMS) from 1996 to 1998[96] and 6 ESC-resistant *E. coli* O157:H7 isolates collected by NARMS from 2000 to 2001[97]. This collection includes representatives from sporadic and outbreak infections[96, 98]. The 4 *Salmonella* and 6 *E. coli* plasmid donors selected for this study were a small sample of epidemiologically unrelated isolates representative of those carrying a bla_{CMY-2} β-lactamase gene on plasmids classified as type A or B on the basis of the bla_{CMY-2} hybridization pattern[99-100]. Since a single PstI restriction site is present within *cmy-2*[101], the *cmy-2* probe hybridized to bands of approximately 12 kb and 800 bp (type A), and 2.5 kb and 800 bp (type B). The PBRT method assigned the A/C and I1 replicons to type A and type B plasmids, respectively (Table 3), which was confirmed by DNA sequencing. The I1-type amplicon sequences were identical to the R64 IncI1 reference plasmid (no. AP005147), whereas the A/C-type amplicon sequences exhibited 26 nucleotide (nt) substitutions with respect to the RA1 IncA/C reference plasmid (no. X73674), which caused 3 amino acid variations. Therefore, the A/C-replicon from the US plasmids may represent a new replicon variant, which we designated repA/C_2 (DNA sequence released under

EMBL accession no. AM087198). The Figure 3 shows conserved *Pst*I restriction profiles obtained for the A/C_2 plasmids that are different from those exhibited by the I1 plasmids.

Table 3: Phenotypic and genetic characteristics of plasmids and transformant/transconjugant strains analyzed in this study

Original strain	Species and serovar	Transferred resistance traits in transconjugants or transformants*	*bla* genes identified on transferred plasmids	Replicons detected by PCR†
USA-4204	*Salmonella enterica* serovar Typhimurium	AmpCazCroCtxFox	CMY-2-type A	A/C_2
USA-2039	*S.* Typhimurium	AmpCazCroCtxFoxGmTo	CMY-2-type A	A/C_2
USA-3977	*S.* Typhimurium	AmpCazCroCtxFox	CMY-2-type A	A/C_2
USA-8401	*Escherichia coli* O157:H7	AmpCazCroCtxFox	CMY-2-typeA	A/C_2
USA-8749	*E. coli* O157:H7	AmpCazCroCtxFox	CMY-2-type A	A/C_2
USA-8868	*E. coli* O157:H7	AmpCazCroCtxFox	CMY-2-type A	A/C_2
USA-1091	*E. coli* O157:H7	AmpCazCroCtxFox	CMY-2-type A	A/C_2
USA-7546	*E. coli* O157:H7	AmpCazCroCtxFox	CMY-2-type A	A/C_2
USA-11371	*E. coli* O157:H7	AmpAtmCazCroCtxFox	CMY-2-type B	I1
USA-1358	*S.* Thompson	AmpAtmCazCroCtxFox	CMY-2-type B	I1
IT-VA416/02	*Klebsiella pneumoniae*	AmpAtmCazCroCtxFoxIpmGmTo	VIM-4, CMY-4	A/C_2
IT-VA417/02	*Enterobacter cloacae*	AmpAtmCazCroCtxFoxIpmGmTo	VIM-4, CMY-4	A/C_2
IT-FI045T	*Enterobacter aerogenes*	AmpAtmCaz	SHV-12	FII
IT-FI008T	*Escherichia coli*	AmpAtmCazTo	SHV-12	FII
IT-BG003T	*Serratia marcescens*	AmpAtmCazTo	SHV-12	FII
IT-NO003T	*K. oxytoca*	AmpAtmCaz	SHV-12	A/C_1
IT-BG017T	*K. pneumoniae*	AmpAtmCazCroCtx	SHV-12	I1
GR-541	*Escherichia coli*	AmpAtmCazCtxCroFoxIpmTo	VIM-1, CMY13	N
GR-116	*E. coli*	AmpAtmCazCroCtxFoxIpmGmTo	VIM-1, CMY13	N
GR-700	*K. pneumoniae*	AmpCazCroCtxFoxIpmGmTo	VIM-1	N
GR-2564	*K. pneumoniae*	AmpCazCroCtxFoxIpmTo	VIM-1	N
GR-1943	*K. pneumoniae*	AmpCazCroCtxFoxIpmTo	VIM-1	N
GR-1955	*K. pneumoniae*	AmpCazCroCtxFoxIpmTo	VIM-1	N
GR-5866	*K. pneumoniae*	AmpCazCroCtxFoxIpmTo	VIM-1	N
GR-51395	*K. pneumoniae*	AmpCazCroCtxFoxIpmTo	VIM-1	N
GR-6/100	*K. pneumoniae*	AmpCazCroCtxFoxIpmTo	VIM-1	N

*Amp, ampicillin; Atm, aztreonam; Caz, ceftazidime; Cro, ceftriaxone; Ctx, cefotaxime; Fox, cefoxtin; Imp, imipenem; Gm, gentamicin; To, tobramycin.

†Discrimination between A/C1 and A/C2 replicons was performed by DNA sequencing of the amplicon obtained by the inc/rep polymerase chain reaction (PCR).

The plasmid donors from Italy consisted of 7 multidrug-resistant isolates of various species of *Enterobacteriaceae* carrying either bla_{SHV-12} or bla_{CMY-4} and bla_{VIM-4} plasmid borne β-lactamase genes (Table 3).These isolates had been collected from 2002 to 2003 at 4 different hospitals in northern or central Italy[98, 102] and were epidemiologically unrelated, except for IT-VA416/02 and IT-VA417/02, which were from the same patient[98]. PCR replicon typing of the 5 bla_{SHV-12}–carrying plasmids detected 3 repFII (100% identical to the reference sequence no. M33752), 1 repI1 (100% identical to the R64 plasmid), and 1 repA/C_1 (99% homologous to the RA1 plasmid) (Table 3), suggesting mobilization of this gene among different plasmid scaffolds. The bla_{SHV-12} plasmids showed different PstI restriction patterns, which confirmed their diversity (Fig. 3). The two plasmids carrying bla_{VIM-4} and bla_{CMY-4} were assigned by PCR replicon typing to the A/C type. The sequence of these replicons showed the same 26 characteristic nucleotide substitutions of the A/C_2- replicon identified in the US plasmids. These 2 A/C_2-plasmids showed an apparently identical PstI restriction profile (data not shown), which was also very similar to that of some USA bla_{CMY-2} plasmids (see the 2039 and 3977 US plasmids and the Italian VA416/02 plasmid in the Fig. 3). The two Italian A/C_2 plasmids, in addition to bla_{CMY-4} (which is a bla_{CMY-2} variant different by only a single nucleotide substitution), also carried the bla_{VIM-4} carbapenemase gene, which has not been reported on bla_{CMY-2}–carrying plasmids from the United States and may represent a novel acquisition. These findings indicate intercontinental spread of these plasmids and novel acquisition of resistance genes.

The plasmid donors from Greece consisted of a collection of seven *K. pneumoniae* isolates carrying the bla_{VIM-1} gene[103] and 2 *E. coli* isolates carrying bla_{VIM-1} and bla_{CMY-13} genes[104]. These isolates, randomly collected from 5 different hospitals in Athens and Piraeus from 2001 to 2003, are representative of the VIM-1–producing isolates circulating in Greece. No repetitive samples were taken from patients. All isolates exhibited decreased susceptibility to carbapenems. Restriction analysis of these plasmids classified them into 6 different groups on the basis of their restriction profi-

les (Fig. 3). By replicon typing, all of these plasmids were assigned to the same repN type replicon, which exhibited two nucleotide point mutations (99% with respect to the EMBL accession no. NC_003292), an indication that they were phylogenetically related and probably evolved from a common ancestor. Although one might expect similar plasmid scaffolds to exist among isolates in Greece and Italy because of geographic proximity, this was not the case. This finding explains the great variability of resistance plasmids carrying different combinations of resistance genes.

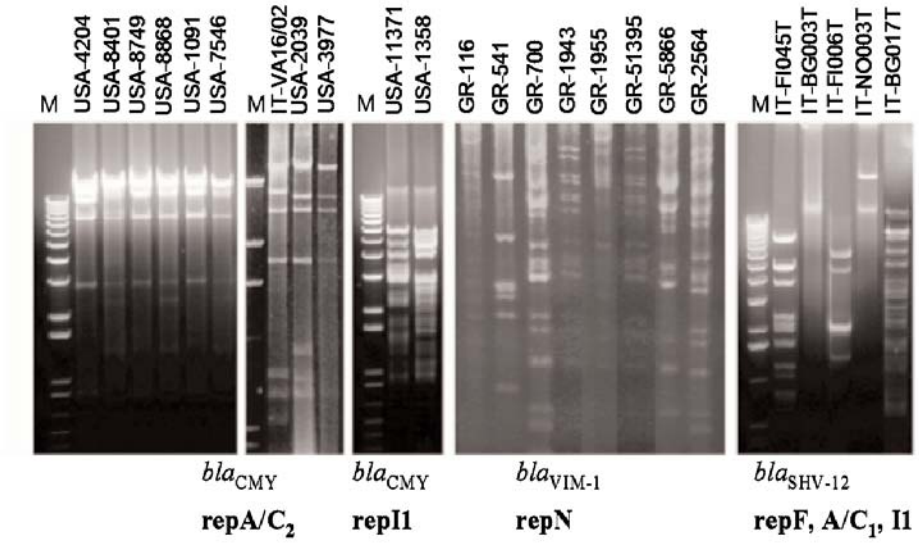

Fig 3: Restiction enzyme analysis of plasmid analyzed in this study. Numbers and letters above each indicate strain reference names as defined in Table 3. M is the 1-Kb Plus DNA ladder (Gibco-BRL, Gaithersburg, MD, USA).

Since the origin of replication is a constant and conserved part of a plasmid, replicon typing focused on this portion of the plasmid is a more sensitive and specific method for identifying phylogenetically related plasmids than restriction-based analysis of the entire plasmid. This fact is probably due to the presence of multiple mobile elements (IS elements, transposons, integrons) that can mediate rearrangements of the plasmid scaffolds, which leads to the formation of apparently divergent plasmids.

4.2. Dissemination of an extended-spectrum-β-lactamase *bla*$_{TEM-52}$ gene-carrying IncI1 plasmid in various *Salmonella enterica* serovars isolated from poultry and humans

In this study several strains of *Salmonella enterica* (serovars Agona, Derby, Infantis, Paratyphi B dT$^+$, and Typhimurium) were analysed for the presence of β-lactamase genes and plasmids. These strains were isolated from poultry and humans in Belgium and France between 2001 and 2005, and showed resistance to extended-spectrum cephalosporines by production of an ESBL not belonging to the CTX-M family. These strains also showed additional resistances to other antibiotic families[91].

Strains studied are shown in . These strains were previously characterized for antibiotic susceptibility testing by the disk diffusion method, and for the Minimal Inibitory Concentration (MIC) of ceftriaxone and ceftiofur. According to the MICs, the levels of resistance to ceftiofur and ceftriaxone were lower in the transconjugant strains than in the parental strains, as expected by the intrinsic lower level of antimicrobial susceptibility of the *E. coli* C1a (*nalA*) recipient strain used in these experiments. PCR assays to detect ESBL genes (TEM, SHV, and CTX-M) were performed on parental and transconjugant strains using previously described primers [105-107], and nucleotide sequencing of the amplicons identified the *bla*$_{TEM-52}$ resistance gene in all strains. Plasmids extracted from the transconjugants were further characterized by PstI restriction analysis showing that they were all identical and greater than 100 kb in size (Fig. 4). Southern blot hybridization experiment with a *bla*$_{TEM-52}$ gene probe was performed. It revealed two PstI fragments of 2.9 and 2.75 kb. In fact, this PstI restriction profile corresponded exactly to that of *bla*$_{TEM-52}$-carrying plasmids isolated in 2002 and 2003 from four isolates of *S. enterica* serovars Typhimurium, Enteritidis, and Panama from French patients with gastroenteritis[107]. A study performed in 2001 and 2002 on *Salmonella* isolated from poultry, poultry products, and human patients in The Netherlands revealed that the TEM-52 variant was the most common ESBL detected in this bacterial collection[108].In particular, TEM-52-producing salmonellae of the serovars Blockley, Virchow, Typhimurium, and Paratyphi B were iden-

Table 4: Characteristics of the *Salmonella* strains and their transconjugants producing TEM-52 used in this study

Strain[a]	Serovar	Geographic origin	Animal or human origin	Yr of isolation	Antibiotic resistance profile[b]	MIC (μg/ml)[c]		SGI1 variant[d]
						Xnl	Cro	
777SA01	Agona	Belgium	Poultry	2001	Ap(Caz)CfCm(Cro)FfSmSpSuTcTm(Xnl)	16	16	SGI1-A
777SA01TC1					Ap(Caz)Cf(Cro)(Xnl)	4	2	
260SA04	Agona	Belgium	Poultry	2004	ApCazCfCmCroFfSmSuTcTmXnl	64	256	—
260SA04TC1					Ap(Caz)Cf(Cro)(Xnl)	2	1	
833SA04	Agona	Belgium	Poultry	2004	ApCazCfCroXnl	32	64	—
833SA04TC1					Ap(Caz)Cf(Cro)(Xnl)	2	1	
977SA01	Derby	Belgium	Poultry	2001	ApCazCfCroXnl	32	16	—
977SA01TC1					Ap(Caz)Cf(Cro)(Xnl)	4	4	
988SA01	Infantis	Belgium	Poultry	2001	ApCazCfCroXnl	32	32	—
988SA01TC1					Ap(Caz)Cf(Cro)(Xnl)	2	1	
2004/10101	Infantis	Belgium	Poultry	2004	ApCazCfCroXnl	32	128	—
2004/10101TC1					Ap(Caz)Cf(Cro)(Xnl)	2	4	
2004/10256	Infantis	Belgium	Poultry	2004	ApCazCfCroXnl	32	64	—
2004/10256TC1					Ap(Caz)Cf(Cro)(Xnl)	2	2	
05-00001	Infantis	Belgium	Human	2005	ApCazCfCroXnl	32	64	—
05-00001TC1					Ap(Caz)Cf(Cro)(Xnl)	4	4	
05-00590	Infantis	Belgium	Human	2005	ApCazCfCroXnl	32	64	—
05-00590TC1					Ap(Caz)Cf(Cro)(Xnl)	2	4	
05-00838	Infantis	Belgium	Human	2005	ApCazCfCroXnl	16	64	—
05-00838TC1					Ap(Caz)Cf(Cro)(Xnl)	4	4	
1043SA04	Paratyphi B	Belgium	Poultry	2004	ApCazCfCroSmSpSuTmXnl	32	64	—
1043SA04TC1					Ap(Caz)Cf(Cro)(Xnl)	4	4	
153SA02	Typhimurium	Belgium	Poultry	2002	ApCazCfCroSmSpSuTmXnl	32	32	—
153SA02TC1					Ap(Caz)Cf(Cro)(Xnl)	2	1	
04-3486	Typhimurium	France	Human	2004	ApCazCfCm(Cro)FfSmSpSuTc(Xnl)	16	32	SGI1
04-3486TC1					Ap(Caz)Cf(Cro)(Xnl)	2	2	

[a]Strains labeled with TC1 are *E. coli* transconjugant strains.

[b] Antibiotics: Ap, ampicillin; Caz, ceftazidime; Cf, cefalothin; Cm, chloramphenicol; Cro, ceftriaxone; Ff, florfenicol; Sm, streptomycin; Sp, spectinomycin; Su, sulfonamide; Tc, tetracycline; Tm, trimethoprim; Xnl, ceftiofur. Parentheses indicate intermediate resistance according to the breakpoints of the CA-SFM (Comité de l'Antibiogramme de la Société Française de Microbiologie) for *Enterobacteriaceae* (i.e., susceptible, >20 mm; resistant, <15 mm).

[c] Cro, ceftriaxone; Xnl, ceftiofur.

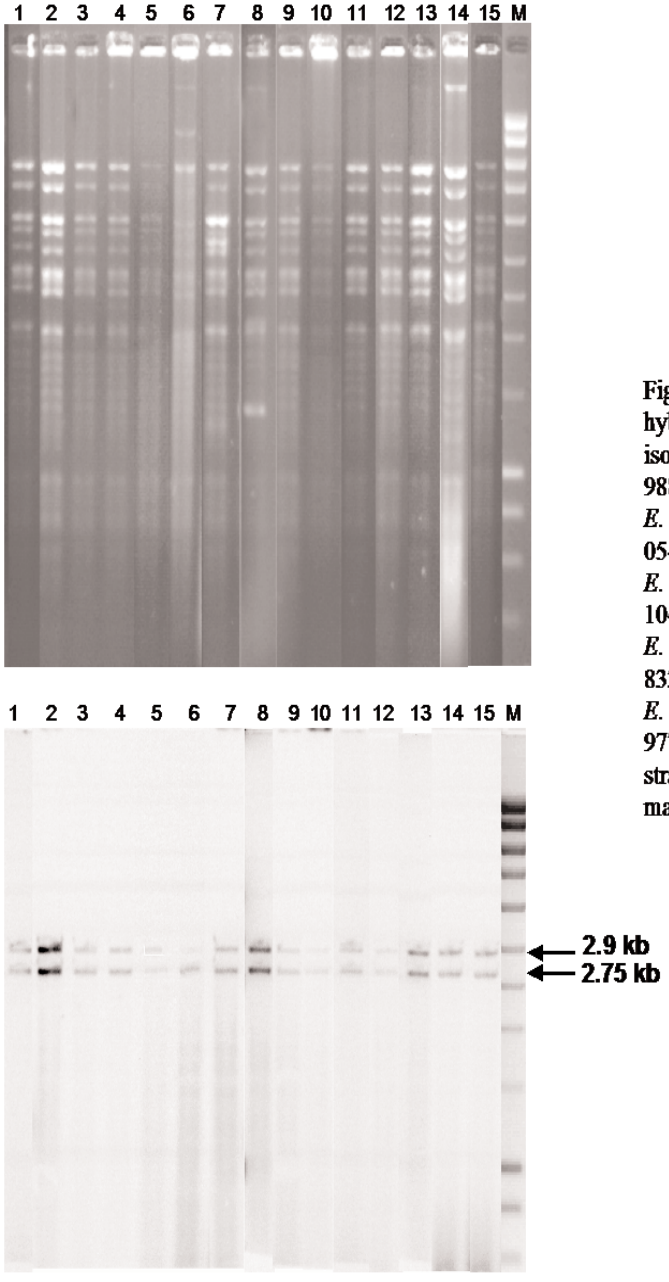

Fig. 4: Restriction analysis (PstI) (upper panel) and Southern blot hybridization with a bla_{TEM-52} probe (lower panel) of plasmid DNAs isolated from *E. coli* transconjugants. Lane 1, *E. coli* transconjugant 988SA01TC1; lane 2, *E. coli* transconjugant 2004/10101TC1; lane 3, *E. coli* transconjugant 2004/10256TC1; lane 4, *E. coli* transconjugant 05-00001TC1; lane 5, *E. coli* transconjugant 05-00590TC1; lane 6, *E. coli* transconjugant 05-00838TC1; lane 7, *E. coli* transconjugant 1043SA04TC1; lane 8, *E. coli* transconjugant 777SA01TC1; lane 9, *E. coli* transconjugant 260SA04TC1; lane 10, *E. coli* transconjugant 833SA04TC1; lane 11, *E. coli* transconjugant 153SA02TC1; lane 12, *E. coli* transconjugant 04-3486TC1; lane 13, *E. coli* transconjugant 977SA01TC1; lane 14, *E. coli* transconjugant pPAN-1 (the latter strain served as control and was previously published [107]); lane M, markers (Smartladder, Eurogentec, Seraing, Belgium).

tified from poultry, and strains of the serovars Thompson, London, Enteritidis, and Blockley were identified from human patients[108]. Several sporadic cases of *E. coli* TEM-52 producers were reported in animals: dogs in Portugal, rabbits in Spain, and beef meat in Denmark. To better identify the molecular mechanism of dissemination of this ESBL, the $bla_{\text{TEM-52}}$-positive plasmids were typed by the PBRT, demonstrating that they all belong to the IncI1 incompatibility group. IncI1 plasmids were recently described in *E. coli* and *Salmonella* strains of different serovars isolated in the United Kingdom associated with relevant β-lactamases such as CMY-2, CMY-7, and CTX-M-15, suggesting a large prevalence of IncI1 plasmids in Europe[109].

4.3. Multilocus sequence typing of IncI1 plasmids carrying extended-spectrum-β-lactamases in *Escherichia coli* and *Salmonella* of human and animal origin

The aim of this work was to analyze and characterize IncI1 plasmids identified in β-lactamase *E. coli* and *Salmonella* producers from animal and human sources in Europe and USA. We set up a new plasmid Multilocus Sequence Typing (pMLST) method to rapidly categorize plasmids belonging to the IncI1 family in different sequence types (STs).

Sixteen IncI1 plasmids were purified from transconjugants/transformants and analyzed by RFLP by PstI restriction. They were categorized in nine different restriction patterns (A-I in Table 5). One strain (2392T) produced no RFLP pattern due to autolysis of the plasmid preparation.

The comparison of three available IncI1 complete DNA sequences was performed (R64-AP005147, colIb-P9-AB021078 and pNF1358-DQ017661). Six genes were selected as potential targets for pMLST because they marked relevant maintenance and replication plasmid functions and were well conserved but also showed significant nucleotide differences potentially useful to

Table 5. Characteristics of the IncI1 plasmids analyzed in this study

IncI1 plasmid[a]	Strain[b]	Country[b]	Year[b]	Origin[b]	β-lactamase	RFLP[c]	pMLST[d]					ST[d]
							repI1	ardA	trbA-pndC	sogS	pilL	
398T	*E. coli*	Italy	2006	Dog	CMY-2	A	1	2	3	2	1	2
3115T	*E. coli*	Italy	2005	Dog	CMY-2	A	1	2	3	2	1	2
18196T	*E. coli*	Italy	2003	Dog	CMY-2	A	1	2	3	2	1	2
1358T	*S.*Thompson	USA	1996	Human	CMY-2	B	1	3	3	4	1	4
C1-VLA	*S.* Anatum	PK	2001	Human	CTX-M-15	C	3	2	6	3	3	8
C12-VLA	*S.* Typhimurium	UK	2002	Human	CTX-M-15	C	3	2	6	3	3	8
C13-VLA	*S.* Typhimurium	UK	2003	Human	CTX-M-15	C	3	2	6	3	3	8
34T	*E. coli*	France	2005	Poultry	CTX-M-1	D	3	2	3	3	3	9
21T	*E. coli*	France	2005	Poultry	CTX-M-1	E	2	1	4	1	2	3
22T	*E. coli*	France	2005	Poultry	CTX-M-1	E	2	1	4	1	2	3
3960T	*E. coli*	Italy	2005	Dog	CTX-M-1	E	2	1	4	1	2	3
2392T	*E. coli*	Italy	2005	Poultry	SHV-12	NA	2	1	4	1	2	3
C10-VLA	*S.* Enteritidis	UK	2001	Human	CTX-M-14	F	1	2	5	4	2	6
05-0001Tc1	*S.* Infantis	Belgium	2005	Human	TEM-52	G	1	2	2	2	3	5
S.82/10	*S.* Enteritidis	Italy	1995	Human	neg	H	2	1	5	4	2	7
R144	*S.* Typhimurium	Reference	NA	NA	neg	I	1	1	1	1	1	1

[a]Plasmids were purified from transconjugants or transformants obtained from the original strains using *Escherichia coli* K12 as recipient strain; [b] Bacterial species, country, year and source of isolation of the organisms where the IncI1 plasmids were identified; NA: Not Available; [c] Plasmid types were determined by restriction fragment length polymorphism (RFLP) by PstI digestion and classified with capitol letters; [d] Plasmid MultiLocus Sequence Typing (pMLST). Allele variants for each sequenced locus (repI1, *pilL, sogS, ardA and trbA-pndC*) were identified and numbered. Different sequence type (ST) were assigned to the different combinations of allele variants observed among the IncI1 plasmids.

the sub-typing of plasmids. In particular, for this analysis we selected *pilL* of the cluster for the type IV pilus biogenesis, *sogS*, encoding the primase that acts in the discontinuous DNA plasmid replication and *ardA*, encoding a type I restriction-modification enzyme. We also analyzed the RNAI antisense regulating the IncI1 replication system (repI1) and the intergenic region of the *trbA* gene *pndC* genes, involved in maintenance and plasmid transfer, respectively (Table 6).

The 16 IncI1 plasmids of our collection were then tested by PCR, DNA sequencing and sequence comparison with the R64 DNA sequence (AP005147). Three allele variants for the repI, *pilL* and *ardA*, 4 alleles for the *sogS* and 6 alleles for the *trbA-pndC* regions were identified. Insertion of the *finQ* gene (encoding the fertility inhibitor) within the 5'end of the *pndC* gene occurred in five strains (C10-VLA S.82/10, C1C-VLA, C12C-VLA and C13C-VLA) and characterized the *trbA-pndC* allele variants 5 and 6, respectively (allele 6 was distinguished from allele 5 by 14 additional nucleotide changes in the *trbA* gene, Table 6).

The assortment of the different alleles defined nine different sequence types (STs) among the 16 IncI1 plasmids. These categories perfectly matched those obtained by RFLP (Table 5), indicating that pMLST has a comparable discriminatory power.

The 3 IncI1 $bla_{\text{CMY-2}}$-plasmids identified in *E. coli* isolated from dogs in Rome (18196T) and in Padua (398T and 3115T) were assigned to the same pMLST and RFLP types (ST2, RFLP-A). They differ from the $bla_{\text{CMY-2}}$-plasmid (1358T) identified by both RFLP (RFLP-B) and pMLST (ST4) in a *Salmonella* Thompson of human origin isolated in USA in 1996

The three plasmids carrying $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1}}$ genes identified in *Salmonella* Anatum (C1-VLA) and Typhimurium (C12-VLA and C13-VLA) in the United Kingdom were identical by both RFLP and pMLST (RFLP-C, ST8). These IncI1 plasmids were characterized by the presence of the *finQ* gene within the *pndC* gene (Table 6).

Table 6. Primers used on the IncI1 plasmids and plasmid MultiLocus Sequence Typing (pMLST) allele variants identified in this study

Primers[a]	Primer sequence	Amplicon size	Primer position in EMBL No. AP005147 R64 plasmid	Allele DNA sequence position	Mismatches in the allele /R64 (mismatches/ sequence length)
repI1 FW	5'-CGAAAGCCGGACGGCAGAA-3'	142	198-216	236-339	repI1-1/R64 (0/104)
repI1 RV	5'-TCGTCGTTCCGCCAAGTTCGT-3'		339-319		repI1-2/R64 (1/104) repI1-3/R64 (2/104)
ardA FW	5'-ATGTCTGTTGTTGCACCTGC-3'	501	61426-61445	61469-61811	ardA1/R64 (0/343)
ardA RV	5'-TCACCGACGGAACACATGACC-3'		61926-61906		ardA2/R64 (11/343) ardA3/R64 (12/343)
trbA FW	5'-CGACAAATGCTTCCGGGGT-3'	883	74198-74216	74221-75032	trbA-pndC1/R64 (7/812) trbA-pndC2/R64 (7/812) trbA-pndC3/R64 (7/812) trbA-pndC4/R64 (10/812)
pndC RV	5'-CGAATCCCTCACCATCCAG-3'	2305[b]	75080-75062	74221-74972[b]	trbA-pndC5/R64 (8/752 + *finQ*) trbA-pndC6/R64 (24/752 + *finQ*)
sogS FW	5'-TTCCGGGGCGTAGACAATACT-3'	291	93088-93108	93125-93378	sogS1/R64 (0/254) sogS2/R64 (2/254)
sogS RV	5'-AACAGTGATATGCCGTCGC-3'	291	93378-93360	93125-93378	sogS3/R64 (8/254) sogS4/R64 (9/254)
pilL FW	5'-CCATATGACCATCCAGTGCG-3'	316	114765-114784	114804-115057	pilL1/R64 (0/254)
pilL RV	5'-AACCACTATCTCGCCAGCAG-3'		115080-115061		pilL2/R64 (5/254) pilL3/R64 (22/254)

[a] DNA sequence of the amplicons is obtained using the forward primer. [b] Amplicon size and allele variant sequence length vary in alleles showing the insertion of the *finQ* gene in the *pndC* gene.

Three of four *bla*$_{CTX-M-1}$-plasmids were classified in the same RFLP-E and ST3 groups (Table 5). Two of them were identified in *E. coli* isolated from poultry in the district of Côtes d'Armor in France in 2005 (21T and 22T)[110] and one was from *E. coli* isolated from a dog in Italy in 2005 (3960T). Interestingly, the ST3 was also assigned to a plasmid identified in *E. coli* isolated from poultry in Italy in the same year (2392T) but this plasmid carried the *bla*$_{SHV-12}$ and *bla*$_{TEM-1}$ genes. The fourth *bla*$_{CTX-M-1}$-plasmid (34T) was different by both restriction analysis (RFLP-D) and pMLST (ST9) and was identified in *E. coli* isolated from poultry in the district of Mayenne in France[110].

Plasmids C10-VLA, 05-0001Tc1, carrying the *bla*$_{CTX-M-14}$ and *bla*$_{TEM-52}$ genes respectively, and plasmids R144 and S.82/10, negative for β-lactamases, showing different RFLP patterns with respect to the other IncI1 plasmids, were assigned to different STs by pMLST (ST6, ST5, ST1 and ST7; Table 5).

4.4. Comparative analysis of IncHI2 plasmids carrying *bla*$_{CTX-M-2}$ or *bla*$_{CTX-M-9}$ from *Escherichia coli* and *Salmonella enterica* strains isolated from poultry and humans

The plasmid-encoded CTX-M family have recently become dominant ESBLs in human isolates in Europe[111]. Significantly, the CTX-M enzymes have emerged in isolates of *E. coli* and *Salmonella* spp. from food, companion, and wild animals, raising a potential public health concern[112-229]. In Belgium and France, *Salmonella enterica* serovar Virchow producing the CTX-M-2 ESBL was firstly identified in poultry flocks, then in poultry meat, and subsequently in humans over the period 2000 to 2003[105]. The chronology of isolation of these strains suggested that these bacteria were transmitted to humans via the food chain, specifi-

cally by poultry meat. In French Guiana in 2004, a *S. enterica* serovar Typhimurium strain producing CTX-M-2 was isolated from a patient with gastroenteritis. In France in 2002, one CTX-M-9-producing *S. enterica* rough strain was isolated from a patient, and in 2003, *S. enterica* serovar Virchow strains were isolated from poultry[106].

The objective of this study was to characterize plasmids carrying $bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-9}}$ from poultry sources and to compare them with those circulating in humans. The following CTX-M-2 and CTX-M-9 transconjugants were selected for this study: six from the *S. enterica* serovar Virchow strain of animal origin, one from the rough isolate, and one from the *S. enterica* serovar Typhimurium strain (Table 7).

Plasmids carrying $bla_{\text{CTX-M-2}}$ or $bla_{\text{CTX-M-9}}$ were analyzed by PBRT method[23]: PBRT indicates that all the strains were positive for the HI2 replicon, thus suggesting the presence of common IncHI2 plasmids in these strains. The HI2 replicon was previously detected on CTX-M-9-positive *Salmonella* and *E. coli* strains of human origin from Spain9[2, 120]. Three transconjugants obtained from representative strains of the Spanish collection (one from an *S. enterica* serovar Virchow strain and two from *E. coli*) were also included in this study for comparison (Table 7).

The HI2 amplicon was sequenced to confirm the PBRT result, and two types of DNA sequences were observed: the R478 type, showing 100% identity with the HI2 replicon of the reference plasmid R478 isolated from *S. marcescens* in the United States in 1969[121]; and the HI2-APEC-O1-R type, showing 100% homology with the HI2 replicon of the pAPEC-O1-R plasmid, recently described in avian pathogenic *E. coli* strains in the United States[122]. HI2-R478 and HI2-pAPEC-O1-R differ by 29 nucleotide substitutions located within the *cis*-repeats (iterons) flanking the replicase (*repA*) gene (accession no. BX664015 and DQ517526, respectively).

Table 7: Plasmid backbone analysis of the IncHI2 plasmids in transconjugants obtained from *Salmonella* and *E. coli* CTX-M-2 and CTX-M-9 producers

Transconjugant (ESBL	repHI2 type	Results of the PCR analysis										
		smr10-11	*smr17-18*	*smr207-208*	*smr239-240*	O1R_160	*terF*	*arsB*	*smr201*	*smr92-93*	*smr136*	*trsD*
p1639-SA-00-1 (CTX-M-2)	HI2 pAPEC-O1-R	+	+	+	+	+	+	-	-	-	-	-
p142-SA-01-1 (CTX-M-2)	HI2 pAPEC-O1-R	+	+	+	+	+	+	-	-	-	-	-
p03-1902-1 (CTX-M-2)	HI2 pAPEC-O1-R	+	+	+	+	+	+	-	-	-	-	-
04-9275-1 (CTX-M-2)	HI2 pAPEC-O1-R	+	+	+	+	+	+	-	-	-	-	-
pROU-1 (CTX-M-9)	HI2 R478	+	+	+	+	+	+	+	+	+	-	-
p2437-1 (CTX-M-9)	HI2 R478	+	+	+	+	+	+	+	+	+	-	-
p2731-1 (CTX-M-9)	HI2 R478	+	+	+	+	+	+	+	+	+	-	-
p3279-1 (CTX-M-9)	HI2 R478	+	+	+	+	+	+	+	+	+	-	-
p3464b-1 (CTX-M-9)	HI2 R478	+	+	+	+	+	+	+	+	+	-	-
p4300-1 (CTX-M-9)	HI2 R478	+	+	+	+	+	+	+	+	+	-	-
112-D-T (CTX-M-9)	HI2 R478	+	+	+	+	+	+	+	+	+	-	-
1185-D-T (CTX-M-9)	HI2 R478	+	+	+	+	+	+	+	+	+	-	-
1406-D-T (CTX-M-9)	HI2 R478	+	+	+	+	+	+	+	+	+	-	-
R478[a]	HI2 R478	+	+	+	+	-	+	+	+	+	+	+
pAPEC-01-R[b]	HI2 pAPEC-O1-R	+[a]	+	+	+	+	+	-	-	-	-	-

[a] Expected PCR result based on the R478 sequence (EMBL accession no. BX664015).

[b] Expected PCR result based on the pAPEC-O1-R sequence (EMBL accession o. DQ517526).

Interestingly, the HI2-R478 replicon type was identified in plasmids carrying the $bla_{\text{CTX-M-9}}$ gene, while the HI2-pAPEC-O1-R replicon type was identified in plasmids carrying the $bla_{\text{CTX-M-2}}$ gene (Table 7).

R478 and pAPEC-O1-R plasmids show many common regions, including the previously mentioned HI2 replicon, but also the Tra1 and Tra2 transfer regions and the genes coding for copper, silver, and tellurite resistance[121, 122]. The two plasmids diverge in terms of insertion sequences, the presence or absence of a class 1 integron, the Tn*10* transposon, and arsenic and mercury resistance loci[121].

To further characterize the CTX-M-2 and CTX-M-9 IncHI2 plasmid backbones, we used a PCR-based scheme, previously applied to the CTX-M-9 producers isolated in Spain (Table 8)[92]. This scheme recognizes five DNA targets unique for IncHI2 plasmids and common between R478 and pAPEC (*smr10-11*, *smr17-18*, *smr207-208*, *smr239-240* and *terF*) and five DNA targets that are specific for R478 but negative in pAPEC (*smr201*, *smr92-93*, *smr136*, *tnsD*, and *arsB*). An additional PCR is also included here to recognize the O1R_160 gene of pAPEC that is interrupted by a Tn*10* insertion in R478 (Table 8). Testing the transconjugants listed in Table 8, we observed that the O1R_160 gene was present in all of the strains, while the *smr136* and *tnsD* genes were absent: these results indicate that both the Tn*10* and Tn*7* transposons are missing in the CTX-M-2 and -9 plasmids analyzed in this study.

All of the plasmids were also positive for the five targets recognizing both R478 and pAPEC plasmids, while the $bla_{\text{CTXM-2}}$-carrying plasmids were negative for the *arsB*, *smr92*, and *smr201* targets, which were positively detected in the $bla_{\text{CTX-M-9}}$-carrying plasmids (Table 7). These results provided evidence that the $bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-9}}$ plasmids show different plasmid scaffolds despite belonging to the same IncHI2 family: the CTX-M-2 pro-

ducers from both human and poultry sources are associated with pAPEC-O1-R derivative plasmids, while the CTX-M-9 producers are associated with R478 derivative plasmids.

Table 8: Primers used in the plasmid backbone analysis[a]

Primer pair	Product of target gene in R478[b]	Primer sequence	PCR gene name (position of amplicon in R478[b] sequence)
10Fw 11Rv	HtdV, putative membrane protein HtdT, putative plasmid transfer protein	5′-AATCGCCTGAATCAGCTGG-3′ 5′-TTCTTTACTACACCAGAGCC-3′	*smr10-11* (6655–7713)
17Fw 18Rv	SMR0017, putative lipoprotein	5′-AACTCTTGAAAATCGTGG-3′ 5′-CTTCAGGCTATCGTTTCG-3′	*smr17-18* (18211–19101)
TerFw TerRv	Tellurium resistance protein	5′-ATGCAGGCTCAAGGAATCGC-3′ 5′-TTCATCGATCCACGGTCTG-3′	*terF* (80270–81163)
92Fw 93Rv	SMR0092, hypothetical protein SMR0093, putative inner membrane protein	5′-CTATGTAAGCAATGATCCTC-3′ 5′-TATAGAGAGCACCGAAGC-3′	*smr92-93* (88861–89862)
TnsDAFw TnsDARv	Tn*7*-like transposition protein	5′-AATCCTTGTTCAGCCGG-3′ 5′-CAAAAGCCAGCCATGCCC-3′	*tnsD* (119360–120825)
136Afw 136Arv	SMR0136, hypothetical protein	5′-TACGAAAATGAATTGTGGC-3′ 5′-AATTTACAATCTGCAGCCC-3′	*smr136* (120906–121768)
ArsBFw ArsBRv	Arsenical pump membrane protein	5′-AGTGAAAGACAGACGAAGCG-3′ 5′-GGCAGATAGTGTGGAATGCG-3′	*arsB* (159735–160870)
201Fw 201Rv	SMR0201 hypothetical protein	5′-TGTCAGGCTAAGTCACTGG-3′ 5′-ATTATACGGTAGATCC-3′	*smr201* (180398–181466)
207Fw 208Rv	SMR0207, conserved hypothetical protein SMR0208, hypothetical protein	5′-TTTCCCAAATAGGCGACGC-3′ 5′-ATGTGAAATTACTATACCGG-3′	*smr207-208* (190238–191131)
239Fw 240Rv	SMR0239, hypothetical protein SMR0240, hypothetical protein	5′-TGGAACGCGTGGTATGTGG-3′ 5′-ATACCTGCCGTTTACCC-3′	*smr239-240* (219372–220364)
O1R_160Fw O1R_160Rv	O1R_160, Tn*10* insertion site	5′-TTATGATGCTGGGCGTACC-3′ 5′-CACCATTACAATCACCTCC-3′	O1R_160 (202719–212075)[c]

[a] The DNA from the reference plasmid R478 of the incompatibility group IncHI2 was used as control for PCR assays. Amplification conditions were initial denaturation at 94°C for 5 min and 30 cycles at 94°C for 1 min, annealing at 60°C for 30 s, and elongation at 72°C for 1 min.
[b] EMBL accession no. BX664015.
[c] Positions 183121 to 182957 in EMBL accession no. DQ517526.

4.5. Worldwide dissemination of expanded-spectrum ß-lactamase VEB-1 and quinolone resistance determinant QnrA1 through acquisition of IncA/C_2 plasmids

Several Qnr determinants (QnrA-like, QnrB-like and QnrS-like proteins) conferring resistance to nalidixic acid and reduced susceptibility to fluoroquinolones, have been identified worldwide in a variety of enterobacterial species and were often associated to ESBLs. In particular, the noticeable association between the ESBL VEB-1 and the QnrA1 determinant was underlined[123, 124]. The ESBL VEB-1 is an Ambler class A ß-lactamase inhibited by clavulanic acid which confers resistance to ESBL but spares carbapenems[125]. The $bla_{\text{VEB-1}}$ gene has been identified in a large variety of Gram negatives, including *Enterobacteriaceae*, *P. aeruginosa*, and *A. baumannii*.

Since the plasmid co-localization of QnrA and VEB-1 encoding genes has been now reported repeatedly from scattered enterobacterial isolates, our objective was to use PBRT in order to trace a possible dissemination of a common plasmid worldwide[94]. The $bla_{\text{VEB-1}}$ and/or *qnrA*-positive plasmids that have been included in our study were from seventeen strains, most of them already reported[86, 123, 124, 126-128]. Except those isolates for which mating-out assays were unsuccessful, most of the strains studied were *E. coli* transconjugants (Tc), these latter allowing an accurate replicon typing as compared to original clinical isolates that may harbour several plasmids. They were collected from 1999 to 2005, from patients hospitalized in different parts of the world (Table 9). The thirteen $bla_{\text{VEB-1}}$-positive isolates were from five countries (France, Turkey, Algeria, Thailand, and Canada), representative of four continents. Among them, two were negative for *qnrA1*. In addition, four *qnrA1*-positive but $bla_{\text{VEB-1}}$-negative isolates recovered from France and Australia were also included in the study.

Table 9: Features of the VEB-1 or QnrA-positive isolates used in this study

Strain †	Species of origin	Country	Year	QnrA1	ESBL	Replicon
TcE1	*Escherichia coli*	Thailand	1999	+	VEB-1	A/C_2
TcE4	*E. coli*	Thailand	1999	+	VEB-1	A/C_2
TcE5	*E. coli*	Thailand	1999	+	VEB-1	A/C_2
TcE7	*E. coli*	Thailand	1999	+	VEB-1	A/C_2
TcE8	*E. coli*	Thailand	1999	+	VEB-1	A/C_2
TcE16	*E. coli*	Thailand	1999	+	VEB-1	A/C_2
TcE18	*Salmonella* spp.	Thailand	1999	+	VEB-1	A/C_2
Tc(p1)	*E. coli*	Canada	2000	+	VEB-1	A/C_2
Tc pQR1	*E. coli*	France	2003	+	VEB-1	A/C_2
TcGOC	*Enterobacter cloacae*	France	2003	+	VEB-1	A/C_2, FIB
LUT	*Citrobacter freundii*	Turkey	2004	+	VEB-1	A/C_2, FIB, K
Ps 15	*Providencia stuartii*	Algeria	2004	-	VEB-1	A/C_2
Tc MAA	*Proteus mirabilis*	Algeria	2004	-	VEB-1	A/C_2
TcK147	*Klebsiella pneumoniae*	Australia	2002	+	SHV-12	HI2, A/C_1, P
A1	*E. cloacae*	France	2004	+	SHV-12	HI2
TcA2	*Enterobacter aerogenes*	France	2005	+	SHV-12	FII
TcA3	*K. pneumoniae*	France	2005	+	-	I1, K

† Tc idicates that this is a transconjugant or a transformant

PBRT was applied to type the resistance plasmids from all the strains. After amplification, replicons were sequenced and used as specific probes to confirm the PBRT result by Southern blot hybridization on purified plasmid DNA. Differentiation between A/C_1 and A/C_2 replicons that differ from 26 nucleotides leading to three amino acids changes can be achieved by subsequent sequencing[90].

PBRT results showed that the thirteen bla_{VEB-1}-positive plasmids (including eleven *qnrA1*-positive) were positive for the A/C-type replicon and sequencing identified the A/C_2 replicon in all cases (Table 9). In two strains (*E. coli* TcGOC and *Citrobacter freundii* LUT), the A/C_2 plasmids were associated to additional replicons, suggesting the presence of multiple plasmids or fusions between plasmids of different backbones. By contrast, all the *qnrA1*-positive but bla_{VEB-1}-negative isolates were negative for the A/C replicon, except transconjugant TcK147 but sequencing identified an A/C_1-type replicon in that strain. These results clearly indicated that the genes encoding QnrA1 and VEB-1, when identified concomitantly in a given isolate, were always localized on plasmids belonging to the same IncA/C_2-incompatibility group that may however vary in size and digestion pattern (Table 9, Fig. 5). In addition, we showed that plasmids carrying the bla_{VEB-1} gene but lacking *qnrA1* were also of the A/C_2 type (Table 9). Plasmids that were *qnrA1*-positive but bla_{VEB-1}-negative were of distinct replicon types, thus suggesting independent acquisition of the *qnrA* gene on different plasmids.

Restriction pattern analysis of plasmid DNAs performed using the PstI endonuclease revealed that all the bla_{VEB-1}-positive plasmids were exhibiting different restriction profiles but also showing common bands, likely corresponding to the common plasmid backbone (Fig. 5). Comparison with an A/C_2-type USA plasmids carrying bla_{CMY-2} ß-lactamase gene revealed also a significant heterogeneity. Hybridization performed with an A/C_2-specific probe as described[90] showed an identical signal thus revealing that the bands carrying the replication control region of the plasmids were of identical size, as expected with plasmids from the same lineage (Fig. 5). By performing hybridization with a *qnrA1*-specific probe, two signals were obtained for each plasmid, as expected due to the presence of a PstI restriction site in the *qnrA1* sequence (Fig. 5). This *qnrA1*-

specific hybridization gave an identical pattern for all the A/C_2 plasmids, which differed from that of the IncFII group (TcA2, Fig. 5), indicating the presence of different structures surrounding the *qnrA1* gene.

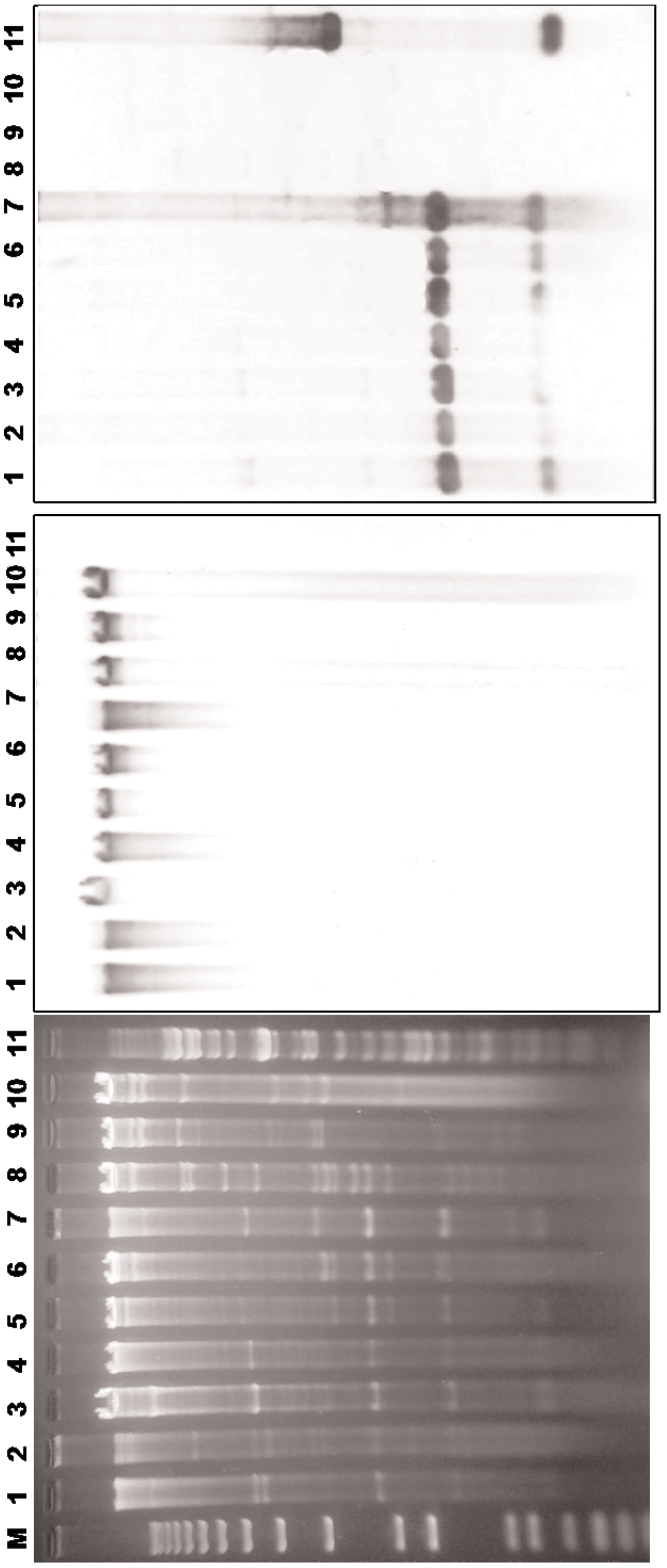

Fig. 5: Restriction and Southern blot hybridization analysis of plasmids analyzed in this study. Lane 1: TcE1; lane 2: TcE5; lane 3: TcE7; lane 4: TcE16; lane 5: Tcp1; lane 6: TcpQR1; lane 7: TcGOC; lane 8: TcMAA; lane 9: 3977 (USA); lane 10: 2039 (USA); lane11: TcA2 (repFII). M is the 1-kb Plus DNA ladder (GibcoBRL).

5. DISCUSSION

The genome sequencing is revolutionizing all aspects of the biological sciences of bacterial pathogens. This has facilitated the discovery of many previously unidentified determinants of pathogenicity and has provided novel insights into what creates a pathogen and showed the role of the horizontal gene transfer in the evolution of the prokaryotic organisms[129].

The horizontal gene transfer is an important mechanism for the evolution of microbial genomes. In the past, a common idea was the evolution of prokaryotic organisms by clonal divergence and selection; actually it is known the importance of the genetic exchange, both acquisition and loss of genetic information[130].

In any collection of bacterial strains it is possible to find strains with more than one species of DNA molecules. In fact, many bacteria carry extrachromosomal, self-replicating genetic elements, called *plasmids*. A plasmid is defined as a double stranded, circular or linear DNA molecule capable of autonomous replication. By definition, plasmids do not carry genes essential for growth of their host under nonstressed conditions but they are vey important molecules since the spectrum of diseases caused by *Enterobacteriaceae* is due to acquisition of a variety of specific resistance and virulence genes harboured on plasmids. Plasmid transfer via conjugation has shown potential for rapid spread through populations. This is a important mechanism for DNA exchange or dissemination, and this presumably accelerate the evolution of bacterial pathotypes, contributing towards the plasticity of bacterial genomes and the diverse combinations of virulence and resistance factors that continue to be reported.

The virulence plasmids of enteric bacterial pathogens have evolved by acquiring either individual genes or block of genes from bacteriophages and other conjugative plasmids by

transposition mechanisms. Many of the remnants of such acquisition events are evident in the sequences of the virulence plasmids of *Shigella*, the pathogenic *E. coli*, and *Yersinia*.

Plasmid-mediated antibiotic resistance is not a new phenomenon; it was discovered and documented quite soon after antibiotics first became commercially available. Plasmids have an uncanny ability to acquire drug resistance determinants from a variety of sources and to constantly evolve new ones, as for example, the recent development of plasmid-mediated resistance to fluoroquinolones. The spread of antibiotic-resistant pathogens is becoming an extremely serious clinical and public health problem worldwide.

In this thesis, resistance plasmids isolated from *Enterobacteriacea* of human and animal origin were fully characterized to highlight the diversity of the molecules responsible for the diffusion of antimicrobial resistance but also to ascertain the impact of horizontally transmitted resistance determinants on recurrent plasmids. The study demonstrated that successful genes, especially those conferring resistance to newer generation cephalosporines are disseminated by conserved plasmids that can therefore being considered as "epidemic plasmids". Typing results provided original insights into the molecular epidemiology of resistance plasmids.

1) The large diffusion of the IncI1 plasmid family:

plasmids belonging to the IncI1 family are considered as virulence plasmids since they are characterized by the presence of a cluster encoding the type IV pili. Type IV pili are appendages produced by gram-negative bacteria such as enteropathogenic and enterotoxigenic *E. coli*. Many type IV pili play important roles in the attachment of bacterial pathogens to membranes of eukaryotic host cells. These peculiar pili are a virulence factor and the association of virulence and resistance determinants may favour the positive selection of

plasmids belonging to the IncI1 family[131]. IncI1 plasmids carrying extended-spectrum and AmpC β-lactamase genes have been described in *E. coli* and *Salmonella*[109] worldwide diffused from animal and human origin.

From the results obtained in this thesis, the spread of an IncI1 plasmid carrying the $bla_{\text{TEM-52}}$ gene among *S. enterica* serovars Agona, Derby, Infantis, Paratyphi B dT^{+} was demonstrated, suggesting a wide dissemination of this ESBLs in Europe in animals and humans. The presence of the $bla_{\text{TEM-52}}$ gene in *E. coli*, as well as in different *Salmonella* serovars, strongly indicated that it is not due to the spread of a single clone but to the horizontal transmission of this resistance trait by IncI1 epidemic plasmids.

IncI1 plasmids are reported in different countries and they are associated to the dissemination of a great assortment of resistance genes such as $bla_{\text{CTX-M}}$, $bla_{\text{CMY-2}}$, bla_{SHV}. Since the discrete variability in terms of restriction profiles and DNA sequences of the different members of the IncI1 plasmid family a further discrimination of plasmid type was necessary to establish the correct epidemiological relationship among them. The pMLST method was suitable for a rapid and easy typing of IncI1 plasmids. The variants can be discriminated by significant and stable nucleotide divergence, matching results obtained by restriction analysis. The pMLST can be applied as a second line of plasmid typing after they have been assigned to incompatibility groups by the PBRT or other methods. A DNA sequence-based method allows to recognize similar plasmids and to detect them among isolates from different countries and laboratories without exchange of strains and direct comparison of the plasmids. pMLST can be developed also for other plasmid families and can contribute to the epidemiological description of plasmid circulation in animal reservoirs and humans by describing the spread of virulence and resistance plasmids. Our results demonstrated that identical IncI1 plasmids carrying the $bla_{\text{CMY-2}}$ gene circulated in

pets living in different towns in Italy in the period 2003-2006 but they were different from the *bla*$_{CMY-2}$-plasmid identified in *Salmonella* in USA. Those carrying the *bla*$_{CTX-M-15}$ gene from *Salmonella* isolated in UK were identical to each other and different from the other IncI1 plasmids. Variable associations among plasmids and β-lactamase genes were observed: the same plasmid scaffold was associated to different genes such as the *bla*$_{CTX-M-1}$ and *bla*$_{SHV-12}$-*bla*$_{TEM-1}$ genes, but also different plasmid scaffolds (ST3 and ST9) were associated to the *bla*$_{CTX-M-1}$ gene. From this study and the current literature, the prevalence of plasmids belonging to IncI1 seems linked to a particular reservoir of *E. coli* and *Salmonella* from poultry. Previous studies demonstrated that IncI1 plasmids are significantly associated to avian pathogenic *E. coli* in poultry populations analyzed in the USA[132]. There could be a particular niche favoring these plasmids in certain specific *E coli* populations, probably due to the contribution of the virulent type IV pili. This characteristic could promote the rapid spread in poultry and other food sources of β-lactamase producers carrying IncI1 plasmids.

2) THE PLASMIDS OF THE INCHI2 FAMILY:

the IncHI2 plasmids are large genetic plasmids (>150 kbp), temperature-sensitive for transfer, frequently associated with human pathogens, encode serologically related pili, transfer at low levels, and carry antibiotic and heavy metal resistance determinants. The first IncHI2 plasmid completely sequenced was R478. This plasmid originally isolated from the gram-negative opportunistic pathogen *Serratia marcescens* in United States in 1969[133], codes also for resistance to tetracycline, chloramphenicol, kanamycin, mercury, silve, copper, arsenic and tellurite.

The regions of similarity among the IncHI plasmids principally encode core plasmid determinants (i.e., replication, partitioning and stability, and conjugative transfer) and a

comparative analysis to define the minimal IncHI plasmid backbone determinants reveal that no resistance determinants are included in the backbone and most of the sequences unique to R478 were contained in a large contiguous region between the two transfer regions. These findings indicate that plasmid evolution occurs through gene acquisition/loss predominantly in regions outside of the core determinants[121].

The association between $bla_{\text{CTX-M-9}}$ and R478 was previously demonstrated for *E. coli* and *S. enterica* [92]. However, in the work of this thesis it has been demonstrated for the first time that the same CTX-M-9-R478 derivative plasmids frequently reported in human isolates was also detected in animal sources such as chicken feces and retail chicken meat.

The only fully sequenced IncHI2 plasmid from *E. coli* isolated from animals is the pAPEC-O1-R plasmid. While IncHI2 plasmids appear to frequently occur among *Salmonella*, *Klebsiella*, and *Serratia* species, they have been found infrequently among *E. coli* isolates. The low rate of occurrence of these plasmids among *E. coli* isolates suggests that these plasmids were likely acquired from other sources. One potential source for such plasmids is the chicken gut, as it is thought that APEC strains that cause disease in poultry originate from the fecal flora[122].

Comparison of the nucleotide sequences of pAPEC-O1-R and R478 plasmids revealed that they were each other extremely similar. Regions common to R478 and pAPEC-O1-R, which might define the IncHI2 backbone, included the Tra1 and Tra2 transfer regions; the copper, silver, and tellurite resistance regions; and the IncHI2 replicon. The DNA not common to both plasmids included insertion sequence elements, different class 1 integrons in both plasmids, and the arsenic and mercury resistance regions in R478. However, few but significant differences were noted between the two plasmid backbones, and specific analysis can be performed to discern them.

In this thesis, the presence of the *bla*$_{\text{CTX-M-2}}$ gene was reported for the first time on pAPEC-O1-R plasmid derivative. pAPEC-01-R was discerned by the R478 thanks to the analysis of 10 different loci along the plasmid backbone. It was important to establish that the pAPEC-O1-R plasmid, identified in avian pathogenic and commensal *E. coli* strains in the United States[122], has acquired the *bla*$_{\text{CTX-M-2}}$ gene in *Salmonella* strains of human and animal origins in Europe. Contamination with ESBL-producing strains of *S. enterica* has been extensively suggested to occur through the food chain, but very few *Salmonella* ESBL producers of animal origin have been reported, while these enzymes are frequently detected in human isolates. In this study, plasmid-mediated horizontal transfer of *bla*$_{\text{CTX-M-2}}$ and *bla*$_{\text{CTX-M-9}}$ genes has been demonstrated between poultry and human *S. enterica* and *E. coli* strains isolated in very different geographical regions. This result is of major concern because food animals may represent a large reservoir for a further dissemination of such genetic determinants to human pathogens.

3) The plasmids of the IncA/C$_2$ family:

the IncA/C plasmids are broad-host-range family able to replicate in a variety of gram-negative bacteria. Since the multidrug resistant (MDR) determinants are often encoded on mobile plasmids, the potential transfer of MDR phenotypes from foodborne pathogens to more virulent human pathogens constitutes a serious public health threat.

Recently a comparative analysis of the DNA sequence of *Yersinia pestis* plasmid pIP1202 revealed a near identical IncA/C plasmid backbone is shared with plasmids isolated from *Salmonella enterica* serotype Newport SL254 and form the fish pathogen *Yersinia ruckeri* YR71.

The discovery of these IncA/C plasmids in evolutionarily distinct pathogens attests to recent genetic exchange, either directly between these bacterial species or through bacterial

intermediates, and it suggests that overlap in the ecological niches of these organisms is sufficient to permit past or future plasmid transmission[134].

In the work done for this thesis IncA/C_2 plasmid derivatives were identified as the main vehicles for the dissemination of the *bla*$_{VEB-1}$ gene on which the QnrA1 determinant may be added. The possibility that both *bla*$_{VEB-1}$ and *qnrA1* genes could be found in a single genetic structure in some isolates has been recently evidenced with their identification in the same *sul1*-type integron[127]. Due to the fact that these experiments provided us a good marker for tracing *bla*$_{VEB-1}$-borne plasmids, and taking in account the broad-host range property of A/C-type plasmids (note that this was never established for the specific A/C_2 subgroup), we tried to amplify the A/C_2-type *rep* gene in a collection of *bla*$_{VEB-1}$-positive *P. aeruginosa* isolates from France, Thailand, India and plasmid, and also ruling out the hypothesis of A/C_2-type plasmid co-integration at the origin of *bla*$_{VEB-1}$ acquisition in *P. aeruginosa*. The current spread of plasmids carrying a large array of resistance determinants among *Enterobacteriaceae* is extremely worrisome since it corresponds to a useful tool for a given strain to become panresistant to antibiotics. In particular, it has been evidenced with the recent identification of the Qnr determinants that plasmids may provide resistance (or at least reduced susceptibility) to quinolones and fluoroquinolones whereas they are already known to carry resistance to ß-lactams, aminoglycosides, chloramphenicol, tetracycline, rifampin, sulfonamides, and disinfectants. The best examples of such plasmid-mediated multidrug resistance are the identifications of pQR1[124] or p1[127] carrying *bla*$_{VEB-1}$ and *qnrA1* together with aminoglycoside resistance genes *aadB*, *aacA1*, and *aadA1*, chloramphenicol resistance gene *cmlA*, rifampin resistance gene *arr2*, disinfectant resistance gene *qacI*, and sulfonamides resistance gene *sul1*. Our study showed that the IncA/C_2-type plasmids may be the source of that worldwide dissemination, what is quite surprising. Indeed, it means that

one plasmid scaffold has brought the same (at least very similar) multidrug resistance to multiple enterobacterial species in different continents. Noteworthy, IncA/C_2-type plasmids were recently identified to be the support of the AmpC-type cephalosporinase CMY-2 encoding gene in USA also to the genes encoding the carbapenemase VIM-4 and the cephalosporinase CMY-4 in Italy[90]. Finally, our study points out the worldwide spread of a plasmid providing multidrug resistance.

4) Epidemic plasmids associated to ESBL genes:

the PBRT was used to study 26 epidemiologically unrelated *Enterobacteriaceae* and demonstrate the dominance of IncA/C or IncN-related plasmids carrying some emerging resistance determinants to extended-spectrum cephalosporins and carbapenems.

The *bla*$_{\text{CMY-2}}$-carrying plasmid circulating in the United States was also detected in Europe in the form of a derivative that also carries the VIM-4 carbapenemase determinant, demonstrating that plasmids carrying resistance to clinically relevant antibiotics can spread worldwide among bacteria responsible for both nosocomial and community-acquired infections. The heterogeneity among Italian plasmids encoding SHV-12 (the most prevalent SHV-type ESBL in this country)[135] suggests a notable potential for this determinant to spread among different plasmid replicons. On the other hand, replicon typing revealed that the VIM-1 encoding plasmids from Greece were all related to each other despite their different restriction profiles, pointing out the common origin of these plasmids. It is interesting that the *bla*$_{\text{CMY-13}}$ gene from Greece is located on the repN plasmid, whereas Italy and the U.S. share the A/C_2 plasmid as vehicle of the *bla*$_{\text{CMY}}$ gene, despite their geographical distance. Further research is necessary to determine the influences on plasmid trafficking, and for these differences and commonalities.

To conclude, the versatility of plasmids together with the usage of antimicrobials in human medicine and animal husbandry, may largely contributed to the spread of antimicrobial resistance. These observations imply that plasmid-mediated antimicrobial resistance is a global problem that does not respect any boundaries, either between animals and humans, or bacterial species and genera, demonstrating the strong capacity of plasmids to be horizontally transmitted. Many questions remain unanswered about mechanisms driving the dissemination of plasmids along the food chain, or mediating the hospital/community exchanges. The exact contribution of antimicrobials use for animal and human therapy, on the one hand, and prevention of infection in humans, on the other, to the positive selection of specific plasmids also remains uncertain. However, further research extending the knowledge of antimicrobial resistance mechanisms will facilitate the development of effective preventive and control strategies against this phenomena.

Replicon identification may provide useful clues as to the evolution of these resistant plasmids. The ability to trace and screen plasmids may facilitate further understanding of the horizontal transfer of antimicrobial resistance.

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