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MOLECULAR EPIDEMIOLOGY OF EMERGING ANTIBIOTIC RESISTANCE MECHANISMS IN COMMENSAL AND PATHOGENIC BACTERIA IN LATIN AMERICA

SUPERVISOR:

PROF. GIAN MARIA ROSSOLINI

PHD STUDENT:

SAMANTA SENNATI

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FOREWORD

This thesis is divided in six sections: general introduction, aim of the PhD project, results and discussion, conclusions and perspectives, bibliography and annexes. The annexes include all the related material that has been published in relation to the thesis work.

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PART I

GENERAL INTRODUCTION

1

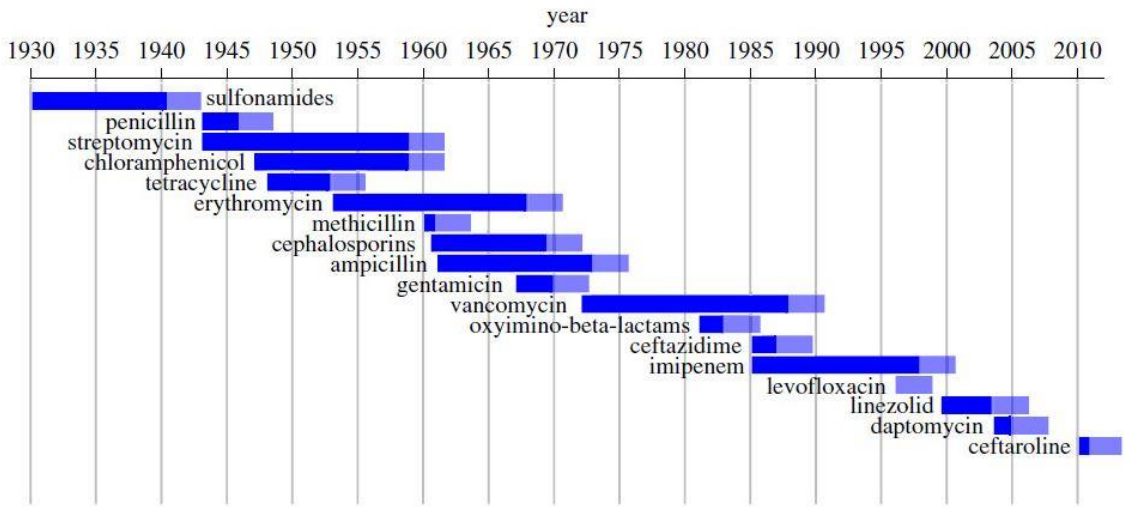
THE PHENOMENON OF ANTIBIOTIC RESISTANCE

The start of the antibiotic era could be associate with the name of Alexander Fleming and his serendipitous penicillin discovery. He noticed that in a culture of *Staphylococcus aureus* a green mould of *Penicillium notatum* was grown, and in the zone immediately around the mould the bacterial cells were lysed. In 1929, he described those results, suggesting a therapeutic use of the substance isolated by the mould, but he couldn't produce it to use as a drug (Fleming A, 1929). Only in 1941, when H. W. Florey studied the pharmacological properties of the penicillin, an efficient drug to be use in therapy became available (Chain E, 1940). Since then, more drugs were found out, the manufacturing processes were simplified and newer formulations were developed (Demain AL, 2009). However, after their discovery, antimicrobials have been largely used and misused not only in the medical field, but also in several non-human applications (agriculture, veterinary medicine, and aquaculture), underestimating the potential problem of antibiotic resistance.

Resistance to an antibiotic occurs when a microorganism is able to grow or survive in the presence of a concentration of antibiotic that is usually sufficient to inhibit or kill organisms of the same species. The terms 'susceptible' and 'resistant' relating to antibiotics are usually used in clinical practice to infer the likely success or failure of treatment. (Abraham EP, 1940). Already in his Nobel Prize speech in 1945, Alexander Fleming, warned that bacteria could become resistant to these remarkable drugs as a normal evolutionary process (Weber JT, 2005). Indeed, the development of each new antibacterial drug has always been followed by the emergence of resistance (Martínez JL, 2014)(Fig. 1).

Although antibiotic resistance can be considered to be an inevitable consequence of antibiotic use, injudicious use of antibiotics in outpatient clinics, hospitalized patients and in the food industry is a major factor facilitating the emergence of resistance worldwide. In many areas, the availability of antibiotics 'over the counter' or via the internet allows the non-prescriber to have free and unrestricted access to these agents. Once resistance has emerged, subsequent dissemination of resistant strains is facilitated by the selection pressure exerted by further antibiotic use, failure to adhere to infection control measures and by poor hygiene (notably in terms of hand hygiene, sanitary conditions and food preparation), which can occur both within and outside healthcare settings (Fig. 2).

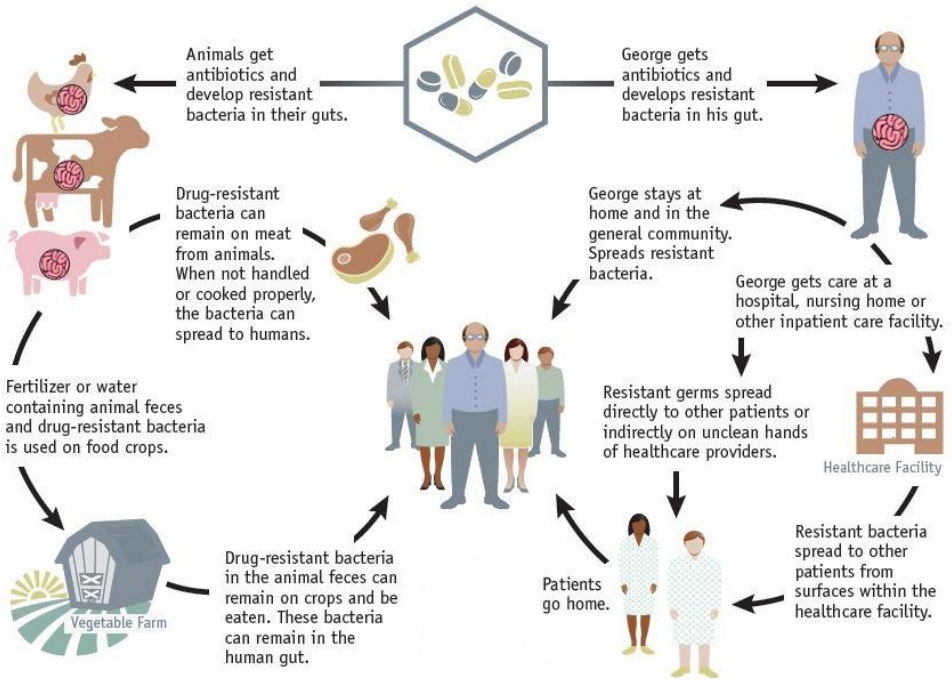
Fig. 1. Timeline of antibiotics introduction and the subsequent evolution of resistance (indicated by the ends of the bars)(McClure NS, 2014).



Antibiotic resistance has significant costs to society in terms of increased mortality, morbidity, use of healthcare resources and time off work.

Globally, the antibiotic resistance problem has been recognized for many years. Although there are many stakeholders in this issue, the World Health Organization (WHO) has a global overview and defines the 21st century as the "post-antibiotic era, far from being an apocalyptic fantasy, in which common infections and minor injuries can kill" (WHO, 2014; Michael CA, 2014).

Fig. 2. How antibiotic resistance spreads (Centers for Disease Control and Prevention (CDC), 2014).



Effective antibiotic stewardship is required globally, together with better diagnostic tests to identify or rule out infection quickly. Multiplex gene detection PCR assays and next generation sequencing (NGS) are other methods that are being utilized to achieve earlier detection of antibiotic resistance. Identification of cultured bacteria through mass spectrometry (e.g. by Matrix-Assisted Laser Desorption Ionization Time of Flight, MALDI-TOF) has reduced the time to identification of organisms compared with conventional biochemical means. Automated susceptibility testing also has the potential to deliver results more quickly (Sabtu N, 2015).

Although resistance has been a steady problem since the first usage of antibiotics, it is the increase in the number, diversity and range of resistant organisms that has become a huge clinical problem. Nowadays a major concern is represented by the emergence and diffusion of multidrug resistant (MDR), extremely drug resistant (XDR), and pandrug resistant (PDR) microorganisms, virtually resistant to all currently available antibiotics (Magiorakos AP, 2012).

These "Superbugs", such as *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter cloacae* (also known as ESKAPE pathogens) represents a major healthcare and economic problem because are routinely isolated in hospital settings and against which we truly have to date no effective treatment, contributing to the "antibiotic resistance crisis" (Rice LB, 2008; Rossolini GM, 2014).

Surveillance of microbial resistance is arguably considered one of the first steps in constraining the antimicrobial chemotherapy crisis (WHO, 2011; Rossolini GM, 2014). Indeed, surveillance is essential for providing information on the magnitude and trends of resistance and may support i) clinicians, in empirical treatment optimization, ii) policy makers, in defining or updating standard treatment guidelines, intervention strategies and infection control measures implementation, but also iii) researchers working in the field of antimicrobial resistance.

2

ANTIBIOTIC RESISTANCE IN LATIN AMERICA

Health care-associated infections are particularly challenging in low resource countries such as those of Latin America owing to their far higher rate of occurrence than in developed countries. For instance, the World Health Organization reported that the pooled prevalence of hospital-wide health care-associated infections between 1995 and 2010 in low- and middle-income countries was 10.1 per 100 patients, which compares unfavorably with the corresponding rate in high-income countries (7.6 episodes per 100 patients) (WHO, 2011). Most infections in developed and low resource countries are by Gram-negative bacteria, predominantly species from the *Enterobacteriaceae* family followed by non-fermenters such as *P. aeruginosa* and *A. baumannii*. The capacity of health care professionals to manage nosocomial infections by *Enterobacteriaceae* has diminished over the last decade due to the emergence and widespread dissemination of multidrug-resistant phenotypes and with the non-production new active drugs (Falagas ME, 2007, Savard P, 2012) .

Populations in low resource countries, living in subnormal agglomerates, are often more susceptible to infections due to increased prevalence of underlying diseases and malnutrition. In addition, the intense cross-transmission of microorganisms in hospitals in conjunction with ineffective healthcare systems, insecure drug supply chains and inconsistent accessibility to newly developed drugs favor the occurrence and dissemination of antimicrobial-resistant infections in clinical settings (Isturiz RE, 2000; Planta, 2007). Moreover, the economy of many developing countries relies on agriculture and livestock keeping, activities known to use large amounts of antimicrobials to increase productivity. The immediate consequence of such practices is the selection of antimicrobial resistant bacteria within the animals' microbiota and among soil and water courses (Capita R, 2013). Contaminated environments together with poor sanitation, crowded living conditions and unsafe drinking water supply, in turn, favor the spread of antimicrobial-resistant bacteria throughout the community. The features mentioned above together with the lack of financial resources and political will to address the problem disastrously made developing countries fertile lands for the evolution of antimicrobial resistance.

Latin America includes 12 nations that altogether comprise approximately 400 million people, which represent nearly 6% of the world population. These countries have experienced rapid economic growth over the last decade, reflected in increased human development indexes.

Nevertheless, enormous income distribution disparities remain ensuing poor living conditions for a significant part of their population.

In Latin American multidrug-resistant *Enterobacteriaceae* is a major concern (Gales AC, 2012; Rosenthal VD, 2010), in fact they play important roles as etiologic agents of both nosocomial and community-acquired infections, even in very remote societies (Bartoloni A, 2009; Woerther PL, 2010).

In these countries antimicrobial resistance in *Enterobacteriaceae* is against the main antimicrobial agents such as beta-lactams, fluoroquinolones and aminoglycosides (Tab. 1) (Bonelli RR, 2014).

Tab. 1. Chronology of resistance mechanisms affecting beta-lactams, fluoroquinolones and aminoglycosides in *Enterobacteriaceae* from Latin America (Bonelli RR, 2014).

Mechanism	Year of isolation	Microorganism	Country
SHV ESBL	1987	*K. pneumoniae*	Chile
FOX	1989	*K. pneumoniae*	Argentina
CTX-M-2-group	1990	*S*. Typhimurium	Argentina
PER	1990	*S*. Typhimurium	Argentina
BES	1996	*S. marcescens*	Brazil
CTX-M-9-group	2000	*Klebsiella* spp.	Brazil
CTX-M-1-group	2002	*E. coli*	Peru
	2002	*K. pneumoniae*	Colombia
CTX-M-8-group	2003	*E. coli*	Brazil
IMP	2003	*K. pneumoniae*	Brazil
TEM ESBL	2004	*K. pneumoniae*	Brazil
GES ESBL	2004	*K. pneumoniae*	Brazil
KPC-2	2005	*K. pneumoniae*	Colombia
	2005	*K. pneumoniae*	Brazil
VIM	2005	*K. pneumoniae*	Venezuela
CMY	2006	*S. flexneri*	Argentina
GES carbapenemase	2008	*K. pneumoniae*	Brazil
KPC-3	2008	*K. pneumoniae*	Colombia
OXA-48	2008	*K. pneumoniae*	Argentina
NDM	2011	*K. pneumoniae*	Colombia
QnrB	2003	*C. freundii*	Brazil
QnrA	2005	*E. cloacae*	Brazil
AAC(6')Ib-cr	2005	*E. coli*	Peru
QnrS	2005	*E. coli*	Peru
		K. pneumoniae	Peru
		K. oxytoca	Peru
		K. pneumoniae	Bolivia
RmtD	1998	*E. cloacae*	Argentina
		C. freundii	Argentina
		S. marcescens	Argentina
RmtB	2005	*E. coli*	Brazil

The limited data on antibiotic resistance for low resource countries are mostly from studies conducted on pathogens isolated during disease outbreaks from community- or from health-care associated infections observed in the few centres where high-quality laboratories are available (Vernet G, 2014).

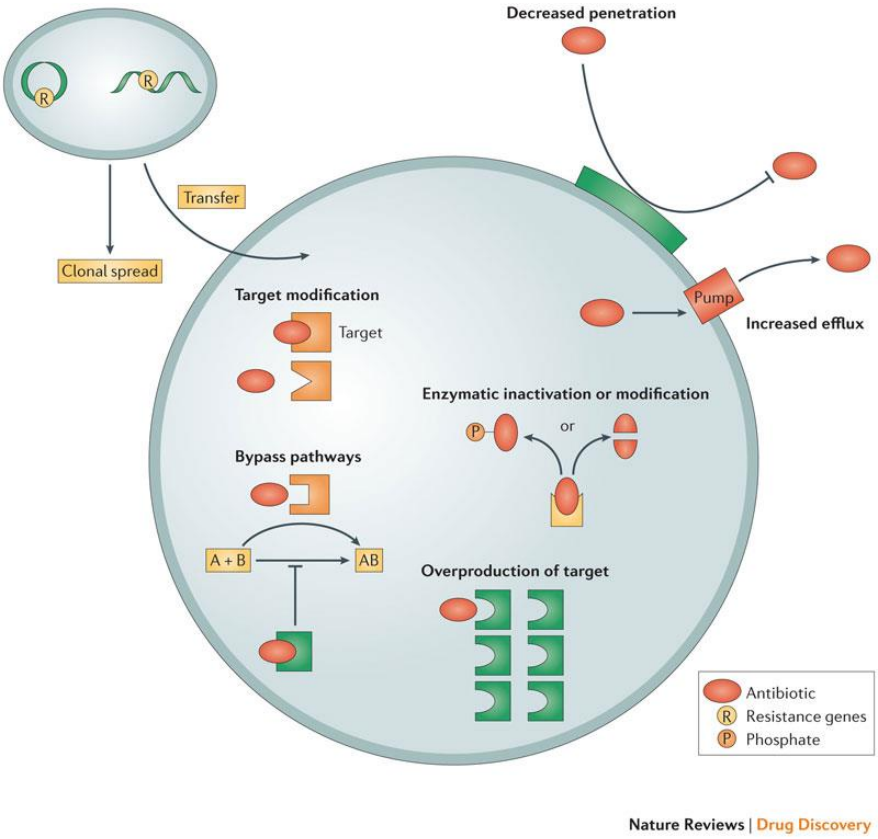
Although several global antibiotic surveillance programs have been proposed over years (e.g., WHONET, SENTRY), in low resource countries effective surveillance programs are difficult to implement because of scarce financial resources, lack of laboratory facilities and, where laboratories do exist, lack of quality control, reliable reagents, adequate supervision, personnel and periodic training (Sosa AJ, 2010; Vernet G, 2014).

3

ANTIBIOTIC RESISTANCE MECHANISMS

Bacteria may manifest resistance to antimicrobial agents exploiting a variety of strategies which are shown in Fig. 3: namely i) inactivation of the drug ii) modification of the site of action (enzyme, ribosome, cell-wall precursor) iii) modification of the permeability of the cell wall iv) overproduction of the target enzyme v) and the bypass of the inhibited steps.

Fig. 3. Mechanisms of genetic resistance to antimicrobial agents (Coates A, 2002).



However, in parallel with the above mentioned mechanisms is known a strategy to go beyond antimicrobial agents called “tolerance”. The main culprit responsible for the tolerance of pathogens to antibiotics is a specialized survivor, a persister. Persisters are not mutants, they are phenotypic variants of actively dividing cells produced stochastically in the population, and their relative abundance rises at the late-exponential phase of growth. Persisters are non-growing dormant cells, which explains their tolerance to bactericidal antibiotics that depend on

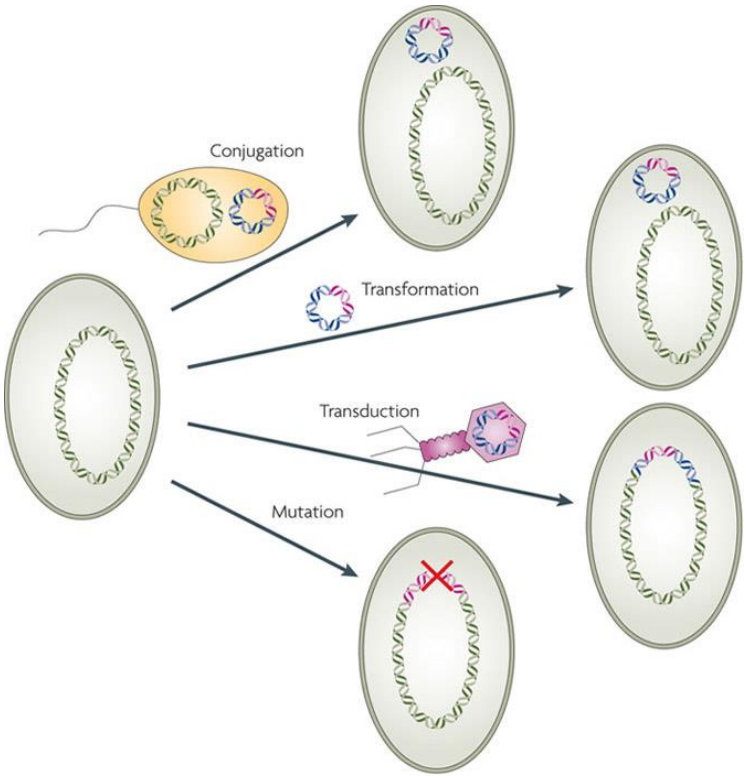
the presence of active targets for killing the cell. Persisters are killed only slowly, if at all, and resume growth when antibiotic concentrations fall. The result is a relapsing infection with a large effective population size that favors the development of resistance. Unfortunately the formation of persisters is still largely unknown.

Resistance to antimicrobial agents could be due to an innate property of the bacterium, defined as "intrinsic resistance", or a consequence of mutation or gene transfer, described as "acquired resistance". The intrinsic resistance depends on chromosomal encoded mechanisms and results from the evolutionary process that led to bacterial speciation. Examples of intrinsic resistance include resistance of all Gram-negative bacteria to glycopeptides, because these large molecules are not able to pass through the Gram-negative outer membrane, or the production of a chromosomal beta-lactamases in all Gram-negative, with the exception of *Salmonella* spp.

The acquired resistance consist in the ability of a naturally susceptible bacteria to get a resistance phenotype by spontaneous mutations in chromosomal genes or by the recruitment of intra- and inter-species exogenous resistance determinants through horizontal gene transfer (HGT) that play an important role in spreading relevant antibiotic resistance determinants and accelerate the dispersal of these genes within major pathogens (Stokes HW, 2011; Jansen G, 2014).

Horizontal gene transfer is defined to be the movement of genetic material between bacteria other than by descent in which information travels through the generations as the cell divides and can results by three mechanisms: i) Transformation - the uptake of naked DNA is a common mode of horizontal gene transfer that can mediate the exchange of any part of a chromosome; this process is most common in bacteria that are naturally transformable; typically only short DNA fragments are exchanged. ii) Conjugation - the transfer of DNA mediated by conjugal plasmids or conjugal transposons; requires cell to cell contact but can occur between distantly related bacteria or even bacteria and eukaryotic cells; can transfer long fragments of DNA. iii) Transduction - the transfer of DNA by phage requires that the donor and recipient share cell surface receptors for phage binding and thus is usually limited to closely related bacteria; the length of DNA transferred is limited by the size of the phage head (Fig. 4).

Fig. 4. Acquired resistance mechanisms: DNA from the biosphere containing an antibiotic resistance gene (pink) can be transferred by horizontal gene transfer into a recipient by several paths: cell-to-cell conjugation; transformation by naked DNA (on plasmids or as linear DNA) that is released by dead cells; or phage-mediated transduction. Resistance can also arise by *de novo* mutation (indicated by a red cross).



Additionally, point mutations in drug target (e.g. PBPs), in regulatory networks (e. g of efflux systems, of uptake pathways, or regulatory genes of chromosomal beta-lactamases) or even in the acquired genes, can contribute in the acquisition of a resistance phenotype by normally susceptible bacteria (Levy SB, 2004; Alekshun MN, 2007).

4

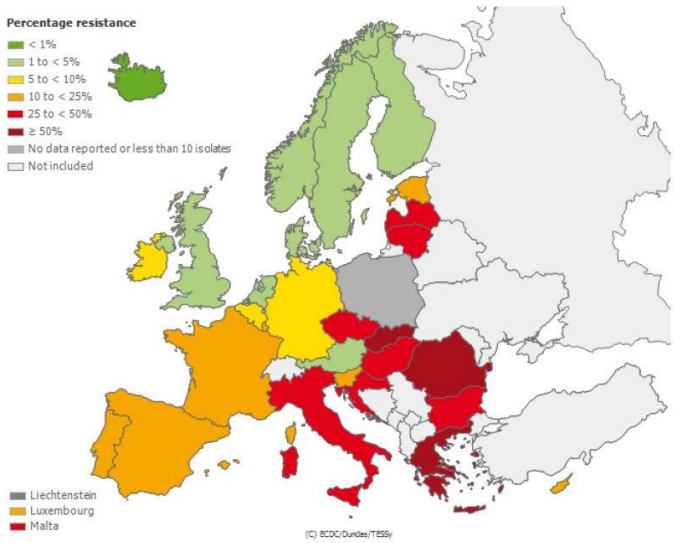
ANTIBIOTIC RESISTANCE IN *ENTEROBACTERIACEAE*

The *Enterobacteriaceae* is a family of Gram-negative bacteria that constitutes a large part of the gut microbiota of humans and animals and includes species, such as *Escherichia coli* and *K. pneumoniae*, that are common causes of nosocomial and community infections (e. g. urinary tract, intra-abdominal, skin and soft-tissue, device-associated infections, septicemia, pneumonia, meningitis).

During the last decade a global and rapid increasing of the antibiotic resistance among *Enterobacteriaceae* has been reported, especially towards aminoglycosides, (fluoro)quinolones, third and fourth generation cephalosporins, cephamycins, and carbapenems. These strains, defined as multidrug-resistant organisms , can be resistant to all currently available antimicrobial agents or remain susceptible only to older, potentially more toxic agents such as the polymyxins, leaving limited and suboptimal options for treatment (Donati V, 2014; Magiorakos AP, 2012).

The European Antimicrobial Resistance Surveillance System (EARSS), an international network of national surveillance systems which collects, comparable and validated antimicrobial susceptibility data for public health action, report the prevalence of multidrug-resistant *K. pneumoniae* in Europe for the year 2014 (Fig. 5).

Fig. 5. Multidrug-resistant *K. pneumoniae* isolates(resistant to third generation cephalosporins, fluoroquinolones and aminoglycosides) in participating countries in 2014. (http://www.ecdc.europa.eu/en/activities/surveillance/EARS-Net/).



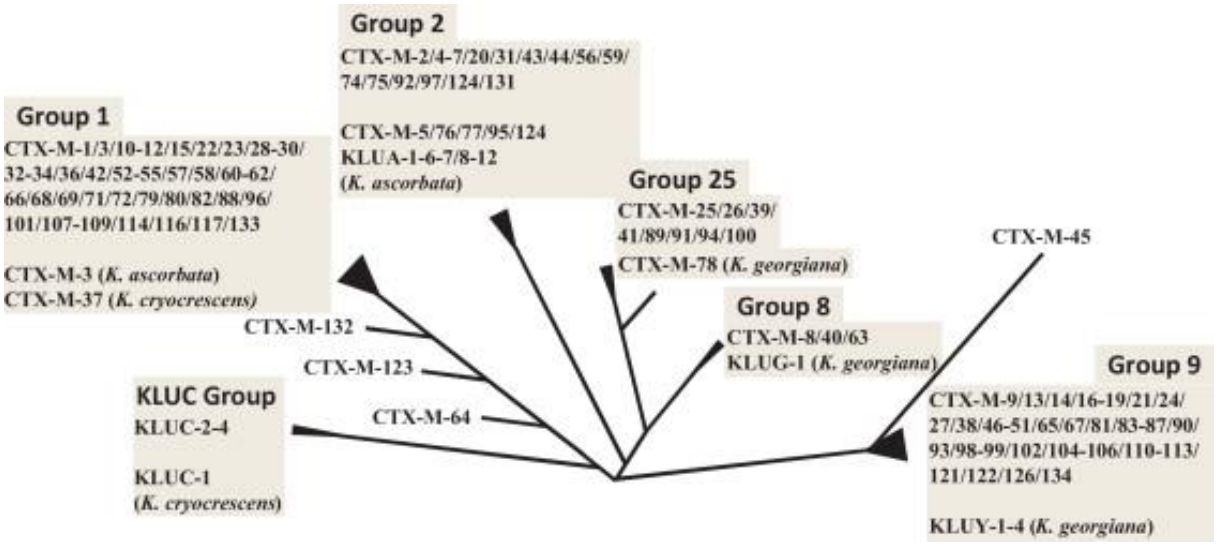
RESISTANCE TO EXPANDED-SPECTRUM CEPHALOSPORINS

Resistance to expanded-spectrum cephalosporins in *Enterobacteriaceae* is principally conferred by the production of extended-spectrum beta-lactamases (ESBL), plasmid mediated AmpC-type enzymes and carbapenemases (Delgado-Valverde M, 2013). The ESBL are able to hydrolyse penicillins, narrow- and expanded-spectrum cephalosporins (except cephamycins) and monobactams, and are usually inhibited by beta-lactamase inhibitors (e. g. clavulanate, tazobactam, sulbactam) (Paterson DL, 2005).

Most ESBL are derivatives of TEM or SHV (enzymes able to confer resistance to penicillins and narrow-spectrum cephalosporins (Bush K, 1995; Jacoby GA, 1991), from which few point mutations at selected loci within the gene give rise to the extended-spectrum phenotype (Bradford P, 2001). Until the end of the 1990s TEM- and SHV-type ESBL were the most detected, and isolates expressed these enzymes were almost associated with nosocomial outbreak and it was very unusual find them in the community (Bradford P, 2001; Paterson DL, 2005). The situation has changed so much dramatically in the last decade, that defined this phenomenon as "the CTX-M pandemic" (Cantón R, 2006). Indeed, the rapid and continuous diffusion of CTX-M-type ESBL has been very efficient and not limited to health-care settings, but has also involved the community, livestock and companion animals (Ewers C, 2012), wildlife (Guenther S, 2011), and river waters (D'Andrea MM, 2013).

Based on their aminoacidic sequence diversity, the 161 CTX-M variants identified so far have been classified into six major phylogenetic groups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTX-M-25 and KLUC. Each group differ from each other by ≥10% amino acid residues and includes a number of minor allelic variants which differ from each other by one or few amino acid substitutions (≤5% amino acid residues). Moreover, there are at least four CTX-M variants that exhibit a hybrid structure, namely CTX-M-45 (formerly Toho-2) which is a hybrid of CTX-M-14 with a protein of unknown origin, and CTX-M-64, CTX-M-123 and CTX-M-132 which are hybrids of CTX-M-15 with different segments of CTX-M-14. The majority of variants are found within the CTX-M groups 1 and 9, especially CTX-M-15 (belonging to group 1) and CTX-M-65 (belonging to group 9) suggesting a higher plasticity for these groups (D'Andrea MM, 2013)(Fig. 6).

Fig. 6. Tree diagram showing the similarity among enzymes of the CTX-M lineage and clustering of members of different CTX-M groups.



The wide dissemination of CTX-M-type ESBL has been moreover promoted by the association of epidemic plasmids with highly successful clonal lineage of *E. coli* and *K. pneumoniae*, the so called high-risk multiresistant and virulent clones circulating worldwide (Woodford N, 2011). The most paradigmatic example is represented by the pandemic *E. coli* ST131 clone (phylogenetic group B2) that has greatly contributed to the global dissemination of CTX-M-15, one of the most widely distributed CTX-M allelic variant in the world (Rogers BA, 2011; Cantón R, 2012; Park SH, 2012).

AmpC-type enzymes are significant contributor to beta-lactams resistance in Gram-negative and are often encoded by resident chromosomal genes mostly in *Enterobacteriaceae* (e.g *E. coli, Citrobacter freundii, Enterobacter* spp., *Yersinia enterocolitica, Morganella morganii*) and other Gram-negative organisms (e.g *P. aeruginosa, Aeromonas* spp., *A. baumannii)* while others are encoded by genes associated with mobile DNA elements that can be acquired by horizontal gene transfer. At low production levels, they can confer resistance to all beta-lactam except carbapenem and zwitterionic oxyimino-cephalosporins (such as cefepime). However they can also show hydrolyzing capacities toward other beta-lactams when produced at high levels. AmpC type beta-lactamases (ACBL) expression is inducible at low level by various beta-lactam antibiotics (strong inducers include cefoxitin, imipenem, clavulanate), while in bacteria showing an acquired ACBL, such as *Salmonella* spp. and *K. pneumoniae*, the production of such enzymes is often (Jacoby GA, 2009). The most common causes of AmpC overexpression in clinical isolates are

mutations in regulatory genes (*ampD, ampE, ampR*) or in the promoter region; intriguingly, there are reports on the *ampC* hyperexpression in *A. baumannii* due to an upstream insertion of an IS that can possibly provide a strong promoter (Corvec S, 2003). The AmpC-type beta-lactamases can be also plasmid located and they have been found worldwide both in nosocomial and non-nosocomial isolates. Most plasmid-borne *ampC* genes are not inducible, and overproduction of AmpC in resistant strains likely results consequently to alteration or mutation in the promoter region. Plasmid encoded AmpC cephalosporinases are closely related in sequence to the chromosomal AmpC enzymes of *E. cloaceae, C. freundii* or *Aeromonas* spp. from which they have been mobilized, and are grouped at least in six different lineages (Tab. 2).

Tab. 2. Major plasmid-encoded AmpC-type β-lactamases (Adapted from Rossolini GM, 2006).

Enzyme Lineage	Geographical distribution	Hosts	Gene source
CMY/LAT	AF/ME, AU, EU, FE, NA	*Escherichia coli* *Klebsiella* spp. *Proteus mirabilis* *Salmonella enterica* *Enterobacter aerogenes*	*Citrobacter freundii*
DHA	AF/ME, EU, FE, NA	*Escherichia coli* *Klebsiella* spp. *Proteus mirabilis* *Salmonella enterica* *Enterobacter aerogenes*	*Morganella morganii*
ACC	AF/ME, EU, NA	*Escherichia coli* *Klebsiella pneumoniae* *Proteus mirabilis* *Salmonella enterica*	*Hafnia alvei*
CMY/FOX	EU/FE	*Escherichia coli* *Klebsiella* spp. *Enterobacter cloacae*	*Aeromonas* spp.?
FOX	EU, NA, SA	*Escherichia coli* *Klebsiella* spp. *Enterobacter cloacae*	*Aeromonas caviae*
ACT/MIR	FE, NA	*Escherichia coli* *Klebsiella pneumoniae*	*Enterobacter* spp.

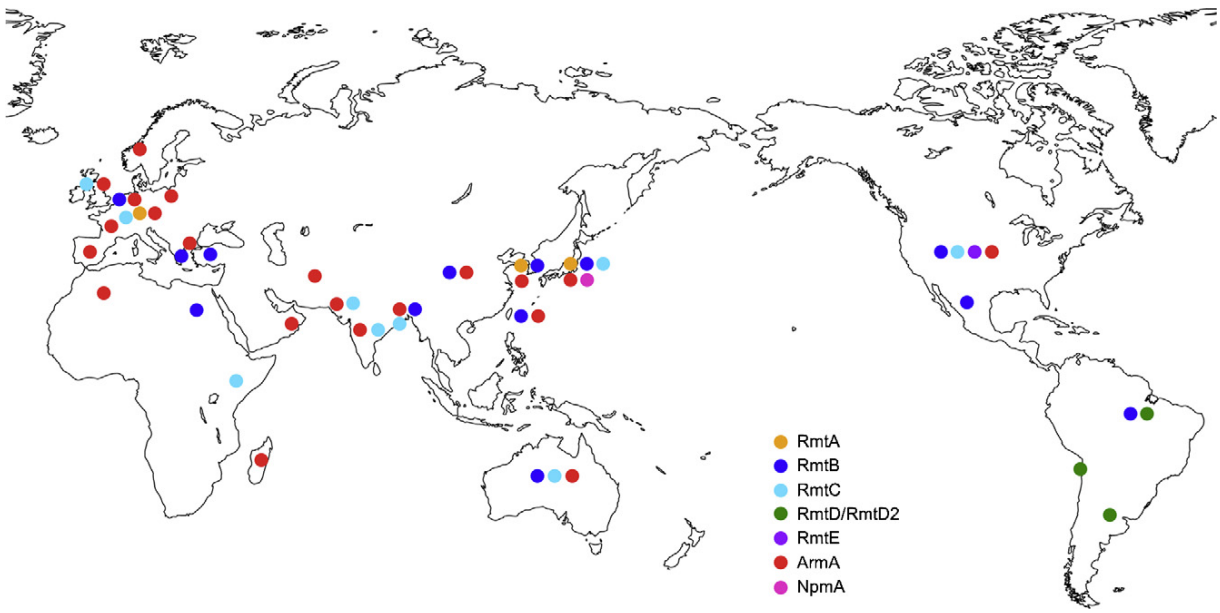
AF/ME: Africa and/or Middle East; AU: Australia; EU: Europe; FE: Far East; NA: North America; SA: South America.

RESISTANCE TO AMINOGLYCOSIDES: 16S RRNA METHYLTRANSFERASES

Despite the development of new beta-lactams and fluoroquinolones, aminoglycosides are still used for the treatment of severe infections caused by gram-negative organisms. Bacterial resistance to these drugs, which act by impairing bacterial protein synthesis through binding to prokaryotic ribosomes, has been reported since their introduction into clinical use. Like other antimicrobial resistance mechanisms, the modes of aminoglycoside resistance in bacteria are

divided into (i) enzymatic modification/inactivation of amino-glycosides, (ii) mutation or modification of aminoglycoside-binding site in target molecule, (iii) decreased permeability of aminoglycosides across bacterial membranes, and (iv) augmented efflux of aminoglycosides from cytosol to outside. Among them, the most prevalent and clinically relevant mechanism of aminoglycoside resistance in both Gram-negative and Gram-positive bacteria is inactivation of the agents by aminoglycoside-modifying enzyme. Based on their molecular mechanisms, the aminoglycoside-modifying enzymes are further divided into 3 groups, acetyltransferases (AACs), nucleotidyltransferases (ANTs), and phosphotransferases (APHs). They are further divided into subclasses that are based on the site of modification and the spectrum of resistance within the class of antimicrobials (Ramirez MS, 2010; Doi Y, 2007). Recently, methylation of the aminoacyl site of the 16S rRNA has been described as an acquired high-level resistance mechanism to clinically important aminoglycosides such as amikacin, tobramycin and gentamicin. Since 2003, eight 16S rRNA methyltransferase genes *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, *rmtF* and *npmA* have been identified in several species of pathogenic bacteria worldwide, being reported in most cases from human clinical isolates (Wachino J, 2012)(Fig. 7).

Fig. 7. Worldwide distribution of aminoglycoside resistance 16S rRNA methyltransferase (Wachino J, 2012).



16S rRNA methyltransferase genes were mostly located on transferable plasmids, and could be easily transferred to other bacterial species. Moreover, the number of 16S rRNA methyltransferase-producing Gram-negative bacteria with virulence potential isolated from human and livestock have gradually increased (Doi Y, 2007).

In recent years, the global spread of 16S rRNA methyltransferase producers has been a concern in association with the rapid worldwide dissemination of the members of *Enterobacteriaceae* that produce NDM-1 metallo-beta-lactamase (MBL), because these two enzymes are often coproduced (Wachino J, 2012; Hidalgo L, 2013).

RESISTANCE TO FLUOROQUINOLONES

Fluoroquinolones are potent, broad-spectrum antibiotics that have been used in medical practice for the treatment of severe or resistant infections since the late 1980. Quinolones are synthetic compounds that enter in bacteria cells by passive diffusion through the transmembrane porins. Fluoroquinolones target DNA gyrase and topoisomerase IV with varying efficiency in different bacteria and inhibit their control of supercoiling within the cell, resulting in impaired DNA replication (at lower concentrations) and cell death (at lethal concentrations). The progenitor of quinolones is nalidixic acid, a drug with limited spectrum to *Enterobacteriaceae,* mainly used for urinary tract infections. Later, new drugs have been developed, and fluoroquinolones (e.g. ciprofloxacin, norfloxacin, levofloxacin, gatifloxacin, moxifloxacin), which differ from quinolones by the addition of a fluorine atom, showed a wider action spectrum against also to *P. aeruginosa*, Gram-positive and mycobacteria (Redgrave LS, 2014). Moreover, some fluoroquinolones, being active against eukaryotic targets, have been studied as a possible novel antiparasitic treatment or explored as potential antineoplastic agents and finally, they have been extensively used in veterinary practice (Ruiz J, 2012). The selective pressure generated by the huge use of these drugs has favored the onset of resistance.

Resistance to fluoroquinolones could be due to a mutational chromosomal mechanisms that cannot be horizontally transferred within a bacterial population: i) accumulation of mutations in the genes encoding DNA gyrase and topoisomerases IV, ii) upregulation of efflux systems and/or downregulation of outer membrane porins, decreasing the intracellular drug accumulation.

However various genes encoding different resistance mechanisms and on mobile genetic elements can decrease susceptibility to quinolone or fluoroquinolone antibiotics; these are often encoded

on plasmids well known as plasmid-mediated quinolone resistance (PMQR) genes (Redgrave LS, 2014).

The first mechanism described was the acquisition of *qnr* genes encoding proteins that bind and protect DNA gyrase and topoisomerase IV from inhibition by fluoroquinolones. Six different lineages of Qnr proteins have been described so far: QnrA, QnrB, QnrS QnrC, QnrD and, more recently QnrVC. The second mechanism is represented by the expression of AAC(6')-Ib-cr, a bifunctional variant of a common acetyltransferase actives on aminoglycosides and also able to acetylate those fluoroquinolones with an amino nitrogen on the piperazinyl ring, such as ciprofloxacin and norfloxacin.

The most recently described fluoroquinolone transferable resistance mechanism consists in a decreased intracellular drug accumulation mediated by two different plasmid mediated efflux pumps: i) QepA, active only on hydrophilic fluoroquinolones (e. g. norfloxacin and ciprofloxacin) and ii) OqxAB, with a wide substrate specificity including not only quinolones (ciprofloxacin, flumequin, norfloxacin, and nalidixic acid), but also chloramphenicol, and trimethoprim (Jacoby GA, 2014). Acquisition of high level resistance to fluoroquinolones in *Enterobacteriaceae* appears to be a multifactorial and multi-step process. In fact, although each single PMQR determinant by itself produces only low level of resistance, its presence seems to facilitate the development of mutations in *gyrA* and *parC* genes, responsible for high level resistance.

Hence, the prevalence of PMQR genes is an important indicator for monitoring fluoroquinolone resistance in *Enterobacteriaceae* (Tab. 3)(Jacoby GA, 2014).

Tab. 3. Summary of the impact of different resistance mechanisms on susceptibility to ciprofloxacin (Redgrave LS, 2014).

Resistance mechanism	Fold change in ciprofloxacin MIC
Gram-negative species[a]	
Topoisomerase substitutions	
gyrA	10–16
parC	0
gyrA (× 2) + *parC*	60
Permeability changes	
Efflux upregulation	4–8
Porin loss	4
PMQRs	
Carriage of *qnr* alleles	>30
Carriage of *qepA*	32
Carriage of *oxqAB*	16
Carriage of *aac(6′)Ib-cr*	4

RESISTANCE TO CARBAPENEMS

Carbapenem-resistant *Enterobacteriaceae* (CRE, mostly contributed by *K. pneumoniae*) represent the most recent and worrisome issue concerning antibiotic resistance.

Carbapenems are considered antimicrobial drugs of "last-resort" for the treatment of severe infections but the overuse of these molecules to fight the emergence of ESBL-producing isolates has led to the selection, especially in nosocomial settings, of pathogens producing carbapenemases. This emergence damages the clinical efficacy of carbapenems, making the future of beta-lactam antibiotics bit uncertain. Colistin, tigecycline and certain aminoglycosides may still retain activity *in vitro* but have insufficient clinical efficacy and higher levels of toxicity than the standard treatment (Tängdén T, 2015).

Two are the main mechanisms responsible for acquired carbapenem resistance in *Enterobacteriaceae*: i) reduced outer membrane permeability by porin alterations in combination with the production of an ESBL or AmpC-type beta-lactamase, (associated with a low-level carbapenem resistance and is not transferable), ii) production of a beta-lactamase with carbapenemase activity, responsible of higher-level carbapenems resistance and it is a transferable mechanism (Rossolini GM, 2014).

Currently, there are at least four types of carbapenemases that are spreading among *Enterobacteriaceae* worldwide, including the KPC and OXA-48 serine carbapenemases (of molecular class A and D, respectively) and the MBLs of the VIM and NDM types, which are structurally and mechanistically different among each other (Tab. 4).

Tab. 4. Commonly detected carbapenemases in *Enterobacteriaceae* (Tängdén T, 2015).

Carbapenemase	Molecular class	Spectrum of hydrolysis	Common species	Geographical epicentres
KPC	A	Cephalosporins and carbapenems	Mainly *Klebsiella pneumoniae*	USA, Greece, Italy, Israel, China
VIM	B	Cephalosporins and carbapenems	Mainly *K. pneumoniae*	Greece
NDM	B	Cephalosporins and carbapenems	*K. pneumoniae* and *Escherichia coli*	Indian subcontinent, Balkans, Middle East
OXA-48	D	Carbapenems	*K. pneumoniae* and *E. coli*	North Africa, Middle East, some Western European countries

KPC, *K. pneumoniae* carbapenemase; NDM, New Delhi metallo-β-lactamase.

5

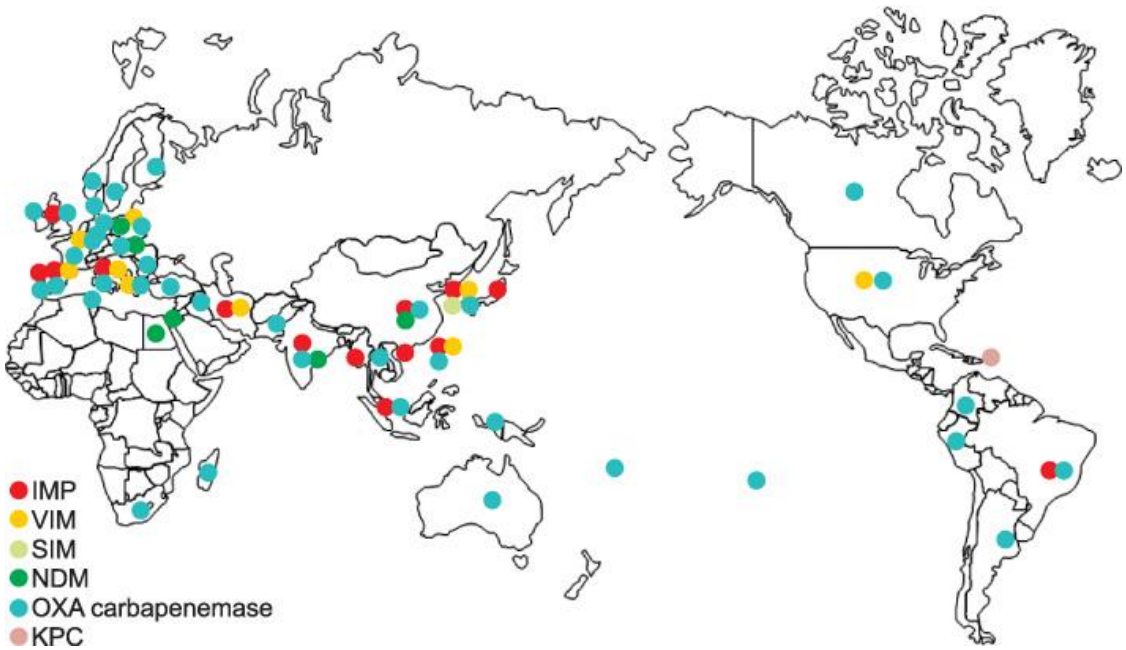
CARBAPENEM-RESISTANT *ACINETOBACTER BAUMANNII*

A. baumannii, a Gram-negative coccobacillus, has emerged as nosocomial pathogen and, in recent years, has also been associated to community-acquired infections, becoming a public health problem of major concern in several countries.

The extensive use of carbapenems, has determined the emergence of carbapenem-resistant *A. baumannii* (CRAb), which usually represent an important clinical threat due to their resistance phenotype usually extending to most or all available antibiotics (Dijkshoorn L, 2007).

Carbapenem resistance in *A. baumannii* could be due to non-enzymatic mechanisms (such as the modification of penicillin-binding proteins and porins or with upregulation of the AdeABC efflux system) or to the expression of metallo-beta-lactamases (MBLs) (such as VIM, IMP, NDM) or, more frequently, to the acquisition of OXA-type carbapenemases (Pogue JM, 2013)(Fig. 8).

Fig. 8. Worlwide dissemination of carbapenemase in *A. baumannii*.



These beta-lactamases represented the most widespread carbapenemases in *A. baumannii*, including the acquired OXA-23, OXA-24, OXA-58, and the intrinsic OXA-51 enzyme. The significant contribution of OXA-type carbapenemases in *A. baumannii* has been emphasized when bla_{OXA} genes are associated with IS*Aba* insertion sequences, which provide strong promoters for their expression. IS*Aba1* play also a key role in the upregulation of chromosomal AmpC beta-lactamase, contributing to the expanded-spectrum cephalosporin resistance (Pogue JM, 2013).

In Latin America OXA-type carbapenemase-producing *A. baumannii* is widely diffused and OXA-58 and OXA-23 are the most described (Labarca JA, 2014).

6

ANTIBIOTIC RESISTANCE IN *STAPHYLOCOCCUS AUREUS*

S. aureus is both a commensal bacterium and a human pathogen. Approximately 30% of the human population is colonized with *S. aureus*, at the level of anterior nares, mouth, groin, vagina and gastrointestinal tract. However, it is also a major human pathogen causing bacteremia and infective endocarditis as well as osteoarticular, skin and soft tissue, pleuropulmonary, and device-related infections (Tong SYC, 2015).

S. aureus can also be responsible for outbreaks of food poisoning and its versatility as pathogen also extends to host range, which includes a lot of animals (Peacock SJ, 2015).

Whereas *S. aureus* is the most significant cause of human disease, several other species belonging to *Staphylococcus* genus are clinically relevant, often referred to collectively as coagulase-negative staphylococci to differentiate them from the coagulase-producing *S. aureus* (although a small number of non–*S. aureus* species do produce coagulase), these species include *Staphylococcus epidermidis*, *Staphylococcus lugdunensis*, *Staphylococcus haemolyticus*, and *Staphylococcus schleiferi.*

The past two decades have witnessed two clear shifts in the epidemiology of *S. aureus* infections: first, a growing number of health care-associated infections, particularly seen in infective endocarditis and prosthetic device infections, and second, an epidemic of community-associated skin and soft tissue infections driven by strains with certain virulence factors and resistance to beta-lactam antibiotics.

Resistance to beta-lactam compounds represents the major resistance trait in *S. aureus*. The so called methicillin-resistant *S. aureus* (MRSA) emerged in the early 1960s and now it is seriously threatening the clinical outcomes of staphylococcal infections (Tsubakishita S, 2011; Stryjewski ME, 2014).

The MRSA phenotype is due to the expression of modified penicillin-binding proteins (PBPs), encoded by the horizontally acquired *mec* genes (*mecA* and *mecC*), which take over the functions of the resident staphylococcal PBPs and are not inhibited by conventional beta-lactams (Hiramatsu K, 2013).

The origin of *mecA* has been dimostrated to be an intrinsic component of the chromosome of *Staphylococcus fleuretti,* one of the oldest staphylococcal species. Thus, the *mecA* gene has been transmitted vertically during the initial steps of staphylococcal speciation, and later has been lost,

as exemplified by the emergence and diffusion of methicillin-susceptible *S. aureus* (MSSA). After the introduction of methicillin in the clinical practice, *mec* genes would have jumped again on *S. aureus*, giving rise to MRSA (Hiramatsu K, 2013).

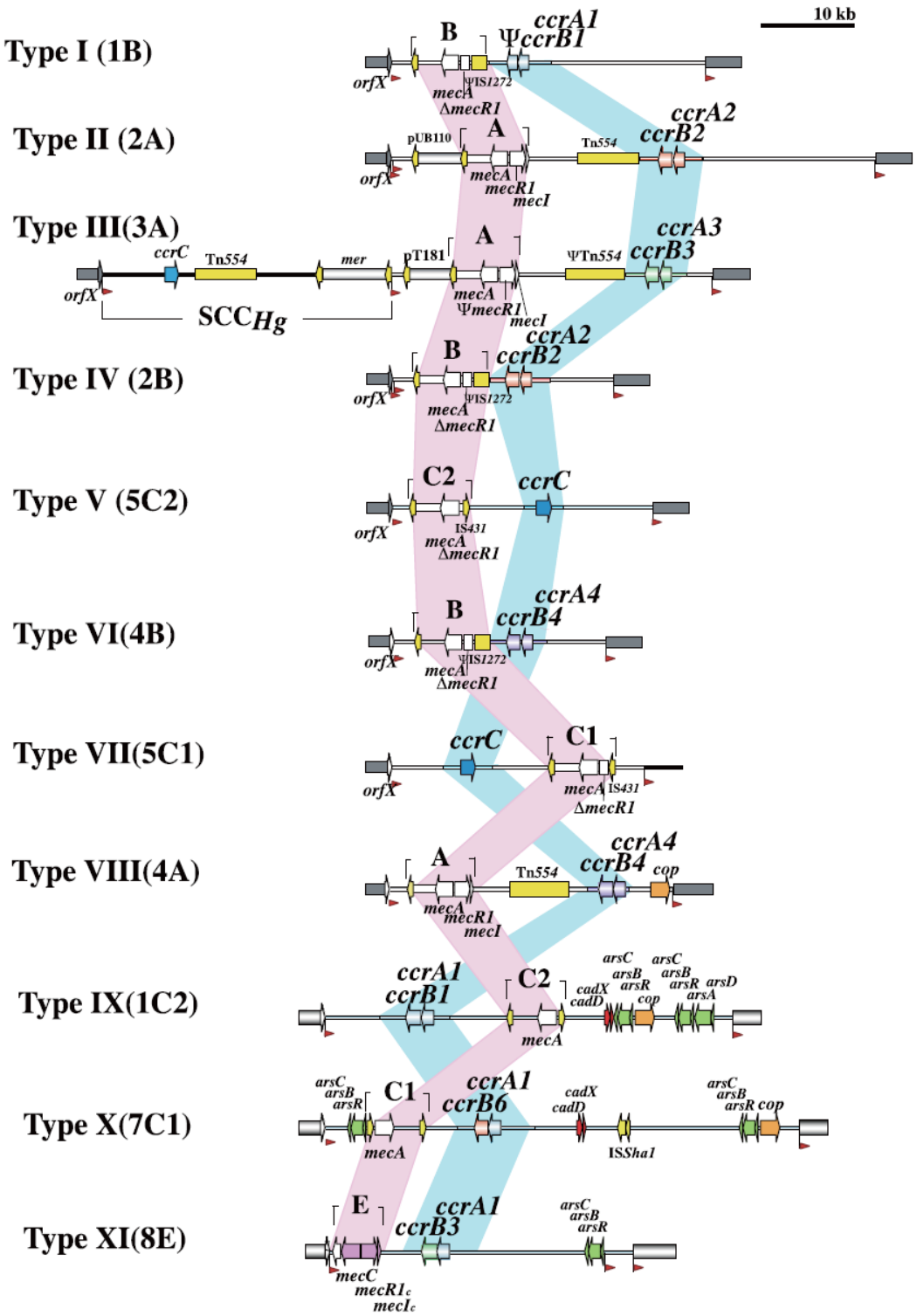
Following the whole-genome sequencing of a phenotypically resistant but *mecA*-negative MRSA strain from bovine mastitis in England, and originally called *mecA*LGA251, was discovered an allele that shares 69% homology with *mecA* and is not detected by *mecA*-based PCR or PBP2a slide agglutination. The designation *mecC* was chosen because another *mecA* variant had already been described in *Macrococcus caseolyticus* (Tsubakishita S, 2010) and was named *mecB* to reflect the order of discovery. *mecC* MRSA strains are relatively rare. Mutagenesis and cloning experiments have confirmed that *mecC* confers methicillin resistance in different *S. aureus* strain backgrounds, but recombinant PBP2a*mecC* has a higher affinity for oxacillin than for cefoxitin, whereas PBP2a*mecA* shows less difference between the two beta-lactams. As with *mecA*, *mecC* is encoded within a SCC*mec* element and the detection of *mecC* in other species of staphylococci (*Staphylococcus xylosus*, *Staphylococcus stepanovicii*, *Staphylococcus sciuri*, and *Staphylococcus saprophyticus)* indicates that, as with *mecA*, *mecC* may also originate in coagulase-negative staphylococci (Peacock SJ, 2015; Paterson GK, 2014).

There are still a number of drugs which retain activity against MRSA, including the glycopeptides (e.g. vancomycin and teicoplanin), linezolid, tigecycline, daptomycin and even some new beta-lactams, such as ceftaroline and ceftobiprole, that are active against the modified PBPs responsible for the methicillin-resistant phenotype. Although resistance to any of these drugs has been reported, the resistance rates remain overall very low, while XDR MRSA strains have not been consistently reported (Rossolini GM, 2014).

SCC*MEC*: THE VEHICLE OF *MEC* GENES

Sequencing of the region containing *mecA* revealed a distinct mobile genetic element named the staphylococcal chromosome cassette (SCC*mec*) that is present in MRSA but absent in MSSA. SCC*mec* elements are highly diverse, with 11 types (I to XI) recognized to date (Hiramatsu K, 2013)(Fig. 9).

Fig. 9. Representation of the eleven types of SCC*mec.* (Hiramatsu K, 2013).



Despite their diversity in size and gene content, they all share important defining characteristics. In all cases, SCC*mec* is integrated into the *S. aureus* genome at an *attB* integration site sequence present at the 3'end of the *orfX* gene.

The second feature shared among the SCC*mec* elements is that they contain a *mec* gene complex comprising *mecA* and its regulatory genes *mecI* and *mecR* (although *mecI* and *mecR* are not intact in some SCC*mec* classes), a cassette chromosome recombinase (*ccr*) gene complex containing one or two site-specific recombinase genes responsible for movement of the SCC*mec*, and typically three J regions. Originally designated junkyard regions due to the presence of pseudogenes and truncated copies of transposons and insertion sequences, these J regions are now commonly referred to as joining regions because they can encode important functions such as resistance to additional antibiotics and to heavy metals.

The third shared feature of SCC*mec* elements is their demarcation by specific inverted repeats and direct repeats containing the insertion site sequence recognized by the *ccr*-encoded recombinases. SCC*mec* typing is widely used for epidemiological surveillance of MRSA and classifies SCC*mec* elements on the basis of their combination of *mec* gene and *ccr* gene complexes, with further subtyping based on the J regions. Two distinct *ccr* complexes have been described to date. The first comprises *ccrA* and *ccrB*, and the second consists of a single *ccrC* gene. The combination of *ccrA* and *ccrB* defines the *ccr* gene complex type, designated as type 1 (*ccrA1B1*), type 2 (*ccrA2B2*), type 3 (*ccrA3B3*), type 4 (*ccrA4B4*), type 7 (*ccrA1B6*), or type 8 (*ccrA1B3*). In contrast, reported *ccrC* variants are very similar, and only one allotype of *ccrC1* has been defined so far that constitutes *ccr* gene complex type 5. In addition to *mecA* and its regulatory genes, the *mec* gene complex includes associated insertion sequences; currently, five *mec* gene complex types are recognized largely on the basis of the presence and location of these insertion sequences (Peacock SJ, 2015).

HA-MRSA, CA-MRSA AND LA-MRSA

For a long time MRSA have been limited to hospital setting (health-care-acquired MRSA, HA-MRSA) where they affected old and/or debilitated patients. HA-MRSA typically show a multidrug-resistance phenotype and are associated with SCC*mec* I-III elements and no production of the virulence factor called Panton-Valentine leukocidin toxin (PVL).

During the last two decades MRSA lineages, unrelated to the existing HA-MRSA strains, have widespread also in community (community-acquired MRSA, CA-MRSA) generally affecting young

healthy people. CA-MRSA are characterized by an overall susceptible phenotype resembling MSSA features, are usually associated with SCC*mec* IV-V elements, and are particularly virulent due to the production of PVL toxin (Pantosti A, 2009).

Nevertheless, the distinction between CA-MRSA and HA-MRSA is beginning to break down and USA300 clone cloud be considered the most emblematic example. In recent year, USA300 clone, which is firstly associated to community-acquired infections has rapidly contributed to an increasing number of hospital-acquired infections, replacing the distinction between CA-MRSA and HA-MRSA in hospital- and community-acquired infections respectively (Otter JA, 2011).

In addition to HA-MRSA and CA-MRSA, recent attention has begun to focus on livestock-associated (LA-) MRSA, typically exhibiting a tetracycline and cotrimoxazole resistance phenotype and harbouring types V SCC*mec* element (Ulheman AC, 2014).

7

THE ROLE OF EPIDEMIC PLASMIDS AND HIGH-RISK CLONES IN THE SPREAD OF MULTIDRUG-RESISTANT *ENTEROBACTERIACEAE*

One of the most urgent areas of antimicrobial drug resistance is the rapid evolution of fluoroquinolone, cephalosporin, and carbapenem resistance among *Enterobacteriaceae*, which has spread globally during the last decade. This phenomenon is mainly due to the global dissemination of international clones and successful plasmids responsible for the diffusion of antimicrobial determinants (Mathers AJ, 2015).

PLASMIDS

Plasmids are defined as double stranded, extra-chromosomal genetic elements able to replicate independently from the chromosome of the host cell. They show an enormous diversity of characteristics, such as size, modes of replication and transmission and host ranges, and they are able to impart a number of phenotype in regard to the repertoire of genes that carry. A basic, general structure can be identified for all plasmids, including an essential backbone of genes involved in replication and maintenance functions; moreover, a considerable variety of accessory genes, of which presence mainly depends on the plasmid type, can be carried by these elements (Carattoli A, 2009).

Plasmids are considered the key vectors of horizontal gene transfer among bacteria of different species, genera and kingdoms, depending on their narrow or broad host range, conjugative properties and efficiency of conjugation.

The ability to recognize and categorize plasmids in homogeneous groups on the basis of their phylogenetic relatedness can be helpful to analyse their distribution in nature, the relationship with the host cell and to discover their evolutionary origin.

In 1971 Datta and Hedges proposed a plasmid classification scheme based on the stability of plasmids during conjugation, a phenomenon called plasmid incompatibility (Inc), defined as the inability of two plasmids belonging to the same Inc group to be propagated stably in the same cell (Carattoli A, 2011). Different strategies have been adopted to recognize and categorize plasmids, such as the original classification based on the stability during conjugation or based on Southern

blot hybridization, using cloned replication regions (replicons) as probes (Couturier M, 1988; Datta N, 1971).

Since 2005, PCR-Based Replicon Typing (PBRT) scheme has been available, targeting in multiplex PCRs the replicons of the major plasmid families occurring in *Enterobacteriaceae*, (A/C, B/O, *colE*, FIA, FIB, FIC, FV, FIIk, HI1, HI2, I1, K, L/M, N, P1α, Q1, U, W, X1, X2, X3 and X4) (Carattoli A, 2005; Johnson TJ, 2012).

As this classification is currently based on known Inc groups and could therefore fails to identify different or novel replicons, the most accurate method to characterize a plasmid is clearly based on the determination of the full length DNA sequence. Together with other specific characteristics of the bacterial strain (i.e. resistance gene content, sequence type by Multi-Locus Sequence Typing (MLST), phylogroup, serotype, etc.), the replicon content is currently used as an additional marker for comparative analysis of unrelated and related strains during epidemiological investigations.

Antimicrobial resistance plasmids can broadly be divided into 2 main groups, namely, the narrow-host-range group, which most often belongs to incompatibility group F (IncF), and the broad-host range group, which belongs to the IncA/C, IncL/M, and IncN. Broad-host-range plasmids can easily be transferred between different species, while narrow-host-range plasmids tend to be restricted to certain species or even clones within species. This concept is important when evaluating plasmids in high-risk clones, as there is a predominance of narrow-range plasmids (specifically IncF with certain beta-lactamases; e.g. $bla_{\text{CTX-M-15}}$, $bla_{\text{KPC-2}}$, and $bla_{\text{KPC-3}}$) in these clones. These plasmids have recently been termed “epidemic resistance plasmids” due to their propensity to acquire resistance genes and rapid dissemination among the *Enterobacteriaceae* (Mathers AJ, 2015).

COLE PLASMIDS

The ColE superfamily includes plasmids found both in Gram-negative and Gram-positive bacteria. ColE1 plasmid, considered the prototype, is a small, multicopy, colicinogenic plasmid, originally found in *E. coli* (Francia MV, 2004).

It is 6,650 bp in size and consists of: i) a region involved in DNA replication and copy number regulation; ii) a 250 bp *cer* site for the Xer system, which is the site-specific recombination system catalyzing conversion of plasmid dimers into monomers; and iii) an immunity region encoding for colicin E1, a bactericidal polypeptide released into the environment by ColE1-harbouring cells to compete with other bacterial strains for an ecological niche (Chan PT, 1985).

ColE1 are non-conjugative plasmids, but they can be mobilized by members of many incompatibility groups, including IncI, IncFI, IncW and IncP. The mobilization machinery consists of *mob* genes encoding relaxosome components and the plasmid origin of transfer (*oriT*) (Francia MV, 2004).

The mobilization and the spreading of ColE-like plasmids are an issue of concern. In fact, ColE-like plasmids harbouring genes responsible for resistance to clinically relevant antibiotics, such as extended-spectrum cephalosporins (CTX-M-type ESBL), fluoroquinolones (*qnr* genes) and carbapenems (bla_{KPC}), have been reported in MDR enterobacteria worldwide (Carattoli A, 2009).

INCF PLASMIDS

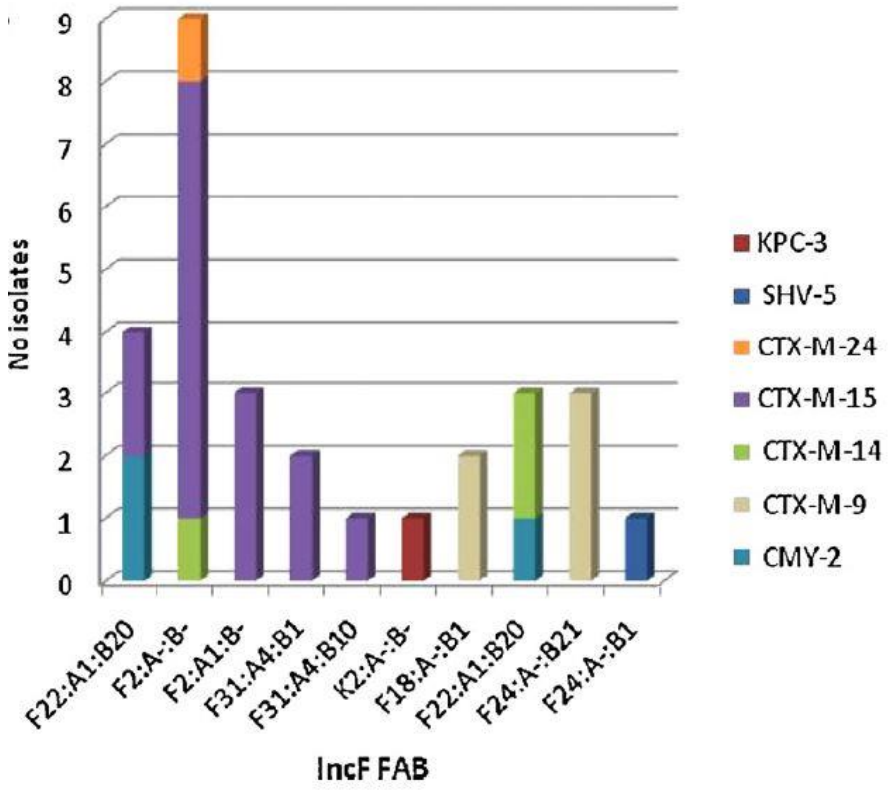
Plasmids belonging to incompatibility group F (IncF) are defined as narrow-host-range because are limited by host range to the family *Enterobacteriaceae*, are usually low copy number plasmids, >100 kb in size, and often carry more than one replicon promoting the initiation of replication (Villa L, 2010).

IncF plasmids are often present in *E. coli*, and up to 70% of plasmids characterized in human and avian *E. coli* isolates belonged to IncF with different replicons. Moreover IncF plasmids use post-segregational killing and addiction systems to ensure their propagation among high-risk clones (Mathers AJ, 2015).

The multi-replicon status can allow the acquisition of plasmids carrying incompatible replicons when replication is driven by a compatible one. The replicon that is not responsible for the control of replication can undergo a succession of genetic alterations. The classic multi-replicon IncF plasmid contains the FII replicon (also designated as FIIA) regulated by CopA, a constitutively synthesized 90 nt antisense-RNA, which is normally silent, and FIA and FIB replicons, which function only in enterobacteria and are regulated by iterons, in cis-negative binding sites of the replication protein RepA (Villa L, 2010).

The IncF plasmids have been associated with the abrupt worldwide emergence of clinically relevant ESBL, such as CTX-M-15, but also with the spread of plasmid-mediated AmpC genes (bla_{CMY} and bla_{DHA}) and quinolone and aminoglycoside resistances encoded by the genes *aac(6')-Ib-cr*, *qnr*, *qepA*, *armA* and *rmtB*. (Carattoli 2011)(Fig. 10).

Fig. 10. Distribution of the major plasmid IncF *Sequence Types* and their association to relevant beta-lactamase genes (Carattoli 2011).



HIGH-RISK CLONES

A 'successful' bacterial strain is an extremely effective vehicle for the dissemination of antibiotic resistance determinants because all of the hosted resistance elements are transmitted vertically (i.e. from mother to daughter cells) by virtue of the strain's spread and its increasing prevalence and because a successful strain has multiple opportunities to act as a donor and to transfer its resistance elements horizontally to other strains, species or genera (Woodford N, 2011).

International multidrug-resistant high-risk clones have a global distribution and can remain viable for prolonged time periods in diverse areas. High-risk clones have acquired certain adaptive traits that increase their pathogenicity and survival skills, which is accompanied by the acquisition of antibiotic resistance determinants. These clones have the tenacity and flexibility to accumulate and then provide resistance and virulence genes to other isolates. High-risk clones have contributed to the spread of global multidrug resistance through the transmission of different

types of genetic platforms, including plasmids, and resistance genes among Gram-negative bacteria (Woodford N, 2011).

MLST has often been used to type several members of the *Enterobacteriaceae*, especially *E. coli* and *K. pneumoniae*. This genotyping method, which measures the DNA sequence variations in a set of housekeeping genes, characterizes strains by their unique allelic profiles using a numerical system (e.g., ST1 and ST2, etc.).

The pandemics caused by multidrug-resistant *E. coli* and *K. pneumoniae* are due mostly to the global dissemination of certain high-risk clones, namely, *E. coli* sequence type 131 (ST131) and *K. pneumoniae* ST258 (Mathers AJ, 2015).

ESCHERICHIA COLI SEQUENCE TYPE 131

E. coli sequence type 131 (ST131) is a recently emerged, extensively antimicrobial-resistant *E. coli* clonal group that has spread explosively throughout the world, driving the rapid increase in prevalence of antimicrobial resistance in *E. coli* (Fig. 11).

Fig. 11. Global dissemination of *E. coli* ST131 clone. Red stars indicate isolates producing ESBL enzymes, and blue stars indicate fluoroquinolone-resistant, non-ESBL-producing isolates (Nicolas-Chanoine MH, 2014).

E. coli ST131 was identified in 2008 as a major clone linked to the spread of the CTX-M-15 ESBL resistance. Since then, *E. coli* ST131 has also been strongly associated with fluoroquinolone resistance, and co-resistance to aminoglycosides and trimethoprim-sulfamethoxazole (Nicolas-Chanoine MH, 2014).

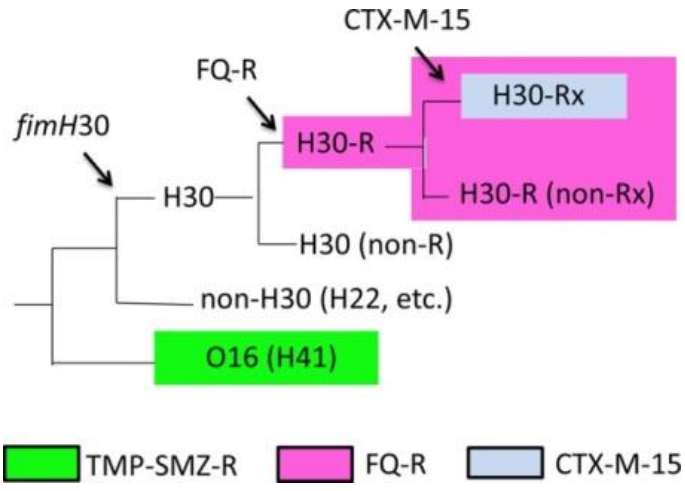
Alarmingly, strains of *E. coli* ST131 resistant to carbapenems have also been reported, further limiting treatment options for this clone (Petty NK, 2014).

E. coli ST131 belongs to phylogenetic group B2, and is considered to be truly pathogenic, due to the spectrum of infections they cause in both community and hospital settings and the large number of virulence-associated genes they contain (Banerjee R, 2013).

Although ST131 first came to attention because of its association with ESBL-producing *E. coli* strains, most ST131 isolates in many locales are ESBL negative, but resistant to fluoroquinolones, and often are coresistant to aminoglycosides and/or trimethoprim-sulfamethoxazole (Banerjee R, 2013).

The most prevalent subclone of ST131, called H30 because it contains the H30 variant of the type 1 fimbrial adhesin gene *fimH*, was identified initially through sub-ST analysis of over 1,000 historical and recent *E. coli* isolates (both ST131 and non-ST131) using a combination of typing strategies, including sequencing of *fimH*, *gyrA*, and *parC*, MLST (Banerjee R, 2014)(Fig. 12).

Fig.12. Schematic dendrogram of ST131 phylogeny reconstructed using whole-genome single nucleotide polymorphism analysis (Banerjee R, 2014).



Investigators observed that the H30 ST131 subclone comprised approximately half of all recent fluoroquinolones-resistant *E. coli* isolates from diverse locales and sources, compared with 1% of fluoroquinolones-susceptible isolates. The close genetic similarity of most H30 strains suggested that they all arose from a single *fimH*30-carrying ancestor. This indicated that the dramatic emergence of fluoroquinolones-resistant ST131 strains has been driven by clonal expansion and dissemination rather than by independent acquisition of fluoroquinolones resistance genes in heterogeneous strains (Banerjee R, 2014).

More recently, an important sublineage within H30, called H30-Rx because of its more extensive antimicrobial resistance profile, was identified by Price et *al*. using whole-genome phylogenetic analysis. These data indicate that the H30 ST131 lineage comprises a series of nested subclones, all derived from a single common fluoroquinolones susceptible H30 ancestor (Price LB, 2013)(Fig. 12).

PART II

AIM OF THE PHD PROJECT

The general objective of the PhD research project was to analyse the molecular epidemiology of emerging antibiotic resistance mechanisms in commensal and pathogenic bacteria in three countries of Latin America: Argentina, Bolivia and Peru.

The research study is part of the follow-up activities of the ANTRES Project (Towards Controlling Antimicrobial Use and Resistance in Low-income Countries—An Intervention Study in Latin America, ICA4-CT-2001-10014) and part of EvoTAR Project (Evolution and Transfer of Antimicrobial Resistance, HEALTH-F3-2011-2011- 282004), both funded by the European Commission. The first one was aimed at investigating antibiotic use and resistance in commensal *Escherichia coli* in two Latin American countries (Bolivia and Peru), the second is aimed at better explaining the evolution and transfer of antibiotic resistance.

In particular, the following specific topics are discussed in the PhD research:

- Analysis of the antimicrobial susceptibility and emerging resistance determinants in clinical isolates in the Bolivian Chaco and complete characterization of the F33:A-:B- RmtB-encoding plasmids.
- Characterization of a carbapenemase-producing *Acinetobacter baumannii* from Bolivia.
- Carriage rates and molecular epidemiology of MRSA in hospitalized patients from the Bolivian Chaco.
- Molecular basis of the emergence and dissemination of quinolone resistance in urban areas of Bolivia and Peru.
- Molecular epidemiology of acquired resistance genes in expanded-spectrum cephalosporin-resistant *Enterobacteriaceae* in Argentina.

PART III

RESULTS AND DISCUSSION

1

ANALYSIS OF THE ANTIMICROBIAL SUSCEPTIBILITY AND EMERGING RESISTANCE DETERMINANTS IN CLINICAL ISOLATES IN THE BOLIVIAN CHACO AND COMPLETE CHARACTERIZATION OF THE F33:A-:B- RMTB-ENCODING PLASMIDS

RELATED PUBLICATIONS:

- Bartoloni A, Sennati S, Di Maggio T, Mantella A, Riccobono E, Strohmeyer M, Revollo C, Villagran AL, Pallecchi L, Rossolini GM. Antimicrobial susceptibility and emerging resistance determinants (bla_{CTX-M}, *rmtB*, *fosA3*) in clinical isolates from urinary tract infections in the Bolivian Chaco. *Int J Infect Dis*. 2015 43:1-6.
- Sennati S, Riccobono E, Di Pilato V, Villagran AL, Pallecchi L, Bartoloni A, Rossolini GM. pHN7A8-related multiresistance plasmids ($bla_{CTX-M-65}$, *fosA3* and *rmtB*) detected in clinical isolates of *Klebsiella pneumoniae* from Bolivia: intercontinental plasmid dissemination?. *J Antimicrob Chemother*. *In press*.

Since very few data on the rates and molecular epidemiology of antibiotic resistance in bacterial pathogens have been reported in Bolivia (Salles MJ, 2013; Guzman-Blanco, 2014; Bonelli RR, 2014), we investigated the antimicrobial susceptibility and resistance determinants of bacterial pathogens responsible for urinary tract infections (UTIs) in the Bolivian Chaco region. The healthcare system of this region, constituted by many rural areas and native villages, depend on small hospitals with limited access to clinical microbiology facilities, which prevents any systematic collection of antimicrobial susceptibility data from the routine microbiological analysis of clinical specimens.

213 non-replicate clinical isolates were collected from June 2010 to January 2014 in the Hospital Basico Villa Montes (Villa Montes, Tarija Department) which is one of the first clinical microbiology

laboratories implemented in the Bolivian Chaco region. They were isolated from urines of both inpatients and outpatients with a clinical diagnosis of UTIs.

Bacterial identification, performed by MALDI-TOF, showed that 209 (98.1%) were *Enterobacteriaceae*, three (1.4%) were *P. aeruginosa* and one (0.5%) was *S. aureus*. (Tab. 5).

Tab. 5. Aetiology of UTIs in the Bolivian Chaco (2010-2014).

Species	No. of isolates	%
Escherichia coli	170	79.8
Klebsiella pneumoniae	19	8.9
Citrobacter spp	6	2.8
Enterobacter spp	5	2.3
Proteus spp	5	2.3
Morganella morganii	3	1.4
Pseudomonas aeruginosa	3	1.4
Providencia rettgeri	1	0.5
Staphylococcus saprophyticus	1	0.5

Antimicrobial susceptibility, tested by disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines, revealed high resistance rates among UTI isolates (Tab. 6).

Considering *E. coli* and *K. pneumoniae*, which were by far the most prevalent pathogens, the most affected drugs were trimethoprim-sulphamethoxazole, tetracycline, nalidixic acid, amoxicillin–clavulanic acid, and ciprofloxacin (Tab. 6). Expanded-spectrum cephalosporins remained active against the majority of isolates (87.3% and 90.5% susceptibility to cefotaxime and ceftazidime, respectively)(Tab. 6). Overall, the most active drugs were meropenem, fosfomycin, amikacin, and nitrofurantoin.

Tab. 6: Antibiotic susceptibility rates (%) of *Escherichia coli* and *Klebsiella pneumoniae* urinary isolates.

Drug[a]	*E. coli* (*n*=170)	*K. pneumoniae* (*n*=19)	Total (*n*=189)
AMC	56.5	47.3	55.6
Cefotaxime	89.4	68.4	87.3
Ceftazidime	92.3	73.7	90.5
Meropenem	100	100	100
SXT	26.5	52.6	28.6
Nalidixic acid	51.2	63.2	52.4
Ciprofloxacin	61.8	68.4	62.4
Gentamicin	72.4	68.4	72.0
Amikacin	95.3	89.5	94.7
Nitrofurantoin	91.8	31.6	85.7
Fosfomycin	98.2	94.7	97.9
Tetracycline	47.1	57.9	47.6

[a]AMC, amoxicillin-clavulanic acid; SXT, trimethoprim-sulphamethoxazole.

Despite a likely overestimation of resistance rates, because in Bolivia microbiological diagnosis represents an extra cost for the patient, and urine specimens for culture are rarely requested by physicians for the diagnosis of uncomplicated UTIs, the high resistance rates observed in clinical isolates from UTIs from the Bolivian Chaco were overall consistent with those reported from other Latin American countries (Salles MJ, 2013; Guzman-Blanco, 2014; Bonelli RR, 2014).

Among 24 isolates that were resistant to expanded-spectrum cephalosporins, screening and confirmatory tests for ESBL identified 22 ESBL producers, which were also subjected to molecular characterization (Tab. 7).

All ESBL producers were found to harbour *bla*$_{\text{CTX-M}}$ genes: CTX-M-1 group variants were the most prevalent (15 isolates, 68%), followed by CTX-M-9 group (7 isolates, 32%) and CTX-M-2 group (1 isolate, 5%), with 1 isolate harbouring variants from two groups (Tab. 7). CTX-M-15 and CTX-M-65 were the most prevalent variants and all four CTX-M-15-producing *E. coli* isolates belonging to phylogenetic group B2 were assigned to the pandemic clone ST131, with two isolates of hospital origin belonging to the H30-Rx subclone (Tab. 7).

Tab. 7. Phenotypic and molecular features of expanded-spectrum cephalosporin-resistant isolates from the Bolivian Chaco.

Isolate	Other resistance traits[a]	CTX-M group	CTX-M variant	Phylogenetic group[b]	Other relevant resistance genes
E. coli VM-72	NAL, CIP, CN, TET	1	CTX-M-15	B2 (H30-Rx ST131)	-
E. coli VM-292	NAL, CIP, CN, FOT, TET	9	CTX-M-65	D	*fosA3*
E. coli VM-77	SXT, NAL, CIP, CN, TET	9	CTX-M-14	D	-
E. coli VM-82	sxt, NAL, CIP, CN, TET	9	CTX-M-14	D	-
E. coli VM-334	SXT, NAL, CIP, CN, NIT, TET	9	CTX-M-65	A	-
E. coli VM-337	SXT, NAL, CIP, CN, NIT, TET	1	CTX-M-15	A	-
E. coli VM-353	FOT, TET	1	CTX-M-55	A	*fosA3*
E. coli VM-364	SXT, NAL, CIP, CN, ak, TET	1	CTX-M-15	B2 (non-H30 ST131)	*aac(6')Ib-cr*
E. coli VM-365	NAL, CIP, ak	1	CTX-M-15	A	*aac(6')Ib-cr*
E. coli VM-366	SXT, NAL, CIP, CN, TET	9	CTX-M-65	A	-
E. coli VM-379	SXT, NAL, CIP, CN, nit, TET	1	CTX-M-15	A	-
E. coli VM-439	SXT, NAL, CIP	1	CTX-M-15	B1	-
E. coli VM-444	SXT, NAL, CIP, CN, AK, TET	1	CTX-M-15	B2 (non-H30 ST131)	*aac(6')Ib-cr*
E. coli VM-474	SXT, NAL, CIP, CN	1	CTX-M-15	D	-
E. coli VM-517	SXT, NAL, CIP, CN, FOT, TET	1	CTX-M-55	D	*fosA3*
E. coli VM-498	NAL, CIP, ak, TET	1	CTX-M-15	B2 (H30-Rx ST131)	*aac(6')Ib-cr*
K. pneumoniae VM-249	SXT, NAL, CIP, CN, NIT, TET	1	CTX-M-15	nd	-
K. pneumoniae VM-354	SXT, NAL, CIP, CN, NIT, TET	1, 2	CTX-M-2, CTX-M-15	nd	-
K. pneumoniae VM-397	NAL, CIP, CN, AK, NIT, FOT, TET	9	CTX-M-65	nd	*fosA3*, *rmtB*
K. pneumoniae VM-419	SXT, NAL, CIP, CN, NIT, TET	1	CTX-M-15	nd	-
K. pneumoniae VM-466	SXT, NAL, CIP, CN, NIT	1	CTX-M-15	nd	-
K. pneumoniae VM-477	SXT, NAL, CIP, CN, AK, NIT, TET	9	CTX-M-65	nd	*rmtB*, *aac(6')Ib-cr*

[a] SXT, trimethoprim-sulphamethoxazole; NAL, nalidixic acid; CIP, ciprofloxacin; CN, gentamicin; AK, amikacin; NIT, nitrofurantoin; FOT, fosfomycin; TET, tetracycline. For antibiotics, upper and lower cases indicate a resistance or intermediate phenotype, respectively.

[b] Nd, not determined.

Interestingly, the urinary *E. coli* isolates analysed in this study exhibited a comparable prevalence of CTX-M producers (9% vs. 17%; p = 0.07) and a similar distribution of CTX-M variants (CTX-M- 15: 56% vs. 38%, p = 0.4; CTX-M-65: 19% vs. 27%, p = 0.8) as those of commensal *E. coli* collected in 2011 from healthy children living in the same urban area (Bartoloni A, 2013), emphasizing the role of commensal enterobacteria as a reservoir of clinically relevant resistance determinants.

Since resistance to aminoglycosides and fosfomycin were unusual in this setting, acquired 16S rRNA methyltransferases and glutathione S-transferases, which have been reported among enterobacterial isolates and often in association with beta-lactamases (Wachino J, 2012; Doi Y, 2007) were investigated in the isolates non-susceptible to these drugs.

The two CTX-M-65-producing *K. pneumoniae* were found to carry 16S rRNA methyltransferases RmtB, suggesting a possible genetic linkage between the two resistance determinants. The other amikacin-non susceptible isolates were found to carry *aac(6')-Ib-cr*, a variant of *aac(6')Ib*, capable to reduce susceptibility to both aminoglycosides and quinolones (Jacoby GA, 2014; Ramirez MS, 2010) (Tab. 7).

Results from the present study add some important information on the epidemiology of 16S rRNA methyltransferases in Latin America (where the dominant RmtD together with RmtB are the only detected)(Bonelli RR, 2014) and confirm the dissemination of *aac(6')-Ib-cr* in this setting and the frequent association with ESBL determinants (Jacoby GA, 2014; Pallecchi L, 2007).

Among the fosfomycin non-susceptible isolates four were identified as producers of glutathione S-transferases and all of them were found to harbor *fosA3* (Tab. 7). Furthermore all the *fosA3*-positive isolates were also CTX-M producers (Tab. 7), confirming the frequent association of *fosA3* with CTX-M determinants (Yang X, 2014; Wachino J, 2010).

This is the first study on the antimicrobial susceptibility of clinical isolates from UTIs of patients in the Bolivian Chaco region (one of the first from Bolivia), and for the first time, the antibiotic resistance determinants in enterobacteria responsible for UTIs have been characterized. These findings indicate an overall high resistance rates, with extremely high levels of resistance to trimethoprim-sulphamethoxazole and fluoroquinolones, and a sizeable presence of ESBL determinants in *Enterobacteriaceae*, suggesting that nitrofurantoin and fosfomycin might represent the first-line treatment options for uncomplicated UTIs in this region. Moreover the emergence of FosA3 and RmtB among ESBL producing isolates compromise the activity of fosfomycin and aminoglycosides which in a similar setting could be important options for complicated UTIs caused by multidrug-resistant pathogens.

Since 16S rRNA methyltransferases is a problem of major concern especially in low-resource countries, we focused the attention on the two *K. pneumoniae* carrying both $bla_{CTX\text{-}M\text{-}65}$ and *rmtB*. *K. pneumoniae* 397Kp and *Klebsiella pneumoniae* 477Kp, isolated from a 75-year-old female outpatient with liver failure and of a 35-year-old female inpatient with acute diarrhoea in June and December 2013 respectively, showed a multiresistance phenotype (Tab. 8).

Tab. 8. Minimum inhibitory concentrations (MICs) of *K. pneumoniae* 397Kp and *K. pneumoniae* 477Kp.

Antibiotic	MIC (mg/L) [a]	
	K. pneumoniae 397Kp	*K. pneumoniae* 477Kp
Ampicillin-sulbactam	>32/16	>32/16
Piperacillin-tazobactam	8/4	8/4
Cefotaxime	>4	>4
Ceftazidime	8	8
Cefepime	2	2
Imipenem	≤1	≤1
Meropenem	≤0.12	≤0.12
Gentamicin	>4	>4
Amikacin	>16	>16
Fosfomycin	>512	16
Ciprofloxacin	>2	>2
Levofloxacin	>4	>4
Nitrofurantoin	>64	>64
Trimethoprim-sulfamethoxazole[b]	≤0.5/9.5	>4/76

[a] MICs were determined by the broth microdilution method according to CLSI guidelines, except for fosfomycin which was tested by the agar dilution method as per CLSI.
[b] The difference observed in the resistance profile to trimethoprim-sulfamethoxazole between the two strains was apparently due to a resistance mechanism not encoded by the p477Kp plasmid.

Genotyping analysis, performed by MLST and pulsed-field gel electrophoresis (PFGE), revealed that *K. pneumoniae* 397Kp and *K. pneumoniae* 477Kp belonged to ST726, a sequence type recently associated with bloodstream infections in Taiwan (Yan JJ, 2015), and that they were possibly related.

Plasmids from *K. pneumoniae* 397Kp (p397Kp) and *K. pneumoniae* 477Kp (p477Kp) were transferred by conjugation at high frequency and were completely sequenced using Illumina next-generation sequencing (NGS) technology.

Plasmids p397Kp (76,863 bp) and p477Kp (74,768 bp) were F33:A-:B- carrying *rmtB*, $bla_{CTX\text{-}M\text{-}65}$, $bla_{TEM\text{-}1b}$ and only in p397Kp *fosA3* resistance genes. They showed 100% nucleotide identity, except that p477Kp lacked the fosfomycin resistance module (Fig. 13).

F33:A-:B- multiresistance plasmids are widespread in *E. coli* isolates from animals in China and pHN7A8, isolated from a dog in 2008 , is a representative of these plasmids (Hou J, 2012; Hou J, 2013; Yang X, 2014; He L, 2013).

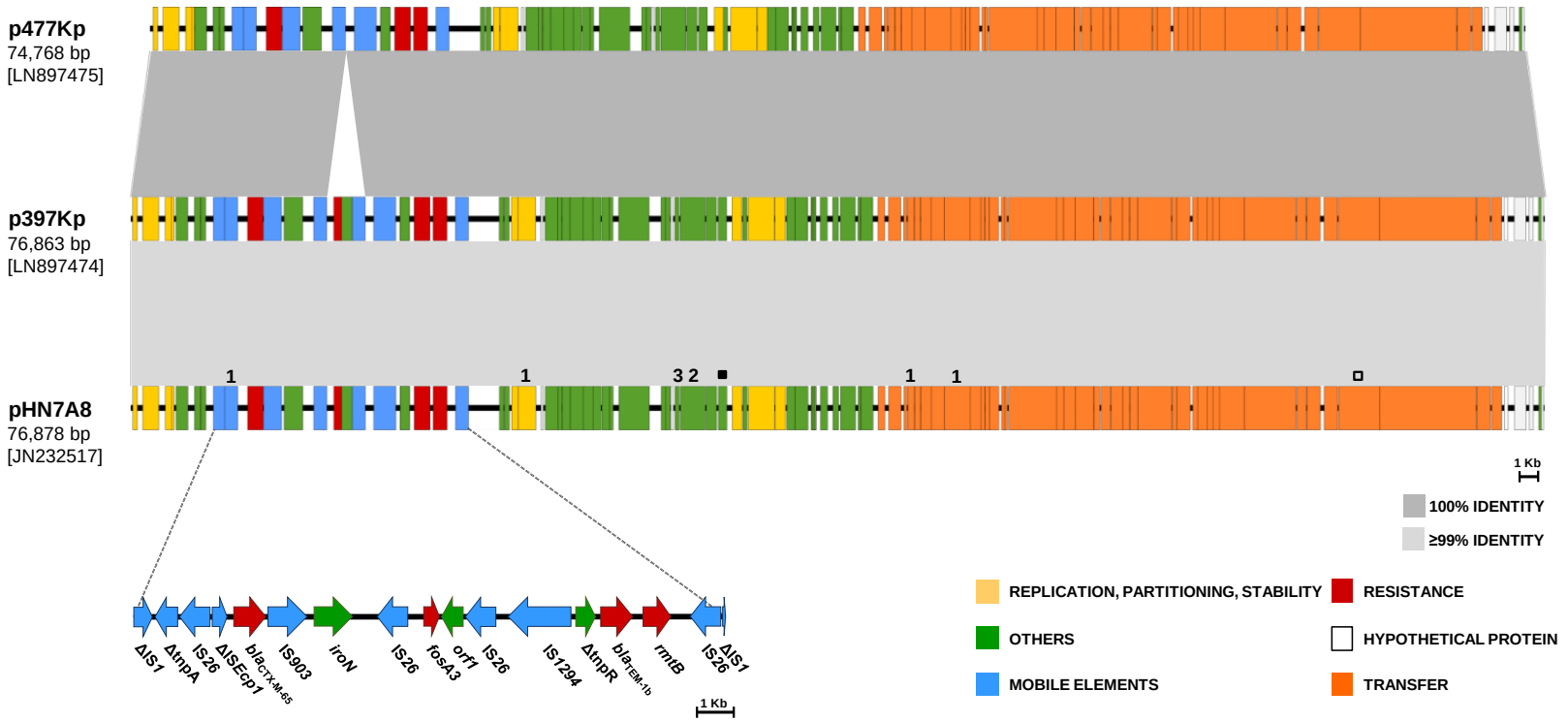
Interestingly p397Kp and p477Kp were found to be highly related to the Chinese epidemic plasmid, from which p397Kp differed only by nine scattered single nucleotide polymorphisms (of which two resulted in amino acid changes in *traA* and *traB*), by the absence of a 9-bp repeat in *traD*, and by the absence of a 6-bp repeat in *yddA* (Fig. 13).

To analyse the plasmids stability and to evaluate the possible loss of *fosA3* resistance module in p477Kp we maintained both plasmids for 60 generations in absence of selective pressure. No evidence of instability of the *fosA3* resistance module was observed and both plasmids were stably maintained.

These results showed that F33:A-:B- multiresistance plasmids, which were previously shown to have undergone an epidemic dissemination in China among *E. coli* strains infecting or colonizing animals, not only may spread also in the clinical setting, but could be responsible for an intercontinental dissemination.

Indeed, p397Kp and p477Kp are the first representatives of such plasmids identified in human clinical isolates and, notably, in a clinically relevant species (*K. pneumoniae*), in which they have been only sporadically described so far (Deng Y, 2011). The routes of spreading of such plasmids in Bolivia are still unknown and would deserve further investigation, in particular to evaluate a possible epidemiological linkage with China, and the role of animals and the food-chain.

Fig. 13**.** Comparative analysis of the two F33:A-:B- plasmids from Bolivia (p397Kp and p477Kp) and the reference F33:A-:B- epidemic plasmid pHN7A8 from China. Genes are represented by bars and are classified by function into different groups. Homologous segments are indicated by grey shading, representing 100% (dark grey) and ≥ 99% (light grey) sequence identity. Number of single nucleotide polymorphisms, and differences involving a 9-bp repeat region in *traD* (open square) and a 6-bp repeat region in *yddA* (filled square) (i.e. both absent in the Bolivian plasmids) are indicated.



2

CHARACTERIZATION OF A CARBAPENEMASE-PRODUCING *ACINETOBACTER BAUMANNII* FROM BOLIVIA

RELATED PUBLICATION:

- Sennati S, Villagran AL, Bartoloni A, Rossolini GM, Pallecchi L. OXA-23-producing ST25 *Acinetobacter baumannii*: First report in Bolivia. *J Glob Antimicrob Resist*. *In press*

Carbapenemase-producing *A. baumannii* has been detected worldwide and is often associated to infections with high morbidity and mortality rates (Peleg AY, 2008; Pogue JM, 2013).

This phenomenon is mainly due to the global dissemination of the so-called European clones I, II and III (also defined as clonal complex CC1, CC2 and CC3) and to the diffusion of carbapenem-hydrolysing class D beta-lactamase among *A. baumannii* species (Higgins PG, 2010).

In Latin America OXA-type carbapenemases have been described since the last two decades with the prevalence of OXA-23 enzyme (Labarca JA, 2014). Moreover the diffusion of *A. baumannii* in this area is associated with the dissemination of CC79 and CC15, while, in contrast with the worldwide epidemiology, the CC2 population has not been detected (Chagas TP, 2014). Furthermore CC25 has been reported in the last few years associated with OXA-23 or NDM-1 carbapenemases (Zarrilli R, 2013).

During a survey on the antimicrobial susceptibility and resistance determinants of bacterial pathogens from the Bolivian Chaco region (Bartoloni A, 2015) we detected a multidrug-resistant *A. baumannii.*

A. baumannii 9495/13, isolated in July 2013 from an infected wound of a 54-year-old male patient from the Hospital Basico of Villa Montes (Villa Montes, Tarija Department, Bolivia), has been identified using both MALDI-TOF and the complete sequencing of $bla_{\text{OXA-51-like}}$ gene.

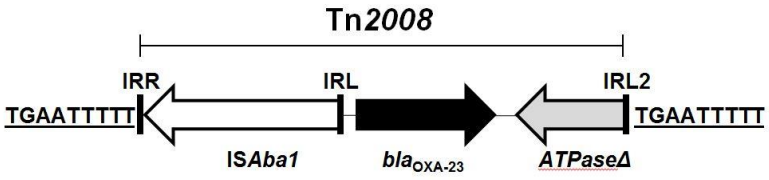
Determination of MICs showed that there was a high level of resistance to all antibiotics tested, with the exception of colistin. *A. baumannii* 9495/13 exhibited resistance to expanded-spectrum cephalosporins, carbapenems, aminoglycosides, fluoroquinolones, tetracycline and trimethoprim/sulfamethoxazole.

Molecular typing assigned *A. baumannii* 9495/13 to the emerging clone ST25 and a copy of IS*Aba1* was detected upstream of the instrinsic *ampC* beta-lactamase (may be responsible for the expanded-spectrum cephalosporins resistance). However, no copy of IS*Aba1* was found out upstream of the intrinsic bla_{OXA-64}. Indeed molecular investigation conducted by PCR revealed that carbapenem resistance was not due to the intrinsic OXA-51-like overexpression, but was found to be associated with the production of OXA-23.

OXA-23 is mainly mobilized by four type of transposons: Tn*2006*, Tn*2007,* Tn*2008* and Tn*2009*; the composite transposon Tn*2006* is the most detected worldwide (Mugnier PD, 2010; Zhou H, 2011).

To investigate the genetic environment of bla_{OXA-23} we performed an inverse PCR and sequencing results showed that in *A. baumannii* 9495/13 OXA-23 is associated with Tn*2008.* Tn*2008* consists in a single copy of IS*Aba1* and has two inverted repeat at the right and left ends of the transposon (Fig. 14). The genetic structure harboring bla_{OXA-23} in *A. baumannii* 9495/13 was find to be inserted in an original chromosomal location between genes encoding an acetolactate synthase catalytic subunit and a hypothetical protein. Moreover Tn*2008* in *A. baumannii* 9495/13 was surrounded by a 9-bp duplication of the target sequence (TGAATTTTT), which, to the best of our knowledge, has not been described before (Nigro S, 2015) (Fig. 14).

Fig. 14. Schematic representation of transposon Tn*2008* in *Acinetobacter baumannii* 9495/13. Sequence of the 9-bp target site duplication is shown, of the 16-bp inverted repeats IRR (reversed and complemented), and IRL and imperfect IRL2 are showed.

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IRR  TTTGTCGTGTACAGAG
IRL  TTTGTCGTGTACAGAG
IRL2 aTTGTCGTGTAttttG
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In Latin America the OXA-23-like enzymes have been found in Brazil, Colombia and Argentina even though OXA-58-like enzymes are the most frequently identified (Opazo A, 2012). Indeed a previous study by Sevillano *et al.* (Sevillano E, 2012) reported that in Bolivia carbapenem-resistant *A. baumannii* was associated to the production of OXA-58 carbapenemase.

To the best of our knowledge, this is the first report of OXA-23-producing *A. baumannii* in Bolivia and the first description of transposon Tn*2008* in Latin America. Our results support previous reports indicating the worldwide spread of the $bla_{\text{OXA-23}}$ gene and highlight its importance in conferring carbapenem resistance among *A. baumannii* isolates from Latin America.

Furthermore, the finding of an OXA-23-producing *A. baumannii* belonging to ST25 in Bolivia confirm recent studies indicating ST25 as a potential emerging clone in Latin America (Chagas TP, 2014; Zarrilli R, 2013). Finally, sequence analysis of the chromosomal location of Tn*2008* in *A. baumannii* 9495/13 supports the recent hypothesis that Tn*2008* could be able to move as a discrete transposable unit (Nigro S, 2015).

In conclusion, this is the first report, to our knowledge, of carbapenem resistance *A. baumannii* isolates from Bolivia associated with the presence of the $bla_{\text{OXA-23}}$ gene. Detection of the antibiotic-inactivating enzyme in a multidrug resistant strain and its expression is of great concern with regard to the spread of carbapenem resistance among hospitals in Bolivia. This fact is alarming, as there are only a few drugs that are clinically available to treat infections caused by this multidrug resistant pathogen.

3

CARRIAGE RATES AND MOLECULAR EPIDEMIOLOGY OF MRSA IN HOSPITALIZED PATIENTS FROM THE BOLIVIAN CHACO

RELATED PUBLICATION:

- Bartoloni A, Riccobono E, Magnelli D, Villagran AL, Di Maggio T, Mantella A, Sennati S, Revollo C, Strohmeyer M, Giani T, Pallecchi L, Rossolini GM. Methicillin-resistant *Staphylococcus aureus* in hospitalized patients from the Bolivian Chaco. 2015. *Int J Infect Dis.* 30:156-60.

Methicillin-resistant *S. aureus* (MRSA) has been disseminating worldwide, arising as a major pathogen in both hospital and community setting and worsening the clinical outcome of staphylococcal infections (Uhlemann AC, 2014). Colonization is known to represent an important risk factor for such infections (Boucher H, 2010).

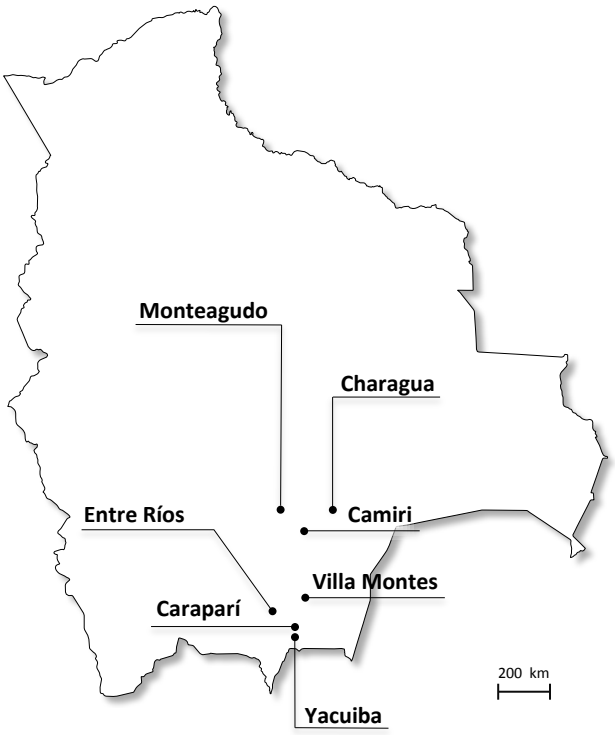
Latin America is not an exception in the global increasing prevalence of MRSA infections and a number of reports have described the epidemiological and molecular features of MRSA clonal lineages circulating in this geographic area over the past two decades (Rodríguez-Noriega E, 2010).

In a previous surveillance study on MRSA nasal carriage a low MRSA prevalence (0-1.5%) has been documented among healthy individuals from the Bolivian Chaco (Bartoloni A, 2013). To investigate, for the first time, the prevalence and molecular epidemiology of *S. aureus* colonization in hospitalized patients from the Bolivian Chaco, we performed two point prevalence studies (in 2012 and 2013) and compared their features with those of few *S. aureus* clinical isolates available from that setting.

A total of 280 inpatients from eight hospitals distributed in the Bolivian Chaco region were enrolled in the study (Fig. 15). All hospitals were small health-care units with 20 to 78 beds,

and together were representative of the organization of the hospital-care system in this area. Facilities for microbiological diagnosis were not available in these hospitals, with the exception of one of them (Villa Montes hospital), in which microbiological analyses has been performed since mid-2010, although a very limited number of samples have been processed.

Fig. 15. Location of the eight hospitals involved in the study.



An overall moderate prevalence (14.6%) of *S. aureus* colonization was detected among the hospitalized individuals included in the study, with no significant differences observed between the two study periods (17.8% and 13.2% in 2012 and 2013, respectively; p=0.4).

Colonization by MRSA isolates was detected with a prevalence of 1.1% in 2012 and 2.1% in 2013, consistently with MRSA colonization rate previously observed among healthy individuals from the rural village of Gutierrez (1.5%) (Bartoloni A, 2013).

The particular health-care system of the Bolivian Chaco, based on very small hospitals, and the fact that 63% of samples were collected within 48 hours of admission, could explain the similar MRSA colonization rates observed in the community and hospital settings in this region.

All *S. aureus* isolates from colonization were subjected to molecular characterization. For comparison purposes, the few (n=9) *S. aureus* clinical isolates, collected in the hospital of Villa Montes since the introduction of the clinical microbiology laboratory, were also included (Tab. 9).

Tab. 9. Population structure of *S. aureus* isolates from colonization and infection in patients from 8 hospitals in the Bolivian Chaco.

Source	Year	MSSA/MRSA	No. of isolates	*Spa*-type (No. of isolates)
Colonization	2012	MSSA	15	t189 (*n*=3); t701 (*n*=3); t359 (*n*=2); t2883 (*n*=2); t002 (*n*=1); t088 (*n*=1); t645 (*n*=1); t1671 (*n*=1); t6907 (*n*=1)
		MRSA	1	t701 (*n*=1)
Colonization	2013	MSSA	21	t024 (*n*=3); t189 (*n*=3); t078 (*n*=2); t645 (*n*=2); t729 (*n*=2); t065 (*n*=1); t319 (*n*=1); t1166 (*n*=1); t1451 (*n*=1); t4710 (*n*=1); t5365 (*n*=1); t6125 (*n*=1); t13417 (*n*=2).
		MRSA	4	t701 (*n*=3); t008 (*n*=1)
Infection[a]	2010-2013	MSSA	4	t002 (*n*=1); t021 (*n*=1); t088 (*n*=1); t645 (*n*=1)
		MRSA	5	t008 (*n*=5)

[a] All clinical isolates except three (t645, t088 and one representative of t008) were from inpatients.

Methicillin-susceptible *S. aureus* (MSSA) isolates revealed a high heterogeneity with clones belonging to several *spa*-type (Tab. 9).

In contrast MRSA were found to belong mostly to *spa*-type t701, harboured SCC*mec* IVc, and were negative for PVL virulence genes. However, a USA300-related isolate was also detected (Tab. 10). The latter isolate showed the characteristics of the USA300 Latin American variant (USA300-LV) (i. e., ST8, *spa*-type t008, SCC*mec* IVc, presence of PVL genes, absence of *arcA*, a gene cluster encoding a complete arginine deaminase pathway), that is one of the most widespread MRSA clones and largely dominant in community and hospital settings in northern countries of Latin America (Tab. 10) (Rodríguez-Noriega E, 2010; Nimmo GR, 2012; Reyes J, 2009; Machuca MA, 2013; Márquez-Ortiz RA, 2014).

Notably, all the available MRSA clinical isolates collected during 2011–2013 (n=5) were also identified as USA300-LV, underscoring the clinical and epidemiological impact of this clone also in the Chaco region (Tab. 10).

Tab. 10. Features of *S. aureus* isolates from colonization and infection belonging to *spa*-type t701 and t008.

Source	Year	Isolate	Origin	Type (population)	MRSA/MSSA	*Spa*-type	PVL	SCC*mec*[c]	PFGE[d]
Colonization	2012	131a	Yacuiba	hospital	MSSA	t701	-	n.a.	A_3
		176a	Monteagudo	hospital	MSSA	t701	-	n.a.	A_3
		188a	Villa Montes	hospital	MSSA	t701	-	n.a.	A_2
Colonization	2013	121a	Camiri	hospital	MRSA	t701	-	IVc	A_1
		272a	Villa Montes	hospital	MRSA	t701	-	IVc	A
		280a	Villa Montes	hospital	MRSA	t701	-	IVc	A
		281a	Villa Montes	hospital	MRSA	t701	-	IVc	A
Infection[a]	2010-2013	284a	Caraparì	hospital	MRSA[e]	t008	+	IVc	B
		304	Villa Montes	hospital	MRSA	t008	+	IVc	B
		306	Villa Montes	hospital	MRSA	t008	+	IVc	B
		233	Villa Montes	hospital	MRSA	t008	+	IVc	B
		393	Villa Montes	hospital	MRSA	t008	+	IVc	B_1
		401	Villa Montes	hospital	MRSA	t008	+	IVc	B
Colonization[b]	2009	140	Gutierrez	community	MRSA	t701	-	IVc	A
		99	Gutierrez	community	MRSA	t701	-	IVc	A_1
		132	Gutierrez	community	MRSA	t701	-	IVc	A_1

[a] All isolates belonging to *spa*-type t008 were from inpatients, with the exception of isolate 304 that was from an outpatient. Isolates 304 and 306 were collected in 2011, isolate 233 in 2012, and isolates 393 and 401 in 2013.
[b] Previously characterized (Bartoloni A, 2013).
[c] n.a., not applicable.
[d] 2 different bands (A/A_1, A_2/A_3); 3 different bands (A/A_2); 5 different bands (A/A_3, A_1/A_2, A_1/A_3).
[e] All MRSA isolates remained susceptible to all non beta-lactam antibiotics tested, with the exception of isolate 284a that showed intermediate susceptibility to erythromycin and ciprofloxacin.

These findings underscore the importance of surveillance to understand the diverse evolutionary trajectories of MRSA lineages in this setting and to implement effective empiric treatment protocols and infection control measures.

4

MOLECULAR BASIS OF THE EMERGENCE AND DISSEMINATION OF QUINOLONE RESISTANCE IN URBAN AREAS OF BOLIVIA AND PERU

RELATED PUBLICATIONS (PUBLISHED BEFORE THE PHD STUDENT PERIOD, BUT IN RELATION TO THE THESIS WORK):

- Pallecchi L, Riccobono E, Mantella A, Bartalesi F, Sennati S, Gamboa H, Gotuzzo E, Bartoloni A, Rossolini GM. High prevalence of *qnr* genes in commensal enterobacteria from healthy children in Peru and Bolivia. 2009. *Antimicrob Agents Chemother*. 53:2632-5.
- Pallecchi L, Riccobono E, Sennati S, Mantella A, Bartalesi F, Trigoso C, Gotuzzo E, Bartoloni A, Rossolini GM. Characterization of small ColE-like plasmids mediating widespread dissemination of the *qnrB19* gene in commensal enterobacteria. 2010. *Antimicrob Agents Chemother*. 54:678-82.

To investigate the role of the intestinal microbiota as reservoir of plasmid-mediated quinolone resistance (PMQR) determinants, was conducted a study to evaluate the faecal carriage of *qnr* genes in healthy children living in urban areas of Latin America (Camiri, Santa Cruz Department, and Villa Montes, Tarija Department, Bolivia; and Yurimaguas, Loreto Department, and Moyobamba, San Martin Department, Peru).

Metagenomes were extracted from pools of commensal enterobacteria obtained by plating 310 faecal samples, selected randomly from the 3,174 collected during the 2005 survey (Bartoloni A, 2008), on MacConkey agar plates supplemented with a low concentration of ciprofloxacin (lower than MICs usually exhibited by enterobacterial isolates harboring *qnr* genes as the sole quinolone resistance mechanism, but higher than the wild-type MIC distribution for *E. coli* and *K. pneumoniae*).

Metagenomes were analysed by a PCR-based approach, which showed a surprisingly high prevalence of *qnrB* (54%) and, at lower level, of *qnrS* (14%), while *qnrA* was not detected.

The presence of *qnr* genes in the 310 selected samples was also investigated by a simplified dot blot DNA-hybridization method, which was found to be a reliable, less labor-intensive and less expensive tool (even tough with an overall lower sensitivity compared to the PCR-approach) to perform large-scale metagenomic analysis (Tab. 11).

Tab. 11. Prevalence of *qnr* genes in commensal enterobacteria from 310 healthy children living in Peru and Bolivia.

Study area	No. of samples	No. of samples grown on MCA-CIP (%)	Any *qnr* (%)[a]		*qnrB* (%)[a]		*qnrS* (%)[a]	
			PCR	dot-blot	PCR	dot-blot	PCR	dot-blot
Peru	164	154 (94)	113 (69)	93 (57)	107 (65)	87 (53)	37 (23)	11 (7)
Bolivia	146	121 (83)	63 (43)	51 (35)	60 (41)	49 (34)	7 (5)	2 (1)
Total	310	275 (89)	176 (57)	144 (46)	167 (54)	136 (44)	44 (14)	13 (4)

[a]The percentage of samples was calculated for the total samples. *qnr* genes were significantly more prevalent in Peru than in Bolivia: $P<0.01$ for any *qnr* and *qnrB*, and $P< 0.001$ for *qnrS*. All positive samples in the dot blot were also positive in the PCR.

Randomly selected enterobacterial pools which gave *qnr*-positive metagenomes, were analysed to detect bacterial hosts of *qnr* genes. Results showed that *qnrB* was mainly carried by *E. coli* and *qnrS* by *K. pneumoniae*.

Among *qnr*-positive enterobacteria, resistance to nalidixic acid was common, while only 32% and 17% of isolates were non-susceptible to ciprofloxacin and levofloxacin, respectively. *qnrB19* was the prevalent *qnrB* gene and was found to be mostly located on low molecular weight plasmids, while all *qnrS* genes were *qnrS1* and were located on high molecular weight plasmids.

These findings represented the first data on the prevalence of *qnr* genes in human commensal bacteria and emphasized the role of commensal enterobacteria as reservoir of similar resistance genes.

A following study was carried out to investigate the presence of *qnr* genes by PCR in 107 ciprofloxacin resistant *E. coli* isolates, randomly selected among 1,053 ciprofloxacin-resistant commensal *E. coli* isolates from the survey performed in Bolivia and Peru in 2005 (Bartoloni A, 2008). Six out of the 107 isolates (6%) were found to be positive for *qnrB* genes, while *qnrA* and *qnrS* genes were not detected. Amplicon sequencing identified the *qnrB19* allele in all of them.

All *qnrB19*-positive isolates exhibited a multidrug-resistance phenotype, belonged to phylogenetic group A (known to be usually associated with commensalism and minor virulence) and were shown to be clonally unrelated, indicating a likely plasmid-mediated dissemination of the *qnrB19* gene. Plasmid characterization by RFLP and Southern blotting demonstrated the dissemination of two small QnrB19-encoding plasmids in clonally unrelated ciprofloxacin resistant commensal *E. coli* from children living in different urban areas of Bolivia and Peru (Tab. 12).

Tab. 12. Features of ciprofloxacin resistant *E. coli* carrying *qnrB19*.

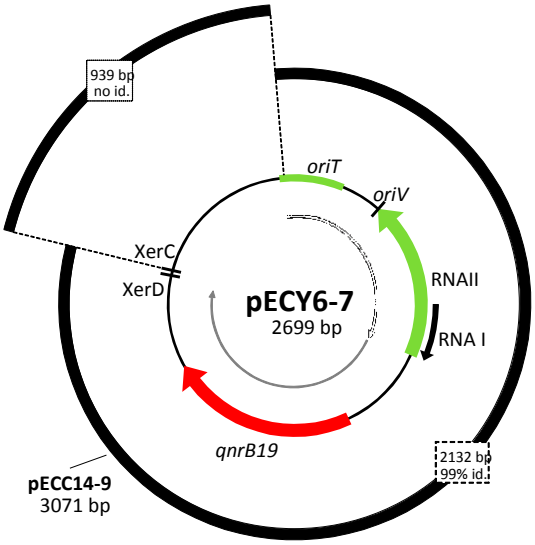
Isolates	Origin	Philogenetic group	RAPD type	Resistance phenotype	Plasmid size (kb)[b]	Transfer in *E. coli* HB101	RFLP	MICs (μg/ml) of transformants[c]			
								Nal	Cip	Lev	Nor
M4-6	Moyobamba, Peru	A	1	Amp, Tet, Tmp, Sul, Str	high mw	no	nd[d]	-	-	-	-
M66-6	Moyobamba, Peru	A	2	Tet, Sul, Str, Kan	2.7	yes	a	16	0.06	0.12	0.25
Y6-7	Yurimaguas, Peru	A	3	Amp, tet, Tmp, sul, Str	2.7	yes	a	16	0.06	0.12	0.25
C14-9	Camiri, Bolivia	A	4	Tet, Tmp, sul, Kan	3.0	yes	b	32	0.12	0.25	0.5
V6-9	Villa Montes, Bolivia	A	5	Amp, Sul, Str, Kan	3.0	yes	b	32	0.06	0.25	0.5
V32-1	Villa Montes, Bolivia	A	6	Amp, Tet, Str, Kan	3.0	yes	b	32	0.06	0.25	0.25

[a] Amp, ampicillin; Tet, tetracycline; Tmp, trimethoprim; Sul, sulfamethoxazole; Str, streptomycin; Kan, kanamycin.
[b] mw, molecular weight. Plasmid sizes were estimated after Southern blotting performed with wild-type isolates and RFLP analysis performed with transformants (except for plasmid from *E. coli* M4-6).
[c] Nal, nalidixic acid; Cip, ciprofloxacin; Lev, levofloxacin; Nor, norfloxacin. MICs of *E. coli* HB101 were as follows: NAL 4 μg/ml, CIP 0.003 μg/ml, LEV 0.0075 μg/ml, NOR 0.003 μg/ml. No other resistance determinant was co-transferred with *qnrB19*.
[d] nd, not determined

Sequence analysis of the two small epidemic plasmids showed that they were closely related ColE-type plasmids (pECY6-7 of 2.7-kb, and pECC14-9 of 3.0-kb) carrying *qnrB19* gene as the sole resistance determinant. In both plasmids, *qnrB19* was located in a conserved genetic context between the RNAII sequence (which controls plasmid replication) and the Xer site (involved in plasmid dimers resolution). Differently from what observed for previously reported *qnrB19* genes, no IS*Ecp1*-like or other putative insertion sequences were present in the *qnrB19*-flanking regions or elsewhere on the plasmids (Fig. 16).

The presence of pECY6-7- and pECC14-9-like plasmids was also evaluated in all the *qnrB*-positive enterobacterial metagenomes previously investigated, using a PCR-mapping approach. Both plasmids were found to be highly prevalent (67% and 16%, respectively), suggesting that dissemination of these small plasmids played a major role in the widespread dissemination of *qnrB* genes observed in commensal enterobacteria from healthy children living in those areas.

Fig. 16. Arrows show direction of transcription of open reading frames and regulatory elements in pECY6-7. The ColE-like plasmid backbone and the *qnr* region are indicated by gray arrows. Comparison between pECY6-7 and pECC14-9 is shown as percentages of sequence identity (id.).



Plasmid pECY6-7 (the smallest and most prevalent one) was found disseminated in different species of enterobacteria (*E. coli*, *K. pneumoniae* and *Escherichia hermannii*). A plasmid identical to pECY6-7 was identified also in a *Salmonella enterica* serovar Typhimurium strain of human origin isolated in the Netherlands (Hammerl JA, 2010), suggesting that the dissemination of this plasmid could be even more widespread.

The reasons accounting for the high prevalence of plasmids pECY6-7 and pECC14-9 in commensal enterobacteria remain unclear. Data collected about consumption of antibiotics excluded the therapeutic use of fluoroquinolones in children from the urban areas investigated. Moreover, the absence of other resistance genes in pECY6-7 and pECC14-9 excluded the possibility that selection of these plasmids could be related to exposure to other antibiotics. Further studies on the mobilization and fitness impact of plasmids pECY6-7 and pECC14-9 could provide important information to understand their remarkable propensity for such a widespread dissemination.

5

MOLECULAR EPIDEMIOLOGY OF ACQUIRED RESISTANCE GENES IN EXPANDED-SPECTRUM CEPHALOSPORIN-RESISTANT *ENTEROBACTERIACEAE* IN ARGENTINA

RELATED PUBLICATIONS:

- Sennati S, Santella G, Di Conza J, Pallecchi L, Pino M, Ghiglione B, Rossolini GM, Radice M, Gutkind G. Changing epidemiology of extended-spectrum β-lactamases in Argentina: emergence of CTX-M-15. *Antimicrob Agents Chemother*. 2012 11:6003-5.
- Cruz GR, Radice M, Sennati S, Pallecchi L, Rossolini GM, Gutkind G, Conza JA. Prevalence of plasmid-mediated quinolone resistance determinants among oxyiminocephalosporin-resistant *Enterobacteriaceae* in Argentina. *Mem Inst Oswaldo Cruz*. 2013 7:924-7.

CTX-M enzymes are the most prevalent ESBL reported worldwide, and CTX-M-15, belonging to group 1, is the most described. The CTX-M scenario in Latin America consist of the predominance of CTX-M-2 group, which is most reported since its first detection (D'Andrea MM, 2013; Cantón R, 2012).

To investigate the current epidemiology of expanded-spectrum cephalosporins resistance determinants in Argentina was conducted a multicenter survey to evaluate the prevalence and nature of ESBL.

1.586 consecutive and non-repetitive enterobacterial clinical isolates were collected in October from both inpatients and outpatients of 15 hospitals of three different regions of Argentina: Buenos Aires (*n*=7), Santa Fè (*n*=4) and Chubut (*n*=4). Among them, 207 (13.1%) exhibited reduced susceptibility to expanded-spectrum cephalosporins (Tab. 13).

Confirmatory tests for ESBL production were performed in all isolates showing reduced susceptibility to expanded-spectrum cephalosporins collected in the first week of October (*n*=55), because they were considered representative of the whole study period: 91% (*n*=50) were ESBL-producing isolates, while the remaining isolates were AmpC producers (9%, *n*=5) (Tab. 13).

Tab. 13. Number of isolates of each species recovered within the study period, expanded-spectrum cephalosporins resistance, and number of resistant isolates that were further studied.

			ESC-resistant isolates recovered within 1 week	
Species	No. of isolates	No (%) of ESC[a]-resistant isolates	No. of isolates	No. of ESBL producers/AmpC producers
Escherichia coli	1120	64 (5.7)	16	14/2
Klebsiella pneumoniae	193	87 (45.1)	22	22/0
Proteus mirabilis	115	14 (12.2)	6	5/1
Enterobacter cloacae	37	11 (29.7)	3	1/2
Morganella morganii	29	11 (37.9)		
Klebsiella oxytoca	20	6 (30)	4	4/0
Citrobacter freundii	18	5 (27.8)		
Serratia spp.	18	5 (27.8)	3	3/0
Providencia spp.	13	2 (15.4)	1	1/0
Citrobacter spp.	8			
Proteus vulgaris	7	2 (28.6)		
Enterobacter aerogenes	3			
Salmonella spp.	2			
Shigella spp.	2			
Proteus penneri	1			
Total	1586	207 (13.1)	55	50/5

[a]ESC, expanded-spectrum cephalosporins.

Results from molecular investigation, conducted by PCR, showed that 85.5% (*n*=47) were CTX-M producers: CTX-M-2 group were detected in 26 isolates (55%), while CTX-M-1 group in 19 (40%), CTX-M-9 group in 3 isolates (6%) and CTX-M-8 group in 1 isolate (2%) (Tab. 14). These prevalence data showed a transformation in the ESBL epidemiology in Argentina with the emergence of other CTX-M groups. Although CTX-M-2 group remained dominant, we observed a notable modification in the prevalence of such enzymes with the emergence of the CTX-M-1 group, in particular of the CTX-M-15 variant, reflecting the worldwide scenery (D'Andrea MM, 2013; Cantón R, 2012) (Tab. 14).

Tab. 14. CTX-M-producing enterobacteria collected during a 1-week study in 15 hospitals distributed in different regions of Argentina.

Species (No. of isolates)	ESBL determinant(s) (No. of isolates)
Klebsiella pneumoniae (21)	$bla_{\text{CTX-M-15}}$ (10)
	$bla_{\text{CTX-M-2}}$ (9)
	$bla_{\text{CTX-M-2}}$ + $bla_{\text{CTX-M-15}}$ (1)
	$bla_{\text{CTX-M-8}}$ (1)
Escherichia coli (13)	$bla_{\text{CTX-M-15}}$ (7)
	$bla_{\text{CTX-M-14}}$ (3)
	$bla_{\text{CTX-M-2}}$ (3)
Proteus mirabilis (5)	$bla_{\text{CTX-M-2}}$ (4)
	$bla_{\text{CTX-M-56}}$ (1)
Klebsiella oxytoca (4)	$bla_{\text{CTX-M-2}}$ (3)
	$bla_{\text{CTX-M-2}}$ + $bla_{\text{CTX-M-15}}$ (1)
Serratia spp. (3)	$bla_{\text{CTX-M-2}}$ (3)
Providencia spp. (1)	$bla_{\text{CTX-M-2}}$ (1)

To better understand the dissemination of the CTX-M-15, we performed a genotyping analysis of the CTX-M-15-producing isolates (7 *E. coli* and 11 *K. pneumoniae*). Results showed a clonal heterogeneity among isolates, and all CTX-M-15-producing *E. coli* belonging to phylogenetic group B2 were identified as ST-131, while CTX-M-15-producing *K. pneumoniae* were assigned to the *sequence type* 11.

Since the association of plasmid-mediated quinolone resistance (PMQR) genes with ESBL and AmpC beta-lactamases is considerable, we investigate the prevalence of PMQR genes in all expanded-spectrum cephalosporins resistant isolates.

While no PMQR genes were detected among AmpC-producing isolates, 66% (*n*=33) of ESBL-producing isolates were positive to at least one PMQR determinant. *qnrB* and *aac(6')-Ib-cr* were detected in 33.3% (*n*=11) and 42.4% (*n*=14) among the PMQR-positive isolates respectively, and 24.3% (*n*=8) were positive for both determinants, while *qnrA*, *qnrC*, *qnrD, qnrS*, and *qepA* genes were not detected. Furthermore *aac(6')-Ib-cr* was detected in 44% (*n*=22) of the ESBL-producing isolates and was found to be associated with CTX-M-15.

These findings demonstrate that the CTX-M epidemiology in Argentina has been changed, and the "pandemic" CTX-M-15 is becoming widespread in this area, also due to the dissemination of successful clones such as *E. coli* ST-131 and *K. pneumoniae* ST-11.

Moreover these results highlights the association between *aac(6')-Ib-cr* and $bla_{\text{CTX-M-15}}$ in multiresistant clones, as reported worldwide (Mathers AJ, 2015).

PART IV

CONCLUSIONS AND PERSPECTIVES

Bacterial resistance to antibiotics is a major public health problem, and the increasing spread of multi-resistant isolates represents an alarming challenge due to the limited treatment options for infections caused by these pathogens.

This phenomenon is an important concern in low resource countries like those of Latin America, where the combination of factors such as inefficient health systems, poor sanitation and uncontrolled use of antimicrobials provide conditions to develop and maintain resistant strains in the hospital settings, at the community level and in the environment.

In this work, was analysed the molecular epidemiology of emerging antibiotic resistance mechanisms in commensal and pathogenic bacteria from Latin America.

In particular, the study on the antimicrobial susceptibility of clinical isolates from urinary tract infections in Bolivia allowed to gather insight into the mechanisms of dissemination of emerging resistance determinants of clinical significance (such as CTX-M, RmtB, FOSA3), emphasizing the role of high-risk clones in the dissemination of antimicrobial resistance determinants.

The same burning phenomenon can be observed in Argentina, where the dissemination of successful clones, responsible for the diffusion of CTX-M and AAC(6')-Ib-cr determinants in clinical settings, has partially contributed to the change of ESBL epidemiology in this country.

Furthermore the finding of the F33:A-:B- epidemic plasmid (widely disseminated among animals in China) in clinical isolates in Bolivia confirm the role of animals as reservoir of multiresistance bacteria. The routes of spreading of such plasmids are unclear and would deserve further investigation, even though the increasing frequency of international travel and the intensive intercontinental commerce could be an easy way for the circulation of resistant strains.

Similarly to pathogenic bacteria, commensals are exposed to the selective pressure of antimicrobial drugs. This exposure may have long-term effects on commensal microbiota, which may evolve in an important reservoir of antibiotic resistant strains potentially transferable to pathogens. Therefore, commensal bacteria are considered indicator in predicting the dissemination of resistance among pathogens. Indeed, surveillance is considered crucial for the implementation of intervention strategies aimed at preserving the efficacy of antimicrobial chemotherapy.

Surveillance studies performed on commensal bacteria in healthy children from urban areas of Bolivia and Peru provided new information regarding the dynamics of emergence and dissemination of clinically relevant resistance phenotypes, included those associated to CTX-M and Qnr determinants.

To investigate the correlation between resistance rates observed in the microbiota of healthy individuals and in the pathogens circulating in the same setting, we have started collaborating with two hospitals that have recently implemented microbiological diagnosis for UTIs (urinary tract infections) and SSTIs (skin and soft-tissues infections).

In this regard, first data concerning the comparison between *S. aureus* from colonization and infection revealed the circulation of the same clones among carriers and patients. These findings underscore that surveillance of antimicrobial resistance on commensal microbiota can be useful to guide empiric management of infections, especially in settings where microbiological diagnosis is not available.

In conclusion, the problem of antimicrobial resistance in Latin America, although a regional challenge, deserves international efforts. Multidisciplinary and collaborative efforts should be made to tackle antibiotic resistance and to achieve “One Health” that is a global health for people, animals and the environment.

Investments in education, public health and sanitation, development of guidelines for strategic antibiotic use, attempts to facilitate the access to rapid diagnostic tools, implementation of effective and internationally connected resistance surveillance programs and strict control and transparency in the antimicrobial supply chain are some examples of measures that could help to decelerate de advance of the antimicrobial resistance in low resource countries.

PART V

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pHN7A8-related multiresistance plasmids (blaCTX-M-65, fosA3 and rmtB) detected in clinical isolates of Klebsiella pneumoniae from Bolivia: intercontinental plasmid dissemination?

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Complete List of Authors:	Sennati, Samanta; University of Siena, Department of Medical Biotechnologies Riccobono, Eleonora; University of Siena, Department of Medical Biotechnologies; University of Florence, Department of Experimental and Clinical Medicine Di Pilato, Vincenzo; University of Florence, Department of Surgery and Translational Medicine, University of Florence Villagran, Ana Liz; Hospital Basico Villa Montes Pallecchi, Lucia; University of Siena, Department of Medical Biotechnologies Bartoloni, Alessandro; University of Florence, Department of Experimental and Clinical Medicine; Careggi University Hospital, Infectious and Tropical Diseases Unit Rossolini, G. M.; University of Siena, Department of Medical Biotechnologies; University of Florence, Department of Experimental and Clinical Medicine; Careggi University Hospital, Clinical Microbiology and Virology Unit
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pHN7A8-related multiresistance plasmids (*bla*$_{CTX-M-65}$, *fosA3* and *rmtB*) detected in clinical isolates of *Klebsiella pneumoniae* from Bolivia: intercontinental plasmid dissemination?

Samanta SENNATI[1], Eleonora RICCOBONO[1,2], Vincenzo DI PILATO[3], Ana Liz VILLAGRAN[4], Lucia PALLECCHI[1], Alessandro BARTOLONI[2,5], Gian Maria ROSSOLINI[1,2,6*]

[1] Department of Medical Biotechnologies, University of Siena, Santa Maria alle Scotte University Hospital, Siena, Italy

[2] Department of Experimental and Clinical Medicine, University of Florence, Careggi University Hospital, Florence, Italy

[3] Department of Surgery and Translational Medicine, University of Florence, Florence, Italy.

[4] Hospital Basico Villa Montes, Villa Montes, Bolivia

[5] Infectious and Tropical Diseases Unit, Careggi University Hospital, Florence, Italy

[6] Clinical Microbiology and Virology Unit, Careggi University Hospital, Florence, Italy

* Corresponding author. Mailing address: Department of Medical Biotechnologies, University of Siena, Santa Maria alle Scotte University Hospital, 53100 Siena, Italy. Phone: +39 0577 233455; fax: +39 0577 233870; e-mail: gianni.rossolini@gmail.com

Keywords

Latin America, 16s rRNA methylase, F33:A-:B- plasmid, epidemic plasmid

Sir,

F33:A-:B- multiresistance plasmids carrying *bla*$_{CTX-M-65}$, *fosA3* and *rmtB* are widespread in *Escherichia coli* isolates of animal origin from China.[1-3] pHN7A8, isolated from a dog in 2008 and completely sequenced, is a representative of these plasmids.[4]

To the best of our knowledge, these epidemic plasmids have not been found in samples of human origin so far. Here we characterized two pHN7A8-related plasmids from clinical isolates of *Klebsiella pneumoniae* from the Bolivian Chaco region.

K. pneumoniae 397Kp and *K. pneumoniae* 477Kp were isolated from urine samples of a 75-year-old female outpatient with liver failure and of a 35-year-old female inpatient with acute diarrhoea, at the Hospital Basico Villa Montes (Villa Montes, Tarija Department, Bolivia) in June and December 2013, respectively. They showed a multiresistance phenotype including expanded-spectrum cephalosporins (cefotaxime MIC >4 mg/L), aminoglycosides (gentamicin MIC >4 mg/L; amikacin MIC >16 mg/L), fluoroquinolones (ciprofloxacin MIC >2 mg/L, levofloxacin MIC >4 mg/L) and, in case of 397Kp, fosfomycin (MIC >512 mg/L) (Supplementary Table S1).

Clonal analysis, performed by MLST (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html) and PFGE, revealed that *K. pneumoniae* 397Kp and *K. pneumoniae* 477Kp belonged to ST726, a sequence type recently associated with bloodstream infections in Taiwan,[5] and that they were possibly related.[6]

Plasmids from *K. pneumoniae* 397Kp (p397Kp) and *K. pneumoniae* 477Kp (p477Kp) were transferred at high frequency (7 x 10^{-2} transconjugant/recipient) in conjugation experiments using *E. coli* J53 Azi^{r} (*met pro* azide resistant) as a recipient, and Mueller Hinton agar plates plus cefotaxime 2 mg/L and sodium azide 100 mg/L for selection of transconjugants.[7]

Complete sequencing of both plasmids was performed using a MiSeq Illumina platform (Illumina Inc., San Diego, CA, USA). The SPAdes assembly algorithm was used to assemble raw sequence data.[8] Gaps were closed by a PCR/sequencing-based strategy, and annotation was manually performed using plasmid pHN7A8 (GenBank accession no. JN232517) as a reference.

Plasmids p397Kp (76,863 bp) (GenBank accession no. LN897474) and p477Kp (74,768 bp) (GenBank accession no. LN897475) showed 100% nucleotide identity, except that p477Kp lacked the *fosA3* module (Figure 1).

Of note, they were found to be highly related to the Chinese epidemic plasmid pHN7A8, from which p397Kp differed only by nine scattered single nucleotide polymorphisms (of which two resulted in amino acid

changes in *traA* and *traB*), by the absence of a 9-bp repeat in *traD*, and by the absence of a 6-bp repeat in *yddA* (Figure 1).

Investigation of plasmid stability in *E. coli* transconjugants, carried out as previously described,[7] revealed that p397Kp and p477Kp were stably maintained up to 60 generations, in absence of selective pressure.

Based on the observation that the most recently identified Bolivian plasmid (p477Kp) lacked the *fosA3* resistance module, experiments were carried out to evaluate the possible loss of this module. For this purpose, *E. coli* J53 carrying p397Kp was maintained for 60 generations in absence of selective pressure, and the rate of *fosA3* loss was determined by replica plating of at least 100 colonies onto Luria Bertani agar plates plus fosfomycin (2 mg/L) and glucose-6-phosphate (25 mg/L), and confirmation of the *fosA3* presence by PCR.[7] No evidence of instability of the *fosA3* resistance module was observed under these experimental conditions.

Results from the present study add some important information on the epidemiology of F33:A-:B- multiresistance plasmids, which were previously shown to have undergone an epidemic dissemination in China among *E. coli* strains infecting or colonizing animals.[1-3] Indeed, p397Kp and p477Kp are the first representatives of such plasmids identified in human clinical isolates and, notably, in a clinically relevant species (*K. pneumoniae*), in which they have been only sporadically described so far.[9] Detection of pHN7A8-related plasmids in clinical isolates from Bolivia not only underscores the ability of these plasmids to spread also in the clinical setting, but also highlights their potential for intercontinental dissemination. The routes of spreading of such plasmids in Bolivia are still unknown and would deserve further investigation, in particular to evaluate a possible epidemiological linkage with China, and the role of animals and the food-chain.

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Transparency declaration

None to declare.

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Figure 1. Comparative analysis of the two F33:A-:B- plasmids from Bolivia (p397Kp, LN897474; p477Kp, LN897475) and the reference F33:A-:B- epidemic plasmid pHN7A8 (JN232517) from China. Physical maps were generated using EasyFig software.[10] Genes are represented by bars and are classified by function into different groups. Homologous segments are indicated by grey shading, representing 100% (dark grey) and ≥ 99% (light grey) sequence identity. Number of single nucleotide polymorphisms, and differences involving a 9-bp repeat region in *traD* (open square) and a 6-bp repeat region in *yddA* (filled square) (i.e. both absent in the Bolivian plasmids) are indicated. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.

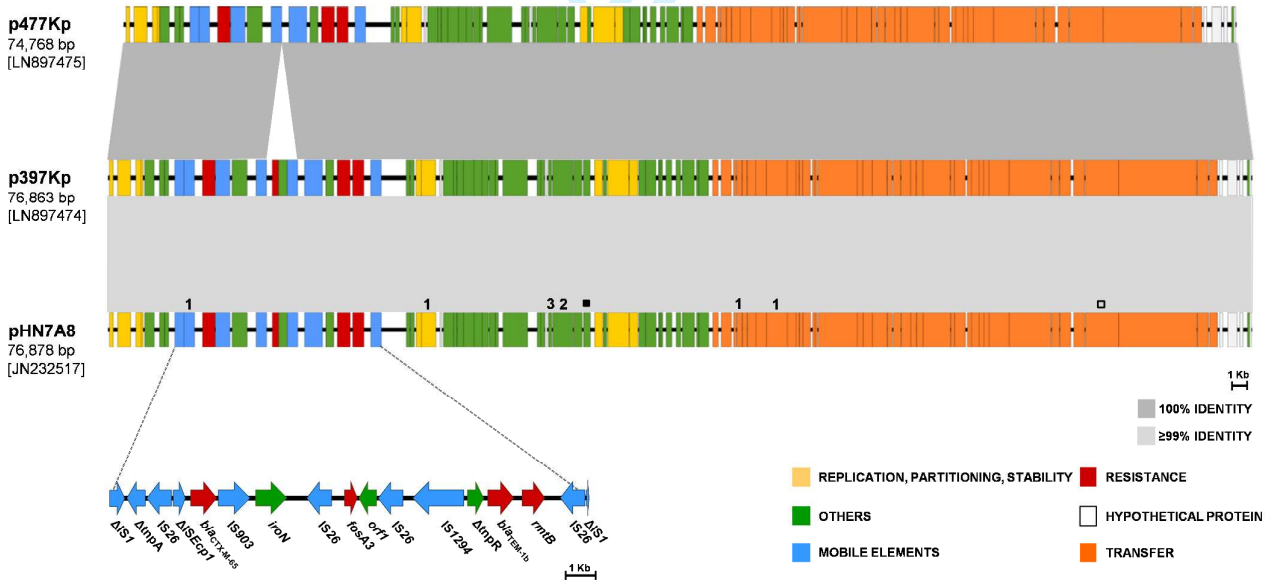

Table S1. Minimum inhibitory concentrations (MICs) of *K. pneumoniae* 397Kp and *K. pneumoniae* 477Kp.

Antibiotic	MIC (mg/L) [a]	
	K. pneumoniae 397Kp	*K. pneumoniae* 477Kp
Ampicillin-sulbactam	>32/16	>32/16
Piperacillin-tazobactam	8/4	8/4
Cefotaxime	>4	>4
Ceftazidime	8	8
Cefepime	2	2
Imipenem	≤1	≤1
Meropenem	≤0.12	≤0.12
Gentamicin	>4	>4
Amikacin	>16	>16
Fosfomycin	>512	16
Ciprofloxacin	>2	>2
Levofloxacin	>4	>4
Nitrofurantoin	>64	>64
Trimethoprim-sulfamethoxazole [b]	≤0.5/9.5	>4/76

[a] MICs were determined by the broth microdilution method according to CLSI guidelines, except for fosfomycin which was tested by the agar dilution method as per CLSI.

[b] The difference observed in the resistance profile to trimethoprim-sulfamethoxazole between the two strains was apparently due to a resistance mechanism not encoded by the p477Kp plasmid.

Title: OXA-23-producing ST25 *Acinetobacter baumannii*: first report in Bolivia

Author: Samanta Sennati Ana Liz Villagran Alessandro Bartoloni Gian Maria Rossolini Lucia Pallecchi

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OXA-23-producing ST25 *Acinetobacter baumannii*: first report in Bolivia

Sir,

Carbapenemase-producing *Acinetobacter baumannii* have been increasingly reported worldwide over the last decade, representing a major clinical threat due to their resistance phenotype usually extending to most or all available antibiotics [1]. A limited number of successful clones [namely international clones I to III, also defined as clonal complexes CC1 to CC3 by multilocus sequence typing (MLST)] have mainly accounted for their global dissemination [1,2]. Carbapenem-hydrolysing class D β-lactamases (CHDLs) are the main mechanism of carbapenem resistance in *A. baumannii*. Apart from the intrinsic OXA-51-like enzymes (responsible for reduced carbapenem susceptibility in the case of overexpression), five groups of acquired CHDLs have been identified in this pathogen, namely OXA-23-like, OXA-40-like, OXA-58-like, OXA-143-like and OXA-235-like enzymes, with OXA-23 being the most widespread worldwide [1]. $bla_{\text{OXA-23}}$ originated from the chromosome of *Acinetobacter radioresistens*, and its dissemination into other *Acinetobacter* spp. has being mainly linked to Tn*2006*, Tn*2007*, Tn*2008* and Tn*2009* transposon variants (where IS*Aba1* or IS*Aba4* insertion sequences account both for $bla_{\text{OXA-23}}$ mobilisation and enhanced expression) [1].

In South America, *A. baumannii* producing OXA-type carbapenemases have been described since the late 1990s ([3] and references therein), with a predominance of OXA-23 and a peculiar population structure characterised by the absence of CC2 (the worldwide predominant clone) and a major role of CC79 and CC15 ([2,3] and

references therein). Furthermore, ST25 (CC25) has recently been identified as an emerging clone, associated either with OXA-23 or with the NDM-1 metallo-β-lactamase ([1–3] and references therein).

Here we describe the first OXA-23-producing ST25 *A. baumannii* from Bolivia, which harboured *bla*$_{\text{OXA-23}}$ associated with Tn*2008*.

Acinetobacter baumannii 9495/13 was isolated in July 2013 from an infected wound of a 54-year-old, male, human immunodeficiency virus (HIV)-positive patient at Hospital Basico de Villamontes (Villa Montes, Tarija Department, Bolivia), who subsequently died for reasons not related to the infection (i.e. congestive heart failure).

Identification was performed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) system (bioMérieux Inc., Marcy l'Étoile, France) and complete sequencing of the *bla*$_{\text{OXA-51-like}}$ gene [3]. Minimum inhibitory concentrations (MICs) were determined by broth microdilution and were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines [4]. MLST was performed according to the Pasteur Institute scheme (http://pubmlst.org/abaumannii/). A PCR sequencing approach was used for detection of CHDLs (i.e. OXA-23, OXA-24 and OXA-58) and other carbapenemases (i.e. VIM, IMP, NDM and KPC) as well as the presence of IS*Aba1* upstream of the intrinsic *bla*$_{\text{OXA-51-like}}$ and *ampC* genes [4]. The genetic environment of *bla*$_{\text{OXA-23}}$ was characterised by inverse PCR [4] and was deposited under GenBank/EMBL accession no. **LN877214**.

Acinetobacter baumannii 9495/13 exhibited resistance to piperacillin/tazobactam (MIC > 128/4 μg/mL), ceftazidime (MIC = 128 μg/mL), cefepime (MIC = 32 μg/mL), imipenem (MIC = 32 μg/mL), meropenem (MIC = 32 μg/mL), gentamicin (MIC > 8 μg/mL), tetracycline (MIC = 16 μg/mL), ciprofloxacin (MIC = 16 μg/mL) and trimethoprim/sulfamethoxazole (MIC = 8/152 μg/mL). It remained intermediate to amikacin (MIC = 32 μg/mL) and was susceptible to ampicillin/sulbactam (MIC ≤ 8 μg/mL) and colistin (MIC = 2 μg/mL). The MIC of tigecycline was ≤0.12 μg/mL.

MLST assigned *A. baumannii* 9495/13 to the emerging clone ST25. Carbapenem resistance was found to be associated with OXA-23, with no contribution of intrinsic OXA-51-like overexpression (i.e. no copy of IS*Aba1* was detected upstream of the intrinsic $bla_{\text{OXA-64}}$). A copy of IS*Aba1* was found upstream of the intrinsic *ampC*, possibly contributing to the expanded-spectrum cephalosporin resistance [1].

In *A. baumannii* 9495/13, $bla_{\text{OXA-23}}$ was found to be associated to a classical Tn*2008* transposon (100% nucleotide identity to **KP780408** and related sequences [5]), inserted in an original chromosomal location (i.e. a spacer region between genes encoding an acetolactate synthase 3 catalytic subunit and a hypothetical protein) and surrounded by a 9-bp duplication of the target sequence (TGAATTTTT) (Fig. 1). Compared with the composite transposon Tn*2006*, which is by far the most prevalent and widespread genetic platform of $bla_{\text{OXA-23}}$, Tn*2008* contains a single copy of IS*Aba1* and has recently been hypothesised to move as a discrete transposable unit, thanks to the presence of an imperfect inverted repeat at the right end of the transposon (Fig. 1) [5].

In Bolivia, carbapenem resistance in *A. baumannii* has been previously associated with OXA-58 [6]. To the best of our knowledge, this is the first report of OXA-23-producing *A. baumannii* in Bolivia and the first description of transposon Tn*2008* in Latin America. The finding of an OXA-23-producing *A. baumannii* belonging to ST25 in Bolivia is consistent with recent studies indicating ST25 as a potential emerging clone in Latin America ([1–3] and references therein). Finally, sequence analysis of the chromosomal location of Tn*2008* in *A. baumannii* 9495/13 supports the recent hypothesis that Tn*2008* might be able to move as a discrete transposable element [5].

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Samanta Sennati [a]

Ana Liz Villagran [b]

Alessandro Bartoloni [c,d]

Gian Maria Rossolini [a,c,e]

Lucia Pallecchi [a,*]

[a] *Department of Medical Biotechnologies, University of Siena, Santa Maria alle Scotte University Hospital, 53100 Siena, Italy*

[b] *Hospital Basico de Villamontes, Villa Montes, Bolivia*

[c] *Department of Experimental and Clinical Medicine, University of Florence, Careggi University Hospital, Florence, Italy*

[d] *Infectious and Tropical Diseases Unit, Careggi University Hospital, Florence, Italy*

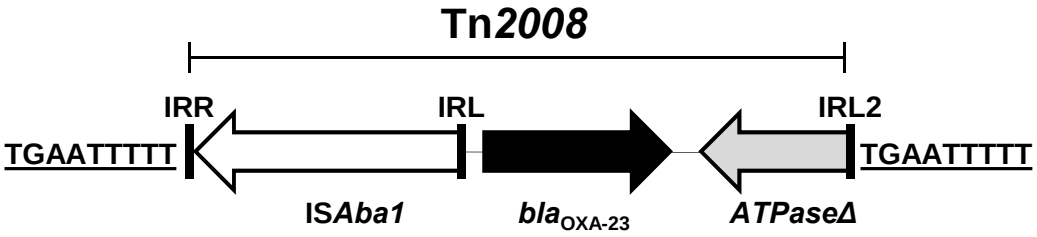
[e] *Clinical Microbiology and Virology Unit, Careggi University Hospital, Florence, Italy*

* Corresponding author. Tel.: +39 0577 233 855

fax: +39 0577 233 870

E-mail address: lucia.pallecchi@unisi.it (L. Pallecchi)

Fig. 1. Schematic representation of transposon Tn*2008* in *Acinetobacter baumannii* 9495/13. Sequence of the 9-bp target site duplication is shown (underlined upper case). Alignment of the 16-bp inverted repeats IRR (reversed and complemented), IRL and imperfect IRL2 is also shown.

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IRR  TTTGTCGTGTACAGAG
IRL  TTTGTCGTGTACAGAG
IRL2 aTTGTCGTGTAtttG
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Antimicrobial susceptibility and emerging resistance determinants (bla_{CTX-M}, *rmtB*, *fosA3*) in clinical isolates from urinary tract infections in the Bolivian Chaco

Alessandro Bartoloni [a,b], Samanta Sennati [c], Tiziana Di Maggio [c], Antonia Mantella [a], Eleonora Riccobono [c], Marianne Strohmeyer [a], Carmen Revollo [d], Ana Liz Villagran [e], Lucia Pallecchi [c], Gian Maria Rossolini [a,c,f,*]

[a] *Department of Experimental and Clinical Medicine, University of Florence, Careggi University Hospital, Largo Brambilla 3, 50134 Florence, Italy*
[b] *Infectious and Tropical Diseases Unit, Careggi University Hospital, Florence, Italy*
[c] *Department of Medical Biotechnologies, University of Siena, Santa Maria alle Scotte University Hospital, Siena, Italy*
[d] *Instituto Nacional de Laboratorios de Salud "Dr. Nestor Morales Villazón" (INLASA), La Paz, Bolivia*
[e] *Hospital Basico Villa Montes, Villa Montes, Bolivia*
[f] *Clinical Microbiology and Virology Unit, Careggi University Hospital, Florence, Italy*

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SUMMARY

Background: Bolivia is among the lowest-resourced South American countries, with very few data available on antibiotic resistance in bacterial pathogens. The phenotypic and molecular characterization of bacterial isolates responsible for urinary tract infections (UTIs) in the Bolivian Chaco are reported here.
Methods: All clinical isolates from UTIs collected in the Hospital Basico Villa Montes between June 2010 and January 2014 were analyzed ($N = 213$). Characterization included susceptibility testing, extended-spectrum beta-lactamase (ESBL) detection, identification of relevant resistance determinants (e.g., CTX-M-type ESBLs, 16S rRNA methyltransferases, glutathione S-transferases), and genotyping of CTX-M producers.
Results: Very high resistance rates were observed. Overall, the lowest susceptibility was observed for trimethoprim–sulphamethoxazole, tetracycline, nalidixic acid, amoxicillin–clavulanic acid, ciprofloxacin, and gentamicin. Of *E. coli* and *K. pneumoniae*, 11.6% were ESBL producers. Resistance to nitrofurantoin, amikacin, and fosfomycin remained low, and susceptibility to carbapenems was fully preserved. CTX-M-15 was the dominant CTX-M variant. Four *E. coli* ST131 (two being H30-Rx) were identified. Of note, isolates harbouring *rmtB* and *fosA3* were detected.
Conclusions: Bolivia is not an exception to the very high resistance burden affecting many South American countries. Optimization of alternative approaches to monitor local antibiotic resistance trends in resource-limited settings is strongly encouraged to support the implementation of effective empiric treatment guidelines.

1. Introduction

South America has long been documented as being affected by high antibiotic resistance rates.[1–5] Complex political and socio-economic factors account for this burden, and the precise quantification at the local, national, and supranational level deserves further attention.[1–4] Bolivia is one of the lowest-resourced countries of South America, and very few data on the rates and molecular epidemiology of antibiotic resistance in bacterial pathogens have been reported from this area.[1–3,5]

Since the late 1990s, cooperation and research activities addressing the phenomenon of antibiotic resistance in the Bolivian Chaco region have been performed by the present investigators, in collaboration with the Bolivian Ministry of Health.[6–13] The healthcare system of this region, which includes many rural areas and native villages, essentially relies upon small hospitals with

* Corresponding author. Tel.: +39 055 7949239; fax: +39 055 7949289.
E-mail address: gianni.rossolini@gmail.com (G.M. Rossolini).

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limited access to clinical microbiology facilities, which prevents any systematic collection of antimicrobial susceptibility data from the routine microbiological analysis of clinical specimens. By using commensal *Escherichia coli* as an indicator for the dissemination of antibiotic resistance in enterobacteria, very high resistance rates to old antibiotics (i.e., ampicillin, trimethoprim–sulphamethoxazole, tetracycline, chloramphenicol, and nalidixic acid) and alarmingly increasing trends of resistance to newer drugs (i.e., expanded-spectrum cephalosporins and fluoroquinolones) have been observed over the last two decades.[8–13] Of note, CTX-M-type extended-spectrum beta-lactamase (ESBL) determinants were first detected in this area in the early 2000s and thereafter underwent rapid dissemination, with an evolution of the dominant CTX-M groups mirroring that observed in other South American countries (i.e., initial dissemination of CTX-M-2, subsequently replaced by CTX-M-1 and CTX-M-9 groups).[1–3,8,14–16]

In this study, data on antimicrobial susceptibility and resistance determinants of bacterial pathogens responsible for urinary tract infections (UTIs) in the Bolivian Chaco region are reported. These data were obtained by analyzing clinical isolates from UTIs collected in the laboratory of the Hospital Basico Villa Montes (one of the first clinical microbiology laboratories implemented in that region) during the first 3 years of activity.

2. Methods

2.1. Bacterial isolates

A total of 213 non-replicate clinical isolates from UTIs were included in the study. They represented all of the isolates from positive urine cultures of patients with a clinical diagnosis of UTI, submitted to the clinical microbiology laboratory of the Hospital Basico Villa Montes (Villa Montes, Tarija Department, Bolivia) since its activation in June 2010, up to January 2014. Clinical isolates from both inpatients and outpatients were included. The isolates were stored in Amies transport medium (Oxoid, Milan, Italy) at 4 °C before being transferred to Italy.

2.2. Bacterial identification and in vitro antibiotic susceptibility testing

Bacterial identification was performed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Vitek MS, bioMérieux Inc., Marcy l'Etoile, France). Antibiotic susceptibility was tested by disk diffusion method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines.[17,18] As the CLSI does not provide interpretative criteria for fosfomycin and *Enterobacteriaceae* other than *E. coli*, results for the former species were interpreted using *E. coli* breakpoints (i.e., susceptible when the inhibition zone diameter is $\geq$16 mm),[18] as also reported in other studies (Endimiani et al.[19] and references therein). Screening and confirmatory tests for ESBL detection were carried out according to CLSI standards.[18] The production of AmpC-like enzymes was suspected on the basis of resistance to cephamycins and inhibition by 3-aminophenylboronic acid.[20] In AmpC producers, the presence of ESBLs was also investigated by modified CLSI confirmatory test, as recently proposed by Poulou et al.[21]Fosfomycin non-susceptible isolates were tested for the production of glutathione S-transferases using the disk potentiation test recently developed by Nakamura et al. (based on the inhibition of glutathione S-transferases by sodium phosphonoformate).[22]

2.3. Molecular analysis techniques

The detection and characterization of $bla_{\text{CTX-M}}$ and bla_{AmpC}-like beta-lactamase genes was performed using a PCR sequencing approach, as described previously.[8,23] PCR amplification was also used for the detection and characterization of the fosfomycin resistance genes *fosA*, *fosA3*, and *fosC2* (encoding glutathione S-transferases),[24] the aminoglycoside resistance genes *armA*, *npmA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, *rmtF*, *rmtG*, and *rmtH* (encoding 16S rRNA methyltransferases), and *aac(6′)Ib* (encoding aminoglycoside acetyltransferases).[25,26] The determination of *E. coli* phylogenetic groups was carried out by multiplex PCR.[27] Established PCR-based methods were used to define the *E. coli* clone B2-O25b-ST131 and its subclones H30 and H30-Rx.[28–30]

2.4. Statistical analysis

Statistical differences were determined by Chi-square test (with Yates' correction) and the unpaired *t*-test, using GraphPad Prism for Windows, version 5.0 (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

3.1. Patient characteristics and aetiology of UTIs

A total of 213 clinical isolates considered responsible for UTIs were collected during the study period (June 2010 to January 2014). The study started upon activation of the clinical microbiology facility. A very low number of urine samples were processed during the first 2 years of activity (due to initial difficulties in implementing the facility), while an increasing trend was observed after 2012. As a consequence, the distribution of clinical isolates over time was as follows: 2010 (7 months), $n = 10$; 2011, $n = 12$; 2012, $n = 73$; 2013, $n = 105$; 2014 (1 month), $n = 13$.

Of the 213 clinical isolates, 71 were from inpatients and 140 were from outpatients; the origin was unknown for two isolates. The overall male to female patient ratio was 40:171, with the sex of two patients unknown. Patients ranged in age from 2 months to 95 years (mean age 44 years, median age 45 years), with the age of 16 patients unknown. Inpatient and outpatient populations differed in age distribution (mean age 54 vs. 39 years, median age 55 vs. 37 years; $p = 0.0003$), while no significant difference was observed in the male to female ratio (12:59 vs. 28:111; $p = 0.57$).

Of the 213 clinical isolates, 209 (98.1%) were *Enterobacteriaceae*, three were *Pseudomonas aeruginosa* (1.4%), and one was a *Staphylococcus saprophyticus* (0.5%) (Table 1). *E. coli* represented the dominant species (79.8%), followed by *Klebsiella pneumoniae* (8.9%) (Table 1), with no differences observed between inpatients and outpatients (data not shown).

3.2. Antibiotic susceptibility

Overall, the UTI isolates collected from the Bolivian Chaco exhibited high rates of antibiotic resistance (Table 2; **Supplementary Material** Table S1).

Table 1
Aetiology of urinary tract infections in the Bolivian Chaco (2010–2014)

Species	No. of isolates	%
Escherichia coli	170	79.8
Klebsiella pneumoniae	19	8.9
Citrobacter spp	6	2.8
Enterobacter spp	5	2.3
Proteus spp	5	2.3
Morganella morganii	3	1.4
Pseudomonas aeruginosa	3	1.4
Providencia rettgeri	1	0.5
Staphylococcus saprophyticus	1	0.5

Table 2
Antibiotic susceptibility rates (%) of *Escherichia coli* and *Klebsiella pneumoniae* urinary isolates

Drug[a]	*E. coli* (*n* = 170)	*K. pneumoniae* (*n* = 19)	Total (*n* = 189)
AMC	56.5	47.3	55.6
Cefotaxime	89.4	68.4	87.3
Ceftazidime	92.3	73.7	90.5
Meropenem	100	100	100
SXT	26.5	52.6	28.6
Nalidixic acid	51.2	63.2	52.4
Ciprofloxacin	61.8	68.4	62.4
Gentamicin	72.4	68.4	72.0
Amikacin	95.3	89.5	94.7
Nitrofurantoin	91.8	31.6	85.7
Fosfomycin	98.2	94.7	97.9
Tetracycline	47.1	57.9	47.6

[a] AMC, amoxicillin–clavulanic acid; SXT, trimethoprim–sulphamethoxazole.

Considering *E. coli* and *K. pneumoniae*, which were by far the most prevalent pathogens, the most affected drugs were trimethoprim–sulphamethoxazole, tetracycline, nalidixic acid, amoxicillin–clavulanic acid, and ciprofloxacin (Table 2). Expanded-spectrum cephalosporins remained active against the majority of isolates (87.3% and 90.5% susceptibility to cefotaxime and ceftazidime, respectively) (Table 2). Of the 24 isolates resistant to cefotaxime, 22 were identified as ESBL producers (11.6% of all *E. coli* and *K. pneumoniae* isolates). The remaining two isolates showed a resistance phenotype suggestive of the production of AmpC-like enzymes. Expanded-spectrum cephalosporin-resistant isolates were significantly more prevalent among *K. pneumoniae* than *E. coli* isolates (31.6% vs. 10.6%; $p = 0.009$). Overall, the most active drugs were meropenem, fosfomycin, amikacin, and nitrofurantoin (the latter only for *E. coli*) (Table 2). *K. pneumoniae* isolates exhibited very low susceptibility to nitrofurantoin, in accordance with previous reports.[31] The few non-enterobacterial pathogens showed susceptible phenotypes typical of wild-type strains, except for resistance to ciprofloxacin exhibited by one of the three *P. aeruginosa* isolates (data not shown). Of note, no significant difference in terms of antibiotic resistance rates and prevalence of ESBL phenotype was observed between clinical isolates from inpatients and outpatients (data not shown), while an increasing trend (statistically significant for expanded-spectrum cephalosporins, quinolones, and nitrofurantoin) was observed with age (**Supplementary Material** Table S2).

The high resistance rates observed in clinical isolates from UTIs from the Bolivian Chaco were overall consistent with those reported from other Latin American countries,[1–4] and with data collected in Bolivia by the Pan-American Health Organization (PAHO).[5,32]

One limitation of the present study, which might have introduced a bias towards an overestimation of resistance rates, is that in Bolivia microbiological diagnosis represents an extra cost for the patient, and urine specimens for culture are rarely requested by physicians for the diagnosis of uncomplicated UTIs. In this scenario, it is likely that a number of the clinical isolates from community-acquired UTIs originated from patients whose initial empiric therapy had failed. The presence of such a selection bias represents a general problem in studies addressing the epidemiology of community-acquired uncomplicated UTIs,[33,34] and is even more complicated in resource-limited countries for issues related to weaknesses of healthcare systems and poverty.

3.3. Characterization of the enterobacterial isolates resistant to expanded-spectrum cephalosporins

All 24 isolates that were resistant to expanded-spectrum cephalosporins exhibited a multidrug resistance phenotype (defined as resistance to at least three different classes of antibiotic agents) (Table 3). In particular, susceptibility rates of expanded-spectrum cephalosporin-resistant *E. coli* ($n = 18$) and *K. pneumoniae* ($n = 6$) were as follows, respectively: meropenem, 100% and 100%; fosfomycin, 83% and 83%; nitrofurantoin, 83% and 0%; amikacin, 72% and 67%; trimethoprim–sulphamethoxazole, 33% and 17%; gentamicin, 22% and 0%; tetracycline, 22% and 17%; ciprofloxacin, 6% and 0%; nalidixic acid, 6% and 0% (Table 3).

All ESBL producers were found to harbour genes encoding CTX-M-type enzymes. CTX-M-1 group variants were the most prevalent (15 isolates, 68%), followed by CTX-M-9 group (seven isolates, 32%) and CTX-M-2 group (one isolate, 5%), with one isolate harbouring variants from two groups (Table 3). Sequencing of $bla_{\text{CTX-M}}$ genes identified $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-65}}$ as the most prevalent variants (Table 3).

The 16 CTX-M-producing *E. coli* isolates belonged to each of the four major phylogenetic groups (A, 38%; D, 31%; B2, 25%; B1, 6%), with no specific association observed between CTX-M allelic variants and phylogenetic groups (Table 3). Of note, all four CTX-M-15-producing *E. coli* isolates belonging to phylogenetic group B2 were assigned to the pandemic clone ST131, with two isolates of hospital origin belonging to the H30-Rx subclone.[28–30,35]

The two isolates producing AmpC-like enzymes were shown to harbour $bla_{\text{CMY-2}}$.

Altogether, the molecular epidemiology of CTX-M enzymes in clinical isolates from the Bolivian Chaco appeared to be coherent with their recent evolution in South America.[1–3,14] The heterogeneity in terms of CTX-M allelic variants and population structure (i.e., diverse *E. coli* phylogenetic groups) would exclude a major role of clonal expansion in the dissemination of CTX-M ESBLs among enterobacteria responsible for UTIs in the study setting, and the investigation of plasmids encoding the most prevalent variant (i.e., CTX-M-15) deserves further attention. Interestingly, the urinary *E. coli* isolates analyzed in this study exhibited a comparable prevalence of CTX-M producers (9% vs. 17%; $p = 0.07$) and a similar distribution of CTX-M variants (CTX-M-15: 56% vs. 38%, $p = 0.4$; CTX-M-65: 19% vs. 27%, $p = 0.8$) as those of commensal *E. coli* collected in 2011 from healthy children living in the same urban area,[8] emphasizing the role of commensal enterobacteria as a reservoir of clinically relevant resistance determinants.

3.4. Detection of emerging aminoglycoside and fosfomycin resistance genes

Acquired 16S rRNA methyltransferases (accounting for high-level and broad-spectrum aminoglycoside resistance) and glutathione S-transferases (accounting for fosfomycin resistance) have been reported increasingly among enterobacterial isolates in recent years, often in association with beta-lactamases, further complicating the management of infections caused by multidrug-resistant isolates.[25,36–39] Although resistance to amikacin and fosfomycin were uncommon in this setting, the isolates non-susceptible to these drugs were screened for the presence of the above resistance determinants.

All isolates exhibiting amikacin non-susceptibility (*E. coli*, $n = 8$; *K. pneumoniae*, $n = 2$) were subjected to a multiplex PCR for the detection of the 10 known 16S rRNA methyltransferase determinants.[25] Positive results for *rmtB* were obtained with the two *K. pneumoniae* isolates. Both isolates also carried $bla_{\text{CTX-M-65}}$, suggesting a possible genetic linkage between the two resistance determinants (not further investigated) (Table 3). The epidemiology of plasmid-encoded 16S rRNA methyltransferases in South America has been poorly investigated so far, with the few data available pointing towards a dominance of RmtD-like enzymes and the occasional occurrence of RmtB (in *E. coli* and *Proteus mirabilis*

Table 3
Phenotypic and molecular features of expanded-spectrum cephalosporin-resistant isolates from the Bolivian Chaco

Isolate	Year	Origin[a]	Other resistance traits[b]	CTX-M group	CTX-M variant[c]	Phylogenetic group[d]	Other relevant resistance genes
E. coli VM-72	2011	H	NAL, CIP, CN, TET	1	CTX-M-15	B2 (H30-Rx ST131)	-
E. coli VM-292	2011	C	NAL, CIP, CN, FOT, TET	9	CTX-M-65	D	*fosA3*
E. coli VM-77	2012	C	SXT, NAL, CIP, CN, TET	9	CTX-M-14	D	-
E. coli VM-82	2012	C	sxt, NAL, CIP, CN, TET	9	CTX-M-14	D	-
E. coli VM-255	2012	C	NAL, CIP, CN	-	-	A	bla_{CMY-2}
E. coli VM-334	2012	H	SXT, NAL, CIP, CN, NIT, TET	9	CTX-M-65	A	-
E. coli VM-337	2012	C	SXT, NAL, CIP, CN, NIT, TET	1	CTX-M-15	A	-
E. coli VM-353	2013	H	FOT, TET	1	CTX-M-55	A	*fosA3*
E. coli VM-363	2013	C	SXT, NAL, CIP, CN, AK, TET	-	-	B1	bla_{CMY-2}, *aac(6′)Ib*
E. coli VM-364	2013	C	SXT, NAL, CIP, CN, ak, TET	1	CTX-M-15	B2 (non-H30 ST131)	*aac(6′)Ib-cr*
E. coli VM-365	2013	C	NAL, CIP, ak	1	CTX-M-15	A	*aac(6′)Ib-cr*
E. coli VM-366	2013	H	SXT, NAL, CIP, CN, TET	9	CTX-M-65	A	-
E. coli VM-379	2013	H	SXT, NAL, CIP, CN, nit, TET	1	CTX-M-15	A	-
E. coli VM-439	2013	H	SXT, NAL, CIP	1	CTX-M-15	B1	-
E. coli VM-444	2013	C	SXT, NAL, CIP, CN, AK, TET	1	CTX-M-15	B2 (non-H30 ST131)	*aac(6′)Ib-cr*
E. coli VM-474	2013	C	SXT, NAL, CIP, CN	1	CTX-M-15	D	-
E. coli VM-517	2013	U	SXT, NAL, CIP, CN, FOT, TET	1	CTX-M-55	D	*fosA3*
E. coli VM-498	2014	H	NAL, CIP, ak, TET	1	CTX-M-15	B2 (H30-Rx ST131)	*aac(6′)Ib-cr*
K. pneumoniae VM-249	2012	C	SXT, NAL, CIP, CN, NIT, TET	1	CTX-M-15	ND	-
K. pneumoniae VM-354	2013	H	SXT, NAL, CIP, CN, NIT, TET	1, 2	CTX-M-2, CTX-M-15	ND	-
K. pneumoniae VM-397	2013	C	NAL, CIP, CN, AK, NIT, FOT, TET	9	CTX-M-65	ND	*fosA3*, *rmtB*
K. pneumoniae VM-419	2013	H	SXT, NAL, CIP, CN, NIT, TET	1	CTX-M-15	ND	-
K. pneumoniae VM-466	2013	C	SXT, NAL, CIP, CN, NIT	1	CTX-M-15	ND	-
K. pneumoniae VM-477	2013	H	SXT, NAL, CIP, CN, AK, NIT, TET	9	CTX-M-65	ND	*rmtB*, *aac(6′)Ib-cr*

[a] H, hospital; C, community; U, unknown.
[b] SXT, trimethoprim–sulphamethoxazole; NAL, nalidixic acid; CIP, ciprofloxacin; CN, gentamicin; AK, amikacin; NIT, nitrofurantoin; FOT, fosfomycin; TET, tetracycline. For the antibiotics, upper case indicates a resistance phenotype and lower case indicates an intermediate phenotype.
[c] All isolates ($n = 24$) were classified as potential extended-spectrum beta-lactamase (ESBL) producers (as per the Clinical and Laboratory Standards Institute (CLSI)) on the basis of inhibition zone diameters of cefotaxime and/or ceftazidime smaller than the screening breakpoints ($\leq$27 mm for cefotaxime and $\leq$22 mm for cefotaxime). The phenotypic confirmatory test for ESBL production (as per CLSI) was positive for 22 isolates using both cefotaxime and ceftazidime, with the exception of the two *E. coli* isolates producing CTX-M-14 (*E. coli* VM-77 and *E. coli* VM-82) for which a $\geq$5 mm increase in inhibition zone diameter in the presence of clavulanic acid was observed only with cefotaxime. The two CMY-2-producing *E. coli* isolates (*E. coli* VM-255 and *E. coli* VM-363) were negative by the modified CLSI confirmatory test for ESBL detection in AmpC producers, as recently proposed by Poulou et al.[21]
[d] ND, not determined.

isolates).[1,40] The present data provide further evidence of the dissemination of RmtB among enterobacterial pathogens from this epidemiological setting.

The amikacin non-susceptible phenotype of the remaining isolates was found to be mostly the result of the presence of *aac(6′)Ib* and its variant *aac(6′)Ib-cr* (detected in one and six isolates, respectively; all but one resistant to expanded-spectrum cephalosporins) (Table 3). These results confirmed the dissemination of *aac(6′)Ib-cr* (clinically relevant for its ability to reduce susceptibility to both aminoglycosides and quinolones) in the study setting,[41] and its frequent association with ESBL determinants.

Among the seven isolates showing a fosfomycin non-susceptible phenotype (*E. coli*, $n = 3$; *Morganella morganii*, $n = 3$; *K. pneumoniae*, $n = 1$), four were identified as producers of glutathione S-transferases by a recently developed disk potentiation test (i.e., all *E. coli* and *K. pneumoniae* isolates),[22] and all of them were found to harbour *fosA3*. Of note, all the *fosA3*-positive isolates were also CTX-M producers (Table 3), confirming the frequent association of *fosA3* with CTX-M determinants.[42–44] The fosfomycin resistance phenotype shown by the three *M. morganii* isolates was not unexpected and confirmed previous reports of a very low susceptibility to this drug.[45]

3.5. Conclusions

In general, antimicrobial chemotherapy for uncomplicated UTIs is selected empirically, based on guidelines that consider the local epidemiology of antimicrobial resistance, which can be very different in diverse geographical settings.[3,33] This is the first study on the antimicrobial susceptibility of clinical isolates from UTIs of patients in the Bolivian Chaco region, and one of the first from Bolivia. The present data indicate an overall high resistance burden, with extremely high levels of resistance to trimethoprim–sulphamethoxazole and fluoroquinolones, and a sizeable presence of ESBL determinants in *Enterobacteriaceae*, suggesting that nitrofurantoin and fosfomycin might represent the first-line treatment options for uncomplicated UTIs in this area.[3,33]

This is also the first study to characterize antibiotic resistance determinants in enterobacteria responsible for UTIs in Bolivia. Among relevant findings, the emergence of FosA3 among ESBL-producing isolates was noted and is of concern, since it could impair the efficacy of fosfomycin. The other alarming finding was the emergence of RmtB, which compromises the activity of all aminoglycosides, which in a similar setting could be important options for complicated UTIs caused by multidrug-resistant pathogens.

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Conflict of interest: The authors declare that they have no competing interests in relation to the results presented in this study.

Authors' contributions: AB and GMR conceived and coordinated the study, analyzed the results, and wrote the manuscript. TDM and AM carried out bacterial identification and susceptibility testing. SS, ER, and LP carried out phenotypic and molecular detection and characterization of resistance determinants, and LP also drafted the manuscript. ALV provided the clinical isolates and helped to draft the manuscript. MS participated in the design of the study, coordinated the field activities, and performed the statistical analysis. CR participated in data analysis and critically revised the manuscript. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2015.12.008.

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Methicillin-resistant *Staphylococcus aureus* in hospitalized patients from the Bolivian Chaco

Alessandro Bartoloni [a,b,*], Eleonora Riccobono [c], Donata Magnelli [a], Ana Liz Villagran [d], Tiziana Di Maggio [c], Antonia Mantella [a], Samanta Sennati [c], Carmen Revollo [e], Marianne Strohmeyer [a], Tommaso Giani [c], Lucia Pallecchi [c], Gian Maria Rossolini [a,c,f,*]

[a] *Dipartimento di Medicina Sperimentale e Clinica, Università di Firenze, Ospedale di Careggi, Largo Brambilla 3, 50134 Firenze, Italy*
[b] *SOD Malattie Infettive e Tropicali, Azienda Ospedaliero-Universitaria Careggi, Firenze, Italy*
[c] *Dipartimento di Biotecnologie Mediche, Università di Siena, Siena, Italy*
[d] *Hospital Basico Villa Montes, Villa Montes, Bolivia*
[e] *Instituto Nacional de Laboratorios de Salud "Dr. Nestor Morales Villazón" (INLASA), La Paz, Bolivia*
[f] *SOD Microbiologia e Virologia, Azienda Ospedaliero-Universitaria Careggi, Firenze, Italy*

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SUMMARY

Objectives: Information is lacking on the methicillin-resistant *Staphylococcus aureus* (MRSA) clonal lineages circulating in Bolivia. We investigated the prevalence and molecular epidemiology of *S. aureus* colonization in hospitalized patients from the Bolivian Chaco, and compared their features with those of the few clinical isolates available from that setting.

Methods: *S. aureus* nasal/inguinal colonization was investigated in 280 inpatients from eight hospitals in two point prevalence surveys (2012, $n = 90$; 2013, $n = 190$). Molecular characterization included genotyping (*spa* typing, multilocus sequence typing, and pulsed-field gel electrophoresis), detection of virulence genes, and SCC*mec* typing.

Results: Forty-one inpatients (14.6%) were *S. aureus* nasal/inguinal carriers, of whom five were colonized by MRSA (1.8%). MRSA isolates mostly belonged to *spa*-type t701, harboured SCC*mec* IVc, and were negative for Panton–Valentine leukocidin (PVL) genes. However, a USA300-related isolate was also detected, which showed the characteristics of the USA300 Latin American variant (USA300-LV; i.e., ST8, *spa*-type t008, SCC*mec* IVc, presence of PVL genes, absence of *arc*A). Notably, all the available MRSA clinical isolates ($n = 5$, collected during 2011–2013) were also identified as USA300-LV.

Conclusions: Overall, MRSA colonization in inpatients from the Bolivian Chaco was low. However, USA300-LV-related isolates were detected in colonization and infections, emphasizing the importance of implementing control measures to limit their further dissemination in this resource-limited area.

1. Introduction

The worldwide emergence and dissemination of methicillin-resistant *Staphylococcus aureus* (MRSA) has significantly reduced the therapeutic options for staphylococcal infections and worsened their clinical outcome.[1] MRSA isolates are resistant to virtually all beta-lactams (except the newer anti-MRSA compounds) due to the expression of low-affinity penicillin-binding proteins (PBPs), encoded by the *mec*A or *mec*C genes, which can overtake the functions of the other PBPs.[1,2] The *mec* genes are carried by particular mobile genetic elements prevalent in staphylococci, named staphylococcal cassette chromosome (SCC) elements, with 11 types of SCC*mec* having been characterized so far.[2]

MRSA has been disseminating across virtually all geographical areas for decades, arising as a major pathogen in both the hospital and community setting, with a limited number of highly successful clonal lineages being responsible for most MRSA epidemics worldwide.[3] The surveillance of MRSA clones (both from infections and colonization) is crucial for the implementation of effective

* Corresponding authors. Tel.: +39 055 7949482; fax: +39 055 7949431. Tel.: +39 055 7949239; fax: +39 055 7949289.
E-mail addresses: alessandro.bartoloni@unifi.it (A. Bartoloni), gianni.rossolini@gmail.com, gianmaria.rossolini@unifi.it (G.M. Rossolini).

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empiric treatment protocols and infection control measures, and to understand the diverse evolutionary trajectories of MRSA lineages on a worldwide scale.[3]

Latin America is not an exception to the global increasing prevalence of MRSA infections, and a number of reports have described the epidemiological and molecular features of MRSA clonal lineages circulating in this geographic area over the past two decades.[4,5] Two major MRSA clones, namely the Brazilian clone MRSA-ST239-IIIA (belonging to sequence type (ST) 239 and harbouring SCC*mec* IIIA) and the Cordobes/Chilean clone MRSA-ST5-I, have accounted for the early emergence and dissemination of MRSA in the hospital setting in several Latin American countries.[4–6] Regarding the community setting, three major MRSA lineages producing the Panton–Valentine leukocidin (PVL) have been described in Latin America: MRSA-ST30-IV and MRSA-ST5-IV, mainly disseminated in southern countries,[4,7,8] and MRSA-ST8-IVc, predominant in northern countries.[4,9–12] The latter is genetically related to the USA300 MRSA pandemic clone, but harbours a different SCC*mec* IV subtype (IVc instead of IVa) and typically lacks the arginine catabolic mobile element (ACME).[9] MRSA-ST8-IVc (sometimes referred to as USA300-LV, for USA300 Latin American variant) has recently been acknowledged as the major clone responsible for both community and hospital MRSA infections in Colombia.[11–13]

Bolivia is one of the poorest countries of Latin America, and in many rural areas the healthcare system relies on small hospitals that have no access to clinical microbiology diagnosis and limited resources for the implementation of infection control measures. An MRSA prevalence of 49% has been reported recently,[14] but data on MRSA clonal lineages circulating in this country are lacking, and very few data are available on the dissemination of MRSA in rural areas.[15,16]

In a previous surveillance study on MRSA nasal carriage, we documented a low MRSA prevalence (range 0–1.5%) among healthy individuals from the Bolivian Chaco, a resource-limited region of Bolivia.[16] In this work we investigated, for the first time, the prevalence and molecular epidemiology of *S. aureus* colonization in hospitalized patients from that region, and compared their features with those of the few *S. aureus* clinical isolates available from that setting.

2. Methods

2.1. Study design and population

S. aureus colonization was investigated in eight hospitals in seven small urban areas of the Bolivian Chaco region (Figure 1 and **Supplementary Material** Table S1). All hospitals are small healthcare units with 20 to 78 beds (**Supplementary Material** Table S1), and together are representative of the organization of the hospital care system in this area. Facilities for microbiological diagnosis of skin and soft tissue infections (SSTIs) were not available at these hospitals, with the exception of one of them – the hospital of Villa Montes. This hospital has performed microbiological analyses since mid-2010, although a very limited number of samples are processed.

The survey was a point prevalence study performed twice (on August 2–3, 2012, and on August 12–17, 2013). All individuals hospitalized during the study periods were considered eligible. After providing written informed consent (obtained from the parents or legal guardians in the case of a minor), samples were obtained from each patient for the detection of *S. aureus* colonization (see below). Full ethical clearance was obtained from the qualified authorities who revised and approved the study design (Convenio de Salud, Ministerio de Salud – Vicariato de Camiri, Camiri, Bolivia).

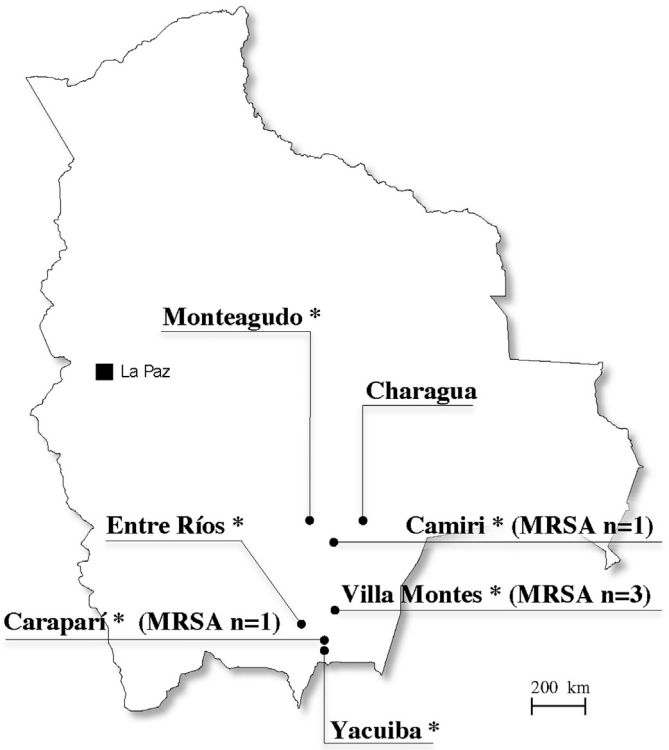

Figure 1. Location of the centres involved in the study. The study involved eight hospitals: Monteagudo (Hospital San Antonio de Los Sauces, Hospital Dermatologico); Charagua (Hospital Mamerto Eguez Soruco); Camiri (Hospital Municipal de Camiri); Villa Montes (Hospital de Villa Montes); Caraparí (Hospital de Caraparí); Entre Rios (Hospital de Entre Rios); Yacuiba (Hospital de Yacuiba). Hospitals with patients colonized by *Staphylococcus aureus* are marked with an asterisk and the number of patients carrying MRSA is reported in brackets.

For comparison purposes, all *S. aureus* clinical isolates collected in the hospital of Villa Montes since the introduction of the clinical microbiology laboratory were included in the present study (see below).

2.2. Screening for carriage of S. aureus

The investigation of *S. aureus* colonization was performed by obtaining two samples from each participant: a nasal swab (a single swab for both nares) and an inguinal swab (a single swab for both groin sides). The nasal and inguinal swabs obtained from each individual were preserved at 4 °C in Amies transport medium (Oxoid, Milan, Italy) and transported to the hospital of Camiri, where the swabs were processed as follows. Each pair of swabs (from each subject) was inoculated overnight at 35 °C in an enrichment medium (2 ml) constituted of tryptic soy broth (TSB) (Oxoid) supplemented with 6.5% NaCl and 25 μg/ml colistin (prepared by adding 1 disk of colistin 25 μg per millilitre of broth). Then, 10 μl of the enriched suspension was plated onto mannitol salt agar (MSA) (Oxoid); the bacterial growth was collected and preserved in Amies transport medium for transfer to Italy. Here, each sample was again plated on MSA, and mannitol-fermenting colonies were subcultured and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS system; bioMérieux Inc., Marcy l'Etoile, France). For each sample, only one *S. aureus* isolate was selected for further analysis.

2.3. In vitro susceptibility testing

Antimicrobial susceptibility testing was performed by disk diffusion method in accordance with the Clinical and Laboratory

Standards Institute (CLSI) guidelines.[17,18] Antibiotic disks were purchased from Oxoid. All isolates were tested for susceptibility to penicillin G, erythromycin, clindamycin, tetracycline, ciprofloxacin, trimethoprim–sulfamethoxazole, gentamicin, chloramphenicol, and rifampin. In addition, they were investigated for the methicillin resistance phenotype by cefoxitin screening disk test, and for inducible clindamycin resistance by D-zone test.[18] *S. aureus* ATCC 25923 was used as quality control.

2.4. Molecular characterization of S. aureus isolates

PVL genes and the *arc*A gene (for ACME) were detected by PCR, as described previously.[10] Genotyping was performed by *spa* typing,[19] multilocus sequence typing (MLST) (http://www.mlst.net),[20] and pulsed-field gel electrophoresis (PFGE) following the CDC protocol (http://www.cdc.gov/HAI/pdfs/labSettings/ar_mras_PFGE_s_aureus.pdf) and interpreted according to the criteria proposed by van Belkum et al.[21] SCC*mec* characterization was achieved following the PCR-based protocols described previously by Kondo et al. and Milheiriço et al. for typing and subtyping, respectively.[22,23]

2.5. Clinical isolates

Clinical isolates were collected in the hospital of Villa Montes, the only one with facilities for the microbiological diagnosis of SSTIs. During the period May 2010 to August 2013, only 10 *S. aureus* clinical isolates were collected from that hospital. This limited number is related to the fact that microbiological diagnosis represents an extra cost for the patient and is rarely requested by physicians. Of the 10 isolates, only nine were available for investigation. Identification was confirmed by MALDI-TOF MS (bioMérieux) and characterization was carried out as described for *S. aureus* isolates from colonization.

2.6. Statistical analysis

Statistical differences were determined by Chi-square test (with Yates' correction) and Fisher's exact test when appropriate. Confidence intervals (95% CI) were calculated using the binomial distribution.

3. Results and discussion

3.1. Characteristics of the study population

Among all individuals admitted to hospital during the point prevalence study periods, 280 (2012, $n = 90$; 2013, $n = 190$) agreed to participate in the survey. In 2013, 80% of inpatients consented to sampling, while no data were available for the 2012 survey.

The male to female ratio of the study participants was 181:99, and they ranged in age from 1 day to 89 years (mean age 31 years and median age 26 years; calculated from all but five patients, for whom age was not available). Inpatients were admitted to maternity ($n = 93$), general medicine ($n = 78$), surgery ($n = 50$), paediatric ($n = 45$), tuberculosis isolation ($n = 7$), first aid ($n = 4$), and intensive care ($n = 3$) wards. At the time of sample collection, 63% of patients had been hospitalized within the previous 48 h and 77% of them reported no hospitalization during the preceding year.

3.2. Colonization by S. aureus among inpatients from eight hospitals in the Bolivian Chaco region

An overall moderate prevalence of *S. aureus* colonization was detected among the 280 hospitalized individuals included in the study ($n = 41$, 14.6%), with no significant differences observed between the two study periods (17.8% (95% CI 10–27%) and 13.2% (95% CI 9–19%) in 2012 and 2013, respectively; $p = 0.4$). Carriers of *S. aureus* were found in all hospitals except in Charagua (where nine inpatients were enrolled in 2012 and 15 in 2013) (Figure 1 and **Supplementary Material** Table S1).

Colonization by MRSA isolates was detected with a rate of 1.1% (95% CI 0–6%) in 2012 and 2.1% (95% CI 0–5%) in 2013 ($p = 1$), and involved five inpatients from three out of the eight hospitals (Camiri, $n = 1$; Caraparí, $n = 1$; Villa Montes, $n = 3$) (Figure 1 and **Supplementary Material** Table S1). No relevant correlation between methicillin-sensitive *S. aureus* (MSSA)/MRSA carriage and clinical/demographic data was observed (data not shown). The observed MRSA colonization rate (1.8% of the total studied population) was overall consistent with the results of a previous study conducted in the same geographic region in 2008 and 2009, which revealed MRSA colonization in three out of 196 (1.5%) healthy individuals from a rural village.[16] A similar MRSA colonization rate was also reported recently in a population-based study performed in a small city of Brazil (0.9%) and among medical students in Colombia (1.6%).[24,25] The particular healthcare system of the Bolivian Chaco, based on very small hospitals, and the fact that 63% of samples were collected within 48 h of admission, could explain the similar MRSA colonization rates observed in the community and hospital settings in this region.

3.3. Characterization of S. aureus isolates from carriers

Susceptibility testing revealed overall low resistance rates in *S. aureus* isolates from carriers. Among MSSA isolates ($n = 36$), most (83%) were non-susceptible to penicillin G, while only a few were non-susceptible to tetracycline (14%), gentamicin (8%), chloramphenicol (6%), and erythromycin (3%, with inducible clindamycin resistance), and all isolates were susceptible to trimethoprim–sulfamethoxazole, ciprofloxacin, linezolid, and rifampin. MRSA isolates ($n = 5$) were susceptible to all of the above non-beta-lactam antibiotics tested (except for one isolate that was non-susceptible to erythromycin and ciprofloxacin).

MSSA isolates tested negative for *mec*A and PVL genes, while all MRSA isolates were found to carry SCC*mec* IVc, and one of them was PLV-positive. *spa* typing analysis revealed a high heterogeneity among MSSA isolates (20 different types, including the new *spa*-type t13417), without a predominance of any *spa*-type (Table 1). In contrast, the five MRSA isolates were found to belong to two *spa*-types: t701 (one isolate in 2012 and three in 2013) and t008 (one isolate in 2013) (Table 1).

Interestingly, besides being the dominant *spa*-type among MRSA isolates in this survey (Table 1), *spa*-type t701 was also found in three MSSA isolates from this survey and all of the MRSA isolates ($n = 3$) detected in a previous study on MRSA nasal carriage in a small rural community in the same geographical setting (Table 2).[16] In order to investigate their clonal relationships, the seven MRSA and three MSSA isolates belonging to t701 were subjected to PFGE analysis, which assigned all isolates to the same clonal lineage (PFGE type A) with a maximum of five different bands (Table 2). These data demonstrate that *spa*-type t701 is endemic in this area (being detected over a period of 5 years in the microbiota of both healthy individuals and inpatients), and suggest the likely local evolution of MRSA from MSSA (or vice versa), as described in other settings.[26] MRSA and MSSA belonging to *spa*-type t701 are described all over the world, from infection and colonization, as also reported in the Ridom SpaServer (http://www.spaserver.ridom.de/). In addition, a recent publication reported that t701 (harbouring different SCC*mec* types) accounted for 30% of MRSA clinical isolates from diverse hospitals in the west of Iran,[27] suggesting a propensity of this lineage to epidemic dissemination.

Table 1
Population structure of *Staphylococcus aureus* isolates from colonization and infection in patients from eight hospitals in the Bolivian Chaco

Source	Year	MSSA/ MRSA	Number of isolates	*Spa*-type (number of isolates)
Colonization	2012	MSSA	15	t189 (*n* = 3); t701 (*n* = 3); t359 (*n* = 2); t2883 (*n* = 2); t002 (*n* = 1); t088 (*n* = 1); t645 (*n* = 1); t1671 (*n* = 1); t6907 (*n* = 1)
		MRSA	1	t701 (*n* = 1)
Colonization	2013	MSSA	21	t024 (*n* = 3); t189 (*n* = 3); t078 (*n* = 2); t645 (*n* = 2); t729 (*n* = 2); t065 (*n* = 1); t319 (*n* = 1); t1166 (*n* = 1); t1451 (*n* = 1); t4710 (*n* = 1); t5365 (*n* = 1); t6125 (*n* = 1); t13417 (*n* = 2)
		MRSA	4	t701 (*n* = 3); t008 (*n* = 1)
Infection[a]	2010–2013	MSSA	4	t002 (*n* = 1); t021 (*n* = 1); t088 (*n* = 1); t645 (*n* = 1)
		MRSA	5	t008 (*n* = 5)

MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *Staphylococcus aureus*.
[a] All clinical isolates except three (t645, t088, and one representative of t008) were from inpatients.

Table 2
Features of *Staphylococcus aureus* isolates from colonization and infection belonging to *spa*-types t701 and t008

Source	Year	Isolate	Origin	Type (population)	MRSA/MSSA	*spa*-type	PVL	SCC*mec*	PFGE[c]
Colonization	2012	131a	Yacuiba	Hospital	MSSA	t701	Neg	NA	A_3
		176a	Monteagudo	Hospital	MSSA	t701	Neg	NA	A_3
		188a	Villa Montes	Hospital	MSSA	t701	Neg	NA	A_2
		121a	Camiri	Hospital	MRSA	t701	Neg	IVc	A_1
Colonization	2013	272a	Villa Montes	Hospital	MRSA	t701	Neg	IVc	A
		280a	Villa Montes	Hospital	MRSA	t701	Neg	IVc	A
		281a	Villa Montes	Hospital	MRSA	t701	Neg	IVc	A
		284a	Caraparí	Hospital	MRSA[d]	t008	Pos	IVc	B
Infection[a]	2010–2013	304	Villa Montes	Hospital	MRSA	t008	Pos	IVc	B
		306	Villa Montes	Hospital	MRSA	t008	Pos	IVc	B
		233	Villa Montes	Hospital	MRSA	t008	Pos	IVc	B
		393	Villa Montes	Hospital	MRSA	t008	Pos	IVc	B_1
		401	Villa Montes	Hospital	MRSA	t008	Pos	IVc	B
Colonization[b]		140	Gutierrez	Community	MRSA	t701	Neg	IVc	A
	2009	99	Gutierrez	Community	MRSA	t701	Neg	IVc	A_1
		132	Gutierrez	Community	MRSA	t701	Neg	IVc	A_1

MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *Staphylococcus aureus*; PVL, Panton–Valentine leukocidin; SCC*mec*, staphylococcal cassette chromosome *mec*; PFGE, pulsed-field gel electrophoresis; NA, not applicable.
[a] All isolates belonging to *spa*-type t008 were from inpatients, with the exception of isolate 304, which was from an outpatient. Isolates 304 and 306 were collected in 2011, isolate 233 in 2012, and isolates 393 and 401 in 2013.
[b] Previously characterized by Bartoloni et al., 2013.[16]
[c] Two different bands (A/A_1, A_2/A_3); three different bands (A/A_2); five different bands (A/A_3, A_1/A_2, A_1/A_3).
[d] All MRSA isolates remained susceptible to all non-beta-lactam antibiotics tested, with the exception of isolate 284a, which showed intermediate susceptibility to erythromycin and ciprofloxacin.

The MRSA isolate belonging to *spa*-type t008 was the only one testing positive for PVL genes and showing non-susceptibility to erythromycin and ciprofloxacin (Table 2). MLST assigned it to ST8 (ST8 clonal complex). As ST8, *spa*-type t008, SCC*mec* IVc, and PLV positivity are among the features of USA300 Latin American variant (USA300-LV), we also tested this isolate for the presence of ACME. The absence of ACME confirmed that this isolate was related to USA300-LV, one of the most widespread MRSA clones and largely dominant in community and hospital settings in northern countries of Latin America.[4,9–13]

3.4. Comparison between S. aureus isolates from colonization and infection

In order to compare *S. aureus* isolates from colonization and infection, nine clinical isolates collected from SSTIs in Villa Montes were also analyzed. They represented all of the *S. aureus* isolates collected in that hospital during the period May 2010 to August 2013, with the exception of one isolate that had not been stored.

Four of them (44.4%) were MSSA, showed a susceptibility phenotype to non-beta-lactam antibiotics, and were negative for the presence of PVL genes. The remaining five isolates (55.6%) were MRSA and were found to share the same features as the MRSA isolate from colonization belonging to *spa*-type t008. Indeed, they were all assigned to *spa*-type t008, showed a similar PFGE pattern (PFGE type B), carried SCC*mec* IVc and PVL genes, and were negative for the presence of ACME. However, differently from the isolate from colonization, they remained susceptible to all non-beta-lactam antibiotics tested.

The low number of *S. aureus* clinical isolates available for investigation and the possibility of obtaining clinical isolates from only one hospital are limitations of the present work. Nonetheless, USA300-LV was isolated over a 3-year period (i.e., first isolate on January 2011, last isolate on June 2013) and accounted for all documented MRSA infections. The identification of USA300-LV clinical isolates in the hospital of Villa Montes over an almost 3-year period, together with the detection of one USA300-LV carrier in a hospital from another urban area, would suggest the dissemination of this relevant clone in the Chaco region.

4. Conclusions

MRSA is a global public health threat in both the hospital setting and in the community.[1]

In this study, we surveyed colonization by *S. aureus* in patients from eight hospitals in the Bolivian Chaco, which is a distinct setting because of the presence of very small hospitals (representative of the local healthcare system), the absence of facilities for microbiological diagnosis, and as a consequence, a lack of microbiological data.

In this context, not studied before, we found a moderate (14.6%) *S. aureus* carriage, comparable to data reported previously

worldwide,[28] and an overall low rate (1.8%) of MRSA carriers. Isolates belonging to *spa*-type t701 seemed to be the most prevalent among MRSA, being disseminated in both the hospital and community setting, but a USA300-LV isolate was also detected. Of note, representatives of this clone were the only type of MRSA detected among clinical isolates of *S. aureus* available for investigation from the study area, underscoring the clinical and epidemiological impact of this clone even in this setting.[9]

Overall, these findings underscore the importance of implementing infection control measures in similar settings and may suggest that alternatives to beta-lactams be considered when empiric antimicrobial therapy is provided for the treatment of an infection compatible with *S. aureus* aetiology. All MRSA isolates were found to be susceptible to several antimicrobials including first-line, oral, and inexpensive drugs such as trimethoprim–sulfamethoxazole and tetracycline. As the prevalence of resistance to non-beta-lactam agents could change over time, susceptibility patterns of *S. aureus* should continue to be monitored, and the information used to guide empiric management decisions.

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Conflict of interest: No conflict of interest to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2014.12.006.

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Prevalence of plasmid-mediated quinolone resistance determinants among oxyiminocephalosporin-resistant Enterobacteriaceae in Argentina

Giovanna Rincon Cruz[1,4], Marcela Radice[1], Samantha Sennati[2], Lucia Pallecchi[2], Gian María Rossolini[2], Gabriel Gutkind[1], Jose Alejandro Di Conza[1,3]/+

[1]Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina [2]Dipartimento di Biotecnologie, Sezione di Microbiologia, Università di Siena, Siena, Italy [3]Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina [4]Escuela de Bacteriología, Universidad Industrial de Santander, Bucaramanga, Colombia

High quinolone resistance rates were observed among oxyiminocephalosporin-resistant enterobacteria. In the present study, we searched for the prevalence of plasmid-mediated quinolone resistance (PMQR) genes within the 55 oxyiminocephalosporin-resistant enterobacteria collected in a previous survey. The main PMQR determinants were aac(6')-Ib-cr and qnrB, *which had prevalence rates of 42.4% and 33.3%, respectively. The* aac(6')-Ib-cr *gene was more frequently found in CTX-M-15-producing isolates, while* qnrB *was homogeneously distributed among all CTX-M producers.*

Key words: PMQR - ESBL-producing Enterobacteriaceae - fluoroquinolone

Quinolone resistance in Gram-negative bacilli is primarily related to mutations in the chromosomal genes encoding for type II topoisomerases, the target site of quinolones (Drlica & Zhao 1997). However, in 1998, the first plasmid-mediated quinolone resistance (PMQR) determinant, *qnrA*, was reported in a *Klebsiella pneumoniae* strain. Since then, four additional *qnr* determinants, *qnrB*, *qnrC*, *qnrD* and *qnrS*, have been identified in Enterobacteriaceae species and some of these determinants have several allelic variants (Rodriguez-Martinez et al. 2011). These determinants encode for a pentapeptide repeat protein that binds to DNA gyrase, protecting the DNA gyrase from quinolone-mediated inhibition and increasing the minimum inhibitory concentrations (MICs) of the quinolones by eight-64-fold (Rodriguez-Martinez et al. 2011).

In addition to the *qnr* genes, various new PMQR genes have been discovered during the past decade, including the modified acetyltransferase *aac(6')-Ib-cr* and the efflux pumps *qepA* and *oqxAB* (Rodriguez-Martinez et al. 2011).

The association of PMQR genes with extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases is noteworthy (Canton & Coque 2006). Although a few studies describing PMQR determinants in selected isolates have been performed, these associations have not been previously studied in Argentina (Quiroga et al. 2007, Jacoby et al. 2009, Andres et al. 2013). This study aimed to investigate the prevalence of PMQR genes (*qnrA*, *-B*, *-S*, *-C* and *-D*, *aac(6)-Ib-cr* and *qepA*) in oxyiminocephalosporin-resistant Enterobacteriaceae recovered during a recent multicentre survey conducted in Argentina (Sennati et al. 2012). In addition, we also examined the coexistence of these determinants with different ESBL and/or AmpC β-lactamases.

The surveillance study was performed during October 2010 in 15 community hospitals distributed in three different regions of Argentina. Samples from both inpatients and outpatients were included. From 1,586 consecutive and non-repetitive enterobacterial clinical isolates recovered during this period, 207 (13.05%) displayed reduced susceptibility to expanded-spectrum cephalosporins (ESC) (Sennati et al. 2012). Antimicrobial susceptibility tests were performed by dilution and diffusion methods according to the Clinical and Laboratory Standards Institute (CLSI) for ampicillin, amoxicillin–clavulanic acid, piperacillin/tazobactam, cephalothin, cefoxitin, cefotaxime, ceftazidime, cefotaxime/clavulanic acid, ceftazidime/clavulanic acid, cefepime, imipenem, meropenem, amikacin, gentamicin, tobramycin, nalidixic acid, ciprofloxacin, levofloxacin and gatifloxacin (CLSI/NCCLS 2010). Molecular epidemiology of PMQR determinants was conducted for all confirmed ESC-resistant isolates (n = 55) collected during the first week of the study (22 *K. pneumoniae*, 16 *Escherichia coli*, 6 *Proteus mirabilis*, 4 *Klebsiella oxytoca*, 3 *Serratia* spp, 3 *Enterobacter* spp and 1 *Providencia* sp.) (Sennati et al. 2012). This sample was considered to be representative of the entire study period because the relative frequency of the most prevalent species was similar throughout the study period.

Molecular detection of *qnrA*, *qnrB, qnrC*, *qnrD* and *qnrS* was carried out by polymerase chain reaction (PCR) amplification using total heat-extracted DNA as a template and primers previously described (Cattoir et al.

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+ Corresponding author: jdiconza@gmail.com
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2007, Cavaco et al. 2009, Wang et al. 2009). For further characterisation of *qnrB* alleles, the following primers were designed (5'-3'): QnrBcF: GTTRGCGAAAAAATTRACAG, QnrBlF: ATGWYGYCATTATGTATA and QnrBcR: CCMATHAYMGCGATRCCAAG. All *qnrB* amplicons were sequenced on both strands using an ABI PRISM 3700 DNA sequencer. Screening for the *aac(6')-Ib* gene was performed using the following primers (5'-3'): aac(6')IbF: CGATCTCATATCGTCGAGTG and aac(6')IbR: TTAGGCATCACTGCGTGTTC. Characterisation of the *aac(6')-Ib-cr* variant was conducted by restriction fragment length polymorfism-PCR using *Bse*GI (Fermentas, Thermo Fisher Scientific Inc, Massachusetts, USA) (Park et al. 2006) and sequencing. The presence of the *qepA* gene was investigated by PCR amplification using the following primers (5'-3'): qepAF: ACATCTACGGCTTCTTCGTCG and qepAR: AACGCTTGAGCCCGTAGATC.

The 55 ESC-resistant isolates investigated in this study included 50 ESBL producers and the remaining five isolates were strong producers of AmpC. Among the ESBL-positive isolates, 47 were CTX-M producers (94%), with the most prevalent enzymes produced being CTX-M-2 (44%) and CTX-M-15 (38%) and to a lesser extent CTXM-14 (3/50), PER-2 (3/50), SHV-12 (2/50), SHV-5 (2/50), CTX-M-8 (1/50) and CTX-M-56 (1/50). Three isolates encoded two different ESBLs simultaneously. Susceptibility to nalidixic acid and ciprofloxacin was 7.3% and the susceptibility rate of isolates to either levofloxacin or gatifloxacin was 23.6%. Gentamicin, amikacin and tobramycin displayed susceptibility rates of 43.6%, 61.8% and 23.6%, respectively. The MIC_{50} and MIC_{90} values of the fluoroquinolones were higher for PMQR-positive *K. pneumoniae* isolates (data not shown). However, no differences in MIC values were observed within *E. coli* isolates.

High diversity of PMQR genes was found among these enterobacteria. Sixty-six percent (33/50) of ESBL-producing isolates had at least one PMQR determinant (Table). In contrast, no PMQR genes were detected in isolates that produced high levels of AmpC (2 *E. coli* and 1 *P. mirabilis* harbouring CMY-2 and 2 *Enterobacter* spp).

Among the PMQR-positive isolates, 42.4% (14/33) and 33.3% (11/33) encoded either *aac(6')-Ib-cr* or *qnrB* as a determinant of quinolone resistance respectively, while 24.3% (8/33) had both determinants. No isolates rendered a positive amplification of *qnrA*, *qnrS*, *qnrC*, *qnrD* or *qepA*.

Five *qnrB* variants were found in this study; *qnrB2-like* was the most prevalent (8/19), followed by *qnrB19-like* (6/19), *qnrB10-like* (3/19), *qnrB1-like* (1/19) and *qnrB6-like* (1/19). A homogeneous distribution of *qnrB* variants among CTX-M producers was observed (Table).

The *aac(6')-Ib-cr* gene was detected in 44% (22/50) of the ESBL-producing isolates, displaying similar percentages for both *E. coli* (56.2%, 9/16) and *K. pneumoniae* (54.5%, 12/22). However, *aac(6')-Ib-cr* was mainly associated with CTX-M-15-producing Enterobacteriaceae (15/19) and to a lesser extent with other CTX-Ms, including CTX-M-2 (5), CTX-M-15/CTX-M-2 (1), CTX-M-14 (1) and CTX-M-8 (1). One of these 22 *aac(6')-Ib-cr*-harbouring isolates (*K. pneumoniae* CV1) also carried the wild-type *aac(6')-Ib* gene coupled to *qnrB19* and both CTX-M-2 and CTX-M-15.

We focused on the relationship between the isolates that harboured both the *aac(6')-Ib-cr* and $bla_{CTX\text{-}M\text{-}15}$ determinants. Different clones were observed among the *E. coli* (7) and *K. pneumoniae* (8) isolates (Table). Phylogenetic analysis (Clermont et al. 2000) grouped the *E. coli* isolates into groups A (2) and B2 (5). Isolates belonging to the phylogenetic group B2 displayed a similar banding profile by REP-PCR and were characterised as ST131 according to the MLST Database (mlst.ucc.ie/mlst/dbs/Ecoli), corresponding with the worldwide pandemic clone known to cause nosocomial and community-acquired infections. Additionally, four/eight *K. pneumoniae* isolates were grouped into the same cluster (Kp1) and two of these isolates also possessed the *qnrB2* allele. According to MLST analysis (Diancourt et al. 2005), seven/eight *K. pneumoniae* isolates were typed as ST11 (Table).

The true prevalence of PMQR genes is underestimated because there are no reliable phenotypic methods to detect their presence; however, previous surveillance reports have shown the prevalence of PMQR determinants among ESBL producers (Cremet et al. 2011, Walsh & Rogers 2012). Reports on contemporary isolates in Latin American countries displayed conflicting results. Nevertheless, comparisons between these studies should be performed carefully due to the different bacterial selection criteria used. In concordance with a multicentre study performed in Mexico (Silva-Sanchez et al. 2011), we observed a high frequency of *qnrB* and *aac(6')-Ib-cr* genes amongst ESBL-producing isolates. However, a very low proportion of these markers were detected in Enterobacteriaceae isolated in a paediatric hospital in Uruguay (Garcia-Fulgueiras et al. 2011). Furthermore, these PMQR genes have also been detected in clinical enterobacteria, with unusual phenotypes of quinolone susceptibility collected in Argentina. Compared to this study, another study reported a different distribution in the *qnrB* allelic variants and the presence of different determinants (Andres et al. 2013).

The present study highlights a putative association between *aac(6')-Ib-cr* and $bla_{CTX\text{-}M\text{-}15}$ and a more homogenous distribution of *qnrB* alleles among ESBL-producing *E. coli* and *K. pneumoniae*.

Notably, some PMQR determinants have been described in multiresistant clones with worldwide distribution (Woodford et al. 2011), such as *E. coli* ST131 and *K. pneumoniae* ST11, which were also detected in the present study, further underscoring the ability of these resistance mechanisms to disseminate.

In conclusion, this study is the first report the prevalence of PMQR genes in ESBL-producing Enterobacteriaceae in Argentina and suggests that the *qnrB* and *aac(6')-Ib-cr* genes are widely dispersed among Enterobacteriaceae, as found in many other countries. These isolates showed high-level quinolone resistance ESC resistance that was mediated by ESBLs; therefore, this study demonstrates the importance of understanding the potential risk associated with empirical treatment using these antibiotic families.

TABLE

Main features of the plasmid-mediated quinolone resistance-harbouring enterobacteria isolated in this study

Species	Isolate	City	Hospital	*qnrB-like* allele	*aac(6')-Ib/ aac(6')-Ib-cr*	ESBL genes	Phylogenetic group/clone	ST	MIC (μg/mL)						
									NAL	CIP	LEV	GAT	GEN	TOB	AMK
Klebisella pneumoniae	CM4	CAB	H6	*qnrB2*	-/+	$bla_{CTX-M-15}$	NC/Kp1	ST11	> 512	> 64	32	16	32	4	4
	CL4	CAB	H1	*qnrB2*	-/+	$bla_{CTX-M-15}$	NC/Kp1	ST11	> 512	> 64	> 64	64	> 64	16	4
	I3	SF	H5	-	-/+	$bla_{CTX-M-15}$	NC/Kp1	ST11	> 512	> 64	16	16	> 64	32	4
	I4	SF	H5	-	-/+	$bla_{CTX-M-15}$	NC/Kp1	ST11	> 512	> 64	16	16	64	16	4
	CL6	CAB	H1	*qnrB2*	-/+	$bla_{CTX-M-15}$	NC/Kp2	ST11	> 512	> 64	16	8	1	16	4
	CL9	CAB	H1	*qnrB1*	-/+	$bla_{CTX-M-15}$	NC/Kp3	ST48	64	4	1	2	32	16	2
	T8	CH	H10	-	-/+	$bla_{CTX-M-15}$	NC/Kp4	ST11	> 512	64	32	16	64	32	4
	CV1	CAB	H7	*qnrB19*	+/+	$bla_{CTX-M-15}/bla_{CTX-M-2}$	NC/Kp5	ST11	> 512	> 64	64	32	> 64	64	32
	L5	CAB	H3	*qnrB19*	-/-	$bla_{CTX-M-15}$	NC/ND	ST392	> 512	> 64	4	4	2	16	8
	B4	CAB	H4	*qnrB2*	-/-	$bla_{CTX-M-15}$	NC/ND	ST11	> 512	64	> 64	64	0.5	1	1
	CV2	CAB	H7	-	-/+	$bla_{CTX-M-2}$	NC/ND	ST15	> 512	64	8	4	< 0.5	4	4
	CM1	CAB	H6	*qnrB19*	-/+	$bla_{CTX-M-2}$	NC/ND	ST11	> 512	> 64	64	32	> 64	> 64	> 256
	B5	CAB	H4	*qnrB19*	-/-	$bla_{CTX-M-2}$	NC/ND	ST11	> 512	64	64	64	> 64	> 64	> 256
	CL7	CAB	H1	-	-/+	$bla_{CTX-M-2}$	NC/ND	ST11	> 512	> 64	16	16	1	8	8
	M2	CAB	H7	*qnrB19*	-/-	$bla_{CTX-M-2}$	NC/ND	ST11	> 512	> 64	> 64	64	2	16	> 256
	CL5	CAB	H1	-	-/+	$bla_{CTX-M-8}$	NC/ND	ST14	64	8	2	4	1	16	8
Escherichia coli	L4	CAB	H3	-	-/+	$bla_{CTX-M-15}$	B2/EC1a	ST131	> 512	> 64	16	16	1	16	8
	T1	CH	H10	-	-/+	$bla_{CTX-M-15}$	B2/EC1a	ST131	> 512	> 64	64	16	> 64	> 64	16
	CM2	CAB	H6	-	-/+	$bla_{CTX-M-15}$	B2/EC1b	ST131	> 512	> 64	8	8	> 64	32	8
	T3	CH	H10	-	-/+	$bla_{CTX-M-15}$	B2/EC1b	ST131	> 512	64	32	8	1	1	2
	SM5	BA	H2	-	-/+	$bla_{CTX-M-15}$	B2/EC1c	ST131	512	2	1	0.5	2	16	4
	M1	CAB	H7	-	-/+	$bla_{CTX-M-15}$	A/EC2	ST410	> 512	> 64	32	16	> 64	64	32
	SM4	BA	H2	-	-/+	$bla_{CTX-M-15}$	A/EC3	ST167	> 512	> 64	64	32	2	16	16
	T2	CH	H10	*qnrB6*	-/+	$bla_{CTX-M-2}$	B1/EC4	ST297	> 512	> 64	> 64	> 64	2	32	4
	SM7	BA	H2	*qnrB2*	-/+	$bla_{CTX-M-14}$	D/EC5	ST68	> 512	> 64	> 64	32	1	1	4
	SM8	BA	H2	*qnrB2*	-/-	$bla_{CTX-M-14}$	D/EC5	ST68	> 512	> 64	64	32	< 0.5	0.5	0,5
Klebsiella oxytoca	C1	SF	H9	-	-/+	$bla_{CTX-M-2}$	NC/ND	ND	128	2	4	2	8	16	2
	T4	CH	H10	*qnrB10*	-/-	$bla_{CTX-M-2}$	NC/ND	ND	> 512	64	32	16	> 64	> 64	16
Proteus mirabilis	SM6	BA	H2	*qnrB2*	-/-	$bla_{CTX-M-2}$	NC/ND	ND	> 512	> 64	> 64	64	32	8	4
	CX2	BA	H8	*qnrB10*	-/-	$bla_{CTX-M-2}$	NC/ND	ND	128	2	4	2	8	16	32
	T12	CH	H10	*qnrB19*	-/-	$bla_{CTX-M-2}$	NC/ND	ND	> 512	32	8	32	64	32	4
Providencia sp.	CL2	CAB	H1	*qnrB2*	-/-	$bla_{CTX-M-2}$	NC/ND	ND	> 512	> 64	> 64	> 64	> 64	64	1
Enterobacter cloacae	CX1	BA	H8	*qnrB10*	-/-	bla_{PER-2}/bla_{SHV-12}	NC/ND	ND	128	2	4	2	8	16	4

AMK: amikacin; BA: Buenos Aires; CAB: Ciudad Autónoma de Buenos Aires; CH: Chubut; CIP: ciprofloxacin; ESBL: extended-spectrum β-lactamases; GAT: gatifloxacin; GEN: gentamicin; H1: Hospital de Clínicas, Universidad de Buenos Aires; H2: Corporación médica San Martín; H3: Hospital Alemán; H4: Hospital Británico; H5: Hospital Iturraspe; H6: CEMIC; H7: Sanatorio Mater Dei; H8: Hospital Eva Perón; H9: Hospital Cullen; H10: Hospital de Trelew; LEV: levofloxacin; MIC: minimum inhibitory concentration; NAL: nalidixic acid; NC: not correspond; ND: not determined; SF: Santa Fe; TOB: tobramycin.

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Changing Epidemiology of Extended-Spectrum β-Lactamases in Argentina: Emergence of CTX-M-15

S. Sennati, G. Santella, J. Di Conza, L. Pallecchi, M. Pino, B. Ghiglione, G. M. Rossolini, M. Radice and G. Gutkind
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Changing Epidemiology of Extended-Spectrum β-Lactamases in Argentina: Emergence of CTX-M-15

S. Sennati,[a] G. Santella,[b] J. Di Conza,[b] L. Pallecchi,[a] M. Pino,[b] B. Ghiglione,[b] G. M. Rossolini,[a,c] M. Radice,[b] and G. Gutkind[b]

Dipartimento di Biotecnologie, Sezione di Microbiologia, Università di Siena, Siena, Italy[a]; Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina[b]; and Dipartimento di Emergenza, Urgenza e dei Servizi Diagnostici, U. O. Microbiologia e Virologia, Azienda Ospedaliera-Universitaria Senese, Siena, Italy[c]

A multicenter survey, carried out in 2010 in Argentina, showed an increased prevalence of extended-spectrum β-lactamase (ESBL)-producing enterobacteria, with some changes in the molecular epidemiology of circulating ESBLs. While enzymes of the CTX-M-2 group remain endemic, the emergence of CTX-M-15 and of enzymes of the CTX-M-8 and CTX-M-9 groups was observed. The CTX-M-15-positive isolates represented 40% of CTX-M producers and included representatives of *Escherichia coli* ST131 and *Klebsiella pneumoniae* ST11.

Extended-spectrum cephalosporin resistance in enterobacteria is mostly mediated by extended-spectrum β-lactamases (ESBLs). Among them, the CTX-M-type ESBLs (initially reported in the second half of the 1980s) are the most prevalent enzymes worldwide (5, 6). To date, the CTX-M family of enzymes comprises at least 124 allotypes, subclassified by amino acid similarities into six sublineages, namely, CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTX-M-25, and CTX-M-45 (http://www.lahey.org/Studies/) (23).

Since its first detection, CTX-M-2 has become the most prevalent ESBL in Argentina, and enzymes of the CTX-M-2 group have been the only CTX-Ms reported in this country (21, 22).

In this work, we report the results of a recent multicenter survey conducted to analyze the prevalence and nature of ESBLs in Argentina, which showed a notable evolution in the molecular epidemiology of circulating enzymes.

A total of 1,586 consecutive and nonrepetitive enterobacterial clinical isolates were recovered during October 2010 from patients at 15 community hospitals distributed in three different regions of Argentina: (i) Ciudad Autónoma de Buenos Aires (CABA) ($n = 5$) and Buenos Aires ($n = 2$), (ii) Santa Fe ($n = 4$), and (iii) Chubut ($n = 4$). Isolates were identified by both conventional and automated methods (Vitek; bioMérieux). Antimicrobial susceptibility tests were performed by the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) (9). ESBL confirmatory tests were performed by synergy tests using cefotaxime (CTX) and ceftazidime (CAZ) compared to CTX-clavulanic acid and CAZ-clavulanic acid-containing disks (10) for all noninducible AmpC-producing enterobacteria. In inducible AmpC producers, ESBL detection was performed using cefepime (FEP) compared to FEP-clavulanic acid-containing disks (M. Quinteros, M. Radice, P. Power, M. Matteo, M. Mollerach, J. Di Conza, N. Costa, and G. Gutkind, presented at the International Congress on Beta-Lactamases, L'Aquila, Italy, 1999). Screening for AmpC β-lactamases was assayed using a 300-μg phenyl boronic acid-containing disk placed 2 cm from the CAZ-containing disks (25).

Two hundred seven isolates exhibiting inhibition zones for CTX of ≤27 mm and/or CAZ of ≤22 mm were collected during the study period (13.1% of all screened enterobacterial isolates) (Table 1). Reduced susceptibility to expanded-spectrum cephalosporins was higher than the 9% observed in a surveillance study performed in Buenos Aires in 2003 ($P < 0.05$) (21), even if in that study only microorganisms recovered from inpatients were considered, while in the present study, samples recovered from both inpatients and outpatients were included.

Confirmatory tests for ESBL production were performed with all of the isolates exhibiting reduced susceptibility to expanded-

TABLE 1 Number of isolates of each species recovered within the study period, extended-spectrum cephalosporin resistance, and number of resistant isolates that were further studied

Species	No. of isolates	No. (%) of ESC[a]-resistant isolates	ESC-resistant isolates recovered within 1 wk	
			No. of isolates	No. of ESBL producers/AmpC producers
Escherichia coli	1,120	64 (5.7)	16	14/2
Klebsiella pneumoniae	193	87 (45.1)	22	22/0
Proteus mirabilis	115	14 (12.2)	6	5/1
Enterobacter cloacae	37	11 (29.7)	3	1/2
Morganella morganii	29	11 (37.9)		
Klebsiella oxytoca	20	6 (30)	4	4/0
Citrobacter freundii	18	5 (27.8)		
Serratia spp.	18	5 (27.8)	3	3/0
Providencia spp.	13	2 (15.4)	1	1/0
Citrobacter spp.	8	–		
Proteus vulgaris	7	2 (28.6)		
Enterobacter aerogenes	3			
Salmonella sp.	2			
Shigella spp.	2			
Proteus penneri	1			
Total	1,586	207 (13.1)	55	50/5

[a] ESC, extended-spectrum cephalosporin.

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Address correspondence to G. Gutkind, ggutkind@ffyb.uba.ar.

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TABLE 2 Primers used in this study

Name	Sequence (5′→3′)	Reference
CTX-M-group-1F	GTTACAATGTGTGAGAAGCAG	17
CTX-M-group-1R	AACGGAATGAGTTTCCCCATT	17
CTX-M-group-2F	ACCAGGCTCAATTGTGGA	This study
CTX-M-group-2R	AGATGAGGGTTCGTTGCAA	This study
CTX-M-group-8F	CACGGATTCAATTTTCAGGAG	3
CTX-M-group-8R	GAGCGCTCCACATTTTTTAG	3
CTX-M-group-9F	GTTACAATGTGTGAGAAGCAG	17
CTX-M-group-9R	CAGCCAGAAAGTTATGGAG	This study
CTX-M-group-25F	GGATGATGAGAAAAAGCGTAAGGC	This study
CTX-M-group-25R	GGACTAATAACCGTCGGTGAC	This study

spectrum cephalosporins collected during the first week of the study ($n = 55$). This sample was considered to be representative of the whole study period, since the relative frequencies of the most prevalent species were similar during the whole month of study (Table 1). The molecular epidemiology of ESBL determinants was investigated in all confirmed ESBL-producing isolates ($n = 50$). The remaining 5 isolates were high-level AmpC producers (Table 1). Molecular detection of ESBL genes was conducted by PCR amplification using alkaline lysis-extracted total genomic DNA as the template and the primers listed in Table 2. Amplicons were sequenced in both strands using an ABI Prism 3700 DNA sequencer.

Of the 50 ESBL producers, 47 were found to carry CTX-M-type determinants (94%) and the simultaneous presence of two different $bla_{\text{CTX-M}}$ determinants have been observed in 2 of them. Among the CTX-M producers, CTX-M-2 group determinants were found in 26 isolates (55%; 25 CTX-M-2 and 1 CTX-M-56), CTX-M-1 group determinants in 19 isolates (40%; all CTX-M-15), CTX-M-9 group determinants in 3 isolates (6%; all CTX-M-14), and CTX-M-8 group determinants in 1 isolate (2%; CTX-M-8) (Table 3).

Although CTX-M enzymes remain the most prevalent ESBL determinants, the dominance of CTX-M-2 reported previously (21) was diluted by the emergence and remarkable spread of CTX-M-15 and, to a lesser extent, by the emergence of other CTX-M groups. The emergence of CTX-M-15 was observed in both *Escherichia coli* and *Klebsiella* spp.

TABLE 3 CTX-M-producing enterobacteria collected during a 1-week study[a] in 15 hospitals distributed in different regions of Argentina

Species (no. of isolates)	ESBL determinant(s) (no. of isolates)
Klebsiella pneumoniae (21)	$bla_{\text{CTX-M-15}}$ (10) $bla_{\text{CTX-M-2}}$ (9) $bla_{\text{CTX-M-2}}$ + $bla_{\text{CTX-M-15}}$ (1) $bla_{\text{CTX-M-8}}$ (1)
Escherichia coli (13)	$bla_{\text{CTX-M-15}}$ (7) $bla_{\text{CTX-M-14}}$ (3) $bla_{\text{CTX-M-2}}$ (3)
Proteus mirabilis (5)	$bla_{\text{CTX-M-2}}$ (4) $bla_{\text{CTX-M-56}}$ (1)
Klebsiella oxytoca (4)	$bla_{\text{CTX-M-2}}$ (3) $bla_{\text{CTX-M-2}}$ + $bla_{\text{CTX-M-15}}$ (1)
Serratia spp. (3)	$bla_{\text{CTX-M-2}}$ (3)
Providencia spp. (1)	$bla_{\text{CTX-M-2}}$ (1)

[a] October 2010.

TABLE 4 Genotypic characterization of CTX-M-15-producing *E. coli* and *K. pneumoniae* isolates

Species and isolate	City	Hospital[a]	Phylogenetic group	Clone	Genetic context of $bla_{\text{CTX-M-15}}$[b]
E. coli					
CM2	Buenos Aires	H6	B2	Ec1	II
L4	Buenos Aires	H3	B2	Ec2	I
M1	Buenos Aires	H7	A	Ec3	II
SM4	Buenos Aires	H8	A	Ec4	II
SM5	Buenos Aires	H8	A	Ec5	I
T1	Chubut	H13	B2	Ec2	I
T3	Chubut	H13	B2	Ec6	I
K. pneumoniae					
B4	Buenos Aires	H4	ND[c]	Kp2	I
CL1	Buenos Aires	H1	ND	Kp3	I
CL4	Buenos Aires	H1	ND	Kp1	I
CL6	Buenos Aires	H1	ND	Kp7	II
CL9	Buenos Aires	H1	ND	Kp4	I
CM4	Buenos Aires	H6	ND	Kp1	I
CV1	Buenos Aires	H7	ND	Kp5	II
I3	Santa Fe	H5	ND	Kp1	I
I4	Santa Fe	H5	ND	Kp1	I
L5	Buenos Aires	H3	ND	Kp6	I
T8	Chubut	H13	ND	Kp8	I

[a] H1, Hospital de Clínicas, Universidad de Buenos Aires; H3, Hospital Alemán, Ciudad Autónoma de Buenos Aires (CABA); H4, Hospital Británico, CABA; H5, Hospital Iturraspe, Santa Fe; H6, CEMIC, CABA; H7, Sanatorio Mater Dei, CABA; H8, Hospital Eva Perón, Buenos Aires; H13, Hospital de Trelew, Chubut.
[b] I, international $bla_{\text{CTX-M-15}}$ genetic environment (GenBank accession no. NC013121.1; II, truncated IS*Ecp1*-$bla_{\text{CTX-M-15}}$ genetic environment (GenBank accession no. HQ157353) (11).
[c] ND, not determined.

The genetic environments surrounding the most prevalent CTX-M determinants, $bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-15}}$, were investigated by PCR mapping and sequencing. The $bla_{\text{CTX-M-2}}$ gene was always located downstream of an IS*CR1* element, as previously described (1, 13). Different genetic environments were found surrounding $bla_{\text{CTX-M-15}}$: in 13 isolates, it was associated with a complete IS*Ecp1* located 48 bp upstream of $bla_{\text{CTX-M-15}}$, in agreement with the worldwide genetic context named "the international $bla_{\text{CTX-M-15}}$ genetic environment" (GenBank accession no. NC013121.1); in 5 isolates, $bla_{\text{CTX-M-15}}$ was associated with a truncated IS*Ecp1* (still conserving a complete promoter), as recently described in the United Kingdom (GenBank accession no. HQ157353) (11) (Table 4).

To investigate the dissemination of CTX-M-15, we performed a genotype analysis of the isolates producing this CTX-M variant (7 *E. coli* and 11 *K. pneumoniae* isolates). Genotyping was performed by determination of the four main *E. coli* phylogenetic groups (7) and by PCR-based fingerprinting using random amplification of polymorphic DNA (RAPD) with the 1290 decamer (19) and repetitive extragenic palindromic PCR (REP-PCR) (16). (Isolates were assigned to a same clone when identical band profiles were obtained with the two PCR-based fingerprinting methods.) Clonal heterogeneity was observed among both *E. coli* and *K. pneumoniae* isolates (Table 4). All of the CTX-M-15-producing *E. coli* isolates belonging to phylogenetic group B2 ($n = 4$) were identified as ST131 by the PCR-based method proposed by Clermont et al. (8) and confirmed by multilocus sequence typing (MLST) [http://mlst.ucc.ie/mlst/dbs/Ecoli/documents/primers

Coli_html] with two *E. coli* isolates (L4 and CM2). Moreover, MLST analysis of the CTX-M-15-producing *K. pneumoniae* isolates (12) assigned the most prevalent clone (Kp1, including 4 isolates circulating in both Buenos Aires and Santa Fe) to sequence type 11 (ST11) (Table 4).

Nowadays, it is worth noting that although some of the CTX-M enzymes have been associated with specific countries, such as CTX-M-9 and CTX-M-14 in Spain (14, 18), CTX-M-1 in Italy (4), and CTX-M-2 in Israel, Japan, and most South American countries (6, 21), others, such as CTX-M-15, have been detected worldwide (2, 4, 15, 20). The present data indicate that the cosmopolitan CTX-M-15 ESBL is becoming widespread also in Argentina and is often associated with clones distributed worldwide, such as *E. coli* ST131 and *K. pneumoniae* ST11 (24), further underscoring the dissemination potential of this enzyme. The new epidemiological scenario may have followed an allodemic rather than an epidemic pattern, reflecting the dissemination of both multiple clones and/or several mobile genetic elements.

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Characterization of Small ColE-Like Plasmids Mediating Widespread Dissemination of the *qnrB19* Gene in Commensal Enterobacteria▽

Lucia Pallecchi,[1] Eleonora Riccobono,[1] Samanta Sennati,[1] Antonia Mantella,[2] Filippo Bartalesi,[2] Christian Trigoso,[3] Eduardo Gotuzzo,[4] Alessandro Bartoloni,[2] and Gian Maria Rossolini[1,5]*

Dipartimento di Biologia Molecolare, Sezione di Microbiologia, Università di Siena,[1] and Dipartimento dei Servizi, U. O. Microbiologia e Virologia, Azienda Ospedaliera-Universitaria Senese,[5] Siena, Italy; Dipartimento Area Critica Medico Chirurgica, Clinica Malattie Infettive, Università di Firenze, Florence, Italy[2]; Instituto Nacional de Laboratorios de Salud INLASA, La Paz, Bolivia[3]; and Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru[4]

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In this work, we have characterized two small ColE-like plasmids (pECY6-7, 2.7 kb in size, and pECC14-9, of 3.0 kb), encoding the QnrB19 quinolone resistance determinant, that were carried by several clonally unrelated quinolone-resistant commensal *Escherichia coli* strains isolated from healthy children living in different urban areas of Peru and Bolivia. The two plasmids are closely related to each other and carry the *qnrB19* gene as the sole resistance determinant, located in a conserved genetic context between the plasmid RNAII sequence (which controls plasmid replication) and the plasmid Xer site (involved in plasmid dimer resolution). IS*Ecp1*-like or other putative insertion sequences are not present in the *qnrB19*-flanking regions or elsewhere on the plasmids. Since we previously observed a high prevalence (54%) of *qnrB* genes in the metagenomes of commensal enterobacteria from the same population of healthy children, the presence of pECY6-7- and pECC14-9-like plasmids in those *qnrB*-positive metagenomes was investigated by PCR mapping. Both plasmids were found to be highly prevalent (67% and 16%, respectively) in the *qnrB*-positive metagenomes, suggesting that dissemination of these small plasmids played a major role in the widespread dissemination of *qnrB* genes observed in commensal enterobacteria from healthy children living in those areas.

Qnr proteins are small pentapeptide repeat proteins that bind and protect type II DNA topoisomerases from inhibition by fluoroquinolones (29–31). They represent the first discovered transferable mechanism of resistance to quinolones, and their dissemination has been associated with the increase of fluoroquinolone resistance rates in clinical isolates of the *Enterobacteriaceae* (17, 24). *qnr*-carrying isolates have been reported worldwide (17, 24), and five different lineages of Qnr proteins have been described so far: QnrA, QnrB, QnrS, and more recently QnrC and QnrD (6, 15, 17, 32).

In previous studies, we have observed a remarkable rate of quinolone resistance in commensal *Escherichia coli* from healthy children living in urban areas of Peru and Bolivia (1) and a high prevalence of *qnrB* genes (mostly *qnrB19*) in commensal enterobacteria from the same population of healthy children (19).

In this work, we have characterized two small ColE-like plasmids encoding QnrB19, carried by several clonally unrelated quinolone-resistant commensal *E. coli* strains isolated from children living in different areas, and we have demonstrated that the dissemination of those plasmids apparently played a major role in the widespread dissemination of *qnrB* genes observed in commensal enterobacteria from that population.

(These results were presented in part at the joint 48th Interscience Conference on Antimicrobial Agents and Chemotherapy/46th Annual Meeting of the Infectious Diseases Society of America, Washington, DC, 2008, and at the 19th European Congress of Clinical Microbiology and Infectious Diseases, Helsinki, Finland, 2009.)

MATERIALS AND METHODS

Bacterial isolates. The 107 *E. coli* isolates investigated in this study for the presence of *qnr* genes represented a random selection of the 1,053 ciprofloxacin-resistant commensal *E. coli* isolates collected during a survey on commensal *E. coli* carried out in 2005 on 3,193 healthy children living in four urban areas of Latin America (Camiri, Santa Cruz Department, and Villa Montes, Tarija Department, Bolivia; and Yurimaguas, Loreto Department, and Moyobamba, San Martin Department, Peru) (1).

Genotyping of *E. coli* isolates. Phylogenetic grouping of *E. coli* isolates was determined by the multiplex PCR-based method, which allows identification of the four major phylogenetic groups (A, B1, B2, and D) (7). Randomly amplified polymorphic DNA (RAPD) genotyping was performed using, separately, the decamer primers 1290 and 1254, as previously described (18). RAPD patterns were considered to be different when the profiles differed by at least one band.

Detection of *qnr* genes. DNA extraction from *E. coli* was performed as described by Sambrook and Russell (27). The presence of *qnr* genes (*qnrA*, *qnrB*, and *qnrS*) was investigated by dot blot hybridization using the DIG system according to the manufacturer's instructions (Roche Diagnostics SpA, Milan, Italy). Specific probes used in hybridization experiments were generated by PCR as described previously (*qnrA* and *qnrS* [25] and *qnrB* [4]). Sequence analysis of *qnrB* genes was determined on both strands of PCR amplification products at an external facility (Macrogen, Seoul, Korea), as described previously (19).

***In vitro* susceptibility testing.** Antimicrobial susceptibility was determined by disk diffusion testing according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (8, 10). MICs were determined by broth microdilution testing according to CLSI guidelines (9–10). Antibiotics were from Sigma-Aldrich (St Louis, MO). *E. coli* ATCC 25922 was always used for quality control purposes.

Plasmid analysis. Plasmid extraction from *E. coli* was carried out by the alkaline lysis method as described by Sambrook and Russell (27). Southern blot analysis was carried out on nylon membranes as described for the dot blot hybridization. *qnrB*-harboring plasmids were transferred into *E. coli* HB101 (F$^-$

* Corresponding author. Mailing address: Dipartimento di Biologia Molecolare, Sezione di Microbiologia, Università di Siena, Policlinico Santa Maria alle Scotte, 53100 Siena, Italy. Phone: 39 0577 233455. Fax: 39 0577 233870. E-mail: rossolini@unisi.it.

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TABLE 1. Features of the six ciprofloxacin-resistant commensal *Escherichia coli* isolates carrying *qnrB19*

Isolate	Origin	Phylogenetic group	RAPD type	Resistance phenotype[a]	Plasmid size (kb)[b]	Transfer in *E. coli* HB101	RFLP	MIC (μg/ml) of drug for transformant[c]			
								Nal	Cip	Lev	Nor
M4-6	Moyobamba, Peru	A	1	Amp Tet Tmp Sul Str	High mw	No	ND[d]				
M66-6	Moyobamba, Peru	A	2	Tet Sul Str Kan	2.7	Yes	a	16	0.06	0.12	0.25
Y6-7	Yurimaguas, Peru	A	3	Amp Tet Tmp Sul Str	2.7	Yes	a	16	0.06	0.12	0.25
C14-9	Camiri, Bolivia	A	4	Tet Tmp Sul Kan	3.0	Yes	b	32	0.12	0.25	0.5
V6-9	Villa Montes, Bolivia	A	5	Amp Sul Str Kan	3.0	Yes	b	32	0.06	0.25	0.5
V32-1	Villa Montes, Bolivia	A	6	Amp Tet Str Kan	3.0	Yes	b	32	0.06	0.25	0.25

[a] Amp, ampicillin; Tet, tetracycline; Tmp, trimethoprim; Sul, sulfamethoxazole; Str, streptomycin; Kan, kanamycin.
[b] mw, molecular weight. Plasmid sizes were estimated after Southern blotting performed with wild-type isolates and RFLP analysis performed with transformants (except for plasmid from *E. coli* M4-6).
[c] Nal, nalidixic acid; Cip, ciprofloxacin; Lev, levofloxacin; Nor, norfloxacin. MICs of *E. coli* HB101 were as follows: NAL, 4 μg/ml, CIP, 0.003 μg/ml, LEV, 0.0075 μg/ml; NOR, 0.003 μg/ml. No other resistance determinant was cotransferred with *qnrB19*.
[d] ND, not determined.

hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 [Str^r] *xyl-5 mtl-1 supE44 leuB6 thi-1*) by electroporation, using a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA) and approximately 500 ng of the plasmid DNA preparation, under the conditions recommended by the manufacturer. Mueller-Hinton agar plates containing nalidixic acid (8 μg/ml) (NAL-MH) were used for selection of transformants. The presence of *qnrB* genes in the transformants was confirmed by PCR. To exclude the occurrence of chromosomal mutations in transformants following selection on NAL-MH, sequence analysis of *gyrA* and *parC* was carried out with selected transformants, as described previously (26). Replicon typing was carried out by the PCR-based method described by Carattoli et al. (3). Plasmid restriction profiles were analyzed by agarose gel electrophoresis after digestion with SacII, HaeIII, EcoRI, and HindIII (Promega, Madison, WI). The nucleotide sequence of pECCY6-7 and pECC14-9 was determined on both strands by using a primer-walking technique with purified plasmid preparations at an external facility (Macrogen). Analysis and comparisons of nucleotide sequence were carried out using programs available at the NCBI web interface (http://www.ncbi.nlm.nih.gov).

The 167 metagenomes of commensal enterobacteria analyzed for the presence of pECY6-7- and pECC14-9-like plasmids represented all the *qnrB*-positive metagenomes detected in a previous study, where the presence of *qnr* genes was investigated in a sample of 310 metagenomic DNAs of commensal enterobacteria from the same population of healthy children from Bolivia and Peru (19). The presence of pECY6-7- and pECC14-9-like plasmids in the metagenomic DNAs was screened for by a PCR mapping approach. PCRs were always carried out in a 50-μl volume, using 30 pmol of each primer, 200 μM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, and 1.5 U of the enzyme GoTaq (Promega) in the reaction buffer provided by the enzyme manufacturer. Reaction parameters were as follows, unless otherwise specified: initial denaturation at 94°C for 5 min; denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and elongation at 72°C for 1.30 min, repeated for 35 cycles; and a final extension at 72°C for 10 min. In the first step of the PCR mapping approach, carried out with all metagenomes, primers designed on *qnrB19* (qnrB19_RV, 5′-CGGCACCTGAAAAATCG CAG) and on the replication origin ($ColE_{PB}$_FW, 5′-CTGACACTCAGTTCC GCGA) were used to target amplification of the conserved region containing *qnrB19* and the ColE-like backbone (qnrB19-$ColE_{PB}$ PCR). In the second step, carried out with metagenomes positive for the qnrB19-$ColE_{PB}$ PCR, primers designed in divergent orientation on *qnrB19* (qnrB19_FW, 5′-TGGATGGGGA CTCAGGTACT, and qnrB19_RV, 5′-CGGCACCTGAAAAATCGCAG) were used to target amplification of the whole plasmids (qnrB19circ PCR) (PCR elongation time of 4 min). The identity of PCR amplicons was confirmed by Southern blotting using a probe targeting the replication origin of pECY6-7 and pECC14-9 ($ColE_{PB}$ probe, generated with primers $ColE_{PB}$_FW [5′-CTGACAC TCAGTTCCGCGA] and $ColE_{PB}$_RV [TGCTGCCAGTGGCGATAAGT]) and restriction profiling with HaeIII. Selected amplicons (representative of amplicons of different sizes and origins) were analyzed by sequencing.

Nucleotide sequence accession numbers. The complete circular nucleotide sequences of pECY6-7 and pECC14-9 have been deposited in the GenBank sequence library and assigned the accession numbers GQ374156 and GQ374157, respectively.

RESULTS AND DISCUSSION

***qnr* genes in ciprofloxacin-resistant commensal *E. coli*.** The presence of *qnr* genes was investigated by PCR in 107 ciprofloxacin-resistant *E. coli* isolates, representing a random sample of the 1,053 ciprofloxacin-resistant commensal *E. coli* isolates collected in a previous survey carried out with 3,193 healthy children living in four urban areas of Peru and Bolivia (1). A total of six out of the 107 isolates (6%) were found to be positive for *qnrB* genes, while *qnrA* and *qnrS* genes were not detected. Amplicon sequencing identified the *qnrB19* allele (5, 23) in all of them. The *qnrB19*-positive isolates were found in each urban area of Peru and Bolivia (Table 1).

Multiplex PCR showed that all the *qnrB19*-positive isolates belonged to phylogenetic group A (Table 1), which is usually associated with commensalism and minor virulence (7, 20), supporting the notion that commensal *E. coli* can act as a reservoir of *qnr* genes. However, RAPD genotyping showed different profiles (Table 1), indicating a likely plasmid-mediated nonclonal dissemination (Table 1). All the *qnrB19*-positive isolates exhibited a multidrug resistance (MDR) phenotype which variably included tetracycline, ampicillin, kanamycin, streptomycin, trimethoprim, and sulfonamides (Table 1).

Genetic support of *qnrB19* genes in ciprofloxacin-resistant commensal *E. coli*. Southern blot experiments, carried out with plasmid preparations from the six *qnrB*-positive isolates, showed that the *qnrB19* genes were located on low-molecular-weight plasmids, with the exception of one isolate from Peru (*E. coli* M4-6) (data not shown). The small *qnrB19*-harboring plasmids were transferred by electroporation into *E. coli* HB101 and further characterized. Restriction analysis with several enzymes (SacII, HaeIII, EcoRI, and HindIII) revealed two different plasmid profiles for the *qnrB19*-harboring plasmids from Peru (estimated size, 2.7 kb) and Bolivia (estimated size, 3.0 kb), respectively (Table 1). Although quinolone MICs were increased, transformants remained susceptible to fluoroquinolones, indicating that the ciprofloxacin resistance phenotype of the donors was contributed by other resistance mechanisms, which were not further investigated in this study (e.g., mutations in the genes encoding DNA topoisomerases II, upregulation of native efflux pumps, and/or decreased expression of outer membrane porins). No other resistance trait present in

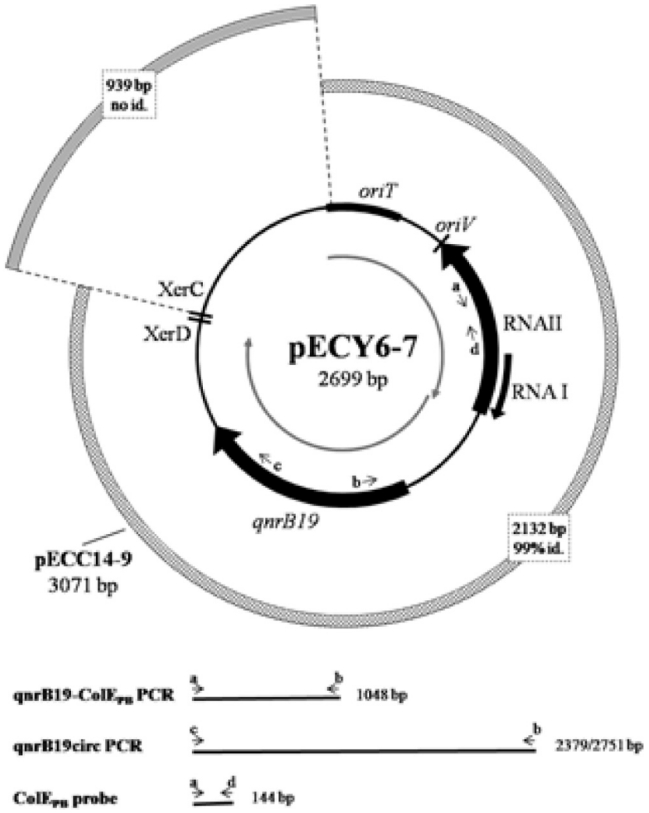

FIG. 1. Genetic organization of plasmid pECY6-7 and comparison with plasmid pECC14-9. Black arrows show the direction of transcription of open reading frames and regulatory elements in pECY6-7. The ColE-like plasmid backbone and the *qnr* region are indicated by gray arrows. Comparison between pECY6-7 and pECC14-9 is shown as percentages of sequence identity (id.). Primers used for plasmid analysis are indicated as short arrows: ColE$_{PB}$_FW, a; qnrB19_RV, b; qnrB19_FW, c; ColE$_{PB}$_RV, d. PCR amplicons are indicated as horizontal lines.

the original isolate was cotransferred. The small *qnrB19*-harboring plasmids could not be assigned to a replicon type using the multiplex PCR-based method (3).

Structure of the small QnrB19-encoding plasmids. A representative of each small *qnrB19*-harboring plasmid, pECY6-7 (from *E. coli* Y6-7) and pECC14-9 (from *E. coli* C14-9) (Table 1), was subjected to complete DNA sequencing.

pECY6-7 and pECC14-9 are small ColE-like plasmids of 2,699 and 3,071 bp, respectively. They share a 2,132-bp common region (99% identical) and differ by a unique region of 567 bp and 939 bp in pECY6-7 and pECC14-9, respectively. The common region contains a ColE-like backbone and the *qnr* region (Fig. 1). The ColE-like backbone includes putative regions for plasmid replication (*oriV*, RNAII, and RNAI [11]) and mobilization (*oriT* [11]) and showed similarity with the corresponding region of the ColE-like plasmids p15A (89%) (28) and p9123 (86%) (a *sul2*-harboring plasmid which has been shown to enhance host fitness) (12). The nature of the plasmid backbone explained why it could not be identified by PCR-based replicon typing. The *qnr* region is located between the putative −35 promoter of RNAII (11) and the plasmid Xer site, which is known to be involved in plasmid dimer resolution (2). The unique regions, located between the Xer site and *oriT*, are not related to each other: the 939-bp region in pECC14-9 showed 93% identity with the corresponding region in p9123 (12), while an internal part of the 567-bp region in pECY6-7 (378 bp) exhibit 85% similarity with plasmid pMGD2 from an environmental isolate of *Klebsiella* spp. (GenBank accession no. AY033498). Homology between pECY6-7 and pECC14-9 abruptly ends in correspondence of the Xer site, suggesting that the Xer site-specific recombination might have played a role in the evolution of these plasmids.

The regions flanking *qnrB19* show 100% identity with those present in the *qnrB19*-harboring transposons Tn*5387* from a *Klebsiella pneumoniae* clinical isolate from the United States (245 bp upstream and 225 bp downstream) (23) and Tn*2012* from an *E. coli* clinical isolate from Colombia (155 bp upstream and 225 bp downstream) (5) (Fig. 2). Upstream of

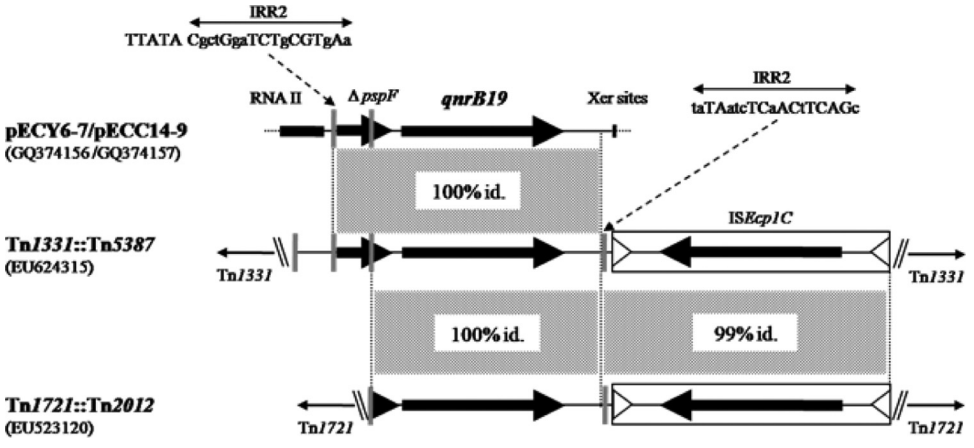

FIG. 2. *qnrB19* region in plasmids pECY6-7 and pECC14-9, compared to that of previously reported *qnrB19* genes. Transposon Tn*5387* is from a *Klebsiella pneumoniae* clinical isolate from the United States (23), and transposon Tn*2012* is from an *Escherichia coli* clinical isolate from Colombia (5). Black arrows show direction of transcription of open reading frames. Vertical gray bars indicate imperfect IRR2 motifs for IS*Ecp1*. Sequences of the putative IRR2 located within the *pspF* gene and in Tn*5387* and Tn*2012* (downstream of the end of homology with the *qnr* region in pECY6-7 and pECC14-9) are reported (uppercase base pairs are identical, whereas lowercase base pairs are different). Homology regions are shown as percentages of sequence identity (id.).

qnrB19, the homology with Tn*5387* ends in correspondence of a putative imperfect IRR2 for IS*Ecp1* located within the *pspF* gene, which has been found in the genetic environment of various *qnrB* genes (22), suggesting that IS*Ecp1*-like elements could have been involved in the mobilization of *qnrB19* to these plasmids. This hypothesis is also supported by the presence, upstream of the putative IRR2, of a 5-bp A+T-rich motif (TTATA) which was shown to represent a potential target site for IS*Ecp1* (21). However, no IS*Ecp1*-like sequence or other insertion sequences are present downstream of *qnrB19* or elsewhere on the plasmids. To explain this finding, one could hypothesize that the IS*Ecp1*-like element involved in *qnrB19* mobilization could have undergone a subsequent excision. The presence of a putative IRR2 in the sequence of Tn*5387* and Tn*2012* immediately downstream of the end of homology with the *qnr* region in pECY6-7 and pECC14-9 would support this hypothesis (Fig. 2).

ColE-like plasmids have been associated with the dissemination of *qnrS* genes in enterobacteria (13, 16), but to the best of our knowledge, this is the first study reporting a *qnrB* variant in such small ColE-like plasmids.

Presence of small *qnrB19*-harboring plasmids related to pECY6-7 and pECC14-9 in *qnrB*-positive metagenomes from commensal enterobacteria. Finding of the closely related pECY6-7 and pECC14-9 plasmids in clonally unrelated strains of commensal *E. coli* from children living in different urban areas of Peru and Bolivia suggested that these plasmids could have a broad diffusion and contribute to the high prevalence of *qnrB* genes that was observed in commensal enterobacteria from the same population of healthy children (19). To investigate this possibility, we screened for the presence of pECY6-7- and pECC14-9-like plasmids in the 167 metagenomes of commensal enterobacteria from children that had previously tested positive for *qnrB* genes (1). Screening was performed by a two-step PCR mapping approach. In the first step, carried out with all metagenomes, amplification of the conserved ColE-like backbone and *qnr* region was targeted by the primers $ColE_{PB}$_FW and qnrB19_RV (qnrB19-$ColE_{PB}$ PCR); in the second step, carried out with the metagenomes that tested positive, amplification of the entire plasmid was targeted by the primers qnrB19_FW and qnrB19_RV (qnrB19circ PCR) (Fig. 1). The identities of amplicons were confirmed by Southern blot hybridization, restriction mapping with HaeIII, and sequencing (with selected samples).

Of the 167 *qnrB*-positive metagenomes analyzed, 128 (77%) were positive with the qnrB19-$ColE_{PB}$ PCR, revealing the presence of pECY6-7- and pECC14-9-like plasmids. Of these 128 metagenomes, 124 (97%) were positive with the qnrB19circ PCR, revealing the presence of pECY6-7 in 97 cases, of pECC14-9 in 12 cases, and of both plasmids in 15 cases (Table 2). Sequence analysis of 4 selected amplicons (2 amplicons of different sizes from each country) confirmed 100% identity with pECY6-7 and pECC14-9, respectively.

Detection of pECY6-7- and pECC14-9-like plasmids in *qnrB19*-positive commensal enterobacteria of species other than *E. coli*. The *qnrB19* genes previously detected in commensal enterobacteria from the population of healthy children were also found in species other than *E. coli* (19). In this work, we investigated the presence of pECY6-7- and pECC14-9-like plasmids in all the *qnrB19*-carrying non-*E. coli* isolates from that study (two *K. pneumoniae* isolates, one *Klebsiella oxytoca* isolate, and one *Escherichia hermannii* isolate) (19) by PCR mapping and sequencing. Plasmids identical to pECY6-7 were found in a *K. pneumoniae* isolate from Peru (*K. pneumoniae* Y1) and in an *E. hermannii* isolate from Bolivia (*E. hermannii* C1). These results confirmed that pECY6-7 is also capable of spreading among enterobacterial species other than *E. coli*.

TABLE 2. Prevalence of plasmids pECY6-7 and pECC14-9 among *qnrB*-positive metagenomes from commensal enterobacteria

Origin	No. of samples	No. of metagenomes positive by:		No. (%) of metagenomes positive for[c]:		
		qnrB19-$ColE_{PB}$ PCR[a]	qnrB19circ PCR[b]	pECY6-7	pECC14-9	Both
Peru	107	84	80	72 (67)	1 (1)	7 (7)
Bolivia	60	44	44	25 (42)	11 (18)	8 (13)
Total	167	128	124	97 (58)	12 (7)	15 (9)

[a] The qnrB19-$ColE_{PB}$ PCR was devised to amplify the conserved region between pEY6-7 and pECC14-9 (performed with primers designed on *qnrB19* and RNAII sequence) (Fig. 1).
[b] The qnrB19circ PCR was devised to amplify entire plasmids (performed with primers designed in divergent orientation on *qnrB19*) (Fig. 1).
[c] Percentages are relative to the total number of samples investigated.

Concluding remarks. Dissemination of plasmid-mediated quinolone resistance determinants, such as the *qnr* genes, has been associated with the worldwide increase in fluoroquinolone resistance rates in clinical isolates of *Enterobacteriaceae* (17, 24). Recently we observed that *qnrB* genes (mainly *qnrB19*) are highly prevalent in the commensal enterobacteria of healthy children living in urban areas of Peru and Bolivia, suggesting that the intestinal microbiota could represent an important reservoir of similar resistance determinants (19). In this work, we demonstrated that two small ColE-like plasmids, closely related to each other and carrying the *qnrB19* gene as the sole resistance determinant, are highly prevalent among commensal enterobacteria in children living in those settings and apparently played a major role in the widespread dissemination of *qnrB* genes observed in that area.

The plasmid pECY6-7 was the most prevalent, and it was found to be disseminated in different species of enterobacteria (*E. coli*, *K. pneumoniae*, and *E. hermannii*). While the manuscript was in revision, a plasmid identical to pECY6-7 was identified in a *Salmonella enterica* serovar Typhimurium strain of human origin isolated in The Netherlands (GenBank accession no. FN428572) (14), suggesting that the dissemination of this plasmid could be even more widespread.

The reasons accounting for the high prevalences of pECY6-7 and pECC14-9 in the commensal enterobacteria of the study population remain unclear. Data collected about household use of antibiotics excluded previous use of fluoroquinolones in the children investigated (1). Moreover, the absence of other resistance genes in pECY6-7 and pECC14-9 excluded the possibility that selection of these plasmids could be related to exposure to other antibiotics. Further studies of the mobilization and fitness impact of the plasmids pECY6-7 and pECC14-9 could provide important information for understanding their remarkable propensity for such widespread dissemination.

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High Prevalence of *qnr* Genes in Commensal Enterobacteria from Healthy Children in Peru and Bolivia▽†

Lucia Pallecchi,[1] Eleonora Riccobono,[1] Antonia Mantella,[2] Filippo Bartalesi,[2] Samanta Sennati,[1] Herlan Gamboa,[3] Eduardo Gotuzzo,[4] Alessandro Bartoloni,[2] and Gian Maria Rossolini[1,5]*

Dipartimento di Biologia Molecolare, Sezione di Microbiologia, Università di Siena,[1] and Dipartimento dei Servizi, U. O. Microbiologia e Virologia, Azienda Ospedaliera-Universitaria Senese,[5] Siena, Italy; Dipartimento Area Critica Medico Chirurgica, Clinica Malattie Infettive, Università di Firenze, Florence, Italy[2]; Distrito de Salud Cordillera, Departamento Santa Cruz, Camiri, Bolivia[3]; and Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru[4]

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A remarkable prevalence of *qnrB* (54%) and, at a lower level, of *qnrS* (14%) was discovered in pools of commensal enterobacteria from 310 healthy children living in Peru and Bolivia, using a metagenomic approach. Analysis of randomly selected enterobacterial pools revealed that *qnrB* was mainly carried by *Escherichia coli* and *qnrS* by *Klebsiella pneumoniae*. Investigation of 11 *qnrB*-positive isolates and 9 *qnrS*-positive isolates revealed the presence of plasmid-borne *qnrB19* ($n = 8$), *qnrB2* ($n = 2$), *qnrB10* ($n = 1$), and *qnrS1* ($n = 9$) genes.

Several plasmid-mediated quinolone resistance (PMQR) mechanisms have been discovered during the past decade, including Qnr proteins, QepA transporters, and the acetyltransferase AAC(6′)-Ib-cr (11, 13). Qnr proteins, the first discovered PMQR mechanism, are small pentapeptide repeat proteins that bind and protect type II DNA topoisomerases from inhibition by fluoroquinolones (17–19). Different lineages of Qnr proteins have been described (QnrA, QnrB, QnrS, and, more recently, QnrC and QnrD), with several allelic variants known for some of them (3, 9, 11, 20). *qnr*-like genes have also been detected on chromosomes from both gram-negative and gram-positive bacteria (9), and, recently, as class 1 integron gene cassettes in the chromosome of *Vibrio cholerae* (7). Although Qnr proteins only determine a moderate reduction of quinolone susceptibility, this may favor the selection of additional resistance mechanisms leading to higher-level quinolone resistance of clinical significance, and the dissemination of *qnr* genes and other PMQR determinants is believed to be an important promoter for evolution of quinolone resistance (11–13).

qnr genes have been reported worldwide, especially in enterobacteria. However, they have mostly been investigated in clinical isolates with specific resistance traits (e.g., quinolone resistance or extended-spectrum β-lactamase phenotype) (11 and references therein), while their prevalence in commensal bacteria remains largely unknown. In this study, we investigated the prevalence of *qnr* genes in commensal enterobacteria from healthy children by a PCR-based metagenomic approach. We also tested a simplified dot blot DNA hybridization method as a less-labor-intensive and expensive tool to perform the metagenomic analysis.

The analysis was carried out on commensal enterobacterial pools from 310 healthy children, ages 6 to 72 months, living in four urban areas of Latin America: two in Peru (Moyobamba, San Martin Department; and Yurimaguas, Loreto Department) and two in Bolivia (Camiri, Santa Cruz Department; and Villa Montes, Tarija Department). The enterobacterial pools, consisting of the bacterial growth obtained by plating fecal samples (one sample per child) onto MacConkey agar (MCA) plates (Oxoid, Milan, Italy), were randomly selected among samples ($n = 3{,}193$) obtained during a survey performed in 2005 in the same areas (1) and stored at −70°C. A loopful of each pool was plated on an MCA plate supplemented with 0.12 μg/ml ciprofloxacin (MCA-CIP). This ciprofloxacin concentration was used for screening purposes since (i) it was lower than MICs usually exhibited by enterobacterial strains harboring *qnr* genes as the sole quinolone resistance mechanism (reference 11 and references therein), while being higher than the wild-type MIC distribution for *Escherichia coli* and *Klebsiella pneumoniae* (http://www.escmid.org/research_projects/eucast/); (ii) a similar ciprofloxacin MIC threshold has previously been used for screening of *qnr*-positive bacteria (4, 8, 14, 15, 21). In case of growth onto MCA-CIP, a loopful of bacteria was directly used for total DNA extraction (10), and about 100 ng of metagenomic DNA was used as template in PCRs (50 μl) to detect the presence of *qnr* genes. Primers and conditions for PCR amplification of *qnr* genes were described previously in references 14 (*qnrA* and *qnrS*) and 2 (*qnrB*). Controls for *qnr* genes were kindly provided by Patrice Nordmann and Laurent Poirel (Université Paris-Sud, K.-Bicêtre, France). The specificity of the PCR products was confirmed by partial sequence analysis of randomly selected amplicons (Macrogen, Seoul, Korea).

* Corresponding author. Mailing address: Dipartimento di Biologia Molecolare, Sezione di Microbiologia, Università di Siena, Policlinico Santa Maria alle Scotte, 53100 Siena, Italy. Phone: 39 0577 233455. Fax: 39 0577 233870. E-mail: rossolini@unisi.it.

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TABLE 1. Prevalence of *qnr* genes in commensal enterobacteria from 310 healthy children living in Peru and Bolivia[a]

Study area	No. of samples	No. (%) of samples grown on MCA-CIP[b]	No. of samples (% [95% CI]) for[c]:					
			Any *qnr*		*qnrB*		*qnrS*	
			PCR	Dot blot	PCR	Dot blot	PCR	Dot blot
Peru	164	154 (94)	113 (69 [61–76])	93 (57 [49–64])	107 (65 [57–72])	87 (53 [45–61])	37 (23 [16–30])	11 (7 [3–12])
Bolivia	146	121 (83)	63 (43 [35–52])	51 (35 [27–43])	60 (41 [33–50])	49 (34 [26–42])	7 (5 [2–10])	2 (1 [0–5])
Total	310	275 (89)	176 (57 [51–62])	144 (46 [40–52])	167 (54 [48–60])	136 (44 [38–50])	44 (14 [11–19])	13 (4 [2–7])

[a] Statistical differences were determined by the chi-square test with the EpiInfo software package, version 6 (Centers for Disease Control and Prevention, Atlanta, GA). The binomial exact 95% confidence interval (95% CI) was calculated by Stata Software release 8.0 (2003; StataCorp LP, College Station, TX).
[b] Rates of growth on MCA-CIP plates were not significantly different ($P > 0.5$).
[c] The percentage of samples was calculated for the total samples. *qnr* genes were significantly more prevalent in Peru than in Bolivia: $P = 0.01$ for any *qnr* and *qnrB*, and $P < 0.001$ for *qnrS*. All positive samples in the dot blot were also positive in the PCR.

The presence of *qnr* genes in the enterobacterial pools was also investigated by dot blot DNA hybridization using a rapid method, essentially as described by Srinivasan et al. (16). Briefly, a loopful of the bacterial growth on MCA-CIP was transferred to 150 μl of lysis solution (0.4 N NaOH, 10 mM EDTA) and incubated at 70°C for 2 h. The bacterial lysate (100 μl) was directly blotted onto Hybond-N+ nylon membranes (Amersham Bioscience, Buckinghamshire, United Kingdom) using a BIO-dot microfiltration apparatus (Bio-Rad Laboratories, Milan, Italy). Nylon membranes were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), fixed by UV light, and hybridized with digoxigenin-dUTP (DIG)-labeled *qnr* probes (generated by PCR using the positive controls, as described above) using the DIG system according to the manufacturer's instructions (Roche Diagnostics SpA, Milan, Italy).

Growth of enterobacterial pools on MCA-CIP was observed with 275 of 310 samples (89%). Analysis of the metagenomes prepared from this bacterial growth by PCR revealed overall positivities of 54% for *qnrB* and 14% for *qnrS*, while *qnrA* was not detected (Table 1). Dot blotting showed signals of variable intensity (Fig. 1) and an overall lower detection sensitivity, but results were fully consistent with those of PCR (i.e., all positive samples in the dot blot, including weak signals, were also positive by PCR) (Table 1). This evidently reflected a variable copy number of target genes in the metagenomic DNA from various samples, with a number of cases in which the amount of target genes could only be detected by the more sensitive gene amplification approach.

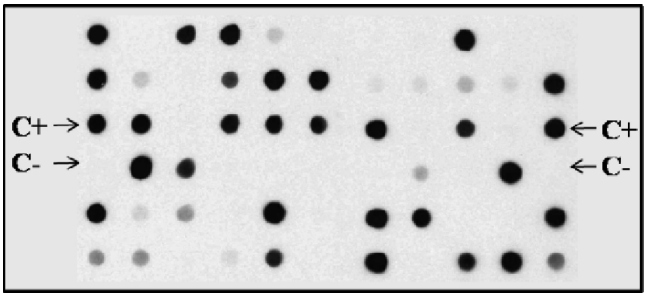

FIG. 1. Nylon membrane prepared with bacterial lysates, hybridized with DIG-labeled *qnrB* probe. All positive signals, including the weakest ones, were positive in PCR experiments. Positive and negative controls (C+ and C−, respectively) are indicated by arrows.

Concerning geographical differences, even though the rates of growth of enterobacterial pools on MCA-CIP were not significantly different, *qnr* genes were significantly more prevalent in Peru than in Bolivia (Table 1). Interestingly, these differences mirrored the higher quinolone resistance rates found in the commensal *E. coli* microbiota of the same population of children from Peru (62% for nalidixic acid and 39% for ciprofloxacin) as compared to Bolivia (51% for nalidixic acid and 26% for ciprofloxacin) (1), which supports an association between dissemination of PMQR determinants and quinolone resistance.

To investigate the nature of the bacterial hosts of *qnr* genes in the commensal microbiota, enterobacterial pools yielding metagenomes positive for *qnrB* ($n = 42$) or *qnrS* ($n = 22$), selected at random, were plated on MCA-CIP to yield isolated colonies. All colonies with a different morphological appearance were collected and subjected to molecular analysis to investigate the presence of *qnrB* and *qnrS* genes by PCR (up to four colonies were analyzed per sample). All *qnr*-positive isolates were identified by the API 20E system (BioMérieux, Marcy l'Etoile, France). When two or more isolates of the same species and carrying the same *qnr* gene were observed in a sample, only one isolate was included in the data analysis.

Isolates carrying *qnrB* or *qnrS* were detected in 36 of 42 (86%) and 14 of 22 (64%) of the selected *qnrB*- and *qnrS*-positive enterobacterial pools, respectively (Table 2). The lack of recovery of *qnr*-positive isolates from some samples was likely due to a lower frequency of such isolates in those enterobacterial pools. In fact, the success in isolating *qnr*-positive isolates from the enterobacterial pools was consistently higher when the corresponding metagenome had been found *qnr* positive also by dot blotting (89% versus 47%) (Table 2), in agreement with the hypothesis that, in those cases, *qnr* genes were carried by a dominant bacterial population.

Identification of the *qnr*-positive isolates showed that *qnrB* was mainly carried by *E. coli* and, more rarely, by *K. pneumoniae* or other enterobacterial species, including *Enterobacter cloacae*, *Klebsiella oxytoca*, *Citrobacter freundii*, and *Escherichia hermannii* (Table 2). On the other hand, *qnrS* was mainly found in *K. pneumoniae* and, more rarely, in *E. coli* and *K. oxytoca*

TABLE 2. Detection and identification of *qnr*-carrying bacterial hosts from enterobacterial pools yielding *qnr*-positive metagenomes

Enterobacterial pool (no. of samples)	No. (%) of samples in which *qnr* hosts were identified	No (%) of *qnr* bacterial hosts		
		E. coli	*K. pneumoniae*	Others[a]
qnrB positive				
PCR and dot blot positive (35)	32 (91)	22 (69)	5 (16)	5 (16)
PCR positive only (7)	4 (57)	4 (100)		
Total (42)	36 (86)	26 (72)	5 (14)	5 (14)
qnrS-positive				
PCR and dot blot positive (12)	10 (83)	3 (30)	7 (70)	
PCR positive only (10)	4 (40)	1 (25)	1 (25)	2 (50)
Total (22)	14 (64)	4 (29)	8 (57)	2 (14)

[a] *qnrB* was detected in *E. cloacae* ($n = 2$), *K. oxytoca* ($n = 1$), *C. freundii* ($n = 1$), and *E. hermannii* ($n = 1$). *qnrS* was detected in *K. oxytoca* ($n = 2$).

(Table 2). In two cases, both *qnr* genes were found to be carried by the same *E. coli* isolate.

MICs of nalidixic acid, ciprofloxacin, and levofloxacin were determined by agar dilution and interpreted according to CLSI (5, 6) for the 48 *qnr*-harboring isolates. Resistance to nalidixic acid was common (77%), while 32% and 17% of isolates were nonsusceptible to ciprofloxacin and levofloxacin, respectively (analytical data are reported in Table S1 in the supplemental material).

The location and nature of *qnr* genes were investigated in 20 selected isolates representative of different species (11 *qnrB* and 9 *qnrS*) by Southern blotting on nylon membranes, as described for dot blot hybridization, and by sequencing of PCR amplicons generated with primers designed on flanking sequences (EU624315 and EU715254 for *qnrB* and EU939771 for *qnrS*). *qnrB* genes were located on either low- or high-molecular-weight plasmids and included mostly *qnrB19* but also *qnrB2* and a new allele of *qnrB10* showing 7 nucleotide differences compared to *qnrB10* DQ631414. All *qnrS* genes were *qnrS1* and were located on high-molecular-weight plasmids (Table 3; and data not shown).

Concluding remarks. This study demonstrated a remarkable dissemination of *qnrB* and, at a lower level, *qnrS* determinants in commensal enterobacteria from healthy children living in urban areas of Peru and Bolivia. The few studies that have investigated the prevalence of *qnr* genes in clinical isolates from South American countries showed that alleles belonging to the *qnrB* are the most frequently reported (11 and references therein), in accordance with the high prevalence of *qnrB* observed in this study. To our best knowledge, this is the first study on the prevalence of *qnr* genes in human commensal bacteria, and present findings suggest that the commensal enterobacterial microbiota could be an important reservoir of similar genes. It will thus be interesting to investigate if a similar situation is also found in the adult population and in other geographical settings.

Since data collected about household use of antibiotics excluded previous use of fluoroquinolones in the children included in this study (1), selection of *qnr* genes could be related to linkage with other resistance genes carried on the same plasmids. Further studies are under way to investigate this issue.

TABLE 3. *qnr* genes in commensal enterobacteria from healthy children in Peru and Bolivia

Isolate[a]	Origin	*qnr* gene	MIC (μg/ml)[b]		
			NAL	CIP	LEV
E. coli Y1	Yurimaguas, Peru	*qnrB19*	32	0.5	1
E. coli M1	Moyobamba, Peru	*qnrB19*	16	0.25	0.5
E. coli C1	Camiri, Bolivia	*qnrB19*	32	0.5	1
E. coli V1	Villa Montes, Bolivia	*qnrB19*	32	0.25	1
K. pneumoniae Y1	Yurimaguas, Peru	*qnrB19*	64	2	1
K. pneumoniae M1	Moyobamba, Peru	*qnrB19*	32	2	2
K. pneumoniae V1	Villa Montes, Bolivia	*qnrB2*	32	1	1
E. hermannii C1	Camiri, Bolivia	*qnrB19*	32	0.25	0.25
E. cloacae V1	Villa Montes, Bolivia	*qnrB2*	32	2	2
K. oxytoca M1	Moyobamba, Peru	*qnrB19*	16	0.25	0.5
C. freundii V1	Villa Montes, Bolivia	*qnrB10*	16	0.25	0.5
E. coli Y2	Yurimaguas, Peru	*qnrS1*	16	0.5	0.5
E. coli Y3	Yurimaguas, Peru	*qnrS1*	16	0.5	0.5
E. coli M2	Moyobamba, Peru	*qnrS1*	16	0.5	0.5
E. coli M3	Moyobamba, Peru	*qnrS1*	32	0.5	2
K. pneumoniae Y2	Yurimaguas, Peru	*qnrS1*	32	1	2
K. pneumoniae M2	Moyobamba, Peru	*qnrS1*	32	4	1
K. pneumoniae C2	Camiri, Bolivia	*qnrS1*	32	2	2
K. pneumoniae V2	Villa Montes, Bolivia	*qnrS1*	16	1	2
K. oxytoca M2	Moyobamba, Peru	*qnrS1*	>128	4	4

[a] The 11 *qnrB*-harboring isolates were selected as follows: four *E. coli* isolates, one from each study area; three *K. pneumoniae* isolates, each from different study areas (no *qnrB*-harboring *K. pneumoniae* isolate detected in Camiri, Bolivia); and four isolates representative of species other than *E. coli* or *K. pneumoniae.* The nine *qnrS*-harboring isolates were selected as follows: all of the *qnrS*-harboring *E. coli* isolates ($n = 4$); four *K. pneumoniae* isolates, one from each study area; and one of the two *qnrS*-harboring *K. oxytoca* isolates.

[b] NAL, nalidixic acid; CIP, ciprofloxacin; LEV, levofloxacin.

Nucleotide sequence accession number. The nucleotide sequence of the new allele of *qnrB10* has been submitted to GenBank and assigned accession no. FJ769283.

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