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REVIEW

Plasmid-mediated quinolone resistance in Enterobacteriaceae: a systematic review with a focus on Mediterranean countries

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Abstract Quinolones are a family of synthetic broad-spectrum antimicrobial drugs. These molecules have been widely prescribed to treat various infectious diseases and have been classified into several generations based on their spectrum of activity. Quinolones inhibit bacterial DNA synthesis by interfering with the action of DNA gyrase and topoisomerase IV. Mutations in the genes encoding these targets are the most common mechanisms of high-level fluoroquinolone resistance. Moreover, three mechanisms for plasmid-mediated quinolone resistance (PMQR) have been discovered since 1998 and include Qnr proteins, the aminoglycoside acetyltransferase AAC(6′)-Ib-cr, and plasmid-mediated efflux pumps QepA and OqxAB. Plasmids with these mechanisms often encode additional antimicrobial resistance (extended spectrum beta-lactamases [ESBLs] and plasmidic AmpC [pAmpC] ß-lactamases) and can transfer multidrug resistance. The PMQR determinants are disseminated in Mediterranean countries with prevalence relatively high depending on the sources and the regions, highlighting the necessity of long-term surveillance for the future monitoring of trends in the occurrence of PMQR genes.

Introduction

Quinolones constitute an important class of broad-spectrum antibacterial drugs and are used for the treatment of various infections. Since the introduction of the first molecule, nalidixic acid, into clinical therapy [1], other molecules have been developed, extending their spectrum of activity [2]. However, increased use of quinolones has led to increasing resistance to these antimicrobials [3]. Indeed, apart from chromosomally encoded quinolone resistance, in the 2000s new mechanisms of quinolone resistance emerged, plasmid-mediated with three mechanisms known so far: Qnr peptides, the aminoglycoside acetyltransferase variant AAC(6′)-Ib-cr, and plasmidic efflux pumps QepA and OqxAB [4]. During the last decade plasmid-mediated quinolone resistance (PMQR) has been increasingly reported in Enterobacteriaceae worldwide [5], including Mediterranean countries that constitute a region with an heterogeneous population from the north of Africa and the south of Europe and the Middle East. This literature review presents a description of the chromosomal and plasmidic fluoroquinolone resistance mechanisms known to date and the epidemiology of occurrence of PMQR determinants in Enterobacteriaceae in Mediterranean countries, with aim of gaining a better understanding of the current situation.

Quinolone structure and clinical use

Quinolones are a family of synthetic broad-spectrum antimicrobial drugs developed by modification of 1-alkyl-1,8-naphthyridin-4-one-3-carboxylic acid [6]. Nalidixic acid was the first molecule introduced into clinical therapy in the early 1960s that was obtained during the synthesis of chloroquine. It had limited clinical use because it was only sufficient for treatment of urinary tract infections and because of the early emergence of

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resistance [1]. Pharmacological improvements on the basic quinolone ring with the addition of fluorine at position C6 and piperazinyl or related rings at position C7 yielded the fluoroquinolones [7]. Thereby, newer class quinolones that expand the spectrum of activity to include Gram-positive bacteria and even anaerobics were developed in the 1980s (Fig. 1).

High bioavailability, relatively low toxicity, and favorable pharmacokinetics have resulted in the clinical success of fluoroquinolones. Thus, these molecules have been widely prescribed to treat respiratory tract infections, including tuberculosis, urinary tract infections (UTIs), intra-abdominal infections, skin and skin structure infections, sexually transmitted diseases, and bone and joint infections [2]. The molecules most frequently used in clinical practice are ciprofloxacin, levofloxacin, and moxifloxacin. They achieve higher serum levels leading to better tissue penetration and greater potency against Enterobacteriaceae and other species [8]. The quinolones that are clinically available have been classified into several generations based on their spectrum of activity (Table 1) [9].

Some quinolone derivatives demonstrated inhibitory potential against eukaryote topoisomerase II and substantial dose-dependent cytotoxic potential against some cancerous cells [10]. Moreover, some quinolones, such as 6-chloro-7-methoxy4(1H)-quinolones, have good antimalarial activity and good activity against multiple stages of *Plasmodium* infection [11]. Fluoroquinolones, such as enrofloxacin, have also been extensively used in veterinary medicine to treat and prevent bacterial infections in food-producing animals, aquaculture, and pets, along with growth promoters [12].

Mechanism of action

Quinolones inhibit bacterial DNA synthesis by interfering with the action of two essential bacterial enzymes, DNA gyrase and topoisomerase IV. Both are heterotetrameric type II topoisomerase enzymes breaking transiently both strands of a duplex and pass another double-helical segment through the break by ATP hydrolysis [13]. The DNA gyrase introduces negative supercoils (or relaxes positive supercoils) into DNA, whereas topoisomerase IV exhibits a potent decatenation activity. Those enzymes are essential for bacterial growth as they control the topological status of the chromosomal DNA to facilitate replication, transcription, recombination, and DNA repair [13].

These enzymes comprise two copies of each of either a GyrA (97 kDa) and GyrB (90 kDa) subunit or a ParC (75 kDa) and ParE (70 kDa; GrlA and GrlB in the *Staphylococcus aureus*) subunit respectively. A few bacteria are able to function with only DNA gyrase, such as *Mycobacterium tuberculosis*, *Helicobacter pylori*, and *Treponema pallidum*, but most bacteria have both enzymes [8]. The targeting of either DNA gyrase or topoisomerase IV as the primary target by fluoroquinolones varies with bacterial species and the specific fluoroquinolone; however, as a broad generalization, the key target in Gram-negative bacteria is DNA gyrase, whereas in Gram-positive microorganisms topoisomerase IV is preferentially targeted [14]. Nevertheless, some recently developed quinolones, such as clinafloxacin and moxifloxacin, have a similar affinity for both targets [15].

Quinolones bind the DNA–enzyme complex of topoisomerase, forming a quinolone–enzyme–DNA complex (known as a ternary complex) in which the type II topoisomerase is trapped with the bound DNA [14]. The drug–enzyme–DNA complexes block cell growth. The bactericidal activity is due to the releasing of double-stranded DNA breaks from those complexes (chromosomal fragmentation process). Furthermore, an indirect route could be responsible for the lethal effect of quinolones, related to reactive oxygen species and/or toxin–antitoxin systems [16, 17]. A bacteriostatic effect

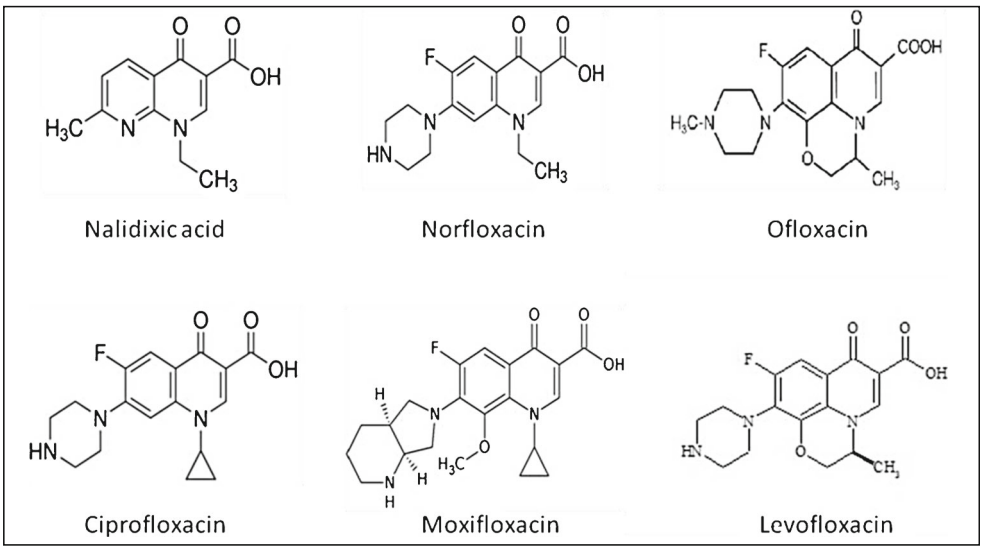

Fig. 1 Chemical structure of some of the quinolones used in clinical practice (http://www.chemspider.com/)

Table 1 Generations and spectrum of activity of quinolones [9]

Generation	Drug	Spectrum of activity
First	Nalidixic acid, oxolinic acid, piromidic acid, pipemidic acid, rosoxacin, cinoxacin (discontinued), flumequine	• Mainly Enterobacteriaceae: *Escherichia coli*, *Proteus* spp., *Klebsiella* spp., *Enterobacter* spp., *Serratia* spp., *Citrobacter* spp., *Salmonella* spp., *Shigella* spp.
Second	Ciprofloxacin (Cipro®), enoxacin, lomefloxacin, nadifloxacin, norfloxacin (Noroxin®), ofloxacin (Floxin®), pefloxacin	• Gram-negative bacilli: *Pseudomonas aeruginosa*, *Moraxella catarrhalis*, *Acinetobacter* spp., *Stenotrophomonas maltophilia*, *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Vibrio cholerae*, *Campylobacter* spp. • Gram-negative cocci: *Neisseria gonorrhea, Neisseria meningitidis* • Gram-positive cocci: *Staphylococcus aureus*, *Staphylococcus epidermidis* • *Mycobacterium tuberculosis* • "Atypical" pathogen: *Chlamydophila trachomatis*, *Mycoplasma* spp.
Third	Levofloxacin (Levaquin®), sparfloxacin (discontinued), gatifloxacin (discontinued).	• Gram-negative bacteria: the same as the first and the second generation • Gram-positive cocci: *Streptococcus pneumoniae* and *Streptococcus pyogenes* • "Atypical" pathogen: *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae*
Fourth	Gemifloxacin (Factive®), Moxifloxacin (Avelox®), trovafloxacin (discontinued).	• Gram-negative bacteria: the same as the third generation • Gram-positive cocci: *Streptococcus pneumoniae*, *Staphylococcus aureus* • Anaerobic: *Bacteroides* spp., *Clostridium* spp. • "Atypical" pathogen: *Legionella pneumophila*, *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae*, *Ureaplasma* spp.

is observed at low quinine concentrations (around the minimum inhibitory concentrations [MICs]), with cleaved complexes blocking DNA replication reversibly and inducing the SOS stress response, which in *Escherichia coli* results in upregulation of various stress response genes that enhance DNA repair capability, leading to the formation of filamentous cells because of the inhibition of cell division [18].

Chromosome-encoded resistance mechanisms

Alterations in target enzymes

The most common mechanism of high-level fluoroquinolone resistance is due to mutations in one or more of the genes that encode the primary and secondary targets of these drugs, the type II topoisomerases (*gyrA*, *gyrB*, *parC*, and *parE*) [19, 20]. These alterations have been localized mainly in the amino terminal domains of GyrA (residues 67 to 106 for *E. coli* numbering) or ParC (residues 63–102) and are in proximity to the active site of tyrosines (Tyr122 for GyrA, Tyr120 for ParC), which are covalently linked to DNA in an enzyme intermediate in both enzymes. This domain has been termed the "quinolone resistance determining region" (QRDR) [19, 21, 22]. However, some mutations localized outside the QRDR region, such as position 51, can also lead to decreased susceptibility to the quinolones [15].

Mutations in the QRDR of the topoisomerase genes, resulting in amino acid substitutions, alter the target protein structure and subsequently the fluoroquinolone binding affinity of the enzyme, leading to quinolone resistance [8]. In *E. coli* and in some other Gram-negative bacteria, such as *Citrobacter freundii*, *Shigella* spp., *Neisseria gonorrhoeae*, and *Acinetobacter baumannii*, mutations in *gyrA* appear most frequently at codon Ser83 and mutations at codon Asp87 are the second most commonly observed [23–25]. These two amino acids (Ser83 and Asp87) likely play a crucial role in mediating quinolone–enzyme interactions. Indeed, a recent study explained that the interaction between quinolones and topoisomerases was due to the formation of a water–metal ion bridge between the oxygen molecules in the amine group of the drug and the hydroxyl residues in conserved serine or acidic residues in the enzyme, mediated by Mg^{2+} ion. Thus, a mutation in the place of serine or nearby another amino residue decreases the affinity of the enzyme for quinolones at the noncatalytic Mg2+ site [26].

Different amino acid substitutions at the same position result in different quinolone susceptibility levels. Furthermore, the presence of a single mutation in the QRDR of *gyrA* usually results in high-level resistance to nalidixic acid only (the MIC of nalidixic acid is greater than 64 mg/L), but to obtain high levels of resistance to fluoroquinolones (ciprofloxacin MICs ranging from 16 to >256 mg/L), the presence of additional mutation(s) in *gyrA* and/or in another target such as *parC* is required (Table 2) [15, 27]. Mutations in specific domains of GyrB and ParE have also been shown to cause quinolone resistance, although they are substantially less common in resistant clinical bacterial isolates than mutations in GyrA or ParC [34, 35].

Reduction in cytoplasmic drug concentrations

As the target of fluoroquinolones is intracellular, these molecules must have the ability to enter the bacterial cell. Quinolones may cross the outer membrane in two different

Table 2 Fluoroquinolone resistance mechanisms associated with changes in MIC of ciprofloxacin in *E. coli*

Quinolone resistance mechanism	Fold change in ciprofloxacin MICs	References
One substitution in GyrA	10–16	[28]
Two substitutions in GyrA + 1 substitution in ParC	60	[28]
Efflux upregulation	4–8	[29]
Porin loss	4	[30]
qnr	32–64	[27]
aac(6')Ib-cr	4	[31]
qepA	32	[32]
oqxAB	16	[33]

ways: through specific porins (e.g., ciprofloxacin) or by diffusion through the phospholipid bilayer (e.g., nalidixic acid). The rate of diffusion of a quinolone is largely associated with its level of hydrophobicity [36].

Gram-negative bacteria can regulate membrane permeability by altering expression of outer membrane porin proteins that form channels for passive diffusion, such as outer membrane proteins OmpF and OmpC in *E. coli*. Downregulation of these channels or mutations in their structural genes can lead to reductions in cytoplasmic drug concentrations that may contribute as a resistance mechanism [36].

Another chromosome-encoded resistance mechanism leading to decreased intracellular drug accumulation of quinolones is the upregulation of native efflux pumps. These efflux systems are largely responsible for the intrinsic susceptibility of many species to fluoroquinolones and other drugs, but are also responsible for increased MICs resulting from derepression of the transporter [37]. Acquired quinolone resistance can occur by mutations in genes encoding regulatory proteins that control the transcription of efflux pump or porin genes. Less often, mutations in efflux pump structural genes have been associated with changes in pump substrate profiles that include quinolones [37, 38].

Several Enterobacteriaceae species possess a chromosomal native AcrAB-TolC efflux pump belonging to the resistance–nodulation–division (RND) family. AcrA is a membrane fusion protein, AcrB is an inner-membrane pump, and TolC is an outer-membrane protein and they build up an efflux pump [39]. Mutations in *acrR* (a repressor of *acrAB*) increase pump activity. On the other hand, mutations that inactivate *marR* (a repressor of *marA*) allow MarA to activate *acrAB*, *tolC*, and a gene that decreases the translation of *ompF*, thus collectively decreasing the influx and increasing the efflux of quinolones [40]. A recent study suggested that *marA* expression is a sensitive marker for early detection of development of levofloxacin resistance [41]. In addition to the Mar regulon, the operon SoxRS regulates the levels of expression of OmpF and some efflux pumps in *E. coli* [42].

A decrease in the level of expression of OmpF is related to an increase in the resistance to some quinolones, such as norfloxacin and ciprofloxacin, and a variety of other antibacterial agents [43, 44], but does not affect the MICs of other quinolones, such as tosufloxacin or sparfloxacin [45]. Despite the fact that both reduced quinolone uptake and the overexpression of efflux pumps lead to low-level quinolone resistance (change in ciprofloxacin MIC of 4- to 8-fold; Table 2), they can have an additive effect, with QRDR mutations contributing to higher-level quinolone resistance. Moreover, they provide a favorable environment for other types of resistance to antibacterial agents [29, 30].

Plasmid-mediated resistance mechanisms

Target protection: qnr proteins

Plasmid-mediated resistance to quinolones was first reported in 1998 by Martínez-Martínez et al. in a *K. pneumoniae* clinical strain that was isolated in July 1994 from the urine of a patient at the University of Alabama at Birmingham Medical Center (UAB) [46]. The gene responsible was termed *qnr* (later named *qnrA1*). This gene was mediated by plasmid pMG252, which could transfer low-level resistance to nalidixic acid, but susceptibility to fluoroquinolones to a variety of Gram-negative recipients (*Citrobacter*, *Salmonella*, and even *P. aeruginosa*) [46].

The PMQR protein QnrA binding to gyrase or topoisomerase IV inhibits the gyrase–DNA interaction, and could account at least in part for the protection against quinolones by minimizing opportunities for these agents to stabilize the lethal gyrase–DNA–quinolone complex [47]. This QnrA1 is a 218-amino-acid protein that belongs to the pentapeptide repeat family, of which more than 500 members are known, distributed in prokaryotic and eukaryotic cells [48]. These proteins are made of tandemly repeated amino acid sequences with a consensus sequence [Ser, Thr, Arg, or Val] [Asp or Asn] [Leu or Phe] [Ser, Thr, or Arg] [Gly] [48].

The pentapeptide repeat proteins also include immunity proteins such as McbG, which, together with McbE and McbF, protects a microcin B17-producing strain from self-inhibition [49]. Microcins are a class of small inhibitory proteins (less than 10 kDa) that are encoded by

bacterial plasmids and differ in their mechanisms of action. Microcin B17, like quinolones, targets DNA gyrase, and McbEFG has been reported to produce low-level resistance to quinolones such as sparfloxacin [50]. The second Qnr relative is MfpA, a protein cloned from the genome of *Mycobacterium smegmatis*, that has a 4-fold effect on susceptibility to ciprofloxacin. Qnr, McbG, and MfpA have < 20% amino acid identity and, thus, are not closely related, but their existence suggests that Qnr could have evolved from an immunity protein designed to protect DNA gyrase and DNA topoisomerase IV from some naturally occurring inhibitor [3].

The description of the *qnrA* gene was subsequently followed by the discovery of plasmid-mediated *qnrS* in *Shigella flexneri* 2b [51], *qnrB* in *K. pneumoniae* [52], *qnrC* in *Proteus mirabilis* [53], and *qnrD* in *Salmonella enterica* [54]. The *qnrVC* (from *Vibrio cholerae*) has been found on plasmids in *Aeromonas punctata* [55] and *Vibrio fluvialis* [56]. It is an atypical member of the Qnr family [57] that differs from the plasmid-mediated *qnr* genes because of the presence of the *attC* site, which is characteristic of integron cassettes [58]. These *qnr* genes generally differ in sequence by 35% or more from *qnrA* and from each other. Furthermore, most of them contain allelic variants differing by 10% or less (*qnrA*: 8, *qnrS*: 9, *qnrB*: 88, *qnrC*: 1, *qnrD*: 2, and *qnrVC*: 7) in which *qnrB* constitutes the most heterogeneous cluster of the *qnr* gene family (http://www.lahey.org/qnrstudies/).

The origin of the plasmid-mediated *qnr* genes has been found on the chromosome of many species, especially in aquatic bacteria, including species of *Aeromonas, Photobacterium, Shewanella*, and *Vibrio* [4]. The progenitor of three QnrA-like determinants (termed QnrA3, QnrA4, and QnrA5) was identified as being the chromosome of the environmental bacterium *Shewanella algae* [59]. *Vibrio splendidus* could be the source of QnrS1-like determinants as chromosomal-encoded Qnr-like proteins shared about 84 and 88% amino acid identity with the plasmid-mediated determinants QnrS1 and QnrS2 respectively [60]. On the other hand, the source of *qnrB* determinants was supposed to be *Citrobacter* spp. including *Citrobacter braakii, Citrobacter werkmanii*, and *Citrobacter youngae* [61]. QnrC is 72% identical to chromosomal Qnr in *V. orientalis* or *V. cholerae* [53]. However, *qnrD* can be found in other Enterobacteriaceae, but are especially likely to be found in *Proteeae*, such as *Proteus*, *Providencia*, and *Morganella* and may have originated there [62]. Vibrionaceae are the source of *qnrVC* genes, the *qnrVC1 attC* site has 89% identity with *V. parahaemolyticus* repeats, and the *qnrVC2* putative *attC* site has 96% identity with a *V. cholerae* repeat sequence [57].

Some *qnr* variants are regulated by the SOS system. The SOS response is triggered by DNA damage, such as that generated by quinolones [63]. The RecA protein is activated by single-stranded DNA and acts as a coprotease to cleave the LexA protein, which otherwise binds as a dimer to LexA boxes, repressing the expression of adjacent genes. Most of these genes are involved in DNA repair or the regulation of cell division [64]. The LexA binding site has been identified in the promoter region of all *qnrB* alleles [65]. This LexA box is also present in *qnrD* and *Smaqnr* (pentapeptide repeat protein reported from the chromosome of *Serratia marcescens*), but not in the other *qnr* variants [66]. SOS regulation could serve to protect the host cell from the potentially toxic effects of QnrB, while allowing augmented production upon exposure to quinolone antimicrobial agents [65]. In the uninduced state, the LexA protein is bound to its site at the promoter region of *qnrB*. Thus, the *qnrB* gene is expressed at a basal level [67]. On induction of the SOS response, by ciprofloxacin for example, single-stranded DNA (ssDNA) is produced and the co-protease activity of the RecA protein is activated by binding to ssDNA [68]. The interaction between LexA and the nucleoprotein filament RecA/ssDNA results in autoproteolytic cleavage of LexA and subsequently in *qnrB* derepression [67]. Induced expression of *qnrB* leads to an increase in the MIC of ciprofloxacin (up to 9-fold). However, a *qnrB*-containing strain does not express quinolone resistance in non-inducing conditions (silent resistance gene); this resistance will be activated only under selective antibiotic pressure [65, 67]. Moreover, it was shown that the direct SOS-dependent regulation of a low-level fluoroquinolone resistance mechanism could be in response to other antimicrobials such as β-lactams and trimethoprim [66].

Enzymatic inactivation: aminoglycoside acetyl transferase AAC(6 ′)-Ib-cr

The aminoglycoside acetyltransferase AAC(6′)-Ib is usually responsible for resistance to tobramycin, amikacin, and kanamycin [69]. Two codon changes, namely Trp102Arg and Asp179Tyr, are found in the variant AAC(6′)-Ib-cr (*c*-iprofloxacin *r*esistance phenotype), which are required to confer reduced susceptibility to certain fluoroquinolones compared with the wild-type AAC(6′)-Ib [31]. The function of the enzyme is to N-acetylate ciprofloxacin at the amino nitrogen on its piperazinyl substituent [70]. Norfloxacin also has free amino nitrogen acting as a substrate of the enzyme, whereas other fluoroquinolones lacking an unsubstituted piperazinyl nitrogen, such as levofloxacin and moxifloxacin, are unaffected [70]. The effectiveness of this enzyme is modest, leading to an increase in the MICs of ciprofloxacin and norfloxacin 3-fold to 4-fold within the range of low-level resistance (Table 2) [31].

Plasmid-mediated efflux pumps

QepA determinant

The *qepA* gene was first discovered in two *E. coli* clinical isolates from Japan and Belgium in 2007 [32, 71]. This gene encodes a 511-amino-acid deduced protein (53 kDa) that shares significant identity with various 14-transmembrane-segment (14-TMS) putative efflux pump belonging to the major facilitator superfamily (MFS) of proton-dependent transporters [32, 71]. The natural reservoir of *qepA* remains unknown. However, it may consist of Actinomycetales species as QepA had significant amino acid identity with likely membrane transporters of the members of the order of Actinomycetales (such as *Streptomyces globisporus*, *Streptomyces coelicolor*, *Nocardia farcinica*, or *Polaromonas* spp.), and its high GC% content (72%) is compatible with this origin [5].

This protein confers significantly decreased susceptibility to the hydrophilic quinolones (e.g., norfloxacin, ciprofloxacin, and enrofloxacin) with an 8- to 32-fold increase in MICs compared with a wild-type susceptibility profile (Table 2) [32]. Since its initial discovery, a variant of *qepA* possessing two amino acid substitutions has been identified and named QepA2 [72]. Wang et al. recently identified a new *qepA* allele, *qepA3*, in Enterobacteriaceae isolates from China [73].

OqxAB determinant

The OqxAB belongs to the RND family, being one of the first plasmid-borne efflux pumps described in 2004 in *E. coli* porcine isolates in Denmark and Sweden, on a conjugative plasmid designated pOLA52 [74, 75]. To our knowledge, this is the first identified genetic resistance mechanism towards olaquindox, an agent used as a growth enhancer in pigs. Moreover, it was shown to mediate resistance to other molecules, such as chloramphenicol, trimethoprim, nalidixic acid, norfloxacin, and ciprofloxacin [33].

The *oqxA* gene codes for the OqxA membrane fusion protein whereas the *oqxB* codes for the OqxB protein, containing 12-transmembrane α-helices for the inner-membrane pump. This system requires the TolC outer-membrane protein to function fully [74].

The OqxAB pump is common (usually 75% or more) on the chromosome of *K. pneumoniae* isolates [76] and it is also prevalent among other species such as *Enterobacter* spp. [77]. It has been reported that the *oqxAB* operon, together with a transcriptional regulator (*orf68*) in the chromosome of *K. pneumoniae*, was captured by IS*26* transposase and transferred to foreign plasmids, which were subsequently disseminated to other bacterial species that do not harbor *oqxAB*-like elements in the chromosome [77].

As for other PMQR, OqxAB alone confers low-level resistance to fluoroquinolones (Table 2). In *K. pneumoniae*, overexpression of the nearby *rarA* gene is associated with increased *oqxAB* expression, whereas increased expression of the adjacent *oqxR* gene downregulates OqxAB production [78]. The overexpression of this efflux pump, resulting from a point mutation in the repressor *oqxR* gene, is responsible for the multidrug resistance phenotype and also for the increased virulence potential in some cases [79].

Impact of PMQR on the clinical resistance level and mutant development

The PMQR genes cause only a modest decrease in ciprofloxacin susceptibility (Table 2). However, it has been shown that PMQR plays an important role in the acquisition of clinical resistance leading to the selection of high-level quinolone resistance [27]. These data indicate that these low-level quinolone-resistant phenotypes may be critical stages (depending on the genotype) in resistance development, including chromosome- and plasmid-mediated mechanisms, at which some low-fitness mutants below the resistance breakpoint are able to evolve clinical resistance with just one or two mutations, and show increased fitness [27].

The clinical importance and the selection of *qnr*-positive cells to high-level fluoroquinolone resistance can be demonstrated by the mutant preventive concentration (MPC), which is the lowest concentration at which no mutants are observed. It represents the MIC of the least-susceptible single-step mutant [80]. The MPC is measured after plating a large inoculum (10^{10} bacteria) on concentrations of a quinolone that are above its MIC. Resistant mutants are selectively enriched only in the concentration range between the MIC and the MPC, which is designated as the mutant selection window. Below the MIC, no mutant will be enriched because selective pressure is absent; above MPC, no mutant will be selected because a double mutation is required for growth [81]. Thus, the best schedule for quinolone administration is one that maintains a quinolone concentration that exceeds the MPC for as much of the dosage interval as possible, thereby minimizing the opportunity for mutant selection [3].

Rodríguez-Martínez et al. showed that the expression of *qnrA* considerably increased the MPC compared with strains without this gene. In the presence of *qnrA*, mutations in *gyrA* and *parC* genes were easily selected to produce high levels of quinolone resistance [82]. Another study reported that the *qepA2* gene also increases the MPCs for ciprofloxacin 4- to 16-fold [83].

Experimental animal models have been used to evaluate the in vivo importance of PMQR genes. Indeed, it has been shown that low-level fluoroquinolone resistance conferred by *aac(6')Ib-cr* is associated with the reduced bactericidal activity of ciprofloxacin in vivo and to lead to ciprofloxacin

therapeutic failure in pyelonephritis. The reduction in bactericidal activity could be explained by in situ N-acetylation of ciprofloxacin [84]. A further work described the significant role of the *aac(6')-Ib-cr* gene in the acquisition of a clinical level of ciprofloxacin and norfloxacin resistance, when combined with three or four chromosomal mutations, both in vitro and in vivo [85].

In addition, several studies used the mouse model of pyelonephritis for in vivo experiments to study the interplay in fluoroquinolone resistance mutations and bacterial fitness. Michon et al. [86] investigated host fitness of *E. coli* isogenic strains after acquisition of the *qnrA3* gene, inserted either alone onto a small plasmid or harbored on a large conjugative native plasmid found in a clinical isolate. Indeed, it was shown that *qnrA3* acquisition enhanced bacterial fitness, which may explain *qnr* emergence and suggests the regulatory role of *qnr*. However, fitness was reduced when *qnrA3* was inserted onto multidrug-resistant plasmids and this can slow down its dissemination without antibiotic exposure. Another study based on in vitro and in vivo models reported that most of the combination of target mutations and *qnrA1* had a significant fitness cost, whereas most of the combinations harboring *qnrS1* had significantly enhanced bacterial fitness. These positive relationships could represent an additional driving force in the development of increased resistance to quinolones [27].

On the other hand, a recent study reported that in *E. coli* J53 carrying *qnrA* and *aac(6')Ib-cr* or *qepA*, *gyrA* mutations are rare, whereas alterations in pumps, porins, and lipopolysaccharides are more common. Mutations upregulating drug efflux (mutations in regulatory genes *marR* or *soxR*) may have a fitness cost, but are relevant to resistance evolution because of their pleiotropic effects on susceptibility to other agents and potential for occurrence at a rate higher than that required for specific changes in the QRDR of *gyrA* [87].

Antibiotic markers, plasmids, and mobilizing elements associated with PMQR genes

Determinants of PMQR have been strongly associated with extended spectrum beta-lactamases (ESBLs) and AmpC ß-lactamases, as shown in Table 3. They have been described in multiresistant clones with worldwide distribution, such as *E. coli* ST131 and *K. pneumoniae* ST11 [119, 120]. Moreover, the PMQR genes have often been transferred on multiresistant conjugative plasmids varying in size, which facilitated their global emergence involving several plasmids with different incompatibility groups [4].

The PMQR *qnrA1*, *qnrA3*, *qnrA6*, *qnrB2*, *qnrB4*, *qnrB6*, and *qnrB10* have usually been associated with the mobilizing element insertion sequence common region (ISCR), whereas *qnrB1* and *qnrB20* have been linked to IS*26* and Orf1005 [4]. The *qnrB19* allele has been found in a transposon comprising IS*Ecp1* [121]. The *qnrS1* has been associated with an upstream Tn*3*-like transposon or upstream of the insertion sequence IS*Ecl2* [122], in several plasmids containing an active TEM-1 gene [4]. Nevertheless, most plasmids carrying *qnrS* genes are relatively smaller than plasmids carrying *qnrA* and *qnrB* and are rarely associated with other antibiotic markers [58]. The *qnrC* gene has been found to be linked to IS*Pmi1* [4], whereas the *qnrD* gene has been found in small nontransmissible plasmids (2.6 to 4 kb) in *Proteeae* and located inside mobile insertion cassette (mic) elements [62].

The *aac(6')-Ib-cr* gene is usually found in a cassette as part of an integron with IS*26* in a multiresistance plasmid, which may contain other PMQR genes such as *qnrB* and also $bla_{CTX\text{-}M\text{-}15}$ gene [58]. Moreover, the *aac(6')-Ib-cr* gene can also be inserted in the chromosome [123].

The *qepA1* determinant has also often been found on plasmids encoding aminoglycoside ribosomal methylase *rmtB*. IS*26* elements and IS*CR3C* have been implicated in mobilizing the *qepA1* gene to plasmids [124], whereas the *qepA2* gene was not associated with the *rmtB* gene and IS*26* elements, but was identified to be flanked by two copies of an IS*CR*-like element [72]. A novel genetic structure surrounding the *qepA3* gene has been described. In some strains, *qepA3* was linked to the non-*rmtB*- or non-ISCR3C-producing and was associated with $bla_{CTX\text{-}M\text{-}14}$ or $bla_{TEM\text{-}12}$ [73].

Besides being commonly intrinsic in *Klebsiella* spp. and *Enterobacter* spp., the OqxAB-encoding genes are often located on conjugative plasmids, within a composite transposon Tn*6010* flanked by IS*26* [75].

Epidemiology of PMQR determinants: Mediterranean countries

The PMQR determinants emerged in various Enterobacteriaceae species, especially *E. coli*, *Enterobacter* spp., *Klebsiella* spp., and *Salmonella* spp. [4] and are less common in other Gram-negative bacteria such as *Aeromonas* spp. [125] *A. baumannii* [126] and *P. aeruginosa* [127].

Currently, these determinants have been increasingly reported in Enterobacteriaceae worldwide, including several Mediterranean countries. Although many reports about PMQR determinants have been published in Spain, Tunisia, Italy, France, Algeria, Egypt, and Turkey, there are no published data yet from Libya, Syria, Lebanon, Albania, Bosnia and Herzegovina, Montenegro, and Malta. The geographic distribution of PMQR determinants in the Mediterranean basin is presented in Fig. 2.

The PMQR genes have been found in different sources, including humans (clinical [96, 111, 117, 128, 129] and community isolates [91, 95, 105, 130]), animals (companion animals [103, 131–133] and food-producing animals [90, 94, 134]), and the environment (waste water treatment plant [135] and hospital effluents [91, 136]).

Table 3 Prevalence of plasmid-mediated quinolone-resistant (PMQR) genes associated with *bla* genes in Mediterranean countries

Reference	PMQR	Rate (%)	Year of isolation	Country	Organism	Origin	Associated *bla* gene
[88]	*qnrB1*	0.7	2003–2007	Algeria	*E. cloacae*	Clinical	CTX-M-15
	qnrB4, *qnrS1*	0.7					SHV-12
	qnrS1	2.1					CTX-M-15, SHV-12
[89]	*qnrB1* *aac(6')Ib-cr*	8 4	2006–2008	Algeria	*E. cloacae*	Clinical	CTX-M-28, TEM-1
[90]	*aac(6')Ib-cr*	22.2	2011	Algeria	*E. coli*	Clinical	CTX-M-15
[19]	*qnrB*	9.1	2012–2013	Algeria	*E. coli*	Wild fish	CTX-M-15/ TEM-24
	qnrS	27.3					CTX-M-15
	aac(6')Ib-cr	22.7					CTX-M-15/ TEM-24
[91]	*qnrB*	3	2010–2014	Algeria	Enterobacteriaceae	Community	CTX-M-15
	qnrS	1.4					TEM-1
	qnrD	1.4					–
	aac(6')Ib-cr *qnrB*	10 6.6					CTX-M-15
[92]	*qnrS*	1.6	2010	Algeria	Enterobacteriaceae	Hospital effluents	CTX-M-15, OXA-1
[93]	*aac(6')Ib-cr* *aac(6')Ib-cr*	3.3 5/11	2002–2005	Croatia	*E. coli*	Clinical	CTX-M-15, OXA-1
	aac(6')Ib-cr	3					
[94]	*qnrA* *qnrB*	1.8 1.4	2007	Egypt	Gram-negative bacteria	Fish farms	TEM-1, TEM 105, SHV-89, OXA-30
	qnrS	2.5					
	aac(6')Ib-cr	1.1					
[95]	*qnrB* *aac(6')Ib-cr*	23.3 23.3	2007	Egypt	*E. coli*	Clinical, community	CTX-M-1, CTX-M-9
[96]	*qnr* *aac(6')Ib-cr*	37.9 41.38	ND	Egypt	*E. coli*	Food-producing animals	AmpC
	qepA	3.45					
[97]	*qnrA*	2.2	2002–2005	France	Enterobacteriaceae	Clinical	VEB-1, SHV-12, CTX-M-1
	qnrS	1.6					TEM-52, SHV-12, CTX-M-1
[98]	*qnrA*	16.3	2002–2005	France	*E. cloacae*	Clinical	SHV-12
[72]	*qepA2*	0.8	2007	France	*E. coli*	Clinical	CTX-M-15
[99]	*qnrA* *qnrB*	12 24	2012	France	*E. cloacae*	Clinical	TEM-1, CTX-M-15, OXA-48
	qnrS	4					
	aac(6')Ib-cr	23					
[100]	*aac(6')Ib-cr*	65.7	2011	Greece	*E. coli*	Clinical	CTX-M-15, OXA-1, TEM-1, KPC-2, VIM-1
[101]	*aac(6')Ib-cr*	13	2004–2006	Israel	*K. pneumoniae*	Clinical	KPC-2, CTX-M-15
[102]	*aac(6')Ib-cr*	11 3.9	2004–2006	Italy	*E. coli*	Community Clinical	CTX-M-15, OXA-1, TEM-1
[103]	*qnrA*	2.1	2006–2012	Italy	*Klebsiella* spp.	Dog	CTX-M-9
	qnrB	4.3					CTX-M-15, DHA-1
	qnrS	2.1					CTX-M-1
	aac(6')Ib-cr	13					CTX-M-15
[104]	*qnrA* *qnrB*	10.25 23	2006–2007	Morocco	*E. coli*, *E. cloacae*, *K. pneumoniae*	Clinical	CTX-M-15, CTX-M-28, DHA-1, SHV-12, TEM-1
	qnrS	2.5					
[105]	*qnrB1*	1	2013	Morocco	Enterobacteriaceae	Community	SHV-12, CTX-M-15, TEM-1
[106]	*qnrB* *qnrS*	1.2 1.8	2011	Morocco	*K. pneumoniae*	Clinical	TEM-1, CTX-M-15, NDM-1, OXA-[1]
	aac(6')Ib-cr	3.6					

Table 3 (continued)

Reference	PMQR	Rate (%)	Year of isolation	Country	Organism	Origin	Associated *bla* gene
[107]	*qnrB* *qnrS*	12.5 31.2	2013	Palestine	*K. pneumoniae*	Clinical	CTX-M, SHV
	aac(6')Ib-cr	43.7					
[108]	*qnrB* *qnrS*	1.8 19	2004–2005	Spain	*Enterobacter* spp.	Clinical	LAP-1
	aac(6')Ib-cr	2.6					
[109]	*qnrA* *qnrB*	0.7 1.3	2006	Spain	*E. coli*, *K. pneumoniae*	Clinical	CTX-M-9, SHV-12
	qnrS	1.5					
	aac(6')Ib-cr	16.2					
[110]	*qnrA1*	0.2	2001–2009	Spain	*Salmonella enterica*	Clinical	CTX-M-9
	qnrB4	0.2					DHA-1
[111]	*qnrB4*	20	2009	Spain	Enterobacteriaceae	Clinical	DHA-1
[112]	*qnrA6* *qnrB1, qnrB2*	16	2005–2007	Tunisia	Enterobacteriaceae	Clinical	CTX-M-15, SHV-28, LAP-1
	qnrS1						
[113]	*qnrA6*	23.4	2005–2006	Tunisia	*Providencia stuartii*	Clinical	CMY-16
[114]	*qnrA6*	9	2011	Tunisia	*Providencia stuartii*	Clinical	OXA-48, PER-1, CMY-4
[115]	*qnrA6*	7	ND	Tunisia	*Proteus mirabilis*, *Morganella morganii*	Clinical	DHA-1
[116]	*qnrS1*	ND	2005–2006	Turkey	*S. enterica*	Clinical	TEM-1
[117]	*aac(6')Ib-cr*	13.6	2006–2010	Turkey	*K. pneumoniae*	Clinical	OXA-48
[118]	*qnrB1*	0.3	2000-2006	Turkey	Enterobacteriaceae	Clinical	CTX-M-15
	qnrS1	0.5					CTX-M-3

ND not determined

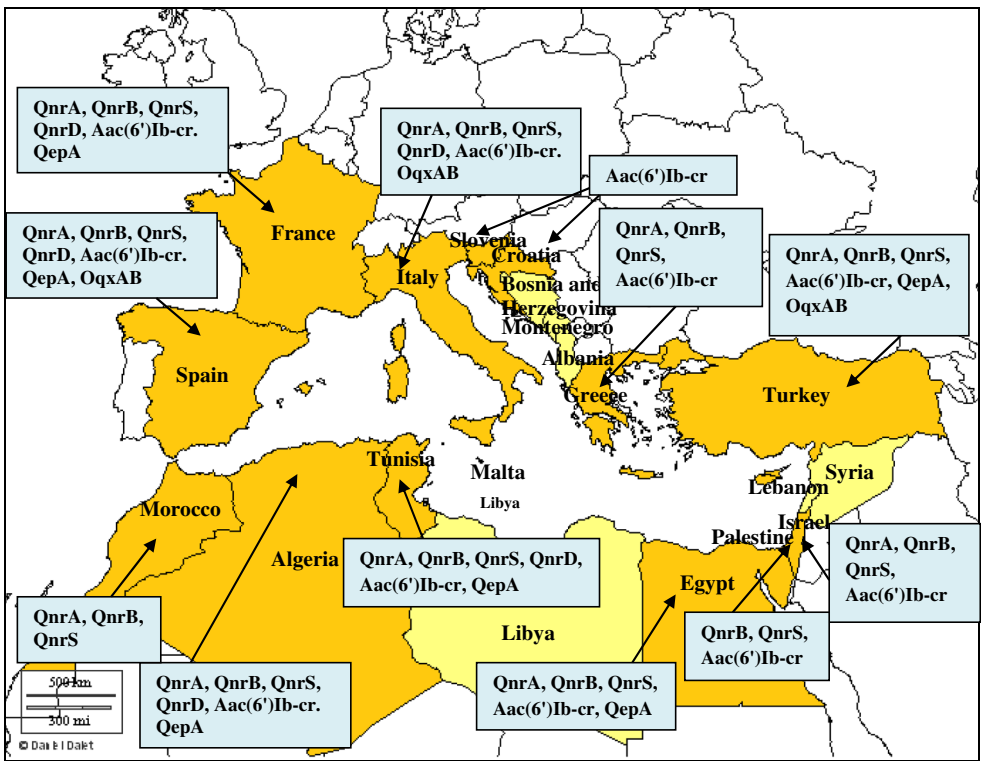

Fig. 2 Geographic distribution of plasmid-mediated quinolone resistance (PMQR) determinants in Mediterranean countries. *Yellow* no case reported, *orange* sporadic or some outbreaks of PMQR-producing isolates

The prevalence of PMQR genes varies by both organism and geographic region and depending on the selection criteria of the strains studied (ESBL-producing, nalidixic acid, fluoroquinolones, etc.) [5]. However, the most commonly reported PMQRs remain the *aac(6')Ib-cr* gene followed by the *qnr* gene. Thus, the *aac(6')-Ib-cr* gene had the highest prevalence at 22%, whereas *qnr* genes were present in 1.3% among a collection of uropathogenic *E. coli* isolated in a Tunisian university hospital [129]. In contrast, *qnr*-related genes (*qnrA*, *qnrB*, *qnrS*) and the *aac(6')-Ib-cr* gene were identified in 14.3 and 3.6% respectively in *Enterobacter cloacae* and *Klebsiella* spp. isolates recovered from cases of clinical and sub-clinical mastitis in Egypt [137]. In many cases, *qnrB* genes seem to be more prevalent overall than other *qnr* genes. In Italy, the prevalence rates of *qnrA*, *qnrB*, and *qnrS* were 1.4, 4.3, and 1.4% respectively in *Klebsiella* spp. isolates from companion animals [103].

An outbreak of *K. pneumoniae* carrying *qnrB1*, *aac(6')Ib-cr*, and $bla_{CTX\text{-}M\text{-}15}$ was reported in a French intensive care unit between 2008 and 2009 [128].

In Spain, the first report of clustered infections caused by a non-ESBL-producing *K. pneumoniae* strain harboring $bla_{OXA\text{-}1}$ and *aac(6')Ib-cr* and belonging to ST14 was identified. The clonal isolates were detected from May 2007 until December 2009 from clinical samples of 38 patients admitted to the University Hospital of Bellvitge, Barcelona, Spain [138]. Dissemination of *qnr* and *aac(6')Ib-cr* genes in *E. coli* is closely associated with the worldwide spread of a single clone, ST131, which is the main cause of clinic- and community-acquired urinary tract infections [139], as described in community *E. coli* strains harboring *aac(6')Ib-cr* and $bla_{CTX\text{-}M\text{-}15}$ isolated in Algeria in 2011 and 2014 [91]. Recent reports have highlighted the Mediterranean Sea as being a potential reservoir of various antibiotic markers, notably PMQR determinants. Indeed, *qnr* (*qnrA*, *qnrB*, and *qnrS*) and *aac(6')-Ib-cr* genes, were identified in 16 (5.8%) and 3 (1.1%) isolates respectively in Gram-negative bacteria isolated from water samples taken from fish farms in the northern part of Egypt at the Mediterranean Sea [94]. Moreover, both *aac(6')Ib-cr* and *qnrB* genes have been identified in CTX-M-15-producing *E. coli* strains belonging to the pandemic clone ST 131 recovered from wild fish in the Mediterranean Sea far from Bejaia (Algeria) [140].

QepA efflux pump is usually identified with a low prevalence. France was the first Mediterranean country to report the QepA determinant (QepA2 variant) in 2008 in one (0.8%) *E. coli* ESBL-producing isolate [72]. In Spain, only one isolate (2.4%) showed the presence of *qepA* among a collection of *Salmonella enterica* clinical isolates [141]. Very recently, QepA1 was identified in two *E. coli* clinical strains isolated in 2011 in Algeria (Yanat et al., unpublished data). This determinant was also described in nalidixic acid-resistant *Salmonella* isolates recovered from various food-animal products in Tunisia, with a rate of 1.16% associated with the *qnr* gene [142].

The identification of OqxAB plasmidic efflux pump is rare in the Mediterranean basin. This determinant is more frequent in China, mainly from animal isolates [143]. Nevertheless, *oqxAB* genes were detected in *E. coli* isolates from animals in Italy with a rate of 15% associated with ST 238 [131] and in *K. pneumoniae* and *E. coli* clinical isolates from Spain and Turkey respectively [76, 144].

Conclusion

In parallel to a high consumption of quinolones, different mechanisms of quinolone resistance were developed in Gram-negative bacteria. PMQR emerged in Enterobacteriaceae isolates with a high prevalence in Mediterranean countries, probably because of the great diversity of the population and the exchange relationship between the regions. As the presence of these determinants is often difficult to detect based on the phenotype observed on a routine basis in the laboratory, the real prevalence of PMQR-positive bacteria could be higher than recorded in the published reports. Therefore, long-term surveillance is needed for the future monitoring of trends in the occurrence of PMQR genes in different regions and in various settings. Also, future studies are required focusing on plasmid analysis to understand more about the successful dissemination of PMQR genes.

Compliance with ethical standards

Conflicts of interest None to declare.

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