Association of plasmid-mediated quinolone resistance and virulence markers in *Escherichia coli* isolated from water

Nuno Mendonça, Joana Ramalho, Pedro Vieira and Gabriela Jorge Da Silva

ABSTRACT

This work aimed to investigate the association of the carriage of plasmid-mediated quinolone resistance (PMQR) genes, the virulence potential encoded in pathogenicity islands (PAIs) and the phylogenetic background in *Escherichia coli* strains isolated from waters of diverse origin. Antimicrobial susceptibilities were determined by the disc diffusion method. Screening for PMQR (*qnr*, *aac*(*6′*)-*Ib*-variant and *qepA*) genes, PAIs and the determination of phylogroup was performed by PCR. Nineteen percent of strains were resistant to nalidixic acid, 11% to ciprofloxacin and 5% to gentamicin. *qnr*A was the only PMQR detected in 16% of strains, susceptible to quinolones and grouped in phylogenetic lineage B1. Sixty-seven percent of the isolates were assigned to the less-virulent groups A and B1. PAIs IV_{536} and II_{CFT073} were detected in 16 and 3% of the isolates, respectively. All PAIs were detected in the phylogroups D and B1. The presence of PAIs in isolates from waters may represent an increased risk for public health, as they were isolated from samples collected from surface and drinking waters. As *E. coli* is an important indicator of microbiological water quality, and also a potential pathogen, routine analysis for its detection could be complemented by screening for virulence factors and antimicrobial genes.

Key words | *Escherichia coli*, pathogenicity islands, phylogeny, plasmid-mediated quinolone resistance, water

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INTRODUCTION

Pathogenic or potentially pathogenic microorganisms enter into aquatic environments mainly through human and animal wastes. Among the human/animal commensal intestinal flora, *Escherichia coli* is a genetically diverse species with the ability to colonize and persist in the environment and within animal hosts (Alexander *et al.* 2010). *E. coli* is also considered a common opportunistic pathogen, depending on the host condition, the route of entry into the host and the virulence determinants, such as adherence factors and siderophores, carried by each strain. *E. coli* strains are a major cause of extra-intestinal infections and represent a major public health burden.

E. coli strains have been grouped in different phylogenetic groups (A, B1, B2, and D) according to their virulence attributes (Herzer *et al.* 1990). While virulent extra-intestinal strains mainly belong to group B2 and, to a lesser extent, to group D, commensal strains have been associated with groups A and B1 (Gordon *et al.* 2008).

Treatment of *E. coli* infection has been increasingly complicated by the emergence of resistance to some antibiotics, including fluoroquinolones. Widespread use of quinolones in human and veterinary medicine has contributed to the selection of quinolone-resistant bacteria. These antibiotics maintain their activity in soil and aquatic environments, which may lead to the development of

doi: 10.2166/wh.2012.153

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resistance among environmental strains (Pena *et al.* 2007). Quinolone resistance is usually mediated by point mutations in the chromosomal genes encoding DNA gyrase and/or topoisomerase IV, leading to target modification, or by mutation in the genes regulating the expression of efflux pumps and/or porins (Jacoby 2005). Recently, plasmid-mediated quinolone resistance (PMQR) determinants have been described, namely *qnr*, *aac*(6′)-*Ib-cr* and *qepA*, conferring low level of resistance (Park *et al.* 2006; Cattoir *et al.* 2007; Ma *et al.* 2009). However, their association with chromosomal mutations may lead to clinically relevant quinolone resistance. Moreover, the insertion of these determinants in mobile elements may allow further dissemination among other bacterial populations and an increase in quinolone resistance in bacterial communities.

Pathogenic isolates harbour specialized virulence factors that may be encoded in pathogenicity islands (PAIs), which are large distinct chromosomal regions (10–200 kb) characterized by a base composition different from core genome. However, they are not exclusively chromosomally encoded, as similar clusters of virulence genes are located on plasmids, which allows the transfer of PAIs between bacteria (Schmidt & Hensel 2004). Moreover, both resistance and virulence determinants may be coded simultaneously in PAIs, as demonstrated for the SGI (Salmonella genomic island 1) in *Salmonella* sp. (Schmidt & Hensel 2004).

Genes inserted in mobile platforms are able to spread among water and soil bacteria communities, altering microbial systems (Schlüter *et al.* 2003). Hence, dissemination to the environment of resistant bacteria simultaneously carrying virulence factors may have unpredictable implications in human and animal health. Thus, the main goal of this study was to screen for the occurrence of PMQR genes and to investigate the association with the virulence potential encoded in PAIs and the phylogenetic background of the *E. coli* strains isolated from water samples of various origins.

METHODS

Between May 2009 and February 2010, 37 water samplings for microbiological analysis were performed according to International Standard Organization (ISO) 19458:2006 (ISO 2006). The detection of bacteria was performed according to ISO 9308-1:2000 (ISO 2000). In order to collect bacteria, 100 ml of the water sample was filtered through a membrane filter with a 0.45 μm pore diameter, under sterile conditions. The membrane was placed on a lauryl sulphate agar plate and incubated at (36 ± 2) °C or (44 ± 0.5) °C for 21 ± 3 h, for the growth of total coliforms or faecal coliforms, respectively. The membranes were observed for bacterial growth. The lactose fermenter isolates were inoculated on to eosine methylene blue agar (Scharlau Chemie S.A., Barcelona, Spain). The characteristic green metallic sheen of colonies suspected to be *E. coli* were further inoculated in CHROMagar™ Orientation (CHROMagar, Paris, France) for confirmation. Only one *E. coli* isolate was considered from each water sample. Overall, 37 isolates of water, distinct in both time and location (19% distribution, 5% recreational, 3% water treatment plant, 24% wells or drills and 49% river), were obtained for further analysis.

The antimicrobial susceptibility to ciprofloxacin (5 μg), nalidixic acid (30 μg), gentamicin (10 μg), cefotaxime (30 μg), ceftazidime (30 μg), imipenem (10 μg) and amoxicillin plus clavulanic acid (30 μg) (Oxoid, Fisher Scientific, Lisbon) were determined by disc diffusion method according to the Clinical and Laboratory Standards Institute guidelines (CLSI 2008).

PMQR determinants (*qnr*, *aac*(6′)-*Ib*-variant and *qepA*) were screened by PCR using specific primers and procedures previously described (Park *et al.* 2006; Cattoir *et al.* 2007; Ma *et al.* 2009). PCR products were purified using the QIAquick PCR Purification kit (QIAGEN, Izasa, Portugal), according to the manufacturer's instructions. Purified PCR products were analysed for DNA sequencing at Stabvida (Lisbon, Portugal). *Klebsiella pneumoniae* 5 (*qnrA* plus *qnrB*), *E. coli* 12HUC (*aac*(6′)-*lb-cr*) and pSTVqepA (*qepA*) were used as control strains for PCR (Yamane *et al.* 2007; Ferreira *et al.* 2010).

Determination of the *E. coli* phylogenetic group was performed by a PCR-multiplex based technique, as previously described (Clermont *et al.* 2000). This triplex-PCR reaction screens for *chuA*, *yjaA* and DNA fragment *tspE4.C2* genes, and allows the classification of the *E. coli* isolates into one of the four phylogenetic groups (A, B1, B2 and D). The clinical *E. coli* strain 12HUC from our laboratory

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collection, which belongs to the phylogenetic group B2, was used as a positive control.

PAI markers were evaluated by a multiplex-PCR technique (Sabaté *et al.* 2006). This triplex-PCR reaction allows the detection of three PAIs, which encoded different virulence determinants: PAI III_{536}, S-fimbriae and an iron siderophore system; PAI IV_{536}, yersiniabactin siderophore system; and PAI II_{CFT073}, P-fimbriae and iron-regulated genes. Amplification products were purified and sequenced as described above.

RESULTS AND DISCUSSION

E. coli strains recovered from water samples were screened for the presence of antimicrobial resistance and virulence factors. This work also allowed a risk assessment regarding the water quality. The water samples were collected from the centre of Portugal between spring 2009 and winter 2010. The water samples that were found to be positive for the presence of *E. coli* were collected from different sources, namely from surface water (49%), groundwater (24%), drinking water (19%), recreational water (5%) and wastewater (3%). It is noteworthy that among the surface water samples, 46% were collected in the district of Coimbra, which is crossed by the Mondego River, where a previous study showed the presence of sub-inhibitory concentrations of fluoroquinolones (Pena *et al.* 2007). Indeed, fluoroquinolones are not completely metabolized in animals and, through human and/or animal wastes, may reach water streams in an active form, with an unpredictable impact on soil and water micro-communities.

Many studies have performed the phenotypic detection of PMQR genes by using mostly nalidixic acid as an indicator, and only a few, the fluoroquinolone ciprofloxacin. Recently, Cavaco & Aarestrup (2009) suggested that nalidixic acid alone did not present the maximum effectiveness for the detection of these resistance determinants. Hence, we used both antibiotics for the detection of PMQR.

Table 1 shows the characteristics of the *E. coli* strains isolated from the water samples. The results obtained for the antimicrobial susceptibility tests showed 19% of resistance to nalidixic acid, 11% to ciprofloxacin and 5% to gentamicin. All strains were susceptible to the combination amoxicillin/clavulanic acid, cefotaxime, ceftazidime and imipenem.

Among the plasmid quinolone resistance determinants, only the *qnrA* was detected in 16% of the isolates tested. The fact that this PMQR was detected in *E. coli* from water may be related to its origin. The *qnrA* reservoir has been identified in *Shewanella algae,* an environmental bacterium from marine and fresh water (Poirel *et al.* 2005). Thus, when in the presence of a quinolone selective pressure, the low level of resistance conferred by *qnrA* may allow the bacterial population to reach a concentration at which secondary chromosomal mutations for higher levels of quinolone resistance may occur, as suggested by Martínez-Martínez *et al.* (1998). Another study detected other PMQR genes (*aac*(6′)-*lb-cr* and *qnrS*) among three multiresistant *E. coli* isolates collected from water (Ishida *et al.* 2010). However, in the present study it was not necessary to search for PMQR in quinolone-resistant isolates as all of our *qnrA* positive strains were susceptible to quinolones. In fact, *qnr* protect bacteria from quinolones at lower concentrations, which could allow the bacteria to develop chromosomal resistance, by mutation of topoisomerase encoding genes (Tran *et al.* 2005a, b). The other PMQR genes (*qnrB*, *qnrS*, *aac*(6′)-*lb-cr* and *qepA*) were not detected in the present study. PMQR genes have been found widely among *E. coli* from clinical and veterinary samples but not in environmental isolates (Rodríguez-Martínez *et al.* 2011). To our knowledge, few reports have searched for and detected these quinolone-resistance determinants in water-isolated *E. coli* (Jakobsen *et al.* 2008; Ishida *et al.* 2010). As aquatic environments may act as reservoirs of PMQR (Poirel *et al.* 2005; Cattoir *et al.* 2008; Picão *et al.* 2008), most studies focus in the detection of PMQR in quinolone-resistant strains which may underestimate the real prevalence of these resistance determinants. However, water is an important vehicle for transmission of infective agents, especially by ingestion, but also by contact with mucosa, highlighting the need for information on the prevalence of antimicrobial resistance determinants, especially genetic elements with capacity to lateral transfer, in potential water-borne infectious agents.

PAI IV_{536} was detected in 16% of *E. coli* isolates and PAI II_{CFT073} in 3% (Table 1). These PAIs have been

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Table 1 | Characteristics of *E. coli* isolates collected from different water samples

Isolate number	Season	Source	Resistance phenotype[a]	PMQRs[c]	Phylogenetic groups	PAIs[d]
Ec 1	Spring 2009	Groundwater	nd[b]	nd	A	nd
Ec 2	Summer 2009	Surface water	nd	nd	D	nd
Ec 3	Summer 2009	Surface water	nd	QnrA	B1	IV536
Ec 4	Summer 2009	Surface water	nd	nd	D	IV536
Ec 5	Summer 2009	Surface water	nd	QnrA	B1	nd
Ec 6	Summer 2009	Surface water	nd	nd	A	nd
Ec 7	Summer 2009	Surface water	nd	nd	B1	nd
Ec 8	Summer 2009	Surface water	nd	QnrA	B1	nd
Ec 9	Summer 2009	Surface water	nd	QnrA	B1	nd
Ec 10	Summer 2009	Surface water	NAL	nd	A	nd
Ec 11	Summer 2009	Surface water	nd	nd	D	nd
Ec 12	Summer 2009	Surface water	nd	nd	A	nd
Ec 13	Summer 2009	Surface water	nd	QnrA	B1	nd
Ec 14	Summer 2009	Surface water	nd	nd	A	nd
Ec 15	Summer 2009	Surface water	NAL, CIP	nd	D	nd
Ec 16	Summer 2009	Surface water	nd	nd	D	IV536
Ec 17	Summer 2009	Surface water	nd	nd	A	nd
Ec 18	Summer 2009	Surface water	nd	nd	D	nd
Ec 19	Autumn 2009	Drinking water	nd	nd	A	nd
Ec 20	Autumn 2009	Drinking water	nd	nd	A	nd
Ec 21	Winter 2010	Drinking water	nd	QnrA	B1	nd
Ec 22	Autumn 2009	Drinking water	nd	nd	D	IV536
Ec 23	Autumn 2009	Drinking water	nd	nd	A	nd
Ec 24	Autumn 2009	Drinking water	NAL, CIP	nd	A	nd
Ec 25	Autumn 2009	Drinking water	nd	nd	D	IV536, IICFT073
Ec 26	Winter 2010	Groundwater	NAL, CIP	nd	B1	nd
Ec 27	Winter 2010	Surface water	nd	nd	B1	nd
Ec 28	Winter 2010	Groundwater	nd	nd	D	nd
Ec 29	Summer 2009	Groundwater	NAL	nd	A	nd
Ec 30	Spring 2009	Recreational water	nd	nd	A	nd
Ec 31	Spring 2009	Recreational water	nd	nd	A	nd
Ec 32	Autumn 2009	Groundwater	GEN	nd	B1	nd
Ec 33	Autumn 2009	Groundwater	NAL	nd	A	nd
Ec 34	Autumn 2009	Groundwater	nd	nd	D	IV536
Ec 35	Autumn 2009	Groundwater	nd	nd	B2	nd
Ec 36	Autumn 2009	Wastewater	NAL, CIP, GEN	nd	D	nd
Ec 37	Winter 2010	Groundwater	nd	nd	A	nd

[a]Resistance to nalidixic acid (NAL), ciprofloxacin (CIP), gentamicin (GEN), amoxicillin/clavulanic acid, ceftazidime and cefotaxime.
[b]nd: not detected.
[c]PMQR: plasmid-mediated quinolone resistance.
[d]PAIs: pathogenicity islands.

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associated with uropathogenic *E. coli* (UPEC) strains (Sabaté *et al.* 2006). Although there have been reports of UPEC detected in water samples (Ramteke & Tewari 2007), to our knowledge this is the first study where specific PAIs were detected among *E. coli* from different water origins. PAI IV_{536} was also the most prevalent (28%) among *E. coli* isolates from wastewaters of different origin, while PAI II_{CFT073} was detected in only 8% of strains (Sabaté *et al.* 2008). In a study focused on the detection of PAIs in clinical samples, PAI IV_{536} was detected in 38% of commensal *E. coli* isolates and 89% in UPEC isolates, while PAI II_{CFT073} was present in 14 and 46% of commensal and UPEC isolates, respectively (Sabaté *et al.* 2006). PAI IV_{536} is reported to be the most ubiquitous PAI found in enterobacteria (Schubert *et al.* 2004). On this basis, it was suggested that it may function as a fitness island rather than a pathogenic one (Karch *et al.* 1999; Oelschlaeger *et al.* 2002). However, *in-vivo* experiments demonstrated its virulence, thus explaining the alternative term of high-PAIs, by which it is known (Sabaté *et al.* 2006). Our results confirmed that PAI IV_{536} was also the more frequent PAI found in water isolates. We found it in isolates from surface, drinking and groundwaters (Table 1). Groundwaters may ultimately be used for private consumption too. This may be of high risk for human and animal health as the detection of these virulence factors and antibiotic resistance determinants as microorganisms in drinking water sources may colonize in gastrointestinal tract (Lee *et al.* 2010). Indeed, Ramteke & Tewari (2007) detected enteropathogenic groups of *E. coli* strains among drinking water samples in India.

Interestingly, all of the PAIs carriers were susceptible to the antibiotics tested. An inverse relationship between the presence of virulence factors and susceptibility to quinolones has been observed by others in uropathogenic *E. coli* (Moreno *et al.* 2006).

According to the phylogenetic groups, we detected 41 and 27% of strains belonging to the less-virulent groups A and B1, while 30 and 3% were from groups D and B2, respectively. *E. coli* strains from phylogenetic groups A and B1 have been considered as potentially less virulent as they tend to produce a lower quantity of virulence factor. The greater presence of *E. coli* from less virulent phylogenetic groups would suggest a lower risk for human health compared to *E. coli* from groups D or B2. Another study on *E. coli* collected from drinking and recreational waters reported 80% of strains belonging to phylogenetic groups A and B1 (Mataseje *et al.* 2009). Among wastewater samples, Sabaté *et al.* (2008) detected 84% of strains from phylogenetic groups A and B1. As observed, our study also founded a higher prevalence of strains with a phylogenetic background compatible with groups A and B1 (68%), although lower than in the studies mentioned. This difference may be related to the water source and the environmental conditions during the sample collection. Although, it was noteworthy that we found a considerable percentage of strains of group D, *E. coli* isolates of groups A and B1 are usually considered as commensal strains, as showed by Sabaté *et al.* (2006) who found 54% of strains belonged to groups A and B1 in *E. coli* isolates from stool of healthy females, while 67% of *E. coli* isolates (UPEC) recovered from urine of females with UTI were from group B2. Furthermore, in the former study, involving wastewaters, 14% of the strains from group D presented PAIs that were not identified by the authors (Sabaté *et al.* 2008), while in the clinical study, 29 and 79% of commensal and UPEC isolates presented PAI IV_{536}, respectively (Sabaté *et al.* 2006). PAI IV_{536} was also found in an isolate belonging to group B1 carrying a QnrA (Table 1). All the *qnrA* genes were detected in group B1 strains collected in waters of different origin, geographical location and season. These different origins imply different acquisition events of *E. coli* strains that could be promoted by the transfer of mobile genetic elements with environmental species, as the *qnrA* reservoir has been detected in marine and freshwater samples (Poirel *et al.* 2005).

CONCLUSIONS

Overall, this study showed the presence of quinolone resistance in microorganisms in the aquatic environment, mostly in strains isolated from consumption, surface and groundwaters, showing that quinolone resistance is present in the environment; the implications of this are difficult to predict. The isolates carrying the *qnrA* genes, apparently an autochthonous gene of the aquatic environment, present an increased survival advantage if under selective pressure,

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and with the potential for lateral transfer to other microorganisms. Moreover, potential pathogenic strains of *E. coli*, mostly from phylogenetic group D, were isolated from drinking, surface and groundwaters and showed different PAIs, which can represent an increased risk for public health.

The presence of *E. coli* in water for human consumption has been an important indicator of the microbiological quality of water. In fact, water from distribution, recreational, river and underground sources showed faecal contamination, as *E. coli* was detected. Adding potential pathogenic bacteria that present resistance and virulence factors that could be transferred, the quality of the water would decrease even more. With the improvement of molecular techniques, such as the use of microarrays, it will be important in the future not only to monitor the presence of *E. coli* in water, but also to unravel their potential virulence and genetic carriage of antimicrobial genes, and consequently, the potential threat to animal and human health.

ACKNOWLEDGEMENTS

All authors contributed equally to this work. The authors would like to acknowledge Ana Tavares, Isabel Cravo and Paula Pereira for sending bacteria. The authors would also like to acknowledge to Professor Sónia Mendo for the gift of *Klebsiella pneumoniae* 5 and Professor Kunikazu Yamane for sending the plasmid pSTVqepA (*qepA*), used as control strains for PCR. N. Mendonça was supported by a grant SFRH/BPD/45815/2008 from Fundação para a Ciência e a Tecnologia, Lisbon, Portugal. This work was supported financially by the ESCMID 2010 Research Grant from the European Society of Clinical Microbiology and Infectious Diseases and by the Center for Pharmaceutical Studies, University of Coimbra.

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First received 1 September 2011; accepted in revised form 16 March 2012. Available online 12 May 2012

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