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

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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).

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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.

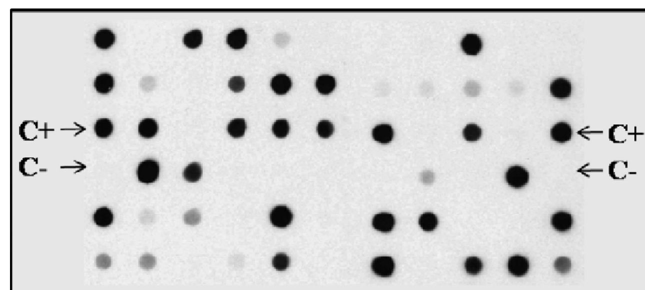

FIG. 1. Nylon membrane prepared with bacterial lysates, hybridized with DIG-labeled *qnrB* probe. All positive signals, including the weakest ones, were positive in PCR experiments. Positive and negative controls (C+ and C−, respectively) are indicated by arrows.

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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