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Full Length Research Paper

Prevalence of plasmid-mediated quinolone resistance qnr genes in Central China

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A total of 179 Gram-negative bacteria strains collected in Wuhan from 2005 to 2006 were screened for *qnrA*, *qnrB* and *qnrS* genes by polymerase chain reaction (PCR). The *qnrA* genes were detected in 3.88% of *Escherichia coli* and 7.69% of *Enterobacter cloacae*, whereas the *qnrB* genes were found in 6.20% of *E. coli* and 7.69% of *E. cloacae*. 2.33% of *E. coli* and 18.92% of *Klebsiella pneumoniae* were positive for the *qnr* gene in PCR. Minimal inhibitory concentrations (MICs) results of ciprofloxacin confirmed that *qnrS* gene could be present in isolates susceptible to quinolones. Furthermore, the results indicated that *qnr* genes were identified on transferable plasmids, and quinolone resistance can be transferred by bacterial conjugation.

Key words: qnr, gram-negative pathogens, plasmid-mediated.

INTRODUCTION

Quinolone resistance has traditionally been attributed to chromosomal mutations, such as DNA gyrase and topoisomerase IV, or activated efflux pumps. However, the plasmid-mediated quinolone resistance associated with *qnr* (now named qnrA1) in *Klebsiella pneumoniae* was firstly found from the United States in 1998 (Martinez-Martinez et al., 1998). The qnr gene encodes a 218-amino-acid protein. The purified qnr protein can protect DNA gyrase and topoisomerase IV activity *in vitro*. Furthermore, the qnr-plasmids are I type integron-associated plasmid and carry multiple resistance determinants providing resistance to several classes of antimicrobials including β-lactams, aminoglycosides and sulfamido (Martinez-Martinez et al., 1998; Tran et al., 2005). The *qnr* gene has already been reported in all populated continents except South America (Robicsek et al., 2006a). And in different geographical areas, the prevalence of *qnr* gene type was different. In Japan, *qnrS* gene was found in *Shigella flexneri* 2b, which showed 59% amino acid identity with the *qnr* gene (Hata et al., 2005). In India, *qnrB* was found in *Citrobacter koseri* with 40% amino acid identity with *qnr* (Jacoby et al., 2006). The present research was conducted to study the prevalence of *qnrA, qnrB* and *qnrS* genes among clinical isolates of Gram negative bacteria in Hubei province of China.

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MATERIALS AND METHODS

Clinical isolates

179 Gram negative bacteria strains (including 129 of *Escherichia coli*, 37 of *K. pneumoniae* and 13 of *Enterobacter cloacae*) were collected from June 2005 to February 2006 in Renmin Hospital of Wuhan University, a District teaching hospital with 1300 ward beds located in central China. All isolates were collected from individual patients who were hospitalized in various types of wards. Species identification was performed using Vitek 32 GNI cards. Additional strains used were *E. coli* C600Lac- SMR (resistance to streptomycin) as a recipient for conjugation.

Susceptibility tests

A disk diffusion susceptibility test was performed on Mueller-Hinton agar by a comparative method. Ampicillin, ampicillin/clavulanic acid, ceftazidime, cefotaxime, cefoxitin and imipenem disks were used.

Minimal inhibitory concentrations (MICs) of the antibiotics were determined according to the Clinical and Laboratory Standards Institute (CLSI) 2007. *E. coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853 were used as quality control reference strains. MICs of ciprofloxacin were determined by agar dilution assay.

Screening for the *qnr* gene in clinical strains

Screening was carried out by polymerase chain reaction (PCR) amplification of *qnrA, qnrB* and *qnrS*. Bacteria isolates were transferred to TE buffer in Eppendorf tubes, and then boiled for 10 min. The supernatant was used as DNA templates for PCR. Primers used were as follows: for *qnrA*, 5'-TCAGCAAGAGGATTTCTCA-3' and 5'-GGCAGCACTATTA CTCCCA-3' to give a 516 bp product,

and the PCR conditions were 94°C for 5 min, 94°C for 45 s , 48℃ for

45 s , and 72°C for 45 s and finally 72°C for 7 min ,with a cycle number of 30; for qnrB, 5'-GATCGTGAAAGCCAGAAAGG-3' and 5'-ACGATG CCTGGTAGTTGTCC-3' to give a 469 bp product, and for *qnrS*, 5'-ACGACATT CGTCAACTGCAA-3',5'-TAAATTGGCACCCTGTAGGC-3'to give a 417 bp product, and the PCR conditions were 94°C for 5 min, 94°C for 45 s, 53°C for 45 s, and 72°C for 1 min, with 32 cycles. Amplification products were detected by electrophoresis on a 2% agarose gel with ethidium bromide and photographed under UV light. All positive products were conformed by direct sequencing of PCR products.

Conjugation experiments

Conjugation experiments were carried out with *E. coli* C600Lac-SMR as the recipient. The recipients were selected on M-H with streptomycin (2000 mg/L) and the donors were selected on M-H with ciprofloxacin (0.06 µg/ml)(BioMérieux, France). Both recipients and donors were added to 2 ml of fresh LB broth and incubated for16 to 24 h. Culture of donor and recipient cells in logarithmic phase (0.5 ml each) were added to 4 ml of fresh LB broth and incubated 16 to 24 h without shaking. Transconjugants were selected on MacConkey agar plates supplemented with streptomycin (2000 mg/L) and ciprofloxacin (0.06 µg/ml). To determine if *qnr* gene was co-transfered, PCR was done to detect *qnr* gene in transconjugants. Then products were checked by electrophoresis on a 1% agarose gel with ethidium bromide and photographed under UV light.

Sequencing

The products were purified with Qiaquick PCR purification kit (QIAGEN, USA). DNA products after amplification were sequenced with an Applied Biosystems sequencer. The nucleotide and deduced protein sequences were analyzed with software available over the Internet at the National Center for Biotechnology Information
website (www.ncbi.nlm.nih.gov). The ClustalW program (www.infobiogen.fr) was used to align multiple protein sequences.

RESULTS

Screening for the qnr gene and sequencing

The *qnr* gene was detected in 25 (13.97%) of 179 Gram negative bacteria (including 129 of *E. coli*, 37 of *K. pneumoniae* and 13 of *E. cloacae*) by PCR (Table 1). Among the 179 isolates, the *qnrA* genes were present in 3.88% of *E. coli* and 7.69% of *E. cloacae*. The *qnrB* genes were present in 6.20% of *E. coli* and 7.69% of *E. cloacae*. The *qnrS* genes were present in 2.33% of *E. coli* and 18.92% of *K. pneumoniae*. We did not find *qnrA* and *qnrB* genes in any of the 37 *K. pneunoniae* and did not find *qnrS* gene in any of the 13 *E. cloacae*. Positive isolates were sequenced with an Applied Biosystems sequencer. All *qnrA* genes were of the *qnrA1* allele, and *E. coli* 175 had a single nucleotide change (CTA→CTG at position
537) (A839G) without amino acid alteration. *qnrB* genes were of the *qnrB4* and *qnrB6* variants, and *qnrS* genes were of the *qnrS1* allele.

Susceptibility tests

Antibiotic susceptibility testing by disk diffusion revealed that the 25 isolates were all susceptible to imipenem but resistant to most β-lactams, aminoglycosides and sulfamido, including cefotaxime, ceftazidime, aztreonam, amikacin and cefoxitin and so on. Among the MICs of all positive isolates (Tables 2, 3 and 4), 2 *qnrA* positive isolates and 4 *qnrB* positive isolates were susceptive to ciprofloxacin.

Transfer of quinolone resistance

Only 10 *qnr* positive isolates were found to transfer resistance to *E. coli* C600Lac-SMR, including 2 *qnrA* positive isolates, 4 *qnrB* and 4 *qnrS* positive isolates. The *qnr* genes from the transconjugants can be detected by PCR.

DISCUSSION

It has been more than 20 years since the fluoroquinolones drugs were introduced into China. Bacterial resistance to quinolones has increased markedly. Some of the national studies suggest that the fluoroquinolone resistance rates in *E. coli* were higher than previously estimated 50 to 60% (Li et al., 2001, 2003). In this study, the prevalence of qnr plasmid-mediated quinolone resistance has been first reported in Central China.

The *qnr* genes were detected in 25 (13.97%) of 179 Gram negative bacteria (including 129 of *E. coli,* 37 of *K. pneumoniae* and 13 of *E. cloacae*). There were 6 strains of *qnrA* genes, 9 strains of *qnrB* genes and 10 strains of *qnrS* genes. Among the 179 isolates, the *qnrA* genes were present in 3.88% of *E. coli* and 7.69% of *E. cloacae*. The *qnrB* genes were present in 6.20% of *E. coli* and 7.69% of *E. cloacae*. The *qnrS* genes were present in 2.33% of *E. coli* and 18.92% of *K. pneumoniae*. The rate

Table 1. Prevalence of qnr genes in gram negative bacteria isolates.

qnr	No. of positive isolates/total no. of isolates (%)		
	***E. coli* (%)**	***E. cloacae***	***K. pneumoniae***
qnrA	5/129(3.88)	1/13(7.69%)	0/37
qnrB	8/129(6.20)	1/13(7.69%)	0/37
qnrS	3/129(2.33)	0/13	7/37(18.92%)
Total	16/129(12.40)	2/13(15.38%)	7/37(18.92%)

Table 2. MICs of qnrA positive isolates.

	Positive isolates					
Ciprofloxacin	17	24	25	62	151	175
MIC (μg/ml)	16	16	64	8	1	0.25

Table 3. MICs of qnrB positive isolates.

	Positive isolates								
Ciprofloxacin	21	75	87	91	95	96	106	128	307
MIC(μg/ml)	32	32	16	8	0.25	1	0.0625	16	0.0625

Table 4. MICs of qnrS positive isolates.

	Positive isolates									
Ciprofloxacin	49	105	114	204	205	207	209	213	226	231
MIC(μg/ml)	32	16	64	8	16	8	32	4	16	4

of *qnrA* positive *E. coli* isolates is lower than that in Shanghai reports in China (Wang et al., 2003). A recent study in the United States found that *qnrA* and *qnrB* genes were detected in 15 (14%) and 6 (6%) of 106 *K. pneumoniae i*solates (Robicsek et al., 2006b). But in our study, *qnrA* and *qnrB* genes were not detected in any of the 37 *K. pneunoniae*. It is probably associated with the geographical distribution of *qnr* genes.

The antibiotic susceptibility test revealed that all of those 25 isolates were susceptible to imipenem but resistant to most β-lactams, aminoglycosides and sulfamido, including cefotaxime, ceftazidime, aztreonam, amikacin and cefoxitin and so on. However, as much to be emphasized in our study is that we find out *qnr* genes can also be found in susceptible organisms (may be due to lack of sufficient chromosomal mutations). 2 *qnrA* positive isolates and 4 *qnrB* positive isolates were susceptive to ciprofloxacin in our study. Among the 6 susceptive isolates, the *E. coli* 96 and *E. cloacae* 307 were also susceptive to ceftazidime. Some reports manifest that these organisms are likely clinically worrisome as resistant organisms (Tran et al., 2005). For under ongoing selective pressure, such organisms, *in vitro*, can readily develop resistance. For instance, Poirel et al. (2006) had reported a sensitive strain of *E. coli* harboring *qnrA* with no classic quinolone resistance mutation, and it was found to develop chromosomal mutations and subsequent high-level resistance after 5 days of norfloxacin therapy (Poirel et al., 2006). Whether it is safe or not to use fluoroquinolone to treat qnr-bearing organisms susceptive

to quinolones is a key issue to be resolved. Therefore, much more work is required to further define the proportion of clinical Enterobacteriaceae harboring such low-level resistance and the effect of these genes on clinical outcomes.

In conjugation tests, quinolone resistance was found to transfer respectively. But only 10 positive isolates were transferred successfully, including 2 *qnrA* positive isolates, 4 *qnrB* and 4 *qnrS* positive isolates. It is probably that the plasmids were lost or broken when they were transferred. In our study, we found that *qnr* genes could also be found in extended-spectrum beta-lactamase (ESBL)-producing isolates and the more associations should be studied in the future. The emergence of plasmid-determined quinolone resistance probably results in the rapid increase in bacterial resistance to quinolones in China. So it is necessary to study the prevalence *qnr* genes as well as the mechanism of *qnr* genes involved in quinolones resistance further in the future in China.

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