Abstract

Background: Quinolone resistance in Enterobacteriaceae results mainly from mutations in type II DNA topoisomerase genes and/or changes in the expression of outer membrane and efflux pumps. Several recent studies have indicated that plasmid-mediated resistance mechanisms also play a significant role in fluoroquinolone resistance, and its prevalence is increasing worldwide. In China, the presence of the qnr gene in the clinical isolates of Enterobacteriaceae has been reported, but this transmissible quinolone resistance gene has not been detected in strains isolated singly from pediatric patients. Because quinolones associated with a variety of adverse side effects on children, they are not authorized for pediatric use. This study therefore aimed to investigate the presence of the qnr gene in clinical isolates of E. coli and K. pneumoniae from pediatric patients in China.

Methods: A total 213 of non-repetitive clinical isolates resistant to ciprofloxacin from E. coli and K. pneumoniae were collected from hospitalized patients at five children's hospital in Beijing, Shanghai, Guangzhou, and Chongqing. The isolates were screened for the plasmid-mediated quinolone resistance genes of qnrA, qnrB, and qnrS by PCR. Transferability was examined by conjugation with the sodium azide-resistant E. coli J53. All qnr-positive were analyzed for clonality by enterobacterial repetitive intergenic consensus (ERIC)-PCR.

Results: The study found that 19 ciprofloxacin-resistant clinical isolates of E. coli and K. pneumoniae were positive for the qnr gene, and most of the qnr positive strains were ESBL producers. Conjugation experiments showed that quinolone resistance could be transferred to recipients. Apart from this, different DNA banding patterns were obtained by ERIC-PCR from positive strains, which means that most of them were not clonally related.

Conclusion: This report on transferable fluoroquinolone resistance due to the qnr gene among E. coli and K. pneumoniae strains indicated that plasmid-mediated quinolone resistance has emerged in pediatric patients in China.
Background
Quinolone resistance in Enterobacteraeae results mainly from mutations in type II DNA topoisomerase genes[1] and/or changes in the expression of outer membrane and efflux pumps[2]. Recently, studies have shown that plasmid-mediated resistance mechanisms also play a significant role in fluoroquinolone resistance, and its prevalence is increasing worldwide [3]. qnrA is the plasmid-mediated quinolone resistance gene encoding a 218 amino acid protein of the pentapeptide family that protects DNA gyrase from quinolone inhibition[4]. The new plasmid-mediated quinolone resistance genes, qnrB and qnrS, have been reported in clinical isolates[5,6]. In China, the presence of the qnr gene in clinical isolates of Enterobacteriaceae from Shanghai [7] and Anhui[8] has been reported, but this transmissible quinolone resistance gene has not been detected in strains isolated singly from pediatric patients. Because quinolones associated with a variety of adverse side effects on children, they are not authorized for pediatric use. Therefore, the objective of this study was to screen for the presence of the qnr gene in clinical isolates of Enterobacteriaceae from five children's hospitals located in Beijing, Shanghai, Guangzhou, and Chongqing from January 2005 to December 2006. Each hospital is a general pediatric hospital locally affiliated to a university. The isolates were collected from five children's hospitals located in Beijing, Shanghai, Guangzhou, and Chongqing from January 2005 to December 2006. Each hospital is a general pediatric hospital locally affiliated to a university. The isolates were collected from five children's hospitals located in Beijing, Shanghai, Guangzhou, and Chongqing from January 2005 to

Methods
Bacterial strains
Three hundred thirty-five Escherichia coli and 392 Klebsiella pneumoniae non-replicate clinical isolates were collected from five children's hospitals located in Beijing, Shanghai, Guangzhou, and Chongqing from January 2005 to December 2006. Each hospital is a general pediatric hospital locally affiliated to a university. The isolates were identified at the participating hospitals by routine methodology at each laboratory, and then were transported to Beijing Children's Hospital for further analysis. All isolates were screened for ciprofloxacin resistance by disk method according to the criteria of NCCLS [9]. The screening showed 146 Escherichia coli and 67 Klebsiella pneumoniae isolates were ciprofloxacin-resistant. Additional strain used was E. coli J53Az8(resistant to azide) as a recipient of the conjugation experiment

Screening for the qnrAM, qnrB, and qnrS gene in clinical strains
The 213 ciprofloxacin-resistant strains of E. coli and K. pneumoniae were screened by multiplex PCR amplification of qnrA, qnrB, and qnrS as previously described[10]. Colonies were transferred to an Eppendorf tube filled with water and boiled to prepare DNA templates for PCR. The primers used for qnrA, qnrB, and qnrS were as follows: 5’-ATTITCTACCGCCAGGATTG-3’ and 5’-GATCCGGAAAGGTAGGTCA-3’ for qnrA for a 516-bp product, 5’-GATCGTGAAACCCAGAAAG-3’ and 5’-ACGATGCGCTGGTAGTTC-3’ for qnrB for a 469-bp product, and 5’-ACGACATTCGTAACGTCAA3’ and 5’-TAAATTGCGACCTGTAGGGC-3’ for qnrB for a 417-bp product. The protocol for the PCR condition was: 94°C for 45 s, 53°C for 45 s, and 72°C for 60 s, with a cycle number of 32, and without DNA template as negative controls in each run. Amplification products were provisionally identified by their size in ethidium bromide-stained agarose gels. The positive amplified PCR product for qnrA, qnrB, and qnrS was analyzed with an automated DNA sequencing system. The results of DNA sequences were compared with the BLAST online search engine from GenBank at the National Center for Biotechnology Information Web site: http://www.ncbi.nlm.nih.gov/blast.

Conjugation experiments
All positive qnr strains were tested for transferred quinolone resistance by conjugation experiments carried out in Luria Broth with E. coli J53Az8 as the recipient. The cultures of donor and recipient cells were incubated via shaking at 37°C in logarithmic phase, and then 0.5 mL of both cultures was added to 4 mL of fresh LB and incubated overnight without shaking. Transconjugants were selected on tryptic soy agar (TSA) plates with sodium azide (100 μg/mL from Sigma Chemical Co., St. Louis, MO) for counter selection and ampicillin (100 μg/mL from Oxydo) to select for plasmid-encoded resistance. To determine if quinolone resistance was cotransferred, colonies were replica-plated onto TSA with and without ciprofloxacin (0.06 μg/mL; Oxoid). The PCR experiments confirmed transconjugants carrying the same qnr gene as their donors.

Antimicrobial susceptibility profiles
MICs for the 19 positive qnr isolates, recipient (J53 Az8), and transconjugant strains were measured by agar dilution in accordance with the guidelines of the NCCLS. The antimicrobials tested were ampicillin/clavulanic acid, cefotaxime, cefazidime, cefoperazone, cefoxitin, cefepime, aztreonam, imipenem, ciprofloxacin, ofloxacin, amikacin, and gentamicin (Oxoid, England). Quality control was performed by testing Escherichia coli ATCC25922. Isolates which showed MICs ≥ 2 μg/mL for cefotaxime and/or ceftriaxone and/or cefazidime and/or aztreonam were considered as ESBL producers. The ESBL phenotype was confirmed by using clavulanic acid (Oxoid, England) according to the manufacturer's recommendation.

Strain typing by ERIC-PCR
The 11 positive qnr Escherichia coli strains and 8 positive qnr Klebsiella pneumoniae strains were typed by ERIC-PCR. Total DNA from qnr positive isolates was analyzed by ERIC sequence PCR with the ERIC1R (5’-ATGTAAGGTCCTGCGGATTCA-3’) and ERIC2 (5’-AAGTAAAGTAC-GGCGTACCG-3’) primers, and PCR was performed as previously described [11]. ERIC-PCR was performed
briefly using the following program parameters: denatur-
ation at 94°C for 1 s, annealing at 52°C for 10 s, and
extension at 72°C for 35 s for 30 cycles, followed by a
final extension at 72°C for 4 min. Amplicons were sepa-
rated on a 1.5% agarose gel containing ethidium bromide
(5 μg/mL) at 60 V for 3 hours. The gels were photo-
graphed and digitized. Isolates were considered different
if their profiles differed by two or more bands[12].

Results
Screening for the qnr genes
Nineteen ciprofloxacin-resistant clinical isolates, includ-
ing eleven E. coli and eight K. pneumoniae strains, were
positive for the qnr gene. Table 1 shows the clinical char-
acteristics of these isolates and the distribution of qnrA,
qnrB, or qnrS. The sequences of qnr genes were all identical
to those of qnrA (EU195836), qnrB (EU443840), and qnrS
(EU391634) with GenBank, respectively.

Conjugation and antimicrobial susceptibility
Quinolone resistance could be transferred by conjugation
in two of eight of Klebsiella pneumoniae qnr-positive
donors. One qnrB-positive and one qnrS-positive
transconjugant were obtained. PCR experiments con-
firmed that the transconjugants harbored the same qnr
gene as their donors. Table 2 shows the susceptibilities of
the qnr-positive clinical isolates for the selected antibiot-
ics. Most isolates were resistant to cephalosporin, aztre-
onam, and aminoglycosides, whereas all of the strains
were susceptible to imipenem. Eight of eleven qnr positive
isolates of Escherichia coli and all eight qnr positive isolates
of Klebsiella pneumoniae were phenotypic ESBL producers,
respectively. Antibiotic susceptibilities for the two
transconjugants showed that the ciprofloxacin resistance
MIC value increased 32- and 1024-fold, respectively;
meanwhile, oflaxacin resistance MIC value increased 2-
and 32-fold, respectively.

Strain typing by ERIC-PCR
The positive qnr isolates of E. coli, and K. pneumoniae
showed different DNA banding patterns, indicating that
they were not clonally related.

Discussion
This study is the first report on the presence of the qnr gene
in clinical isolates from pediatric patients in China. The
prevalence rates of qnr among ciprofloxacin-resistant iso-
lates of E. coli and K. were 7.5% (11 of 146), and 11.9%
(8 of 67), respectively. The qnr rate in isolates of
Escherichia coli analyzed in this study was similar to the
prevalence in Shanghai, China, wherein there was 8% (6
of 78 strains) of ciprofloxacin-resistant clinical isolates of
E. coli [7]. These rates are lower than the prevalence of
qnrA detected among ceftazidime-resistant or cipro-

Table 1: Clinical characteristics and qnr genotype of the qnr-positive isolates

| Number of Strains | Specimen      | Sex | Age    | Diagnoses       | qnr         | ESBL |
|-------------------|---------------|-----|--------|-----------------|-------------|------|
| E. coli           |               |     |        |                 |             |      |
| 05B239           | sputum        | m   | 2 months | Pneumonia      | qnrA, qnrB, qnrS | -    |
| 05C2795          | sputum        | f   | 15 days | Pneumonia      | qnrA      | +    |
| 05G1844          | vulvar secretions | f | 5 years | Vulvitis      | qnrA, qnrS | -    |
| 05G12889         | urine         | m   | 2 months | Hydronephrosis | qnrA, qnrB | +    |
| 05SB14           | sputum        | f   | 24 days | Pneumonia      | qnrS      | +    |
| 05SB25           | urine         | f   | 5 months | UTI         | qnrA, qnrB | +    |
| 05SB47           | tracheal      | f   | 12 months | Pneumonia | qnrA, qnrB | +    |
| 06G60            | blood         | m   | 3 years | ALL          | qnrA      | +    |
| 06G62            | sputum        | f   | 24 days | Pneumonia      | qnrS      | +    |
| 06G99            | sputum        | f   | 4 months | Pneumonia      | qnrA, qnrB | -    |
| 06SA30           | sputum        | f   | 2 months | Pneumonia      | qnrS      | +    |
| K. pneumoniae    |               |     |        |                 |             |      |
| 05C2978          | sputum        | m   | 9 months | Pneumonia      | qnrB      | +    |
| 05G44            | sputum        | f   | 1 months | Pneumonia      | qnrB      | +    |
| 05SA32           | sputum        | f   | 21 months | Pneumonia | qnrS      | +    |
| 06B295           | sputum        | m   | 11 days | Pneumonia      | qnrB      | +    |
| 06B700           | urine         | f   | 4 years | UTI         | qnrB      | +    |
| 06C3889          | sputum        | f   | 4 days | Pneumonia      | qnrS      | +    |
| 06C5524          | sputum        | f   | 3 months | Pneumonia | qnrB      | +    |
| 06SB60           | sputum        | m   | 3 months | Pneumonia      | qnrS      | +    |

*UTI, Urinary tract infection;  
*ALL, Acute lymphoblastic leukemia.
floxacin-resistant strains (24%) of Enterobacter in USA[13]. Among the three groups of the qnr gene, qnrA was more prevalent (8 of 11) in Escherichia coli strains, whereas qnrB was more prevalent (5 of 8) in the Klebsiella pneumoniae isolates. As there are differences in the criteria for testing strains, evaluating the precise prevalence of plasmid-mediated quinolone resistance was difficult.

It should be noted that all the positive qnr isolates were distributed in pediatric patients: more than one-third were isolated from children younger than one year of age, and nearly one-fourth were isolated from neonates. Because of the variety of adverse side effects of quinolones in children, hospitals are not advised to use them on children. In China, quinolones are not used on children younger than 16 years old. Presumably, the source of the qnr gene might not be directly associated with the selective pressure caused by the quinolones used in pediatrics, but it could be related to horizontal transmission from adults or other reservoir. Recent findings showed that these genes come from environmental gram-negative bacterial species, such as Shewanella algae, the progenitor of the qnrA genes [14], and Vibrio splendidus or Aeromonas spp., the progenitor of qnrS genes[15,16]. This shows that the aquatic environment is an important reservoir of novel antibiotic resistance determinants. Quinolones are antimicrobial agents extensively used in aquaculture and are stable molecules in water [17]. Exposure to lower concentrations of quinolones increases the chance for selection of resistance. Therefore, they may be the source of an important driving force for the selection of quinolone resistance. Another study showed that qnrS genes were identified from E. coli and E. cloacae isolated from zoo animals in Japan, suggesting that animals could be a potential reservoir of quinolone-resistant bacteria[18]. Quinolones are the most common antimicrobial used in animals, the annual consumption of which is about 470 tons in China [19]. In the present study, more than two-thirds of qnr positive strains were isolated from sputum sample. These strains may be confined in children's respiratory tract, which come from the contaminated environment.

This study showed that most qnr positive strains were ESBL producers, indicating the relationship between the

### Table 2: Resistance profiles of the qnr positive strains and transconjugants

| Strain     | MIC(μg/ml)       | AMC | CFP | CTX | CAZ | FOX | FEP | IPM/CS | ATM | AMK | GEN | CIP | OFX |
|------------|------------------|-----|-----|-----|-----|-----|-----|--------|-----|-----|-----|-----|-----|
| E. coli    |                  |     |     |     |     |     |     |        |     |     |     |     |     |
| 05B239     | 32 | >512 | 512 | 32  | 32  | 512 | 0.25 | 64    | 4   | 128 | 64  | 32  |     |
| 05C2795    | 128 | 512 | 64  | 256 | 512 | 16  | 0.5  | 256   | 16  | 256 | 16  | 32  |     |
| 05G1844    | 32  | 512 | 64  | 8   | 32  | 8   | 0.125| 16    | 4   | 128 | 64  | 32  |     |
| 05G1889    | 32  | 512 | >512| 32  | 32  | 128 | 0.25 | 32    | 8   | 128 | 64  | 64  |     |
| 05SB14     | 16  | >512| 128 | 8   | 32  | 16  | 0.125| 8     | 8   | 256 | 64  | 16  |     |
| 05SB25     | 32  | >512| >512| 128 | 32  | 128 | 0.125| 64    | 4   | 128 | 64  | 32  |     |
| 05SB47     | 32  | >512| >512| 512 | 16  | >512| 0.25 | 256   | 8   | 128 | 256 | 64  |     |
| 06G60      | 32  | 512 | 512 | 32  | 16  | 128 | 0.25 | 64    | 4   | 128 | 32  | 16  |     |
| 06G62      | 16  | 512 | 512 | 8   | 32  | 32  | 0.125| 8     | 8   | 128 | 64  | 32  |     |
| 06G99      | 64  | >512| >512| 512 | 128 | 0.25| 256  | 4     | 128 | 64  | 64  |     |     |
| 06SA30     | 32  | >512| >512| 8   | 32  | 32  | 0.25 | 16    | 16  | 256 | 4   | 32  |     |
| K. pneumoniae |
| 05C2978b   | 32  | 128 | 128 | 512 | 16  | 64  | 0.125| 128   | 4   | 32  | 256 | 128 |     |
| 05SA32b    | 32  | 256 | 256 | 512 | 16  | 128 | 0.125| 128   | 8   | 2   | 4   | 8   |     |
| 05G44      | 128 | >512| >512| >512| 512 | 1   | 512  | 8     | 32  | 4   | 4   |     |     |
| 06B295     | 32  | >512| 128 | 64  | 16  | 64  | 1    | 64    | 2   | 4   | 8   |     |     |
| 06B700     | 32  | 512 | 512 | 256 | 256 | 128 | 0.25 | 128   | 8   | 128 | 32  | 8   |     |
| 06C3899    | 32  | >512| 256 | 512 | 32  | 64  | 0.125| 256   | >512| >512| 256  | 256 |     |
| 06C5524    | 32  | 256 | 128 | 512 | 16  | 64  | 64   | 6     | >512| >512| 64  | 32  |     |
| 06SB60     | 32  | >512| >512| 64  | 32  | 512 | 0.25 | 256   | 4   | 128 | 4   | 32  |     |

| Recipients | MIC(μg/ml)       | AMC | CFP | CTX | CAZ | FOX | FEP | IPM/CS | ATM | AMK | GEN | CIP | OFX |
|------------|------------------|-----|-----|-----|-----|-----|-----|--------|-----|-----|-----|-----|-----|
| J53        | 16   | 2   | 0.125 | 0.25 | 2   | 1   | 0.0625| 4     | 8   | 0.25| 0.125| 0.25|     |

| Transconjugants |
|-----------------|
| Tc 05C2978b     | 32  | 128 | 128 | 512 | 64  | 32  | 0.125| 128   | 8   | 512 | 128 | 8   |     |
| Tc05SA32b      | 32  | 512 | 512 | 512 | 128 | 0.25| 256  | 16    | 128 | 4   | 0.5 |     |     |

a AMC, amoxicillin/clavulanic acid; CFP, cefoperazone; CTX, cefotaxime; CAZ, cefotaxime; FOX, cefoxitin; FEP, cefepime; IPM/CS, imipenem/ cilastatin; ATM, aztreonam; AMK, amikacin; GEN, gentamicin; CIP, ciprofloxacin; OFX, ofloxacin.
b domor.
c Tc, transconjugants
qnr gene and ESBL. Other studies have shown that several qnrA-positive isolates express ESBL [7,20,21]. A qnrS gene on a plasmid encoding ESBL in a K. pneumoniae strain also has been reported in Taiwan, China [22]. Therefore, the fact that expanded-spectrum cephalosporins are widely used among Chinese children may also contribute to the presence of the qnr gene. Another study by the present researchers suggested that third-generation cephalosporins are commonly used in pediatric patients (data not published).

Conjugation experiments showed that only two qnr-positive K. pneumoniae strains were able to transfer the qnr gene in transconjugants. This transferability was similar to the findings of other studies, which also showed that not all qnr-positive strains were able to transfer quinolone resistance [23,24]. The lower resistance to ciprofloxacin and oflaxacin in these two transconjugants than their donor strains may imply the presence of additional chromosomal resistance mutations. The results of ERIC-PCR showed that the positive qnr strains have different types of DNA band, thereby suggesting that the qnr in clinical isolates was not caused by the spread of identical strains.

Although the number of data points is limited, this report confirms the spread of the plasmid-mediated quinolone resistance qnr gene in E. coli and K. pneumoniae strains isolated from pediatric patients. Still, however, further research is needed to understand the influence of this gene on quinolone resistance for pediatric patients in China.

Conclusion
The prevalence rates of qnr among the clinical isolates of ciprofloxacin resistance in E. coli and K. pneumoniae were 7.5% (11 of 146) and 11.9% (8 of 67), respectively. The transferability of fluoroquinolone resistance because of the qnr gene among E. coli and K. pneumoniae strains shows that plasmid-mediated quinolone resistance has been spread in pediatric patients in China.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
All authors of the present study contributed substantially to the conception and design of the study, were involved in the writing and in the critical revision of the manuscript, and read and approved the final version of the manuscript submitted for publication. AW was mainly responsible for data analysis and preparation of the manuscript; XS and YY conceived of the study, participated in its design and coordination, and helped in drafting the manuscript; QL, YW, YC, LD, QD, HZ, CW, LL, and XX were mainly responsible for the collection of clinical isolates and information about the patients; and HD and LW facilitated the PCR and conjugation experiments on the samples.

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