

## Prevalence and characteristics of $\beta$ -lactamase and plasmid-mediated quinolone resistance genes in *Escherichia coli* isolated from farmed fish in China

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**Objective:** To determine the molecular epidemiology of extended-spectrum  $\beta$ -lactamases (ESBLs) and plasmid-mediated quinolone resistance (PMQR) in *Escherichia coli* isolated from farmed fish in China.

**Methods:** *E. coli* was isolated from fish gut samples from fish farmed throughout Guangdong province and tested for the presence of the  $\beta$ -lactamase genes and PMQR-encoding genes using PCR and DNA sequence analysis. Co-transfer of plasmids encoding for ESBLs as well as PMQR determinants was explored by conjugation into *E. coli*.

**Results:** A total of 218 non-duplicate *E. coli* were recovered from fish gut samples.  $\beta$ -Lactamase genes were identified in 19 (17%) of 112 strains with reduced susceptibility to ampicillin, and PMQR genes were identified in 59 (73.8%) of 80 strains with reduced susceptibility to ciprofloxacin. Only three ESBL genes were identified in three isolates: *bla*<sub>CTX-M-14</sub>, *bla*<sub>CTX-M-79</sub> and *bla*<sub>SHV-27</sub>. PMQR gene screening identified *qnr* genes ( $n=59$ ) as the most common, including *qnrB* ( $n=33$ ), *qnrS* ( $n=21$ ) and *qnrD* ( $n=5$ ), with *aac(6')-Ib-cr* ( $n=6$ ) being rarely found. The co-carriage of two or three PMQR genes in one strain was found in 7 (11.9%) isolates. The ESBL gene *bla*<sub>CTX-M-79</sub> was found to be co-carried with *qnrS*. Co-transfer of *qnrS* was observed with *bla*<sub>CTX-M-79</sub>.

**Conclusions:** Our study is the first to demonstrate the existence of high levels of mobile genes conferring reduced susceptibility to fluoroquinolones as well as the presence of ESBL genes in fish produced in China, and identifies a significant reservoir of antibiotic resistance genes relevant to human medicine.

**Keywords:** plasmid-mediated quinolone resistance, ESBL, aquaculture, molecular epidemiology

### Introduction

Strains of Enterobacteriaceae that produce extended-spectrum  $\beta$ -lactamases (ESBLs), particularly *Escherichia coli* and *Klebsiella* spp. producing CTX-M, have emerged as significant antibiotic-resistant human pathogens across the world.<sup>1</sup> A recent study of the acquisition of faecal carriage of CTX-M-type ESBL-producing *E. coli* by travellers to different parts of the world from Sweden showed an acquisition rate of 32% when travelling to Asia (excluding India).<sup>2</sup> This high rate of acquisition possibly reflects exposure to food and water contaminated with ESBL *E. coli*, as the incidence of ESBL production in clinical isolates of *E. coli* in China is 65%.<sup>3</sup> Antibiotic-resistant *E. coli* from animal sources in countries with high human carriage rates should not

be ignored, as there is considerable evidence they contribute to the burden of human microbial resistance, particularly in parts of the world with variable regulation of antibiotic use.<sup>4,5</sup> A study from China demonstrated high rates of plasmid-mediated quinolone resistance (PMQR) genes [*aac(6')-Ib-cr* and *qepA*] predominantly in companion animals.<sup>6</sup> An alarmingly high rate of ESBL carriage has recently been reported in food animals from Hong Kong (63% in pigs; 58% in chickens; 33% in cattle); almost all being co-resistant to ciprofloxacin.<sup>7</sup>

Fish farming (aquaculture) is growing rapidly, particularly in Asia. China is the world's biggest producer of farmed fish, producing 32.7 million tons of the total world production of 52.5 million tons in 2008.<sup>8</sup> The consequences of the extensive use of antibiotics in aquaculture are the selection in the fish gut

flora of multiresistant bacteria that are passed to the human gut commensal flora when the fish are eaten.<sup>9</sup> The heavy use of agents such as oxolinic acid, tetracycline, florfenicol and nitrofurantoin has selected resistance in fish pathogens, so quinolones are increasingly the preferred agents.<sup>9</sup> Although China is the world's largest producer of farmed fish, no studies of the prevalence of multiresistant Enterobacteriaceae with resistance to human medicine critical antibiotics (e.g. quinolones and extended-spectrum cephalosporins) in farmed fish exist to our knowledge in the published literature. We have undertaken a prevalence study in retail fish farmed across Guangdong province in southern China to identify the extent and molecular characteristics of the quinolone and ESBL resistance gene reservoir that has the potential to transfer to the human gut flora.

## Materials and Methods

### Bacterial strains

A total of 15 different fish markets, which were widely dispersed across the city of Guangzhou, were selected for the study in 2010. Each market received farmed freshwater fish from farms throughout Guangdong province, which is the largest centre for aquaculture in China. Twenty fish were sampled at each market, each from a different vendor, by opening the gut using a sterile scalpel following washing the gut surface with sterile saline and then swabbing the contents. The swabs were plated on MacConkey agar incubated for 18 h at 37°C and a single colony of *E. coli* was selected for further study. *E. coli* were identified using API20E (BioMerieux, Beijing, China).

### Antimicrobial susceptibility testing

The MICs of ampicillin, ceftiofur, cefotaxime, chloramphenicol, florfenicol, spectinomycin, kanamycin, tetracycline, co-trimoxazole, nalidixic acid and ciprofloxacin were determined by agar dilution using CLSI methods.<sup>10</sup> We tried to comply with recent recommendations<sup>11</sup> and have used breakpoints applicable to human infections rather than animals, as we are interested in the effect of the movement of resistance genes from fish to humans rather than the prediction of therapeutic outcome in fish. All isolates demonstrating reduced susceptibility to ampicillin (MIC<sub>50</sub> ≥ 8 mg/L) and reduced susceptibility to ciprofloxacin (MIC<sub>50</sub> ≥ 0.06 mg/L) were retained for molecular studies. We chose a ciprofloxacin MIC<sub>50</sub> ≥ 0.06 mg/L in order to stand the maximum probability of identifying PMQR-carrying isolates.<sup>12</sup>

### Characterization of ESBL and PMQR determinants

All ampicillin-resistant *E. coli* were screened using PCR as described previously for *bla*<sub>CTX-M</sub> genotype groups 1, 2, 8, 9 and 25,<sup>13</sup> *bla*<sub>TEM</sub>,<sup>14</sup> *bla*<sub>SHV</sub>,<sup>14</sup> *bla*<sub>OXA</sub>,<sup>15</sup> *bla*<sub>LEN</sub>,<sup>16</sup> and *bla*<sub>CMY</sub>, *bla*<sub>MOX</sub>, *bla*<sub>DHA</sub>, *bla*<sub>ACC</sub> and *bla*<sub>MIR</sub>-*bla*<sub>FOX</sub>.<sup>17</sup>

All ciprofloxacin-resistant isolates were characterized by PCR for PMQR determinants *aac*(6')-*Ib-cr* (identified by BtsCI digestion), *qepA*, *qnrA*, B, S, and D as previously described.<sup>18</sup>

All PCR products from ESBL and PMQR genes including *qnr* variants were confirmed and analysed by DNA sequencing. The presence of *E. coli* sequence type (ST) 131 was determined in ESBL isolates by a PCR screening method.<sup>19</sup>

Conjugative transfer of plasmids encoding ESBLs was studied by mating with azide-resistant *E. coli* J53, as described previously.<sup>18</sup> Transconjugants were detected by plating mating mixtures on Luria-Bertani agar supplemented with 150 mg/L sodium azide and 2 mg/L cefotaxime. Co-transfer of resistance determinants was explored by amplifying the relevant genes in the transconjugants.

## Results

### Bacterial strains and antimicrobial susceptibility testing

A total of 218 *E. coli* isolates were recovered from the 300 fish gut samples. The MIC results are shown in Table 1. High rates of resistance to ampicillin, florfenicol, tetracycline and co-trimoxazole and reduced susceptibility to ciprofloxacin were detected. Reduced susceptibility to ampicillin was found in 112 isolates (51%) and reduced susceptibility to ciprofloxacin in 80 isolates (37%).

### Characterization of ESBL and PMQR determinants

Among the 112 *E. coli* isolates with reduced susceptibility for ampicillin, 19 (17%) carried β-lactamase genes: *bla*<sub>TEM</sub> (10), *bla*<sub>SHV</sub> (5), *bla*<sub>CTX-M</sub> (2) and *bla*<sub>LEN</sub> (2). The two *bla*<sub>CTX-M</sub> genes were *bla*<sub>CTX-M-14</sub> and *bla*<sub>CTX-M-79</sub>. Among the five SHV-type β-lactamases, four were non-ESBL genes (*bla*<sub>SHV-1</sub>, *bla*<sub>SHV-11</sub>, *bla*<sub>SHV-25</sub>, *bla*<sub>SHV-26</sub>) and one ESBL (*bla*<sub>SHV-27</sub>). The LEN-type β-lactamase genes were found in two isolates. These genes encode narrow-spectrum β-lactamases that have escaped onto plasmids and are increasingly recognized in humans. The molecular variants found were *bla*<sub>LEN-17</sub> and a novel variant, *bla*<sub>LEN-26</sub> (GenBank accession number JQ067123). Among 80 ciprofloxacin isolates with reduced susceptibility, 59 (73.8%) harboured PMQR genes: *qnr* (59), *aac*(6')-*Ib-cr* (6). Of the 59 *qnr*

**Table 1.** Percentage resistant, MIC<sub>50</sub> and MIC<sub>90</sub> of 218 *E. coli* to 11 antimicrobial agents (breakpoints used are shown in the footnotes)

Antimicrobial agents	AMP	CTF	CTX	CHL	FFC	SPC	KAN	TET	SMX/TMP <sup>a</sup>	CIP	NAL
Percentage resistant (n)	78.9 (172) <sup>b</sup>		2.3 (5) <sup>a</sup>	23.4 (51) <sup>a</sup>			6.9 (15) <sup>a</sup>	39 (85) <sup>a</sup>	41.7 (90) <sup>a</sup>	4.1 (9) <sup>a</sup> /36.7 (80) <sup>c</sup>	16.0 (35) <sup>a</sup>
MIC <sub>50</sub>	32	0.25	0.25	8	16	32	4	4	256	0.03	4
MIC <sub>90</sub>	256	1	1	128	256	512	32	64	>512	2	256

Antibiotic breakpoints used (in mg/L) are shown in parentheses. AMP, ampicillin (8); CTF, ceftiofur; CTX, cefotaxime (8); CHL, chloramphenicol (32); FFC, florfenicol; SPC, spectinomycin; KAN, kanamycin (64); TET, tetracycline (16); SMX/TMP, co-trimoxazole (4); CIP, ciprofloxacin (4); NAL, nalidixic acid (32).

<sup>a</sup>CLSI resistance breakpoint.

<sup>b</sup>Reduced susceptibility MIC<sub>50</sub> ≥ 8 mg/L.

<sup>c</sup>Isolates with reduced susceptibility, MIC<sub>50</sub> ≥ 0.06 mg/L.

genes, 33 were *qnrB*, 21 *qnrS*, and only 5 *qnrD*. No *qnrA*, *qnrC* or *qepA* genes were detected. Two or three PMQR genes co-existing in one strain were found in 7 (11.9%) of 59 PMQR gene-positive isolates, *qnrB*+*qnrD* (2), *qnrB*+*aac(6')-Ib-cr* (2), *qnrS*+*qnrD* (1) and *qnrS*+*aac(6')-Ib-cr* (1).

The ESBL gene *bla*<sub>CTX-M-79</sub> was found in combination with *qnrS*, while the isolate with *bla*<sub>CTX-M-14</sub> carried no PMQR genes. The co-transfer of *qnrS* was observed with *bla*<sub>CTX-M-79</sub>, and none of the other plasmids carrying other ESBL genes mobilized PMQR genes by conjugation. *bla*<sub>CTX-M-14</sub> genes were also transferred alone by conjugation. None of the ESBL-producing strains belonged to the ST131 clone.

## Discussion

As the world's largest producer of farmed fish (32.7 million tons; 62% of world production in 2008), understanding the incidence and composition of the antibiotic resistance reservoir in farmed fish in China is important. We chose farmed fish, as there are no published data on the incidence of different antibiotic resistances in *E. coli* from this very important animal food source in China. The range of antibiotics that has been recorded as being used in aquaculture is wide, including aminopenicillins, amphenicols, macrolides, aminoglycosides, nitrofurans, fluoroquinolones, sulphonamides and tetracyclines.<sup>9</sup> Historically amphenicols (florfenicol and chloramphenicol), sulphonamides and tetracyclines have been used, usually by incorporation into fish food. A substantial body of data exist on the occurrence of tetracycline resistance in aquaculture, particularly but not exclusively in fish pathogens.<sup>9</sup> Increasing rates of resistance to these older heavily used antibiotics in fish pathogens such as *Aeromonas salmonicida* has resulted in a shift to increased usage of quinolones. While high-level resistance to quinolones arises from mutations in the chromosomal *gyrA* and *parC* genes, it is the lower-level plasmid-mediated resistance conferred by the *qnr* and *aac(6')-Ib-cr* genes that creates an environment in bacteria for the rapid selection of high-level resistance.<sup>12</sup> Prior to our study there is only one study from Egypt looking at the prevalence of these genes together with ESBL genes in Gram-negative bacteria from water samples from fish farms.<sup>20</sup> The quinolone and the third-generation cephalosporin (3GC) classes of antibiotics are important for the treatment of serious infections in humans caused by Enterobacteriaceae. The consequences for human health are more serious than from resistance to older antibiotics such as tetracycline and chloramphenicol. Because of the scale of fish farming in China, the impact on the prevalence of resistance in the gut flora of the general community is likely to be highly significant. The rates of resistance (using human clinical breakpoints) seen in the *E. coli* from fish guts in our study was generally high, particularly for tetracycline (39%) and co-trimoxazole (42%), with reduced susceptibility to ciprofloxacin (37%) and ampicillin (51%).

The ESBL rate was comparatively low (3/218, 1.5%) in the present study; however, 3GCs are unlikely to be widely used in aquaculture at the moment. The Egyptian study also found only 11/274 (4%) isolates carrying ESBL genes.<sup>20</sup> In both China and Egypt, the rate of carriage of ESBLs in humans is high, so these isolates were most likely derived from contamination of the fish in the farm from human sewage, probably via river

**Table 2.** Distribution of PMQR genes in 80 ciprofloxacin-resistant *E. coli* identified from 218 *E. coli* from farmed fish in Guangdong province collected at 15 markets

PMQR genes	No. of isolates positive for gene(s)	No. of markets at which fish were positive
<i>qnrB</i>	30	11
<i>qnrS</i>	16	8
<i>qnrD</i>	2	2
<i>qnrB</i> + <i>qnrD</i>	2	2
<i>qnrS</i> + <i>qnrD</i>	1	1
<i>qnrB</i> + <i>aac(6')-Ib-cr</i>	2	2
<i>qnrS</i> + <i>aac(6')-Ib-cr</i>	2	2
<i>aac(6')-Ib-cr</i>	2	2

water. CTX-M-14 is the most common CTX-M genotype in China.<sup>1</sup> CTX-M-79, which was also identified, is a double-nucleotide variant of CTX-M-15 that has been reported from human infections and faecal carriage in China.<sup>21</sup>

The distribution of PMQR genes was interesting (Table 2), with *qnrB* and *qnrS* dominant, and just a small number of *qnrD* and *aac(6')-Ib-cr*. The frequency was much higher than in Egypt (55/218 in China, 15/274 in Egypt), although the distribution was similar. The relative frequency of PMQR genotypes is similar to reports from food animals in China<sup>22</sup> and human clinical isolates from China.<sup>23,24</sup>

The very high rates of resistance in the *E. coli* in our study to agents that have and most probably continue to be used (i.e. tetracyclines, florfenicol and co-trimoxazole) most probably reflects a strong selective pressure for continuing antibiotic resistance in *E. coli* on the fish farms. The recent change to increased usage of quinolones should result in increased resistance, which we have demonstrated with a very high rate (55/218 isolates, 25.2%) of PMQR in *E. coli*. This rate is higher than recent studies from China have reported for poultry and swine (14%),<sup>25</sup> chickens (22.2%)<sup>22</sup> and human clinical isolates (12.8% and 17.9%).<sup>18,25</sup>

The increasing industrialization of food production in Asia is resulting in greater usage of antimicrobials. A recent survey of faecal carriage of *E. coli* with resistance to 'critically important' antibiotics in food animals in Hong Kong showed a substantial increase in resistance in 2008 compared with an earlier study in 2002.<sup>7</sup>

Our study is the first to demonstrate the existence in fish produced in China (the world's largest aquaculture environment) of high levels of PMQR genes as well as the presence of ESBL genes. Efforts should be made worldwide to more closely monitor and introduce control of antibiotic resistance in aquaculture, as this represents a major reservoir of resistance genes likely to threaten the human use of critical antibiotics in the future.

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## Transparency declarations

None to declare.

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