Molecular mechanisms of fluoroquinolone and expanded-spectrum cephalosporin resistance in *Vibrio parahaemolyticus*: abridged secondary publication

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KEY MESSAGES

1. We investigated the molecular mechanisms of cephalosporin resistance and fluoroquinolone resistance in *Vibrio parahaemolyticus* strains isolated from food and clinical specimens.
2. Cephalosporin resistance in *V parahaemolyticus* was due to expression of β-lactamase gene, bla_{PER-1} and bla_{CMY-2}, harboured by various conjugative plasmids.
3. The complete sequence of bla_{PER-1}-bearing plasmid, pVPH1, was depicted and found to belong to the $MOB_{(H12)}$ group of self-transmissible plasmids, which is prevalent in *Enterobacteriaceae* and *Vibrionaceae*.
4. Single amino acid substitution $Ser^{83}Ile$, in GyrA and the $Ser^{85}Leu$ change in ParC, were found to be associated with fluoroquinolone-resistance in *V parahaemolyticus*.
5. Three novel *qnrVC* genes, *qnrVC5*, *qnrVC6*, and *qnrVC7*, were detectable in these strains and their structure and functions were characterised.5. Three novel qnrVC genes, qnrVC5, qnrVC6, and qnrVC7, were detectable in these strains and their structure and functions were characterised.

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Introduction

Vibrio parahaemolyticus is a major causative agent of gastroenteritis, particularly in areas with high seafood consumptions; *V parahaemolyticus* infection has increased owing to emergence of the serotype O3:K6.[1] Our recent study reported an increasing trend of resistance in *V parahaemolyticus* against new front-line antibiotics such as fluoroquinolones and extended-spectrum-cephalosporins. In Hong Kong, the increasing prevalence of multidrug-resistant *V parahaemolyticus* strains may impact public health because it is the leading cause of foodborne illnesses. It is important to understand the molecular mechanisms that cause *V parahaemolyticus* resistance against fluoroquinolones and extended-spectrum-cephalosporins. The current study aimed to delineate the molecular mechanisms of fluoroquinolone and extended-spectrum-cephalosporin resistance in *V parahaemolyticus*. Results may provide guidance of clinical treatment of multidrug resistant *V parahaemolyticus* and insights to developing new approaches to prevent further dissemination of multidrug resistance-encoding elements in *V parahaemolyticus*.

Methods

The isolation of *V parahaemolyticus* from food samples was performed as previously described.[2] All *V parahaemolyticus* strains were cultured in LB broth or agar containing 3% salt.

Susceptibilities to 13 antimicrobials were determined by an agar dilution method according to the guidelines of Clinical and Laboratory Standards Institute.[3]

PCR assays for screening most of the reported β-lactamases and plasmid mediated quinolone resistance (PMQR) were performed as previously described.[4]

Bacterial genome sequencing was performed using the Illumina platform in Beijing Genome Institute. Bacterial plasmid sequencing was performed using both Illumina and PacBio platforms as previously described.[5]

Results and Discussion

Bacterial isolation

A total of 385 *V parahaemolyticus* isolates were collected from raw shrimp samples purchased in markets in four different locations (Hong Kong Island, Hung Hom, Tsuen Wan, and Sai Kung) in Hong Kong from January to April in 2010 and May to September in 2011. A total of 98 clinical *V parahaemolyticus* strains isolated from stool

samples of diarrhoeal patients collected from the Prince Wales Hospital and Beijing CDC from 2009 to 2011 were also included. A total of 54 (17%) *V parahaemolyticus* strains were isolated from various food samples in Shenzhen from 2012.

Antimicrobial resistance profile of clinical and food *V parahaemolyticus* isolates

The 385 *V parahaemolyticus* strains isolated from shrimps in Hong Kong were highly resistant to ampicillin (88%), amikacin (52%), and tetracycline (51%). The isolates also exhibited resistance to sulfamethoxazole (43%), nalidixic acid (40%), streptomycin (35%), kanamycin (32%), and chloramphenicol (30%). Surprisingly, about 9% of the *V parahaemolyticus* isolates also exhibited resistance to cefotaxime, ceftriaxone, and aztreonam, and 9% of them were resistant to ciprofloxacin. The *V parahaemolyticus* strains isolated from clinical specimens were mostly susceptible to most of the antibiotics tested. They were highly resistant to ampicillin (92%), followed by sulfamethoxazole (20%), nalidixic acid (16%), streptomycin (10%), kanamycin (8%), chloramphenicol (8%), tetracycline (6%), and ciprofloxacin (2%). No isolate was resistant to cephalosporins. The 54 *V parahaemolyticus* isolates collected from Shenzhen exhibited a high rate of resistance to ampicillin (100%) and tetracycline (11%). Five isolates (9%) were resistant to sulfamethoxazole/trimethoprim, one isolate was resistant to chloramphenicol. Importantly, two isolates were found to be resistant to amoxicillin/clavulanic acid, cefoxitin, and ceftazidime.

Mechanisms of extended-spectrum cephalosporin resistance in *V parahaemolyticus*

Identification of β-lactamase genes

Among the *V parahaemolyticus* stains isolated from Hong Kong and Shenzhen, 21 non-repeated (maximum one isolate per sample) cephalosporin-resistant *V parahaemolyticus* strains were obtained, including 12 isolated in 2010 in Hong Kong, 7 isolated in 2011 in Hong Kong, and two isolated from Shenzhen in 2012. These cephalosporin-resistant *V parahaemolyticus* strains were further characterised to delineate their mechanisms of cephalosporin resistance. The bla_{PER-1} gene was identified in all these 12 isolates. For two cephalosporin-resistant *V parahaemolyticus* strains (V43 and V4) isolated from food in Shenzhen, bla_{TEM-1} and bla_{PER-1} were detected in V43, whereas the β-lactamase gene bla_{CMY-2} was detected for the first time in strain V4 (Table).

Characterisation of bla_{PER-1}-bearing plasmid

Three bla_{PER-1} positive isolates (V43, 2010V36, and 2011V1) that exhibited unique PFGE patterns were

TABLE. Antimicrobial susceptibility profile of *V parahaemolyticus* strains or *Escherichia coli* carrying various antimicrobial resistance genes

Strain	β-lactamase gene	Plasmid mediated quinolone resistance	Minimal inhibitory concentration, mg/L									
			AMP (32)*	CTX (4)	CRO (4)	CAZ (16)	GEN (16)	NAL (32)	CIP (4)	CHL (32)	KAN (64)	TRI (16)
J53			1	≤0.125	≤0.125	≤0.125	1	2	≤0.125	2	0.25	≤0.125
V4	bla_{CMY-2}		128	8	32	8	1	0.5	≤0.125	2	8	16
V4-J53	bla_{CMY-2}		>128	16	64	64	1	2	≤0.125	64	1	>64
V43	bla_{PER-1}		>128	>128	>128	>128	8	1	≤0.125	2	8	32
V43-J53	bla_{PER-1}		128	128	128	>128	8	2	≤0.125	32	0.5	>64
2010V36	bla_{PER-1}		>128	>128	>128	>128	1	0.25	≤0.125	0.25	2	16
2010V36-J53	bla_{PER-1}		>128	>128	>128	>128	1	2	≤0.125	2	2	>64
2011V1	bla_{PER-1}		>128	>128	>128	>128	4	0.5	≤0.125	16	64	>64
2011V1-J53	bla_{PER-1}		>128	>128	>128	>128	4	4	≤0.125	16	64	>64
E coli TG.1(pCR2.1)								4	<0.05			
TG.1(pCR2.1-*qnrVC1*)		*qnrVC1*						32	0.125			
TG.1(pCR2.1-*qnrVC6*)		*qnrVC6*						64	0.25			
TG.1 (pCR2.1-*qnrVC5*)		*qnrVC5*						32	0.1			
TG1(pCR2.1-*qnrVC7*)		*qnrVC7*						16	0.06			
TG1(pCR2.1-*qnrVC5-S100A*)		*qnrVC5*						32	0.25			
TG1(pCR2.1-*qnrVC5-A152T*)		*qnrVC5*						8	0.06			
TG1(pCR2.1-*qnrVC7-A100S*)		*qnrVC7*						16	0.06			
TG1(pCR2.1-*qnrVC7-T152A*)		*qnrVC7*						32	0.25			

selected for further characterisation. Conjugation experiments showed that plasmids carrying bla_{PER-1} in these three *V parahaemolyticus* strains could be transferred to *Escherichia coli* J53. S1 nuclease PFGE and Southern hybridisation showed that the bla_{PER-1} gene in V43 and 2011V1 was located in a plasmid of ~200kb in size, and the bla_{PER-1} gene in strain 2010V36 was located in a ~175kb plasmid. The complete sequence of plasmid from a *V parahaemolyticus* strain V36, designated as pVPH1, was obtained. The complete plasmid sequence of pVPH1(KP688397) was 183,730bp in size and had an average G+C content of 45.2%, which is similar to that of the chromosome of *V parahaemolyticus* (45.1% to 45.6%). Annotation results revealed that pVPH1 harboured 114 predicted coding sequences; among them, several functional regions could be identified. After conducting the BLASTN alignment search against NCBI database, the sequence organisation of pVPH1 was found to be similar to that of pAQU1 (72%) and pAQU2 (76%) [Fig 1]. The MDR regions of pVPH1 are centralised in a region of ~40kb in size, which is flanked by two different Tn*3* family transposase genes. There were many different genetic mobile elements in this mosaic region, such as IS*26*, Class 1 Integron, IS*CR1*, IS*4321* and IS*6100*. A mercuric resistance operon (*mer* operon) was also found between the Tn*3* and the TniA transposase elements, which was similar to that of plasmid pR148 from *Aeromonas hydrophila*. Downstream of the mercuric resistance operon was a ParDE type II toxin-antitoxin system. A Class 1 Integron with an *arr3-dfrA23* cassette flanked by IS*26* and IS*CR1* was also found with bla_{PER-1} being located downstream of IS*CR1* in an area including *gst* (encoding a glutathione S-transferase), *abct* (encoding an ABC-type transporter) and three hypothetical genes, followed by the *qacEΔ1* and *sul1* genes. This mobile element can be circularised, and it was speculated that IS*CR1* may act like IS*91* to generate circular intermediates and mobilise genes immediately upstream of IS*CR1*.

Characterisation of bla_{CMY-2}-bearing plasmid by S1-PFGE and Southern hybridisation

Conjugation experiments showed that the plasmid borne bla_{CMY-2} gene in V4 strain could be transferred to *E coli* J53. S1 nuclease PFGE and Southern hybridisation analysis showed that the V4 strain harboured 4 plasmids of different sizes, ranging from ~45kb to ~150kb. The conjugative plasmid detected in transconjugant V4J53 was ~150kb in size. Southern hybridisation confirmed that the bla_{CMY-2} gene was located in this transmissible plasmid in both V4 and V4J53. Plasmid typing indicated that the transmissible plasmid belonged to IncA/C type plasmid.

The flanking sequence of bla_{CMY-2} was amplified by PCR mapping according to published sequences and confirmed by sequencing. It was found that the arrangement of genetic environment of bla_{CMY-2} was *traB-traV-traA-ISEcp1-*bla_{CMY-2}*-blc-sugE-encR-orf1-orf2-orf3-orf4-dsbC-traC* (Fig 1). The β-lactamase gene bla_{CMY-2} gene is the most commonly reported plasmid-encoded AmpC β-lactamase gene in *Salmonella* spp, *E coli*, and other

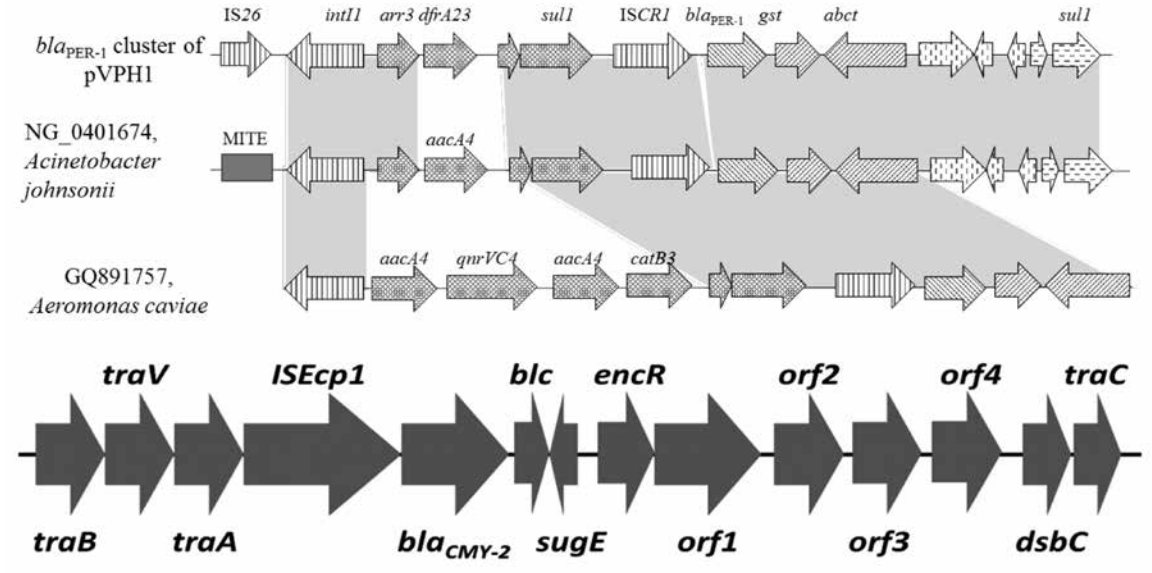

FIG 1. Genetic structure of bla_{CMY-2} and bla_{PER-1}: (a) annotated coding sequences are displayed as arrows. The shadow quadrangles indicate the consensus sequences among different organisms. The arrows without gene names represent the hypothetical open reading frames. (b) Genetic structure of bla_{CMY-2} -borne IncA/C conjugative plasmid in *V Parahaemolyticus* strain V4.

species of *Enterobacteriaceae* worldwide. This gene renders the host organisms resistant to a variety of β-lactams, including oxyimino-cephalosporins and cephamycins.

Mechanisms of quinolone/fluoroquinolone resistance in *V parahaemolyticus*

Mechanisms of quinolone resistance

A total of 65 nalidixic-acid-resistant, ciprofloxacin-susceptible *V parahaemolyticus* strains isolated from Hong Kong from 2010-2011 were selected to investigate the mutations in *gyrA* and *parC* genes and the presence of plasmid-mediated quinolone resistance (PMOR) genes. Most quinolone-resistant isolates were found to possess a single mutation which resulted in the S83I amino acid change in GryA (77%), among which 62% harboured another single mutation which resulted in the S85F substitution in ParC. Around 23% of the nalidixic acid-resistant did not possess any target mutations, suggesting that other mechanisms of quinolone resistance were involved. However, none of the known PMQR genes including *qnr, qeqA, oqxAB*, or *qnrVC* was detectable in any of these strains.

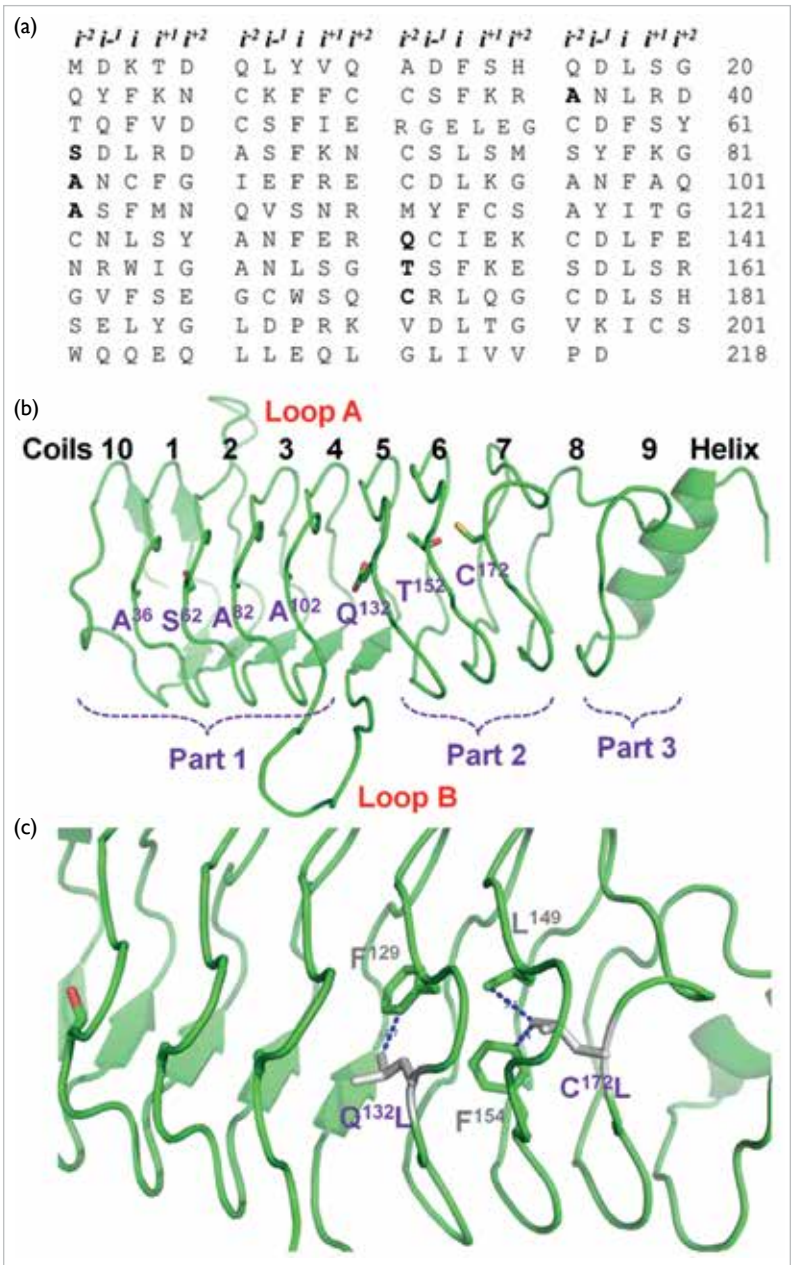

FIG 2. Amino acid sequence and structural representation of QnrVC7 and specific mutant proteins. (a) Tabular array of QnrV7 amino acids grouped by pentapeptide repeats with coils along the vertical axis and faces along the horizontal axis, i^{-2} residues characterised in this study are highlighted; (b) Overall structure of QnrVC7 and its organisation. The sites at the i-2 position of face 4 are indicated; and (c) Amino acid substitutions that led to an increase in protective activity of QnrVC7. The potential hydrophobic interactions that may enhance the stability of QnrVC7 are indicated.

Mechanisms of fluoroquinolone resistance and novel PMQR genes in V parahaemolyticus

Fifteen non-repeated (maximum one isolate per sample) ciprofloxacin-resistant *V parahaemolyticus* strains (minimal inhibitory concentration [MIC]≥4) isolated from shrimp samples in 2010 were selected to characterise their molecular mechanism of fluoroquinolone resistance. All isolates except for one were found to harbour the single amino acid substitution Ser[83]Ile, in GyrA and Ser[85]Leu change in ParC (Table). The actual contribution of single amino acid substitution in GyrA and ParC to fluoroquinolone resistance in *V parahaemolyticus* needs further confirmation since mutations in both genes have also been found to be associated with only intermediate resistance to ciprofloxacin in *V cholera* isolates. One *V parahaemolyticus* isolate was resistant to norfloxacin, but did not harbour any mutations in both *gyrA* and *parC* genes, or any known PMQR genes.

A new *qnrVC* gene with a novel substitution, Ala[100]Ser, compared to *qnrVC4*, was identified. We designated this novel *qnrVC* gene as *qnrVC5*. To further confirm its functional role, the whole coding region of *qnrVC5* and its natural promoter region were cloned into the pCR2.1 vector using primers. The MIC of *E coli* carrying the pCR2.1-*qnrVC5* exhibited reduced susceptibility to ciprofloxacin (MIC=0.1) and resistance to nalidixic acid (MIC=32) [Table].

Another novel *qnrVC* gene, *qnrVC6*, was detected in a ~200kb conjugative plasmid that also carried a bla_{PER-1} gene recovered from a *V parahaemolyticus* strain isolated from food. Sequence analysis of the full length *qnrVC* gene revealed that this gene differed from *qnrVC1* by one single amino acid, Asp[71]Asn. Cloning of this novel *qnrVC* allele as *qnrVC6* mediated decreased susceptibility to ciprofloxacin and nalidixic acid, suggesting a role of *qnrVC6* in mediating nalidixic acid resistance (Table).

A novel *qnrVC*-like gene was identified in one *V cholerae* isolate. Sequence analysis of the

full-length of this gene showed that it differed from qnrVC5 by three amino acids substitution ($M^{18}L$, $S^{100}A$ and $A^{152}T$). We designated this novel *qnrVC* allele as *qnrVC7*. The activities of the *qnrVC* gene product were investigated by determining the MICs to fluoroquinolones/quinolones of the clone. *E coli* TG1 carrying pCR2.1–*qnrVC7* exhibited reduced susceptibility to ciprofloxacin and nalidixic acid (Table). However, compared to *qnrVC5*, *E coli* carrying *qnrVC7* exhibited ~2-fold and 4-fold lower MIC toward nalidixic acid and ciprofloxacin respectively. Mutational analysis of various residues between revealed that substitution of A^{152} by T reduced the activity of the *qnrVC7* product and A^{152} was critical for the activity of QnrVC proteins.

Structural and functional characterisation of QnrVC7 protein

Sequence comparison and mutational analysis showed that amino acid T^{152}, located at the i^{-2} position on face 4, was responsible for the reduced protection activity of QnrVC7. This study extended the characterisation to all residues located at the i^{-2} position of face 4 to elucidate the structure / activity relationship of QnrVC7 as well as other Qnr proteins. These data suggested that Ala, Ser and in some cases Thr were the best fit residues at i^{-2} position of face 4 on coils 1, 2, 3 and 4, and that the bulky side chain residues such as Leu and Asp would disrupt the well-organised tight stacks of these coils. The requirement of small side chain residues at i^{-2} position was consistent with the finding in other Qnrs such as QnrA and QnrC. However, substitution with Gly was not tolerated either, suggesting the small side chain is required for forming the stable stack between coils (Fig 2).

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Disclosure

Results from this research project have been published in:

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