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Characterisation of novel mutations involved in quinolone resistance in *Escherichia coli* isolated from imported shrimp

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ABSTRACT

Fifty-five nalidixic acid-resistant *Escherichia coli* strains were isolated from imported shrimp. Purified PCR amplicons of *gyrA*, *gyrB*, *parC* and *parE* from the template DNA of all isolates were sequenced and analysed for point mutations that confer resistance to nalidixic acid and ciprofloxacin. Point mutations in the quinolone resistance-determining regions (QRDRs) of GyrA at positions 68, 83 and 87 and in ParC at positions 80 and 84 as well as in the non-QRDR of GyrA at positions 112, 127, 128 and 154 along with point mutations in *parE* at position 476 conferred resistance to these antibiotics. Computational modelling and analysis of the different point mutations and their role in the enhanced resistance to these antibiotics indicated that only mutation at codons 83 (Ser→Ile) and 87 (Asp→Asn) played a vital role in increasing the minimum inhibitory concentration (MIC) to these drugs compared with other mutations. Ethidium bromide experiments indicated higher efflux pump activities in quinolone-resistant *E. coli* strains compared with their quinolone-sensitive counterparts. Class 1 integrons measuring 0.7–2.3 kb were amplified and sequenced from the template DNA of the isolates. Sequence analysis of the 2.0 kb and 1.7 kb integrons indicated the presence of resistance determinants for trimethoprim (*dfrA12* and *dfrA17*) and aminoglycosides (*aadA2* and *aadA5*). These results indicate that use of nalidixic acid, ciprofloxacin and other antibiotics in shrimp aquaculture ponds may select *E. coli* resistant to these antibiotics and that imported shrimp is a reservoir of multiple antibiotic-resistant *E. coli*.

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1. Introduction

Shrimp aquaculture is a low-cost, highly profitable, rural industry in several Asian countries such as Thailand, Vietnam, Indonesia, China, India and Bangladesh [1]. These Asian nations are turning to farm-raised shrimp as an attractive source of international trade revenue and they currently produce nearly 80% of the world's farmed shrimp [2]. The USA imports more than 1.3 billion pounds of farmed shrimp, with a market value of more than US$5 billion, from Asia [3]. The increased global demand and high rates of return in shrimp aquaculture have led to the development and implementation of technologies that improve production and profits [2]. However, production can be curtailed by diseases caused by a variety of bacteria [4]. Large quantities of antibiotics have been used to limit these diseases associated with overstocked aquaculture ponds and to stimulate shrimp growth rates [1,5,6]. However, this use of antibiotics may select for bacteria resistant to multiple antibiotics [5]. In addition, the residues of many of these antibiotics have been found incorporated in shrimp tissues [7]. Therefore, minimising the use of antibiotics in aquaculture may help to limit the prevalence of antibiotic-resistant bacteria in food-producing ecosystems [8].

Quinolones are a class of powerful broad-spectrum antimicrobial agents effective in the treatment of selected community-acquired and nosocomial infections. These antibiotics are administered orally or intravenously for the treatment of serious human infections [9]. They show excellent broad-spectrum activity both against Gram-negative and Gram-positive bacteria, good oral absorption and tissue penetration, and a low incidence of serious side effects [9]. However, the indiscriminate use of quinolones and fluoroquinolones in shrimp farms to prevent the outbreak of diseases may select bacteria resistant to these antibiotics and reduce the efficacy of these drugs in clinical treatment of diseases [10].

Several mechanisms of resistance to quinolones have been documented [11–16], primarily attributed to chromosomal mutations in DNA gyrase and/or topoisomerase IV genes [13]. Additional mechanisms, such as altered cell membrane permeability and

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overexpression of multidrug efflux pumps, also contribute to high-level bacterial resistance to these antibiotics [11]. However, limited information is available on the occurrence and prevalence of quinolone-resistant *Escherichia coli* in imported shrimp. In this report, we describe the isolation of 55 quinolone-resistant *E. coli* isolates from imported shrimp and characterise their mechanisms of quinolone resistance.

2. Materials and methods

2.1. Isolation, characterisation and identification

Unless otherwise stated, bacteria were isolated from 330 farm-raised, frozen, imported shrimp (*Penaeus monodon*) samples purchased from retail grocery stores. These samples were purchased between March 2008 and July 2009 from six different retail grocery stores. Typically, 2 g of thawed shrimp samples was homogenised with 10 mL of Luria broth (LB) (USB Corporation, Cleveland, OH) in a stomacher (Tekmar Co., Cincinnati, OH) for 5 min. One loopful of the enriched sample was streaked on MacConkey agar plates (Thermo Fisher Scientific, Lenexa, KS) and incubated at 37 °C overnight. Presumptive positive colonies for *E. coli* were biochemically characterised and identified by the Vitek GNI+ card with VTK-R07-01 software (bioMérieux Vitek, Hazelwood, MO) and by fatty acid methyl ester analysis (MIDI, Newark, DE). All isolates were stored in LB containing 20% glycerol at −70 °C and were grown overnight at 37 °C in LB or on trypticase soy agar (TSA) plates supplemented with 5% sheep blood (Thermo Fisher Scientific).

2.2. Determination of antibiotic susceptibility and the minimum inhibitory concentration (MIC) of the isolates

The antibiotic susceptibility of each *E. coli* isolate was determined using a disc diffusion assay [17]. The susceptibility of each isolate was determined as per the criteria specified by the Clinical and Laboratory Standards Institute (CLSI) [18]. A sensitive strain of *E. coli* (in-house) was used as a control. MICs for the antibiotics ciprofloxacin and nalidixic acid were determined by the broth dilution method using Mueller–Hinton broth (Oxoid Ltd., Basingstoke, UK) [18]. The concentration ranges were 0.125–128 μg/mL for ciprofloxacin and 4–512 μg/mL for nalidixic acid.

2.3. Genomic DNA extraction

Genomic DNA was extracted from cells grown overnight at 37 °C using a QIAamp DNA Mini Prep Kit (QIAGEN, Valencia, CA).

2.4. Primer design and detection of quinolone resistance genes by PCR

The presence of quinolone resistance genes (*qnrA*, *qnrB*, *qnrS*, *gyrA*, *gyrB*, *parC* and *parE*) was investigated in the template DNA by PCR (Table 1). Detection and amplification of the quinolone resistance genes were carried out as detailed elsewhere [19,20].

2.5. Detection of mutations in the quinolone resistance-determining regions (QRDRs)

The target genes (*qnrA*, *qnrB*, *qnrS*, *gyrA*, *gyrB*, *parC* and *parE*) were amplified by PCR and were purified using a QIAquick PCR Purification Kit (QIAGEN). Both strands of the purified PCR amplicons were sequenced using the primers used for amplification of the QRDR.

Table 1
Oligonucleotide primers used in the amplification of fluoroquinolone resistance genes from *Escherichia coli* isolated from imported shrimp.

Primer	Nucleotide sequence	Target gene	Size (bp)
gyrAF	5′-AAATCTGCCCGTGTCGTTGGT-3′	*gyrA*	344
gyrAR	5′-GCCATACCTACGGCGATACC-3′		
gyrBF	5′-GAAATGACCCGCCGTAAA-3′	*gyrB*	272
gyrBR	5′-ACGACCGATACCACAGCC-3′		
parCF	5′-CTGAATGCCAGCGCCAAATT-3′	*parC*	168
parCR	5′-GCGAACGATTTCGGATCGTC-3′		
parEF	5′-CTGAACTGCTGGCGCAGATG-3′	*parE*	483
parER	5′-GCGGTGGCAGTGCGACGTAA-3′		
qnrAF	5′-TCAGCAAGAGGATTTCTCA-3′	*qnrA*	627
qnrAR	5′-GGCAGCACTATTACTCCCA-3′		
qnrBF	5′-GATCGTGAAAGCCAGAAAGG-3′	*qnrB*	469
qnrBR	5′-ACGATGCCTGGTAGTTGTCC-3′		
qnrSF	5′-ACGACATTCGTCAACTGCAA-3′	*qnrS*	417
qnrSR	5′-TAAATTGGCACCCTGTAGGC-3′		
IntF	5′-GGCATCCAAGCAGCAAG-3′	Class 1 integron	Variable
IntR	5′-AAGCAGACTTGACCTGA-3′		

The quinolone resistance genes and integrons were amplified after an initial denaturation at 94 °C for 2 min. PCR was performed with 35 cycles and, after completion of the 35 cycles, a final extension step of 10 min at 72 °C was included in all protocols.

2.6. Accumulation of ethidium bromide (EtBr) in fluoroquinolone-sensitive and -resistant strains of Escherichia coli

Accumulation of EtBr was monitored as described elsewhere [12]. Fluorescence was read on a Synergy 2 Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT). Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (Sigma–Aldrich, St Louis, MO), an efflux pump inhibitor, was added to the assay mixture at a final concentration of 100 μM. The natural fluorescence of the cells was subtracted and the fluorescence intensity was expressed in relative fluorescence units. All experiments were performed in triplicate.

2.7. Homology modelling

The crystal structures of DNA gyrase [Protein Data Bank (PDB) ID 2Y3P] from *E. coli* and topoisomerase IV from *Streptococcus pneumoniae* (3FOE and 3FOF) and *Staphylococcus aureus* (2XCT) were used as templates for computational modelling and analysis of type IIA topoisomerases from *E. coli* ECIS18 and ECIS803 [21–23]. The homology three-dimensional models for wild-type and mutant proteins were generated using SWISS-MODEL [24]. The experimentally determined PDB structure files and their at-a-glance structural overview information were obtained from the databases RCSB PDB (http://www.rcsb.org/pdb/) and PDBsum (http://www.ebi.ac.uk/pdbsum/). To predict changes in protein stability and contacts of structural units after point mutation, CUPSAT and CSU were adopted [25,26]. PyMOL (0.99RC6) was used for visualisation of structural figures [27].

2.8. Amplification and sequencing of integrons

Integrons were amplified as detailed elsewhere [28]. Both strands of the purified amplicons were sequenced using M13 primers with an ABI Prism® BigDye® Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA).

3. Results

3.1. Isolation and identification of fluoroquinolone-resistant Escherichia coli from imported shrimp samples

In total, 163 bacterial isolates that fermented lactose on MacConkey agar were picked for further characterisation and

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