Letter to the Editor

Seafood as a Reservoir of Gram-negative Bacteria Carrying Integrons and Antimicrobial Resistance Genes in Japan*

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PCR and DNA sequencing were used to screen and characterize integrons and resistance genes in Gram-negative bacteria isolated from seafood products in Japan. A total of 215 Gram-negative bacteria were isolated from local and imported seafood samples collected from retail markets in Hiroshima Prefecture. Class 1 integrons containing gene cassettes encoding resistance to trimethoprim (*dfrA12* and *dfrA17*), aminoglycosides (*aadA2*), and β-lactams (bla_{PSE-1}) were identified in six bacterial isolates. Four different β-lactamase-encoding genes including bla_{TEM-1}, bla_{CMY-2}, bla_{CMY-13}, and bla_{CMY-39} were identified in seven isolates. A novel gene bla_{CMY-39} was isolated from a strain of *Citrobacter freundii*. Plasmid-mediated quinolone resistance genes, including *qnrB2*, *qnrB6*, and *qnrS1*, were also identified in 10 isolates. This study highlights the presence of antimicrobial resistance genes in seafood-associated bacteria in Japan and indicates that seafood could be a reservoir and route of transmission of antibiotic-resistant bacteria to humans.

At present, antimicrobial resistance is a global public health crisis, with increasing rates of morbidity and mortality attributed to therapeutic failure of bacterial infections. Worldwide, food safety challenges include the emergence of new pathogens, re-emergence of known pathogens, and transfer of antibiotic-resistant bacteria to humans via the food chain[1]. There is currently a worldwide growth of aquaculture with a rapid increase in therapeutic and prophylactic usage of antimicrobials, including those important in human medicine. The intensive use of antimicrobial agents in aquaculture is the principal selective pressure for antimicrobial resistance in bacteria, as significant concentrations of antimicrobials remain for prolonged time periods in sediments and the overlying water column[2]. Mobile genetic elements, including plasmids, transposons, and integrons, which disseminate antibiotic resistance genes by horizontal gene transfer, play an important role in the evolution and dissemination of multidrug resistance in the aquatic environment[1]. The transfer of plasmids containing resistance genes between fish pathogens and other aquatic bacteria indicates that these bacteria can act as reservoirs of resistance genes that can be further disseminated to humans. Although the presence of integrons and resistance genes in the bacteria of animal-food origin has been well documented, there is scarcity of information about resistant bacteria inseafood. Therefore, the aim of this study was to screen for the presence of integrons and resistance genes in Gram-negative bacteria isolated from local and imported seafood in Japan.

A total of 215 Gram-negative bacteria were isolated from 14 local and imported seafood samples randomly purchased from different retail markets in Hiroshima Prefecture, between May and July 2006. The seafood samples were immediately transported at 4 °C conditions to the laboratory and were used for bacterial isolation within 24 h. Then, 25 g of each sample was placed into a sterile plastic bag containing 225 mL of sterilized phosphate buffer (pH 7.2) and homogenized for 1 min using a stomacher. From the pre-enriched homogenate, 1 mL was incubated overnight in 5 mL Luria-Bertani broth and then plated on MacConkey and thiosulfate-citrate-bile salt-sucrose agar and incubated at 37 °C for 24-48 h. The isolation and identification of bacteria were conducted by conventional techniques. Importantly, all bacterial isolates were confirmed by the API 20E system (BioMérieux, Marcy-l'Étoile, France).

The antimicrobial susceptibility phenotypes of bacterial isolates were determined using a Kirby-Bauer disk diffusion assay according to the

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standards and interpretive criteria described by the Clinical and Laboratory Standards Institute. The following antibiotics were used: ampicillin (AMP), 10 μg; amoxicillin-clavulanic acid (AMC), 20/10 μg; aztreonam (ATM), 30 μg; cefoperazone (CFP), 30 μg; cefotetan (CTT), 30 μg; cefoxitin (FOX), 30 μg; cefpodoxime (CPD), 10 μg; ceftazidime (CAZ), 30 μg; ceftriaxone (CRO), 30 μg; chloramphenicol (CHL), 30 μg; ciprofloxacin (CIP), 5 μg; doripenem (DOR), 10 μg; gentamicin (GEN), 10 μg; imipenem (IMP), 10 μg; kanamycin (KAN), 30 μg; meropenem (MEP), 10 μg; nalidixic acid (NAL), 30 μg; norfloxacin (NOR), 10 μg; oxacillin (OXA), 30 μg; sulfamethoxazole-trimethoprim (SXT), 23.75/1.25 μg; streptomycin (STR), 10 μg and tetracycline (TET), 30 μg. The reference strain *E. coli* ATCC 25922 was included as a quality control.

DNA was prepared using boiled lysates, and conserved primers were used to detect and identify class 1 and class 2 integrons, as previously described[3]. PCR screening for TEM, SHV, CTX-M, OXA, and CMY β-lactamase-encoding genes was performed using universal primers for the respective gene families, as described previously[3]. PCR was also used to screen for plasmid-mediated quinolone resistance (PMQR) genes, *qnrA*, *qnrB*, *qnrS*, and *aac(6')-Ib-cr*, as described previously[3]. Both strands of the PCR product were then sequenced using an ABI automatic DNA sequencer (Model 373; PerkinElmer). All PCR primers are listed in Table 1.

The purified PCR fragment corresponding to bla_{CMY-39} was cloned into *Eco*RV-digested pBluescript vector using a Takara Ligation Kit. Bluescript contains anampicillin resistance gene. *E. coli* TG1 was transformed with the resulting recombinant plasmid, pBCMY-39, and the transformants were selected on Luria-Bertani agar medium supplemented with cefotetan (10 mg/L). Positive colonies containing expressed CMY-39 were confirmed by PCR. A similarity search was conducted

Table 1. Primers Used for PCR and DNA Sequencing

Primer	Sequence (5′-3′)	Target	Reference
Integrons			
5′-CS	GGCATCCAAGCAGCAAG	Class 1 integron	[3]
3′-CS	AAGCAGACTTGACCTGA		
hep74	CGGGATCCCGGACGGCATGCACGATTTGTA	Class 2 integron	[3]
hep51	GATGCCATCGCAAGTACGAG		
β-Lactamases			
TEM-F	ATAAAATTCTTGAAGACGAAA	bla_{TEM}	[3]
TEM-R	GACAGTTACCAATGCTTAATC		
SHV-F	TTATCTCCCTGTTAGCCACC	bla_{SHV}	[3]
SHV-R	GATTTGCTGATTTCGCTCGG		
OXA-F	TCAACTTTCAAGATCGCA	bla_{OXA}	[3]
OXA-R	GTGTGTTTAGAATGGTGA		
CTX-M-F	CGCTTTGCGATGTGCAG	bla_{CTX-M}	[3]
CTX-M-R	ACCGCGATATCGTTGGT		
CMY-F	GACAGCCTCTTTCTCCACA	bla_{CMY}	[3]
CMY-R	TGGAACGAAGGCTACGTA		
CMY-F-2	ACGGAACTGATTTCATGATG	bla_{CMY} (whole gene)	[3]
CMY-R-2	GAAAGGAGGCCCAATATCCT		
Quinolone esistance genes			
qnrA-F	ATTTCTCACGCCAGGATTTG	*qnrA*	[3]
qnrA-R	GATCGGCAAAGGTTAGGTCA		
qnrB-F	GATCGTGAAAGCCAGAAAGG	*qnrB*	[3]
qnrB-R	ACGATGCCTGGTAGTTGTCC		
qnrS-F	ACGACATTCGTCAACTGCAA	*qnrS*	[3]
qnrS-R	TAAATTGGCACCCTGTAGGC		
aac(6')-Ib-F	TTGCGATGCTCTATGAGTGGCTA	*aac(6')-Ib-cr*	[3]
aac(6')-Ib-R	CTCGAATGCCTGGCGTGTTT		

using the BLAST program, available from the NCBI BLAST homepage (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

PCR and DNA sequencing identified class 1 integrons in six bacterial isolates: three *Aeromonas hydrophila*, one *Citrobacter freundii*, one *Enterobacter cloacae*, and one *Klebsiellaoxytoca*. The identified resistance gene cassettes within the class 1 integrons were dihydrofolate reductase types *dfrA12* and *dfrA17*, aminoglycoside adenyl transferase type 2 *aadA2*, which confers resistance to streptomycin and spectinomycin, and β-lactamase-encoding gene typebla_{PSE-1}, which confers resistance to β-lactams (Table 2). Most of the resistance genes captured by class 1 integrons had observable phenotypes (Table 2). All class 1 integron-positive strains were isolated from seafood produced locally in Japan. Class 1 integrons harboring similar gene cassettes were previously identified in *Aeromonas* spp. isolated from rainbow trout farms in Australia[4] and from fresh fish in Mexico[5]. Our group previously identified class 1 integrons in Gram-negative bacteria (including *A. hydrophila*, *C. freundii*, *E. cloacae*, and *Klebsiella* spp.) isolated from fish farms in Egypt[6]. Class 1 integrons were also identified in *E. coli* strains isolated from commercial fish and seafood in Korea[7] and from *Salmonella enterica* strains isolated from imported seafood in the United States[1]. Importantly, all isolates were negative for class 2 integrons.

Penicillin derivatives (β-lactams) are broad-spectrum antibacterial agents widely used in human and veterinary medicine. PCR and DNA sequencing identified β-lactamase-encoding genes in

Table 2. Occurrence of Integrons and Resistance Genes in Gram-negative Bacteria Isolated from Seafood

No	Species	Source[a]	Country	Phenotype[b]	Genotype Integrons/ESBLs/Qnr
2	*Enterobacter cloacae*	*Pagrus major*	Japan	AMP, CHL, STR, SXT	1) Class 1 integron (*aadA2*) 2) bla_{TEM-1}
4	*Aeromonas hydrophila*	*Pagrus major*	Japan	AMP, CHL, KAN, NAL, NOR, STR, SXT, TET	1) Class 1 integron (*dfr12-orf-aadA2*) 2) Class 1 integron (*dfr17*)
16	*Enterobacter cloacae*	SM1	Indonesia	AMP, FOX, STR, SXT	1) bla_{TEM-1}
21	*Enterobacter cloacae*	SM1	Indonesia	AMP, CHL, FOX, STR, SXT, TET	1) bla_{TEM-1}
28	*Citrobacter freundii*	*Pagrus major*	Japan	AMP, CHL, FOX, STR, TET	1) bla_{TEM-1} 2) bla_{CMY-13} 3) *qnrB2*
29	*Citrobacter koseri*	*Pagrus major*	Japan	AMP	1) *qnrB2*
79	*Pantoea* spp.	Shrimp	Indonesia	AMP	1) *qnrS1*
89	*Aeromonas hydrophila*	Alfonsin	Japan	DOR, FOX, IMP, MEP, NAL, STR, TET	1) Class 1 integron (bla_{PSE-1})
92	*Klebsiella oxytoca*	*Pagrus major*	Japan	AMP, NAL, STR, SXT, TET	1) Class 1 integron (*dfr17*)
94	*Citrobacter freundii*	*Pagrus major*	Japan	AMP, CFP, CHL, FOX, STR, TET	1) Class 1 integron (bla_{PSE-1}-*aadA2*) 2) bla_{CMY-13} 3) *qnrB2*
103	*Enterobacter cloacae*	Salmon	Chile	FOX, STR, SXT, TET	1) *qnrB6*
151	*Citrobacter freundii*	Salmon	Chile	AMP, CFP, FOX, STR	1) bla_{CMY-2} 2) *qnrB2*
153	*Enterobacter cloacae*	Shrimp	Indonesia	AMP	1) *qnrS1*
160	*Aeromonas hydrophila*	Shijimi	Japan	AMP, DOR, IMP, MEP, STR	1) Class 1 integron (bla_{PSE-1})
176	*Citrobacter freundii*	SM3	India, North Pacific, China	AMC, AMP, CAZ, CFP, CPD, CRO, CTT, FOX, STR	1) bla_{CMY-39} 2) *qnrB2*
205	*Citrobacter freundii*	SM3	India, North Pacific, China	STR	1) *qnrB2*
211	*Citrobacter freundii*	Alfonsin	Japan	AMP	1) *qnrB2*

Note. [a]SM, seafood mix; [b]AMC, amoxicillin-clavulanic acid; AMP, ampicillin; CAZ, ceftazidime; CFP, cefoperazone; CHL, chloramphenicol; CPD, cefpodoxime; CRO, ceftriaxone; CTT, cefotetan; DOR, doripenem; FOX, cefoxitin; IMP, imipenem; MEP, meropenem; NAL, nalidixic acid; NOR, norfloxacin; STR, streptomycin; SXT, sulfamethoxazole-trimethoprim; TET, tetracycline.

seven isolates (four *C. freundii* and three *E. cloacae*) (Table 2). The narrow-spectrum β-lactamase-encoding gene bla_{TEM-1} was identified in four isolates (three *E. cloacae* and one *C. freundii*). bla_{TEM-1} was previously detected in Gram-negative bacteria isolated from fish farms in Egypt[6] and also in *E. coli* strains isolated from commercial fish and seafood in Korea[7]. AmpC β-lactamase-encoding genes bla_{CMY-2}, bla_{CMY-13}, and bla_{CMY-39} were identified in four *C. freundii* isolates (Table 2). bla_{CMY-39} is a novel gene isolated from a *C. freundii* strain 10a-1. bla_{CMY-39} has a unique threonine-to-lysine mutation at position 210 and encodes resistance to cephamycins (cefotetan and cefoxitin) and third-generation cephalosporins (cefpodoxime, ceftazidime, and ceftriaxone) (Table 2). This resistance phenotype was confirmed by the cloning and expression of bla_{CMY-39} in *E. coli* TG1 (data not shown). This novel bla_{CMY} gene was designated as bla_{CMY-39} in the β-lactamase database (http://www. lahey. org/Studies/other. asp# Table 1). The nucleotide sequence of bla_{CMY-39} is available in the EMBL/GenBank/DDBJ databases under accession no. AB372224. bla_{CMY-2} was previously identified in *A. salmonicida* isolated from juvenile Atlantic salmon aquaculture facilities in Canada[8]. bla_{CMY-2} was more recently identified in an *E. coli* strain isolated from freshwater fish from two lakes in Switzerland[9].

Quinolones are broad-spectrum antimicrobial agents and are among the most widely used antimicrobial compounds in humans and animals. Multiplex PCR screening and DNA sequencing identified PMQR genes in 10 isolates (six *C. freundii*, two *E. cloacae*, one *C. koseri*, and one *Pantoea* spp.), including *qnrB2*, *qnrB6*, and *qnrS1* (Table 2). *qnrA*, *qnrB*, and *qnrS* were previously identified in Gram-negative bacteria (including *C. freundii*, *C. koseri*, and *E. cloacae*) isolated from fish farms in Egypt[6]. *qnrB* and *qnrS* were the most common PMQR genes in *E. coli* isolated from farmed fish in China[10].

In summary, this study characterized class 1 integrons and resistance genes in several Gram-negative bacteria isolated from local and imported seafood in Japan. Seafood is a possible route of transmission of resistant bacteria to humans. Therefore, with increasing global seafood consumption and trade, the continuous monitoring of antibiotic resistance genes in the marine environment is particularly important to ensure seafood safety, particularly for those countries with a heavy reliance on seafood.

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