NOTE *Bacteriology*

Detection of *aac(6')-Ib-cr* in Avian Pathogenic *Escherichia coli* Isolates in Japan

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ABSTRACT. We investigated the prevalence of plasmid-mediated quinolone resistance (PMQR) genes in avian pathogenic *Escherichia coli* (APEC) strains in Japan. A total of 117 APEC strains collected between 2004 and 2007 were examined for PMQR genes (*qnrA, qnrB, qnrC, qnrD, qnrS*, *aac(6')-Ib*-cr, *qepA* and *oqxAB*) by polymerase chain reaction. None of the APEC strains carried *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA or oqxAB*, but one of the isolates was identified as an AAC (6')-Ib-cr producer. Phylogenetic grouping, multi-locus sequence typing and serotyping showed that this isolate belonged to phylogenetic group A, sequence type 167 and untypable serogroup. To our knowledge, this is the first report of the *aac (6')-Ib-cr* gene in bacteria from food-producing animals in Japan.
KEY WORDS: *aac(6')-Ib-cr,* APEC, PMQR, quinolone resistance.

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Avian pathogenic *Escherichia coli* (APEC), the etiological agent of colibacillosis, causes local or systemic infections including colisepticemia, coligranuloma, air sac disease, pericarditis and swollen head syndrome [9]. Colibacillosis is responsible for large economic losses in the poultry industry throughout the world. In Japan, about 2,500,000 chickens were condemned with APEC by poultry meat inspection in 2011 according to a government report [17].

Of fluoroquinolone drugs, enrofloxacin (ERFX) was first approved for avian colibacillosis in 1991; subsequently, ofloxacin (OFLX), danofloxacin (DNFX) and norfloxacin (NFLX) were approved in Japan. Previously, resistance to all quinolones in *Enterobacteriaceae* was believed to be chromosome-encoded, i.e., amino acid substitution of DNA gyrase and topoisomerase IV (multiple mutations in the quinolone resistance-determining region), decreased outer membrane permeability (porin defect) or activation of naturally occurring efflux pumps [12]. In addition, the plasmid-mediated quinolone resistance (PMQR) genes, *qnr, aac(6')-Ib-cr*, *qep* and *oqx*, have been reported in bacteria isolated from humans and food-producing animals worldwide [24].

In Japan, *qnrS1* was first identified in human isolates of *Shigella flexneri* in 2003 [11]. Clinical isolates of *E. coli* harboring *qepA* and *oqxAB* were found in 2002 and 2008, respectively [23, 29]. In addition, *qnrS1* has been detected in *Salmonella* isolated from food-producing animals [1, 3]. In these reports, the source of PMQR genes in *Enterobacteriaceae* in Japan is described. APEC strains are considered to be one of the most exposed to fluoroquinolones among the pathogenic bacteria in food-producing animals based on the sales of volume of broilers and the period of approval of the antibiotics. The aim of this work was to assess the prevalence of PMQR genes in APEC strains in Japan and characterize the PMQR-gene-positive strain.

A total of 117 APEC strains were collected from chickens with colibacillosis (one isolate per chicken) between 2004 and 2007 in Japan. Isolates obtained between 2004 and 2006 were reported in our previous study [2, 19]. The presence of the *qnrA, qnrB* and *qnrS* genes was determined by polymerase chain reaction (PCR) [5]. The *qnrC* and *qnrD* genes were detected using previously described primers [6, 27]. The *qepA*, *oqxAB* and *aac(6')-Ib* genes were detected as previously described [7, 16, 20]. Of the 117 APEC isolates, two isolates (18-PLEc-C-36 collected in 2006 and 19-PLEc-C-53 collected in 2007) were positive for *aac(6')-Ib*, and none of the isolates was positive for the other PMQR genes.

AAC(6')-Ib-cr differs from AAC(6')-Ib by two amino acids, Trp102Arg and Asp179Tyr, and these substitutions allow it to reduce the antibacterial activities of not only aminoglycoside but also NFLX and ciprofloxacin through acetylation of their piperazinyl substituent [26]. The *aac(6')-Ib* gene sequences were determined by direct sequencing of the PCR products from the two positive isolates. The *aac(6')-Ib* gene from18-PLEc-C-36 showed complete identity to the *aac(6')-Ib-cr* gene from a strain of *Klebsiella pneumoniae* (GenBank accession number EU195449), while the 19-PLEc-C-53 sequence was identified as wild-type *aac(6')-Ib* (accession number AF322577).

We next confirmed the production of the fluoroquinolone-modifying enzyme AAC(6')-Ib-cr in 18-PLEc-C-36 strain using a disk-based method reported by Wachino [26]. Briefly, the 18-PLEc-C-36 strain was grown in LB broth (Becton, Dickinson and Co., Sparks, MD, U.S.A.) containing NFLX (8mg/*l*) with shaking for 18 hr at 35°C. The broth containing the same concentration of NFLX in the other tube was used as the control. Fifty microliters of each culture medium was applied on the blank disk set on a Mueller-Hinton agar

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Table1. Characteristics of *aac (6')-Ib-cr*-positive strain, 18-PLEc-C-36

ST	phylogenetic type[a)]	serogroup[a)]	β-lactamase[a)]	virulence gene			Antimicrobial susceptibility[a)]				
				afa/draBC	*iutA*	other virulence genes[b)]	MIC for fluoroquinolones (mg/*l*)				Resistance type
							ERFX	DNFX	NFLX	OFLX	
ST167	group A	untypable	CTX-M-15	+	+	–	128	256	128	64	ABPC-CEZ-KM-OTC-NA-ERFX-DNFX-NFLX-OFLX

a) Previously reported by Asai *et al*. or Ozawa *et al*. [2, 19]. b) Other virurences were: *papA*, *sfaS*, *focG*, *cnf1*, *hlyA* and *neuC*. Absence of *papC*, *tsh*, *cvaC* and *iss* in 18-PLEc-C-36 was previously reported by Ozawa *et al*. [19].

(Becton, Dickinson and Co.) plate inoculated with *E. coli* ATCC 25922 and incubated for 18 hr at 35°C. The growth-inhibitory zone of the 18-PLEc-C-36 culture medium significantly decreased compared with the control.

Sequence typing and virulence gene detection were then investigated in 18-PLEc-C-36 strain. The following 12 virulence genes were detected by PCR, as described previously: *afa/draBC, papA, papC, sfaS* and *focG*(adhesins); *cnf1* and *hlyA*(toxins); *iutA*(siderophore); *neuC*(KI antigen); *tsh*(autotransporter); *cvaC*(colicin V) and *iss*(increased serum survival) [8, 14, 18]. Absence of four virulence genes (*papC, tsh*, *cvaC* and *iss*) of 18-PLEc-C-36 strain was already reported [19]. ST was determined using the MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli) [28]. In addition, bacterial conjugation was performed using 18-PLEc-C-36 strain as the donor strain and *E. coli* DH5α (rifampicin and nalidixic acid (NA) resistance) as the recipient, using broth mating method. Plasmids were extracted from 18-PLEc-C-36 strain and the transconjugant using Qiagen mini-kit (Qiagen, Gaithersburg, MD, U.S.A.) according to the manufacturer's instructions. Plasmid incompatibility grouping was performed using the transconjugant and a PCR-based replicon typing method [4].

18-PLEc-C-36 strain was resistant to ampicillin (ABPC), cefazoline (CEZ), kanamycin (KM), oxytetracycline (OTC) and fluoroquinolone drugs (ERFX, DNFX, NFLX and OFLX) (Table1) [2, 19]. Therefore, the susceptibility of 18-PLEc-C-36 transconjugant for ABPC, CEZ, KM, OTC, ERFX, DNFX, NFLX and OFLX was determined by the same method [2, 19]. The transconjugant was resistant to ABPC (MIC, 128mg/*l*) and CEZ (MIC, 64 mg/*l*), but susceptible to KM (MIC, ≤4 mg/*l*), OTC (MIC, 1 mg/*l*), ERFX (MIC, 0.125 mg/*l*), DNFX (MIC, 0.25 mg/*l*), NFLX (MIC, 0.5 mg/*l*) and OFLX (MIC, 0.25 mg/*l*). MICs of the transconjugant for fluoroquinolone drugs including NFLX were the same with those of the recipient. 18-PLEc-C-36-transconjugant was *aac(6')-Ib-cr* and $bla_{\text{CTX-M-15}}$ positive by PCR and had an approximately 48-kbp plasmid. The transconjugant belonged to Inc.FI incompatibility group.

The characteristics of *aac(6')-Ib-cr* positive strain are summarized in Table 1. It was reported that a specific genotype of fluoroquinolone-resistant O78 APEC may be widely distributed and that 80% of APEC strains have the *iss* virulence gene, and more than half of those have *tsh* and *cva* in Japan [19]. The characteristics of the AAC(6')-Ib-cr producer are different from those of predominant fluoroquinolone-resistant APEC strains already reported in Japan.

In human clinical field, the serotype O25b-ST131-B2 clone harboring both $bla_{\text{CTX-M-15}}$ and *aac(6')-Ib-cr* has spread worldwide, however, there are no reports of the ST167 clone harboring both these genes. The complete sequence of the pEK499 plasmid (strain A: 117536bp) from one O25b-ST131-B2 isolate has been reported and appears to be a fusion of type FII and FIA replicons, harbored resistance genes $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, $bla_{\text{TEM-1}}$, *aac(6')-Ib-cr, mph (A)*, *cat*B4 and *tet (A)*. These were responsible for cephalosporin, β-lactamase inhibitor, aminoglycoside, chloramphenicol and tetracycline resistance. And, FII replicon and multireplicons FIA, FIB and FII have been described in $bla_{\text{CTX-M-15}}$-carrying plasmids of ST131 *E. coli* [22]. The size, plasmid replicon type and antimicrobial resistance patterns differed between O25b-ST131-B2 and 18-PLEc-C-36. With regard to ST167, clonal relatedness was identified between a Mongolian avian extended-spectrum β-lactamase ($bla_{\text{CTX-M-9}}$) producing *E. coli* isolate and a clinical isolate that originated from a hospitalized patient in Europe [10].

Since *aac(6')-Ib-cr* was first detected in clinical isolates in China in 2000, it has been found in clinical *Enterobacteriaceae* isolates worldwide including in Japan [21, 26]. Isolates harboring *aac(6')-Ib-cr* have also been detected in food-producing animals in Korea, China and Europe [7, 15, 16, 25]. Particularly in China, a clear trend of increase in the prevalence of *aac(6')-Ib-cr* among the isolates from chicken was observed from 2001 to 2007 [13]. In contrast, this is the first report of an isolate harboring *aac(6')-Ib-cr* from food-producing animals in Japan. Furthermore, *qnrA, qnrB, qnrC, qnrD, qnrS, qepA* and *oqxAB* were not detected in any of the APEC isolates in this study. To date, no PMQR genes other than *qnrS1* have been detected in food-producing animals in Japan [1, 3], suggesting a low prevalence of PMQR in this country.

In conclusion, an *aac(6')-Ib-cr* positive isolate was first detected from food-producing animal in Japan. The characters, serotype, phylogenetic type, virulence-associated gene and ST of isolate are different from those of predominant fluoroquinolone-resistant APEC in Japan and of *E. coli* harboring *aac(6')-Ib-cr* and $bla_{\text{CTX-M-15}}$ previously reported in human and animal. Although PMQR genes confer only low-level resistance, they spread horizontally and facilitate the selection of additional chromosome-encoded quinolone resistance mechanisms [21]. In addition, PMQR is frequently found in the isolates from food-producing animals in the world, and it would be difficult to prevent the invasion of resistance genes from foreign countries to Japan [13]. Con-

tinuous monitoring of PMQR in food-producing animals is essential to assess the risk of transmitting fluoroquinolone-resistant bacteria and resistance determinants to humans.

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