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Acquisition of *mcr-1* and Cocarriage of Virulence Genes in Avian Pathogenic *Escherichia coli* Isolates from Municipal Wastewater Influents in Japan

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ABSTRACT This study focused on the detection of the plasmid-mediated *mcr* colistin resistance gene in *Escherichia coli* isolates from wastewater treatment plants (WWTPs). Seven influent samples were collected from three WWTPs in Nagano Prefecture, Japan, during August and December 2018. Colistin-resistant *E. coli* isolates were selected on colistin-supplemented CHROMagar ECC plates. *mcr-1*-positive isolates were subjected to whole-genome sequencing (WGS) analysis. From six influent samples, seven *mcr-1*-positive but extended-spectrum β-lactamase (ESBL)-negative isolates belonging to different genetic lineages, namely, B2-O25:H4-ST131-*fimH*22, B2-O2:H1-ST135-*fimH*2, B1-O8:H9-ST764-*fimH*32, B1-O23:H16-ST453-*fimH*31, A-O81:H27-ST10-*fimH*54, A-O16:H5-ST871-*fimH*25, and F-O11:H6-ST457-*fimH*145, were detected. The MICs of colistin for these isolates ranged from 4 to 16 mg/liter. The *mcr-1* genes were located on plasmids belonging to IncX4 and IncI2 in five and two isolates, respectively. Four IncX4 plasmids with the same size (33,309 bp) showed high sequence similarity (4 single-nucleotide variations). The remaining one IncX4 plasmid, with a size of 33,858 bp, carried the *mcr-1* gene with the single synonymous nucleic substitution T27C. Two IncI2 plasmids with sizes of 60,710 bp and 60,733 bp had high sequence similarity (99.9% identity; 100% query coverage). Two of five isolates carrying IncX4 plasmids and both of the isolates carrying IncI2 plasmids harbored ColV plasmids carrying virulence-associated genes of avian pathogenic *E. coli* (APEC). In addition, another isolate of the B2-O25:H4-ST131-*fimH*22 lineage had those APEC-associated virulence genes on its chromosome. In conclusion, *mcr-1*-positive *E. coli* environmental isolates were mostly characterized as positive for APEC-associated virulence genes. The copresence of those genes may suggest the existence of a common source in animals and/or their associated environments.

IMPORTANCE Colistin is considered a last-line therapeutic option in severe infections due to multidrug-resistant Gram-negative bacteria, in particular carbapenemase-producing *Enterobacteriaceae* and multidrug-resistant *Acinetobacter baumannii*. An increasing prevalence of *mcr* genes in diverse *Enterobacteriaceae* species, mainly *Escherichia coli* and *Klebsiella pneumoniae* from humans and food animals, has become a significant concern to public health all over the world. In Japan, *mcr* genes have so far been detected in food animals, raw meat, wastewater, and human clinical samples. This study reports the copresence of *mcr-1* and avian pathogenic *E. coli* (APEC)-associated virulence genes in five of seven *E. coli* isolates recovered from aquatic environments in Japan. Our study highlights the importance and urgency of action to reduce environmental contamination by *mcr* genes that may likely occur

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due to exposure to untreated wastewater through combined sewer overflow by recent unusual weather.

KEYWORDS WWTPs, *mcr-1*, APEC, ColV

Colistin is an old antibiotic discovered in 1949 in Japan, which is considered a last-line therapeutic option in severe infections due to multidrug-resistant Gram-negative bacteria, in particular carbapenemase-producing *Enterobacteriaceae* and multidrug-resistant *Acinetobacter baumannii*. The acquisition of colistin resistance in *Enterobacteriaeae* is attributed mostly to amino acid substitutions in the chromosome-encoded PmrA/PmrB and PhoP/PhoQ two-component systems or the *mgrB* gene, a PhoP/PhoQ negative regulator, which is not transferable through horizontal gene transfer (1). These mechanisms are expected to work as innate resistance mechanisms against bactericidal cationic peptides that are widely distributed in the lysosome of phagocytes, including neutrophils and macrophages of host animals (2). However, exogenous acquisition of the plasmid-mediated colistin-resistance *mcr-1* gene in *Enterobacteriaceae* has been newly identified in patients, pigs, and retail meat (pork and chicken) from China in 2015 (3). Since then, the *mcr-1* gene has been detected in diverse *Enterobacteriaceae* species, mainly *Escherichia coli* and *Klebsiella pneumoniae* from humans, food animals, and foods all over the world, followed by the discovery of *mcr* gene variants *mcr-1* to *-9* (4–7). A higher prevalence of *mcr-1*-carrying isolates from food-producing animals than that from humans has been recognized in Asia, Europe, Africa, and North America (4, 8). Colistin has been widely used as a veterinary medicine for decades to prevent and treat infections with some Gram-negative bacteria, including verotoxin 2e-producing *E. coli* that causes edema disease in young porcine (9). In addition, colistin has been extensively used as one of the feed additives in food animals for growth promotion, especially in Asian countries, South Africa, and Brazil (7, 10). Such a sustained selection pressure exerted on colistin may have been an important driving force that led the development of reservoirs of colistin resistance in food animals, which can increase the risk of transmission of colistin-resistant bacteria as well as *mcr* genes to humans via the food chain. Based on human health risk assessment results, the Japanese government announced the ban of colistin usage as a feed additive in food animals in July 2018.

For several years, the *mcr* genes have been increasingly reported in water environments, including wastewater, rivers, seawater, and hospital sewage, in many different countries (5, 11–14). Particularly, the frequent detection of *mcr* genes has been recognized in municipal wastewater (15, 16). In Japan, *mcr* genes have so far been detected in both healthy and diseased food animals, in raw meat samples, including retail domestic chicken meat documented by us, and in human clinical samples, although their epidemiology has been sparsely studied (17–19). However, the occurrence of *mcr* genes in aquatic environments remains largely unknown in Japan, except for a very recent report on an *E. coli* sequence type 393 (ST393) strain carrying *mcr-3.1* (20). This study focused on the detection of *mcr* genes in municipal wastewater, which is considered important in the spread of antibiotic-resistant bacteria and genes (21, 22).

RESULTS AND DISCUSSION

Detection of *mcr-1*-harboring *E. coli* isolates and their antimicrobial susceptibilities. A total of seven colistin-resistant *E. coli* isolates were detected from wastewater samples of six different inlets. Namely, one isolate each was from two different inlets of wastewater treatment plant A (WWTP A) (strains A1 and A2), two isolates were from the same inlet of WWTP B (strains B1 and B2), and one isolate each was from three different inlets of WWTP C (strains C1, C2, and C3). Colistin-resistant *E. coli* was not detected from the remaining one of two inlets of WWTP B. PCR screening revealed that all strains carried *mcr-1* genes. The MICs of antimicrobial agents for the seven colistin-resistant *E. coli* strains A1, A2, B1, B2, C1, C2, and C3 carrying *mcr-1* are listed in Table 1. These parental strains exhibited resistance to colistin with MICs from 4 to 16 mg/liter. All

TABLE 1 MICs of antimicrobials for seven *E. coli* strains harboring *mcr-1* detected in inflow samples from three municipal WWTPs

	MIC (mg/liter) by *E. coli* strain and WWTP:						
	A		B		C		
Antimicrobial agent	A1	A2	B1	B2	C1	C2	C3
Piperacillin	1	1	>64	>64	1	1	1
Ampicillin-sulbactam	≤4-2	≤4-2	16-8	16-8	≤4-2	≤4-2	≤4-2
Cefazolin	2	2	2	2	2	1	1
Cefotaxime	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Ceftazidime	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Cefpodoxime	≤1	≤1	≤1	≤1	≤1	≤1	≤1
Cefepime	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Flomoxef	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Aztreonam	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5	≤0.5
Imipenem	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
Meropenem	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
Amikacin	4	2	2	≤1	2	2	2
Gentamicin	1	1	0.5	≤0.25	≤0.25	0.5	0.5
Minocycline	2	2	>8	4	0.5	0.5	1
Levofloxacin	0.25	0.25	0.5	≤0.25	≤0.25	≤0.25	≤0.25
Sulfamethoxazole-trimethoprim	≤9.5-0.5	≤9.5-0.5	≤9.5-0.5	≤9.5-0.5	≤9.5-0.5	≤9.5-0.5	≤9.5-0.5
Fosfomycin	≤32	≤32	≤32	≤32	≤32	≤32	≤32
Colistin	8	8	8	8	16	4	8

strains were susceptible to β-lactams, aminoglycosides, levofloxacin, sulfamethoxazole-trimethoprim, fosfomycin, and minocycline except that strains B1 and B2 were resistant to piperacillin and strain B1 also displayed resistance to minocycline.

Other colistin-resistant strains, including colistin-resistant *Klebsiella pneumoniae* mediated by chromosomal mutations and intrinsically resistant *Serratia marcescens*, *Providencia stuartii*, and *Edwardsiella tarda*, were also detected.

We initially used MacConkey agar plates supplemented with colistin, which is a commonly used screening medium, resulting in the failure to detect colistin-resistant *E. coli* isolates except *E. coli* strain A1. All seven colistin-resistant *E. coli* strains in this study were able to grow normally on MacConkey agar plates without colistin. Similarly, a CTX-M-1-producing *E. coli* harboring the *mcr-1* gene on an IncI2 plasmid recovered from retail chicken meat reported previously could not grow on MacConkey agar with colistin but grew well on MacConkey agar with cefotaxime (18). Thus, it seems likely that those bacteria with outer membrane damage caused by colistin cannot grow on MacConkey agar containing bile salts. The frequency of occurrence of colistin-resistant *E. coli* harboring the *mcr-1* gene that mediates low-level colistin resistance might be underestimated due to using MacConkey agar supplemented with colistin that possibly causes detection failure.

Location of *mcr-1* genes. Colistin-resistant transformants possessing the *mcr-1*-carrying plasmids pA1, pA2, pB1, pB2, pC1, pC2, and pC3 were successfully obtained for all seven *E. coli* parental strains A1, A2, B1, B2, C1, C2, and C3, respectively. For those transformants, colistin MICs were 8 mg/liter, which is 32-fold higher than that (0.25 mg/liter) for *E. coli* DH10B without a plasmid, and they were susceptible to other antimicrobials, as listed in Table 1 (data not shown). pA1, pA2, pB1, pB2, and pC3 belonged to IncX4 plasmids, while pC1 and pC2 belonged to IncI2 plasmids.

Genetic characterization of *mcr-1*-carrying IncX4/IncI2 plasmids. The complete circular nucleotide sequences of the *mcr-1*-carrying IncX4 plasmids pA1, pA2, pB1, pB2, and pC3 and IncI2 plasmids pC1 and pC2 were determined. Those IncX4 and IncI2 plasmids were found to have no antimicrobial resistance genes other than *mcr-1*.

Four IncX4 plasmids, namely, pA1, pA2, pB2 and pC3, with the same size of 33,309 bp, G+C contents of 41.8%, and 39 protein-coding genes showed very high sequence similarity (4 single-nucleotide variations), where pA2 had 100% sequence identity to pC3. Those plasmids aligned very well (>99.9% identity; 100% query coverage) with *mcr-1*-carrying IncX4 plasmids, such as pRYU3223C-1 (GenBank accession number AP018411) harbored by *K. pneumoniae* RYU3223 from a human urine

sample in Japan, pICBEC72Hmcr (GenBank accession number CP015977) harbored by *E. coli* ICBEC72H from a human soft tissue sample in Brazil, and pCSZ4 (GenBank accession number KX711706) harbored by *E. coli* CSZ4 from a pork sample in China (Fig. 1a). The remaining one IncX4 plasmid pB1, which carried the *mcr-1* gene with single synonymous nucleotide substitution T27C, was 33,858 bp, with a G+C content of 41.6% and 42 protein-coding genes. It had 100% sequence identity with 100% query coverage to pGZ49269 (GenBank accession number MG210939) harbored by *E. coli* GZ49269 from a human urine sample in China and pMR0617mcr1 (GenBank accession number CP024462) harbored by *K. pneumoniae* QS17-0161 from a human sputum sample in Thailand. The genetic context of *mcr-1* in those IncX4 plasmids was characterized by the presence of the insertion sequence IS*26* upstream of the *mcr-1-pap2* element in pA1, pA2, pB2, and pC3 and generating an 8-bp duplication (TCACACAG), as described previously (23), whereas we found the absence of IS*26* and an inverted orientation of the *mcr-1-pap2* element in pB1. Those IncX4 plasmids had a *virB* gene cluster encoding a type IV secretion system.

Two IncI2 plasmids, pC1 and pC2, with sizes of 60,710 bp and 60,733 bp, respectively, G+C contents of 42.5%, and 71 protein-coding genes had high sequence similarity (99.9% identity; 100% query coverage). Those pC1 and pC2 plasmids were closely related (>99.9% identity, query coverage of 96% to 100%) to *mcr-1*-carrying IncI2 plasmids pMRY15-131_2 (60,722 kb; GenBank accession number AP017622) harbored by *E. coli* MRY15-131 from a cow with mastitis in Japan, pRYU2912C-1 (60,732 bp; GenBank accession number AP018412) harbored by *E. coli* RYU2912 from a human fecal sample in Japan, p6383 (61,198 bp; GenBank accession number MG594798) harbored by *E. coli* 6383 from a human urine sample in Argentina, and pAF23 (61,177 bp; GenBank accession number KX032519) harbored by *E. coli* Af23 from a human blood sample in South Africa (Fig. 1b). For the plasmids pC1 and pC2, the *mcr-1-pap2* element, without an upstream IS*Apl1*, was located immediately downstream of the *nikB* gene encoding relaxase of the plasmid. Those IncI2 plasmids possessed *virB* and *pil* gene clusters.

IncX4 plasmids pA1, pA2, pB1, pB2, and pC3 and IncI2 plasmids pC1 and pC2 did not have an IS*Apl1* insertion sequence upstream of the *mcr-1* gene. The IS*Apl1* is presumably involved in the initial transposition of the *mcr-1* element and then is lost for stabilization of *mcr-1* on those plasmids (24). However, right inverted repeat (IRR)-like sequence IRR2 of IS*Apl1* proposed by Sun et al. was identified within the 3′-terminal region of *pap2* in all those plasmids, suggesting a possible linkage of IS*Apl1* with the transposition of the *mcr-1* (25). The *mcr-1*-carrying IncX4 and IncI2 plasmids characterized in this study were highly homologous among themselves and to those from humans and food animals of different geographical origins, suggesting their important role in spreading the *mcr-1* gene.

Genetic features of *E. coli* harboring *mcr-1*. Diversity in clonal lineages and serotypes within *E. coli* strains was observed (Table 2). Namely, phylogroup B2 strains A1 and C1 belonged to O25:H4-ST131-*fimH*22 and O2:H1-ST135-*fimH*2, respectively. Phylogroup A strains A2 and B2 belonged to O16:H5-ST871-*fimH*25 and O81:H27-ST10-*fimH*54, respectively. Phylogroup B1 strains B1 and C3 belonged to O8:H9-ST767-*fimH*32 and O23:H16-ST453-*fimH*31, respectively. The remaining phylogroup F strain C2 belonged to O11:H6-ST457-*fimH*145. *E. coli* B1 and B2 from WWTP B contained the $bla_{\text{TEM-1}}$ gene, and the latter strain, B2, also carried multiple antimicrobial resistance genes, including aminoglycoside adenylyltransferase genes *aadA1* and *aadA2* (Table 2). Those B1 and B2 strains were positive for the *astA* gene, encoding the enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1). *E. coli* A1, C1, C2, and C3 were categorized as extraintestinal pathogenic *E. coli* (ExPEC) and/or uropathogenic *E. coli* (UPEC).

The STs of *E. coli* strains identified in this study have not been reported previously among *E. coli* isolates harboring *mcr-1*-positive IncI2 or IncX4 plasmids derived from human, food animals, and retail meats except ST457 in Japan (17). It was noteworthy

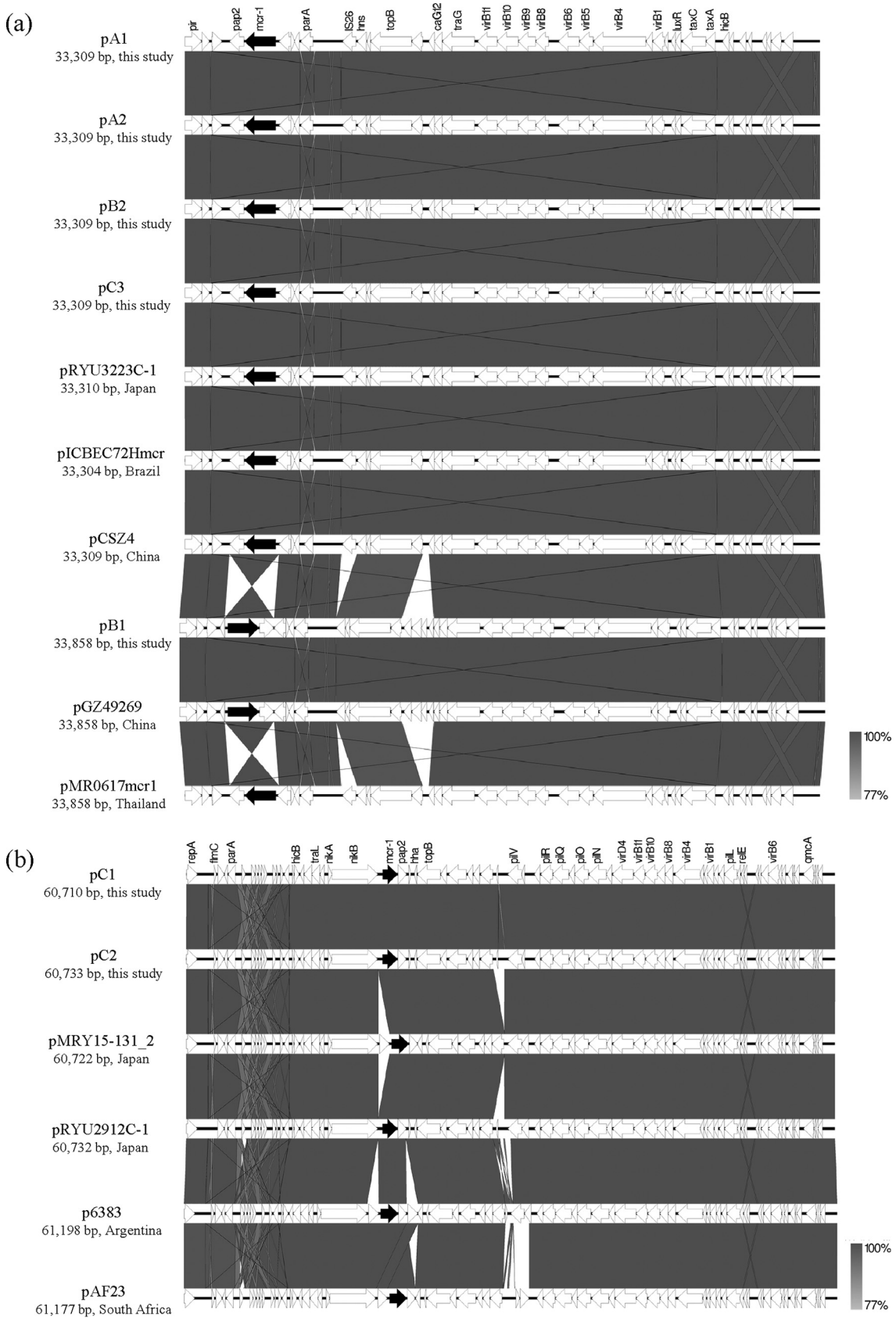

FIG 1 Linear comparison of complete plasmid sequences of *mcr-1*-carrying plasmids. (a) Comparison of IncX4 plasmids pA1, pA2, pB1, pB2, and pC3 in this study with plasmids pRYU3223C-1 (GenBank accession number AP018411), pICBEC72Hmcr (GenBank accession

(Continued on next page)

that our *E. coli* STs included clinically important clonal lineages ST131 (strain A1) and ST10 (strain B2). The highly successful epidemic clone ST131 is a major contributor to ExPEC infections in humans, where B2-O25:H4-ST131-*fimH*30 is the current globally dominant ST131 subclone responsible for fluoroquinolone resistance and ESBL production (26, 27). The B2-O25:H4-ST131-*fimH*22 lineage, the evolutionary precursor of the B2-O25:H4-ST131-*fimH*30 lineage, is mostly composed of fluoroquinolone-susceptible populations. However, this lineage has been associated with poultry, human bloodstream infections, and carriage of *mcr-1* and *mcr-3* (3, 28, 29). ST10, which has been frequently reported in human fecal samples and food samples, is recognized as the most common ST among *mcr-1*-carrying *E. coli* isolates (30). The remaining STs, namely, ST871, ST767, ST135, ST457, and ST453, have also been identified in humans and animals.

The fitness advantage of host *E. coli* DH5α by the acquisition of those IncI2- and IncX4-type plasmids due to their enhanced fitness of hosts, competition advantage over other plasmid types, and plasmid stability has been described (31). Thus, the ability of non-lineage-specific horizontal gene transfer of IncI2 and IncX4 plasmids may pose an increased risk for disseminating *mcr* genes across a wide range of bacterial lineages, including clinically important lineages, becoming a threat to both human and animal health through the discharge of untreated wastewater.

Virulence genes associated with APEC, NMEC, and resistance genes. APEC- and neonatal meningitis *E. coli* (NMEC)-associated virulence genes were detected in *E. coli* A1, B1, C1, C2, and C3 among seven *mcr-1*-harboring strains (Table 2). B2-O25:H4-ST131 *E. coli* A1 harbored 12 chromosomally located genes, *sitABCD*, *iroBCDEN*, *iss*, *mat*, and *kpsMT*II. In contrast, *E. coli* C1, C2, and C3 had more than 17 virulence genes associated with IncF-type ColV plasmids and chromosomes. With regard to *E. coli* C1 of the B2-O2:H1-ST135 clone, 13 genes, such as *sitABCD*, *iroBCDEN*, *iss*, and *hlyF*, were identified within the 124,394-bp ColV plasmid pColV-C1 on a RepFIB/RepFII plasmid backbone (Fig. 2A), while *ibeA*, *vat*, *mat*, and *kpsMT*II were located on the chromosome. In *E. coli* B1, a 40,920-bp sequence, including 18 APEC-associated virulence genes and repFIB; a 15,444-bp sequence, including tetracycline resistance gene *tet*(B) and class I integron containing trimethoprim resistance gene *dfrA17* downstream of chloramphenicol resistance gene *catA1*; and a 29,259-bp sequence, including *iroBCDEN*, *cva/cvi*, and *iss*, were identified. Those three sequences, exhibiting >99.99% nucleotide identities to the corresponding sequences of the IncF-type ColV plasmid *E. coli* strain D3 plasmid A (GenBank accession number CP010141), were presumed to be located on a ColV plasmid. Notably, *E. coli* B1 also had the IncI1-type ColBM plasmid pColBM-B1 (84,497 bp in size) carrying *cba* and *cma* genes (Fig. 2B).

E. coli B2 harbored the tetracycline resistance gene *tet*(M) downstream of the class 1 integron containing the gene cassette array *dfrA12-orfF-aadA2-cmlA1-aadA1-qacH*-IS*440* on an IncF plasmid.

Most of the virulence genes associated with APEC are commonly located on IncF-type ColV or ColBM virulence plasmids, as was observed among strains B1, C1, C2, and C3. The remaining strain A1 belonged to the B2-O25:H4-ST131-*fimH*22 lineage. This lineage has been linked to poultry products and carried the ColV plasmid with high frequency, serving as a vehicle for poultry-to-human transmission and infection (32). However, the strain A1 did not have ColV plasmid, and *sitABCD*/*iroBCDEN* and *iss* genes located on ColV plasmid of the above four strains were found on the chromosome instead. Strain C1 of the B2-O2:H1-ST135-*fimH*2 lineage was characterized by possessing NMEC virulence-associated genes, including *ibeA* located on the genomic island

FIG 1 Legend (Continued)
number CP015977), pCSZ4 (GenBank accession number KX711706), pGZ49269 (GenBank accession number MG210939), and pMR0617mcr1 (GenBank accession number CP024462). (b) Comparison of IncI2 plasmids pC1 and pC2 in this study with plasmids pMRY15-131_2 (GenBank accession number AP017622), pRYU2912C-1 (GenBank accession number AP018412), p6383 (GenBank accession number MG594798), and pAF23 (GenBank accession number KX032519). The arrows show the translation orientation of the coding genes. The *mcr-1* genes are indicated by black arrows.

TABLE 2 Genetic features of *mcr-1*-harboring *E. coli* isolates recovered from WWTPs[a]

WWTP	*E. coli* strain	Sequence type	Serotype	Phylogroup	*fimH* type	Replicon type		Other antimicrobial resistance genes in:		Virulence-associated genes[b] in:		Presumed pathotypes
						mcr-1-carrying plasmid (study plasmid, bp)	Other plasmids	Chromosome	Plasmid	Chromosome	Plasmid	
A	A1	131	O25:H4	B2	*fimH*22	IncX4 (pA1, 33,309)		*mdf*(A)		*iroB, iroC, iroD, iroE, iroN, iss, sitA, sitB, sitC, sitD, mat, kpsMT*II, *sfa/focDE, fyuA, chuA, yfcV, gad, mchB, mchC, mchF, mcmA*		APEC, NMEC, ExPEC, UPEC
	A2	871	O16:H5	A	*fimH*25	IncX4 (pA2, 33,309)		*mdf*(A)		*yfcV, gad*		
B	B1	767	O8:H9	B1	*fimH*32	IncX4 (pB1, 33,858)	IncFIB/IncFIC (ColV), IncI1 (ColBM)	*mdf*(A)	bla_{TEM-1}, *catA1*, *tet*(B), *dfrA17*	*fyuA, astA, gad, lpfA*	*cba, cma, iroB, iroC, iroD, iroE, iroN, iss, sitA, sitB, sitC, sitD, cva/cvi, iucA, iucB, iucC, iucD, hlyF, ompT, iutA*	APEC, NMEC
	B2	10	O81:H27	A	*fimH*54	IncX4 (pB2, 33,309)	IncFIA, IncFIB, IncX1, p0111	*mdf*(A), *mef*(B)	bla_{TEM-1}, *aadA1*, *aadA2*, *tet*(M), *cmlA1*, *dfrA12*	*astA, gad*		
C	C1	135	O2:H1	B2	*fimH*2	IncI2 (pC1, 60,710)	IncFIB/IncFII (ColV)	*mdf*(A)		*ibeA, mat, kpsMT*II, *vat*, *fyuA, chuA, gad*	*iroB, iroC, iroD, iroE, iroN, iss, cva/cvi, sitA, sitB, sitC, sitD, hlyF, ompT*	APEC, NMEC, UPEC
	C2	457	O11:H6	F	*fimH*145	IncI2 (pC2, 60,733)	IncFIB/IncFII (ColV)	*mdf*(A)	*aph(3′)-Ia*, *dfrA5*	*irp2, bor, kpsMT*II, *fyuA*, *chuA, yfcV, air, eilA, gad, lpfA*	*iroB, iroC, iroD, iroE, iroN, iss, cva/cvi, sitA, sitB, sitC, sitD, iucA, iucB, iucC, iucD, hlyF, ompT, iutA*	APEC, NMEC, ExPEC, UPEC
	C3	453	O23:H16	B1	*fimH*31	IncX4 (pC3, 33,309)	IncFIB/IncFIC (ColV), p0111	*mdf*(A)	*tet*(A)	*bor, kpsMT*II, *papC*, *gad*, *lpfA*	*iroB, iroC, iroD, iroE, iroN, iss, cva/cvi, sitA, sitB, sitC, sitD, iucA, iucB, iucC, iucD, hlyF, ompT, tsh, iutA*	APEC, NMEC, ExPEC

[a]*iroB*, *C*, *D*, *E*, and *N*, salmochelin siderophore system; *iss*, increase serum survival lipoprotein; *sitA*, *B*, *C*, *and D*, ferrous iron transport system; *mat*, meningitis-associated and temperature-regulated fimbriae; *kpsMT*II, group II capsule antigen; *sfa/focDE*, s-fimbriae adhesin/F1 fimbriae; *fyuA*, yersiniabactin receptor; *chuA*, *E. coli* heme-utilization; *yfcV*, fimbrial protein; *gad*, glutamate decarboxylase; *mchB*, microcin H47 part of colicin H; *mchC*, putative microcin H47 biosynthesis protein *mchF*, microcin H47 secretion ATP-binding protein; *mcmA*, microcin M part of colicin H; *astA*, enteroaggregative *E. coli* heat-stable enterotoxin; *lpfA*, long polar fimbriae; *cba*, colicin B activity protein; *cma*, colicin M activity protein; *cva/cvi*, colicin V operon; *iucABCD*, aerobactin synthesis; *hlyF*, avian hemolysin; *ompT*, outer membrane protease; *iutA*, ferric aerobactin receptor; *ibeA*, invasion of brain endothelium; *vat*, vacuolating autotransporter toxin; *irp2*, iron-repressible protein; *bor*, bacteriophage lambda bor protein; *air*, enteroaggregative immunoglobulin repeat protein; *eilA*, salmonella HilA homolog; *papC*, pilus associated with pyelonephritis; *tsh*, temperature-sensitive hemagglutinin; APEC, avian pathogenic *E. coli*; NMEC, neonatal meningitis *E. coli*; ExPEC, extraintestinal pathogenic *E. coli*; UPEC, uropathogenic *E. coli*.
[b]APEC- and NMEC-associated virulence genes are underlined.

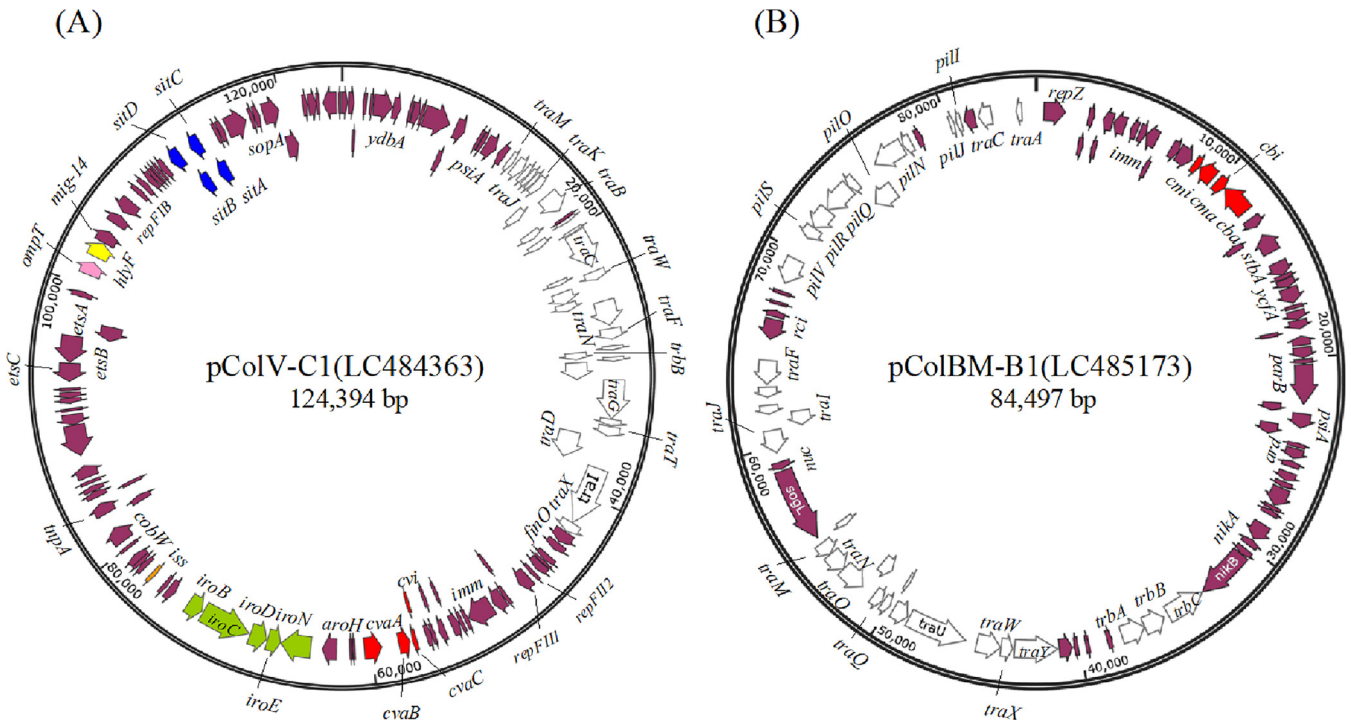

FIG 2 Circular genetic maps of plasmids carrying APEC-associated virulence genes. pColV-C1 (GenBank accession number LC484363) harbored by *E. coli* C1 (A). The APEC-associated virulence genes *cva/cvi*, *iroBCDEN*, *sitABCD*, *hlyF*, *ompT*, and *iss* are shown in red, green, blue, yellow, pink, and orange, respectively. The *tra/trb* conjugation genes are marked in white. pColBM-B1 (GenBank accession number LC485173) harbored by *E. coli* B1 (B). The colicin B and M operon, including *cba* and *cma* genes, is shown in red. The *tra/trb* and *pil* conjugation genes are marked in white.

GimA and *mat* on the chromosome in addition to the ColV plasmid. In contrast, *mcr* genes were not detected among 73 bla_{CTX-M}-harboring *E. coli* isolates containing no ColV plasmid recovered from untreated inflow samples of the same WWTPs, namely, A, B and C, and an additional WWTP (unpublished data). Thus, in an environment of municipal wastewater, the *mcr-1* gene was found to be frequently associated with a non-ESBL-producing *E. coli*-carrying ColV plasmid.

A limitation of our study is that our approach cannot show the prevalence of *mcr* genes in wastewater sources since colistin-containing medium was used for selecting colistin-resistant bacteria. Thus, colistin-susceptible *mcr*-positive isolates were not detected (33). Our sample size is also small; however, the strength of this study was that seven *mcr-1*-positive *E. coli* isolates were detected from six different inlets of three different WWTPs in a country where *mcr* genes are not endemic. Notably, APEC-associated virulence genes were identified in five of those seven isolates.

Recent extremely unusual weather conditions due to climate change have resulted in frequent torrential rains and floods, which can trigger combined sewer overflow (CSO) events. The presence of antimicrobial residues, antimicrobial-resistant bacteria, and antimicrobial resistance genes has been reported in the municipal sewage system (34, 35). Thus, the CSOs discharge raw sewage containing resistant bacteria directly from manholes onto surface streets or into rivers, posing serious environmental and public health risks (36). This study highlights the importance and urgency of action to reduce environmental contamination by *mcr-1*-harboring *E. coli* as well as *mcr-1*-harboring plasmids that may likely occur due to CSOs in Japan, whereas *mcr*-type genes have been detected rarely in isolates from human and food animals, and in only one strain from wastewater (17, 19, 20).

Conclusion. This study reports the copresence of *mcr-1* and APEC-associated virulence genes in five of seven *E. coli* isolates recovered from aquatic environments in Japan. Our study revealed that the *mcr-1* gene resided exclusively on the two leading Inc types of plasmids harbored by genetically diverse lineages of *E. coli*. Those *E. coli*

strains were characterized negative for ESBL genes but positive for APEC-associated virulence genes. The wastewater environment could also become a potential incubator for *mcr*-carrying microbes, although livestock have been considered their important reservoir.

MATERIALS AND METHODS

Sampling of wastewater influents. One influent sample each was collected from different inlets of three municipal wastewater treatment plants (WWTPs), namely, A (2 inlets), B (2 inlets), and C (three inlets), in Nagano Prefecture, Japan, during August and December 2018. Those WWTPs, A, B, and C, serve approximately 134,000 people in Nagano City, 95,000 people in Azumino City, and 125,000 people in Matsumoto City, respectively, for treating wastewater from household and industrial sources but not from livestock farms. About 500 ml each of WWTP influent samples was collected in sterile glass bottles, which were transported rapidly under cooling conditions to our laboratory and were stored at 4°C until being processed within 3 hours after obtaining the samples.

Detection of colistin-resistant *E. coli* and screening of *mcr* genes. One milliliter of each well-mixed influent sample was inoculated in a tube containing 9 ml of brilliant green lactose bile broth (BGLB broth; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 2 mg/liter colistin (Fujifilm Wako Pure Chemical Co., Osaka, Japan), which was incubated overnight at 37°C. Then, 10 μl of the broth was plated onto 2-mg/liter colistin-supplemented CHROMagar ECC agar (Kanto Chemical Co., Tokyo, Japan), and incubated at 37°C for 18 to 24 hours. For each influent sample, all colonies exhibiting the typical *E. coli* morphological appearance were subjected to matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany) using the Bruker BioTyper database and software, version 3.1 (Bruker Daltonics), for confirming the bacterial species. The score cutoff of $\geq$2.000 was applied for species-level identification according to the manufacturer's recommendation. Those *E. coli* isolates were screened for the presence of *mcr-1*, *mcr-2*, and *mcr-3* by PCR as described previously (3, 37, 38).

Antimicrobial susceptibility testing. This testing was performed by the broth microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI) using dry plate DP31 (Eiken Chemical Co., Tokyo, Japan), and the results were interpreted according to CLSI M100 28th edition guidelines except for colistin (39). The MIC of colistin was determined by an in-house-prepared panel according to the CLSI broth microdilution method and was interpreted using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines, namely, $\leq$2 and $>$2 mg/liter for colistin susceptible and resistant, respectively (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_9.0_Breakpoint_Tables.pdf).

Transformation experiments. Transformation experiments were conducted by electroporation with *E. coli* DH10B as the recipient strain, and plasmid DNA was extracted using a Kado and Liu method (40). Transformants were selected on LB agar plates supplemented with 2 mg/liter colistin. The antimicrobial susceptibility of the transformants was determined by the broth microdilution method, and the presence of the *mcr-1* gene was confirmed by PCR and sequencing.

***E. coli* strain typing.** Phylogenetic grouping was performed by multiplex PCR targeted at four genetic markers, namely, *chuA*, *yjaA*, TspE4.C2, and *arpA*, allowing the isolates to be classified into seven main phylogenetic groups, namely, A, B1, B2, C, D, E, and F, and cryptic clade I as described previously (41).

Multilocus sequence typing (MLST) was carried out using seven housekeeping genes of *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*, and ST was assigned according to the *E. coli* MLST website (http://enterobase.warwick.ac.uk/species/ecoli/allele_st_search). If *E. coli* isolates sharing the same phylogroup and ST were detected from the same sample, one representative isolate was included in the study analysis.

A multiplex PCR was used to detect virulence factors for ExPEC and UPEC (42). Pathotypes of *E. coli* isolates were presumed to be ExPEC if positive for $\geq$2 of 5 genetic markers, including *papAH* and/or *papC*, *sfa/focDE*, *afa/draBC*, *kpsM*II, and *iutA*, and to be UPEC if positive for $\geq$3 of 4 genetic markers, including *chuA*, *fyuA*, *vat*, and *yfcV*.

WGS analysis. Genomic DNA was extracted using the Wizard genomic DNA purification kit (Promega, Madison, WI). After library preparation using the NEBNext Ultra DNA library prep kit (New England BioLabs, Ipswich, MA), whole-genome sequencing (WGS) analysis of 150-bp paired-end sequencing was performed on the HiSeq platform (Illumina, San Diego, CA). The raw reads were *de novo* assembled into contigs using the A5-miseq assembly pipeline (43). Those assembled contigs were subsequently queried with MLST 2.0, SeroTypeFinder 2.0, FimTyper 1.0, PlasmidFinder 2.0, ResFinder 3.1, and VirulenceFinder 2.0 available from the Center for Genomic Epidemiology (http://www.genomicepidemiology.org) for MLST, serotyping, *fimH* typing, plasmid replicon typing, antimicrobial resistance gene identification, and virulence gene identification, respectively. Annotation of the resulting scaffolds was performed by using the DDBJ Fast Annotation and Submission Tool (DFAST) (https://dfast.nig.ac.jp/) (44). An in-depth exploration of APEC- and NMEC-associated virulence genes was performed manually on WGS data.

Plasmid sequencing. Plasmid DNA was extracted from *E. coli* DH10B transformants harboring the *mcr-1* plasmid with the PureYield plasmid midiprep kit (Promega) and was subjected to pulsed-field gel electrophoresis. Plasmid DNA was excised from the gel and purified using the Wizard SV gel and PCR clean-up system (Promega), which was used to prepare sequencing libraries using the Nextera XT DNA sample preparation kit (Illumina). Next-generation sequencing analysis of 150-bp paired-end sequencing

was performed on the MiSeq platform (Illumina). The contigs obtained after *de novo* assembly as described above were subjected to gap-closing PCR and Sanger sequencing to determine the complete nucleotide sequence. The obtained sequence data were submitted to the DFAST for annotation. Easyfig (http://mjsull.github.io/Easyfig/) was used for the genetic comparison of plasmids.

Accession number(s). The complete nucleotide sequences of plasmids pA1, pA2, pB1, pB2, pC1, pC2, and pC3 harboring *mcr-1* genes described in this paper have been deposited at GenBank under the accession numbers LC477138, LC477294, LC479452, LC479085, LC469775, LC473131, and LC479086, respectively. The complete nucleotide sequences of plasmids pColV-C1 and pColBM-B1 have been deposited under the GenBank accession numbers LC484363 and LC485173, respectively.

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We declare no conflicts of interest.

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