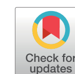
Environment International 130 (2019) 104708

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Integrated aquaculture contributes to the transfer of *mcr-1* between animals and humans via the aquaculture supply chain



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ARTICLE INFO

Keywords:
Colistin
Prevalence
Transmission
mcr-1
E. coli
Aquaculture

ABSTRACT

Background: Since its discovery in 2015, the mobile colistin resistance gene *mcr-1* has been reported in bacteria from > 50 countries. Although aquaculture-associated bacteria may act as a significant reservoir for colistin resistance, systematic investigations of *mcr-1* in the aquaculture supply chain are scarce.
Objectives: We investigated the presence of colistin resistance determinants in the aquaculture supply chain in south China and determined their characteristics and relationships.
Methods: A total of 250 samples were collected from a duck-fish integrated fishery, slaughter house, and market in Guangdong Province, China, in July 2017. Colistin-resistant bacteria were isolated on colistin-supplemented CHROMagar Orientation plates, and the species were identified by matrix-assisted laser desorption/ionization time-of-flight assay. The presence of *mcr* genes was confirmed by polymerase chain reaction analysis. We examined the minimum inhibitory concentrations (MICs) of 16 antimicrobial agents against the isolates using agar diffusion and broth microdilution methods. Whole-genome sequencing (WGS) was used to explore the molecular characteristics and relationships of *mcr-1*-positive *Escherichia coli* (MCRPEC).
Results: Overall, 143 (57.2%) colistin-resistant bacteria were isolated, of which, 56 (22.4%, including 54 *Escherichia coli* and two *Klebsiella pneumoniae*) and four *Aeromonas* species were positive for *mcr-1* and *mcr-3*, respectively. The animal-derived MCRPEC were significantly more prevalent in integrated fishery samples (40.0%) than those in market (4.8%, $P < 0.01$) samples but not in slaughter house (28.0%, $P = 0.164$). All MCRPEC were highly resistant to ampicillin, tetracycline, and compound sulfamethoxazole (> 90%) but were susceptible to carbapenems and tigecycline. WGS analysis suggested that *mcr-1* was mainly contained on plasmids, including IncHI2 (29.6%), IncI2 (27.8%), IncX4 (14.8%), and IncP (11.1%). Genomic analysis suggested *mcr-1* transmission via the aquatic food chain.
Conclusions: MCRPEC were highly prevalent in the aquaculture supply chain, with the isolates showing resistance to most antibiotics. The data suggested *mcr-1* could be transferred to humans via the aquatic food chain. Taking the "One Health" perspective, aquaculture should be incorporated into systematic surveillance programs with animal, human, and environmental monitoring.

1. Introduction

Since first being reported in *Escherichia coli* from China, the plasmid-mediated colistin resistance determinant *mcr-1* has been identified worldwide in various Enterobacteriaceae from humans, animals, and the environment (Liu et al., 2016; Wang et al., 2017a). Subsequently, an additional seven colistin resistance genes, *mcr-2* to *mcr-8*, have been reported, and are currently the subject of substantial international

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https://doi.org/10.1016/j.envint.2019.03.056
Received 2 January 2019; Received in revised form 25 March 2019; Accepted 25 March 2019
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attention (AbuOun et al., 2017, 2018; Borowiak et al., 2017; Carattoli et al., 2017; Partridge et al., 2018; Wang et al., 2018; Xavier et al., 2016; Yang et al., 2018; Yin et al., 2017). Despite the origins of the *mcr* genes remaining mysterious, the aquatic environment, particularly the aquaculture industry, is the leading contender for being the reservoir of colistin resistance determinants (Cabello et al., 2017; Shen et al., 2018b). Several studies have isolated *mcr-1*-positive *E. coli* (MCRPEC) and *mcr-3*-positive *Aeromonas* from the aquatic environment (Shen et al., 2018b), while a comprehensive resistome analysis has revealed a possible transmission route for *mcr-1* via the broiler chicken supply chain (Wang et al., 2017b). Recently, a significant correlation between aquaculture and aquatic food products and a high prevalence of MCRPEC in human intestinal samples was identified (Shen et al., 2018c). However, biological evidence supporting the transmission of *mcr-1* between animals and humans via the aquaculture supply chain is lacking.

Colistin is a last-line antibiotic for treating Gram-negative bacterial infections in a clinical setting; however, since the late 1980s it has also been used in massive quantities as a feed additive in food-animal production in China. Given the high mobility of *mcr* genes under the selective pressure provided by colistin use, the Chinese Ministry of Agriculture banned colistin as a feed additive (e.g., growth promoter) in food animals in April 2017 (Walsh and Wu, 2016). While colistin is not officially used in aquaculture in China, the aquatic environment can be contaminated with colistin through farm runoff carrying animal feces containing residual colistin (Shen et al., 2018c). A previous study isolated seven (3.65%, 7/192) MCRPEC strains from grass carp purchased from fish markets in Guangdong Province, one of the main aquaculture areas in China, and the *mcr-1* genes were located in five conjugative plasmids (2 IncI2, 2 IncP and 1 IncX4), both IncI2 and IncX4 plasmids were dominant carriers of *mcr-1* of various origin (Lv et al., 2018). However, systematic investigations of colistin resistance among bacteria in the aquaculture supply chain, along with analyses of the relationships among isolates from different sectors, including human carriage, are still lacking. Notably, the unique duck-fish integrated fishery system is prevalent in Guangdong Province, which allows for direct transfer of antibiotic-resistant bacteria between ducks and fish.

Therefore, we investigated the prevalence of colistin resistance among bacteria isolated from the aquaculture supply chain, including a duck-fish integrated fishery operation, a slaughter house, and a market in Boluo County, Guangdong Province, China. Furthermore, to decipher the molecular epidemiology of *mcr-1*-positive isolates, the genomic characteristics of these isolates were defined by whole-genome sequencing (WGS). In addition, we assessed the possibility of *mcr-1* transfer from animals to humans via the aquaculture supply chain.

2. Methods

2.1. Sample collection and identification

We undertook an observational study to investigate the prevalence of colistin resistance determinants in the aquaculture supply chain. A schematic diagram of our study design is shown in Fig. S1. We first targeted a slaughter house, then traced its upstream integrated fishery and its downstream market. Between the 20th and the 25th July 2017 we collected 250 non-duplicate biological samples from a duck-fish integrated fishery (duck rectal swabs, $n = 66$; fish intestinal contents, $n = 14$; soil, $n = 10$; water, $n = 10$; duck fodder, $n = 10$; flies, $n = 28$), a slaughter house (duck carcasses, $n = 50$), and a market (duck meat, $n = 39$; fish, $n = 23$) in Boluo County, an area rich in fisheries located about 120 km from Guangzhou, the capital city of Guangdong Province (Fig. S2). The samples were enriched in Luria-Bertani broth (Luqiao, Beijing, China) at 37 °C for 24 h without shaking before being inoculated onto CHROMagar Orientation plates (CHROMagar, Paris, France) containing 2 mg/L colistin and incubated at 37 °C for a further 24 h. Single colistin-resistant clones representing each of the observed colony morphologies of Enterobacteriaceae ($n = 143$) were collected from the plates and screened for the presence of *mcr-1* to *mcr-8* by polymerase chain reaction (PCR) using previously described primers listed in Table S1 (AbuOun et al., 2017; Borowiak et al., 2017; Carattoli et al., 2017; Liu et al., 2016; Wang et al., 2018; Xavier et al., 2016; Yang et al., 2018; Yin et al., 2017). Species identification was carried out by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonik, Bremen, Germany). Due to the small obtained numbers of *mcr-1*-positive *Klebsiella pneumoniae* ($n = 2$) and *mcr-3*-positive *Aeromonas* species ($n = 4$), our further analyses exclusively focused on *mcr-1*-positive *E. coli*. For genomic analyses, we downloaded the whole-genome sequences of MCRPEC isolates of human origin collected from Guangzhou City from the BioProject database (No. PRJNA354216, $n = 70$) (Zhong et al., 2018), along with the WGS of human MCRPEC isolates from Guangzhou City previously isolated by our laboratory (BioProject number PRJNA400107, $n = 12$) (Shen et al., 2018c).

2.2. Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of 16 antimicrobial agents (ampicillin, amoxicillin/clavulanic acid, ceftiofur, ceftriaxone, cefepime, imipenem, meropenem, gentamicin, polymyxin B, colistin, ciprofloxacin, florfenicol, fosfomycin, tetracycline, tigecycline, and trimethoprim/sulfamethoxazole) against all 54 MCRPEC isolates were determined using the agar dilution except polymyxin B, colistin, and tigecycline were tested by broth microdilution methods, with *E. coli* ATCC 25922 used as a quality control strain. For agar dilution, MHA (Mueller-Hinton Agar, Luqiao, Beijing) plates containing appropriate concentration of antimicrobial agents were prepared immediately before using, and approximate 0.5 McFarland of strains were inoculated onto plates by adaptors for 16–20 h in 35 °C ± 2 °C under ambient air condition. For broth microdilution method, about 50 μL fresh CA-MHB (Cation-Adjusted Mueller-Hinton Broth, Luqiao, Beijing) containing appropriate concentration of antimicrobial agents was serially diluted by two folds in the 'U'-plate, and 50 μL approximate 0.5 McFarland of strains were added into broth in total 100 μL volume, subsequently the mixture was cultured for 16–20 h in 35 °C ± 2 °C under ambient air condition. The breakpoints were interpreted according to the European Committee on Antimicrobial Susceptibility Testing clinical breakpoints (version 8.0) (EUCAST, 2018) and the Clinical and Laboratory Standards Institute document M100 (28th edition) (CLSI, 2018a). Results for the veterinary medicines, including florfenicol and ceftiofur, were interpreted according to the CLSI document VET08 (CLSI, 2018b).

2.3. Genomic analysis

Genomic DNA was extracted from the 54 MCRPEC isolates using a Wizard Genomic DNA Purification Kit (Promega, Beijing, China) according to manufacturer's instructions. Indexed DNA libraries were constructed using a KAPA HyperPrep Kit for Illumina platforms (Roche, Basel, Switzerland) with standard protocols, and were sequenced on the Illumina HiSeq X Ten platform (Annoroad, Beijing, China). All 54 draft MCRPEC genomes, along with short reads downloaded from BioProject (BioProject number PRJNA354216), were assembled using SPAdes version 3.9.0 (Bankevich et al., 2012). In silico phylotyping was carried out using the ClermonTyping method (Beghain et al., 2018), while multilocus sequence typing (MLST) and identification of resistance genes and virulence-associated genes were conducted using SRST2 Toolkit version 0.2.0 (Inouye et al., 2014). The plasmid type (Inc type) of *mcr-1*-carring contigs was determined using PlasmidFinder version 1.3 (Carattoli et al., 2014), with very short contigs and those not containing a replicon marker classified as having an unknown type. Comparison of genetic context in the different *mcr-1*-carrying plasmids was undertaken using BLAST Ring Image Generator (Alikhan et al., 2011). A minimum spanning tree was generated in BioNumerics version 7.0

(Applied Maths, Sint-Martens-Latem, Belgium) using the BURST algorithm to identify related sequence types in the different backgrounds. A phylogenetic tree based on the core genome sequences of the isolates was constructed using Harvest version 1.1.2 (Treangen et al., 2014), with the corresponding characteristics of each isolate visualized using online tool iTOL version 4 (Letunic and Bork, 2016). The population structure of the phylogenetic tree was defined using hierBAPS version 6.0 (Cheng et al., 2013). All WGS data obtained in this study have been deposited in the BioProject database under BioProject number PRJNA503777.

2.4. Statistical analyses

Statistical analyses were performed using R 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria). The prevalence of MCRPEC in different sectors or from different origins was compared using a chi-square test or Fisher's exact test, with significance accepted at $P < 0.05$. The confidence intervals (CI) of prevalence were computed using an Exact binomial test.

3. Results

3.1. High detection rate of MCRPEC

To investigate the prevalence of colistin resistance determinants in the aquaculture supply chain, we collected a total of 250 samples, including ducks, fish, and environmental samples, from a duck-fish integrated fishery (referred to hereafter as "fishery"), a slaughter house, and a market in Guangdong Province, from which 143 (57.2%, 95% CI: 50.8–63.4) colistin-resistant isolates were obtained including 78 (54.5%) *E. coli*, 21 (14.7%) *K. pneumoniae*, 16 (11.2%) *Aeromonas* sp., and 28 (19.6%) other Enterobacteriaceae (Table S2). These isolates were individually cultured and screened for the presence of *mcr-1* to *mcr-8*, with 56 (22.4%, 95% CI: 17.4–28.1) isolates, including 54 *E. coli* and two *K. pneumoniae* isolates, testing positive for *mcr-1.1*. A further four (1.6%, 95% CI: 0.4–4.0) isolates, all identified as *Aeromonas* sp., were positive for *mcr-3*, while none of the other *mcr* variant genes was detected in this study (Table 1). Among the fishery samples, MCRPEC were significantly more prevalent in samples collected from ducks compared with those from fish (45.5% vs. 14.3%, $P < 0.01$). MCRPEC were also significantly more prevalent in animal-based samples from the fishery compared with those from the market (40.0% vs. 4.8%, $P < 0.01$), while no significant differences in prevalence were observed between fishery and slaughter house samples (40.0% vs. 28.0%, $P = 0.164$). Interestingly, although the percentage of colistin-resistant isolates from fish was quite high (92.9% in the fishery and 60.9% in the market), the incidence of *mcr* genes in these isolates was almost zero (only two MCRPEC isolated from farmed fish and one *mcr-3*-positive *Aeromonas* isolate from a market sample).

3.2. Minimum inhibitory concentration profiles of MCRPEC

Antimicrobial susceptibility testing using 16 antimicrobial agents showed that almost all MCRPEC isolates were resistant to ampicillin, tetracycline, trimethoprim/sulfamethoxazole, and ciprofloxacin with resistance rates of 96.3%, 96.3%, 98.1%, and 81.5%, respectively. This finding is consistent with the antibiotics used in Chinese aquaculture (Standard No. SC/T 1083-2006, 1084-2006, and 1085-2006, issued by the Ministry of Agriculture of China). Similarly, high rates of resistance to florfenicol (74.1%), a drug used only in the animal husbandry industry, were identified among the MCRPEC isolates. Rates of resistance to the fourth-generation cephalosporin cefepime were significantly lower than those to the third generation cephalosporins such as ceftiofur and ceftriaxone (25.9% vs. 75.9% and 72.2%, respectively, $P < 0.01$). Only three MCRPEC isolates were resistant to amoxicillin/clavulanic acid. All isolates were susceptible to carbapenems and tigecycline (Table 2).

3.3. Genomic epidemiology of MCRPEC

To determine the genotype of the MCRPEC isolates, we performed MLST and ClermontTyping (also known as phylogroup) using the WGS data. MLST data for the 54 MCRPEC isolates was used to generate a minimum spanning tree, which separated the isolates into 34 clades. Distinct sequence types (STs) were associated with the different sectors, with a high level of genetic diversity observed overall (Fig. 1 and Table S3). ST93 ($n = 5$), ST162 ($n = 4$), and ST156 ($n = 3$) were the most prevalent STs among the fishery isolates and were unique to isolates from this sector. ST48 and ST648 isolates were found in both fishery and market samples, indicating a possible association between the isolates from these two sources (Fig. 1). ClermontTyping classified 31 (57.4%) and 12 (22.2%) of the isolates into groups A and B1, respectively, which are usually associated with commensal strains (Fig. 2 and Table S3). Isolate 772 was assigned to a subspecies of *E. coli*, classified as clade I, making it highly divergent from the other strains (Fig. 2).

A phylogenetic tree based on the core genome was constructed to assess the relationships among the MCRPEC isolates in the aquaculture supply chain. Bayesian analysis of the population structure (BAPS) was used to define the lineages of the 54 MCRPEC isolates, and revealed four distinct lineages. The main lineage, designated lineage IV, included 31 (57.4%) isolates all belonging to phylogroup A from fishery, slaughter house, and market samples. The next most prominent lineage was lineage III, containing 16 (29.6%) isolates (Fig. 2). Except for one isolate, which belonged to lineage I, all of the MCRPEC isolates from the different sectors fell into the three main lineages, indicating no obvious correlation between lineages and sectors. Notably, three small clusters, C3, C4, and C5, contained several duck-derived MCRPEC isolates with a high degree of whole-genome sequence similarity (pairwise single nucleotide polymorphisms (SNPs) ≤ 7, as shown in Table S4). Similarly,

Table 1
Prevalence of colistin-resistant isolates and *mcr-1/3*-positive isolates in samples from the aquaculture supply chain.

Sample site	Sample type	Number (%, 95 CI)			
		Number of Samples	Colistin resistant isolates	*mcr-1*-positive isolates	*mcr-3*-positive isolates[c]
Integrated fishery	Duck	66	45 (68.2, 55.6–79.1)	32 (48.5, 36.0–61.1)[b]	0
	Fish	14	13 (92.9, 66.1–99.8)	2 (14.3, 1.8–42.8)	0
	Environment[a]	58	32 (55.2, 41.5–68.3)	5 (8.6, 2.9–19.0)	1 (1.7, 0.0–9.2)
Slaughter house	Carcass of duck	50	28 (56.0, 41.3–70.0)	14 (28.0, 16.2–42.5)	2 (4.0, 0.5–13.7)
Market	Duck meat	39	11 (28.2, 15.0–44.9)	3 (7.7, 1.6–20.9)	0
	Fish	23	14 (60.9, 38.5–80.3)	0	1 (4.3, 0.1–21.9)
Total		250	143 (57.2, 50.8–63.4)	56 (22.4, 17.4–28.1)	4 (1.6, 0.4–4.0)

[a] Environmental samples included soil, flies, water, and fodder. Three *mcr-1*-positive and one *mcr-3*-positive isolates were obtained from water. Two *mcr-1*-positive isolates were obtained from soil.
[b] The two *mcr-1*-positive isolates were identified as *Klebsiella pneumoniae*.
[c] All *mcr-3*-positive isolates were identified as *Aeromonas* species.

Table 2
Minimum inhibitory concentration profiles of *mcr-1*-positive *Escherichia coli* isolates from the aquaculture supply chain (n = 54).

Antibiotics	Range	MIC50	MIC90	Resistance isolates (%, 95%CI)
Ampicillin	2, ≥512	128	256	52 (96.3, 87.3–99.5)
Amoxicillin/clavulanic acid	1/0.5, 64/32	8/4	16/8	3 (5.6, 1.2–15.4)
Ceftiofur	0.5, ≥512	32	512	41 (75.9, 62.4–86.5)
Ceftriaxone	0.06, ≥512	64	512	39 (72.2, 58.4–83.5)
Cefepime	0.03, ≥512	2	32	14 (25.9, 15.0–39.7)
Imipenem	0.125, 0.25	0.125	0.125	0
Meropenem	0.015, 0.03	0.015	0.03	0
Polymyxin B	4, 8	4	4	54 (100)
Colistin	4, 8	4	8	54 (100)
Gentamicin	0.125, ≥512	16	128	27 (50.0, 36.1–63.9)
Ciprofloxacin	0.125, 128	64	64	44 (81.5, 68.6–90.7)
Florfenicol	4, 256	128	128	40 (74.1, 60.3–85.0)
Fosfomycin	1, ≥512	16	> 512	17 (31.5, 19.5–45.6)
Tetracycline	2, ≥512	128	256	52 (96.3, 87.3–99.5)
Tigecycline	0.06, 2	0.25	1	0
Trimethoprim/sulfamethoxazole	1/19, 8/152	4/76	8/152	53 (98.1, 90.1–100)

isolates from slaughter house samples within clusters C1, C6, and C7 showed very few differences (with 1, 5 and 0 SNPs, respectively). Intriguingly, in cluster C2, two MCRPEC isolates from duck and fish samples were classified as ST156 and phylogroup B1, and showed 100% identity to each other (SNPs = 0) (Fig. 2, Table S4).

3.4. Genomic characteristics of MCRPEC

Acquired resistance genes (ARGs) were screened using WGS data to examine the resistance genotypes of the MCRPEC isolates from the aquaculture supply chain. All 54 isolates harbored at least one category of β-lactam resistance gene, including *ampC*, bla_{SHV}, bla_{TEM}, bla_{OXA}, and bla_{CTX-M}. In contrast, all MCRPEC isolates were negative for carbapenemase-producing genes. Tetracycline resistance genes *tet*(A) and *tet*(B) were detected in 52 (96.3%) isolates, which was consistent with phenotype data. Similarly, 49 (90.7%) and 51 (94.4%) isolates carried trimethoprim and sulfonamide-family resistance genes *dfrA* and *sul*, respectively. Furthermore, only 10 (18.5%) MCRPEC isolates were negative for plasmid-mediated quinolone resistance genes *qnr* or *oqxA/B* (Fig. 2 and Table S5).

Virulence-associated factors (VAGs) play an important role in fitness and pathogenicity of *E. coli*. Therefore, we analyzed all VAGs-ARGs combinations to access the pathogenicity of MCRPEC isolates from aquaculture. VAGs from pathogenic *E. coli*, such as enterotoxigenic, enteropathogenic, enteroaggregative, enteroinvasive, enterohemorrhagic, and diffusely adherent *E. coli*, were rarely found in the aquaculture-derived MCRPEC, and were also absent in the two phylogroup D isolates, which is usually considered a pathogenic phylotype (Salipante et al., 2015). Moreover, very few toxin-related genes were identified in these isolates (Fig. 2 and Table S6).

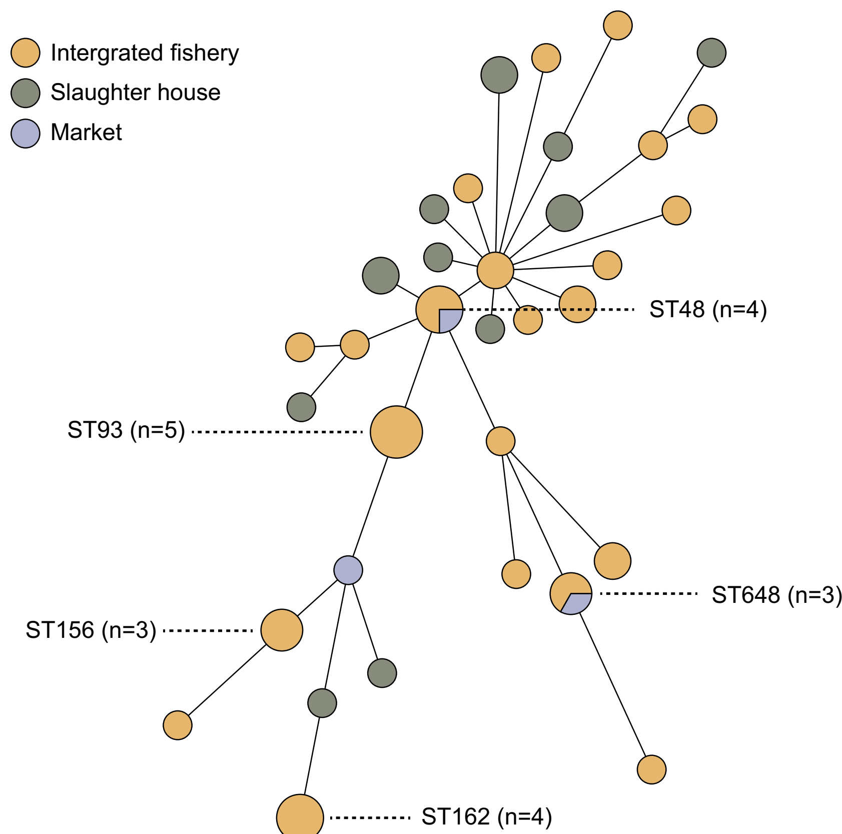


Fig. 1. Minimum spanning tree of MCRPEC from the aquaculture supply chain generated from multilocus sequence typing data. Each node represents a distinct sequence type (ST). Different colors indicate different origins of isolates. Node size is proportional to the number of isolates represented by said node. Selected nodes are labeled with corresponding ST and number of isolates (at least three).

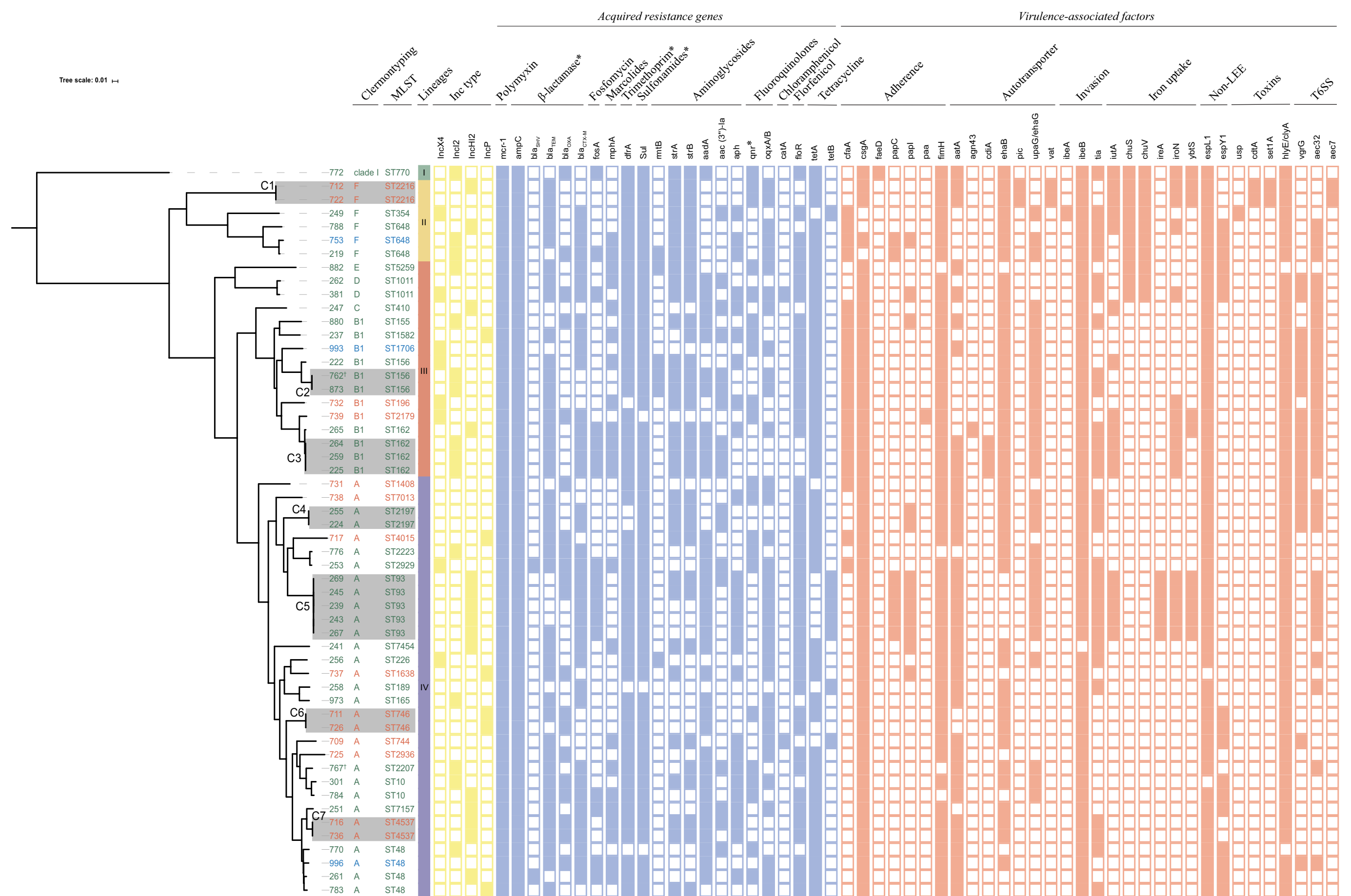


Fig. 2. Phylogenetic tree of all MCRPEC isolates from the aquaculture supply chain. The tree was constructed using core-genome single nucleotide polymorphism (SNP) data and is rooted by midpoint. The sources of the isolates are differentiated by the color of the name label (green, integrated fishery; red, slaughter house; blue, market), and the isolate names are marked with a dagger indicating fish origin. Clermont type and multilocus sequence type are shown in text after the corresponding isolate name, and lineages are denoted by colored stripes. The molecular characteristics of each isolate, including Inc. type, resistance genes, and virulence-associated genes, are denoted by filled squares for presence and empty squares for absence. Each column is annotated with the corresponding genes and their functions, and genes or antibiotics marked with an asterisk represent a family of genes. Closely related isolates within a sub-cluster are highlighted with a grey background and clusters are labeled C1–C7. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The presence and genetic location of *mcr-1* was determined by BLAST analysis and mapping using the WGS data. As expected, *mcr-1* was mainly contained on plasmids (49/54, 90.7%) in the aquaculture-derived isolates, similar to other strains from humans or poultry (Shen et al., 2018a; Shen et al., 2018c; Wang et al., 2017b). However, *mcr-1* was found on the chromosome in three isolates, while the location could not be determined for four isolates, most likely as a result of very short *mcr-1*-carrying contigs (< 3 Kb) that were too short for reliable analysis. Among the *mcr-1*-carrying plasmids, IncHI2 (16/54, 29.6%) and IncI2 (15/54, 27.8%) and were the most prevalent types, followed by IncX4 (8/54, 14.8%), IncP (6/54, 11.1%), and Incp0111 (2/54, 3.7%) (Fig. 2 and Table S3).

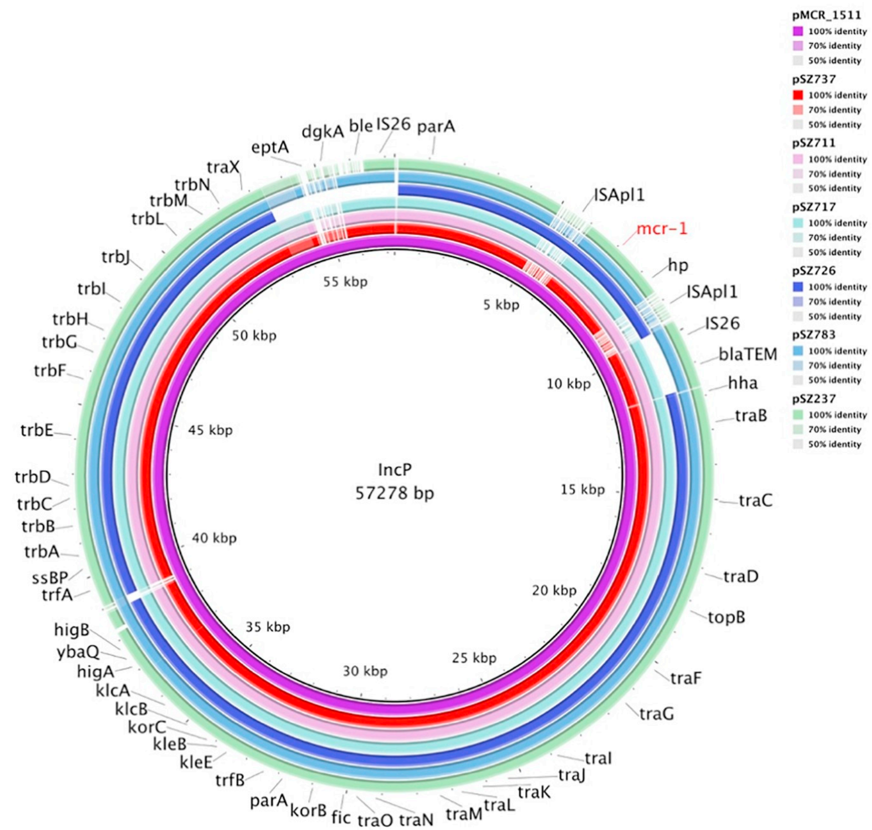
3.5. Genetic context of *mcr-1*-carring plasmids

The genetic context of *mcr-1* was conserved within each plasmid type (IncHI2, IncI2, IncX4, IncP, and Incp0111) depending on its backbone structure (Fig. 3). The insertion sequence IS*Apl1* was located upstream of *mcr-1* in six IncI2-plasmids, which is similar to the first-reported *mcr-1*-carring plasmid, pHNSHP45, found in a pig-derived *E. coli* isolate (GenBank accession number KP347127) (Fig. 3A). Likewise, a single IS*Apl1* element was identified upstream of *mcr-1* in 15 IncHI2-type plasmids (Fig. 3B). Only two IncP plasmids contained two copies of IS*Apl1* flanking *mcr-1*, while β-lactam resistance gene bla_{TEM} was found along with *mcr-1* in five IncP plasmids (Fig. 3C). Eight IncX4-plasmids showed almost 100% nucleotide sequence identity to pECGD-8-33 (GenBank accession number KX254343) from an *E. coli* isolate from pig feces in China (Fig. 3D). *mcr-1* was also adjacent to an IS*Apl1* element in an Incp0111-type plasmid, and the complete sequence of this plasmid was identical to that of pZR78 (GenBank accession number MF455226) from an *E. coli* isolate discovered in pig feces (Fig. 3E). IS*Apl1* was the only insertion sequence flanking *mcr-1* in the examined plasmids.

3.6. Associations between aquaculture and human gut isolates

To assess possible human transmission via the aquatic food chain, we downloaded the whole-genome sequences of MCRPEC isolates originating from human gut samples from Guangzhou and constructed a phylogenetic tree of MCRPEC from the various sources based on SNPs within core-genome genes (total ~320.4 Mb aligned, ~2.4 Mb per strain). In general, isolates from the same origin belonged to closed clusters exhibiting homologous genome characteristics (Fig. 4). Interestingly, related strains from aquaculture and human samples were observed in sub-clusters C8–C16, suggesting the transfer of MCRPEC between animals and human via the aquatic food chain. We detected 516–608 SNPs (mean = 539.5, 0.02%) between human isolate SYSU0007 and the genomes of four isolates (264, 265, 259, and 225) from duck samples from the fishery within sub-cluster C11, indicating a

C



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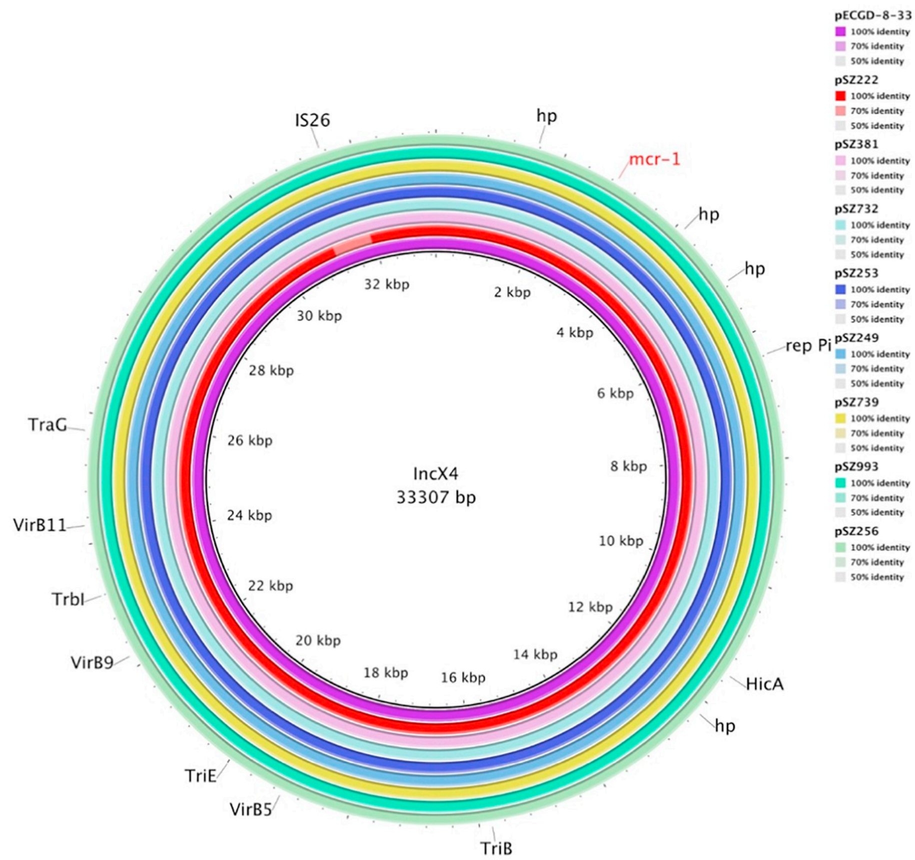
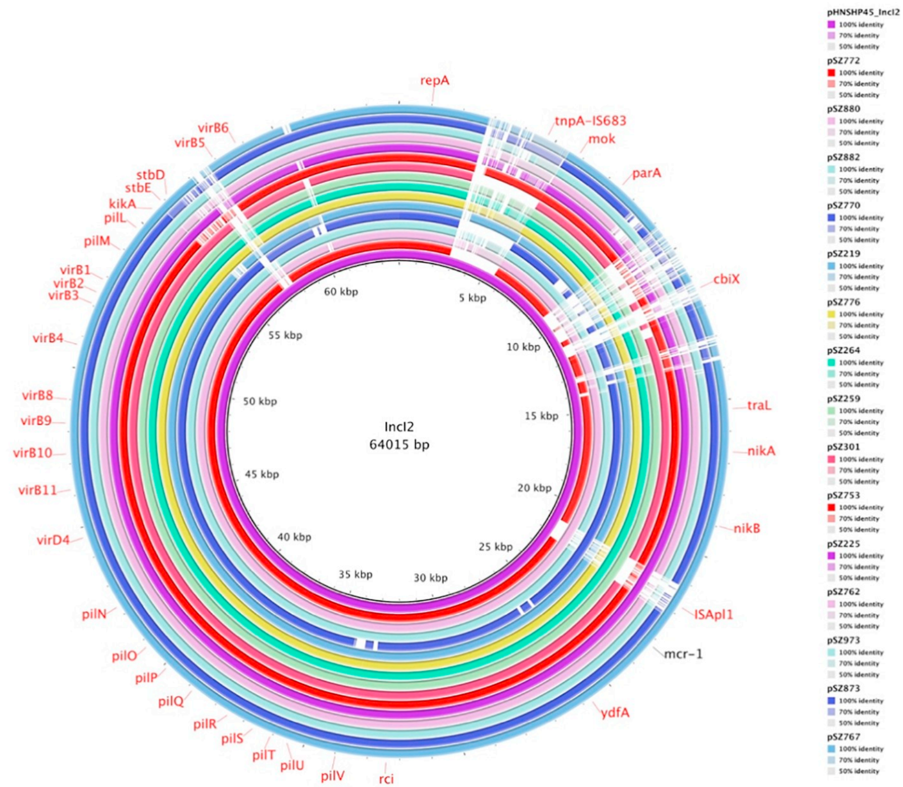


Fig. 3. (*continued*)

A



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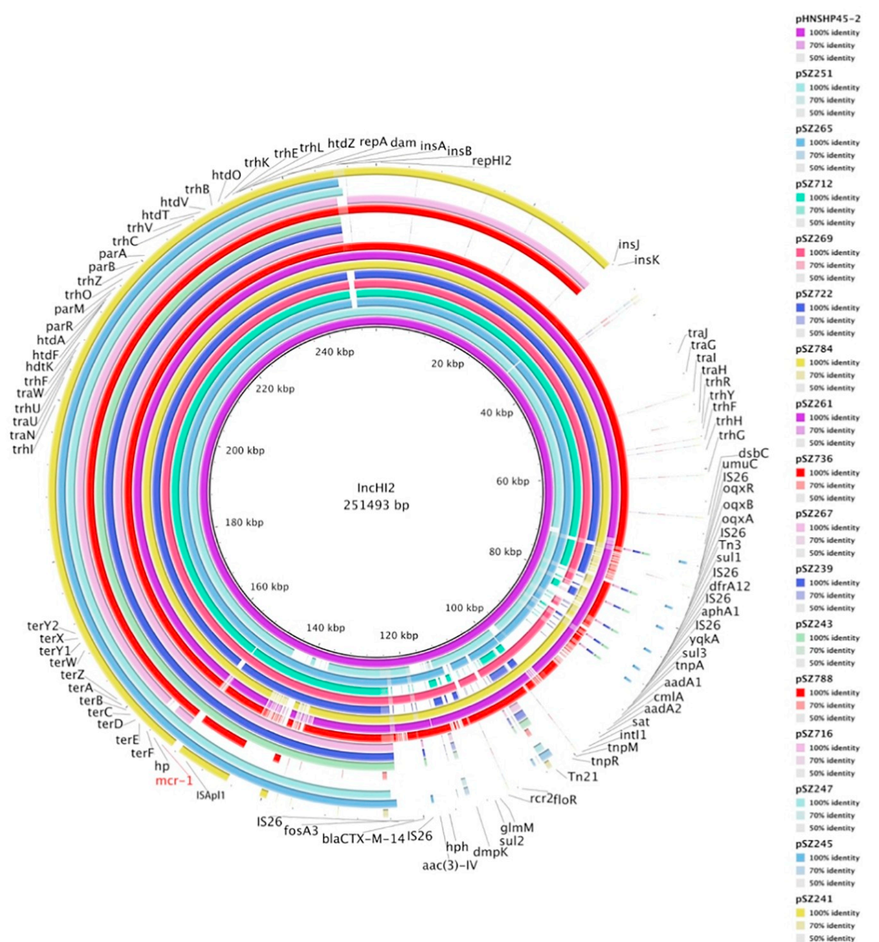

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Fig. 3. Circular alignments of reference plasmid sequences with homologous contigs from MCRPEC isolated in this study. Each ring represents the corresponding plasmids shown in each figure, and plasmid types are given in the center of the rings. (A) IncI2 plasmid (reference plasmid pHNSHP45, accession number KP347127). (B) IncHI2 plasmid (reference plasmid pHNSHP45-2, accession number KU341381). (C) IncP plasmid (reference plasmid pMCR_1511, accession number KX377410). (D) IncX4 plasmid (reference plasmid pECGD-8-33, accession number KX254343). (E) Incp0111 plasmid (reference plasmid pZR78, accession number MF455226).

high degree of similarity. Likewise, within sub-cluster C13, strain 222 from a duck sample from the fishery was highly homologous to five human isolates (SNPs ranging from 444 to 795, mean = 621, 0.03%). All isolates in C13 were classified as ST156, including isolates 762 and 873, which were slightly separate from the other strains in the same sub-cluster (Fig. 4 and Table S4). Close relationships between isolates from aquaculture samples and humans were found in several other sub-clusters, including C8, C9, C10, C12, C14, and C15, with a minimum SNP number of 504 (0.02% difference between isolates 381 and SYSU0093 in C9) and a maximum SNP number of 1601 (0.07% difference between isolates SYSU0062 and 249 in C8). Notably, in the slightly larger C16 sub-cluster, correlations were observed among isolates from the fishery, slaughter house, market, and human samples (SNPs ranging from 850 to 2690, mean = 1842 and difference ranging from 0.04%–0.11%) (Fig. 4 and Table S4).

4. Discussion

Although *mcr-1*-positive Enterobacteriaceae have been isolated from aquatic environments in several countries (Shen et al., 2018b), the datasets in these studies are relatively small and there has been no systematic analysis of MCRPEC in the aquaculture supply chain. In addition, while one publication reported seven (3.65%, 7/192) MCRPEC isolates from retail fish (Lv et al., 2018), the study did not describe the prevalence and association of the isolates in relation to the full aquaculture supply chain. However, the current study indicates that MCRPEC are highly prevalent in the aquaculture supply chain (averaging 21.6% of isolates from all samples), and that *mcr-1* is the predominant colistin resistance determinant in these samples. A previous study identified a strong association between the high prevalence of MCRPEC in aquaculture and human gut carriage (Shen et al., 2018c), with the current study providing biological evidence to support this. The prevalence of MCRPEC in duck samples was significantly higher than that in fish samples (45.5% vs. 14.3%, $P < 0.01$), indicating that the duck cloaca is a more favorable environment for MCRPEC than the fish intestine, which is consistent with the previously-observed low prevalence of MCRPEC in retail fish (Lv et al., 2018) but high prevalence in broiler chickens (Shen et al., 2016). However, we observed a high prevalence of colistin-resistant isolates in fish (92.9% in fishery and 60.9% in market samples), indicating the likelihood of additional resistant mechanisms other than *mcr* genes presented in the fish microbiota.

In terms of aquaculture culture modes, a previous study has shown a higher abundance of ARGs for tetracycline, β-lactam, sulfonamide, and erythromycin in sediments from integrated fishery operations compared to those from fish or duck ponds, suggesting a higher

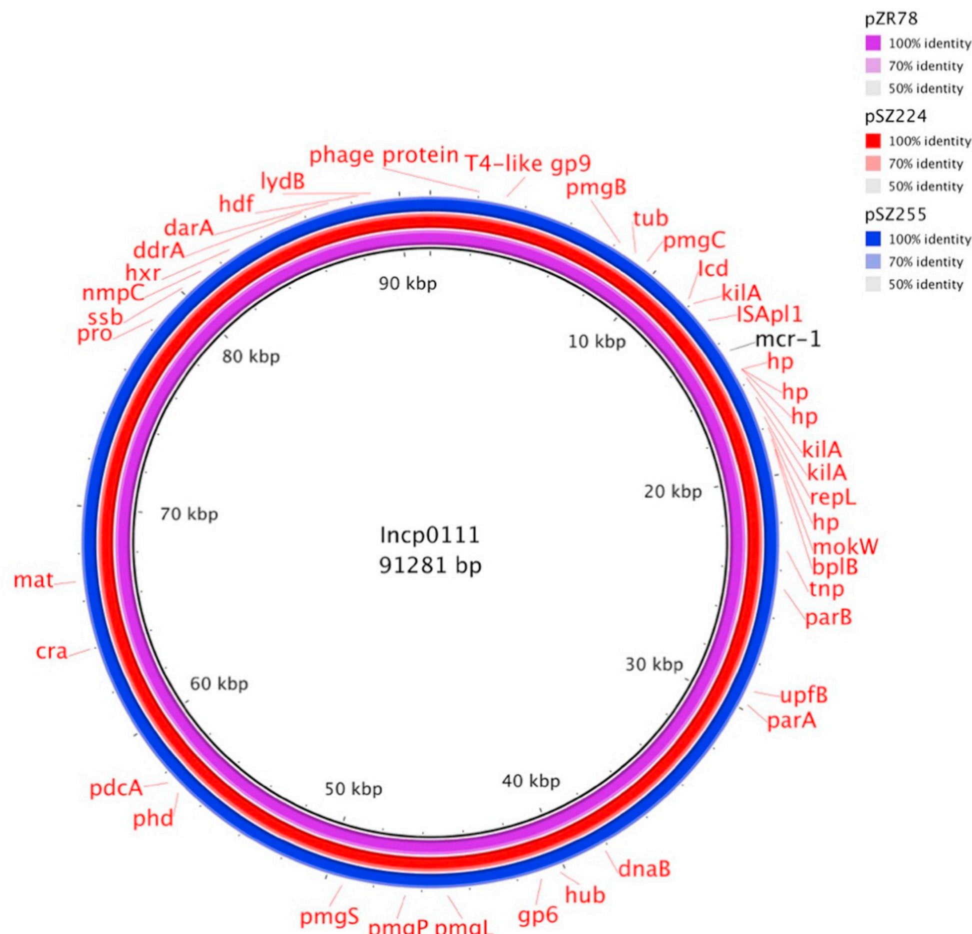

Fig. 3. (*continued*)

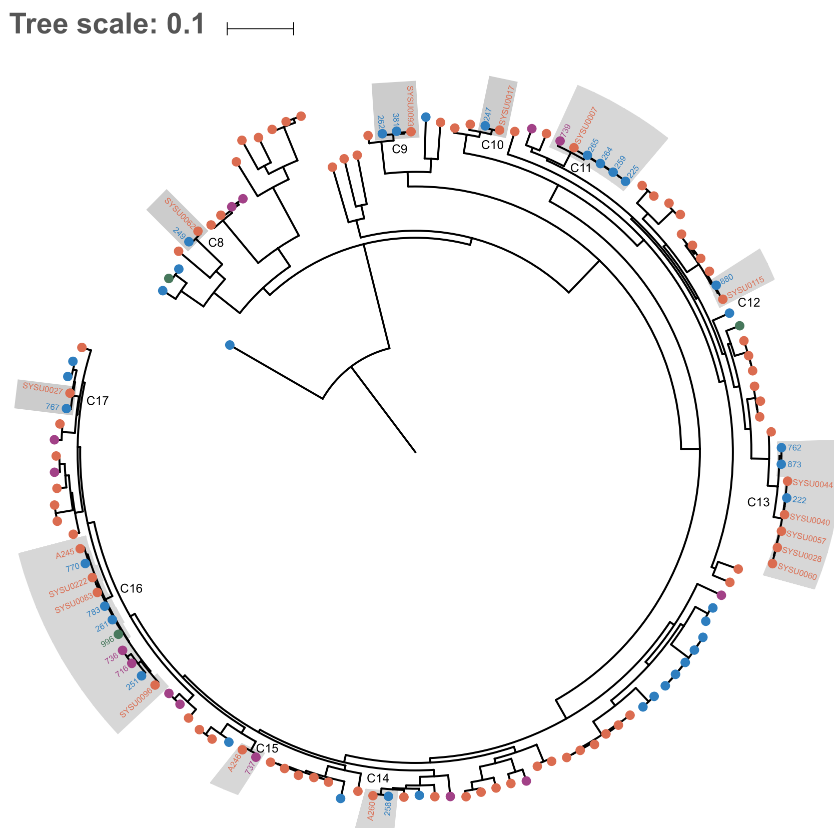

Fig. 4. Phylogenetic tree of MCRPEC isolates from aquaculture and human samples. The midpoint tree was generated based on core-genome single-nucleotide polymorphism (SNP) data. Colored dots indicate the origins of the MCRPEC (red, human; blue, integrated fishery; purple, slaughter house; green, market). Homologous isolates are indicated using grey shading and are labeled with the isolate name and sub-cluster (C8–C17). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

dissemination of ARGs in aquaculture systems (Huang et al., 2017). Likewise, two additional studies have provided further evidence for the transmission of ARGs and contamination of the aquatic environment at integrated fish culture mode sites (Dang et al., 2011; Shah et al., 2012). Our data suggests the transfer of colistin resistance determinants between duck and fish isolates at one integrated fishery (Fig. 2). Because antibiotics and ARGs may be stable in aquatic environments for long periods of time (Xu et al., 2015), the use of numerous antibiotics in aquaculture and in the rearing of waterfowl, especially ducks, would result in a situation in which high levels of ARGs and residual antibiotics are present in the environment. Such a circumstance could accelerate the exchange of ARGs between bacteria under selective pressure.

The mobile colistin resistance has attracted worldwide attentions, the impacts of aquaculture on the dissemination and accumulation of *mcr* genes has recently been identified as important as livestock farming (Shen et al., 2018c). Therefore, aquaculture, which is gradually being recognized as a previously-neglected ARG dissemination hotspot (Cabello et al., 2016), should be incorporated into systematic surveillance programs in accordance with the "One Health" perspective. Moreover, integrated culture methods, not only duck-fish but also animal-fish, are considered economical and efficient farming modes in most developing countries. As such, greater assessment and supervision of the use of antimicrobial agents and the transmission of antimicrobial resistance determinants is needed within the industry.

The *mcr-1* gene is generally mediated by various plasmids (Sun et al., 2018), with IncI2, IncX4, and IncHI2 being the most prevalent plasmid types in MCRPE from humans and animals (Shen et al., 2018a; Shen et al., 2018c; Sun et al., 2018; Zhong et al., 2018). Accordingly, IncHI2 (16/54, 29.6%), IncI2 (15/54, 27.8%), and IncX4 (8/54, 14.8%) were the predominant replicon types of *mcr-1*-carring plasmids in this study. However, this result is inconsistent with the finding that in isolates derived from retail fish, *mcr-1* is predominantly located on IncP (2/7) and IncI2 (2/7) plasmids or on the chromosome (2/7) (Lv et al., 2018). Notably, the high prevalence of IncHI2 plasmids bearing *mcr-1* in the aquaculture supply chain could be attributed to the presence of various resistance genes and heavy metal resistance systems also located on these plasmids, which would allow the host to better survive the pressures of the aquatic environment. Furthermore, the close relationships among the MCRPEC isolates from the aquaculture supply chain and from humans indicates the possible transfer of *mcr-1* via the aquatic food chain, which is concordant with our hypothesis developed following correlation analysis of anthropogenic factors (Shen et al., 2018c).

Although the number of SNPs between the closely-related MCRPEC isolates from different origins was rarely < 40 (ranging from 0 to 3019, 0–0.13%), which is usually considered the cutoff for strain designation, but the isolates could in fact belong to the same strain because the SNPs represented a very small fraction of the genome of *E. coli* (~2.4 Mb per strain in alignment). Although colistin is not officially used in aquaculture in China, it was approved for the treatment of severe infection in a clinical setting in September 2017. Our study revealed the possible transmission of colistin resistance determinants through the aquaculture supply chain, which can consequently impact human health.

5. Conclusions

We demonstrated a high prevalence of MCRPEC isolates in the aquaculture supply chain, with the majority of *mcr-1* genes were plasmid-borne. In addition, comprehensive genomic analyses revealed possible transfer of colistin-resistance determinants between integrated fisheries and humans via the aquatic food chain. Based on the concept of "One health via whole chain", further investigation of the impact of aquaculture supply chain from fisheries, slaughter houses, and markets on the community, and even to hospitals, is urgently needed. Such investigations should include particular emphasis on sample size, sampling time, sample sites, and traceability of the samples along the supply chain. In addition, data on the presence of antibiotics actually

used and its residues, especially critical drugs like colistin, in aquaculture could be combined with metagenomic data to provide a more precise assessment of the impacts of colistin use in aquaculture. Finally, a comparative analysis of colistin resistance among integrated fisheries, monoculture ponds, and unmanaged ponds should also be conducted to access the impacts of these different culture methods on the evolution and transmission of antibiotic resistance determinants.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.03.056.

Acknowledgments

This work was supported in part by the National Key R&D Program of China (2018YFD0500300), the National Natural Science Foundation of China (81661138002 and 31370046), the Sanming Project of Medicine in Shenzhen (SZSM201611068) and the Shenzhen Science and Technology Planning Project (JCYJ20170413101841798). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Data availability

All WGS data obtained in this study have been deposited in the BioProject database under BioProject number PRJNA503777.

Competing interests

All authors declare that they have no conflicts of interest.

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