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Alkaline Peptone Water-Based Enrichment Method for *mcr-3* From Acute Diarrheic Outpatient Gut Samples

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A third plasmid-mediated colistin resistance gene, *mcr-3*, is increasingly being reported in Enterobacteriaceae and *Aeromonas* spp. from animals and humans. To investigate the molecular epidemiology of *mcr* in the gut flora of Chinese outpatients, 152 stool specimens were randomly collected from outpatients in our hospital from May to June, 2017. Stool specimens enriched in alkaline peptone water or Luria-Bertani (LB) broth were screened for *mcr-1*, *mcr-2*, and *mcr-3* using polymerase chain reaction (PCR)-based assays. Overall, 19.1% (29/152) and 5.3% (8/152) of the stool samples enriched in alkaline peptone water were PCR-positive for *mcr-1* and *mcr-3*, respectively, while 2.7% (4/152) of samples were positive for both *mcr-1* and *mcr-3*. Strains isolated from the samples that were both *mcr-1*- and *mcr-3*-positive were subjected to antimicrobial susceptibility testing by broth microdilution. They were also screened for the presence of other resistance genes by PCR, while multilocus sequence typing and whole-genome sequencing were used to investigate the molecular epidemiology and genetic environment, respectively, of the resistance genes. *mcr-3*-positive *Aeromonas veronii* strain 126-14, containing a *mcr-3.8*-*mcr-3*-like2 segment, and *mcr-1*-positive *Escherichia coli* strain 126-1, belonging to sequence type 1485, were isolated from the sample from a diarrheic butcher with no history of colistin treatment. *A. veronii* 126-14 had a colistin minimum inhibitory concentration (MIC) of 2 μg/mL and was susceptible to antibiotics in common use, while *E. coli* 126-1 produced TEM-1, CTX-M-55, and CTX-M-14 β-lactamases and was resistant to colistin, ceftazidime, and cefotaxime. Overall, there was a higher detection rate of *mcr-3*-carrying strains with low colistin MICs from the samples enriched in alkaline peptone water than from samples grown in LB broth.

Keywords: *mcr-3*, *Aeromonas veronii*, diarrheic outpatient, alkaline peptone water, enrichment method

INTRODUCTION

Since the identification of a third plasmid-mediated colistin resistance gene, *mcr-3*, in a porcine *Escherichia coli* isolate from China in 2017 (1), several *mcr-3* variants have been detected in clinical *E. coli* and *Salmonella* isolates from Denmark, Spain, and China (2–5). The amino acid sequence

of MCR-3 is highly similar to that of phosphoethanolamine transferases from various *Aeromonas* and Enterobacteriaceae species (1). Ling et al. reported that chromosomally located *mcr-3* variants, including *mcr-3.3* and *mcr-3-like*, which were identified in *Aeromonas veronii* from chicken meat, showed 95.2 and 84.2% nucleotide sequence identity, respectively, to *mcr-3* from *E. coli* of porcine origin (6). Interestingly, the reported minimum inhibitory concentration (MIC) of colistin for the *mcr-3*-carrying *A. veronii* isolate from chicken meat was 2 µg/mL while colistin MICs for the *mcr-3*-positive Enterobacteriaceae were in the range of 4–8 µg/mL. Thus, *mcr-3*-positive *Aeromonas* spp. strains are likely to go undetected by routine clinical tests. Our previous studies have established an optimized enrichment method for the screening of *mcr-1* from human gut and environmental water sources (7, 8), in which the *mcr-1*-carrying strains demonstrated MICs for colistin of 1–32 µg/mL. As *Aeromonas* spp. generally prefer an alkaline pH, we improved the enrichment method using alkaline peptone water. In this study, we used the newly developed enrichment method to investigate the epidemiology of *mcr* in the gut flora of outpatients treated in our hospital.

MATERIALS AND METHODS

Stool Specimens and Microbial Enrichment

A total of 152 stool specimens were randomly collected from outpatients suffering from acute diarrhea at the Intestinal Clinic of the Second Affiliated Hospital of the Zhejiang University School of Medicine from May to June 2017. Aliquots (~1 g) of each stool sample were individually inoculated into 5 mL of alkaline peptone water (Binhe, Hangzhou, China) and 5 mL of Luria-Bertani (LB) broth for enrichment overnight at 35°C. The alkaline peptone water was adjusted to a pH of 8.4–9.2 and contained 15.0 g/L of tryptone, 4.0 g/L of beef extract, and 10.0 g/L of NaCl.

Detection of *mcr*-Positive Isolates by Enrichment Culture

Following incubation, each enrichment culture tube was inverted 10 times to resuspend the cells and a 1-mL aliquot of suspension was transferred to a fresh 1.5-mL tube. The suspension was centrifuged for 3 min at 8,000 rpm, after which the supernatant was discarded and 1 mL of 0.9% (w/v) saline was added to wash and resuspend the cell pellet. The centrifugation step was then repeated, and 70 µL of ultra-pure water was added to the pellet, which was then boiled for 5 min. Following centrifugation, a 3-µL aliquot of the supernatant was used as template for polymerase chain reaction (PCR) amplification of *mcr-1*, *mcr-2*, and *mcr-3* as described previously (1, 9, 10).

Following initial PCR-based screening, four of the alkaline peptone water enrichment cultures tested positive for both *mcr-1* and *mcr-3* and were therefore selected for colony isolation. A 10-µL aliquot of suspension from the enrichment cultures was inoculated onto *Salmonella–Shigella* agar plates and incubated at 37°C overnight. Resultant colonies were selected for further purification and confirmation of the presence of the *mcr* genes using the PCR-based method described above. Final identification of

the *mcr*-positive colonies was performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Daltonik GmbH, Bremen, Germany) analysis. As this method cannot distinguish between *E. coli* and *Shigella* spp., Kligler Iron Agar and Motility-indole-Urea medium were added to help identify *E. coli* strains. An *mcr-3*-positive *A. veronii* isolate and a *mcr-1*-positive *E. coli* isolate were identified from one of the four *mcr-1*- and *mcr-3*-positive alkaline peptone water enrichment cultures, and were tested for antimicrobial susceptibility and screened for the presence of other common β-lactamase-encoding genes using further PCR.

Antimicrobial Susceptibility Testing

The MICs of eight antibiotics against *mcr*-positive isolates were determined using a broth microdilution procedure. The susceptibilities of each of the isolates to meropenem, ceftazidime, cefotaxime, cefoperazone–sulbactam, amikacin, and ciprofloxacin were determined according to the Clinical and Laboratory Standards Institute guidelines (11). The breakpoints for colistin and tigecycline against *E. coli* were obtained from the European Committee on Antimicrobial Susceptibility Testing breakpoint tables (12). *E. coli* ATCC25922 was used as a quality control strain for broth microdilution assays.

Detection of Other Common β-Lactamase-Encoding Genes

Additional β-lactamase-encoding genes, including bla_{TEM}, bla_{SHV}, $bla_{CTX-M-1-group}$, and $bla_{CTX-M-9-group}$ were detected by PCR using previously described primers and conditions (13).

Multilocus Sequence Typing (MLST)

Molecular typing of the *mcr-1*-positive *E. coli* isolate was performed by MLST using conditions and primers described on the MLST website (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). The sequences of the seven housekeeping genes were compared with those in the *E. coli* MLST database.

Whole-Genome Sequencing

The selected *mcr-3*-positive *A. veronii* isolate was submitted for 300-bp paired-end whole-genome sequencing using the Illumina Hiseq 2500 system (Annoroad, Beijing, China). The raw Illumina reads were assembled into a draft genome sequence using CLC Genomics Workbench 9.0 (CLC Bio, Aarhus, Denmark). Antibiotic resistance genes were analyzed using SRST2 (14), with reference sequences for the antibiotic resistance genes obtained from the ARG-ANNOT database (15).

RESULTS

Detection of *mcr*-Positive Isolates Following Enrichment Culture

Following enrichment in alkaline peptone water, 19.1% (29/152) and 5.3% (8/152) of the stool samples were PCR-positive for *mcr-1* and *mcr-3*, respectively, while 18.4% (28/152) of the samples enriched in LB broth were positive for *mcr-1*. None of the LB enrichment samples tested positive for *mcr-3* (**Table 1**), and none

of the samples from either enrichment method were positive for *mcr-2*.

An *mcr-3*-positive *A. veronii* isolate (strain 126-14) and a *mcr-1*-positive *E. coli* isolate (strain 126-1) were simultaneously isolated from the same alkaline peptone water-enriched stool sample. The sample was collected from a 42-year-old male pork butcher with no medication history of colistin. He was admitted to the gastroenterology clinic for 2 days suffering from acute abdominal pain and diarrhea following ingestion of watermelon. He developed a fever (38.4°C), and stool analysis showed the

yellow loose stool did not contain any leukocytes or erythrocytes. Levofloxacin and viable *Lactobacillus acidophilus* tablets were administrated, and the patient attained complete remission.

Antimicrobial Susceptibility Testing

Identified as sequence type 1485 by MLST, *mcr-1*-positive *E. coli* isolate 126-1 showed resistance to colistin, ceftazidime, and cefotaxime, and additional PCR analyses confirmed the co-existence of bla_{TEM-1}, $bla_{CTX-M-55}$, and $bla_{CTX-M-14}$ in this strain. *mcr-3*-positive *A. veronii* isolate 126-14 was susceptible to all tested antibiotics, and had MICs for colistin and tigecycline of 2 and 1 μg/mL, respectively (**Table 2**).

Whole-Genome Sequencing

Whole-genome sequencing of *mcr-3* positive *A. veronii* isolate 126-14 produced 146 contigs. Two adjacent *mcr-3* variants, the novel upstream variant termed *mcr-3.8* and the downstream variant termed *mcr-3*-like2, were located on 5,338-bp contig 85 and were separated by only 66 bp. The sequences of these two
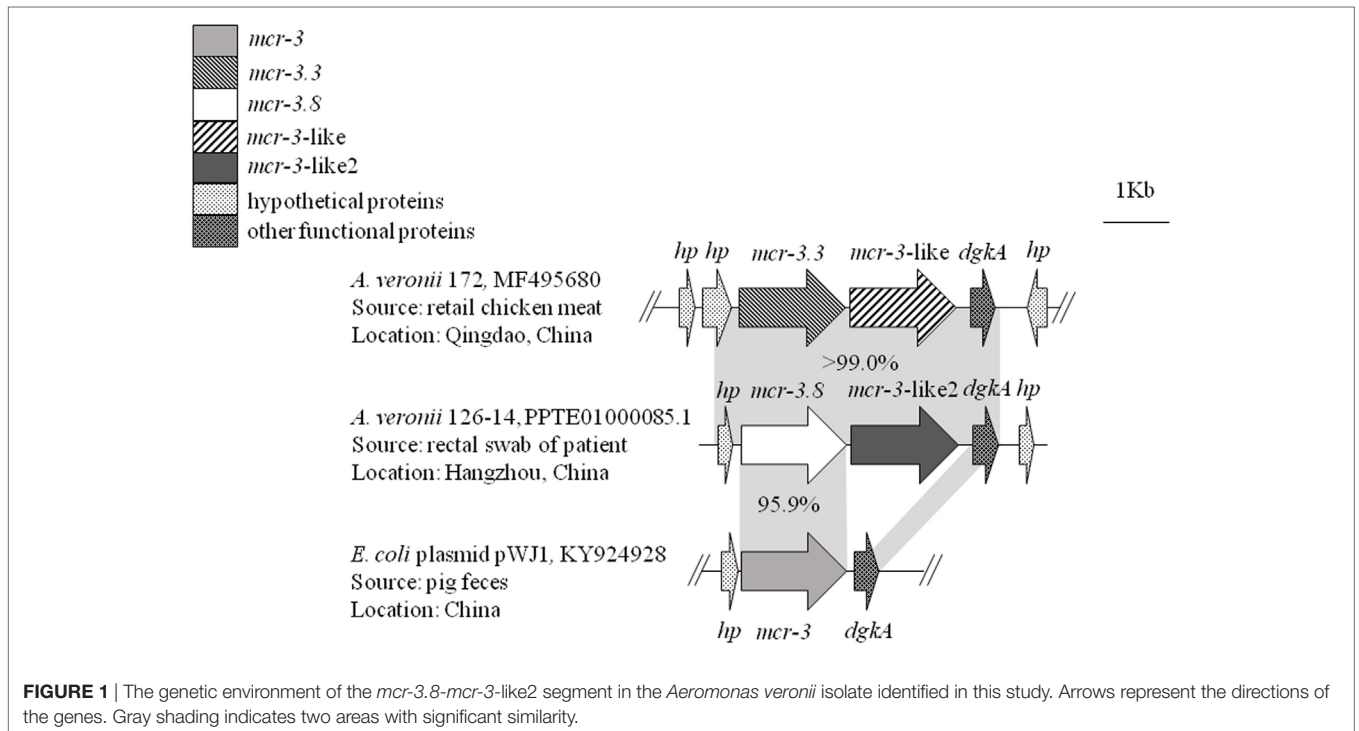
TABLE 1 | Initial polymerase chain reaction screening results for the presence of *mcr* genes in enrichment stool cultures.

Enrichment culture type	Sample size	*mcr-1* (*n*, %)	*mcr-2* (*n*, %)	*mcr-3* (*n*, %)	*mcr-1 + mcr-3* (*n*, %)
Alkaline peptone water	152	29, 19.1	0, 0	8, 5.3	4, 2.7
Luria-Bertani broth	152	28, 18.4	0, 0	0, 0	0, 0

TABLE 2 | MICs and resistance gene profiles of *mcr-3*-positive *Aeromonas veronii* 126-14 and *mcr-1*-positive *Escherichia coli* 126-1.

Isolate	MICs of (μg/mL)								Resistance gene(s)
	CL	MEM	CAZ	CTX	SCF	AMK	CIP	TIG	
A. veronii 126-14	2	≤0.25	≤0.5	≤0.25	≤1/0.5	≤8	≤0.25	1	*mcr-3*
E. coli 126-1	8	≤0.25	>32	>32	8/4	≤8	0.5	1	*mcr-1*, bla_{TEM-1}, $bla_{CTX-M-55}$, $bla_{CTX-M-14}$

MIC, minimum inhibitory concentration; CL, colistin; MEM, meropenem; CAZ, ceftazidime; CTX, cefotaxime; SCF, cefoperazone-sulbactam; AMK, amikacin; CIP, ciprofloxacin; TIG, tigecycline.

FIGURE 1 | The genetic environment of the *mcr-3.8-mcr-3*-like2 segment in the *Aeromonas veronii* isolate identified in this study. Arrows represent the directions of the genes. Gray shading indicates two areas with significant similarity.

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MCR-3        1   MPSLIKIKIVPLMFFLALYFAFMLNWRGVLHFYEILYKLEDFKFGFAISLPILLVAALNF    60
MCR-3.3      1   ************************************************************    60
MCR-3.8      1   ************************************************************    60
MCR-3-like   1   *F*AVR**V**FVLL***V***L***PVL****D**SNI*H**I**VV*I*F********    60
MCR-3-like2  1   *F*AVR**V**FVLL***V***L***PVL****D**SNI*H**I**VV*I*F********    60

MCR-3       61   VFVPFSIRYLIKPFFALLIALSAIVSYTMMKYRVLFDQNMIQNIFETNQNEALAYLSLPI   120
MCR-3.3     61   ************************************************************   120
MCR-3.8     61   ************************************************************   120
MCR-3-like  61   **M*****F*M*****F*FVTGS*A***********GD*********S**F**VNA**   120
MCR-3-like2 61   **M*****F*M*****F*FVTGS*A***********GD*********S**F**VNA**   120

MCR-3      121   IVWVTIAGFIPAILLFFVEIEYEEKWFKGILTRALSMFASLIVIAVIAALYYQDYVSVGR   180
MCR-3.3    121   *G**********************************************************   180
MCR-3.8    121   *G**********************************************************   180
MCR-3-like 121   *I**ILT*LL**A*I***K***AST*Y***AQ*L****F**VIVGI*********A*I**   180
MCR-3-like2121   *I**ILT*LL**A*I***K***AST*Y***AQ*L****F**VIVGI*********A*I**   180

MCR-3      181   NNSNLQREIVPANFVNSTVKYVYNRYLAEPIPFTTLGDDAKRDTNQSKPTLMFLVVGETA   240
MCR-3.3    181   ************************************************************   240
MCR-3.8    181   ************************************************************   240
MCR-3-like 181   **QT*N*********MY**S**L*R**M******V******T*V*KKD**********   240
MCR-3-like2181   **QT*N*********MY**S**L*R**M******V******T*V*KKD**********   240

MCR-3      241   RGKNFSMNGYEKDTNPFTSKSGGVISFNDVRSCGTATAVSVPCMFSNMGRKEFDDNRARN   300
MCR-3.3    241   ************************************************L***   300
MCR-3.8    241   ************************************************L***   300
MCR-3-like 241   ************************************Y***********************   300
MCR-3-like2241   ************************************★***********************   300

MCR-3      301   SEGLLDVLQKTGISIFWKENDGGCKGVCDRVPNIEIEPKDHPKFCDKNTCYDEVVLQDLD   360
MCR-3.3    301   **************V*********************K***Y***************E**   360
MCR-3.8    301   **************V*********************K***Y***************E**   360
MCR-3-like 301   ************************************************************   360
MCR-3-like2301   ************************************************************   360

MCR-3      361   SEIAQMKGDKLVGFHLIGSHGPTYYKRYPDAHRQFTPDCPRSDIENCTDEELTNTYDNTI   420
MCR-3.3    361   ************************************************************   420
MCR-3.8    361   ************************************************************   420
MCR-3-like 361   ************************************************************   420
MCR-3-like2361   ************************************************************   420

MCR-3      421   RYTDFVIGEMIAKLKTYEDKYNTALLYVSDHGESLGALGLYLHGTPYQFAPDDQTRVPMQ   480
MCR-3.3    421   *******A*************************************K***********   480
MCR-3.8    421   *******★*************************************K***********   480
MCR-3-like 421   *********************************************K***********   480
MCR-3-like2421   *********************************************K***********   480

MCR-3      481   VWMSPGFTKEKGVDMACLQQKAADTRYSHDNIFSSVLGIWDVKTSVYEKGLDIFSQCRNVQ  541
MCR-3.3    481   *******I*****MN*E****KN***AN******************AI**QE****K****N*  541
MCR-3.8    481   *******IT****MN*E****KN***AN******************AI**QE****K****N*  541
MCR-3-like 481   ************************************************************  541
MCR-3-like2481   ************************************************************  541
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FIGURE 2 | Alignment of the MCR-3, MCR-3.3, MCR-3-like, MCR-3.8, and MCR-3-like2 sequences from *Escherichia coli* (GenBank accession no. KY924928) and *Aeromonas* isolates (GenBank accession no. MF495680 and PPTE01000085.1).

variants have been deposited in GenBank under accession no. PPTE01000085.1. Both variants showed >99.0% nucleotide and amino acid sequence identity to the *mcr-3.3* and *mcr-3-like* genes in *A. veronii* isolated from chicken meat, and 95.9 and 87.2% nucleotide sequence identity and 95.8 and 84.8% amino acid sequence identity, respectively, to the original *mcr-3* gene (**Figures 1** and **2**). Similar to the *mcr-3.3-mcr-3-like* segment in a previously identified *A. veronii* isolate (GenBank accession no. MF495680), the *mcr-3.8-mcr-3-like2* segment in *A. veronii* 126-14 was surrounded by both hypothetical genes and diacyl-glycerol kinase alpha-encoding gene *dgkA* but lacked transfer elements (**Figure 1**).

DISCUSSION

To the best of our knowledge, this study is the first report of the co-occurrence of a *mcr-3*-positive *A. veronii* isolate and a *mcr-1*-positive *E. coli* isolate from the gut of a diarrheic outpatient. Gram-negative *Aeromonas* spp. cause various infections in both humans and animals, with *Aeromonas*-associated diarrhea and gastroenteritis the most frequent manifestations of infection in humans. *Aeromonas* infections affect all age groups, including both healthy and immunocompromised individuals (16). The transferable colistin resistance gene *mcr-1* has been reported in Enterobacteriaceae isolated both from food-producing animals and humans, with carriage rates of 5.1 and 6.2%, respectively (17, 18). These carriage rates indicate that the emergence and spread of *mcr-1* probably occurred first in isolates of animal origin, which then spread to humans. In this study, the *mcr-3*-positive *A. veronii* strain isolated from the fecal sample might have been the cause of the acute diarrhea. The patient was employed as a pork butcher and had no history of colistin use, which indicated that the *mcr-3* and the *mcr-1* genes likely originated from isolates of food–animal origin. This suggests that foodstuffs of farm animal origin may act as a critical transmission vehicle in the dissemination of mobile colistin resistance genes from animal-associated with human-associated bacteria.

In this study, 19.1% (29/152) and 5.3% (8/152) of the stool samples enriched in alkaline peptone water were PCR-positive for *mcr-1* and *mcr-3*, respectively, while only 18.4% (28/152) of the LB broth-enriched samples tested positive for *mcr-1*. This suggests that the alkaline peptone water enrichment step before direct sample testing resulted in higher sensitivity of the *mcr* screening from human stool samples compared with LB broth cultivation. In addition, the detection rate of *mcr-1* in this study (approximately 19%) was much higher than that reported previously (about 6%) (18), probably due to the enrichment step and direct enriched sample testing by PCR. Furthermore,

mcr-3-positive *A. veronii* isolate 126-14 had a colistin MIC of 2 µg/mL, which was in agreement with the previously reported colistin MIC for a *mcr-3*-carrying *A. veronii* isolate from chicken meat (6). Therefore, these results suggest that unsupplemented *Salmonella–Shigella* agar rather than medium supplemented with colistin is better for selection of *mcr-3*-carrying *Aeromonas* spp. strains. Overall, only one *mcr-3*-positive *A. veronii* isolate was recovered from the four PCR-positive alkaline peptone water-enriched stool samples. Although *Aeromonas* spp. are widely distributed in soil (19), foodstuffs (20), and aquatic environments (21), they usually constitute a small percentage of the human gut flora (22). As such, the number of *Aeromonas* spp. present in an enrichment culture may still be too low to detect *via* direct culture plating methods.

In conclusion, the alkaline peptone water enrichment method was optimal for detection of *mcr-3*-carrying strains with low colistin MICs from the human gut microbiota. The method is simple to perform and can be used in any laboratory that is equipped to perform PCR assays and can obtain alkaline peptone water at the proper pH. In addition, as the human intestine may serve as a reservoir for antibiotic resistance genes, including *mcr-3*, and play an important role in horizontal gene transfer, the rapid horizontal spread of mobile colistin resistance genes between and within Enterobacteriaceae and *Aeromonas* spp. in the human gut should be closely monitored.

ETHICS STATEMENT

Written informed consent was obtained from the patient for the publication of this research. The Ethics Committee of the Second Affiliated Hospital of Zhejiang University, School of Medicine, already approved this work.

AUTHOR CONTRIBUTIONS

RZ and HZ designed and supervised the study. HW participated in stool sample collecting. QS, LS, HW, and ZH conducted the microbial enrichment, PCR, antimicrobial susceptibility testing, and strain characterization. YH analyzed the whole-genome sequencing. RZ, QS, YH, HZ, and LS contributed to the data interpretation and manuscript writing. All the authors have approved the final version and have agreed to be accountable for all aspects of the work.

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