Infection and Drug Resistance

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ORIGINAL RESEARCH

Colistin Resistance Mediated by *Mcr-3*-Related Phosphoethanolamine Transferase Genes in *Aeromonas* Species Isolated from Aquatic Environments in Avaga and Pakro Communities in the Eastern Region of Ghana

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Purpose: Colistin is classified by the World Health Organization (WHO) as a critically important and last-resort antibiotic for the treatment of infections caused by carbapenem-resistant bacteria. However, colistin resistance mediated by chromosomal mutations or plasmid-linked mobilized colistin resistance (*mcr*) genes has emerged.

Methods: Thirteen *mcr*-positive *Aeromonas* species isolated from water samples collected in Eastern Ghana were analyzed using whole-genome sequencing (WGS). Antimicrobial susceptibility was tested using the broth microdilution method. Resistome analysis was performed *in silico* using a web-based platform.

Results: The minimum inhibitory concentration (MIC) of colistin for all except three isolates was >4 µg/mL. Nine new sequence types were identified and whole-genome analysis revealed that the isolates harbored genes (*mcr-3*-related genes) that code for Lipid A phosphoethanolamine transferases on their chromosomes. BLAST analysis indicated that the amino acid sequences of the *mcr-3*-related genes detected varied from those previously reported and shared 79.04–99.86% nucleotide sequence identity with publicly available *mcr-3* variants and *mcr-3*-related phosphoethanolamine transferases. Analysis of the genetic context of *mcr-3*-related genes revealed that the genetic environment surrounding *mcr-3*-related genes was diverse among the different species of *Aeromonas* but conserved among isolates of the same species. *Mcr-3*-related-gene-IS-*mcr-3*-related-gene segment was identified in three *Aeromonas caviae* strains.

Conclusion: The presence of *mcr-3*-related genes close to insertion elements is important for continuous monitoring to better understand how to control the mobilization and dissemination of antibiotic resistance genes.

Keywords: *Aeromonas* spp, colistin, *mcr-3*-related gene, phosphoethanolamine transferases, whole genome analysis

Introduction

Since their discovery and introduction into clinical use, antibiotics have significantly reduced mortality and continue to be indispensable in medical care.[1] Therefore, preserving their efficacy is of utmost importance.[2] Despite this, antimicrobial resistance (AMR) has emerged and increased at an alarming rate, implicated in more than one million deaths in 2019, and is considered a serious public health threat.[3,4] Several studies have reported the emergence of multi-drug resistant bacterial

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pathogens of different origins and have provided important information on the severity of this global health crisis and the need to promote the responsible use of antibiotics.[4,5] Concerns regarding antibiotic resistance are further deepened by the increased prevalence of bacteria resistant to last-tier antibiotics such as carbapenems. In instances where severe infections are caused by carbapenem-resistant strains, treatment has been limited to tigecycline, colistin, aminoglycosides and fosfomycin,[6] but the extensive use of colistin has resulted in the unavoidable emergence of resistance.[7]

Colistin, also called polymyxin E, is an important last line antibiotic discovered in 1947 and approved for clinical use in 1959. The medical gains from colistin use had previously been unsatisfactory and were abandoned by the 1980s due to reports of nephrotoxicity and neurotoxicity associated with its use and the introduction of safer antibiotics.[8,9] However, the increased incidence of multidrug resistant organisms necessitated the reintroduction of colistin in clinical practice for systemic treatment.[10,11]

Colistin functions by binding to the negatively charged phosphate group of lipid A in the lipopolysaccharide (LPS) of bacterial outer membranes, disrupting the membrane, causing leakage of cytoplasmic contents, and ultimately causing cell death.[12,13] The clinical efficacy of colistin has been eroded by the recent rise and continuous reports of plasmid-mediated mobilized colistin resistance (*mcr*) genes encoding colistin resistance, in addition to previously known mechanisms emerging from chromosomal mutations involving several two-component systems.[14] To date, ten *mcr*-genes (*mcr-1* to *mcr-10*) with many variants have been identified in diverse Gram-negative bacterial species from different sources. Nevertheless, it has been demonstrated that some variants do not confer phenotypic resistance.[15]

To control the spread of colistin resistance, regular investigations into the occurrence of resistant bacteria and studies of the genetic context of the detected genes are necessary to improve our knowledge of how the genes are transferred or spread, to serve as a guide for the development of interventions.

Although like in many parts of the world the study of antibiotic resistance in Ghana has largely been within the ambit of hospital environments,[16] the extensive use of antibiotics in human and veterinary medicine and their successive release into water bodies have greatly contributed to the global distribution of antibiotic-resistant strains.[14,17,18] Thus, water bodies represent a reservoir of mobile genetic elements associated with antibiotic resistance. Therefore, our study aimed to analyze *mcr*-gene-carrying colistin resistant Gram-negative bacteria isolated from water samples collected from two communities in Eastern Ghana.

Materials and Methods

Sample Collection, Processing and Bacterial Isolation

Water samples (10mL −1000mL) were collected from rivers, wells, mud water, surface water of a reservoir and irrigation pond, at six and nine sites in the Avaga and Pakro Adesa communities (Figure 1), respectively, far from animal farms and

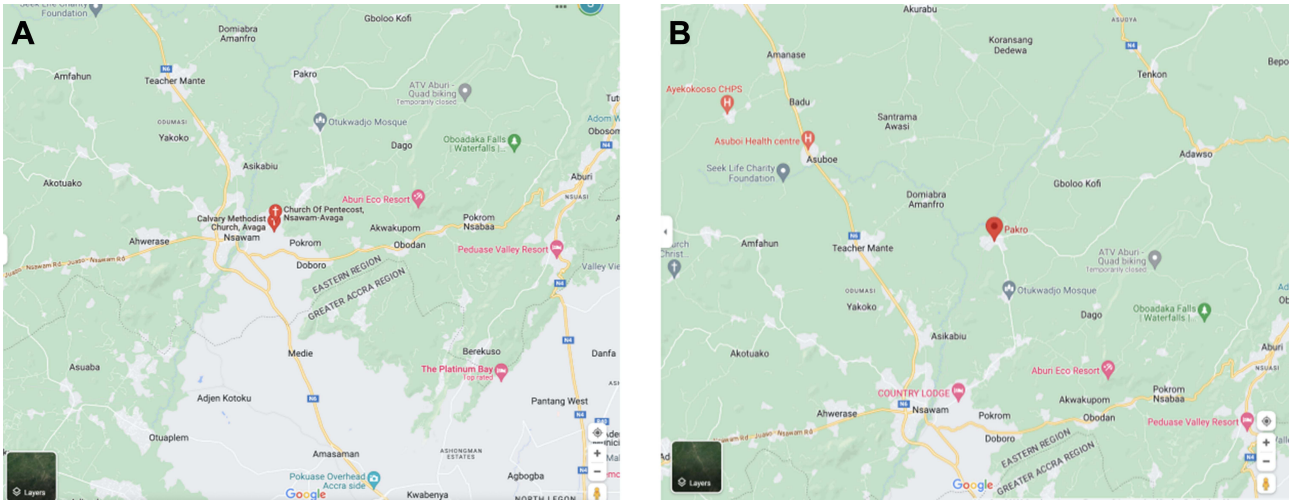

Figure 1 Overview of sampling sites in Eastern region of Ghana. (**A**) Map showing area of sample collection in Avaga community. (**B**) Map showing area of sample collection in Pakro Adesa community. Maps were generated using maps.google.com (https://www.google.com/maps) last accessed 17/11/2023.

medical facilities where colistin may be used, at Nsawam, in the Eastern region of Ghana, in June 2021. These samples were collected to investigate the status of colistin resistance under the low selective pressure of colistin. The samples were transported to the Bacteriology Department of the Noguchi Memorial Institute for Medical Research for initial processing. The samples were filtered serially through different filter dimensions and then through a 0.45µm filter to remove large particles. The filtrates were aliquoted into 5 mL tubes and transported in cold chain (in ice packs) to the Tokyo Medical and Dental University for further analysis. After vigorous vortexing, portions (100µL) of the samples were enriched in 2mL of tryptic soy broth (TSB) at 37°C overnight. Twenty microliters (20µL) of the overnight cultures were spread on antibiotic-supplemented bromothymol blue agar plates (colistin 2µg/mL, cefotaxime 4µg/mL, ampicillin/sulbactam 16/8µg/mL, tigecycline 4µg/mL) to suppress the growth of most Gram-positive bacteria and increase the chances of selecting colistin or tigecycline resistant isolates. Approximately five to eight morphologically different colonies from each plate were passaged onto fresh agar plates and stored in skimmed milk for further analysis.

16S rRNA Species Identification

The isolated bacteria were identified using 16S rRNA sequencing. Crude DNA was extracted from the isolates as described earlier.[19] Ten (10µL) microliters PCR reaction mix were prepared with 5µL of 2x Emerald premix (Takara, Japan), 0.5µL each of forward (16S-8UA: AGAGTTTGATCMTGGCTCAG) and reverse (16S-1485B: ACGGGCGGTGTGTRC) primers,[20] 3µL of nuclease-free water and 1µL of DNA template. The PCR was run with an initial denaturation at 98°C for 1 min, followed by 30 cycles of 98°C for 5s, 57°C for 10s, and 72°C for 1 min, and a final extension at 72°C for 3 min. The expected band size was ~1500bp. PCR products were purified with EXOSAP IT (Applied Biosystems, Thermo Fisher Scientific, Tokyo, Japan) and sequenced on a 3730xl DNA Analyzer (Thermo Fisher Scientific, Tokyo, Japan) with BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Tokyo, Japan). Identification was performed using a BLAST search of sequencing results.

Antimicrobial Susceptibility Testing and *Mcr*-Genes Screening

The minimum inhibitory concentrations (MICs) of antibiotics were determined by broth microdilution using DP45 plates (Eiken Chemical Co., Tokyo, Japan). Antibiotic activity was interpreted according to the Clinical and Laboratory Standards Institutes (CLSI) guidelines (CLSI M100, 2022, 32nd edition). Multiplex PCR detection of *mcr* (*1–10*) genes was performed using primer sets designed by Masato Suzuki (unpublished data), as listed in Table 1. The PCR products of the positive isolates were purified using the FastGene Gel/PCR Extraction Kit (NIPPON Genetics Co. Ltd., Tokyo, Japan) and subjected to Sanger sequencing. The results were analyzed using BLAST searches.

Genome Sequencing and Analysis

For short-read sequencing, genomic DNA was extracted from *mcr*-positive isolates using the Magattract HMW DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Paired-end libraries were prepared using

Table 1 List of Primers for *Mcr-1-10* Detection in This Study

mcr Group	Target	Primer Name	Primer Sequence	Size (bp)
mcr-1 group	*mcr-1, mcr-2, mcr-6*	mcr-1G_722F	TGTTCGTCGTCGGTGAGACG	205
		mcr-1G_926R	TGGTATTTGGCGGTATCGAC	
mcr-4	*mcr-4*	mcr-4_116	AACAACCAGAAGTTGATCCC	272
		mcr-4_387R	CCCAGTCAGCAGTAGATTGG	
mcr-5	*mcr-5*	mcr-5_112F	TGGAATGCCCTTCTTGCTGG	310
		mcr-5_421R	ACACGGATACGGCTGCAACC	
mcr-3 group	*mcr-3, mcr-7*	mcr-3G_848	GCATGTTCTCCAATATGGGG	427
		mcr-3G_1275	GAAATCGGTGTAGCGGATGG	
mcr-8	*mcr-8*	mcr-8_89F	CACTTTGGCAAACACTATGG	528
		mcr-8_616R	GGTAACCATTCGTATTACGC	
mcr-9 group	*mcr-9, mcr-10*	mcr-9G_702F	GGTGATTGGCGAAACGGCAC	635
		mcr-9G_1337R	AGCAGCACGGTGTTGTACTG	

Illumina DNA prep with the IDT for Illumina DNA/RNA UD Indexes and sequenced on an Illumina Miniseq (Illumina Inc., San Diego, USA) generating reads of length 149bp. Short reads were assessed for quality using fastqc v0.11.9 (https://github.com/s-andrews/FastQC). Reads were subjected to quality trimming and filtering with fastp[21] v0.23.1 (https://github.com/OpenGene/fastp). Libraries for long-read sequencing were prepared using native barcoding and ligation sequencing kits EXP-NB104 and SQK-LSK109, respectively, in accordance with the manufacturer's instructions. Libraries were loaded onto FLO-MIN106 R9.4.1 flow cells, and sequencing was conducted using MinION Mk1B (Oxford Nanopore Technologies, Oxford, United Kingdom). Barcoded Fast5 reads generated were basecalled using Guppy v1.1.4 (https://community.nanoporetech.com/protocols/Guppy-protocol/). Demultiplexing of reads and adapter sequence trimming were performed using the Porechop[22] v0.2.4. Long reads with low quality (MinION Q < 10) and long reads (<1000 bp) were filtered out using filtlong (https://github.com/rrwick/Filtlong). Hybrid assembly of long-read and short-read sequences was performed using Unicycler[23] v0.4.8 (https://github.com/rrwick/Unicycler). The quality of the assembled genomes was checked using QUAST[24] v5.2.0 (https://github.com/ablab/quast). Genomes were uploaded to the RAST[25] online server (https://rast.nmpdr.org/) for annotation. The assembled genomes were screened for acquired antibiotic resistance genes and sequence types (ST) using the web-based Center for Genomic Epidemiology (CGE) databases, ResFinder[26] v4.1 (https://cge.cbs.dtu.dk/services/ResFinder/) and MLST[27] (https://cge.cbs.dtu.dk/services/MLST/), respectively. Plasmids and other extra-chromosomal structures were confirmed using BLAST. The insertion elements were determined using the ISFinder[28] (isfinder.biotoul.fr/blast.php). Virulence factors were identified using an online virulence factor database[29] (VFDB) (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi). The identities of the isolates were confirmed using FastANI[30] (https://github.com/ParBLiSS/FastANI). The genetic relatedness of STs was assessed using a minimum spanning tree generated by PHYLOViZ Online[31] (https://online.phyloviz.net/index). Amino acid sequences of publicly available *mcr-3* variants were retrieved from NCBI and aligned with Multiple Sequence Comparison by Log- Expectation (MUSCLE) integrated within megaX, along with sequences from this study and based on the alignment generated, a maximum likelihood phylogeny with 1000 bootstrap replicates was constructed with megaX v10.2.6.[32] Accession numbers of all the genomes used in this study are listed in Supplementary Data 1. Comparison and visualization of the genetic contexts of the identified *mcr-3*-related genes were performed using EasyFig v.2.2.2.[33]

The complete genomes of all sequenced isolates were deposited in GenBank under Bioproject PRJNA473419. Sequences of all *mcr-3*-related genes have been deposited in GenBank under the accession numbers OP176034-OP176041.

Results

Detection and Prevalence of *Mcr*-Positive Colistin-Resistant Strains

A total of 112 non-duplicate Gram-negative isolates from 12 genera were obtained from all the water samples. These included *Acinetobacter* spp. (n = 36), *Aeromonas* spp. (n = 27), *Bordetella hinzii* (n = 1), *Chryseobacterium* spp. (n = 1), *Enterobacter* spp. (n = 2), *Escherichia coli* (n = 2), *Herbaspirillum* spp. (n = 2), *Klebsiella* spp. (n = 2), *Providencia rettgeri* (n = 1), *Pseudomonas* spp. (n = 34), *Rheinheimera tangshanensis* (n = 1), *Vogesella perlucida* (n = 3).

Isolates were classified as resistant to colistin if their colistin MIC was ≥4 µg/mL.[14,34] Thirteen *Aeromonas* spp. were resistant to colistin, namely *Aeromonas dhakensis* (n = 5), *Aeromonas veronii* (n = 1), *Aeromonas hydrophila* (n = 4), *Aeromonas jandaei* (n = 1), *Aeromonas caviae* strain (n = 1), and *Aeromonas* spp. (n = 1). Nine (9) out of the 13 resistant isolates (*A. dhakensis*, n = 4, *A. hydrophila*, n = 3, *A. jandaei*, n = 1, *Aeromonas* spp. n = 1) were positive for *mcr-3*, as determined by PCR and Sanger sequencing. Four other isolates, three of which had colistin MIC ≤1µg/mL and identified as *A. caviae* were positive for *mcr-3* target by PCR, and the remaining one isolate (*A. dhakensis*) with colistin MIC >4µg/mL was PCR negative. At the end, the nine PCR-positive isolates and four isolates aforementioned (n = 13) were selected for whole-genome sequencing. Further analysis focused on the selected *Aeromonas* species designated BC01, BC02, BC03, BC04, BC05, BC11, BC13, BC14, BC15, BC16, BC17, BC18, and BC19. The proportions of resistance to colistin and other tested antibiotics among all *Aeromonas* species including the selected 13 are shown in Table 2. The MIC values for the selected 13 *Aeromonas* spp. are provided in Supplementary Data 2.

Table 2 Antibiotic Resistance Profiles of All *Aeromonas* Species (n = 27)

Antimicrobial Agents	Breakpoint for Resistance (μg/mL)	% Resistance	MIC (μg/mL)		
			Range	MIC_{50}	MIC_{90}
Piperacillin	≥128	3.7	≤2->64	≤2	8
Tazobactam/Piperacillin	≥4/128	3.7	≤4/2->4/64	≤4/2	4/8
Cefepime	≥32	0.0	≤0.5–2	≤0.5	≤0.5
Ceftazidime	≥32	3.7	≤1- >32	≤1	2
Gentamicin	≥16	0.0	≤1-2	≤1	2
Amikacin	≥64	0.0	≤4-16	≤4	≤4
Minocycline	≥16	3.7	≤1->8	≤1	2
Levofloxacin	≥8	0.0	≤0.5–2	≤0.5	≤0.5
Meropenem	≥16	0.0	≤0.5–4	≤0.5	≤0.5
Imipenem	≥16	0.0	≤0.5–2	1	1
Aztreonam	≥32	3.7	≤2->16	≤2	≤2
Colistin	≥4	48.1	≤1->4	≤1	>4
Tobramycin	≥16	0.0	≤1-4	≤1	4
Ciprofloxacin	≥4	0.0	≤0.25–1	≤0.25	0.5
Sulfamethoxazole/Trimethoprim	≥76/4	37.0	≤9.5/0.5->38/2	≤9.5/0.5	>38/2

Characterization of *Mcr*-Positive Isolates and *Mcr-3*-Related Genes

Hybrid *de novo* assembly of reads from illumina and nanopore generated complete genomes of sizes ranging from 4.7Mbp to 5Mbp with GC contents between 58.92% and 61.72% (Supplementary Data 4). The genomes of BC01, BC02 and BC11 were composed of chromosomes of size 4.6Mbp, three plasmid sequences and one uncharacterized sequence of length 1712bp. These plasmid/extra-chromosomal sequences did not contain any identifiable antibiotic-resistance genes. Sequence type prediction based on the allele numbers of six housekeeping genes of *Aeromonas* spp. (*gyrB, groL*, *gltA, metG*, *ppsA, recA*) using the MLST web server on the CGE platform yielded indeterminate results (unknown STs) for all but one isolate. The genomes of the isolates were submitted to PubMLST for assignment of new STs (https://pubmlst.org/bigsdb?db=pubmlst_aeromonas_isolatesandpage=submitandgenomes=1). MLST analysis yielded nine (9) new STs: ST1417, ST1426, ST1427, ST1428, ST1429, ST1430, ST1431, ST1432, ST1433, and a previously identified ST340 (Table 3). Most STs occurred as singletons and shared no genetic relationship with each other or with STs from other countries (Figure 2, Supplementary Data 4). A clonal complex (CC) was identified, comprising *A. dhakensis* ST1427 from this study and *A. dhakensis* ST1298 isolated in Bangladesh, and linked by a single

Table 3 Characteristics of Thirteen (13) *Mcr* Positive *Aeromonas* Species Used in Further Analysis

Isolate ID	Isolation Source	Site of Sample Collection	Species	mcr Gene	Colistin MIC (μg/mL)	Accession No.	ST
BC01	Water samples	Avaga	*A. caviae*	*mcr-3-related*	≤ 1	CP102315 - CP102319	1417
BC02	Water samples	Avaga	*A. caviae*	*mcr-3-related*	≤ 1	CP102320 - CP012324	1417
BC03	Water samples	Avaga	*A. dhakensis*	*mcr-3-related*	>4	CP102325	1426
BC04	Water samples	Avaga	*A. dhakensis*	*mcr-3-related*	>4	CP102326	340
BC05	Water samples	Avaga	*A. dhakensis*	*mcr-3-related*	>4	CP102327	1427
BC11	Water samples	Avaga	*A. caviae*	*mcr-3-related*	≤ 1	CP102334 - CP102339	1417
BC13	Water samples	Pakro	*A. hydrophila*	*mcr-3-related*	>4	CP102364	1428
BC14	Water samples	Pakro	*Aeromonas spp*	*mcr-3-related*	>4	CP102454 - CP102455	1429
BC15	Water samples	Pakro	*A. dhakensis*	*mcr-3-related*	>4	CP102365	1430
BC16	Water samples	Pakro	*A. hydrophila*	*mcr-3-related*	>4	CP102366	1428
BC17	Water samples	Pakro	*A. jandaei*	*mcr-3-related*	>4	CP102367	1431
BC18	Water samples	Pakro	*A. dhakensis*	*mcr-3-related*	>4	CP102368 - CP102369	1432
BC19	Water samples	Pakro	*A. hydrophila*	*mcr-3-related*	>4	CP102370	1433

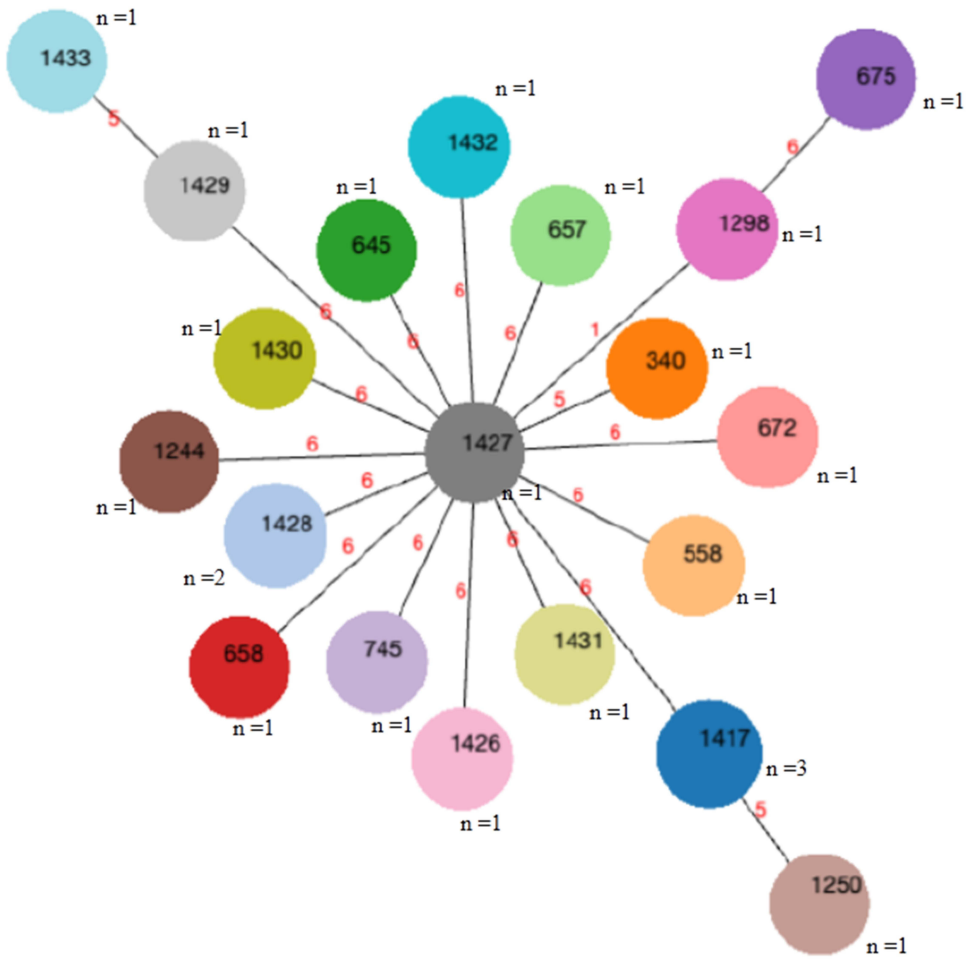

Figure 2 Minimum spanning tree based on the allelic profile of 10 STs identified in *Aeromonas* species in this study (ST1417, ST1426, ST340, ST1427, ST1428, ST1429, ST1430, ST1431, ST1432, ST1433) and others from PubMLST database generated with PHYLOViZ online. The different STs are represented by a unique node color. Node size is indicative of the number of isolates for each ST. Number of isolates forming node size has been illustrated (n). Red numbers on connecting lines represent the number of locus variants between the STs.

difference in allele number. Further genome mining identified open reading frames (*orf*) encoding *mcr-3*-related lipid A phosphoethanolamine transferases of variable sequence lengths in all 13 isolates. Two different coding sequences of the *mcr-3*-related lipid A phosphoethanolamine transferase (*EptA*), one of length 1398bp and made of 465 amino acids (aa), was identified in the genomes of BC01 (*A. caviae*), BC02 (*A. caviae*), and BC11 (*A. caviae*), and shared 99.57% amino acid identity with *mcr-3.27* (WP_017778762.1). The second sequence which was 1626bp in length in BC11 with 541aa, appeared to be truncated in BC01 and BC02 with a length of 1044bp, made of 347 aa (Figure 3). The sizes of *mcr-3*-related *EptA* genes in all the isolates are listed in Supplementary Data 3.

Resistome analysis revealed that the *Aeromonas* spp not only carried *mcr-3*-related genes but also carried sulphonamide resistance genes *sul*1, aminoglycoside resistance genes *aadA1*, tetracycline resistance genes *tet*E, $bla_{\text{OXA-2}}$-like, $bla_{\text{MOX-6}}$-like, *qacE*-like, *ampH*-like, *cphA1*-like, *imiH*-like, *cphA7, amp5*-like, $bla_{\text{CEPH-A3}}$-like genes on their chromosomes (Supplementary Data 3). Virulome assessment identified virulence factors, such as flp type IV pili, lateral flagella, polar flagella, mannose-sensitive hemagglutinin pilus (MSH), type IV pili, type 1 fimbriae, type II, III, and VI secretion systems (T2SS, T3SS, and T6SS, respectively), aerolysin *AerA*/cytotoxic enterotoxin, hemolysin HlyA and hemolysin III toxins in *Aeromonas* spp. Details of virulence factors and their related genes are available in Supplementary Data 1. Virulence genes were more abundant in *A. dhakensis* species than in other species, and *A. caviae* carried the least number of virulence genes. Flp type IV pili were only present in *A. hydrophila* species, whereas lateral flagella were only present in *A. dhakensis* and *A. jandaei* species. The

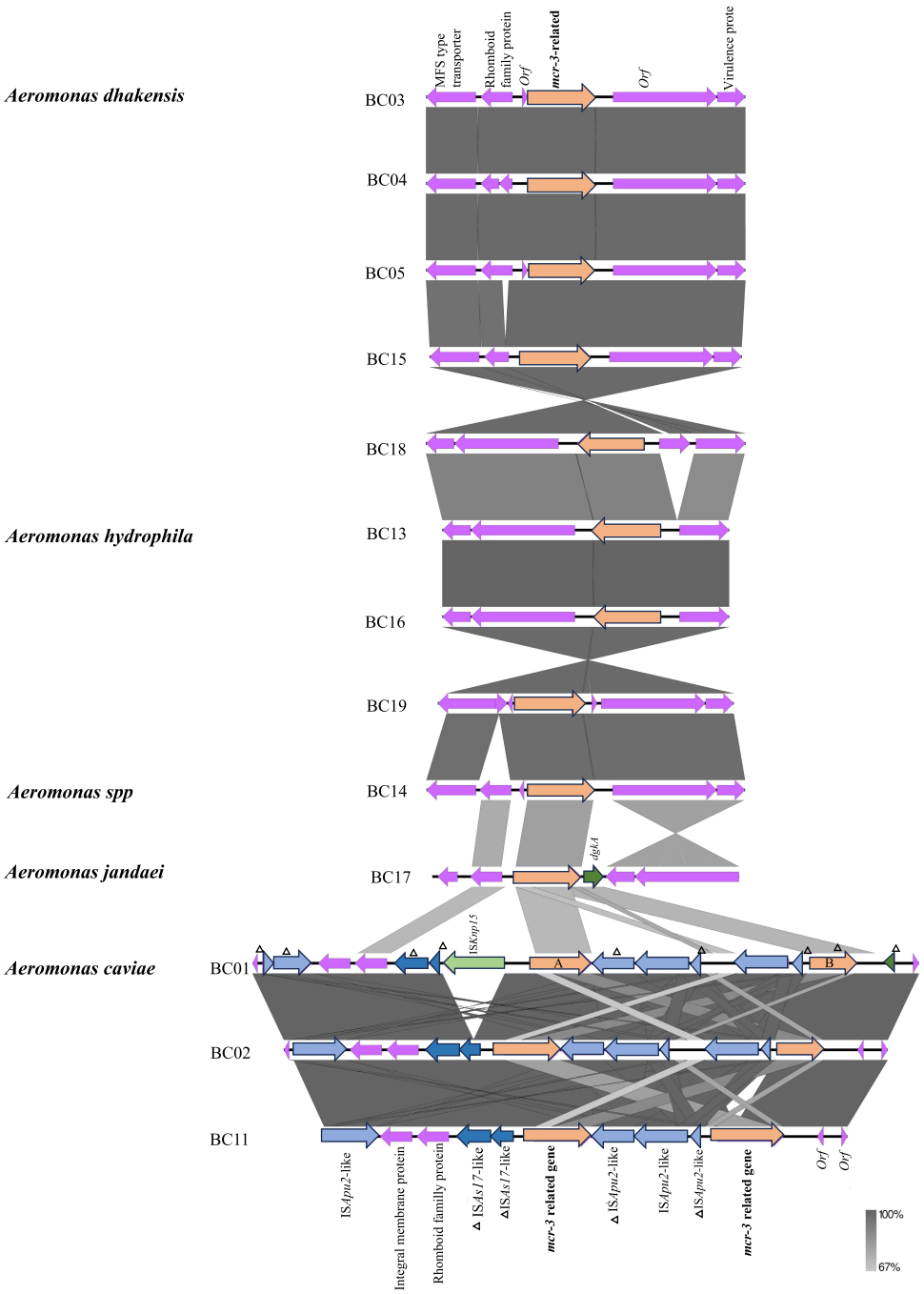

Figure 3 Linear comparison of genetic context of chromosomal *mcr-3*-related genes in isolates from Ghana generated with EasyFig v2.2.2. All genetic structures surrounding *mcr-3*-related genes are represented by arrows. Dark grey color gradient represents BLAST hits with the maximum (100%) identity and light grey area shows BLAST hits with the minimum identity. *Mcr-3*-related genes are shown in purple color. Insertion sequences are shown in sea-blue/blue color. Same genetic structures are shown in the same color. Truncated sequences are indicated with small triangles.

mannose-sensitive hemagglutinin, polar flagella, tap type IV pili, and T2SS were carried in all species, but the genes encoding these factors were more copious in some species than in others.

Location, Genetic Context of *Mcr-3*-Related Genes and Phylogeny

The *mcr-3*-related genes were located on the chromosomes of all the 13 isolates. Similar genetic contexts of *mcr-3*-related genes were observed in the five *mcr*-positive *A. dhakensis* (BC03, BC04, BC05, BC15, and BC18),

characterized by the presence of an *orf*, rhomboid family proteins, and a major facilitator superfamily type transporter protein (MFS) in the upstream region, and an *orf* and virulence proteins in the downstream region. In *A. hydrophila* species (BC13, BC16, and BC19), an MFS transporter was found upstream, with an *orf* and a virulence protein located downstream. In the upstream region of *mcr-3*-related gene in *Aeromonas* spp. BC14, an *orf,* rhomboid family protein and an MFS transporter were identified, with an *orf* and virulence proteins downstream of the gene. Three *A. caviae* species (BC01, BC02, and BC11) harbored a *mcr-3*-related-gene-IS*Apu2*-like-IS*Apu2*-like-IS*Apu2*-like-*mcr-3*-related-gene segment. Notably, the second copy of the *mcr-3*-related gene labelled "B" (Figure 3) was ostensibly truncated in BC01 and BC02. Immediately upstream of the first *mcr-3*-related gene (A) in BC01 was the insertion sequence IS*Kpn15*, followed by two copies of truncated insertion elements that shared 99% similarity to IS*As17* belonging to the IS*2* family of insertion sequences, a rhomboid family protein, integral membrane protein, and an IS*4* family insertion element IS*Apu2*-like. The same structures were found in BC02 and BC11 except for the absence of IS*Kpn15*. Three truncated and two full-length copies of insertion elements that shared 99% similarity with IS*Apu2* were located downstream of A (Figure 3).

To explain the evolutionary relationship of *mcr-3*-related genes identified in this study with other previously described *mcr-3* variants, a maximum likelihood tree was constructed comprising amino acid sequences of 52 publicly available colistin resistance gene variants and those from this study. The *mcr* variants were divided into subgroups. The 465aa *mcr-3*-related *EptA* in BC01, BC02, and BC11 was differentiated into the same subgroup, and the sequence appeared to be more homologous to *mcr-3.15, mcr-3.27* and *mcr-3.38*. BC17 *mcr-3*-related (538aa) seemingly shared an evolutionary relationship with *mcr-7.1*. However, the remaining *mcr-3*-related genes were differentiated into distinct subgroups, which diverged from previously reported *mcr-3* variants (Figure 4).

Discussion

Aeromonas species are distributed across many different habitats, despite being native to aquatic environments[35] and notwithstanding that *A. hydrophila* has been demonstrated to be an efficient organism for degrading polycyclic aromatic hydrocarbons (PAHs) with good prospects for bioremediation of PAHs polluted river systems,[36] the *Aeromonas* genus is also known to cause human infections ranging from meningitis, respiratory, and genitourinary infections to gastroenteritis and soft skin tissue infections in both immunocompetent and immunocompromised persons.[37,38] These species were identified as the most prevalent colistin-resistant organisms carrying *mcr-3*-related genes in this study, similar to the results of a study in South Africa.[14] The species carrying the *mcr-3*-related genes were isolated from well, surface water of reservoir, pond water and more abundantly from mud water, and even though they were not obtained from clinical sources, they constitute a threat in the effort to confront the antimicrobial resistance situation because resistance can spread between different bacterial species across environments by horizontal gene transfer, and the aquatic environment could potentially be a reservoir for the distribution of colistin resistance.[39] Furthermore, the detection of colistin-resistant organisms in these water sources poses a human health risk, as some of these serve as water sources for domestic activities for the inhabitants of Pakro and Avaga.

As opposed to other bacterial species, *Aeromonas* MLST scheme applies to the whole genus[40] (http://pubmlst.org/Aeromonas/). MLST analysis produced 10 distinct STs, with most occurring as singletons, suggesting that they are distantly related, as expected. The analysis also revealed *A. dhakensis* ST1427 from this study and *A. dhakensis* ST1298 isolated from drinking water in Dakar, Bangladesh (s://pubmlst.org/bigsdb?page=infoanddb=pubmlst_aeromonas_isolatesandid=1283) were clonal, sharing five out of the six alleles, suggesting that they may have evolved from a similar ancestor. Additionally, the appearance of ST1417 three times (Figure 2) raises concerns regarding the possibility of clonal dissemination of the *mcr*-3-related genes. *A. dhakensis* ST340 was identified as similar to that isolated from a stool sample in a previous study in Malaysia.[41] Isolation of ST340 from both environmental and clinical sources suggests that ST340 can cause human infections through ingestion of contaminated water.

Various genes encoding virulence factors have been identified in *Aeromonas* spp., some of which are markedly specific to different species. *A. dhakensis* is described to exhibit extensive virulence among *Aeromonas* spp.,[41,42] and causes infections, with some leading to fatalities.[43] As expected, *A. dhakensis* carried the most virulence-determining genes. Hemolysin Ahh1 is a major virulence factor of *A. dhakensis* and *A. hydrophilia*, responsible for severe tissue

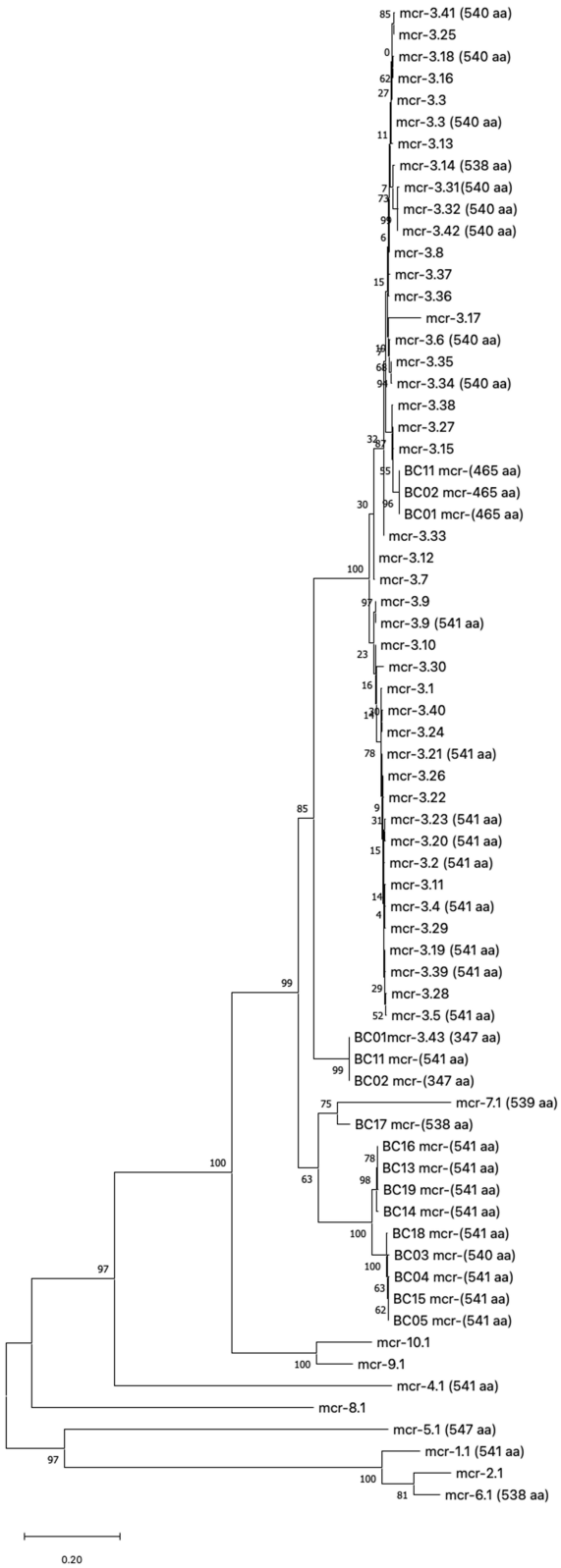

Figure 4 Maximum likelihood phylogeny of *mcr-3* variants based on amino acid sequences. Tree was constructed using the JTT matrix-based model with 1000 bootstrap repeats. Sequences of *mcr-3*-related genes from this study are labelled with the prefix BC. BC01-BC11 were identified in samples collected from Avaga and BC13-BC19 from Pakro-Adesa communities in Ghana.

necrosis during severe soft tissue infections.[42] Consistent with our findings, this gene was found mostly in *A. dhakensis* and *A. hydrophila*. Pili are well-characterized virulence factors in pathogenic bacteria that aid in adherence to surfaces.[44] Three pili types were identified: the flp type IV, the tap type IV, and the MSH. The flp types have been found to affect biofilm formation, autoaggregation, and rough colony morphology.[45] While some MSH- and tap type IV-related genes were carried by all 13 species, the flp type was found only in the *A. hydrophila* species. The T2SS plays a role in releasing aerolysin-related cytotoxic enterotoxins (*Act*), which can potentially cause diarrhea and wound infections. While T3SS and T6SS contribute to *Aeromonas* infections, T6SS in particular has been described to secrete an effector involved in cell apoptosis.[46] All these virulence factors suggestively contribute to the virulence of some *Aeromonas* species as medically important pathogens.

Although plasmid-mediated resistance is problematic, the cost associated with chromosomally-mediated resistance makes it a preferable option, as plasmids can easily be lost through evolutionary events or in the absence of antibiotic selection pressure. In contrast, chromosomally-mediated resistance is irreversible, and bacteria can maintain the genes in perpetuity, provided that their carriage does not incur a fitness cost.[47] *Mcr-3*-related genes identified in this study were borne on chromosomes, which is a cause for concern because these genes will likely persist with or without the availability of colistin.

Insertion elements belonging to the IS*2* (IS*As17*-like) and IS*4* (IS*Apu2*-like) families were identified in multiple copies, and IS*Kpn15* in a single copy, in the flanking regions of *mcr-3*-related genes in *A. caviae* species, but not in other *Aeromonas* species. This suggests that the insertion sequences may be involved in the mobilization of *mcr*-genes and also a requisite for insertion of *mcr-3*-related genes in *A. caviae*[48] and given that *Aeromonas* spp. are normal microflora in fish and poultry, the transfer of mobile *mcr* genes could be facilitated among other bacterial species in the same environment.[34,49]

Biofilm formation is characteristic of *Aeromonas* spp. It enables the pathogens to persist in water distribution systems as well as in the human body.[49] The process of biofilm formation is controlled by quorum sensing.[50] The rhomboid family proteins, which are reported to be involved in bacterial quorum sensing, were located upstream of *mcr-3*-related genes in all species except *A. hydrophila* species.[51] Although the quorum sensing or biofilm formation ability of the species was not explored in this study, it is plausible that this protein acts to enhance quorum sensing, thereby affecting biofilm formation ability, as demonstrated in other studies.[49] In addition, a *dgkA* gene, which encodes diacylglycerol kinase and catalyzes the conversion of diacylglycerol to phosphatic acid, was identified immediately downstream of *mcr-3*-related gene in the *A. jandaei* strain, which is consistent with previous reports where *dgkA* was found immediately downstream of *mcr-3* genes on both chromosomes and plasmids.[34,52,53]

Phylogenetic analysis based on the amino acid sequences indicated that the amino acid sequences of BC01, BC02, and BC11, encoding a protein of 465 amino acids, were mostly similar to those of *mcr-3.27* and *mcr.3.15,* whereas the sequences coding for proteins of 347 or 541 amino acids were more divergent from other sequences, suggesting distinct evolutionary origins.[54] The similarity in sequences between BC17 *EptA* and *mcr-7.1* is not surprising, as *mcr-7.1* is reported to have originated from *Aeromonas* species and shares sequence similarity with *mcr-3*.[55]

Conclusion

We isolated colistin-resistant *Aeromonas* species with wide array of virulence determinants from water samples collected from communities in Eastern Ghana, and whole-genome analysis revealed the presence of *mcr-3*-related genes close to insertion elements with potential for mobilization. This suggests that environmental water samples could be reservoirs of colistin-resistant bacterial species and supports the call for investigation of antibiotic resistance to transcend hospital environments. Also, findings from the study implied clonal dissemination of *mcr-3*-related gene-carrying *Aeromonas* spp. and highlight the potential risk posed by their presence in the aquatic environments. The isolation of colistin-resistant bacteria from water samples represents a threat to human health, and the findings of this study offer a rationale to reinforce the implementation of control measures to restrict the spread of antibiotic resistance and improve human health.

Data Sharing Statement

Dataset generated from the study is available from the corresponding authors upon reasonable request.

Ethical Considerations

The ethical committee and review board of the Noguchi Memorial Institute for Medical Research, University of Ghana (FWA00001824) and the Faculty of Medicine, Tokyo Medical and Dental University (M2017-208) reviewed and approved the protocols for this study.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no financial or personal interests that could be misconstrued as potential conflicts of interest.

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