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Posted Date: 29 March 2024

doi: 10.20944/preprints202403.1830.v1

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Mobile Colistin-Resistant Gene; *mcr-1*, *mcr-2*, *mcr-3* Identified in Diarrheal Pathogens among Infants, Children, and Adults in Bangladesh: Implications for the Future

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Abstract: Colistin is a last-resort antimicrobial for treating multidrug-resistant Gram-negative bacteria. Phenotypic colistin resistance is highly associated with plasmid-mediated mobile colistin resistance (*mcr*) genes. *mcr*-bearing *Enterobacteriaceae* have been detected in many countries, with the emergence of colistin-resistant pathogens a global concern. This study assessed the distribution of *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5* genes with the phenotypic colistin resistance in isolates from diarrheal infants and children in Bangladesh. Bacteria were identified using the API-20E biochemical panel and 16s rDNA gene sequencing. Polymerase chain reactions detected *mcr* gene variants in the isolates. Their susceptibilities to colistin were determined by agar dilution and E-test by minimal inhibitory concentration (MIC) measurements. Over 30.0% (69/225) of isolates showed colistin resistance by agar dilution assessment (MIC> 2.0 µg/mL). Overall, 15.5% of isolates carried *mcr* genes (7, mcr-1; 17, mcr-2; 13, mcr-3; and co-occurrence occurred in 2 isolates). Clinical breakout MIC values (≥ 4 µg/mL) were associated with 91.3% of *mcr*-positive isolates. The *mcr*-positive pathogens include twenty *Escherichia spp.*, five *Shigella flexneri*, five *Citrobacter spp.*, two *Klebsiella pneumoniae*, and three *Pseudomonas parafulva*. *mcr*-genes appeared to be significantly associated with phenotypic colistin resistance phenomena (p=0.000), with 100% colistin-resistant isolates showing MDR phenomena. Age and sex of patients showed no significant association with detected *mcr* variants. Overall, *mcr*-associated colistin-resistant bacteria have emerged in Bangladesh, which warrants further research to determine their spread and instigate activities to reduce resistance.

Keywords: mobile colistin-resistance; *mcr* gene; human-*mcr*; diarrheal infant patients; bangladesh; MDR; antimicrobial stewardship programs

1. Introduction

In recent years, emerging antimicrobial resistance (AMR) has been recognized as a significant public health concern that transcends international boundaries [1]. In 2019, it was estimated there were 4.95 million deaths globally associated with bacterial AMR, including 1.27 million deaths directly attributable to bacterial AMR, with the highest mortality currently seen in South Asian and sub-Saharan African countries [2]. There is also considerable morbidity and costs associated with AMR [3–6]. If AMR is not adequately tackled, this could reduce gross domestic product per country

 doi:10.20944/preprints202403.1830.v1

by up to 3.8% [5,6]. As a result, AMR is increasingly seen as the next pandemic unless urgent measures are introduced to reverse the rising rate [7]. The overuse and misuse of antibiotics either prophylactically or therapeutically in either humans or animal husbandry generates selection pressure, increasing the development of AMR in pathogens and other diverse commensal microbial populations [8–11]. Whilst AMR is a universal phenomenon, the burden among low- and middle-income countries (LMICs) is appreciably higher due to economic, political, and environmental factors, including poor governance and infrastructures, as well as a limited number of national initiatives [12–15]. This is now changing with increasing recognition of the clinical and economic consequences of AMR. Global initiatives include the Global Action Plan (GAP) by the World Health Organization (WHO) to reduce AMR [1], as well as initiatives from the World Bank and OECD [6,16]. The GAP has been translated into the National Action Plans in Bangladesh, with countries at different stages of their implementation due to resource and other issues [17–20].

Other important global initiatives include dividing antibiotics into three different categories based on their resistance potential, which includes the 'Access', 'Watch', and 'Reserve' categories [21,22]. 'Access' antibiotics should typically be prescribed to treat commonly encountered infections with lower resistance rates and include penicillins. Those in the 'Watch' group, which includes azithromycin, quinolones, and a number of cephalosporins, should ideally only be prescribed in critical conditions as they have a greater chance of resistance development. Antibiotics in the 'Reserve' category, which include fifth-generation cephalosporins, some carbapenems, and linezolid, should only prescribed in multidrug resistance cases, with the aim of curbing rising AMR rates [21,23–25]. Reducing the extent of 'Watch' antibiotics being prescribed or dispensed can help to appreciably reduce the extent of multidrug-resistant organisms [26]. More recently, the WHO has launched the AWaRe book, giving prescribing guidance for approximately 35 clinical infections, including the choice of drug [24,27], dose, and duration for both children and adults, to reduce the extent of inappropriate antibiotic use. The target is that at least 60% of antibiotic use in given settings should be 'Access' antibiotics [23,24].

Colistin is also classified as a 'Reserve' antibiotic following the global increase in the prevalence of carbapenem-resistant *Enterobacteriaceae*, [22,28]. However, there are now concerns with its over use and resultant resistance development [29,30]. This includes in animals where there has been appreciable use of colistin as a growth promoter in recent years [30], with studies now showing that 96% or more of total worldwide use of colistin is still in poultry and pig farming [31,32]. Concerns with the overuse of colistin including both animals and humans, and resultant resistance development via zoonotic gene transfers, coupled with its importance in treating resistant gram-negative infections to reduce morbidity and mortality, has resulted in the World Health Organization and others classifying colistin as an antibiotic of very high importance for use in humans with its use reserved [21,33–39]. Alongside this, many countries now ban the use of colistin as a growth promoter in animal feeds and prophylactically to prevent bacterial infections [40–42]. Such measures have shown to be effective in reducing resistant strains [41–43]. This is important in Bangladesh, given extensive colistin-resistant *Escherichia coli* in broiler meat and chicken feces [44–46], exacerbating resistance among patients to colistin in Bangladesh [47–50]. Over-the-counter dispensing of antibiotics is also common in Bangladesh and a concern, especially when this involves 'Watch' and 'Reserve' antibiotics [51–54]

Overall, activities to enhance the appropriate use of colistin in both animals and humans are essential as colistin still remains the antibiotic of choice for multiple drug-resistant gram-negative bacterial infections (MDR-GNB). This includes carbapenem-resistant *Acinetobacter baumannii* (CRAB) as well as other pathogens resistant to the new antimicrobial agents [55–57]. However, the use of colistin as a last resort antibiotic is greatly threatened by the rise of plasmid-borne mobile colistin resistance gene [58–60], spreading rapidly via horizontal gene transfer [61]. Resistance to colistin is generated by the chromosomally mediated modification of lipopolysaccharide (LPS) [62]. Acquisition of colistin resistance by a novel plasmid-mediated gene, *mcr-1*, was first described in *Enterobacteriales* from both farm-animal products and humans [63]. Earlier studies have shown the genotypic linkage of the mobile colistin resistance genes, *mcr-1*, to the phenotypic colistin resistance [64,65]. Since then,

 doi:10.20944/preprints202403.1830.v1

the variants of *mcr*-carrying multiple species of *Enterobacteriales* have been detected in many countries from environments, animals, and humans [66–69]. Subsequently, more variants of transferable colistin resistance *mcr* gene (*mcr-1* to *mcr-9*) have been described in *Enterobacteriaceae* [70,71].

In general, a substantial part of *Enterobacteriales* is normal flora; however, a few of those microbial pathogens can cause systemic bacteremia [72], community-acquired infections [73,74], and healthcare-associated infections (HAI) [75]. A recent outbreak with colistin-resistant pathogens in China ended a very high case-fatality rate in humans [76]. Consequently, the identification of the root cause, transmission, and trajectories of colistin-resistant infection is an increasing priority globally. We are aware that *mcr*-gene variants can be detected in the environment, animals, human fecal samples, and food products [77]. However, only a limited number of studies have also showed the dissemination of plasmids carrying these variants in infants with acute diarrhea [78]. This is important in Bangladesh, with diarrhea being a major cause of childhood mortality in the country, combined with the increasing prevalence of resistant genes in children exacerbated by the overuse of antibiotics [79–81].

Consequently, this study was designed to investigate different variants of the *mcr* gene and their association with colistin resistance among diarrheal pathogens in infants, children and adults in Bangladesh and the demographic factors associated with the identified *mcr* variants. The findings can be used to suggest future policies and initiatives where there are concerns.

2. Results

2.1. Study Patients

We collected a total of 179 diarrheal stool samples from infants, children, and adults in different locations in Bangladesh (Figure 1) throughout our study and isolated 228 distinct bacteria from 168 culture-positive diarrheal patients. The study patients comprised of 102 (57%) males and 77 (43%) females.

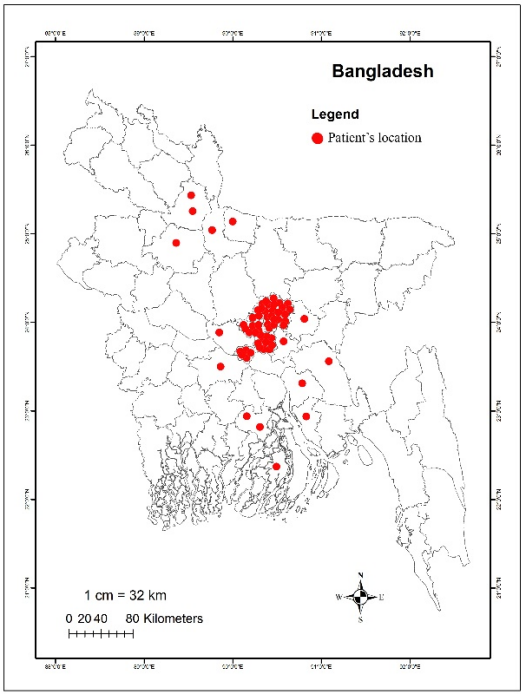

Figure 1. Sampling Location. Different sites were marked, indicating the spatial distribution of diarrheal patients from whom samples were collected. The map displays sampling sites across various cities in Bangladesh.

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In eleven stool samples, no bacterial growth appeared. The majority of study patients were infants and children who needed hospitalization (admitted to Uttara Medical College, Dhaka), and the median and interquartile range (IQR) age was 1.17 (0.75-2.5) years. Additionally, 78.1% of patients were middle class, 21.1% were poor, and 0.8% were rich (Figure 2A). The duration of diarrhea among all patients ranged from 3- 40 days, and the mean duration (standard deviation) was 7.2± 4.78 days.

The ages of the patients were categorized into five groups, namely <1 year, 1-5 years, 6-10 years, 11-15, and >15 years. This revealed that disease occurrence was higher in the 1-5 years age group (48.0%). Figure 2B provides further information regarding the incidence of diarrhea associated with each age group. Among all the patients, 179 (100%) appeared with watery stools, 4 (2.2%) presented with abdominal cramps, 39 (21.8%) with vomiting, and 2 (1.10%) patients had blood in their stools (Figure 2C).

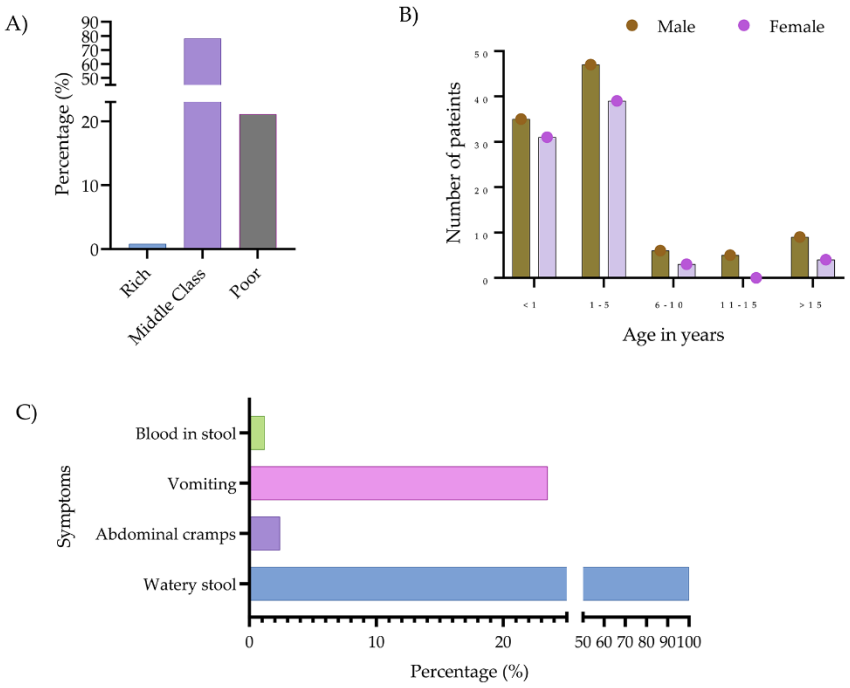

Figure 2. Socioeconomic status of patients (A), as well as their age, sex (B), and clinical symptoms associated with diarrhea (C).

2.2. Identification of Diarrheal Pathogens and Their Phenotypic Colistin Susceptibility

Of the 228 isolates, 140 were categorized as Escherichia spp. (61.40%), 20 as Citrobacter spp. (8.77%), 18 as Klebsiella spp. (7.89%), 13 as Shigella flexneri (5.70%), 10 Enterobacter spp. (4.39%), and seven as Stenotrophomonas maltophi (3.07%). Figure 3 contains details of all the pathogens identified. One Proteus sp and two staphylococci were excluded from further study since colistin resistance is natural in Proteus and Staphylococcus species [82,83].

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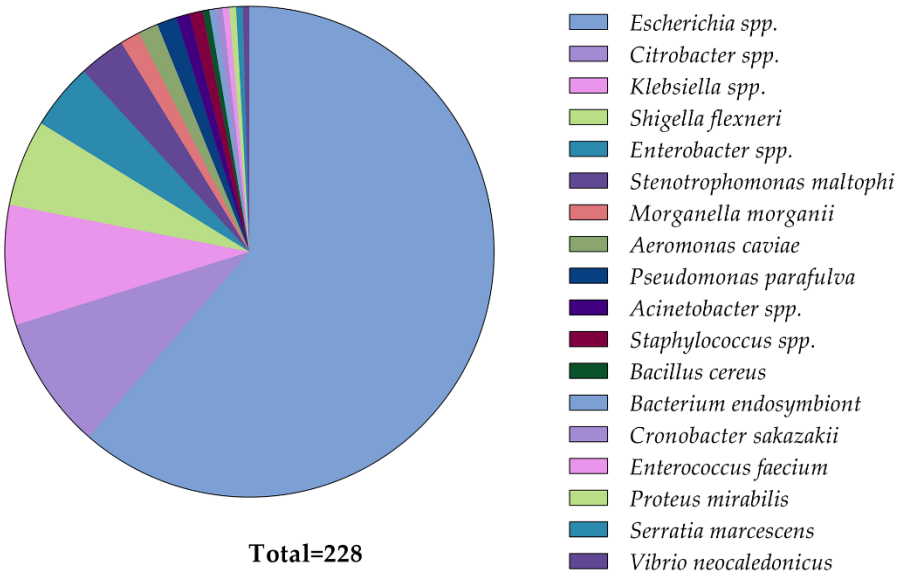

Figure 3. Distribution of different bacteria identified from diarrheal patients. The pie chart illustrates the relative numbers of various bacteria identified among diarrheal patients. Each segment represents a specific bacterial species, with the size of each segment corresponding to the frequency of its occurrence in the sampled population.

The agar dilution method determined the test bacteria as susceptible (S) when there is no growth at ≤2 μg/mL colistin sulfate concentrations and resistant (R) when growth appeared at >2 μg/mL. In addition, the disc diffusion method was used to evaluate the antibacterial potency of colistin sulfate (25 μg) in vitro for the 225 isolates, and bacteria were considered colistin-resistant (R) if ≤10 mm diameter zone of inhibition was recorded. Of the 225 isolates, the agar dilution test revealed that 69 (30.7%) isolates were resistant to colistin sulfate. However, the colistin disc diffusion method showed that 180 (80.7%) isolates were resistant. No significant relationship was observed between agar dilution and the disc diffusion method.

2.3. Prevalence of mcr Genes in Diarrheal Isolates

All 225 isolates were subjected to polymerase chain reaction (PCR) to find mcr-1 to mcr-5 genes. Three types of mcr genes (mcr-1, mcr-2, mcr-3) were detected in 35 isolates. These included 20 for Escherichia spp., five for Shigella flexneri, five for Citrobacter spp. and two Klebsiella pneumoniae and Enterobacter hormaechei, and one Pseudomonas parafulva. Bacteria identified from ten other genera did not appear with mcr genes (Table 1).

Table 1. Identified diarrheal pathogens carrying *mcr* gene variants[a].

Bacteria type[b]	Number of isolates carrying *mcr* genes		Percentage of *mcr*-positive isolates
	Positive	Negative	
Escherichia spp.[c]	20	120	14.3
Shigella flexneri	5	8	38.5
Citrobacter spp.[d]	5	15	25.0
Klebsiella pneumoniae	2	16	11.1
Enterobacter hormaechei	2	8	20.0
Pseudomonas parafulva	1	2	33.3
Aeromonas caviae.	0	3	-

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Acinetobacter spp	0	2	-
Bacillus cereus	0	1	-
Bacterium endosymbiont	0	1	-
Morganella morganii	0	3	-
Serratia marcescens	0	1	-
Stenotrophomonas maltoph	0	7	-
Vibrio neocaledonicus	0	1	-
Cronobacter sakazakii	0	1	-
Enterococcus faecium	0	1	-
Total	35	190	

Note : [a]12 mcr-1 and two mcr-2 genes were identified by polymerase chain reaction (PCR). [b]Bacteria were primary identified based on their growth on selective culture media followed by biochemical tests. Further, identifications were confirmed by API 20E and 16s rDNA sequencing. [c]Escherichia spp. includes three Escherichia fergusonii and seventeen Escherichia coli. [d]Citrobacter spp. includes one Citrobacter europaeus, two Citrobacter portucalensis and two Citrobacter portucalensis

Of the 35 mcr positive isolates, co-occurrence was identified in 2 isolates, one contained mcr-1, mcr-2 and the other contained mcr-2, mcr-3. The harborage of mcr-1, mcr-2 and mcr-3 were identified as 3.1% (7 isolates), 7.6% (17 isolates), 5.8% (13 isolates) respectively. Combined, the presence of mcr variants was 15.56% (35/225).

2.4. Phenotypic-Genotypic Association

Of the 35 mcr positive isolates, the agar dilution test identified 32 (91.4%) resistant isolates and 3 (8.6%) sensitive isolates, which revealed high statistical significance of mcr variants gene associations (p= 0.000). Further separate analyses showed very significantly high statistical associations of mcr-1, mcr-2, and mcr-3 with phenotypic colistin resistance (p = .000, for all the three gene variants) (Table 2). All of the test isolates grew well on the control plate without colistin sulfate. As a susceptible control, Escherichia coli ATCC25922 strain with MIC of 2 μg/mL was employed.

Table 2. Phenotypic genotypic association of *mcr* genes and colistin resistance.

Presence of *mcr* gene varients		**Phenotypic susceptibility**		**P value**
		sensitive	**resistance**	
mcr-1	Positive (7)	1	6	.001
	Negative (218)	155	63	
mcr-2	Positive (17)	3	14	.000
	Negative (208)	153	55	
mcr-3	Positive (13)	0	13	.000
	Negative (212)	156	56	
combined	Positive (35)	3	32	.000
	Negative (190)	152	38	

The MIC was determined by E-test and agar dilution test separately using a range of ≤.5 μg/mL to >256 μg/mL. The median and IQR MIC for mcr-positive isolates was 32.0 (8.0-128) μg/mL (Table 3). One isolate (Escherichia coli) with co-carriage of mcr-1 and mcr-2 exhibited MIC values of 128 μg/mL. The other co-carrying bacterium (Escherichia coli) with mcr-2 and mcr-3 showed a MIC value of 8 μg/mL. The three mcr-positive bacteria that showed phenotypic susceptibilities to colistin-sulfate were Citrobacter portucalensis, Citrobacter freundii, and Escherichia coli (Table 3). Alongside, the median and IQR MIC for mcr-negative isolates was 1.0 (0.5-2.0) μg/mL. Whilst 20% of the mcr-negative isolates (38/190) exhibited resistance to colistin sulfate in agar dilution with the MIC range from 4 μg/mL to >128 μg/mL (Figure 4).

 doi:10.20944/preprints202403.1830.v1

Table 3. Diarrheal isolates with identified mcr-gene variants and associated minimum inhibitory concentration of colistin.

mcr-possitive isolate ID	Identified bacteria[a]	Identified *mcr* gene varient	Phenotypic colistin susceptibility by MIC (μg/mL)[b]
PBD009	*Shigella flexneri*	*mcr-3*	256
PBD014	*Klebsiella pneumoniae*	*mcr-2*	128
PBD018	*Escherichia coli*	*mcr-3*	8
PBD021	*Escherichia coli*	*mcr-3*	128
PBD022	*Escherichia coli*	*mcr-3*	32
PBD027	*Citrobacter portucalensis*	*mcr-2*	.5
PBD028	*Escherichia coli*	*mcr-3*	256
PBD033C2	*Pseudomonas parafulva*	*mcr-1*	128
PBD35	*Citrobacter portucalensis*	*mcr-2*	8
PBD35C1	*Citrobacter freundii*	*mcr-2*	128
PBD35C2	*Citrobacter freundii*	*mcr-2*	1
PBD039	*Escherichia fergusonii*	*mcr-2*	256
PBD040	*Citrobacter europaeus*	*mcr-2*	64
PBD043	*Klebsiella pneumoniae*	*mcr-2*	16
PBD058	*Escherichia coli*	*mcr-2, mcr-3*	8
PBD062	*Escherichia fergusonii*	*mcr-2*	32
PBD072	*Escheril chia fergusonii*	*mcr-2*	64
PBD077	*Escherichia coli*	*mcr-1*	8
PBD077C1	*Shigella flexneri*	*mcr-1*	32
PBD077C2	*Escherichia coli*	*mcr-1,mcr-2*	128
PBD080C2	*Escherichia coli*	*mcr-1*	2
PBD081C3	*Escherichia coli*	*mcr-2*	32
PBD081C4	*Enterobacter hormaechei*	*mcr-3*	128
PBD081C1	*Escherichia coli*	*mcr-2*	32
PBD082	*Shigella flexneri*	*mcr-1*	128
PBD083C1	*Shigella flexneri*	*mcr-3*	32
PBD083C2	*Escherichia coli*	*mcr-2*	64
PBD084C1	*Enterobacter hormaechei*	*mcr-2*	128
PBD84C2	*Escherichia coli*	*mcr-2*	4
PBD090	*Escherichia coli*	*mcr-3*	64
PBD096	*Escherichia coli*	*mcr-3*	8
PBD107	*Escherichia coli*	*mcr-1*	8
PBD114	*Escherichia coli*	*mcr-3*	8
PBD116	*Escherichia coli*	*mcr-3*	64
PBD117	*Shigella flexneri*	*mcr-3*	64

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Note: [a]Bacteria were identified by rapid biochemical test kit API 20E system (BioMe ´rieux, Durham, NC) followed by16s rDNA sequencing. [b]The minimum inhibitory concentration (MIC) measurement was conducted by agar dilution method following the EUCAST guidelines.

MIC analyses at each specific value between mcr-positive and mcr-negative isolates showed that most mcr-carrying isolates were identified with higher MIC levels of colistin sulfate ranging from >8 µg/mL to >128 µg/mL (Figure 4). At the same time, the vast majority of the mcr-negative isolates exhibited lower levels MIC, ranging from ≤.5 µg/mL to 2.0 µg/mL. A fraction of mcr-negative isolates demonstrated MIC values from 32 µg/mL to >128 µg/mL (Figure 4).

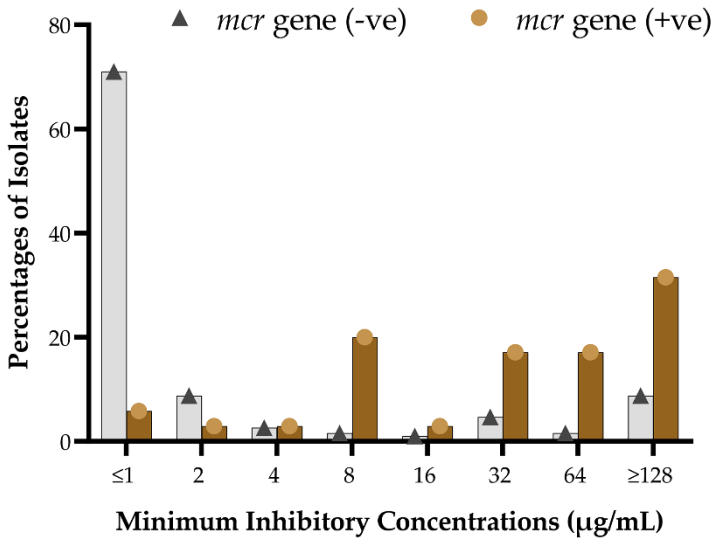

Figure 4. Distribution of MIC levels of colistin sulfate among mcr-positive and mcr-negative isolates.

2.5. Multi-Drug Resistance and mcr-Carriage

All mcr-positive isolates (carrying at least one mcr gene) were examined for the presence of MDR. The disk diffusion test was conducted to identify whether the 35 mcr-positive isolates exhibited susceptibility to the other 17 different antibiotics from 8 different groups or not. Remarkably, 100% of mcr-positive organisms exhibited MDR outcomes (Table 4), and mcr-negative isolates revealed a comparatively lower frequency of MDR outcomes.

Table 4. Multi-drug resistance (MDR) phenomena of mcr-positive isolates.

List of antibiotics tested (n = 17, from eight drug-classes)		**Phenotypic susceptibilities of *mcr*-positive diarrheal isolates**																		
Drug class	**Antibiotic name**	PBD009	PBD014	PBD018	PBD021	PBD022	PBD027	PBD028	PBD033C2	PBD035	PBD035C1	PBD035C2	PBD039	PBD040	PBD043	PBD058	PBD062	PBD072	PBD077
β-lactam with β-lactamase inhibitor	Amoxi-clav[a]	R	R	R	R	R	R	I	R	R	R	S	R	I	R	I	S	S	S
Cephalosporins	Cefuroxime-G2	R	R	R	R	I	I	I	R	R	R	R	R	R	R	S	R	R	S

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	Cefixime-G3	R	R	R	R	I	R	R	R	R	R	R	R	R	R	S	R	R	S
	Cefepime-G4	R	R	R	R	R	I	R	R	I	I	I	R	I	R	I	R	R	S
Carbapenems	Imipenem	R	R	I	R	R	I	I	R	I	R	S	I	I	R	S	I	R	S
	Meropenem	R	R	S	R	R	S	R	S	S	S	S	I	S	S	S	S	R	S
Quinolone and fluoroquinolones	Nalidixic acid	I	S	R	I	I	I	I	R	I	R	R	R	I	R	R	R	R	R
	Ciprofloxacin	R	S	R	S	S	S	R	R	I	R	R	R	R	S	I	R	I	R
	Levofloxacin	R	S	R	S	S	S	I	R	S	I	S	R	S	R	S	R	S	R
	Lomefloxacin	S	S	R	S	I	S	R	R	I	R	R	R	S	I	R	R	R	R
Aminoglycosides	Gentamicin	S	S	I	S	R	S	I	S	S	S	S	S	S	R	S	R	R	R
	Amikacin	R	S	I	S	S	S	R	S	S	I	I	R	I	R	S	I	I	I
	Netilmicin	S	S	I	I	S	S	R	S	S	S	I	I	R	S	R	S	R	I
	Tobramycin	S	S	S	S	R	S	R	I	S	S	S	R	S	R	S	R	R	S
Polymyxins	Colistin	R	S	R	S	R	R	R	R	S	R	R	R	R	R	S	S	R	R
Nitrofuran	Nitrofurantoin	I	R	I	R	R	I	R	R	S	I	S	S	I	R	I	R	I	I
Trimethoprim	Trimethoprim-sulfamethoxazole	S	S	R	S	R	I	S	R	S	R	R	S	S	S	R	R	R	R
MDR status[e]		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Note: R, Resistant; S, Sensitive; I, Intermediate; MDR, multidrug resistant. [a]Amoxicillin-clavulanic acid. [b]PBD033C2 is Pseudomonas spp. which is naturally resistant to Amoxicillin-clavulanic acid, therefore, AST results have been excluded from MDR calculation. [c]PBD081C4 and PBD084C1 are Enterobacter spp., which is naturally resistant to amoxicillin-clavulanic acid, therefore, AST results has been excluded from MDR calculation. [d]different generations of cephalosporin. [e]Multidrug-resistant isolate is defined by center for disease control (CDC) as an isolate that is resistant to at least one antibiotic in three or more drug classes (https://www.cdc.gov/narms/resources/glossary). '+', indicates MDR-positive; and '-', indicates MDR-negative.

2.6. Demographic Factors Associated with the mcr-Carriage

More than one type of bacterial isolate was isolated and analyzed from each stool sample. Consequently, 130 bacteria were evaluated from 95 male stool samples and the other 95 from 73 female stool samples. Bacteria isolated from female stool samples carried more mcr genes (16.8%, 16/95) in comparison to male-origin isolates (14.6%, 19/130). In the five different age groups of the study population, almost uniform gene distributions were reported. Overall, the results showed that sex and age groups were not significantly associated with the presence of mcr-1, mcr-2, and mcr-3 (p= 0.445 to 0.781) (Table 5).

Table 5. Demographic factors associated with mcr genes (n = 225).

Demography		**Number (%) of different *mcr*-gene variants**		***p*-value**
		***mcr-1* positive (n = 7)**	***mcr-1* negative (n = 218)**	
Gender	Male	5 (3.8)	125 (96.2)	0.702*
	Female	2 (2.1)	94 (97.9)	

 doi:10.20944/preprints202403.1830.v1

Age group (years)	<1	4 (4.6)	83 (95.4)	0.707*
	1-5	3 (3.3)	88 (96.7)	
	6-10	0	12 (100)	
	11-15	0	5 (100)	
	>15	0	30 (100)	
		***mcr-2* positive (n = 17)**	***mcr-2* negative (n = 208)**	
Gender	Male	8 (6.2)	122 (93.8)	0.445*
	Female	9 (9.5)	87 (90.5)	
Age group (years)	<1	8 (9.2)	79 (90.8)	0.480*
	1-5	4 (4.4)	87 (95.6)	
	6-10	1 (8.3)	11 (91.7)	
	11-15	0	5 (100)	
	>15	4 (13.3)	26 (86.7)	
		***mcr-3* positive (n = 13)**	***mcr-3* negative (n = 212)**	
Gender	Male	7 (5.4)	123 (94.6)	0.779*
	Female	6 (6.3)	89 (93.7)	
Age group (years)	<1	5 (5.7)	82 (94.3)	0.781*
	1-5	4 (4.4)	87 (87)	
	6-10	1 (8.3)	11 (91.7)	
	11-15	0	5 (100)	
	>15	3 (10.0)	27 (90.0)	
		***mcr-1 to mcr-3* positive (n = 35)**	***mcr-1 to mcr-3* negative (n = 190)**	
Gender	Male	19 (14.6)	111 (85.4)	0.711*
	Female	16 (16.8)	79 (83.2)	
Age group (years)	<1	15 (17.2)	72 (82.8)	0.503*
	1-5	11 (12.1)	80 (87.9)	
	6-10	2 (16.7)	10 (83.3)	
	11-15	0	5 (100)	
	>15	7 (23.3)	23 (76.7)	

Note: %, row percentage; *Fisher's Exact test.

3. Discussion

We believe this is one of the first studies in Bangladesh to investigate colistin-resistant genes in human diarrheal pathogens among infants and children. Our data clearly showed the association of phenotypic colistin resistance and the *mcr* genes (*mcr-1* to *mcr-5*), similar to other published studies on colistin resistant bacteria isolated from diarrhea [78,84,85]. This builds on earlier studies in Bangladesh including among children and adults [68,86].

Isolates harboring *mcr* genes have been detected with high MIC values, showcasing disparities between the agar dilution test and the disk diffusion method [87]. This variation can be attributed to the slow diffusion of colistin disks on agar medium for the complex and large molecular structure of colistin sulfate. In parallel, some *mcr*-negative isolates exhibited resistance to colistin. Several potential reasons may account for this phenomenon. Firstly, mutations in the *mgrB* gene [88], responsible for binding polymyxin antibiotics in the gram-negative cell wall, are particularly prevalent in *Klebsiella pneumoniae* [89,90]. Secondly, the absence of testing for other variants of *mcr* genes including *mcr-6*, *mcr-7*, *mcr-8*, *mcr-9*, and *mcr-10*, which could also attribute phenotypic colistin resistance [91,92]. Thirdly, some resistant bacteria may develop capsules, a polysaccharide coating on the outer surface of the cell wall [93–95]. Fourthly, overexpression of efflux pump systems could contribute to resistance development [96,97]. Fifthly, modulation in the bacterial cell surface, including alterations in the structure of lipopolysaccharides (LPS) of the cell membrane, might affect

 doi:10.20944/preprints202403.1830.v1

the binding of polymyxin antibiotics [98]. Additional investigations will be necessary to uncover potential molecular explanations for the observed differences between phenotypic and genotypic colistin resistance.

In this study, male children had a higher incidence rate of diarrhea than female children, similar to previous studies [99,100]. However, the reason for this difference is unclear. We also found that 57.14% of *mcr*-positive isolates were resistant to amoxicillin/clavulanic acid, and 57 to 71% of *mcr*-carrying isolates resistant to all generations of cephalosporins, although higher generation were more susceptible. This is a concern with clinicians now prescribing more carbapenems due to the decreased potency of cephalosporins, with meropenem showing more susceptibility among the 17 different antibiotics in the eight groups studied. However, recent studies showed carbapenem-resistant *Enterobacteriaceae* are now a global threat [101–103]. The MDR status of all *mcr*-positive isolates is positive, which is a threat to public health in Bangladesh and beyond, given the ensuing rise in untreatable infectious diseases [104–106].

This study underscores the clinical significance of establishing comprehensive surveillance systems for priority antibiotics such as colistin. In addition, we urge the Government of Bangladesh to ban the use of colistin as a growth promoter in animal feeds and prophylactically to prevent bacterial infections, similar to other countries [40–42]. This is because such measures have been shown to be effective in reducing resistant strains [41–43]. Alongside, this instigates antimicrobial stewardship programs (ASPs) in ambulatory care similar to other important antibiotics in Bangladesh where there are concerns about resistance development [107–110]. This includes ASPs among community pharmacists and drug sellers building on ASPs in other sectors in Bangladesh [52,111].

We are aware that there are several limitations with this study. Firstly, it was challenging to accurately estimate the real-world scenario since adults experiencing diarrheal problems could readily seek treatment at a healthcare center, whereas children relied on their parents' decisions and assistance to access medical care. Secondly, the small sample size posed significant obstacles to conducting fully powered statistical analyses. However, our research spanned fifteen distinct districts across Bangladesh, and the outcomes are anticipated to be applicable if similar studies are conducted in other districts in the country. Thirdly, while this study examined *mcr* gene variants up to *mcr-5*, newer variants such as *mcr-6*, *mcr-7*, *mcr-8*, and *mcr-9* were not investigated. Having said this, efforts were made to maintain internal validity by conducting independent trials when necessary. Despite these limitations, we believe our findings are robust, providing guidance to all key stakeholders in Bangladesh to enhance future sensitivity to colistin.

4. Materials and Methods

4.1. Study Design and Sampling

A prospective cross-sectional study was conducted between January 2020 and December 2020 among diarrheic children visiting the outpatient Department of Uttara Adhunik Medical College Hospital, Dhaka, Bangladesh. A total of 179 children and adults having acute diarrhea participated in this study prior to treatment with any prescribed antibiotics.

Acute diarrhea was defined as three or more liquid, loose, mucus, or bloody stools within 24 h, lasting no longer than 14 days. Fever was defined as a temperature of ≥37.5 °C. Demographic data was taken from each child, and informed consent was obtained from the parents or guardians before sample collection. All relevant demographic, clinical, and laboratory data were recorded and transferred to the questionnaire prepared for this study.

The ages of the children and patients were categorized into five groups, namely <1 year, 1-5 years, 6-10 years, 11-15, and >15 years, based on previous studies [112]. The income of parents was classified into rich, middle class, or poor, and as we are aware, this can make a difference [113].

A sterilized cotton swab was dipped in the mucus, purulent or bloody part of the freshly passed stool sample, placed immediately in CaryBlair Medium (Oxoid, Hampshire, UK), and transported to the laboratory for further analysis within six hours of collection.

 doi:10.20944/preprints202403.1830.v1

4.2. Isolation and Identification of Bacteria

Collected samples were pre-enriched in buffered peptone water (Oxoid®, Hampshire, UK) at a dilution ratio of 1:10 and were incubated overnight at 37 ◦C. A loopful of each culture was streaked on MacConkey agar (Liofilchem Inc, Italy) and cysteine-, lactose-, and electrolyte-deficient (CLED) agar (Liofilchem Inc, Italy) and subsequently incubated at 37 ◦C for 24 h in aerobic condition simultaneously. MacConkey agar supports gram-negative diarrheal pathogens (Supplementary Figure S1A), while CLED agar aids in the growth of gram-negative bacteria and gram-positive cocci if present in diarrheal samples. Colony counts of 10^3 or 10^5 CFU/mL were considered for a cut-off value for a probable diarrheal sample [114].

Gram's staining and biochemical tests were initially performed to identify growth-positive bacteria. A rapid biochemical-test kit API 20E (BioMe´rieux, Durham, NC), consisting of carbohydrate batteries and enzymatic substrates in a set of chromogenic panels, was used to verify the isolated identity (Supplementary Figure S1B) [115]. A part of the bacterial identity was confirmed by the polymerase chain reaction (PCR) amplification and sequencing of the 16S rDNA gene [116]. In total, 228 different isolates were generated from all the diarrheal samples. Three bacteria (one Proteus and two staphylococci) were excluded from the study for the next level analyses since colistin resistance is a natural phenomenon of the excluded isolates. The remaining 225 isolates were subjected to assessment of colistin susceptibility and *mcr-1* to *mcr-5* carriage. The isolates were preserved in 30% glycerol in Trypticase Soy Broth (TSB) at – 80°C until further use.

4.3. Phenotypic Colistin Susceptibility Testing

The phenotypic antibiogram profiles of diarrheal isolates against colistin were determined primarily using the Kirby–Bauer disk diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical & Laboratory Standards Institute (CLSI) guidelines (Supplementary Figure S1C) [117,118]. A 3-hour bacterial suspension in Mueller–Hinton broth was prepared to a concentration of McFarland 0.5 equivalent and then streaked on Mueller–Hinton agar (MHA, Oxoid, Basingstoke, UK) plates using a cotton swab to ensure consistent growth. The susceptibility of the isolate to the following discs of antibiotics (Oxoid, Basingstoke, UK) was evaluated: colistin (25 μg), Amoxycillin + Clavulanic acid (30 μg), Cefuroxime Sodium (30 μg), Cefixime (30 μg), Cefepime (30 μg), Imipenem (10 μg), Meropenem (10 μg), Nalidixic acid (10 μg), Ciprofloxacin (5 μg), Lomefloxacin (10 μg), levofloxacin (5 μg), Gentamicin (30 μg), Amikacin (30 μg), and Netilmicin (30 μg), Tobramycin (10 μg), Nitrofurantoin(300 μg), Trimethoprim-sulfamethoxazole (25 μg); by placing them on the bacterial lawns and incubating at 37°C overnight. A clear zone was developed around the disc for sensitive bacteria, and zone diameter was measured and evaluated to categorize Bacteria as susceptible (S), intermediate (I), and resistant (R) from the CLSI guideline charts for the appropriate antibiotics tested [118].

The isolates' phenotypic antimicrobial susceptibilities were further tested by the agar dilution method [119]. The lowest concentration of colistin adequate to inhibit the visible growth of bacterial test isolates was determined by minimal inhibitory concentration (MIC) measurement by agar dilution method [119]. For agar dilution MIC, different concentrations of colistin-sulfate powder (Santa Cruz Biotechnology Inc, TX) from .50 μg/mL to 256.0 μg/mL in a two-fold dilution order were incorporated into MHA medium accordingly. One pure culture colony was inoculated into Mueller-Hinton broth to prepare each test inoculum and incubated for three hours at 37°C that develops a density of inoculum equivalent to 10^4 colony-forming units (CFU) per spot to drop on the MHA. The inoculum density was periodically compared to a 0.5 McFarland standard, equivalent to approximately 10^8 CFU/mL. The plates were incubated at 37°C in the air for 18-20 hours. Agar dilution MICs were performed in duplicates. The experiments were repeated when some single colonies or a thin haze growth was observed within the inoculated spot.

The epsilometer test (E-test) was performed parallel partly using a commercial strip containing a predefined gradient of colistin concentrations (Liofilchem Inc, Italy) to validate colistin MIC determination by the agar dilution method [87,120]. Concordant results were found in independent MIC assessment and E-test (Supplementary Figure S1D,E). *E. coli* ATCC 25922 strain was used as the

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quality control strain for disc diffusion and MIC testing. Besides, a control plate without colistin-sulfate was examined for the growth of both test and control isolates. Following EUCAST and CLSI guidelines, isolates were considered susceptible (S) when the MIC values exhibited ≤2 μg/mL and resistant (R) when MICs appeared >2 μg/mL [117]. Multidrug-resistant (MDR) isolates were described as those isolates that were found to be resistant to at least three different classes of antibiotics [121].

4.4. Detection of the Colistin Resistance mcr Genes

All 225 isolates were subjected to a single plex polymerase chain reaction (PCR) to detect the *mcr-1* gene, yielding a 309 bp DNA band, using primers described elsewhere [63], and confirmed by sequencing. Amplicons were visualized under UV light after 1.2% agarose gel electrophoresis. The other four primer pairs to detect *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5* gene amplicons were obtained from a recently published original study [122]. Multiplex polymerase chain reaction (PCR) was conducted to detect the *mcr-1* to *mcr-5* genes in the isolates. In brief, the modified protocol was as follows: prepared bacterial DNA (2.0 μL) was added to a 2× PCR premixture (15 μL, GeneON, Germany), and five pmol of each primer (1 μL), and deionized water was added to obtain a final volume of 30 μL. Reactions went through an initial denaturation at 94 °C for 15 min followed by 25 cycles of amplification (Applied Biosystems 2720 Thermal Cycler, Singapore), consisting of denaturation for 30 s at 94 °C, annealing for 90 s at 55 °C, and extension for 1 min at 72 °C, and a final 10 min elongation at 72 °C. Expected amplicons for *mcr-1* (309 bp), *mcr-2* (715 bp), *mcr-3* (929 bp), *mcr-4* (1116 bp), and *mcr-5* (1644 bp) underwent electrophoresis through 1.2% agarose gel followed by staining with ethidium bromide and were visualized under UV light (Supplementary Figure S1E). Lastly, the obtained results were validated by separate single plex PCR analyses of the *mcr* genes.

4.5. Statistical Analysis

Using IBM SPSS statistics data editor (version 21) and GraphPad prism software (version 9.5), verified data were entered and then examined. The bivariate analysis did not include missing data. The *mcr* gene variations carried by diarrheal pathogens and their phenotypic traits were described using both descriptive and inferential statistical methods. Any associations between categorical data were examined using Pearson's chi-square test, with the appropriate use of Yate's continuity correction. Fisher's Exact test results of a 2 x 2 contingency table were presented in place of the chi-square test results if the predicted frequency of the test cannot be assumed. Two-tailed p-values were computed, with a significance level of 0.05.

4.6. Ethics Statements

This study was authorized [No. UAMC/ERC/Recommend-62/2018, dated 09.07.2018] by the Ethics Review Committee of Uttara Adhunik Medical College. All research protocols complied with the Declaration of Helsinki regarding the use of human beings in research.

Each adult study participant provided written informed consent before the collection of their urine samples. For patients under the age of 18, parents or legal guardians were separately asked for written informed permission. Patient's identities were anonymized.

5. Conclusions

Our findings indicate that multidrug-resistant pathogenic bacteria containing *mcr* genes are a major reservoir in the guts of young Bangladeshi children and adults. The *mcr-1*, *mcr-2* and *mcr-3* variants predominate in the Bangladeshi diarrheal bacteria over other variants, such as *mcr-4* and *mcr-5*. We did not find any association between phenotypic colistin resistance and age, sex.

The advent of clinical MDR pathogens resistant to colistin intended as an antibiotic for last resort may spread diseases and illnesses that are subsequently incurable. The findings require immediate monitoring and action for both national and international antimicrobial stewardship. This includes limiting the use of colistin as a growth promoter agent in animal husbandry in Bangladesh, similar

to other countries. This especially as AMR is increasingly being transferred from animals to people as a result of improper antibiotic use in animal feeding. There also needs to be increased patient education to address hygiene levels as well as seek measures to improve the availability of safe drinking water in the country. Alongside this, ASPs aim to help physicians, pharmacists, and drug sellers reduce the inappropriate prescribing and dispensing of colistin. We will continue to monitor the situation.

Supplementary Materials: The following supporting information on detailed procedures for bacterial isolation, antibiotic susceptibility testing, and *mcr* detection via Polymerase Chain Reaction (PCR), as outlined in Supplementary Figure S1.

Author Contributions: S.S and R.M.N.: Sample collection, Methodology, Investigation, Formal analysis, and Manuscript drafting; M.B.H: Data acquisition, Investigation, Data validation, Visualization; U.L.U and M.A.A.: Methodology, Data curation, Writing-Reviewing, Validation and Editing; A.S.M.M and M.R.K.K: Clinical and demographic data acquisition, Resources, Conceptualization, Visualization and Validation; SN: Project administration, Resources, Methodology, Validation, and Visualization; B.G.: Conceptualization, Methodology, Re-writing and Editing, Visualization; SI: Conceptualization, Supervision, Resources, Data curation and analysis, Writing-Reviewing, and Editing, Study coordination. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: This study was authorized [No. UAMC/ERC/Recommend-62/2018, dated 09.07.2018] by the Ethics Review Committee of Uttara Adhunik Medical College, Dhaka, Bangladesh.

Informed Consent Statement: Informed consent was obtained from parents before the start of sample collection.

Data Availability Statement: Data is contained within the article and available upon request.

Conflicts of Interest: The authors declare no conflict of interest.

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