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ORIGINAL ARTICLE



# Resistome and microbial profiling of pediatric patient's gut infected with multidrug-resistant diarrhoeagenic *Enterobacteriaceae* using next-generation sequencing; the first study from Pakistan

Ome Kalsoom Afridi[a], Johar Ali[b,c,d] and Jeong Ho Chang [a]

[a]Department of Biology Education, Kyungpook National University, Daegu, Republic of Korea; [b]Center of Genome Sciences, Rehman Medical Institute Peshawar, Pakistan; [c]Executive Development Center, Sukkur Institute of Business Administration University, Pakistan; [d]Department of Biotechnology, Institute of Integrative Biosciences, CECOS University of IT & Emerging Sciences, Peshawar, Pakistan

**ABSTRACT**

A high prevalence of multidrug-resistant (MDR) pathogens has been reported in adult and pediatric populations of Pakistan. However, data describing the effect of MDR microbes on the gut microbiota is scarce. We designed a cross-sectional pediatric study to investigate the effect of MDR microbes' infection on the gut microbiome and its resistome of children using high-throughput next-generation sequencing (NGS). A cross-sectional study was conducted at a tertiary health care hospital in Peshawar Pakistan, between 5 September 2019 to 15 February 2020. Pediatric patients with acute gastroenteritis (n = 200) were enrolled. All the enrolled pediatric patients underwent initial antimicrobial resistance (AMR) screening using the disk diffusion method. Children with MDR infections were identified and selected for gut microbiome and its resistome profiling using NGS. Out of 200 enrolled pediatric patients, 80 (40%) were found infected with MDR diarrheagenic *Enterobacteriaceae* consisting of 50 (62.5%) infections caused by extended-spectrum beta-lactamase (ESBL) producing *E. coli* while 30 (37.5%) by MDR *Enterobacter* specie. A total of 63 and 17 antibiotic-resistant genes (ARGs) conferring resistance to 7 and 5 classes of antibiotics were identified in the resistomes of MDR diarrheagenic *Enterobacteriaceae* infected and healthy children, respectively. NGS-based gut microbial profiling of MDR *Enterobacter spp*., ESBL producing *E. coli* infected pediatric patients and healthy controls revealed the predominance of *Proteobacteria* and *Actinobacteria*, respectively. An increased abundance of several pathogenic gram-negative bacteria namely *E. coli, Enterobacter cloacae*, and *Salmonella enterica* was observed in the gut microbiota of children infected with MDR bacterial infections than that of the healthy controls. This work indicates that children with MDR infections have reduced microbial diversity and enriched ARGs than healthy controls. The emergence of MDR bacterial strains and their association with gut dysbiosis needs immediate attention to regulate antibiotics usage in Pakistani children.

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## 1. Introduction

Antimicrobial resistance (AMR) is one of the serious health threats across the globe. It is estimated that AMR could lead to 10 million annual deaths by 2050 [1]. AMR acquired an endemic status in Pakistan due to the unsafe usage of antibiotics [2]. Although Pakistan endorsed the national action plan for AMR control in line with World Health Organization (WHO), its implementation is still in the pipeline. The 'superbug' such as extensively drug-resistant (XDR) *Salmonella typhi* has been reported in Pakistan [3]. Presently, the lack of national antimicrobials regulatory authorities, excessive antibiotics usage, self-medication, unsafe water, and poor sanitation and hygiene are the leading causes of AMR in Pakistan. Multidrug-resistant (MDR) bacteria are an emerging health issue in developing countries. Among various MDR bacteria, *Enterobacteriaceae* is the leading cause of nosocomial and community-acquired infections resulting in serious bloodstream, respiratory, urinary tract, and wound infections [4]. Among the various members of *Enterobacteriaceae*, β-lactam resistance is one of the most common patterns of AMR acquisition. Globally, extended-spectrum beta-lactamases (ESBL) producing *Enterobacteriaceae* is potentially known for hospital and community-acquired infections [5].

Recently, an alarming increase has been reported in infection caused by MDR *Enterobacteriaceae* by the US Centers for Disease Control and Prevention [6]. Clinically important MDR-*Enterobacteriaceae* in children is AmpC beta-lactamase resistance, carbapenem-resistant beta-lactamases, or ESBL. The high

**CONTACT** Johar Ali Johar.ali1@rmi.edu.pk Center of Genome Sciences, Rehman Medical Institute Peshawar, Khyber Pakhtunkhwa 25000, Pakistan. Executive Development Center, Sukkur Institute of Business Administration University, Sindh 65200, Pakistan. Department of Biotechnology, Institute of Integrative Biosciences, CECOS University of IT & Emerging Sciences, Peshawar 25000, Pakistan; Jeong Ho Chang jhcbio@knu.ac.kr Department of Biology Education, Kyungpook National University, 80 Daehak-ro, Buk-gu, Daegu 41566, Korea.

prevalence of MDR- *Enterobacteriaceae* in children poses a serious global threat due to the approval of a small number of broad-spectrum antibiotics. ESBL producing *Enterobacteriaceae* has been reported to be highly prevalent in Pakistan [7]. Among the various members of *Enterobacteriaceae, Enterobacter cloacae* complex (ECC), *Escherichia coli* (*E. coli), Klebsiella spp., Yersinia spp., Salmonella spp*., and *Shigella spp*. are the most common nosocomial pathogens causing various serious infections. The emergence of high throughput next-generation sequencing technologies (NGS) escalated the characterization of AMR genes (ARGs) and enabled the genetic profiling of non-cultivable bacteria. In Pakistan and other developing countries, traditional culture-based antibiotic susceptibility testing is still in routine practice for AMR diagnosis and surveillance. The superiority of NGS over the traditional culture-based methods makes it a robust method for the accurate characterization of AMR bacteria and ARGs [8]. In developing countries, acute gastroenteritis potentially leads to the use of antibiotics which is implicated to cause the emergence of MDR microbes. However, data describing the effect of these MDR microbes on the gut microbiota is scarce. We designed a cross–sectional pediatric study to investigate the effect of MDR microbe's infection on the gut microbiome and its resistome of children using NGS. To the best of our knowledge, the effect of MDR microbes on the gut microbiota has never been investigated in both adult and pediatric population of Pakistan.

## 2. Materials and methods

### 2.1. Selection of subjects on the basis of major bacterial infection

Initially, pediatric patients (n = 1000) were screened for various bacterial infections using the records of Pathology Laboratory of a Tertiary Health Care, Hospital of Peshawar. The preliminary screening based on conventional phenotypic methods, revealed the prevalence of ESBL *E. coli* (60%; n = 600) and *Enterobacter* (31%; n = 310) in pediatric patients infected with acute gastroenteritis. Initially, patients infected with *Enterobacter* and ESBL producing *E. coli* were selected for further biochemical and antibiotic susceptibility testing (AST).

### 2.2. Sample collection, biochemical and antibiotic susceptibility testing

Hospitalized pediatric patients (n = 200, mean age 2.8 ± 0.4) with acute gastroenteritis who did not respond to the standard antimicrobial therapies and were found to be infected with AMR bacteria by initial hospital-based AST screening were selected for further investigation. Informed consents were signed from the parents/guardians of the enrolled patients. The selected pediatric patients were already on antibiotic treatment, however these patients did not respond positively to the prescribed antibiotics. These patients were not recovering and were initially tested and declared to be mostly infected with antimicrobial resistant (AMR) ESBL *E. coli* and *Enterobacter* spp. The antibiotics administration was stopped up to 72 h following the standard guidelines prior to sample collection for subsequent biochemical investigation.

Stool samples of healthy children (n = 2, mean age 2.8 ± 0.4) without any history of previous AMR bacterial infections and antibiotics administrations for the last 6 months were also collected. Using a sterile wire loop, all the collected (n = 200) diarrheal stool samples under aseptic measures were streaked onto the MacConkey (Oxoid, Thermo Scientific, UK). The streaked plates were incubated for 18–48 h at 35–37°C. Following incubation, *E. coli* and *Enterobacter* spp colonies were identified based on morphological characteristics. Bright pink to red colonies were presumed to be the characteristic colonies of *E. coli* and *Enterobacter* (lactose fermenter pink colonies). Colonies (n=3) morphologically resembling *E. coli* and *Enterobacter* were then streaked onto the freshly prepared blood agar plates. The streaked blood agar plates were incubated at 37°C for 18–24 h. For further confirmation, 1-5 colonies from the blood agar plates were subjected to standard biochemical tests [9]. The identified bacterial strains (*Enterobacter* and *E. coli*) were subjected to Kirby-Bauer disc-based antibiotic susceptibility testing using Mueller Hinton Agar. For AST, standard first-line antibiotic discs [10] were used according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [11] .

### 2.3. Selection of diarrheal stool samples for NGS

Phenotypically confirmed cases of MDR *Enterobacter species* and ESBL producing *E. coli* were identified through AST and their diarrheal stool samples were analyzed for microbial and its resistome profiling using high throughput shotgun metagenomic sequencing. Stool samples of healthy children were sequenced as controls. The consistencies of collected stool specimens were identified using the Bristol Stool Form Scale (BSFS).

### 2.4. DNA extraction

DNA from the pediatric diarrheal stool samples was extracted using PureLink™ Microbiome DNA Purification Kit (Invitrogen, ThermoFisher Scientific; Cat. no. A29790) following the manufacturer's instruction with modifications made to optimize the protocol

according to the simple laboratory benchtop instruments. Genomic DNA concentration was determined using a Qubit fluorometer following the manufacturer's instructions (Qubit™ fluorometer, Invitrogen, CA 92,008, USA).

### 2.5. NGS library preparation and data analysis

NGS libraries were prepared and loaded onto the flow cell for shotgun metagenome sequencing (Illumina MiSeq) using the protocol as described earlier [8]. NGS data analysis was carried out using a variety of publicly available bioinformatics pipelines [8].

## 3. Results

### 3.1. Prevalence of MDR diarrheagenic *enterobacteriaceae*

Out of 200 enrolled pediatric patients, 80 children (40%) were found infected with MDR diarrheagenic *Enterobacteriaceae*. Among these MDR diarrheagenic *Enterobacteriaceae*, MDR *Enterobacter species* constituted for 37.5% (n = 30) while ESBL producing *E. coli* constituted for 62.5% (n = 50). To study that whether MDR diarrheagenic *Enterobacteriaceae* infections have any profound effect on the gut microbiota of children, we collected diarrheal stool samples of pediatric patients infected with MDR *Enterobacter* strain (n = 1) and ESBL producing *E. coli* (n = 1) for NGS (Figure 2). The diarrheal stool samples used for NGS analysis have been identified as type 7 (watery diarrhea) by BSFS indicating severe diarrhea.

### 3.2. Resistome analysis

Based on shotgun metagenome sequencing reads, the percentage abundance of different ARGs were estimated for both diseased (Table 1) and healthy controls (Table 2). The resistome analysis identified diverse ARGs in the gut microbiota of pediatric patients infected with MDR *Enterobacter* and ESBL producing *E.coli*. Efflux pumps associated ARGs ($acr_A$, $acr_B$, $emr_A$, $emr_B$, $mdt_A$, $mdt_B$) were most abundantly detected in patients infected with MDR *Enterobacter* (n = 19, 55.8%) and ESBL producing *E. coli* (n = 16, 66.6%). The other abundantly detected ARGs in the gut resistome of ESBL producing *E. coli* infected patient were aminoglycoside (n = 4, 16.6%), beta-lactam (n = 2, 8.3%), and peptide antibiotics associated ARGs (n = 2, 8.3%). In addition to efflux pumps associated ARGS, the other abundantly detected ARGs in the gut resistome of MDR *Enterobacter* infected patient were beta-lactam (n = 7, 20.5%), tetracycline (n = 3, 8.8%), peptide antibiotics (n = 2, 5.8%), rifampin (n = 2, 5.8%), and quinolone (n = 1, 2.9%) Table 1. A total of 17 ARGs conferring resistance to tetracyclines (n = 3, 86.7%), macrolide-lincosamide-streptogramin (n = 6, 12%), beta-lactam (n = 1, 0.5%), MDR (n = 5, 0.4%), and aminoglycoside (n = 2, 0.4%) were detected in the resistome of healthy children. The various ARGs found in the resistome of healthy controls are listed in Table 2.

### 3.3. NGS-based gut bacterial profiling at the phylum level

NGS-based gut profiling of healthy controls (n = 2) revealed *Actinobacteria* (64.5%) and *Firmicutes* (33.1%) as the most abundant phyla (Figure 1). Minor phyla identified in control samples were *Proteobacteria* and *Verrucomicrobia* (<2%). In contrast, *Proteobacteria* (45.6%) was found to be the most abundant phylum in the gut microbiota of pediatric patients infected with MDR *Enterobacter* and ESBL producing *E. coli* followed by *Bacteroidetes* (36.6%), *Firmicutes* (12.5%), *Actinobacteria* (5%), and *Tenericutes* (0.3%). The percentage abundance of various bacterial phyla in both healthy controls and patients infected with MDR *Enterobacter* and ESBL producing *E. coli* (Figure 1A).

### 3.4. NGS-based gut bacterial profiling at the family level

A total of 14 bacterial families were identified in patients infected with MDR *Enterobacter* and ESBL producing *E. coli* while10 bacterial families were identified in healthy controls. The most abundant bacterial families in healthy controls were *Bifidobacteriaceae* (54.9%), *Lachnospiraceae* (12.6%), and *Coriobacteriaceae* (8.8%) while *Enterobacteriaceae* (40%), *Bacteroidaceae* (32%), and *Ruminococcaceae* (8.8%) were found abundantly in

**Table 1.** List of antibiotic-resistant genes identified in the gut resistome of children infected with multidrug-resistant bacteria.

| Antibiotic classes | Antibiotic resistance genes |
|---|---|
| Efflux pump system | $acr_A$, $acr_B$, $acr_D$, $acr_E$, $acr_F$, $bac_A$, $bae_R$, $bae_S$, $Cpx_A$, *CRP*, $emr_A$, $emr_B$, $emr_R$, *H-NS*, $Kdp_E$, $mar_A$, $mdt_A$, $mdt_B$, $mdt_C$, $mdt_F$, $mdt_G$, $mdt_H$, $mdt_K$, $msb_A$ |
| Beta-lactam | $bla_{TEM-116}$, $bla_{AZECL-25}$, $bla_{CMH-2}$, $bla_{CMH-3}$, $bla_{CTX-M-101}$, *CTX-M-107*, *MIR-13*, *bla*-Penicillin Binding Protein |
| Aminoglycoside | $AGly_{RmtB}$, $AGly_{StrB}$, $rmt_{B1}$, *APH [6]*-Id |
| Tetracycline | $tet_A$, $tet_{34}$, $tetA_{46}$ |
| Quinoline | *QnrS1* |
| Rifampin | $rpo_B$, $rpo_{B2}$ |
| Peptide antibiotics | *pmrF*, *ugd*, *yojl* |

**Table 2.** List of antibiotic-resistant genes identified in the resistome of healthy children.

| Antibiotic class | Antibiotic resistance genes |
|---|---|
| Tetracyclines | $tet_M$, $tet_W$, $tet_O$ |
| MLS | $erm_B$, $erm_X$, $mef_A$, $Msr_C$, $Msr_D$, $lnu_C$ |
| beta-lactam | $bla_{TEM}$ |
| MDR-efflux pump | $emr_B$, $emr_Y$, $mdt_B$, $mdt_E$, $emr_R$ |
| Aminoglycoside | $acr_F$, $APH_6$ |

MLS = macrolide-lincosamide-streptogramin, MDR = multidrug resistance

patients infected with MDR *Enterobacter* and ESBL producing *E. coli*. The other families identified in the gut microbiota of patients infected with MDR *Enterobacter* and ESBL producing *E. coli* are *Clostridiaceae* (5.1%), *Bifidobacteriaceae* (4.4%), *Enterococcaceae* (3.3%), *Selenomonadaceae* (1.8%), *Sphingobacteriaceae* (1.3%), *Streptococcaceae* (0.9%), and *Aeromonadaceae* (0.7%). Minor families identified in healthy controls were *Lactobacillaceae, Erysipelotrichaceae, Eubacteriaceae, Streptococcaceae, Akkermansiaceae, Ruminococcaceae, Eggerthellaceae, Enterobacteriaceae, Leuconostocaceae*, and *Clostridiaceae* (Figure 1B).

### 3.5. NGS-based gut bacterial profiling at the genus level

The total number of bacterial genera identified in the gut microbiota of healthy controls and pediatric patients infected with both MDR *Enterobacter* and ESBL producing *E. coli* were 16 and 7, respectively. The most abundant genera identified in healthy controls were *Bifidobacterium* (56%), *Collinsella* (9.5%), *Blautia* (6.2%), and *Dorea* (4.1%). The other minor genera in the gut microbiota of healthy controls were *Enterococcus* (4.7%), *Eubacterium* (3.2%), *Holdemanella* (2.6%), *Streptococcus* (2.3%), *Leuconostoc* (2.2%), *Anaerostipes* (1.3%), *Catenibacterium* (1.1%), *Akkermansia* (1.1%), *Escherichia* (0.8%), *Ruminococcus* (0.4%), and *Eggerthella* (0.2%). On the other hand, the predominantly abundant genera in the gut microbiota of ESBL producing *E. coli* and *Enterobacter* infected patients were *Enterobacter* (46.2%), *Salmonella* (18.1%), *Escherichia* (21%), *Shigella* (5.2%), *Klebsiella* (3.5%), *Raoultella* (3%), and *Arsenophonus* (3%; Figure 1 C).

### 3.6. NGS-based gut bacterial profiling at the species level

Microbial analysis at the species level revealed the abundance of commensal symbiotic bacteria in control samples while the predominance of pathogenic bacteria associated with opportunistic infections was observed in the gut microbiota of patients infected with MDR *Enterobacter* and ESBL producing *E. coli*. A total of 30 bacterial species were identified in control samples, among which *Bifidobacterium longum* (35%) was the most abundantly detected followed by *Bifidobacterium breve* (10.5%), *Collinsella aerofaciens* (8.8%), *Bifidobacterium bifidum* (4.8%), *Ruminococcus torques* (3.7%), *Enterococcus faecium*

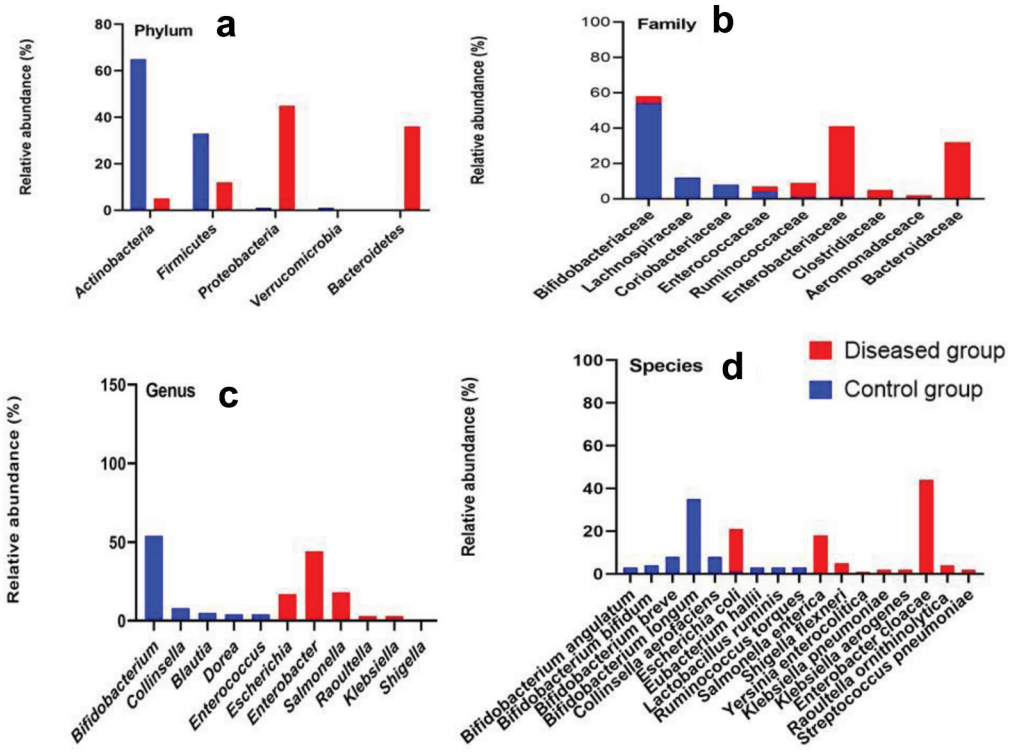


**Figure 1. Relative percentage abundance of gut microbiota of multi drug-resistant** diarrheagenic *Enterobacteriaceae* **infected patients and negative controls at various taxonomic levels (A)** Relative abundance at the phylum level **(B)** relative abundance at the family level **(C)** relative abundance at the genus level **(D)** relative abundance at species level.

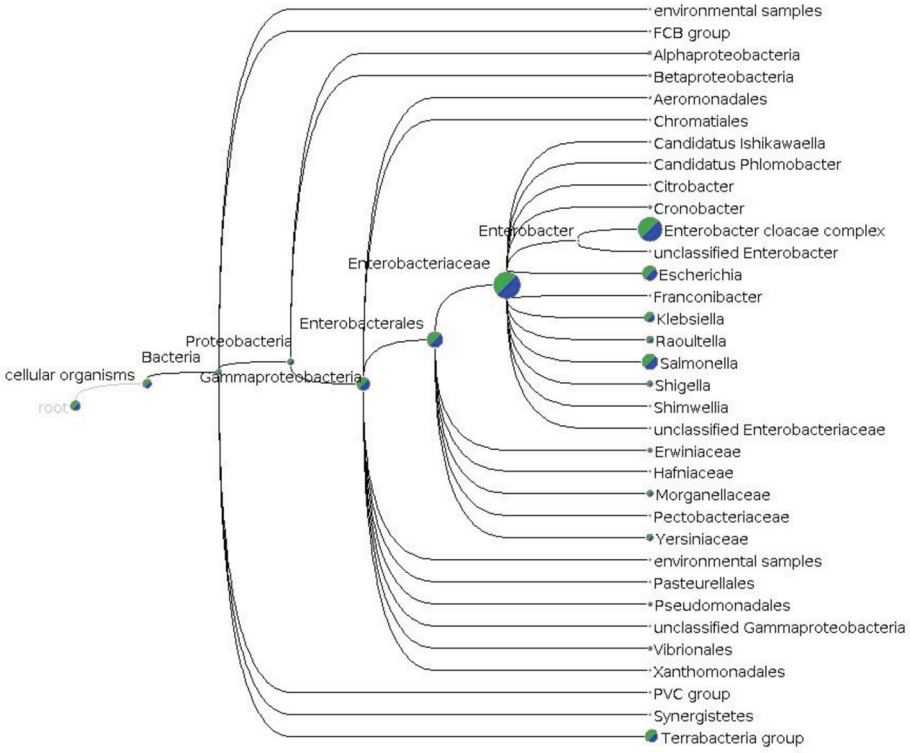


**Figure 2.** Cladogram computed by representing the bacterial community profiles of pediatric patients infected with multi-drug resistant *Enterobacter* spp (blue dots) and extended-spectrum beta-lactamase-producing *E. coli* (green dots), respectively.

(3.5%), *Bifidobacterium angulatum* (3.1%), *Enterococcus faecium* (3.5%), *Eubacterium hallii* (3.2%), *Lactobacillus ruminis* (3.2%), *Holdemanella biformis* (2.7%), *Catenibacterium mitsuokai* (2.4%), *Bifidobacterium catenulatum* (2.5%), *Dorea longicatena* (2.3%), *Streptococcus salivarius* (2%), *Dorea formicigenerans* (1.8%), *Eubacterium rectale* (1.5%), *Anaerostipes hadrus* (1.3%), *Blautia obeum* (1.2%), and *Akkermansia muciniphila* (1.1%). The abundance levels of opportunistic pathogenic bacterial species in healthy controls were found to be <1%. On the contrary, the most abundant bacterial species detected in the gut microbiota of patient infected with MDR *Enterobacter* and ESBL producing *E. coli* were *Enterobacter cloacae* (44.2%) followed by *E. coli* (25%), *Salmonella enterica* (18.5%), *Klebsiella pneumoniae* (2.4%), *Klebsiella aerogenes* (2.4%), *Yersinia enterocolitica* (2%), *Arsenophonus nasoniae* (2%), *Shigella flexneri* (1.5%), *Raoultella ornithinolytica* (1%), and *Streptococcus pneumoniae* (1%; Figure 2). The percentage abundance of various bacterial species in both pediatric healthy controls and diseased groups are shown in Figure 1D.

## 4. Discussion

The emergence of MDR infections poses serious health issues to public health worldwide. MDR infections have been associated with poor clinical and therapeutic outcomes, longer hospitalizations, and increased risk of relapses. Lack of validated treatment for MDR infections is currently one of the serious challenges of the global health sector. Microbial genomics and metagenomics played a key role in the identification of these MDR microbes which helped in designing effective strategies to mitigate or manipulate resistant microbes [12]. The high prevalence of MDR infections in Pakistan has been evidenced by numerous reports [13].

Previous studies in Pakistan mainly investigated the prevalence of various ARG types in humans and food animals using methods other than NGS. Studies exploring the association of MDR infection and gut microbiota are scarce in Pakistan and to the best of our knowledge, none of the earlier studies investigated that whether MDR microbial infection has any profound effect on the gut microbiota of children. Acute gastroenteritis potentially leads to excessive use of antibiotics in developing countries which is implicated to cause the emergence of MDR. In the present study, all the enrolled pediatric patients infected with acute gastroenteritis were screened for the presence of MDR microbes. The initial disk diffusion-based AMR screening revealed that 40% of children were found infected with MDR diarrheagenic *Enterobacteriaceae*. The presence of MDR diarrheagenic *Enterobacteriaceae* in children with acute gastritis has been in agreement with a recent study [14]. A recent study in Qatar revealed the high prevalence of antimicrobial-resistant children hospitalized with acute gastroenteritis [14]. Similarly, another recent

study from Iran also supports our results indicating the presence of MDR diarrheagenic *Enterobacteriaceae* in children with gastroenteritis [10]. Among the various members of the family *Enterobacteriaceae, Enterobacter species*, and ESBL producing *E. coli* were identified as the major infecting bacterial strains.

To explore the effect of MDR *Enterobacteriaceae* on the gut microbiota, each of MDR *Enterobacter specie* and ESBL producing *E. coli* strains were selected for gut microbiome and its resistome profiling using NGS. In addition to the diseased children, the gut microbiota of healthy children was also profiled to ascertain how they differ from each other. The resistome analysis of MDR *Enterobacteriaceae* (*Enterobacter specie* and ESBL producing *E. coli*) infected children revealed the high abundance of various ARGs conferring resistance to multiple classes of antibiotics (Table 1). Among the various genes identified in the resistome of MDR *Enterobacteriaceae* infected children, efflux pumps associated ARGs were found most abundantly in both *Enterobacter species* and ESBL producing *E. coli* infected pediatric patients. The high prevalence of antibiotic efflux pumps and their association with MDR *Enterobactericeae* in our study is supported by a recent study indicating its presence in the pediatric population [15]. The abundant presence of various ARGs in the resistome of MDR *Enterobactericeae* infected children could be attributed to the use of antibiotics administered routinely during acute gastroenteritis. Parental self-medication is an important factor justifying the high prevalence of MDR genes in pediatric patients [16]. In Pakistan, antibiotics are commonly administered by children and infant's mothers without a physician's prescription [17]. Furthermore, antibiotics are available over the counter and are freely available to everyone in Pakistan due to the absence of any legislation unlike developed countries [17]. In contrast to the diseased children, the gut resistome of healthy controls exhibited lower prevalence and diversity of various ARGs (Table 2). Tetracyclines associated ARGs were found to be the most abundant genes in the resistome of healthy children. Although, antibiotic therapy potentially acts as a selective pressure for the emergence of various ARGs in the human resistome. However, studies provide evidence for the presence of various ARGs in human resistome even in the absence of any selective pressure [18]. Furthermore, the presence of various ARGs in the resistome of healthy children in our results are in agreement with a previous study indicating the existence of diverse ARGs in the resistome of healthy infants and children [18]. Literature provides evidence that ARGs appear as the inherent features of the human microbiome albeit, excessive antibiotic administration upsurges the acquisition, transmission, and dissemination of various ARG types in the different gut resistomes [19]. The abundance of tetracycline-associated ARGs in healthy controls could be justified by the fact tetracycline has been reported to be the most commonly used group of antibiotics worldwide [20,21]. Moreover, the use of tetracycline has been reduced in recent years especially its therapeutic usage has been stopped in pregnant women or children aged less than 8 years [20]. However, in developing countries, it is still commonly used for therapeutic purposes and to promote growth in food animals, and in this way, the intestinal bacteria remained exposing to it.

NGS-based gut microbial profiling of children infected with MDR diarrheagenic *Enterobacteriaceae* revealed a severely dysbiotic microbiota characterized by increased abundance of phylum *Proteobacteria* (45.6%), family *Enterobacteriaceae* (40%), and enrichment of pathogenic bacteria compared to the healthy controls. The higher abundance of *Proteobacteria* in MDR diarrheagenic *Enterobacteriaceae* infected children indicates antibiotics mediated gut dysbiosis. Our suspicion is supported by a previous report linking the predominance of *Proteobacteria* to be associated with the continuous antibiotic intake [22]. With increasing age the exposure of the microbiome to various antibiotics increases which eventually leads to an altered microbiota. A study revealed that the administration of ciprofloxacin for up to five days in three healthy adults causes abrupt alterations in the structure of major bacterial taxa such as *Lachnospiraceae, Faecalibacterium*, and *Ruminococcaceae* [23,24]. Similarly, another study reported that antibiotic exposure in premature infants reduced microbial richness, diversity, and causes the enrichment of various ARGs [25,26]. These findings indicate that antibiotic exposures cause profound effects on the richness and diversity of the gut microbiome and often lead to the expansion of various ARG types.

Our results also indicated that children with MDR infections have reduced microbial diversities and enriched ARGs than healthy controls. Furthermore, an increased abundance of several pathogenic gram-negative bacteria namely *E. coli, Enterobacter cloacae*, and *Salmonella enterica* was observed in the microbiota of children infected with MDR microbes than that of the healthy controls. The underlying mechanism of microbial colonization by particular pathogens is not completely clear yet [27]. The dysbiotic gut and abundance of ARGs in the resistome of children is supported by a recent study indicating microbial dysbiosis and ARGs enrichment in diseased Pakistani adult subjects infected with MDR *E. coli* [28].

The predominance of *Bifidobacteriaceae* associated non-pathogenic bacterial species in healthy controls reflects their intact gut microbiota while the abundance of *Enterobacteriacea* associated opportunistic pathogens in MDR microbes infected patients indicates the

association of AMR with gut microbial dysbiosis. Our results are supported by a previous study indicating that diarrhea and various doses of antibiotics increase the likelihood of children's gut microbiota enrichment with MDR bacteria in children [29]. The various gram-negative bacteria identified in the gut microbiota of MDR infected patients in the present study have been reported to be the potential causative agents of diarrhea in children [30]. Bacterial species belonging to the genus *Yersinia* and *Klebsiella* identified in the gut microbiota of MDR diarrheagenic *Enterobacteriaceae* infected patients have been associated with severe diarrheal diseases such as yersiniosis [31].

## 5. Conclusion

Conclusively, using the shotgun metagenome sequencing approach, we demonstrated that the resistome and microbiome of MDR-infected children differ considerably from the healthy children. An altered gut microbiota with low microbial diversity and enriched ARGs was identified in children infected with MDR infections. High throughput sequencing-based studies for AMR are mostly lacking in Pakistan due to limited resources. To the best of our knowledge, we are the first to profile microbial and its resistome composition of children infected with MDR infection and compared it with that of healthy controls. Furthermore, this NGS-based method offers robust resistome and microbial characterization. The emergence of MDR bacterial strains and their association with gut dysbiosis needs immediate attention to regulate antibiotics usage in Pakistani children. We recommend that NGS-based genomic characterization studies with a larger sample size are needed to ascertain the complete molecular picture of MDR-gram negative bacterial strains in Pakistani pediatric patients. Moreover, this study will help the clinicians to mitigate the gut microbiome of children and provide personalized microbial supplementations to reverse dysbiosis. Legislations by the Pakistani government banning parental self-medication can potentially help to reduce the burden of MDR infections in children.

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## Ethics approval and consent to participate

Ethical approval was obtained from the Rehman Medical Institute-Research Ethics Committee (Ref: RMI/RMI-REC /Approval/33) Peshawar, Pakistan. Informed consent was obtained from the patient's guardians who participated in this study.

## Consent for publication

Not applicable

## Availability of data and materials

The metagenomic raw reads used for this study were deposited in publicly accessible NCBI's Sequence Read Archive (SRA) under the accession number: PRJNA612780.

## Disclosure statement

The authors declare that they have no competing interests.

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## Declarations

## Authors' contributions

OKA performed experiments, analyzed data, wrote the paper. JHC supervised the study, provided advice in study design, and co-edited the paper. JA supervised and designed the study, provided advice in data analysis, critically discussed results, and edited the paper.

## ORCID

Jeong Ho Chang http://orcid.org/0000-0002-2126-0317

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