Shotgun metagenomics unravels higher antibiotic resistome profile in Bangladeshi gut microbiome

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of

Master of Science in Biotechnology

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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing a degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
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Ethics Statement

This work is completely original and has never been published. It is based on my own sincere study and careful analysis. The study gives due acknowledgment to all of its sources (correct citation).

Dedicated to my

beloved father

for his unconditional love and never ending support

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Abstract

Antibiotic resistance management is a challenging task in Low and Middle-Income Countries (LMICs) such as Bangladesh. Improper regulation and uncontrolled spreading of Antibiotic Resistant Genes (ARGs) from LIMCs pose a significant threat to global public health. The human gut microbiome is a massive reservoir of Antibiotic Resistant Genes (ARGs). In this study, we unraveled the ARGs in the gut microbiome of the Bangladeshi population and compared them with several other countries around the world. Here, 31 fecal samples from different ethnic groups living in Bangladesh namely Bengali (n=9), Chakma (n=6), Khyang (n=5), Marma (n=6), and Tripura (n=5) were collected. Shotgun metagenomic sequencing method was implemented for revealing the ARGs. The resistome profiling was executed on three levels- the total microbiome, the plasmidome, and the virome. In all three levels, samples from Bangladeshi cohorts showed higher ARG profiles compared to foreign samples. On average, the number of ARGs in the Bangladeshi samples ranged between 75.11 and 88. Among them, class C beta-lactamases, quinolone resistance genes, and tetracycline efflux pumps were relatively more abundant. Additionally, the MexPQ-OpmE drug resistance pathway was found to be more prevalent. Findings from our study suggest that the spread of antibiotic resistance within the Bangladeshi population is being facilitated by the gut microbiome, especially via the mobilome. Therefore, strict regulation on antibiotic usage is necessary to halt the spread of ARGs.

Keywords: Antibiotic resistance, resistome, shotgun metagenomics, mobilome, LMIC.

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Introduction

1.1 The Emergence of Antibiotic Resistance

Infectious diseases are currently a significant cause of morbidity and mortality worldwide (1). The most noteworthy, and probably the most costly regarding morbidity and mortality, concerns bacteria. Following its discovery in the late 19th century, there arose a necessity for suitable preventative and therapeutic measures, similar to those required for other pathogens that affect a wide range of living organisms, such as humans, animals, fish, plants, insects, and other species. Yet, it wasn't until the discovery and use of antibiotics half a century later that successful therapy became possible. With the discovery of antibiotics, the healthcare community thought that the battle with infectious diseases was won (2,3). They are the most potent life-saving drugs developed in the twentieth century, saving millions of lives from bacterial infections and reducing significant public health concerns such as infectious diseases, and maternal and infant mortality (4). Based on worldwide pharmaceutical sales statistics in 76 countries, antibiotic usage in defined daily doses increased by 65 percent from 2000 to 2015, with predicted consumption to increase by more than 200 percent by 2030 (5). Antibiotic treatment was formerly more common among high-income countries; but, due to an increase in revenue, antibiotic consumption increased dramatically in low and middle-income countries including India, China, Pakistan and Bangladesh (6,7). Unfortunately, the use of these wonder drugs has been accompanied by the rapid emergence of resistant strains, prompting medical experts to fear a return to the pre-antibiotic era (8). Antibiotic resistance in pathogenic bacteria has appeared as a major public health concern in recent decades addressing economic loss, treatment failure, and challenges in global health security (9). Despite the fact that antibiotics are a new addition to humanity's overall history, the capability of bacteria to resist various antibiotics is ancient (10). Recent data reveals the existence of over 20,000 potential resistance genes of nearly 400 different types, derived mainly from bacterial genome sequences (11). In the United States, about 35,000 people die each year from antibiotic-resistant diseases (12).

1.2 Mechanism and Acquisition of Antibiotic Resistance in Bacteria

The complexity of the processes that lead to the emergence and dissemination of resistance cannot be overstated (13). A wide range of biochemical and physiological mechanisms can be responsible for antimicrobial resistance. The main mechanisms of resistance include limiting drug uptake, modifying drug targets, inactivating drugs, and actively pumping out drugs (10,14,15). These mechanisms can either be intrinsic to the microorganisms or acquired from other microorganisms (16). Antibiotics are classified into two groups based on their functions: bactericidal antibiotics kill bacterial cells while bacteriostatic antibiotics inhibit the growth of microbial cells. Some of the broadly used bacteriostatic antibiotic groups are Tetracyclines (e.g. doxycycline, minocycline) whereas common bactericidal antibiotics are Aminoglycosides (e.g.gentamicin, neomycin), Beta-lactams (e.g. penicillins, carbapenems, cephalosporins) or Fluoroquinolones (e.g. Ciprofloxacin) (17). Despite the diversity of these mechanisms, control over the organisms remains elusive (18). This is due to inadequate management of antimicrobial agents, such as increased consumption by humans and animals and improper prescribing (19). The continuous growth of microorganisms even in the presence of antimicrobial agents, has been one of the most important and persistent driving forces for antibiotic discovery. The potency of the bacteria to change its physical appearance and genetic makeup has led resistance to almost all available antibiotics in clinical practice. Gram negative bacteria can utilize their outer cell membrane to block antibiotic entrance, while other microorganisms employ efflux pumps, manufacture various proteins and modify their targets to get rid of the opponent molecule (20). In the process, they obtain antibiotic resistance genes (ARGs) that encodes intrinsic antibiotic resistance mechanisms. Resistance is considered as a natural phenomenon of bacteria; however, the overuse/misuse of antibiotics, as well as the slow pace at which new medicines are discovered has expedited the dissemination of the resistance gene. Overuse of antimicrobials can occur because the choice of drug is based on a combination of low cost and low toxicity, leading to improper prescribing of broad-spectrum drugs that may be ineffective against the specific organism causing the infection (21). ARGs can be shared

among bacterial organisms via Horizontal Gene Transfer (HGT) through conjugation or transduction commencing rapid development of multi drug resistance in bacteria.Plasmid, transposons and mobile integrons act as a vital medium in gene transfer. Studies on lateral gene transmission demonstrated gene transfer to be 25 times more in human associated bacteria than other bacteria in the environment (22,23).

1.3 Antibiotic Resistance in Low- and Middle-Income Countries (LMICs)

The impact of antibiotic resistance genes (ARGs) is not limited by geographical boundaries and affects all areas and populations worldwide (24). The consequences of ARGs are particularly critical in low- and middle-income countries (LMICs), where 80% of the estimated 10 million deaths caused by the high burden of infectious diseases are expected to occur(25). In densely populated areas where hygiene and sanitation are often problematic, and access to quality healthcare is limited, the unregulated use of antibiotics in humans, animals, and crops creates an ideal environment for ARGs to rapidly spread and thrive. In high-income countries (HICs), well-established surveillance systems provide a comprehensive picture of the prevalence of drug-resistance among common bacterial pathogens across settings, enabling the monitoring of the evolution of ARGs. However, most LMICs lack such well-structured and comprehensive systems (26–28). According to the World Health Organization (WHO), antimicrobial resistance is the most significant threat to Southeast Asian countries (29). Almost all Asian countries, including Sri Lanka, China, South Korea, Japan, Hong Kong, Taiwan, Thailand, Singapore, and India, harbor methicillin and erythromycin-resistant bacteria. The dissemination of antibiotic resistant bacteria from Asia to other nations is a regular occurrence. New Delhi metallo-beta-lactamase-1 (NDM-1) generated in India spread to the United Kingdom, Sweden, Austria, Belgium, France, the Netherlands, Germany, the United States, Canada, Japan, China, Malaysia, Australia, and Korea (30). Colistin-resistant Enterobacteriaceae originated in China in 2016 and have subsequently spread to more than 30 nations (31).

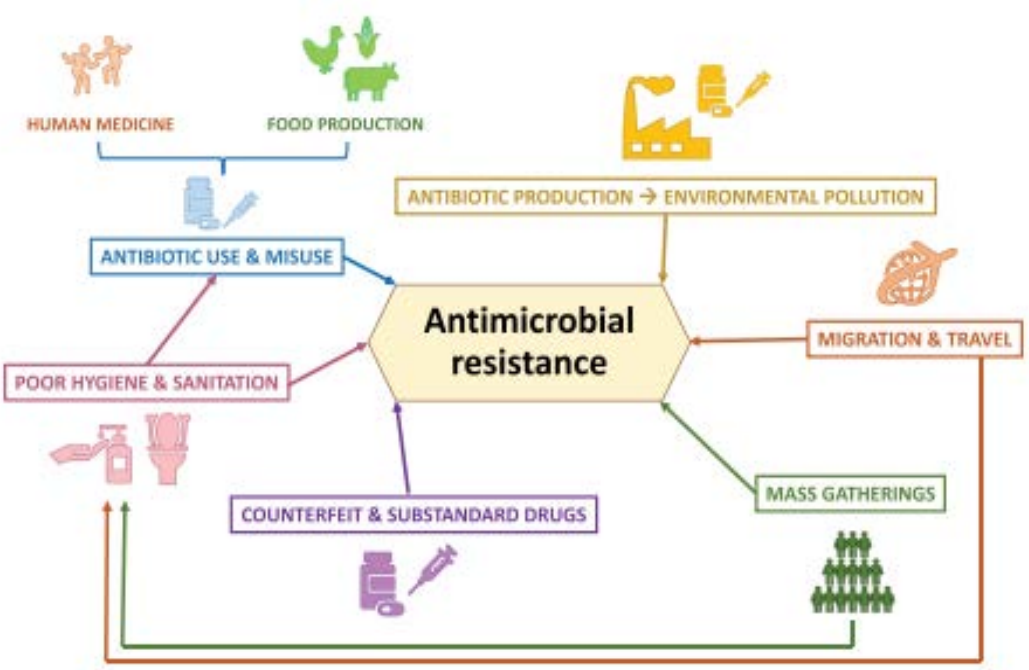

Figure 1.1: Key drivers of antimicrobial resistance in low- and middle-income countries (27).

1.4 Antimicrobial Resistance in Bangladesh

Bangladesh is highly susceptible to antimicrobial resistance being in close proximity to India. Besides, proper maintenance of hygiene cannot be assured all the time in this country, and therefore, antibiotics are an important resource to control different epidemics, nosocomial infections and post-surgery sepsis (32). Concurrently, the availability of antimicrobial medicine as an over-the-counter drug with influential unethical pharmaceutical practices and irresponsible antibiotic usage in agriculture and farming has escalated its exploitation in the nation (7). A two fold increase in multidrug resistance has been observed between 2015 and 2019, amid which *E. coli* and *Staphylococcus aureus* were found to be the most prevalent multidrug resistant pathogens (33). In another study, typhoid patients were found to be resistant to second-line therapy (ciprofloxacin) conducted in Chittagong division in 2003 (34). Efficacy of various antibiotics like ampicillin, sulfamethoxazole-trimethoprim and tetracycline no longer show efficacy as before. Chittagong division encompasses three districts of Chittagong Hill Tracts, considered as the only extensive hill area in Bangladesh. It consists of 1.6 million people, of which about 50% belong to small ethnic

groups (35). The prevalence of antimicrobial resistance in tribal people has been largely ignored. A recent study stated that these indigenous groups self-prescribe various antibiotics which is boosting the increasing rate of antibiotic resistance (36).

1.5 Impact of Antibiotics on the Human Gut Microbiota

The human gut is a reservoir of interdependent microorganisms who participates in digestion, immune system maintenance, metabolic reactions and protections from pathogens (37). Several studies showed that antibiotics can disrupt the taxonomic compositions of the human microbiota. The gut viral components are highly dominated by the bacteriophages or phages known to have significant roles in shaping microbial composition (38). Polymerase chain reaction on viral DNA showed ARGs to be resistant to various antibiotics including lactamase, quinolone and vancomycin. ARGs were found to be present in the human gut for several years even after short-term antibiotic intake (39). These ARGs, which can be transferred between bacteria through horizontal gene transfer (HGT), are particularly susceptible to exchange within the gut due to the high density of microorganisms (40). The gut provides an ideal environment for the spread of resistance genes, as many bacteria remain in close proximity, facilitating the transfer of ARGs (41). The use of prophylactic or therapeutic antibiotics creates selective pressure on these microbes, further fueling the transfer of resistance genes (42) . In addition, the use of antibiotics in animals, particularly in pigs, chickens, and cattle, also contributes to the development of antibiotic-resistant organisms (43). These organisms can then be transmitted to humans through the consumption of contaminated food or exposure to contaminated wastewater and animal feces. Once introduced, these microbes and/or ARGs can integrate into the gut microbial community (44).

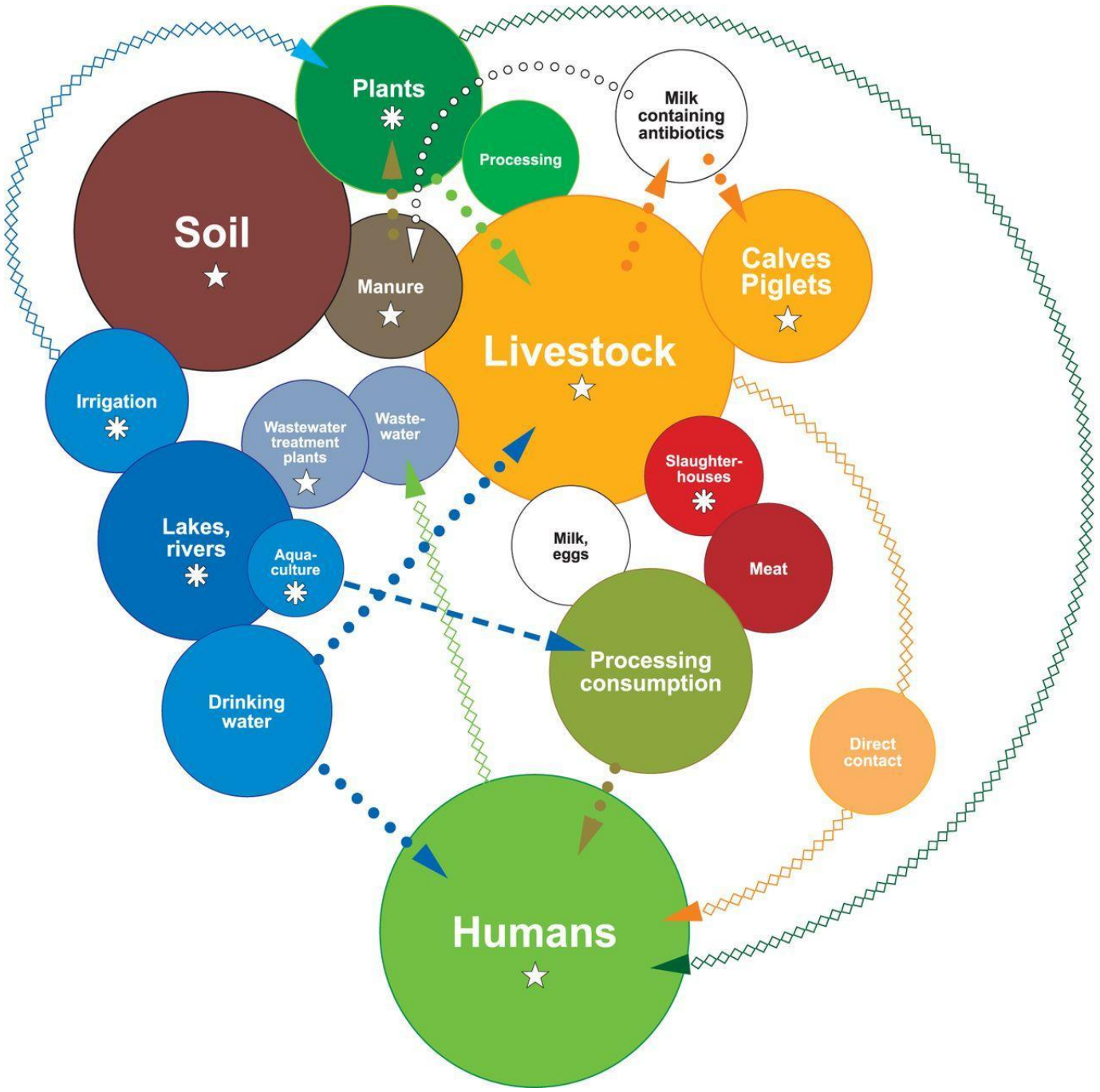

Figure 1.2: Pathway map of Antimicrobial agents (AMA) and antimicrobial reistance (AMR) dissemination within agriculture, the environment, and the food processing industry. Movement of AMA or AMR is indicated by overlapping circles and arrows, respectively; different colors define different groups of reservoirs. Stars indicate the hot spots of antimicrobial genes (ARG) and antimicrobial-resistant bacteria (ARB) with high bacterial densities, nutrient availability, and selective pressure in the digestive tract of livestock and humans, in manure storage facilities, wastewater treatment plants, and in the rhizosphere. Asterisks indicate possible hot spots of ARG

and ARB in water, sediments, and biofilms in aquaculture, rivers, lakes, and irrigation systems, as well as in slaughterhouse facilities and on plant surfaces (45).

1.6 Aim of the study

In this study, the resistome profile of different indigenous groups of Bangladesh and the mainstream Bengali population using shotgun metagenomics was explored. Afterward, the analysis was compared with the healthy population from 11 countries across the world. The aim of the study was to determine if the resistome profile of the Bangladeshi population, including different indigenous groups and the mainstream Bengali population, was similar with other populations worldwide or if it displayed significant differences from other cohorts.

Methodology

2.1 Sample Collection

A total of 31 fecal samples were collected from four ethnic groups (all applicable international, national, and/or institutional guidelines regarding human samples were followed; ethical approval was given by the Ethical review board of National Institute of Biotechnology) including Chakma, Marma, Khyang, and Tripura from Rangamati, Chittagong hill tracts (22.6533° N, 92.1789° E) and Bengali from Dhaka (23.9536° N, 90.1495° E), Bangladesh. Participants did not have any recent records of antibiotic consumption. The samples were transported in an ice box from the collection site to the National Institute of Biotechnology and immediately stored at -80°C before DNA extraction.

2.2 Data Collection from other studies

To compare our samples with other studies around the world, we obtained raw metagenomic reads from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) that were submitted after sequencing. Samples/reads were collected from Australia (n=8), Cameroon (n=10), China (n=10), Denmark (n=9), Germany (n=10), Hong Kong (n=9), India (n=10), Italy (n=5) and Korea (n=10), their NCBI Bioproject numbers are PRJNA503909, PRJEB27005, PRJNA624763, PRJEB2054, PRJEB48605, PRJEB48269, PRJNA39711, PRJNA386422, PRJNA743718, PRJNA48479 respectively. Moreover, reads were obtained from NIH Human Microbiome Project (HMP) (https://hmpdacc.org/). Six healthy samples (from the USA) were collected from the HMP1 portal.

2.3 DNA extraction and metagenomics Sequencing

DNA was extracted from fecal samples using the Invitrogen™ PureLink™ Microbiome DNA Purification Kit. Here, 0.2±0.05 g stool samples were transferred to special beads containing tubes and went through PureLink™ spin-column technology. DNA concentration and the purity were measured using Thermo Scientific™ NanoDrop™ 2000/2000c and stored in -20°C. For

metagenomics sequencing, library preparation was done using TruSeq® DNA Library Prep Kits. Afterward, sequencing was performed in Illumina HiSeq 2500.

2.4 Raw sequence processing and assembly

The Fastq raw reads were aligned with Burrows-Wheeler Aligner (BWA) against *Homo sapiens* (assembly GRCh38.p13) to remove human DNA contaminants (46). The unaligned reads were trimmed using Trimmomatic (Bolger et al., 2014). The trimmed reads underwent *de novo* assembly using MEGAHIT (47).

2.5 Virome identification

To sort out the viral sequences, the assembled contigs were screened using DeepVirFinder (https://github.com/jessieren/DeepVirFinder). DeepVirFinder implemented a reference and alignment-free machine learning method to differentiate the viral sequences in the metagenomic contigs via convolutional neural networks (CNN) based deep learning (48). DeepVirFinder gave a score to every contig. Contigs with ≥ 0.9 score and p value with ≤ 0.05 were considered as viral contigs.

2.6 Classification of plasmidome

The plasmidal contigs were classified using PlasClass (https://github.com/Shamir-Lab/PlasClass). PlasClass can classify short plasmidal sequences from metagenomic samples with improved and more reliable methods. Contigs with ≥ 0.9 score were selected as plasmidal contigs.

2.7 Antibiotic resistome detection

The resistant genes were identified from viral, plasmidal and total contigs via ABRicate 0.5 (https://github.com/tseemann/abricate). This tool can use databases such as NCBI, CARD, ARG-ANNOT, Resfinder, MEGARES, EcOH, PlasmidFinder, Ecoli_VF and VFDB. Here, Resfinder database was implemented to find out the antibiotic resistance genes. Afterward, the contigs were translated using Prodigal (49). The gene count data from ABRicate went under log 10 transformation. The transformed datasets were implemented for Kruskal Wallis test followed by Pairwise Wilcoxon test and Spearman's rank correlation. R version 4.2.2 (https://www.R-project.org) and ggplot2 package (https://ggplot2.tidyverse.org) were utilized for all statistical analyses and visualization (50). The antibiotic resistant causing proteins were determined by fARGene (51). fARGene applies optimized gene models hence it can identify previously uncharacterized resistance genes with higher accuracy, even if their sequence similarity is low to known ARGs.

2.8 Pathway analysis

To identify the drug resistance related pathways, Prodigal annotated proteins were classified using Micorbeannotator. MicrobeAnnotator is a comprehensive functional annotation for microbial genomes that merges results utilizing KEGG Orthology (KO), Enzyme Commission (E.C.), Gene Ontology (GO), Pfam, and InterPro (52). From all the pathway tables, drug resistant pathways were sorted out. Linear discriminant analysis (LDA) of the metabolic pathways were conducted using Linear discriminant analysis Effect Size (LEfSe) (53).

Results

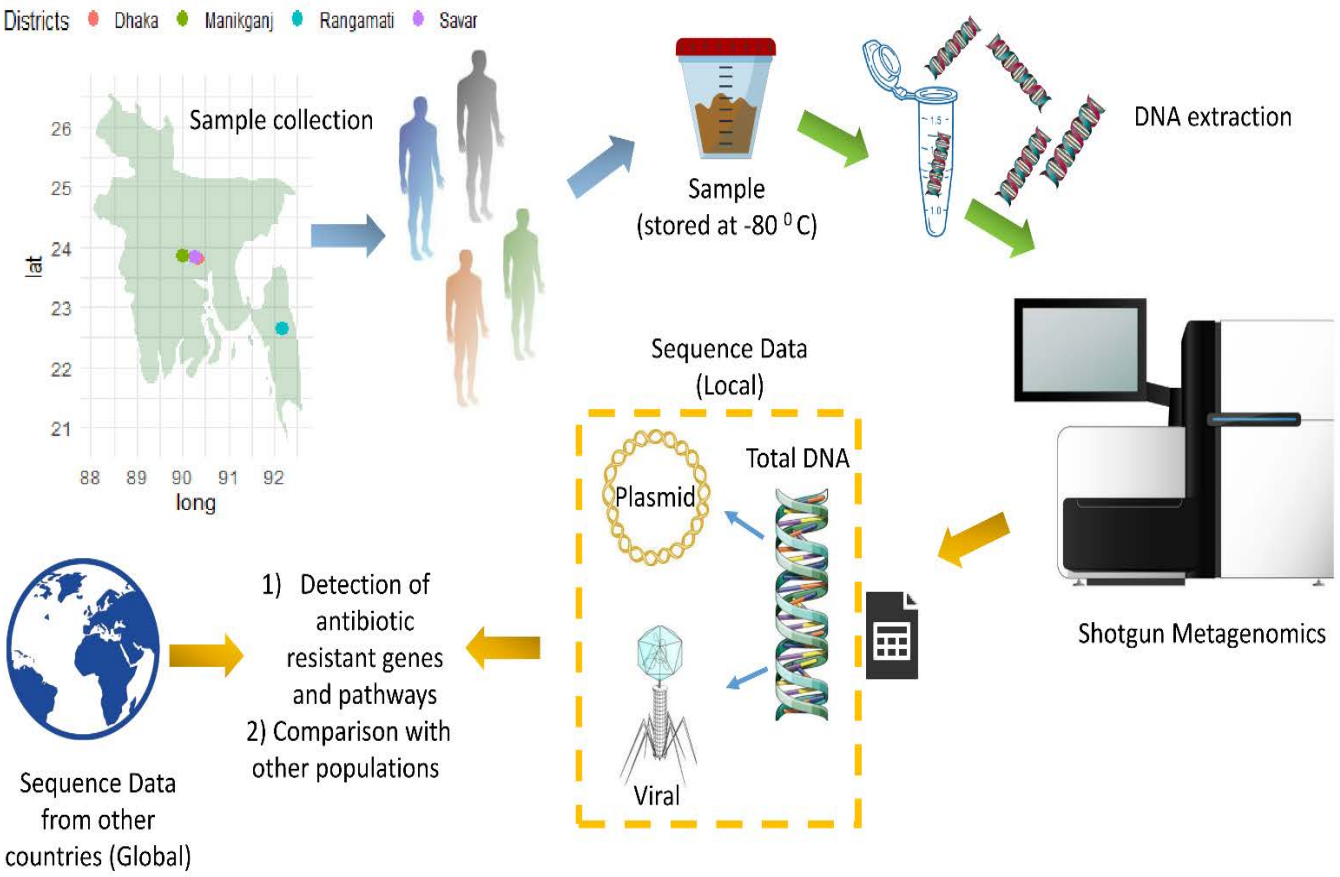

Figure 3.1: Schematic representation of the whole study.

3.1 Prevalence of ARGs were higher in the samples of Bangladesh

Antibiotic resistant gene (ARG) homologs were detected by ABRicate. ARGs were present in every Bangladeshi sample. The highest number of ARG were identified in the Marma population (**Table 3.1: SM1-SM6**).

Table 3.1: Bangladeshi ethnic cohorts and the distribution of antibiotic resistant gene homologs in their total microbiome, plasmidome and viraome as detected by ABricate.

Serial No.	Sample No.	Country(ethnicity)	Gender	Antibiotic Resistant Genes	Total Plasmidal Antibiotic Resistant Genes	Viral Antibiotic Resistant Genes
1	SB4	Bangladesh(Bengali)	Female	97	14	2
2	SB7	Bangladesh(Bengali)	Female	72	11	5
3	SB8	Bangladesh(Bengali)	Female	84	7	4
4	SB14	Bangladesh(Bengali)	Female	72	9	0
5	SB15	Bangladesh(Bengali)	Male	74	7	2
6	SB21	Bangladesh(Bengali)	Male	66	7	2
7	SB24	Bangladesh(Bengali)	Male	82	9	4

8	SB25	Bangladesh(Bengali)	Male	63	8	0
9	SB33	Bangladesh(Bengali)	Male	66	8	2
10	SC2	Bangladesh(Chakma)	Female	103	17	1
11	SC3	Bangladesh(Chakma)	Female	70	7	2
12	SC4	Bangladesh(Chakma)	Male	71	9	4
13	SC5	Bangladesh(Chakma)	Male	86	4	4
14	SC6	Bangladesh(Chakma)	Female	72	5	5
15	SC25	Bangladesh(Chakma)	Female	89	8	2
16	SK1	Bangladesh(Khyang)	Male	126	20	2
17	SK2	Bangladesh(Khyang)	Female	62	11	1
18	SK3	Bangladesh(Khyang)	Female	70	11	6
19	SK4	Bangladesh(Khyang)	Male	86	13	1
20	SK17	Bangladesh(Khyang)	Male	96	5	3
21	SM1	Bangladesh(Marma)	Female	107	16	1
22	SM2	Bangladesh(Marma)	Female	79	8	4
23	SM3	Bangladesh(Marma)	Male	80	9	1
24	SM4	Bangladesh(Marma)	Female	94	8	2
25	SM5	Bangladesh(Marma)	Male	92	13	6

26	SM6	Bangladesh(Marma)	Female	71	12	4
27	ST1	Bangladesh(Tripura)	Male	83	7	8
28	ST3	Bangladesh(Tripura)	Female	66	4	2
29	ST4	Bangladesh(Tripura)	Female	98	20	3
30	ST6	Bangladesh(Tripura)	Female	93	9	7
31	ST15	Bangladesh(Tripura)	Female	62	8	2

The average ARGs were 87.17± standard deviation (SD) 12.98. The second and third highest number of ARGs were found in Chakma (81.83± SD 13.2) and Khyang (88± SD 25.06) respectively. Tripura samples showed 80.4± SD 15.98 ARGs and Bengali had 75.11 ± SD 10.83 ARGs.Interestingly, in Bangladeshi samples, Bengali groups had the lowest ARGs.

The heatmap analysis depicted in **Figure 3.2** demonstrated clusters where tet(W), tet(37), catB4, tet(32), ctxA2, VanR_G, tet(Q), oqxA, tet(44), blaOXA-347, dtrF, aadA1, and many other ARGs were highly abundant in Bangladeshi population whereas lower abundance was observed in other population.

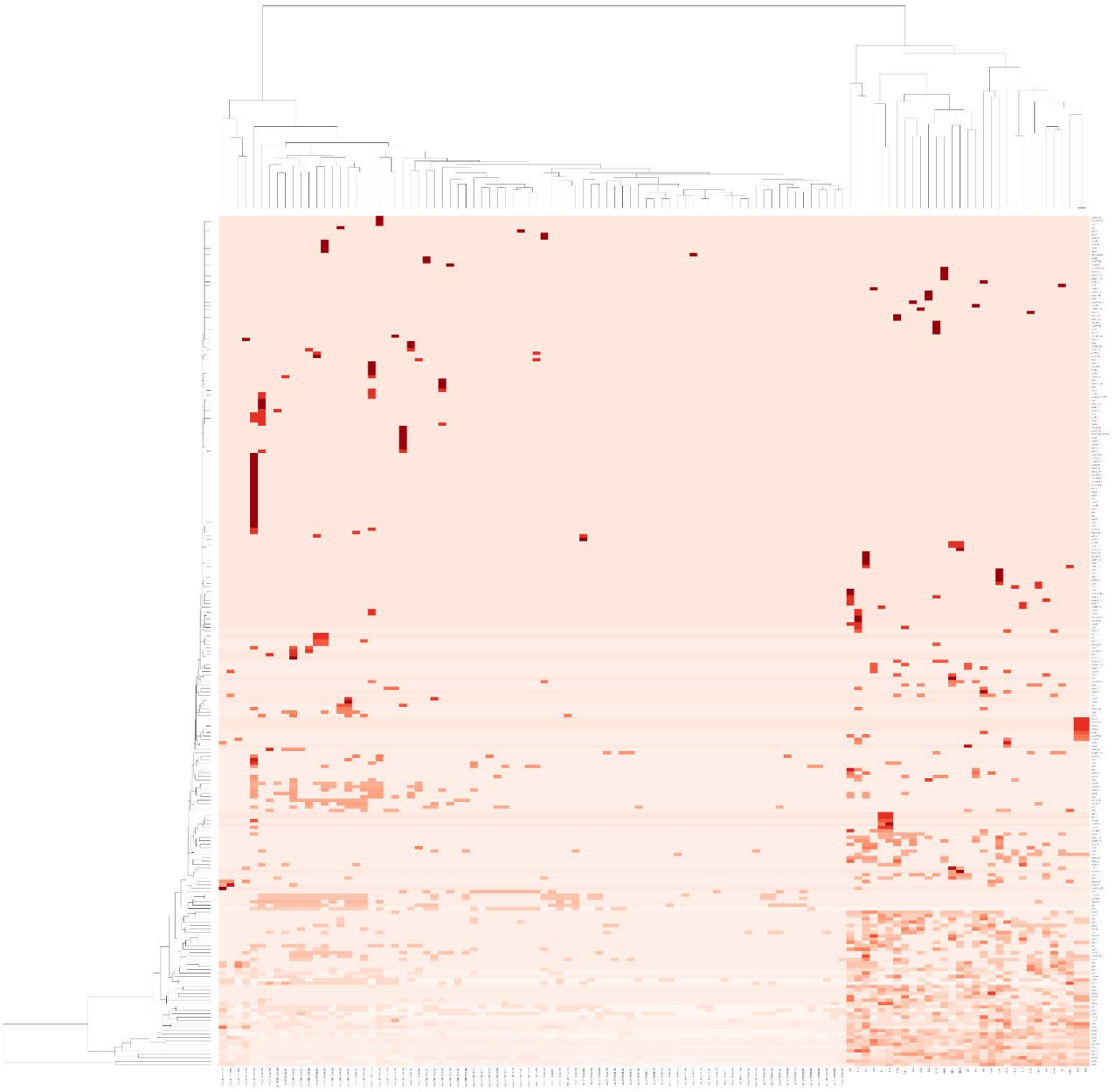

Figure 3.2: Heat map analysis of the ARG abundances in different samples.

According to the Kruskal-Wallis test, the local and global groups have different distributions of ARG abundance. In general, Bangladeshi samples possessed higher abundance of ARGs compared to foreign samples **(Figure 5) (Table 3.2).**

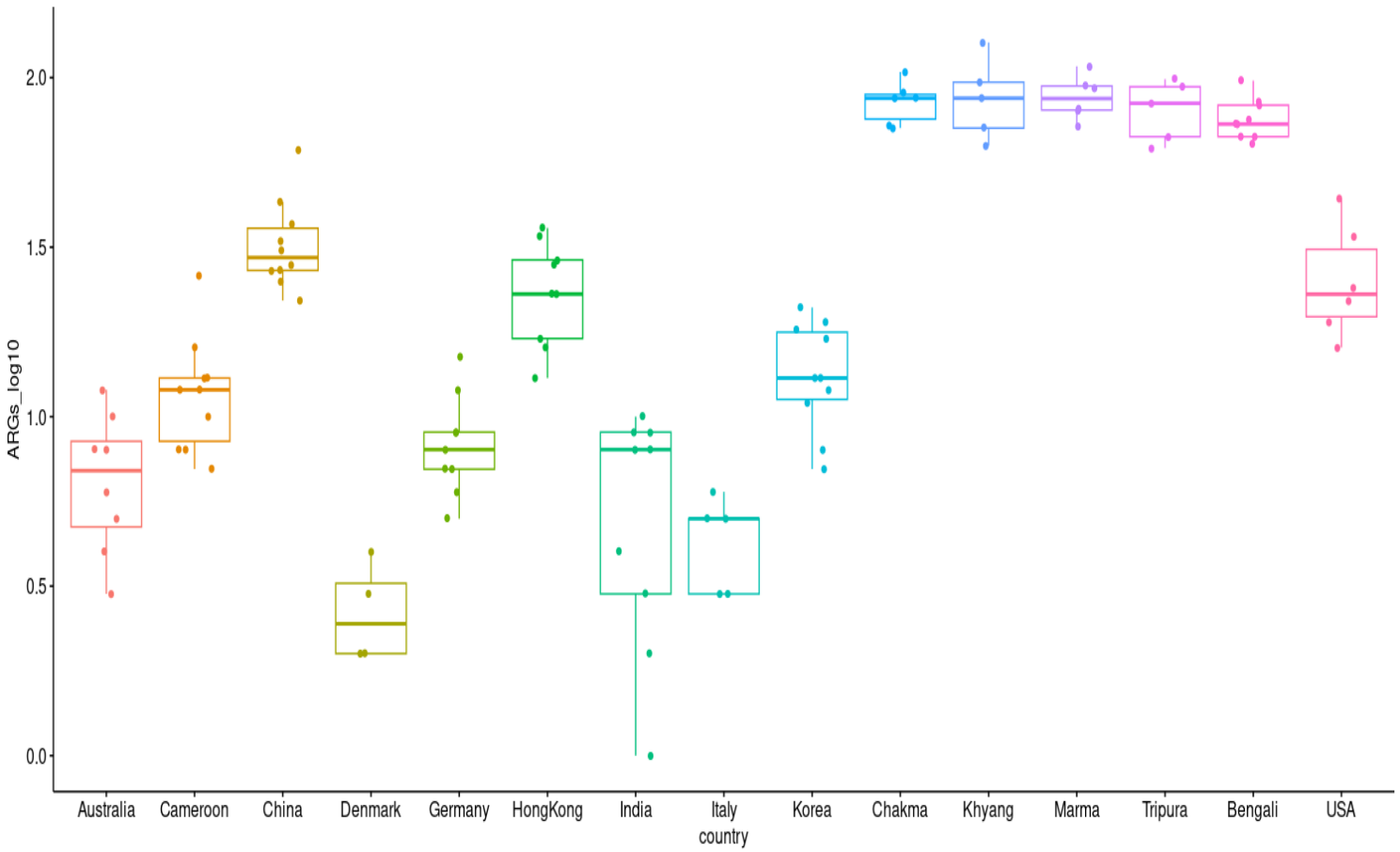

Figure 3.3: Distribution of antibiotic-resistant genes in each group was visualized using boxplot. Their Kruskal-Wallis test score indicates differences between the cohorts.

Table 3.2: International cohorts and the distribution of antibiotic resistant gene homologs in their total microbiome, plasmidome and virome as detected by ABricate.

Serial No.	Sample No.	Country	Total Antibiotic Resistant Genes	Plasmid Antibiotic Resistant Genes	Viral Antibiotic Resistant Genes
1	SRR8164235	Australia	3	3	0
2	SRR8164239	Australia	7	1	1

3	SRR8164240	Australia	4	4	0
4	SRR8164241	Australia	7	2	0
5	SRR8164242	Australia	9	2	2
6	SRR8164243	Australia	5	1	0
7	SRR8164244	Australia	11	11	1
8	SRR8164245	Australia	2	3	0
9	ERR2619707	Cameroon	11	3	0
10	ERR2619708	Cameroon	11	10	0
11	ERR2619709	Cameroon	12	2	1
12	ERR2619710	Cameroon	7	4	1
13	ERR2619711	Cameroon	12	5	1
14	ERR2619713	Cameroon	25	0	1
15	ERR2619714	Cameroon	6	0	0
16	ERR2619715	Cameroon	7	0	1
17	ERR2619716	Cameroon	9	0	1
18	ERR2619717	Cameroon	15	0	0
19	SRR11649931	China	26	13	3
20	SRR11649932	China	27	8	2

21	SRR11649933	China	30	20	5
22	SRR11649934	China	36	1	1
23	SRR11649935	China	26	16	7
24	SRR11649936	China	21	6	4
25	SRR11649937	China	42	24	2
26	SRR11649938	China	60	37	5
27	SRR11649939	China	24	12	0
28	SRR11649940	China	32	20	0
29	ERR011298	Denmark	1	2	0
30	ERR011299	Denmark	2	1	1
31	ERR011341	Denmark	3	2	2
32	ERR011344	Denmark	1	1	0
33	ERR7439145	Germany	14	10	1
34	ERR7439146	Germany	7	3	0
35	ERR7439148	Germany	4	1	0
36	ERR7439149	Germany	6	6	0
37	ERR7439151	Germany	11	1	1
38	ERR7439155	Germany	6	3	1

39	ERR7439157	Germany	5	3	0
40	ERR7439159	Germany	8	0	1
41	ERR7439160	Germany	8	0	3
42	ERR7162883	HongKong	16	7	4
43	ERR7162884	HongKong	28	19	3
44	ERR7162885	HongKong	35	16	1
45	ERR7162886	HongKong	22	4	3
46	ERR7162887	HongKong	27	14	7
47	ERR7162888	HongKong	12	5	3
48	ERR7162889	HongKong	33	24	0
49	ERR7162890	HongKong	15	5	5
50	ERR7162891	HongKong	22	10	4
52	SRR5898911	India	2	1	0
53	SRR5898912	India	0	1	2
54	SRR5898914	India	1	2	0
55	SRR9108915	India	3	4	0
56	SRR9108943	India	7	3	2
57	SRR9108944	India	9	2	1

58	SRR9108945	India	8	3	0
59	SRR9108946	India	7	1	2
60	SRR9108947	India	8	4	3
61	SRR5533705	Italy	5	1	0
62	SRR5533706	Italy	2	1	0
63	SRR5533711	Italy	2	1	1
64	SRR5533712	Italy	4	0	1
65	SRR5533713	Italy	4	0	1
66	SRR15040928	Korea	10	3	1
67	SRR15040929	Korea	12	4	0
68	SRR15040930	Korea	6	7	0
69	SRR15040931	Korea	17	2	2
70	SRR15040932	Korea	7	5	0
71	SRR15040933	Korea	16	1	1
72	SRR15040934	Korea	18	11	1
73	SRR15040935	Korea	20	3	3
74	SRR15040936	Korea	12	7	4
75	SRR15040937	Korea	11	0	0

76	SRS011271	USA	43	3	1
77	SRS012902	USA	21	4	1
78	SRS012969	USA	33	0	0
79	SRS013158	USA	18	4	0
80	SRS015190	USA	23	2	0
81	SRS013829	USA	15	0	0

However, the Pairwise Wilcoxon test shown in **Figure 3.4** indicated that samples from Bangladesh are not statistically different (p value > 0.5).

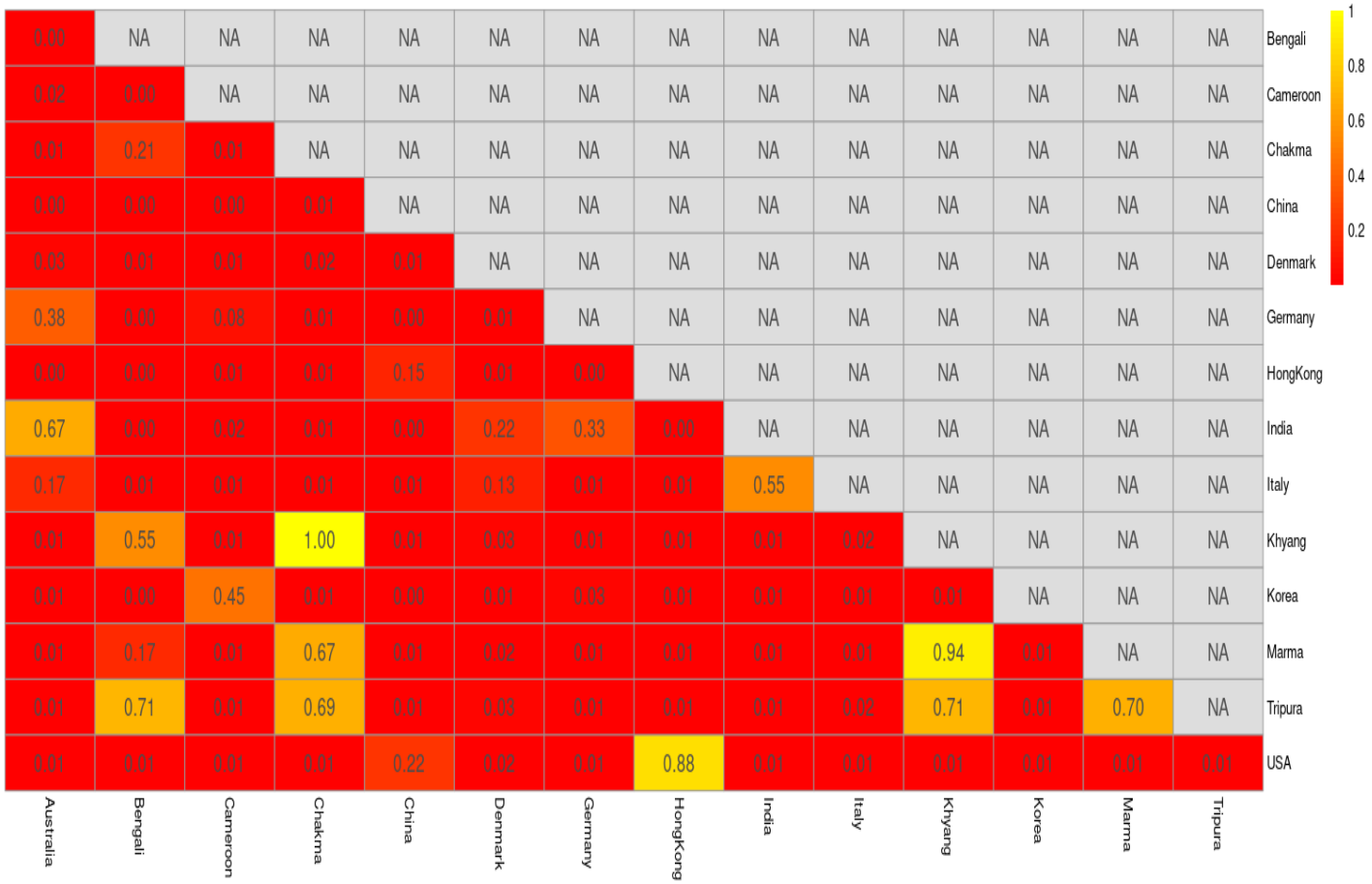

Figure 3.4: Distribution of antibiotic-resistant genes in each group visualized using Pairwise

Wilcoxon test showing statistically significant p values within the populations. The p values are depicted as a heatmap (score 1 to 0).

The protein level identification from fARGene analysis (**Figure 3.5)** showed Class_a and class_tet_rpg were highly prevalent in the Bangladeshi population. Interestingly, Class_b_1_2 was less abundant in the Bangladeshi groups compared to other populations and class_d_1 was absent.

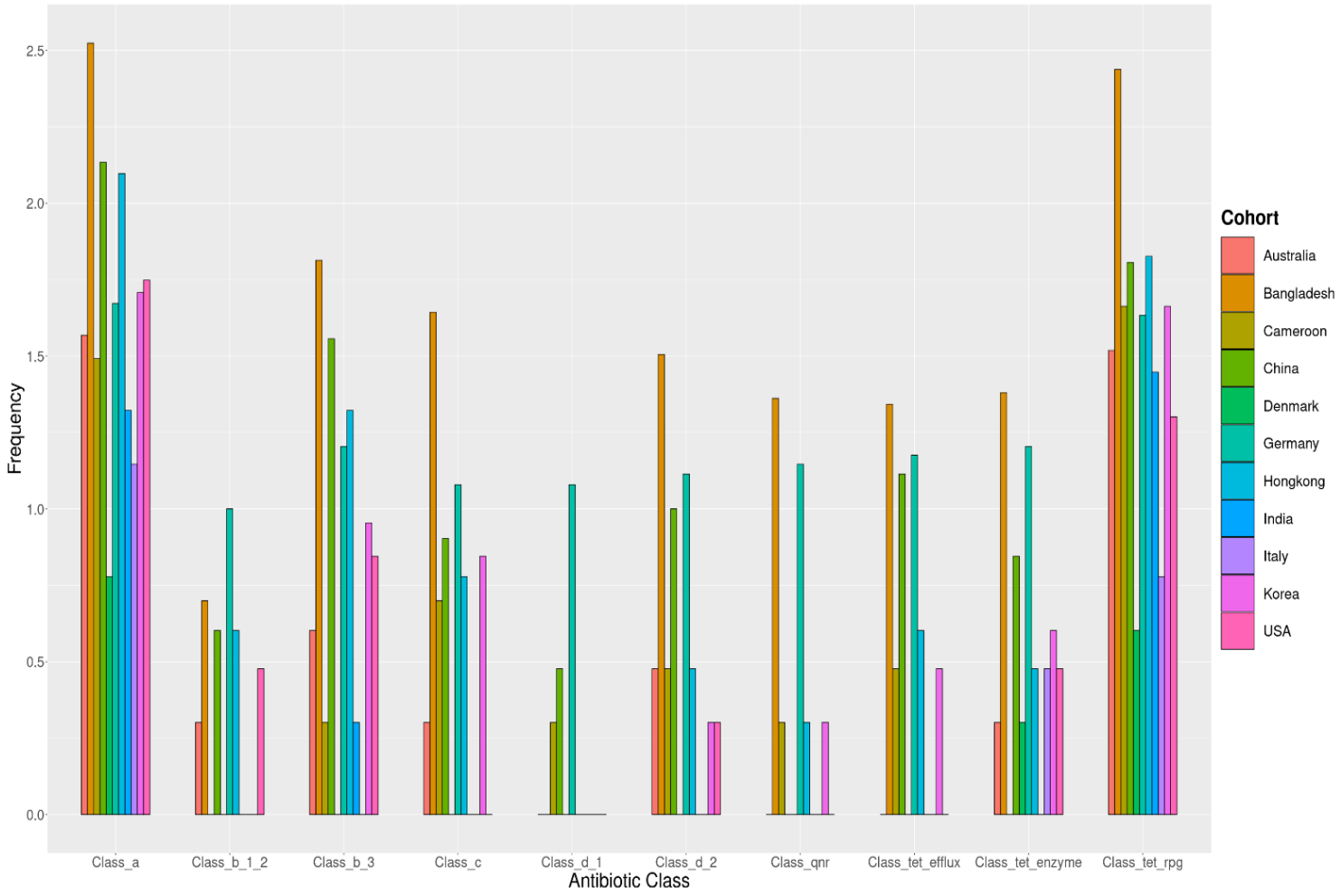

Figure 3.5: Various antibiotic resistance-causing proteins with their distributions.

3.2 ARGs are more frequent in the mobilome of Bangladeshi samples

The mobile carriers of ARGs are mainly plasmids and viruses (Bacteriophage). Here, the plasmidal and viral DNA sequences were deciphered using several Machine Learning (ML)/ Deep Learning based tools. The detected viral genomes from Bengali, Chakma, Marma, Tripura and Khyang samples had ARGs on average 4.4-2.33. The average ARGs of plasmid sequences from Bengali, Tripura, Marma, Khyang and Chakma were 12-8.33 (**Table 3.3**).

Table 3.3: Mean, median and standard deviation of antibiotic resistant gene homologs in total microbiome, plasmidal and viral genomes from Table 3.1.

	Chakma	**Marma**	**Tripura**	**Khyang**	**Bengali**
Total Antibiotic Resistant Genes					
Mean	81.83	87.17	80.4	88	75.11
Median	79	86	83	86	72
Standard Deviation	13.2	12.98	15.98	25.06	10.83
Plasmid Antibiotic Resistant Genes					
Mean	8.33	11	9.6	12	8.88
Median	7.5	10.5	8	11	8
Standard Deviation	4.63	3.22	6.11	5.39	2.32
Viral Antibiotic Resistant Genes					

Mean	2.83	3	4.4	2.6	2.33
Median	2.5	3	3	2	2
Standard Deviation	1.47	2	2.88	2.07	1.73

In plasmidal sequences catB4, tet(A), tet(W), erm(F), QnrS1, tet(32), tet(O), sul1, vanR-G were highly abundant in plasmidome (**Figure 3.6).**

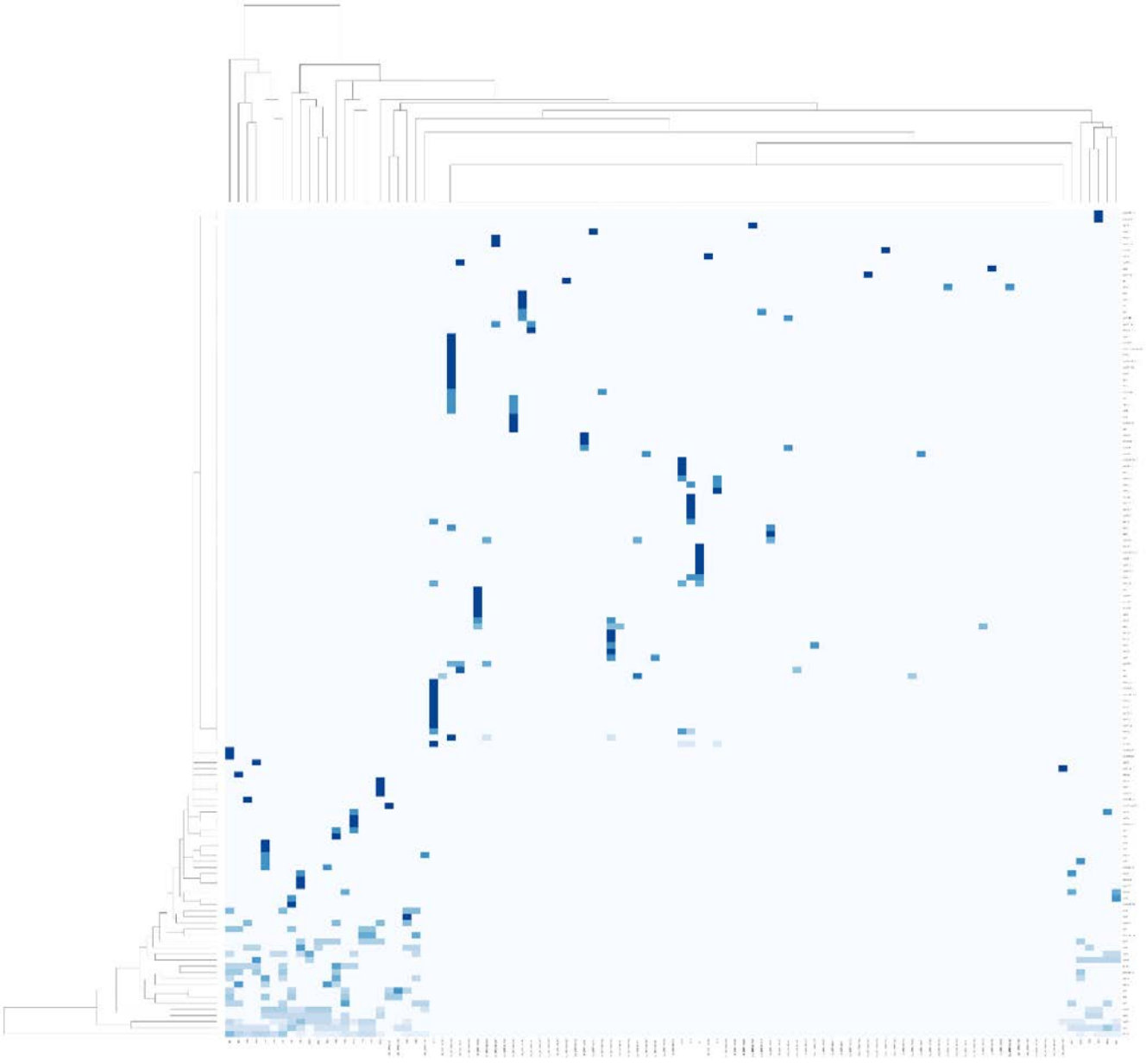

Figure 3.6: Antibiotic Resistance Gene (ARG) homolog abundances in the gut plasmidome.

In viral sequences tet(A), cfxA6, aadA1, blaTEM-1B, tet(32), tet(O), tet(Q), ermB, were more enriched in Bangladeshi virome than other countries (**Figure 3.7**)

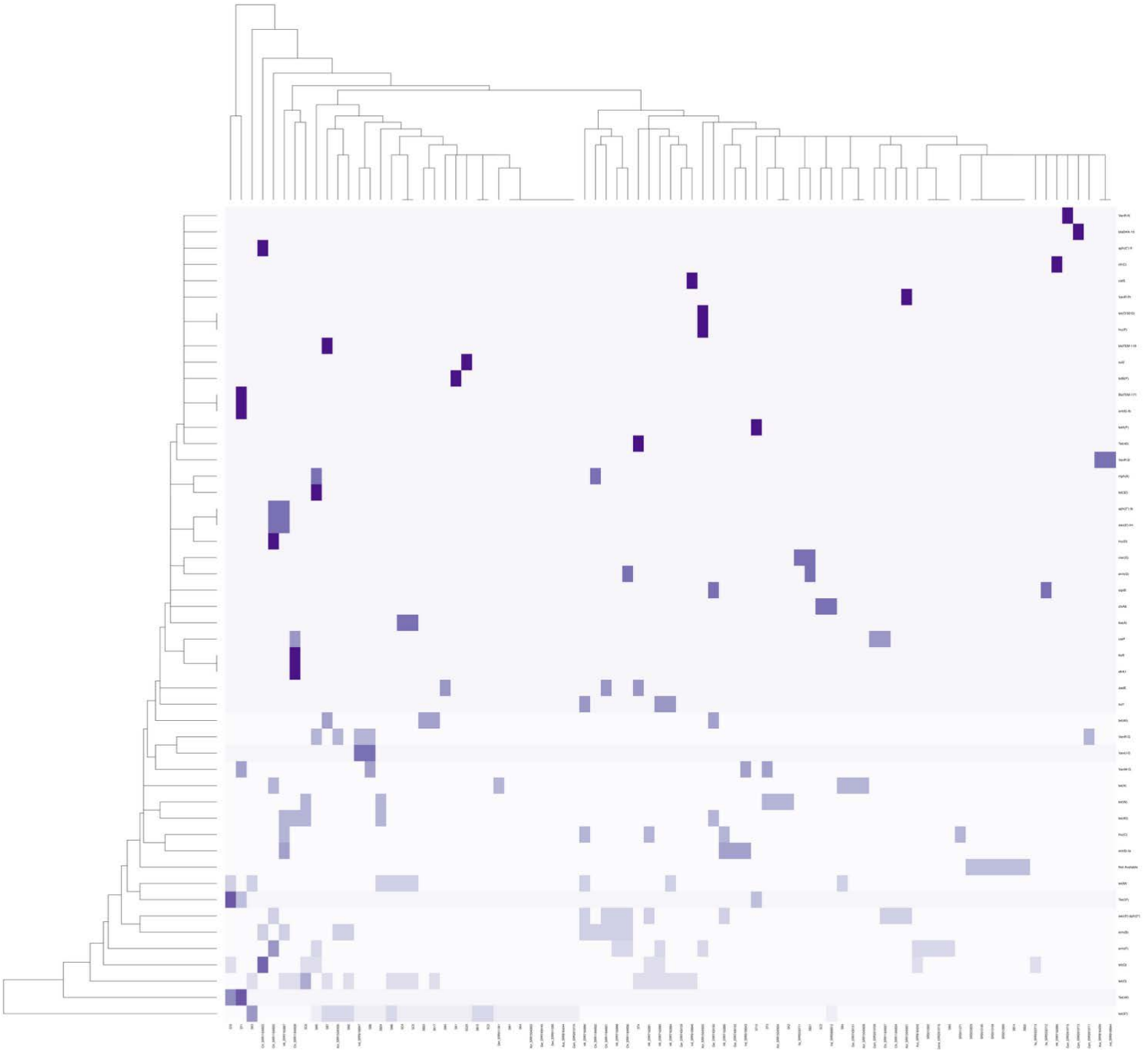

Figure 3.7: Antibiotic Resistance Gene (ARG) homolog abundances in the gut virome.

(**Figure 3.8a) , (Figure 3.8b)** shows the abundance of total, plasmids and viral ARGs were relatively higher in local samples. Number of total ARGs, viral ARGs and plasmidal ARGs have shown a positive correlation (Spearman's ρ value: 0.63 & 0.68, p value < 0.05).

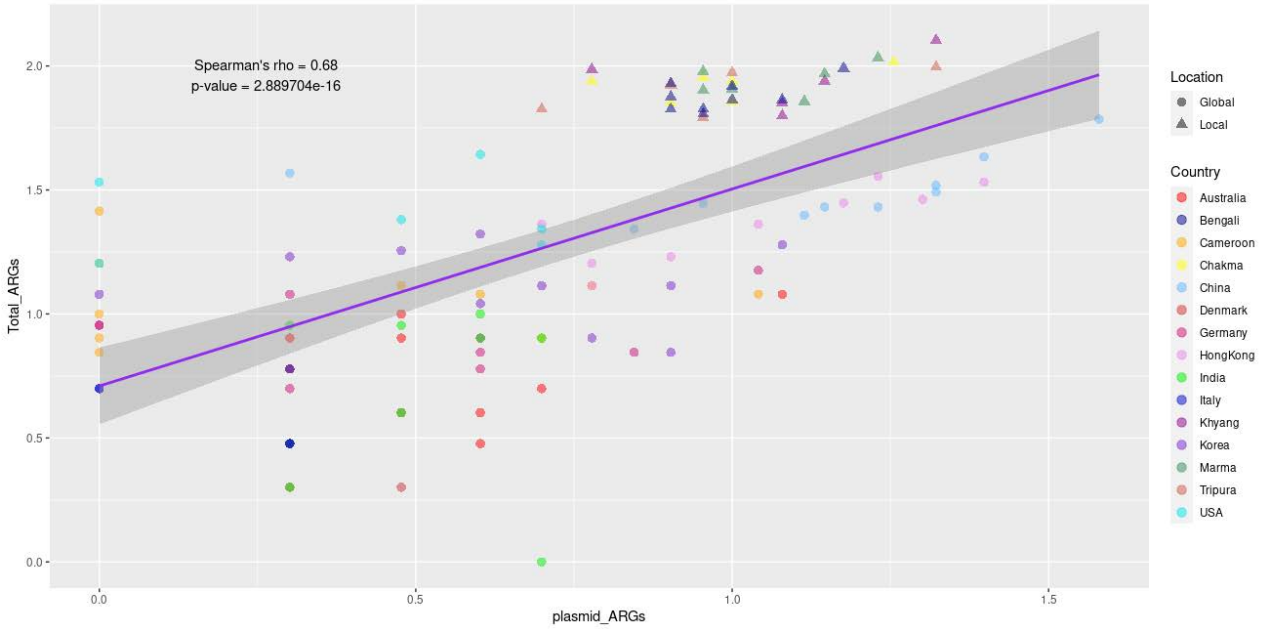

Figure 3.8a: Correlation between total antibiotic-resistant genes with Plasmidal antibiotic-resistant genes (Spearman's rho = 0.68, p-value < 0.05).

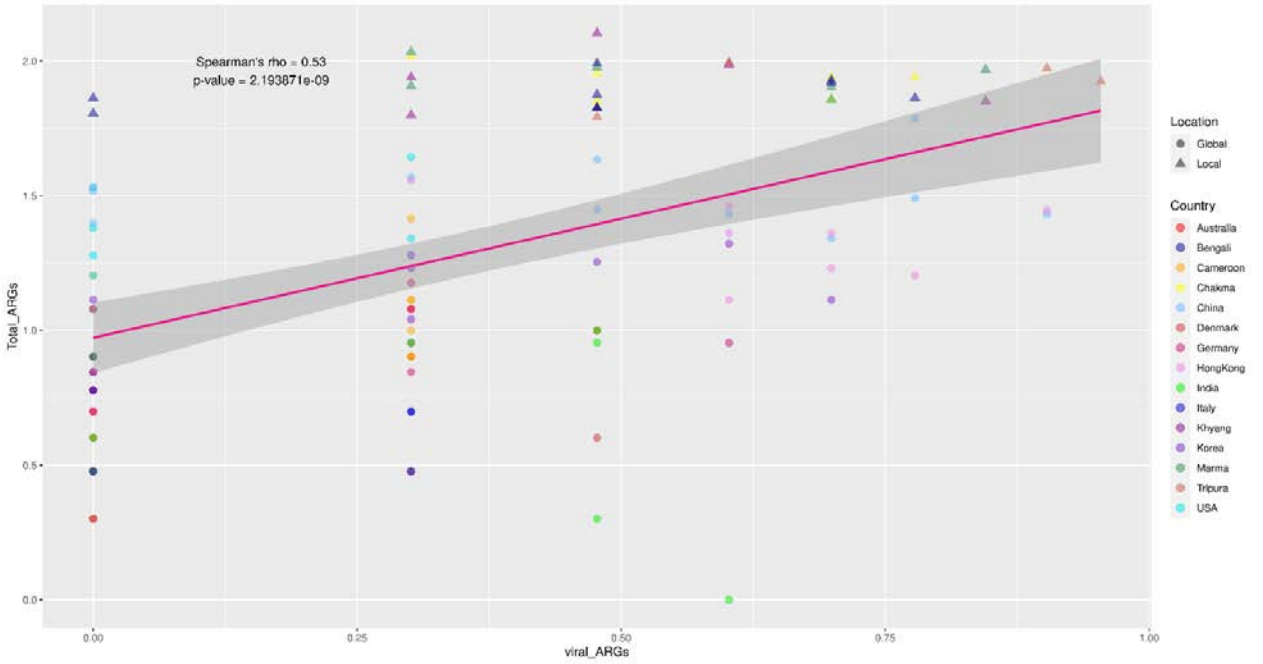

Figure 3.8b: Correlation between total antibiotic-resistant genes with Viral antibiotic-resistant genes (Spearman's rho = 0.68, p-value < 0.05)

3.3 Pathway abundance analysis of the selected cohorts

Antibiotic resistance related pathways were detected using MicrobeAnnotator. The pathways were distributed differently in between the cohorts as depicted in **Figure 3.9.**

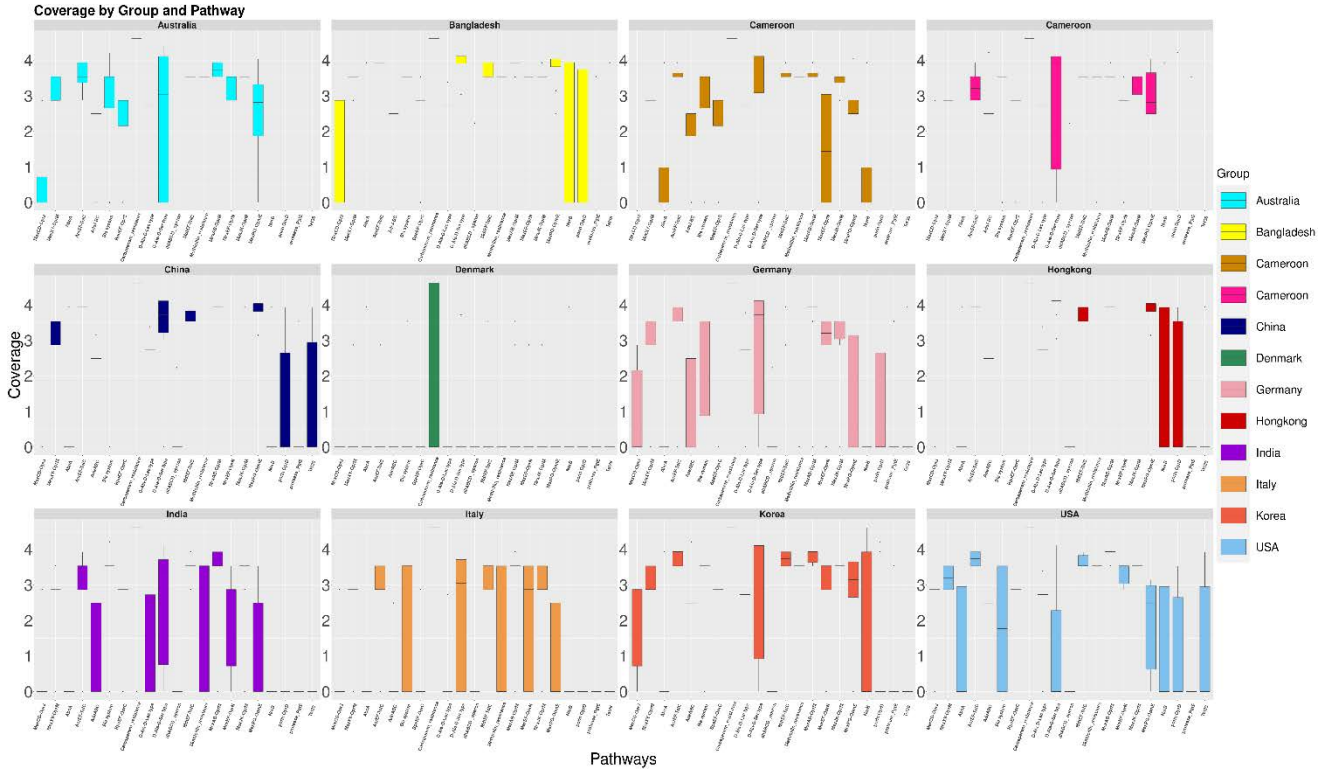

Figure 3.9: Drug resistance pathway distributions among the groups. Each facet grid represents one group and the distributions of different resistant pathways in those groups.

To identify the differentially abundant pathways across the all cohorts and each group with Bangladeshi samples, LEFSe was implemented. LEFSe demonstrated differentially abundant pathways in Chakma and Bengali groups (**Figure 3.10a).** Chakma and Bengali populations have relatively higher abundance of NorB and MexPQ-OpmE pathways respectively. However, when all the samples from Bangladesh were joined together and compared with each group, MexPQ-OpmE showed higher abundance in each group except China (**Figure 3.10b).**

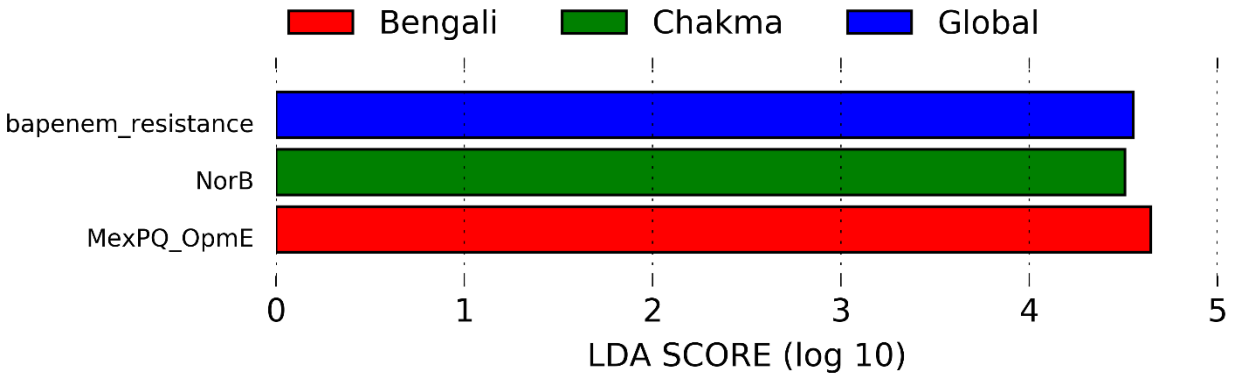

Figure 3.10a: Linear Discriminant Analysis (LDA) of the pathways from all the samples.

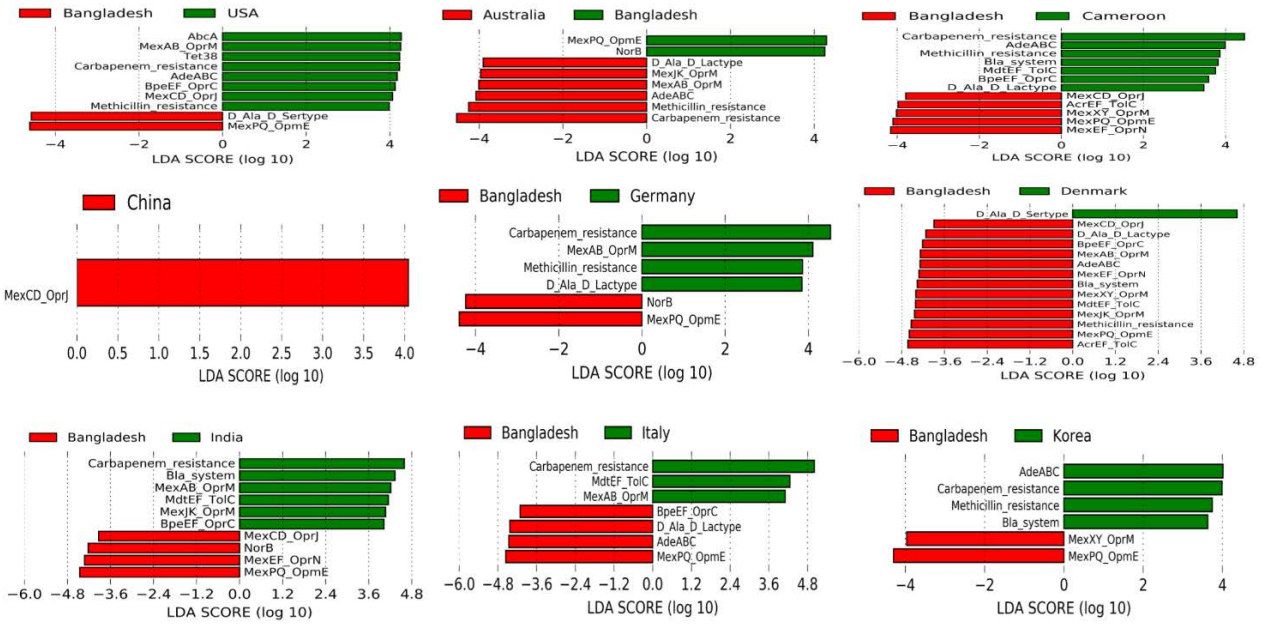

Figure 3.10b: Differentially abundant pathways were also detected for each group with Bangladesh.

Discussion

Management of Antibiotic pollution in Low and middle income countries (LMICs) is challenging due to low resources, poor management and improper infrastructure of sewage systems. In Bangladesh, prevalence of antibiotic resistant microorganisms are increasing at an alarming rate (7). In Bangladesh, where industrial development is going in an enormous force, the Bengali and indigenous people are getting used to modern lifestyles for several decades. Since different antibiotics are randomly given over the counter (OTC) in Bangladesh, resistance is booming in this place (54). Moreover, usage of antibiotics in poultry and animal farms, improper hygiene, industrial and untreated hospital wastewater are fueling this problem. Such incidents will facilitate the generation of multidrug resistant pathogens which will be hard to manage (55,56).

Antibiotic resistance and the availability of antimicrobial agents are natural phenomena that have existed from ancient times. Through the episodes of evolution and natural selections, microbes produce antibiotics and bacteria have developed antibiotic resistance to survive against these antimicrobials (57). However, in nature, microbes produce antibiotics at minute level and antibiotic resistance does not develop rapidly. Enhanced elevation of antibiotics from artificial sources such as medical wastewater, human/ animal manure and urine can quickly increase abundance of antibiotic resistance in the environment. Under higher concentration of antibiotics, a selection pressure will promote to turn an ARG like homolog into a full functional ARG (57,58). Hence, reservoirs of these ARGs (or resistomes) disseminate ARGs or ARG homologs in the environment and pose a massive threat for public health (58). Human gut microbiome is a vast reservoir of antibiotic resistome (59). Analyzing the human gut resistome is critical to decrypt the spreading of ARGs and their types or prevalence (60). ARGs in non-pathogenic microorganisms is not a threat and sometimes protective for the microbiome against antibiotics, however, these genes can be obtained by pathogenic bacteria through mechanisms such as DNA conjugation, transformation and transduction (58,61). These processes transmit viral (bacteriophage) elements and mobilomes like plasmids. Therefore, elevated presence of ARGs in plasmids and viruses indicates more chances of ARG spreading.

In this study, we analyzed the gut resistome of 31 samples from five Bangladeshi cohorts and compared them with different studies. First resistant genes and proteins were detected for the total

microbiome. Afterward, the plasmidome and virome were separated. Using ABricate, the resistant genes were identified in these mobilomes. Total abundance of ARGs were higher in local samples. ARGs were also higher in viral and plasmidal elements. The heatmap analysis showed that tet(W), tet(37), catB4, tet(32), VanR_G, oqxA and many other ARGs were highly abundant in the Bangladeshi population. These types of diversity and elevated abundances create a higher possibility of multi drug resistant pathogens. However, for other samples (where antibiotics are not available in OTC), these diverse ARGs were less frequent. Log abundance was significantly higher in the local samples and their difference was not statistically significant between them. However, they were different from other countries. Hence, to reduce the ARG abundance proper regulation on antibiotic usage is required. .

In plasmidal DNA catB4, tet(A), tet(W), erm(F), QnrS1, tet(32), tet(O), sul1, vanR-G were more frequent in the Bangladeshi cohorts. Same type of pattern was also observed in viral sequences where tet(A), cfxA6, aadA1, blaTEM-1B, tet(32) were highly enriched in the samples from Bangladesh. The presence of these types of various genes in the mobilome increases the probability of transduction, transformation and conjugation into pathogenic bacteria. Elevated mobile elements with resistant genes will enhance the generation of drug resistant pathogens. This pattern was observed in Figure 5 where we have seen a positive correlation between total resistome and resistant genes in the mobilome.

fARGene detected resistance causing proteins in the whole microbiome and found elevated frequency of tetracycline efflux pump and tetracycline resistant related enzymes in Bangladeshi groups. These elevations indicate that tetracycline resistant genes are spreading at a higher rate in the environment. From the previous studies in Bangladesh, researchers and clinicians have observed that the median tetracycline resistance of *Eschericia coli* and *Staphylococcus aureus* are 65 and 43.5 respectively. For Doxycycline, the value is 61.1 in *E. coli* (62). Hence, this type of broad spectrum antibiotics might not work in the near future. The fARGene analysis also showed prevalence of Class b_1_2 beta-lactamase was relatively lower in Bangladeshi samples and Class_d_1 was absent in

Bangladeshi samples. Interestingly, Quinolone resistant genes (Class_qnr) were very high in local samples. Class_qnr was only present in 5 out of 11 groups. Fluoroquinolone such as ciprofloxacin can be found in sewage sludge at milligram/ kilogram concentrations but ciprofloxacin sensitive strains are very uncommon in sewage (63). Moreover, administration of Ciprofloxacin did not enrich quinolone resistance (qnr) genes in gut microbiome (64). However, presence of qnr type genes in locales most plausibly indicates prolonged exposure of quinolone antibiotics. This exposure might come from environment, direct consumption of antibiotics or consumption of foods that contain antibiotics (e.g., Broiler or Layer Chicken) (65). The LEfSe analysis showed a higher abundance of MexPQ-OpmE pathway in Bangladeshi samples than other groups. MexPQ-OpmE pathway may provide fluoroquinolone resistance in Bangladeshi gut microbiome (66).

Our study suggests controlling the exposure of antibiotic pollution using different regulations from the government and proper authority. Antibiotics in OTC must be halted in Bangladesh to reduce the antibiotic based selection pressure in gut microbiome. Moreover, industrial use of antibiotics should be governed by the authorities properly. We hope our study will elevate consciousness among the people of Bangladesh and other South Asian LMICs.

Conclusion

Antibiotic resistant genes are more prevalent in the gut microbiota of Bangladeshi people than other groups/ countries. This higher prevalence was observed in both urban, sub-urban and rural areas. It was detected within different indigenous and Bengali cohorts. The higher ARG frequency in gut mobilome is increasing the possibility of generating multi-drug resistant pathogens using transduction, transformation and conjugation. Therefore, proper regulation is mandatory in future to improve this situation.

References

1. Michaud CM. Global Burden of Infectious Diseases. In: Encyclopedia of Microbiology [Internet]. Elsevier; 2009 [cited 2023 Mar 15]. p. 444–54. Available from: https://linkinghub.elsevier.com/retrieve/pii/B9780123739445001851

2. Davies J, Davies D. Origins and Evolution of Antibiotic Resistance. Microbiol Mol Biol Rev. 2010 Sep;74(3):417–33.

3. Aminov RI. A Brief History of the Antibiotic Era: Lessons Learned and Challenges for the Future. Front Microbiol [Internet]. 2010 [cited 2023 Mar 15];1. Available from: http://journal.frontiersin.org/article/10.3389/fmicb.2010.00134/abstract

4. Calhoun C, Wermuth HR, Hall GA. Antibiotics. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 [cited 2023 Feb 28]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK535443/

5. Global antibiotic consumption increased by 65% between 2000 and 2015, study finds. Pharm J [Internet]. 2018 [cited 2023 Feb 28]; Available from: http://www.pharmaceutical-journal.com/news-and-analysis/news/global-antibiotic-consumption-increased-by-65-between-2000-and-2015-study-finds/20204603.article

6. Klein EY, Van Boeckel TP, Martinez EM, Pant S, Gandra S, Levin SA, et al. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. Proc Natl Acad Sci [Internet]. 2018 Apr 10 [cited 2023 Feb 28];115(15). Available from: https://pnas.org/doi/full/10.1073/pnas.1717295115

7. Hoque R, Ahmed SM, Naher N, Islam MA, Rousham EK, Islam BZ, et al. Tackling antimicrobial resistance in Bangladesh: A scoping review of policy and practice in human, animal and environment sectors. Klein EY, editor. PLOS ONE. 2020 Jan 27;15(1):e0227947.

8. Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. P T Peer-Rev J Formul Manag. 2015 Apr;40(4):277–83.

9. Murray CJL, Ikuta KS, Sharara F, Swetschinski L, Robles Aguilar G, Gray A, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. The Lancet. 2022 Feb;399(10325):629–55.

10. Peterson E, Kaur P. Antibiotic Resistance Mechanisms in Bacteria: Relationships Between Resistance Determinants of Antibiotic Producers, Environmental Bacteria, and Clinical Pathogens. Front Microbiol. 2018 Nov 30;9:2928.

11. Liu B, Pop M. ARDB--Antibiotic Resistance Genes Database. Nucleic Acids Res. 2009 Jan 1;37(Database):D443–7.

12. CDC. Antibiotic Resistance Threatens Everyone [Internet]. Centers for Disease Control and Prevention. 2022 [cited 2023 Feb 28]. Available from: https://www.cdc.gov/drugresistance/index.html

13. von Wintersdorff CJH, Penders J, van Niekerk JM, Mills ND, Majumder S, van Alphen LB, et al. Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. Front Microbiol [Internet]. 2016 Feb 19 [cited 2023 Mar 15];7. Available from: http://journal.frontiersin.org/Article/10.3389/fmicb.2016.00173/abstract

14. Munita JM, Arias CA. Mechanisms of Antibiotic Resistance. Kudva IT, Zhang Q, editors. Microbiol Spectr. 2016 Mar 25;4(2):4.2.15.

15. Uddin TM, Chakraborty AJ, Khusro A, Zidan BRM, Mitra S, Emran TB, et al. Antibiotic resistance in microbes: History, mechanisms, therapeutic strategies and future prospects. J Infect Public Health. 2021 Dec;14(12):1750–66.

16. C Reygaert W, Department of Biomedical Sciences, Oakland University William Beaumont School of Medicine, Rochester, MI, USA. An overview of the antimicrobial resistance mechanisms of bacteria. AIMS Microbiol. 2018;4(3):482–501.

17. Nemeth J, Oesch G, Kuster SP. Bacteriostatic versus bactericidal antibiotics for patients with serious bacterial infections: systematic review and meta-analysis. J Antimicrob Chemother. 2015 Feb 1;70(2):382–95.

18. Schroeder M, Brooks B, Brooks A. The Complex Relationship between Virulence and Antibiotic Resistance. Genes. 2017 Jan 18;8(1):39.

19. Llor C, Bjerrum L. Antimicrobial resistance: risk associated with antibiotic overuse and initiatives to reduce the problem. Ther Adv Drug Saf. 2014 Dec;5(6):229–41.

20. Wright GD. Molecular mechanisms of antibiotic resistance. Chem Commun. 2011;47(14):4055.

21. Serwecińska L. Antimicrobials and Antibiotic-Resistant Bacteria: A Risk to the Environment and to Public Health. Water. 2020 Nov 25;12(12):3313.

22. Modi SR, Collins JJ, Relman DA. Antibiotics and the gut microbiota. J Clin Invest. 2014 Oct 1;124(10):4212–8.

23. Sun D, Jeannot K, Xiao Y, Knapp CW. Editorial: Horizontal Gene Transfer Mediated Bacterial Antibiotic Resistance. Front Microbiol. 2019 Aug 27;10:1933.

24. Aslam B, Khurshid M, Arshad MI, Muzammil S, Rasool M, Yasmeen N, et al. Antibiotic Resistance: One Health One World Outlook. Front Cell Infect Microbiol. 2021 Nov 25;11:771510.

25. Iskandar K, Molinier L, Hallit S, Sartelli M, Hardcastle TC, Haque M, et al. Surveillance of antimicrobial resistance in low- and middle-income countries: a scattered picture. Antimicrob Resist Infect Control. 2021 Mar 31;10(1):63.

26. Iskandar K, Molinier L, Hallit S, Sartelli M, Catena F, Coccolini F, et al. Drivers of Antibiotic Resistance Transmission in Low- and Middle-Income Countries from a “One Health” Perspective—A Review. Antibiotics. 2020 Jul 1;9(7):372.

27. Sulis G, Sayood S, Gandra S. Antimicrobial resistance in low- and middle-income countries: current status and future directions. Expert Rev Anti Infect Ther. 2022 Feb 1;20(2):147–60.

28. Sharma A, Singh A, Dar MA, Kaur RJ, Charan J, Iskandar K, et al. Menace of antimicrobial resistance in LMICs: Current surveillance practices and control measures to tackle hostility. J Infect Public Health. 2022 Feb;15(2):172–81.

29. Chereau F, Opatowski L, Tourdjman M, Vong S. Risk assessment for antibiotic resistance in South East Asia. BMJ. 2017 Sep 5;j3393.

30. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. Br Dent J. 2010 Oct;209(7):353–353.

31. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis. 2016 Feb;16(2):161–8.

32. Hart CA, Kariuki S. Antimicrobial resistance in developing countries. BMJ. 1998 Sep 5;317(7159):647–50.

33. Safain KS, Bhuyan GS, Tasnim S, Hasib SH, Sultana R, Islam MS, et al. Situation of antibiotic resistance in Bangladesh and its association with resistance genes for horizontal transfer [Internet].

Microbiology; 2020 Apr [cited 2023 Feb 28]. Available from: http://biorxiv.org/lookup/doi/10.1101/2020.04.06.027391

34. Asna SMZH, Haq JA, Rahman MM. Nalidixic acid-resistant Salmonella enterica serovar Typhi with decreased susceptibility to ciprofloxacin caused treatment failure: a report from Bangladesh. Jpn J Infect Dis. 2003 Feb;56(1):32–3.

35. CHT-report-LR-August20-website.pdf [Internet]. [cited 2023 Feb 28]. Available from: https://www.unicef.org/bangladesh/sites/unicef.org.bangladesh/files/2019-09/CHT-report-LR-August20-website.pdf

36. Saha A, Marma KKS, Rashid A, Tarannum N, Das S, Chowdhury T, et al. Risk factors associated with self-medication among the indigenous communities of Chittagong Hill Tracts, Bangladesh. Pegoraro C, editor. PLOS ONE. 2022 Jun 13;17(6):e0269622.

37. Thursby E, Juge N. Introduction to the human gut microbiota. Biochem J. 2017 Jun 1;474(11):1823–36.

38. Sutton TDS, Hill C. Gut Bacteriophage: Current Understanding and Challenges. Front Endocrinol. 2019 Nov 29;10:784.

39. Jakobsson HE, Jernberg C, Andersson AF, Sjölund-Karlsson M, Jansson JK, Engstrand L. Short-Term Antibiotic Treatment Has Differing Long-Term Impacts on the Human Throat and Gut Microbiome. Ratner AJ, editor. PLoS ONE. 2010 Mar 24;5(3):e9836.

40. Lerner A, Matthias T, Aminov R. Potential Effects of Horizontal Gene Exchange in the Human Gut. Front Immunol. 2017 Nov 27;8:1630.

41. Crits-Christoph A, Hallowell HA, Koutouvalis K, Suez J. Good microbes, bad genes? The dissemination of antimicrobial resistance in the human microbiome. Gut Microbes. 2022 Dec 31;14(1):2055944.

42. BARRON M. The Gut Resistome and the Spread of Antimicrobial Resistance. 2022 Jun 13; Available from: https://asm.org/Articles/2022/June/The-Gut-Resistome-and-the-Spread-of-Antimicrobial

43. Economou V, Gousia P. Agriculture and food animals as a source of antimicrobial-resistant bacteria. Infect Drug Resist. 2015 Apr;49.

44. He Y, Yuan Q, Mathieu J, Stadler L, Senehi N, Sun R, et al. Antibiotic resistance genes from livestock waste: occurrence, dissemination, and treatment. Npj Clean Water. 2020 Feb 19;3(1):1–11.

45. Thanner S, Drissner D, Walsh F. Antimicrobial Resistance in Agriculture. Baquero F, editor. mBio. 2016 May 4;7(2):e02227-15.

46. Li H, Durbin R. Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics. 2009 Jul 15;25(14):1754–60.

47. Li D, Liu CM, Luo R, Sadakane K, Lam TW. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct *de Bruijn* graph. Bioinformatics. 2015 May 15;31(10):1674–6.

48. Ren J, Song K, Deng C, Ahlgren NA, Fuhrman JA, Li Y, et al. Identifying viruses from metagenomic data using deep learning. Quant Biol. 2020 Mar;8(1):64–77.

49. Hyatt D, Chen GL, LoCascio PF, Land ML, Larimer FW, Hauser LJ. Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics. 2010 Dec;11(1):119.

50. Chen Z, Yu D, He S, Ye H, Zhang L, Wen Y, et al. Prevalence of Antibiotic-Resistant Escherichia coli in Drinking Water Sources in Hangzhou City. Front Microbiol. 2017 Jun 16;8:1133.

51. Berglund F, Österlund T, Boulund F, Marathe NP, Larsson DGJ, Kristiansson E. Identification and reconstruction of novel antibiotic resistance genes from metagenomes. Microbiome. 2019 Dec;7(1):52.

52. Ruiz-Perez CA, Conrad RE, Konstantinidis KT. MicrobeAnnotator: a user-friendly, comprehensive functional annotation pipeline for microbial genomes. BMC Bioinformatics. 2021 Dec;22(1):11.

53. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol. 2011;12(6):R60.

54. Mannan A, Shohel M, Rajia S, Mahmud NU, Kabir S, Hasan I. A cross sectional study on antibiotic resistance pattern of Salmonella typhi clinical isolates from Bangladesh. Asian Pac J Trop Biomed. 2014 Apr;4(4):306–11.

55. Al Amin Md, Hoque MN, Siddiki AZ, Saha S, Kamal MdM. Antimicrobial resistance situation in animal health of Bangladesh. Vet World. 2020;13(12):2713–27.

56. Hasan B, Faruque R, Drobni M, Waldenström J, Sadique A, Ahmed KU, et al. High Prevalence of Antibiotic Resistance in Pathogenic Escherichia coli from Large- and Small-Scale Poultry Farms in Bangladesh. Avian Dis. 2011 Dec;55(4):689–92.

57. Blair JMA, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJV. Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol. 2015 Jan;13(1):42–51.

58. Larsson DGJ, Flach CF. Antibiotic resistance in the environment. Nat Rev Microbiol. 2022 May;20(5):257–69.

59. Feng J, Li B, Jiang X, Yang Y, Wells GF, Zhang T, et al. Antibiotic resistome in a large-scale healthy human gut microbiota deciphered by metagenomic and network analyses. Environ Microbiol. 2018 Jan;20(1):355–68.

60. van Schaik W. The human gut resistome. Philos Trans R Soc B Biol Sci. 2015 Jun 5;370(1670):20140087.

61. Connelly S, Fanelli B, Hasan NA, Colwell RR, Kaleko M. SYN-007, an Orally Administered Beta-Lactamase Enzyme, Protects the Gut Microbiome from Oral Amoxicillin/Clavulanate without Adversely Affecting Antibiotic Systemic Absorption in Dogs. Microorganisms. 2020 Jan 22;8(2):152.

62. Ahmed I, Rabbi MdB, Sultana S. Antibiotic resistance in Bangladesh: A systematic review. Int J Infect Dis. 2019 Mar;80:54–61.

63. Reinthaler FF, Galler H, Feierl G, Haas D, Leitner E, Mascher F, et al. Resistance patterns of Escherichia coli isolated from sewage sludge in comparison with those isolated from human patients in 2000 and 2009. J Water Health. 2013 Mar 1;11(1):13–20.

64. Willmann M, Vehreschild MJGT, Biehl LM, Vogel W, Dörfel D, Hamprecht A, et al. Distinct impact of antibiotics on the gut microbiome and resistome: a longitudinal multicenter cohort study. BMC Biol. 2019 Dec;17(1):76.

65. Imam T, Gibson JS, Foysal M, Das SB, Gupta SD, Fournié G, et al. A Cross-Sectional Study of Antimicrobial Usage on Commercial Broiler and Layer Chicken Farms in Bangladesh. Front Vet Sci. 2020 Dec 16;7:576113.

66. Mima T, Sekiya H, Mizushima T, Kuroda T, Tsuchiya T. Gene Cloning and Properties of the RND-Type Multidrug Efflux Pumps MexPQ-OpmE and MexMN-OprM from *Pseudomonas aeruginosa*. Microbiol Immunol. 2005 Nov;49(11):999–1002.