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ORIGINAL ARTICLE

Characterization, Molecular Identification and Antimicrobial Susceptibility Testing of Diarrheagenic *Escherichia coli* Isolated from Diarrhea Infected Patients Around Dhaka City

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ABSTRACT

Background: Mostly *Escherichia coli* is an innocuous intestinal inhabitant and does not cause infection or disease in a healthy individual. Nevertheless, Shiga toxigenic *Escherichia coli* (STEC) is one of the few strains that causes severe diarrheal disease in humans all over the world, where tropical and developing countries have a higher incident rate.

Objective: This research aimed to determine and evaluate the vulnerability of diarrheagenic *E. coli* to certain antibiotics isolated from hospitalized diarrhea-infected patients in Dhaka city.

Methodology: After initial cultural isolation and identification through BAM, *E. coli* species were identified automatically via VITEK 2 COMPACT ID-GN card. Molecular identification was performed through detection of 16S rRNA, *stx1*, and *stx2* genes by means of PCR. VITEK 2 COMPACT was used to perform the Antimicrobial Susceptibility Test (AST). Agreement and error were analyzed based on Clinical & Laboratory Standards Institute (CLSI) guidelines.

Results: With the VITEK 2 COMPACT system, 218 (97.75%) of the 223 isolates were accurately identified to the species level. 32.7% (73 out of 218) of the isolates in the research we conducted were classified as very good, and 45.7% (102 out of 218) of the isolates were recognized at the excellent level. The percentages of individuals with good and acceptable level identification were 10.8% (24 out of 218) and 8.5% (19 out of 218), respectively. More importantly, 2.2% (5 out of 218) isolates were unidentified by the system. In this experiment, from 218 isolates of *E. coli*, *stx1* and *stx2* were detected in 86 (39.4%) and 122 (56.0%), respectively. In total, 218 organisms were tested for antibiograms with AST-GN72 card after identification. We found that 81.6 % of isolated strains (178 out of 218) exerted at least resistance to one antibiotic.

Conclusion: Multi-resistant serovars most frequently showed resistance to ampicillin, chloramphenicol, trimethoprim, and sulphonamide. Ongoing research is an evidence for researchers to evaluate the etiology of emerging multi-drug resistance Shiga-toxin producing *E. coli*.

Keywords	*Address of Correspondence	Article info.
Automatic Identification, VITEK 2 COMPACT ID & AST Card, Shiga Toxin, stx Genes, 16S rRNA, Polymerase Chain Reaction.	*shamim.uzzaman@dgda.gov.bd, shamimzaman45@gmail.com*	*Received: April 30, 2024* *Accepted: June 25, 2024*

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INTRODUCTION

Diarrheal disease is one of the primary factors behind childhood mortality in developing countries[1]. Each year, diarrhea kills over 525,000 children under the age of five. Around 1.7 billion children worldwide have diarrheal sickness each year. It lasts for a few days and might cause dehydration because of fluid loss. The first signs of dehydration are irritation and a lack of the customary stretchiness of the skin[2].

The primary etiological agents of diarrheal diseases include enteric bacteria (*Vibrio cholera*, *Shigella*, *Salmonella*, and *Escherichia coli*) and viruses (i.e., rotavirus). *E. coli* is a part of the intestinal flora and a normal inhabitant of the human gut. However, certain *E. coli* types can be pathogenic to humans and cause diarrhea, particularly in children[3]. Certain combinations of virulence factors categorized diarrheagenic *E. coli* (DEC) into six different prevalent pathotypes[4]. Virulence factor genes of enteric *E. coli* pathotypes are distinguished into four main virulence classes, such as colonization (*bfp, eae, tir*), fitness (*sdiA, iutA, iucB, yjaA*), toxins (*stx1, stx2, estA, estB, LT I, LT II, eatA, astA*), and effectors (*espA, espB, espC, espD, espF*)[5].

E. coli that produces Shiga toxin prevents host cells from synthesizing proteins, which results in cell death. *E. coli* isolates that are particularly virulent may express only *stx1*, *stx2*, or both toxins. *Stx2* strains are known to be more toxic than *stx1* strains, and they are often associated with hemorrhagic colitis (HC) or hemolytic uremic syndrome (HUS) in human infection[6].

Notably, DEC strains and normal fecal flora cannot be easily differentiated by applying conventional phenotypic methods, including basic biochemical tests or culture. One of the most effective molecular methods for identifying genes encoding virulence elements in DEC pathotypes is the polymerase chain reaction (PCR)[7]. Quick and sophisticated molecular methods are insufficient in lower-income countries for detecting *E. coli* from contaminated food and water[8]. Paton *et al.* (1998) demonstrated the use of multiplex PCR assays for determining the presence and type of Shiga toxigenic (STEC) *E. coli* for *stx1, stx2, rfb*, O157:H7*, rfbO11, eaeA*, and enterohemorrhagic (EHEC) *E. coli hlyA*[9]. An improved approach for quantifying and detecting bacteria in environmental samples is direct PCR (DPCR).

Rapid antibiogram is now time-consuming due to the massive sample load. We solved this issue with VITEK 2 compact, having outstanding accuracy and precision, which considerably cuts down on handling time[10]. Antimicrobial resistance poses a threat to animal and public health worldwide and has detrimental effects on the individual, environment, people, and economy[11]. O157 or non-O157 STEC is most fearsome because of its capability of transferring multi-drug resistance genes horizontally, and to our knowledge, there is no information available on this issue in Bangladesh[12].

The goal of our research was to figure out the extent of EHEC/STEC and assess the magnitude of multidrug-resistant *Escherichia coli* found in patients with diarrhea in the Dhaka city, which currently has the most disease-causing resistant bacteria in Bangladesh[13]. Implicitly, we have shown how *Escherichia coli* becomes impregnable while treating with antibiotics. By reducing the risks posed by superbugs and providing information for worldwide antibiotic resistance prevention initiatives, this research will support national and international public health organizations. Our work will be helpful to extrapolate the emergence resistance pattern and also be helpful to determine the vigilant activity.

MATERIALS AND METHODS

Sampling and Pure Culture Preparation

Two hundred and twenty-six stool samples of diarrhea-infected patients were collected from eight hospitals in and around Dhaka, Bangladesh. We collected these samples in a stool sample container from pathology laboratory with patients' information from eight different government hospitals. We maintained cold-chain during transport and preserved our samples in laboratory refrigerator. Analyses were carried out in microbiology laboratory of National Regulatory Authority (NRA) under the Ministry of Health and Family Welfare, Bangladesh, from March 2022 to April 2023. BAM (Bacteriological Analytical Manual) method was applied for cultural identification[14]. Briefly, samples were pre-enriched aerobically for 24 hours in Bacteriological Peptone Water (BPW) at 37°C. Following that, 1 ml of the pre-enriched culture was added to the

MacConkey Broth (MCB). After being streaked on MacConkey Agar (MCA), the enriched culture was incubated for 24 hours in an aerobic environment at 37°C. Red and pink colonies on MCA had streaked onto Eosin Methylene Blue (EMB) agar media. Purple colonies with a metallic green sheen on EMB medium were subsequently streaked on Soybean Casein Digest Agar (SCDA) and incubated at 37°C for 18 to 24 hours[15]. Then this pure cultures were used in Gram's staining, automated biochemical identification using ID-GN CARD through VITEK 2 compact machine, and molecular identification (PCR) based on targeted primers. The bacteria were tested for antibiotic susceptibility using the AST-GN72. The AST-GN72 card contains 18 antimicrobial agents, which span several classes and generations.

Identification of Bacteria with VITEK 2 Compact

Red and pink colonies on MCA and metallic green sheen colonies on EMB, which were then streaked on SCDA, underwent Gram's staining pursuing method developed by Merchand IA *et al.*[15]. After a 24-hour incubation period at 37°C, colonies were examined to ensure cell morphology - size, shape, color, and opacity.

BioMérieux's VITEK 2 compact is a completely automated identification system. The VITEK 2 compact Gram-Negative identification card (GN) is intended for Gram-negative bacteria that are both fermenting and non-fermenting[16]. For suspension preparation, a single colony from non-selective TSA media was taken. For the preparation of the suspension, a 12 ×75 mm polystyrene tube was filled with 3 ml of half-strength saline water (liquid solution of 0.45% - 0.5% sodium chloride, pH was adjusted to 4.5 - 7.0). Dens Check Plus was used to regulate the turbidity so that it was within the range of 0.5 to 0.63 McFarland. After the turbidity was adjusted, the freshly made solution was loaded upon the cassette and placed inside the filling section[16,17]. Reference culture, *Escherichia coli,* ATCC no. 25922, were used to check the quality of analysis.

Molecular Detection of Pathogenic Genes in *E. coli* Isolates by PCR Assays

PCR assays were applied to all 218 isolates to identify *E. coli* strain. The heat lysis method was used to take out the genomic DNA of *E. coli*[18]. Briefly, Eppendorf tubes containing 200 µl of PBS and pure *E. coli* colonies were heated for exactly ten minutes. After that, the tubes were promptly placed on ice and left around 10 minutes to give them a cold shock. Following that, the supernatant was collected and centrifuged at 10,000 rpm for ten minutes in order to form a DNA template for PCR.16S rRNA, a highly conserved area, was selected to conform *E. coli*. The primer set for the PCR test conducted is shown in (Table **1**)[19]. A 25.00 µL reaction mixture comprising 6.5 ml of nuclease-free water, 12.5 ml of 2X PCR Master Mix (Novagen, Merck Millipore, Germany), 1.0 ml of forward primers, 1.0 ml of reverse primers, and 4.0 ml of template (extracted DNA) was designed as the master mix for a single sample. The first denaturation at 94°C for 5 minutes ensued by 35 cycles of denaturation for 30 seconds at 94°C, annealing for 2 minutes at 52°C, and extension for 45 seconds at 72°C in the PCR amplification process. The last extension was then performed at 72°C for 5 minutes. The temperature of the heat cycler was kept at 4°C until the electrophoresis process was completed[20]. One percent agarose gel was used for molecular sieving the obtained PCR outcomes, and a system for recording the gel samples was used to visualize the results. Additionally, PCR was performed with primers specific to the *stx1* and *stx2* genes in order to identify *E. coli* that produces Shiga toxin. Positive control was used to prove the validity of our test, and negative control helped to determine the effect of external factors.

Table 1. Primers Used in PCR for 16S rRNA, *stx1* Gene and *stx2* Gene.

Primer Names	Target Genes	Primer Sequences (5'-3')	Size (bp)	References
EC 16S rRNA (F)	16S rRNA	5'-GACCTCGGTTTAGTTCACAGA-3'	585	(19)
EC 16S rRNA (R)		5'-CACACGCTGACGCTGACCA-3'		
stx1 (F)	*stx1*	5'CACAATCAGGCGTCGCCAGCGCACTTGCT3'	606	(21)
stx1 (R)		5'TGTTGCAGGGATCAGTCGTACGGGGATGC3'		
stx2 (F)	*stx2*	5'CCACATCGGTGTCTGTTATTAACCACACC3'	372	(21)
stx2 (R)		5'GCAGAACTGCTCTGGATGCATCTCTGGTC3'		

Antibiotic Susceptibility Test with VITEK 2 Compact

VITEK 2 compact AST-GN72 card (bioMérieux, Inc.) was used to assess *E. coli's* antibiotic resistance against 18 different antibiotics (n = 218). There are 18 antibiotics on this card, divided into 11 different classes (Table **5**)[17]. Furthermore, 145 µl of the obtained and modified turbid solution was put into a vial with 3 ml of a saltwater solution that was half its original concentration. The preparation process for the inoculum suspension and card insertion was similar to that for the ID-GN card[16].

Analysis of Identification Results

The identification findings are analyzed in four different ways: (i) Correct identification, in which strains are figured out that exhibit low discrimination (the VITEK 2 compact mechanism suggests at least two species, a single of which is alike to the reference method, and its determination can be settled by conducting few more basic tests), or strains that are accurately identified to the species level; (ii) low discrimination, which requires extensive testing to prove final strain; (iii) misidentification, wherein VITEK 2 compact displays inconsistent results that deviate from the reference method; and (iv) no identification, because the system does not recommend any species names[16]. The mean time for generating results was also determined for each identification.

Assessments of Susceptibility

The proportion of MICs within the two-fold dilution of the pertinent CLSI or other reference data is known as essential agreement (EA). Regarding the susceptibility test analysis, there are two possible approaches may occur: (i) Category agreement (CA) and (ii) Discrepancies. According to NCCLS reasoning criteria, the MICs obtained using both methods in CA classified the category of microbial susceptibility as susceptible, intermediate, or resistant[22]. Three categories of errors are applied to discrepancies: very major (VME), major (ME), and minor (mE). Considered major errors (ME) when the VITEK 2 compact system exhibited resistance and the reference method indicated susceptibility; considered minor errors (mE) when the VITEK 2 compact system showed intermediate susceptibility and the reference method indicated susceptibility or resistance; or when the VITEK 2 compact system stated susceptibility or resistance and the reference method suggested intermediate susceptibility[23,24].

RESULTS

E. coli Identification Using Cultural and Cell Morphological Attributes

An overall of 223 *E. coli* isolates came forth from 226 specimens using MCA and EMB agar media. *E. coli* cultures obtained from patient samples were purple colonies on EMB agar with a metallic green sheen and red and pink colonies with black centers on MCA. Under a light microscope, Gram-negative rods in pairs or singles were visible after *E. coli* was stained with Gram stain.

Identification of *E. coli* with VITEK 2 Compact System

Table **2** excerpts the efficacy of the VITEK 2 compact ID-GN card in accurately identifying *E. coli* according to cultural and morphological traits. Of 223 isolates, 102 were accurately identified at the species level with excellent (96-99% probability) confidence levels. Furthermore, the VITEK 2 compact system achieved a very good confidence level (93-95% probability) in identifying 73 strains up to the species level. Additionally, the algorithm classified 19 isolates as acceptable level (85-88% probability) and 24 isolates as good level (89-92% probability). There were 5 isolates that couldn't be identified by the system. The strains, categorized as "low discrimination," were distinguished through single and/or double conventional confirmatory analysis, such as motility and/or serum antibody tests.

Molecular Detection of 16S rRNA and Proportion of STEC

The PCR primers designed to target the 16S rRNA gene of *Escherichia coli* successfully amplified 585 base pair DNA fragments, thereby confirming the existence of *Escherichia coli* in 218 isolates (Figure **1**). Figure **2** is the presentation of electrophoresis band of *stx1* gene against a standard positive control. Figure **3** is the electrophoresis band of PCR amplified *stx2* gene commensurate with 300 base pair positive control.

PCR assays also show that the prevalence of isolates with the *stx2* genotype (24 cases, 11.00%) was higher than

those with the *stx1* genotype (19 cases, 8.72%) (Figure **2** & **3**). Also, 32 (14.68%) had both *stx1* and *stx2* genes (Table **3**).

Table 2. Reliability of the VITEK 2 Compact Platform for Identification of *E. coli* by ID-GN Card.

	Level of Identification					
	Excellent[a] (96 - 99) % Probability	**Very Good[a] (93 - 95) % Probability**	**Good[a] (89 - 92) % Probability**	**Acceptable[a] (Low Discrimination) (85-88) % Probability**	**Unidentified**	**Total no. of Strain Tested**
No. of Strain	102	73	24	19	5	223
Percentage	45.7%	32.7%	10.8%	8.5%	2.2%	
[a] Level of identification is a measurement of probability expressed in percent.						

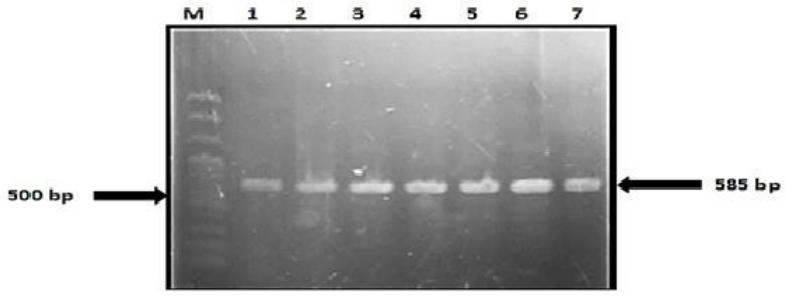

Figure 1. PCR assay for amplification of 16S rRNA of *E. coli*. In the left (lane M): 100 base pair of a standard DNA ladder marker (Novagen, Merck Millipore, Germany); lane (1-7): DNA derived from *Escherichia coli* culture.

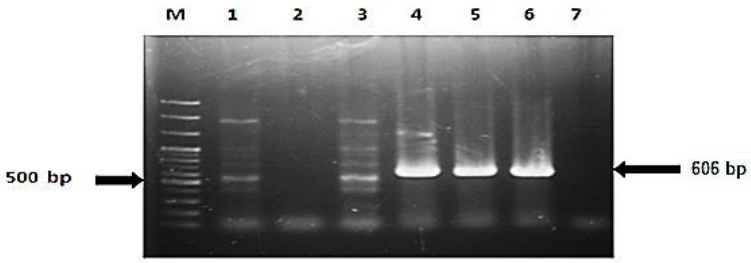

Figure 2. Amplification of 606 bp fragment of *stx*1 gene of *E. coli* by PCR assay. In the left (lane M): 100 base pair of a standard DNA ladder marker (Novagen, Merck Millipore, Germany); lanes 1-5: DNA derived from *Escherichia coli* culture; lane 6: positive control and lane 7: Negative control.

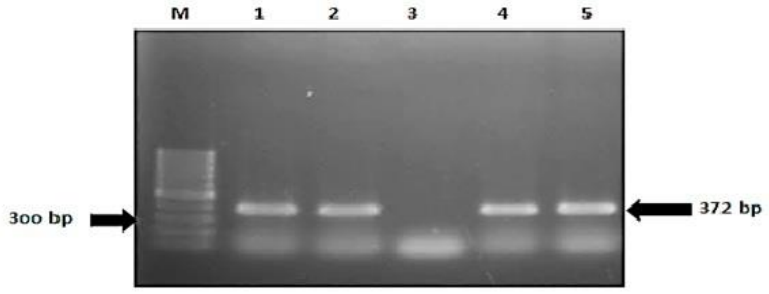

Figure 3. Agarose gel electrophoresis depicting PCR amplicons of *stx2* genes of *Escherichia coli*. In the left (lane M): 100 base pair of a standard DNA ladder marker (Novagen, Merck Millipore, Germany); lanes 1-5: DNA derived from *Escherichia coli* culture; Lanes 1, 2, 4 and 5: *stx2* positive *E. coli*. Lane 3: Negative control.

Table 3. Stx Profile of Isolated *E. coli*

Type of Shiga Toxin	No. of Strain (%)
stx1[a]	19 (8.72)
stx2[a]	24 (11.00)
*stx*1 and *stx2*[a]	32 (14.68)
[a] All isolates are different, there is no common ETEC/STEC in any group.	

Table 4. Distribution of Age and Sex for the Diarrhea Infected Patients (N=218) Around Dhaka City.

Variables		Total no. of Patients	Age in Years			Statistics
			<18 (39.4%)	18-60 (52.3%)	>60 (8.3%)	
Gender	Male	98 (45.0%)	41 (41.8%)	46 (46.9%)	11 (11.2%)	$\chi^2=3.13$
	Female	120 (55.0%)	45 (37.5%)	68 (56.7%)	7 (5.8%)	$p=0.21$

Table 5. Breakpoints and Concentration Levels for Antibiotics Employed in AST

Antibiotics	MIC (µg/ml) Extent Found by VITEK 2 Compact Advance Expert System		Breakpoint Standard (CLSI 2020)[1, 2]		
	VITEK 2 Compact	MIC Calling Range	Susceptible	Intermediate	Resistant
Amoxicillin/Clavulanic Acid	0.015-16	2/1-32/16	≤8/4	16/8	≥32/16
Ampicillin	0.015-32	2 - 32	≤8	16	≥32
Piperacilllin/ Tazobactam	0.03-32	4/4 - 128/4	≤16	32-64	≥128
Cephalothin[1]	0.03-32	2 - 64	≤8	16	≥32
Cefazolin	0.015-16	4 - 64	≤2	4	≥8
Cefoxitin	0.12-32	4 - 64	≤8	16	≥32
Cefuroxime	0.015-16	1 - 64	≤8	16	≥32
Cefpodoxime	0.015-32	0.25 - 8	≤16	-	≥32
Ceftazidime	0.015-32	1 - 64	≤8/4	-	≥16/4
Ceftriaxone	0.015-16	1 - 64	≤1	2	≥4
Aztreonam	0.015-16	1 - 64	≤4	8	≥16
Gentamicin	0.12-32	1-16	≤4	8	≥16
Tobramycin	0.015-32	1-16	≤4	8	≥16
Ciprofloxacin	0.12-32	0.25 - 4	≤0.25	0.5	≥1
Levofloxacin	0.015-16	0.12 - 8	≤0.5	1	≥2
Tetracycline	0.015-32	1-16	≤4	8	≥16
Nitrofurantoin	0.015-16	16 - 512	≤32	64	≥128
Trimethoprim /Sulfamethoxazole	0.03-32	20(1/19)-320(16/304)	≤2/38	NA	≥4/76

[1] CLSI (2020)-M100, 30th edition, was used as the standard breakpoint for all antibiotics except cefalotin. Because of the unavailability in CLSI (2020), CLSI 2009 was used as the breakpoint standard for cefalotin.
[2] R=resistant; I=intermediate; S=susceptible.

Association of the Socio-Demographic Characteristics of the Patients with the Precipitation of Diarrhea

Age-related data were available for 218 diarrhea-infected patients around Dhaka city, while gender information was available for all the patients (Table **4**). The majority of the cases (52.3%) were found in the age range between 18 and 60. Individuals under 18 years of age accounted for 39.4% of cases, while the lowest percentage (8.3%) was observed in those aged 60 years or older. (Table **4**). Although the gender distribution across the four age groups was statistically the same ($p > 0.05$), the majority (55.0%) of all diarrhea cases were found in females.

Antibiotic Susceptibility Tests

Every antibiotic's minimum inhibitory concentration (MIC) determined by the VITEK 2 compact technology was contrasted against CLSI (2020), M11-S19 and M100, 30th edition[25] (Table **5**). For each MIC, three categories (resistant, intermediate, and susceptible) were taken into account using the most recent CLSI breakpoint criteria (Table **5**). Calling range and breakpoint were also used to determine category agreement (CA).

Table **6** provides an overview of *E. coli*'s general susceptibility pattern for each of the 18 antibiotics that were studied, including EA, CA, VME, ME and mE values. Out of the 218 isolates in the AST, in total EA was 98.87%, and the aggregate CA was 96.0%. For thirteen antibiotics, the EA was 100 percent; however, the CA was 100% for four particular antibiotics and 98.6% for another set of four drugs. Furthermore, very major errors were identified in Cefoxitin, Ceftriaxone, Gentamicin, Tobramycin, Levofloxacin and Nitrofurantoin. Major errors were reported for Nitrofurantoin, followed by Ciprofloxacin, Aztreonam, Ceftazidime, Ceftriaxone, Piperacillin/Tazobactam, Gentamicin, Tobramycin, and Levofloxacin. Nitrofurantoin, followed by Piperacillin/Tazobactam, were the most commonly reported minor errors.

When 19 antibiotics were used against 75 STEC isolates, above 50% of bacteria had been resistant to Cefoxitin, Tetracycline, Cephalothin, Cefazolin, Ampicillin, Amoxicillin/ Clavulanic Acid (Figure **4**)

Table 6. Performances of AST-GN72 Card for *Escherichia coli* Considering CLSI Reference Method.

Antimicrobial	Quantity of Isolates with Susceptibility[2]				Count (%) of Error[3]				
	Total	R	I	S	EA	CA	VME	ME	mE
Amoxicillin/Clavulanic Acid	218	196	13	9	218(100)	215(98.6)	0(0)	0(0)	3(1.4)
Ampicillin	218	191	11	16	218(100)	218(100)	0(0)	0(0)	0(0)
Piperacillin / Tazobactam	218	126	41	51	200(91.7)	182(83.5)	0(0)	2(0.91)	16(7.4)
Cephalothin[1]	218	192	2	24	218(100)	218(100)	0(0)	0(0)	0(0)
Cefazolin	218	191	1	26	218(100)	218(100)	0(0)	0(0)	0(0)
Cefoxitin	218	140	32	46	218(100)	213(97.7)	2(0.91)	0(0)	3(1.4)
Cefuroxime	218	133	35	50	215(98.6)	215(98.6)	0(0)	0(0)	0(0)
Cefpodoxime	218	78	57	83	208(95.4)	205(94.0)	0(0)	0(0)	3(1.4)
Ceftazidime	218	69	38	111	213(97.7)	207(95.0)	0(0)	2(0.91)	4(1.8)
Ceftriaxone	218	60	61	97	218(100)	207(95.0)	3(1.4)	2(0.91)	6(2.8)
Aztreonam	218	52	37	129	210(96.3)	204(93.5)	0(0)	3(1.4)	3(1.4)
Gentamicin	218	44	29	145	218(100)	213(97.7)	2(0.91)	2(0.91)	1(0.46)
Tobramycin	218	50	11	157	218(100)	215(98.6)	1(0.46)	1(0.46)	1(0.46)
Ciprofloxacin	218	31	17	170	218(100)	209(95.9)	0(0)	5(2.3)	4(1.8)
Levofloxacin	218	48	24	146	218(100)	213(97.7)	1(0.46)	1(0.46)	3(1.4)
Tetracycline	218	159	44	15	218(100)	215(98.6)	0(0)	0(0)	3(1.4)
Nitrofurantoin	218	92	70	56	218(100)	182(83.5)	6(2.8)	8(3.7)	22(10)
Trimethoprim /Sulfamethoxazole	218	70	5	143	218(100)	218(100)	0(0)	0(0)	0(0)
Total	3924				3880 (98.87)	3767(96.0)	15(0.38)	26(0.66)	72 (1.83)

[3] EA=essential agreement; CA=categorical agreement; VME=very major error; ME=major error; mE=minor error.

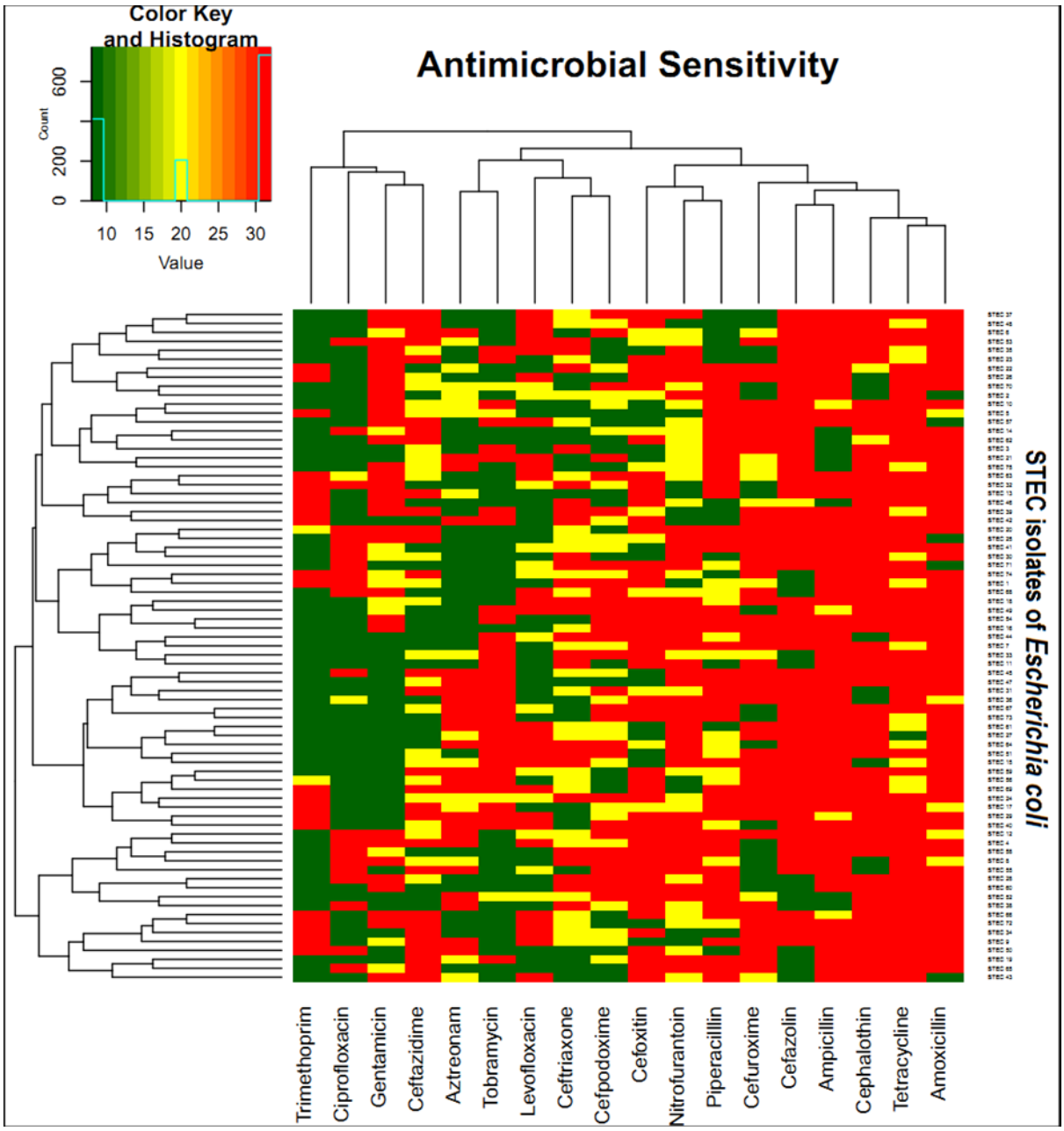

Figure 4. This heat map illustrates the antibiotic susceptibility of STEC isolates. Antibiotics are denoted by rows, bacterial strains by columns; resistance is shown by red blocks, sensitivity to antibiotics is displayed by green units, and the antibiotics' intermediate effect is exhibited by yellow brick.

DISCUSSION

Adults and older people are especially vulnerable to the disease's development to more serious sequelae, as shown by Smith-Palmer *et al.* (2005); however, in people who were relatively in well condition, the illness may just cause loose stool or even mild[26]. Of the participants in this research, 52.29% were between the ages of 18 and 60, and 39.45% were between the ages of 0 and 18. Furthermore, adults and young children are more prone to diarrhea or enteric infections in this locality which is also common in different areas of the world[27]. A higher proportion of females (55.5%) is also observed in our study. The obtained result is in conformity with a prior

investigation conducted by Olowe OA *et al.* (2014), in which 125 (61.9%) of the 202 *E. coli* isolates concerned female patients, while 77 (38.1%) came from male patients[28]. In a recent study conducted by Deneke W *et al.* (2024), they found an overall rate of 11.5% DEC isolates in Ethiopia from stool specimens collected from admitted patients in primary healthcare facilities[29].

Suardana *et al.* have effectively employed the polymerase chain reaction (PCR)-driven technique in 2014 for identifying 16S rRNA, *stx1* and *stx2* genes in *E. coli* species isolation and identification[30]. In our study, the expanded size of the selected nucleotide was 585 bp, indicating or reconfirming 218 *E. coli* strains, where 19 (8.72%) were positive for *stx1*, 24 (11.00%) for *stx2* gene and 32 (14.68%) for both *stx1* and *stx2*. In contrast, Tahamtan Y *et al.* (2010) found the presence of *stx1* (10.27%) and *stx2* (53.42%) genes, respectively, in 146 *E. coli* isolated from cattle using multiplex PCR[31]. Moreover, Shridhar *et al.* (2017) found 56 (29.2%) of the 192 STEC strains positive for both *stx1* and *stx2*, 93 (48.4%) for *stx1*, and 43 (22.4%) for *stx2* only[32]. The frequency of *stx1* and *stx2* virulence genes presented by Rubab M *et al.* is higher than any other study mentioned here, it is 76.4% and 86.27%, respectively[33].

Antimicrobial susceptibility testing and bacterial identification are commonly conducted using the VITEK 2 compact system. In a study by Caroline *et al.* (2003), the VITEK 2 compact ID-GNB identification card showed that 414 (85.9%) enteric strains, including gram-negative bacilli and Enterobacteriaceae, were accurately identified at probability levels of excellent to good, and an additional 34 (7.1%) strains had a low level of discrimination but were correctly identified[10]. His investigation also revealed that 15(3.1%) strains were misidentified, and 19 (3.9%) strains were unidentified. ID-GN cards were also used in our investigation, and the identification quality is comparable. In this instance, 199(89.2%) strains were identified accurately with an excellent to good probability level, while 19(8.5%) strains were accurately identified with a low level of discrimination. Furthermore, 5(2.2%) of the strains could not be identified, possibly due to improper MacFarland turbidity adjustment or more than single colony picked while taking from culture plate. The VITEK 2 compact ID-GN card has a species-level identification accuracy of 97.8% (218 out of 223) in this study. However, a study conducted by Funke *et al.* (2004) evaluated Gram-negative rods by VITEK 2 compact demonstrated 100% accuracy for *E. coli* identification[34].

For evaluating AST using VITEK2, the Food and Drug Administration has set lowest value requirements for its proficiency[35]. The recommended values are CA ≥90%, ME ≤3%, VME ≤1.5%, and acceptable mE rates ≤10%. Because of the high level of agreement (EA and CA) from the reference requirements and the extremely low rates of discrepancies (VME and ME) for all antibiotic-microorganism combinations, except Nitrofurantoin, where VME and ME ranged from 0 to 2.8% and 0 to 3.7%, our investigation suggests that the susceptibility test results using VITEK2 was accurate. In this investigation, the category agreement was 96% and the essential agreement was 98.87%, both of which were greater than in Munoz-Dávila *et al.*'s (2013) study[36]. The analysis reported major errors and very major errors of 0.7% and 0.4%, respectively. Munoz-Dávila *et al.*, meanwhile, observed 0.4% major error rates and 0.2% VM errors[36]. The combined major errors and minor errors percentage was 2.49%, but Bazzi *et al.* found 1.04% combined ME and mE in their study[37]. Contrariwise, Sanjana M *et al.* showed very little ME but a high percentage of VME in her analysis for most of the antimicrobial groups[38].

The Kirby-Bauer disc diffusion or broth dilution method is the most popular form of antimicrobial susceptibility testing. But this approach is not flawless due to some probable errors, including subjectivity, improper preparation of inoculum, inconsistent results, and analyst errors, which are easily replaced by VITEK 2 compact, an error-free automated fluorescence-based technology[39]. The current experiment revealed 89.91 percent resistance to amoxicillin-clavulanic acid. Konaté A *et al.* conducted a research investigation in Burkina Faso in 2017 and noted substantial rates of amoxicillin-clavulanic acid susceptibility (77.4%)[40]. On the other hand, a 2011 experiment conducted in Dar es Salaam, Tanzania, revealed that *E. coli* only had a 7.8% resistance to amoxicillin-clavulanic acid[41]. Additionally, this study demonstrated the spatial variance in resistances and the rise in resistance toward penicillin. Furthermore, the Diarrheagenic *E. coli* isolates showed over 70% resistance to cephalothin (88.07%),

Ampicillin (87.61%), Cefazolin (87.61%) and Tetracycline (72.94%) in this study. In another study conducted by Yihunie FB *et al.*, they found amoxicillin (89.5%), gentamycin (57.9%), tetracycline (57.9%), and ampicillin (52.6%) are the most common resistant antibiotics which resonate with our study[42]. Albeit our MIC values are slightly different from those of Amézquita-López BA *et al.* but, the MIC calling range is almost same for the common antibiotics[43]. According to another research, *E. coli* strains show extreme resistance to a wide range of antimicrobial agents, such as ampicillin (100%), tetracycline (73.3%), and cephalothin (76.7%)[44]. On the contrary, Afum T *et al.*, 39 observed isolates of *E. coli* showed the highest resistance to tetracycline (79.1%) and cefazolin (58.3%)[45]. We found 27.5% ceftriaxone resistant isolates comparing to Mukta D G *et al.*, they found only 13.0 % resistant isolates, in their case they counted *stx1* and *stx2* genes in a lower percentage[46]. Another cephalosporin of third-generation, Ceftazidime has a 95.8 % resistant shown by Tula M Y *et al.* comparatively greater than our 87.6 % resistant STEC[47].

In our investigation, *E. coli* was resistant to trimethoprim-sulfamethoxazole also. Trimethoprim was once thought to be a reliable defense against invasive *Escherichia coli* that also causes diarrhea, but currently it is not considered an effective prophylaxis due to the growing resistance of diarrheagenic *E. coli* to sulfamethoxazole-trimethoprim[44]. Currently, ampicillin and sulfamethoxazole-trimethoprim resistant strains are treated with cephalosporins and fluoroquinolones on a prescription basis[48]. Interestingly, the current study has revealed over 66% sensitivity to some antibiotics, such as Ciprofloxacin (77.98%), Tobramycin (72.01%), Levofloxacin (66.98%), and Gentamicin (66.51%). Likewise, recent research conducted by Kibret *et al.* (2011) revealed, most *E. coli* strains (> 60%) were sensitive to levofloxacin, ciprofloxacin and gentamicin[49]. Also, a study conducted by John B K *et al.* (2024) in Africa where they showed that DEC exposed a heightened resistance to carbapenems, quinolones and cephalosporins[50].

During the course of this research, we encountered certain constraints. Although RT-PCR is more precise and quantitative than conventional PCR, we were able to conduct our analysis using conventional PCR, which isn't even a multiplex PCR. Our restricted accessibility prevented us from getting samples from a few additional hospitals. Data would be more accurate and versatile if we would able to collect more samples from additional hospitals. We are limited in our ability to seek out additional antibiotics in our antibiogram by the finite quantity of antibiotics that VITEK 2 compact EST cards supply. Our results would be more comparable and statistically significant if we could classify our sample based on age, gender, and race, but the manuscript is already close to its word limit.

In summary, VITEK 2 compact reliably performed to identify *E. coli* isolates. The study revealed that the diarrheagenic *E. coli* was more immune to the medications studied than to those that are often provided in Bangladesh, including cephalothin, trimethoprim/sulfamethoxazole combinations, ampicillin, tetracycline, amoxicillin-clavulanic acid, and cefazolin. This might be the result of their widespread availability, irrational use, and illicit sales of antibiotics without a valid prescription or recommended dosage.

CONCLUSION

In conclusion, multiplex PCR and hybridization are the better choices for their accuracy in identification. We minimized our limitations by using an automated identification system. The number of instances of *E. coli* that form ESBLs and STEC was relatively higher. The study's findings revealed multi-drug-resistant *E. coli* in diarrhea-infected patients, which presents a significant therapeutic challenge to human medicine. Therefore, it is necessary to control the use of antibiotics and mandate AST monitoring in hospital patients.

CONFLICT OF INTEREST

We affirm that there is no conflict of interest associated with this paper's publication.

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