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Original Research

# Molecular detection and antibiogram profiles of *Escherichia coli* and *Vibrio cholerae* isolated from raw vegetables in the northern district of Bangladesh

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**Abstract**

Raw vegetables are essential for a well-balanced diet as they provide vitamins, minerals, dietary fiber, and phytochemicals. This study aimed to isolate, identify, and evaluate the microbial loads of *Escherichia coli* and *Vibrio cholerae* in raw vegetables sold at local markets in the Dinajpur district of Bangladesh. A total of 35 vegetable samples were collected from four markets in Dinajpur district. The isolates were identified using cultural, staining, biochemical, and molecular tests. Microbial loads were enumerated (TVC) using the pour plate technique. Molecular detection of bacterial species was confirmed targeting the *16S rRNA* and *groEL* genes of *E. coli* and *V. cholerae*, respectively. The amplification was done on 704 bp fragments of the *16S rRNA* gene of *E. coli* and 1117 bp fragments of *Vibrio* spp. For the confirmation of *V. cholerae*, amplification of a 418 bp fragment of the *groEL* gene was performed through multiplex PCR. An antimicrobial susceptibility test was conducted on all isolates of bacteria against eleven and eight antibiotics by disc diffusion. The total viable count (TVC) in potato, carrot, cabbage, cauliflower, tomato, green chili, cucumber, mustard sak, and coriander leaves were 2.4 ± 0.37, 2.2±0.14, 2.1±0.26, 1.8±0.14, 1.7±0.27, 1.5±0.33, 1.5±0.33, and 1.4±0.25 mean log colony forming units ± standard deviation/mg, respectively. Out of 35 raw vegetable samples, 16 (45.71%) and 13 (37.14%) isolates were culture positive for *E. coli* and *V. cholerae*. Subsequently, 5 (31.25%) and 4 (30.76%) isolates of *E. coli* and *V. cholerae* were confirmed positive molecularly. All 16 and 13 isolates of *E. coli* and *V. cholerae* were subjected to antibiogram testing against 11 and 8 antibiotics. *E. coli* isolates were highly resistant to ceftazidime, cefixime, ampicillin, and oxytetracycline, but sensitive to gentamycin, ceftriaxone, colistin, and enrofloxacin. Similarly, *V. cholerae* isolates were highly resistant to nalidixic acid, trimethoprim, and polymyxin, but highly sensitive to kanamycin, gentamicin, and streptomycin. The study's findings indicate that raw vegetables pose a significant public health risk due to MDR *E. coli* and *V. cholerae*. To achieve safer levels of these bacteria in raw vegetables, good production practices and hygiene awareness are essential.

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## 1. Introduction

Raw vegetables make up a significant portion of a person's diet in many parts of the world and are beneficial to nutrition, especially in terms of phyto-nutrieuticals, which include minerals, dietary fiber, vitamins C, A, B1, B6, B9, and E, and phytochemicals (Silva Dias, 2022). Consuming vegetables on a daily basis has been linked to improved gastrointestinal health, clear vision, and a decreased risk of heart disease, stroke, chronic illnesses including diabetes, and multiple cancers. Vegetables contain powerful antioxidant phytochemicals that can reduce the risk of developing chronic illnesses through preventing damage from free radicals, altering the metabolic and detoxification processes of carcinogens, or even enacting mechanisms that alter the growth of tumor cells (Rudzińska *et al.*, 2023; Pinto *et al.*, 2021; Sotler *et al.*, 2019; Dias, 2012).

In Bangladesh total vegetable consumption in 2013 was 4,049 kt, according to Faostat (Haque and Hoque, 2021). This represents a 0.226% increase over the prior year. This is 0.226 percent higher than it was the year before (Aalm and Nase, 2020).

Despite their many health advantages, eating fresh vegetables has also been linked to risks for consumers (Bekele *et al.*, 2017). Vegetables are typically eaten raw because they are high in fiber, vitamins, minerals, antioxidants, and carbs. Phytonutrients have the potential to function as efficient carriers of infections (Desiree *et al.*, 2021; Said, 2012). Pathogenic microorganisms can contaminate raw vegetables when they are grown in fields, harvested, handled after harvest, processed, and distributed. Vegetables can become contaminated at different times by different agronomic techniques. Most contamination occurs before harvesting, either directly from domestic and wild animals or through the use of sewage, irrigation water, effluent from livestock operations, or contaminated manure. It can also happen during the harvesting, transport, processing, distribution, and marketing processes, or even at home (Alegbeleye

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*et al.*, 2023; Łepecka *et al.*, 2022). The quality of raw vegetables and human health are impacted when wastewater is used for irrigation. It might be the potential source of harmful microbes on vegetables. Bacteria such as *Escherichia coli* O157:H7, *Shigella* spp., *Vibrio* spp., *Campylobacter* spp., *L. monocytogenes*, and *Salmonella* spp. (Osafo *et al.*, 2022). It can infect raw vegetables when it comes into touch with dung and sewage. In addition, contaminations can happen after harvest through unclean wash water, cross-contamination from an infected food handler, and eating of contaminated food, whether raw or cooked, can be a significant risk factor for the spread of pathogens (Machado-Moreira *et al.*, 2019; Said, 2012). Contamination and growth of spoilage microorganisms usually limit the shelf life of vegetables (Alegbeleye *et al.*, 2022). Food-borne illnesses are caused by a variety of microbe types and the toxins they produce, including *Salmonella* spp., Shigella spp., *E. coli*, *Bacillus anthracis*, *Klebsiella* spp., *Brucella* spp., *L. monocytogenes*, *Yersenia enterolytica*, and *Eubacteriaceae* (Islam *et al.*, 2023; Julqarnain *et al.*, 2022).

*E. coli* may be a common facultative resident of the human environment and the large intestine. It is one of the most prevalent causes of a number of common bacterial infections, including as newborn meningitis, cholecystitis, bacteremia, cholangitis, tract infections (UTI), traveler's diarrhea, and other clinical illnesses (Hossain *et al.*, 2024; Ema *et al.*, 2022). *E. coli* spreads by the oral and fecal routes. It is frequently found in food, soil, and water due to its adaptability and versatility. The potential of bacterial contamination is very significant when using raw manure as fertilizer (Ongeng *et al.*, 2015).

*V. cholerae* is a gram-negative, highly motile, curved or comma-shaped rod bacterium that generates cholerae enterotoxin and causes life-threatening secretory diarrhea (Weil and Harris, 2015). Usually, the bacteria are found in freshwater lakes and rivers and other aquatic habitats. The most common way that cholera spreads to humans or animals is through contaminated water sources (Abioye *et al.*, 2021). The source of contamination is the feces of diseased people. In humans and certain animals, cholera can result in severe diarrhea, vomiting, dehydration, and shock. Death may come within hours if treatment is not received (Chowdhury *et al.*, 2022). Man may consume the germs and become exposed to it (Baker-Austin *et al.*, 2018). This could happen from drinking contaminated water, eating raw vegetables, or coming into contact with the excrement of diseased animals or humans (Rebaudet *et al.*, 2013).

*E. coli* bacteria can be found in food, the environment, and the intestines of both people and animals. *E. coli* is a very large type of bacteria. Most strains of *E. coli* are toxic, while some can make you unsound. While some strains of *E. coli* can cause pneumonia, lung illnesses, tract infections, and other illnesses, others can cause diarrhea (Fleckenstein and Kuhlmann, 2019).

*E. coli* and *V. cholerae* have been documented to be responsible for outbreaks connected to the intake of fresh but raw vegetables and fruits (such as lettuce, spinach, carrots) (Aurin *et al.*, 2020). Pathogenic *E. coli* and *V. cholerae* can infect raw vegetables when they are growing in the field, when fertilizer (cow dung) is applied, or when the veggies are harvested, transported, processed, stored, and distributed. Thus far, no reports of outbreaks or sporadic illnesses linked to *V. cholerae* and *E. coli* from fresh raw vegetables have come from Bangladesh. In order to isolate *E. coli* and *V. cholerae* raw vegetables were gathered near Dinajpur city for this purpose. Therefore, this study was conducted to isolate, identify, and evaluate the microbial loads along with the antimicrobial susceptibility patterns of *E. coli* and *V. cholerae* in raw vegetables sold in local markets in the Dinajpur district of Bangladesh, with the aim of assessing the public health risks associated with these pathogens.

## 2. Materials and Methods

### *2.1 Ethical approval*

No ethical approval is required for this study.

### *2.2 Study area*

The present study was conducted during the period from December 2019 to March 2020 in the Bacteriology Laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200. The study purposed used samples were collected from 4 different markets (Basher hat, Gopalgonj, Liliar Mor, and Kalitola) located in the district of Dinajpur Sadar area (Figure 1).

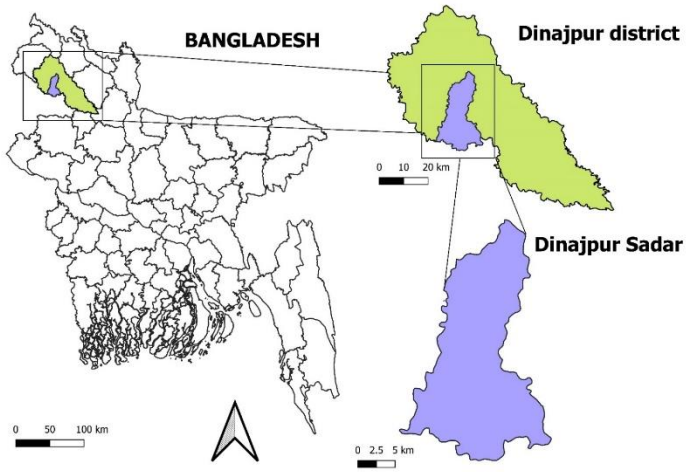



**Figure 1.** Map of the study area, from where the samples were collected.

### *2.3 Sample collection*

A total 35 samples comprising potato (6), cucumber (4), carrot (2), green chili (06), mustard sak (1), cabbage (4), cauliflower (2), coriander leaves (4), and tomato (6) were collected from 4 different markets (Basher hat, Gopalgonj, Liliar Mor, and Kalitola) located in the district of Dinajpur area (Figure 1) using hand gloves, sealed poly bags (transparent zip lock poly; thickness: 30-100 mic; size: 175 mm* 100mm). The samples were labeled with an identification mark and quickly moved to the proper containers. The samples were handled with great care, placed in an ice box at 4°C, and then brought right away to bacteriology laboratory, Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200, Bangladesh our lab for examination. The samples are handled with care and stored in a box. Because of aseptic care was taken during transportation and also the samples were kept in sterile container until they're prepared for bacteriological analysis.

### *2.4 Sample processing*

In a sterile mortar and pestle, 10 g of materials were mixed. To create a 10% sample suspension, the samples were then completely homogenized in 90 milliliters of sterile phosphate-buffered saline (PBS; pH 7.4, Merck KGaA, Germany) solution. The sample was diluted ten times, from $10^{-1}$ to $10^{-10}$, in accordance with the guidelines provided by Qamar *et al.* (2023) and the International Organization for Standardization (ISO, 1995). Serial dilution was then performed by repeatedly using the previous dilution as the input for the next step, resulting in a reduced number of bacterial colonies to obtain pure colonies. 1 mL of a homogenized sample and 9 mL of sterile PBS were combined in this process. For the isolation of bacteria from vegetables, homogenized samples were incubated at 37°C for 24 hours in order to enrich them in nutrient agar.

### *2.5 Total viable count enumeration*

The manufacturer's instructions were followed while preparing the nutrient agar media (HiMedia, India), and it was autoclaved for 15 minutes at 121°C to achieve sterilization. Using a micropipette, 0.1 mL of each tenfold dilution was spread out in duplicate onto nutrient agar plates (HiMedia). Using a sterile spreader, the diluted samples were applied to the plate's surface as soon as possible. The plates were incubated for 24 hours at 37°C. The TVC was computed using the ISO methodology (ISO, 1995). Counts were performed on plates

showing between 30 and 300 colonies after incubation. The following formula was used to determine CFU/g: number of colonies × dilution factor/volume of culture plated.

### 2.6 Isolation and identification of E. coli and V. cholerae

The overnight enriched culture was streaked onto MacConkey (MC) agar, Eosin Methylene Blue (EMB) agar, and Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar (Hi-media), followed by 24 hour incubation at 37°C. MacConkey (MC) agar medium was used for the identification of organism under the family Enterobacteriaceae through studying fermentation characteristics. Eosin methylene blue (EMB) agar medium was used for the purpose of selective growth of *E. coli*. Thiosulphate citrate bile salt sucrose (TCBS) agar was used for the growth of *V. cholerae* (Bolinches *et al.*, 1988; Cheesbrough, 1985).

The colonies which produced bright pink colonies on to MacConkey (MC) agar that were selected as *E. coli*. For the more confirmation of *E. coli* isolated colonies were sub-cultured onto EMB agar. The colonies that displayed a dark core and a metallic green gloss were chosen. In contrast, the colonies appeared onto yellow and shiny color onto Thiosulphate citrate bile salt sucrose (TCBS) agar that were selected of *V. cholerae* (Cheesbrough, 2006). The colony characteristics (size, shape, edge, color, and opacity), morphological characteristics by Gram's staining, the sugar fermentation test, and a battery of biochemical tests (methyl red, Voges-Proskauer (VP), catalase, triple sugar iron (TSI) agar slant test, and Simmon's citrate) were used to identify the bacteria (Cheesbrough, 2006).

### 2.7 Polymerase chain reaction (PCR)

For the molecular characterization of *E. coli* and *V. cholerae* by PCR, the PCR mixture was prepared according to the composition and quantities. The boiling process was utilized to extract deoxyribonucleic acid (DNA) (Aworh *et al.*, 2023). All 16 isolates and 13 isolates, respectively, were subjected to PCR tests for *E. coli* and *V. cholerae* confirmation. The highly conserved region of *16S rRNA and groEL were* selected for the identification of *E. coli* and *vibrio* spp.; the primer list utilized for the PCR experiment is shown in Table 1 (Sarker *et al.*, 2018; Hossain *et al.*, 2014). Multiplex PCR was used to identify the type of bacterium (*V. cholerae*) (Hossain *et al.*, 2014).

A 20:1 PCR reaction mixture including 5:1 ribonuclease free water, 10:1 PCR master mixture (Thermo Scientific, EU), 3:1 genomic DNA, and 2:1 primer was used for a single sample. The PCR amplification process involved five minutes of initial denaturation at 95°C, thirty cycles of denaturation at 94°C for forty seconds, primer annealing at 56°C for thirty seconds, and primer extension at 72°C for thirty seconds. For *E. coli*, the final extension was performed at 72°C for ten minutes. The PCR reaction profile for *V. cholerae* was also set up with the following temperature parameters: five minutes of initial denaturation at 94°C, thirty cycles of denaturation at 94°C for thirty seconds, primer annealing at 69°C for thirty seconds, and thirty seconds of primer extension at 72°C, culminating in a final extension at 72°C for seven minutes (Sarker *et al.*, 2018).

Another for *V. cholerae* the PCR reaction profile was set with the following thermal conditions: an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at 69°C for 30 seconds, and primer extension at 72°C for 30 seconds, with a final extension at 72°C for 7 minutes. Using a primer-specific PCR reaction that targeted the *16S rRNA* and *groEL* gene of *E. coli* and *V. cholerae* were molecularly identified (Table 1) (Hossain *et al.*, 2014).

**Table 1.** Primers used for the molecular detection *E. coli* and *Vibrio* spp.

| Name of primers | Sequence (5′-3′) | Amplicon size (bp) | reference |
|---|---|---|---|
| ***16S rRNA*** | F: AATTGAAGAGTTTGATCATG<br>R: CTCTACGCATTTCACCGCTAC | 704 bp | Sarker *et al.* (2018) |
| ***groEL*** | F: TCCARAACATGGGCGCACAA<br>R: ACGTTTTGYTCTTCGTTGTCRC | 1117 bp | Hossain *et al.* (2014) |

### 2.8 Antibiogram study

Antimicrobial discs with various concentrations were employed to assess the sensitivity of the isolated bacteria. Mueller-Hinton agar (MHA) (HiMedia) was subjected to an antibiotic susceptibility test utilizing the Kirby-Bauer disk diffusion method, as reported by Bauer *et al.* (1966). The following antibiotic disc was employed for *E. coli*, ampicillin (30 µg/disc), gentamycin (10 µg/disc), kanamycin (30 µg/disc), colistin (30 µg/disc), nalidixic Acid (30 µg/disc), enrofloxacin (5 µg/disc), oxytetracycline (30 µg/disc), ceftriaxone (30 µg/disc), ceftazidime (30 µg/disc), cefixime (5 µg/disc), and cephalexin (30 µg/disc) (HiMedia).

On the contrast, these antibiotics is applied against *V. cholerae* gentamycin (10 µg/disc), streptomycin (10 µg/disc), kanamycin (30 µg/disc), trimethoprim (25µg/disc), ciprofloxacin (5 µg/disc), ampicillin (30 µg/disc), nalidixic acid (30 µg/disc), and polymyxin B (5 µg/disc) (HiMedia). The Clinical and Laboratory Standards Institute's guidelines for sensitivity, intermediate, and resistance were compared to the zone sizes of the bacteria (CLSI, 2018).

### 2.9 Statistical analysis

Using Duncan's multiple range test (Statistical Package for the Social Sciences, version 11.5, IBM Corp., NY, USA), the findings of TVC of bacteria detected in the raw vegetables sold at local markets were examined for statistical significance. A statistically significant result was defined as $p < 0.05$.

## 3. Results

### 3.1 Total Viable Count (TVC) of raw vegetable samples

Total Viable Count (TVC) of vegetable samples from markets in Dinajpur district, revealing varying levels of microbial loads. To determine TVC, all thirty-five vegetable samples were tested. The results of the bacteria isolates are shown in Table 2, where the mean values are given in log10 CFU/gm. Based on these findings, the average number of bacteria was calculated from samples of various vegetables and varied from 1.4 ± 0.25 to 2.4 ± 0.37 CFU ± standard deviation (SD)/gm. There were 2.4 ± 0.37 CFU ± SD/gm of bacteria isolated from potato samples, which was the largest number, and 2.4 ± 0.37 CFU ± SD/gm of bacteria recovered from coriander leaves, which was the lowest number (Table 2; Figure 2).

**Table 2.** Total Viable Count (TVC) of vegetable samples from different markets in Dinajpur district.

| Name of the samples | Number of the samples | TVC (mean log CFU ± SD/gm) |
|---|---|---|
| **Potato** | 6 | 2.4±0.37 |
| **Carrot** | 2 | 2.2±0.14 |
| **Cabbage** | 4 | 2.1±0.26 |
| **Cauliflower** | 2 | 1.8±0.14 |
| **Tomato** | 6 | 1.7±0.27 |
| **Green chili** | 6 | 1.5±0.33 |
| **Cucumber** | 4 | 1.5±0.22 |
| **Mustard sak** | 1 | ND |
| **Coriander leaves** | 4 | 1.4±0.25 |

*ND= Not detected

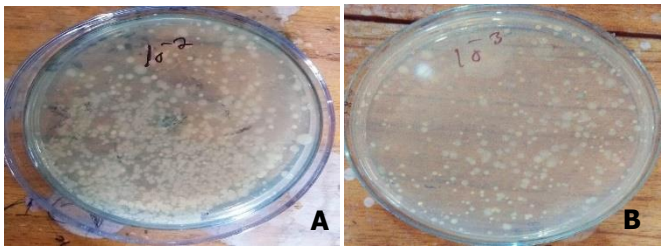


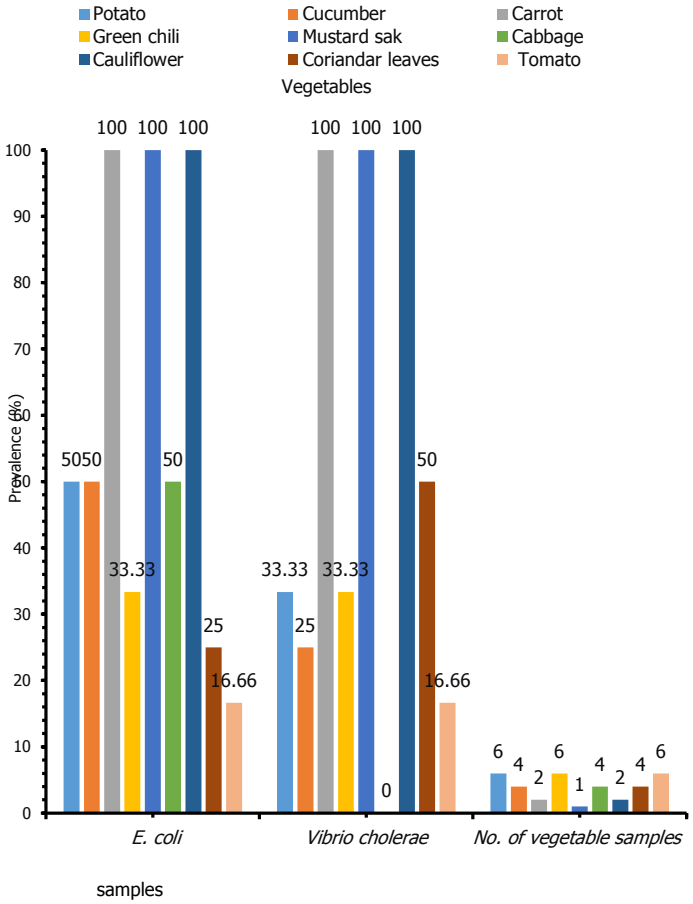

**Figure 2.** (A and B). Total viable count (TVC) of various vegetable samples.

### 3.2 Prevalence of E. coli and V. cholerae

From 35 vegetable samples, 29 distinct bacterial isolates were found in this investigation on the frequency of *E. coli* and *V. cholerae*. A total of 16 isolates of *E. coli* were found in 3 (50%), 2 (50 %), 2 (100%), 2 (33.33%), 1 (100%), 2 (50%), 2 (100%), 1 (25%), and 1 (16.66%) potato, cucumber, carrot, green chili, mustard sak,

cabbage, cauliflower, coriander leaves, and tomato vegetables samples (Figure 3). Conversely, 13 isolates of *V. cholerae* were detected in 2 (33.33%), 1 (25%), 2 (100%), 2 (33.33%), 1 (100%), 0 (0%) 2 (100%), 2 (50%), and 1 (16.66%) potato, cucumber, carrot, green chili, mustard sak, cabbage, cauliflower, coriander leaves, and tomato. Six samples showed no microbial growth. These findings underscore the widespread contamination of raw vegetables by *E. coli* and *V. cholerae*, posing potential health risks (Figure 3).

**Figure 3.** Prevalence of *E. coli* and *Vibrio cholerae* in different types of vegetable




### 3.3 Cultural, staining, and biochemical characteristics

The cultural characteristics of bacterial isolates obtained from vegetable samples. *E. coli* colonies on MC agar exhibited a distinctive bright pink color colonies and EMB agar showed a blue-green metallic sheen color colonies. In contrast, *V. cholerae* colonies on TCBS agar appeared yellow and shiny (Figure 4 and 5). Gram staining showed that *E. coli* produces short, pink, rod-shaped, single or paired Gram-negative bacteria. On the other hand, gram staining *V. cholerae* expressed gram negative, curved rod, single or paired and motile. Most of the isolates of *E. coli* showed positive results for methyl red (MR), indole, and TSI (with yellow on butt and slant, and positive for $H_2S$ production), but negative for Voges-Proskauer (VP) and citrate utilization (CT). Similarly most of the isolates *V. cholerae*, exhibited positive results for MR, indole, TSI (yellow on butt and slant), and citrate utilization, while showing negative results for VP and gas production in TSI.

### 3.4 Molecular detection

#### 3.4.1 Molecular confirmation of E. coli targeting 16S rRNA gene by PCR

All 16 culture positive isolates of *E. coli* were tested for PCR targeting the *16S rRNA* gene for amplification of 704 bp of DNA fragment and 16 of 5 (31.25%) isolates showed positive result in PCR that isolated from vegetable (Table 3 and Figure 6).

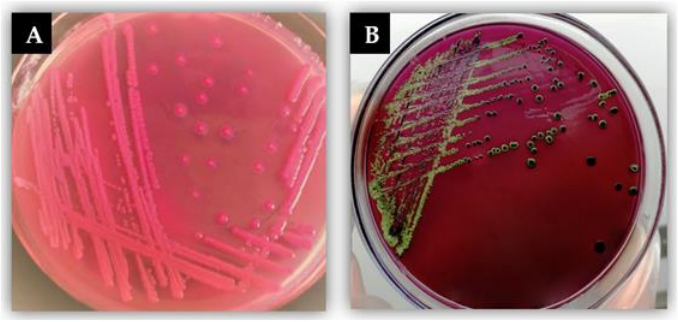



**Figure 4.** *E. coli* produced bright pink color colonies on MC agar (Figure 4A) and greenish colonies with metallic sheen on EMB agar (Figure 4B).

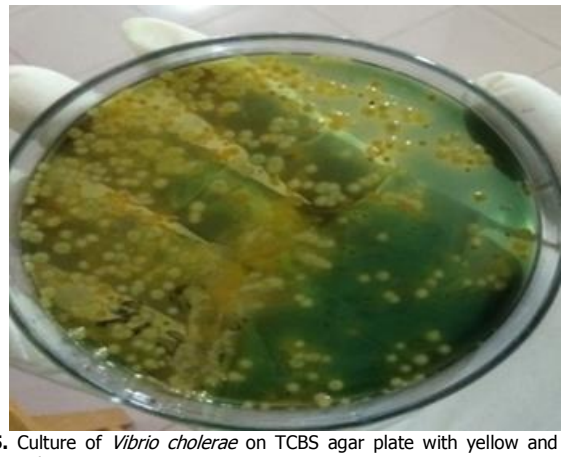


**Figure 5.** Culture of *Vibrio cholerae* on TCBS agar plate with yellow and shiny colonies.

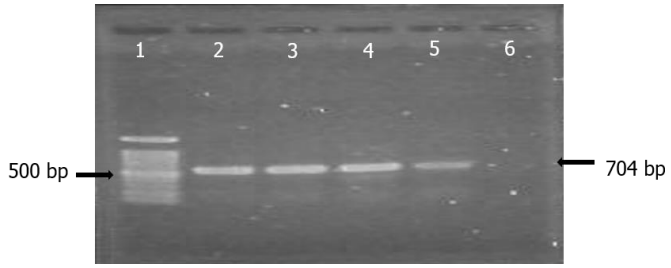



**Figure 6.** PCR amplification of *E. coli* genus specific gene primer (*16S rRNA*) of *E. coli*. Lane 1: 100bp DNA Marker; Lane 2-4: Representative *E. coli* isolates; Lane 5 positive control; Lane 6: Negative control.

#### 3.4.2 Molecular confirmation of Vibrio spp. and V. cholerae targeting groEL gene and multiplex PCR

Among the 13 positive suspected *Vibrio* isolates, 4 (30.76%) were confirmed as *Vibrio* spp. when genus specific *groEL* primers were used and a positive band appeared at 1117 bp (Table 3 and Figure 7). All the four *Vibrio* spp. were confirmed as *V. cholerae* when multiplex PCR was performed using species specific *groEL* primers and positive band was appeared at 418 bp (Figure 8). No isolate was detected as *V. parahaemolyticus, V. alginolyticus* and *V. vulnificus* by multiplex PCR.

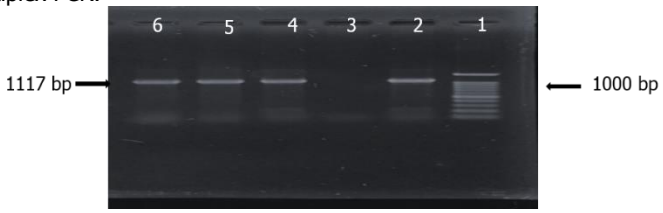



**Figure 7.** PCR amplification of *groEL* gene for specific detection of the genus *Vibrio* Lane 1: 100bp DNA Marker, Lane 2: Positive control. Lane 3: Negative control, Lane 4-6: Representative *Vibrio* isolates.

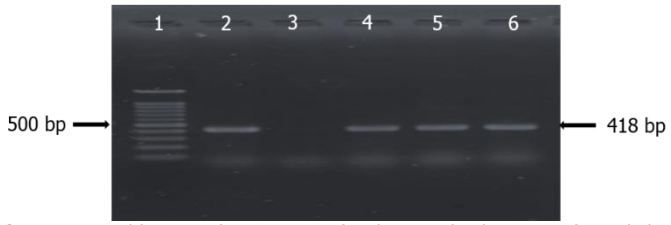



**Figure 8.** Amplification of *groEL* gene for the specific detection of *V. cholerae* (418bp) where, Lane 1: 100bp DNA Marker, Lane 2: positive control; Lane 3: negative control, Lane 4-6: representative *Vibrio* isolates.

**Table 3.** Overall Molecular prevalence of *16S rRNA* gene of *E. coli* and *groEL* gene of *V. cholerae* isolated from raw vegetables.

| Name of the organisms | Name of the genes | Number of detected isolates | Prevalence of detected gene of *E. coli* (n=5) and *V. cholerae* (n=4) | 95% CI |
|---|---|---|---|---|
| *E. coli* | *16S rRNA* | 5 | 31.25% | 16.3-61.2 |
| *V. cholerae* | *groEL* | 4 | 30.76% | 12.7-57.6 |

### *3.5 Antibiogram profiles*

*E. coli* and *V. cholerae* isolates tested positive for antibiotics, showing that they were resistant, intermediate and susceptible. Antibiotic susceptibility tests were performed on all 16 and 13 culture-positive isolates of *E. coli* and *V. cholerae*, respectively, using 11 and 8 different antibiotics under 7 and 5 distinct antibiotic classes (Figure 9 and 10).

All *E. coli* isolates (n=16) showed various resistance pattern against of all antibiotics. But *E. coli* isolates were highly resistant to 93.75% against ceftazidime, 87.25% against cefixime, 81.25% against ampicillin, 75% against oxytetracycline, respectively. Besides, highly sensitive isolates were exhibited 87.50%, 81.25%, and 68.75% against gentamycin, ceftriaxone, colistin, and enrofloxacin (Figure 9).

Another, 13 *V. cholerae* isolates 92.30% resistant to nalidixic acid, 76.92% against trimethoprim, 69.23% against polymyxin, 53.84% against ciprofloxacin and amoxicillin. Conversely, highly sensitive isolates were exhibited 92.30%, 84.61%, and 53.84% against kanamycin, gentamicin, and streptomycin (Figure 10).

### *3.6 MDR profile of E. coli and V. cholerae isolated from vegetable sample*

Out of 16 culture positive *E. coli* isolates, 10 (62.50%) were MDR in terms of phenotype. A total of 11 resistance patterns were observed where six were MDR patterns. The MDR prevalence was 62.50%. The MDR pattern numbers 4 (AMP-OTC-CAZ-CFX-SEF), 2 (AMP-K-OTC-CAZ-CFX-SEF), and 2 (OTC-CAZ-CFX-SEF) were observed in the highest number of MDR *E. coli* isolates. The resistance pattern number 2 (OTC-CAZ-CFX-SEF), 1 (AMP-CAZ-CFX-SEF), 1 (AMP-CAZ-CFX), 1 (AMP-CAZ-CFX), and 1 (AMP-CAZ) was not phenotypically MDR in nature. Every *E. coli* isolate had a different profile of antibiotic resistance, with MAR indices ranging from 0.27 to 0.72 (Table 4).

Other 13 culture positive *V. cholerae* isolates, 12 (92.30%) were phenotypically MDR in nature. A total of 10 resistance patterns were observed where 9 were MDR patterns. The MDR prevalence was 92.30%. The MDR pattern numbers 2 (W-CIP-AMP-NA-PB), 2 (W-AMP-NA-PB), and 2 (W-CIP-NA) were observed in the highest number of MDR *V. cholerae* isolates. The resistance pattern number 1 (W-PB) was not phenotypically MDR in nature. Each *V. cholerae* isolate's profile of antibiotic resistance was found to be unique, with MAR indices ranging from 0.25 to 1. (Table 5).

## 4. Discussion

Fresh vegetables are widely recognized for their nutritional benefits, providing essential vitamins, minerals, and fiber crucial for maintaining good health. However, concerns about potential health hazards associated with their production and consumption persist. A significant issue arises from the use of chemical fertilizers in vegetable farming, which can introduce harmful toxins into the produce, thereby posing risks to consumers (Mostafidi *et al.*, 2020). Additionally, several multidrug resistance isolates associated with fresh vegetables which are responsible for different infectious diseases in human health worldwide. Customers are more concerned with maintaining good eating habits and their health as they are becoming more aware of the health benefits of fresh fruits and vegetables. However, concurrently with the rise in fresh produce-related foodborne illness outbreak (Nagyová *et al.*, 2019).

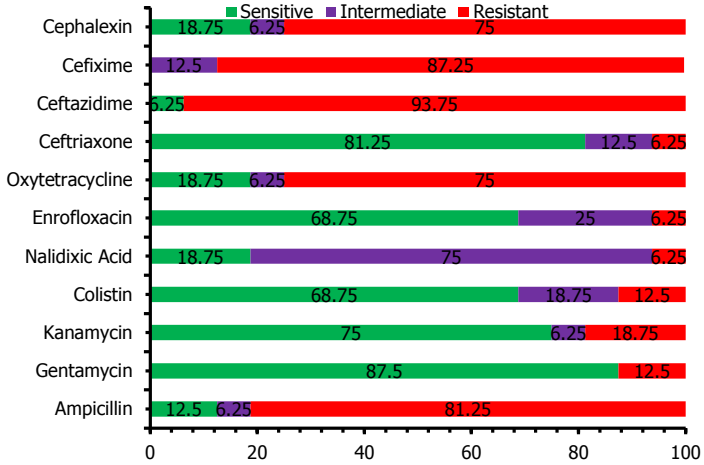



**Figure 9.** Graphical presentation of the antimicrobial susceptibility profiles of *E. coli* isolates from different types of vegetable samples, 11 different antibiotics under 7 different classes. Bar diagram showing respectively the number of isolates sensitive /intermediate /resistance and percentage pattern against Ampicillin (AMP), GEN: Gentamycin (GEN), Kanamycin (K), Colistin (COL), Nalidixic Acid (NAL), Enrofloxacin (ENO), Oxytetracycline (OTC), Ceftriaxone (CRO), Ceftazidime (CAZ), Cefixime (CFX), and Cephalexin (SEF) antibiotic.

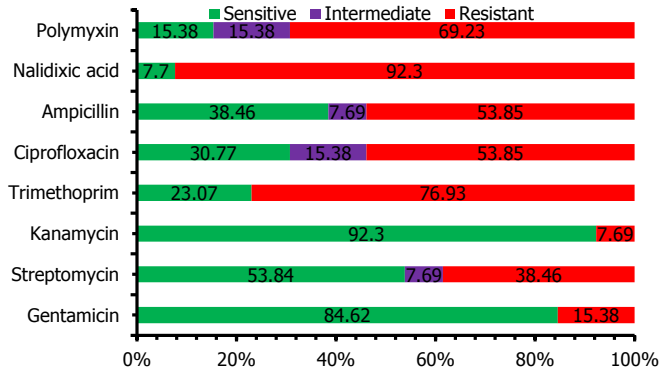



**Figure 10.** Graphical presentation of the antimicrobial susceptibility profiles of *V. cholerae* isolates from different types of vegetable samples, 8 different antibiotics under 5 different classes. Bar diagram showing respectively the number of isolates sensitive /intermediate /resistance and percentage pattern against Gentamicin (GEN), Streptomycin (S), Kanamycin (K), Trimethoprim (W), Ciprofloxacin (CIP), Ampicillin (AMP), Nalidixic acid (NA), and Polymyxin B (PB) antibiotic.

**Table 4.** Multidrug resistant profile of *E. coli* isolated from raw vegetable samples.

| No. of Pattern | No. of antibiotic classes | Multidrug resistance profiles of *E. coli* | No. of isolates showed resistant n (%) | Overall MDR prevalence n (%) | MAR Index |
|---|---|---|---|---|---|
| 1 | 6 | AMP-GEN-NAL-ENO-OTC-CAZ-CFX-SEF | 1 (6.25) | | 0.72 |
| 2 | 4 | AMP-GEN-K-OTC-CRO-CAZ-CFX-SEF | 1 (6.25) | | 0.72 |
| 3 | 4 | AMP-K-OTC-CAZ-CFX-SEF | 2 (12.50) | | 0.54 |
| 4 | 3 | AMP-OTC-CAZ-CFX-SEF | 4 (25) | | 0.45 |

| | | | | | |
|---|---|---|---|---|---|
| 5 | 3 | COL-OTC-CAZ-CFX-SEF | 1 (6.25) | 10 (62.50) | 0.45 |
| 6 | 3 | AMP-COL-CAZ-CFX | 1 (6.25) | | 0.36 |
| 7 | 2 | OTC-CAZ-CFX-SEF | 2 (12.50) | | 0.36 |
| 8 | 2 | AMP-CAZ-CFX-SEF | 1 (6.25) | | 0.36 |
| 9 | 2 | AMP-CAZ-CFX | 1 (6.25) | | 0.27 |
| 10 | 2 | AMP-OTC | 1 (6.25) | | 0.27 |
| 11 | 2 | AMP-CAZ | 1 (6.25) | | 0.27 |

AMP: Ampicillin; GEN: Gentamycin; K: Kanamycin; COL: Colistin; NAL: Nalidixic Acid; ENO: Enrofloxacin; OTC: Oxytetracycline; CRO: Ceftriaxone; CAZ: Ceftazidime; CFX: Cefixime; SEF: Cephalexin; MDR: Multidrug Resistance; MAR: Multiple Antibiotic Resistance.

**Table 5.** Multidrug resistant profile of *V. cholerae* isolated from raw vegetable samples.

| No. of Pattern | No. of antibiotic classes | Multidrug resistance profiles of *V. cholerae* | No. of isolates showed resistant n (%) | Overall MDR prevalence n (%) | MAR Index |
|---|---|---|---|---|---|
| 1 | 5 | GEN-S-K-W-CIP-AMP-NA-PB | 1 (7.69) | 12 (92.30) | 1 |
| 2 | 4 | W-CIP-AMP-NA-PB | 2 (15.38) | | 0.62 |
| 3 | 4 | W-AMP-NA-PB | 2 (15.38) | | 0.50 |
| 4 | 4 | S-AMP-NA-PB | 1 (7.69) | | 0.50 |
| 5 | 4 | S-W-CIP-NA | 1 (7.69) | | 0.50 |
| 6 | 3 | CIP-AMP-NA | 1 (7.69) | | 0.37 |
| 7 | 3 | W-CIP-NA | 2 (15.38) | | 0.37 |
| 8 | 3 | W-NA-PB | 1 (7.69) | | 0.37 |
| 9 | 3 | S-NA-PB | 1 (7.69) | | 0.37 |
| 10 | 2 | W-PB | 1 (7.69) | | 0.25 |

GEN: Gentamicin; S: Streptomycin; K: Kanamycin; W: Trimethoprim; CIP: Ciprofloxacin; AMP: Ampicillin; NA: Nalidixic acid; PB: Polymyxin B; MDR: Multidrug Resistance; MAR: Multiple Antibiotic Resistance.

Recent research has highlighted the presence of multidrug-resistant bacteria such as *E. coli* and *V. cholerae* in fresh vegetables, posing global public health challenges due to their potential to cause infectious diseases (Sultana *et al.,* 2021). The study conducted in northern Bangladesh have specifically identified these pathogens in vegetable samples, revealing patterns of resistance to antibiotics commonly used in clinical settings. In this molecular study, we isolated two most important multidrug resistance isolates including *E. coli* and *V. cholerae* from different vegetables samples.

Many research have looked into the possible pre and post-harvest (in the field) and post-harvest causes of product contamination within the supply chain. Pathogen communities have the ability to establish themselves on developing crops throughout the pre-harvest stage. After harvest, the likelihood will increase due to either further direct contamination or the expansion of already-existing pathogen populations during processing and handling methods. In the field, water will most likely be a major source of contamination. Runoff from neighboring animal pastures and irrigation from a contaminated source are potential contributors (Iwu and Okoh, 2019). The risk associated with using water from resources that change in microbiological quality for irrigation is the cause of surface and ground water contamination in Bangladesh, indicating the need for stronger regulations (Bilal *et al.*, 2023; Jeong *et al.,* 2016; Uyttendaele *et al.,* 2015). There are several documented pathways of water contamination than irrigation. One potential source of contamination is insects. Contaminated et al flies have been demonstrated to directly transfer bacteria to plant leaves or fruits in scientific settings (Yin *et al.,* 2022; Osafo *et al.,* 2022; Wasala *et al.,* 2013).

The Total Viable Count (TVC) analysis of vegetable samples from markets in Dinajpur district revealed significant variations in bacterial contamination levels. The results indicated an average bacterial count ranging from 1.4 ± 0.25 to 2.4 ± 0.37 CFU ± standard deviation (SD)/g, with potatoes showing the highest contamination (2.4 ± 0.37 CFU ± SD/g) and coriander leaves the lowest (2.4 ± 0.37 CFU ± SD/g). These findings are critical in understanding the microbial safety of fresh produce in the region. Aurin *et al.* (2020) observed a similar spectrum of microbial loads in fresh vegetables from Dhaka markets, with TVC ranging from $8\times10^7$ to $1.70\times10^8$ CFU/g. Further, Mahfuza *et al.* (2016) and Sultana *et al.* (2021) reported substantial ranges in TVC ($8\times10^3$ to $2.1\times10^8$ CFU/ml) and total coliform counts (TCC) ($1.5\times10^4$ to $2.2\times10^8$ CFU/ml) in various fruits, salad vegetables and juices different areas in Dhaka city, Bangladesh. Similarly, Mrityunjoy *et al.* (2013) reported that the *V. cholerae* was found $5.1\times10^6$, $4.1\times10^5$, $4.2\times10^5$, $1.1\times10^7$, $7.6\times10^6$, $2.5\times10^5$, and 8.25×105 cfu/g or cfu/ml in cucumber, carrot, tomoto, cauliflower, cabbage, kolmisak, and helenchasak in the Malibag market of Dhaka city, Bangladesh. These studies collectively underscore the widespread nature of microbial contamination, reflecting ongoing challenges in maintaining food safety standards. The quality of irrigation water, a significant factor, can introduce pathogens from animal waste and environmental runoff (Jeong *et al.,* 2016; Uyttendaele *et al.,* 2015). Insects, particularly flies, have also been identified as vectors that can transfer bacteria directly to vegetable surfaces during growth and handling (Osafo *et al.,* 2022; Wasala *et al.,* 2013). These vectors contribute to the contamination observed across different studies and regions.

In our study, we found that *E. coli* exhibited the highest prevalence, with 35 of 16 (45.71%) strains detected accounting for of the tested samples. and *V. cholera* was also notably present, with 35 of 13 (37.14%) strains identified, making up the samples analyzed. The prevalence of *E. coli* in our study was higher than the finding of Ratshilingano *et al.* (2022), who observed out of 51 *E. coli* isolates, including 28 *E. coli* were obtained from 17.4% (11 of 63) of the water samples, 11.8% (16 of 136) leafy green samples. In our study, the prevalence of *V. cholerae* was lower than the findings of Sultana *et al.* (2021), who conducted a study where out of 20, 8 (40%) isolates were found to be positive for *V. cholerae* in street fruits and juices from different areas in Dhaka City, Bangladesh. These findings underscore the significant presence of these pathogenic bacteria in fresh vegetable samples, highlighting potential risks associated with their consumption if not properly handled and cooked.

Out of sixteen culture positive *E. coli*, *16S rRNA* gene was confirmed to be present in of 5 (31.25%) isolates in our study. In another study, the similar report supported by Datta *et al.* (2024) reported that the prevalence of *E. coli* was found 32.0% (96/300) in selected vegetables and herbs in Bangkok, Thailand. Besides, in our research, among the thirteen culture positive suspected *Vibrio* isolates, 4 (30.76%) were confirmed as molecular-positive *Vibrio* spp. All the four *Vibrio* spp. were confirmed as *V. cholera* through multiplex PCR. The molecular prevalence of *V. cholerae* in our study supported by Rivera *et al.* (2001), who observed that out of 40 suspected *Vibrio* isolates, 12 (30%) were confirmed as *Vibrio* spp. using genus-specific *groEL* primers and further multiplex PCR analysis identified 8 of these 12 isolates as *Vibrio cholerae.* On the contrary, the prevalence of *groEL* gene-positive *V. cholerae* was higher than that of Singh *et al.* (2002), who revealed that out of 50 samples, 10 (20%) were suspected to be *Vibrio* spp. through initial screening. When tested with *groEL* primers, 3 (30%) of these suspected isolates were confirmed as *Vibrio* spp. by the appearance of a positive band at 1117 bp. Further analysis using species-specific primers confirmed all 3 isolates as *Vibrio cholerae*, showing a positive band at 418 bp.

In our study, *E. coli* isolated from vegetable samples exhibited high resistance rates to ceftazidime (93.75%), cefixime (87.25%), and ampicillin (81.25%), while demonstrating relatively higher sensitivity to gentamycin (87.50%), ceftriaxone (81.25%), and enrofloxacin (68.75%). In contrast, findings from another study by Aurin *et al.* (2020) showed the comparable resistance trends but with different antibiotics who reported high resistance rates among *E. coli* strains to ceftriaxone (100%), nitrofurantoin (94%), erythromycin

(89%), and amoxicillin (83%) had the highest resistance against the isolated organisms along with Imipenem showed the highest sensitivity (86%) in fresh leafy and salad vegetable samples. The other study conducted by Alabi *et al.* (2022) found that the most susceptible antibiotics to *E. coli* isolated from water leaves were imipenem (83.3%), then gentamicin (77.8%), ciprofloxacin (66.7%), ceftazidime (61.1%), ampicillin (55.6%), amoxicillin-clavulanic acid (50.0%), cefotaxime (44.4%), cefoxitin (33.3%), septrin (38.8%), and streptomycin (25.9%). Another study coducted by Habib *et al.* (2023), who examined the *E. coli* (n=145) was isolated from fresh salad vegetables, the isolates with the highest phenotypic resistance to ampicillin (20.68%), tetracycline (20%), and trimethoprim-sulfamethoxazole (10.35%) were investigated for antimicrobial resistance.

We isolated 13 *Vibrio cholerae* isolates where 92.30% resistant to nalidixic acid, 76.92% against trimethoprim, 69.23% against polymyxin, 53.84% against ciprofloxacin and amoxicillin. Conversely, highly sensitive isolates were exhibited 92.30%, 84.61%, and 53.84% against kanamycin, gentamicin, and streptomycin. The another finding conducted by Dalsgaard *et al.* (2000) reported that isolates of all *Vibrio cholerae* from raw vegetable samples found that 90% of the isolates were resistant to nalidixic acid, 80% to trimethoprim, and 70% to polymyxin B. Resistance to ciprofloxacin and amoxicillin was observed in 60% and 50% of the isolates, respectively. In contrast, high sensitivity was observed against kanamycin (95%), gentamicin (85%), and streptomycin (60%). Budiman *et al.* (2022) reported that the antibiotic resistance analysis showed 4.35% isolates of *V. cholera*e were resistant to gentamicin, streptomycin (17.39%), trimethoprim (52.17%), ciprofloxacin (30.43%), ampicillin (13.04%), nalidixic acid (82.61%), and polymyxin B (91.30%). This highlights the significant challenge of antibiotic resistance in foodborne pathogens and underscores the need for effective surveillance and intervention strategies.

The findings from this study reveal significant concerns regarding multidrug-resistant (MDR) *E. coli* and *V. cholerae* isolates in vegetable samples. Out of the 16 culture-positive *E. coli* isolates, 10 (62.50%) exhibited MDR phenotypes, with a total of 11 resistance patterns observed, including 6 MDR patterns. The prevalence of MDR among *E. coli* isolates was notably high at 62.50%, with patterns such as AMP-OTC-CAZ-CFX-SEF, AMP-K-OTC-CAZ-CFX-SEF, and OTC-CAZ-CFX-SEF being most frequent.

In contrast, all 13 culture-positive *V. cholerae* isolates were phenotypically MDR, accounting for 92.30% prevalence across 10 observed resistance patterns, 9 of which were MDR. Common MDR patterns included W-CIP-AMP-NA-PB, W-AMP-NA-PB, and W-CIP-NA. Another study noted significant antimicrobial resistance in foodborne pathogens including *E. coli* and *V. cholerae*, with moderate levels of multi drug resistance patterns included common antibiotics like ciprofloxacin and ampicillin (Yu *et al.,* 2019). Notably, each isolate of *E. coli* and *V. cholerae* exhibited a unique antibiotic resistance profile, with multiple antibiotic resistance (MAR) indices ranging from 0.25 to 1. The presence of high percentage MDR bacteria in raw vegetables in the northern district of Bangladesh compared to others study, possibly due to differences in local agricultural practices or environmental factors. These findings underscore the serious health risks associated with the presence of MDR *E. coli* and *V. cholerae* in vegetable samples, highlighting the urgent need for enhanced surveillance and mitigation strategies to ensure food safety and public health.

Both studies highlight the urgent need for enhanced surveillance and control measures to mitigate the spread of antibiotic-resistant bacteria through vegetables. The similarities in resistance profiles across studies indicate a consistent challenge in managing bacterial contamination in agricultural settings, emphasizing the importance of rigorous food safety protocols and antibiotic stewardship to protect public health.

Given these findings, it is vital to follow food safety practices when handling and consuming fresh vegetables. Proper washing and boiling of vegetables are critical steps in reducing the danger of bacterial illnesses. Furthermore, there is an urgent need for proper agricultural laws and monitoring to protect public health against microbial contamination in fresh produce (Nagyová *et al.,* 2019). This study emphasizes the necessity of ongoing efforts to improve food safety procedures and raise consumer awareness about the safe handling and eating of fresh vegetables worldwide.

## 5. Conclusions

The current study provided thorough information about the prevalence, characterization, and antibiotic resistance profiles of *Escherichia coli* and *V. cholerae* isolated from raw vegetables in Dinajpur district, Bangladesh. The study revealed the presence of MDR strains, which demonstrated considerable resistance to important antibiotics such as ampicillin, ceftazidime, and nalidixic acid. These findings highlight the critical public health concern posed by MDR bacteria in raw vegetables, indicating potential health concerns associated with their use. Addressing these difficulties would necessitate coordinated efforts to implement strict food safety rules, promote healthy agricultural practices, and raise awareness among producers and consumers alike. Furthermore, constant monitoring of antibiotic usage in agricultural and clinical contexts is critical to minimize the rise of antimicrobial resistance pattern.

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## Data availability

The data generated from this study will be available on the valid request from the corresponding author.

## Informed consent statement

No informed consent was required to conduct the study.

## Conflict of interest

The authors declare no conflict of interest.

## Authors' contribution

**Conceptualization**: Anwar Hossain Rana, Md. Khaled Hossain, and Farzana Afroz; Data collection, **study conduct, and manuscript write up:** Anwar Hossain Rana; **Methodology preparation, manuscript write up, molecular data analysis, formatting, and reviewed the final version manuscript:** Palash Bose; **Data analysis, first draft develop, and result section interpretation:** Kazi Abdus Sobur, Sakib Mowdood, and Md. Mosharraf Hossen; **Figure preparation and formal analysis**: Nazmi Ara Rumi, Mahmudul Hasan, and Nusrat Jahan. **Reviewed and revised the final version**: Atikur Rahman Titas, Palash Bose, and Md. Aoulad Hosen. All authors critically reviewed the manuscript and agreed to submit final version of the manuscript.

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