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*Article*

# *Escherichia coli* in betel leaves: prevalence, virulence characterization and antibiogram

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**Abstract:** *Escherichia coli* is a significant foodborne pathogen, frequently linked to hemorrhagic diarrhea, especially in developing nations, where it presents considerable public health concerns. This study was conducted to examine the virulence gene profiles and antibiotic resistance patterns of *E. coli* strains isolated from piper betel leaves. A total of 100 betel leaf samples, including fresh (n = 60) and ready-to-eat (n = 40) specimens, were collected and tested for the presence of *E. coli* using standard diagnostic techniques, such as selective culture methods, staining, latex agglutination, and polymerase chain reaction (PCR) assays. Further, the identified *E. coli* isolates underwent PCR-based testing for virulence genes and disk diffusion assays to assess antibiotic susceptibility. Among the 100 samples screened, 4% (n = 4/100; 95% CI: 1.57–9.84; P = 0.1126) were identified as *E. coli* O157, and 33% (n = 33/100; 95% CI: 24.56–42.69; P = 0.4011) were classified as non-O157 isolates. The virulence gene *stx*$_1$ was found in 10.81% of isolates, while *stx*$_2$, *eae*A, and *hly*A genes were not detected in any samples. Antibiotic resistance analysis showed that all isolates (100%, 37/37) were resistant to amoxicillin and erythromycin, with 75.68% (28/37) demonstrating resistance to tetracycline. Notably, all isolates were fully susceptible to ceftriaxone and ciprofloxacin. A majority (72.97%, 27/37) of isolates were sensitive to streptomycin, and 67.57% (25/37) were sensitive to gentamicin. Additionally, 86.48% of the *E. coli* isolates exhibited multidrug resistance (MDR), showing 10 resistance patterns, including 8 MDR patterns. The most common MDR pattern was AMX-TE-E, observed in 56.76% (21/37) of isolates. One isolate demonstrated resistance to six of the eight tested antibiotics across four distinct classes, with the resistance pattern AMX-TE-GEN-S-E-AZM. The MAR indices for *E. coli* isolates ranged between 0.25 and 0.75. These findings highlight the significant threat posed to global public health by multidrug-resistant shiga toxin-producing *E. coli* found on piper betel leaves in urban environments.

**Keywords:** *E. coli*; prevalence; virulence factors; antibiotic resistance; multidrug resistance; betel leaf contamination

## 1. Introduction

The betel leaf is derived from a vine that belongs to the Piperaceae family, and it is commonly referred to as "paan" in Bangladesh. It is widely consumed in Bangladesh, India, Pakistan, and several other Asian nations such as Myanmar, Vietnam, Malaysia, Singapore, Thailand, and the Philippines. The plant is an evergreen perennial climber that thrives in shaded environments (Iswariya and Uma, 2017). In South and Southeast Asia, betel leaf is commonly chewed and holds important sociocultural and ceremonial significance. In Bangladesh, its cultivation is a key source of income for farmers, particularly in the districts of Bagerhat, Jhenaidah, Natore, Satkhira, Rajshahi, Kushtia, Khulna, Barishal, Chittagong and Sylhet (Mahfuza *et al*., 2020).

Contamination can happen at various stages of production, including both pre-harvest and post-harvest processes (Golberg *et al*., 2011). Studies have also documented the existence of shiga toxin-producing *Escherichia coli* in water sources and in the urine of hospitalized patients in Bangladesh (Talukdar *et al*., 2013; Islam *et al*., 2015). Betel leaf is often consumed fresh and raw, making it challenging to effectively decontaminate. Consequently, similar to other fresh produce, it can serve as a common vehicle for transmitting enteropathogenic bacteria (Berger *et al*., 2010).

*E. coli* O157:H7 is a specific serotype of *Escherichia coli* known for its link to foodborne infections, typically arising from consuming contaminated or improperly cooked foods (Gally and Stevens, 2017). Shiga toxin-producing *E. coli* (STEC) strains can lead to severe health issues by releasing toxins that damage the intestinal and kidney linings. Infections with STEC can result in serious conditions which can sometimes be fatal (Karch *et al*., 2005). Numerous studies conducted in Bangladesh have documented the isolation of shiga toxin-producing *E. coli* from individuals suffering from diarrhea, as well as from various food sources and animals, particularly in the Dhaka region (Islam *et al*., 2007; Islam *et al*., 2008; Fazle *et al*., 2014).

Enterotoxigenic *E. coli* is the leading cause of childhood diarrhea in Bangladesh, responsible for nearly 20% of all cases (Qadri *et al*., 2005). The export of betel leaves from Bangladesh faces significant challenges attributable to adulteration with *Salmonella* and *E. coli* O157:H7 (Husna *et al*., 2015). Factors contributing to this issue include improper food handling practices, inadequate sanitation, weak food safety regulations, limited funding for safer equipment, and insufficient training for food handlers (Haileselassie *et al*., 2013). Although there have been a few studies focused on the isolation and molecular categorization of *E. coli* O157:H7 and *Salmonella* spp. in betel leaves in Bangladesh, further research is needed (Haque *et al*., 2017; Kamal *et al*., 2018; Ripon *et al*., 2021).

Multiple antibiotic resistance (MAR) indexing aids as a valued and economical apparatus for tracing the origins of bacterial contamination in various environments. A MAR index value exceeding 0.2 is particularly significant, as it suggests that the bacteria likely originate from high-risk sources where the use of antibiotics is prevalent. Such sources can include agricultural settings where antibiotics are administered to livestock, as well as healthcare facilities where antibiotics are frequently prescribed. Understanding the MAR index is crucial for public health, as it can help identify potential contamination pathways and guide efforts to mitigate the spread of antibiotic-resistant bacteria. Moreover, the MAR index can assist regulatory bodies in establishing guidelines for antibiotic usage in agriculture and medicine, ultimately contributing to more effective infection control measures (Osundiya *et al*., 2013). In developing countries, antimicrobial resistance is a persistent concern, particularly due to the high prevalence of infectious diseases and limited financial resources, which restrict access to newer and more expensive treatments (Okeke *et al*., 2005).

Betel leaves, widely consumed in Bangladesh and several Asian countries, are often eaten raw, which increases the risk of contamination by harmful pathogens. *E. coli*, particularly the shiga toxin-producing strain (STEC), poses a serious threat to public health as it can cause severe foodborne illnesses. The lack of proper hygiene, inadequate food safety measures, and improper handling of betel leaves have been linked to contamination by *E. coli*, making them a potential vehicle for the transmission of enteropathogenic bacteria. Despite the known risks, limited studies have investigated the occurrence, virulence, and antibiotic resistance patterns of *E. coli* in betel leaves in Bangladesh. Understanding these factors is crucial for addressing food safety concerns and protecting public health.

The current study hypothesized betel leaves in Bangladesh are frequently contaminated with *E. coli*, including virulent and multidrug-resistant strains, which pose a significant risk for foodborne illness and antibiotic resistance spread. Considering the above discussion, the present study investigated the prevalence, virulence characterization, and antibiotic resistance patterns of *E. coli* isolated from betel leaves in Bangladesh.

The findings provide valuable insights into the safety of betel leaves in Bangladesh, highlighting the public health risks associated with *E. coli* contamination. The findings will help inform policy makers, health officials, and food safety authorities on the necessity of improving food handling practices, implementing stricter regulatory frameworks, and enhancing public awareness about foodborne pathogens.

## 2. Materials and Methods

### 2.1. Ethical approval

The study was approved by the Ethical Committee of Bangladesh Agricultural University in Mymensingh, Bangladesh, with the reference number AWEEC/BAU/2020(12).

### 2.2. Collection and preparation of samples

A total of 100 betel leaf samples were randomly collected in sterilized polybags containing 0.1% peptone water from various markets in Mymensingh city, Bangladesh, between January 2022 and December 2022 (Figure 1). This included 60 fresh betel leaves (FBL) and 40 ready-to-eat betel leaves (RTE-BL). The samples were promptly transported in a cool thermos to the Bacteriology Laboratory at Bangladesh Agricultural University, Mymensingh, for the isolation and identification of both *E. coli* O157:H7 and non-O157:H7 strains. All samples were processed in the laboratory within 24 hours, following strict hygienic protocols throughout the collection, transportation, and processing stages.

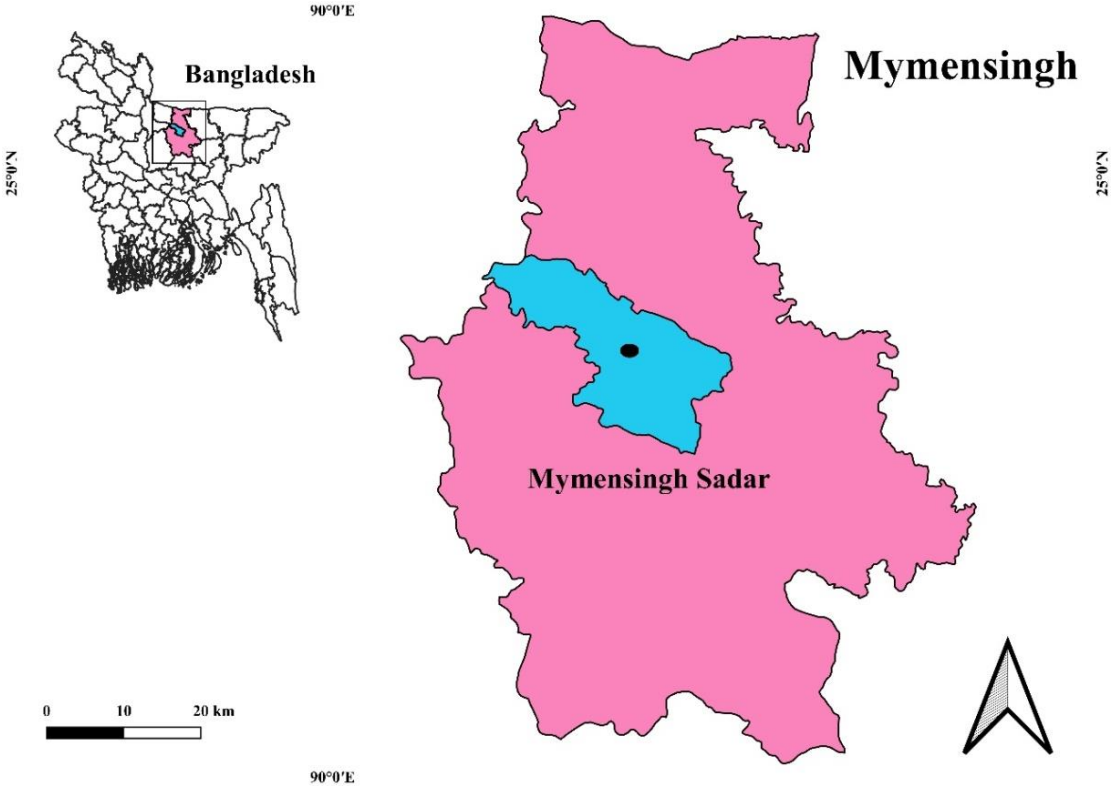


**Figure 1. Map showing the location of the study area.**

### 2.3. Isolation and identification of *E. coli* O157:H7

*E. coli* O157:H7 was isolated and identified from betel leaves following the methodology described by Islam *et al*. (2007). Initially, fresh and ready-to-eat betel leaf samples were rinsed using 10 ml of 0.1% peptone water to eliminate surface contaminants. After rinsing, 200 µl of the peptone water was inoculated into Tryptone Soy Broth (Oxoid, UK) and incubated overnight at 37°C to promote the growth of any *E. coli* present.

After enrichment, a tenfold serial dilution was prepared to aid in isolating and quantifying *E. coli*. From the fifth dilution, 50 µl was spread onto sorbitol-MacConkey agar (Oxoid, UK) supplemented with cefixime (0.05 mg/L) and tellurite (2.5 mg/L), also known as CT-SMAC. These selective agents help distinguish *E. coli* O157:H7, which forms colorless colonies due to its inability to ferment sorbitol. The plates were nurtured at 37°C for 18 to 24 hours, allowing the colonies to form. Up to 12 colorless colonies from CT-SMAC plates were nominated and marked onto Tryptone Soy Agar (TSA) plates for further analysis. To verify the identity of *E. coli* O157:H7, agglutination testing was accompanied by the Wellcolex® *E. coli* O157:H7 Rapid Latex Agglutination Test (Oxoid, UK). This test uses latex beads covered with antibodies specific to *E. coli* O157:H7, allowing for fast and accurate identification. Furthermore, all isolates were permitted to undergo PCR amplification directed at the *rfb*E O157 gene and *mal*B promoter in order to verify the existence of both O157:H7 and non-O157:H7 strains. Tables 1 and 2, respectively, provide a summary of the prerequisite primers and conditions.

### 2.4. DNA extraction

Bacterial DNA extraction was accomplished by a straightforward boiling technique, based on the method outlined by Kabir *et al*. (2011). In this process, three to five pure colonies from Tryptone Soya Agar plates were picked and transferred into an Eppendorf tube containing 250 µl of deionized water. The tubes were placed in a boiling water bath for 10 minutes to lyse the bacterial cells and release DNA. Following boiling, they were immediately transferred to ice for 10 minutes to induce cold shock, aiding in further protein denaturation and DNA stabilization. The samples were then centrifuged at 12,000 rpm for 10 minutes to separate cellular debris, leaving DNA in the supernatant. A 100 µl volume of the supernatant was carefully collected and used as the DNA template for PCR in subsequent experiments.

### 2.5. Amplification of virulence genes

PCR amplification was conducted with a reaction volume of 20 µl to detect specific genes in the *E. coli* isolates. The reaction mixture included 10 µl of master mix (Promega, USA), which provided essential components such as enzymes, nucleotides, and buffer for efficient amplification. Additionally, 1.0 µl of every forward and reverse primer (10 pmol each) was supplemented to ensure appropriate annealing to the target DNA regions. The DNA template, 3.0 µl of bacterial DNA extracted earlier, was incorporated to supply the genetic material needed for amplification. To adjust the reaction to the final volume of 20 µl, 5.0 µl of deionized water was added. The PCR amplification was accomplished by a Thermocycler (2720 Thermal Cycler, Applied Biosystems, USA), which enabled precise temperature control during the different stages of the reaction. The primers used for the identification of virulence genes in the *E. coli* isolates, including their sequences and target genes, are outlined in Table 1. The thermal cycling conditions, such as denaturation, annealing, and extension, are presented in Table 2. These parameters perform a vital role in ensuring the successful amplification of the target genes and ensuring the reproducibility of the procedure.

**Table 1. The primers used for the identification of virulence genes in the *E. coli* isolates along with their sequences and corresponding target genes.**

| Primer | Sequence (5'-3') | Target gene | Amplicon size | Reference |
|---|---|---|---|---|
| *rfb*O157F | CGGACATCCATGTGATATGG | *rfb*$E_{O157}$ | 259 bp | Paton and Paton (1998) |
| *rfb*O157R | TTGCCTATGTACAGCTAATCC | | | |
| ECO-1 | GACCTCGGTTTAGTTCACAGA | *mal*B | 585 bp | Wang *et al*. (1996) |
| ECO-2 | CACACGCTGACGCTGACCA | | | |
| $Stx_1$F | CACAATCAGGCGTCGCCAGCGCACTTGCT | $stx_1$ | 606 bp | Heuvelink *et al*. (1996) |
| $Stx_1$R | TGTTGCAGGGATCAGTCGTACGGGGATGC | | | |
| $Stx_2$F | CCACATCGGTGTCTGTTATTAACCACACC | $stx_2$ | 372 bp | Heuvelink *et al*. (1995) |
| $Stx_2$R | GCAGAACTGCTCTGGATGCATCTCTGGTC | | | |
| *eae*F | TGCGGCACAACAGGCGGCGA | *eae*A | 629 bp | Heuvelink *et al.* (1995) |
| *eae*R | CGGTCGCCGCACCAGGATTC | | | |
| *hly*EHECF | GAGCGAGCTAAGCAGCTTG | *hly*A | 889 bp | Wieler *et al.* (1996) |
| *hly*EHECR | CCTGCTCCAGAATAAACCACA | | | |

### 2.6. Agarose gel electrophoresis of PCR products

The PCR products were run on 1% and 1.5% agarose gels (Invitrogen, USA) and stained with ethidium bromide at a concentration of 0.5 µg/ml (Sigma-Aldrich, USA). The gels were subsequently visualized on an ultraviolet transilluminator (BDA digital, Biometra GmbH, Germany).

**Table 2. Thermal cycling conditions used for PCR amplification of various genes.**

| Thermal profile | Target genes | | | | | |
|---|---|---|---|---|---|---|
| | ***rfb*E$_{O157}$** | ***mal*B** | ***stx*$_1$** | ***stx*$_2$** | ***eae*A** | ***hly*EHEC** |
| Initial denaturation | 94°C/10min | 95°C/5min | 95°C/5min | 95°C/5min | 95°C/5min | 95°C/5min |
| Denaturation | 94°C/1min | 94°C/45sec | 95°C/30sec | 95°C/30sec | 95°C/1min | 95°C/30sec |
| Annealing | 56°C/1min | 52°C/45sec | 61°C/30sec | 58°C/30sec | 56°C/1min | 57°C/30sec |
| Extension | 72°C/1min | 72°C/1min | 72°C/30sec | 72°C/30sec | 72°C/40sec | 72°C/30sec |
| Final extension | 72°C/7min | 72°C/5min | 72°C/7min | 72°C/7min | 72°C/7min | 72°C/7min |

**Table 2. Contd.**

| Thermal profile | Target genes | | | | | |
|---|---|---|---|---|---|---|
| | *rfb*$E_{O157}$ | *mal*B | *stx*$_1$ | *stx*$_2$ | *eae*A | *hly*EHEC |
| Number of cycles | 35 | 30 | 35 | 35 | 35 | 35 |
| Amplicon size (bp) | 259 | 585 | 606 | 372 | 629 | 889 |

### 2.7. Antimicrobial susceptibility test

The antimicrobial resistance profiles of the *E. coli* isolates were determined using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar plates (Oxoid, UK), following the protocol established by Bauer *et al*. (1966). This involved assessing susceptibility to various antibiotics through disks with concentrations of Amoxicillin (30 µg), Azithromycin (30 µg), Ciprofloxacin (5 µg), Erythromycin (30 µg), Gentamicin (10 µg), Ceftriaxone (10 µg), Streptomycin (10 µg), and Tetracycline (30 µg). Results were interpreted according to Clinical and Laboratory Standards Institute (CLSI, 2018) guidelines, categorizing isolates as susceptible (S), intermediate (I), or resistant (R). Isolates resistant to three or more antibiotic classes were classified as multidrug-resistant (MDR), in accordance with the criteria defined by Sweeney *et al*. (2018).

### 2.8. Multiple antibiotic resistance index (MARI)

The MAR index for *E. coli* isolates was determined based on the formula provided by Msolo *et al*. (2020), defined as,

$$\text{MAR index} = a/b,$$

Where "a" represents the number of antibiotics that the specific isolate is resistant to, and "b" indicates the total number of antibiotics tested for each isolate.

### 2.9. Data management and statistical analysis

Data were systematically recorded using Microsoft Excel 2016 (Microsoft Office 2016, Microsoft, Los Angeles, CA, USA), providing a structured format for managing the information. Statistical analysis was performed using SPSS version 20, a reliable tool for conducting various statistical evaluations. Descriptive statistics were utilized to carry out frequency and percentage analyses, allowing for a comprehensive overview of the data distribution. To determine statistical significance, *P*-values were calculated, with a threshold set at less than 0.05 ($P<0.05$) indicating significant results.

## 3. Results

In this study, 100 betel leaves samples were evaluated initially based on growth characteristics in culture media (CT-SMAC and TSA agar media) and found Gram-negative small rods, separately arranged under microscope (100X) for the tentative identification of *E. coli* isolates. The tentative identified *E. coli* isolates were then tested by applying rapid latex agglutination test for the confirmation of O157:H7 (Figure 2) and PCR assays for the confirmation of both O157:H7 and non-O157:H7 (Figures 3 and 4). In this study, the overall prevalence of *E. coli* in the betel leaf samples was found to be 4% (4/100; 95% CI = 1.57-9.84; $P = 0.1126$) for *E. coli* O157:H7 and 33% (33/100; 95% CI = 24.56-42.69; $P = 0.4011$) for non-O157:H7 strains (Table 3). Among the *E. coli* O157:H7 isolates, 5% (3/60; 95% CI = 1.71–13.70) were obtained from fresh betel leaves, while 2.5% (1/40; 95% CI = 0.44–12.88) were found in ready-to-eat betel leaves samples. For non-O157:H7 isolates, 38.33% (23/60; 95% CI = 27.09-50.98) were isolated from fresh betel leaves, and 25% (10/40; 95% CI = 14.19-40.19) were isolated from ready-to-eat samples.

**Table 3. Prevalence of *E. coli* in betel leaf samples.**

| Name of sample | No. of sample | Prevalence [No. (%)] | | 95% CI | | *P* value | |
|---|---|---|---|---|---|---|---|
| | | O157:H7 | non-O157:H7 | O157:H7 | non-O157:H7 | O157:H7 | non-O157:H7 |
| FBL | 60 | 3 (5.0) | 23 (38.33) | 1.71-13.7 | 27.09-50.98 | | |
| RTE-BL | 40 | 1 (2.5) | 10 (25) | 0.44-12.88 | 14.19-40.19 | 0.1126 | 0.4011 |
| Total | 100 | 4 (4.0) | 33 (33) | 1.57-9.84 | 24.56-42.69 | | |

FBL: Fresh betel leaves; RTE-BL: Ready-to-eat betel leaves

Out of the 37 *E. coli* isolates examined, the *stx*$_1$ gene was found to be the most common, occurring in 10.81% of the isolates (Figure 5). However, the isolates were found to be devoid of the *stx*$_2$, *eae*A and *hly*A genes in this study (Table 4).

**Table 4. Prevalence of virulence genes in *E. coli* from betel leaf samples.**

| Isolates (n) | No. (%) of isolates positive for virulence genes | | | |
|---|---|---|---|---|
| | ***stx*$_1$** | ***stx*$_2$** | ***eae*A** | ***hly*A** |
| *E. coli* (37) | 4 (10.81) | - | - | - |

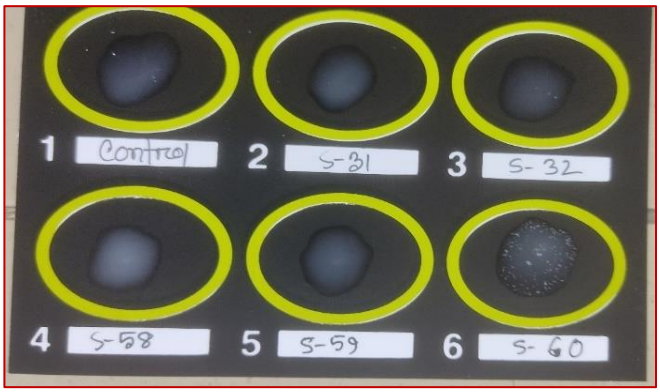


**Figure 2. Latex agglutination test results. 1 = negative control; 2, 3, 4, 5 = negative samples; 6 = positive confirmation for *E. coli* O157:H7.**

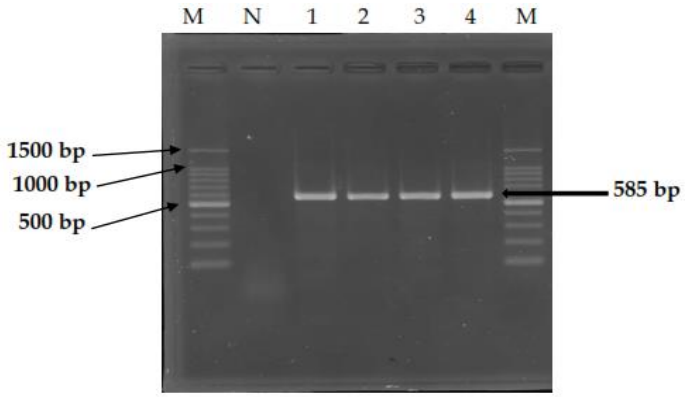


**Figure 3. Amplification of the *mal*B promoter gene (585 bp). Lane M = 100 bp DNA ladder (Takara, USA); Lane N = negative control; Lanes 1-4 = *E. coli* isolates positive for the *mal*B promoter gene.**

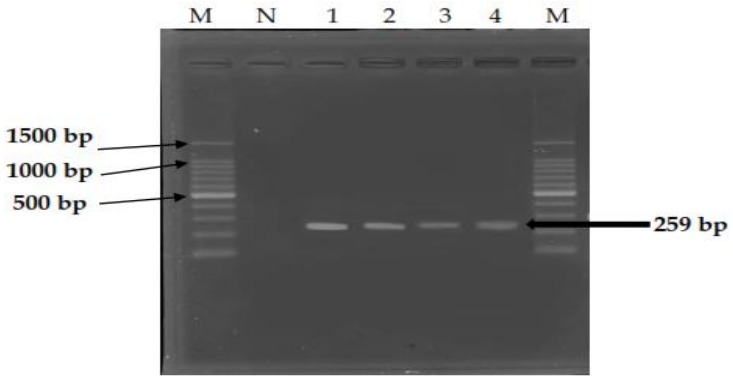


**Figure 4. Amplification of the *rfb*EO157 gene (259 bp). Lane M = 100 bp DNA ladder (Takara, USA); Lane N = negative control; Lanes 1-4 = *E. coli* isolates positive for the *rfb*EO157 gene.**

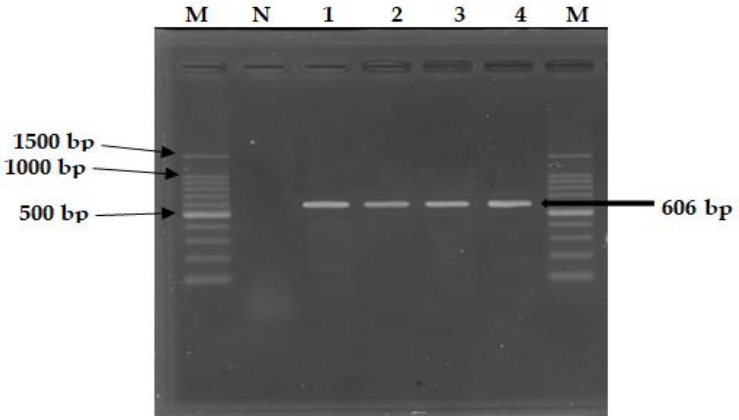


**Figure 5. PCR assay for the amplification of the *stx*$_1$ gene (606 bp) specific to shiga toxin-producing *E. coli*. Lane M = 100 bp DNA ladder (Takara, USA); Lane N = negative control; Lanes 1-4 = *E. coli* isolates positive for the *stx*$_1$ gene.**

The antibiogram analysis showed that all *E. coli* isolates (100%, 37/37) exhibited complete resistance to both erythromycin and amoxicillin. Furthermore, 75.68% (28/37) of the isolates showed resistance to tetracycline. In contrast, every isolate (100%, 37/37) was entirely sensitive to ceftriaxone and ciprofloxacin. Additionally, a significant proportion of *E. coli* isolates (72.97%, 27/37) were susceptible to streptomycin, while over half (67.57%, 25/37) were susceptible to gentamicin (Figure 6).

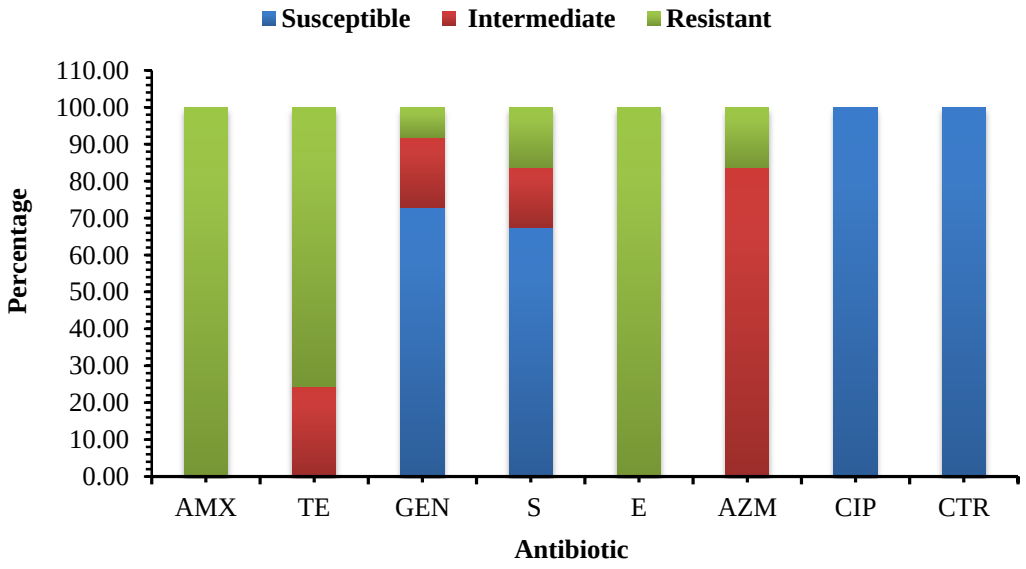


**Figure 6. Antimicrobial susceptibility profiles of *E. coli* isolates in response to various antibiotics. AMX = Amoxicillin, TE = Tetracycline, GEN = Gentamicin, S = Streptomycin, E = Erythromycin, AZM = Azithromycin, CIP = Ciprofloxacin, and CTR = Ceftriaxone.**

The results of this study revealed that 86.48% (32 out of 37; 95% CI: 40.74-76.60) of the *E. coli* isolates displayed multidrug-resistant (MDR) characteristics. These isolates exhibited a total of 10 distinct antibiotic resistance patterns, with 8 classified as MDR. Notably, the AMX-TE-E pattern was the most prevalent, found in 56.76% (21 out of 37) of the isolates. One isolate was resistant to six of the eight antibiotics evaluated, representing four different antibiotic classes (AMX-TE-GEN-S-E-AZM). Furthermore, the MAR indices for the *E. coli* isolates ranged between 0.25 and 0.75 (Table 5).

**Table 5. The MDR and MAR profiles of *E. coli* isolates from betel leaves in Mymensingh.**

| No. of pattern | Antibiotic resistance patterns | No. (%) of isolates | No. (class) of antibiotics | No. (%) of MDR isolates | Overall no. (%) of MDR isolates | MAR index |
|---|---|---|---|---|---|---|
| 1 | AMX-E | 4 (10.81) | 2 (2) | - | 32 (86.48) | 0.25 |
| 2 | AMX-S-E | 3 (8.11) | 3 (3) | 3 (8.11) | | 0.38 |
| 3 | AMX-TE-E | 21 (56.76) | 3 (3) | 21 (56.76) | | 0.38 |
| 4 | AMX-GEN-E | 1 (2.70) | 3 (3) | 1 (2.70) | | 0.38 |
| 5 | AMX-E-AZM | 1 (2.70) | 3 (2) | - | | 0.38 |
| 6 | AMX-TE-S-E | 1 (2.70) | 4 (4) | 1 (2.70) | | 0.50 |
| 7 | AMX-TE-GEN-E | 1 (2.70) | 4 (4) | 1 (2.70) | | 0.50 |
| 8 | AMX-TE-E-AZM | 3 (8.11) | 4 (3) | 3 (8.11) | | 0.50 |
| 9 | AMX-TE-S-E-AZM | 1 (2.70) | 5 (4) | 1 (2.70) | | 0.63 |
| 10 | AMX-TE-GEN-S-E-AZM | 1 (2.70) | 6 (4) | 1 (2.70) | | 0.75 |

## 4. Discussion

In this study, multiple culture media, including selective media, were utilized concurrently to isolate the organism, which is a crucial aspect of microbiological research aimed at identifying specific bacterial strains. The selection of culture media was informed by the experiences of previous researchers working in related fields (Islam *et al*., 2017; Nahar *et al*., 2018). This approach not only enhances the likelihood of successful isolation of pathogens but also allows for a more comprehensive understanding of the microbial landscape present in the samples. The use of varied media types can help differentiate between different bacterial species and strains, ensuring that researchers can accurately identify potential health risks connected with foodborne pathogens. The colonies of both *E. coli* O157:H7 and non-O157:H7 presented as gram-negative, pink rod-shaped bacteria arranged in singles, pairs, or short chains, aligning with the observations made by Momtaz *et al*. (2012), Uddin *et al*. (2019). This morphological characteristic is significant as it supports the initial identification process and provides a basis for further confirmatory tests. The distinct appearance of these colonies on selective media highlights the effectiveness of the chosen culture techniques in isolating specific *E. coli* serotypes, which is essential for understanding their prevalence and potential pathogenicity. To further validate the identification of these isolates, the Wellcolex *E. coli* O157:H7 latex agglutination test was employed on colonies grown on Tryptone Soy Agar (TSA) plates. This test is a reliable method for confirming the presence of *E. coli* O157:H7, a strain associated with severe foodborne illness. The successful identification of *E. coli* O157:H7 in this study, consistent with results described by Ababu *et al*. (2020), emphasizes the importance of utilizing both morphological characteristics and biochemical tests to ensure accurate identification of pathogenic strains.

The study found that the prevalence of *E. coli* in betel leaves was 37% (37 out of 100), which is similar to the 44% (44 out of 100) prevalence reported by Ripon *et al*. (2021). This similarity suggests that environmental factors and agricultural practices in Bangladesh may contribute to a consistent level of *E. coli* contamination in betel leaves across different studies. The presence of *E. coli* in fresh produce is concerning, as it indicates potential pathways for pathogen transmission to consumers, particularly since betel leaves are often consumed raw. Furthermore, these findings are higher than those of Haque *et al*. (2017) and Nahar *et al*. (2018), who reported prevalence rates of 17.34% and 21.73%, respectively. The increase in prevalence observed in this study could be attributed to several contributing factors. Changes in agricultural practices, such as the increased use of fertilizers and pesticides, can lead to the contamination of crops, including betel leaves, with pathogenic bacteria. Additionally, deteriorating water quality, influenced by industrial discharge and inadequate sanitation, can facilitate the spread of harmful microorganisms in agricultural settings. Furthermore, shifts in food handling processes, such as inadequate washing, improper storage, and cross-contamination during preparation, may exacerbate the risk of contamination. As urbanization increases, the demand for fresh produce, including betel leaves, may lead to more intensive farming methods that can enhance the risk of contamination.

The occurrence of virulence genes in *E. coli* isolates is a crucial factor in understanding the pathogenic potential of this bacterium. In this study, the finding of the $stx_1$ gene at a occurrence of 10.81% (4 out of 37 isolates) is significant, particularly as it indicates a higher occurrence compared to previous findings by Ripon *et al*. (2021), where $stx_1$ was identified in only 4.55% (2 out of 44 isolates). The presence of the $stx_1$ gene is particularly concerning, as it is associated with shiga toxin production, which can result in serious gastrointestinal disorders and complications, including hemolytic uremic syndrome (HUS). In contrast, the $stx_2$ gene was not spotted in any of the isolates in this investigation, suggesting a lower risk associated with this specific virulence factor among the tested samples. The absence of the *eae*A and *hly*A genes in all isolates indicates that these strains may be

deficient in certain virulence factors typically linked to higher levels of pathogenicity in *E. coli*. The *eae*A gene is recognized to perform a part in the attachment and invasion of intestinal cells, while *hly*A is associated with hemolysin production, which can enhance the bacteria's ability to cause disease. Therefore, while the presence of $stx_1$ is noteworthy, the overall virulence profile of the isolates appears to be less concerning due to the absence of these additional genes.

The findings from the antibiogram analysis revealed that all tested *E. coli* isolates (100%, 37/37) exhibited complete resistance to erythromycin and amoxicillin. This alarming level of resistance aligns with the observations made by Munaomar *et al*. (2018), as well as Mohamed *et al*. (2019) and Kamal *et al*. (2018), who also documented 100% resistance to these antibiotics in their respective studies. The persistent resistance of *E. coli* to commonly used antibiotics, particularly erythromycin and amoxicillin, raises significant concerns regarding the effectiveness of these medications in treating infections caused by this bacterium. The implications of such widespread resistance are critical, especially considering that erythromycin and amoxicillin are often prescribed for treating various bacterial infections. The complete lack of susceptibility observed in this study could reflect a broader trend of antibiotic misuse and overuse in both clinical and agricultural settings. These factors can lead to the selection of resistant strains, complicating treatment options and increasing the risk of treatment failure. Moreover, the complete resistance to these antibiotics suggests that alternative treatment strategies may need to be developed to effectively manage infections triggered by resilient *E. coli* strains.

The investigation revealed that a significant proportion of *E. coli* isolates, specifically 75.68% (28 out of 37), demonstrated resistance to tetracycline. This conclusion is dependable with the clarifications of Ali Almahdi and Kumar (2019), who noted that some *E. coli* isolates exhibited moderate resistance to tetracycline, while Sarker *et al*. (2019) reported instances of complete resistance to this antibiotic. The high level of tetracycline resistance among the isolates raises important questions about the potential underlying factors contributing to this phenomenon. One possible explanation is the inappropriate use of antibiotics, which may have led to the selection of resistant strains. In many regions, tetracycline and other antibiotics are often available without prescription, leading to self-medication and misuse. This misuse can accelerate the development of antibiotic resistance, as bacteria are exposed to suboptimal doses that do not fully eradicate the infection, allowing resistant variants to survive and proliferate. Moreover, the emergence of resistant *E. coli* strains poses significant challenges for public health, as tetracycline has been a commonly used antibiotic for treating various bacterial infections, particularly in both human and veterinary medicine. The presence of tetracycline-resistant *E. coli* not only complicates treatment options for healthcare providers but also raises concerns about the potential for these resistant strains to spread within communities or through the food chain.

Our analysis indicated that all *E. coli* isolates (100%, 37 out of 37) exhibited complete sensitivity to ceftriaxone and ciprofloxacin. This finding aligns with the observations made by Ripon *et al*. (2021), who also conveyed that *E. coli* strains were entirely susceptible to these two antibiotics. Furthermore, comparable outcomes were documented by Kamal *et al*. (2018) and Nahar *et al*. (2018), both of whom found that 100% of *E. coli* isolates were sensitive to ciprofloxacin. The high sensitivity rates to ceftriaxone and ciprofloxacin suggest that these antibiotics remain effective treatment options against *E. coli* infections in the studied population, which is encouraging given the rising prevalence of antibiotic resistance globally. In addition to these findings, the study demonstrated that 72.97% (27 out of 37) of *E. coli* isolates were susceptible to streptomycin, while over half (67.57%, 25 out of 37) showed susceptibility to gentamicin. These results highlight the potential effectiveness of these aminoglycoside antibiotics in managing infections triggered by *E. coli*. However, it is important to note that the susceptibility rates for streptomycin and gentamicin were lower compared to the complete sensitivity observed for ceftriaxone and ciprofloxacin. In contrast, research conducted by Faruque *et al*. (2019) reported susceptibility rates of 60% for streptomycin and 50% for gentamicin among *E. coli* isolates. The discrepancies between these studies may be attributed to various factors, including geographical differences, the specific population studied, and local antibiotic usage practices. The variation in susceptibility profiles emphasizes the need for ongoing monitoring of antibiotic resistance patterns, as these can evolve rapidly due to factors such as increased antibiotic pressure and changes in bacterial genetics. The implications of these findings are significant for clinical practice, particularly in terms of selecting appropriate empirical treatment regimens for *E. coli* infections.

The outcomes of this investigation revealed that a considerable majority, precisely 86.48% (32 out of 37; 95% CI: 40.74-76.60%), of the *E. coli* isolates demonstrated multidrug-resistant (MDR) characteristics. This aligns with the work of Haque *et al*. (2017), who reported similar MDR profiles among bacterial isolates from betel leaves. The presence of such high rates of MDR in *E. coli* isolates is concerning and highlights a potential public health issue, as these bacteria can complicate treatment options for infections. Among the identified MDR patterns, the "AMX-TE-E" pattern was the most prevalent, observed in 56.76% (21 out of 37) of the isolates. This finding is

consistent with the results reported by Hassan *et al*. (2014), which further validates the significance of this resistance pattern in *E. coli*. The dominance of the "AMX-TE-E" pattern suggests that these isolates may have developed resistance through selective pressure exerted by the use of amoxicillin (AMX), tetracycline (TE), and erythromycin (E), underscoring the importance of monitoring antibiotic use in agricultural and clinical settings. Additionally, the study found that all isolates had a MAR index bigger than 0.2, which parallels the findings of Osundiya *et al*. (2013). A MAR index above this threshold often indicates contamination from high-risk sources, where antibiotic usage is prevalent. The high MAR index values suggest that a considerable proportion of these isolates may originate from environments where antibiotics are extensively employed, further contributing to the problem of antibiotic resistance. The findings are further supported by Beshiru *et al*. (2022) and Fallah *et al*. (2022), who reported MAR index values exceeding the acceptable threshold of 0.2, with values reaching 0.60 and 0.75, respectively. These elevated MAR index values across various studies indicate a worrying trend of increasing antibiotic resistance and suggest that *E. coli* in these environments are likely exposed to multiple antibiotics, which may enhance their resistance mechanisms.

## 5. Conclusions

The global challenge of antimicrobial resistance (AMR) is intensified by the excessive application of antibiotics in food and food products. Strict regulations and policies are to be needed to mitigate health risks. Hygienic practices and avoiding antimicrobials are crucial in processing betel leaves. The findings of this study suggest that ciprofloxacin and ceftriaxone are more effective treatment alternatives for foodborne illnesses associated with *E. coli* contamination in betel leaves.

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## Data availability

All relevant data are presented within the manuscript.

## Conflict of interest

None to declare.

## Authors' contribution

Nasrin Islam: Investigation, Methodology; M. Rafiqul Islam: Investigation, Methodology, Writing-original draft; Razany Akter Liza: Investigation; Md. Ashiquen Nobi: Investigation; Sk Shaheenur Islam: Investigation, Writing-original draft; Mohammad Arif: Investigation; Mohammad Ferdousur Rahman Khan: Supervision; Seksun Samosornsuk: Data analysis, Writing-review & editing; Worada Samosornsuk: Data analysis, Writing-review & editing; S. M. Lutful Kabir: Conceptualization, Funding acquisition, Supervision. All authors have read and approved the final manuscript.

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