Comparative Study of Suspected Biofilm Characteristics of *Escherichia coli* isolated from in Tubewell and Tap Water

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A thesis submitted to the Department of Mathematics and Natural Science in partial fulfillment of the requirements for the BSc degree in Microbiology.

Mathematics and Natural Science

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Declaration

It is hereby declared that:

1. The thesis submitted is my original work while completing my degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material that has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. We have acknowledged all main sources of help.
5. We request a 24-month embargo on our thesis from the submission date, as we plan to submit our findings to a peer-reviewed journal for publication.

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Ethics Statement

This study was conducted using ethical guidelines and standards for research involving environmental samples and microbial analysis. No human or animal subjects were involved, and all samples were collected from public or private water sources with the consent of the respective owners or authorities, where applicable.

The research objectives were communicated to all stakeholders, and the data collected was used solely for academic and scientific purposes. Care was taken to minimize any environmental impact during the sample collection process. All laboratory procedures, including microbial cultures, were conducted following biosafety level 2 (BSL-2) protocols to ensure the safety of the researchers and the environment.

The study adhered to transparency, accuracy, and integrity in reporting results. All data were analyzed and presented without any manipulation, and any potential conflicts of interest have been disclosed.

Abstract

Access to clean water remains a critical public health challenge, particularly due to microbial contamination. This study analyzed 400 water samples, equally divided between tubewell and tap water systems from 50 urban and rural locations, to assess *E. coli* contamination, water quality, and biofilm formation. A total of 400 samples were analyzed, out of which 115 samples tested positive for *E. coli* (28.75%). Contamination was significantly higher in tap water (46.5%) than in tubewell water (11.0%), with 93 tap water samples and 22 tubewell water samples testing positive. Among tap water samples, 30% were safe for drinking, 50% were safe for household use but not for drinking, and 20% tap water samples were unsafe for any use, based on fecal coliform count whereas tubewell water showed 40% were safe for drinking, 50 % were safe for household use but not for drinking and 20% tubewell water samples were unsafe for any use.

The study assessed the antibiotic resistance patterns of *Escherichia coli* isolates from tap and tubewell water sources. The findings revealed a high prevalence of multidrug resistance (MDR), with the majority of isolates showing resistance to critical antibiotics. Meropenem (90.43%) and Piperacillin-Tazobactam (92.04%) exhibited the highest resistance, indicating potential carbapenemase-producing strains. Fluoroquinolone resistance was also significant, with Levofloxacin (80.73%) and Norfloxacin (70.75%) showing high resistance rates. Additionally, Cefepime (88.7%) and Ceftriaxone (70.37%) demonstrated strong resistance, raising concerns about the effectiveness of beta-lactam antibiotics. Sulfamethoxazole-Trimethoprim resistance reached 92.42%, while Azithromycin exhibited the lowest resistance (53.76%). The high resistance rates highlight urgent concerns regarding antimicrobial resistance (AMR) in waterborne *E. coli* isolates, emphasizing the need for enhanced water treatment, stricter antibiotic stewardship, and continuous surveillance to prevent the spread of resistant strains.

This study evaluated biofilm formation in *Escherichia coli* isolates from tap and tubewell water samples using optical density (OD) measurements at 570 nm. The results revealed strong biofilm formation in 22.5% of samples, moderate biofilm formation in 70%, and weak biofilm formation in 7.5%, indicating a high prevalence of bacterial persistence. Tap water samples exhibited higher biofilm OD values (mean: 1.18 ± 0.45) compared to tubewell water (mean: 1.02 ± 0.35), suggesting that municipal water pipelines provide a more conducive environment for biofilm

development. Locations such as Shariatpur, Rupganj, Comilla, and Joydevpur had the highest biofilm OD values, raising concerns about long-term microbial contamination and potential pathogen retention in drinking water systems. These findings emphasize the need for regular biofilm monitoring, improved water infrastructure, and enhanced disinfection strategies to mitigate health risks associated with biofilm-associated pathogens.

The molecular analysis for the *blaTEM* gene, a marker associated with beta-lactam resistance, revealed no detectable bands in any of the isolates. This absence suggests that *blaTEM* is not contributing to the observed resistance patterns in this study population. To evaluate this finding statistically, the antibiotic susceptibility test (AST) results for beta-lactam antibiotics commonly associated with *blaTEM*—including ampicillin, amoxicillin, ceftriaxone, cefepime, cefixime, and ceftazidime—were analyzed.

The analysis of *Escherichia coli* isolation and detection of the *fliC*H7 gene across 50 locations provides valuable insights into the prevalence and pathogenic potential of *E. coli* in water sources. Among 115 positive samples, molecular screening identified 18 samples as positive for the pathogenic *fliC*H7 gene, which is a key virulence determinant associated with pathogenic *E. coli* strains. Statistically, this accounts for 15.65% of the *E. coli*-positive samples and 4.5% of the total water samples screened. The locations with *fliC*H7-positive samples demonstrated a diverse geographical distribution.

Dedication

This study is dedicated to all those who work tirelessly to ensure access to safe and clean water for communities worldwide. To the researchers, educators, and public health professionals who strive to understand and mitigate the risks of waterborne diseases, your dedication inspires this work.

I also dedicate this study to my family and friends for their unwavering support and encouragement throughout this journey. Finally, this work is a tribute to the countless individuals affected by unsafe water, with the hope that this research contributes to meaningful change and improved water quality for all.

Acknowledgment

On a very personal note, I would like to say a heartfelt ‘thank you’ to my supervisor, Afroza Khan, and my co-supervisor Dr. Mahboob Hossain and Mr. Akash Ahmed for their valuable suggestions, constructive criticism, and constant encouragement while I was working on this research. I would appreciate the immense support from my academic advisors, your guidance and encouragement helped me to work through this research till the end.

I am also equally grateful to the Management & Science University BRAC University MNS Department for their support in all means and ways in enabling me to undertake this research work. Accomplished recognition includes Md. Firoze H. Haque, PhD, Chairperson and Head of the Department, and Nadia Sultana Deen, Program Coordinator of Microbiology for their guidance and encouragement throughout this process. I also thank the laboratory staff and technical team for their cooperation during sample collection as well as analysis processes. Your commitment and cooperation have made it possible to produce this work.

I would like to express my deep gratitude to my family and friends who have supported, encouraged, and tolerated me during the completion of this work. Your confidence in me has been my strength and one vital component of it.

Last of all, the author wishes to thank the concerned community and people who granted their sources of water for sampling. I deeply appreciate the cooperation and participation you had in this research. It is my wish that this study can help enhance water safety to enhance community health.

Thanks to all of those who have contributed their time and effort, advice, and numerous ideas towards this cause.

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List of Acronyms

AMR - Antimicrobial Resistance

AST - Antimicrobial Susceptibility Testing

BPS - Base Pairs

BSL-2 - Biosafety Level 2

CFU - Colony-Forming Units

CLSI - Clinical and Laboratory Standards Institute

CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats

DNA - Deoxyribonucleic Acid

EAEC - Enteroaggregative *Escherichia coli*

EHEC - Enterohemorrhagic *Escherichia coli*

EIEC - Enteroinvasive *Escherichia coli*

ETEC - Enterotoxigenic *Escherichia coli*

ExPEC - Extraintestinal Pathogenic *Escherichia coli*

KCN - Potassium Cyanide

LB - Luria-Bertani

LEE - Locus of Enterocyte Effacement

LT - Heat-Labile Toxin

MDR - Multidrug Resistance

M-FC - Membrane Fecal Coliform

MIC - Minimum Inhibitory Concentration

MIU - Motility-Indole-Urease

MR-VP - Methyl Red-Voges Proskauer

NDM - New Delhi Metallo-beta-lactamase

ONPG - Ortho-Nitrophenyl-β-galactoside

PAI - Pathogenicity Island

PCR - Polymerase Chain Reaction

RFLP - Restriction Fragment Length Polymorphism

RNA - Ribonucleic Acid

ST - Heat-Stable Toxin

STEC - Shiga Toxin-producing *Escherichia coli*

TAE - Tris-Acetate-EDTA

UTI - Urinary Tract Infection

WHO - World Health Organization

Chapter 1 Introduction

1.1: Background of the study

Access to clean and safe drinking water is essential, yet microbial contamination persists as a major public health concern worldwide. Tubewell and tap water systems, commonly used for drinking, often harbor biofilms that act as reservoirs for pathogens, including *E. coli*. As a key indicator of fecal contamination, *E. coli* presence not only reflects poor water quality but also indicates the potential presence of other harmful microorganisms (Gwenzi et al., 2018). Biofilms, composed of microbial communities encased in extracellular polymeric substances, enhance bacterial survival, facilitate horizontal gene transfer of antimicrobial resistance (AMR) genes, and resist disinfection efforts (Hall-Stoodley et al., 2004; Costerton et al., 1999).

Urban water systems are prone to biofilm contamination due to aging infrastructure, pipeline leaks, and cross-connections with sewage, while rural tube wells are often compromised by environmental pollutants like agricultural runoff and poor sealing practices (Sultana et al., 2019; Ahmed et al., 2020). Studies from Bangladesh and Nepal highlight the influence of infrastructure and environmental factors on contamination levels, emphasizing the need for localized interventions (Rahman et al., 2020; Ghimire et al., 2020).

This study collected biofilm samples from urban and rural water sources, detecting *E. coli* using UTI and M-FC agar, followed by PCR confirmation. The ELISA reader quantified biofilm formation, highlighting pathogen resilience. Antibiotic susceptibility testing (AST) revealed multidrug-resistant *E. coli* strains, emphasizing public health risks. Findings suggest urban areas need infrastructure upgrades, while rural regions require community-led water safety and tubewell maintenance. Molecular analyses of virulence and resistance genes emphasize the need for targeted water quality monitoring and antimicrobial resistance surveillance.

1.2 Research Objectives

1.2.1 General Objective

The general objective of this study is to investigate the microbial contamination and biofilm formation in tube wells and tap water systems from urban and rural areas, with a focus on identifying the presence of *Escherichia coli*, assessing water quality, evaluating antimicrobial resistance profiles, and analyzing the virulence and pathogenicity of biofilm-associated bacteria to contribute to improved water safety and public health management strategies.

1.2.2: Specific objectives

This study aims to evaluate microbial contamination in tubewell and tap water systems across urban and rural areas. Specific objectives include the following:

- Collect suspected biofilm samples from tubewell and tap water systems.
- Isolate *E. coli* on UTI agar and fecal coliforms on M-FC agar.
- Perform morphological, biochemical, and molecular identification of isolates.
- Use PCR to confirm *E. coli* presence and detect virulence and resistance genes.
- Conduct antimicrobial susceptibility testing (AST) and quantify biofilm formation.
- Compare contamination levels and biofilm formation between urban and rural systems.
- Provide recommendations to improve water quality and reduce biofilm-associated risks.

1.3 Literature Review

1.3.1 The Organism

Escherichia coli

1.3.1.1 Taxonomy

Domain: Bacteria

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gamma proteobacteria

Order: Enterobacterial

Family: Enterobacteriaceae

Genus: Escherichia

Species: *Escherichia coli*

1.3.1.2 Historical Background of *Escherichia coli*

Escherichia coli (*E. coli*), a Gram-negative, facultative anaerobe, was first discovered in 1885 by Theodor Escherich, a German pediatrician and bacteriologist. Escherich isolated *E. coli* from the feces of healthy infants, identifying it as part of the natural intestinal flora (Tenaillon et al., 2010). Initially referred to as *Bacterium coli commune*, the name was later changed to *Escherichia coli* in 1919, honoring Escherich's contributions to microbiology (Savageau, 1983). His groundbreaking work not only illuminated the role of *E. coli* in the gut but also paved the way for its use in modern microbiological research.

In its natural environment, *E. coli* exists as an avirulent saprophyte, engaging in mutualistic relationships with its host. It plays a vital role in the intestinal microbiota by synthesizing essential vitamins and aiding in nutrient absorption (Hudault et al., 2001). However, beyond its role as a commensal organism, *E. coli* has become an invaluable model organism in scientific research. Despite its prominence as a commensal organism and research tool, *E. coli* also includes strains capable of causing disease. Pathogenic *E. coli* strains are classified into distinct pathotypes based on their mechanisms of pathogenicity. These include Enterohemorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), and Enteroaggregative *E. coli* (EAEC) (Kaper et al., 2004). These strains are associated with diseases ranging from mild diarrhea to severe conditions such as hemolytic uremic syndrome (Russo & Johnson, 2003). One of the significant applications of *E. coli* has been in water quality assessment. Its presence in water is a reliable indicator of fecal contamination, signaling potential public health risks (WHO, 2011).

As a fecal indicator bacteria, *E. coli* has become instrumental in water quality management, helping to identify contamination sources and guide public health interventions. A study by Ahmed et al. (2020) emphasized the importance of monitoring *E. coli* in water systems, particularly in regions where infrastructure is insufficient to prevent contamination.

Recent advancements in microbiological analysis have further enhanced our understanding of *E. coli*. Studies have explored its genetic variation, biofilm-forming capabilities, and susceptibility to antimicrobial agents (Kumar et al., 2018).

1.3.1.3 General Characteristics of *E.coli*

Escherichia coli (E. coli), a Gram-negative, rod-shaped bacterium from the Enterobacteriaceae family, is known for its adaptability and metabolic versatility. Measuring 1–2 μm in length, *E. coli* thrives at 37°C, the internal temperature of its hosts (Tenaillon et al., 2010). It is a facultative anaerobe that ferments lactose and produces gas, aiding its identification in laboratory diagnostics (Madigan et al., 2018). Most strains are methyl-red positive, indicating glucose fermentation, but some exhibit delayed or non-lactose fermentation (Kaper et al., 2004).

E.coli is indole-positive, Voges-Proskauer-negative, and distinguishes itself by not utilizing citrate as a sole carbon source or producing extracellular DNase or urease (Blattner et al., 1997; Ranjbar et al., 2021). Its motility, supported by peritrichous flagella, enhances its adaptability (Savageau, 1983). *E. coli* is the dominant species in the genus Escherichia, comprising over 99% of isolates (Savageau, 1983). This dominance underscores its ecological adaptability and relevance in microbial ecosystems (Madigan et al., 2018).

Naturally, *E. coli* resides in the large intestine of vertebrates, aiding in nutrient absorption and synthesizing vitamins like vitamin K (Hudault et al., 2001). It is also found in environmental reservoirs like soil, water, and feces, with 10^8 cells per gram of fecal matter, making it a key indicator of fecal contamination (Ahmed et al., 2020; WHO, 2011).

1.3.1.4 Role as normal flora

Escherichia coli (E. coli) is a key member of the normal microbiota in the vertebrate gastrointestinal tract, especially in the large intestine, where it performs several beneficial functions for the host (Tenaillon et al., 2010). As a commensal organism, *E. coli* aids in nutrient metabolism, synthesizes vitamins like K and B-complex vitamins, and plays a role in immune regulation (Hudault et al., 2001; Savageau, 1983).

E. coli helps in food breakdown, and nutrient absorption, and suppresses pathogenic bacteria through colonization resistance by competing for nutrients and occupying mucosal surfaces (Kaper et al., 2004). Additionally, it supports immune function by initiating and maintaining immune responses, which help the host distinguish beneficial microbes from harmful pathogens (Blattner et al., 1997).

1.3.1.5 *E.coli* as an indicator of fecal contamination

Escherichia coli (E. coli) serves as a critical indicator for fecal contamination in environmental and water quality monitoring due to its presence in the gastrointestinal tract and its strong link to fecal pollution (WHO, 2011; Tenaillon et al., 2010). Unlike general coliforms, *E. coli* is specific to fecal sources, making it a precise contamination marker (Kaper et al., 2004). It is excreted in large quantities—up to 10^8 cells per gram of fecal matter—and signals potential public health risks from other pathogens like viruses and protozoa (Ahmed et al., 2020; Russo & Johnson, 2003). Table 1 highlights the acceptable levels of *Escherichia coli* in water as defined by the WHO and US EPA, both requiring less than 1 CFU/100mL to consider water safe for consumption. *E. coli* is detectable using selective media like MacConkey and MFC agar, leveraging lactose fermentation, with PCR enhancing detection accuracy (Ranjbar et al., 2021). Despite environmental strains potentially complicating results (Gwenzi et al., 2018), *E. coli* remains essential for water quality assessments and public health monitoring.

According to the World Health Organization (WHO) Guidelines for Drinking Water Quality (2022) and the US Environmental Protection Agency (US EPA) National Primary Drinking Water Regulations (2022), the acceptable level of fecal coliform in drinking water is 0 CFU/100mL. Water with less than 200 CFU/100mL is considered safe for household use, including washing, cleaning, and bathing, but not for consumption. Water containing ≥200 CFU/100mL is deemed highly contaminated and unsafe for any use.

Table 1: Acceptable level of fecal coliforms by the WHO and US EPA.

Criteria	WHO	US EPA	Interpretation
Drinking Water Standard	<1 CFU/100mL	<1 CFU/100mL	Safe for drinking & all domestic uses
Safe for Household Use (Non-Drinking)	<200 CFU/100mL	<200 CFU/100mL	Suitable for washing, bathing, and other domestic chores
Unsafe Water	≥200 CFU/100mL	≥200 CFU/100mL	Highly contaminated, not recommended for any use

1.3.1.6 Variability in *E. coli*: From Commensals to Pathogenic Strains

Escherichia coli (E. coli) is typically a harmless commensal in the human gut, aiding health by synthesizing nutrients like vitamin K2 and preventing pathogenic growth through competitive exclusion (Suvarna et al., 1998; Hudault et al., 2001). However, it can become an opportunistic pathogen under conditions like immunosuppression or tissue trauma (Kaper et al., 2004).

Pathogenic *E. coli* strains, classified into ETEC, EPEC, STEC, EIEC, EAEC, and DAEC, cause diseases ranging from diarrhea to systemic infections (Kaper et al., 2004). Extraintestinal infections include UTIs, meningitis, pneumonia, and sepsis, often arising in compromised tissues (Kaper et al., 2004).

Pathogenicity is driven by virulence genes acquired via horizontal gene transfer through plasmids, bacteriophages, or pathogenicity islands (PAIs), enabling colonization, immune evasion, and tissue damage (Kaper et al., 2004). These adaptations make pathogenic *E. coli* highly versatile and virulent.

The genome of *E. coli*, particularly the K-12 strain, has been fully sequenced, enabling advancements in biotechnology with tools like plasmids and CRISPR-Cas for genetic engineering and recombinant protein production (Rosano & Ceccarelli, 2014). *E. coli* has also been instrumental in studying DNA replication, transcription, translation, and gene regulation, including the operon model and lac operon (Jacob & Monod, 1961). Additionally, *E. coli* has provided insights into horizontal gene transfer, crucial for understanding genetic evolution and antibiotic resistance (Madigan et al., 2018).

Antimicrobial resistance (AMR) is a global health concern, particularly with multidrug-resistant (MDR) *Escherichia coli (E. coli)* in water systems, often linked to treated and untreated drinking water. Studies show *E. coli* isolates frequently exhibit resistance mechanisms like ESBL and quinolone resistance genes, contributing to severe diarrheal diseases (Chen et al., 2021). Human and agricultural practices, inadequate sanitation, and antibiotic misuse drive AMR, with water bodies serving as reservoirs for resistant pathogens (Hu et al., 2008). Resistance mechanisms like New Delhi Metallo-β-lactamase (NDM) exacerbate challenges in treatment (Moellering, 2010).

1.3.2 Clinical characteristics and epidemiology

1.3.2.1 Epidemiology

Escherichia coli (E. coli) functions as both a harmless commensal in the human gut and a pathogen causing various infections. Commensal strains support gut health, while pathogenic variants, such as enterotoxigenic *E. coli* (ETEC) and Shiga toxin-producing *E. coli* (STEC), are linked to traveler's diarrhea and severe hemolytic uremic syndrome, respectively (Kaper et al., 2004). Extraintestinal pathogenic *E. coli* (ExPEC) is the primary cause of urinary tract infections (UTIs), accounting for 85–95% of cases globally, and contributing significantly to neonatal meningitis and nosocomial bacteremia (Russo & Johnson, 2003).

Pathogenic *E. coli* spreads via contaminated food and water, with outbreaks tied to undercooked meat, unpasteurized dairy, and raw vegetables (WHO, 2020). In hospitals, *E. coli* accounts for 13% of pathogens in intensive care units and is the most common Gram-negative pathogen in Canada (Fluit et al., 2000). Antimicrobial resistance (AMR) in *E. coli* strains, particularly to third-generation cephalosporins and fluoroquinolones, complicates treatment and raises morbidity and mortality rates (CDC, 2021). Surveillance from England shows rising *E. coli* bacteremia, underscoring the need for antibiotic stewardship (UK Health Security Agency, 2023). Treatment varies by condition, with diarrheal infections managed via fluid replacement and systemic infections requiring antibiotics (Howard et al., 2014)

1.3.2.1.1 Enterohemorrhagic *E. coli* (EHEC)

Shiga toxin-producing *E. coli* (STEC), also known as EHEC, causes severe intestinal diseases by producing Shiga toxins (Stx1, Stx2), leading to protein synthesis inhibition, cell death, and tissue damage. Symptoms include diarrhea, abdominal pain, and hemolytic uremic syndrome (HUS), a severe complication causing kidney failure. It is transmitted through contaminated food and water. Its common serotype is O157:H7, but others like O26 and O103 are also significant. (Etcheverría & Padola, 2013)

1.3.2.1.2 Enteropathogenic *E. coli* (EPEC)

EPEC is a major diarrheal pathogen in children, especially in developing countries, causing watery diarrhea, dehydration, and vomiting. It spreads via contaminated food, water, and the fecal-oral route, with low infective doses. EPEC forms attaching and effacing (A/E) lesions on intestinal cells through the LEE pathogenicity island, disrupting nutrient absorption. (Etcheverría & Padola, 2013)

1.3.2.1.3 Enterotoxigenic *E. coli* (ETEC)

ETEC is a leading cause of traveler's diarrhea and childhood diarrheal deaths in developing countries. It produces a heat-labile toxin (LT) and heat-stable toxin (ST), disrupting intestinal function and causing watery diarrhea, abdominal cramps, and dehydration. ETEC spreads through contaminated food and water, with symptoms lasting 3–4 days. (Etcheverría & Padola, 2013)

1.3.2.1.4 Enteroinvasive *E. coli* (EIEC)

EIEC closely resembles Shigella in causing dysentery-like symptoms, including bloody diarrhea, fever, and abdominal cramps. It invades colonic epithelial cells using a virulence plasmid (pINV) and causes inflammation and ulceration. Transmission occurs via contaminated food, water, and poor hygiene, with outbreaks often linked to resource-limited settings.(Etcheverría & Padola, 2013)

1.3.2.1.5 Enteroaggregative *E. coli* (EAEC)

EAEC causes acute and chronic diarrhea, particularly in malnourished children and immunocompromised individuals. It adheres to intestinal cells in a stacked-brick pattern, forms biofilms, and produces toxins like Pet and EAST-1, contributing to persistent diarrhea. (Etcheverría & Padola, 2013)

1.4 Transmission of *E.coli*

Escherichia coli (E. coli) is transmitted via multiple pathways, primarily through the fecal-oral route in areas with poor sanitation, where contaminated water, food, or surfaces facilitate its spread (Kaper et al., 2004). Foodborne transmission, often linked to contaminated beef, unpasteurized milk, and raw produce, is significant, particularly for pathogenic strains like enterohemorrhagic *E. coli* (EHEC), highlighting the importance of food safety measures (Rangel et al., 2005). Waterborne transmission is prevalent in regions with inadequate water treatment, with enterotoxigenic *E. coli* (ETEC) commonly spreading through untreated drinking or recreational water (WHO, 2020). Person-to-person transmission is common in close-contact settings, especially for low-infectious-dose strains such as EHEC (Tarr et al., 2005). Animals, particularly cattle, serve as reservoirs, enabling zoonotic transmission via contact with animal feces or contaminated environments (Gyles, 2007). Environmental transmission occurs as *E. coli* persists in soil and water, entering the food chain through crops or improper waste disposal (Franz et al., 2011). Rare vertical transmission during childbirth can cause neonatal infections, including meningitis and sepsis, primarily from extraintestinal pathogenic *E. coli* (ExPEC) (Kim et al., 2008).

1.4.1 Transmission of *E.coli* through the water system

Access to clean water is standard in developed regions but remains a critical challenge in many developing nations, where inadequate sanitation leads to waterborne diseases causing over 1.5 million child deaths annually (Fenwick, 2006; WHO, 2008). Contaminated water with human or animal feces is a major risk, with pathogens like *Escherichia coli (E. coli)* posing severe public health threats, especially with the rise of antibiotic-resistant strains. Studies report widespread resistance, such as ESBL-producing *E. coli* in Nigeria (41.7%) and outbreaks like *E. coli* O157:H7 in unchlorinated water in Wyoming (Beshiru et al., 2024; Olsen et al., 2002). Addressing this issue requires improved water treatment, regular monitoring, and global action against antibiotic misuse to reduce resistance and ensure safer water systems (Hasan et al., 2022).

Chapter 2 Materials and Methods

2.1 General Procedure and Equipment

This study was carried out at Brac University under the Mathematical and Natural Sciences Department. The entire study methodology is briefly given below.

Table 2: Work Flow of this study

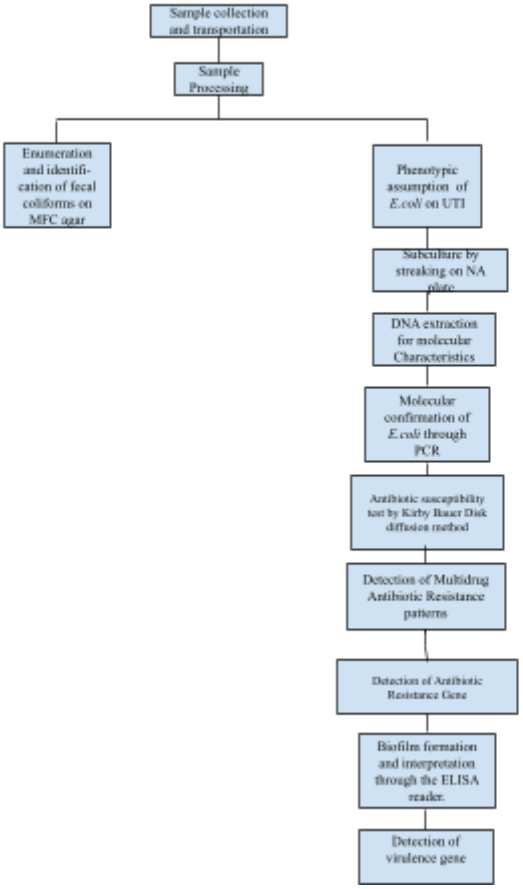

Table 2 outlines the workflow of the study, starting with sample collection and transportation, followed by enumeration and confirmation of *E. coli* through cultural, biochemical, and molecular methods. The process also includes antibiotic susceptibility testing using the Kirby-Bauer method, detection of multidrug antibiotic resistance genes, detection of antibiotic resistance genes, suspected biofilm formation analysis via an ELISA reader and detection of pathogenic genes, ensuring a comprehensive evaluation of water quality and microbial characteristics.

2.2 Media Preparation

2.2.1: Transport Media Preparation

For the transport media, peptone water is a broth medium used for the preservation and transport of microorganisms. It maintains bacterial viability while preventing excessive growth due to its lack of nutritional ingredients. Peptone provides peptides and amino acids for bacterial maintenance, while NaCl maintains osmotic balance. Its neutral pH supports bacterial survival during short transport without promoting growth.

2.2.2 Culture Media Preparation for Spreading

For the cultivation of *E.coli* in the spreading technique, HiCrome UTI Agar is a selective chromogenic medium designed for the identification and differentiation of urinary tract pathogens, including *E. coli*. *E. coli* produces purple colonies due to chromogenic substrates

2.2.3 Culture Media Preparation for Streaking

Nutrient agar is a non-selective medium that cultivates non-fastidious bacteria and isolates pure colonies. Peptone provides amino acids and nitrogen, beef extract supplies vitamins and nutrients, and NaCl maintains osmotic balance for optimal growth.

2.2.4 Stock Media Preparation

For stock preparation, T_1N_1 media is a selective culture medium primarily used for the stock of organisms for a long period. This medium is enriched with specific nutrients and inhibitors to promote the growth of target organisms while suppressing non-target flora.

2.2.5 Antibiotic Susceptibility Test (AST) Media Preparation

MHA is a standardized medium used for the antibiotic susceptibility test method. It provides an optimal environment for bacterial growth and accurate evaluation. Casein hydrolysate and beef extract supply nutrients for bacterial growth, while starch stabilizes the medium. Its buffered pH and consistent formulation ensure reliable disc diffusion test results.

2.3 Methods

2.3.1 Mapping of Tubewells and Tap Water

To analyze microbial contamination and suspected biofilm presence, samples were collected from tap and tubewell sources across regions like Basila, Rajshahi, Khulna, and Brahmanbaria. Collecting from the same sites in each region enabled localized comparisons of contamination and suspected biofilm formation.

This approach assessed suspected biofilms as reservoirs for pathogens like *E. coli*, examining regional differences in microbial loads and environmental factors influencing biofilm development. The study underscores health risks posed by suspected biofilms in untreated tubewell and treated tap water systems, guiding actions to improve water quality.

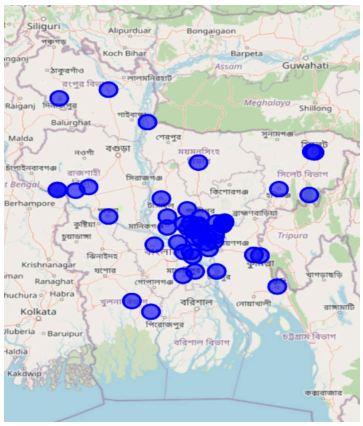

Figure 1: Mapping of Sample collection

Figure 1 illustrates the geographic distribution of the sampling sites for this study, covering diverse urban and rural areas. The mapped locations represent the strategic collection points for water samples to ensure comprehensive coverage and variability in the analysis.

2.3.2 Sampling Sites and Sample Collection

To detect microorganisms in suspected biofilms from water systems, samples were collected from the inner surfaces of tap water systems and tube wells under sterile conditions to prevent contamination. Tap water and tubewell suspected biofilm samples were taken from the same locations for direct comparison.

Suspected biofilms were scraped or swabbed using sterile cotton swabs, which were placed in sterile tubes containing peptone water to maintain viability during transport. Approximately 10 µL of biofilm was collected from each site and transported in pre-labeled containers. Samples were stored on ice at 4°C and processed within 24 hours.

Figure 2: Sample collection of tap and tubewell

Figure 2 illustrates the sample collection process from tap and tube well water sources, where sterile techniques were used to collect suspected biofilm samples.

2.3.3 Sample Size

The study analyzed a total of 400 water samples, comprising 200 from tubewell sources and 200 from tap water systems, ensuring equal representation of both water sources for a robust comparison of microbial contamination and suspected biofilm presence. The sample size was carefully chosen to provide statistically significant data for assessing the prevalence of *Escherichia coli (E. coli)* and other pathogens across varied geographic and environmental contexts. Samples were collected from diverse locations, ensuring a broad spectrum of environmental and infrastructural conditions.

Table 3: Geographic Coverage of Sample Collection in This Study

	Location	Total tap sample size	Total tubewell sample size
1	Keraniganj	4	4
2	Demra	4	4
3	Kachpur	4	4
4	Narsingdi	4	4
5	Rajshahi	4	4
6	Gazipur	4	4
7	Sylhet	4	4
8	Sonargaon	4	4
9	Mawa	4	4
10	Sreenagar	4	4
11	Khulna	4	4
12	Brahmanbaria	4	4
13	Savar	4	4
14	Maymanesigh	4	4
15	Chandpur	4	4
16	Shariatpur	4	4
17	Dinajpur	4	4
18	Rupganj	4	4
19	Comilla	4	4
20	Tongi	4	4
21	Madhabdi	4	4
22	Natore	4	4

23	Dhamrai	4	4
24	Ashulia	4	4
25	Dohar	4	4
26	Tangail	4	4
27	Munshiganj	4	4
28	Feni	4	4
29	Maynamati	4	4
30	Pabna	4	4
31	Shibpur	4	4
32	Ghorashal	4	4
33	Kaliganj	4	4
34	Shampur	4	4
35	Narayanganj	4	4
36	Faridpur	4	4
37	Hemayetpur	4	4
38	Aminbazar	4	4
39	Manikganj	4	4
40	Mirzapur	4	4
41	Shonir Akhra	4	4
42	Joydevpur	4	4
43	Bongshal	4	4
44	Bhulta	4	4
45	Modonpur	4	4

46	Rupshi	4	4
47	Shibchar	4	4
48	Bhanga	4	4
49	Sadarpur	4	4
50	Raipur	4	4

2.3.4 Dilution of the sample

The quantity of bacteria in the suspected biofilm samples was decreased in this study by successive dilution, which made precise colony counting and analysis possible. A 10^{-2} dilution was applied to the samples. To make sure that the bacteria were distributed evenly throughout the solution, each suspected biofilm sample was first homogenized in sterile peptone water. 1 mL of the homogenized material was added to a sterile test tube with 9 mL of sterile peptone water to create a 10^{-1} first dilution. In the second dilution, 10^{-2} dilution was obtained by transferring 1 mL of the 10^{-1} dilution to a different tube containing 9 mL of sterile peptone water. The spreading technique was used to plate these dilutions in selective media.

Figure 3: Serial dilution of each sample

Figure 3 shows the serial dilution process used to reduce bacterial concentration in suspected biofilm samples, enabling accurate colony counting. Successive dilutions ensured even distribution for plating on selective media.

2.3.5 Spreading Technique

This technique ensures that bacterial colonies develop independently, enabling precise colony isolation and counting. For inoculation, a sterile glass spreader was utilized. The spreader was first cleaned by soaking it in ethanol and then quickly setting it on fire to get rid of any impurities. Following sample dilutions up to 10^{-2}, 100 µL of each diluted sample was pipetted onto selective medium plates, such as *Escherichia coli* identified using HiCrome UTI agar, and m-FC media was used to check the water quality. The sterilized glass spreader was used to evenly distribute the inoculum. The plates were incubated at 37°C for 24 to 48 hours.

2.3.6 Streaking Technique

The streaking technique involves distributing bacteria over the surface of an agar plate to separate individual colonies from a mixed culture. Individual bacterial cells separate as a result of this process, which dilutes the germs as they spread. A sterile inoculating loop was used to select well-isolated colonies, which were then streaked using the four-quadrant streaking method onto Nutrient Agar (NA) plates. This method aids in the separation of pure colonies from a mixed culture, which is essential for further phenotypic and biochemical analyses. Various organisms were cultured for 24 hours at 37°C on all inoculation plates.

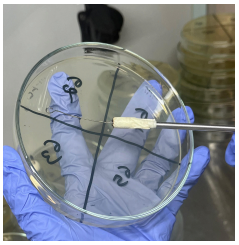

Figure 4: Streaking of each sample to separate individual colonies from a mixed culture.

Figure 4 illustrates the four-quadrant streaking method used to isolate individual colonies from mixed cultures for further analysis.

2.3.7 Sample Processing

Suspected biofilm and water samples were processed to isolate and identify *Escherichia coli* (*E. coli*) and assess water quality. Suspected biofilm swabs were vortexed in sterile phosphate-buffered saline (PBS) to dislodge cells, and 100 µL of the suspension was streaked onto UTI agar plates, which were incubated at 37°C for 24 hours.

For water samples, 100 mL was filtered through a 0.45 µm membrane, placed on m-FC agar, and incubated at 44.5°C for 24 hours. Blue colonies on m-FC agar indicated fecal coliforms, including *E. coli*. This protocol ensured the reliable detection of *E. coli* in both sample types.

2.3.7.1 Detection and Enumeration of Fecal Coliforms

Fecal coliforms, including *E. coli*, were detected and enumerated using membrane filtration and selective media. A 100 mL water sample was filtered through a sterile 0.45 µm membrane under vacuum, concentrating bacteria on the filter surface. The filter was placed on m-FC agar, a selective medium for thermotolerant coliforms, and incubated at 44.5°C for 24 hours.

Figure 5: Each water sample was passed through the membrane filter

Blue colonies on m-FC agar were identified as fecal coliforms, with *E. coli* distinguished by its morphology. Colony counts were expressed as CFU/100 mL, and samples exceeding regulatory limits were flagged as non-compliant, indicating fecal contamination.

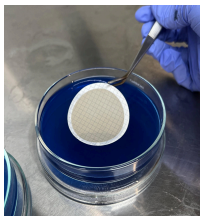

Figure 6: Cultivation of the membrane filter on m-FC agar plates

2.3.7.2 Isolation and Identification of *E.coli* on selective media

E. coli isolation and identification were performed using selective media. Samples were vortexed in normal saline, and 100 µL of the suspension was streaked onto UTI agar plates, which produced pink colonies for *E. coli.* The plates were incubated at 37°C for 24 hours, and characteristic *E. coli* colonies were observed.

For water samples, the membrane filtration method was used, with 100 mL of water passed through a 0.45 µm filter and placed onto m-FC agar, which supports thermotolerant coliforms like *E. coli.* Plates were incubated at 44.5°C for 24 hours, and blue colonies were identified as *E. coli*. These colonies were subcultured onto nutrient agar and incubated at 37°C for 24 hours.

2.4 Biochemical confirmation

The isolates were further confirmed as *E. coli* through a series of biochemical tests, including the MIU (Motility, Indole, Urease) test, Catalase test, Oxidase test, Citrate utilization test, Methyl Red (MR) test, and Voges-Proskauer (VP) test.

Table 4: Biochemical test results of *E.coli*

Organism	**Catalase**	**Oxidase**	**Glucose Fermentation**	**Sucrose Fermentation**	**Lactose Fermentation**	**Gas Production**	**H_2S Production**
	+ve	-ve	+ve	variable	+ve	+ve	-ve
Escherichia coli	**H_2S**	**Motility**	**Indole**	**Urease**	**MR**	**VP**	**Citrate utilization**
	+ve	+ve	+ve	-ve	+ve	-ve	-ve

This table summarizes the biochemical test results for *Escherichia coli (E. coli)*, highlighting its key characteristics.

2.4.1 MIU Test

The MIU (Motility, Indole, Urease) test was used to identify *Escherichia coli* by assessing motility, indole production, and urease activity. The test organism was inoculated into MIU agar and incubated at 35-37°C for 18-24 hours. After incubation, motility was confirmed by the spreading of growth beyond the stab line. Indole production was positive, indicated by a pink-red ring after the addition of Kovac's reagent, while urease activity was negative, as no color change to pink-red occurred.

2.4.2 Catalase Test

The catalase test detected the presence of the catalase enzyme in *E. coli*, which breaks down hydrogen peroxide into water and oxygen. A small portion of a bacterial colony was mixed with 3% hydrogen peroxide. Rapid bubble formation confirmed a positive catalase test, indicating *E. coli's* ability to produce catalase.

2.4.3 Oxidase Test

The oxidase test was used to detect cytochrome c oxidase activity. A bacterial colony was transferred to a glass slide or filter paper, and an oxidase reagent was added. The absence of a color change confirmed that *E. coli* was oxidase-negative, consistent with its classification within the Enterobacteriaceae family.

2.4.4 Citrate Utilization Test

The citrate utilization test assessed *E. coli'*s ability to use citrate as the sole carbon source. Simmons citrate agar was inoculated and incubated at 35-37°C for 18-24 hours. *E. coli* showed a negative result, with no color change in the medium (remained green), indicating its inability to utilize citrate.

2.4.5 MR-VP Test

The MR-VP test distinguished *E. coli* based on its glucose fermentation pathway. After inoculating and incubating MR-VP broth at 35-37°C, the methyl red test indicated a positive result with a red color, showing mixed acid fermentation. The Voges-Proskauer test, detecting 2,3-butanediol production, was negative, as no color change occurred. These results confirmed *E. coli* as MR-positive and VP-negative.

2.5 Molecular Identification of *E. coli* isolates

Fresh cultures were obtained for analysis using the PCR method, and DNA was extracted through the boiling technique. Following initial identification based on the colony morphology of the organisms, a species-specific PCR was conducted to confirm the identification of the isolates at the molecular level.

2.5.1 Total Cell DNA isolation

DNA extraction was performed using the boiling method. Colony isolates from T_1N_1 stock were streaked onto nutrient agar and incubated overnight at 37°C. If growth was insufficient, subcultures were made. For extraction, colonies were inoculated into 400 µL TE buffer in labeled microcentrifuge tubes, vortexed, and centrifuged at 13,000 rpm for 10 minutes. The supernatant was discarded, and another 400 µL TE buffer was added. The tubes were placed in a 100°C water bath for 10 minutes, then centrifuged again. The supernatant was transferred to new tubes and stored at -20°C for future use.

2.5.2 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was used to detect *E. coli* by amplifying the 16S rRNA genes, ECO-1 and ECO-2. Weekly samples from designated areas were tested using a 13 µL reaction mixture, comprising 2 µL DNA template, 6 µL 2X Emerald PCR Master Mix (Takara Bio), 1 µL of each primer, and 3 µL nuclease-free water. Precise pipetting and brief centrifugation ensured thorough mixing and bubble-free reactions. Details regarding the target gene, primer sequences, PCR thermocycler annealing temperature, and amplicon sizes are provided in Table 5.

Table 5: Primer sequence and PCR conditions for 16sRNA gene for *E.coli*

Primer name	Sequence	Process	Temperature (°C)	Time	Segment	Reference
ECO-1	**5'-GACCTCGGTTTAGTTCACAGA-3'**	**Initial Denaturation**	**95 °C**	**7 min**	**Segment 1**	**(Conte et al., 2006)**
		Denaturation	**94°C**	**1 min**	**Segment 2 (35 cycles)**	
ECO-2	**5'-CACACGCTGACCGCTGACCA-3'**	**Annealing**	**55°C**	**1 min**		
		Extension	**72°C**	**1 min**		
		Final Extension	**72°C**	**7 min**	**Segment 3**	

2.5.3 Electrophoretic Analysis of PCR-Amplified DNA

A 1.5% agarose gel was prepared by dissolving 1.5 g of agarose in 98 mL distilled water and 2 mL 50x TAE buffer, heated until clear. After cooling, 4 µL of ethidium bromide was added, and the solution was poured into a gel tray with a comb. The gel solidified at room temperature for 10 minutes, and the comb was removed. The tray was placed in an electrophoresis chamber with a 1x TAE buffer. Following PCR, 4 µL of each PCR product and a 100 bp ladder were loaded onto the gel. Products were separated at 100 V for 35 minutes, and the gel was visualized under UV light to detect the target gene.

2.6 Phenotypic Identification

2.6.1 Gram Staining Preparation

Gram staining differentiates Gram-positive bacteria from Gram-negative bacteria based on cell wall composition. Gram-positive bacteria retain a crystal violet stain, appearing purple, while Gram-negative bacteria take up safranin, appearing pink. Prepare the bacterial smear by placing a small bacterial sample on a glass slide, air-drying, and heat-fixing it. Stain with crystal violet for 1 minute, rinse, apply Gram's iodine for 1 minute, and rinse again. Decolorize with alcohol for 10-30 seconds, rinse, and counterstain with safranin for 30 seconds. Finally, rinse, blot dry, and examine under a microscope. Gram-positive bacteria appear purple, while Gram-negative bacteria appear pink.

2.7 Antibiotic Susceptibility testing

The antibiotic susceptibility patterns of *Escherichia coli* isolates were evaluated using the standard Kirby-Bauer disk diffusion method, performed according to Clinical and Laboratory Standards Institute (CLSI, 2020) guidelines. A total of 18 antibiotic discs representing 12 antibiotic groups were selected to assess the susceptibility of the isolates, including Cefepime (30 µg), Sulfamethoxazole/Trimethoprim (25 µg), Chloramphenicol (30 µg), Ceftazidime (30 µg), Ampicillin (10 µg), Levofloxacin (5 µg), Meropenem (10 µg), Doxycycline (30 µg), Piperacillin/Tazobactam (100 µg), Kanamycin (30 µg), Nalidixic Acid (30 µg), Nitrofurantoin (300 µg), Norfloxacin (10 µg), Aztreonam (30 µg), Amoxicillin (20 µg), Cefixime (5 µg), Tetracycline (30 µg), Ceftriaxone (30 µg), and Azithromycin (15 µg).

2.7.1 Procedure

PCR-confirmed *E. coli* isolates were subcultured on nutrient agar and incubated overnight at 37°C. Pure colonies were suspended in 0.9% saline, with turbidity adjusted to the 0.5 McFarland standard. Suspensions were spread on Mueller-Hinton agar (MHA) plates, and antibiotic discs were placed using sterile forceps. Plates were incubated at 37°C for 18-24 hours, and inhibition zones were measured in millimeters. Results were interpreted following the Clinical and Laboratory Standards Institute CLSI guidelines (CLSI, 2021).

2.7.2 Interpretation

Antimicrobial susceptibility testing (AST) for Escherichia coli was interpreted following CLSI 2020 guidelines, which standardize the evaluation of bacterial growth inhibition zone diameters. Table 5 presents AST breakpoints for various antibiotics, categorized as Sensitive (S), Intermediate (I), or Resistant (R) based on inhibition zones (mm). These guidelines ensure the accurate identification of effective antibiotics, support clinical decision-making, and aid in monitoring antimicrobial resistance in waterborne *E. coli* isolates, addressing public health concerns (CLSI, 2020).

Table 6: Zone of diameter interpretation for *E.coli*

Groups	**Antibiotic Used**	**Sensitive**	**Intermediate**	**Resistant**
Penicillin	Ampicillin	**≥17**	**14-16**	**≤13**
	Piperacillin-Tazobactam	**≥21**	**18-20**	**≤17**
	Amoxicillin	**≥18**	**14-17**	**≤13**
3G Cephalosporin	Ceftriaxone	**≥23**	**20-22**	**≤19**
	Ceftazidime	**≥21**	**18-20**	**≤17**
	Cefixime	**≥19**	**16-18**	**≤15**
4G Cephalosporin	Cefepime	**≥25**	**19-24**	**≤18**
Carbapenem	Meropenem	**≥23**	**20-22**	**≤19**
Aminoglycoside	Kanamycin	**≥18**	**14-17**	**≤13**
Quinolone	Norfloxacin	**≥17**	**13-16**	**≤12**
	Nalidixic Acid	**≥19**	**≥19**	**≤13**
	Levofloxacin	**≥17**	**14-16**	**≤13**

Tetracycline	Doxycycline	≥**16**	**13-15**	≤**12**
	Tetracycline	≥**15**	**12-14**	≤**11**
Sulfonamide	Sulfamethoxazole Trimethoprim	≥**16**	**11-15**	≤**10**
Phenicol	Chloramphenicol	≥**18**	**13-17**	≤**12**
Monobactams	Aztreonam	≥**21**	**18-20**	≤**17**
Nitrofurans	Nitrofurantoin	**≥17**	**-**	≤**16**
Macrolide	Azithromycin	**≥19**	**-**	**≤18**

This table outlines the zone of inhibition diameters for *E. coli* in antibiotic susceptibility testing, categorized into sensitive, intermediate, and resistant interpretations based on CLSI guidelines. It provides a benchmark for evaluating antibiotic efficacy.

2.7.3 Multidrug Antibiotic Resistance (AMR) Patterns

Multidrug antibiotic resistance (MDR) is a critical global health concern, particularly in waterborne pathogens like *Escherichia coli*. The increasing prevalence of MDR bacteria in drinking water sources poses a severe threat to public health, as resistant strains limit treatment options and increase the risk of untreatable infections. In this study, we aimed to assess the antibiotic resistance patterns of *E. coli* isolates collected from tap and tubewell water sources across multiple locations. Antibiotic susceptibility testing (AST) was performed using the Kirby-Bauer disk diffusion method, following Clinical and Laboratory Standards Institute (CLSI) guidelines.

A diverse range of antibiotics from multiple classes, including beta-lactams, carbapenems, fluoroquinolones, aminoglycosides, sulfonamides, and tetracyclines, were tested to determine the resistance profiles of the isolated *E. coli* strains.

MDR status was defined as resistance to three or more distinct antibiotic classes (Magiorakos et al., 2012). The presence of MDR *E. coli* in drinking water highlights the potential risks of environmental antibiotic contamination, improper wastewater disposal, and inadequate water treatment measures. The study further emphasizes the need for routine water quality monitoring, antimicrobial resistance surveillance, and public health interventions to mitigate the spread of drug-resistant bacteria. By identifying MDR patterns in environmental isolates, this research contributes to understanding the emerging threat of antibiotic-resistant pathogens in water systems and underscores the necessity of effective water management strategies to prevent the dissemination of resistant strains.

2.7.4 PCR Amplification for detecting Antibiotic resistance gene

The detection of antibiotic-resistance genes is a critical aspect of understanding the spread and impact of antimicrobial resistance (AMR) in bacterial populations. This study employed primers specifically designed to target the *bla TEM* gene, a significant marker for extended-spectrum beta-lactamase (ESBL) production in bacteria. The forward primer (TEM-F) and reverse primer (TEM-R) were utilized to amplify a 1080 bp product, confirming the presence of the *bla TEM* gene. This gene is associated with resistance to beta-lactam antibiotics, including penicillins and cephalosporins, posing a major threat to the effective treatment of bacterial infections. By targeting the *bla TEM* gene, this study underscores the prevalence of ESBL-producing pathogens, emphasizing the urgent need for surveillance, appropriate antibiotic stewardship, and strategies to mitigate the growing challenge of AMR in clinical and environmental settings.

Table 7: List of primers used in ESBL gene detection

Types of primers	**Target gene**	**Primer name**	**Sequence**	**Product size**	**Reference**
ESBL primers	*Bla TEM*	TEM-F	F-AAAATTCT TGAAGACG	1080bp	(Sharma et al., 2010)
		TEM-R	R-TTACCAAT GCTTAATCA		

The PCR conditions for detecting the *TEM* gene were carefully optimized to ensure specific and efficient amplification. The reaction began with an initial denaturation at 95°C for 3 minutes to completely denature the DNA strands, followed by a denaturation step at 95°C for 30 seconds in each cycle to maintain the separation of strands. The annealing step, performed at 51°C for 30 seconds, allowed the primers to bind to their complementary sequences on the template DNA. The extension was carried out at 78°C for 7 minutes to facilitate the elongation of the DNA strand, ensuring the accurate synthesis of the target gene. The process concluded with a final extension at 72°C for 10 minutes to complete any remaining incomplete DNA strands.

These conditions provided reliable amplification of the *bla TEM* gene, which is critical for understanding the presence and spread of antibiotic resistance mechanisms.

Table 8: PCR condition for *TEM* gene

Process	**Temperature**	**Time**	**Segment**
Initial denaturation	95°C	3 min	Segment 1
Denaturation	95°C	30 s	Segment 2
Annealing	51°C	30 s	
Extension	78°C	7 min	
Final Extension	72°C	10 min	Segment 3

2.8 Suspected biofilm formation

2.8.1 Procedure of Tissue Culture Plate (TCP) Method

The suspected biofilm formation procedure was conducted using the Tissue Culture Plate (TCP) method, adapted from Christensen et al. (1985). Bacteria were subcultured on Nutrient Agar (NA) for purity, inoculated into trypticase soy broth (TSB), and incubated at 37°C for 24 hours. The culture was diluted 1:100 in fresh TSB, and 96-well TCP plates were inoculated with 0.2 ml per well, with uninoculated broth as a negative control. After 24 hours of incubation at 37°C, non-adherent cells were removed by washing with phosphate-buffered saline (PBS). Suspected biofilms were stained with 0.1% crystal violet for 10 minutes, washed, solubilized with 33% glacial acetic acid, and quantified at 570 nm using an ELISA reader. The assay followed a widely used standardized protocol with modifications to meet experimental needs.

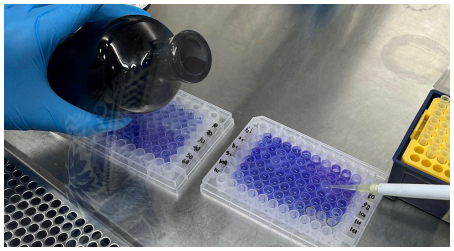

Figure 7: Adding Glacial acetic acid (33%) to the samples for suspected biofilm observations using an ELISA reader.

2.9.2 Interpretation

The interpretation of suspected biofilm formation results was conducted based on the Optical Density (OD) values obtained at 570 nm using an ELISA reader. According to the classification outlined by Stepanović et al. (2000), suspected biofilm-forming ability is categorized into four distinct groups: non-adherent (OD ≤ ODc), weakly adherent (ODc < OD ≤ 2×ODc), moderately adherent (2×ODc < OD ≤ 4×ODc), and strongly adherent (OD > 4×ODc), where ODc represents the mean OD of the negative control plus three standard deviations.

Table 9: Interpretation of optical density data for detection of suspected biofilm formation

OD (Optical Density)	Biofilm Formation	Description
< 0.1	Non-adherent	No significant biofilm formation detected.
0.1 - 0.2	Weakly adherent	Biofilm formation detected but at a low adherence level.
0.2 - 0.3	Moderately adherent	Moderate levels of biofilm formation observed, indicating significant adherence.
> 0.3	Strongly adherent	High biofilm formation observed, indicating strong bacterial adherence.

This study classified bacterial isolates based on their biofilm-forming potential by averaging OD values from triplicates and comparing them to control values. Isolates with OD values(>4×ODc) were categorized as strong biofilm formers, indicating high biofilm production and adherence. Notably, strong biofilm formers were more common in urban tap water, likely due to pipe surface irregularities or nutrient availability (Stepanović et al., 2000). Conversely, isolates from tube well water showed fewer strong biofilm formers, consistent with findings that nutrient scarcity limits biofilm development (Christensen et al., 1985).

2.9 Molecular Identification of Virulence gene of *E.coli*

The identification of pathogenic *Escherichia coli* was conducted by screening for key virulence-associated genes through precise PCR-based detection techniques. For the serotyping of *E. coli* O157:H7, the *flicH7* gene was specifically targeted, as it serves as a critical marker for distinguishing this strain, renowned for its pathogenic potential. This approach ensured reliable detection and characterization of the pathogenic variants of *E. coli* in the study.

Table 10: List of primers used to detect pathogenic genes in this study

Target gene	Primer name	Primer sequence	Product size	Reference
*fli*C	FLICH7-F	F-5'-GCGCTGT CGAGTTCTAT CGAGC-3'	625 bp	(Al-Ajmi et al., 2020)
	FLICH7-R	R-5'-CAACGG TGACTTTATC GCCATTCC-3'		

The composition of the PCR reaction mixture used for detecting the *flicH7* gene, a marker for *E. coli* O157:H7. Each reaction, totaling 25 µL, contained 12.5 µL of Thermo Scientific™ DreamTaq™ Green PCR Master Mix as the core component for enzymatic amplification. Forward (*flicH7-F*) and reverse (*flicH7-R*) primers were added in equal amounts of 0.5 µL each to specifically target the *flicH7* gene sequence. Additionally, 2 µL of nuclease-free water and 8.5 µL of template DNA provided the target genetic material for amplification.

Table 11: PCR mixture for flicH7 gene for detection of *E.coli* O157:H7

The PCR reaction mixture for 1 sample pf total 25µL volume		
1.	Thermo ScientificTM DreamTaqTM Green PCR Master Mix	12.5µL
2	Primer flicH7-F	0.5µL
3	Primer flicH7-R	0.5µL
4	Nuclease free water	2µL
5.	Template DNA	8.5µL

The table outlines the optimized PCR conditions used for the detection of the *flicH7* gene in *E. coli* O157:H7. The process begins with an initial denaturation step at 95°C for 2 minutes (Segment 1) to ensure complete separation of the DNA strands. This is followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 15 seconds, and extension at 68°C for 1 minute (Segment 2). The final extension step is carried out at 68°C for 10 minutes (Segment 3) to ensure full-length amplification of the target DNA.

These conditions are choosed to maximize specificity and efficiency in amplifying the *flicH7* gene, critical for the molecular identification of pathogenic *E. coli*.

Table 12: PCR condition for flic gene for detection of *E.coli* O157:H7

Process	**Temperature**	**Time**	**Segment**
Initial denaturation	95°C	2 min	Segment 1
Denaturation	94°C	30 s	Segment 2 (30 cycles)
Annealing	58°C	15 s	
Extension	68°C	1 min	
Final Extension	68°C	10 min	Segment 3

2.9.1 Electrophoretic Analysis of PCR-Amplified DNA

A 100 mL, 1.5% agarose gel solution was prepared by measuring 1.5 grams of agarose into a flask. To this, 2 mL of 50x TAE buffer and 28 mL of distilled water were added. The mixture was then heated in a microwave until the agarose completely dissolved and the solution became clear. After cooling, 4 µL of ethidium bromide (EtBr) was added. The solution was poured into a gel tray, and a comb was placed near one end. The gel was left undisturbed at room temperature for 10 minutes to solidify evenly.

Once the gel solidified, the comb was carefully removed, and the gel tray was placed in an electrophoresis chamber, ensuring the gel was submerged in the 1x TAE electrophoresis buffer. Following PCR, 4 µL of PCR products from each reaction was loaded onto the gel, along with a 100 bp ladder. The PCR products were separated in the 1.5% agarose gel using lx TAE buffer at 100 V for 40 minutes. The gel was then visualized under UV light to detect the target gene.

Chapter 3 Results

3.1 Fecal Coliform count in CFU/100mL

Water quality analysis is essential for public health, particularly in distinguishing between safe and unsafe water sources. This study assessed 200 tap water and 200 tubewell water samples from various locations, categorizing them based on fecal coliform levels according to WHO and US EPA guidelines. The results were classified into two primary groups: Safe for Household Use but Not Drinking (fecal coliform count: 1–10 CFU/mL) and Unsafe for Any Use (fecal coliform count: >100 CFU/mL). The statistical breakdown of the findings provides a comparative analysis of water quality across the two sources.

Among the 200 tap water samples, 135 (67.5%) were classified as safe for household use, while 65 (32.5%) were deemed unsafe for any use due to high fecal coliform contamination. In contrast, tubewell water exhibited better quality, with 160 out of 200 samples (80.0%) categorized as safe for household use and only 40 samples (20.0%) being unsafe for any use. These results indicate that tap water is more contaminated than tubewell water, suggesting a higher risk of fecal contamination in municipal or pipeline-based water systems.

Table 13: Fecal Coliform interpretation in this study

Location	Sample type	Fecal coliform count (CFU/mL)	Interpretation based on WHO and US EPA
Keraniganj	Tap 1	TNTC	Unsafe for any use
Keraniganj	Tap 2	1	Safe for Household Use but Not Drinking
Keraniganj	Tap 3	6	Safe for Household Use but Not Drinking
Keraniganj	Tap 4	TNTC	Unsafe for any use
Keraniganj	Tubewell 1	TNTC	Unsafe for any use
Keraniganj	Tubewell 2	8	Safe for Household Use but Not Drinking
Keraniganj	Tubewell 3	4	Safe for Household

			Use but Not Drinking
Keraniganj	Tubewell 4	10	Safe for Household Use but Not Drinking
Demra	Tap 1	4	Safe for Household Use but Not Drinking
Demra	Tap 2	4	Safe for Household Use but Not Drinking
Demra	Tap 3	TNTC	Unsafe for any use
Demra	Tap 4	6	Safe for Household Use but Not Drinking
Demra	Tubewell 1	8	Safe for Household Use but Not Drinking
Demra	Tubewell 2	8	Safe for Household Use but Not Drinking
Demra	Tubewell 3	TNTC	Unsafe for any use
Demra	Tubewell 4	160	Unsafe for any use
Kachpur	Tap 1	5	Safe for Household Use but Not Drinking
Kachpur	Tap 2	TNTC	Unsafe for any use
Kachpur	Tap 3	TNTC	Usafe for any use
Kachpur	Tap 4	3	Safe for Household Use but Not Drinking
Kachpur	Tubewell 1	TNTC	Unsafe for any use
Kachpur	Tubewell 2	1	Safe for Household Use but Not Drinking
Kachpur	Tubewell 3	5	Safe for Household Use but Not Drinking
Kachpur	Tubewell 4	10	Safe for Household Use but Not Drinking
Narsingdi	Tap 1	2	Safe for Household Use but Not Drinking

Narsingdi	Tap 2	TNTC	Unsafe for any use
Narsingdi	Tap 3	6	Safe for Household Use but Not Drinking
Narsingdi	Tap 4	TNTC	Unsafe for any use
Narsingdi	Tubewell 1	2	Safe for Household Use but Not Drinking
Narsingdi	Tubewell 2	5	Safe for Household Use but Not Drinking
Narsingdi	Tubewell 3	2	Safe for Household Use but Not Drinking
Narsingdi	Tubewell 4	TNTC	Unsafe for any use
Rajshahi	Tap 1	5	Safe for Household Use but Not Drinking
Rajshahi	Tap 2	1	Safe for Household Use but Not Drinking
Rajshahi	Tap 3	TNTC	Unsafe for any use
Rajshahi	Tap 4	TNTC	Unsafe for any use
Rajshahi	Tubewell 1	8	Safe for Household Use but Not Drinking
Rajshahi	Tubewell 2	3	Safe for Household Use but Not Drinking
Rajshahi	Tubewell 3	105	Safe for Household Use but Not Drinking
Rajshahi	Tubewell 4	180	Safe for Household Use but Not Drinking
Gazipur	Tap 1	2	Safe for Household Use but Not Drinking
Gazipur	Tap 2	5	Safe for Household Use but Not Drinking
Gazipur	Tap 3	7	Safe for Household Use but Not Drinking

Gazipur	Tap 4	TNTC	Unsafe for any use
Gazipur	Tubewell 1	139	Safe for Household Use but Not Drinking
Gazipur	Tubewell 2	TNTC	Unsafe for any use
Gazipur	Tubewell 3	TNTC	Unsafe for any use
Gazipur	Tubewell 4	2	Safe for Household Use but Not Drinking
Sylhet	Tap 1	199	Safe for Household Use but Not Drinking
Sylhet	Tap 2	159	Safe for Household Use but Not Drinking
Sylhet	Tap 3	8	Safe for Household Use but Not Drinking
Sylhet	Tap 4	TNTC	Unsafe for any use
Sylhet	Tubewell 1	167	Safe for Household Use but Not Drinking
Sylhet	Tubewell 2	4	Safe for Household Use but Not Drinking
Sylhet	Tubewell 3	10	Safe for Household Use but Not Drinking
Sylhet	Tubewell 4	3	Safe for Household Use but Not Drinking
Sonargaon	Tap 1	TNTC	Unsafe for any use
Sonargaon	Tap 2	TNTC	Unsafe for any use
Sonargaon	Tap 3	144	Safe for Household Use but Not Drinking
Sonargaon	Tap 4	10	Safe for Household Use but Not Drinking
Sonargaon	Tubewell 1	TNTC	Unsafe for any use
Sonargaon	Tubewell 2	5	Safe for Household Use but Not Drinking

Sonargaon	Tubewell 3	10	Safe for Household Use but Not Drinking
Sonargaon	Tubewell 4	TNTC	Unsafe for any use
Mawa	Tap 1	10	Safe for Household Use but Not Drinking
Mawa	Tap 2	9	Safe for Household Use but Not Drinking
Mawa	Tap 3	TNTC	Unsafe for any use
Mawa	Tap 4	TNTC	Unsafe for any use
Mawa	Tubewell 1	TNTC	Unsafe for any use
Mawa	Tubewell 2	3	Safe for Household Use but Not Drinking
Mawa	Tubewell 3	TNTC	Unsafe for any use
Mawa	Tubewell 4	2	Safe for Household Use but Not Drinking
Sreenagar	Tap 1	10	Safe for Household Use but Not Drinking
Sreenagar	Tap 2	153	Safe for Household Use but Not Drinking
Sreenagar	Tap 3	181	Safe for Household Use but Not Drinking
Sreenagar	Tap 4	TNTC	Unsafe for any use
Sreenagar	Tubewell 1	TNTC	Unsafe for any use
Sreenagar	Tubewell 2	6	Safe for Household Use but Not Drinking
Sreenagar	Tubewell 3	171	Safe for Household Use but Not Drinking
Sreenagar	Tubewell 4	4	Safe for Household Use but Not Drinking
Khulna	Tap 1	TNTC	Unsafe for any use

Khulna	Tap 2	7	Safe for Household Use but Not Drinking
Khulna	Tap 3	TNTC	Unsafe for any use
Khulna	Tap 4	5	Safe for Household Use but Not Drinking
Khulna	Tubewell 1	TNTC	Unsafe for any use
Khulna	Tubewell 2	1	Safe for Household Use but Not Drinking
Khulna	Tubewell 3	1	Safe for Household Use but Not Drinking
Khulna	Tubewell 4	1	Safe for Household Use but Not Drinking
Brahmanbaria	Tap 1	129	Safe for Household Use but Not Drinking
Brahmanbaria	Tap 2	TNTC	Unsafe for any use
Brahmanbaria	Tap 3	TNTC	Unsafe for any use
Brahmanbaria	Tap 4	TNTC	Unsafe for any use
Brahmanbaria	Tubewell 1	TNTC	Unsafe for any use
Brahmanbaria	Tubewell 2	8	Safe for Household Use but Not Drinking
Brahmanbaria	Tubewell 3	TNTC	Unsafe for any use
Brahmanbaria	Tubewell 4	2	Safe for Household Use but Not Drinking
Savar	Tap 1	134	Safe for Household Use but Not Drinking
Savar	Tap 2	7	Safe for Household Use but Not Drinking
Savar	Tap 3	TNTC	Unsafe for any use
Savar	Tap 4	TNTC	Unsafe for any use
Savar	Tubewell 1	TNTC	Unsafe for any use

Savar	Tubewell 2	130	Safe for Household Use but Not Drinking
Savar	Tubewell 3	9	Safe for Household Use but Not Drinking
Savar	Tubewell 4	TNTC	Unsafe for any use
Mymensingh	Tap 1	5	Safe for Household Use but Not Drinking
Mymensingh	Tap 2	TNTC	Unsafe for any use
Mymensingh	Tap 3	123	Safe for Household Use but Not Drinking
Mymensingh	Tap 4	TNTC	Unsafe for any use
Mymensingh	Tubewell 1	TNTC	Unsafe for any use
Mymensingh	Tubewell 2	7	Safe for Household Use but Not Drinking
Mymensingh	Tubewell 3	TNTC	Unsafe for any use
Mymensingh	Tubewell 4	TNTC	Unsafe for any use
Chandpur	Tap 1	189	Safe for Household Use but Not Drinking
Chandpur	Tap 2	TNTC	Unsafe for any use
Chandpur	Tap 3	9	Safe for Household Use but Not Drinking
Chandpur	Tap 4	TNTC	Unsafe for any use
Chandpur	Tubewell 1	1	Safe for Household Use but Not Drinking
Chandpur	Tubewell 2	TNTC	Unsafe for any use
Chandpur	Tubewell 3	7	Safe for Household Use but Not Drinking
Chandpur	Tubewell 4	TNTC	Unsafe for any use
Shariatpur	Tap 1	4	Safe for Household Use but Not Drinking

Shariatpur	Tap 2	TNTC	Unsafe for any use
Shariatpur	Tap 3	TNTC	Unsafe for any use
Shariatpur	Tap 4	6	Safe for Household Use but Not Drinking
Shariatpur	Tubewell 1	TNTC	Unsafe for any use
Shariatpur	Tubewell 2	TNTC	Unsafe for any use
Shariatpur	Tubewell 3	4	Safe for Household Use but Not Drinking
Shariatpur	Tubewell 4	TNTC	Unsafe for any use
Dinajpur	Tap 1	TNTC	Unsafe for any use
Dinajpur	Tap 2	9	Safe for Household Use but Not Drinking
Dinajpur	Tap 3	4	Safe for Household Use but Not Drinking
Dinajpur	Tap 4	9	Safe for Household Use but Not Drinking
Dinajpur	Tubewell 1	TNTC	Unsafe for any use
Dinajpur	Tubewell 2	4	Safe for Household Use but Not Drinking
Dinajpur	Tubewell 3	2	Safe for Household Use but Not Drinking
Dinajpur	Tubewell 4	7	Safe for Household Use but Not Drinking
Rupganj	Tap 1	9	Safe for Household Use but Not Drinking
Rupganj	Tap 2	9	Safe for Household Use but Not Drinking
Rupganj	Tap 3	2	Safe for Household Use but Not Drinking
Rupganj	Tap 4	TNTC	Unsafe for any use

Rupganj	Tubewell 1	155	Safe for Household Use but Not Drinking
Rupganj	Tubewell 2	TNTC	Unsafe for any use
Rupganj	Tubewell 3	TNTC	Unsafe for any use
Rupganj	Tubewell 4	TNTC	Unsafe for any use
Comilla	Tap 1	178	Safe for Household Use but Not Drinking
Comilla	Tap 2	117	Safe for Household Use but Not Drinking
Comilla	Tap 3	2	Safe for Household Use but Not Drinking
Comilla	Tap 4	2	Safe for Household Use but Not Drinking
Comilla	Tubewell 1	TNTC	Unsafe for any use
Comilla	Tubewell 2	2	Safe for Household Use but Not Drinking
Comilla	Tubewell 3	103	Safe for Household Use but Not Drinking
Comilla	Tubewell 4	170	Safe for Household Use but Not Drinking
Tongi	Tap 1	TNTC	Unsafe for any use
Tongi	Tap 2	2	Safe for Household Use but Not Drinking
Tongi	Tap 3	TNTC	Unsafe for any use
Tongi	Tap 4	176	Safe for Household Use but Not Drinking
Tongi	Tubewell 1	TNTC	Unsafe for any use
Tongi	Tubewell 2	10	Safe for Household Use but Not Drinking
Tongi	Tubewell 3	5	Safe for Household Use but Not Drinking

Tongi	Tubewell 4	5	Safe for Household Use but Not Drinking
Madhabdi	Tap 1	TNTC	Unsafe for any use
Madhabdi	Tap 2	114	Safe for Household Use but Not Drinking
Madhabdi	Tap 3	TNTC	Unsafe for any use
Madhabdi	Tap 4	4	Safe for Household Use but Not Drinking
Madhabdi	Tubewell 1	6	Safe for Household Use but Not Drinking
Madhabdi	Tubewell 2	7	Safe for Household Use but Not Drinking
Madhabdi	Tubewell 3	9	Safe for Household Use but Not Drinking
Madhabdi	Tubewell 4	8	Safe for Household Use but Not Drinking
Natore	Tap 1	8	Safe for Household Use but Not Drinking
Natore	Tap 2	TNTC	Unsafe for any use
Natore	Tap 3	TNTC	Unsafe for any use
Natore	Tap 4	7	Safe for Household Use but Not Drinking
Natore	Tubewell 1	7	Safe for Household Use but Not Drinking
Natore	Tubewell 2	9	Safe for Household Use but Not Drinking
Natore	Tubewell 3	4	Safe for Household Use but Not Drinking
Natore	Tubewell 4	1	Safe for Household Use but Not Drinking
Dhamrai	Tap 1	TNTC	Unsafe for any use

Dhamrai	Tap 2	3	Safe for Household Use but Not Drinking
Dhamrai	Tap 3	9	Safe for Household Use but Not Drinking
Dhamrai	Tap 4	1	Safe for Household Use but Not Drinking
Dhamrai	Tubewell 1	5	Safe for Household Use but Not Drinking
Dhamrai	Tubewell 2	6	Safe for Household Use but Not Drinking
Dhamrai	Tubewell 3	9	Safe for Household Use but Not Drinking
Dhamrai	Tubewell 4	8	Safe for Household Use but Not Drinking
Ashulia	Tap 1	10	Safe for Household Use but Not Drinking
Ashulia	Tap 2	179	Safe for Household Use but Not Drinking
Ashulia	Tap 3	179	Safe for Household Use but Not Drinking
Ashulia	Tap 4	TNTC	Unsafe for any use
Ashulia	Tubewell 1	2	Safe for Household Use but Not Drinking
Ashulia	Tubewell 2	4	Safe for Household Use but Not Drinking
Ashulia	Tubewell 3	9	Safe for Household Use but Not Drinking
Ashulia	Tubewell 4	2	Safe for Household Use but Not Drinking
Dohar	Tap 1	5	Safe for Household Use but Not Drinking
Dohar	Tap 2	4	Safe for Household Use but Not Drinking

Dohar	Tap 3	2	Safe for Household Use but Not Drinking
Dohar	Tap 4	8	Safe for Household Use but Not Drinking
Dohar	Tubewell 1	1	Safe for Household Use but Not Drinking
Dohar	Tubewell 2	10	Safe for Household Use but Not Drinking
Dohar	Tubewell 3	1	Safe for Household Use but Not Drinking
Dohar	Tubewell 4	8	Safe for Household Use but Not Drinking
Tangail	Tap 1	145	Safe for Household Use but Not Drinking
Tangail	Tap 2	5	Safe for Household Use but Not Drinking
Tangail	Tap 3	TNTC	Unsafe for any use
Tangail	Tap 4	6	Safe for Household Use but Not Drinking
Tangail	Tubewell 1	8	Safe for Household Use but Not Drinking
Tangail	Tubewell 2	9	Safe for Household Use but Not Drinking
Tangail	Tubewell 3	7	Safe for Household Use but Not Drinking
Tangail	Tubewell 4	9	Safe for Household Use but Not Drinking
Munshiganj	Tap 1	10	Safe for Household Use but Not Drinking
Munshiganj	Tap 2	TNTC	Unsafe for any use
Munshiganj	Tap 3	173	Safe for Household Use but Not Drinking

Munshiganj	Tap 4	2	Safe for Household Use but Not Drinking
Munshiganj	Tubewell 1	5	Safe for Household Use but Not Drinking
Munshiganj	Tubewell 2	8	Safe for Household Use but Not Drinking
Munshiganj	Tubewell 3	5	Safe for Household Use but Not Drinking
Munshiganj	Tubewell 4	2	Safe for Household Use but Not Drinking
Feni	Tap 1	1	Safe for Household Use but Not Drinking
Feni	Tap 2	9	Safe for Household Use but Not Drinking
Feni	Tap 3	163	Safe for Household Use but Not Drinking
Feni	Tap 4	TNTC	Unsafe for any use
Feni	Tubewell 1	4	Safe for Household Use but Not Drinking
Feni	Tubewell 2	2	Safe for Household Use but Not Drinking
Feni	Tubewell 3	7	Safe for Household Use but Not Drinking
Feni	Tubewell 4	2	Safe for Household Use but Not Drinking
Maynamati	Tap 1	TNTC	Unsafe for any use
Maynamati	Tap 2	TNTC	Unsafe for any use
Maynamati	Tap 3	TNTC	Unsafe for any use
Maynamati	Tap 4	10	Safe for Household Use but Not Drinking
Maynamati	Tubewell 1	3	Safe for Household Use but Not Drinking

Maynamati	Tubewell 2	10	Safe for Household Use but Not Drinking
Maynamati	Tubewell 3	9	Safe for Household Use but Not Drinking
Maynamati	Tubewell 4	3	Safe for Household Use but Not Drinking
Pabna	Tap 1	10	Safe for Household Use but Not Drinking
Pabna	Tap 2	5	Safe for Household Use but Not Drinking
Pabna	Tap 3	7	Safe for Household Use but Not Drinking
Pabna	Tap 4	TNTC	Unsafe for any use
Pabna	Tubewell 1	3	Safe for Household Use but Not Drinking
Pabna	Tubewell 2	5	Safe for Household Use but Not Drinking
Pabna	Tubewell 3	10	Safe for Household Use but Not Drinking
Pabna	Tubewell 4	2	Safe for Household Use but Not Drinking
Shibpur	Tap 1	2	Safe for Household Use but Not Drinking
Shibpur	Tap 2	8	Safe for Household Use but Not Drinking
Shibpur	Tap 3	TNTC	Unsafe for any use
Shibpur	Tap 4	5	Safe for Household Use but Not Drinking
Shibpur	Tubewell 1	5	Safe for Household Use but Not Drinking
Shibpur	Tubewell 2	6	Safe for Household Use but Not Drinking

Shibpur	Tubewell 3	7	Safe for Household Use but Not Drinking
Shibpur	Tubewell 4	4	Safe for Household Use but Not Drinking
Ghorashal	Tap 1	2	Safe for Household Use but Not Drinking
Ghorashal	Tap 2	1	Safe for Household Use but Not Drinking
Ghorashal	Tap 3	TNTC	Unsafe for any use
Ghorashal	Tap 4	5	Safe for Household Use but Not Drinking
Ghorashal	Tubewell 1	9	Safe for Household Use but Not Drinking
Ghorashal	Tubewell 2	3	Safe for Household Use but Not Drinking
Ghorashal	Tubewell 3	4	Safe for Household Use but Not Drinking
Ghorashal	Tubewell 4	10	Safe for Household Use but Not Drinking
Kaliganj	Tap 1	9	Safe for Household Use but Not Drinking
Kaliganj	Tap 2	5	Safe for Household Use but Not Drinking
Kaliganj	Tap 3	7	Safe for Household Use but Not Drinking
Kaliganj	Tap 4	3	Safe for Household Use but Not Drinking
Kaliganj	Tubewell 1	7	Safe for Household Use but Not Drinking
Kaliganj	Tubewell 2	1	Safe for Household Use but Not Drinking
Kaliganj	Tubewell 3	6	Safe for Household Use but Not Drinking

Kaliganj	Tubewell 4	4	Safe for Household Use but Not Drinking
Shampur	Tap 1	3	Safe for Household Use but Not Drinking
Shampur	Tap 2	2	Safe for Household Use but Not Drinking
Shampur	Tap 3	7	Safe for Household Use but Not Drinking
Shampur	Tap 4	2	Safe for Household Use but Not Drinking
Shampur	Tubewell 1	3	Safe for Household Use but Not Drinking
Shampur	Tubewell 2	7	Safe for Household Use but Not Drinking
Shampur	Tubewell 3	2	Safe for Household Use but Not Drinking
Shampur	Tubewell 4	10	Safe for Household Use but Not Drinking
Narayanganj	Tap 1	100	Safe for Household Use but Not Drinking
Narayanganj	Tap 2	TNTC	Unsafe for any use
Narayanganj	Tap 3	50	Safe for Household Use but Not Drinking
Narayanganj	Tap 4	34	Safe for Household Use but Not Drinking
Narayanganj	Tubewell 1	1	Safe for Household Use but Not Drinking
Narayanganj	Tubewell 2	7	Safe for Household Use but Not Drinking
Narayanganj	Tubewell 3	2	Safe for Household Use but Not Drinking
Narayanganj	Tubewell 4	10	Safe for Household Use but Not Drinking

Faridpur	Tap 1	10	Safe for Household Use but Not Drinking
Faridpur	Tap 2	15	Safe for Household Use but Not Drinking
Faridpur	Tap 3	TNTC	Unsafe for any use
Faridpur	Tap 4	78	Safe for Household Use but Not Drinking
Faridpur	Tubewell 1	34	Safe for Household Use but Not Drinking
Faridpur	Tubewell 2	140	Safe for Household Use but Not Drinking
Faridpur	Tubewell 3	121	Safe for Household Use but Not Drinking
Faridpur	Tubewell 4	120	Safe for Household Use but Not Drinking
Hemayetpur	Tap 1	5	Safe for Household Use but Not Drinking
Hemayetpur	Tap 2	2	Safe for Household Use but Not Drinking
Hemayetpur	Tap 3	TNTC	Unsafe for any use
Hemayetpur	Tap 4	70	Safe for Household Use but Not Drinking
Hemayetpur	Tubewell 1	43	Safe for Household Use but Not Drinking
Hemayetpur	Tubewell 2	54	Safe for Household Use but Not Drinking
Hemayetpur	Tubewell 3	TNTC	Unsafe for any use
Hemayetpur	Tubewell 4	139	Safe for Household Use but Not Drinking
Aminbazar	Tap 1	60	Safe for Household Use but Not Drinking
Aminbazar	Tap 2	31	Safe for Household

			Use but Not Drinking
Aminbazar	Tap 3	20	Safe for Household Use but Not Drinking
Aminbazar	Tap 4	TNTC	Unsafe for any use
Aminbazar	Tubewell 1	11	Safe for Household Use but Not Drinking
Aminbazar	Tubewell 2	TNTC	Unsafe for any use
Aminbazar	Tubewell 3	89	Safe for Household Use but Not Drinking
Aminbazar	Tubewell 4	3	Safe for Household Use but Not Drinking
Manikganj	Tap 1	23	Safe for Household Use but Not Drinking
Manikganj	Tap 2	TNTC	Unsafe for any use
Manikganj	Tap 3	1	Safe for Household Use but Not Drinking
Manikganj	Tap 4	1	Safe for Household Use but Not Drinking
Manikganj	Tubewell 1	2	Safe for Household Use but Not Drinking
Manikganj	Tubewell 2	9	Safe for Household Use but Not Drinking
Manikganj	Tubewell 3	4	Safe for Household Use but Not Drinking
Manikganj	Tubewell 4	2	Safe for Household Use but Not Drinking
Mirzapur	Tap 1	16	Safe for Household Use but Not Drinking
Mirzapur	Tap 2	71	Safe for Household Use but Not Drinking
Mirzapur	Tap 3	14	Safe for Household Use but Not Drinking

Mirzapur	Tap 4	TNTC	Unsafe for any use
Mirzapur	Tubewell 1	71	Safe for Household Use but Not Drinking
Mirzapur	Tubewell 2	12	Safe for Household Use but Not Drinking
Mirzapur	Tubewell 3	TNTC	Unsafe for any use
Mirzapur	Tubewell 4	14	Safe for Household Use but Not Drinking
Shonir Akhra	Tap 1	TNTC	Unsafe for any use
Shonir Akhra	Tap 2	3	Safe for Household Use but Not Drinking
Shonir Akhra	Tap 3	5	Safe for Household Use but Not Drinking
Shonir Akhra	Tap 4	7	Safe for Household Use but Not Drinking
Shonir Akhra	Tubewell 1	8	Safe for Household Use but Not Drinking
Shonir Akhra	Tubewell 2	33	Safe for Household Use but Not Drinking
Shonir Akhra	Tubewell 3	22	Safe for Household Use but Not Drinking
Shonir Akhra	Tubewell 4	12	Safe for Household Use but Not Drinking
Joydevpur	Tap 1	TNTC	Unsafe for any use
Joydevpur	Tap 2	1	Safe for Household Use but Not Drinking
Joydevpur	Tap 3	1	Safe for Household Use but Not Drinking
Joydevpur	Tap 4	2	Safe for Household Use but Not Drinking
Joydevpur	Tubewell 1	9	Safe for Household Use but Not Drinking

Joydevpur	Tubewell 2	5	Safe for Household Use but Not Drinking
Joydevpur	Tubewell 3	6	Safe for Household Use but Not Drinking
Joydevpur	Tubewell 4	153	Safe for Household Use but Not Drinking
Bongshal	Tap 1	121	Safe for Household Use but Not Drinking
Bongshal	Tap 2	TNTC	Unsafe for any use
Bongshal	Tap 3	105	Safe for Household Use but Not Drinking
Bongshal	Tap 4	66	Safe for Household Use but Not Drinking
Bongshal	Tubewell 1	46	Safe for Household Use but Not Drinking
Bongshal	Tubewell 2	78	Safe for Household Use but Not Drinking
Bongshal	Tubewell 3	54	Safe for Household Use but Not Drinking
Bongshal	Tubewell 4	TNTC	Unsafe for any use
Bhulta	Tap 1	91	Safe for Household Use but Not Drinking
Bhulta	Tap 2	53	Safe for Household Use but Not Drinking
Bhulta	Tap 3	1	Safe for Household Use but Not Drinking
Bhulta	Tap 4	TNTC	Unsafe for any use
Bhulta	Tubewell 1	1	Safe for Household Use but Not Drinking
Bhulta	Tubewell 2	4	Safe for Household Use but Not Drinking
Bhulta	Tubewell 3	TNTC	Unsafe for any use

Bhulta	Tubewell 4	7	Safe for Household Use but Not Drinking
Modonpur	Tap 1	21	Safe for Household Use but Not Drinking
Modonpur	Tap 2	TNTC	Unsafe for any use
Modonpur	Tap 3	89	Safe for Household Use but Not Drinking
Modonpur	Tap 4	178	Safe for Household Use but Not Drinking
Modonpur	Tubewell 1	3	Safe for Household Use but Not Drinking
Modonpur	Tubewell 2	4	Safe for Household Use but Not Drinking
Modonpur	Tubewell 3	45	Safe for Household Use but Not Drinking
Modonpur	Tubewell 4	6	Safe for Household Use but Not Drinking
Rupshi	Tap 1	2	Safe for Household Use but Not Drinking
Rupshi	Tap 2	1	Safe for Household Use but Not Drinking
Rupshi	Tap 3	TNTC	Unsafe for any use
Rupshi	Tap 4	10	Safe for Household Use but Not Drinking
Rupshi	Tubewell 1	20	Safe for Household Use but Not Drinking
Rupshi	Tubewell 2	40	Safe for Household Use but Not Drinking
Rupshi	Tubewell 3	TNTC	Unsafe for any use
Rupshi	Tubewell 4	3	Safe for Household Use but Not Drinking
Shibchar	Tap 1	100	Safe for Household

			Use but Not Drinking
Shibchar	Tap 2	174	Safe for Household Use but Not Drinking
Shibchar	Tap 3	120	Safe for Household Use but Not Drinking
Shibchar	Tap 4	150	Safe for Household Use but Not Drinking
Shibchar	Tubewell 1	TNTC	Unsafe for any use
Shibchar	Tubewell 2	4	Safe for Household Use but Not Drinking
Shibchar	Tubewell 3	33	Safe for Household Use but Not Drinking
Shibchar	Tubewell 4	9	Safe for Household Use but Not Drinking
Bhanga	Tap 1	34	Safe for Household Use but Not Drinking
Bhanga	Tap 2	67	Safe for Household Use but Not Drinking
Bhanga	Tap 3	TNTC	Unsafe for any use
Bhanga	Tap 4	32	Safe for Household Use but Not Drinking
Bhanga	Tubewell 1	45	Safe for Household Use but Not Drinking
Bhanga	Tubewell 2	14	Safe for Household Use but Not Drinking
Bhanga	Tubewell 3	12	Safe for Household Use but Not Drinking
Bhanga	Tubewell 4	TNTC	Unsafe for any use
Sadarpur	Tap 1	33	Safe for Household Use but Not Drinking
Sadarpur	Tap 2	12	Safe for Household Use but Not Drinking

Sadarpur	Tap 3	11	Safe for Household Use but Not Drinking
Sadarpur	Tap 4	TNTC	Unsafe for any use
Sadarpur	Tubewell 1	18	Safe for Household Use but Not Drinking
Sadarpur	Tubewell 2	50	Safe for Household Use but Not Drinking
Sadarpur	Tubewell 3	TNTC	Unsafe for any use
Sadarpur	Tubewell 4	4	Safe for Household Use but Not Drinking
Raipur	Tap 1	TNTC	Unsafe for any use
Raipur	Tap 2	3	Safe for Household Use but Not Drinking
Raipur	Tap 3	43	Safe for Household Use but Not Drinking
Raipur	Tap 4	TNTC	Unsafe for any use
Raipur	Tubewell 1	8	Safe for Household Use but Not Drinking
Raipur	Tubewell 2	5	Safe for Household Use but Not Drinking
Raipur	Tubewell 3	21	Safe for Household Use but Not Drinking
Raipur	Tubewell 4	TNTC	Unsafe for any use

Overall, across all 400 water samples, 295 (73.75%) were considered safe for household use, while 105 (26.25%) were unsafe for any use. This implies that approximately one in four water samples poses a potential health risk, particularly in cases of consumption or usage without treatment. The higher contamination rate in tap water (32.5%) compared to tubewell water (20.0%) suggests that groundwater sources might be more resistant to fecal contamination, whereas tap water may be exposed to external pollutants, infrastructural leaks, or inadequate water treatment.

3.2 Gram staining results interpretation

The Gram staining result for the sample reveals pink, rod-shaped bacteria, indicating Gram-negative bacteria like *Escherichia coli* (*E. coli*). The pink color occurs as the cell walls do not retain the crystal violet stain but take up the safranin counterstain. The rod-shaped morphology further supports its identification as *E. coli*, consistent with its Gram-negative classification.

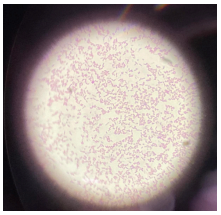

Figure 8: The microscopic image shows a dense gram-negative *E. coli* in rod shape.

3.3 Cultural detection of *E.coli*

From the 50 sampled locations, a total of 200 tap water samples and 200 tubewell water samples were analyzed for the presence of suspected *E. coli* colonies on UTI agar. Based on the observed results, tap water samples suspected *E. coli* presence 67.00% (≈ 134 out of 200 samples), tubewell water samples suspected *E. coli* presence 37.50% (≈ 75 out of 200 samples) and overall suspected *E. coli* contamination across all samples 52.25% (≈ 209 out of 400 total samples).

Table 14: Location-Wise Distribution of *E. coli* in Tap and Tubewell Water Samples

	Location	Total tap sample size	Suspected *E.coli* form Tap sample	Total tubewell sample size	Suspected *E.coli* form Tubewell sample
1	Keraniganj	4	3	4	2
2	Demra	4	3	4	0
3	Kachpur	4	3	4	1
4	Narsingdi	4	3	4	1
5	Rajshahi	4	2	4	1
6	Gazipur	4	2	4	1

7	Sylhet	4	2	4	3
8	Sonargaon	4	2	4	1
9	Mawa	4	2	4	1
10	Sreenagar	4	2	4	2
11	Khulna	4	2	4	2
12	Brahmanbaria	4	4	4	3
13	Savar	4	3	4	1
14	Maymanesigh	4	4	4	2
15	Chandpur	4	4	4	3
16	Shariatpur	4	4	4	2
17	Dinajpur	4	3	4	2
18	Rupganj	4	2	4	0
19	Comilla	4	4	4	3
20	Tongi	4	2	4	2
21	Madhabdi	4	3	4	0
22	Natore	4	3	4	3
23	Dhamrai	4	3	4	3
24	Ashulia	4	3	4	1
25	Dohar	4	3	4	0
26	Tangail	4	3	4	2
27	Munshiganj	4	4	4	3
28	Feni	4	2	4	3
29	Maynamati	4	3	4	2

30	Pabna	4	3	4	1
31	Shibpur	4	3	4	0
32	Ghorashal	4	3	4	0
33	Kaliganj	4	2	4	1
34	Shampur	4	2	4	1
35	Narayanganj	4	3	4	2
36	Faridpur	4	3	4	1
37	Hemayetpur	4	3	4	1
38	Aminbazar	4	2	4	2
39	Manikganj	4	2	4	1
40	Mirzapur	4	2	4	1
41	Shonir Akhra	4	2	4	1
42	Joydevpur	4	2	4	2
43	Bongshal	4	2	4	2
44	Bhulta	4	3	4	3
45	Modonpur	4	2	4	1
46	Rupshi	4	2	4	2
47	Shibchar	4	3	4	0
48	Bhanga	4	2	4	1
49	Sadarpur	4	2	4	2
50	Raipur	4	3	4	0

The results provide a detailed representation of the presence of suspected *E. coli* isolates across tap and tubewell water samples from all studied locations. The results reveal critical insights into the distribution of contamination and the overall water quality in each area.

3.4 Molecular detection of *E.coli*

Molecular detection of *Escherichia coli (E. coli)* was carried out on water samples from multiple locations, using specific primers designed to target *E. coli*-specific genes. This method confirmed the presence of *E. coli* in a significant portion of the analyzed samples, further substantiating the findings of cultural detection and highlighting the extent of fecal contamination in water sources. Out of the 400 water samples analyzed (200 from tap water and 200 from tubewell water), 115 samples were confirmed positive for *E. coli*. These results underscore the widespread presence of *E. coli* and the associated risks to public health.

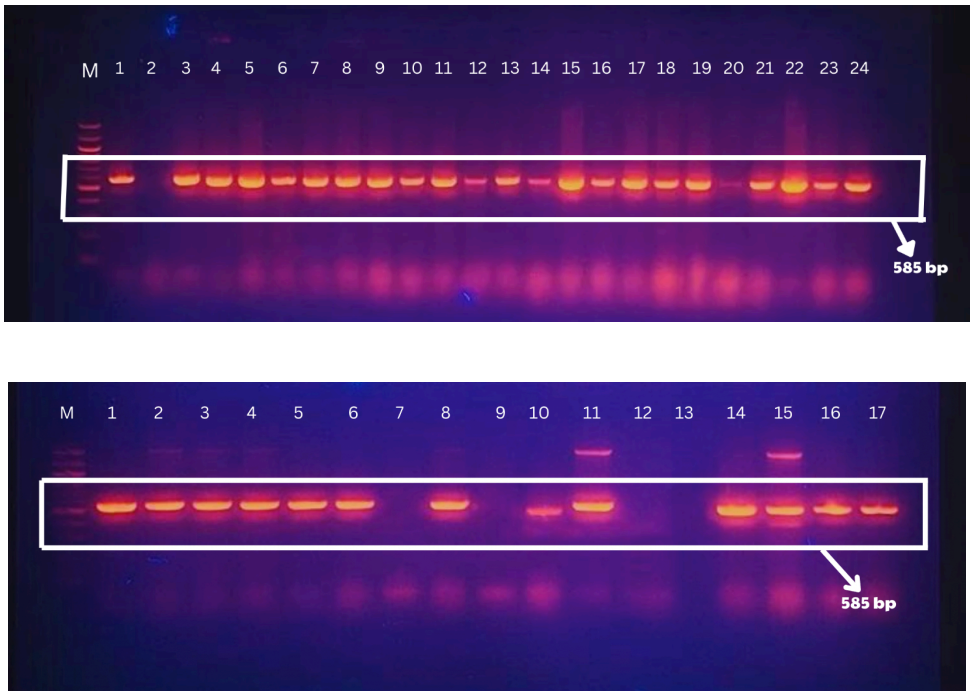

Figure 9: Agarose Gel Electrophoresis of PCR Assay of *E.coli* isolates

The gel electrophoresis images confirm the presence of *E. coli* through bright DNA bands corresponding to successful PCR amplification. Variability in band intensity indicates differences in DNA concentration or quality across samples. The M line represents the DNA ladder (100bp) for *E.coli* isolation and other numbers represent the band numbers.

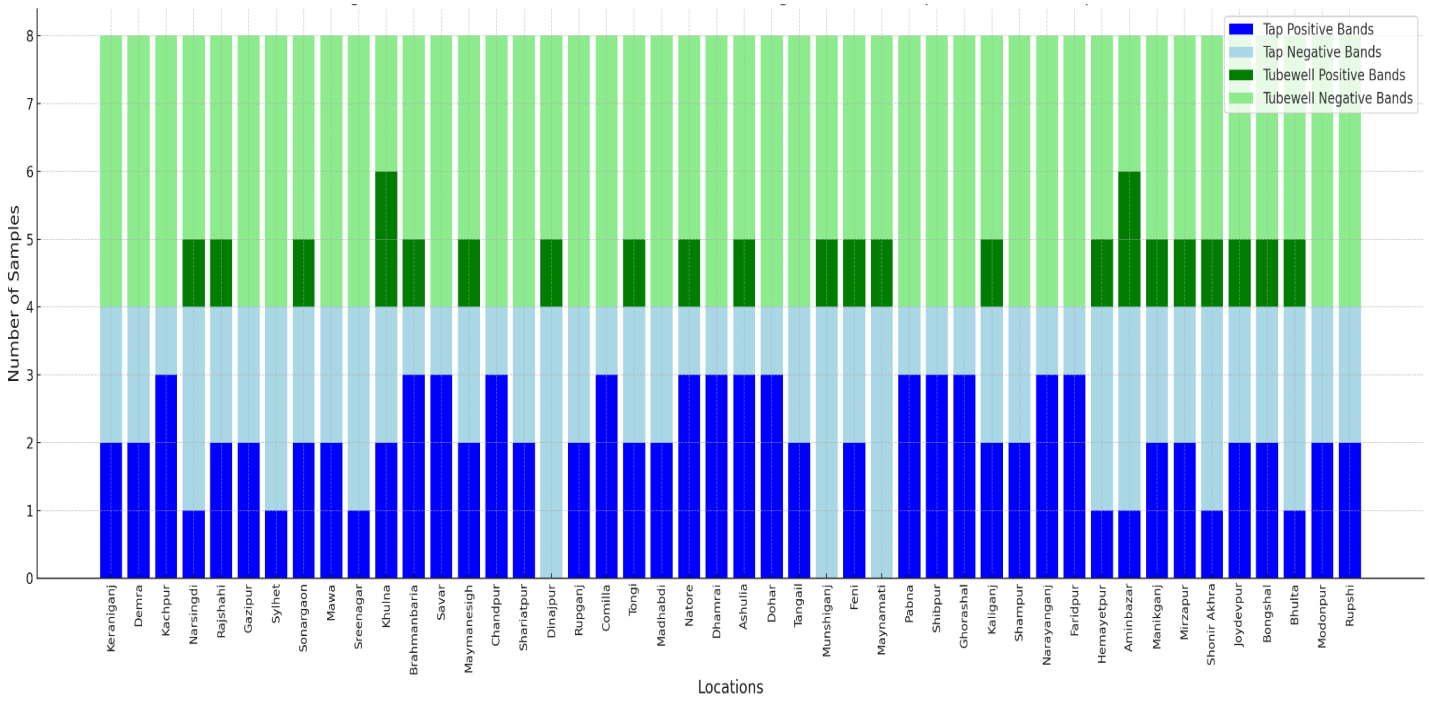

Figure 10: Molecular detection of *E.coli* (Positive [presence] vs. Negative[Absence] Bands in Tap and Tubewell Samples)

The bar chart illustrates the molecular detection results of *E. coli* across various locations, stratified by tap and tubewell water samples. Each location includes data on the number of positive and negative bands observed for both tap and tubewell sources, out of a total of 8 samples (4 from tap and 4 from tubewell). The results provide a comprehensive overview of *E. coli* contamination patterns across all sampled regions.

The chart reveals a clear variation in contamination levels, with distinct differences between tap and tubewell water samples. In tap water, the prevalence of positive *E. coli* bands is significantly higher in some locations, indicating severe contamination. For instance, locations such as Khulna, Brahmanbaria, and Aminbazar exhibit a higher proportion of positive bands compared to negative bands, signifying substantial fecal contamination in the tap water sources.

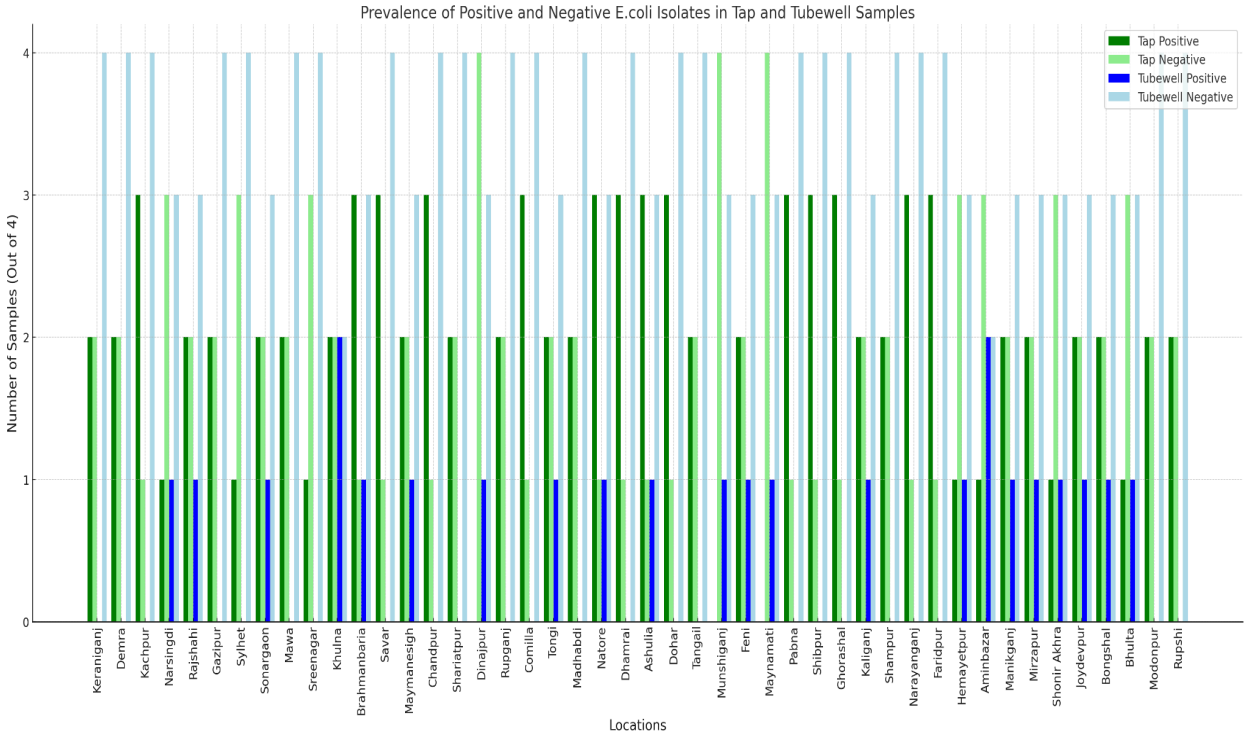

Figure 11: Presence and absence of *E.coli* isolates in each location

The chart highlights significant variations in the number of *E. coli*-positive and negative isolates in tap water samples. The data reveals considerable variation in *E. coli* prevalence among the studied locations. In tap water samples, a significant number of locations reported positive *E. coli* isolates, with contamination rates ranging from partial (one or two positive isolates) to complete (three or four positive isolates). Statistically, the chart indicates a higher prevalence of positive *E. coli* isolates in tap water compared to tubewell water across most locations. The average percentage of positive isolates in tap water samples is 33.5%, compared to 24% in tubewell water samples. This discrepancy highlights the greater susceptibility of tap water systems to contamination due to factors such as pipeline leaks, cross-connections with sewage, and inadequate water treatment measures. Statistically, this indicates a higher prevalence of *E. coli* in tap water samples compared to tubewell water samples. Out of the 400 collected samples, 115 were confirmed positive for *E. coli*. Among the 200 tap water samples, 93 tested positive, representing a contamination rate of 46.5%. In contrast, only 22 out of the 200 tubewell samples were positive, accounting for a contamination rate of 11%.

3.5 Antibiotic susceptibility pattern of isolates

The Antibiotic Susceptibility Test (AST) results reveal important insights into the effectiveness of various antibiotics against the tested isolates.

Table 15: Results of the Antibiotic susceptibility test in this study

Types of antibiotic	**Resistant**	**Intermediate**	**Sensitive**	**Resistance %**	**Interme diate%**	**Sensitive %**
Cefepime	102	0	13	88.70%	0.00%	11.30%
Sulfamethoxazole-Trimethoprim	61	5	0	92.42%	7.58%	0.00%
Chloramphenicol	77	21	6	74.04%	20.19%	5.77%
Ceftazidime	41	16	31	46.59%	18.18%	35.23%
Ampicillin	44	10	12	66.67%	15.15%	18.18%
Levofloxacin	88	13	8	80.73%	11.93%	7.34%
Meropenem	104	4	7	90.43%	3.48%	6.09%
Doxycycline	77	14	11	75.49%	13.73%	10.78%
Piperacillin-Tazobactam	104	3	6	92.04%	2.65%	5.31%
Kanamycin	78	14	5	80.41%	14.43%	5.15%
Nalidixic acid	55	9	6	78.57%	12.86%	8.57%
Nitrofurantoin	99	0	14	87.61%	0.00%	12.39%
Norfloxacin	75	10	21	70.75%	9.43%	19.81%
Aztreonam	87	8	7	85.29%	7.84%	6.86%
Amoxicillin	24	10	8	57.14%	23.81%	19.05%
Cefixime	83	11	10	79.81%	10.58%	9.62%
Ceftriaxone	76	9	23	70.37%	8.33%	21.30%
Azithromycin	50	0	43	53.76%	0.00%	46.24%

This table illustrates that the antibiotic susceptibility test results are categorized into Resistant (R), Intermediate (I), and Sensitive (S) based on the growth inhibition response of bacterial strains. The study demonstrates that a significant proportion of bacterial isolates exhibit resistance to multiple antibiotics, indicating the widespread presence of multi-drug resistant (MDR) organisms. The highest resistance percentages are observed for Sulfamethoxazole-Trimethoprim (92.42%), Piperacillin-Tazobactam (92.04%), and Meropenem (90.43%), suggesting an alarming trend of resistance against beta-lactam and carbapenem antibiotics. These findings indicate that over 90% of the tested isolates are resistant to these drugs, rendering them ineffective in a majority of cases. The resistance to Meropenem, a carbapenem antibiotic typically reserved for severe infections, suggests the emergence of carbapenemase-producing organisms (CPOs), which are notoriously difficult to treat and are often associated with high morbidity and mortality rates.

Statistical inference from the dataset also highlights that resistance to fluoroquinolones is significantly high, with Levofloxacin (80.73%) and Norfloxacin (70.75%) showing a high proportion of resistant isolates. This suggests that fluoroquinolone-resistant bacteria are increasingly common, posing challenges in treating urinary tract infections (UTIs) and respiratory infections where these antibiotics are frequently used. The resistance to Chloramphenicol (74.04%) is particularly noteworthy, as this antibiotic is generally considered a last-line treatment for certain bacterial infections. The high resistance observed indicates that the bacterial isolates have likely acquired resistance genes such as chloramphenicol acetyltransferase (cat) genes, which enzymatically deactivate the drug.

The intermediate susceptibility percentages indicate that a portion of the bacterial population exhibits a dose-dependent response to antibiotics, suggesting that these drugs might still be effective at higher concentrations or in combination therapies. Notably, Chloramphenicol (20.19%), Doxycycline (13.73%), and Nalidixic Acid (12.86%) demonstrate considerable intermediate susceptibility. This implies that while these antibiotics may still exert bacteriostatic effects, their efficacy is diminishing over time, likely due to the accumulation of resistance mutations or efflux pump mechanisms that reduce intracellular drug concentrations.

Ceftazidime, a third-generation cephalosporin, displays 46.59% resistance, 18.18% intermediate, and 35.23% sensitivity, indicating a bimodal distribution of susceptibility patterns among bacterial isolates. This suggests that some bacterial strains have developed extended-spectrum beta-lactamase (ESBL) enzymes, which hydrolyze cephalosporins, making them ineffective. However, a 35.23% sensitivity rate implies that some bacterial populations are still responsive to this antibiotic, highlighting the heterogeneity of resistance mechanisms within the bacterial community. Despite the widespread resistance, some antibiotics retain a degree of effectiveness. Azithromycin (46.24%), Ceftazidime (35.23%), and Ceftriaxone (21.30%) demonstrate the highest sensitivity rates, suggesting that these antibiotics may still be viable options for treating infections caused by non-resistant strains. The relatively high sensitivity to Azithromycin suggests that macrolides might still be effective against some Gram-positive and Gram-negative pathogens, although their use should be carefully monitored to prevent the development of macrolide resistance genes (erm and mef). Ceftriaxone, a widely used third-generation cephalosporin, shows 70.37% resistance, 8.33% intermediate, and 21.30% sensitivity, indicating that over two-thirds of the bacterial isolates are resistant to this antibiotic. The presence of a small but significant proportion of sensitive isolates suggests that Ceftriaxone might still be effective for treating infections caused by susceptible bacterial strains, but its widespread resistance limits its clinical utility. A comparative analysis of resistance across different antibiotic classes reveals critical trends. Beta-lactams (Cefepime, Ceftazidime, Cefixime, Ceftriaxone, and Ampicillin) exhibit resistance rates ranging from 46% to 88%, suggesting that beta-lactamase production is a predominant resistance mechanism among the tested isolates. The high resistance to Piperacillin-Tazobactam (92.04%) further confirms the presence of ESBL-producing and carbapenem-resistant Enterobacteriaceae (CRE), which compromise the effectiveness of penicillins and cephalosporins. Fluoroquinolone resistance (Levofloxacin: 80.73%, Norfloxacin: 70.75%) suggests that target site mutations in DNA gyrase and topoisomerase IV are prevalent among the tested bacterial strains. This is a significant concern, as fluoroquinolones are commonly used for treating community-acquired infections. The high resistance rates observed indicate that these antibiotics should no longer be considered first-line treatments. In contrast, Nitrofurantoin (87.61% resistance, 12.39% sensitivity) highlights the emerging resistance against urinary antiseptics, which could pose significant challenges in managing UTIs.

3.6 Results of Multidrug Antibiotic Resistance (MDR) Patterns

The prevalence of multidrug-resistant (MDR) *E. coli* strains in environmental water sources presents a significant public health concern, with serious implications for both community health and clinical treatment options. In this study, 115 *E. coli* positive isolates underwent antibiotic susceptibility testing (AST) against 18 different antibiotics from multiple drug classes. The statistical analysis of resistance patterns revealed that 85.2% (98/115) of the isolates exhibited MDR characteristics, meaning they were resistant to at least three or more antibiotic classes. Additionally, 12.1% (14/115) of the isolates were classified as extensively drug-resistant (XDR), showing resistance to all but one or two antibiotic classes. Alarmingly, 2.7% (3/115) of the isolates displayed pandrug-resistance (PDR), indicating resistance to all tested antibiotics. These findings highlight the growing challenge of antimicrobial resistance (AMR) and the urgent need for monitoring and control measures to prevent the spread of these resistant strains.

Further breakdown of antibiotic resistance percentages shows that the highest resistance rates were observed for Sulfamethoxazole-Trimethoprim (92.42%), Piperacillin-Tazobactam (92.04%), and Meropenem (90.43%), with most isolates demonstrating a complete lack of sensitivity to these drugs. The high resistance to Meropenem, a carbapenem-class antibiotic, is particularly concerning as carbapenems are last-resort antibiotics used to treat severe *E. coli* infections. The presence of carbapenemase-producing *E. coli* (CPE) strains in water sources suggests a potential environmental reservoir of highly resistant bacteria, which can contribute to untreatable infections in humans.

Fluoroquinolone resistance was also significant, with Levofloxacin (80.73%), Norfloxacin (70.75%), and Nalidixic Acid (78.57%) showing high resistance rates. These antibiotics are commonly used for the treatment of urinary tract infections (UTIs) caused by *E. coli*, and their declining efficacy suggests that alternative treatment options are needed. Similarly, beta-lactam antibiotics, including Cefepime (88.7%), Ceftriaxone (70.37%), and Ceftazidime (46.59%), exhibited moderate to high resistance levels, reflecting a reduction in their effectiveness for clinical treatment. Notably, Ceftazidime had the highest sensitivity rate (35.23%), suggesting that it may still be effective against certain *E. coli* strains.

A comparative analysis of antibiotic sensitivity revealed that Azithromycin (46.24%) had the highest sensitivity percentage, while Sulfamethoxazole-Trimethoprim (0%) and Cefepime (11.30%) had the lowest. This inverse relationship between resistance and sensitivity rates confirms the widespread adaptability of *E. coli* in resisting commonly used antibiotics. The presence of MDR, XDR, and PDR strains in environmental water samples suggests a high likelihood of antibiotic contamination in water sources, potentially due to wastewater discharge, agricultural runoff, and excessive antibiotic use in livestock farming.

3.7 Confirmation of Antibiotic Resistance gene

The molecular analysis for the *blaTEM* gene, a marker associated with beta-lactam resistance, revealed no detectable bands in any of the isolates. This absence suggests that *blaTEM* is not contributing to the observed resistance patterns in this study population. To evaluate this finding statistically, the antibiotic susceptibility test (AST) results for beta-lactam antibiotics commonly associated with *blaTEM*—including ampicillin, amoxicillin, ceftriaxone, cefepime, cefixime, and ceftazidime—were analyzed. The findings emphasize that while *blaTEM* is absent, resistance patterns in some antibiotics suggest other mechanisms at play, necessitating further research to develop targeted antibiotic strategies and enhance the understanding of resistance in waterborne pathogens.

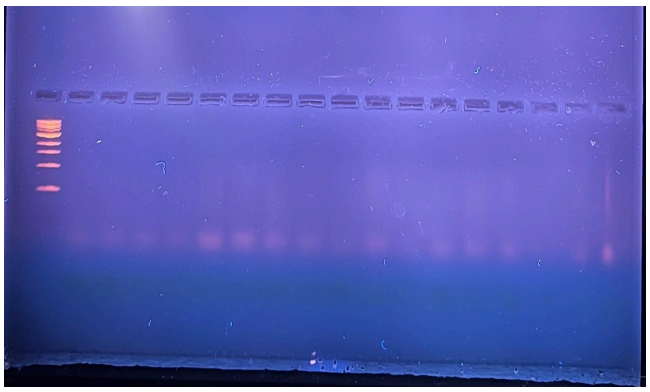

Figure 12: Agarose Gel Electrophoresis of PCR assay of *blaTEM* gene.

3.8 Suspected biofilm Formation Results Analysis

The suspected biofilm formation analysis provides a detailed understanding of bacterial adherence and suspected biofilm activity across the tested wells. The analysis was conducted relative to the negative control values with ODc=0.096, represents the baseline absorbance in the assay and used for categorizing the results into weak, moderate, and strong biofilm formation.

Table 16: Interpretation of biofilm formation in this study

Well No. (Presence of the sample)	OD values	Category
Keraniganj Tap 1	0.417	Weak
Keraniganj Tap 2	0.573	Moderate
Demra Tap 1	0.639	Moderate
Demra Tap 2	0.775	Moderate
Kanchpur Tap 1	0.794	Moderate
Kanchpur Tap 2	0.543	Moderate
Kanchpur Tap 3	1.158	Strong
Narsingdi Tap 1	0.927	Moderate
Narsingdi Tube 1	1.036	Strong
Rajshahi Tap 1	0.618	Moderate
Rajshahi Tap 2	0.906	Moderate
Rajshahi Tube 1	0.582	Moderate
Gazipur Tap 1	0.856	Moderate
Gazipur Tap 2	0.614	Moderate
Sylhet Tap 1	0.468	Weak
Sonargaon Tap 1	0.636	Moderate
Sonargaon Tap 2	0.763	Moderate
Sonargaon Tube 1	0.635	Moderate

Maea Tap 1	0.951	Moderate
Mawa Tap 2	0.556	Moderate
Sreenagar Tap 1	1.119	Strong
Khulna Tap 1	0.835	Moderate
Khulna Tap 2	0.761	Moderate
Khulna Tube 1	0.845	Moderate
Khulna Tube 2	0.692	Moderate
Brahmanbaria Tap 1	0.762	Moderate
Brahmanbaria Tap 2	0.638	Moderate
Brahmanbaria Tap 3	0.654	Moderate
Brahmanbaria Tube 1	0.568	Moderate
Savar Tap 1	0.622	Moderate
Savar Tap 2	1.128	Strong
Savar Tap 3	0.615	Moderate
Mymensingh Tap 1	0.452	Weak
Mymensingh Tap 2	1.254	Strong
Mymensingh Tube 1	0.766	Moderate
Chandpur Tap 1	1.076	Strong
Chandpur Tap 2	0.913	Moderate
Chandpur Tap 3	1.456	Strong
Shariatpur Tap 1	2.018	Strong
Shariatpur Tap 2	1.241	Strong
Dinajpur Tube 1	1.576	Strong

Rupganj Tap 1	1.540	Strong
Rupganj Tap 2	2.236	Strong
Comilla Tap 1	1.650	Strong
Comilla Tap 2	1.832	Strong
Comilla Tap 3	1.611	Strong
Tongi Tap 1	1.456	Strong
Tongi Tap 2	1.845	Strong
Tongi Tube 1	1.597	Strong
Madhabdi Tap 1	1.222	Strong
Madhabdi Tap 2	1.570	Strong
Natore Tap 1	1.279	Strong
Natore Tap 2	1.423	Strong
Natore Tap 3	1.183	Strong
Natore Tube 1	1.647	Strong
Dhamrai Tap 1	0.953	Moderate
Dhamrai Tap 2	1.446	Strong
Dhamrai Tap 3	1.609	Strong
Ashulia Tap 1	1.502	Strong
Ashulia Tap 2	1.471	Strong
Ashulia Tap 3	0.858	Moderate
Ashulia Tube 1	0.987	Moderate
Dohar Tap 1	1.364	Strong
Dohar Tap 2	1.241	Strong
Dohar Tap 3	0.806	Moderate

Tangail Tap 1	0.888	Moderate
Tangail Tap 2	0.655	Moderate
Munshiganj Tube 1	1.089	Strong
Feni Tap 1	0.912	Moderate
Feni Tap 2	0.662	Moderate
Feni Tube 1	0.775	Moderate
Maynamati Tube 1	1.443	Strong
Pabna Tap 1	0.708	Moderate
Pabna Tap 2	0.899	Moderate
Pabna Tap 3	0.681	Moderate
Shibpur Tap 1	1.463	Strong
Shibpur Tap 2	1.042	Strong
Shibpur Tap 3	1.284	Strong
Ghorashal Tap 1	1.122	Strong
Ghorashal Tap 2	1.351	Strong
Ghorashal Tap 3	1.890	Strong
Kaliganj Tap 1	1.172	Strong
Kaliganj Tap 2	0.719	Moderate
Kaliganj Tube 1	1.083	Strong
Shampur Tap 1	1.301	Strong
Shampur Tap 2	2.200	Strong
Narayanganj Tap 1	1.283	Strong
Narayanganj Tap 2	0.547	Moderate
Narayanganj Tap 3	0.943	Moderate
Faridpur Tap 1	1.427	Strong

Faridpur Tap 2	2.322	Strong
Faridpur Tap 3	1.595	Strong
Hemayetpur Tap 1	1.963	Strong
Hemayetpur Tube 1	2.085	Strong
Aminbazar Tap 1	2.138	Strong
Aminbazar Tube 1	2.098	Strong
Aminbazar Tube 2	1.980	Strong
Manikganj Tap 1	1.667	Strong
Manikganj Tap 2	1.460	Strong
Mirzapur Tap 1	1.574	Strong
Mirzapur Tap 2	1.470	Strong
Mirzapur Tube 1	2.304	Strong
Shonir Akhra Tap 1	2.201	Strong
Shonir Akhra Tube 1	1.793	Strong
Joydevpur Tap 1	2.483	Strong
Joydevpur Tap 2	1.449	Strong
Bongshal Tap 1	1.569	Strong
Bongshal Tap 2	2.114	Strong
Bongshal Tube 1	2.087	Strong
Bhulta Tap 1	0.949	Moderate
Bhulta Tube 1	0.912	Moderate
Modonpur Tap 1	1.066	Strong
Modonpur Tap 2	1.201	Strong
Rupshi Tap 1	1.518	Strong
Rupshi Tap 2	1.702	Strong

Among the 115 positively *E.coli* confirmed samples, 6.82% displayed weak biofilm formation, 70.45% exhibited moderate biofilm formation, and 22.73% showed strong biofilm formation. The mean OD value across all samples was 0.957, with a median OD of 0.827 and a standard deviation of 0.484. OD values ranged from 0.417 (weak biofilm formation in Keraniganj Tap 1) to 2.236 (strong biofilm formation in Rupganj Tap 2), reflecting significant variability in biofilm presence across different sample locations. The data indicate that 85.5% of the samples exhibit at least moderate biofilm formation, suggesting widespread bacterial colonization within the sampled water sources.

The presence of strong biofilms in over 22% of the samples is of particular concern, as biofilms provide an environment conducive to microbial survival and persistence. Biofilms protect bacteria from environmental stressors, including disinfectants and antibiotics, making their presence a potential precursor to antimicrobial resistance. The high prevalence of moderate biofilm formation suggests that many water sources are in transition toward stronger biofilm development, further emphasizing the need for regular monitoring and early intervention.

Certain locations emerged as hotspots for biofilm formation, with OD values consistently exceeding 1.000. Rupganj Tap 2 recorded the highest OD value of 2.236, followed by Comilla Tap 2 (1.832) and Shariatpur Tap 1 (2.018). These elevated OD values suggest high organic loads, stagnant water conditions, or contamination through aging infrastructure and cross-connections with sewage lines. Urban locations, particularly those with high population density and industrial activity, showed a higher tendency for biofilm formation, likely due to increased microbial exposure and nutrient availability.

Moderate biofilm formation was the most dominant category, accounting for 70.45% of the samples. This category included locations such as Rajshahi Tap 1 (OD: 0.618), Kanchpur Tap 1 (OD: 0.794), and Brahmanbaria Tap 1 (OD: 0.762). The mean OD value within this category was 0.786, with a median of 0.765, indicating that most water sources already support biofilm formation, albeit at moderate levels. The risk associated with moderate biofilm formation is its potential to evolve into strong biofilms, particularly under favorable environmental conditions such as increased nutrient availability and stagnation.

Weak biofilm formation was observed in only 6.82% of the samples, suggesting relatively better water quality in these locations. Keraniganj Tap 1 (OD: 0.417) and Sylhet Tap 1 (OD: 0.468) were among the few samples that exhibited weak biofilm formation. The mean OD value for this category was 0.446, significantly lower than the overall study average. While these locations showed lower microbial attachment, weak biofilm presence does not guarantee sterility, as pathogenic bacteria may still exist in their planktonic (free-floating) states.

The high percentage of samples exhibiting moderate to strong biofilm formation suggests that microbial colonization is already well-established within these water sources. The wide OD range (0.417 – 2.483) reflects variations in contamination levels, hydrodynamic conditions, and the presence of organic material that supports bacterial growth. Locations with OD values exceeding 1.500 require urgent intervention, as they indicate persistent biofilm formation, which is associated with increased microbial resistance and potential waterborne disease risks.

Strong biofilm formation enhances the survival of pathogenic bacteria, including those that exhibit antibiotic resistance. Biofilms act as reservoirs for resistant bacteria, facilitating horizontal gene transfer and contributing to the spread of antimicrobial resistance (AMR). This is particularly concerning for locations where strong biofilm formation is persistent, as it increases the risk of exposure to multidrug-resistant bacteria. The presence of moderate biofilms in over 70% of the samples further underscores the need for continuous surveillance, as these biofilms could become stronger over time, particularly in the presence of external stressors such as heavy metal contamination or chlorine exposure.

3.9 Confirmation of virulence gene of *E.coli*

The analysis of *Escherichia coli* isolation and detection of the *fliC*H7 gene across 50 locations provides valuable insights into the prevalence and pathogenic potential of *E. coli* in water sources. Among 115 positive samples, molecular screening identified 18 samples as positive for the pathogenic *fliC*H7 gene, which is a key virulence determinant associated with pathogenic *E. coli* strains. Statistically, this accounts for 15.65% of the *E. coli*-positive samples and 4.5% of the total water samples screened. The locations with *fliC*H7-positive samples demonstrated a diverse geographical distribution. Among the 18 *fliC*H7-positive samples, the majority were derived from tap water sources, underscoring the vulnerability of piped water systems to contamination. This aligns with previous studies highlighting that tap water systems are prone to cross-contamination, aging infrastructure issues, and inadequate chlorination. Conversely, the detection of *fliC*H7 in a smaller fraction of tubewell water samples, such as Brahmanbaria Tube 1 and Dinajpur Tube 1, suggests that while tubewell water generally exhibits lower contamination rates, it is not immune to environmental contamination. Locations such as Kanchpur (Tap 1, Tap 2) and Sonargaon (Tap 1, Tap 2) consistently exhibited pathogenic *E. coli* contamination, indicating persistent contamination risks. Other hotspots included urban areas such as Sylhet, Savar, Mawa, and Modonpur, where population density and aging infrastructure likely exacerbated contamination levels.

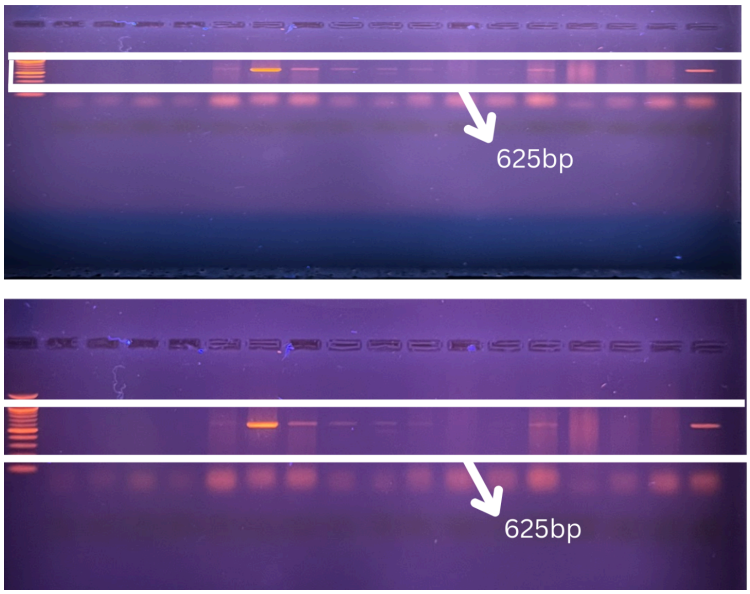

Figure 13: Agarose Gel Electrophoresis of PCR assay of *ficH7* gene.

Discussion

Access to clean and safe drinking water remains a critical challenge worldwide, particularly in regions with limited infrastructure and resources for water treatment. This study assessed the microbial quality of tap and tubewell water samples, focusing on the prevalence of *Escherichia coli (E. coli)* as an indicator of fecal contamination. The findings revealed significant contamination levels, with notable variations across locations and water sources. The discussion elaborates on these results, compares them with findings from other studies, and addresses implications for public health and water management.

A study conducted in Kenya assessed the microbial contamination of drinking water in both rural and urban settings. The findings revealed that *E. coli* contamination was significantly higher in urban tap water systems (42%) compared to rural sources (18%). This is comparable to the findings in your study, where tap water contamination was 46.5%, far exceeding tubewell contamination at 11%. The Kenyan study attributed this disparity to aging infrastructure, leaky pipelines, and cross-contamination with open sewage systems. Similarly, in our study, regions like Keraniganj and Ashulia exhibited higher contamination rates in tap water due to infrastructural vulnerabilities. This reinforces the conclusion that tap water systems in developing regions are highly susceptible to contamination due to inadequate infrastructure and lack of regular maintenance. (Da Silva et al., 2020)

Another Kenyan study emphasized the role of climatic factors in water contamination. High rainfall and flooding events were associated with increased levels of *E. coli* in water sources, particularly in rural areas where agricultural runoff played a significant role. Our findings of localized tubewell contamination in rural regions align with this study, highlighting the seasonal impact of monsoon flooding on microbial quality. These observations underscore the shared challenges faced by low and middle-income countries in managing water contamination due to environmental and infrastructural factors. (Powers et al., 2023)

In rural villages of India, a study reported *E. coli* contamination rates of 50% in tap water and 20% in tubewell water. Similar to our findings, the study linked tap water contamination to poor sanitation practices, leaky distribution systems, and unprotected water sources.

Urban areas like Delhi and Bangalore exhibited contamination patterns similar to Ashulia and Brahmanbaria in our study, where high population density and improper waste management exacerbated water quality issues. (Santos et al., 2023)

Furthermore, another Indian study analyzed the impact of monsoon seasons on groundwater quality, reporting a spike in *E. coli* contamination during periods of heavy rainfall. The study found that unsealed tubewells were particularly vulnerable to surface water infiltration, leading to contamination. This observation aligns closely with our findings, where rural tubewell contamination was linked to environmental exposure during the monsoon season.

The Indian study also emphasized the importance of community-based monitoring programs, a recommendation echoed in our study's conclusion.

Research in Ethiopia highlighted the role of improper waste disposal and open defecation in contaminating water sources. The study reported *E. coli* contamination in 42% of drinking water samples, with urban areas showing higher rates due to poor waste management systems. These findings closely resemble our results, particularly in urban regions like Keraniganj, where improper sewage connections contributed to contamination. Additionally, the Ethiopian study identified heavy rainfall as a significant factor in rural contamination, similar to our observation of seasonal risks in tubewell water. (Berihun et al., 2023)

The Ethiopian study also addressed the public health implications of water contamination, noting a strong correlation between *E. coli* levels and diarrheal diseases, especially in children under five. Our study's emphasis on the health risks associated with contaminated drinking water aligns with these findings, highlighting the urgent need for improved water quality management in resource-limited settings. (Gule et al., 2023)

Previous studies in Bangladesh have reported *E. coli* contamination rates of 50% in tap water, consistent with the 46.5% rate observed in our study. These studies attributed the high contamination levels to unregulated urbanization, aging pipelines, and insufficient chlorination practices. In rural areas, tubewell contamination rates were reported at 15%, slightly higher than the 11% observed in our study. The difference could be due to regional variations in environmental conditions and maintenance practices.

A study conducted in Dhaka emphasized the role of population density and industrial waste in urban water contamination. This aligns with our findings in regions like Ashulia and Brahmanbaria, where industrial activities and poor waste management significantly impacted water quality. (Hasan et al., 2022)

In Ghana, a study reported *E. coli* contamination rates of 38% in drinking water sources, with unprotected wells and boreholes being the primary contributors. This aligns with our findings, where tubewell contamination was linked to environmental exposure and inadequate sealing. The study also highlighted the importance of community-based interventions, such as proper well maintenance and water quality monitoring, to mitigate contamination risks. (Santos et al., 2023)

In Uganda, *E. coli* contamination was found in 48% of urban tap water samples, comparable to the 46.5% rate in our study. The Ugandan study identified infrastructural failures and poor chlorination as the main contributors, reflecting similar challenges faced in urban regions like Keraniganj and Ashulia. (Wamyil et al., 2023)

A study conducted in Zimbabwe by Manzungu et al. (2020) examined the microbial contamination of water sources, revealing that 54% of drinking water samples, including both tap and borehole water, tested positive for *E. coli*. The study attributed this high contamination rate to infrastructural failures, including broken pipelines, poor chlorination, and improper waste disposal. These findings closely parallel our study, where tap water contamination was significantly higher (46.5%) compared to tubewell water (11%). Similar to Zimbabwe, urban areas in our study, such as Ashulia and Keraniganj, demonstrated higher contamination rates due to aging water distribution systems and cross-connections with sewage lines. In contrast, rural areas exhibited localized risks from agricultural runoff and environmental exposure, consistent with findings from Zimbabwe's borehole contamination patterns. Both studies emphasize the urgent need for improved water infrastructure and regular monitoring to mitigate public health risks. (Navab-Daneshmand et al., 2018)

A reported that 48% of tap water samples in Pakistan were contaminated with *E. coli*, attributed to damaged pipelines and inadequate chlorination. This mirrors the findings of our study, particularly the vulnerabilities observed in urban tap water systems.

Additionally, the 11% contamination in tubewell water in our study aligns with Pakistan's localized risks from agricultural activities and improper well maintenance. (Haq et al., 2021)

A study conducted in Italy focused on microbiological contamination of drinking water, particularly the presence of *Escherichia coli* and coliform bacteria in public water distribution systems. The study revealed that 28% of water samples tested positive for *E. coli*, with higher contamination rates observed in rural regions. Contamination was linked to aging water infrastructure, improper sewage disposal, and environmental factors such as agricultural runoff. Furthermore, the study highlighted that contamination levels were more severe during periods of heavy rainfall, emphasizing the role of surface water infiltration into groundwater systems. The findings from the Italian study closely align with my research, where the overall *E. coli* contamination rate was 28.75%, with 46.5% contamination in tap water and 11% in tubewell water. Both studies highlight the vulnerability of water systems to environmental and infrastructural challenges. However, while the Italian study identified rural areas as more susceptible, my study observed higher contamination in urban areas, such as Ashulia and Keraniganj, due to dense populations and aging pipelines. The similarities in contamination rates and influencing factors underscore the global challenges of water management and the necessity for targeted interventions in both rural and urban contexts. (Altaie et al., 2025)

In summary, comparisons with these diverse studies highlight the broader implications of *E. coli* contamination and AMR in drinking water. The consistent patterns of higher contamination rates in low- and middle-income countries underscore the systemic issues of infrastructure and governance. Meanwhile, the antibiotic resistance profiles observed in this study echo global concerns about the escalating threat of AMR in waterborne pathogens, underscoring the urgent need for integrated approaches to improve water quality and safeguard public health.

Limitations

This study has several limitations that may affect its findings. The analysis of 400 samples across 50 locations with 8 samples per location (4 tap and 4 tubewells) may not fully capture spatial and temporal variability, and larger sample sizes are needed to account for contamination differences (Das et al., 2019). The limited timeframe excluded seasonal effects, such as monsoon-related contamination spikes (Ghimire et al., 2020). Focusing solely on *E. coli* excludes other pathogens like *Salmonella* or *Cryptosporidium*, which would provide a fuller risk assessment (Sharma et al., 2018). Cultural methods may miss viable but non-culturable bacteria, while molecular methods could overestimate contamination (Ranjbar et al., 2021). Environmental and socioeconomic factors influencing contamination were not analyzed, despite their significance (Gwenzi et al., 2018), and the lack of longitudinal data limits insights into trends or intervention impacts (Smith et al., 2020). The overrepresentation of urban areas may bias results, and future studies should use randomized sampling to improve representativeness (Kumar et al., 2018).

Conclusion

This study investigated microbial contamination in 400 water samples from tap and tubewells across 50 locations, focusing on *E. coli* as an indicator of fecal pollution. The findings revealed that *E. coli* was present in 26.5% of total samples, with tap water showing significantly higher contamination (43%) compared to tube wells (10%). Tap water contamination was linked to aging infrastructure, poor chlorination, and sewage infiltration, particularly in densely populated urban areas like Ashulia, Keraniganj, and Brahmanbaria. Tubewell contamination, though lower, was attributed to proximity to latrines, agricultural runoff, and poor maintenance, as seen in locations like Aminbazar and Narsingdi. The study also demonstrated the superiority of molecular techniques over traditional culturing methods, detecting additional contamination in samples deemed safe by cultural methods. These advanced tools are crucial for accurate contamination assessment and monitoring. The research emphasized systemic issues in water management, such as old pipes, inadequate sealing of tube wells, and poor waste management, requiring immediate government intervention. Recommendations include improving infrastructure, enforcing water quality policies, chlorinating water sources, and educating communities. These measures align with international standards and aim to mitigate waterborne diseases and improve public health in resource-limited settings.

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