DETECTION AND CHARACTERIZATION OF DIARRHEAGENIC *E. COLI* ISOLATED FROM RIVER OF TERAI BELT OF PROVINCE NO. 1, NEPAL

A Dissertation Submitted to the Department Of Microbiology, CENTRAL CAMPUS OF TECHNOLOGY, Tribhuvan University, Dharan, Nepal, in Partial Fulfillment of the Requirements for the Award of Degree of Master of Science in Microbiology (**Public Health**)

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CERTIFICATE OF APPROVAL

On the recommendation of **Asst. Professor Mr. Hemanta Khanal,** this dissertation work by **MS. KABITA DHAKAL** entitled **"Detection and Characterization of Diarrheagenic *E. Coli* Isolated From River of Terai Belt of Province No. 1, Nepal"** has been approved for the examination and is submitted to the Tribhuvan University in partial fulfillment of the requirements for M.Sc. Degree in Microbiology (**Public Health**).

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ABSTRACT

The most frequent human gut pathogens are *E. coli* strains. The majority of known *E. coli* strains are an etiologic cause of diarrhea in developing nations. The spread of enteric pathogens into flowing water is a significant issue linked with the disposal of these wastes. The study aimed to determine and characterize the diarrheagenic *E. coli* isolated from river water of terai belt of Province no.1. Altogether 43 river water sample were collected aseptically and transported to the laboratory, then the collected sample were enriched and cultured in EMB agar plate and incubated for 24 hrs at 37ºC. Furthermore, gram staining and biochemical test were performed for the colony having greenish metallic sheen. 18 positive isolated strains were subjected to antibiotic susceptibility tests, biofilm assays, MIC. The data were statistically analyzed with the help of SPSS version 25.

From the study, the overall prevalence of *E. coli* was 41.86%. The sensitivity of *E. coli* isolates showed a significant difference in Amikacin, Cefotaxime, Nalidixic acid, and Chloramphenicol whereas Ciprofloxacin was most susceptible. Moreover, 72.22% were weak biofilm producers, followed by moderate biofilm producers i.e., 5.56%, and 22.22% of isolates were non-biofilm producers. According to the study, 44.44% of *E. coli* isolates were sensitive to ciprofloxacin while 22.22% were resistant, 83.33% of isolates were resistant to Ampicillin while 11.11% were sensitive. The 7-gene multiplex PCR assays indicated that 0.14% (1/7) of isolates were harboring the stx2 gene and 57.14% (4/7) of isolates were harboring escV and invE genes. Virulent gene ent and pic was present in 14.28% (1/7) isolates. Atypical bfpB and bla AmpC gene harboring were detected in 42.85(3/7) isolates.

Keywords: water resources, pathogenic *E. coli*, minimum inhibitory concentration, biofilm production, PCR.

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LISTOF ABBREVIATIONS

NA	Nutrient Agar
EMB	Eosin Methylene Blue
AST	Antibiotic Susceptible Test
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standard Institute
µg	Micro gram
ESBL	Extended Spectrum Beta Lactamase
OD	Optical Density
MDR	Multi-Drug Resistant
MHA	Muller Hinton Agar
MIC	Minimum Inhibitory Concentration
MRVP	Methyl Red Voges Proskauer
TSB	Tryptic Soya Broth
CDC	Centers for Disease Control and Prevention
WHO	World Health Organization
TSI	Triple Sugar Iron
HC	Hemorrhagic Colitis
HUS	Hemolytic Uremic Syndrome

CHAPTER I

INTRODUCTION

1.1 Background

The type species of the genus Escherichia, which includes largely motile Gram-negative bacilli and belongs to the Enterobacteriaceae family, is *Escherichia coli*.

It's the most common facultative anaerobe in human colonic flora, and it colonizes the infant's gastrointestinal system in a matter of hours. Within a few hours of birth, *E. coli* colonizes human newborns' gastrointestinal tracts. In most cases, *E. coli* and its human host co-habitat for decade as a key element of the normal intestinal microflora of human and other mammals. Because commensal *E. coli* niche is the mucous layer of the mammalian colon, it may be able to take use of its ability to use gluconate in the colon more effectively than other resident species (Conway T et al 2015).

Virulence factors that impact a wide range of cellular processes are used by several different *E. coli* strains to induce a variety of intestinal and extraintestinal illnesses. These pathogens have been divided into two major groups: enteric pathogens and extraintestinal pathogens. The enteric pathogens are agents of diarrhea in humans and animals, with the exception of Shiga toxin-producing *Escherichia coli*, which also indirectly affects body areas other than the intestine (Kuhnert 2000).

Except in immunocompromised hosts or when the normal gastrointestinal barriers are overcome, these commensal *E. coli* strains rarely cause disease. Only the most effective combinations of virulence factors have survived to form particular *E. coli* pathotypes that can cause disease in healthy people. Enteric/diarrheal disease, urinary tract infections (UTIs), and sepsis/meningitis are three common clinical syndromes that can develop from infection with one of these pathotypes. Enteroaggregative *E. coli* (EAEC), Enterohemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC), Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), and Diffusely Adherent *E. coli* (EDEC) are

the six pathotypes currently recognized that can cause diarrhea in humans, based on phenotypic traits and specific virulence factors (Ndlovu et al 2014).

E. coli strains are the most common human gut pathogens. Enterotoxigenic *E. coli*

(ETEC), Enteroinvasive *E. coli* (EIEC), Vero cytotoxin-producing *E. coli* (VTEC), and Enteropathogenic *E. coli* (EPEC) are pathogenic strains that are specifically associated with diarrhea (EPEC). In the third world, ETEC is linked to newborn and adult watery diarrhea, as well as travelers' diarrhea. Major waterborne bacterial infections are caused by *Escherichia coli*, which is successfully transferred through contaminated water sources that are regularly shed into water sources *via* faces. Both contaminated drinking water and recreational waters have been recognized as sources of waterborne transmission (WHO). The contamination of the water supply with pathogenic *E. coli* strain is a serious risk factor for spreading waterborne infections in human (Swedan et al 2019).

All daily activities, including drinking, domestic use, food production, and recreation, require water. *E. coli* in water is a significant signal of pollution from sewage or animal waste. The presence of pathogenic *E. coli* in the environment is a major public health problem because it raises the risk of waterborne illness. Although water borne diarrheal infections may accumulate in river water and cause pollution of drinking and irrigation water (Otazo et. al 2018). They are generally found in very low concentrations in ambient waters (Hus et al 2009). *E. coli* strains that produce Shiga toxins, which cause diarrhea, are most usually connected with food poisoning and other serious human disorders. Hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) are fatal infections caused by *E. coli* strains, and many people have died as a result.

In underdeveloped countries, the majority of identified *E. coli* strains are an etiologic cause of diarrhea (Sweden et al 2019). The etiological agents of diarrhea, *Escherichia coli* strains, are one of the most important of the different etiological agents of diarrhea, where strains have developed by acquiring a specific set of features that have effectively persisted in the host

(Tania et al 2016). In Angola and Malawi, EHEC was suspected of causing bloody diarrhea and deaths among refugees.

The distribution of antibiotic-resistant Enterobacteriaceae strains in the aquatic environment has been examined in several parts of the world in recent years. Because of its practical importance, the majority of the studies focused on transferrable drug resistance. Only after strains have been shown to be treatment resistant are species recognized. Antibiotic resistance in enteric bacteria has been found in a variety of water sources, and these water sources could help spread resistant bacteria to a larger population of people and animals. This is especially true in low-income nations like Tanzania, where animal and human water supplies are regularly shared (Lyimo et al 2016).

Antimicrobial resistance has been observed to be on the rise in enteropathogenic bacteria, especially *E. coli*, in recent years, occasionally leading to a point-break situation when no antibiotic treatment options exist. In the underdeveloped nations, enteropathogens are often seen and can cause life-threatening illnesses, especially in children (Taldukar et al 2013**).**

Survival of bacteria outside of the host under famine and diverse environmental conditions has resulted in the development of two basic bacterial strategies: dormancy or the establishment of sessile communities in close proximity to surfaces. Bacteria generally do not exist freely in suspension, but rather in intricate colonies known as biofilms. Biofilms can be thought of as a universal strategy for bacterial survival that allows them to make the best use of the nutrients available.

Desiccation, bacteriophages, amoeba, and biocides employed in industrial processes are among challenges that biofilm protects microorganisms from resistance can be caused by the synthesis of antibiotic inactivating enzymes, such as beta-lactamase, which accumulates within the glycocalyx and creates concentration gradients that protect underlying cells (Ponnusamy et al 2012).

The production of biofilms by *E. coli* contributes to the spread of infections and makes their removal harder. Different extracellular appendages that aid *E. coli* surface colonization, as well as their finely regulated expression and

activity, all contribute to the creation of mature biofilms. Biofilms of *Escherichia coli* have been discovered to be the primary cause of many intestinal illnesses. Biofilms play a role in up to 60% of human infections, and antibiotic treatment is difficult to eliminate them biofilm in word (Ponnusamy et al 2012).

The minimum inhibitory concentration (MIC) is the gold standard for determining antimicrobial susceptibility in organisms, and it is used to evaluate the efficacy of all other susceptibility testing procedures. The minimum inhibitory concentration (MIC) of a medicine is defined as the lowest concentration that will prevent observable growth of an organism following an overnight incubation period (Andrews 2001).

A multiplex PCR for detection of three categories of diarrheagenic *E. coli* was developed. With this method, enterohemorrhagic, enteropathogenic and enterotoxigenic, enteroinvasive and enteroaggregative *E. coli* were identified in water samples from patients with hemorrhagic colitis, watery diarrhea, or hemolytic-uremic syndrome and from food-borne outbreaks.

For the detection of the following virulence markers: *eaeA* for the structural gene of intimin of EPEC and EHEC, *bfpA* for the structural gene of the bundle forming pilus of EPEC, *hlyA* for the plasmid encoded enterohemolysin of EHEC, *elt* and stla for the enterotoxins of ETEC, *ial* for the invasion associated locus of the invasion plasmid found in EIEC, CVD432 for the nucleotide sequence of the *aggR* DNA fragment of EAEC (Aranda et al 2004, Brandal et al, 2007, Kimata et al 2005). The main aim of this study is to identify the diarrheagenic *E. coli* by cultural and PCR methods.

1.2 Rationale of the study

Water is a necessity for all living things to survive. Water is used for a variety of purposes, and the waste water left over is known as sewage, which is made up of 99% water and 1% solids. River water may have a high bacterial load, including coliforms and other microbes. Sewage may enter into the river water as a result of a drainage system leak. Drainage from surrounding houses is disposed of in river water as a result of urbanization as well as due to the open

area people defecate nearby water sources which is the main source for *E. coli* contamination. Different living organisms, as well as humans, use these water sources for drinking and a variety of other reasons. *E. coli*, which is found in the water, enters the body during this procedure. There are several strains of *E. coli* that are very pathogenic and responsible for diarrheagenic and extraintestinal diseases in humans. Pathogenic strains such as VTEC, EPEC, EIEC, and ETEC are particularly to blame.

E. coli was previously thought to be unable to survive in the environment and grow in secondary habitats such as water, sediment, and soil, but recent research has shown that *E. coli* can survive in the environment for long periods of time and potentially replicate in water, algae, and soils in tropical, subtropical, and temperate climates. *E. coli*'s aptitude in the environment is most likely related to its energy acquisition adaptability.

E. coli is an excellent bioindicator model for antimicrobial resistance surveillance research since it may easily develop resistance and is found in many different animal species. Antibiotic therapy of infection has become less successful due to the advent of dangerous bacteria that are resistant to various antibiotics. Bacteria in the guts of humans and animals are exposed to various types, concentrations, and frequencies of antimicrobial agents, which leads to the development of resistance.

Water is used for various purposes and waste water after its use is known as sewage which consists of approximately 99% water and 1% solid. Sewage consists of heavy bacterial load like coliforms as well as other microorganisms. Due to the leakage in the drainage system, sewage may get access into the different water sources. These water sources are used by different living organisms as well as human beings for drinking as well as other various purposes. During this process *E. coli* present in the water enters into the body.

Different *E. coli* pathogenic strains cause various diseases. It's because of some variations in their genomic structure. As a result, this research aids in the identification of *E. coli* pathogenic genes that cause human diarrhea.

There are various strains of *E. coli* which are highly pathogenic and are responsible to cause diarhogenic disease in human beings. Especially pathogenic strains like VTEC, EPEC, EIEC and ETEC are responsible (Kaper J B et al 2004).

1.3 Objectives

1.3.1 General objectives

- Detection and characterization of diarrheagenic *E. coli* isolated from rivers flowing in the Terai belt of Province No. 1

1.3.2 Specific objectives

- To isolate and identify the *Escherichia coli* from water sample.
- To perform antibiotic sensitivity testing of *Escherichia coli*.
- To identify Biofilm producing *Escherichia coli*.
- To perform minimum inhibitory concentration of Ampicillin and Gentamycin against *Escherichia coli*.
- To diagnose diarrheagenic *E. coli* by PCR.

CHAPTER II

LITERATURE REVIEW

2.1 Water

All living species, even dangerous bacteria, require water to survive. Access to safe drinking water is a basic human need and thus a fundamental right, but many people lack access to clean and safe drinking water, and many people die as a result of water-borne bacterial diseases. Contaminated water endangers everyone's physical and mental health, and it's an insult to human dignity (WHO 2003).

E. coli is mostly associated with human feces; it is a useful indicator of human fecal contamination of water as well as the appropriate focus of monitoring for indicators of potential enteric pathogens in environmental and potable waters. Surface water *E. coli* comes from a variety of sources, including municipal wastewater discharge, septic leachate, agricultural or storm water run-off, wildlife, and non-point sources of human and animal waste (Ndlovu et al 2015). Feces entering water through direct pollution of surface run-off or sewage can introduce a wide range of diseases. There was a substantially higher probability of major aquatic disease outbreaks in intensive aquaculture. It has higher demands for water treatment chemicals and medications for illness prevention and treatment, which could result in the spread of resistant human pathogen strains in nearby waters (Obasohan et al 2010).

E. coli is a common cause of extraintestinal infections, and it has various virulence factors, including as adhesins, fimbriae, hemolysin, and aerobactin, that can aid in the bacterium's pathogenesis (Sabate et al 2008). Water and food contamination by feces is a prevalent and chronic problem that has a negative influence on public health as well as local and national economies (Satoshi et al 2008).

According to the World Health Organization (WHO), water is responsible for nearly 80% of all disease, particularly in developing nations (Cheesbrough

2000). For the disposal of both animal and human wastes of fecal origin, land application is becoming a very regular routine procedure. The spread of enteric pathogens into the running water is a serious problem associated with the disposal of such wastes. Animals raised for food are known to be reservoirs for enteric diseases, which can spread through the food chain and infect humans (Ronand et al 2002).

Food was responsible for 52% of *Escherichia coli* O157:H7 outbreaks between 1982 and 2002, with fruit and vegetables accounting for 21% of these cases (Rangel et al 2005). Irrigation water, soil, poorly composted manure, and human handling are all important sources of microbial contamination before harvest (Beuchat 1995). Contaminated water in fields is widely assumed to be a primary cause of *E. coli* O157:H7 epidemics (Fonseca et al 2011). According to the WHO, the annual death toll from water-related diseases surpasses 5 million people. More than half of these are microbial gut illnesses (Cabral 2010).

2.2 Escherichia coli

Escherichia coli is a Gram-negative bacterium that is naturally facultatively anaerobic. It's a rod-shaped, nonsporulating bacterium that can be simply and cheaply produced in the laboratory. It has been used as a model organism in a variety of biological engineering and industrial microbiology research (Lee 1996). The majority of *E. coli* strains are found in the intestines of warm-blooded creatures, where they aid the host by preventing harmful bacteria from colonizing (Singleton 1999).

Escherichia coli (*E. coli*), a member of the fecal coliform group of bacteria that is often found in the gastrointestinal system and feces of warm-blooded animals, is characterized by its inability to break down urease. *E. coli* levels in freshwater are a recommended indication for freshwater recreation, and their presence in ambient and drinkable waters gives direct proof of fecal contamination from warm-blooded animals (Bej et al 1990).

E. coli, on the other hand, is more than a laboratory worker or an innocuous intestine dweller; it may also be a very adaptable and usually lethal pathogen.

Virulence factors that impact a wide range of cellular processes are used by several different *E. coli* strains to induce a variety of intestinal and extraintestinal illnesses. In tiny children and the elderly, a newly found strain of *E. coli* (*E. coli* 0157:H7) can cause serious illness and even death. However, there are some highly adapted *E. coli* clones that have gained unique virulence properties, allowing them to adapt to new environments and cause a broad spectrum of disease (Kaper 1998).

Meningitis, septicemia, urinary tract infections, and intestinal infections are all ailments caused by *E. coli*. *E. coli* strains that cause diarrhea are less likely to cause extraintestinal infections, whereas those that do cause extraintestinal illnesses are less likely to cause diarrhea (Russo and Johnson, 2003). Extraintestinal pathogenic *E. coli* (ExPEC) are *E. coli* strains that cause extraintestinal illnesses (Johnson 2000). Intestinal pathogenic *E. coli* strains are infrequently seen in the feces of healthy people and are rarely the cause of extraintestinal illness (Smith et al 2005).

2.2.1 Diarrheagenic *Escherichia coli*

Escherichia coli, which is extensively employed as a microbiological quality indicator for water, is also a major cause of diarrhea and other enteric disorders (Talukdar et al 2013; Gomes et al 2016). E coli strains implicated in diarrheal disorders are one of the most important of the different etiological agents of diarrhea, where strains have developed by acquiring a specific set of features that have effectively persisted in the host through horizontal gene transfer (Gomes et al 2016). Lateral gene transfer from harmful microbes to *E. coli* strains, on the other hand, could result in the creation of new pathogenic strains. Due to the presence of a specific gene associated with pathogenicity, colonization factors that are often missing in non-pathogenic strains of this characteristic indicator organism, they cause various types of diarrheal disorders (Ndlovu et al 2014).

Because of the presence of unique genes important for pathogenicity, colonization, and virulence factors, pathogenic *E. coli* strains can cause distinct types of diarrhea infections (Prescott et al 2005). A certain strain of *Escherichia coli* has been linked to a variety of diseases that affect both

animals and humans all over the world (Matthew et al 2009). Enterotoxigenic *E. coli* (ETEC), Enterohemorrhagic *E. coli* (EHEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), and Diffusion adherent *E. coli* (DAEC) are now identified *E. coli* pathotypes that cause diarrhea in people (Talukdar et al 2013).

In developing nations, acute microbial diarrheal illnesses represent a major public health issue. The people who are most afflicted by diarrheal infections are those who have the fewest financial resources and the least sanitary conditions. More than 1.5 million children die each year from diarrheal infections because they do not have access to appropriate sanitation (Cabral 2010).

Diarrheal diseases are significant infectious diseases that cause a high rate of morbidity and mortality over the world (Sweden et al 2019). Diarrheal disease accounts for 4.1 percent of the total daily global burden of disease and kills 1.8 million people per year, 90 percent of whom are children under the age of five (Talukdar et al 2013).

EHEC was identified from 5.1 percent of children with diarrhea in a study of pediatric diarrhea in Nigeria. In Angola and Malawi, EHEC was suspected of causing bloody diarrhea and mortality among refugees (Relly WHO 1998). Diarrhea is still a major public health issue for children in northeastern Brazil's developing districts. *Escherichia coli* is the most prevalent bacterial pathogen linked to endemic forms of infantile diarrhea (Scaletsky et al 2002).

The countries of Africa, Asia, and Latin America in the low and middle east are the most afflicted, with diarrheal disease happening more frequently and with lethal consequences (Gomes et al 2016). Bangladesh has a high prevalence of diarrheal illnesses. In Bangladesh, an estimated 20,000 children under the age of five died from diarrheal infections in 2008 (Talukdar et al 2013). Diarrhea is still the most common ailment among tourists and foreign residents in Kathmandu, as well as travelers in general. Nepal has the highest risk among 28 nations studied in a multicenter study reporting rate ratios for gastrointestinal travel (Pandey et al 2010).

In underdeveloped nations, diarrhea attack rates (episodes per child per year) in children under the age of five range from 6.0 to 11.9 per 1,000 children (Ono et al 2014). Diarrhea kills an estimated 2.2 million people worldwide each year, the majority of them are children (WHO 1999). Every year, 76 million cases of foodborne disease are reported in the United States, resulting in 325,000 hospitalizations and 5000 fatalities. *E. coli* is also to blame for these illnesses (Ishii et al 2008). In Africa, studies reveal that diarrhea is responsible for almost 20% of all deaths in children under the age of five who live in settlements with limited access to water and sanitation (Momba et al 2006).

2.3 Pathotypes of *Escherichia coli*

Due to the presence of particular genes responsible for pathogenicity, colonization, and virulence factors that are not found in non-pathogenic *E. coli* strains, pathogenic *E. coli* strains can cause unique types of diarrhea infections (Prescott et al 2005). These strains are divided into two categories: intestinal and extra-intestinal. Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* EPEC), Enteroinvasive *E. coli* (EIEC), Enterohaemorrhagic *E. coli* (EHEC), Enteroaggregative *E. coli* (EAEC), Diffusely adherent *E. coli* (DAEC), Uropathogenic *E. coli* (Ndlovu et al 2014**).** Extraintestinal pathogenic *E. coli* are a distinct group of bacteria that mostly cause infections of the urinary tract in people of all ages, as well as sepsis and meningitis in small children and animals (Kuhnert et al 2000).

2.3.1 Enterotoxigenic *Escherichia coli* (ETEC)

ETEC is a heterogeneous family of lactose fermenting *E. coli* that produces enterotoxins that can be heat labile or heat stable, as well as colonization factors that allow the organism to colonize the small intestine and induce diarrhea (Qadri et al 2005). ETEC strains are thought to be a pathogenic prototype because they colonize the surface of the small bowel mucosa and produce enterotoxins, resulting in a net secretory condition (Narato et al 1998).

ETEC is the most prevalent cause of travelers' diarrhea in children under the age of five, and it can have fetal effects (Matthew et al 2009). ETEC travelers' diarrhea is particularly common during hot and humid months, as well as among first-time visitors to underdeveloped nations. Travelers' diarrhea is caused by contaminated food and water and is characterized by frequent, watery diarrhea that lasts several days and frequently results in dehydration and malnutrition in small children (Narato et al 1998).

ETEC serotype can cause infantile gastroenteritis. The number of reports of their occurrence in developed countries is comparatively small, but it is an extremely important cause of diarrhea in the developing world, where there is no adequate clean water and poor sanitation (Cabral 2010).

These strains are the most common isolated bacterial entero-pathogens in children under the age of five in underdeveloped nations, accounting for hundreds of millions of cases of diarrhea and tens of thousands of fatalities each year (Cabral 2010). In Bangladesh, *E. coli* is one of the most common causes of enteric infection, with ETEC being the most common pathotype (Talukdar et al 2013).

2.3.2 Enteropathogenic *Escherichia coli* (EPEC)

EPEC is a kind of diarrheagenic *E. coli* that has been associated to baby diarrhea in low-income countries. This pathovar is part of a group of infections that cause A/E lesions on intestinal epithelial cells (Matthew et al 2009). EPEC is a major cause of potentially fatal diarrhea in infants in underdeveloped countries, however multiple outbreaks of EPEC diarrhea in healthy inoculum from a common source have been described. It can be spread by the fecal-oral route with contaminated hands, infected weaning foods or formula, or contaminated fomites (Narato et al 1998).

EPEC was previously a common cause of newborn diarrhea outbreaks in the United States and the United Kingdom. These nosocomial and communityaccepted outbreaks were frequently explosive, with up to 50% mortality. EPEC strains have also been linked to intermittent diarrhea outbreaks in the US and other developing nations. EPEC infection has been

linked to 30-40% of baby diarrhea in studies in Brazil, Mexico, and South Africa, and EPEC infection has been reported to outnumber rotavirus infection in several investigations (Narato et al 1998).

2.3.3 Enterohemorrhagic *Escherichia coli* (EHEC)

Enterohemorrhagic *E. coli* pathogenesis is shared by clones of zoonotic *E. coli* belonging to several O serogroups. These bacteria have developed and acquired particular virulence characteristics that allow them to colonize and infect the human colon without infecting the bloodstream. EHEC induce bloody diarrhea, severe colitis, and HUS if they've been consumed. When infection is linked to severe colonic and/or renal illness, these bacteria are referred to as EHEC (Goldwater et al 2012).

In 1983, two cases of acute crampy stomach discomfort, watery diarrhea followed by severely bloody diarrhea, and little or no fever were studied. Shiga toxin is a significant virulence factor and a defining feature of EHEC. This powerful cytotoxin is the cause of death and a variety of other symptoms in EHEC patients. Humans are mainly infected by contaminated food and drink (Matthew et al 1998). Healthy cattle have also been discovered to be the bacterium's principal reservoir. EHEC has also been reported to survive for months in manure and water trough sediment after being isolated from bodies of water (pond, stream), wells, and water troughs (Goldwater et al 2012).

The CDC believes that *E. coli* 0157:H7 causes more than 20,000 infections and up to 250 deaths each year in the United States, although the lack of clinical laboratories to screen for this bacterium makes any figures difficult. EHEC is a significant disease in Europe and Japan, in addition to its importance in North America (Narato et al 1998).

2.3.4 Enteroinvasive *Escherichia coli* (EIEC)

Lysin decarboxylase negative, non-motile, and lactose negative EIEC strains are common. EIEC strains are closely linked to Shigella spp. biochemically, genetically, and pathogenetically (Narato et al 1998). Because EIEC and Shigella have similar pathogenic processes, it is often assumed that they should form a single pathovar. Shigella germs are very contagious and

produce bacillary and bloody diarrhea. This pathovar is distinct from other *E. coli* pathovars in that it contains obligatory intracellular bacteria that lack flagella and adhesion factor (Matthew et al 1998).

A period of watery diarrhea precedes the beginning of sparse dysenteric stool with blood and mucus in both Shigella and EIEC infections. In fact, only watery diarrhea is seen in the majority of EIEC patients and many Shigella patients (Narato et al 1998). Dysentery was more common in people over the age of 50. Any meal contaminated with human feces from an ill person, either directly or by polluted water, could make others sick (Cabral 2010).

Although the frequency of EIEC in affluent nations is assumed to be low, foodborne outbreaks can occur on occasion, such as one restaurant-related incident in Texas that affected 370 people (Narato et al 1998). In a 1985 study in Bangkok, Thailand, 410 children without diarrhea and an equal number of control children without diarrhea were tested for the presence of Shigella, EIEC, and other pathogens. It was discovered that 17 of the children with diarrhea and six of the youngsters without diarrhea had yielded (Cabral 2010).

2.3.5 Enteroaggregative *E. coli* (EAEC)

EAEC is increasingly becoming recognized as a cause of endemic and epidemic diarrhea around the world. EAEC is the second most prevalent cause of travelers' diarrhea after ETEC in both developed and developing countries, despite the fact that it is considered an emerging disease. EAEC colonization occurs in the mucosa of both the small and large intestines, causing minor colon inflammation. It damages mucosal tissue by secreting cytotoxins; however, not all toxins are present in all cases, and mucus or blood may be present (Croxen et al 20010).

In Mexico, Chile, Bangladesh, and Iran, EAEC has been linked to intermittent diarrhea. In India, studies have found that the sickness is most commonly indicated by watery, secretory diarrhea without fever or coarse blood (Narato et al 1998).

2.4 Antibiotic Resistance

Antibiotic resistance is a worldwide problem that is increasing morbidity and mortality (Port et al 2014). Antibiotic resistance is on the rise, owing to the overuse of antibiotics in human medicine. Among gram-negative bacteria, multidrug resistance has been on the rise (Pathak et al 2017).

Infectious disease experts are increasingly concerned about the spread of antibiotic resistance genes among bacterial strains (Maynard et al 2003). When bacteria evolve under selective pressure to confer resistance to antibiotics used to treat their infection, this is known as resistance (Port et al 2014). Many antibiotic resistance genes are found on plasmids and/or transposons, allowing them to be passed across bacteria. B-lactamase enzymes are bacteria-produced enzymes that hydrolyze the amide bond of antibiotics with four members (penicillin, cephalosporin, monobactam, and carbapenems) (Pathak et al 2017). They hydrolyze -lactam antibiotics, causing penicillin, cephalosporin, and aztreonam resistance (Reinthaler et al 2010).

Resistance to fluoroquinolone drugs, such as ciprofloxacin, levofloxacin, moxifloxacin, norfloxacin, and nalidixic acid, has been increasing in *E. coli* since the 1090s. The use of ciprofloxacin or other fluoroquinolones increases the chance of isolating resistant *E. coli* germs from patients receiving long-term hospital care, and resistance is linked to treatment failure (Smith et al 2007).

Antibiotics are virtually always present in the surface waters of populated areas, exerting evolutionary pressure on the microorganisms they encounter. Apart from medications, there is a direct entry of antibiotic-resistant bacteria into the environment through animal husbandry via liquid and solid manure, as well as human excretion via wastewater (Reinthaler et al 2010). The increased resilience of pathogenic bacteria in the environment, as well as their ecotoxic impacts, are causing considerable worry. This involves both the ecology of the resistance gene as well as the ecology of the resistant bacteria (Aziz et al 2014).

Since the first report of antibiotic resistance in the late 1930s, shortly after its usage in medicine, approximately 20,000 possible resistance genes have been uncovered in genome sequencing data repositories (Aslan et al 2018). Antimicrobials can reduce the duration of bacterial enteritis, but they have little effect on viral and non-infectious diarrheas, which account for 10 to 70% of all episodes. Antimicrobial abuse is unfortunately all too often linked to enteric illnesses (Okeke et al 2005).

2.5 Biofilm

Microbial communities that live in a self-produced extracellular polymeric matrix made up of exopolysaccharides (ESP), proteins, and DNA adhering to abiotic or biotic surfaces are known as biofilms (Schiebel et al 2017). Bacteria in a biofilm are shielded against a variety of stresses, including nutrition scarcity and sanitizers (Vogeleer et al 2021). Desiccation, bacteriophages, amoeba, and biocides used in industrial operations are among threats that biofilms defend bacteria from. Resistance to antibiotics can be caused by the development of inactivating enzymes, and there is evidence that the relatively large levels of antibiotic inactivating enzymes such as -lactamase that accumulate within the glycocalyx form concentration gradients that can protect underlying cells (Ponnusamy et al 2012).

Biofilms play a part in up to 60% of human infections, and they're tough to get rid of with antibiotics (Ponnusam et al 2012). In the case of *E. coli*, biofilmassociated bacteria can be up to 1000 times more resistant to antimicrobial therapy than their planktonic counterparts. Biofilm formation (BF) contributes to the occurrence of various infections and makes eradication difficult (Theeb et al 2021).

Bacterial biofilms can cause persistent, nosocomial, and device-related infections in clinical settings because of their great resistance to medications and the host immune system. It's becoming more widely accepted that the creation of microbial communities is linked to bacterial pathogenicity, and that bacteria that survive in biofilms are the primary cause of recurrent and chronic illnesses (Schiebel et al 2017).

Biofilms are a major public health concern because of their link to antibiotic resistance in bacteria (Theeb et al 2021). Biofilm-mediated sanitizer resistance obstructs contamination management, particularly in food processing plants. This allows biofilms to survive and persist in hostile environments such as water, nutrient-limited environments, and environments where sanitizers are used (Vogeleer et al 2021). Several studies have shown that pathogenic or opportunistic pathogenic bacteria in biofilms can survive longer and have stronger chlorine resistance than those in drinking water. The nutrient supply for bacteria is better in biofilms, and bacteria are shielded from disinfection (Markku et al 2007). Due to insufficient washing or sanitizing treatment, *E. coli* 0157:H7 may survive in infected fresh cut produce (H Olmezet et al 2010).

2.6 Treatment

When a patient is diagnosed with diarrhea, it is critical to avoid dehydration since dehydration can be fatal, especially in youngsters. This is done to replenish the fluids lost in the intestines as well as replace the electrolytes that have been lost (Welkos et al 1997). Controlling diarrheal symptoms can help you avoid serious illnesses like hemolytic uremic syndrome and hemorrhagic colitis. Antibiotics were once the primary treatment for bacterial infections; however, the use of these antibiotics in the treatment of illnesses caused by diarrheagenic *E. coli* strains is now being closely scrutinized. Antibiotic resistance to treatment has been causing significant problems in medical institutions, clinics, and scientific studies (Seti et al 2014). As a result, it's more important than ever to be cautious while choosing antibiotics for therapy (Qudri et al 2005).

Antibiotics can be used to treat certain strains of diarrheagenic *E. coli*, such as ETEC, EPEC, and EAEC; however, antibiotics cannot be used to treat infections caused by the EHEC strain. Through the stimulation of the bacterial SOS response, this strain causes difficulties such as the generation of toxins (Kimmitt et al 2000). This is a reaction that damages DNA caused by infection, potentially worsening the condition (Huang et al 2018).

2.7 Prevention

Because surface water is frequently contaminated with this bacterium, the majority of *E. coli* illnesses are acquired through water. The precipitating variables are poor sanitation and hygiene, overcrowding, and a lack of access to safe drinking water (Talukdar et al 2013). Coliform bacteria in water sources such as rivers, lakes, and groundwater have also necessitated poor control because these microbes can directly or indirectly affect human health. Through dirty water from untreated sewage, septic tanks, and other sources, coliform bacteria can cause significant sickness such as gastroenteritis and diarrhea (Mijin et al 2019). Percentage of presumptive E coli positive samples must be validated for the Examination of Water and Wastewater. These microbes may survive for long periods of time in the environment, and humans are exposed to the bacteria through contaminated feed, handling, and drinking water, as well as farm irrigation. They're also employed as a fecal contamination indicator in water.

Due to global water scarcity, it is vital to address the issues that arise from water reuse. One possible issue with reuse is the introduction of chemical and biological contaminants into the environment from WWTP effluents. Targeting fecal coliform bacteria has been used to monitor recycled water, but antibiotic-resistant indicator bacteria have not been included (Aslan et al 2018).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

A complete list of materials, types of equipment, chemicals, reagent, antibiotics and media used for the study are listed in Appendix 1.

3.2 Methods

3.2.1 Study Design and study area

Along the Mahendra highway, the study area was expanded from the Mechi River in the east to the Saptakoshi River in the west. Altogether, 43 big and small rivers pass within a total distance of 188.7 km. The research was laboratory based cross sectional study conducted in the microbiology laboratory of the central campus of technology in Hattisar, Dharan, Sunsari, Nepal.

Photo 1: study area from Mechi River to Saptakoshi River

3.2.2 Sample size and types

The Study was carried out from October 2020 to march 2021. During the study, A total of 43 water samples were collected asceptically and transported to the laboratory. The sample size was calculated by using the standard formula:

Sample size = $4pq/e^2$

Where:

p = the prevalence rate from previous research

q = 1-p

e = standard error

3.3 Sample collection and transportation

River water samples were collected from a number of rivers that flow through the Mahendra Highway in Province No. 1. Water samples were collected aseptically in sterile 250ml Biological Oxygen Demand bottles.

The samples were subsequently transported aseptically from the collection site to the laboratory. The samples were preserved in biological oxygen demand bottles that were kept on dry ice kept at 4^oC to maintain optimal microbiological conditions (Shrestha et al 2019).

3.4 Isolation of E. *coli*

In the laboratory, the collected water sample was enriched in lactose broth for 6 hours. Then *E. coli* was isolated by inoculating 0.1ml of water sample by the spread plate technique using a sterile dolly rod on Eosin Methylene Blue agar medium (HiMedia, India). The media plates were incubated at 37°C for 24 hours. After incubation, the culture was studied by its colony characteristics and it was sub-cultured on Nutrient Agar (HiMedia, India) for further identification (Cheesbrough 2006).

3.4.1 Storage of isolated organisms

The isolated organism was maintained and kept at -20^oC after being inoculated in nutritional broth containing 15% glycerol (Angshumanjana et al 2016).

3.4.2 Identification of organism

The organism was identified by performing various biochemical tests. A Gram staining test was performed. Biochemical tests such as the Catalase test, Oxidase test, IMVIC test, and urease test, as well as carbohydrate fermentation tests were performed (AL-Baer a s et al 2017).

3.5 Antibiotic Susceptibility test

All *E. coli* isolates from river water samples were subjected to in-vitro susceptibility tests by Kirby-Bauer disc diffusion techniques using Muller Hinton agar (HiMedia, India) as recommended by CLSI guidelines (2012). After 24 hours, fresh cultures were selected and transferred into NB to obtain turbidity equivalent to 0.5 McFarland barium sulfate standards. MHA plates were inoculated with sterile cotton swabs, then antibiotics were placed with sterile forceps and allowed to stand at room temperature for 15 minutes for prediffusion, and then incubated at 37°C for 16–18 hours. The zone of inhibition was interpreted as susceptible, intermediate, and resistant according to the CLSI "Diffusion Supplement Table" (2012).

3.6 Biofilm formation test

The quantification of biofilm was performed according to (Christensen et al 1985). In this method, 5ml of overnight culture of *E. coli* was prepared in Tripticase soya broth. Then, 100 µl of diluted culture was inoculated in a sterile 96-well polystyrene tissue culture plate well containing tryptic soya broth (HiMedia, India) additionally supplemented with 1% glucose. The plate was incubated at 37° C for 24 hours for biofilm formation. The unbound cell was discarded and washed several times with sterile Phosphate Buffer Saline (HiMedia, India) (pH-7.2). About 125 µl of 0.1% crystal violet solution was added and left inverted to dry at 60 °C for 30 minutes to fix the biofilms. The quantitative determination was performed by solubilizing the biofilm by adding 125 µl of 30% acetic acid (HiMedia, India) to each well and incubating the plate for 15 minutes at room temperature. Later, it was transferred to another microtitre plate for reading the absorbance at 570 nm by an ELISA plate reader (Loncare LR-620 microplate reader, Medical Technology Co.,

Ltd). The optical density (OD) of test wells was interpreted. The experiment was performed in triplicate. The OD of each strain was obtained by the arithmetic mean of the absorbance of negative controls (ODnc). The following classification was used for the determination of biofilm formation: no biofilm production (ODs≤ODnc), weak biofilm production (ODnc<ODs≤2.ODnc), moderate biofilm production (2.ODnc < ODS ≤ 4.ODnc), and strong biofilm production (4.OD<ODs) as described by (Stepanovic et al 2007).

3.7 Determination of Minimum Inhibitory Concentration

The minimum concentrations of Ampicillin and Gentamicin (HiMedia, India) for *E. coli* isolates were screened by the microdilution method as suggested by CLSI (2012) guidelines. The Ampicillin and Gentamicin powder were accurately weighed and a stock solution of 256 µg/ml and 512 µg/ml, respectively, was prepared. A known volume of 0.5 McFarland suspension of bacterial culture was added to each well containing the TSB broth. From the stock solution, a different concentration of drug ranging from 64 µg/ml to 0.125 µg/ml was made in roundbottom microtiter plates by serial dilution. The wells for positive and negative controls were even maintained in the plates. The microtiter plates were incubated at 37°C for 24 hours. The well with the highest concentration of drugs in which the growth of bacteria is inhibited is known to be the MIC.

3.8 DNA Extraction

The bacterial culture (1.5 ml) was grown overnight in Luria Bertani (LB) broth at 37^0C. After the overnight growth, the culture was diluted 10fold in 10mM Tris HCl, ph-8 buffer containing 1mM ethylene diamine tetra acetic acid disodium salt (EDTA), and it was boiled for 10 min. Centrifugation was done at 12000 rpm for 10 min at 4^0C and the supernatant was used as a template DNA for PCR (Sezonov G et al 2007).

3.9 Polymerase Chain Reaction (PCR)

The PCR assay utilized the primer pairs and the presence of... genes was detected.

Analysis of the primer of the targeted gene was performed on the online available BLAST programs (http://www.ncbi.nlm.nih.gov/blast/), BLASTN, BLASTx, and BLASTP. As described previously, DNA was extracted by boiling the sample followed by storage at -20°C until use. A bacterial cell lysate was used as the source of DNA. To ensure that each individual primer pair was adequate for amplification, a single-target PCR assay was conducted with our control strains. After confirmation of the specificity of each primer by monoplex PCR, the optimized protocol was carried out with a 35 µl reaction mixture that contained 10X PCR amplification buffer (100 mM Tris [pH 9.0], 500 mM KCl, 0.1% gelatin), 2.5 µl of magnesium chloride (25 mM); 2.5 µl each of 2.5 mM dATP, dCTP, dGTP, and dTTP; 90 pmol of primer gene; 1.2 U of Taq DNA polymerase; Milli-Q water to a final volume of 29.5 l; and 5.5 µl of cell lysate (template DNA). Finally, the reaction mixture was overlaid with a drop of sterile mineral oil (HiMedia, India). Amplification was carried out for 4 min at 94°C for the initial denaturation, followed by 30 cycles of 1.5 min at 94°C, 1.5 min at 55°C, and 1.5 min at 72°C, with a final round of 7 min at 72°C in a thermal cycler. The PCR product (12 l) was visualized by using a gel documentation after electrophoresis in 2% agarose gels in Trisborate-EDTA buffer at 100 V for 45 min and ethidium bromide staining (0.5 µg/ml) (Khuntia et al. 2008).

Primers used for the differentiate different strains of E. coli

SN	Target gene	Primer	Sequence	Product size
1	stx2	F R	GTTTTGACCATCTTCGTCTGATTATTGAG AGCGTAAGGCTTCTGCTGTGAC	324
2	*escV*	F R	ATTCTGGCTCTCTTCTTCTTTATGGCTG CGTCCCCTTTTACAAACTTCATCGC	544
3	*ent*	F R	TGGGCTAAAAGAAGACACACTG CAAGCATCCTGATTATCTCACC	629
4	*aggR*	F R	AGCCGTTTCCGCAGAAGCC AAATGTCAGTGAACCGACGATTGG	1111
5	*invE*	F R	CGATAGATGGCGAGAAATTATATCCCG CGATCAAGAATCCCTAACAGAAGAATCAC	766
6	*eae* intimin	F R	ACC CGG CAC AAG CAT AAG CGTAAAGCGRGAGTCAAT RTA	741

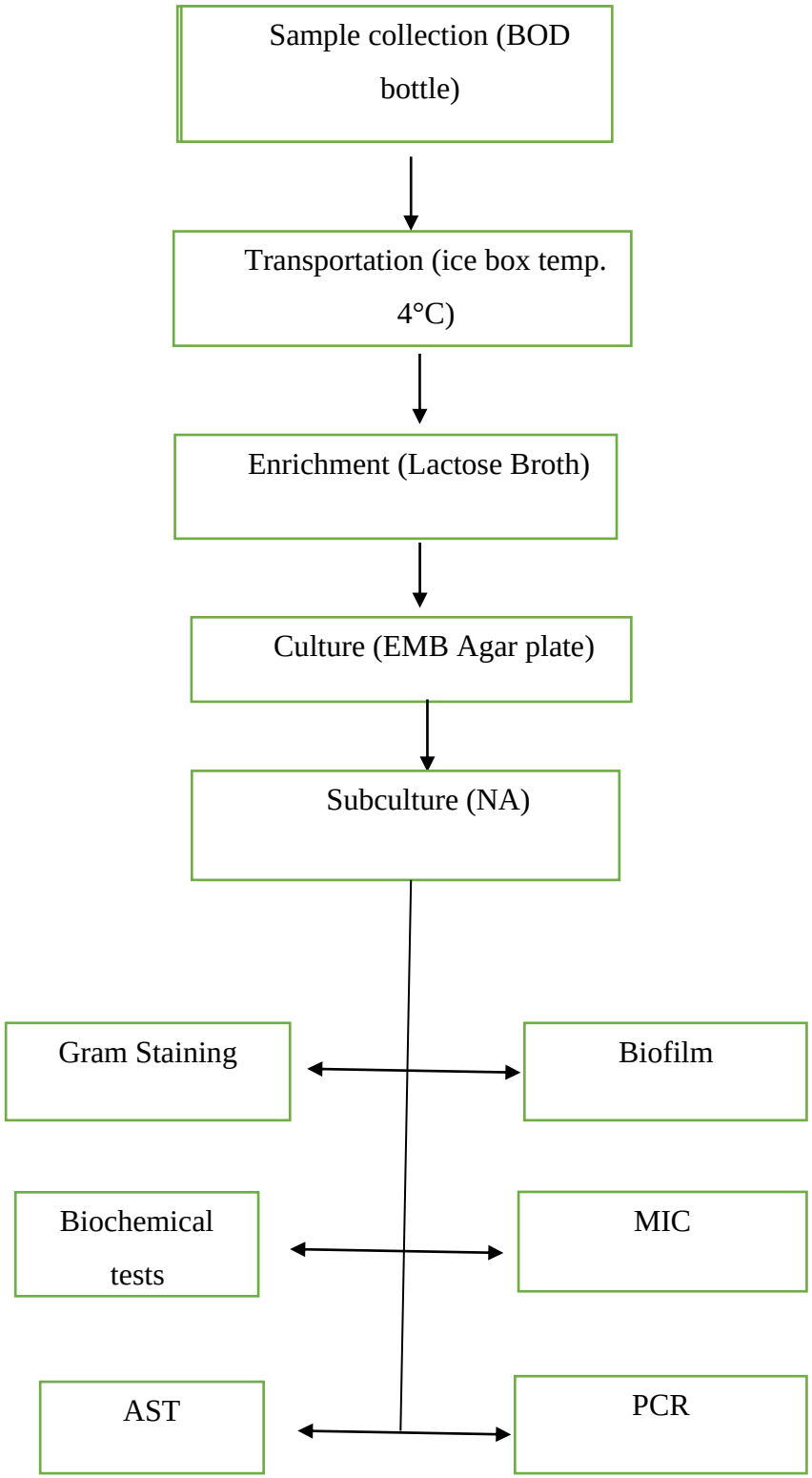
3.10 Quality Control for Tests

During the study, standard procedures were followed for the collection, isolation, and identification in order to maintain the quality and accuracy of all tests. All the media, antibiotics, and reagents were prepared, stored, and utilized as per the recommendation by the manufacturing company. Antibiotic discs were stored at refrigerator temperature.

3.11 Data management and Analysis

The data was documented in MS Excel 2010. Statistical analysis was performed using SPSS software version 16.0 and the hypothesis was assumed to be significant if the P-value was less than 0.05 at a 95% confidence level.

3.12 Flow Chart for Methodology



CHAPTER IV

RESULTS

4.1 Sample Distribution result

A total of 43 river water samples were collected from different big and small rivers that pass across the Mahendra highway from the Mechi river to the Saptakoshi river. Among them, 15 water samples were taken from Jhapa district. Similarly, 17 from Morang district and 11 from Sunsari were taken as shown in figure 4.1.

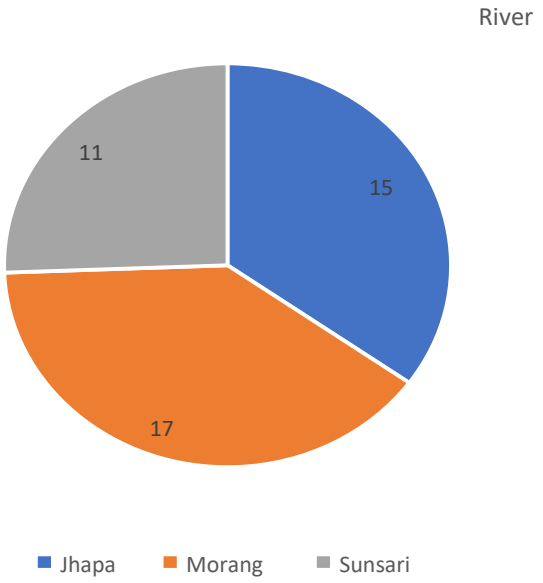


Fig 4.1: sample distribution

4.2 Occurrence and Distribution of *E. coli*

The presence of *E. coli* is shown in Figure 4.2, and it was discovered that 18 (41.8%) of the 43 water samples tested positive for *E. coli*, whereas the remaining 25 (58.2%) tested negative for *E. coli*.

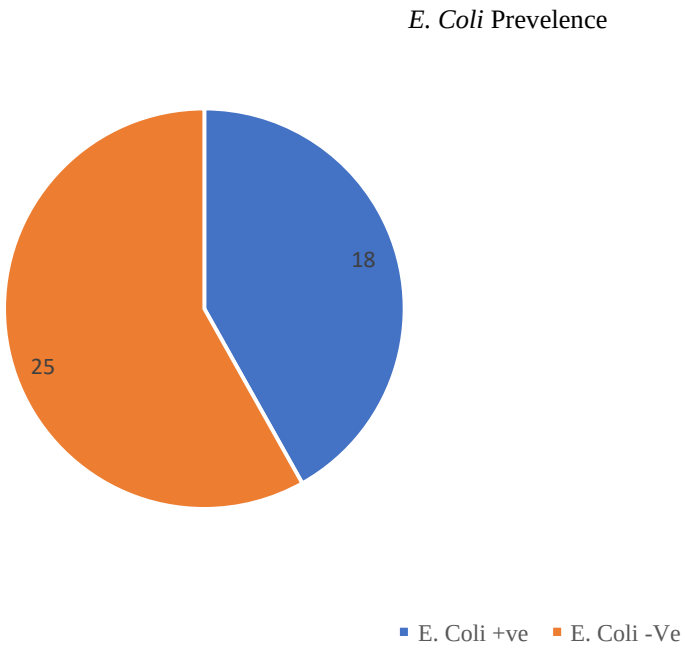


Fig. 4.2: Occurrence of *E. coli*

4.3 Location wise distribution

Out of 15 rivers in Jhapa, 7 were tested positive for *E. coli*, and 8 were found to be E. coli negative. Similarly, from a total of 17 rivers, 5 were tested positive for E. coli while the remaining 12 were tested negative in Morang district, and from 11 total samples from Sunsari district, 6 were tested E. coli positive while 5 were E. coli negative as shown in figure 4.3.

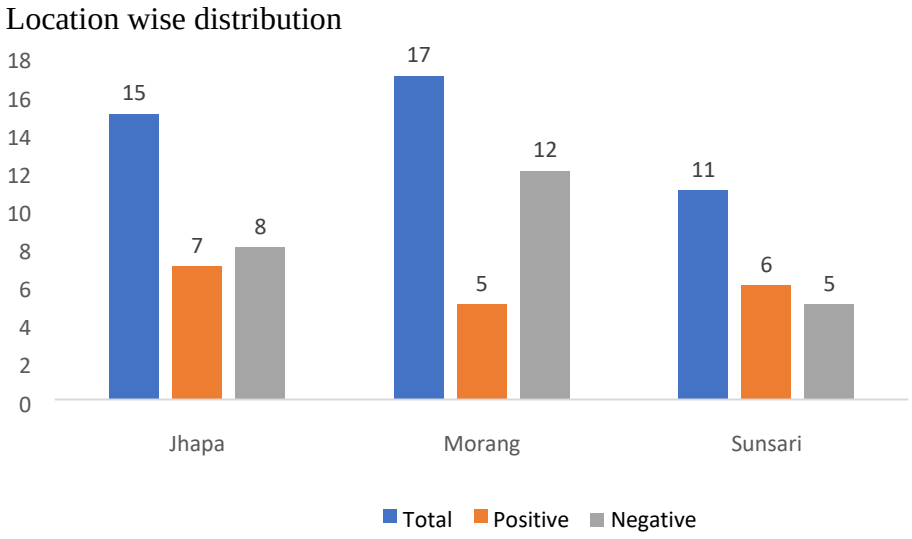


Figure 4.3: Location wise distribution of *E. coli*

4.4 Antibiotic Susceptibility Pattern of *E. coli* Isolates

The sensitivity, intermediate, and resistance patterns of isolated *E. coli* samples against several antibiotics are shown in the table below. Ciprofloxacin 12 (66.67%) was determined to be the most sensitive medicine, followed by Amikacin 11 (68.75 %), Nalidixic acid 7 (38.88 %), Chloramphenicol, and Cefoxitin 6 (37.5 %) and 6 (33.34 %), respectively. Azithromycin was shown to be 4 (22.23 %) sensitive, while Ampicillin was found to be 1 (33.34 %). While Nalidixic acid and ciprofloxacin 6 (33.34 %) were discovered to be the most intermediate drugs, they were followed by Amikacin and Cefoxitine 4 (22.23 %), Ciprofloxacin and Azithromycin 3 (16.67 %). whereas Ampicillin is the most resistant drug among all, i.e., 17 (94.45%), followed by Azithromycin, 11 (61.1%)

Table 1: Antibiotic susceptibility test of *E. coli*

SN.	Antibiotics	Resistance	Intermediate	Sensitive	P-value
1	AZM	11 (61.1%)	3 (16.67%)	4 (22.23%)	0.078
2	NA	5 (27.78%)	6 (33.34%)	7 (38.88%)	0.001
3	AK	3 (18.75%)	4 (22.22%)	11 (68.75%)	0.037
4	AMP	17 (94.45%)	-	1 (5.56%)	0.453
5	C	6 (33.33%)	6 (33.33%)	6 (33.33%)	0.001
6	CX	8 (44.44%)	4 (22.23%)	6 (33.34%)	0.001
7	CIP	3 (16.66%)	3 (16.66%)	12 (66.66%)	0.201

4.5 Biofilm Production of *E. coli*

Out of 18 positive samples, 13 (72.22%) isolates were weak biofilm forming producers, followed by 1 (5.56%) was moderate biofilm producer, while 4 (22.22%) isolates were non biofilm producers.

Table 2: biofilm formation of *E. coli*

Isolates	Biofilm formation	Total	Percentage
	High (%)	---	--
E. coli	Moderate (%)	1	5.56%
	Weak (%)	13	72.22%
	None (%)	4	22.22%

4.6 Minimum Inhibitory Concentration of *E. coli* against Ampicillin

15 *E. coli* isolates were resistant to ampicillin, while 2 were sensitive and 1 was intermediate. The numbers of the *E. coli* isolates having different MICs of Ciprofloxacin are shown by the microtiter plate method given in the table 3, which is performed according to the CLSIM guidelines.

Table 3: Minimum Inhibitory Concentration of *E. coli* against Ampicillin

Isolates	MIC (µg/ml) of Ampicillin		
E. coli	≤ 8 (Sensitive)	16 (Intermediate)	≥ 32 (Resistant)
	2 (11.11%)	1 (5.55%)	15 (83.33%)

4.7 Minimum Inhibitory Concentration of *E. coli* against Gentamycin

Out of 18 positive samples, 8 *E. coli* strains were sensitive, 6 were intermediate, and 4 were resistant to Gentamycin. The numbers of the *E. coli* isolates having different MICs of Gentamycin are shown by the microtiter plate method and are given in the table below, which is performed based on the CLSIM guideline.

Table 4: Minimum Inhibitory Concentration of *E. coli* against Gentamycin

Isolates	MIC (µg/ml) of Gentamycin		
E. coli	≤ 4 (Sensitive)	8 (Intermediate)	≥ 16 (Resistant)
	8 (44.44%)	6 (33.33%)	4 (22.22%)

4.8 PCR Detection of E. coli

From the 18 isolates of E. coli isolated from the study, the PCR run was successful and at least 7 isolates were positive for genes and confirmed as E. coli. The 7-gene multiplex PCR assays indicated that 0.14% (1/7) of isolates was harboring the stx2 gene and 57.14% (4/7) of isolates were harboring escV and invE genes. Virulent gene ent and pic was present in 14.28% (1/7) isolates. Atypical bfpB and bla AmpC gene harboring were detected in 42.85(3/7) isolates.

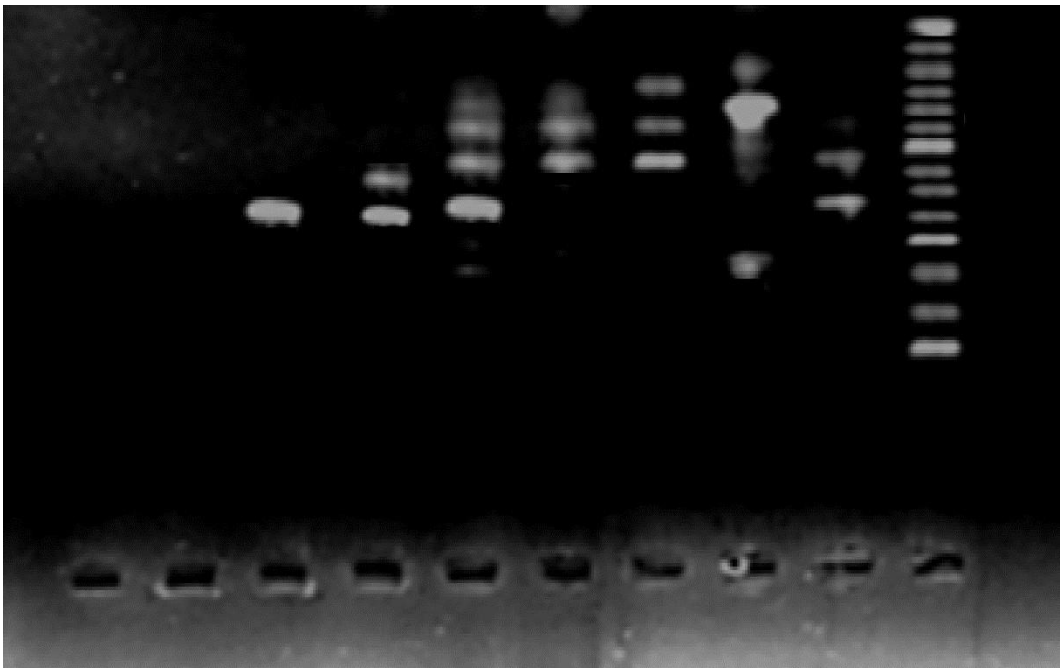


Photo 2: PCR Detection of *E. coli*

Photographs

Photo 3: Water sample collection

Photo 4: *E. coli* isolates on EMB agar plate

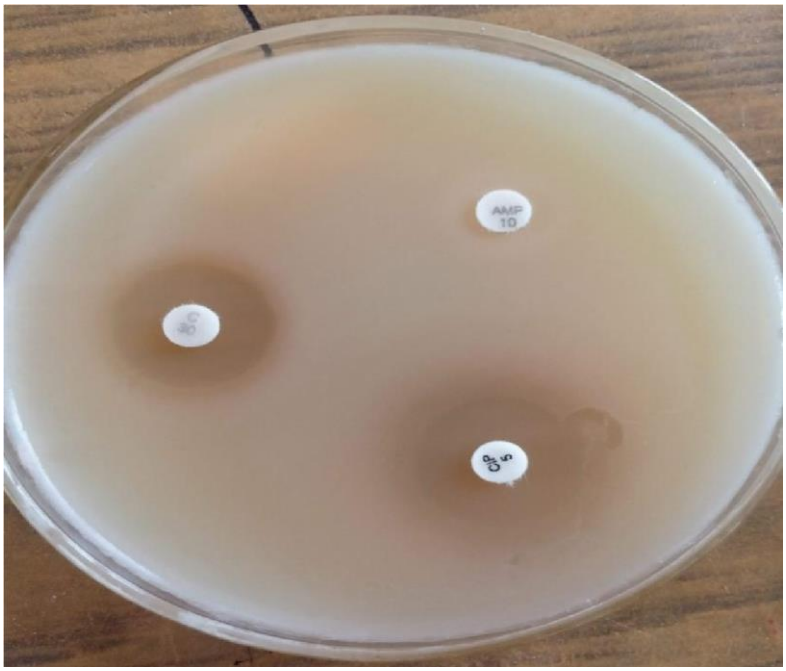


Photo 5: AST of *E. coli*

Photo 6: AST of *E. coli*

Photo 7: Biochemical Tests

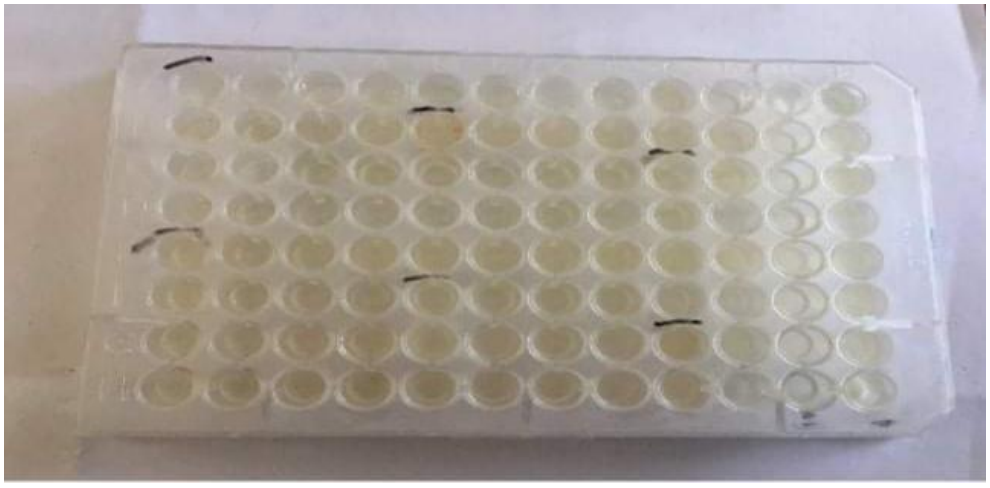


Photo 8: Minimum Inhibitory Concentration test

CHAPTER V

DISCUSSION

Water supplies and quality are critical fsor urban growth and the environment, particularly in areas where there is a severe water deficit (Jun Xiao et al 2012). Human activities have an impact on water quality, which is deteriorating as a result of urbanization, population increase, industrial production, climate change, and other causes. The ensuing water contamination poses a major threat to the planet's and people's health (Joshua Nizel Halder et al 2015). Water contamination from excessive nutrient loading, plastic debris, antibiotics, and other pollutants is another danger to river ecosystem integrity. As a result, approximately 80% of the world's population faces increased water security threats, and one out of every three people does not have access to safe drinking water, according to the United Nations' Sustainable Development Goal (SDG) (Jiacong Huang et al 2021).

This research was carried out at Microbiological Department Laboratory of Central Campus of Technology, Hattisar Dharan. Out of 15 samples from Jhapa, 17 samples from Morang, and 11 samples from Sunsari, 7 samples, 5 samples, and 6 samples were tested to be *E. coli* positive, respectively. A similar study conducted in the river water of the Kathmandu valley showed the highest prevalence of *E. coli* (Shrestha et al., 2016). Similarly, *E. coli* samples were recovered from drinking, which is similar to our findings, which revealed that 41.8 % of the samples tested positive for *E. coli* water (Sadaf Tariq et al 2021). Other investigations have shown virulent *E. coli* strains in freshwater locations in impoverished countries as fecal indicator bacteria and animal fecal contact. In general, these findings revealed that the risk of contamination may increase over time, signaling that it is past time to take proper precautions.

Contamination of environmental waterways by pathogens poses a serious health risk and jeopardizes future water supplies for living and recreational purposes. *E. coli* is found in the intestines of both humans and animals, and it is released into the environment through feces. Fecal bacteria are commonly

employed as markers of contamination in rivers, sea beaches, lakes, ground water, surface water, recreational water, and the many and varied activities linked with these since fecal matter is the principal source of disease-causing agents in water. Contact with contaminated water can lead to gastrointestinal disease, skin infections, ear infections, respiratory infections, eye infections, brain infections, and wound infections. Stomach pains, diarrhea, nausea, vomiting, and a lowgrade fever are the most commonly reported symptoms. To improve environmental health, effective monitoring of water quality and early detection of contamination, as well as more sustainable ways and a reduction in drug use, will be required (Price Wildeboer 2017).

Escherichia coli is a bacterium that has evolved to exist in a beneficial symbiosis with humans and many other animals, where it either causes sickness or lives in a beneficial symbiosis. The fact that there is *E. coli* in the environment, especially in water requires the great attention. While Non-pathogenic *E. coli* can be found in water and food. The presence of harmful bacteria is a clear symptom of poor hygiene. *E. coli* strains must be regarded as a direct threat to humans as well as animal welfare. *E. coli* is an indicator for fecal contamination of water sources. It's a well-known potential pathogen that can cause a range of human diseases via water, including gastrointestinal illness.

The prevalence of *E. coli* in this study is 41.86% which is much lower than the 99.3% reported by (Cho et al 2018). The prevalence is also lower than the 59% reported by (Gwirrbi et al 2019), whereas only 18.7% of the *E. coli* isolated from stream water by (Odonkar and Oddo 2018) showed less prevalence. This showed that the widespread fecal contamination within the watershed.

In this study, the antibiotic susceptibility test of *E. coli* reported that the most effective antibiotic was Ciprofloxacin, Amikacin, Nalidixic acid, Chloramphenicol, Cefotaxime, Azithromycin and Ampicillin. Out of 18 positive samples, Ciprofloxacin was 70.59%, Amikacin was 68.75%, Nalidixic acid was 38.88%, Chloramphenicol was 37.5%, Cefotaxime was

33.34%, Amikacin was 22.23% and Ampicillin was 5.56% sensitive against *E. coli*.

The study conducted by (Aziz RJ et al 2014) showed that Cefotaxime and Ampicillin were 23% and 16% sensitive to *E. coli* which was somehow similar to our study. Similarly in the study conducted by (Swedan S et al 2019) Ampicillin was 2.7% Sensitive to *E. coli* which was near to this study, and Ciprofloxacin was 83.5% sensitive which was considerable but, Cefotaxime was found to be 86.2% sensitive which was very high as compared to this study. Similarly, Amikacin 91.75%, Nalidixic acid 89.65% and Chloramphenicol 69.07% were highly susceptible while Ciprofloxacin 74.2% (Odonkar and Addo 2018) which was almost similar.

Likewise, the resistant drug was Ampicillin (94.45%), Azithromycin (61.1%), Cefotaxime (44.45%), Chloramphenicol (31.25%), Nalidixic acid (27.78%) and
Ciprofloxacin (11.77%). The study conducted by (Swedan S et al 2019) showed Ampicillin was 93.6% and Ciprofloxacin was 16.5% resistant to *E. coli* which was similar to our study but, Cefotaxime was 12.9% resistant which is less as compared to this study. Antibiotic-resistant pathogenic *E. coli* strains found in water sources may contribute to the spread of antimicrobial resistance and virulence genes among other bacteria in the environment.

The Minimum Inhibitory Concentration test was done using two drugs against *E. coli*. These two drugs were Ampicillin and Gentamycin. In this study, Ampicillin was found to be 83.33% resistant and Gentamycin was found to be 22.22% resistant. The highest level of non-susceptibility to Ampicillin (60.2%) where low level of non-susceptibility was observed among isolates to Gentamycin in the study conducted by (Kldsley AK et al 2018).

The biofilm formation test was completed, and 72.22 % of the 18 positive samples were weak biofilm producers, 5.56 % were moderate biofilm producers, and 22.22 % were non biofilm producers. In a biofilm formation test performed on positive water samples gathered from various sources, *E. coli* was found to be a weak biofilm producer (13.51%) (Mahapatra et al,

2015), which is a relatively low number in this study. Similarly, all of the samples were positive in a quantitative biofilm analysis conducted over three time periods of 24 hours, 48 hours, and 72 hours (Tariq et al 2021). (Barros et al 2014) identified 31 (64.58%) strains of biofilm *E. coli* in water of rivers nearby the Atlantic Ocean which is higher in comparision to this study.

PCR was carried out with the isolated E. coli, and 0.14% were positive for stx2. A similar study showed 2.5% positive (Alfinete NW et al., 2022) which is somehow nearer to this study. (Antikamen J et al 2009) showed 21% positive for eae and escV gene from EPEC which is less in comparision to this study which showed 57.4% positive for escV and invE, similarly a study showed 36% positive for eaeA and/or escV (Kagambega A et al 2012).
As a result, our findings suggest that the risk of contamination may increase over time, and that it is past time to take proper precautions.

CHAPTER VI

CONCLUSION AND RECOMMENDATION

6.1. Conclusion

In the study we found that 41.86% of water samples were contaminated with pathogenic strains of *E. coli*. Therefore, strict quality control measures should be implemented to ensure proper treatment of water and wastewater drainage that may lead to river. This would ensure the discharge of adequately treated wastewater into water bodies to prevent the occurrence and spread of water- and food-borne diseases as well as fatal infections. Isolates were most sensitive to drug Ciprofloxacin which was statistically significant. MIC of Gentamycin showed sensitivity of drug against *E. coli*. Mostly weak biofilm production was seen. Different pathogenic genes of E. coli were seen which are prone to cause diarrhea and can be very fetal.

The medical fraternity in this study shows that the river water is being polluted which needs to be minimized so Province No.1 may take cues from our study to improvise the protocols for the treatment of susceptible infections with pathogenic strains of *E. coli*.

6.2 Recommendation

- Regular monitoring of antimicrobial susceptibility and rates of ESBL and MBL production along with multidrug resistance among clinical isolates is very necessary.
- Further research is required in order to evaluate the health risks of using reclaimed water harbouring antibiotic resistant bacteria for drinking, agricultural and recreational purposes.

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APPENDICES

APPENDIX I

MATERIALS

Glasswares	**Equipments**	**Microbiological and Biochemical media**	**Chemicals and reagents**	**Materials**	**Sample**	**Others**
B.O.D bottles	Autoclave	Eosin methylene blue Agar	Alcohol	Test tube rack	Water	Cotton swab
Pipettes	Hot air oven	Mueller hinton agar	Lysol	Wash bottle		Inoculating loop
Petri plates	Microscope	Nutrient agar	Glycerol	Burner		Labelling tape
Conical flasks	Incubator	Nutrient broth	Sodium chloride			
Test tubes	Ice box	Simmons citrate media	Sulfuric acid			
L-Shaped dolly rod		MR-VP broth	Barium chloride			
Forcep		Glucose	Catalase reagent (3% H_2O_2)			
Beakers		Fructose	Alpha-napthol (5%)			
Glass rods		Sucrose	Crystal violet			
Slides			Gram's Iodine			
Cover slips			Safranin			
			Kovacs reagent			
			Methyl red			
			Potassium hydroxide			

APPENDIX II

COMPOSITION OF MEDIA

1. Eosin Methylene Blue Agar:

Ingredients	Gms / Litre
Peptone	10 g
Lactose	5 g
Sucrose	5 g
Dipotassium, PO_4	2 g
Agar	13.5 g
Eosin Y	0.4 g
Methylene blue	0.065 g
Distilled water	1 L
Final pH	7.1

2. Nutrient Agar:

Ingredients	Gms/ Litre
Beef Extract	3 g
Peptone	5 g
Agar	15 g
Distilled Water	1000ml
Final pH	6.8+/-0.2

3. Nutrient Broth

Ingredients	Gms / Litre
Beef extract	1
Yeast extract	2
Peptone	5
Sodium chloride	5

3. Mueller Hinton Agar:

Ingredients	Gms / Litre
Beef extract	2
Acid hydrolysate of casein	17.5
Starch	1.5
Agar	17

4. MR-VP media:

Ingredients	Gms/ Litre
Peptone	7
Dextrose	5
Dipotassium phosphate	5

5. Simmons citrate:

Ingredients	Gms/ Litre
Magnesium sulfate	0.2
Ammonium dihydrogen phosphate	1
Dipotassium phosphate	1
Sodium citrate	2
Sodium chloride	5
Bromothymol blue	0.08
Agar	15

APPENDIX III

STAINS AND REAGENTS

1. Crystal violet:

Crystal violet	20g
Ethyl alcohol	95ml
Ammonium oxalate	9g
Distilled water	905ml

2. Gram's iodine:

Iodine	1g
Potassium iodide	2g
Distilled water	300ml

3. 95% ethyl alcohol:

Ethyl alcohol	95ml
Distilled water	5ml

4. Safranin:

Safranin	10ml
(2.5% safranin in 95% ethyl alcohol)	
Distilled water	100ml

5. Kovacs reagent:

Dimethyl amino benzaldehyde	5g
Amyl alcohol	75ml
Conc. Hydrochloric acid	25ml

6. Methyl red solution:

Methyl red	0.05g
Ethyl alcohol	28ml
Distilled water	22ml

7. VP reagent:

VP reagent –I

α- Napthol	5g
Ethyl alcohol	100ml

VP reagent –II

Potassium hydroxide	40g
Distilled water	100m

8. Hydrogen Peroxide Solution

Hydrogen peroxide	3ml
Distilled water	97ml

APPENDIX-IV

Procedure of isolation of bacteria

1. Isolation of *E. coli*

The plates were incubated at 37°c for 24 hours. *E. coli* colonies were identified on the basis of colony characteristics on Nutrient Agar, Gram's reaction and biochemical tests.

2. Subculture on NA

Green metallic sheen colonies from EMB were sub cultured on NA and incubated for 24 hours at 37°c. Large, round, greyish white colonies having raised, entire, opaque surface were indicative of *E. coli*.

3. Gram's staining

Isolated colony selected for staining:

1. Smear was made from pure culture by emulsifying a colony in normal saline and heat fixed.

2. Smear flooded with crystal violet for 1 mint.

3. Wash with water

4. Add Gram's Iodine for 1minute.

5. Wash with water.

6. Decolorize with absolute alcohol for 10-15secs.

7. Wash with water

8. Flood with safranin for 1minute.

Wash with water, blot dry and examine under oil immersion objective of the microscope.

4. Indole test:

The bacterial colony was inoculated on tryptone broth and then incubated at 37^0C for 24 hours. After 24 hours of incubation, 1ml of Kovac's reagent was added. Appearance of red color (red ring) on the top of media indicates positive indole test.

Principle:

This test is used to determine the ability of bacteria to oxidize the tryptophan by producing tryptophanase enzyme.

5. MR-VP test:

The bacterial colonies were inoculated into MR and VP broth and incubated at 37^0C for 24 hours. After incubation, 5 drops of methyl red indicator were added to MR broth and mixed well for MR test. The positive test was indicated by the development of red color, and negative with yellow color. For VP test, 5 drops of Barritt's reagent was added to VP broth and shaken well. Positive test is indicated by the development of pink red color.

Principle of MR test:

The principle of this test is to detect the ability of bacteria to produce and maintain sufficient stable acid from glucose fermentation which is indicated by MR indicator.

Principle of VP test:

This test detects the ability of bacteria to produce a neutral end product, acetyl methyl carbinol (acetoin) from glucose fermentation.

6. Citrate utilization test:

A bacterial colony was stabbed on the butt of the Simmons citrate agar and then streaked on slant by a sterile inoculating needle. Then the inoculated media were incubated at 37^0C for 24 hours. A positive test was indicated by the growth of organism and change of color of media from green to blue. Bromothymol blue is green acidic (pH 6.8 and below) and blue when alkaline (pH 7.6 and higher).

7. Catalase test:

3% H_2O_2 was taken in a clean and dry test tube (3ml). A small amount of culture from nutrient agar plate was added and mixed with the help of

glass rod. Positive test is indicated by the formation of bubbles of oxygen gas.

Principle:

Catalase acts as a catalyst in the breakdown of hydrogen peroxide to oxygen and water. Bubbles of oxygen are released if the organism is catalase producer.

8. Carbohydrate fermentation test

Procedure:

I. Preparation of Carbohydrate Fermentation Broth

Weigh and dissolve trypticase, Sodium chloride, and Phenol red in 100 ml distilled water and transfer into conical flasks. Add 0.5% to 1% of desired carbohydrate into all flasks. Insert inverted Durham tubes into all tubes, the Durham tubes should be fully filled with broth. Sterilize at 115^0 C for 15 minutes. Do not overheat the Phenol red Carbohydrate fermentation broth. The overheating will result in breaking down of the molecules and form compounds with a characteristic color and flavor. The process is known as caramelization of sugar (the browning of sugar). Transfer the sugar into screw capped tubes or fermentation tubes and label properly.

II. Inoculation of Bacterial Culture into the Phenol Red Carbohydrate Broth

Aseptically inoculate each labeled carbohydrate broth with bacterial culture (keep uninoculated tubes as control tubes). Incubate the tubes at 18-24 hours at 37^oC.Observe the reaction.

Principle:

When microorganisms ferment carbohydrate an acid or acid with gas are produced. Depending up on the organisms involved and the substrate being fermented, the end products may vary. Common end-products of bacterial fermentation include lactic acid, formic acid, acetic acid, butyric

acid, butyl alcohol, acetone, ethyl alcohol, arbon dioxide and hydrogen. The production of the acid lower the pH of the test medium, which is detected by the color change of the pH indicator. Color change only occurs when sufficient amount of acid is produced, as bacteria may utilize the peptone producing alkaline by products.

9. Antibiotic susceptibility test

In vitro susceptibility of the pure bacterial species to fifteen different antibiotics was determined using Kirby- Bauer disk diffusion technique using Muller-Hinton agar and antibiotic discs as described by the National Committee for Clinical Laboratory Standards (CLSI, 2006). One ml of each bacterial isolates prepared directly from an overnight incubated agar plates adjusted to 0.5 McFarland Standard was inoculated using sterile swab into each of the Petri-dishes containing Mueller Hinton agar and were allowed to stand for 30 minutes for pre-diffusion of the inoculated organisms.

Antibiotic discs were seeded into the petri dishes containing Mueller-Hinton agar (MHA) for each bacterial isolate. The AST of the isolates towards various antimicrobial discs was done by modified Kirby-Bauer M2-A9 disc diffusion method as recommended by Clinical Laboratory Standard Institute (CLSI) using MHA as follows:

1. MHA was prepared and sterilized as instructed by the manufacturer.
2. The pH of the medium was adjusted to 7.2-7.4 and the depth of the medium at 4mm (about 25 ml per plate) was maintained in petri dish.
3. Using a sterile wire loop, a single isolated colony whose susceptibility pattern is to be determined was touched and inoculated into MHB tube and was incubated at 37oC for 2-4 hrs.
4. After incubation, the turbidity of the suspension was matched with the McFarland standard tube number 0.5 (which is equivalent to 10 to power 4 organisms).
5. Using a sterile swab, an MHA plate was inoculated with the matched suspension using a carpet culture technique.

6. The plate was then allowed to stand for 20-30 minutes for the pre-diffusion of the inoculated organisms.

7. Using clean and sterile forceps, the above-mentioned antibiotic discs (6 mm) were placed on the MHA. The discs were placed at the considerable distance apart from each other on a 90 mm Petri-dish. Then the plate was incubated at 37 ºC for 24 hrs.
8. After incubation, the plates were observed for zone of inhibition and the diameters of inhibition zones were measured in millimeters (mm). The measurement was interpreted as sensitive and resistant according to the manufacture's standard zone size interpretative manual of CLSI (2006).

The percentage resistance was calculated using the formula *PR=a/b×100, where 'PR'* was percentage resistance, '*a'* was the number of resistant isolates and '*b'* was the number of isolates tested with the antibiotic. The percentage sensitivity was calculated using the formula *PS=c/d×100, where 'PS'* was percentage sensitivity, '*c'* was the number of sensitive isolates and '*d'* was the number of isolates tested with the antibiotic.

10. Biofilm Production

Microtiter plate (MtP) assay is a quantitative method to determine biofilm production by microplate reader.

1. Bacterial suspension was prepared in MHB supplemented with 1% glucose and adjusted to 0.5 McFarland (1.108 cfu/ml). This bacterial suspension was 20-fold (1/20) diluted to reach 5.106 cfu/ml.

2. Then 180 µl of Mueller-Hinton Broth (MHB) supplemented with 1% glucose and 20 µl of bacterial suspensions were inoculated into 96-well flat-bottomed sterile polystyrene microplate to obtain 5.105 cfu/ml as a final concentration (tenfold dilution (1/10)).

3. Microplates were incubated at 24 h at 37°C. The sessile isolates of which biofilms formed on the walls of wells of microplate were stained with only 150 µl of safranine for 15 min, after planktonic cells in wells

of microplate were discharged by washing twice with phosphate-buffered saline (PBS) (pH 7.2) and wells are dried at 60°C for 1 h.

4. Before staining with safranine, fixation of biofilms was done by subjecting to 150 µl of methanol for 20 min or drying at 60°C for 1 h.
5. Then safranine-stained wells of microplates were washed twice with PBS to discharge safranine stain.
6. After air drying process of wells of microplate, dye of biofilms that lined the walls of the microplate was resolubilized by 150 µl of 95% ethanol or 33% glacial acetic acid or methanol.
7. Then microplate was measured spectrophotometrically at 570 nm by a microplate reader. The studies are repeated in triplicates.
8. Uninoculated wells containing sterile MHB supplemented with 1% glucose were considered to be the negative controls are used as blanks. The blank absorbance values were used to identify whether biofilm formation of isolates exists or not.
9. The wells of isolates of which OD values higher than blank well are considered to be biofilm producers.

Categorization can be done as no biofilm production (0), weak (+ or 1), moderate (++ or 2), and strong biofilm production (+++ or 3) by the calculation of cutoff value (ODc) shown below;

$OD \leq ODc$ no biofilm production

$ODc < OD \leq 2 \times ODc$ weak biofilm production

$2 \times ODc < OD \leq 4 \times ODc$ moderate biofilm production

$4 \times ODc < OD$ strong biofilm production.

APPENDIX-V

BIOCHEMICAL RESULT

Table 5: Biochemical test results:

S.N.	Tests	Result
1	Indole	Positive
2	MR (Methyl red)	Positive
3	VP (Voges-Proskauer)	Negative
4	Citrate utilization test	Negative
5	Catalase	Positive
6	Carbohydrate Fermentation Test	Positive
7	Gram Staining	Negative (Rod)

Table 6: McFarland standard:

S.N.	McFarland turbidity standard no.	0.5	1	2	3	4
1	1% Barium chloride (ml)	0.05	0.1	0.2	0.3	0.4
2	1% sulfuric acid (ml)	9.95	9.9	9.8	9.7	9.6
3	Approx. cell density (1×1^8 CFU/ml)	1.5	3	6	9	12

Appendix VI

AST

Table 7: Antibiotic Susceptibility Test

Antibiotic used	Symbol	Disc Content (mcg)	Diameter of zone inhibition (mm)		
			Resistance	Intermediate	Susceptible
Ampicillin	AMP	10	13	14-16	17
Amikacin	AK	30	14	15-16	17
Cefoxitine	CX	30	22	23-25	26
Azithromycin	AZM				
Nalidixic acid	NA	30	13	14-18	19
Chloramphenicol	C	30	12	13-17	18
Ciprofloxacin	CIP	30	20	21-30	31

(Source: Product information Guide, HiMedia Laboratories Pvt. Limited, Mumbai, India)