MOLECULAR CHARACTERIZATION OF *Escherichia coli* PATHOTYPES FROM IRRIGATION WATER SAMPLES COLLECTED FROM OJO IBA

BY

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A PROJECT SUBMITTED TO THE DEPARTMENT OF BIOLOGICAL SCIENCES, COLLEGE OF BASIC AND APPLIED SCIENCES, MOUNTAIN TOP UNIVERSITY, MAKOGI, IBAFO, OGUN STATE, NIGERIA.
IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD DEGREE OF BACHELOR OF SCIENCE (B.Sc.) IN MICROBIOLOGY.

SEPTEMBER, 2022

DECLARATION

I hereby declare that this project was under the supervision of Dr. MOSES ABIALA is a product of my own research work. Information derived from various sources have been duly acknowledged in the text and a list of references provided

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Oyediran Oluwasegun D. DATE

CERTIFICATION

This is to certify that this project write-up titled **"MOLECULAR CHARACTERIZATION OF *ESCHERICHIA COLI* STRAINS FROM WATER SAMPLES COLLECTED FROM OJO IBA"** was compiled and written by **OYEDIRAN OLUWASEGUN DANIEL** with Matriculation number 18010101032 of the Department of Biological Sciences, College of Basic and Applied Sciences, Mountain Top University, Ogun State, under the supervision of Dr. M.A Abiala.

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Dr. (Mrs) C.I. AYOLABI DATE

(Ag. HEAD OF DEPARTMENT)

DEDICATION

I hereby dedicate this project to God who helped me through my studies.

ACKNOWLEDGEMENT

My utmost gratitude goes to the Almighty GOD, who in his infinite mercies inspired the conception of this seminar report and also made it possible to be a great success. I appreciate the Head of Department, Biological Sciences Dr. (Mrs) C.I. Ayolabi.

I also sincerely wish to use this opportunity to thank every member of my family for their moral, spiritual and financial support, and my friends Ajide Esther, Ahmed Precious, Akinayo Sunday, Joseph Favour, Akinriade Blessing, and Oluwatoyin Precious for their support. I also thank my supervisor Dr Moses Abiala who put me through with my project work. I sincerely wish to express my sincere gratitude to the Department of Biological Sciences.

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ABSTRACT

E. coli is a pathogen that causes both simple and complex food related infections and is a pathogen of concern. It is also a common source of diarrheagenic sicknesses worldwide. Water used in irrigation of lettuce plants were collected from various farms around Ojo-Iba, Lagos state. Biochemical, cultural and molecular characterization of the irrigation water was carried out. A total of twelve samples was with code ranging from W1L1- W4L12 collected. These twelve samples were identified to have potentially harbored pathogenic *E. coli* strains on SMAC and MAC based on the morphological characteristics observed. Biochemical tests were also carried out for further confirmation. Multiplex PCR, identified that six out of twelve samples had Enterotoxigenic *E. coli* present in them. The positive samples which showed the presence of Enterotoxigenic *E. coli* are: W1LW, W2L4 W2L5 W2L6 W3L9 W4L10 W4L11

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Water is a fundamental need for all human, animal, and plant life. Water is used in a variety of ways by diverse organisms for biological processes including growth. Water is essential to plant growth and productivity. Farmers disregard the necessity to safeguard the safety of irrigation water and how it can influence consumer life because water is easily available everywhere. The quality of water utilized in pre-planting and post-harvest procedures has decreased as a result of population growth and various human activities. Due to inadequate infrastructure, improper farming methods, and the careless discharge of garbage into water, access to high-quality water is quickly turning into a mirage in emerging nations like Nigeria. According to reports, pathogenic E. coli can be found in water bodies that have surface or subsurface dumping of human and animal waste (Christina *et al*., 2018). This water introduces the pathogenic E when it is employed in planting procedures. enters the plant, coli. Vegetables contain E. When vegetable leaves are not thoroughly cleaned or cooked, coli has the ability to stick to them and enter the human gastrointestinal tract. Additionally, fruits and vegetables that are produced from plants get polluted when harvesting activities are not conducted hygienically. Unclean hands and utensils may be the source of this infection. Diseases brought on by EPEC are a factor in some nations' premature mortality rates. Food security, or the availability of enough food for consumption to feed an expanding population, is still a problem in developing countries. It is believed that more than 200 different diseases brought on by microorganisms are waterborne and foodborne, posing risks to the elderly, young children, and pregnant women. (Favian et al., 2020) Warm-blooded mammals' gastrointestinal tracts are home to the bacterium Escherichia coli, which is known to be the cause of diarrhea. after consuming E. coli, the organism has the capacity to stick to the intestine's epithelial cells. As a result, this creates diarrhea that is often watery and occasionally bloody. The microorganism is linked to the physical changes in the intestine's structural integrity. Bloody diarrhea is associated with attachment and an acute tissue destruction process. Low grade fever and vomiting are also associated with infection. (*John et al.,* 2007).The rod-shaped bacteria *Escherichia coli* (*E. coli*) is widely found in the gastrointestinal system and excrement of warm-blooded mammals (Katouli, 2010). It belongs to the fecal coliform bacteria group and is differentiated by its inability to

degrade urease. The quantity of yellow and yellow brown colonies growing on a 0.45-micron filter placed on m-TEC media and incubated at 35.0^{o} C for 22-24 hours is used to determine *E. coli* levels in freshwater. The presence of *E. coli* colonies can be confirmed by the addition of urea substrate. This bacterium is a favored indicator for freshwater recreation, and its presence indicates warm-blooded animal feces contamination. *E. coli* can cause disorders like meningitis, septicemia, urinary tract infections, and intestinal infections, despite the fact that it is normally innocuous. In children and the elderly, the strain of *E. coli* (*E. coli* 0157:H7) can cause serious illness and even death. (school, 2018)While many people identify *E. coli* with food poisoning, various strains of the bacteria can cause pneumonia and urinary tract infections. *E. coli* is responsible for 75 percent to 95 percent of urinary tract infections. *E. coli* is a normal intestinal resident, which is how it gets into the urinary tract. Some strains of *E. coli* cause illness by producing a toxin known as Shiga. The gut lining is damaged by this toxin. STEC stands for "Shiga toxin-producing *E. coli*," and refers to the strains of *E. coli* that produce the toxin (Paton & Adrienne, 1998).

Within a few hours of birth, *Escherichia coli* colonizes the gastrointestinal system of human newborns. *E. coli* and its human host usually cohabit for decades in good health and mutual benefit. Except in immuno-compromised hosts or when the typical gastrointestinal barriers are overcome — as in peritonitis, for example — these commensal *E. coli* strains rarely cause disease. The mucous layer of the mammalian colon serves as a home for commensal *E. coli*. The bacteria are fierce competitor at this crowded site, containing the human gut microflora's most abundant facultative anaerobe. Despite a large amount of knowledge on *E. coli*'s genetics and physiology, the processes by which *E. coli* maintains this beneficial symbiosis in the colon remain little understood. One intriguing theory proposes that *E. coli* uses its capacity to use gluconate in the colon more efficiently than other resident species, allowing it to occupy a metabolic niche that is extremely specialized (James, James, & Harry, 2004). The *E. coli* O157:H7 infection outbreaks connected to romaine are more frequently associated with lettuce commercially grown and harvested at the end of the growing seasons in California and Arizona has been recognized for several years. although contamination of lettuce products is rare, between 1998 and 2019, 36 outbreaks that traced back to lettuce were recorded by the centers for disease control and prevention. most of these outbreaks involved romaine lettuce harvested in the fall on the California central coast such as in Salinas, and in late winter in southern California and Arizona. these two states are the major lettuce growing areas in the United States with farm production valued at

nearly $2.7 billion in 2021. Diarrhoeagenic *Escherichia coli* (DEC) account for about 40% of episodes of acute diarrhea in children in developing countries. They also play a significant causative role in diarrhea in Nigeria, in both adults and children. Currently, there are eight pathotypes of DEC strains: enterotoxigenic, enterohaemorrhagic, enteroinvasive, enteropathogenic, enteroaggregative, diffusely adherent, cytolethal distending toxin-producing and cell detaching *E. coli*. Each pathotype of DEC has a distinct set of virulence factors encoded in the plasmids or chromosome. The genes that encode these factors are conserved among strains that are isolated from diverse sources in different parts of the world (Paul and Patricia, 1990).

DEC strains are usually transmitted via a fecal-oral route which involves contaminated sources of water or food and may be involved in outbreaks of waterborne diarrhea. *Escherichia coli* can enter drinking water via inadequate or failing septic or sewer systems, runoff from land applied with animal wastes or animal feeding operations and wildlife. Identification of the source of pollution is a high priority in order to protect source water quality and to assess the public health risk associated with contamination from a particular host source. Consequently, much progress has been made over the years to develop many phenotypic and genotypic microbial source tracking (MST) methods which are recommended components of fecal pollution reduction strategies. Nigeria is one of the countries in the world where about 90 million people don't have access to potable water as well as access to safe food and 130,000 children under the age of five die each year from avertable waterborne diseases due to uncoordinated efforts of various agencies of government. The larger part of the population, particularly those in the rural and suburban communities' resort to water from wells and streams for domestic purposes (Kaur *et al*., 2010).

Those wells which are hand dug are usually around 4–15 ft in diameter and about 25 ft deep. In most parts of Nigeria, most of the wells are shallow because of the high-water table. Shallow wells are more prone to contamination due to their proximity to the soil surface and potential source of contamination. These alternative sources of water are largely untreated and might harbor waterborne pathogens. Therefore, the use of these sources of water is a health risk for this population. In Ojo-Iba where farmers are giving contract to grow diverse number of vegetables such as Lettuce, Spring onions, Ewedu, etc., water used in planting and post planting operations are gotten from digging large holes called wells, which allows access to water from underneath the earth. The water is untreated and open, giving room to contamination by diverse pathogenic

microorganisms including pathogenic *E. coli*. (Babatunde *et al.,* 2022). Despite the risk posed by exposure to *E. coli* contaminated water, very little data is available on this in OJO-IBA, and the pathogenic potential, diversity of implicated isolates and factors associated with their presence in well water remain unknown. Therefore, this study determined the prevalence, diversity and factors associated with the presence of DEC in well water used in planting practices in OJO-IBA, Lagos, Nigeria.

1.2 JUSTIFICATION OF STUDY

Escherichia coli are microorganisms that can be found in the gastro-intestinal tract of mammals through the consumption of vegetables such as lettuce. However, some strains have become pathogenic by acquiring virulence factors. Somes of these are strains Enterotoxigenic *E. coli*, Enterohemorragic *E. coli*, Enteropathogenic *E. coli*, Enteroinvasive *E. coil*, and Enteroaggregative *E. coli*, are among the pathogenic strains of *E. coli*. In Nigeria especially Lagos, in order to maintain adequate watering of the lettuce plant for proper growth and yield, contaminated water is mostly used by the lettuce farmers which directly or indirectly introduce pathogenic *E. coli* to the lettuce plants and subsequently consumed by the consumers. This study therefore unraveled the identity of possible pathogenic *E. coli* associated with irrigation water used in watering lettuce in Ojo-Iba, Lagos state.

1.3 OBJECTIVES OF STUDY

The objectives of this study are;

- Enumerate possible *Escherichia coli* and associated Enterobacteriaceaee in the irrigation water of vegetable farms.
- To determine the presence of pathogenic *E. coli*, isolated by using Sorbitol-MacConkey Agar, Nutrient Agar, and MacConkey Agar.
- To identify *Escherichia coli* isolates isolated from the irrigation water through a series of biochemical test and PCR.

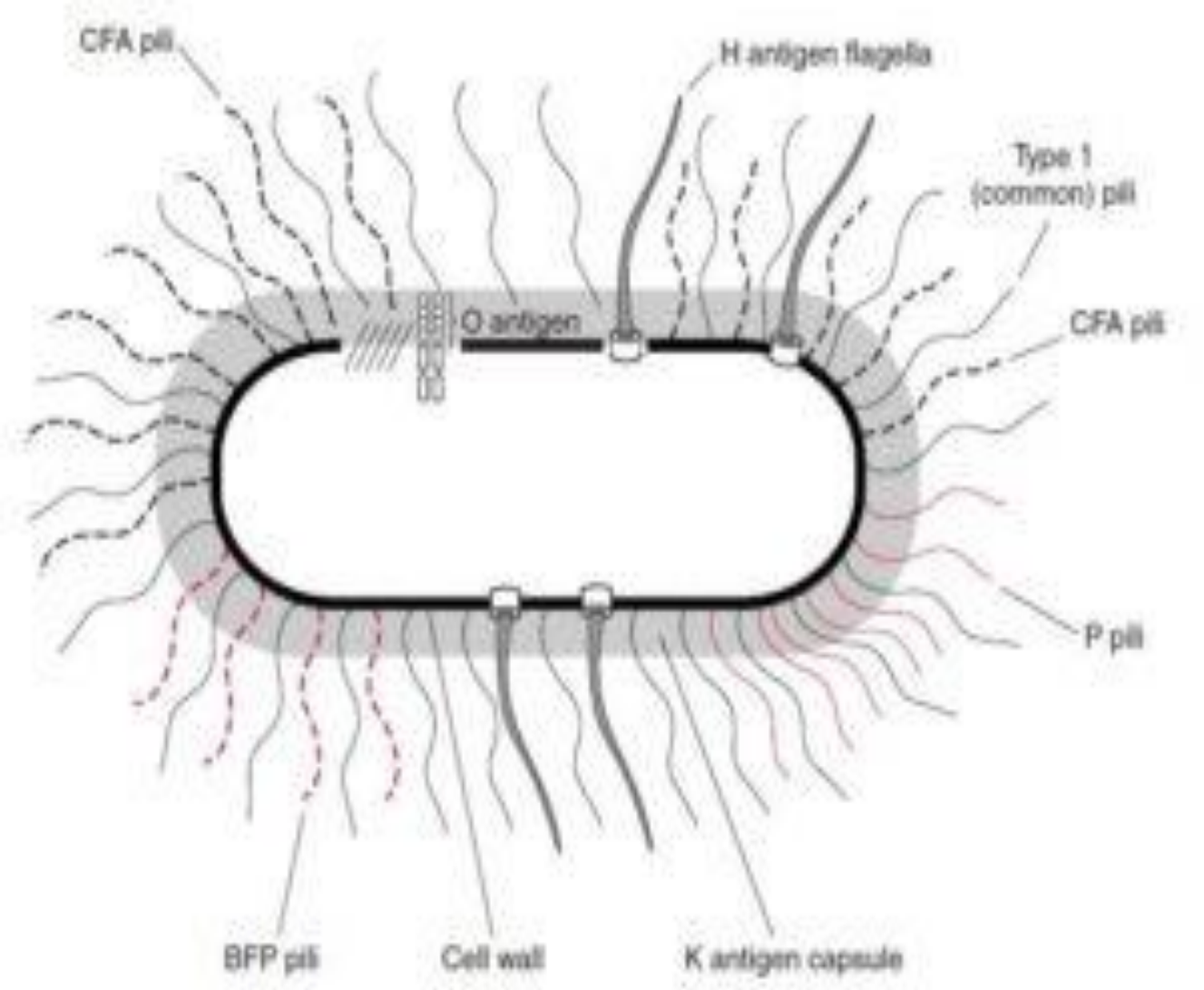

Figure 1.1: Diagram of *Escherichia coli* Source*:* (Kumari, 2021)

CHAPTER TWO

LITERATURE REVIEW

The bacteria Escherichia coli (E. coli) typically dwells in the intestines of healthy humans and animals. This bacterium is typically not harmful. It facilitates food digestion. Certain E. coli strains, though, Symptoms of coli infection can include low-grade fever, stomach pains, and diarrhea. Some E. Infections with E. coli can be harmful. E. A rod-shaped member of the Enterobacteriaceae family, coli is a bacterium. It can survive in both air-filled and airless conditions. The intestines of healthy people and warm-blooded animals are home to these microorganisms. the majority of E. Coli are safe and even support the health of your digestive system. However, if you consume infected food or drink tainted water, some types can make you throw up. (Felson, 2020) An infrequent but harmful cause of gastroenteritis is *Escherichia coli* O157. This bacterium is remarkable because it causes the hemolytic uraemic syndrome, which is the most common cause of acute renal failure in children in the Americas and Europe, in a small but considerable percentage of infected individuals. The more efficient use of evidence-based techniques could prevent many *E. coli* O157 infections, which is particularly crucial because once an infection has been confirmed, there are no treatment therapies available to reduce the risk of developing the hemolytic uraemic syndrome. (Pietrangelo, 2021). Physicians should be aware of whether laboratories in their region frequently test for E. coli O157 in stool specimens due to variations in testing procedures. Antimicrobial therapy is still debatable; some research suggest that it could trigger hemolytic uraemic syndrome, while others show no effect or even a protective one. By warning patients about the risks of consuming undercooked ground meat or unpasteurized milk products and juices, stressing the value of handwashing to stop the spread of diarrheal illness, and alerting public health authorities to unusually high rates of hemolytic uraemic syndrome or bloody diarrhea, doctors can help to prevent E coli O157 infections (Paul and Patricia, 1990).

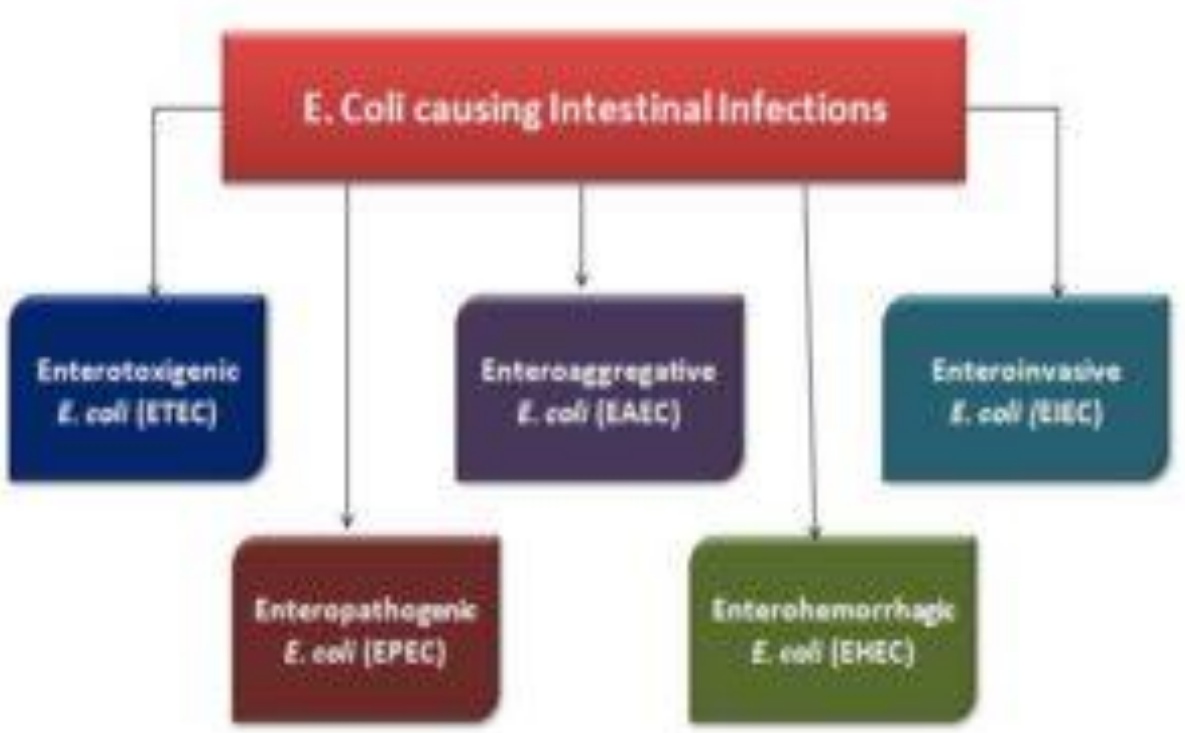

Figure 2.1: *Escherichia coli* pathotypes Source: (Kumari, 2021)

2.1 Shiga toxin-producing *Escherichia coli* (STEC):

E. coli are a diverse group of bacteria that normally live in the intestines of humans and animals. Although most strains of these bacteria are harmless, some produce toxins that can make you sick and cause diarrhea (loose stool/poop) such as Shiga toxin-producing *E. coli* (STEC) it is also known as Verotoxin producing *E. coli*. People become infected with STEC when they eat any product contaminated with the bacteria. Infection can also occur from consumption of contaminated raw or improperly cooked food products and also from unwashed hands. (School, 2018). These strains are called STEC/VTEC (Shiga toxin or verotoxin-producing *E. coli*) or EHEC (enterohaemorrhagic *E. coli*), and their toxins have the potential to cause bloody diarrhoea and Haemolytic Uremic Syndrome (HUS), a serious complication that can be fatal. In the EU and as reflected in EFSA's work on zoonoses, Shiga-toxin producing *Escherichia coli* is referred to as

VTEC (verotoxin-producing *E. coli*) but the term STEC was used for this outbreak as it is in line with terminology used by WHO and other organizations. (Paul SMead, 1990)

Shiga toxin-producing *Escherichia coli* (STEC), especially of serotype O157:H7, cause a zoonotic food or waterborne enteric illness that is often associated with large epidemic outbreaks as well as the hemolytic uremic syndrome (HUS), the leading cause of acute renal failure in children. After ingestion, STEC colonize enterocytes of the large bowel with a characteristic attaching and effacing pathology, which is mediated by components of a type III secretion apparatus encoded by the LEE pathogenicity island. (Obrig, 2010)

Shiga toxins are translocated from the bowel to the circulatory system and transported by leukocytes to capillary endothelial cells in renal glomeruli and other organs. After binding to the receptor globotriaosylceramide on target cells, the toxin is internalized by receptor-mediated endocytosis and interacts with the subcellular machinery to inhibit protein synthesis

2.2 Enterotoxigenic *Escherichia coli* (ETEC):

Enterotoxigenic *Escherichia coli* (ETEC) infection is the most common type of colibacillosis of young animals (primarily pigs and calves), and it is a significant cause of diarrhoea among travellers and children in the developing world. The main virulence attributes of ETEC are adhesins and enterotoxins, which are mostly regulated on large plasmids. Almost all ETEC bacteria are known to adhere to receptors on the small intestinal epithelium by their proteinaceous surface appendages (fimbriae, pili) or by afimbrial proteins without inducing significant morphological changes. (Béla & Péter, 2005).

Similar to cholera, ETEC-induced diarrhea has a pathogenesis that involves the formation of enterotoxins and components that promote colonization. ETEC infection can cause clinical symptoms that vary from mild diarrhea to a serious cholera-like illness. Antibiotics are not typically required for treatment, with the exception of traveler's diarrhea, because rehydration is an effective treatment for ETEC diarrhea. The frequency and characterization of ETEC on a worldwide scale are inadequate because of the difficulty in recognizing the organisms; no simple diagnostic tests are presently available. Protection strategies, as for other enteric infections, include improvements in hygiene and development of effective vaccines.

Increases in antimicrobial resistance will dictate the drugs used for the treatment of traveler's diarrhea. Efforts need to be made to improve our understanding of the worldwide importance of ETEC. (Firdausi *et al.*, 2005)

2.3 Enteroaggregative *Escherichia coli* (EAEC):

Diarrhea caused by EAEC is watery, often with the presence of mucus, with or without blood and abdominal pain, vomiting and low fever. Acute self-limiting diarrhea is the usual pathology, but some patients may develop protracted diarrhea, *i.e.*, lasting more than 14 days. Prolonged diarrhea occurs depending on the host's immunity, nutritional status and genetic susceptibility. Genetic susceptibilities associated with EAEC diarrhea were identified in North American travelers to Mexico. Recently, it was showed that EAEC is a cause of acute diarrheal illness among children residing in both developing and developed regions, adults and persons with HIV infection residing in developing regions, and travelers to developing regions in both developing and industrialized regions, showing that EAEC strains are relatively heterogeneous, and limited numbers of studies were available that examined the independent roles of the many putative EAEC virulence genes in acute diarrheal illness (Kaur *et al*., 2010).

EAEC induces diarrheal illness, although it is typically less severe than that caused by EHEC and EPEC. Despite the reduced severity in disease, this bacteria group has gained more attention in recent years as a cause for persistent diarrhea, a condition that can lead to dehydration. This activity is associated with EAEC's ability to form biofilms. EAEC isolates exhibit high genomic variability. EAEC strains has yet to be identified, although some isolates are known to carry HPI. Most virulence genes appear to be plasmid-encoded. (Kelly and Beth, 2015). Diarrhea caused by EAEC is watery, often with the presence of mucus, with or without blood and abdominal pain, vomiting and low fever. Acute self-limiting diarrhea is the usual pathology, but some patients may develop protracted diarrhea, *i.e.*, lasting more than 14 days. Prolonged diarrhea occurs depending on the host's immunity, nutritional status and genetic susceptibility (Tânia *et al.*, 2016).

2.4 Enteroaggregative *Escherichia coli* (EAEC):

EAEC was first described in 1987 in a child with acute diarrhea in Lima, Peru, and has since been linked with persistent diarrhea in children living in areas where EAEC is endemic, individuals with human immunodeficiency virus infection, and as a cause of diarrhea in travelers from

industrialized countries visiting less-developed areas of the world. (Laurence, 2022). In several regions of the world, EAEC surpasses enterotoxigenic *E. coli* (ETEC) as the most common bacterial pathogen identified in diarrheal stool samples and in the United States; this emerging pathogen is becoming increasingly recognized as a leading cause of sporadic diarrhea in otherwise healthy adults and children. Contaminated food appears to be the main source of EAEC infection and has been implicated in several foodborne outbreaks of diarrhea. (Pablo, 2010)

2.5 Enteroinvasive *Escherichia coli* (EIEC):

The gut pathogen enteroinvasive Escherichia coli (EIEC) causes enteritis. Similar to Shigella, it has a pathogenic mechanism that causes inflammation, mucosal ulceration, and epithelial invasion of the large bowel. Patients typically display bacillary dysentery symptoms. The metabolic processes of the EIEC strains are abnormal; they may ferment lactose slowly or not at all, they lack lysine decarboxilase, and they are non-motile. Furthermore, the majority of EIEC bacteria produce somatic antigens that are either very similar to or identical to Shigella antigens. (Prats, 1995). A large (140 MDa) plasmid that codes for the synthesis of many outer membrane proteins implicated in invasiveness mediates EIEC invasion. These strains have been isolated with some regularity in South America, the Extreme Orient, and Eastern Europe. In Spain the incidence of enteroinvasive *E. coli* is extraordinarily low (0.2%), the serogroup O124 being the most frequently isolated. EIEC enteritis has been associated to sporadic cases occurring in travelers. Occasional outbreaks related to ingestion of contaminated water or food and person to person have been reported. (Prats, 1995).

Clinically, EIEC infections often present with fever, systemic toxicity, crampy stomach discomfort, tenesmus, and urgency, associated with watery diarrhea or a dysentery syndrome with blood, mucus, and leukocytes in the stools. Considering Shigella spp. The disorder resembles bacillary dysentery, and the two diseases share virulence genes. The results of the sequencing of several housekeeping genes indicate that EIEC is more closely linked to Shigella than to noninvasive E. coli. Despite the fact that endemic disease is more prevalent in developing countries, EIEC diarrhea mostly happens in outbreaks. EIEC may be to fault for up to 5% of sporadic diarrhea episodes and 20% of instances of bloody diarrhea in some areas of the developing world. EIEC cause a diarrheal illness that is indistinguishable from Shigellosis and

appear to be a less common cause of diarrheal disease than the other *E. coli* pathogens, although this may be related to the methods that are used to detect these organisms. EIEC appears to be mostly responsible for occasional episodes of diarrheal illness (Martina et al., 2017). Shigella spp. and the EIEC have a close relationship. can employ Shigella-like genetic material to encode virulence proteins like the TTSS. The EIEC enters the intestinal cell, multiplies intracellularly, and extends into neighboring intestinal cells via virulence proteins known as "invasins." Cell death and occasionally bloody diarrhea result from this process. The majority of EIEC disease manifests clinically as watery diarrhea and is difficult to recognize at the bedside from the many other causes of diarrhea (Poolman, 2017).

2.6 Enteropathogenic *Escherichia coli* (EPEC):

A number of other species, including cattle, swine, rabbits, and nonhuman primates, such as common marmosets and cottontop tamarins, have also been documented to contract EPEC, making it one of the most prevalent bacterial causes of diarrhea in people worldwide. The molecular underpinnings of EPEC pathogenesis and virulence have been discovered via studies over the past 30 years. The bundle-forming pilus (bfp), which is encoded by a gene in typical EPEC, is in charge of the bacteria's initial attachment to enterocytes. More than 25 bacterial proteins can be directly inserted into the cytoplasm of the target cell by the organisms' type 3 secretion system (T3SS), which is present in them (Puente et al, 1996). The enzootic illness is spread by the fecal-oral pathway, and it is probably widespread in most marmoset colonies. As described below, unless specialized diagnostic approaches are used, these infections may go unrecognized and undiagnosed. While enzootic infection is common, clinical signs and more severe disease occur sporadically in colonies. (Alberto *et al.,* 2022)

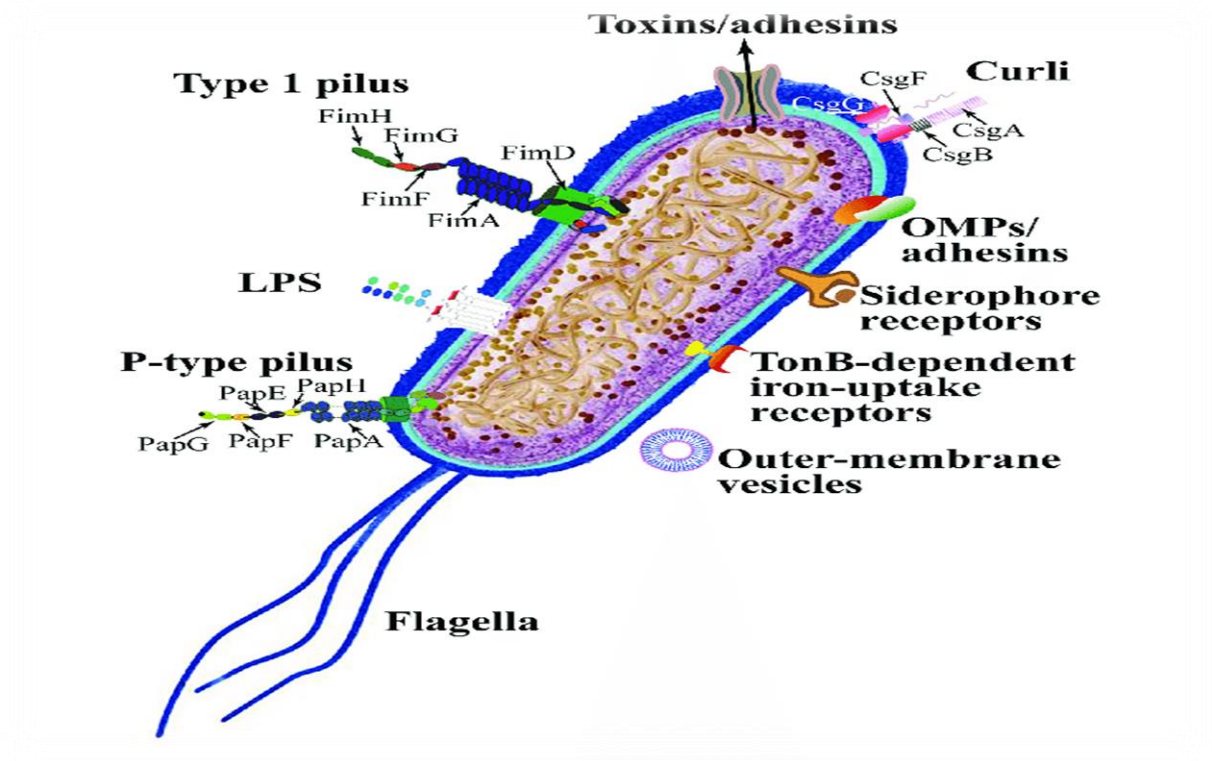

Figure 2.2: Mechanism of pathogenicity of *Escherichia coli*. Source: (Maria *et al*., 2017)

CHAPTER THREE

MATERIALS AND METHODS

3.1 SAMPLING

Water samples were collected from (Ojo, Iba) Lagos state Nigeria. Samples were collected in a sterile McCartney bottle, kept in ice-pack and transported to the laboratory for analysis.

3.2 MATERIALS

Materials used include: petri-dishes, beakers, conical flasks, test tubes (with rack), glass spreader, 70% ethanol. Micro pipette (with tips), Eppendorf tubes, PCR tubes, spatula

3.3 REAGENTS AND EQUIPMENTS USED

Equipment used include: Autoclave, weighing balance, distiller, wash bottles, water bath (set at 50°C and 100°C), incubator (set at 37°C), Bunsen burner, vortex, oven, inoculating loop, gel electrophoresis tanks.

3.4 MEDIA USED:

Media used were Nutrient Agar, MacConkey Agar and Sorbitol-MacConkey Agar, Buffer peptone water, Brain Heart Infusion broth.

PEPTONE WATER:

Peptone water is a microbial growth medium composed of peptic digest of animal tissue and sodium chloride. The pH of the medium is 7.2± 0.2 at 25 °C and is rich in tryptophan. Peptone water is also a nonselective broth medium which can be used as a primary enrichment medium for the growth of bacteria.

Preparation

1. The dehydrated medium was dissolved in the appropriate volume of distilled water to make up 0.1% peptone water based on manufacturers instruction's instructions in a conical flask and mixed thoroughly. The conical flask is then closed in cotton wool that is wrapped in aluminum foil.

2. The mixture was heated for a while to dissolve the powder completely and dispensed by pipetting into various test tubes for serial dilution.

3. It was then sterilized by autoclaving at 121°C for 15mins.

Three types of media were used for the isolation of *Escherichia coli*; MacConkey agar (MAC), 23 Nutrient agar (NA), Sorbitol-MacConkey Agar (SMAC)

SORBITOL-MACCONKEY AGAR (SMAC)

Sorbitol MacConkey agar was prepared according to the manufacturer's instruction for isolation and detection of *E. coli* O157:H7.

Preparation

1. The dehydrated medium was dissolved in the appropriate volume of distilled water i.e. 51.5g of SMAC in 1000 ml distilled water based on manufacturers instruction's instructions in a conical flask and mixed thoroughly. The conical flask is then closed in cotton wool that is wrapped in aluminum foil.

2. The mixture was heated for a while to dissolve the powder completely and was then sterilized by autoclaving at 121°C for 15minutes.

3. The medium was then allowed to cool to a range of 45-50°C and poured aseptically into sterile petri dishes and left to solidify. This medium is reddish-purple in color.

MACCONKEY AGAR:

MacConkey Agar is used for gram-negative enteric bacteria isolation and lactose fermentation differentiation from non-lactose fermenting bacteria and lactose fermenting bacteria but provides pink colonies on MacConkey Agar as *Escherichia coli*.

Preparation

1. The dehydrated medium was dissolved in the appropriate volume of distilled water then 48.5g of MacConkey in 1000 ml distilled water based on manufacturers instruction's 24 instructions in a conical flask and mixed thoroughly. The conical flask was then closed in cotton wool that is wrapped in aluminum foil.

2. The mixture was heated for a while to dissolve the powder completely and was then sterilized by autoclaving at 121°C for 15minutes. Avoid overheating.

3. The medium was then allowed to cool to a range of 45-50°C and poured aseptically into sterile petri dishes and left to solidify. The medium is neutral red in color

NUTRIENT AGAR

Nutrient agar was prepared according to the manufacturer's instruction.

Preparation

1. The dehydrated medium was dissolved in the appropriate volume of distilled water i.e. 28g of Nutrient agar in 1000 ml distilled water based on manufacturers instruction's instructions in a conical flask and mixed thoroughly. The conical flask is then closed in cotton wool that is wrapped in aluminum foil.

2. The mixture was heated for a while to dissolve the powder completely and was then sterilized by autoclaving at 121°C for 15minutes.

3. The medium was then allowed to cool to a range of 45-50°C and poured aseptically into sterile petri dishes and left to solidify. The medium appears opalescent and is light amber in color.

3.5 ISOLATION OF *Escherichia coli*

3.5.1 Sample preparation

1ml of samples (irrigation water) was pipetted into an already prepared 1% BPW (Enrichment broth) and then homogenized using a vortex, after which serial dilution was carried out and appropriate dilutions were plated on SMAC and MacConkey agar plates.

3.5.2 Serial dilution

One milliliter (1ml) of samples were pipetted using the micro-pipette (set at 1000µl) into test tubes containing 9ml of BPW (0.1 %) to obtain 10^{-1}, followed by transfer of 1ml from10^{-1} into a new test tube (containing 9ml BPW) to create 10^{-2} dilution, the test tubes are then put in the vortex mixer for even mixing. The test tubes were labelled for easy identification.

3.5.3 Plating

The plates were labeled accordingly after which 0.1ml from the dilution factors (10^{-1} and 10^{-5}), was plated onto SMAC Agar, MAC Agar and Nutrient Agar for isolation of enteropathogenic *E. coli*, and the total viable count using the spread plate technique (the glass rod before every use was dipped into ethanol and then flamed in the Bunsen burner so as to maintain aseptic conditions). The plates were incubated at 35°C -37°C for 18-24 hours and counted.

3.5.4 Sub culturing

This is done to purify the isolated bacterial colonies from a mixed culture to a new and single culture. The bacteria colonies that were sub cultured were those which were differentiated on the basis of colony morphology (size, shape, color, elevation, appearance, surface and opacity). The colonies gotten from the previously incubated SMAC plate were sub cultured into nutrient agar.

The inoculating loop was heated using the Bunsen burner until red hot and allowed to cool, it was then used to take a loop-full from the mixed culture and then streaked onto the new petri dish (Nutrient agar plate). The plates were then inverted and incubated at 37°C for 18-24 hours.

3.5.5 Cryopreservation of isolates

A loop-full of each isolate was taken from the nutrient agar plate and was inoculated into 5ml of the brain heart infusion (BHI) broth each in a test tube, it was then incubated at 37°C for 18-24 hours. After incubation 750µl of each inoculum was added into a Eppendorf tube containing 750µl of sterile 20% glycerol which acts as a cryo-protectant and was stored in a freezer at -4°C.

3.6 MOLECULAR IDENTIFICATION

3.6.1 Activation of Isolates

1ml of pure BHI was prepared into 2ml Eppendorf tubes and sterilized using the autoclave at 121°C for 15 minutes. 100µl of each thawed stock culture was added into the various Eppendorf tubes containing sterile BHI after it was allowed to cool, it was then incubated at 37°C for 48hours.

3.6.2 Prewashing

Each isolate was centrifuged in Eppendorf tubes at 5000rpm for 3 minutes. The BHI supernatant was discarded into a waste container, leaving the pellet in the tubes. 1.5ml of sterilized distilled

water was added into the tubes, vortexed and then centrifuged at 5000rpm for 3 minutes. The supernatant was discarded and 200 µl of sterilized distilled water was added to the tubes and vortexed.

3.6.3 DNA Extraction by Boiling Using Heating Block

The heating block was switched on and allowed to reach 100°C. The Eppendorf tubes containing the prewashes isolates were placed into the heating block and the lid was gently placed over it to prevent the tubes from popping open.

It was allowed to boil for 15 minutes; the boiled DNA were then placed into ice to cool for 5 minutes. The already cooled DNA was centrifuged at 7000rpm for 6 minutes after which 150 µl of the DNA supernatant was carefully transferred into an already properly coded fresh Eppendorf tube.

3.6.4 Polymerase Chain Reaction (PCR)

The component of the PCR used for the characterization of *E. coli* pathotypes is shown in table 3.2 below. After the PCR cocktail has been prepared it was placed in a thermocycler. The PCR was carried with initial denaturation at for , cycles of for , for and for , and finally for cycles. The PCR product were confirmed by gel electrophoresis and visualized under UV light with a gel documentation system.

3.6.5 Agarose Gel Electrophoresis

The agarose gel was prepared using dry agarose powder, 1g of agarose powder was dissolved in 50ml od TAE buffer the mixture was then boiled until a clear solution was gotten 3µl of ethidium bromide was added to the mixture using a micropipette. The mixture was swirled and allowed to cool slightly but not left to solidify. The mixture was then poured into a gel cast with the combs in place and left to solidify. The gel is gently removed and transferred in an electrophoresis tank and TAE buffer was poured over it. 4µl of the PCR products are pipetted into each well of the already well-formed gel after removing the comb. The tank is connected to a power source and allowed to run. The gel is viewed under the UV transilluminator.

Table 3.1: Multiplex PCR reaction components

TREATMENT 1

No.	**Reagents**	**Initial concentration**	**Final concentration**	**Volume per rxn(v/r)**	**n=20**
1	Master mix	5x	1x	2	40
2	*Stfh*	20 µm	0.4	0.2	4
3	*StRh*	20 µm	0.4	0.2	4
4	*Vtxif*	20 µm	0.25	0.125	2.5
5	*VtxiR*	20 µm	0.25	0.125	2.5
6	*Vtx2f*	20 µm	0.5	0.25	5
7	*Vtx2R*	20 µm	0.5	0.25	5
8	*Ipahf*	20 µm	0.1	0.05	10
9	*IpahR*	20 µm	0.1	0.05	10
10	$Mgcl_2$	25 mm	1.5	0.6	12
11	dH_20		4.15		83
12	DNA				

Table 3.2: Multiplex PCR reaction components

TREATMENT 2

No.	**Reagents**	**Initial concentration**	**Final concentration**	**Volume per rxn(v/r)**	**n=20**
1	Master mix	5x	1x	2	40
2	*StRp*	20 µm	0.5	0.25	5
3	*StRp*	20 µm	0.5	0.25	5
4	*Eltaf*	20 µm	0.45	0.225	4.5
5	*EltaR*	20 µm	0.45	0.225	4.5
6	*Eaep*	20 µm	0.15	0.075	1.5
7	*EaeR*	20 µm	0.15	0.075	1.5
8	Mgcl2	20 µm	1.5	0.6	12
9	dH_20	20 µm		4.3	86
10	DNA	20 µm			

Table 3.3: Multiplex PCR reaction components

TREATMENT 1

No.	**Reagents**	**Initial concentration**	**Final concentration**	**Volume per rxn(v/r)**	**n=59**
1	Master mix	5x	1x	2	118
2	Stfh	20 µm	0.4	0.2	11.8
3	StRh	20 µm	0.4	0.2	11.8
4	Vtxif	20 µm	0.25	0.125	7.375
5	VtxiR	20 µm	0.25	0.125	7.375
6	Vtx2f	20 µm	0.5	0.25	14.75
7	Vtx2R	20 µm	0.5	0.25	14.75
8	Ipahf	20 µm	0.1	0.05	2.95
9	IpahR	20 µm	0.1	0.05	2.95
10	$Mgcl_2$	25 mm	1.5	0.6	35.4
11	dH_20		4.15		244.85
12	DNA				

Table 3.4: Multiplex PCR reaction components

TREATMENT 2

No.	**Reagents**	**Initial concentration**	**Final concentration**	**Volume per rxn(v/r)**	**n=59**
1	Master mix	5x	1x	2	118
2	*StFp*	20 µm	0.5	0.25	14.75
3	*StRp*	20 µm	0.5	0.25	14.75
4	*Eltaf*	20 µm	0.45	0.225	13.275
5	*EltaR*	20 µm	0.45	0.225	13.275
6	*Eaep*	20 µm	0.15	0.075	4.425
7	*EaeR*	20 µm	0.15	0.075	4.425
8	Mgcl2	20 µm	1.5	0.6	35.4
9	dH_20	20 µm		4.3	253.7
10	DNA	20 µm			

Table 3.5: Protocol for Thermocycler

No of cycles	Steps	Temperature (°C)	Time
1	Initial denaturation	95°C	15 minutes
35	Denaturation	95°C	50 seconds
35	Annealing	57°C	40 seconds
35	Extension	72°C	50 seconds
1	Final Extension	72°C	3 minutes

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 RESULTS

Twelve samples of code W1L1-W4L12 were collected from a farm at the Ojo-Iba area of Lagos state where lettuce is grown, *Escherichia coli* on SMAC (Sorbitol MacConkey agar and MacConkey agar showed pink and white colors. Week one with code W1, showed colonies to be small in size, circular shaped with butyrous appearance. They appeared with smooth surface, low convex elevation and opaque. Multiplex PCR showed one(W1L1) out of four samples in week one to possess Enterotoxigenic *E. coli*. Week two also showed colonies to be small in size, circular shaped with butyrous appearance, with smooth surface, low convex elevation and opaque. W2L5and W2L6 were positive for carrying Enterotoxigenic genes out of four other samples. Week three with code W3, showed colonies to be small in size, circular shaped with butyrous appearance. They also appeared with smooth surface, low convex elevation and opaque. After multiplex PCR was carried out, one out of four samples

The biochemical tests were carried out to identify the bacterial species by differentiating them on the basis of biochemical activities. Based on the outcomes of the biochemical tests in Table 4.3. Isolates were thought to be *Escherichia coli* based on the selective medium that were utilized for identification. The isolates are all oxidase- and Gram-negative. Catalase is present in the majority of the isolates.

Multiplex PCR was done to determine how many samples were pathogenic, and Gel electrophoresis using agarose gel was used to determine the pathotype of *E. coli* present. Multiplex PCR revealed that six out of twelve isolates were pathogenic, and Gel electrophoresis using agarose gel confirmed that they were all Enterotoxigenic *E. coli*.

The primers used were *StFp, StRp, EaeR, Eaep, EltaR, Eltaf, StRp, StFp.*

Table 4.1: Morphological characteristics of isolates on SMAC

Isolate	Sample	Sample ID	Colour	Shape	Size	Elevation	Appearance	Surfaces	Opacity
1st sampling	Water	W1L1	White Pink	Circular	Small	Low convex	Butyrous	Smooth	Opaque
	Water	W1L2	White Pink	Circular	Small	Low convex	Butyrous	Smooth	Opaque
	Water	W1L3	White Pink	Circular	Small	Raised	Butyrous	Smooth	Opaque
2nd sampling	Water	W2L4	White Pink	Circular	Medium	Low convex	Butyrous	Smooth	Opaque
	Water	W2L5	White Pink	Circular	Small	Low convex	Butyrous	Smooth	Opaque
	Water	W2L6	White Pink	Circular	Small	Low convex	Butyrous	Smooth	Opaque
3rd Sampling	Water	W3L7	White Pink	Circular	Small	Low convex	Butyrous	Smooth	Opaque
	Water	W3L8	White Pink	Circular	Small	Low convex	Butyrous	Smooth	Opaque
	Water	W3L9	White Pink	Circular	Medium	Low convex	Butyrous	Smooth	Opaque
4th Sampling	Water	W4L10	White Pink	Circular	Small	Convex	Butyrous	Smooth	Opaque
	Water	W4L11	White Pink	Circular	Large	Low convex	Butyrous	Smooth	Opaque
	Water	W4L12	White Pink	Circular	Large	Raised	Butyrous	Smooth	Opaque

Table 4.2: Biochemical testing for *E. coli* organism

CODE	GRAM STAINING	CATALASE	OXIDASE
W1L1	Negative (-ve)	Positive (+ve)	Negative(-ve)
W2L4	Negative (-ve)	Positive (+ve)	Negative (-ve)
W3L7	Negative (-ve)	Positive (+ve)	Negative (-ve)
W4L10	Negative (-ve)	Positive (+ve)	Negative (-ve)

4.2 PCR AMPLIFICATION IMAGE

The genetic characterization shows DNA band on agarose gel of the presumptive pathogenic *E. coli* after PCR amplification. This confirms the results from the morphological and biochemical test.

Figure 4.1: Sorbitol MacConkey agar plate showing growth of *Escherichia coli*

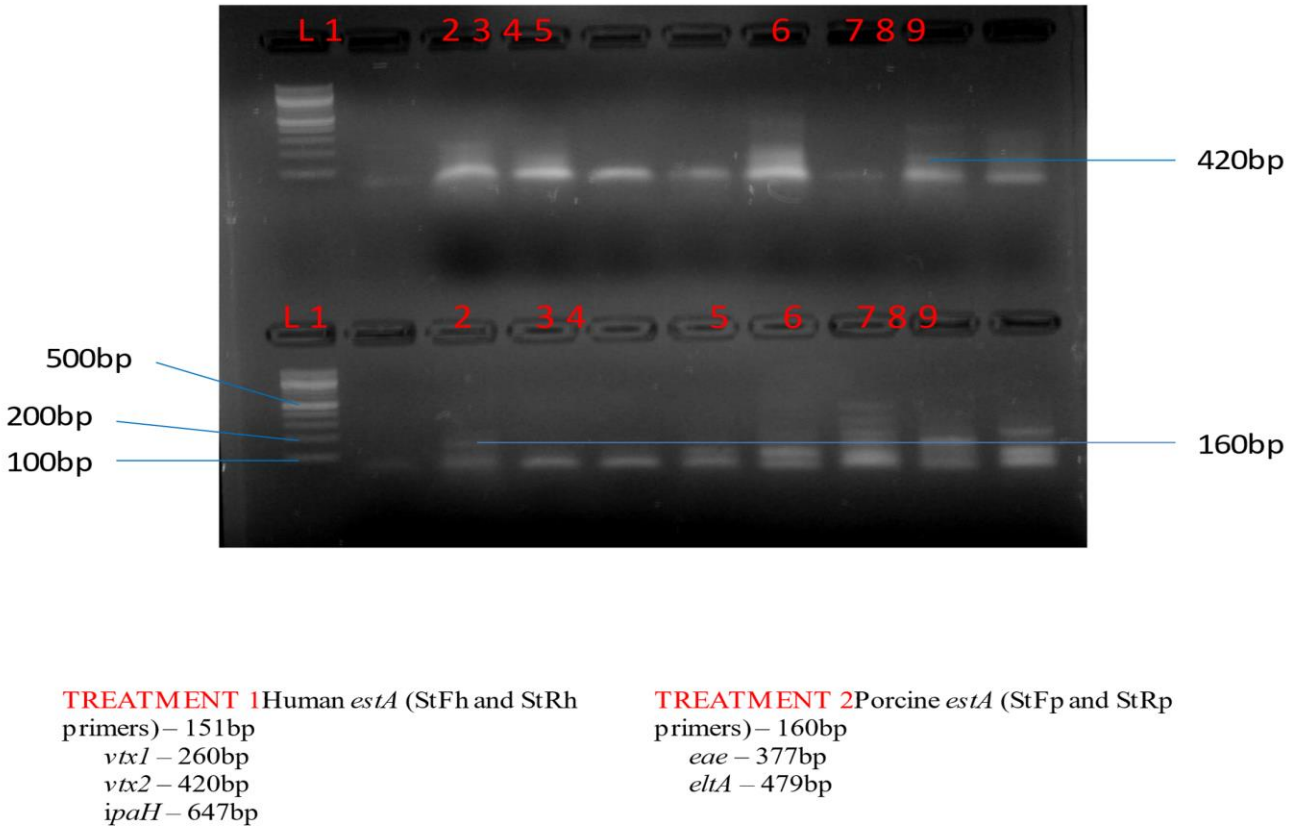

Figure 4.2: Agarose gel electrophoresis multiplex PCR for *E. coli* pathotypes

Lanes L=DNA Ladder, Lane 1-9 fragments of isolates from soil samples. 8 has vtx2 (420bp), 2 and 9 has Porcine estA (160bp) genes.

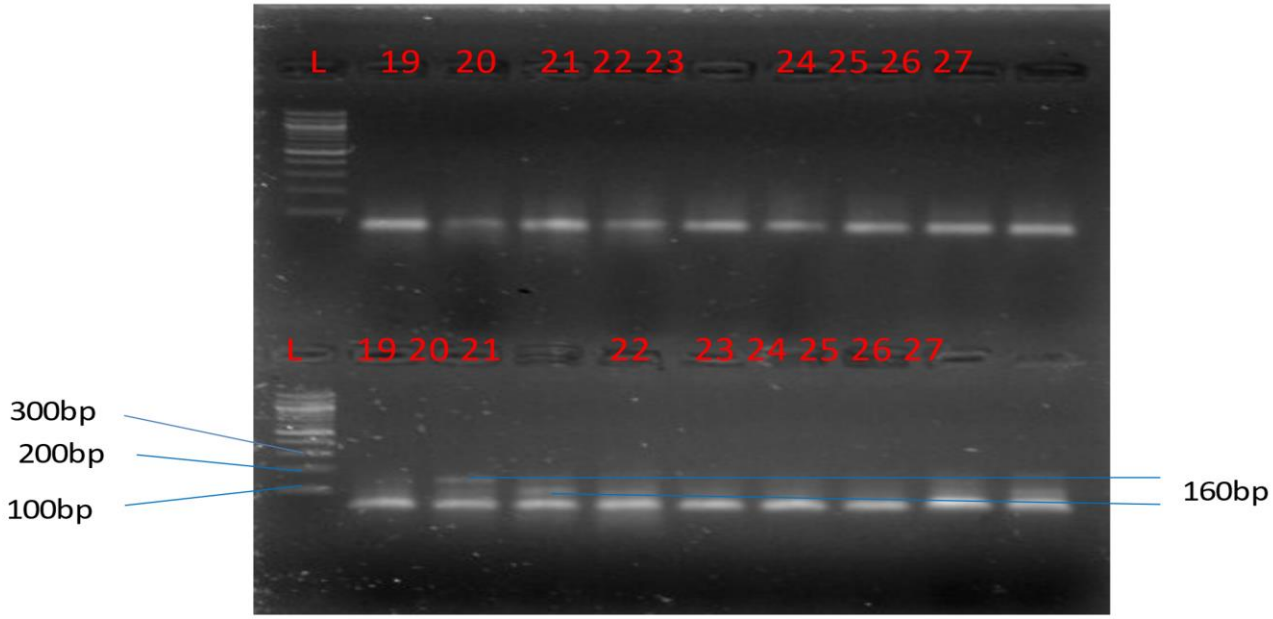

Figure 4.3: Agarose gel electrophoresis multiplex PCR for E. coli pathotypes

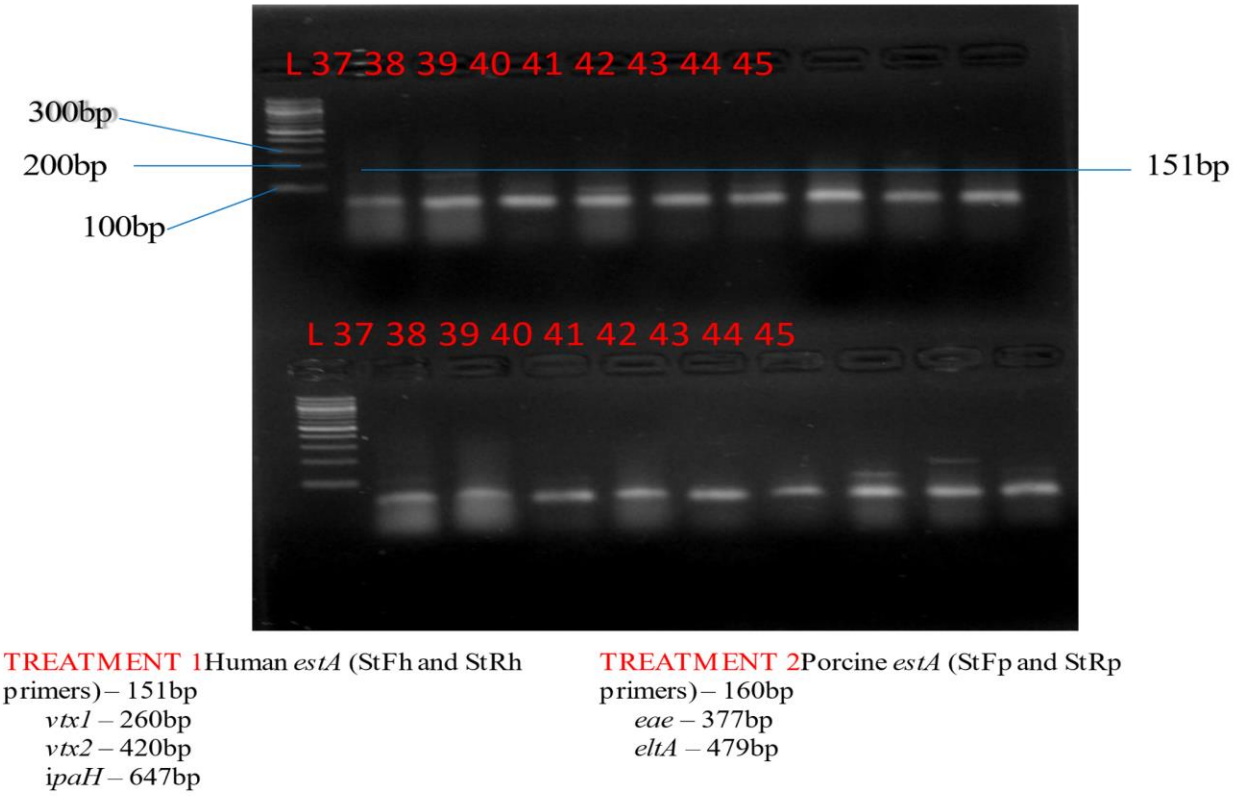

Figure 4.4: Agarose gel electrophoresis multiplex PCR for E. coli pathotypes

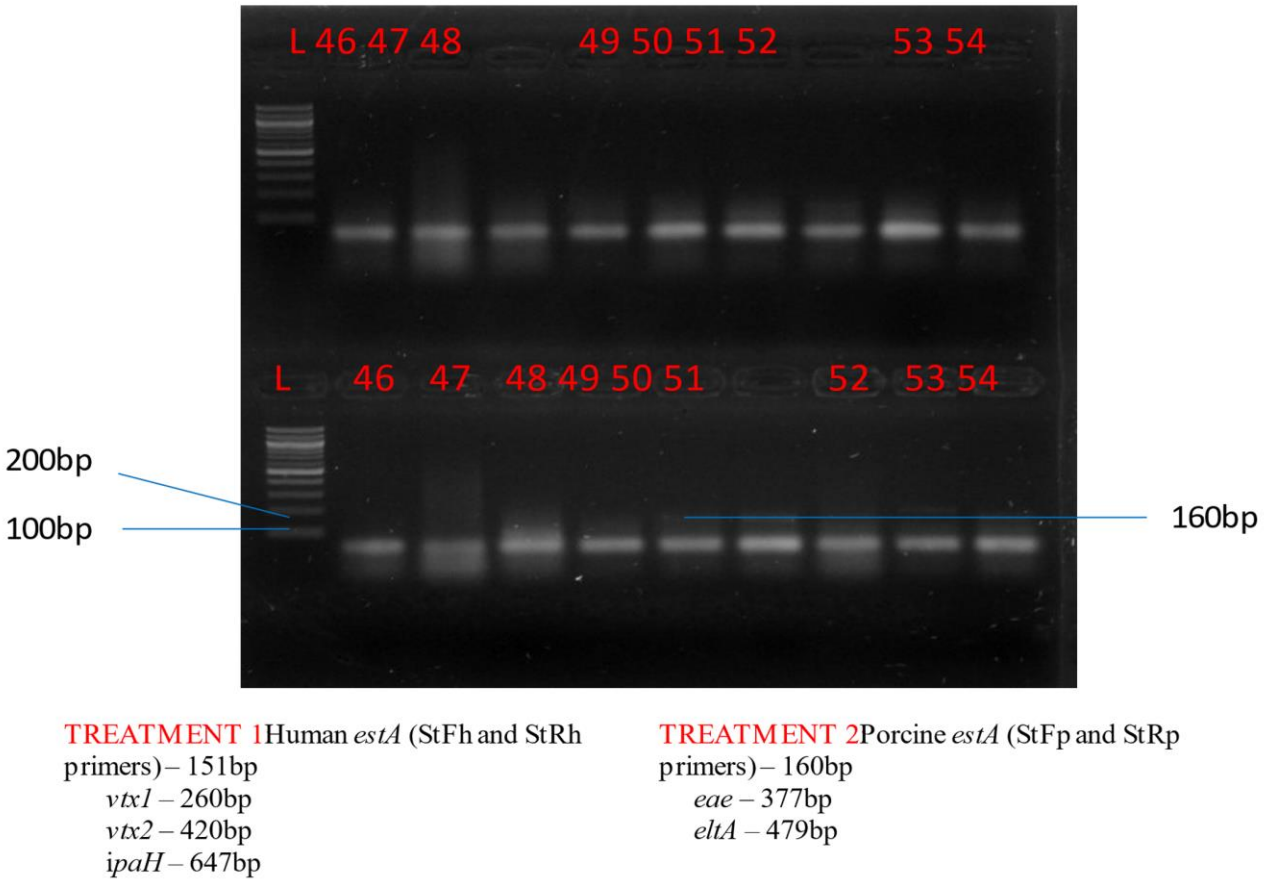

Figure 4.5: Agarose gel electrophoresis multiplex PCR for E. coli pathotypes

4.3 DISCUSSION

E. coli can be found in both human and animal stomachs. It is a multipurpose pathogen, though, and is linked to intestinal illnesses like diarrhea and extraintestinal infections like urinary tract infections (Ebenezer & Mark, 2022). They are present in water, soil, plant leaves, equipment and utensil surfaces, and can contaminate food and water through various channels (Sangshin et al., 2013). Agricultural water, soil amendments, growing and harvesting techniques, animal infiltration, nearby land usage, and employee health and hygiene practices were areas of attention for contamination on agricultural products like lettuce. Other factors of contamination of lettuce fruits as observed are: facility and equipment sanitary design, cleaning and sanitizing practices, washing and drying of fresh-cut romaine lettuce, and storage of romaine lettuce (U.S Food Administration, 2018).

Twelve samples of code W1L1-W4L12 were collected from a farm at the Ojo-Iba area of Lagos state, where lettuce was grown. Morphological confirmation of *E. coli* was shown on MAC and SMAC agars, where the colonies appeared to be pink and white colors respectively. They were also small in size, circular shaped with butyrous appearances. They appeared with smooth surface, low convex elevation and opaque Further biochemical tests were carried out to identify the bacterial species by differentiating them on the basis of biochemical activities which showed that the bacteria isolates were all oxidase- and Gram-negative. Catalase is present in the majority of the isolates. Multiplex PCR revealed that six out of twelve isolates were pathogenic, and Gel electrophoresis using agarose gel confirmed that they were all Enterotoxigenic *E. coli* (Zita *et al*., 2000).

It was observed that good agricultural practices were not carried out at the Ojo, Iba area, where farmers are given contracts to grow and harvest a targeted amount of vegetables, such as lettuce, spring onions, Corchorus (Ewedu), spinach, etc. Farmers exposed the vegetables to various contamination by pathogenic *E. coli*. (Felson, 2020). For instance, the water used for irrigation was dirty. Farmers went into the wells to get the water used to water the plants thereby introducing pathogenic *E. coli* from their bodies. Furthermore, a dump site was observed close to the farm which could be another likely source of irrigation water contamination as erosion could sweep pathogenic *E. coli*, into the wells were the irrigation water is gotten.

Easily harvestable crops that were handled carelessly and with dirty utensils. Plants that are gathered are not thoroughly washed. Kumari (2002). E. By mixing cut lettuce in wash systems at fresh-cut produce manufacturing/processing facilities, E. coli contamination of the lettuce from some of the growing fields mentioned in the traceback may have been increased. Washing lettuce at a facility that produces or

processes fresh-cut food or at home by customers may lessen but not completely get rid of viruses, including STEC, from romaine lettuce. (2018) (U.S. Food and Drug Administration). The elder plant may get contaminated when young and old plants are harvested, maintained together, or combined after harvesting. E. coli infection is also a potential if plants are uprooted during harvest. coli that is rhizosphere-dwelling (Leo et al., 2021). Additionally, the area is home to livestock like cows and goats, who consume some of these vegetables. Additionally, animal waste was mishandled and used as fertilizer. The potential transfer of E. coli from produce to humans must be addressed in order to better safeguard consumers from exposure to such food. It is important to comprehend how bacteria from manure or irrigation water affects plants (Wallheimer, 2010).

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

The irrigation water used in planting process at the Ojo-Iba area of Lagos, was confirmed to possess pathogenic *E. coli* strains. Six samples out of twelve tested positive with *estA* genes and this concludes c that the water used in watering the lettuce plant is contaminated and is a source of adverse health effect to consumers.

5.2 RECOMMENDATION:

The use of clean tools and utensils during all stages of planting and harvesting, the use of untreated compost manure should be abolished, the strict enforcement of proper hand washing, the separation of animals' feeding areas from farms were lettuce are grown for human consumption. The proper washing of food items, and thorough cooking are all examples of preventive measures. To protect the safety of the food supply, government agencies should implement Good Agricultural Practices (GAP) policies.

Producers should apply manure to fields well in advance of planting and harvesting, a wait of 90-120 days between manure application and harvesting, with a minimum of 40 days between planting and harvesting, should minimize the chance of *E. coli* contamination

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