MOLECULAR CHARACTERIZATION OF *Escherichia coli* PATHOTYPES ASSOCIATED WITH THE SOIL OF LETTUCE PLANTS FROM OJO-IBA AREA, LAGOS STATE.

BY

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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 has been written by me under the supervision of Dr Moses Abiala and is a record of my own research work. It has not been presented in any previous application for a higher degree or any other university. All citations and sources of information are clearly acknowledged by means of reference.

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     JOSEPH, FAVOUR GIFT   DATE

CERTIFICATION

This is to certify that this research project titled "**MOLECULAR CHARACTERIZATION OF** *Escherichia coli* **PATHOTYPES ASSOCIATED WITH THE SOIL OF LETTUCE PLANTS FROM OJO-IBA AREA, LAGOS STATE**" was carried out by **JOSEPH FAVOUR GIFT**, with matriculation number **18010101038**. This project meets the requirements governing the award of **Bachelor of Science (B.Sc.)** in **Microbiology** Department of Biological Sciences of Mountain Top University, Ogun State, Nigeria and is approved for its contribution to knowledge and literary presentation.

_____   _____

   DR. M.A ABIALA   DATE

  (Project supervisor)

_____   _____

   DR. (MRS) C.I AYOLABI   DATE

  (Ag. Head of Department)

DEDICATION

I dedicate this project to God Almighty for giving me life, good health and all I needed to make this work a success and secondly to my dear mum Mrs Joseph Judith and my sister for their guidance, understanding, provision and sacrifice. And also, to my dad Mr Martins Joseph may his soul rest in peace. Amen

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ABBREVIATION

ml   Millilitre

µm   Microliter

%   Percent

Mm   Micrometre

Min   Minute

et al., And others

bp   Base pair

E. coli   *Escherichia coli*

No.   Number

ABSTRACT

Among leafy green vegetables, lettuce is consumed fresh and, in most cases, not washed by consumers before consumption. It is presumed that the soil borne pathogenic *E. coli* associated with vegetable forms are usually from organic manure such as cow dung, poultry manure and other faecal contaminants are known to be of public health concern to both vegetable farmers as well as consumers of such vegetables. The major objective of this study was to determine the molecular characterization of *Escherichia coli* pathotypes associated with the soil of lettuce plant. The soil of lettuce fields, on the other hand, has not been tested for the presence of pathogenic *E. coli*. As a result, this research is based on soil from lettuce fields that has been routinely treated with poultry manure. A molecular method was used, specifically the utilization of multiplex PCR. All twelve isolates were identified as potential Enterobacteriaceae containing *E. coli*, on Sorbitol MacConkey Agar, MacConkey Agar and also biochemically identified. Further identification with multiplex PCR revealed that out of the twelve samples, one was positive with *E. coli*. Using multiplex PCR, the *E. coli* was found to be pathogenic. In conclusion, the agricultural soil samples collected from Ojo-Iba area, Lagos state harbour pathogenic *E. coli*, which could be transferred to fresh Produce (Lettuce) intended for human consumers. Some of the isolates harboured either the Human *est A* and Porcine *est A* genes making them enterotoxigenic *Escherichia coli* (ETEC) and highly virulent, thus capable of causing severe forms of infection in human beings.

Keywords: *Escherichia coli*, lettuce, PCR, virulence gene, Soil.

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Vegetables are crucial components of the majority of healthy diets, and demand for them has grown recently as a result of sugg*est*ions for leading healthy lif*est*yles (CDC, 2010). The use of animal manure in soil for cultivation is common and is still considered to be better than chemical fertilizers (Dutta *et al*., 2014). Inter*est*ingly, there have been more worries regarding the safety of utilizing animal manures as fertilizer in vegetable cultivation due to the recent increase in fresh vegetable contamination with fecal pathogenic bacteria like *Escherichia coli* (Verma *et al*., 2013). *Escherichia coli* and other zoonotic pathogens are found in animal dung and feces (McGarvey *et al*., 2004; Dhama *et al*., 2013). The demand for fresh vegetables with minimum processing has led to a growth in the variety and quantity of items available to customers. A rise in *Escherichia coli* incidents, particularly those involving lettuce, has also been recorded. This poses a significant health risk to consumers and may have a negative financial impact on the agriculture sector. There have been reports of the int*est*inal bacteria *Escherichia coli* infecting a broad range of hosts, including humans. Infected calves may excrete up to 50,000 CFU of *Escherichia coli* per gram of feces, which is why it is assumed that cattle in particular are natural carriers for these infections in animals. When plants produce come into touch with contaminated manure or irrigation waters, the leaves of the plants (produce) get exposed (before harv*est*) to these organisms. Public health professionals are particularly concerned about *Escherichia coli* because only a small number of cells can result in sickness when consumed. Soil preparation and planting are the stages of the production process where pathogen contamination is most likely to occur (Islam *et al*., 2004). Using laser scanning confocal microscopy, epifluorescence microscopy, and the recovery of live cells from the interior tissues of plants, Solomon *et al*., 2002 demonstrated the transmission of *Escherichia coli* from manure-contaminated soil and irrigation water to lettuce plants. They concluded that *Escherichia coli* entered the edible part of the plant directly through the conducting tissues of the root system (Franz *et al*., 2008).

Numerous studies have shown that *Escherichia coli* and other bacteria can contaminate vegetables, posing a serious threat to human health. The use of raw animal manure, contamination,

and cross-contamination by harv*est*ing and packing equipment, worker health and hygiene, and contamination are all recognized as causes of produce contamination in the manufacturing and sale of vegetables. Additionally, irrigation water is thought to be a significant factor in crop contamination because of the frequent microbial contamination (Leimbach *et al*., 2013).

1.3   JUSTIFICATION OF STUDY

In most circumstances, people do not wash lettuce before eating it, making it one of the leafy green vegetables that are consumed fresh. The soil-borne pathogenic *Escherichia coli* linked to lettuce to associate with organic manures which include; cow dung, poultry manure, and other faecal pollutants that are known to be hazardous to the public's health for both lettuce growers and consumers of such lettuce.

1.4   OBJECTIVES OF STUDY

The objectives of this study is;

- Enumerate possible *Escherichia coli* and associated Enterobacteriaceae in the soil of vegetable farms.
- To identify *Escherichia coli* isolates isolated from the soil through a series of biochemical t*est* and PCR.
- To determine the presence of EPEC isolated by using Sorbitol-MacConkey Agar, Nutrient Agar, and MacConkey Agar.

CHAPTER TWO

LITERATURE REVIEW

2.1 ETIOLOGY OF *Escherichia coli*

The first person to describe *Escherichia coli* was a Bavarian doctor, Theodor Escherich on the 14th of July 1885 in a lecture. According to Shulman *et al*. (2007), the Bacterium coli commune, also known as *E. coli*, was one of 19 bacteria that Escherich studied and isolated over 15 months. The organism was characterized by Escherich as a Gram-negative bacillus measuring roughly 1.1–1.5 m 2.0–6.0 m. Castellani and Chalmers sugg*est*ed the name *E. coli* in 1919 as a tribute to Escherich, and the Judicial Commission of the International Committee on Systematic Bacteriology formally adopted it in 1958 (Foster-Nyarko and Pallen, 2022). In the wild, E. coli is a typical inhabitant of the guts of vertebrates as well as other non-host environments such as water, soil, manure, and food (Van Elsas *et al*., 2011, Blount 2015). As a result, E. coli is a very adaptable species that can thrive in a variety of ecological settings (Leimbach *et al*., 2013). *Escherichia coli* is also a versatile pathogen, causing a wide range of illnesses and at least 2 million deaths in humans annually (Kosek *et al*., 2003). Not shortly after its discovery, the organism's contribution to int*est*inal and extraint*est*inal disease was understood (Friedmann, 2014). The bacterium being able to grow in both aerobic and anaerobic environments is called a facultative anaerobe. In animals' lower int*est*ines, it is the most prevalent aerobe (Tenaillon *et al*., 2010).

Escherichia coli is a rod-shaped, Gram-negative bacterium, and classified as a member of the family Enterobacteriaceae within the Gamma proteobacteria class. *Escherichia coli* is among one well-studied bacteria (Jang *et al*.,2017). *Escherichia coli* has been used as the host bacterium in a variety of gene-manipulation systems that produce a huge number of enzymes and other commercial goods. The first study on *Escherichia coli's* genome sequence analysis was published in 1997. Over 4800 *Escherichia coli* genomes have been sequenced since then (Jang *et al*., 2017). The fast growth characteristics of *Escherichia coli* make it suitable to study the evolution of micro-organisms and a long-term experimental evolution study of *Escherichia coli* involving more than 50 000 generations is ongoing (Tenaillon *et al*., 2016). Due to the presence of certain colonization factors, virulence factors, and genes linked with pathogenicity that are typically absent in other *Escherichia coli*, pathogenic strains of *Escherichia coli* can cause several different diarrhoeal illnesses in hosts (Bolton *et al*., 2007). *Escherichia coli* is a common bacterial species. In addition

to being one of the most widely distributed bacterial species in the environment, it is a component of the gut microflora of both humans and warm-blooded animals. The microbiome is defined as the sum of microorganisms, their genomes, and environmental interactions in both human and animal hosts. Bacteria belonging to the *Escherichia coli* species comprise an essential component of the microbiome. This bacterial species colonizes the gastroint*est*inal system throughout the early stages of life, developing favorable interactions with the host and being crucial in preserving the balance of the many other bacterial species that make up the gut microflora. *Escherichia coli* is also one of the most widely distributed bacterial species in the environment, found in practically all environmental niches, including soil and water. Due in large part to their great genetic flexibility, *Escherichia coli* strains have the unusual capacity to colonize a variety of hosts and habitats. This ability to successfully form partnerships with other microbes is also a result of their remarkable ability to interact with them. Different phenotypic and genotypic variations can be seen in both commensal and pathogenic *Escherichia coli* strains (Van Elsas *et al*., 2011).

2.2 *ESCHERICHIA COLI* AS A HUMAN PATHOGEN

Escherichia coli includes not only commensal strains but also pathogenic ones that cause a variety of human diseases resulting in more than 2 million deaths each year (Kaper *et al*., 2004). Six *Escherichia coli* pathotypes, including;

- Shiga toxin-producing *Escherichia coli* (STEC)
- Enteropathogenic *Escherichia coli* (EPEC)
- Enterotoxigenic *Escherichia coli* (ETEC)
- Enteroaggregative *Escherichia coli* (EAEC)
- Diffusely adherent *Escherichia coli* (DAEC)
- Enteroinvasive *Escherichia coli* (EIEC)

These strains are categorized based on their virulence characteristics and pathogenicity mechanisms that they use to cause gastroint*est*inal illnesses like diarrhea (Kaper *et al*., 2004). Pathogenic E. coli strains have been associated with several waterborne epidemics, and STEC and EPEC have been held responsible for several outbreaks worldwide (Chandran and Mazumder 2015). According to Baliere *et al.,* 2015 pathogenic E. coli contamination in the environment can result from animal wastes such as manure and other animal wastes, wastewater from slaughterhouses, and effluent from wastewater treatment facilities.
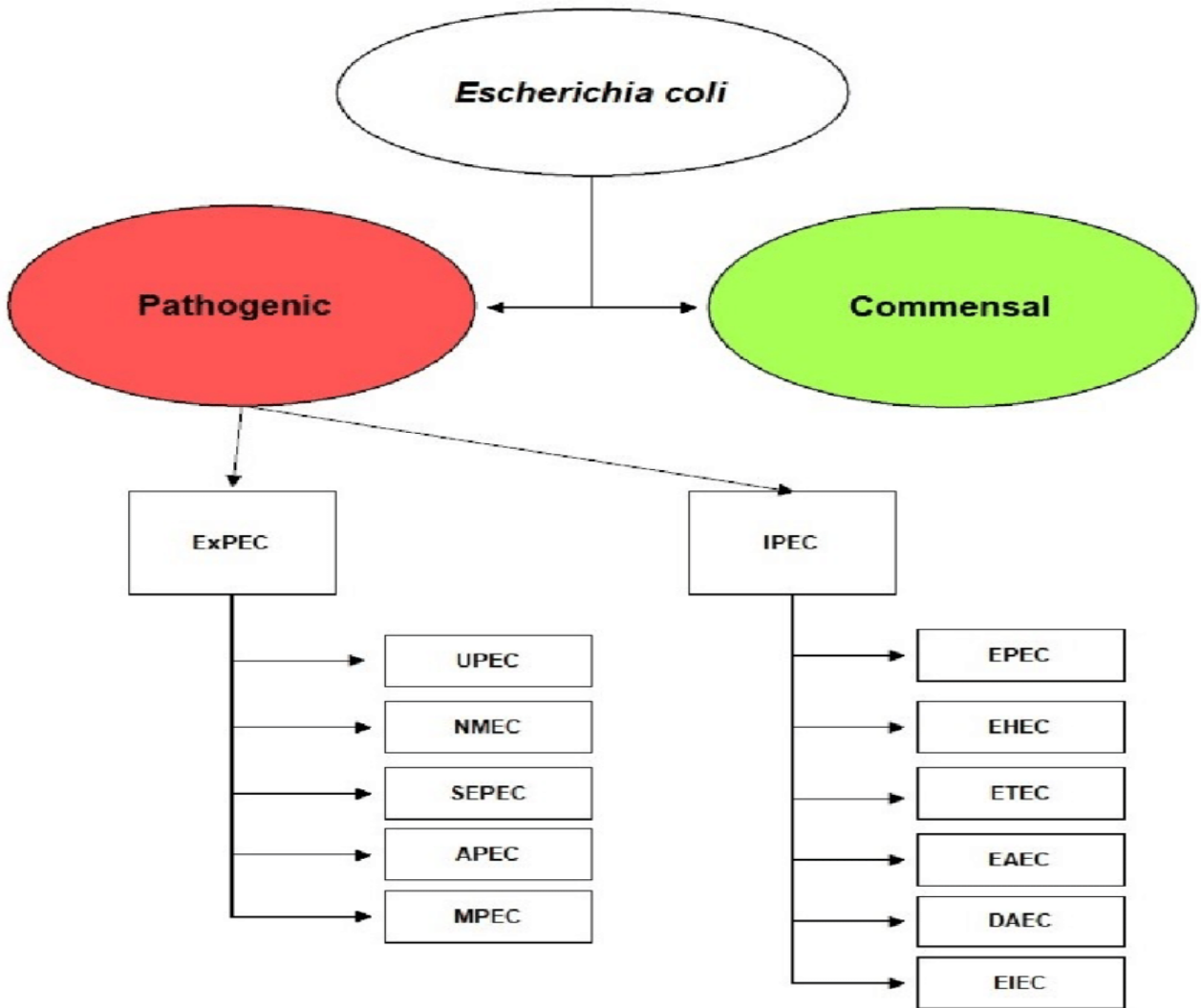
Figure 2.1: Classification of pathogenic *Escherichia coli* (Sora *et al*., 2021).

2.2.1   ENTEROINVASIVE *Escherichia coli* (EIEC)

In 1944, a bacterium known as the "paracolon bacillus" was initially recognized as the source of EIEC; however, a prototype EIEC was subsequently shown to be an O124 serogroup of *Escherichia coli*. A research team discovered a group of *Escherichia coli* that can cause

experimental keratoconjunctivitis in guinea pigs during the Serény-t*est* in the 1950s. These *Escherichia coli* were initially categorized under the species *Shigella* as *Shigella manolovi*, *S. sofia, Shigella* strain 13, and *S. metadysenteriae*. The ailment starts with severe abdominal spasms, weakness, watery stools, dysuria, and fever. This condition rarely gets worse and turns into loose stools that contain blood or mucus. Fecal leukocytes seen in shigellosis can also be seen in the mucus of a person infected with EIEC (Ekici, 2019). The distinguishing attribute of EIEC from several other *E. coli* is the development of watery diarrhea in infected individuals in most cases. EIEC could also occasionally cause dysentery and what is known as invasive inflammatory colitis (Nataro *et al.,* 1998).

2.2.2   ENTEROTOXIGENIC *Escherichia coli* (ETEC)

The symptoms of an ETEC infection include frequent, watery diarrhea that lasts for many days with little to no fever, which can cause dehydration and malnutrition in young children. The disease is brought on by consuming contaminated food or drink. One of the foods linked to ETEC infections is raw vegetables, along with soft cheeses. Among impoverished nations with inadequate hygiene and infrequently implemented water sanitation measures, ETEC is a prominent cause of diarrhea in babies. Furthermore, they are one of the main reasons why people from developed nations who visit developing nations have traveler's diarrhea (Northey *et al*., 2007). ETEC colonizes the gastroint*est*inal tract of the host by adhering to certain receptors on the enterocytes in the int*est*inal lumen by the action of hair-like fimbriae. Colonization factor antigens (CFAs), also known as *Escherichia coli* surface antigens or fimbrial antigens, are more than 20 different kinds of antigens that specify the antigenicity of a particular strain (Isidean *et al*., 2011).

The capacity to express one or more heat-stable (ST) or heat-labile (LT) enterotoxins is a characteristic of ETEC. One of these toxins, called LTI, is plasmid-encoded and mimics the cholera toxin produced by Vibrio cholerae strains. Despite being chromosomally encoded, LTII is identical to LTI in both structure and manner of action. The STa/STI and STb/STII groups of the plasmid-encoded heat-stable enterotoxins (Isidean *et al*., 2011).

2.2.3   ENTEROAGGREGATIVE *Escherichia coli* (EAEC)

EAggEC (sometimes referred to as EAEC) is linked to acute or chronic diarrhea, particularly in underdeveloped nations. A watery, mucoid, diarrheal disease, usually with little to no fever and no vomiting, follows an infection. The description of this *Escherichia coli* pathotype was first put out

towards the end of the 1980s, when research on the interactions between different *Escherichia coli* strains and monolayers of cultivated HEp-2 cells permitted the diversification of enteropathogenic *Escherichia coli* based on their adhesion pattern. While other *Escherichia coli* strains displayed a more diffuse adherence phenotype later diverged into "true Diffuse Adherence" (DA) and "Aggregative Adherence" (AA), the researchers could see that while EPEC displayed the typical Localized Adhesion (LA), other *Escherichia coli* strains displayed a more localized adherence phenotype (Nataro *et al*., 1987). It is unclear exactly how EAggEC produces diarrhea and what impact the different pathogenicity variables play. The unusual "stacked, brick-like" aggregative adhesion of EAggEC strains to tissue culture cells is one of their defining characteristics. Infant foodstuffs and formulae, milk, and water have all been implicated in EAggEC outbreaks (O'Sullivan *et al*., 2007).

2.2.4   DIFFUSELY ADHERENT *Escherichia coli* (DAEC)

Cultures of Hep2 or HeLa cells are called DAEC because of their diffuse adhesion characteristics (Taddei, 2003). DAEC serotypes are known to cause chronic diarrhea in children aged 1 to 5 years (Poole *et al*., 2007). They induce degeneration of the int*est*inal epithelium by binding to proteins that accelerate degradation. Mild diarrhea with white blood cells in the stool is a sign of infection (Taddei, 2003).

2.2.5   ENTEROHAEMORRHAGIC *Escherichia coli* (EHEC)

EHEC is a human disease that was first identified in 1982 as being the cause of haemorrhagic colitis, non-bloody diarrhoea, and hemolytic uremic syndrome (HUS). The primary reservoir for EHEC is the int*est*inal system of cattle, and early outbreaks were linked to eating hamburgers that weren't fully cooked. Sausages, unpasteurized milk, lettuce, cantaloupe melon, apple juice, and radish sprouts, which were to blame for an outbreak of 8,000 cases in Japan, are just a few of the dietary items that have since been linked to sickness (Kaper *et al*., 2004). The key virulence factor for EHEC is *Stx*, which is also known as verocytotoxin (VT). EHEC has also been responsible for multiple outbreaks linked to the petting zoo and farm visits, recreational and municipal drinking water, and person-to-person transmission. According to a recent inv*est*igation, being exposed to a contaminated building could result in airborne transmission (Varma *et al*., 2003).

The 60 megadaltons enterohemorrhagic *Escherichia coli* (EHEC) virulence plasmid secretes verotoxins or Shiga-like toxins. The Shiga-like toxin was given this name because it resembles the

Shigella dysenteriae type 1 toxin in both structure and function and is likewise neutralized by the Shiga toxin antiserum. *Escherichia coli* O157:H7 produces two different forms of Shiga-like toxins: Shiga-like toxin 1 and Shiga-like toxin 2. The two toxins are antigenically distinct, with the Shiga-like toxin 2 producing hemorrhagic colitis in adult rabbits but both toxins are known to be cytotoxic, causing fluid buildup in rabbit ligated ileal loops, paralysis, and death in mice and rabbits (O'Brien and Holmes, 1987).

2.2.6   ENTEROPATHOGENIC *Escherichia coli* (EPEC)

The first pathotype of *Escherichia coli* to be described was EPEC. To distinguish certain *Escherichia coli* serotypes linked to epidemics of infantile diarrhea, the term enteropathogenic *Escherichia coli* (EPEC) was first introduced by Neter *et al*., (1955). It is the oldest known serotype of E. coli that causes diarrhea, and its most crucial characteristic is compliance. Along with watery diarrhea, EPEC infection also causes vomiting and low body temperature (Poole *et al*., 2007). However, EPEC is passed from person to person; Rarely, it is also known to be spread through contaminated food and water (Gerba, 2014). Around Europe and the US, significant outbreaks of EPEC infections with fatality rates of up to 50% occurred often in the middle of the 20th century. Due to higher levels of general cleanliness, these infections are now only sometimes recorded in developed nations, although in low-income nations, this pathotype continues to be one of the most common causes of diarrhoea. In 1987, EPEC serotypes of 12 distinct O serogroups (O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158) were acknowledged by the World Health Organization (Okunbi, 2019).

2.2.6.1   PATHOGENESIS OF ENTEROPATHOGENIC *Escherichia coli* (EPEC)

Historically, the negative traits of EPEC strains, particularly their inability to create enterotoxins or to exhibit Shigella-like invasiveness, were used to define them (Clarke *et al*., 2003). The pathogenesis of EPEC infection occurs in four stages. In the initial stage, bundle-forming pili (Bfp), the intimate adhesin intimin, and short, surface-associated filaments (EspA filaments), all of which are determined by both plasmid and chromosomal genes, are expressed by EPEC cells when the appropriate environmental circumstances are met. The translocated intimin receptor (Tir) and an as of yet unspecified number of effector molecules are directly injected into the host cell by a type III secretion system in the second step, which also involves the attachment of EPEC cells to the epithelial cell via Bfp and EspA filaments. Effector molecules trigger cell-

signaling pathways, altering the host cell's cytoskeleton and causing actin to depolymerize and microvilli to disappear. Protein kinase A and tyrosine-protein kinase both operate to modify Tir before it inserts into the host membrane. The third stage involves the loss of EspA filaments from the bacterial cell surface, intimate attachment caused by the bacterial adhesin intimin binding to the modified Tir, and buildup of actin and other cytoskeletal components below the site of bacterial adherence. The fourth phase results in the construction of the ped*est*al structure that is unique to EPEC as cytoskeletal components have gathered close to the attachment site. The cell processes are disrupted by the effector molecules (translocated from the bacteria), which ultimately results in cell death (Lapointe *et al.,* 2009, Croxen *et al*., 2013).

Table 2.1: Summary of incidence and epidemiology of *E. coli* serotype (Gerry, 2021).

Pathogenic *Escherichia coli*	Site of infection	Associated disease	Incidence	Target population	Significant transmission routes
ETEC	Small intestine	Traveler's diarrhoea, chronic childhood diarrhoea (in developing countries)	16 U.S. outbreaks (1996-2003); Prevalence 1.4% in Patients with diarrhoea 79,420 cases of traveller's diarrhoea each year (in the USA)	International travellers and children in developing countries	Food (raw produce, street vendors) and water
EPEC	Small intestine	Infant diarrhea	Hundreds of thousands of deaths worldwide	Children in developing countries	Water, infant formula
EHEC	Large intestine	Haemorrhagic Colitis (HC), Haemolytic uremic syndrome (HUS)	110,000 cases and 61 deaths annually in the USA	All ages	Food (Beef produce), person-to-person, water, animals
EIEC	Large intestine	Dysentery	Low in developed countries	Children in developing countries	Water(rare), person-to-person
EAEC	intestine	Watery diarrhoea with or without blood in the stool, acute and chronic	Developed and developing countries	Children and adults' travellers	Food, water, person-to-person

2.3 PUBLIC HEALTH HAZARD OF FRESH PRODUCE (LETTUCE)

The term "vegetable" refers to any part of a plant that can be eaten, including the stems and leaves of celery, the roots and tubers of carrots, the bulbs of onions, the leaves of spinach and lettuce, the flowers of globe artichokes, the fruits of apples, cucumbers, pumpkins, strawberries, and tomatoes, and the seeds of beans and peas (Gupta *et al*., 2003). According to research by Garg *et al*. (2010), a variety of vegetables have been epidemiologically related to outbreaks of disease brought on by bacteria, viruses, and parasites. Consumption of vegetable products is frequently considered a potential risk factor for infection with enteropathogens like *Salmonella* and *Escherichia coli*, as the presence of *Escherichia coli* is a sign of fecal contamination (Hanson *et al*., 2012).

Given that fresh produce (lettuce) is a part of the world food system, concerns about its safety are also becoming more widespread. Unfortunately, many of these fresh produce (lettuce) stores are located next to trash bins. (Mritunjay and Kumar 2015). The microbiological safety of vegetable products is receiving more attention as the intake of vegetables grows due to their health advantages (Callejón *et al*., 2015). Vegetable produce, especially lettuce, is of particular concern because they are either eaten raw or just a little processed without a "kill-step" or deactivation stage. Having a diet rich in nutrients and is widely acknowledged as being beneficial (Berger *et al.,* 2010). In Nigeria, the consumption of ready-to-eat lettuce has significantly increased due to its established health and nutritional advantages. Consuming raw or hardly processed lettuce has been linked to disease since research has shown that bacterial outbreaks are primarily caused by *Escherichia coli* (Burnett and Beuchat 2000; Olaimat and Holley, 2012).

When you consume contaminated food, microorganisms infect your dig*est*ive system and cause food-borne illnesses. These microorganisms need to be viewed under a microscope since they are too small to see without one. Since contamination only impacts a small fraction of the crop, it is difficult to detect. Visual contamination detection is difficult because bacteria are hard to see. Lettuce has rough surfaces, which leads to stem scars where the reduced area creates a location for bacteria to hide. (DuPont and LaBorde, 2015). Each year, 125,000 people die from food-borne illnesses, which affect 40% of children under the age of five. (WHO 2020).

2.4   LETTUCE AS PART OF A HEALTHY DIET

Around the world, lettuce (Lactuca sativa L.) is a widely cultivated and commonly consumed vegetable. China is the world's great*est* producer of lettuce. Lettuce is frequently used in salad mixes, and the popularity of salads is rising. So, according to Kenny and O'Beirne (2009), lettuce may greatly increase the nutritional value of meals. When opposed to other vegetables that are cooked or processed, such as potatoes, lettuce is often consumed fresh, which preserves more nutrients. Lettuce has a minimal calorie, fat, and salt content. It is a good source of fiber, iron, folate, and vitamin C. Additionally, lettuce is a wonderful source of many other bioactive chemicals that have positive health effects. Studies conducted in both vitro and in vivo have demonstrated that the bioactive substances in lettuce have anti-inflammatory, chol*est*erol-lowering, and anti-diabetic properties. However, there are differences in the nutrient makeup and bioactive substances of various lettuce varieties. The most popular lettuce in the US, crisphead lettuce, has a relatively low concentration of vitamins, minerals, and bioactive substances. Leaf-type lettuce and romaine are more nutrient-dense lettuces with folate levels equivalent to other rich leafy vegetable sources. Green lettuce has fewer phenolic chemicals than red-colored lettuce. The nutritional value of baby lettuce was also looked at due to its rising popularity (Kim *et al*., 2016).

2.5   OUTBREAKS ASSOCIATED WITH THE CONSUMPTION OF LETTUCE

In the United States between 1973 and 1997, lettuce (Lactuca sativa) was one of the fresh produce items most frequently linked to outbreaks of food-borne disease (Sivapalasingam *et al*., 2004). The majority of outbreaks linked to this product and other leafy greens are caused by *Escherichia coli* O157:H7 bacterium (Brandl, 2006; Rangel *et al*., 2005; Sivapalasingam *et al*., 2004). 20 outbreaks and 634 cases of *Escherichia coli* O157:H7 illness were linked to lettuce alone from 1998 to 2005, according to the U.S. Centers for Disease Control and Prevention (Lynch, 2007).

Table 2.2 Outbreaks linked with consumption of lettuce (FDA, 2022; Carstens *et al.*, 2001)

Date	Vehicle	Pathogen	Confirmed cases	States
May 2008 October 2008 November 2008	Romaine lettuce Lettuce Lettuce	*E. coli* O157:H7 *E. coli* O157:H7 *E. coli* O157:H7	9 59 130	United states Multistate and Canada Canada
September 2009	Lettuce	*E. coli* O157:H7	39	Multistate
April 2010	Romaine lettuce	*E. coli* O145	33	Multistate
October 2011	Romaine lettuce	*E. coli* O157:H7	60	Multistate
April 2012 June 2012	Romaine lettuce Romaine lettuce	*E. coli* O157:H7 *E. coli* O157:H7	28 52	Canada Multistate
April 2013	Leafy greens	*E. coli* O157:H7	14	Multistate
April 2014	Romaine lettuce	*E. coli* O126	4	United states
April 2015	Leafy greens	*E. coli* O145	7	Multistate
2016	Lettuce	*E. coli* O157:H7	11	United states
December 2017	Romaine lettuce	*E. coli* O157:H7	2	Canada
November 2018	Romaine lettuce	*E. coli* O157:H7	43	Canada

2. 6 POTENTIAL SOURCE OF LETTUCE CONTAMINATION

Fresh produce cultivation is open, which makes it susceptible to contamination from several sources. This is because each farm has its particular collection of environmental risk factors, such as topography, relationships between different land uses, and climate. A combination of these distinct environmental risk factors affects the frequency and spread of foodborne pathogens as well as the risk of produce contamination (Strawn *et al*., 2013).

2. 6. 1 ROUTES OF CONTAMINATION IN LETTUCE AT PREHARV*EST*

At the preharv*est* stage, several sources of *Escherichia coli* contamination have been discovered, including animals and insects, manure and soil supplemented by manure, water, seeds, and dust.

Manure and manure-amended soil

Escherichia coli O157:H7 can persist for a long time in soil (Islam *et al*., 2004). Lettuce plants can develop infected edible parts when cultivated on soil with polluted manure. Because lettuce's outer leaves are more likely to be exposed to the elements and come into contact with contaminated soil (Oliveira *et al*., 2012). In contrast to the inner leaves of romaine lettuce, Escherichia coli O157:H7 moves from soil supplemented with infected compost primarily to the outside leaves. Depending on the kind of soil, humidity, and temperature, Escherichia coli O157:H7 can persist in the soil for 7 to 25 weeks (Solomon *et al*., 2002).

Because of the advantages liv*est*ock manure offers, it has been used as fertilizer in agriculture for hundreds of years. These advantages include improving soil physical properties (aggregation) so that it can hold more nutrients and water and become more fertile, encouraging soil microbial activity, which helps the soil's supply of trace minerals, and improving plant nutrition. Producing liv*est*ock dung is also less expensive than chemical fertilizers, which can be up to four times as expensive as manure (Gagliardi and Karn, 2002). According to *est*imates, the well-*est*ablished poultry and cattle industries in Nigeria produce about 932,5 metric tons (MT) of manure annually, and the use of bovine manure (cow dung) as fertilizer, either fresh, untreated, or after composting, is common (Adejinmi, 2000). The human int*est*inal bacteria in the soil are introduced to agricultural soils by animal waste and irrigation with sewage water. Amoah *et al*., (2007)

discovered unacceptably high levels of fecal coliforms in lettuce grown in two major areas in Ghana. The presence of the same enterohemorrhagic *E. coli* strains in edible vegetable sections has been confirmed. Although experimental comparison with commensal, non-pathogenic *E. coli* failed to find any traits that would give the serotype such an advantage, these observations indicate that *E. coli* O157:H7 is an ecologically fit microorganism with superior survival and growth capability in non-pathogenic environments (Chung *et al.,* 2009).

Irrigation water

One of the main causes of lettuce contamination is considered to be contaminated water (Zhang *et al*., 2018). *Escherichia coli* O157:H7 outbreaks connected to lettuce have been attributed to contaminated irrigation water as a source. In Sweden, a large number of Escherichia coli O157 (H-antigen not identified) illnesses associated with iceberg lettuce have been linked to the use of contaminated irrigation water from a small creek (Leifert et al., 2008). The most likely source of contamination for the Escherichia coli O157:H7 disease outbreaks linked to romaine lettuce in Canada and the United States in spring 2018 and autumn 2018 was irrigation water. But there is a chance of groundwater pollution, especially in areas with a lot of livestock output and/or manure application to the land (Jamieson et al., 2002). From safest to least safe, the likelihood of pathogen contamination, such as Escherichia coli O157:H7, often increases (FAO, 2008).

Animals and insects

Animals both dom*est*ic and wild can excrete *Escherichia coli* O157:H7 in their feces (Langholz *et al*., 2013). When animals enter the field and drop faeces on the leafy greens, this can directly cause contamination of the leafy greens or indirectly through pollution of the soil and water. Ruminant animals, including cattle, are recognized *Escherichia coli* O157:H7 reservoirs (Hancock *et al*., 2001). In California, *Escherichia coli* O157:H7 was found in 2.7% and 2.6%, respectively, of the cow fecal samples taken from 8 and 20 cattle ranches (Benjamin *et al*., 2015). The spread of *Escherichia coli* O157:H7 may potentially include insects and animals. *Escherichia coli* may be spread to a clean surface by flies carrying it on their body surfaces. P*est* flies can transport *Escherichia coli* O157:H7 up to 180 meters, according to research on fields of leafy greens close to a cattle feedlot (Berry *et al*.,2019).

Seeds

Pathogens can be present on plants like leafy greens due to contaminated seeds and seedlings, however, when seeds are grown under controlled circumstances, the likelihood of contamination is thought to be low (FAO 2008). The bacteria *Escherichia coli* O157:H7 may adhere to lettuce seedlings and endure for a very long period (Gagliardi and Karn, 2002).

2.6.2   ROUTES OF CONTAMINATION IN LETTUCE AT POST HARV*EST*

Lettuce can also be contaminated during post-harv*est* through various routes.

Humans

In several foodborne disease outbreaks linked to leafy greens, food handlers have been thought to be the source of contamination. Fecal-oral transmission of *Escherichia coli* O157:H7 by infected individuals is possible. When lettuce is being harv*est*ed, contamination may happen if the employees handling the crop don't follow proper hygiene procedures. Several stages of lettuce production, such as harv*est*, removing the outer leaves, coring, and packaging, might include direct human interaction (FAO 2008). The home, retail stores, and r*est*aurants are other places where lettuce contamination can occur *Escherichia coli* O157:H7 can spread to lettuce from sources like uncooked ground beef when it is not handled correctly. This can happen when infected hands and/or cutting boards are used to handle the meal (Wachtel *et al*., 2003).

Equipment

It has been shown that *Escherichia coli* can be easily transferred to and persist on various surfaces used in lettuce production such as shredder, flume and tank, shaker table and then contaminate a significant amount of lettuce that are not contaminated during processing. Equipment and machinery may potentially contribute to the infection of lettuce with *Escherichia coli* O157:H7. Processed lettuce was infected when field trimming and coring tools were contaminated with *Escherichia coli* O157:H7 (Taormina *et al*., 2009). In one research, at least 19 lettuce heads were infected with *Escherichia coli* O157:H7 with a single contaminated coring knife (McEvoy *et al*., 2009).

2.6.3 SURVIVAL AND PERSISTENCE OF *ESCHERICHIA COLI* IN SOIL

The ability of pathogens to survive in environments other than the animal host has been connected to their capacity to colonize lettuce crops. The persistence and population dynamics of human diseases in extraint*est*inal settings, particularly soil, have been the subject of numerous research. Early studies by Islam *et al*. (Islam *et al*., 2004) showed that *E. coli* O157:H7 survived in soils modified with infected compost for more than 200 days. *E. coli* O157:H7 survived for 211 and 332 days, respectively, in two independent inv*est*igations utilizing soils treated with manure (Franz *et al*., 2011; You *et al*., 2006). The life periods of these pathogens in soil, however, were observed in other research to be shorter (Bolton *et al*., 2011). These variations in results have been linked to various experimental setups, inoculation techniques, and microbial quantification methodologies. Additionally, a number of variables, such as the edaphic characteristics of the soil, the weather, interactions with other microbes, and physiological variations across bacterial strains, may have an impact on the survival of e. coli in soils. For instance, Franz *et al*., (2011) discovered that *E. coli* O157:H7 lifespan in soil differed across various strains and depended on the bacterial origin and their oxidative capacities. O157:H7 isolates from humans have a longer survival rate than those from animals. Additionally, compared to strains that only lasted a short time, strains that lasted longer oxidized propionic acid, -ketobutyric acid, and -hydroxybutyric acid at higher rates. *E. coli* depends critically on their environment to survive. The frequency of isolates and the prevalence of e. coli in lettuce fields were both impacted by a number of external factors (Strawn *et al*., 2013). The likelihood of isolating e. coli from lettuce fields was raised by the altitude, wetness, and access to water sources of the soil. This was in line with the results of a prior inv*est*igation, which showed that E. coli O157:H7 survived longer in wet soil than in dry soil (Ohtomo *et al*., 2004).
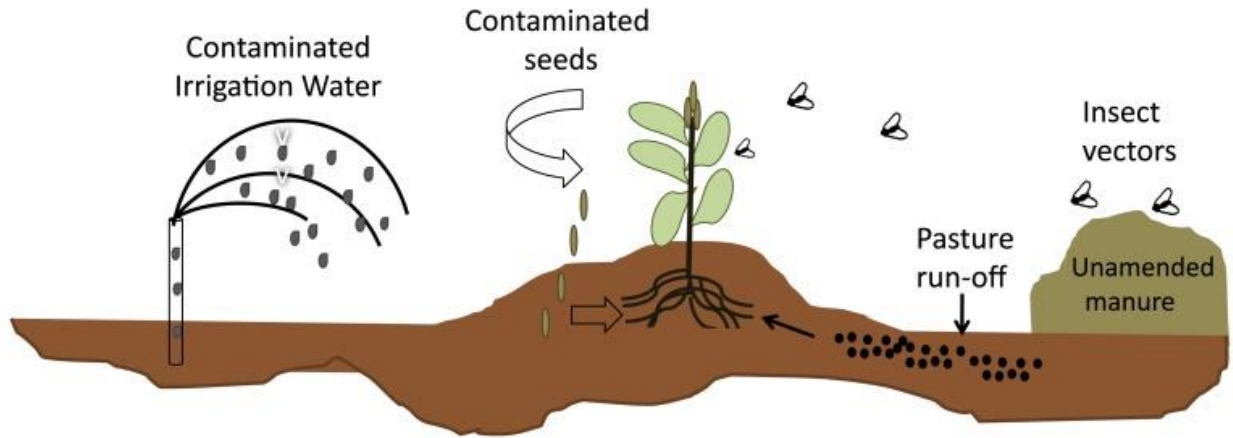
Figure 2.2: *Escherichia coli* pathogens in lettuce: routes of contamination. Representation of the conditions that can cause the contamination of lettuce with Enterobacteria in the pre-harvest environment (Betsy *et al*., 2014).

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.1 SAMPLE COLLECTION

In this study, a total number of 12 soil samples were collected from Ojo-Iba local government, Lagos state farmland between the 13th of May to 24th of May 2022 in Lagos. All samples were packed in separate sterile plastic bags in ice pack and taken immediately to the laboratory for further microbiological examination.

3.2 MATERIALS AND EQUIPMENT

The following materials and equipment were used; Petri-dishes, beakers, conical flasks, glass spreader, 70% ethanol, cryo tubes, micropipette (with their tips), test tubes (with their racks), PCR tubes, autoclave, weighing balance, incubator (set at 37°C), Bunsen burner, inoculating loop, spatula, cotton wool, hockey stick, 90% ethanol, aluminum foil, measuring cylinder, water bottle, distiller, gel electrophoresis tank, gel documentation, heating block, vortex mixer, centrifuge.

3.3 CULTURE MEDIA USED

Media used for *Escherichia coli*; Nutrient Agar, MacConkey Agar, and Sorbitol- MacConkey Agar (SMAC), Buffer peptone water (0.1 and 1%), brain heart infusion broth (BHI), distilled water, 20% glycerol.

3. 3.1 BUFFER PEPTONE WATER (BPW)

NaCl and the peptic dig*est* of animal tissue are the main ingredients of peptone water, a substrate for microbial development. The medium is tryptophan-rich and naturally alkaline at a temperature of 25 °C. The nonselective A broth medium peptone water can also be employed as the main enrichment medium for bacterial growth.

Preparation

1. In a conical flask, the dehydrated medium was well mixed after being dissolved in 1 litre of distilled water to create 0.1% and 1% peptone water following the manufacturer's instructions. Finally, a cotton wool swab covered in aluminum foil is used to seal the conical flask.

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

3. 9ml of the 0.1% was then dispensed into various test tubes for serial dilution.

3.3.2 SORBITOL MacConkey AGAR(SMAC)

It's for the isolation and detection of *Escherichia coli* O157:H7, sorbitol MacConkey agar was prepared following the manufacturer's instructions.

Preparation

1. The dehydrated medium was dissolved in the appropriate volume of distilled water that is 51.5g of SMAC in 1000 ml distilled water based on the manufacturer's instructions in a conical flask and mixed thoroughly. The conical flask is then closed with 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 15 minutes.

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.

Note: This medium is reddish-purple.

3.3.3 NUTRIENT AGAR

Nutrient agar was prepared according to the manufacturer's instruction for isolation and detection of the total count of mesophilic organism.

Preparation

1. The dehydrated medium was dissolved in the appropriate volume of distilled water i.e., 28g of Nutrient agar in 1 litre of distilled water based on manufacturer's instructions in a conical flask and mixed thoroughly. The conical flask is then closed in cotton wool that is wrapped in aluminium 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.

Note: The medium appears opalescent and is light amber in colour.

3.3.4 BRAIN HEART INFUSION (BHI) BROTH

BHI is recommended for the cultivation of fastidious pathogenic microorganisms.

1. The dehydrated medium was dissolved in the appropriate volume of distilled water based on manufacturer's instructions in a conical flask and mixed. The conical flask was then corked with a foil cork.

2. The mixture was stirred for a while using the magnetic stirrer to completely dissolve the powder.

3. 5ml of the media was then dispensed into various t*est* tubes and covered with foil cork and was then sterilized by autoclaving at 121°C for 15minutes.

3.3.5 MacConkey AGAR

MacConkey agar is the type of agar medium that is most frequently used to isolate *Escherichia coli*. This selective media prevents the growth of other gram-positive bacteria by containing lactose as sugar, peptone, sodium chloride, and bile salt. To comprehend the nature of fermentation, other colors including neutral red, crystal violet, and Enterobacteriaceae are also used. MacConkey agar should be used to find and isolate gram-negative organisms in clinical, dairy, food, water, pharmaceutical, and industrial source samples. The sources of nitrogen and vitamins in MacConkey Agar are the enzymatic dig*est*ion of gelatin, casein, and animal tissue.

Preparation
1. The prepared medium was suspended in the appropriate volume of distilled water i.e. 48.5g of MacConkey in 1 litre of distilled water based on the manufacturer's instructions in a conical flask and mixed thoroughly. The conical flask is then closed in cotton wool that is wrapped in aluminium 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 Agar was then allowed to cool to a range of 45-50°C and poured aseptically into sterile Petri dishes and left to solidify.

NOTE: The medium is neutral red in colour.

3.4 ISOLATION AND IDENTIFICATION OF *ESCHERICHIA COLI*

3. 4.1 SAMPLE PREPARATION

Exactly 1g of the soil samples were weighed and poured into a sterile stomacher bag containing 9ml of 1% BPW (enrichment broth) and then homogenized using the stomacher at 180 rpm for 2 minutes after which serial dilutions were performed and appropriate dilutions were plated on SMAC and MacConkey agar plates.

3. 4.2 SERIAL DILUTIONS

One milliliter (1ml) of the samples (soil) was pipetted using the micro-pipette (set at 1000ul) into the t*est* tubes containing 9ml of BPW (0.1%) to obtain 10^{-2}, followed by the transfer of 1ml from 10^{-2} into a new t*est* tube (containing 9ml of BPW) to create 10^{-3} dilution, the t*est* tubes are then put in the vortex mixer for even mixing. The dilution factor was repeated for 10^{-3} to 10^{-5}. The t*est* tubes were labeled for easy identification.

3.4.3 PLATING

After labelling the agar plates accordingly, 0.1 ml from the appropriate dilutions (10^{-3} and 10^{-5}), was plated onto SMAC Agar and MAC Agar, for the isolation of enteropathogenic *E. coli*, and the Total viable count using the spread plate technique (the glass rod was dipped into alcohol and then flamed in the Bunsen burner before spreading to maintain aseptic conditions). The plates were incubated at 35℃- 37℃ for 18- 24 hours and counted.

3.4.4 SUBCULTURING

Subculturing is done to purify the isolated bacterial colonies from a mixed culture to a new and single culture, the bacterial isolates transferred or sub-cultured were those which were differentiated based on their colony morphology, shape, color, elevation and other physical characteristics. The colonies gotten from the previously incubated SMAC plates (white and pink) were subcultured into Nutrient agar.

A loopful of the isolate was taken using the inoculating loop (the inoculating loop is heated using the Bunsen burner and allowed to cool before taking the loop from the original mixed culture and streaked onto the new petri-dish). For sub-culturing, the isolate containing loop is transferred to

the new petri-dish using the streaking method procedure. The plates were inverted and incubated at 37°C for 18- 24 hours.

3.4.5   CRYOPRESERVATION OF ISOLATE

A loopful of each isolate (two white, two pink) from the incubated nutrient agar was inoculated into 5ml of BHI broth each in a t*est* tube and incubated at 37°C for 18- 24 hours. After incubating, 750ul of the inoculum was added into a sterile Eppendorf tube containing 750ul of sterile 20% glycerol (duplicated) which acts as a cryoprotectant and was stored in a freezer at -4 ℃.

3.5   BIOCHEMICAL IDENTIFICATION

Biochemical t*est*s were performed with pink and white producing isolates according to the methods described in Bergey's Manual of bacteriology. The following biochemical t*est* was carried out for the identification of bacterial isolates; Catalase t*est*, indole t*est*, and oxidase t*est*.

3.5.1   GRAM STAINING

On a clean, grease-free slide, a smear of suspension was created with a loopful of the isolate. It was air dried and heat fixed. Drops of crystal Violet were poured and kept for about 30 seconds and rinsed with water. It was then flooded with gram's iodine for 1 minute and rinsed with water. 95% alcohol was added for about 10-20 seconds and rinsed with water. Safranin was added for about 1 minute and rinsed with water. It was then air dried and Observed under a Microscope.

3.5.2   CATALASE T*EST*

Put the microscope slide in the Petri dish. Keep the Petri dish's lid handy. Collect a small bit of the isolate and put it on a microscope slide using a clean inoculation loop or wooden applicator. Take care not to gather any agar. Place one drop of 3% H2O2 using an eyedropper on the spacer on the microscope slide. Don't mix things up. As soon as bubbles start to appear (O2 + water = bubbles), immediately place a lid on the petri dish to contain the aerosol. Observing bubble formation on a dark background can improve readability.

3.5.3   OXIDASE T*EST*

Dry a small piece of filter paper after soaking it in 1% Kovács Oxidase Reagent. Pick a well-separated colony from a brand-new bacterial plate (18–24 hours of incubation) using a ring, and then rub it on the filter paper that has been treated. Observe how the color changes. The

microorganism is oxidase positive when the color turns deep purple in 5 to 10 seconds. The microorganisms postpone the oxidase positive when the color changes to purple between 60 to 90 seconds. The bacterium is oxidase negative if the color is the same or persists for more than two minutes.

3.6   MOLECULAR IDENTIFICATION

3.6.1 ACTIVATION OF ISOLATES

The autoclave was used to sterilize the 1ml of pure BHI in 2ml Eppendorf tubes for 15 minutes at 121°C. After being given time to cool, 100 l of each thawed stock culture was put into several Eppendorf tubes containing sterile BHI. The cultures were then cultured for 48 hours at 37 °C.

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 A 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 15minutes, the boiled DNA was 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 95 for 15mins, followed by 35 cycles of 94°C for 50s, 57°C for 40s and 72°C for 50s, and finally 72°C for 3 min 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	*Vtx1f*	20 µm	0.25	0.125	2.5
5	*Vtx1R*	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 µm	1.5	0.6	12
11	dH$_2$0		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$_2$0	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	*Vtx1f*	20 µm	0.25	0.125	7.375
5	*Vtx1R*	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 µm	1.5	0.6	35.4
11	dH_2O		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$_2$0	20 µm		4.3	253.7
10	DNA	20 µm			

Table 3.5: Protocol for Thermocycler

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

Table 3.6: Primers used for multiplex PCRs (Persson *et al*., 2007)

Gene target	Virulence factor	Sequence (5´)	Final concentration (μM)	Amplicon size (bp)
Human *est A*	STIh	TTTCGCTCAGGATGCTAAACCAG CAGGATTACAACACAATTCACAGCAGTA	0.4 0.4	151
Porcine *est A*	STIp	CTTTCCCCTCTTTTAGTCAGTCAACTG CAGGATTACAACAAAGTTCACAGCAG	0.4 0.4	160
Vtx1	VT1	GTTTGCAGTTGATGTCAGAGGGA CAACGAATGGCGATTTATCTGC	0.25 0.25	260
eae	Intimin	GGYCAGCGTTTTTTCCTTCCTG TCGTCACCARAGGAATCGGAG	0.15 0.15	377
Vtx2	VT2	GCCTGTCGCCAGTTATCTGACA GGAATGCAAATCAGTCGTCACTC	0. 5 0. 5	420
eltA	LTI	AAACCGGCTTTGTCAGATATGATGA TGTGCTCAGATTCTGGGTCTCCT	0.45 0.45	479
ipaH	IpaH	TTGACCGCCTTTCCGATACC ATCCGCATCACCGCTCAGAC	0.1 0.1	647

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 RESULTS

The microbial analysis of the samples gotten from Ojo-Iba local government, Lagos state. The microbial analysis was carried out for total viable counts, general *Escherichia coli*, and pathogenic *Escherichia coli*. The results of the findings were summarized in the Table 4.1 and Table 4.2 showing the morphological characteristics of samples cultured on Sorbitol MacConkey agar and MacConkey agar using the spread plate method with bacterial isolates collected from soil sampling in Ojo-Iba, Lagos state. All the samples had pink and white, raised, circular and smooth colonies on selective media SMAC and MAC. While in the Table 4.3, the results of the biochemical characterization of the twelve (12) isolates using catalase and oxidase *test*s alongside Gram stain results from using the spread plate method with bacterial isolates collected from soil sampling from Ojo-Iba, Lagos state.

Based on the biochemical tests results in Table 4.3. In the selective media used for identification, isolates were suspected to be *Escherichia coli.* All the isolates are Gram negative, oxidase negative, and catalase positive.

4.2 GENOTYPIC CHARACTERIZATIONS OF THE ISOLATES

Template DNA was prepared from cellular DNA of biochemically identified isolate by boiling method and 5μl of template DNA was subjected to PCR for the detection of *E. coli* specific virulent genes using specific primers. Isolates that gave bands of expected size were suspected to carry these genes in their chromosomes.

For PCR multiplex

The 12 presumptive *Escherichia coli* isolates were further examined by multiplex PCR for the detection of *vtx1*, and/or *vtx2, eltA, ipaH, eae*, Human *est A* and Porcine *est A* genes. The result revealed that one isolate from soil purchased from Ojo-Iba contained Human *est A* and Porcine *est A* gene, which makes it Enterotoxigenic *E. coli* positive. Agarose gel electrophoresis of PCR products for *eae, vtx1*and/or *vtx2, ipaH, Human est A, Porcine est A, and eltA* as shown in **Plate 4.2**

Table 4.1: Morphological characteristics of isolates on Sorbitol MacConkey agar

Isolate	Sample	Isolate ID	Color	Number of growths	Shape	Size	Elevation	Surface	Opacity	Margin
1st sampling	Soil	S1L1	White and pink	TNTC	Circular	Small	Low convex	Smooth	Translucent	Entire
		S1L2	White	TNTC	Circular	Small	Low convex	Smooth	Translucent	Entire
		S1L3	Pink and white	TNTC	Circular	Small	Low convex	Smooth	Translucent	Entire
2nd sampling	Soil	S2L4	Pink and white	TNTC	Circular	Small	Low convex	Smooth	Translucent	Entire
		S2L5	Pink and white	TNTC	Circular	Small	Low convex	Smooth	Translucent	Entire
		S2L6	White	TNTC	Circular	Small	Low convex	Smooth	Translucent	Entire
3rd sampling	Soil	S3L7	Pink and white	4	Circular	Small	Low convex	Smooth	Translucent	Entire
		S3L8	Pink and white	TNTC	Circular	Small	Low convex	Smooth	Translucent	Entire
		S3L9	Pink and white	97	Circular	Small	Low convex	Smooth	Translucent	Entire
4th sampling	Soil	S4L10	White	2	Circular	Small	Raised	Smooth	Translucent	Entire
		S4L11	Pink and white	84	Circular	Small	Low convex	Smooth	Translucent	Entire
		S4L12	Pink and white	19	Circular	Small	Low convex	Smooth	Translucent	Entire

Table 4.2: Morphological characteristics of isolates on MacConkey agar

Isolate	Sample	Isolate ID	Color	Number of growths	Shape	Size	Elevation	surface	Opacity	Margin
1st sampling	Soil	S1L1 SIL2 S1L3	White and pink	TNTC	Circular	Small	Low convex	Smooth	Translucent	Entire
2nd sampling	Soil	S2L4 S2L5 S2L6	White and pink	TNTC	Circular	Small	Low convex	Smooth	Translucent	Entire
3rd sampling	Soil	S3L7 S3L8 S3L9	White White and pink White and pink	TNTC	Circular	Small	Low convex Raised Low convex	Smooth	Translucent	Entire
4th sampling	Soil	S4L10 S4L11 S4L12	White and pink White and pink white	TNTC	Circular	punctiform	Low convex	Smooth	Translucent	Entire

Table 4.3: Biochemical t*est* performed on isolates

S/N	Code	Gram staining	Catalase	Oxidase
1	S1L1	Negative (-)	Positive (+)	Negative (-)
2	S1L2	Negative (-)	Positive (+)	Negative (-)
3	S1L3	Negative (-)	Positive (+)	Negative (-)
4	S2L4	Negative (-)	Positive (+)	Negative (-)
5	S2L5	Negative (-)	Positive (+)	Negative (-)
6	S2L6	Negative (-)	Positive (+)	Negative (-)
7	S3L7	Negative (-)	Positive (+)	Negative (-)
8	S3L8	Negative (-)	Positive (+)	Negative (-)
9	S3L9	Negative (-)	Positive (+)	Negative (-)
10	S3L10	Negative (-)	Positive (+)	Negative (-)
11	S3L11	Negative (-)	Positive (+)	Negative (-)
12	S3L12	Negative (-)	Positive (+)	Negative (-)

Plate 4.1: Sorbitol MacConkey agar plate showing growth of *Escherichia coli*
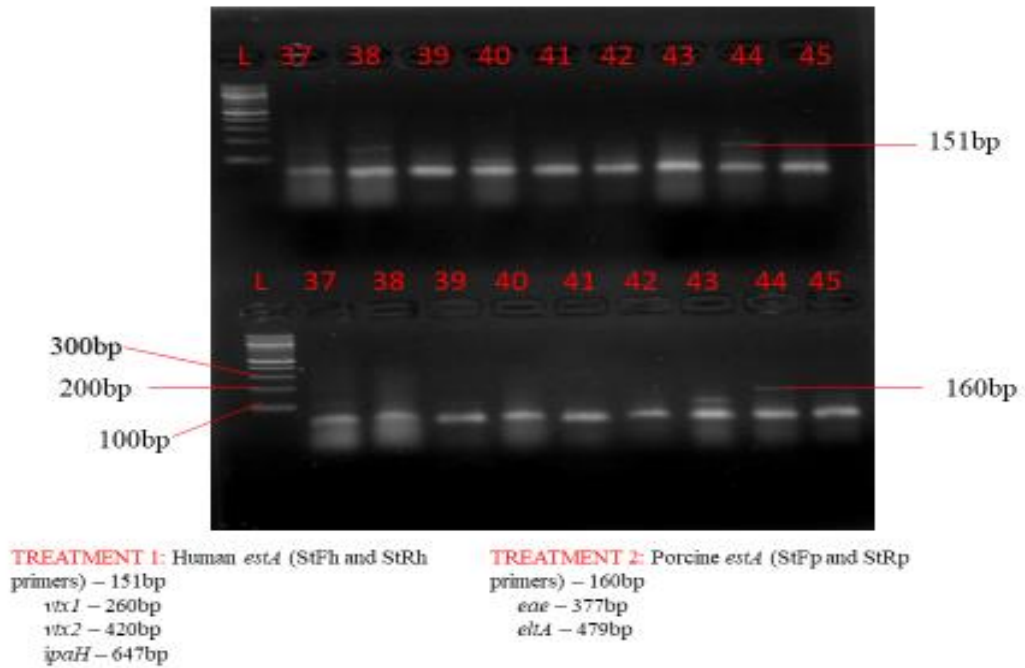
Plate 4.2: Agarose gel electrophoresis multiplex PCR for E. coli pathotypes

Lanes L=DNA Ladder, Lane 37-45 fragments of isolates from soil samples. 44 has StFh and StRh (151bp), StFp and StRp (160bp) genes.

4. 3 DISCUSSION

In this study, out of all the 12 *Escherichia coli* isolates collected from Ojo-Iba area, one (S4L12) was tested positive for the Human *est A* (Treatment 1) and Porcine *est A* gene (Treatment 2), indicating that the soil was contaminated with pathogenic *Escherichia coli*. *Escherichia coli* are pervasive, or present almost everywhere in nature. They can contaminate food through these channels and can be found in soil. It was also observed that good agricultural practices were not carried out at Ojo-Iba area and farmers are given contracts to grow and harvest a targeted amount of lettuce. The sample, S4L12 which is reported in figure 4.2, lane 44 produced bands within the range of 100bp-200bp. The band produced is mostly consistent with the Human *est A* and Porcine *est A* gene which is 151bp (Treatment 1) and 160bp (Treatment) long, a gene associated with the pathotype ETEC (Persson *et al.,* 2007).

Data on the prevalence of *E. coli* in soil from earlier studies conducted in numerous nations have been widely varied. Additionally, the strain must be isolated to confirm the presence of Human *est A* and Porcine *est A* genes in addition to relevant virulence factors in the same live cell while excluding the presence of free DNA phages in the enrichment culture because the presence of virulence genes presumptively determines the presence of bacteria in the case of ETEC (EFSA, 2013). This step may delay identification because it is challenging to design culture media that specifically or differentially permit the formation of ETEC, but it is required because molecular techniques may exaggerate the genuine ETEC contamination (EFSA, 201M).

E. coli O157:H7 counts in agricultural soil are not well known. Unfortunately, because agricultural soil is naturally exposed to direct and indirect sources of microbial contaminants like animal dung, runoff, contaminated irrigation water, free-ranging animals, municipal sewage, and effluents, it is almost unavoidable that it will contain pathogens like *E. coli* O157:H7 (Alegbeleye *et al*., 2018; Iwu and Okoh 2019). Again, our findings are not entirely unexpected given that the majority of the sampling locations employed inadequately composted animal dung of animal origin to amend the soil during the primary production of fresh produce. This demonstrates that the presence of organic materials promotes the survival and persistence of *E. coli* O157:H7 in the soil. This is demonstrated in the study of Gagliardi and Karns, who found that *E. coli* O157:H7 persisted in fallow soil for 21 to 45 days after dairy manure treatment. Before being applied to soil, animal-based manure must be properly treated (Gagliardi and Karns, 2002) Despite the fact that

competition with soil microorganisms and unfavorable environmental factors might affect pathogen survival, little is known about how long E. coli O157:H7 cells can survive in manure-amended soils (Jiang *et al*., 2002). it was observed during sample collection that the irrigation water used on the farm was from a well dug by the farmers close to the farm. Also, refuse dump was identified close to the farm. The presence of this refuse dump is a fundamental factor to the likely presence of pathogenic *E. coli* in the soil samples. It can be inferred that the probability that this contamination occurred as a result of the close proximity to a refuse dump is very high. Further interaction with the farmers also led to the belief that the manure used could also be a source of contamination. They stated that cow dung was applied to the farm. It is possible that the manure used was not treated properly causing soil contamination.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

The agricultural soil samples collected from Ojo-Iba area of Lagos state harbour *Escherichia coli*, which could be transferred to fresh Produce (Lettuce) intended for human consumption. Some of the isolates harboured either Human *est A* and Porcine *est A* gene making them Enterotoxigenic *Esherichia coli* (ETEC) and highly virulent, thus capable of causing severe forms of infection in human beings.

5.2 RECOMMENDATION

In this study, the incidence of *E. coli* O157:H7 on the farm will be reduced through better cattle management techniques, fencing around farms and irrigation water sources to keep ruminants out, as well as proper treatment of manure from ruminants and irrigation water before it is applied on soil for planting.

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