ISOLATION AND MOLECULAR CHARACTERIZATION OF *Escherichia coli* PATHOTYPES FROM LETTUCE PLANTS ISOLATED FROM OGUDU, LAGOS STATE, NIGERIA

BY

AJIDE, ESTHER OLUWAPELUMI

18010101033

A PROJECT SUBMITTED TO THE DEPARTMENT OF BIOLOGICAL SCIENCES, COLLEGE OF BASIC AND APPLIED SCIENCES, MOUNTAIN TOP UNIVERSITY, MAKOGI, IBAFO, OGUN STATE,

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IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF A BACHELOR OF SCIENCE DEGREE (B.Sc. Hons.) IN MICROBIOLOGY.

SEPTEMBER, 2022.

DECLARATION

I hereby declare that this project written under the supervision of Dr. M.A Abiala is a product of my own research work. Information derived from various sources has been duly acknowledged in the text and a list of references provided. This project has not been previously presented anywhere for the award of any degree or certificate.

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AJIDE, ESTHER OLUWAPELUMI

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DATE

CERTIFICATION

This is to certify that this project titled "**Isolation and Molecular Characterization of *Escherichia coli* Pathotypes from Lettuce Plants Isolated from Ogudu, Lagos State**" was carried out by **AJIDE, ESTHER OLUWAPELUMI**, with matriculation number 18010101033. This project meets the requirements governing the award of Bachelor of Science (B.Sc.) Degree in Microbiology, Department of Biological sciences of Mountain Top University, Ogun State, Nigeria and is approved for its contribution to knowledge and literary presentation.

__________________________________ (Signature and Date)

DR. M. A. ABIALA

(Project Supervisor)

__________________________________ (Signature and Date)

DR. (MRS) C. I. AYOLABI

(Ag. Head of Department)

DEDICATION

I dedicate this seminar report to the Almighty God, my wonderful family and my friends.

ACKNOWLEDGEMENT

I Appreciate the Almighty God for strengthening me, for directing my path, for his continuous guidance, and for giving me the grace to complete this project.

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ABBREVIATIONS

A/E	Attaching and effacing
BHI	Brain heart infusion
BPW	Buffered peptone water
ETEC	Enterotoxigenic *Escherichia coli*
DAEC	Diffusely adherent *Escherichia coli*
EAEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
HGT	Horizontal gene transfer
MAC	MacConkey agar
NA	Nutrient agar
PAA	Plasmid of aggregative adherence
PAI	Pathogenicity Island
PCR	Polymerase chain reaction
SMAC	Sorbitol MacConkey agar
STEC	Shiga toxigenic *Escherichia coli*
STX	Shiga toxin
VT	Verocytotoxin
VTEC	Verotoxigenic *Escherichia coli*

ABSTRACT

Recently, there has been an increase in the consumption of vegetables such as lettuce worldwide in a bid to maintain a healthy diet. However, along with this increase is an increase in reports of foodborne outbreaks. The common pathogen associated with these outbreaks reported is the bacteria, *Escherichia coli.* Consumption of lettuce contaminated with this pathogenic *E. coli* could result in several manifestations of Diarrhea and in severe cases Hemorrhagic colitis. Research has been ongoing in the isolation and characterization of this pathogen in lettuce samples and samples from infected patients in various parts of the world. Nevertheless, there has not been notable research done in the isolation and characterization of this pathogen in Africa, specifically, Nigeria considering the severity of infections caused by it. Data has also shown that most cases reported in Nigeria have not been related to the ingestion of food products from non-bovine origin such as lettuce. As a result, this study was aimed at the isolation and characterization of *E. coli* pathotypes from a farm in Ogudu, Lagos state, Nigeria. First, the morphological characteristics of the isolates were observed on MacConkey agar and Sorbitol MacConkey agar to provide a primary means of identification. Subsequently biochemical tests were done for further characterization which were all consistent with the characteristics of *E. coli*. However, its pathogenicity was not well defined. The characterization proceeded with the use of Multiplex Polymerase chain reaction (PCR) to give an insight into the specific pathotypes based on the amplified genes. The results produced indicated that some of the lettuce samples were contaminated with the pathotypes, Enterotoxingenic *E. coli* (ETEC) and Enterohaemorrhagic *E. coli* (EHEC). The pathotypes are very dangerous when consumed due to the toxins they secrete. This is a very serious issue and it must be treated with utmost importance, as it poses great hazard to all members of the population especially old people and children.

Keywords: Diarrhea, *Escherichia coli*, Lettuce, Multiplex Polymerase chain reaction (PCR), *E. coli* Pathotypes.

CHAPTER ONE
INTRODUCTION

1.1 BACKGROUND OF STUDY

In recent times, vegetables have been considered essential ingredients of most healthy diets. As a result, there has been an increase in the demand for these vegetables based on healthy lifestyle recommendations. However, most vegetables, like lettuce plants are usually consumed directly with or without washing which has resulted in multiple health issues worldwide. Solomon (2002) demonstrated that due to layering of leaves and wide surface area observed in lettuce, they are able to house bacteria even after washing. This poses great and immense hazard to the health of people as there is a potential for contamination with gastrointestinal pathogens. There are numerous points at which lettuce can become contaminated throughout food production and distribution, including water for irrigation used on the farm, soil type, method of harvest, washing, procedures for packaging, and kitchen use (Beuchat, 1996; Froder *et al.,* 2007; Gorny *et al.,* 2006). According to research, the primary pathogens involved in the outbreaks are *Salmonella spp and Escherichia coli,* they make up about 72% of the pathogens involved in these contaminations (Gravani, 2009). However, intestinal diarrheagenic *E. coli* strains are known to be the major contributor of diarrheal diseases worldwide (Croxen *et al.,* 2013).

Escherichia coli is part of the regular microflora of the gastrointestinal microflora of humans and animals. However, a few strains are pathogenic and cause distinct diarrheal syndromes (Padhye and Doyle 1992; Buchanan and Doyle, 1992). The diarrheagenic *E. coli* that have been linked with foodborne illness have been grouped into several categories based on virulence properties, clinical syndromes, differences in epidemiology, and distinct 0:H serogroups (Levine, 1987). The main categories include enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC), Diffusely Adherent *E. coli* (DAEC) and Adherent invasive *E. coli* (AIEC). As foodborne pathogens that have caused multiple fatal outbreaks in both developing and industrialized nations, several of these pathotypes are public health problems (Yang *et al.,* 2017; Alegbeleye *et al*., 2020). Shiga-toxin or Vero toxin producing (STEC/VTEC) EHEC is unique and notable for causing human infections out of all diarrheagenic *E. coli* found. (Wani *et al.,* 2003). The EHEC serotype O157; H7 is one of numerous serotypes seen in STEC.

Many diseases that developed due to *E. coli* infections have been connected to the intake of contaminated lettuce and have been reported (CDC, 2003). In July 1998, contaminated leaf lettuce was related to an *E. coli* O157:H7 disease outbreak, that affected 40 people in Montana (Ackers *et al*., 1998). At least four more outbreaks of *E. coli* O157:H7 illnesses occurred after this and was related to the ingestion of contaminated lettuce. In 1997, approximately 15% of the people in a village located in India was affected by pathogenic *E. coli*. Subsequently, diarrheagenic *E. coli* cases were also reported in, Libya (Dow *et al.,* 2006), Mali (Boisen *et al.,* 2011), sub-Saharan Africa (Kotloff *et al.,* 2013) and Nigeria (Okeke *et al*., 2010). Over 900 people were diagnosed with HUS, and it eventually brought to notice the fact that hybrid pathogen infections that had both EAEC and STEC virulence genes (Mellmann *et al.,* 2011; Estrada-Garcia and Navarro-Garcia, 2012).

In light of these foodborne outbreaks, it is evident that there is dearth of information regarding microbiological quality, safety of vegetables like lettuce especially in Nigeria. As a result, this study investigates the prevalence as well as to unfold the *E. coli* pathotypes associated with ready to eat lettuce plants from farmers market produce obtained from a farm in Ogudu, Lagos, Nigeria.

1.2 AIMS AND OBJECTIVES

This study was carried out to isolate and determine the possible presence of *E. coli* in ready to eat lettuce. In addition, the study also characterizes the *E. coli* possibly present in the lettuce samples isolated from Ogudu, Lagos State, Nigeria.

1.3 JUSTIFICATION OF STUDY

Lettuce plants (*Lactuca sativa*) are usually consumed directly with or without washing which has resulted in multiple health issues worldwide. In addition, these health issues have been connected to pathogenic *E. coli* on these lettuce plants. However, the presence of pathogenic *E. coli* associated with freshly harvested and ready to eat lettuce has not been properly established in Lagos, Nigeria. Knowing fully well that pathogenic *E. coli* remains a huge public health hazard, this study therefore investigates the *E. coli* pathotypes associated with ready to eat lettuce from Ogudu in Lagos state.

CHAPTER TWO
LITERATURE REVIEW

2.1 ETIOLOGY OF *Escherichia coli*

Escherichia coli can be referred to as a widely studied genus of bacteria with more than 700 identified serotypes, it belongs to the family Enterobacteriaceae and was first described by Dr. Theodor Escherich in 1885 (Escherich, 1989). This gram-negative, facultative, anaerobic bacterium is rod-shaped, and are about 0.25–1.0 µm in diameter and 2.0 micrometers (µm) long. It is considered to be part of the microbiota of the gastrointestinal tract of humans and other warm-blooded animals aiding in food absorption (Drasar, 1974). The mucous layer of the mammalian colon is specifically the niche of commensal *E. coli*. The bacterium, which is the most prevalent facultative anaerobe of the human intestinal microflora, is a very successful competitor at this congested site (Sweeney *et al*., 1996). Typically, a few hours after delivery, *E. coli* colonize and live in the gastrointestinal system of infants. Commensal *E. coli* strains and humans coexist without any adverse consequences. However, in patients with impaired gastrointestinal barriers or immunocompromised hosts, interactions with commensal *E. coli* can lead to serious diseases. (Tenaillon *et al.,* 2010). Notably, specific *E. coli* strains are the causative agents for quite a number of illnesses, including extraintestinal and intestinal diseases in humans all around the world. In 1982, cases of acute bloody diarrhea related to the intake of hamburgers at a popular fast-food business led to the first identification of *E. coli*, as a pathogen. (Riley *et. al*., 1983). Since then, there has been several reports of outbreaks from developing and developed countries.

2.1.1 PATHOTYPES OF *E. coli*

There are a number of *E. coli* strains that have undergone evolution and developed particular virulence factors, giving them a greater capacity to evolve and adapt to different environments and the capacity to cause a variety of diseases. These virulence traits are commonly encoded on plasmids (mobile genetic elements) that can be transferred to other strains to produce novel virulence factor combinations or plasmids (genetic elements) that may have previously been mobile but have now undergone evolution to become "locked" within the genome. However, only the most effective virulence sequences last enough to produce unique "PATHOTYPES" of *E. coli* that may infect healthy people. (Nataro and Kaper, 1998). Nine pathovars have been

described for *E. coli* strains identified in humans, causing diarrheagenic and extraintestinal diseases (Nash *et al*., 2010). Of these, six major pathotypes have been described as enteric pathogenic *E. coli*, including Enterohaemorrhagic *E. coli* (EHEC), Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAEC), Diffusely adherent *E. coli* (DAEC), and, a new pathotype, Adherent-Invasive *E. coli* (AIEC), causing mostly diarrhea and intestinal disorders.

2.1.1.1 ENTEROPATHOGENIC *E. coli* (EPEC)

The first pathotype of *E. coli* to be identified was EPEC. It can be broken down further into other serogroups which are categorized as EPEC strains. In 1945, John Bray initially described EPEC as the agent that causes frequent infantile diarrhea outbreaks in the USA and the United Kingdom in the 1940s and 1950s. (Robins-Browne, 1987). Although its occurrence disappeared in developed countries after 1950s, it then became the principal pathogen causing infantile diarrhea in growing countries during the 20th century (Ochoa *et al.,* 2011). In developing nations like Brazil, Chile, Peru, and Iran, infantile diarrhea caused by EPEC infection ranged from 5% to 10%. (Ochoa *et al*., 2008). EPEC is still considered a pathogen of great public health issue for infants and adults in developing countries with a high death (morbidity) rate in children younger than six months. An identified intestinal histopathology of EPEC infections has been defined; known as 'attaching and effacing' (A/E) (McDaniel *et al.,* 1995). An A/E lesion occurs by unique sequences ultimately leads to the polymerization of actin beneath the attached EPEC resulting in diarrhea (Clarke *et al.,* 2003; Nataro and Kaper., 1998).

2.1.1.2 ENTEROHAEMORRHAGIC *E. coli* (EHEC)

This pathotype of *Escherichia coli* was identified initially as the principal pathogen that caused human disease in 1982, with two outbreaks of hemorrhagic colitis that occurred in the states of Oregon and Michigan (Riley *et al*., 1983). EHEC includes *E. coli* 0157:H7 and *E. coli* 026:H11. The most important serotype playing a role in EHEC outbreaks is O157: H7, which is still considered a serious health concern all around the world. Outbreaks of EHEC serotypes usually have occurred through the fecal-oral route by person-to-person transmission, animal contact, and consumption of under-thermally processed foods as well as undercooked meat products, unpasteurized apple juice, raw milk, or cross-contaminated raw vegetables such as bean sprouts and lettuce (Pakbin *et al.,* 2020). Infection of EHEC which is an attaching/effacing pathogen

results in bloody diarrhoea (haemorrhagic colitis), non-bloody diarrhoea and haemolytic uremic syndrome (HUS) (Kaper *et al.,* 2004). The characteristic virulence trait exhibited by key EHEC are Shiga toxins (Stx), also referred to as verocytotoxin (VT). The shiga-like toxin (SLT), encoded by Stx genes, belongs to the shiga-toxin producing *E. coli* group and is responsible for pathological manifestations leading to specific disease symptoms caused during EHEC infections (Joseph *et al.,* 2020).

2.1.1.3 ENTEROTOXIGENIC *E. coli* (ETEC)

ETEC has been described as the fundamental pathogen causing watery diarrhoea, which can range from a mild, self-limiting disease to severe purging disease as well as travelers and children's diarrhea by releasing enterotoxins in the human small intestine. The highest infection and mortality rates of ETEC are found in children under the age of two (Nataro *et al.,* 1998). Approximately 280 million children aged 0 to 4 years were reported to experience ETEC induced diarrhea (Wenneras and Erling, 2004). The largest cases of ETEC infections reported occurred in 1998, in the state of Illinois, Unites States where approximately 3,300 people were believed to have become ill through consumptions of foods prepared by infected personnel (Beatty *et al.,* 2006). Generally, the two main virulence traits exhibited by ETEC that lead to diarrhea in humans are colonization factors and enterotoxins (Crofts *et al.,* 2018). The secretions of the enterotoxins, heat-stable toxins (STs), and heat-labile toxins (LTs), on the other hand, are the most notable virulence factors. Upon secretion, these enterotoxins activate the production of cyclic nucleotides, which contributes to intestinal net water, salt, and fluid loss and causes secretory diarrhea in humans. (Mirhoseini *et al*., 2018).

2.1.1.4 ENTEROINVASIVE *E. coli* (EIEC)

Upon research, EIEC have been proven to be very similar to *Shigella spp* based on its pathogenic mechanisms, genetic and biochemical composition (Pupo *et al.,* 2000). EIEC and the bacteria *Shigella* possess certain similarities which includes their inability to ferment lactose, lack of motility, and they both are lysine decarboxylase negative. These characteristics serve as markers in differentiating the pathogens from other bacteria. EIEC and *Shigella* exhibit similar virulence factors, but it is possible to distinguish these pathogens using some biochemical tests. Once localized in the epithelial cell cytoplasm, EIEC suppresses the host immune response and counteracts the immune defense system by using protein effectors to persist and survive inside

the colonocytes. 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). The distinguishing trait of EIEC from the other types of *E. coli* is that it is an obligate intracellular pathogen without either adhesion or flagella factors (Van den Beld and Reubsaet, 2012).

2.1.1.5 ENTEROAGGREGATIVE *E. coli* (EAEC)

EAEC has been recognized as the second leading cause of severe and recurring travelers' diarrhea after ETEC infections occurred in developed and developing nations. It also serves as part of the main causes of enteric infections in patients with HIV/AIDS (Donnenberg, 2013). The ability of EAEC strains to aggregately bind to tissue culture cells is one of their defining characteristics. This pathotype of *E. coli* continue to be recognized as the principal cause of recurring or constant diarrhea in children and adults causing several outbreaks worldwide. In 2011, a hybrid strain of EAEC/STEC (serotype O104: H4) caused a large outbreak in Germany, resulting in more than 4300 diarrhea cases and 50 deaths (Rogawski *et al.,* 2017). The watery diarrhea caused by EAEC is at times accompanied with blood or mucous in stool without fever or vomiting. The basic mode of pathogenicity of EAEC infection comprises of the invasion and establishment of pathogen in the intestinal mucosa, especially that of the colon, before the secretion of enterotoxins and cytotoxins (Nataro *et al.,* 1998). The plasmid of aggregative adherence (pAA), which refers to a family of virulence plasmids, and pathogenicity islands dispersed across the chromosome are where the virulence components of EAEC are encoded (Nataro, 2005).

2.1.1.6 DIFFUSELY ADHERENT *E. coli* (DAEC)

DAEC are known to cause various diseases including diarrhea in children between 1 to about 5 years of age, urinary tract infection (UTI) in adults, pregnancy complications (Le Bouguénec *et al.,* 2006). Sources implicated in outbreaks of DAEC include contaminated food, especially undercooked ground beef, contaminated water and contact with livestock and other animals. Diffusely Adherent *Escherichia coli* are so named because of a diffuse pattern of adherence to HEp-2 and HeLa cell monolayers (Croxen *et al.,* 2013). According to research, strains of this pathogen have been identified and isolated in stools of adults who show no symptoms which

indicates that adults are asymptomatic carriers of DAEC strains. This has contributed to chronic inflammatory intestinal diseases, including Crohn's, coeliac, and inflammatory bowel diseases (Mansan-Almeida *et al.,* 2013).

Table 2.1: Classification of Pathogenic *E. coli* (LDHP, 2016)

E. coli	**Epidemiology**	**Diarrhea**	**Mechanism**
EHEC	Hemorrhagic colitis and hemolytic uremic syndrome in all ages and thrombotic thrombocytopenic purpura in adults	Bloody or non-bloody diarrhea	Cytotoxin production and adherence
EPEC	Acute and chronic endemic and epidemic diarrhea in infants	Watery	Adherence effacement
ETEC	Infantile diarrhea in developing countries and travelers' diarrhea	Watery	Adherence, enterotoxin production
EIEC	Diarrhea with fever in all ages	Bloody or not	Adherence, invasion of mucosa
EAEC	Chronic diarrhea in infants	Watery	Adherence
DAEC	Diarrhea in infants and adults	Watery	Diffused adherence

2.2 MOLECULAR MECHANISMS OF *E. coli* PATHOGENICITY

All *E. coli* strains known to cause diarrheal diseases can be regarded as the major agents that result in diarrheal infections and strains are able to thrive in the environment as a result of the traits obtained from horizontal gene transfer (Kaper and Nataro, 2004; Croxen *et al*., 2013). These pathogenic *E. coli* possess similar virulence strategies in many areas and the virulence factors associated with *E. coli* mediated disease have been known for several years. All pathovars, with the exception of EIEC, require host cell adhesion, which is typically accomplished by means of lengthy appendages known as pili or fimbriae. Following adhesion, *E. coli* must minimize processes that occur in the host cell, by the use of certain proteins secreted. Seizing and altering host cell signaling pathways would then result in an organised

invasion of host cells, evasion of host immune responses, and effective colonization which will most likely result in infections. (Bhavsar, 2007). Although they frequently attack the same host machinery, each pathotype has its own distinctive mechanisms for adhering to and taking advantage of the host cell. The genomes of diarrheagenic bacteria are significantly shaped by the loss and gain of mobile genetic components (plamids). HGT, or horizontal gene transfer, is a crucial technique for quickly transferring novel features to recipient species also allowing its coevolution with the host (Shames *et al.,* 2009). Pathogenic bacteria have large collections of virulence genes called pathogenicity islands (PAIs), which are only present in pathogenic bacteria and cannot be found in non-pathogenic bacteria. Importantly, another notable fact is that each pathovar's evolution may not necessarily occur through the transfer of genes from one lineage to the other; for instance, it was discovered that various phylogenies of *E. coli* independently acquired EHEC virulence proteins. Research studies have also pointed to the fact that, due to the diversity exhibited by *E. coli*, their genomes can be as large as 1MB, which is even larger than commensal isolates attributed to the loss of certain genetic materials and pathogenicity islands (PAIs (Rasko *et al.,* 2008; Touchon *et al.,* 2009).

2.3 VEGATABLES AS A SOURCE OF *E. coli*

A notable increase in the intake of vegetables and plants products had been observed and it can be attributed to large amounts of contributions like nutrients and other functional properties (Liu, 2003; Luna-Guevara *et al.,* 2019). However, simultaneously, these fresh produce continue to cause a growing number and cases of foodborne outbreaks as a result bacterial contamination of these products (Callejon *et al.,* 2015). The most frequently observed vegetables that result in bacterial outbreaks are fresh herbs, spinach and most importantly, lettuce (Jay-Russell *et al.,* 2014). According to multiple reports, the principal pathogens that cause outbreaks linked to this product and other leafy greens is *E. coli* (Brandl, 2006; Rangel *et al*., 2005). Generally, leaf surfaces are not as abundant as animal intestines in nutrients availability but studies have indicated that these surfaces possess the sugars sucrose and fructose distributed heterogeneously (Delaquis *et al.,* 2007; Miller *et al.,* 2001) Therefore, this uneven allocation of physical and chemical components on one leaf at a time and among the leaves of the same plant may create microsites that are hospitable to bacteria, including enteric pathogens like *E. coli*. In addition, the ability of these microorganisms to thrive also depends on other factors, including the specific features of the microorganism, fruit ripeness, environmental conditions, plant development,

bacterial resistance to the plant metabolic processes, plus harvest, and postharvest processes (Matthews, 2014). Unfortunately, existing methods of industrially sanitizing and washing produce do not ensure the complete eradication of microorganisms that could cause an infection (Abadias *et al.,* 2012). Also, it has been established epidemiologically that the consumption of vegetables contaminated with as low as 10 cells could result in significant health problems (Jinneman *et al.,* 1995).

2.4 LETTUCE PLANTS

The lettuce plant, Lactuca sativa L., belongs to the successful and varied Asteraceae (Compositae) family of plants, which has a wide geographic range. (Funk *et al.,* 2005). Lettuce are one of the major vegetables with an increased demand due to the change of diets in most parts of the world. Initially, the lettuce market was controlled by Europe and North America, however, around the late 20th century, lettuce consumption had spread to other parts worldwide. Today, most lettuce plants are cultivated for their leaves, although one type is cultivated primarily for its stem and one for its seeds, which are made into an oil. Lettuce is a particularly perishable product with a short shelf life. Antioxidants, such as flavonols, are found in appreciable amounts in lettuce plants (Hohl *et al*. 2001). Whether alone or in conjunction with other greens, vegetables, meats, and cheeses, salads are where lettuce is most frequently utilized. While the stems are consumed both fresh and cooked, lettuce leaves could be used to make soups, sandwiches, and wraps. Likewise, it has been discovered that these lettuce might not be the richest source of nutrients but most times it is consumed raw which helps to retain its nutritional value. However, this also poses a hazard such that washing of the lettuce plants without further processing may not significantly reduce the microbial load present.

Figure 2.1: Lettuce plant (Lactuca sativa) (Source: Zenz, 2006)

2.5 EPIDEMIOLOGY AND PREVALENCE OF *E. coli* IN LETTUCE PLANTS

Worldwide, *E. coli* outbreaks have mostly been related to the ingestion of food products of bovine origin such as dairy products, undercooked ground beef (Griffin, 1995), but recently outbreaks linked to the ingestion of food products of non-bovine origin are being reported more frequently (Beauchat, 1999). Shiga-toxin or Vero toxin-producing (STEC/VTEC) *E. coli* has been found to be the most significant pathotype in human infections out of all diarrheagenic *E. coli* investigated (Wani *et al.,* 2003). Additionally, it has been discovered that, of all the STEC serotypes, the EHEC serotype O157:H7 is the most virulent and is to blame for outbreaks of bloody diarrhea that have occurred all over the world. Since the initial *E. coli* O157:H7 infection epidemic in 1982, there have been numerous outbreaks in the USA, with estimates of 73,000 illnesses caused by this strain of *E. coli* and 61 related deaths happening each year (CDC, 2003). Numerous epidemiology studies carried out found that various factors contribute to the shift of prevalence based on different geographical areas, population, age distribution, socioeconomic class and detection methods (Ochoa *et al.,* 2008). Most of the outbreaks during 1996 to 2008 were associated with leafy green vegetables, where *E. coli* O157:H7 was the primary pathogen (Gravani, 2009). There have been numerous reports of *E. coli* O157:H7 outbreaks linked to contaminated lettuce and a sizable outbreak linked to the ingestion of contaminated unpasteurized apple juice (CDC, 2003). Also, between 1998 and 2005, lettuce alone was implicated for 20 outbreaks and 634 instances of *E. coli* O157:H7 infection, according to the U.S. Centers for Disease Control and Prevention (Lynch, 2007). In July 1998, contaminated leaf lettuce was linked to an *E. coli* O157:H7 infection outbreak, that affected 40 people in Montana (Ackers *et al.,* 1998). At least four more outbreaks of *E. coli* O157:H7 illnesses occurred after this and were related to the intake of contaminated lettuce (Buck *et al.,* 2003). These outbreaks show how crucial fresh produce is becoming as a source of the transmission of foodborne illness. From 2008 to 2018, 57 cases of *E. coli* infection related to the intake of leafy greens were identified in Canada and the United States through multiple sources (CDC, 2018; CDC, 2020). The major vegetables associated with these outbreaks were romaine lettuce, iceberg lettuce, leaf lettuce, spinach, mesclun mix, and spring mix. Upon further testing, out of the 57 *E. coli* infections identified, 48 were caused by *E. coli* 0157:H7 while the other 9 were caused by non-O157 *E. coli*. Also of the 48, 18 were linked to the intake of lettuce. These cases show the severity and continual increase in infections caused by pathogenic *E. coli* around the world.

2.6 PATHOGENICITY OF *E. coli* IN HUMANS

The diversity of *E. coli* has been recognized, and it has led to the discovery that, different pathotypes have different methods of pathogenicity. Specific virulence factors encoded by gene clusters are responsible for these mechanisms. The genes involved in pathogenicity may encode processes like invasion, motility, attachment, iron acquisition, toxin activity, and others. Also noteworthy is the possibility that these *E. coli* pathotypes have similar virulence characteristics and methods (Mainil, 2013). The typical attaching and effacing lesion is caused by EPEC, which attach to small bowel enterocytes but disrupt the usual microvillar structure. Cytoskeletal abnormalities are accompanied with diarrhea and an inflammatory reaction (Kaper *et al.,* 2004). The ability to synthesize shiga toxins—of which there are two main varieties, shiga toxin 1 and shiga toxin 2—is a characteristic shared by EHEC/STEC. (Melton-Celsa, 2014). The capacity of EHEC/STEC to adhere to epithelial cells in the intestine is another crucial step in the disease's etiology. In the colon, they possess the ability to cause the adhering and effacing lesion. The synthesis of Shiga toxin (Stx), whose systemic absorption results in potentially fatal consequences, is the defining characteristic of EHEC. (Kaper *et al.,* 2004). ETEC strains are known to produce adhesins, also known as colonization factors, which are proteinaceous complexes that can form fimbrial or nonfimbrial structures on the surface of the bacterial cell. These adhesins, which ETEC strains produce, help the bacteria attach to the intestinal mucosa and increase host specificity for various strains. (Qadri *et al.,* 2005). They release enterotoxins that are both heat-labile and heat-stable and cause diarrhea. (Kaper *et al.,* 2004). Virulence factors for the pathotype EAEC include adhesins, toxins, and secreted proteins. However, these virulence factors, which are plasmid-borne, are not present in all strains (Czeczulin *et al.,* 1999). EAEC forms a thick biofilm on the small and large bowel epithelia and attaches to them, producing secretory enterotoxins and cytotoxins that cause secretory diarrhea with a lot of mucus production but no blood in the stool (Kaper *et al.,* 2004). EIEC attaches primarily to the large intestine mucosa and enters cells via endocytosis. (Levine, 1987). Plamsidial and chromosomal genes are just two examples of the many bacterial genes that may be involved in the infectious process. On HeLa and HEp-2 cells, DAEC strains are heterogeneous groups that produce a widespread adherence pattern. This phenomenon is facilitated by Fimbrial and afimbrial adhesins, which populate the small intestine and have been related to both recurrent urinary tract

infections (UTIs) in adults and diarrhea in children. These adhesins are expressed by a family of similar operons. (Servin, 2005).

2.7 SOURCES OF PATHOGENIC *E. coli* CONTAMINATION IN LETTUCE

Fresh produce may be contaminated at any point in the production chain between farm and table. Studies carried out show that produce contamination is high during three periods: in the field, during initial processing, and in kitchens (Ailes *et al.,* 2008). There are three types of factors that affect microbiota present in fresh products: physical, chemical, and biological. Physical factors, such as pH, temperature, and moisture, affect the growth and some metabolic activities of microbiota. The presence of nutrients in lettuce that microbes might exploit is one chemical factor. The presence of competitive microbiota and bacterial-plant interactions round out the biological components. (Sela, 2009). Contamination of lettuce could emerge at two major stages from the farm to fork, the preharvest and postharvest stage, these contaminations in turn leads to the ingestion of pathogenic microorganisms resulting in diseases

2.7.1 PREHARVEST CONTAMINATION

The soil and inadequately composted manure are the two main causes of lettuce contamination during the preharvest stage. After application of these improperly treated manure to the soil, there is a large probability of pathogens travelling to other parts of the plant. Due to the addition of animal feces, soil is naturally thought to be a reservoir for a variety of pathogens that can cause human diseases, including pathogenic *E. coli* (Whipps *et al.,* 2008). Additionally, domestic animals and wildlife pose a risk as sources of pathogenic bacteria, especially for lettuce and most vegetables in the preharvest period. Berger provided evidence for this by demonstrating how animal waste contributes to the contamination of produce and can result in *E. coli* O157:H7 epidemics. (Berger *et al.,* 2010). Insects could also be a source of plant contamination. Contaminated flies have also demonstrated the capacity to serve as vehicles, transferring *E. coli* to various plant parts (Berger *et al*., 2010). The irrigation water used is also a fundamental factor in the possible presence of pathogens in lettuce. The risk of using contaminated water for irrigation, however, is dependent on the type of irrigation system employed. There have been recent instances of epidemics brought on by eating lettuce that had been irrigated with water polluted with *E. coli* O157:H7. (CDC, 2018). The probability for pathogenic microorganisms such as *E. coli* to spread in contaminated water is lower with the use of drip irrigation compared

to other sprinkler systems (Mitra, 2009). Additionally, another factor that could cause contamination especially in the process of handling and harvesting of crops are the workers' hands. They could also serve as sources for transferring pathogens during preharvest due to the lack of access to latrines or handwashing stations (Lynch *et al.,* 2009).

2.7.2 POSTHARVEST CONTAMINATION

Findings show that the levels of *E. coli* in crops, such as lettuce, are much greater at the end of the handling process than at the beginning. (Frank, 2009). This is attributed to the direct contamination due to handling in the postharvest stage or multiplication of the pathogen in lettuce. Generally, vegetables have a minimal shelf life and as a result, lack of cooling during storage could result in the growth of these pathogens. Water is also employed in many steps, such as washing, chill tanks, sprays during the postharvest process (Duffy *et al.,* 2005). Vegetables must be washed in order to remove soil, debris, and some bacteria. However, this does not entirely guarantee safety. Some outbreaks are related to the process of cutting of vegetables during salad preparation with unclean implements in restaurants or home kitchens and cross contamination by the food handler's hands due to poor hygiene (Lynch *et al.,* 2009).

CHAPTER THREE
MATERIALS AND METHODS

3.1 SAMPLING

Ready to eat lettuce samples were obtained directly from a farm in Ogudu, Lagos state, Nigeria for four consecutive weeks. A total of 12 samples were obtained using Ziploc bags and transported quickly to the laboratory for analysis.

3.2 MATERIALS

Petri dish, bunsen burner, wire loop, eppendorf tubes, test tubes, spreader, beaker, conical flask, foil paper, micropipettes, distilled water, paper tape, marker, cotton wool, latex hand gloves, gel electrophoresis tanks, sterile tips, wash brush, measuring cylinder, test tube rack,

3.3 EQUIPMENTS

UV tranilluminator, water bath, gel electrophoresis tanks, distiller, weighing balance, incubator stomacher, ice maker.

3.4 MEDIA USED

Buffered peptone water (BPW), Nutrient agar, MacConkey agar, Sorbitol MacConkey agar (SMAC), Brain heart infusion (BHI) broth

Buffered peptone water (BPW)

BPW is an enrichment media which specifically improves the detection of bacteria from raw ingredients

Preparation

1. The dehydrated BPW was dissolved in a conical flask containing distilled water according to the manufacturer's instruction and sealed properly with an aluminum foil
2. The mixture was then mixed properly by heating to allow even distribution and autoclaved for sterilization at 121°c for 15 minutes
3. The sterilized BPW was then distributed into appropriate test tubes.

Nutrient agar

This is a basal media that supports the growth of a wide range of bacteria

Preparation

1. The powdered nutrient agar was dissolved in a conical flask containing distilled water according to the manufacturer's instruction (28g of nutrient agar powder in 1L of distilled water) and sealed properly with an aluminum foil
2. The mixture was then mixed properly by heating to allow even distribution and autoclaved for sterilization at 121°c for 15 minutes
3. The sterilized media was allowed to cool to temperatures between 45°c -50°c before being poured aseptically into labelled petri dishes and allowed to solidify.

MacConkey agar

This is a media mainly used to identify gram negative, enteric bacteria based on their ability to ferment lactose.

Preparation

1. The powdered MacConkey agar was dissolved in a conical flask containing distilled water according to the manufacturer's instruction (49.5g of MacConkey agar powder in 1L of distilled water) and sealed properly with an aluminum foil
2. The mixture was then mixed properly by heating to allow distribution and autoclaved for sterilization at 121°c for 15 minutes
3. The sterilized media was allowed to cool to temperatures between 45°c -50°c before being poured aseptically into labelled petri dishes and allowed to solidify.

Sorbitol MacConkey Agar (SMAC)

SMAC is a variant of MacConkey agar which is used to distinguish pathogenic strains of *E. coli* from non-pathogenic strains.

Preparation

1. The powdered SMAC was dissolved in a conical flask containing distilled water according to the manufacturer's instruction (50.03g of powdered SMAC in 1L of distilled water) and sealed properly with an aluminum foil
2. The mixture was then mixed properly by heating to allow distribution and autoclaved for sterilization at 121°c for 15 minutes

3. The sterilized media was allowed to cool to temperatures between 45°c -50°c before being poured aseptically into labelled petri dishes and allowed to solidify.

Brain Heart Infusion (BHI) broth

BHI broth is a media used for the isolation, cultivation and maintenance of a variety of bacteria

Preparation

1. The dehydrated BHI was dissolved in a conical flask containing distilled water according to the manufacturer's instruction (52g in 1L of distilled water), the mixture was then sealed with an aluminum foil and mixed properly by heating to allow even distribution.
2. The media was dispensed into labelled Eppendorf tubes using a micropipette and autoclaved for sterilization at 121°c for 15 minutes.

3.5 ISOLATION OF *E. coli*

3.5.1 Sample preparation

Primary enrichment

Precisely 16g of the lettuce sample was weighed and then chopped using a sterile knife before being poured into a conical flask containing 150ml of 1% BPW which is an enrichment broth, serving as the first dilution, 10^{-1}. Subsequently, serial dilutions consisting of 3 different dilution factors was carried out, progressing from 10^{-2}.to 10^{-4}.

3.5.2 Serial dilution

One milliliter (1ml) of the samples were pipetted using the micropipette set at 1000µl into test tubes containing 9ml of 0.1% BPW resulting in the dilution factor 10^{-2}, this is followed by the transfer of another 1ml into the next test tube from the 10^{-2} to obtain the dilution factor of 10^{-3} ,this process is repeated again until the dilution factor, 10^{-4} is obtained. The test tubes are labelled appropriately for easy identification.

3.5.3 Plating

SMAC and MAC agar plates were labelled accordingly and 0.1ml from dilutions 10^{-3} and 10^{-4} were plated onto the SMAC plates while 0.1ml from the 10^{-1} dilution factor were plated on MAC plates using the spread plate technique, this involves the use of a glass rod to spread the

sample evenly on the agar surface. In order to maintain aseptic conditions, the glass rod was flamed before and after spreading. The inoculated plates were then incubated at 37°c for 18-24 hours, then counted.

3.5.4 Sub culturing

This is procedure carried out to isolate and purify samples from a mixed culture to a new culture plate, the selected colonies to be sub cultured are colonies which are differentiated based on their shape, colour, colony morphology.

A loopful of the selected colonies (white colonies) were obtained from the SMAC plate using the wire loop. To maintain aseptic conditions the wire loop was flamed using the Bunsen burner before and after subculturing. The colonies contained on the wire loop were then sub cultured onto nutrient agar plates that have been labelled accordingly using streaking method. In order to preserve the colonies, the wire loop was allowed to cool with each flaming process. The inoculated plates were inverted and placed in the incubator for 18- 24 hours at 37°c.

3.5.5 Cryopreservation of the isolate

A loopful of the isolates from the previously incubated nutrient agar was inoculated into a test tube containing 5ml of Brain heart infusion (BHI) broth and incubated at 37°c for 18-24 hours. After incubation, 750µl of the inoculum was added to a sterile Eppendorf tube containing 750µl of sterile 20% glycerol which acts as a cyroprotectant and it was stored in the freezer at -4°c.

3.6 BIOCHEMICAL TESTS

3.6.1 Gram staining

This is a test done to determine if a sample is gram negative or positive based on the thickness of the peptidoglycan layer

A smear of selected colony was made from the culture plate on a clean, grease-free glass slide. Afterwards, the smear was heat fixed by passing the slide over a Bunsen burner flame briefly. The slide was then flooded with crystal violet (primary stain) and allowed to stand for 1 minute. The stain was washed with water and stained with lugol's iodine (mordant) for 1 minute. After this, the stain was washed with water and decolourised with 70% alcohol (decolouriser) for 20 seconds. The stain was washed with water and counter stained with safranin (Secondary stain)

for 1 minute and washed with water. The slide was then allowed to air dry and observed under the microscope.

3.6.2 Catalase test

This is a test used to differentiate bacteria that produce the enzyme catalase from those that do not.

Procedure

The selected colony was transferred using a wire loop to the surface of clean and dry glass slide. A drop of 3% hydrogen peroxide (H_2O_2) was added and observed for the production of gas bubbles.

3.5.3 Oxidase test

This is also a test used to determine the presence of the enzyme cytochrome oxidase. Positive samples result in a purple colour change. For this test an oxidase test strip was used.

Procedure

The isolated colony to be tested was obtained using a sterile wire loop and rubbed on the test strip. The sample was observed for a colour change

3.7 MOLECULAR IDENTIFICATION

3.7.1 DNA Extraction

The samples were activated using BHI broth, this was done by the addition of 100µl of each isolate from E1- E4 (Pulling method) to a 2ml cryotube containing 1ml of BHI which had been autoclaved. The pulled isolates were centrifuged at 500g for 3 minutes and the supernatant was decanted. Next, 750µl of distilled water was added to the pellet, vortexed and the samples were centrifuged again at 500g for 3 minutes. Afterwards, the supernatant was decanted and the previous process was repeated. After discarding the supernatant, 200µl of distilled water was added to the pellet, vortexed and placed in a heating block for 15 minutes at 100°c. After heating, the samples were transferred into ice to cool for 5 minutes. The samples were then centrifuged at 7000 RPM for 6 minutes. After centrifugation, the supernatant of the samples which contained the extracted DNA were transferred into newly labelled Eppendorf tubes and stored in the freezer at -20°c.

3.7.2 Polymerase chain reaction (PCR)

The components used in the identification of *E. coli* are shown in Tables 3.3, 3.4, 3.5 and 3.6 below.

After preparation of the PCR cocktail and addition of the extracted DNA, the samples were placed in the thermal cycler for analysis. The PCR began with initial denaturation at 95°c for 5 minutes; 35 cycles of 95°C for 2 min; 42°C for 30 s and 72°C for 4 min; and the final elongation step at 72°c for 10 minutes. In addition, for negative controls a template DNA was replaced with sterile water. The PCR products were confirmed by electrophoresis and visualized under UV light with a Gel documentation system.

3.7.3 Agarose gel electrophoresis

Dry agarose powder was used in the preparation of the agarose gel. 1g of the agarose gel was dissolved in 50ml of TAE buffer and microwaved until a clear solution was formed, 3μl of ethidium bromide was carefully added to the mixture using a micropipette. It dissolved into the mixture which was left to cool but not solidify, the appropriate quantity was then transferred into the gel cast with the gel combs in place and allowed to solidify. After solidifying, the comb was taken out and TAE buffer was added. 4μl of the of the PCR products were pipetted into the wells formed by the combs, after pipetting the gel dock was connected to the power pack and left to run for 30 minutes. After this, the gel was viewed under the UV transilluminator.

Table 3.1: Showing sample ID

Sampling week	**Sample ID**
Week 1	F1K
Week 2	F2K
Week 3	F3K
Week 4	F4K

Table 3.2: Gene targets, virulence factors, sequences and amplicon sizes for PCR (Persson *et al.,* 2007)

Gene targets	Virulence factor	Sequence 5’	Amplicon size	*E. coli* pathotypes
Human *estA* (StFh)	STIh	F- TTTCGCTCAGGATGCTAAACCAG	151	ETEC
StRh		R- CAGGATTACAACACAATTCACAGCAGTA		
Porcine *estA* (StFp)	STIp	F- CTTTCCCCTCTTTTAGTCAGTCAACT	160	
StRp		R- CAGGATTACAACAAAGTTCACAGCAG		
vtx1	VT1	F- GTTTGCAGTTGATGTCAGAGGGA	260	EHEC
		R- CAACGAATGGCGATTTATCTGC		
Eae	Intimin	F- GGYCAGCGTTTTTTCCTTCCTG	377	EPEC
		R- TCGTCACCARAGGAATCGGAG		
vtx2	VT2	F- GCCTGTCGCCAGTTATCTGACA	420	EHEC
		R- GGAATGCAAATCAGTCGTCACTC		
EltA	LT1	F- AAACCGGCTTTGTCAGATATGATGA	429	ETEC
		R- TGTGCTCAGATTCTGGGTCTCCT		
IpaH	IpaH	F- TTGACCGCCTTTCCGATACC	647	EIEC
		R- ATCCGCATCACCGCTCAGAC		

Table 3.3:Components of multiplex PCR for *E. coli*, Treatment 1 (Batch 1)

No.	**Reagents**	**Initial concentration**	**Final concentration**	**Volume/rxn**	**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	*vtx1*F	20 µm	0.25	0.125	2.5
5	*vtx1*R	20 µm	0.25	0.125	2.5
6	*vtx2*F	20 µm	0.5	0.25	5
7	*vtx2*R	20 µm	0.5	0.25	5
8	*Ipah*F	20 µm	0.1	0.05	1.0
9	*Ipah*R	20 µm	0.1	0.05	1.0
10	Mgcl2	25 µm	1.5	0.6	12
11	dH_2O		4.15		83
12	DNA				

Table 3.4:Components used for multiplex PCR for *E. coli*, Treatment 2 (Batch 1)

No.	Reagent	Initial concentration	Final concentration	Volume/rxn	N=20
1	Master mix	5x	1x	2	40
2	StrF	20 µm	0.5	0.25	5
3	StrR	20 µm	0.5	0.25	5
4	*Elta*F	20 µm	0.45	0.225	4.5
5	*Elta*R	20 µm	0.45	0.225	4.5
6	*Eae*F	20 µm	0.15	0.075	1.5
7	*Eae*R	20 µm	0.15	0.075	1.5
8	$Mgcl_2$	25 µm	1.5	0.6	12
9	dH_20			4.3	86
10	DNA				

Table 3.5: Components used for multiplex PCR for *E. coli*, Treatment 1 (Batch 2)

No.	Reagents	Initial concentration	Final concentration	Volume/rxn	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	*vtx1*F	20 µm	0.25	0.125	7.375
5	*vtx1*R	20 µm	0.25	0.125	7.375
6	*vtx2*F	20 µm	0.5	0.25	14.75
7	*vtx2*R	20 µm	0.5	0.25	14.75
8	*Ipah*F	20 µm	0.1	0.05	2.95
9	*Ipah*R	20 µm	0.1	0.05	2.95
10	Mgcl2	25 µm	1.5	0.6	35.4
11	dH_2O		4.15		244.85
12	DNA				

Table 3.6: Components used of Multiplex PCR for *E. coli*, Treatment 2 (Batch 2)

No.	Reagent	Initial concentration	Final concentration	Volume/rxn	N=59
1	Master mix	5x	1x	2	118
2	StrF	20 µm	0.5	0.25	14.75
3	StrR	20 µm	0.5	0.25	14.75
4	*elta*F	20 µm	0.45	0.225	13.275
5	*elta*R	20 µm	0.45	0.225	13.275
6	*eae*F	20 µm	0.15	0.075	4.425
7	*eae*R	20 µm	0.15	0.075	4.425
8	$Mgcl_2$	25 µm	1.5	0.6	35.4
9	dH_20			4.3	253.7
10	DNA				

Table 3.7: Protocol for the thermal cycler

Analysis	Step	Temperature (°C)	Time
1x	Initial denaturation	95	5 min
35x	Denaturation	95	2 min
	Annealing	42	30 sec
	Polymerization	72	4 min
1x	Final polymerization	72	10 min
1x	Hold	4	∞

CHAPTER FOUR
RESULTS AND DISCUSSION

4.1 RESULTS

This project was aimed at the isolation and characterization of *E. coli* pathotypes from a farm in ogudu, Lagos state. For this study, 12 samples were obtained for a total of four weeks and 48 isolates obtained; the samples were evaluated for the presence of *E. coli.* The samples were phenotypically characterized based on their growth on SMAC and MacConkey agar. The agar mediums were based on the ability of *E. coli* to ferment lactose, a characteristic common to the family Enterobacteriaceae which *E. coli* belongs. All observed characteristics are present in Tables 4.1 and 4.2 respectively. Most samples reported pink and white, raised and circular colonies, which can be observed in Plate 4.1. These characteristics are consistent with the typical morphologies exhibited by *E. coli* on these differential agar mediums. The following biochemical tests, Gram staining, oxidase test and catalase test were also carried out for further characterization, the results for each sample are reported in Plate 4.3. The results show that the samples generally were gram negative, catalase positive and oxidase negative. Likewise, these are consistent with *E. coli*. In addition, PCR amplification was also carried out using appropriate primers targeted at the genes, Human *estA*, Porcine *estA*, *vtx1*, *vtx2*, *eae*, *eltA*, *ipaH*. The PCR process allowed the characterization of the *E. coli* present in the lettuce, taking into consideration the fact that different pathotypes of *E. coli* vary in the virulence genes they possess. Each gene was specifically used to detect pathotypes of *E. coli* present in the sample. The results for PCR amplification can be observed in Plate 4.2, 4.3, 4.4 which shows three positive samples from the 12 samples obtained. The positive samples were obtained from week 3 and week 4 of sampling and are represented specifically by the sample ID F3K7, F4K10 and F4K11. The sample F3K7, which can be observed in Plate 4.2 showed bands of 260bp consistent with the *vtx1* genes, indicating the pathotype EHEC. The samples F4K10 and F4K11 present in Plate 4.3 and 4.4 respectively, showed bands of 160bp consistent with the Porcine *estA* genes, this on the other hand indicates the presence of the ETEC pathotype.

Table 4.1: Morphological characteristics of isolates on Sorbitol MacConkey Agar

Sampling week	Sample	Isolate ID	No of Colonies	Colour	Shape	Elevation	Appearance	Surface	Opacity
Week 1	Lettuce	F1K1	TNTC	Pink and white	Circular	Raised	Smooth	Smooth	Opaque
		F1K2	38	Pink and white	Circular	Convex	Smooth	Smooth	Translucent
		F1K3	TNTC	Pink and white	Circular	raised	Smooth	smooth	Opaque
Week 2	Lettuce	F2K4	TNTC	Pink and white	Circular	Low convex	Smooth	smooth	Opaque
		F2K5	6	Pink and white	Circular	raised	Smooth	smooth	Opaque
		F2K6	TNTC	Pink and white	Circular	raised	Smooth	smooth	Opaque
Week 3	Lettuce	F3K7	TNTC	Pink and white	Circular	Low convex	Smooth	smooth	Opaque
		F3K8	40	Pink and white	Circular	raised	Smooth	smooth	Opaque
		F3K9	7	Pink and white	Circular	Low convex	Smooth	smooth	Opaque
Week 4	Lettuce	F4K10	TNTC	White	Circular	raised	Smooth	smooth	Opaque
		F4K11	TNTC	Pink and white	Circular	raised	Smooth	smooth	Opaque
		F4K12	TNTC	Pink and white	Circular	raised	Smooth	Small	Opaque

Plate 4.1: Plate showing *E. coli* Colonies on SMAC

Table 4.2: Morphological characteristics of isolates on MacConkey agar

Sampling week	Sample	Isolate ID	No of colonies	Colour	Shape	Elevation	Appearance	Surface	Opacity
Week 1	Lettuce	F1K1	TNTC	Pink	Circular	Convex	Smooth	Smooth	Opaque
		F1K2	40	Pink	circular	Low convex	Smooth	smooth	Opaque
		F1K3	TNTC	Pink	circular	convex	Smooth	smooth	Opaque
Week 2	Lettuce	F2K4	TNTC	Pink	circular	Convex	Smooth	smooth	Opaque
		F2K5	26	Pink	circular	Low convex	Smooth	smooth	Opaque
		F2K6	TNTC	Pink	circular	Convex	Smooth	smooth	Opaque
Week 3	Lettuce	F3K7	TNTC	Pink	Circular	convex	Smooth	smooth	Opaque
		F3K8	36	Pink	circular	Low convex	Smooth	smooth	Opaque
		F3K9	29	Pink	circular	Convex	Smooth	smooth	Opaque
Week 4	Lettuce	F4K10	TNTC	Pink and white	circular	Convex	Smooth	smooth	Opaque
		F4K11	TNTC	Pink	circular	convex	Smooth	smooth	Opaque
		F4K12	TNTC	Pink	circular	Low convex	Smooth	smooth	Opaque

Table 4.3: Biochemical testing for *E. coli* bacteria

CODE	GRAM STAINING	CATALASE	OXIDASE
FIKI	Negative	Positive	Negative
FIK2	Negative	Positive	Negative
FIK3	Negative	Positive	Negative
F2K4	Negative	Positive	Negative
F2K5	Negative	Positive	Negative
F2K6	Negative	Positive	Negative
F3K7	Negative	Positive	Negative
F3K8	Negative	Positive	Negative
F3K9	Negative	Positive	Negative
F4K10	Negative	Positive	Negative
F4K11	Negative	Positive	Negative
F4K12	Negative	Positive	Negative

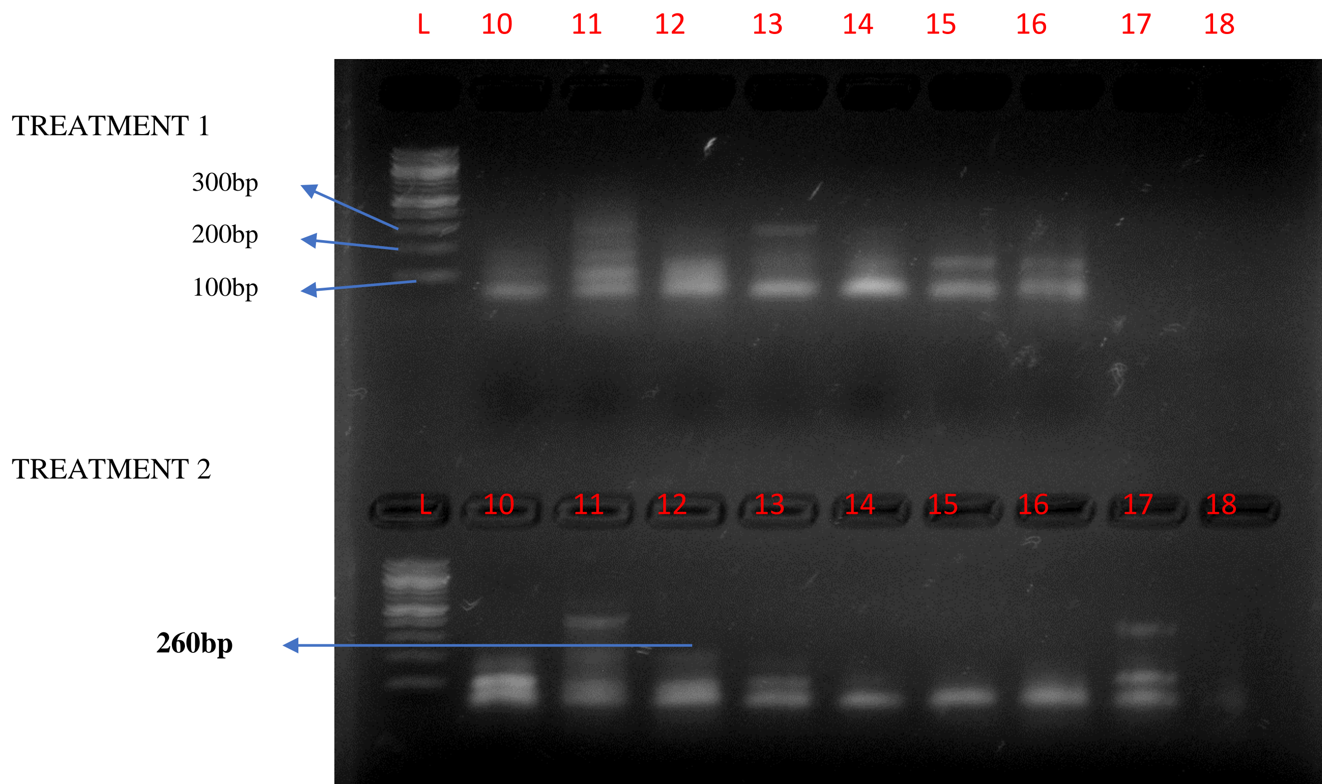

Plate 4.2: Agarose gel electrophoresis Multiplex PCR for detection and characterization of *E. coli*. Lane M= DNA marker, Lane 12 = isolate shows band size of 260bp which indicates that it is most likely positive for *vtx1* gene which indicates the likely presence of EHEC.

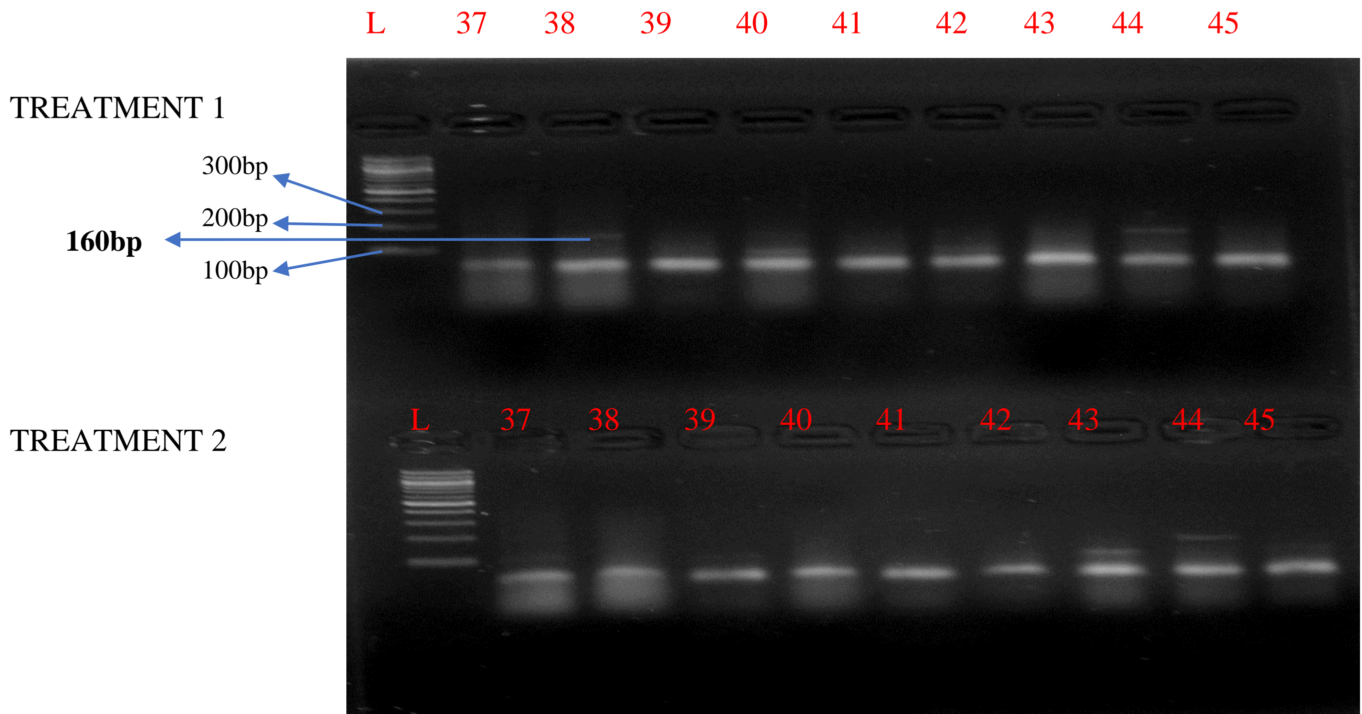

Plate 4.3: Agarose gel electrophoresis Multiplex PCR for detection and characterization of *E. coli*. Lane M= DNA marker, Lane 38 = isolate shows the band size of 160bp which means it is positive for Porcine *estA* gene which indicates the likely presence of ETEC

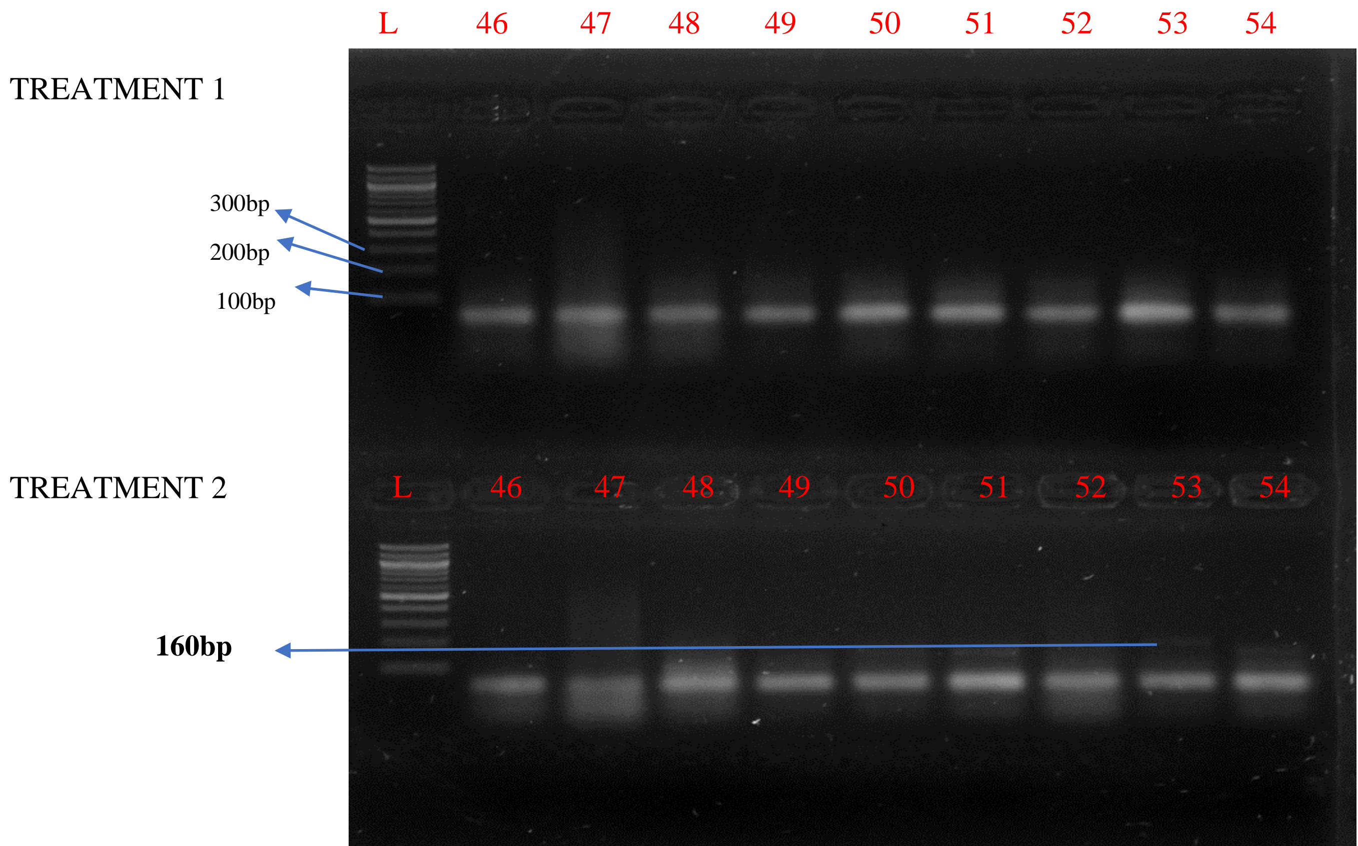

Plate 4.4: Agarose gel electrophoresis Multiplex PCR for detection and characterization of *E. coli*. Lane M= DNA marker, Lane 53 = isolate shows the band size 160bp indicating it is positive for Porcine *estA* (StFp and StRp) gene which indicates the likely presence of ETEC.

4.2 DISCUSSION

This study was aimed at the isolation and characterization of *E. coli* from lettuce which continues to be an increasing component of healthy diets around the world. The results obtained show the likely presence of pathogenic *E. coli* in the samples obtained which requires serious attention. The pathotypes EHEC and ETEC were identified constituting 25% of the samples. The presence of this pathogen in ready to eat lettuce isolated from a farm poses serious threat to the health of humans across Nigeria. Countries such as the United States have reported outbreaks associated with lettuce in times past (Lynch, 2007). Canada has also reported cases of infections caused by the intake of lettuce contaminated with pathogenic *E. coli* (CDC, 2018). However, in Africa, specifically Nigeria, very minimal amount of research has been done in this area. Cases of infection traced to the consumption of other food products contaminated with diarrheagenic *E. coli* has been reported in Nigeria but no case has been associated with the ingestion of contaminated lettuce (Okeke *et al.,* 2010). This however, can be attributed to the lack of accurate means of gathering data in Nigeria, as it is difficult to quantify or epidemiologically link infections caused as a result of the consumption of food contaminated with pathogenic *E. coli* to any source.

Generally, most infections from diarrheagenic *E. coli* have been related to the ingestion of food from non-bovine origin, but recently the cases reported from the infections caused by the consumption of vegetables like lettuce continue to rise. As such, this study was done in order to identify the possible presence and characterize *E. coli* present in ready to eat lettuce. The results obtained show that all the samples were positive for *E. coli* based on the initial results obtained on MacConkey agar and SMAC. Consequently, this gave rise to suspicions of the presence of pathogenic *E. coli* in the lettuce samples. Upon further characterization using biochemical tests, all samples showed characteristics which are all typical characteristics exhibited by *E. coli*. The characterization proceeded further with the use of Multiplex PCR. This method, aimed at the amplification and identification of possible pathogenic pathotypes of *E. coli*, showed that 3 (F3K7, F4K10 and F4K11) out of the twelve samples reported, showed bands within the range of 150-300bp which indicates the likely presence of pathogenic *E. coli*. Specifically, the sample, F4K10 which is reported in Plate 4.2, lane 12 produced bands within the range of 200-300bp. The band produced is mostly consistent with the *vtx1* gene which is 260bp long, a gene associated with the pathotype EHEC (Persson *et al.,* 2007). Also, the sample F3K7 reported in

Plate 4.3, lane 38 produced a band between 150-200bp demonstrating the likely presence of the Porcine *estA* gene which is 160bp long. This gene is characteristic to the pathotype ETEC (Persson *et al.,* 2007). The sample F4K11 also produced a band within the range of 150-200bp pointing to the pathotype, ETEC (Persson *et al.,* 2007). These pathotypes identified in the lettuce samples serve as a major hazard to humans as ETEC could result in the secretion of enterotoxins which leads to watery diarrhea (Mirhoseini *et al.,* 2018). EHEC on the other hand results in the secretion of shiga toxins also known as verocytotoxins, which are toxins capable of causing bloody diarrhea (Hemorrhagic colitis) in humans (Kaper *et al.,* 2004). This should be regarded as a public health issue based on the severity. It should also be noted that this study has added to the claims that lettuce could serve as a vehicle for the transfer of pathogens

The likely source of contamination of these lettuce samples could have occurred at various points of cultivation; from the use of uncomposted manure, contaminated irrigation water or general farm management practices like other studies have reported (Buck *et al.,* 2003). However, it was observed upon sampling that the irrigation water utilized on the farm was from a canal close to the farm. In addition, refuse dump was identified close to the canal. The presence of this refuse dump is a fundamental factor to the likely presence of pathogenic *E. coli* in the lettuce 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 fresh manure was applied to the farm during week three of sampling, and based on the results of the study, the positive isolates were from samples obtained in week three and four of sampling. It is possible that the manure used was not treated properly causing contamination.

This study clearly shows the significance of data collection, location of farms and farm management practices in the transmission of pathogenic *E. coli* from lettuce to humans.

CHAPTER FIVE
CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

The results obtained from this study indicates the possibility of contamination of lettuce plants by diarrheagenic *E. coli*. In this study two pathotypes of *E. coli* were also characterized which leads to the understanding that the risk associated with the consumption of ready to eat lettuce from the lettuce farm in Ogudu, Lagos State is very high. Importantly, these lettuce samples continue to be sold in the market posing great hazard to unassuming consumers. Consumption of these lettuce plants could lead to infections resulting from the activities of ETEC and EHEC when ingested.

5.2 RECOMMENDATIONS

In view of the results obtained from this study, it is recommended that: There should be more effort into the collection, report and storage of data from individuals suffering from diarrheagenic infections. Accurate data would allow epidemiologists draw epidemiologic links between other similar infections, enhancing the ability to trace these infections to a particular source. In addition, this would lead to investigations that accurately isolate and characterize the causative agent.

Governmental agencies concerned with food safety should also implement stricter policies for farm owners around Nigeria concerning location, farm management practices and farming necessities.

Consumers should also be educated on the possibility of infections from contaminated lettuce plants. This would also urge consumers to take extra precaution such as thorough washing before consumption of vegetables like lettuce.

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