PREVALENCE OF SHIGA TOXIN PRODUCING *E. COLI* IN FRESH PRODUCE FROM ROAD SIDE FRUIT VENDORS AROUND LAGOS AND OGUN STATE.

By

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A PROJECT SUBMITTED TO THE DEPARTMENT OF BIOLOGICAL SCIENCES, COLLEGE OF BASIC AND APPLIED SCIENCES,

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DECLARATION

I hereby declare that this project was carried out by me and is a report of my own research work. It has not been presented in any previous application for a higher degree of this or any other University. All citations and sources of information are clearly acknowledged by means of reference.

………………………………………….. ………………………………

KAREEM MISTURA OLUWATIMILEYIN Date

CERTIFICATION

This is to certify that the content of this project entitled '**Prevalence of Shiga Toxin producing *E. coli* in fresh produce in Lagos and Ogun State**' was prepared and submitted by **KAREEM MISTURA OLUWATIMILEYIN** in partial fulfillment of the requirements for the degree of **BACHELOR OF SCIENCE IN MICROBIOLOGY**. The original research work was carried out by her under my supervision and is hereby accepted.

____________________________ ____________________________

Dr. O. E Fayemi Supervisor Date

____________________________ ____________________________

Dr. O.T. Kayode (Head of Department) Date

DEDICATION

I dedicate this work to the Almighty God, the source of knowledge, wisdom, understanding and Life. I also dedicate this work to my supervisor Dr. O. E. Fayemi and also Dr. G.B Akanni and Ms Joy and also to my parents Mr & Mrs Kareem for their support.

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ABSTRACT

Fresh produce constitutes a necessary part of the human diet, it provides essential vitamins, minerals, and fiber. However, the rising consumption of fresh produce has added to an elevated number of illness outbreaks around the world. Fresh produces are often consumed uncooked, therefore; there is increased risk if contaminated with pathogenic microorganisms like *E. coli* O157:H7 causing foodborne illness and in extreme cases death especially in children. In developing countries like Nigeria, fresh produce is purchased both in closed stores and road side open kiosk and on most occasions are not processed hygienically. This study evaluated the prevalence of Shiga toxin producing *E. coli* in varieties of fresh produce (Lettuce, Water Melon, Pineapple, Cucumber, Cabbage, Carrot and Pawpaw) from various roadside fruit vendors at different locations around the South-west region of Nigeria precisely Lagos and Ogun state. A total of 64 samples were tested for presence of *E. coli* using Sorbitol MacConkey Agar and MacConkey Agar plates. All samples had presumptive STEC, molecular identification of selected isolates (n = 21) for STEC virulence genes (stx_1 stx_2 and eae) using multiplex PCR was done. The total viable count (TVC) for presumptive STEC in the samples was in the range of 4.5 to 7.8 log_{10}cfu/g. Lettuce from Ibafo had the highest total viable count. Using Multiplex PCR, stx1 gene was detected in some of the isolates which confirmed the presence of STEC in the fresh produce. The prevalence of STEC among the isolates (n = 21) was 4.8%. The presence of STEC and a very high microbial count of presumptive STEC poses a threat to public health which could lead to foodborne illnesses including hemorrhagic colitis (HC) or hemolytic uremic syndrome (HUS).

Key words: STEC, fresh produce, food borne disease, virulence gene, HUS.

CHAPTER ONE

1.0 INTRODUCTION

Freshly cut produce are “any fresh fruit or vegetable or any combination thereof that has been minimally processed” (IFPA, 2001). Freshly-cut produce offer consumers a ready-to-eat, affordable, convenient, nutritious and fresh-like tasting food product and does not need much processing before consumption. Fresh produce consumption has expanded dramatically as a result of its numerous nutritional and functional benefits in past years (Liu, 2003). Fresh vegetables and fruits are key sources of vitamins like vitamins B, C, and K, as well as minerals like calcium, potassium, and magnesium, and also dietary fibre (Yahia *et al*., 2019). However, Fresh produce has been connected to outbreaks of foodborne illness, implying the presence of virulent bacteria such Shiga toxin-producing *Escherichia coli* (STEC), Listeria monocytogenes, Salmonella and others (Callejón *et al*., 2015). There have been several outbreaks of foodborne illness linked to fresh produce, the most notable of which include sprouting seeds, tomatoes, and leafy greens. (Doyle and Erickson., 2008).

Fresh produce contamination can occur throughout the pre-harvest, harvest, postharvest, transportation and during processing stages including washing with water that has been contaminated. Since, fresh fruits and vegetables are frequently eaten uncooked, there is a higher risk of infection if pathogens are present. Across the globe, outbreaks of illness linked to fresh produce have been connected to a wide range of bacteria, viruses, and parasites, despite their physiological differences, these bacteria share similar characteristics. The majority of foodborne pathogens connected with fresh produce come from enteric habitats. (Harris *et al*., 2003).

1.1 STATEMENT OF PROBLEM

Fresh produce is a ready-to-eat food products that doesn’t go through vigorous processing before consumption, as a result of this, it poses serious health risk to consumers if contaminated with pathogens. Consumption of fresh produce contaminated with pathogenic microorganisms will cause foodborne illnesses and if not treated immediately can lead to death especially in children. Foodborne illness will also affect consumers economically because of the amount of money that will be spent on prescribed drugs and hospital bills for the illness.

1.2 JUSTIFICATION OF THE STUDY

It is important to investigate the microbiological safety of road side fresh produce to know the level of pathogens present in it and identify the most prevalent microorganism. Samples of

various fresh produce from different locations would be examined for the presence of STEC in them which if present poses the risk of mortality due to consumption of the fresh produce.

1.3 OBJECTIVES OF THE STUDY

- To isolate and identify pathogenic STEC in fresh produce sold by road-side vendors.
- To determine the possibility of survival of pathogenic bacteria in fresh produce samples.
- To determine the presence of virulence genes/ factors in the associated STEC.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 FRESH PRODUCE IMPORTANCE

Fresh Produce is a general term for a variety of farm-grown Fruits and vegetables. Fruits and vegetables are high in vitamins, particularly vitamins C and A; minerals, particularly electrolytes; and, lately phytochemicals, particularly antioxidants, and are proposed as a source of dietary fiber (Slavin *et al.,*2012). Fresh fruits and vegetables contribute to a nutritious diet, as well as the prevention of chronic illnesses such as heart disease, cancer, diabetes, and obesity, and several micronutrient shortages, particularly in developing nations (Septembre-Malaterre *et al*., 2018).

Previous research has linked an increased intake of fruits and vegetables to a lower risk of death from cardiovascular disease, with an average reduction in risk of 4% for each additional serving of fruit and vegetables consumed per day. (Wang *et al*., 2014). Green leafy vegetables like lettuce and spinach were found to be the most strongly linked to a lower risk of cardiovascular disease. (Hung *et al*., 2004). (Appel *et al*., 2005) revealed that eating a diet rich in fruits and vegetables lowers blood pressure even more than replacing some of the carbohydrate with healthy unsaturated fat or protein. A daily diet of 400 grams of fresh vegetables is recommended by the World Health Organization. Fruits and vegetables add nutrients to your diet that help protect you from heart disease, stroke, and some cancers (WHO*,* 2003).

Fresh produce consumption has significantly increased since the 1980s, due to consumer demand for a healthy diet and more nutritious foods, particularly in high-income countries (WHO*,* 2003). However, Fruits and vegetables, particularly fresh leafy greens, are rapidly becoming recognized as important carriers for the transmission of human pathogens formerly linked to animal-derived foods. (Berger *et al*., 2010). Foodborne disease caused by consuming contaminated fresh produce is a regular occurrence that has serious health consequences as well as huge economic and social consequences. (Alegbeleye *et al.,* 2018). As fresh produce consumption increases, the incidence of related illnesses and outbreaks caused by microbial pathogens is bound to grow (Carstens *et al*., 2019).

2.2 MICROBIAL CONTAMINATION OF FRESH PRODUCE

The contamination of fresh produce microbes can occur through different routes from the pre-harvesting stage to its post harvesting handling. The most serious food safety concern for

fresh produce is pathogenic bacteria, followed by foodborne viruses, mycotoxins, and pesticide residues. (Van Boxstael *et al*., 2013). Several microorganisms, from various locations have been isolated from different varieties of fresh produce. According to (Berger *et al*., 2010), it was established that in the USA from 1973-1997, viruses accounted for 20% of outbreaks, parasites accounted for 16% and bacteria accounted for 60%.

Some of the human pathogens commonly linked to fresh vegetables are *Hepatitis A, Aeromonas spp., Norovirus, Cyclospora cayetanensis, Staphylococcus spp., Campylobacter spp., Clostridium botulinum, E. coli, L. monocytogenes, Shigella spp., Salmonella and Yersinia enterocolitica* (Harris *et al*, 2003). Table 2.1 shows some of the human pathogens often linked with outbreaks induced by fresh produce and the nature of the illnesses. The organism, produce item, and environmental circumstances in the farm and afterward, including storage conditions, can influence pathogen survival and/or growth on fresh produce. Pathogens will generally survive but not thrive on the unharmed exterior surface of fresh fruits and vegetables, due to the protective nature of the plant's natural barriers (for example, cell walls and wax layers) (Harris *et al*., 2003).

Table 2.1 Human pathogens associated with fresh produce (Aworh, 2020).

Pathogen	**Nature of disease**
Norovirus	Vomiting and diarrhoea
Hepatitis A virus	Hepatitis A liver infection
Enteropathogenic *Escherichia coli* (EPEC)	Diarrhoea
Campylobacter spp	Inflammatory and sometimes bloody diarrhoea, Guillain-Barre syndrome
Shiga toxin-producing *E. coli* (STEC) e.g. O157:H7 and other non-O157 STEC	Diarrhoea, haemolytic uremic syndrome, end-stage renal disease
Staphylococcus aureus	Acute intoxication
Shigella spp	Bloody Diarrhoea
Yersinia enterocolitica	Diarrhoea
Cyclospora cayetanensis	Diarrhoea
Salmonella typhi	Typhoid fever, liver abscesses and cysts
Listeria monocytogenes	Sepsis, central nervous system infection, neurological sequelae

2.3 *Escherichia coli*

Escherichia coli (*E. coli*) is a bacterium that belongs to the Enterobacteriaceae family and was named after German paediatrician Theodor Escherich (Ewing, 1986). It is the most common facultative anaerobe found in humans and warm-blooded animals' large intestines (Conway, 1995). They are gram negative rods with a length of up to 3 µm that ferment glucose and a variety of carbohydrates. On Macconkey agar, these lactase fermenters create pink colonies. Certain strains of *E. coli* have haemolytic activity on blood agar. coli. It has peritrichous flagella and is frequently fimbriate (Jay, 2000). They can be motile or nonmotile, producing lateral instead of polar flagella when motile. Many strains develop other appendages besides flagella, such as fimbriae or pili, which are proteinaceous structures (or appendages or fibres) that move freely from the bacterial surface and aid in adhesion to other cells or host tissues (Desmarchelier and Fegan, 2016).

majority of *E. coli* strains acts as normal flora, and inhabit the gastrointestinal tracts of *E. coli* by using humans and animals. However, certain strains of *E. coli* have developed into pathogenic *E. coli* by using plasmids, transposons, bacteriophages, and/or pathogenicity islands to acquire virulence factors. Serogroups, pathogenicity mechanisms, clinical signs, and virulence factors can all be used to classify pathogenic *E. coli* (Nataro and Kaper, 1998). *E. coli* strains engaged in diarrheal disorders are one of the most important of the different etiological agents of diarrhoea, with strains evolving by acquiring a specific set of features that have successfully survived in the host through horizontal gene transfer (Kaper *et al*., 2004). Pathotypes of *Escherichia coli* linked to diarrhoeal illnesses have attracted people's interest all over the world.

2.4 Classification of pathogenic *E. coli*

The pathotypes of *E. coli* that cause diarrhoea are known as diarrheagenic *E. coli* (DEC) (Nataro *et al*., 1998). The DEC pathotypes are classified as enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), and enterhemorrhagic (Shiga toxin producing) *E. coli* (EHEC/STEC) based on their preferred host colonization sites, virulence mechanisms, and signs and symptoms and consequences (Todar, 2011). Enterotoxigenic (ETEC), enteroinvasive (EIEC), enteropathogenic (EPEC), and Vero cytotoxigenic (VTEC) are the virulence types of *E. coli* (Percival *et al*., 2014).

Another pathotype, the diffusely-adherent *E. coli* (DAEC) pathotype, has been reported, which consists of strains that stick to epithelial cells in a disseminated pattern (Nataro *et al*., 1998). Furthermore, certain *E. coli* strains categorized as the adherent invasive *E. coli* (AIEC) pathotype are one of the possible Crohn's disease agents (CD). CD is an inflammatory bowel disease (IBD) believed to be caused by a combination of variables (genetics, intestinal microbiota, environment, and enteric infections) (Rolhion and Darfeuille-Michaud, 2006).

Table 2.2: Intestinal pathogenic *E. coli* pathotypes defined on the basis of the presence of specific virulence genes (genetic identifiers), virulence characteristics and symptoms/illness/disease (Kostas et al., 2019).

Pathotype	**Colonization site**	**Defining virulence genes/factors**	**Symptoms/illness/disease**
Shiga toxin-producing *E. coli* (STEC)	Distal, ileum, colon	stx^+	Mild to severe bloody diarrhea through to HC, HUS and thrombocytopaenia
Enteropathogenic *E. coli* (EPEC)Typical (tEPEC)	Small intestine	eae^+, $bfpA^+$	Profuse watery diarrhea especially in children < 5 years old
Atypical (aEPEC)	Small intestine	eae^+	Profuse watery diarrhea especially in children < 5 years old
Enterotoxigenic *E. coli* (ETEC)	Small intestine	CFAs, LT, ST	Acute watery diarrhea (< 5 years old)Traveller's diarrhea
Enteroinvasive *E. coli* (EIEC)(Shigella)	Colon	Ial^+, $IpaH^+$	Shigellosis/bacillary dysentery
Enteroaggregative *E. coli* (EAEC)	Small intestine and/or colon	$aatA^+$, $aggR^+$,	Persistent diarrhoeaTraveller's diarrhea
Diffusely adherent *E. coli* (DAEC)	Intestine	$afaC^+$	Acute watery diarrhea in children. Speculated to contribute to Crohn's disease in adults
Adherent invasive *E. coli* (AIEC)	Small intestine	Uncharacterised	Speculated to contribute to Crohn's disease in adults

2.5 SHIGA TOXIN PRODUCING *Escherichia coli* (STEC)

Shiga-toxin-producing *Escherichia coli* (STEC) are a genus of Gram-negative bacteria that live in the guts of ruminant animals like cows and sheep as normal microflora. STEC colonization causes no symptoms in these animals, but it can result in severe illness in people (Kintz *et al*., 2017). Shiga toxins (Stx) are produced during STEC infections and are named after the toxin produced by *Shigella dysenteriae* serotype 1 (*Shigella dysenteriae*). Because of their cytotoxicity for Vero cells, these are also known as verocytoxins (VT) (Kostas *et al*., 2019).

STEC are zoonotic pathogens that are transmitted to humans through contaminated food, particularly meat products, water, as well as direct animal contact (e.g. farm visits), environmental contamination, and human-to-human transmission via the faecal-oral route (Kostas et al., 2019). Shiga-toxin-producing (enterohemorrhagic) Escherichia coli (STEC/EHEC) are a major cause of acute gastrointestinal disease and a major cause of morbidity and mortality in industrialized countries, with serotype O157:H7 being the most common and severe infection (Kaper et al., 2014).

When compared to other bacterial causes of gastroenteritis, STEC is linked to more severe diseases and complications (Hall *et al*., 2008). STEC outbreaks are commonly characterized by symptoms such as vomiting, abdominal pain, and diarrhea, which can proceed to haemorrhagic colitis. About 10% of cases develop to haemolytic uremic syndrome (HUS), which is marked by anaemia, renal failure, and low platelet counts (Gould *et al*., 2009), and about 30% of confirmed cases necessitate hospitalization (Gould *et al*., 2009) (Byrne *et al*., 2015).

The feature that distinguishes STEC from other classes of pathogenic *E. coli* was described by (Konowalchuk and Speirs,1977) specifically the production of a toxin that has an irreversible cytopathic effect on Vero (African green monkey kidney) cells. Though it is now established that STEC strains from a wide range of serotypes can cause significant human disease, O157:H7 is the most common STEC serotype in many regions of the world and has historically been the one most frequently connected with major outbreaks (Kim *et al*., 2001).

Serotype O26, O103, O91, O146, and O145 are the most common non-O157 serogroups associated with human illness in Europe, while O26, O45, O103, O111, O121, and O145 are the most common non-O157 serogroups associated with human illness in the United States (Brooks *et al*., 2005). The presence of any of these STEC serogroups in food has been believed to indicate the possibility of serious illness if the food is eaten without being treated to *kill E. coli.*

The major virulence component of STEC is Shiga toxin (Stx), which is encoded by the stx gene (CDC, 2012). The stx toxin genes are carried by lambdoid bacteriophages incorporated into the *E. coli* genome, and there are two forms of shiga toxins (Stx1 and Stx2). chromosomal of *E. coli* Stx1 has four subtypes (a, c, d, and e), while stx2 has twelve (a to l). The presence of the stx2 gene has been associated to the emerging of bloody diarrhoea and HUS (Ethelberg *et al*., 2004). Many STEC are attaching and effacing (A/E) bacteria, meaning they have the eae gene on the locus of enterocyte effacement (LEE) and cause unique lesions on the surfaces of intestinal epithelial cells(enterocytes). Furthermore, the outcome of infection is influenced by a number of strain and host characteristics (Kostas *et al*., 2019). It is known that once Shiga toxins enter the bloodstream, they can induce HUS, a triad of symptoms that include thrombocytopenia, microangiopathic haemolytic anaemia, acute renal failure (Smith *et al*., 2014), and/or changes in the central nervous system (Rivas *et al*., 2016).

The name “Shiga toxin” is gotten from Kiyoshi Shiga, a Japanese microbiologist who was the first to describe the bacteria S. dysenteriae in 1898 (Felsenfeld, 1957). These bacteria produce a toxin that is physically and antigenically similar to *E. coli*'s Shiga toxin 1 (Stx1). Shiga toxins are an AB5 toxin type that consists of a monomeric, enzymatically active A subunit that is non-covalently linked to a pentameric B subunit that attaches to the glycosphingolipid globotriaosylceramide (Gb3, also known as CD77 or Pk blood group antigen), the cell's unique binding site (Johannese *et al*., 2010). Stxs are created during colonization and proliferation) and are released as free proteins expelled from the Gram-negative cell wall's periplasmic region or encased in outer membrane vesicles released by the bacteria (Bauwens *et al.,* 2017).

The pertussis and diphtheria toxins, as well as the cholera toxin family, are all members of the AB class of bacterial toxins, while Shiga toxins appear to have a different evolutionary relationship with them (Ling *et al*., 1998). Shiga toxins are members of the ribosome-inactivating protein family. Shiga toxins are divided into two immunologically distinct types, Stx1 and Stx2, which have the same physiology but cannot be neutralized by heterologous antibodies due to their only 50% homology and ten subtypes (Stx1a, Stx1c, Stx1d, and Stx2a to Stx2g). Each subtype is then separated into variations by one or more amino acid differences from the prototype (Scheutz *et al.,* 2012). At the nucleotide sequence level, Stx1c and Stx1d are 91–95 % identical to Stx1. The prototypic Stx2 (also known as Stx2a) and its other variants Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g are only around 56% similar to Stx1 at the nucleotide sequence level (Christian, 2020).

The causal agent of edema in piglets, Stx2e, is the most distantly related to the other Stx2 variants in terms of protein sequence, biological activity, and receptor usage. (DeGrandis *et*

al.,1989). Stx1, Stx2, and Stx2 variant genes are encoded in the STEC genome, which is part of the lambdoid bacteriophage genome, because STEC strains frequently contain numerous stx-converting phages, they can produce a variety of toxin kinds (Schmidt, 2001).

2.5.1 Sources and routes of infection of STEC

STEC strains, especially those belonging to serotype O157:H7, had long been thought to be mostly found in cattle. According to epidemiological research, STEC strains have been found in the gastrointestinal tracts of numerous domestic animals, including sheep, pigs, cattle, dogs, and cats (Beutin *et al*., 1993). Serological research has shown that the vast majority of cattle have been exposed to STEC at some stage during their life (Clarke *et al*.,1994). As a result, raw milk consumption (Adams *et al*., 2019) is recognized as a possible source of STEC infection in humans, but environmental contamination of drinking water and vegetables, direct contact with animals, and person-to-person propagation have also been identified as important modes of transmission (Karch *et al*., 1999).

2.5.2 Pathogenesis of STEC

STEC stays in the intestine after ingestion, colonizing and attaching to the gut epithelium of the distal ileum and colon. Resistance to stomach acidity is a significant property of STEC strains that may affect their ability to colonize the human gut, particularly at low infectious doses (Paton and Paton, 1998). The infectious dosage for some STEC strains (O111:H2 and O157:H7) is estimated to be in the range of 1 to 100 CFU (Griffin *et al*., 1994). Fimbriae provide the initial attachment, after which a filamentous type III secretion system (T3SS) injects effector proteins (Esp proteins) (Gaytan *et al*., 2016). Injection of the translocated intimin receptor (Tir), which incorporates into the host cell's plasma membrane and communicates with the outer membrane protein intimin of the bacteria, causes bacterial adhesion to the host cell and effacement of the brush border microvilli (Erdem *et al*., 2007). The connection between intimin and Tir causes the bacteria to create an intimate attachment, which causes actin polymerization as well as the continuous formation of attaching and effacing (A/E) lesions (Kenny *et al*., 1997). On the chromosomal "locus of enterocyte effacement" (LEE) pathogenicity island, the genes for Tir, intimin, and the T3SS are located (Kostas *et al*., 2019). Following attachment, the STEC produces Shiga toxin, which attaches to the host cell's Gb3 or Gb4 receptor. The toxin then penetrates the cell and inhibits protein synthesis, potentially resulting in cell death (Legros *et al*., 2018).

2.5.3 Clinical symptoms of STEC

STEC has a 72–120-hour incubation period (Smith *et al.*, 2014). Many STEC-infected patients experience watery diarrhoea at first, but this can quickly progress to bloody diarrhoea (Thorpe, 2004). Severe abdominal discomfort is also a common complaint. STEC infection can lead to HUS, a life-threatening condition marked by a triad of acute renal failure, microangiopathic haemolytic anaemia, and thrombocytopenia (Smith *et al.*, 2014). Some people with HUS experience neurological issues such as drowsiness, severe headache, convulsions, and encephalopathy (Oakes *et al.*, 2006). HUS affects people of all ages; however, it is more common in infants, young children, and the elderly. It is, in fact, a leading cause of acute renal failure in children (Noris and Remuzzi, 2005). The age distribution of HUS could be due to young children's immunological naivety and the elderly's deteriorating immune system function (Karmali, 1989).

2.5.4 Haemolytic uraemic syndrome (HUS)

The Shiga toxin-producing Escherichia coli-associated hemolytic uremic syndrome (STEC-HUS) is a type of thrombotic microangiopathy defined by a triad of symptoms: thrombocytopenia, mechanical hemolytic anaemia with schistocytosis, and ischemic organ destruction (George and Nester, 2014). It is caused by a Shiga toxin-producing *E. coli* infection in the gastrointestinal tract, and is referred to as "typical" HUS, as opposed to "atypical" HUS, which is caused by a malfunction of the alternative complement pathway, and "secondary" HUS, which is as a result of a variety of co-existing illnesses. The kidney and brain are the organs most prone to STEC-HUS (Trachtman *et al.*, 2012), however other organ involvements have been documented and should be taken into account while evaluating STEC-HUS patients. The bulk of patient deaths are caused by neurological issues (Oakes *et al.*, 2006). Coma and seizures are the two most prevalent neurologic symptoms. STEC-HUS seldom recurs, and patients generate antibodies that may be protective in part (Karmali *et al.*, 2003).

In STEC-HUS patients, acute kidney injury (AKI) can vary from asymptomatic urine sediment abnormalities to severe renal failure and renal disease at the end-stage. Proteinuria is usually mild when accompanied by haematuria and leukocyturia (Siegler *et al.*, 1991).

For O157:H7 infection, however, the percentage of individuals whose case is complicated by HUS is around 10% (Gould *et al.*, 2013), and it varies based on the patient and strain characteristics. During the course of STEC-HUS, some patients need renal replacement therapy (RRT), and 15% of children experience hypertension (Siegler *et al.*, 1991).

2.6 FACTORS CONTRIBUTING TO THE CONTAMINATION OF FRESH PRODUCE

There are multiple routes and causes of food contamination, and extensive research has been conducted to fully comprehend the methods by which infections are transferred into fresh produce (Kotzekidou *et al*., 2016). For different agricultural zones, the sources and channels of produce contamination varies (Strawn, 2013). Pathogenic bacteria can infect fruits and vegetables while they are growing in fields or orchards, or during harvesting, postharvest handling, processing, transportation and marketing (Luna-Guevara *et al*., 2019). Contamination sources can be grouped into two categories: preharvest and postharvest sources of contamination (Gil *et al*., 2015). Table 2.3 shows the factors before harvest and after harvest that contributed to the contamination of fresh produce.

Table 2.3: Factors contributing to the contamination of fresh produce. (Beuchat and Ryu, 1997; Steele and Odumeru, 2004; Johnston, 2006; Beuchat, 2006)

Pre-harvest	Post-harvest
• Soil • Irrigation water • Untreated manure • Air (dust) • Wild and domestic animals (including fowl and reptiles) • Insects • Human handling	• Human handling (workers, consumers) • Harvesting equipment • Transport containers (field to packing shed) • Wild and domestic animals (including fowl and reptiles) • Insects • Air (dust) • Wash and rinse water • Sorting, packing, cutting, and further processing equipment • Ice • Transport vehicles • Improper storage (temperature, physical environment) • Improper packaging (including new packaging technologies) • Cross-contamination (other foods in storage areas)

2.7 OUTBREAKS INVOLVED WITH FRESH PRODUCE AND STEC

As indicated by the isolation of these species from various fruits and vegetables, several pathogenic bacterial species are predominantly responsible for the contamination of fruits and vegetables (Zilelidou *et al*., 2015). According to the Centre for Science in the Public Interest (CSPI), from 1998 to 2007, there were 4,638 outbreaks (117,136 cases) of foodborne illnesses in the United States. Vegetables were responsible for 33% (228 outbreaks) and roughly 50% (345 outbreaks) of these outbreaks (CSPI, 2009). In 1995, *E. coli* O157:H7 was found in 40 individuals in the United States (13 of whom were hospitalized), and 70% of these patients said they had eaten leaf lettuce (Ackers *et al*., 1998). In 1999, 72 cases of *E. coli* O157:H7 poisoning were reported in the United States. (Wachtel and Charkowski, 2002).

Table 2.4: Produce related foodborne outbreaks associated with Shiga toxin producing *E. coli* between 1997 and 2018. (Alegbeleye et al., 2018) (Iwu and Okoh , 2019).

Food vehicle	Country	Pathogen	Number of cases	Number of hospitalizations	Deaths
Sprouts (Alfalfa, raw clover)	US	*Escherichia coli*	59	17	0
Radish sprout	Japan	*E. coli* O157:H7	126	Not Specified	13
Lettuce (romaine)	Canada	*E. coli* O157:H7	29	Not Specified	Not Specified
Lettuce, cucumber	UK	*E. coli* 096	50	Not Specified	Not Specified
Leafy green	USA	*E. coli* 0157:H7	25	9	1
Alfalfa sprouts	*USA*	*E. coli* 0157:H7	11	2	0
Raw clover sprouts	USA	*E. coli* 0157:H7	19	8	0
Ready-to-eat salads-	USA	*E. coli* 0157:H7	33	7	0
Imported cucumber	*USA*	*E. coli* 0157:H7	84	17	0
Vegetable sprouts	*Europe*	*E. coli* O104:H4	3911	Not specified	47

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 SAMPLE COLLECTION

Samples of seven different fresh produce, (Lettuce, Cucumber, Pineapple, Watermelon, Carrot, Cabbage, Pawpaw) were gotten randomly from road side fruit vendors from different locations around Lagos State and Ogun State. The fresh produce samples were then taken immediately to the laboratory in sterile polyethylene bags and separately for microbial analysis.

Table 3.1 Fresh produce samples and their corresponding location

Fresh produce sample	Location		
Lettuce (*n* = 9)	Jakande (L) 3	Ibafo (O) 3	Magboro (O) 3
Cabbage (*n* = 9)	Yaba (L) 3	Ibafo (O) 3	Magboro (O) 3
Pine apple (*n* = 9)	Magodo (L) 3	Ibafo (O) 3	Magboro (O) 3
Water melon (*n* = 9)	Magodo 3	Yaba (O) 3	Magboro (O) 3
Cucumber (*n* = 9)	Jakande(L) 3	Ibafo (O) 3	Magboro (O) 3
Carrot (*n* = 9)	Yaba (L) 3	Ibafo (O) 3	Magboro (O) 3
Pawpaw (*n* = 9)	Yaba (L) 3	Ibafo (O) 3	Magboro (O) 3

Key notes: (L)- Lagos state. (O)- Ogun state.

3.2 MATERIALS AND EQUIPMENT USED

Materials used include: stomacher bags, petri-dishes, measuring cylinder, glass pipettes, beakers, conical flasks, glass spreader, inoculating loop, scotch bottles, Eppendorf tubes, micro pipette (with their tips), test tubes and foil corks (with their racks), PCR tube, glass slide, oxidase test disc.

Equipment used: weighing balance, stirrer Hot plate, Autoclave, vortex mixer, water distiller, water bath (set at 50°C and 100°C), incubator (set at 37°C), Bunsen burner, oven, centrifuge, heating block, gel electrophoresis tanks, gel documentation system.

3.3 MEDIA AND REAGENTS USED

For isolation of *E. coli*: 70% ethanol, 20% Glycerol, Buffer Peptone Water, Sorbitol Maconkey Agar, Maconkey Agar, Brain Heart infusion (BHI) Broth, Nutrient Agar.

For molecular identification: 1x TAE buffer, distilled water, Nuclease free water, Ethidium Bromide.

For biochemical test: Crystal Violet, Iodine, alcohol (95%), Safranin, 3% Hydrogen Peroxide.

3.3.1 Buffer peptone water (BPW)

Peptone water is a microbial growth medium composed of peptic digest of animal tissue and sodium chloride. The pH of the medium is 7.2±0.2 at 25 °C and is rich in tryptophan. Buffered Peptone Water is a pre-enrichment medium used for food samples before selective enrichment and isolation.

Preparation

1. 1g of the dehydrated medium was dissolved in 1 litre of distilled water to make 0.1% peptone water and for 1% peptone water, 10g of the dehydrated medium was dissolved in 1litre of distilled water in a conical flask and was mixed thoroughly. The conical flask is then closed with a cotton wool that is wrapped in aluminium foil.
2. The mixture was then stirred for a while using the magnetic stirrer hot plate to dissolve the powder completely.
3. 9ml of the 0.1% was then dispensed into various test tubes for serial dilution.
4. 225ml of the 1% was then dispensed into conical flask.
5. The test tubes and conical flasks containing the media was then autoclaved at 121°C for 15mins.

3.3.2 Sorbitol-macconkey agar (SMAC)

(SMAC) Sorbitol MacConkey agar is a selective and differential media used for detecting sorbitol non-fermenting *Escherichia coli* O157: H7. It was prepared based on the manufacturer's instruction for isolation and detection of *E. coli* O157:H7. SMAC is reddish-purple in colour after preparation.

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 manufacturer's instructions in a conical flask and mixed thoroughly. The conical flask is then closed with a foil cork.

2. The mixture was stirred for a while using the magnetic stirrer hot plate to dissolve the powder completely and was then sterilized by autoclaving at 121°C for 15mins.
3. The medium was then left to cool and poured in an aseptic manner into sterile petri dishes and left to solidify.

3.3.3 Macconkey agar

MacConkey Agar is used for gram-negative enteric bacteria isolation and lactose fermentation differentiation from non-lactose fermenting bacteria and lactose fermenting bacteria but provides pink colonies on MacConkey Agar as *Escherichia coli*. The medium is neutral red in colour after preparation.

Preparation

1. The dehydrated medium was dissolved in the appropriate volume of distilled water i.e. 48.5g of MacConkey in 1 litre of distilled water based on manufacturer's instructions in a conical flask and mixed thoroughly. The conical flask is then closed with a foil cork.
2. The mixture was stirred for a while using the magnetic stirrer hot plate to dissolve the powder completely and was then sterilized by autoclaving at 121°C for 15mins.
3 The medium was then left to cool and poured in an aseptic manner into sterile petri dishes and left to solidify.

3.3.4 Nutrient agar

Nutrient agar is a general-purpose nutrient medium used for the cultivation of microbes supporting the growth of a wide range of non-fastidious organisms. It was prepared based on the manufacturer's instruction for isolation and detection of 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 with a foil cork.
2. The mixture was stirred for a while using the magnetic stirrer hot plate to dissolve the powder completely and was then sterilized by autoclaving at 121°C for 15mins.
3. The medium was then left to cool and poured in an aseptic manner into sterile petri dishes and left to solidify.

3.3.5 Brain heart infusion (BHI) Broth

BHI is recommended for the cultivation of fastidious pathogenic microorganisms.

Preparation

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 dissolve the powder completely.
3. 5ml of the media was then dispensed into various test tubes and covered with foil cork and was then sterilized by autoclaving at $121^{0}C$ for 15minutes.

3.4 ISOLATION OF *E. coli*

3.4.1 Sample preparation

Twenty-five (25g) of the samples were weighed and poured aseptically into a sterile stomacher bag containing 225ml 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 Nutrient agar, (Sorbitol MacConkey Agar) SMAC and MacConkey agar plates.

3.4.2 Serial dilution

One millilitre (1ml) of the samples were pipetted using the micro-pipette (set at 1000ul) into test tubes containing 9ml of BPW (0.1%) to obtain 10^{-2}, followed by the transfer of 1ml from 10^{-2} into a new test tube (containing 9ml of BPW) to create 10^{-3} dilution, the test tubes are then put in the vortex mixer for even mixing. The dilution factor was repeated for 10^{-3}, 10^{-4}. The test tubes were labelled for easy identification.

3.4.3 Plating

After labelling the agar plates accordingly, 0.1 ml from the appropriate dilutions (10^{-1}-10^{-4}), was plated onto SMAC Agar, MAC Agar, and Nutrient Agar for the isolation of *E. coli* 0157:H7, 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 so as to maintain aseptic conditions). The plates were incubated at 35°C- 37°C for 18- 24 hours and counted.

3.4.4 Sub culturing

Sub culturing 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 on the basis of their colony morphology, shape, colour, elevation and other physical characteristics. The colonies gotten from the previously incubated SMAC plates 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 test tube and incubated at 37°C for 18- 24 hour. After incubating, 750µl of the inoculum was added into a sterile Eppendorf tube containing 750µl of sterile 20% glycerol (duplicated) which acts as a cryoprotectant and was stored in a freezer at -4 ºC.

3.5 BIOCHEMICAL TEST

3.5.1 Gram staining

A smear of suspension was formed with a loopful of the isolate on a clean slide that is grease-free. It was allowed to air dry before being heat fastened. Drops of crystal violet were poured, held for 30 seconds, and then washed using 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 Microscope.

3.5.2 Catalase Test

Using an inoculating loop, a small amount of the isolate was transferred to the surface of a clean, dry glass slide, a drop of 3% H_2O_2 was added and observed for the evolution of oxygen bubbles.

3.5.3 Oxidase Test

An oxidase disc was used. An isolated colony to be tested was picked and rubbed on the disc. It was observed for colour change within 10 seconds.

3.6 MOLECULAR IDENTIFICATION

3.6.1 DNA Extraction

Isolates from the same sample were pulled (1ml of BHI was added to a cryotube and autoclaved. 50µl of each isolate E1-E4 (*E. coli* 1-4) was added into the cryotube to activate). The pulled isolates were centrifuged at 10,000RPM for 5minutes and the supernatant was decanted, 1ml of sterile distilled water was added to the Eppendorf tube, vortexed and centrifuged again at 10,000 RPM for 5 minutes the supernatant was discarded and the process was repeated, 200µl of sterile nuclease free water was then pipetted into the Eppendorf tube containing the pellets, vortexed and then it was placed in the heating block to boil at 100°C for 15minutes, it was then placed in ice to cool, the content of the Eppendorf tube was then centrifuged finally at 14,000RPM for 5 minutes, a new set of Eppendorf tubes were labelled and the supernatant containing the extracted DNA was then transferred into the new Eppendorf tubes and they were placed in racks and stored in the freezer at -20°C for further analysis.

3.6.2 Polymerase chain reaction (PCR)

The components of the PCR used for *E. coli* identification is shown in table 3.2 below. After preparing the PCR cocktail It was placed into the thermocycler. The PCR was carried with initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 2 min; 42°C for 30 s and 72°C for 4 min; and a final elongation step at 72°C for 10 min. negative control reactions was included. For negative controls, template DNA was replaced with sterile water. The PCR products were confirmed by electrophoresis and visualized under UV light with a Gel Documentation system. Table 3.**2** shows the PCR reaction components used for 16s rRNA amplification.

Table 3.2: PCR reaction components used for 16s rRNA amplification

No.	Component	Initial concentration	Final concentration	Volume/rxn
1	Master mix	5x	1x	2µl
2	16sf	20µm	0.25µm	0.125µl
3	16sr	20µm	0.25µm	0.125µl
4	DNA			2µl
5	dH_2O			5.75µl
6	Total			10µl

Table 3.3: Protocol condition for thermal cycler

Analysis	Step	Temperature	Time
1x	Initial denaturation	95^0c	5 min
35x	Denaturation	95^0c	2 min
	Annealing	42^0c	30 sec
	Polymerization	72^0c	4 min
1x	Final polymerization	72^0c	10 min
1x	Hold	4^0c	∞

Table 3.4: Primers used for multiplex PCR for STEC

Gene	Oligonuceotide sequence (5'-3)	Size	Reference
Stx1	GAAGAGTCCGTGGG ATTACGAGCGATGCAGCTATTAATAA	130bp	Paton and Paton, 1999
Stx2	ACCGTTTTTCAGATTTTGACACATA TACACAGGAGCAGTTTCAGACAGT	298bp	Paton and Paton, 1999
EaeA	GTGGCGAATACTGGCGAGACT CCCCATTCTTTTTCACCGTCG	890bp	Paton and Paton, 1999

Table 3.5: Components used for Multiplex PCR for STEC

No.	Reagents	Initial concentration	Final concentration	Volume/rxn
1	Master mix	5x	1x	5µl
2	Stx1F	20µm	0.25µm	0.3125µl
3	Stx1R	20µm	0.25µm	0.3125µl
4	Stx2F	20µm	0.25µm	0.3125µl
5	Stx2R	20µm	0.25µm	0.3125µl
6	EaeAF	20µm	0.25µm	0.3125µl
7	EaeAR	20µm	0.25µm	0.3125µl
8	dH_20			15.125µl
9	DNA			3µl

3.6.3 Gel Electrophoresis Using Agarose

The agarose was prepared using dry agarose powder, 1.8g of the agarose powder was dissolved in 100ml of 1x 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. It is then swirled and left to cool but not solidify, the content of the flask is then transferred into the gel container with the combs in place, after, it is left to solidify and the comb is gently removed. 1x TAE buffer is poured into the gel container. 3µl of DNA ladder as added to the fist well and 4µl of the amplicon (one sample per well) were then pipetted into each well that was formed after removing the comb. The tank is connected to the power pack and left to run at 100 volts for 45mins and the gel is viewed using the gel documentation system for results.

3.7 PRECAUTIONS

- Aseptic techniques were observed at every stage of work.
- Cross contamination of the samples was avoided.
- Ensured that the samples were always properly labelled.
- Personal protective technique was observed, such as wearing of covered shoe, nose cover, gloves, lab coat, etc.
- Ensured that the inoculating loop cooled before picking the organism when sub-culturing in order not to kill organism of interest.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 RESULTS

The microbial analysis of the fresh produce samples gotten from Lagos and Ogun state were reported. All samples had pink (non-0157) and white (O157) raised, circular and smooth colonies on SMAC and MAC. Which indicates the presence of *E. coli* in the samples.

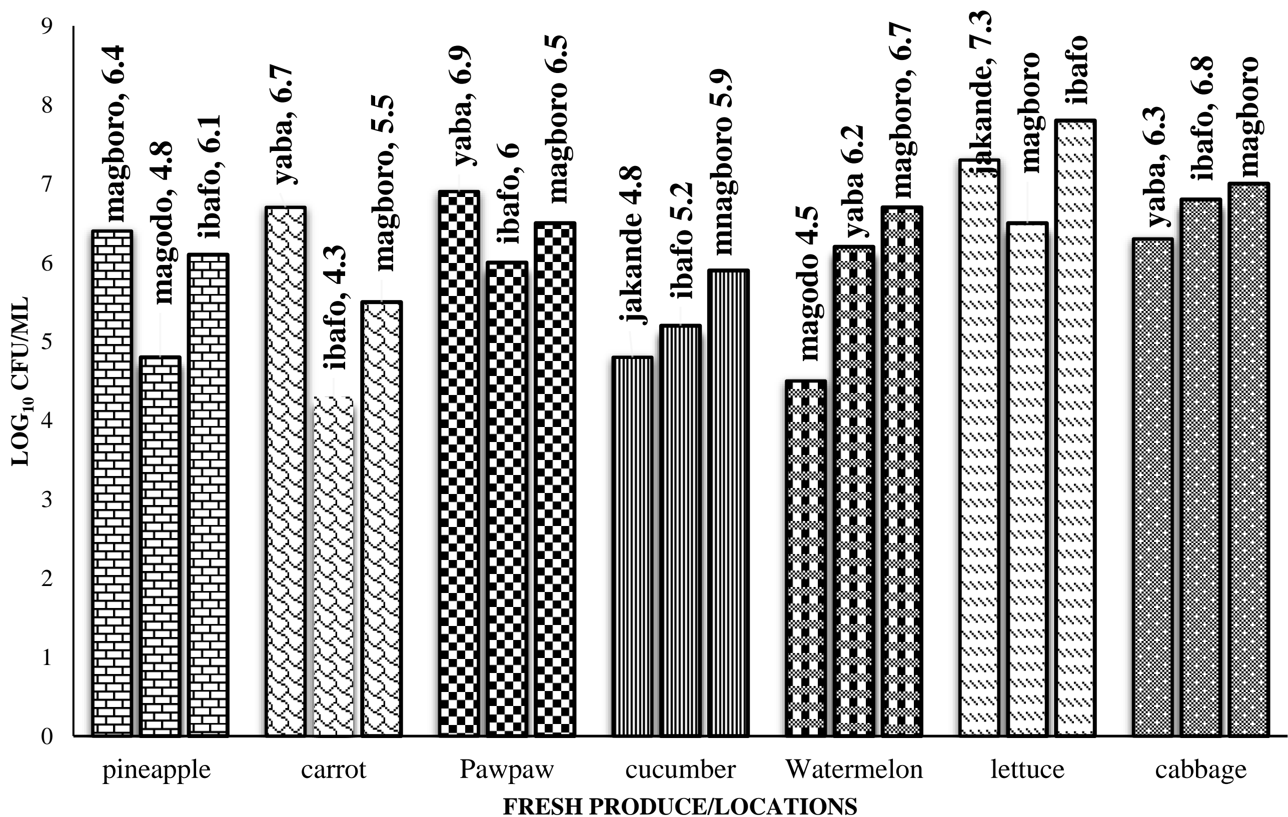
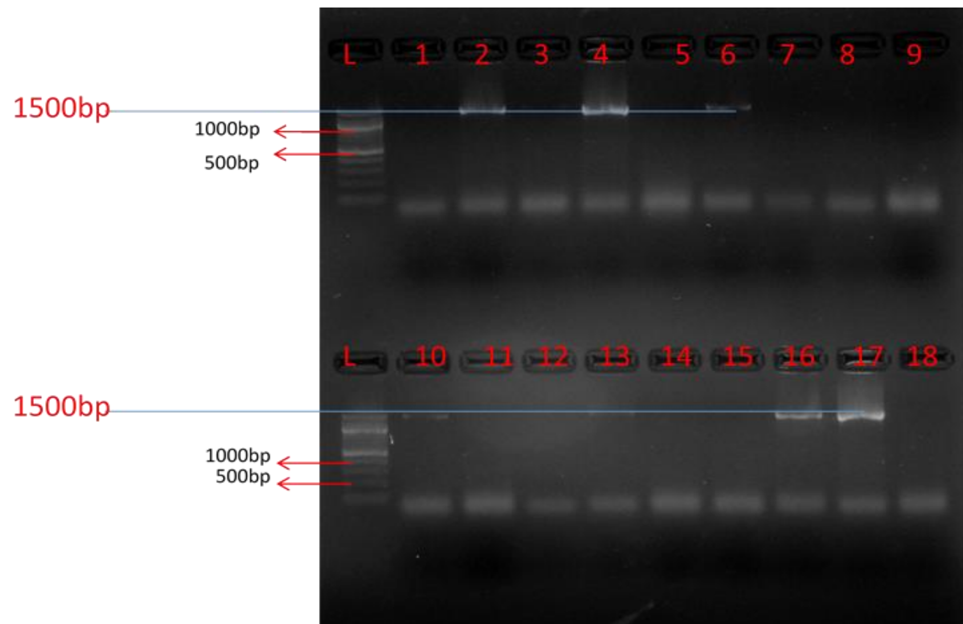


Fig 4.1: Total viable count in fresh produce from different locations in Lagos and Ogun State.

Lettuce had the highest TVC range of 6.5- 7.8 $\log_{10}$ cfu/g followed by cabbage with a TVC range of 6.3- 7.0 $\log_{10}$ cfu/g. The TVC in Pawpaw had ranged between 6.0- 6.9 $\log_{10}$ cfu/g, while, Water melon had TVC range of 4.5- 6.7 $\log_{10}$ cfu/g, lower TVC was recorded in cucumber which ranged between 4.8 and 5.9 $\log_{10}$ cfu/g.

Lettuce and carrot sample from Ibafo had TVC of 7.8log 10cfu/g and 4.3 log10 cfu/g respectively.

In Magboro, Lettuce and Pawpaw had the same count (6.5 log_{10}cfu/g) while Carrot had the lowest count of 5.5 log_{10}cfu/g. In Magodo the TVC difference wasn't significant. Pineapple and watermelon had 4.8 log_{10}cfu/g and 4.5 log_{10}cfu/g respectively. In Jakande lettuce had a significantly higher TVC of 7.3 log_{10}cfu/g, while cucumber had TVC of 4.8 log_{10}cfu/g.



16S RDNA for E.COLI 38-58 no 51
52 53

Figure 4.2 Agarose Gel electrophoresis of PCR amplicon for (16 rRNA amplification)

For Multiplex PCR

21 isolates were randomly picked for further examination by PCR for the detection of stx_1, and/or stx_2 and eae genes. It showed that one isolate (Magboro Watermelon) contained stx_1 gene (130 bp), which makes it Shiga toxin producing *E. coli* positive. Agarose gel electrophoresis of PCR products for *eaeA*, stx_1, stx_2 and are shown in figure 4.2

Figure 4.3 Agarose gel electrophoresis for Multiplex

FOR BIOCHEMICAL TEST: all samples had the same results

Isolate	Biochemical tests	Result and observation
All isolates	Catalase test	**Positive-** presence of bubbles
	Gram staining	Gram negative positive (red colour)
	Oxidase test	Negative- no colour change observed

4.2 DISCUSSION

This study reported the isolation of STEC in fresh produce sold by the road side using selective medium SMAC, PCR amplification using 16s, stx1, stx2, eaeA forward and reverse primers. Total viable count was a great indicator of the microbiological quality of the produce examined, which ranged from 4.3 to 7.0 log10cfu/g, indicating that all of the samples had a high microbial load that was greater than the World Health Organization's standard bacterial count (WHO, 2006).Hazard Analysis and Critical Control Points Total Quality Management (HACCP-TQM) Technical Guidelines lay down the microbial quality for raw foods (Aycicek *et al*., 2006), where produce with TVC < 10^4 are regarded as good, 10^4 - 5× 10^6 is average, 5× 10^6 - 5× 10^7 is bad and > 5× 10^7 CFU/g is regarded as spoiled food. Based on that, 66% are categorized as average, 28% are poor quality and 4.8% is spoilt.

There have been other reports of fresh produce with a high aerobic count (Viswanathantan and Kaur, 2000). The TVC of Ibafo Lettuce was the highest. The high average bacterial count is probably due to unhygienic procedures from farm to market, as well as exposure to possible microbial contamination at each stage (Heaton and Jones, 2008). According to the results, all samples had presumptive *E. coli* isolates in them. This shows the probability of an un hygienic cultivating, storing and marketing environment. *E. coli* presence in these samples suggests faecal contamination from manures, as well as poor post-harvest cleaning by processors to remove dirt and debris (Jawetz, 2007).

From the samples, 21 isolates were picked randomly for STEC genes identification by Multiplex PCR and 4.8% of the presumptive *E. coli* isolates (n=21) was positive for stx1 virulence gene. This indicates that the STEC isolated from the sample (Magboro Watermelon) is dangerous and capable of causing human infections (Abong'o *et al*., 2007). also reported that the stx1 gene had a greater incidence than the stx2 gene. Although the two proteins have comparable biological activity, the Stx1 toxin has been reported as being less cytotoxic than Stx2 in terms of clinical symptoms (Zschöck *et al*., 2000). STEC stx1 subtypes such as Stx1c tend to cause asymptomatic to mild diarrhea and Stx1e do not really affect humans, (Friedrich *et al*., 2003), nevertheless, they can be regarded as low or minimal risk, but not risk free. The low prevalence of STEC in this study's samples is similar to previous research from other areas of the world (Abong'o *et al*., 2007), which had prevalence of 10.3% and 7.3% respectively. In a fresh produce microbiological survey, Mukherjee and his co-workers could not isolate STEC from the vegetables (Mukherjee *et al*., 2004). Another study in Norway, which tried to identify and isolate STEC in lettuce did not succeed (Loncarevic *et al*., 2005).

The virulence genes that were used for this study to detect STEC isolate have been successfully used in previous researches to discover the identity of these bacteria (Khalil and Goma, 2016, Mazaheri *et al*., 2014). The presence of STEC shows that there has been a recent contamination by faecal matter and possible presence of other enteric pathogens known to be agents that causes food borne gastroenteritis and bacterial diarrhoea disease e.g. *Salmonella, Shigella, Campylobacter* etc. (Luna-Guevara *et al*., 2019). STEC can cause infections even at unnoticeable levels, because of its low infective dose therefore it is necessary that the microbial quality of these fresh produce should be known (Wilshaw *et al*.,1994). The fresh produce in these locations is displayed carelessly on top of surfaces; Sometimes fresh produce is split into halves for customer's financial affordability. In most cases the split surfaces are not covered to protect them from getting contaminated from the surrounding environments. Occasionally, fresh produce is sold packed in polyethylene bags; however, most of the time, they are not packaged and are left opened in the air thus making them to be easily contaminated by airborne pathogens. It is therefore important that the government should implement rules concerning general hygiene and proper food handling.

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

The study showed possible public health hazard related with consuming fresh produce gotten from different roadside fruit vendors in Lagos and Ogun States, Nigeria. A high microbial load and the presence of STEC in fresh produce is a risk to public health and can cause life-threatening foodborne illnesses. Therefore, Control measures aimed at preventing any practice that potentially contaminate fresh produce should be implemented to avoid any kind of contamination. Consumers should be educated about the potential risk of consuming uncooked fresh vegetables. Regulatory and educational efforts from the government officials and academic community also are needed to improve the safety of fresh farm produce that are intended for use as ready-to-eat food products in Nigeria. More precautions are needed for the processing and handling of fresh produce. The hygienic environments and handling can largely influence the cross contamination of other products not prone to contamination by STEC. Therefore, efforts should be made to control this bacterium in Nigerian food products in order to avoid illness or death as a result of eating STEC-contaminated food.

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