OCCURRENCE OF *Escherichia coli* O157:H7 ON CABBAGE, LETTUCE AND CARROT GROWN ON FARMS USING CATTLE MANURE AS FERTILIZER IN ZARIA, NIGERIA

BY

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DECLARATION

I Ernest Chinedu AJAEGBU, hereby declare that this Thesis entitled "Occurrence of *Escherichia coli* O157:H7 on cabbage, lettuce and carrot grown on farms using cattle manure as fertilizer in Zaria, Nigeria" is my personal effort and a true record of my research work in the Department of Veterinary Public Health and Preventive Medicine under the supervision of Dr. E.C. Okolocha and Prof. J.K.P. Kwaga. To the best of my knowledge, no part of this project was previously presented for another degree or diploma at this or any other institution and all sources of information have been acknowledged by means of references.

_____ _____ _____

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CERTIFICATION

This Thesis title "Occurrence of *Escherichia coli* O157:H7 on cabbage, lettuce and carrot Grown on Farms using Cattle Manure as Fertilizer in Zaria, Nigeria" meets the regulation governing the award of the degree of Masters in Science of the Ahmadu Bello University, Zaria and is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

I dedicate this work first of all to God, Almighty for giving me life, good health and all I needed to make this work a success and secondly to my dear parents, Mr. Sunday and Mrs. Love Ajaegbu for their guidance, understanding and sacrifice. It is also dedicated to my brothers, Stanley and Kingsley Ajaegbu, to my fiancé, Mercy Emmanuel and to a friend indeed Pharm. Koko Ita Ewa, for their contributions and support.

May the Almighty God bless you all! Amen

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ABSTRACT

There have been a number of outbreaks associated with *E. coli* O157:H7, most of which are the result of consumption of contaminated fresh produce. In the present study, the occurrence of *E. coli* O157:H7 on fresh produce cultivated in farms using cattle manure as fertilizer was determined. Questionnaires were administered to 18 farmers growing cabbage, lettuce and carrot to determine some of their farming practices that may contribute to contamination of these vegetables during cultivation up to the point of harvest. A total of 360 samples of cabbage, lettuce and carrot and soils were collected over a period of 3 months from 18 farms, out of which 300 samples (50 cabbage, 50 lettuce, 50 carrot and 50 each of their respective soils) were collected from farms using organic fertilizer (experimental farms) and 60 samples (10 cabbage, 10 lettuce, 10 carrot and 10 each of their respective soils) from farms using inorganic fertilizer (control farms). The samples were placed in sterile polythene bags, labeled and transported to the laboratory for analysis. These samples were enriched in modified tryptone soya broth and cultured on sorbitol MacConkey agar supplemented with cefixime and tellurite (CT-SMAC). Colourless isolates were picked and stored on nutrient agar slants. Conventional biochemical tests, Microbact 12E and latex agglutination test kit (Wellcolex) were used to screen, confirm as *E. coli* and serotype the stored isolates respectively. The results obtained showed that of the 150 vegetables and 150 soil samples collected from the experimental farms, 4 (2.7%) *E. coli* O157:H7 isolates were obtained from vegetables and 4 (2.7%) from soil. But of the 60 samples collected from the control farms, no *E. coli* O157:H7 isolate was obtained from the vegetables and soil samples. The eight *E. coli* O157:H7 isolates obtained were subjected to Enzyme Immuno-Assay (EIA) using Ridascreen verotoxin ELISA kit to test for the

production of verotoxins. The result obtained showed that of the eight isolates tested, verotoxin was detected in only two of the isolates. The antibiotic sensitivity results showed that all the isolates were resistant to at least one antibiotics especially cloxacillin but were all susceptible to gentamicin and ciprofloxacin. From the questionnaires, it was deduced that the use of cattle manure as fertilizer is a major risk factor associated with the occurrence of *E. coli* O157:H7 on cabbage, lettuce and carrot grown on such farms. The study concluded that the major risk factor associated with on-farm contamination of fresh produce is the use of cattle manure and that the use of cattle manure as fertilizer in these farms was the major source of contamination of vegetables grown on them with *E. coli* O157:H7. Farmers need to be educated on the risk associated with the use of cattle manure as fertilizer and on ways to reduce contamination of fresh produce in the farm. Adequate precautions need to be taken in the course of preparing these vegetables for human consumption. Also, there is the need to curb the abuse and misuse of antibiotics in animal production, human and veterinary medicine.

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ABBREVIATIONS AND SYMBOLS

'F'	=	Fimbrial antigen
'H'	=	Flagellar antigen
'K'	=	Capsular antigen
'O'	=	Somatic antigen
CT-SMAC	=	Sorbitol MacConkey agar supplement with Cefixime and Tellurite
DAEC	=	Diffuse-adherent *E. coli*
EAEC	=	Enteroaggregative *E. coli*
EHEC	=	Enterohaemorrhagic *E. coli*
EIA	=	Enzyme Immunoassay
EIEC	=	Enteroinvasive *E. coli*
EPEC	=	Enteropathogenic *E. coli*
ETEC	=	Enterotoxigenic *E. coli*
H-	=	Means the isolate lacks the Flagellar antigen/Non-motile
HC	=	Haemorrhagic Colitis
HUS	=	Haemolytic Ureamic Syndrome

LGAs	=	Local Government Areas
LT	=	Heat Labile toxin
MAR	=	Multiple Antibiotic Resistance
MDR	=	Multi-Drug Resistance
MR	=	Methyl Red
mTSB	=	Modified Tryptone Soya Broth
PCR	=	Polymerase Chain Reaction
SIM	=	Sulphide, Indole, Motility medium
ST	=	Heat Stable toxin
TDA	=	Tryptophan Deaminase
TTP	=	Thrombotic Thrombocytopenic Purpura
VP	=	Voges Proskauer
VT	=	Verotoxin
VTEC	=	Verocytotoxigenic *E. coli*
hrs	=	Hours
ppm	=	Parts per million
rpm	=	Revolution per minute

et al	=	And others
mm	=	Millimetres
ml	=	Millilitres
g	=	Grams
µl	=	Microlitres
µg	=	Micrograms
S/N	=	Serial Number
No.	=	Number
^{0}C	=	Degrees Celcius
+	=	Plus/positive reactions
-	=	Negative reactions
>	=	Greater than
<	=	Less than
=	=	Equals to
%	=	Percentage

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Escherichia coli are gram negative, motile, facultative anaerobic bacillus of the family *Enterobacteriaceae* and are classified into different serotypes based on their somatic and flagellar antigens (Doyle and Padhye, 1989). Most strains of *E. coli* are harmless but a small proportion can cause clinical symptoms in humans and other mammals (Kaper *et al*., 2004). The pathogenic strains of *E. coli* cause enteric diseases and are grouped into six categories which include; enterohaemorrhagic (EHEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteropathogenic (EPEC), enteroaggregative (EAEC), and diffuse-adherent *E. coli* (DAEC) (Chin, 2000). In terms of zoonoses, the most important category is the enterohaemorrhagic *E. coli*, which causes severe disease. There are typical and atypical EHEC strains belonging to a large number of serogroups. The most important of these serogroups are *E. coli* O157, O26, O111, O103, O145, O91 and O22 (Chin, 2000).

According to present knowledge, typical EHEC of serogroups O157, O111, O26 and O103 are the most virulent ones. In total, more than 200 different serovars have been described for EHEC but *E. coli* O157:H7 is the best known and therefore, often named the prototype EHEC strain (Baljer and Wieler, 1999). It is one of the most significant food borne pathogens that has gained increased attention (Riley *et al*., 1983). In the last two decades, the organism has become a global health problem; in 1996 alone, major outbreaks were

reported in Germany and Scotland; and the largest recognized outbreak, affecting approximately 5000 persons, occurred in Japan (Izumiya *et al*., 1997).

Typical illness as a result of an *E. coli* O157:H7 infection can be life-threatening and clinical symptoms shown by susceptible individuals range from mild diarrhoea and haemorrhagic colitis to complicated infections such as haemolytic uraemic syndrome, and thrombotic thrombocytopenic purpura (Law, 2000; Willshaw *et al*., 2001; Meng *et al*., 2007).

The sources of *E. coli* O157:H7 are domestic and wild animals, but ruminants are regarded as the main natural reservoir. Although *E. coli* O157:H7 has been isolated from sheep (Kudva *et al*., 1998), pigs (Chapman *et al*., 1997), deer (Fischer *et al*., 2001), and goats (Pritchard *et al*., 2000), generally, it is accepted that cattle are the primary reservoir of this pathogen (Rasmussen *et al*., 1993; Chapman *et al*., 1997).

E. coli O157:H7 is known to persist in the rumen and colon once ingested by the animal, contaminating the faeces, which subsequently acts as a vehicle in the horizontal transmission to uninfected animals (Faith *et al*., 1996). It has a worldwide distribution and is known to persist on and contaminate fresh vegetable produce at pre-harvest stage (Beuchat, 1996; Buck *et al*., 2003).

Pre-harvest contamination sources include faeces, soil, irrigation water, inadequately composted manure, environmental conditions, wild and domestic animals, insects and human handling (Beuchat and Ryu, 1997).

Manure is a natural by-product of livestock production with several possible uses (Gagliardi and Karns, 2002). A survey of 48 sites in Asia, Latin America and Africa

showed that a majority of farmers use manure as fertilizers to grow their crops (Baltenweck *et al*., 2002). Overall, an estimated 70% of total fertilizer inputs, in developing countries, are derived from animal manure (Fresco and Steinfeld, 1998). In Nigeria, about 932.5 metric tons (MT) of manure is produced annually from the well established poultry or livestock industries which keep expanding at the rate of 8% per year, with bovine manure being commonly used as fertilizer, applied either fresh (untreated) or after composting it for some time (Adejinmi, 2000).

The increased consumption of fresh, raw and sparsely cooked vegetables, coupled with the importation of fresh vegetable produce from countries where produce handling is compromised by lower sanitation standards, has increased the focus on how the microenvironment of these kinds of foods affect food safety (NACMCF, 1999). Produce can become contaminated with *E. coli* O157:H7 at any point along the path from farm to the consumer's table: during cultivation and harvesting, post-harvest handling, processing, packaging or distribution (Beuchat and Ryu, 1997). One of the recognized avenues for contamination is through the use of improperly treated manure as fertilizers (Khandaghi, *et al*., 2010). There have been a number of outbreaks associated with *E. coli* O157:H7, most of which are the result of consumption of minimally processed and contaminated salad vegetables (e.g. lettuce, cabbage, and carrot) (Cieslak *et al*., 1993; CDC, 1996; 1999; 2001). It is well documented that cattle is the major reservoir for *E. coli* O157:H7 and the use of its faeces as fertilizer in organic farming has been implicated in several food borne outbreaks of *E. coli* O157:H7 infection (Morgan *et al*., 1998).

1.2 Statement of Research Problem

As a result of nationwide and global programs which promote fruit and vegetable consumption, there has been a significant increase in the consumption of fresh vegetable produce in many countries of the world over the past two decades (Tauxe *et al*., 1997). The projection is that the consumption of fresh fruits and vegetable should increase even more in the years to come, making it essential to address produce safety (NCI, 2003).

E. coli O157:H7 is a zoonotic pathogen of public health concern in most countries of the world and it is the cause of many food borne outbreaks which have been associated with the consumption of sparsely cooked or minimally processed contaminated vegetables (Cieslak *et al*., 1993; CDC, 1996; 1999; 2001). In the United States, from 1973 to 1997, 29% of all lettuce-related outbreaks were due to *E. coli* O157:H7 and 38% of all *E. coli* O157:H7 outbreaks with fresh vegetable produce were associated with lettuce (Sivapalasingani *et al*., 2004)

Outbreaks have been known to occur in the developing world with the first reported outbreak of *E. coli* O157:H7 infection occurring in South Africa in 1992 (Effler *et al*., 2001). Ever since, according to Raji *et al*. (2003), there have been reports of outbreaks occurring in Central African Republic in 1996 and in Cameroon in 1997 (Cunin *et al*., 1999), in Uganda in East Africa, and in Egypt in North Africa. In West Africa, *E. coli* O157:H7 illness has been reported in Nigeria since 1994 (Ogunsanya *et al*., 1994; Olorunshola *et al*., 2000).

An important factor in the emergence of this pathogen in Africa has been indicated by available information on the carriage of *E. coli* O157:H7 in cattle (Effler *et al*., 2001; Renter *et al*., 2003).

Illness caused by *E. coli* O157:H7 is associated with symptoms like bloody diarrhoea and abdominal cramps with little or no fever. However, young children and the elderly are more likely to have severe symptoms like haemolytic uraemic syndrome which is a leading cause of acute kidney failure in the United States (Law, 2000; Willshaw *et al*., 2001; Meng *et al*., 2007). Cattle manure contaminated with *E. coli* O157:H7 can come into contact with produce directly through its use as fertilizers (Doyle, 2000).

It has been shown that *E. coli* O157:H7 can become internalized in lettuce by entering the plant through the root system from a planting mixture of manure and soil and migrate throughout the edible part of the plant (Solomon *et al*., 2002). Also, a study has proven that the rinsing of lettuce twice with water is unable to remove significant amounts of *E. coli* O157:H7 (Boyer *et al*., 2007).

In Nigeria, some works have been done on the isolation of *E. coli* O157:H7 from animals (Tekdek *et al*., 1995) and animal products (Olatoye, 2010), water (Chigor *et al*., 2010) and vegetables sold in markets (Enabulele and Uraih, 2009). However, there appears to be no known documented studies on the contamination of vegetables with *E. coli* O157:H7 in the farms in Nigeria.

1.3 Justification

There has been concern on pathogens in vegetables which has risen due to increasing number of outbreaks of food borne illnesses caused by consumption of fresh and minimally processed vegetables (Tauxe *et al*., 1997). Recently, a strain of verotoxin producing EAEC

(*E. coli* O104:H4) caused a serious outbreak of food borne illness in Germany (FIRA, 2011).

Urban farming is an emerging phenomenon in Nigerian cities of which vegetable cultivation is known to be the most widely practiced. This is probably because of its contribution to balanced diet such as; reduced risk for some forms of cancer, heart diseases, stroke and other chronic diseases (Hung *et al*., 2004). Cattle manure contaminated with *E. coli* O157:H7 can contaminate fresh vegetable produce directly through its use as fertilizers (Doyle, 2000).

This study is imperative because of the potential risks associated with the use of cattle faeces in farming, the reports on the persistence of *E. coli* O157:H7 in manure and its transmission through fresh vegetable produce (Doyle, 2000) as well as absence of reports on the prevalence of *E. coli* O157:H7 on vegetables grown on manure-fertilized farms in Zaria.

Hence, an investigation on the occurrence of *E. coli* O157:H7 on vegetables in this study is of great importance as it would contribute to the understanding of the epidemiology of the infection caused by this pathogen. It is therefore, against this background that this study on the occurrence of *E. coli* O157:H7 on salad vegetables grown on animal manure-fertilized farms in Zaria was carried out.

1.4 Aim of the Study

To determine the occurrence of *E. coli* O157:H7 on salad vegetables (cabbage, lettuce and carrot) obtained from farms that use animal manure as fertilizers in Zaria, Kaduna State, Nigeria.

1.5 Objectives

The objectives of this study are to:

1. Isolate, identify and characterize *E. coli* O157:H7 on cabbage, lettuce, carrot and soil samples obtained from farms using animal manure as fertilizers in Zaria, Nigeria.
2. Determine antimicrobial susceptibility of *E. coli* isolates to eight commonly used antimicrobials.
3. Determine the risk factors involved in the occurrence of *E. coli* O157:H7 on cabbage, lettuce and carrot grown on farms using animal manure as fertilizer in Zaria, Nigeria.

1.6 Research Hypotheses

H_0: salad vegetables grown on farms in Zaria using animal manure as fertilizers are not contaminated with *E. coli* O157:H7.

There is no significant association between the distributions of *E. coli* O157:H7 in farms that use animal manure as fertilizers and farms that use inorganic fertilizers.

There are no risk factors associated with the occurrence of *E. coli* O157:H7 on salad vegetables grown on farms fertilized with animal manure.

CHAPTER TWO

LITERATURE REVIEW

2.1 Historical Background

Escherichia coli which is commonly abbreviated as *E. coli*, is a bacterium that was discovered in the faeces of healthy individuals in 1885 by a German bacteriologist Theodore von Escherich (Escherich, 1885). It is a normal inhabitant of the gastrointestinal tract of animals and humans and although the majority of its strains are harmless, a few have become highly adapted to cause diarrhoea and a range of extra-intestinal diseases. *Escherichia coli* are routinely characterized by serological identification of somatic (O), flagellar (H) and capsular (K) antigens (Guler and Gunduz, 2007).

However, while some serotypes correlate closely with certain clinical syndromes, differentiation of pathogenic strains from the normal flora depends on the identification of virulence characteristics (Vogt and Dippold, 2005). It has been recognized since 1977 by Konowalchuk that some of the strains of *E. coli* that have adapted to cause diarrhoea, produce toxins that have an irreversible cytopathic effect on cultured Vero cells (Konowalchuk *et al*., 1977). Such verocytotoxigenic *E. coli* (VTEC) have been shown to belong to over 200 different serotypes (Johnson *et al*., 1996; Strockbine *et al.*, 1998). They are also described as Shiga toxin-producing *E. coli* (EHEC) due to the similarity demonstrated between verocytotoxins (VT) and Shiga toxins (Stx) of *Shigella dysenteriae* (O'Brien and Laveck, 1983). In Nigeria, the serotypes O45:H19, O132:H32, O6:H21,

O80:H26, O86:H3, O168:H8 and O6:H4 have been isolated from diarrhoeic and non diarrhoeic calves with most of them shown to produce verotoxins (Tekdek *et al*., 1995).

In the past two decades, VTEC O157:H7 has increased in importance world-wide as a public health problem. *Escherichia coli* O157:H7 is the predominant and most virulent serotype in a pathogenic subset of VTEC and designated enterohaemorrhagic *E. coli* (EHEC). This designation is based on its capacity to cause haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) in humans, its ability to produce VT, ability to cause attaching and effacing lesions on epithelial cells and their possession of a characteristic large plasmid (Nataro and Kaper, 1998). *Escherichia coli* O157:H7 was first recognized as a food borne pathogen when associated with an outbreak of HC in 1982 (Jay, 2000). The strain was isolated from patients in Oregon and Michigan exhibiting bloody diarrhoea and severe abdominal cramping after eating undercooked hamburger at a fast food restaurant (Jay, 2000).

The most common symptom of HC is bloody diarrhoea while other symptoms of the disease include fever, vomiting and nausea. Haemorrhagic colitis caused by *E. coli* O157:H7 was directly linked to HUS in 1985 (Jay, 2000). It has been reported that between 2-7% of children infected with *E. coli* O157:H7 develop HUS which causes haemolytic anaemia and thrombocytopenia followed by acute renal failure (Jay, 2000). Another complication related to HC is thrombotic thrombocytopenic purpura (TTP); a condition similar to HUS, except it is associated with brain damage and primarily affects the elderly (Doyle and Cliver, 1990).

Other non-O157 serotypes, *E. coli* O26:H11, O104:H21, O111:H– and O145:H–, have been associated with occasional outbreaks of human disease and others still with sporadic cases (Johnson *et al*., 1996).

2.2 Aetiology and Physiopathogenesis of *E. coli*

Escherichia coli are a gram negative, motile, facultative anaerobic bacillus of the family *Enterobacteriaceae* and are classified into different serotypes based on the scheme originally developed by Kauffmann, which is based primarily on the serological identification of their somatic 'O' and flagellar 'H' antigens (Doyle and Padhye, 1989). The somatic 'O' antigens (polysaccharide) are known to be thermostable and differentiate *E. coli* into more than 170 serogroups while the flagellar 'H' antigen, which is thermolabile and proteinic, distinguishes the serotypes (56) of each serogroup. It is important to know that the K (capsular) and F (fimbrial) antigens are also important (Doyle and Padhye, 1989).

The pathogenic strains, which cause enteric disease, are grouped into six categories and are known to differ in their pathogenesis and virulence properties, with each comprising of a distinct group of O:H serotypes. These categories include the enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enterohaemorrhagic (EHEC), enteroaggregative (EAEC) and diffuse-adherent (DAEC). In terms of zoonoses, the most important category is the enterohaemorrhagic *E. coli* (Chin, 2000).

2.2.1 Enteropathogenic *E. coli* (EPEC)

EPEC was the first pathotype of *E. coli* to be described. The term enteropathogenic *E. coli* (EPEC) was proposed by Neter *et al*. (1955) to designate certain serotypes of *E. coli* that were associated with outbreaks of infantile diarrhoea. In 1987, the World Health Organization recognized EPEC serotypes of 12 different O serogroups (O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142 and O158). The disease occurs primarily in nursing babies under 1 year, in whom it can cause a high mortality rate (Benenson, 1990). The disease is characterized by watery diarrhoea containing mucus but no visible blood; fever; and dehydration with a short incubation period. EPEC occurs primarily in developing countries and has practically disappeared in Europe and the US. It occurs mostly in the warm seasons (summer diarrhoea). The sources of infection are formula milk and weaning foods that become contaminated due to poor cleaning of bottles and nipples, or deficient hygiene on the mother's part. Children in poor socioeconomic groups are frequently exposed to EPEC and generally acquire immunity after the first year of life (Benenson, 1990). In epidemic diarrhoea in newborns in nurseries, airborne transmission is possible through contaminated dust. Some outbreaks have also been described in adults (Benenson, 1990). Through volunteer feeding studies, the infectious dose of EPEC in healthy adults has been estimated to be 10^6 organisms. The pathogenesis of EPEC involves intimin protein (encoded by ***eae*** gene) that causes attachment and effacing lesions (Hicks *et al*., 1998) and a plasmid-encoded protein referred to as EPEC adherence factor (EAF) that enables localized adherence of bacteria to intestinal cells (Tobe *et al*., 1999). EAF production can be demonstrated in Hep-2 cells and the presence of ***eae*** gene can be tested by PCR assays (Nataro and Kaper, 1998).

2.2.2 Enterotoxigenic *E. coli* (ETEC)

This category has been most intensely studied in recent years of which a lot of knowledge has been added about the physiopathogenic action mechanisms of these bacteria and the prevention of diarrhoeal disease in several animal species through research. ETEC strains are known to synthesize various types of toxins which are heat-labile (LT) (that is very similar in size (86 kDa), sequence, antigenicity and function to the cholera toxin) and heat-stable (ST) toxin (that is of low molecular size (4 kDa) and resistant to boiling for 30 mins) (Harvey *et al*., 2007). There are several variants of ST, of which *ST1a* or *STp* is found in ***E. coli*** isolated from both humans and animals, while *ST1b* or *STh* is predominant in human isolates only. Production of LT can be detected by Y-1 adrenal cell assays (Nataro and Kaper, 1998) or serologically by commercial reverse passive latex agglutination assay and ELISA. The toxins are plasmid-dependent and such plasmids can be acquired by ETEC strains that lack them from strains that possess them (i.e. may be transferable). Enterotoxigenic strains are distributed heterogeneously among the different O:H serotypes. ETEC strains make use of fimbriae or pili (non-flagellar, proteinic, filamentous appendices) to adhere to specific enterocytes, multiply and produce one or more toxins. These pili interact with epithelial cells; they are very important virulence elements and are called colonization factors (WHO, 1991).

Man and animals are the main reservoir of which the source of infection is the faeces of patients and carriers. The route of transmission is faecal-oral while the vehicle of infection may be food and water contaminated by human faeces. ETEC produce enterotoxins when the organisms multiply in the intestine. These strains are commonly responsible for "traveler's diarrhoea". They have been responsible for illness in India, in U.S. soldiers in Vietnam and in travelers in Mexico. This is a problem for travelers from developed

countries with good hygiene who visit countries with poor hygiene standards (Doyle and Padhye, 1989).

The illness is characterized by severe diarrhoea, often leading to dehydration. The diarrhoea may last up to 19 days usually with no fever. Onset of the illness can occur 8 to 44 hrs after ingestion. The infective dose, as determined by a human study, is 10^8 to 10^{10} microorganisms (Doyle and Padhye, 1989). Outbreaks as a result of ETEC have been reported to affect many people in both developing and developed countries. Primarily, this category affects children under 2 or 3 years of age with the incidence of the disease declining and remaining low after the four years of age. There have been outbreaks among adults that affected hundreds of people and were attributed to specific foods and contaminated water. One of the largest outbreaks, affecting more than 2,000 people, occurred in 1975 in a national park in Oregon (USA) (Doyle and Padhye, 1989).

2.2.3 Enteroinvasive *E. coli* (EIEC)

This category represents a small group of *E. coli*. Many isolates are non-motile (lacking the H antigen) and they are slow to ferment lactose or are non-lactose fermenting. They are the cause of a disease similar to bacillary dysentery caused by *Shigella* and possess somatic antigens that may cross-react with those of *Shigella* (DuPont *et al*., 1971). Like ***Shigella***, there are no known animal reservoirs; hence the primary source for EIEC seems to be humans infected (DuPont *et al*., 1971). Although the infective dose of ***Shigella*** is low and in the range of 10 to few hundred cells, volunteer feeding studies showed that at least 10^6 EIEC organisms are required to cause illness in healthy adults. EIEC can invade and multiply in the cells of the intestinal mucosa, especially in the colon. The invasion phenotype of EIEC is encoded by a high molecular weight plasmid, which can be detected

by invasion assays using HeLa or Hep-2 tissue culture cells (DuPont *et al*., 1971; Mehlman *et al*., 1982) or by PCR and probes specific for invasion genes. The illness as a result of this pathogen occurs 8 to 24 hrs after ingestion of food or water containing this organism. Symptoms as a result of Enteroinvasive *E. coli* include abdominal pain, fever, malaise, myalgia, headache and watery faeces containing mucus and blood. In the United States in 1981, an outbreak of this type occurred which was traced to imported French Brie and Camembert cheese. Bacterial counts of the cheese revealed that there were 10^5-10^7 *E. coli*/gram (Doyle and Padhye, 1989). These strains are biochemically and culturally different from other strains of *E. coli*. Unlike typical ***E. coli***, EIEC are non-motile, do not decarboxylate lysine and do not ferment lactose, so they are anaerogenic. EIEC is endemic in the developing countries and accounts for 1% to 5% of all patients with diarrhoea who see a doctor (Benenson, 1990).

2.2.4 Enteroaggregative *E. coli* (EAEC)

This name is given to a group of *E. coli* that shows an aggregative adherence pattern in a HEp-2 assay rather than a localized (as in EPEC) or diffused one. This category is considered to be provisional until it is better defined. Enteroaggregative *Escherichia coli* (EAEC) was first described in 1987 as *E. coli* that adhered to HEp-2 cells in culture in a characteristic, stacked-brick aggregative phenotype (Benenson, 1990). Within the first few years after its description, this pathotype became associated with diarrhoeal illness in Chile, India and Mexico. A study was done on 42 cultures, 40 from children with diarrhoea in Santiago (Chile), 1 from Peru and 1 from a North American student who had visited Mexico. All these strains tested negative for enterohaemorrhagic, enterotoxigenic, enteropathogenic and enteroinvasive *E. coli* with DNA probes. They also failed to fit in one

of these categories on the basis of serotyping. This group causes characteristic lesions in rabbit ligated ileal loops and mice (Levine *et al.*, 1988; Vial *et al.*, 1988). EAEC causes persistent diarrhoea in nursing babies with an incubation period estimated at one to two days (Benenson, 1990).

2.2.5 Diffuse-adherent *E. coli* (DAEC)

Diffuse adhering *E. coli* (DAEC) have been recognized as the sixth category and appears as a heterogeneous group (Nataro and Kaper, 1998). Strains of *E. coli* in this category are known to bind to host cells in a pattern of diffuse adherence, in which the bacteria uniformly cover the entire cell surface (Scaletsky *et al*., 1984). The implication of DAEC strains in diarrhoea remains controversial, since some studies have reported that these strains are found similarly in children with and without diarrhoea (Giron *et al*., 1991; Jallat *et al*., 1993). Tacket *et al*. (1990) suggested that DAEC may cause disease in immunologically naive or malnourished children due to their inability to conclusively induce diarrhoea with DAEC in health adult volunteers. However, in Northern Brazil, Diffuse adherent *E. coli* has been shown in study to be the cause of acute diarrhoea in children (Scaletsky *et al*., 2002). Discrepancies among epidemiological studies could be explained by age-dependent susceptibility to diarrhoea or by the use of an inappropriate detection method such as DNA probing (Levine *et al*., 1993).

2.2.6 Enterohaemorrhagic *E. coli* (EHEC)

The main etiologic agent of this category is *E. coli* O157:H7 which has been a public health problem with a lot of involvements in major outbreaks globally ever since it was first recognized in 1983 (Riley *et al.*, 1983). It was concluded by Griffin and Tauxe (1991) that *E. coli* O157:H7 is an emerging and new pathogen, because they felt that such a distinctive

illness which often has serious consequences (haemolytic uremic syndrome) would have attracted attention at any period. Later, O26:H11, O45:H2 and three non-motile *E. coli* (O4, O111 and O145) were added to this group of organisms.

Escherichia coli O157:H7 is a non-invasive organism, which produces verotoxin as its primary virulence factor (Doyle and Cliver, 1990) which are named for their cytotoxicity to African green monkey kidney cells called Vero cells (Meng *et al*., 2001). The enterohaemorrhagic *E. coli* (EHEC) are characterized by a 60 megadalton virulence plasmid and by its secretion of shiga-like toxins or verotoxins. The Shiga-like toxin was thus named because it is similar both in structure and activity to the toxin produced by *Shigella dysenteriae* type 1 and is also neutralized by the Shiga toxin antiserum. The shiga-like toxins produced by *E. coli* O157:H7 are of two types which are shiga-like toxin 1 and shiga-like toxin 2. The two toxins are antigenically different with both toxins known to be cytotoxic, causing fluid accumulaton in rabbit ligated ileal loops and paralysis and death in mice and rabbits while the shiga-like toxin 2 produces haemorrhagic colitis in adult rabbits (O'Brien and Holmes, 1987).

Escherichia coli O157:H7 differs from other strains of *E. coli* because it fails to ferment D-sorbitol within 24 hrs (Willshaw *et al*., 1997; Meng *et al*., 2001). Differentiation of *E. coli* O157:H7 is typically done with Sorbitol MacConkey agar; where positive colonies are clear (Hitchens *et al*., 1995). *Escherichia coli* O157:H7 are also unable to hydrolyze 4-methylumbelliferone glucuronide (MUG) or grow well at temperatures greater than 44.5°C in *E. coli* broth which sets it apart from other strains (Meng *et al*., 2001). Dupont *et al.* (1971) determined on the basis of a human study that ingestion of 10^6 to 10^8 cells of some pathogenic strains of *E. coli* was needed to cause diarrhoeal illness in a healthy individual,

but for *E. coli* O157:H7 the infectious dose was previously unknown. However, from a compilation of outbreak data, it has been reported to be as low as 10 organisms, which indicates that it takes a low number of microorganisms to cause illness in young children, the elderly and immune-compromised people (Meng *et al*., 2001).

Illness as a result of this organism is characterized by severe abdominal cramps usually, but not always, followed by bloody diarrhoea (haemorrhagic colitis). Some individuals exhibit only watery diarrhoea. Vomiting may occur but there is usually little or no fever. The incubation period is usually about 3 to 9 days. This microorganism can also cause haemolytic uremic syndrome in children. This is the leading cause of kidney failure in children, which often requires dialysis and may ultimately lead to death. Other manifestations of illness due to this microorganism include a central nervous system involvement in which patients develop blood clots in the brain and death frequently results (CDC, 1993).

The enterohaemorrhagic *E. coli* (EHEC) are geographically distributed worldwide of which the serotype O157:H7 has been isolated in outbreaks in Canada, Britain and the United States. It has also been isolated from faecal samples taken from sporadic cases of haemorrhagic diarrhoea submitted to public health or hospital laboratories for examination in Argentina, Australia, Belgium, the former Czechoslovakia, China, Germany, Holland, Ireland, Italy and Japan (Griffin and Tauxe, 1991).

From 1982 to 1992, 17 outbreaks occurred in the US; the smallest affected 10 people and the largest 243. In November 1992, an outbreak occurred among people who had eaten undercooked hamburgers at a fast food restaurant chain. The same *E. coli* serotype was

isolated from the ground beef found in these restaurants with seventeen more outbreaks occurring in 1993 (CDC, 1993). In the developing world, outbreaks have been known to occur with the first reported outbreak of *E. coli* O157:H7 infection occurring in South Africa in 1992 (Effler *et al*., 2001), in Central African Republic in 1996 and in Cameroon in 1997 (Cunin *et al*., 1999).

In Nigeria, *E. coli* O157:H7 illness has been reported since 1994 (Ogunsanya *et al*., 1994; Olorunshola *et al*., 2000).

2.3 Food Borne Outbreaks as a Result of *E. coli* O157:H7

Food safety is a major public health concern in many countries today of which an estimate of 76 million people contract food-borne diseases, 325,000 are hospitalized and 5,000 die each year in the United States (Mead *et al*., 2004). *Escherichia coli* O157:H7 which is one of the food borne pathogens of greatest interest in recent times, has been estimated to be responsible for approximately 73,000 cases of haemorrhagic colitis, 2,168 hospitalizations and 61 deaths each year in the United States while other EHEC strains account for an additional approximately 37,740 illnesses, 1,048 hospitalizations and 30 deaths annually (Mead *et al*., 1999; Meng *et al*., 2001).

Outbreaks as a result of *E. coli* O157:H7 tend to be seasonal with 86% occurring between May and October and this may be due to greater consumption of ground beef during these months, a greater prevalence of transmission among herds during the summer; or greater rates of poor food handling practices (Meng *et al*., 2001). The majority of food borne outbreaks of haemorrhagic colitis originates from meat products, specifically ground beef.

A multi-state outbreak of *E. coli* O157:H7 during the summer of 2002 resulted in 38 confirmed cases; 6 cases of HUS and 1 death. The meat was recalled and accounted for one of the largest recalls (19 million pounds) of meat in U.S. history (CDC, 2002).

However, fresh and minimally processed produce and juice products have been increasingly implicated as vehicles of food borne illness over the last three decades (Sivapalasingam *et al*., 2004). Researchers estimate that between 1973 and 1997 outbreaks increased from two per year in the 1970s to seven per year in the 1980s and sixteen per year in the 1990s with *E. coli* O157:H7 being increasingly related to produce outbreaks (Sivapalasingam *et al*., 2004). Rangel *et al*. (2005) reported that between 1982 and 2002, 21% of all *E. coli* O157:H7 outbreaks were linked to produce (Rangel *et al*., 2005). In southeastern Massachusetts, an outbreak of *E. coli* O157:H7 associated with unpasteurized apple cider in 1991occurred resulting in 23 cases (Meng *et al*., 2001). The cider was made from unwashed pressed apples which were contaminated by cattle that grazed in fields adjacent to the orchard, or deer which had been spotted nearby, coupled with the fact that juice was not pasteurized and no preservatives were added (Meng *et al*., 2001). Since that outbreak, there have been two other outbreaks in which apple cider or juice had been linked to *E. coli* O157:H7 infection (CDC, 1996; 1997). Vegetables have also been associated with outbreaks in which lettuce has been reported to be the most common, accounting for 34% of the outbreaks (Rangel *et al*., 2005). In 1995, there were two lettuce-associated outbreaks which occurred in Montana and in Maine with 74 cases and 37 cases respectively (Ackers *et al*., 1998; Meng *et al*., 2001). In 1996 an outbreak of *E. coli* O157:H7 resulting in 47 cases occurred in Illinois and Connecticut, where mesclun lettuce was the vehicle of infection (Hilborn *et al*., 1999; Meng *et al*., 2001). The source of contamination was

thought to be similar to apple cider outbreaks where cattle were grazing close to the crop. In 1999 alone, lettuce or other salad ingredients were confirmed to be the sources in 5 separate outbreaks that occurred resulting in a total of 163 cases of haemorrhagic colitis (CDC, 2002).

Other produce types have also been associated with *E. coli* O157:H7 outbreaks since 1993 and they include alfalfa sprouts, coleslaw, fruit salad, pea salad, cantaloupe and grapes (CDC, 2002). In Minnesota, a pre-packaged lettuce (Dole lettuce), was linked to an outbreak of *E. coli* O157:H7 in 2005 which resulted in 23 confirmed cases, with 8 of them needing hospitalization and one suffering from kidney failure. The company involved in its production had to recall over 245,000 bagged lettuce and salad mixes. The Food and Drug Administration which by law oversees U.S. food safety and regulate the food industry with inspection and enforcement, had to release a letter in 2004 because of the high numbers of outbreaks as a result of lettuce, urging all firms that grow, pack or ship fresh lettuce, to re-evaluate their procedures and to refer to the FDA's Guide to Minimize Microbial Food Safety Hazards for fruits and vegetables (CFSAN, 2004).

2.4 Use of Cattle Manure in Farming and its Associated Risk

Cattle manure has been used as fertilizer in farming for centuries and this is because of the benefits derived from it which include the fact that it improves the soil structure (aggregation), so that it holds more nutrients and water and becomes more fertile, it encourages soil microbial activity thus promoting the soil's trace mineral supply and improving plant nutrition. Also the fact that cattle manure requires less effort to produce

makes it less expensive than chemical fertilizers which can cost as much as four times more than cow manure (Gagliardi and Karn, 2002).

A survey of 48 sites in Asia, Latin America and Africa showed that majority of farmers use animal manure on their crops (Baltenweck *et al*., 2002). Overall, an estimated 70% of total fertilizer inputs, in developing countries, are derived from animal manure (Fresco and Steinfeld, 1998). In Nigeria, it has been estimated that about 932.5 metric tons (MT) of manure is produced annually from the well established poultry/livestock industries which keep expanding at 8% per year, with bovine manure being commonly used as fertilizer and is applied either fresh/untreated or after composting (Adejinmi, 2000).

Despite the benefits derived from the use of cattle manure in farming it still has been implicated in several food borne outbreaks. This is due to the fact that many potential pathogens of livestock as well as humans can be found in cattle manure. This has raised concerns over health risks from manure handling which include; direct transfer of zoonotic pathogens to farm staff and neighbours, the cause and spread of disease affecting farm animals, the contamination of water and food crops (Böhm, 2004). These concerns over the health risks from manure handling can often be the main reason for revising manure management practices (Guan and Holley, 2003).

E. coli O157:H7 is one of the pathogens known to contaminate the faeces of cattle due to the fact that it persists in the rumen and colon once it is ingested by the animal (Faith *et al*., 1996). It has been reported that the greatest prevalence of *E. coli* O157:H7 is usually found in heifers and calves under 24 months of age. Calves that have been recently weaned off milk appear to have the highest prevalence, while the prevalence in milk fed calves is very

low. Even though faecal shedding is sporadic, it does seem to occur at the same time in clusters of animals (Rahn *et al*., 1997).

Feed deprivation may cause animals to increase their shedding with the amount of *E. coli* O157:H7 shed in the manure estimated to be between 3 – $X10^4$ CFU/gram of faeces. It is important to note that the *E. coli* O157:H7 infective dose for humans is about 10 CFU which is the lowest colony forming unit of the common human food-borne pathogens (Hancock *et al*., 1997). Although cattle are considered to be the main reservoir for *E. coli* O157:H7, it should be realized that this pathogen can cycle through the environment and food chain via manure, insects, water, soil and food (Fig. 2.1). This point is further buttressed by reports of *E. coli* O157:H7 (EHEC) detection from various on-farm sources like water, feed and bedding material (Hancock *et al*., 1998; Davis *et al*., 2005), from wild birds and rodents (Nielsen *et al*., 2004), insects (Alam and Zurek, 2004) and from environmental sources like water (Schets *et al*., 2005) and soil (Looper *et al*., 2006).

2.5 Significance of Produce Consumption in the Transmission of *E. coli* O157:H7

The importance of fruits and vegetables in nutritious, healthy diets cannot be over emphasized and in recent years consumers have been encouraged to eat more of these products. For many countries, particularly developing countries, these products have become a valuable commodity (WHO, 2011). In 1991 the National Cancer Institute (NCI), along with the Produce for Better Health Foundation (PBH) launched a five-a-day campaign encouraging Americans to consume at least five servings of fruits and vegetables per day to reduce cancer and ensure lasting health.

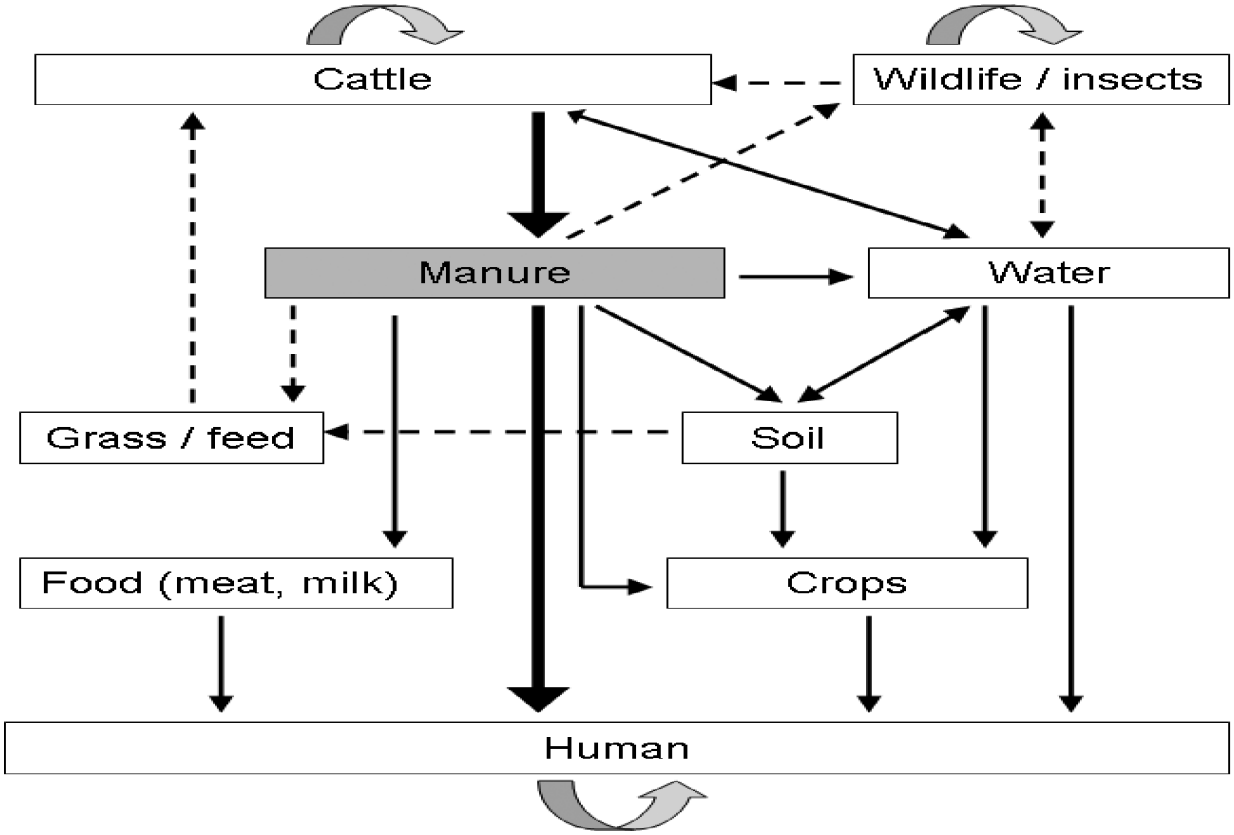


Fig. 2.1**:** Reservoirs and Modes of Transmission of EHEC.

Solid lines represent direct or indirect transmission routes between cattle and humans, dashed lines represent transmission lines back to cattle (Franz, 2007).

As a result of this campaign which was backed up by several advertisements stressing the health benefits associated with the consumption of fresh fruits and vegetables, the average adult consumption of produce increased significantly within three years of its implementation (NCI, 2003). The result of this program seen after its evaluation in 1999 resulted in the recommendation that more emphasis be placed on the program. Partnership to this program increased to include the American Cancer Society (ACS), the Centers for Disease Control and Prevention (CDC), the United States Department of Agriculture

(USDA), United Fresh Fruit and Vegetable Association, Produce Marketing Association and National Alliance for Nutrition and Activity (NCI, 2003).

The U.S. Center for Nutrition Policy and Promotion reported that per capita consumption of fresh vegetable produce is steadily increasing of which the per capita consumption of fresh fruits increased by 19% and consumption of vegetables increased by 29% between 1980 and 2001 (CARD, 2004). Tauxe *et al*. (1997) reported that the geographic sources and distribution of fresh fruits and vegetables has expanded rapidly, including increased global trade of these products which eliminates the seasonality associated with some produce types. But more recently, new technologies which extend shelf life and new trade agreements have increased the imports of fresh vegetable produce in order to fill gaps where domestic supplies are too small to meet demands and domestic products are out of season. As a result, between 1980 and 2001, fresh fruit imports increased by 155 percent and fresh vegetable imports increased by 265 percent (CARD, 2004). The USDA made forecasts which brought to light that the trend toward increased consumption of fresh fruits and vegetables will continue with per capita expenditures on fruits and vegetables expected to have the highest increases among all types of foods through 2020. These increases would be as a result of higher incomes, the large number of aging baby boomers, a gradually increasing population, increasing consumption of ethnic foods and higher levels of education among consumers (CARD, 2004). This is to give a little insight to the role fresh vegetable produce play in the health and diet of humans in the twenty first century. At the same time, food safety problems linked to the consumption of fresh fruits and vegetables contaminated with microorganisms are increasing. Recent food borne outbreaks linked to the consumption of leafy greens, tomatoes, sprouts and green peppers clearly demonstrate

that the consumption of contaminated fruits and vegetables represents an important source of food borne diseases (WHO, 2011).

There are several different ways that consumers may purchase fresh vegetable produce in grocery stores or markets with most produce purchased in bulk as raw vegetables. However, pre-harvest contamination can occur through the use of raw manure or slurry (liquid manure) on or near fruit and vegetable crops, particularly those produce intended to be eaten raw. This is a potential health hazard since *E. coli* O157:H7 can be present in animal faeces (ACIAR, 2001).

Fresh vegetable produce are mainly composed of water, giving microorganisms an environment to thrive. A research by Li *et al*. (2001) proved that when cut lettuce was inoculated with 3.2 log CFU/ml of *E. coli* O157:H7 and stored at refrigeration temperatures for 7 days, growth occurred to reach levels of approximately 6 log CFU/ml. This demonstrates that bacteria can receive required growth nutrients from produce and grow during refrigeration. Also the leakage of fluids from lettuce as a result of transportation and storage practices could provide nutrients to support growth of bacteria (Li *et al*., 2001). Lettuce production practices commonly include a rinse step in which the leaves are sanitized using tap water containing 100 to 200 ppm of free chlorine (Beuchat *et al*., 1998). This level of chlorine has been shown to be only marginally effective at reducing the level of ***E. coli*** O157:H7 on lettuce tissue surfaces (Beuchat**,** 1999). The ineffectiveness of chlorine and other surface-sanitizing agents is likely dependent on whether the target organisms are readily accessible. However, cells of ***E. coli*** O157:H7 have been shown to penetrate into the stomata and junction zones of cut lettuce leaves, becoming entrapped 20 to 100 μm below the surface of the cut edge (Seo and Frank, 1999), which were protected

from sanitation with chlorine. Research has also demonstrated that lettuce grown in soil containing contaminated manure or irrigated with contaminated water results in contamination of the edible portion of the lettuce plant. Moreover, the results of the study suggest that edible portions of a plant can become contaminated without direct exposure to a pathogen but rather through transport of the pathogen into the plant by the root system (Solomon *et al*., 2002).

2.6 Diagnoses of *E. coli* O157:H7 Infection

E. coli O157:H7 has become the predominant zoonotic VTEC and for this reason, diagnostic methods have been developed to detect selectively this serotype in human clinical cases (Strockbine *et al*., 1998) and in food sources (Vernozy-Rozand, 1997). The diagnosis of *E. coli* O157:H7 infection needs to be considered for all patients who are presented with diarrhoea, especially bloody diarrhoea or haemolytic uremic syndrome (HUS) (Griffin and Tauxe, 1991). Ideally, samples should be examined as soon as they are received in the laboratory. If samples will not be processed immediately, they should be either refrigerated or frozen at -70 C as soon as possible after collection. Refrigerated specimens should be examined within 1-2 hrs but if samples cannot be examined within this time, they should be placed in transport medium. The isolation of *E. coli* O157:H7 from positive samples involves some procedures which include the following;

2.6.8 Liquid enrichment

Clinical samples are routinely plated directly on to solid media for isolation of *E. coli*, but the number of target *E. coli* O157:H7 organisms in faeces from healthy carriers is usually low and enrichment in liquid media improves recovery. Some of the enrichment media

commonly used are buffered peptone water either unsupplemented (which gives good recovery) or supplemented with 8 mg/litre vancomycin, 10 mg/litre cefsulodin and 0.05 mg/litre cefixime in order to suppress the growth of Gram-positive organisms, *Aeromonas* spp. and *Proteus* spp.; modified trypticase–soy broth (mTSB) supplemented with 20 mg/litre novobiocin or 10 mg/litre acriflavin to reduce the growth of Gram-positive organisms; or modified *E. coli* broth with 20 mg/litre novobiocin (mEC+n). EHEC grow poorly at 44°C of which 24 hours incubation may increase recovery if the organisms are stressed (OIE, 2008).

2.6.9 Selective culture for *Escherichia coli* O157

There are no biochemical characteristics that distinguish the majority of EHEC from other *E. coli*, however, *E. coli* O157:H7 can be distinguished from other *E. coli* by their inability to ferment D-sorbitol rapidly and their lack of beta-glucuronidase activity; these can be exploited in the isolation and identification of this organism. It is important to note that less common sorbitol fermenting and beta-glucuronidase positive *E. coli* O157:H– variants (non-motile due to lack of expression of the H7 antigen), will not be identified by isolation in such selective media chosen for these biochemical characteristics (Karch and Bielaszewska, 2001). MacConkey agar containing 1% D-sorbitol instead of lactose (SMAC) is a useful and inexpensive medium on which non-sorbitol-fermenting *E. coli* grows as small, round grayish white colonies. Reports have shown that the addition of 0.5% rhamnose and addition of 0.05 mg /litre cefixime (CR-SMAC) to MacConkey agar containing 1% D-sorbitol improves its selectivity by inhibiting overgrowth by *Proteus* spp. Rhamnose is known to be an expensive supplement, however, an alternative modification which has a greater inhibitory effect against non- O157 *E. coli* and other non-sorbitol

fermenters, such as *Aeromonas*, *Plesiomonas*, *Morganella* and *Provindencia*, is the addition of 2.5 mg/litre potassium tellurite in addition to cefixime (CT-SMAC) (O'Brien and Laveck, 1983).

It is important to know that sorbitol-fermenting (SF) *E. coli* O157:H– have been isolated from patients with diarrhoea and haemolytic uremic syndrome, but the epidemiology of this infection is poorly understood and only rarely has the organism been isolated from animals, including cattle. The majority of SF *E. coli* O157:H– isolates are susceptible to tellurite and cannot be identified on CT-SMAC (Lee and Choi, 2006).

2.6.10 Identification and characterization of suspect colonies

Colonies growing on selective media that are suspected to be EHEC must be confirmed biochemically or genotypically to be *E. coli* while the somatic 'O' and flagellar 'H' antigens are identified serologically. Not all EHEC isolated from animals are thought to be pathogenic for people as some isolates of *E. coli* O157, particularly from pigs, are non-verocytotoxigenic and non-pathogenic for humans. Therefore diagnosis must include the demonstration of known virulence factors in the isolates. These include the verocytotoxins *VT*1 (*stx*1) and *VT*2 (*stx*2) and their genes and an outer membrane adhesion protein associated with attaching and effacing lesions, intimin, which is encoded by the *eae* gene (Law, 2000).

2.6.11 Biochemical tests

Biochemically, EHEC are similar to other *E. coli*. However, *E. coli* O157:H7 strains differ in failing to ferment sorbitol, failing to produce beta-glucuronidase and fermenting

raffinose and dulcitol. *Escherichia coli* can be distinguished from *E. hermanii* by lack of growth in the presence of potassium cyanide and failure to ferment cellobiose. *Escherichia hermanii* is positive for both tests. Ninety-eight per cent of *E. hermanii* strains have a characteristic yellow pigment on nutrient agar that is not seen in EHEC. *Escherichia coli* may be confirmed by demonstration of the use of tryptophan and beta-galactosidase activity or by commercially available biochemical test strips (OIE, 2008).

2.6.12 Serological tests

Commercial latex kits are available for the following antigens; O157, O26, O91, O103, O111, O128, O145 and H7. Tests should be carried out according to the manufacturer's instructions and should incorporate positive and negative control organisms and control latex. Presumptive diagnosis may also be made using slide or tube agglutination tests with anti-O lipopolysaccharide antiserum (antisera to 181 O– antigens are available). The O157 antiserum has been shown to cross-react with other organisms such as *E. hermanii* which is frequently found in foods, *Salmonella* O group N, *Yersinia enterocolitica* serotype O9 and *Citrobacter freundii* (Bettelheim *et al*., 1993; Lior and Borczyk, 1987), indicating the need to confirm putative EHEC colonies as *E. coli.* Isolates can be tested for the presence of flagellar antigen of which antisera have been raised to 56 H (flagellar) antigens, but this may require passage through motility medium as some pathogens are non-motile (*E. coli* O157:H–) (OIE, 2008).

2.6.13

2.6.14

2.6.15 Subtyping using polymerase chain reaction (PCR) based-methods

A variety of methods are available in reference laboratories to help discriminate between strains of *E. coli* O157:H7 to aid epidemiological investigations of outbreaks of human disease (Hopkins and Hilton, 2000; Strockbine *et al*., 1998). These methods vary in technical complexity and more than one technique is required to provide useful differentiation. Techniques include phage typing, biotyping and antimicrobial sensitivity testing, plasmid profiling, restriction fragment length polymorphism analysis, ribotyping, pulsed field gel electrophoresis (PFGE) and various PCR-based analyses (Random Amplification of Polymorphic DNA; Repetitive DNA Element PCR; Amplified Fragment Length Polymorphism Analysis). Of these, only phage typing and PFGE are widely used.

Many PCR protocols and primers are described in the literature for detection of *stx*1, *stx*2 and *stx*2 variant genes (Nataro and Kaper,1998; Paton and Paton,1998; Strockbine *et al*., 1998) and a number of these toxin-typing PCR methods have recently been compared (Ziebell *et al*., 2002). Demonstration of the genes associated with VT-production does not confirm gene expression and hence production of toxin.

PCR can be used on pure or mixed plate or broth cultures and extracts from food or faeces. It can also be used to detect genes in non-viable organisms. As well as its role in diagnosis, PCR has the potential to be used to screen samples for EHEC in epidemiological studies. It is also important to note that amplification of target genes in bacterial DNA extracts from faeces is less successful than from pure cultures and careful preparation of the sample is required to improve sensitivity. In addition, DNA probes, PCR assays and microarrays have also been developed to detect other genes in EHEC shown to be associated with virulence in humans, which include *eae* (encoding for intimin), *ehx* (encoding for

enterohaemolysin production), *fliC* (encoding the H7 antigen), O157 *rfb* (encoding O157 LPS), *uidA* (the mutant glucuronidase gene in beta-glucuronidase-negative *E. coli* O157:H7) and *katP* (a gene carried on the large plasmid of *E. coli* O157:H7 encoding a novel catalase peroxidase) (Nataro and Kaper,1998; Paton and Paton,1998; Strockbine *et al*., 1998; Bekal *et al*., 2003).

2.6.16 Diagnosis using ridascreen verotoxin enzyme immune-assay (Ridascreen EIA)

Many assays have been developed for the diagnosis of VTEC which include cultural, immunological and genetical methods. In order to identify almost all types of VTEC in any kind of test sample, reports have shown that the most straightforward way is by detection of the production of Shiga toxin or by detecting the gene sequences encoding for Shiga toxin production (*stx*) (Bettelheim and Beutin 2003).

In the evaluation of immunological tests for shiga toxin which usually do not discriminate between biologically active or inactive toxins, the vero cell toxicity test which detects functionally active toxin is often used as a gold standard. The *stx*-specific PCR are known to detect gene sequences of VTEC irrespective of whether these genes are expressed or not. The need for tests for the routine diagnosis of these pathogenic organisms to be rapid, reliable and easy to perform has brought about the introduction of standardized, quality-controlled commercially-obtainable immunological test kits with a growing number of test kits for the detection of shiga toxin being offered by different companies (Scheutz *et al*. 2001).

The Ridascreen verotoxin test kit is one of such enzyme immunoassay for the qualitative determination of verotoxins 1 and 2 (Shiga-like toxins I and II) of *Escherichia coli* in stool

samples and enrichment cultures owing to its high specific (Beutin *et al*., 2007). This test involves specific antibodies which are used in a sandwich-type method. These specific antibodies against verotoxins 1 and 2 are applied to the surface of the well in the microwell plate and the suspension of the sample to be tested and the controls are pipette into the well in the microwell plate at ambient temperature (20-25^{0}C) for incubation. After a wash step, peroxidase-conjugated specific antibodies against the verotoxins are added and the plate incubated at room temperature (20-25^{0}C). In the presence of verotoxin, a sandwich complex is formed made up of the immobilized antibodies, the verotoxins and the conjugated antibodies. Unattached enzyme-labelled antibodies are removed during a further washing phase. After adding the substrate, the attached enzyme changes the colour of the previously colourless solution in the wells of the microwell plate to blue when the test is positive. On adding the stop reagent, the colour changes from blue to yellow.

The evaluation of diagnostic test kits by reference laboratories has been shown to be valuable for the assessment of the specificity and sensitivity of commercially obtainable products (Bettelheim and Beutin, 2003). However, in the case of Ridascreen, the specificity and sensitivity of this commercially available test kit in the detection of different variants of *stx*1 and *stx*2 with EHEC reference strains was evaluated in a study carried out by Beutin *et al*. (2007) with the result obtained showing that the Ridascreen-EIA is suitable to detect all known variants of the *stx*1 and *stx*2 with a relative sensitivity of 95.7% and a relative specificity of 98.7%.

2.7 Antimicrobial Susceptibility of *E. coli*

The use of antibiotics began in the 1940s and shortly after it became widespread in that period, strains of bacteria began to emerge that were resistant to them (Dibner and Richards, 2005). The selection procedures caused by the inappropriate and careless use of antibiotics, the overuse of antibiotics in humans and its use as growth promoters in food of animals, tended to increase the prevalence of resistant populations of microorganisms which is now the reason for the current concerns about hospital-acquired infections, a heightened awareness of the need for cleanliness and attempts to more strictly control the use of antibiotics (WHO, 1997).

Antibiotic resistance is an adaptive response in which microorganisms begin to tolerate an amount of drug (antibiotics) that would ordinarily be inhibitory. The development of mechanisms for circumventing or inactivating antimicrobic drugs is largely due to genetic versatility and adaptability of microbial populations. The property of antibiotic resistance can be intrinsic as well as acquired where the intrinsic antibiotic resistance exists naturally and is not acquired through specific genetic changes. The genetic events most often responsible for antibiotic resistance are chromosomal mutations or extrachromosomal DNA that are transferred from a resistant species to a sensitive one.

The resistance encountered in bacterial infection today can be said to be a contribution of chromosomally-mediated resistance arising from mutation during therapy or spread of a single organism. Chromosomal drug resistance usually results from spontaneous random mutations in bacterial populations (Kathleen and Arthur, 2001). Chromosomal mutations may change membrane permeability, reducing the efficacy of chromosomally encoded

enzymes such as beta- lactamases, or alter the target enzyme, e.g., dihydrofolate reductase (DHFR). These mechanisms which are chromosomally mediated are such that they can only be passed on vertically to the progeny of the resistant organisms and as a result account for only a small proportion of resistant isolates and generally mediate only low-level resistance.

On the other hand, plasmid-mediated resistance accounts for most high-level resistance found in bacteria resulting in the rapid spread and accumulation of the most efficient resistance mechanisms. Plasmids are also passed on vertically to the cell's progeny during cell division. However, many resistance plasmids are also capable of mediating their transfer to other organisms and indeed to organisms of other species by conjugation, transformation, or transduction. This tends to greatly increase the potential for the spread of resistance genes carried on these plasmids. Resistance plasmids seem to be stable in bacterial populations even when there is no selective pressure (Bennett and Linton, 1986). Studies have shown that plasmids encoded with drug resistance are naturally present in microorganisms before they have been exposed to the drug. Such traits are "lying in wait' for an opportunity to be expressed and to confer adaptability on the species (Kathleen and Arthur, 2001). Most resistance plasmids mediate resistance to more than one, sometimes as many as seven antibiotics (Foster, 1983).

Transposons have contributed to the evolution of resistance plasmids, providing genetic variation and allowing the dissemination of accumulated genes between plasmids and their incorporation into the chromosome. Since the first recognition of transposon, by Hedges and Jacob (1974), transposition has been shown increasingly to be a major contribution to plasmid development owing to the fact that complete and functional resistance genes can be

transferred from one replicon to another by this mechanism. Two major classes of transposon have been identified; composite transposons which are heterogeneous group of elements, carrying a variety of resistance genes that share similar structural and functional properties, but share little DNA homology (Schmitt, 1986) and the complex transposons which are more homogeneous, comprising three different but related "families", the Tn*3*, Tn*21* and Tn*2501* groups (Lafond *et al*., 1989).

These transposons and plasmids carry similar resistance mechanisms. For example, R388 and R46, highly conserved regions are found in sequences immediately surrounding the structural gene. Flanking the structural gene are GTTA sequences which mark the insertion points for the resistance gene and represent recombination hot-spots necessary for crossover of resistance genes. At the 5′-end of the resistance gene there is an open reading frame which codes for a protein that shares homology with both transposon resolvases and phage site-specific integrases. This integrase has been designated *int* and is responsible for the recombination of the resistance genes (Stokes and Hall, 1989). At the 3′-end of the resistance gene is an imperfect palindromic region of 59 bp that is essential for control of integration and contains the GTTA sequence which marks the point of excision. A gene "cassette" containing the resistance gene, the GTTA sequence and all but the last seven bases of the 59-bp region, has been proposed as the unit of recombination. Circularization of the element, after excision from its original location, precedes its integration in the new replicon (Collins and Gutman, 1992). This small mobile genetic element has been called an integron (In). Integrons have been implicated in antibiotic resistance and its evolution also seems to have been the result of antibiotic pressure (Stokes and Hall, 1989).

In *Enterobacteriaceae* antimicrobial resistance poses a critical public health threat, especially in the developing countries (Karlowsky *et al*., 2003) which has been reported to be due to the presence of transferable plasmids encoding multidrug resistance and their dissemination among different enterobacterial species (Blake *et al*., 2003; Carattoli, 2009). Reports have warned that antibiotic resistance in *E. coli* continues to increase with the fear that this problem could become as big as that of methicillin resistance *Staphylococcus aureus* (MRSA) as healthy non-hospitalized people have become infected with the antibiotic resistant strains of this pathogen. *E. coli* are able to produce enzymes called Extended-Spectrum Beta-Lactamases (ESBLs) that stop certain antibiotics from working, among which are some of the most widely used in hospitals (Pitout and Laupland, 2008). Also, Meng *et al*. (1998) who worked on antibiotic resistance of *Escherichia coli* O157:H7 and O157:NM isolated from animals, food and humans reported that cattle isolates had the highest rate of antibiotic resistance with the most frequent resistance type overall being streptomycin-sulfisoxazole-tetracycline. *E. coli* O157:NM isolates from cattle were resistant to six antibiotics: ampicillin, kanamycin, sulfisoxazole, streptomycin, tetracycline and ticarcillin.

2.7.1 Disk diffusion test

In order to guide the appropriate antibiotic treatment of bacterial infections, bacterial pathogens isolated from clinical specimens are usually tested against a selection of antibiotics to assess their degree of susceptibility. This is usually done with bacteria that have been grown on solid media. In general, these tests involve exposing a pure culture of the bacterium to several different drugs and observing the effects of the drugs on growth (Will *et al*., 2005).

Currently the disk diffusion test is most often used in the Kirby-Bauer Method, which was developed in the early 1960ʻs at the University of Washington Medical School by William Kirby, A.W. Bauer and their colleagues (Bauer *et al*., 1966).

In this method, an inoculating loop or needle is used to touch four or five colonies of the pathogen growing on agar and then used to inoculate a tube of culture broth. The culture is incubated for a few hrs at 37°C until it becomes slightly turbid and is diluted to match a turbidity standard. A sterile cotton swab is dipped into a standardized bacterial test suspension and used to evenly inoculate the entire surface of the Mueller Hinton agar plate. After the agar surface has dried for about five minutes, the appropriate antibiotic test disks are place on it, either with sterilized forceps or with a multiple applicator device. The plate is immediately placed in a 37°C incubator which after 16 to 18 hrs of incubation the antibiotic diffuses out of the disk into the agar, along a concentration gradient. If the bacterial strain is sensitive to the antibiotic, then a zone of inhibition (no growth) occurs around the disk. The diameters of zones of inhibition are measured to the nearest millimeter and compared with a standard for each drug. This allows the organism to be described as sensitive, intermediately susceptible or resistant. The profile of antimicrobic sensitivity, or antibiogram, provides data for drug selection. The Kirby-Bauer procedure is less effective for bacteria that are anaerobic, highly fastidious, or slow growing (*Mycobacterium*) (Kathleen and Arthur, 2001).

2.8 Treatment for *E. coli* Infections in Animals or Humans

A lot of research has been undertaken while others are still ongoing with regards to the treatment of infections caused by pathogenic *E. coli*. In the light of some research, it is

evident that not all infections caused by pathogenic *E. coli* require treatment as many of the infections are self- limiting with most people recovering without specific treatment in five (5) to ten (10) days. However prior to treatment, it is very important that proper diagnosis be made in order to rule out other common infections that produce similar signs and symptoms to those of *E. coli* infections such as salmonellosis and shigellosis. Considering the pathophysiology of pathogenic *E. coli* infections, treatment should consist of correcting fluid and electrolyte imbalances (Farthing *et al*., 1996) due to the fact that the noninvasive diarrhoea (caused by enterotoxigenic or enteropathogenic strains of *E. coli*) results from the production of two toxins that interact with intestinal juices and promote excessive loss of chloride and water, while the invasive form directly attacks the intestinal mucosa without producing enterotoxins, causing local irritation, inflammation and diarrhoea. Infants or immunocompromised patients with such infection could be given intravenous (I.V.) antibiotics based on the organism's drug sensitivity with salicylates or opium tincture administered for cramping and diarrhoea (Lippincott, 2010).

E. coli O157:H7 enteric infection has been recognized as the most common cause of HUS in the United States, which causes acute renal failure in children. It is reported that such children require dialysis, which is a way of removing built up toxins in the blood while the kidneys heal even though 5% of patients who survive have long-term renal impairment (Siegler *et al*., 1994). No cure is known to exist for HUS and although management beyond intensive support with needed renal replacement therapy remains controversial, there appears to be a growing consensus that plasma exchange therapy brings benefit and should be used (Elliott and Nichols, 2001). Therapeutic plasma exchange as an early phase

treatment for *E. coli* O157:H7 associated with haemolytic uremic syndrome had also been reported by Nakatani *et al.* (2002) to be effective.

The use of antibiotics for treatment of *Escherichia coli* O157:H7 infection has also become controversial since a previous small study found that it may increase the risk of haemolytic uremic syndrome (HUS) (Proulx and Seidman, 1999). There is no clear consensus also on whether antibiotics should be administered as treatment of *E. coli* O157:H7, but since the report by Wong *et al*. (2000), avoidance of antibiotic therapy for any presumably infectious enteritis appears to be a growing practice. However, other larger studies have reported a protective effect or no association (Ostroff *et al*., 1989; Bell *et al*., 1997; Proulx *et al*., 1992; Cimolai *et al*., 1994; Slutsker *et al*., 1998).

2.8.1 Prevention and control of pathogenic *E. coli* infection

Infections caused by pathogenic *E. coli* have been reported to be transmitted through consumption of foods contaminated with these pathogens. In the United States alone, an estimated $6 billion each year is spent on medical care and lost productivity due to food borne diseases, most of which cause diarrhoea (IDSA, 2001). According to the Centers for Disease Control and Prevention, it takes several steps (the food production chain) to get food from the farm to the dining table and contamination can occur at any point along the chain; which can be during production, processing, distribution, or preparation. Prevention would therefore require control measures at all stages of the food chain, from agricultural production on the farm to processing, manufacturing and preparation of foods in both commercial establishments and household kitchens (CAC/RCP, 2009).

It is important to note that no single treatment exists to eliminate all potentially harmful microorganisms in all fresh fruits and vegetables or foods of animal origin. Indeed, risk cannot be minimized or controlled at any single point in the production-processing-distribution-preparation chain (CAST, 2009), only a comprehensive food safety system, from farm to table, will minimize the risk of food borne illness and this cannot be achieved in isolation but through collaboration between the individual consumers, food industries and the government.

Many food borne diseases could be avoided if those who prepare meals follow simple steps needed to prepare food safely (WHO, 2012). Preventive measures for infection caused by *E. coli* O157:H7 is similar to those recommended for other food borne diseases. Basic good food hygiene practices, as described in the WHO five keys to safer food in 2012, can prevent the transmission of pathogens responsible for many food borne diseases and also protect against food borne diseases caused by EHEC. The core messages of the WHO five keys to safer food include: keep clean, which has to do with proper washing of hands with soap and water; separation of raw and cooked food; cook thoroughly so that the centre of the food reaches at least 70°C; keep food at safe temperatures which involve storing foods or leftovers at temperatures below 5^0C; and use of safe water and raw materials (WHO, 2012).

Another strategy to improve food safety and reduce the impact of food borne diseases is the education of food handlers, including consumers with the education of mothers and care-givers on food safety principles seen as vital if there is to be a substantial improvement in prevention of diarrhoeal disease in infants and children. High risk group (for EHEC are the young children and the elderly) which are known to have increased vulnerability to food

borne diseases need to be given greater emphasis in food safety education programmes (WHO, 2012). It is important to note that food safety education is most effective when messages are targeted toward changing behaviours in people that are most likely to result in food borne illness (Medeiros *et al*., 2001).

Most outbreaks caused by pathogenic *E. coli* have been traced back to animals such as cattle which have served as sources of contamination to the environment and foods (CDC, 1993). Since pathogenic microorganisms found on or in fresh vegetable produce are found throughout the natural environment and contamination can occur at any point in the food production-processing-distribution-preparation chain from field to table, it is therefore vital that good agricultural practices (GAP) (which include proper site selection, water quality testing, run-off control, manure and compost management, domestic animal and wildlife control, worker health and hygiene monitoring, field sanitation of harvest equipment and safe harvesting practices) be implemented by the fresh vegetable produce farmers to decrease the risk of in-field contamination (CAST, 2009).

There are currently no existing knowledge and technologies that can eliminate all food safety problems associated with the microbial contamination of fruits and vegetables. However, farmers of fresh vegetable produce can adopt practices to reduce rather than eliminate contamination of fruits and vegetables from microorganisms. In 2011, the World Health Organization published a manual which describes five key practices that can be adopted by farmers to reduce microbial contamination of fresh fruits and vegetables during planting, growing, harvesting and packaging. These practices including; practicing of good personal hygiene, using of safe water to irrigate farms, protecting fields from faecal contamination by animals (cattle) including birds, use of treated manure or treated faecal

waste and finally by keeping harvest equipment, containers and storage facilities clean and dry (WHO, 2011).

Hazard Analysis Critical Control Point (HACCP) principles, which is a food safety system based on prevention that is concerned with identifying food safety hazards that are reasonably likely to occur in a food process, creating controls to prevent the hazards and then monitoring those controls, can be adopted by food industries especially those that are involved in processing, packaging, storing, transportation and distribution of fresh produce.

It is the role of governments to protect their subjects' health and interests as consumers of publicly marketed food. This can be done by implementing an effective and transparent food control system which considers all aspects of the food chain, farm-to-table, food processing, storage and transport and retail sale. Governments have key role in setting policy and providing legislation that lays down minimum food safety or marketing standards that food businesses must meet and also ensure that they comply with these requirements (www.eolss.net). Food safety is a responsibility shared at all levels of government of which the federal, state and local government have distinct but interconnected roles to play. The local, state and federal food safety regulators can use the FDA Food Code as a model to develop or update their own food safety rules and to be consistent with national food regulatory policy and emphasize prevention.

In Nigeria, the mandate for food safety is shared by various government ministries, departments and agencies (MDAs) and also cuts across different tiers of government: Federal, State and Local. The agencies include; National Agency for Food and Drug Administration and Control (NAFDAC), Standards Organization of Nigeria (SON) and the

Consumer Protection Council. NAFDAC is the lead food safety authority and operates at the Federal and State levels along with State Government Agencies. At the Local Government level there are the LGAs and the Primary Healthcare Development Agency which are responsible for street food vending, catering establishments and traditional markets (Ifenkwe, 2012).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study on the occurrence of *E. coli* O157:H7 on cabbage, lettuce and carrot grown on farms in Zaria fertilized with animal manure was carried out in Zaria located in Kaduna State, within latitudes 11^0 $7^/$**,** 11^0 $12^/$ N and longitudes 07^0 $41^/$ E. Zaria is a medium sized city divided into Sabon Gari and Zaria City Local Government Areas (LGAs) with an estimated population of 547,000 and a growth rate of 3.5% per annum (Ministry of Economic Development, 1996). It is approximated that about 40-75% of its working population derive their principal means of livelihood from agriculture (ABU, 2000). Agricultural activity in Zaria can be divided into two types: rainy season (from May to October) and irrigation farming during the dry season (from November to April). It is characterized by a tropical climate, a monthly mean temperature ranging from 13.8 to 36.7^0 C and an annual rainfall of 1092.8mm (Agbogu *et al*., 2006). Horticulture is the second most prevalent agricultural activity in Zaria with vegetables being mostly produced, but in some cases fruits are sandwiched among cereal crops (Chuo, 2009).

3.2 Cultivation of Cabbage, Lettuce and Carrot

In Zaria, the three farm produce studied were cabbage (*Brassica oleracea var capitata*), called “cork” in Hausa, lettuce (*Lactuca sativa*), called “ganye salad” in Hausa and carrot

(*Daucus carota var sativa*) called “karas” in Hausa. They are all commonly grown between the period of September and March by irrigation farming method.

Prior to cultivation, the farmers till and divide their farm lands into several ridged rectangular segments with the ability to hold some amount of water. The prepared farm lands were then heavily fertilized using manure from dustbins, burnt ashes or organic manure from animal faeces and followed by watering using water from streams flowing along or away from their farm area. The farmers draw water from the stream using a pumping machine connected to pipes joined together. These pipes aid in conveying the water pulled to the farms through dug path ways that allow the water to flow gently into the farms.

For cabbage and lettuce, the preparation of the farm lands began after many seeds of the products to be cultivated had been grown on a small portion of land where they germinated in clusters for a period of less than two weeks. This served as a source from which they were transplanted to the already prepared land at uniform distance from each other. Carrots are cultivated by directly spreading the seeds on the already prepared land. Subsequently, the farmers watered and fertilized the farms using organic manure (burnt ashes, dustbin manure or animal faeces) or inorganic fertilizer until 3-4 months when the plants were ready for harvesting.

3.3 Questionnaire

Before and during the course of sample collection, 18 structured questionnaires prepared (see Appendix 1) were tailored to farmers growing cabbage, lettuce and carrot which took into consideration the demographic data of respondents, their farming practices during

cultivation and harvest and the markets where these vegetables were supplied. Oral interviews were conducted for farmers that could not read or write.

3.4 Pilot Study

A pilot study was conducted due to the fact that we did not find any work in Nigeria that has been published with regards to the isolation of *E. coli* O157:H7 from salad vegetables on farms. One hundred and twenty (120) samples of cabbage, lettuce, carrot and soil samples were collected from 6 farms in Zaria City and Sabon Gari LGAs. The samples collected were immediately transported to the laboratory for analyses.

3.4.1 Sample size determination

Using the probable prevalence (1.7%) obtained from the pilot study, the sample size for the study was determined as described by Mugo (2008).

Calculation:

Using the formula $n = \frac{Z^2 P(1-P)}{d^2}$

Where:

n= sample size

P= probable prevalence = 0.017

Z= Z-score = 1.96 (assuming a 95% confidence interval)

d= desire absolute precision= 0.05

$$n = \frac{1.96^2 \times 0.017(1-0.017)}{0.05^2}$$

n= 26

The sample size calculated above as 26, gave the least sample that should be collected. However, because of the purpose and the design of this study for effective investigation and coverage, a total number of 360 samples were collected.

3.5 Sample Collection and Isolation

The samples were selected as probability samples of which the simple random sampling method was used to select samples for this study. With the permission of the farmer, a round wire was thrown across the farm blindly and any vegetable trapped by the wire was selected for the study.

A total of one hundred and twenty (120) samples each of cabbage, lettuce, carrot and their respective bed soils (50 g collected from around each randomly selected plants approximately 2 cm deep from the surface) were collected over a period of three months (November, 2012 to January, 2013) from eighteen (18) farms, chosen in such a way so as to cover Zaria City and Sabon Gari LGAs.

Of the 18 farms visited, 150 vegetable samples (50 of cabbage, 50 of lettuce and 50 of carrot) and 50 soil samples each from the respective vegetable farms were collected from 15 farms fertilized with animal manure (experimental farms) to give a total of 300 vegetables and soil samples, while 30 vegetable samples (10 of cabbage, 10 of lettuce and 10 of carrot) and 10 soil samples each from the respective vegetable farms were collected from three farms fertilized with inorganic fertilizer (control farms) to give a total of 60 samples. Each sample collected was immediately placed in a sterile polythene bag which was labeled (taking into consideration the LGAs, the farm, the type of vegetable and the sample number) and transported to the laboratory within 1 hour after collection and immediately processed for enrichment.

3.5.1 Isolation of *E. coli* O157:H7

A flow chart for the isolation of *E. coli* O157:H7 in this study is shown in Appendix 2.

Each sample collected was treated according to the protocol described by Lejeune *et al*. (2004) as described below.

3.5.2 Enrichment of samples

Samples (cabbage, lettuce and carrot) to be enriched were first of all shredded with the aid of sterile knives, scissors and forceps. Ten (10) grams of each sample was homogenized in 90 ml of modified Tryptone Soya Broth (mTSB) for 1minute using a Stomacher (Thermo Laboratory Blender 400, Tekmer, Ohio) and incubated at 37^0C for 24 hrs. Ten grams of the respective soil samples from each vegetable sample was collected and enriched with 90 ml of mTSB in a 100 ml conical flask and incubated for 24 hrs at 37^0C.

3.5.3 Isolation by selective plating

Following 24 hrs incubation period, using sterile wire loop, enriched samples were quadrant streaked on sorbitol-MacConkey agar supplemented with cefixime and tellurite (CT-SMAC) which was prepared in accordance with the manufacturer's instruction and then incubated at 37^0C for 24 hrs.

3.5.4 Colony identification

The colourless colonies on CT-SMAC were considered as presumptive *E. coli* O157 because O157 do not ferment sorbitol (see Appendix 3). Non-sorbitol fermenters were thereafter picked and inoculated onto nutrient agar slants, incubated at 37^0C for 24 hrs and stored in the refrigerator for further tests.

3.5.5 Biochemical testing of isolates

Stored isolates suspected to be *E. coli*, were further verified using conventional biochemical tests on the bases of hydrogen sulphide, indole production and motility with (SIM) medium (Merk, Germany), citrate utilization with simmons citrate agar (Merk, Germany), methyl red and voges-proskauer using MR-VP medium (Merk, Germany) and urease production using urea agar (Oxoid, UK) (Harrigan,1998).

a) H_2S, Indole and Motility test

Sulphide Indole Motility (SIM) medium (Oxoid, UK) which was prepared according to the manufacturer's instruction was used for these tests. The suspected isolates were inoculated into the medium using a sterile straight wire followed by incubation at 37^0C for 24 hrs. On 24 hrs incubation, the upper layer of the inoculated medium and line of stabbing was observed for the presence of dark colouration which was indicative of a

positive H_2S test. Also, the stabbed area of the inoculated medium was observed for diffused growth of the organism which was indicative of a positive motility test. Thereafter, 4-5 drops of Kovac's reagent were added to the inoculated SIM medium using a sterile pasture pipette in order to determine the production of indole by the organism which was evident by a red colour in the reagent layer within one minute. The result was considered negative by the appearance of a colourless layer on top of the test culture.

b) Citrate utilization test

In this test a sterile needle was used to inoculate the suspected isolates on the surface of the Simmon's citrate agar slant which contains a pH indicator (Bromothymol blue) and incubated at 35^0C for 24 hrs. At neutral pH as a result of the organism present not utilizing citrate a green colouration of the indicator was observed, thus indicating a negative test. A blue colouration was indicative of positive test (Harrigan, 1998).

c) Methyl Red-Voges Proskauer test

Non-sorbitol fermenters were inoculated into 5 ml of MR-VP broth and incubated for 48 hrs at 37^0C. After incubation, about 1ml of the broth was transferred to a small sterile serological tube followed by the addition of 2-3 drops of methyl red and the colour on the surface of the medium was read immediately. A red colouration on the addition of the indicator signified a positive methyl red test. To the rest of the broth in the original tube, 5 drops of 40% potassium hydroxide (KOH) was added followed by the addition of 5 drops of 5% of alcoholic (ethanol) alpha-Naphthol and shaken gently. The cap of the tube was loosened and the tube placed in a sloping position. The

development of a red colour starting from the liquid-air interface within 15-30 minutes was indicative of a positive test (Harrigan, 1998).

d) Urease production test

Urea agar slants were prepared according to the manufacturer's instruction and inoculated with the suspect isolates using sterile pasteur loop followed by incubation at 37^0C for 24 hrs. The development of a pink colouration within 18-24 hrs of incubation was indicative of positive test while a yellow colouration was considered negative (Harrigan, 1998).

3.5.6 Confirmation of isolates as *E. coli*

A commercially available biochemical test strip (Microbact 12E, Oxoid, UK) (see Appendix 4) was used in accordance with the manufacturer's instructions to confirm isolates suspected to be *E. coli* based on the result of the biochemical test (OIE, 2008).

3.5.6.1 Procedure for Microbact 12E

The isolate to be inoculated was grown on a selective media (CT-SMAC). Using a sterile straight wire, 2 to 3 colonies were picked and emulsified in 5.0 ml of peptone water and incubated at 37^0C for 4 hrs. A sterile pipette was then used to transfer one drop of the peptone water culture into 2.5 ml of sterile normal saline solution (0.85%). The wells of the individual substrate sets were exposed by cutting the end tag of the sealing strip and slowly peeling it back. Using a micropipette with sterile micropipette tips, 100 µl of the bacterial suspension was added to each well in the set. Mineral oil was then used to overlay the substrates in wells 1, 2 and 3, using a pipette. The inoculated rows were then re-sealed and

well labeled at the end of the tag with the specimen identification number followed by incubation at 37^0C for 24 hrs. On 24 hrs incubation period, the sealing tape on the test strips were peeled back and evaluated. Results were recorded on a report form as positive or negative by comparing them with a colour chart and making reference to the table of reactions provided. However, to well 8 (Indole production), two (2) drops of indole (Kovac's) reagent was added and the result evaluated within 2 minutes of addition of the reagent. To well 10 (Voges-Proskauer reaction), 1 drop each of VP1 and VP2 reagent were added and the result evaluated within 15 to 30 minutes after the addition. To well 12 (Tryptophan Deaminase), 1 drop of TDA reagent was added and the result evaluated immediately.

3.5.6.2 Interpretation of Microbact 12E result

In Microbact 12E, an octal coding system has been adopted of which each group of three reactions produce a single digit of the code. Using the results recorded on the report forms, the indices of the positive reactions are circled and the sum of these indices in each group of three reactions formed a code of four numbers. This code obtained was then entered into the computer-aided identification package and the resulting organism and its percentage probability recorded (see Appendix 5).

3.5.7 Serotyping for *E. coli* O157:H7

The isolates that were confirmed as *E. coli* using Microbact 12E, were further subjected to serotyping by bacterial agglutination using a commercial latex kit for *E. coli* O157:H7 (Wellcolex *E. coli* O157:H7 kit, Oxoid, UK) (see Appendix 6) which is a rapid latex agglutination test for confirming non-sorbitol fermenting colonies as possessing the O157 somatic antigen and H7 flagellar antigen.

3.5.7.1 Test procedure for O157 detection

Fresh cultures grown at 37^0C for 24 hrs on sorbitol MacConkey agar (Oxoid, UK), were used for this test. For each test sample, 40 µl of saline was placed in two circles on a reaction card using a micropipette. With the help of a mixing stick provided, sufficient growth just enough to cover the blunt end of the stick was removed from the plate and emulsified in the saline by rubbing with the flat end of the stick, mixing it thoroughly without damaging the surface of the card. This was also done for the second circle on the reaction card already containing saline after which the mixing sticks were immediately discarded. For each of the test sample, one drop of O157 Test Latex was placed in one circle (with the emulsified culture) and one drop of O157 Control Latex in the other circle by holding the dropper bottles vertically in order to dispense an accurate drop. A mixing stick was then used to mix the contents of the circles, spreading the latex carefully over the entire area of the circle. The card was then rocked slowly for 30seconds and observed for agglutination. The used reaction card was then discarded for safe disposal.

The agglutination of the O157 Test Latex accompanied by a lack of agglutination of the Control Latex on observation was used to indicate the presence of O157 antigen in the culture under test. Absence of agglutination in both O157 test and control reagents was used to indicate the absence of O157 antigen in the test culture (see Appendix 7).

3.5.7.2 Test procedure for H7 detection

Test cultures that were positive for O157 antigen were further tested for H7 antigen by subculture in Tryptone Soya Broth (TSB) (Oxoid, UK) at 37^0C for 24 hrs. For each test sample 40 µl each of TSB-growth cultures were placed in two circles on a reaction card, followed by placing a drop of the H7 Test Latex in one circle (with the broth culture) and a

drop of H7 Control Latex in the other circle. The contents of the circles were then thoroughly mixed using a mixing stick, carefully spreading the latex over the entire areas of the circle. The card was then slowly rocked for 30 seconds and observed for agglutination after which the card was discarded. Agglutination of the H7 Test Latex accompanied by a lack of agglutination of the Control Latex on observation was used to indicate the presence of H7 antigen while the absence of agglutination in both reagents was indicative of the absence of H7 antigen in the test culture (see Appendix 8).

3.5.8 Ridascreen verotoxin test

Isolates that were confirmed as *E. coli* O157:H7 were subjected to the ridascreen verotoxin test (an enzyme immunoassay) for the qualitative determination of verotoxins using commercial Elisa kit (Ridascreen® Verotoxin ELISA Kit, R-Biopharm, Darmstadt, Germany) in order to determine their potential virulence for humans (see Appendix 9). The procedure which was followed complies with the recommendations of the RKI (Robert Koch Institute) and the BfR (German Institute of Risk Evaluation).

3.5.8.1 Procedure for verotoxin test using ELISA

Isolates to be tested were inoculated into 4 ml modified tryptone soya broth (mTSB) with Mitomycin C and incubated at 37^0C for 24 hrs. After incubation, the entire samples were centrifuged at 3000 rpm for 15 minutes. Using sterile micropipette tips, 100 µl of the supernatant was transferred into the wells and 2 drops of the positive control and the negative control (sample dilution buffer) were also transferred into separate wells followed by incubation at room temperature (20 -25^0C) for 60 minutes. The plate was carefully washed using wash buffer provided which was prepared by mixing 1 part of the wash buffer concentrate with 9 parts distilled water with the plate blotted by knocking it onto a

clean absorbent paper in order to remove residual moisture. Two (2) drops of the enzyme conjugate were thereafter, added to the previously inoculated wells and incubated at room temperature (20 -25^{0}C) for 30 minutes. This was also followed by washing and blotting as described above. Thereafter, 2 drops of substrate provided were added to each well and incubated in the dark at room temperature (20 -25^{0}C) for 15 minutes. This was followed by the addition of 1 drop of stop reagent to each well in order to stop the reaction (see Appendix 10). Using an ELISA machine, the extinction was measured at 450 nm.

3.5.8.2 Evaluation and interpretation of Ridascreen test result

The test was evaluated to be correct by comparing the extinction for the positive and negative control obtained to that specified by the producers as the standard extinction for a correct test. Next, a cut-off was established by adding 0.1 extinction units to the measured extinction for the negative control as described by the manufacturer. The samples were considered positive if their extinction was greater than or equal to the calculated cut-off for the negative control.

3.6 Antimicrobial Susceptibility Testing of Isolates

Antimicrobial susceptibility testing was carried out on the isolates that were confirmed as *E. coli* using the agar disk diffusion method as described by Bauer *et al*. (1966) according to the guidelines recommended by the Clinical and Laboratory Standards Institute (CLSI, 2011). The test isolates were inoculated into a sterile tryptone soya broth (Oxoid, UK) that was prepared according to the manufacturer's instruction and incubated at 37^{0}C for 24 hrs.

Using sterile saline, the broth cultures were adjusted to obtain turbidity optically comparable to 0.5 McFarland standards after which sterile Mueller Hinton agar plates were

then inoculated with 0.1 ml of the TSB culture and spread over the entire sterile agar surface using sterile swap sticks. The drug impregnated discs that were used (Oxoid, UK) containing Sulfamethoxazole/Trimethoprim (25µg), Ciprofloxacin (5µg), Chloramphenicol (30µg), Ampicillin (10µg), Gentamicin (30µg), Tetracycline (30µg), Streptomycin (10µg) and Cloxacillin (5µg) were placed on the surface of the inoculated agar plates using a dispenser (Oxoid, UK) and incubated at 37^0C for 18 hrs.

The mean diameters of zones of inhibition of the test antibiotics exhibited by each test isolate were measured to the nearest millimeter using a graduated ruler (see Appendix 11). The isolates tested were classified as either sensitive (S), intermediate (I) or resistant (R) based on the zone diameter breakpoints to the nearest whole number in millimeters (mm) for each drug used (CLSI, 2011).

Multi Drug Resistance (MDR) character of the isolates obtained which is defined as resistance to all the tested antibiotics in at least two of the classes (Dominic *et al*., 2005) was determined in this study by observing the resistance pattern of the isolates to the antibiotics. In addition, the spread of bacterial resistance in the population which can be revealed by a tool known as Multiple Antibiotic resistance (MAR) index was determined for the bacterial isolates obtained by dividing the number of antibiotics to which each isolate was resistant to (a) by the total number of antibiotics tested (b). An MAR index greater than 0.2, indicates that the strains of such isolates originated from an environment where several antibiotics are used (Tambekarm *et al*., 2006).

3.7

3.8

3.9 Data Analyses

Data obtained were analyzed using the statistical package for social sciences (SPSS) version 17.0. The results were presented in percentages, tables and charts and Chi-square test and Fisher's exact test were used to compare the presence of the organism in the types of vegetable and soil samples from the experimental and control farms. Values of $P < 0.05$ were considered significant. Prevalence of *E. coli* O157:H7 in vegetables and soil was determined using the formula

$$\textit{Prevalence} = \frac{\textit{Positive samples}}{\textit{Total number of samples analyzed}} \times 100\%$$

CHAPTER FOUR

RESULTS

The occurrence of *E. coli* O157:H7 on cabbage, lettuce and carrot grown on farms using cattle manure as fertilizer in Zaria, Nigeria was studied.

4.1 Result of the Pilot Study

A total of 120 samples of cabbage, lettuce, carrots and the soils from vegetable farms were collected from Zaria City and Sabon Gari LGAs for the pilot study, out of which only four presumptive *E. coli* isolates were isolated based on their phenotypic and biochemical characteristics. Of these four isolates, one (1) was obtained from a vegetable sample while the remaining three were obtained from the soil samples (Table 4.1).

The four presumptive isolates were tested by Microbact 12E and for O157 and H7 antigens leading to the confirmation of two isolates (Table 4.2). This gave an *E. coli* O157:H7 prevalence of 1.7% which was used to determine overall sample size.

Table 4.1: Isolation of *E. coli* from Cabbage, Lettuce, Carrot and Soil Samples on which they were grown during a Pilot Study

Vegetables	**No. of Samples**	**No. of Positive *E.coli***	**(%) Positive**
Cabbage	20	0	0
CS	20	1	5
Lettuce	20	1	5
LS	20	2	10
Carrot	20	0	0
CrS	20	0	0
Total	**120**	**4**	**3.33**

Legend: CS represents soil obtained from cabbage farm; LS represents soil obtained from lettuce farm, while CrS represents soil obtained from carrot farm.

Table 4.2: Summary of Microbact and Latex Agglutination Test Result for Pilot Study

Tests	No. of Isolates Tested	No. Positive	(%) Positive
Microbact	4	2	50
O157 Test	2	2	100
H7 Test	2	2	100

4.2 Conventional Biochemical, Microbact 12E and Serotyping Test Results

Based on the phenotypic and biochemical characteristics of the isolates obtained from the vegetable and soil samples (see Appendix 12), 10 presumptive *E. coli* isolates were identified from the vegetable samples from the experimental farms (farms using animal manure as fertilizer) out of which 3 (30%) of the presumptive *E. coli* isolates were from cabbage, 2 (20%) from carrots and 5 (50%) from lettuce, while one (1) presumptive isolate was identified from cabbage from a control farm (farms using inorganic fertilizer) giving a total of 11 vegetable isolates (Table 4.3). Furthermore, a total of 12 *E. coli* isolates were identified from soil samples obtained from the experimental farms out of which 1 (6.7%) was isolated from soil obtained from cabbage farm, 7 (14%) were isolated from soil obtained from lettuce farm, and 4 (33.3%) from soil obtained from carrot farm. No isolate was obtained from soil samples collected from the control farms (Table 4.4).

Table 4.3: Isolation of *E. coli* from Cabbage, Lettuce and Carrot Samples in Experimental and Control Farms in Zaria, Nigeria

Vegetable Samples	Experimental farm (EM)		Control farm (CM)	
	No. of samples	No. +ve (%) for *E. coli*	No. of samples	No. +ve (%) for *E. coli*
Cabbage	50	3 (6)	10	1 (10)
Lettuce	50	5 (10)	10	0 (0)
Carrot	50	2 (4)	10	0 (0)
Total	**150**	**10 (6.67)**	**30**	**1 (3.33)**

Legend: EM implies farms fertilized with animal manure; CM implies farms fertilized with inorganic fertilizer.

Table 4.4: Isolation of *E. coli* from Soil Samples in Experimental and Control Farms in Zaria, Nigeria

Soil Samples from	Experimental farm (EM)		Control farm (CM)	
	No. of samples	No. +ve (%) for *E. coli*	No. of samples	No. +ve (%) for *E. coli*
Cabbage farm	50	1 (2)	10	0 (0)
Lettuce farm	50	7 (14)	10	0 (0)
Carrot farm	50	4 (8)	10	0 (0)
Total	**150**	**12 (8)**	**30**	**0 (0)**

Legend: EM implies farms fertilized with animal manure; CM implies farms fertilized with inorganic fertilizer.

These presumptive isolates gave a total of 23 which were subjected to Microbact 12E testing for confirmation as *E. coli*. Table 4.5 shows that out of the 11 vegetable isolates obtained from the experimental and control farms that were tested by Microbact 12E, a total number of six isolates were confirmed as *E. coli.* Also, out of the 12 soil isolates obtained from the experimental farms (farms using manure as fertilizer) a total number of seven isolates were confirmed as *E. coli* (Table 4.6). There was none from soils collected from the control farms (farms using inorganic fertilizer).

Table 4.5: Microbact 12E Confirmation of *E. coli* Isolates from Cabbage, Lettuce and Carrot Samples in Experiment and Control Farms in Zaria, Nigeria

	Experimental farm (EM)		Control farm (CM)	
Vegetable Samples	**No. of Isolates**	**No. +ve (%) Confirmed *E. coli***	**No. of Isolates**	**No. +ve (%) Confirmed *E. coli***
Cabbage	3	2 (66.6)	1	1 (100)
Lettuce	5	1 (20)	0	0 (0)
Carrot	2	2 (100)	0	0 (0)
Total	**10**	**5 (50)**	**1**	**1**

Legend: EM implies farms fertilized with animal manure; CM implies farms fertilized with inorganic fertilizer.

Table 4.6: Microbact 12E Result of *E. coli* Isolates from Soil Samples in Experimental and Control Farms in Zaria, Nigeria

Soil Samples from	Experimental farm (EM)		Control farm (CM)	
	No. of samples	No. +ve (%) for *E. coli*	No. of samples	No. +ve (%) for *E. coli*
Cabbage farm	1	0 (0)	0	0 (0)
Lettuce farm	7	4 (57)	0	0 (0)
Carrot farm	4	3 (75)	0	0 (0)
Total	**12**	**7 (58.3)**	**0**	**0**

Legend: EM implies farms fertilized with animal manure; CM implies farms fertilized with inorganic fertilizer.

The result of latex agglutination test in Table 4.7 shows that out of the 13 *E. coli* isolates tested for the presence of O157 somatic antigen, 5 (38.5%) of the vegetable isolates and 7 (53.9%) of the soil isolates were positive. The resulting 12 *E. coli* O157 isolates were further tested for the presence of H7 flagella antigen out of which eight (four of each were from vegetables and soil) tested positive, while four were *E. coli* O157 lacking H7 flagella antigen. One of the 13 isolates was negative for both O157 and H7 antigens.

Table 4.7: Latex Agglutination Test Results of *E. coli* Isolates from Cabbage, Lettuce, Carrot and Soil where they were grown in Zaria, Nigeria

S/N	Source of Isolates	Isolate No.	Agglutination with	
			O157	H7
1	Cabbage	SBCb8	-	-
2	Cabbage	ZACb10	+	+
3	Cabbage	ZCCb3	+	-
4	Lettuce	ZBLt7	+	+
5	LS	ZBLtS7	+	-
6	LS	SCLtS3	+	+
7	LS	SCLtS4	+	-
8	LS	SCLtS5	+	-
9	Carrot	ZCCr1	+	+
10	CrS	ZCCrS1	+	+
11	CrS	ZCCrS6	+	+
12	Carrot	ZACr10	+	+
13	CrS	SBCrS10	+	+

Legend: LS represents soil obtained from lettuce farm, while CrS represents soil obtained from carrot farm.

The results in Table 4.8 shows that all the eight (8) *E. coli* O157:H7 isolates were obtained from vegetables and soil samples from the experimental farms (farms using animal manure as fertilizer) and none from vegetables and soil samples from the control farms (farms using inorganic fertilizers).

Table 4.8: Comparison of *E. coli* O157:H7 Isolation from Cabbage, Lettuce, Carrot and Soil in Experimental and Control Farms where they are grown in Zaria, Nigeria

	No. of Sample		No. Positive		% Positive	
Farm	**Vegetables**	**Soil**	**Vegetables**	**Soil**	**Vegetables**	**Soil**
Experimental	150	150	4	4	2.7	2.7
Control	30	30	0	0	0	0
Total	**180**	**180**	**4**	**4**	**2.2**	**2.2**

OR= 1.874 (CI=0.0982 to 35.74) P= 0.49

Prevalence rates of *E. coli* O157:H7 for the individual vegetable samples analyzed in Table 4.9, shows that carrot had the highest prevalence rate of 4% compared to cabbage and lettuce with 2% each even though the difference was not statistically significant. Also, pooling all the vegetables together resulted in a pooled prevalence rate of 2.7% for vegetables and 2.7% as the pooled prevalence rate for soils (Table 4.10).

Table 4.9: Prevalence of *E. coli* O157:H7 Isolated from Cabbage, Lettuce and Carrot Cultivated on farms in Zaria, Nigeria

Vegetables	No. of Samples Analyzed	No. of *E. coli* O157:H7	(%) Positive	x^2	P-value
Cabbage	50	1	2.0	0.514	0.774
Lettuce	50	1	2.0		
Carrot	50	2	4.0		
Total	**150**	**4**	**2.7**		

Table 4.10: Prevalence of *E. coli* O157:H7 Isolates from Cabbage, Lettuce, Carrot and Soil where they were grown in Zaria, Nigeria

Samples from	No. of Samples Analyzed	No. of *E. coli* O157:H7	(%) Positive
Vegetables	150	4	2.7
Soil	150	4	2.7
Total	**300**	**8**	**2.7**

4.3 Result of Ridascreen Verotoxin Test

The results obtained showed that of the 8 *E. coli* O157:H7 isolates tested for the production of verotoxins, only 2 (25%) of the isolates were positive for the verotoxins. One was obtained from carrot in Zaria City Local Government Area (ZACr10) and the other from soil fertilized with cattle manure on which lettuce was grown in Sabon Gari Local Government Area (SCLtS3) (Table 4.7).

4.4 Results of Antibiotic Susceptibility Patterns of the *E. coli* Isolates

The results obtained from the 13 *E. coli* isolates tested for antibiotic susceptibility revealed that these isolates showed varied and multi drug resistance (MDR) to the 8 antibiotics tested with high multiple antibiotic resistance (MAR) index. These isolates can be grouped within the range of those that are resistant to at least 2 antibiotics (25%) to those resistant to at most 6 antibiotics (75%) per isolate (Table 4.11). Also, observing the patterns of percentage resistance of the isolates to individual antibiotics, the isolates can be classified as showing 0% resistance and 100% resistance (Fig 4.1). All the isolates were resistant to cloxacillin (5µg), but were susceptible to ciprofloxacin (5µg) and gentamicin (30µg).

In addition, the frequency of resistance patterns (Table 4.10), showed that 4(30.8%) of the isolates were resistant to cloxacillin, ampicillin and sulfamethoxazole/trimethoprim, 4(30.8%) were resistant to cloxacillin, ampicillin, sulfamethoxazole/trimethoprim and streptomycin, while only 2(15.4%) of the isolates was resistant to cloxacillin and ampicillin. However, one of the isolates was observed to have a high percentage resistance (75%) in that it was resistant to almost all the antimicrobial drugs tested except ciprofloxacin and gentamicin.

Table 4.11: Antibiotic Susceptibility Patterns of *E. coli* Isolates from Cabbage, Lettuce, Carrot and Soil where they were grown in Zaria, Nigeria

		Antibiogram									(%)
Sample	**Isolate**	**OB**	**C**	**AMP**	**TE**	**RL**	**ST**	**CIP**	**CN**	**Resistance patterns**	**Resistance**
Lettuce and soil	ZBLt7	R	I	R	S	R	R	S	S	OB,AMP,RL,ST	50
	ZBLtS7	R	S	R	S	S	S	S	S	OB,AMP	25
	SCLtS3	R	I	S	I	R	S	S	S	OB,RL	25
	SCLtS4	R	S	R	I	R	R	S	S	OB,AMP,RL,ST	50
	SCLtS5	R	S	R	I	R	I	S	S	OB,AMP,RL	37.5
PR(%) of isolates to single Antibiotics	n=5	100	0	80	0	80	40	0	0		
Cabbage and soil	SBCb8	R	I	R	S	R	I	S	S	OB,AMP,RL	37.5
	ZACb10	R	S	R	S	I	I	S	S	OB,AMP	25
	ZCCb3	R	R	R	R	R	R	I	S	OB,C,AMP,TE,RL,ST	75
PR(%) of isolates to single Antibiotics	n=3	100	33.3	100	33.3	66.7	33.3	0	0		
Carrot	ZCCr1	R	R	I	R	R	R	S	S	OB,C,TE,RL,ST	62.5
	ZCCrS1	R	S	R	S	R	R	S	S	OB,AMP,RL,ST	50

and soil	ZACr10	R	S	R	S	R	R	S	S	OB,AMP,RL,ST	50
	ZCCrS6	R	S	R	S	R	S	S	S	OB,AMP,RL	37.5
	SBCrS10	R	I	R	S	R	I	S	S	OB,AMP,RL,	37.5
PR(%) of isolates to single Antibiotics	n=5	100	20	80	20	100	60	0	0		

Legend: OB-Cloxacillin, C- Chloramphenicol, AMP- Ampicillin, TE- Tetracyclin, RL- Sulfamethoxazole/Trimethoprim, ST- Streptomycin, CIP- Ciprofloxacin, CN- Gentamicin, R- Resistant, I- Intermediate, S- Susceptible, PR- Percentage Resistance, n- Number of isolate.

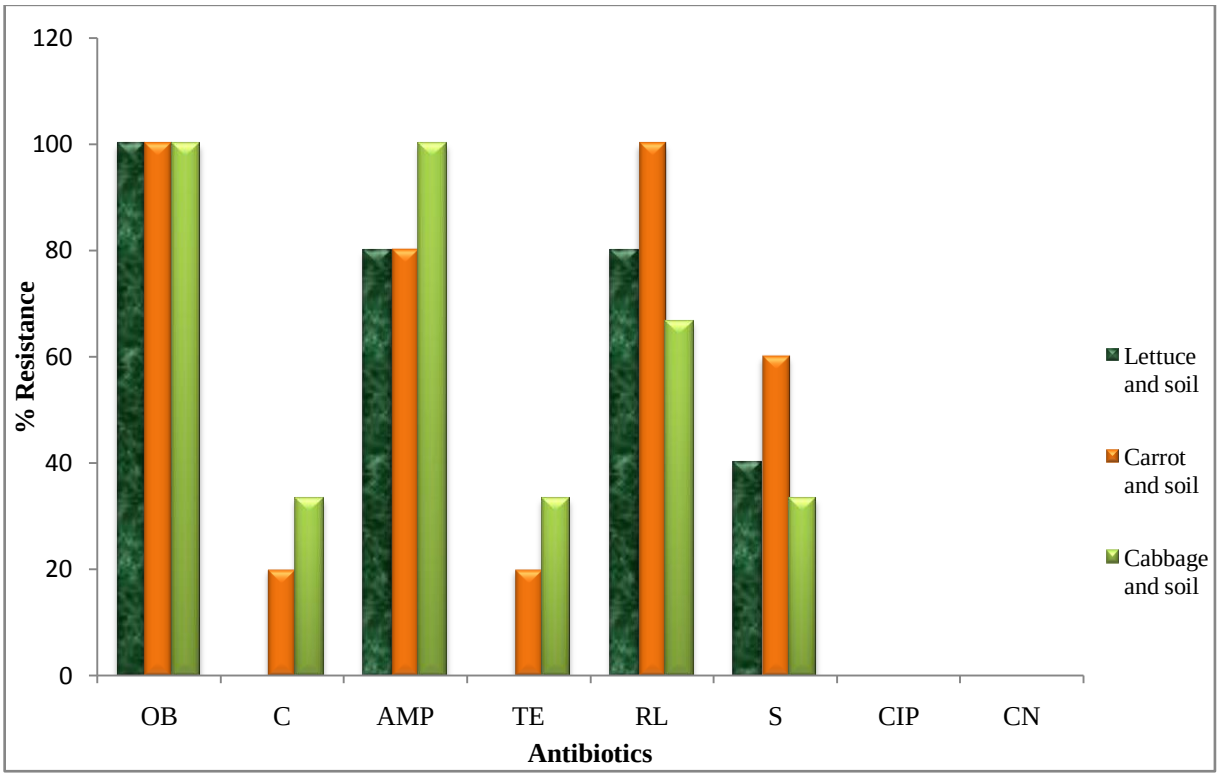


Fig 4.1: Percentage Frequency of Resistance in *E. coli* Isolates Obtained from Cabbage, Lettuce, Carrot and Soil where they were grown in Zaria, Nigeria

Legend: OB-Cloxacillin, C- Chloramphenicol, AMP- Ampicillin, TE- Tetracyclin, RL- Sulfamethoxazole/Trimethoprim, ST- Streptomycin, CIP- Ciprofloxacin, CN- Gentamicin

Fig 4.2 shows the frequency of 6 different antibiograms of which the resistance patterns (cloxacillin, ampicillin, sulfamethoxazole/trimethoprim and cloxacillin, ampicillin, sulfamethoxazole/trimethoprim, streptomycin) had the highest frequency of all the patterns observed.

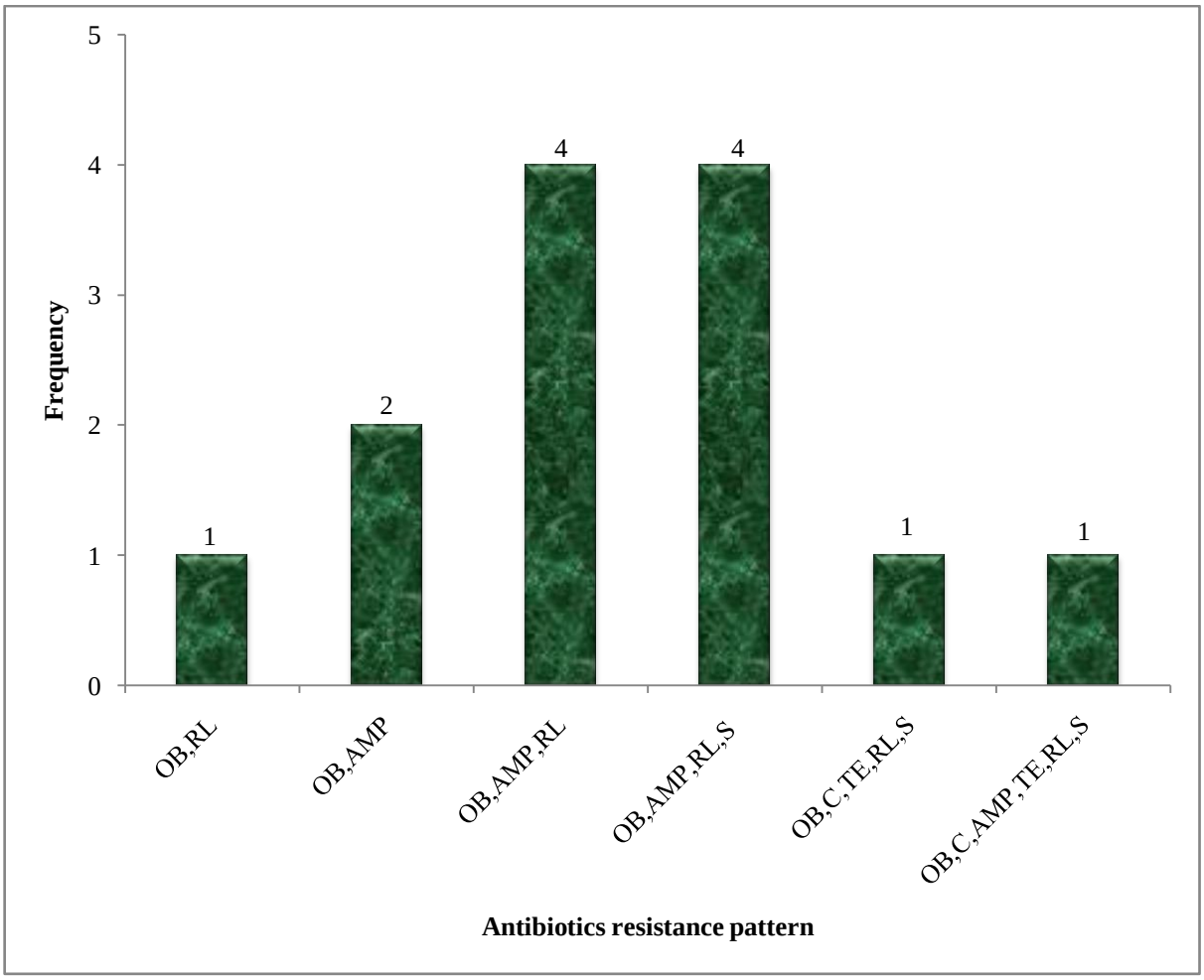


Fig 4.2: Frequency of 6 different Antibiograms of *E. coli* isolates from Cabbage, Lettuce, Carrot and Soil where they were grown in Zaria, Nigeria

Legend: OB-Cloxacillin, C- Chloramphenicol, AMP- Ampicillin, TE- Tetracyclin, RL- Sulfamethoxazole/Trimethoprim, ST- Streptomycin, CIP- Ciprofloxacin, CN- Gentamicin

Table 4.12 shows the result of MAR Index analysis of the isolates based on their antibiotics resistance patterns showing that all the isolates tested against the 8 antibiotics showed MAR index greater than 0.2.

Table 4.12: MAR Index Analysis of the *E. coli* Isolates from Cabbage, Lettuce, Carrot and Soil where they were grown in Zaria, Nigeria

S/N	Isolate	No. of antibiotics to which isolate was resistant (a)	MAR Index (a/b)
1	ZBLt7	4	0.50
2	ZBLtS7	2	0.25
3	SCLtS3	2	0.25
4	SCLtS4	4	0.50
5	SCLtS5	3	0.38
6	SBCb8	3	0.38
7	ZACb10	2	0.25
8	ZCCb3	6	0.75
9	ZCCr1	5	0.63
10	ZCCrS1	4	0.50
11	ZCCrS6	4	0.50
12	ZACr10	3	0.38
13	SBCrS10	3	0.38

Legend: Total No. of antibiotics to which the isolates were subjected =8 **(b)**

MAR means multiple antibiotics resistance

4.5 Results of Questionnaire Analyses

Questionnaires were administered to 18 farmers from whose farms samples were collected. The distribution of *E. coli* O157:H7 in Sabon Gari and Zaria City LGAs is shown in Table 4.13. Four out of 9 farms in Zaria City Local Government Area were positive for *E. coli* O157:H7. Relating this to the questionnaire results, it was observed that these 4 farms contaminated with *E. coli* O157:H7 isolates were among the farms that used cattle manure as fertilizer. In Sabon Gari Local Government Area, 2 farms among the farms using organic manure were also considered positive for *E. coli* O157:H7 out of 9 farms.

Table 4.13: Distribution of *E. coli* O157:H7 in Sabon Gari and Zaria City Local Government Areas by Farms in Zaria, Nigeria

Local Govt. Area	No. of farms	Frequency of O157:H7	(%) Positive	P-value
Sabon Gari	9	2	22.2	0.31
Zaria City	9	4	44.4	
Total	**18**	**6**	**33.3**	

Table 4.14 shows the distribution of *E. coli* O157:H7 by the types of organic manure used in farms that cultivated fresh vegetables in Zaria. Cattle manure was the most widely used organic manure by farmers in Zaria of which out of the 15 farms using organic manure, 8 (53.3%) used cattle faeces. Each of the remaining seven organic manure fertilized farms used goat, poultry, sheep faeces, a combination of cattle manure with others and manure from garbage dumps. Five of the 8 farms using cattle manure were found to be contaminated with *E. coli* O157:H7 while one farm that used poultry manure was contaminated with *E. coli* O157:H7. On the other hand, farms using goat, sheep faeces and dustbin/garbage dumps as manure were not contaminated with *E. coli* O157:H7.

Table 4.14: Distribution of *E. coli* O157:H7 in Sabon Gari and Zaria City LGAs by Types of Organic manure in Zaria, Nigeria

Type of Organic Manure	No. of Farms	Frequency of Isolation	(%) Frequency of Isolation
Cattle faeces	8	5	63
Goat faeces	1	0	0
Poultry faeces	1	1	100
Sheep faeces	1	0	0
Cattle + others	3	0	0
Dustbin/Garbage dumps	1	0	0
Total	**15**	**6**	**40**

Table 4.15 shows the post harvest handling of cabbages, lettuce and carrots by farmers in Zaria. Three farms washed their fresh vegetable produce before packaging while 15 farms packaged without washing. *E. coli* O157:H7 was isolated from three of the farms whose farmers washed their vegetables before packaging and from three of the farms whose farmers packaged their produce without washing.

Table 4.15: Distribution of *E. coli* O157:H7 in Sabon Gari and Zaria City LGAs by Post-Harvest Handling

Handling/Care	No. of farms	Frequency of Isolation	(%) Frequency of Isolation
Wash before packaging	3	3	100
Package without washing	15	3	20
Total	**18**	**6**	**33.3**

Figure 4.3 shows a pie chart of where farmers sold their cabbage, lettuce and carrots. Fifty-six (56%) of the farmers sold their vegetables at Tudun Wada and Sabon Gari markets, while 22% of the farmers sold theirs at Samaru and Sabon Gari markets. Only 17% of the farmers sold their produce to only Samaru market and 6% of the vegetables were sold out of Zaria.

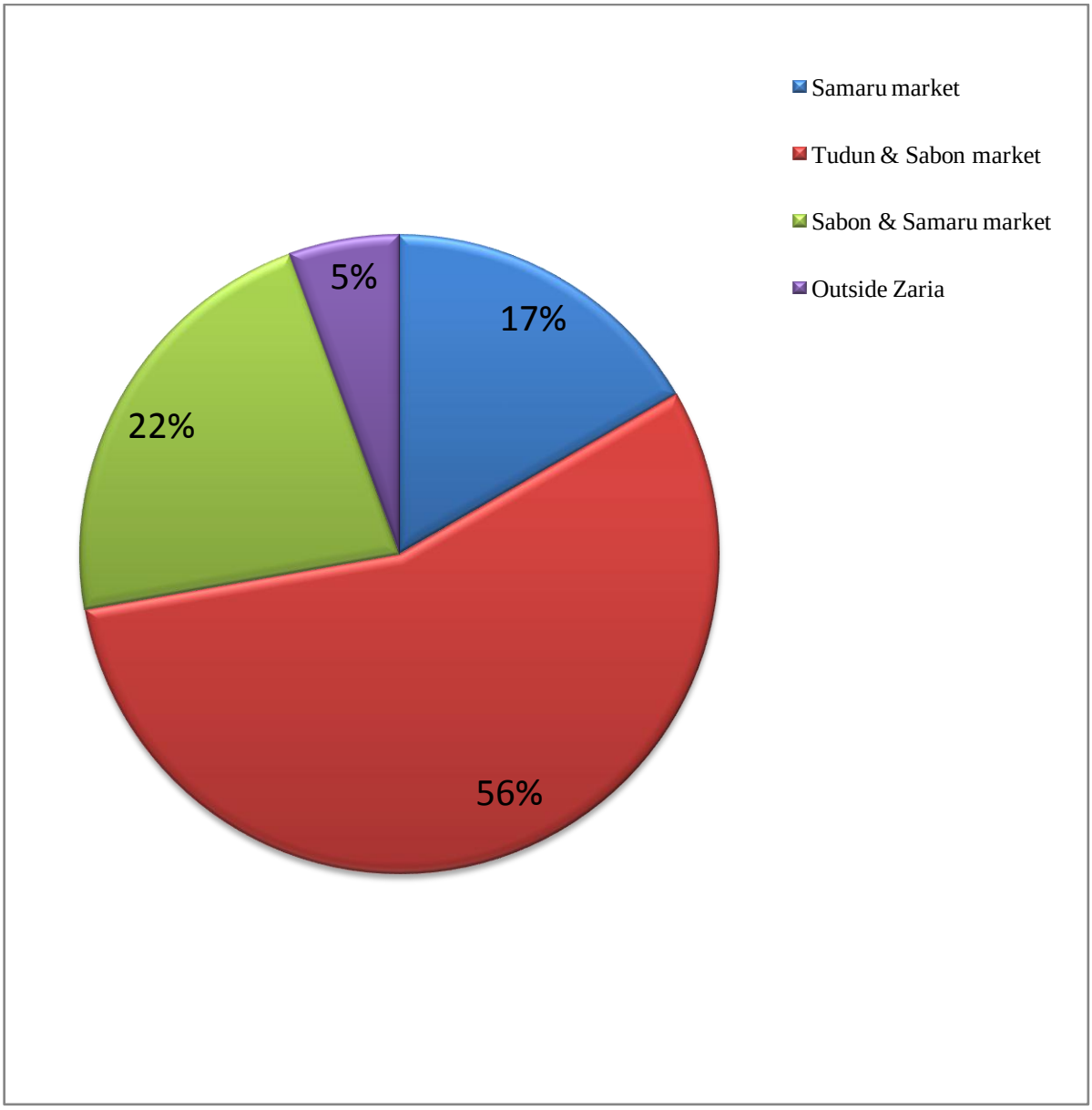


Fig 4.3: Distribution of Markets in Zaria and Percentage of Produce Received from Farmers

CHAPTER FIVE

DISCUSSION

The occurrence of *E. coli* O157:H7 on cabbage, lettuce and carrots were determined in Zaria, among farms that used animal manure as fertilizer. The possibility of leafy vegetables and root crops such as lettuce, cabbage and carrots to be contaminated with *E. coli* O157:H7 is of growing concern since these farm products can be eaten raw without cooking.

Considering the occurrence of *E. coli* O157:H7 from cabbage, lettuce and carrots and their corresponding soils on which they were grown, there was isolation in cabbage though there was no isolation from the soil on which it was grown. This may indicate that soil may not be the source of *E. coli* O157:H7 in cabbage. The occurrence of *E. coli* O157:H7 in lettuce was from both the leaves and the soil on which it was grown. This may be an indication that the source of contamination of lettuce with *E. coli* O157:H7 was from the soil. Also, considering the isolation of *E. coli* O157:H7 from carrot and the soil on which it was grown which was twice more in soil than in carrot, it may be an indication that contamination of carrot by *E. coli* O157:H7 was very high showing that the source of *E. coli* O157:H7 in carrot was absolutely from the soil which is of public health significance. The data obtained revealed that culturable *E. coli* O157:H7 were present on vegetables and in soil samples from animal manure-applied farms in Zaria. Isolation rates of 2.7% on both vegetables and soil samples respectively were obtained, giving a pooled vegetable and soil prevalence rate of 2.7% out of a total 300 samples collected. However, it is not surprising that these results

were obtained, the previous studies have shown that cattle are the principal reservoir of *E. coli* O157:H7 (Renter *et al*., 2003; Lejeune *et al*., 2004). Also, since in the present study, most of the farmers were found to use organic manure of cattle faeces to fertilize their farms.

From the low prevalence of 2.7% obtained in this study, it may be postulated that farms in Zaria using organic manure have a low distribution of *E. coli* O157:H7. One possible reason may be the fact that most farmers in Zaria generally applied cattle manure on the land intended for fresh vegetable produce cultivation during the early harmattan period (i.e. between October and November). Considering the time when these produce were ready for harvesting (March the next year), it can be stated that there is a rather long time interval between incorporation of manure into soil and harvesting of the vegetables and as a result the build-up of pathogens may have been greatly reduced. This is thought to be so considering the United States Department of Agriculture (USDA) organic certification program held in the United States. In the program, it was recommended as a risk mitigation strategy that farmers growing edible crops that do not come in contact with the soil to incorporate manure into the soil at least 90days before harvesting and 120days before harvesting for edible crops that come in contact with soil (Beuchat, 1996). It has also been well documented that vegetables in the field can become contaminated with pathogenic microorganisms by internalization via the root system (Tauxe *et al*., 1997; Welinder-Olsson *et al*., 2004; Jablasone *et al*., 2005) and splash dispersal from the soil surface (Franz, 2007). On the bases of previous studies on splash dispersal, the low frequency of *E. coli* O157:H7 isolated in this study could be due to the way in which these farms were watered using the irrigation farming method. As a result, the possibility of an *E. coli* O157:H7 organism

being transported from the contaminated soil to the vegetables (cabbage and lettuce) by splash dispersal may be rather low.

Comparing the prevalence rates obtained in cabbage, lettuce and carrot, the reason why the prevalence rate in carrot was higher than that in cabbage and lettuce could be due to the fact that in a farm setting, carrot is embedded in the soil hence the possibility of sufficient contact of carrot tubers with the soil is higher than the contact of leaves of cabbage and lettuce with soil; thus, giving a higher likelihood of contamination in carrots. This is contrary to works done on vegetables sold in markets where lettuce was reported to have higher contamination rate than cabbage and carrots (Abdul-Raouf *et al*., 1993).

Another reason for the low prevalence of *E. coli* O157:H7 may be due to the presence of interfering bacteria during isolation (e.g. *Pseudomonas* species) (Szabo *et al*., 1986; Vernozy-Rozand, 1997; LeJeune *et al*., 2001). Also, the isolation of *E. coli* O157:H7 can be hampered by the presence of other gram negative facultative anaerobic rods which also colonize fresh vegetable produce and do not ferment sorbitol, appearing as colourless colonies on CT-SMAC agar such as *Aeromonas, Plesiomonas, Morganella and Providencia* species (Fujisawa *et al*., 2002). Hence, there is a possibility that the actual prevalence may be higher than reported in this study. On the other hand, it is also possible that the prevalence of *E. coli* O157:H7 may be low in vegetables and soils in the study area.

The prevalence of 2.7% of *E. coli* O157:H7 in vegetables and 2.7% in soil obtained in this study are similar to the report of Khandaghi *et al*. (2010), who detected *E. coli* O157:H7 in 2.1% of 282 of vegetables and soil samples collected.

In this study, the isolation rate of *E. coli* O157:H7 was higher in experimental than control farms even though there was no statistical significance (P = 0.49). However, considering the odds ratio value (OR=1.874), it could be stated that the chances of cabbage, lettuce and carrot to be contaminated with *E. coli* O157:H7 is higher in farms using cattle manure as fertilizer than in farms using inorganic fertilizer.

Out of the eight *E. coli* O157:H7 isolates subjected to the Ridascreen verotoxin ELISA test, only two (25%) were positive for the production of verotoxin. Verotoxin was not detected in the six isolates possibly due to the fact that they might still possess the *stx*1 or *stx*2 genes but were unable to express them as Tekdek *et al*. (1995) similarly detected verotoxins in 25% of VTEC isolates from calves or they might have lost the *stx*1 and *stx*2 genes as losses of the genes prior to testing by PCR have been documented (Karch *et al.,* 1998; Feng *et al.,* 2001; Blanco *et al.,* 2003). Also, Raji *et al*. (2006) was unable to detect the presence of *stx*1 or *stx*2 genes in 11 *E. coli* O157 strains tested by PCR. It may be variants of shiga toxins 1 and 2 (*stx*1 and *stx*2) that the isolates possessed.

Antimicrobial resistance in *Enterobacteriaceae* poses a critical public health threat, especially in the developing countries where the indiscriminate use of antibiotics as prophylactic or therapeutic measures to control bacterial infections is on the increase. This has a direct clinical implication which involves the development and transfer of resistance genes by these bacteria, thereby making it difficult to effectively control the increasing range of human bacterial diseases (WHO, 1997; Karlowsky *et al*., 2003).

In this study, the antibiotic sensitivity results showed that all the isolates were resistant to at least one antibiotics especially cloxacillin but were all susceptible to gentamicin and

ciprofloxacin. The antibiotic susceptibility results obtained in this study is similar to the report by Okolocha *et al.* (2006) and Walsh *et al*. (2006) who reported in their studies that the *E. coli* isolates tested were highly susceptible to gentamicin and ciprofloxacin. The susceptibility exhibited by all the isolates with respect to gentamicin and ciprofloxacin could be seen as the result of the mode of action of the drugs (Brown, 1988), complemented by less frequent prescription of these drugs by physicians. The high cost of these drugs and route of administration (intramuscular or intravenous) could have restricted misuse thereby reducing the emergence of resistant bacterial strains. The observed resistance of the isolates tested to ampicillin, chloramphenicol, streptomycin sulfamethoxazole/trimethoprim and tetracycline, is probably an indication of the fact that these isolates have had previous exposure to these antibiotics before or had acquired the resistance genes from other drug resistant organisms which may have led to the development of resistant strains (Wickens and Wade, 2005; Madden, 2009).

The identification of MDR isolates in this study based on the definition of MDR revealed that all the isolates with the exception of two (ZBLtS7 and ZACb10) were resistant to at least two of the classes of antibiotics tested which qualify them to be designated as Multi Drug Resistant. Isolates ZBLtS7 and ZACb10 were resistant to cloxacillin and ampicillin only which both belong to the same class of antibiotics.

Since bacteria with MAR Index > 0.2 are thought to have originated from an environment where several antibiotics are used (Tambekarm *et al*., 2006), the result of this study show that all the isolates had MAR index greater than 0.2, with 5 isolates having considerably higher MAR index. This result implies that even though two isolates were not Multi Drug Resistant, all the isolates would have spread from an environment of high antibiotic use.

From the questionnaire analyses, the result of the distribution of *E. coli* O157:H7 by LGAs revealed that more farms in Zaria City LGA were contaminated with *E. coli* O157:H7 than in Sabon Gari LGA. This could be attributed to the fact that farmers growing cabbage, lettuce and carrots in Zaria City LGA used more of animal manure than inorganic fertilizer as opposed to farmers in Sabon Gari LGA even though there was no statistical significance ($P = 0.31$). Considering the type of organic manure used, farmers in this study used more of cattle manure as fertilizer than other animal faeces and most of the farms (63%) fertilized with cattle manure were contaminated with *E. coli* O157:H7. This is an indication that cattle manure is the major risk factor associated with contamination of fresh vegetables grown on farms with *E. coli* O157:H7. Most farmers (56%) in this study including those whose vegetables were contaminated with *E. coli* O157:H7 supplied their cabbage, lettuce and carrots to major markets in Zaria (Tudun Wada and Sabon Gari market) which could be of public health significance. Considering post-harvest handling of the farmers, *E. coli* O157:H7 was isolated from vegetables and soils obtained from farms in which farmers washed their fresh vegetable produce before packaging and from farms in which farmers' do not wash theirs before packaging. The implication is that for those farmers who package without washing, their produce could serve as a source of contamination to other uncontaminated fresh vegetable produce they come in contact with when mixed together in the market. On the other hand, washing already contaminated fresh vegetable produce in the stream could serve as source of re-contamination to the stream.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Based on the findings of this study and the deductions derived there from, it could be concluded that cabbage, lettuce and carrot grown on farms in Zaria using cattle manure as fertilizer are contaminated with *E. coli* O157:H7 although the frequency of contamination was found to be low. In this study, cattle manure is considered as the major risk factor associated with the occurrence of *E. coli* O157:H7 on farm produce. In addition, the study has demonstrated that lettuce, cabbage and carrot may contribute to the overall prevalence of *E. coli* O157:H7 in Zaria.

6.2 Recommendations

In the light of the findings of the study, the following recommendations are made;

1. There is a need for farmers to be educated on the risk involved in using cattle manure as fertilizer; and on ways to reduce or eliminate contamination of fresh vegetable produce in the farm such as composting of the manure and applying the USDA principle of 90-day interval between application of manure and harvesting of food crops that do not come in contact with the soil and 120-day interval for those that the food crops come in contact with the soil.

2. Considering the low infective dose of this organism and the fact that these fresh vegetable produce can be consumed raw without any form of cooking, the general public need to be educated on the risk involved in the consumption of these fresh produce.
3. It is also recommended that operators of establishments such as restaurants where these fresh vegetable produce are prepared in the form of "salads" or other forms of products to be consumed by the public, should be educated to always wash these vegetables properly with clean water and avoid the use of contaminated water or utensils in the course of the preparation.
4. In order to have accurate judgment about the possibility and range of transmission of *E. coli* O157:H7 in fresh vegetable produce cultivated on farm lands using cattle manure, it is recommended that further studies be conducted to determine the prevalence and control of *E. coli* O157:H7 in cattle herds in Zaria.
5. Given the findings that most of the *E. coli* isolates were multidrug resistant with high MAR index, there is need to curb abuse and misuse of antibiotics in animal production and veterinary medicine.
6. Government agencies (e.g. NAFDAC, CDC) should set policies that would encourage farmers to treat animal manure before use as fertilizer. They should also provide legislation that lays down minimum marketing standards that food establishments must meet and ensure that they comply with these requirements through training, inspection and enforcement.

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APPENDICES

APPENDIX 1: SAMPLE QUESTIONNAIRE FOR SALAD VEGETABLE FARMS

DEPARTMENT OF VETERINARY PUBLIC HEALTH and PREVENTIVE MEDICINE AHMADU BELLO UNIVERSITY, ZARIA

Dear Respondent,

This questionnaire is designed as part of a scientific study of the occurrence of *E. coli* O157:H7 on salad vegetables grown on cattle manure-fertilized farms in Zaria. This study is of importance in addressing produce safety in Nigeria. With all sincerity, personal information would not be required and all information obtained will be treated confidentially and solely for the purpose of this study.

Thank you for your cooperation.

Ernest C. Ajaegbu.

Date……

Serial number……

Location……

L.G.A……

Family size ranges i) single [ ] ii) 2-5 [ ] iii) 5-10 [ ] iv) 10-15 [ ] v) >15 [ ]

Age (a) 10 – 20 (b) 21 – 30 (c) 31 – 40 (d) 41 – 50 (e) >50

1. The type of vegetables grown
   i) Cabbage [ ] ii) Lettuce [ ] iii) Carrot [ ] iv) mixed vegetables [ ]
2. What type of fertilizer is used?
   i) Inorganic fertilizer [ ] ii) Organic manure [ ] iii) Both [ ]
3. If organic farming is practiced what type of fertilizer is applied? i) Cattle faeces [ ] ii) Goat faeces [ ] iii) Poultry faeces [ ] iv) sheep faeces [ ] v) Dust bin ("boilah") [ ]
   vi) Mixture of any two of the four mention i and ii [ ] I and iii [ ] I and iv [ ]
4. Where is the source of manure?
   i) Commercial sellers [ ] ii) Directly from animal raisers [ ]
5. Manure applied on farm
   i) Compost manure [ ] ii) Fresh manure [ ] iii) Dry manure
6. How are the crops in the farm watered?
   i) Rain-fed system [ ] ii) Irrigation system [ ]
7. Post-harvesting practice
   i) Wash and package vegetables [ ] ii) Package vegetables without washing [ ]
8. Distribution of vegetables
   i) Sell directly to consumers [ ] ii) Supply to retailers [ ] iii) Supply to wholesalers [ ]
9. Markets in Zaria and Sabon gari that receive supply
   i) Tudun wada market [ ] ii) Sabon gari market [ ] iii) Samaru market [ ]
10. Do you consume your vegetables or sell only
    i) Consume and supply [ ] ii) Supply only [ ]

11. Have you or any member of your family ever had diarrhoea?

Yes [ ] or No [ ]

12. If yes, which time of the year?

i) Between November and April (year)……..

ii) Between May and October (year)……...

13. Was there blood in your stool?

Yes [ ] or No [ ]

14. Did you go to the hospital?

Yes [ ] or No [ ]

15. Do you know whether cattle manure can carry pathogenic microorganisms?

Yes [ ] or No [ ]

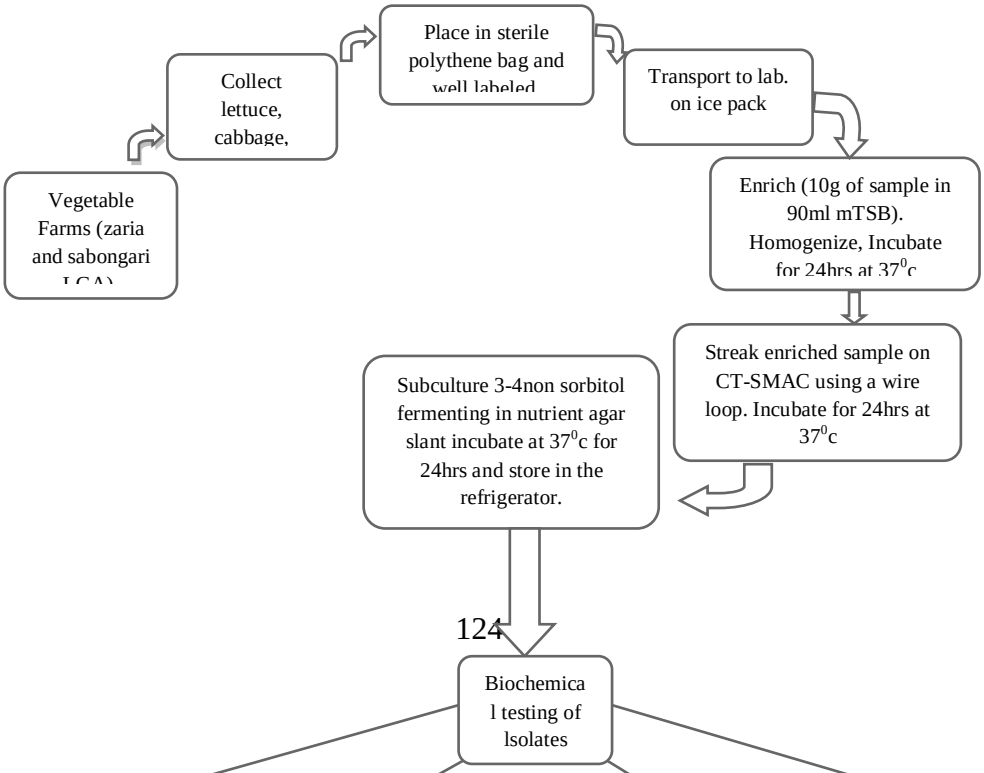
16. Do you know whether vegetables can serve as sources of infection that can cause diarrhoea?

Yes [ ] or No [ ]

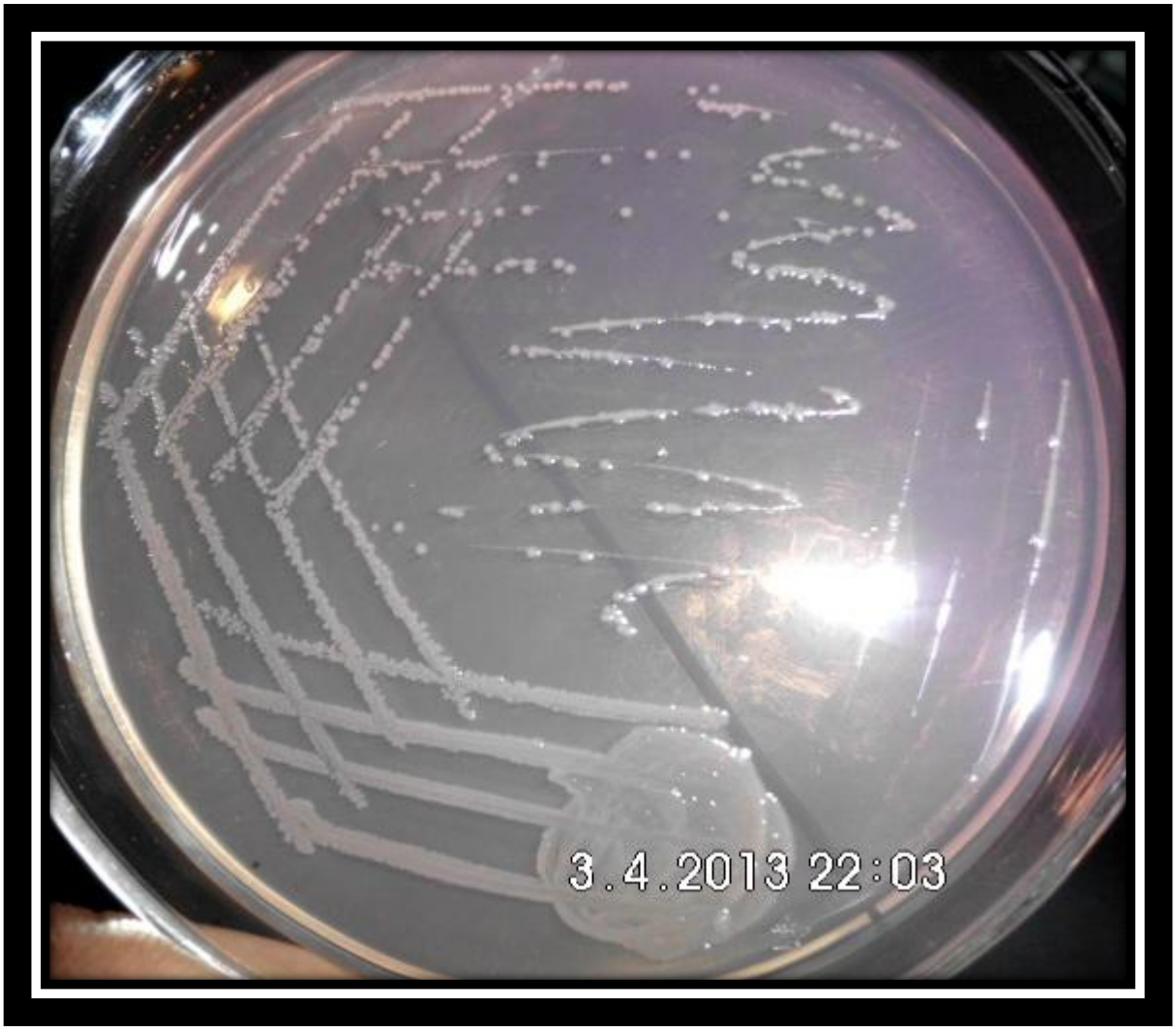
17. Have you heard of *E. coli*?

Yes [ ] or No [ ]

APPENDIX 13: A "QUESTION MARK" Flow Chart for Isolation of *E. coli* O157:H7 Used in this Study



(-) (+) (+) (-) (+) (-) (+)
(-)



APPENDIX 3: Plate Showing Non-Sorbitol Fermenting Presumptive *E. coli* Colonies on CT-SMAC



APPENDIX 4: Plate Showing an Example of Microbact 12E Test Result

(Note microtitre plate with four groups of three reactions each)

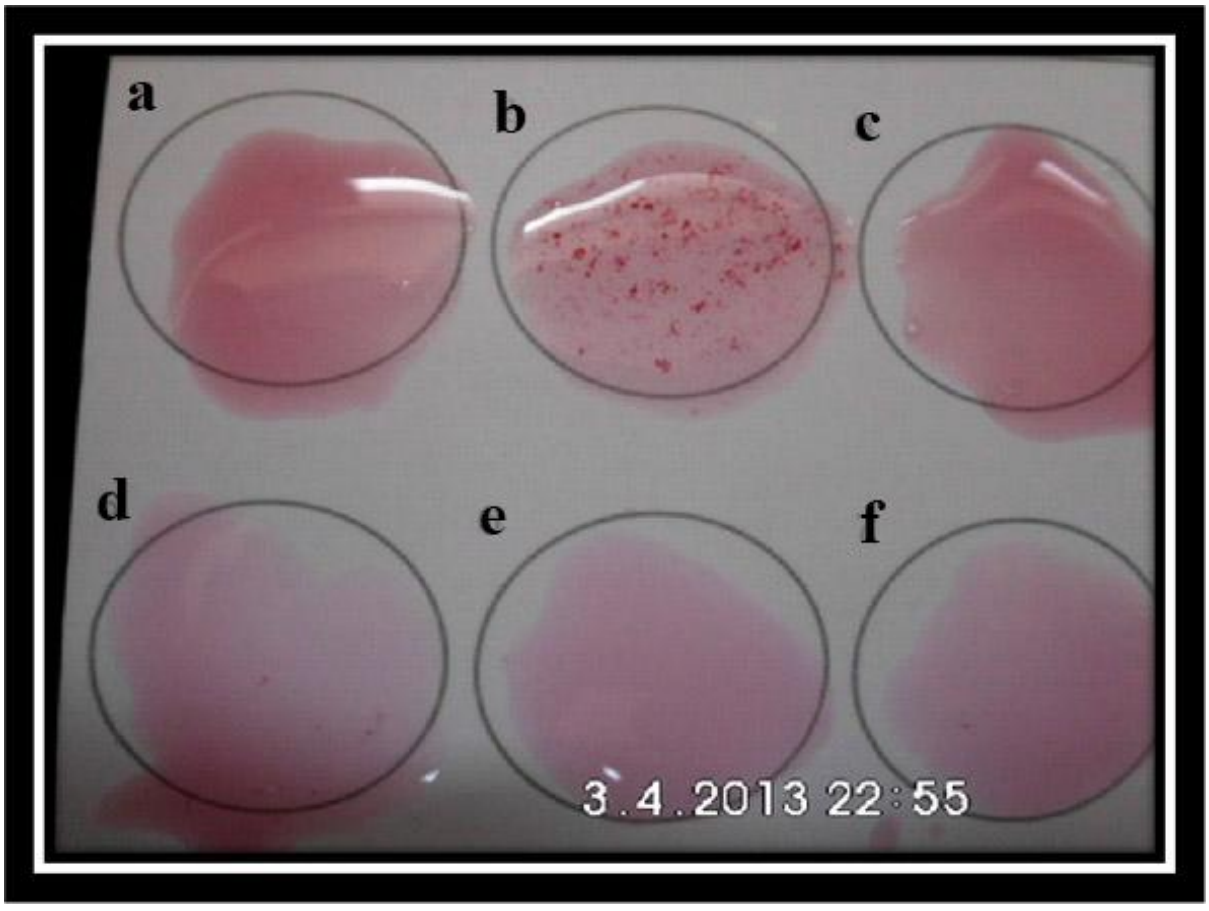
APPENDIX 5: Microbact 12E Result of *E. coli* Isolates from Vegetable and Soil Samples in Experimental and Control Farms in Zaria

S/N	Sample	Code of Isolates	Microbact	Resulting Organism	(%) Probability
1	Lettuce soil	SBLtS4	-	*P. stuartii*	54
2	Lettuce soil	SCLtS3	+	*E. coli*	71
3	Lettuce soil	SCLtS4	+	*E. coli*	84
4	Lettuce soil	SCLtS5	+	*E. coli*	69
5	Lettuce	ZALt6	-	*S. marcescens*	72
6	Lettuce	ZBLt7	+	*E. coli*	76

7	Lettuce soil	ZBLtS7	+	*E. coli*	80
8	Cabbage	SBCb8	+	*E. coli*	68
9	Cabbage	ZACb10	+	*E. coli*	91
10	Carrot	ZCCr1	+	*E. coli*	73
11	Carrot soil	ZCCrS6	+	*E. coli*	72
12	Cabbage soil	SACbS8	-	*P. stuartii*	84
13	Carrot soil	SBCrS10	+	*E. coli*	70
14	Cabbage soil	SCCrS10	-	*C. freundii*	43
15	Cabbage	ZCCb3	+	*E. coli*	65
16	Carrot	ZACr10	+	*E. coli*	87
17	Carrot soil	ZCCrS1	+	*E. coli*	79
18	Lettuce	SBLt10	-	*P. stuartii*	49
19	Lettuce	SCLt3	-	*P. rettgeri*	72
20	Lettuce soil	ZALtS3	-	*P. stuartii*	53
21	Lettuce	ZCLt1	-	*P. stuartii*	66
22	Lettuce soil	ZCLtS8	-	*C. diversus*	48
23	Cabbage	ZCCb9	-	*Y. enterocolitica*	71

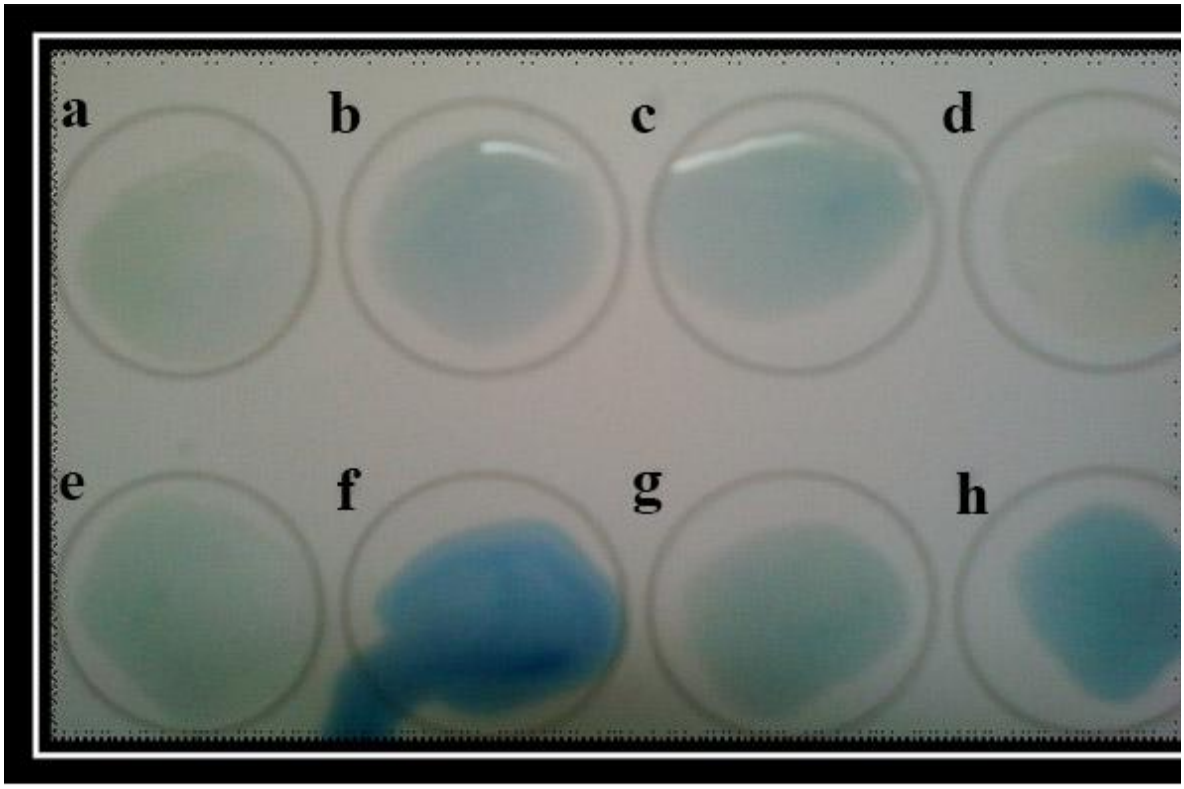


APPENDIX 6: Plate Showing Latex Agglutination Reagents used for serotyping


a
b
c
d
e
f
3.4.2013 22:55

APPENDIX 7: Plate Showing O157 Latex Agglutination Test Result

(Circle 'b' showing positive plate)

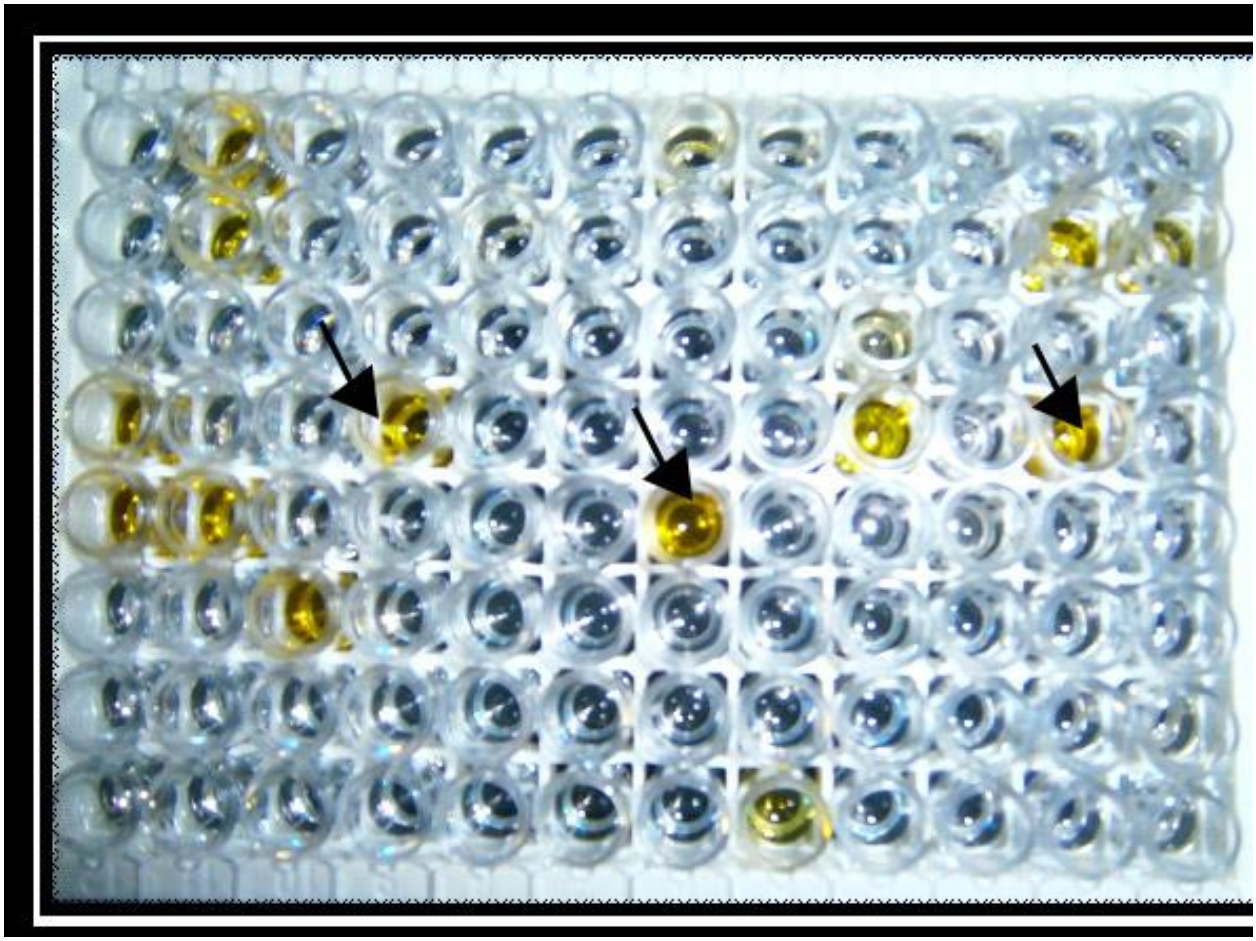


APPENDIX 8: Plate Showing H7 Latex Agglutination Test Result

(Circle 'a' & 'e' showing negative result while 'f' & 'h' showing positive)

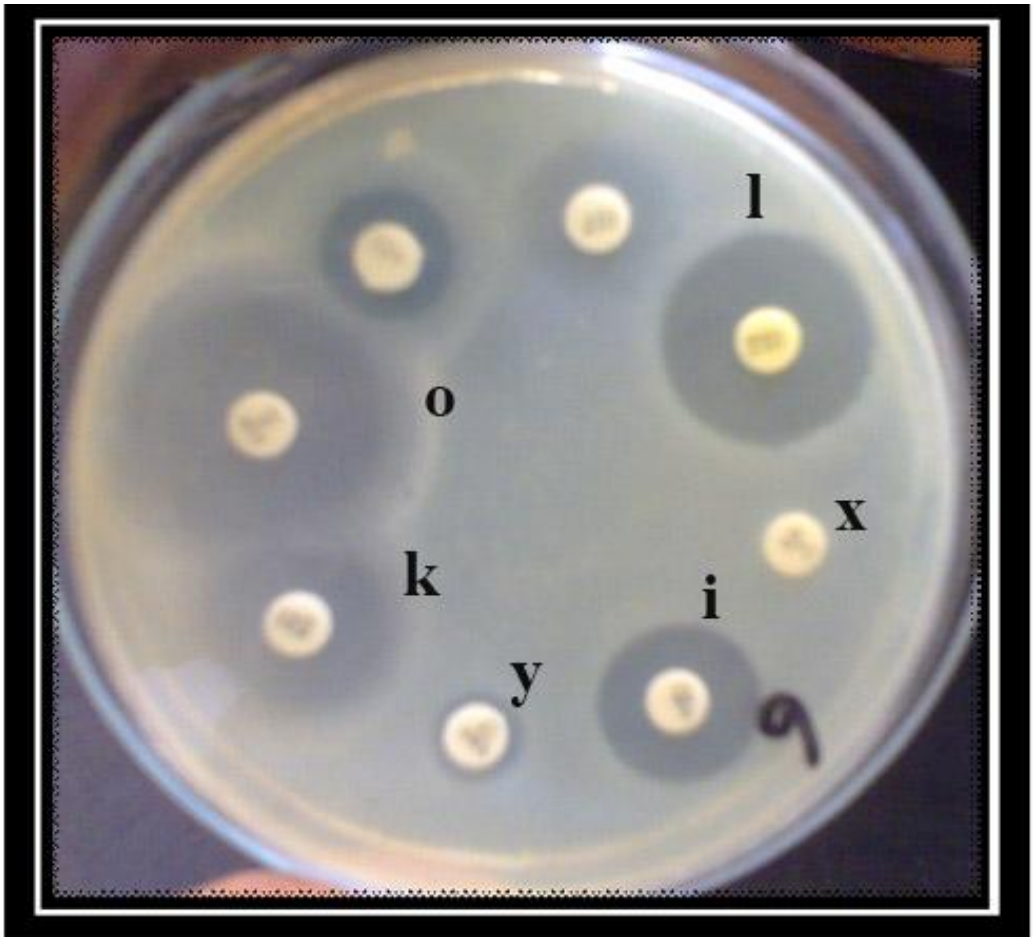

Diluent 2
100 ml
Wash
Wash buffer 10x conc.

APPENDIX 9: Plate Showing Ridascreen® Verotoxin Enzyme Immunoassay Test Reagents



APPENDIX 10: Plate Showing Ridascreen® Verotoxin Enzyme Immunoassay Test Result

(Note the arrow heads showing colour change)



APPENDIX 11: Mueller Hinton agar Plate Showing Antibiotics Susceptibility Test Result

('x and y' are resistant while 'o, k, i, l' are showing varying degrees of susceptibility)

APPENDIX 12: Phenotypic and Biochemical Characteristics of Isolates obtained from Cabbage, Lettuce, Carrot and Soil on which they were grown in Zaria, Nigeria

Samples	Type	No. of Samples	No. of Samples with colonies that were		
			Sorbitol negative	Motile, H_2S(-ve) and Citrate(-ve)	MR and Indole (+ve), VP and Urease (-ve)
Cabbage	Vegetable	60	26	4	4
	Soil	60	27	2	1
Lettuce	Vegetable	60	38	5	5
	Soil	60	45	13	7
Carrot	Vegetable	60	49	6	2
	Soil	60	48	6	4
Total		**360**	**233**	**36**	**23**

APPENDIX 13: Composition of Media Used for Conventional Biochemical Test

1. Sorbitol MacConkey Agar:

Typical formular (g/l)

Peptone 20.0; Sorbitol10.0; Bile salts No. 31.5; Crystal violet 0.001; Agar 15.0.

(Oxoid, Hampshire, UK).

2. Tryptone Soya Broth: (Casein soya bean digest broth)

   Typical formula (g/l)

   Pancreatic digest of casein 17.0; Enzymatic digest of soya bean 3.0; Sodium chloride 5.0; Di-Potassium hydrogen phosphate 2.5; Glucose 2.5.

   (Oxoid, Hampshire UK).

3. Simmons Citrate Agar:

   Typical formula (g/l)

   Magnesium sulphate 0.2; Ammonium dihydrogen phosphate 0.2; Sodium ammonium phosphate 0.8; Sodium citrate, Tribasic 2.0; Sodium chloride 5.0; Bromothymol blue 0.08; Agar 15.0. (Oxoid, Hampshire UK).

4. SIM Medium:

   Typical formula (per litre)

   Tryptone 20g; Peptone P 6.1g; Ferrous ammonium sulphate 0.2g; Sodium thiosulphate 0.2g; Agar 3.5g. (Oxoid, UK).

5. M.R.V.P Medium:

   Typical formula (g/l)

   Peptone 7.0; Glucose 5.0; Phosphate buffer 5.0.

   (Oxoid, Hampshire UK).

6. Mueller-Hinton Agar:

   Typical formula (g/l) Beef dehydrated infusion from 300.0; Casein hydrolysate 17.5; Starch 1.5; Agar 17.0 (Oxoid, Hampshire UK).

7. Modified Tryptone soya broth:

   Typical formula (g/l)

   Pancreatic digest of casein 17.0; Papaic digest of soya bean meal 3.0; Sodium chloride 5.0; Di-potassium hydrogen phosphate 4.0; Glucose 2.5; Bile salts 1.5

   (Oxoid, Hampshire UK).

8. Urea Agar Base:

   Typical formula (g/l)

   Peptone 1.0; Glucose 1.0; Sodium chloride 5.0; Di-sodium phosphate 1.2; Potassium dihydrogen phosphate 0.8; Phenol red 0.012; Agar 15.0.

   (Oxoid, Hampshire UK).

9. Cefixime Tellurite, Selective Supplement.

   (SR0172E) (Oxoid, UK).