HAZARDS ASSOCIATED WITH ENTERIC AGENTS ON VEGETABLES GROWN IN IRRIGATED FIELDS TREATED WITH MANURE IN PARTS OF KANO AND PLATEAU STATES, NIGERIA.

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FACULTY OF SCIENCE

AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA

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Declaration

I hereby declare that the work in this dissertation titled 'HAZARDS ASSOCIATED WITH ENTERIC AGENTS ON VEGETABLES GROWN IN IRRIGATED FIELDS TREATED WITH MANURE IN PARTS OF KANO AND PLATEAU STATES, NIGERIA' has been carried by me in the Department of Microbiology under the supervision of Prof V.J. Umoh., Prof J.B. Ameh, and Prof. S.E. Yakubu. The molecular study was undertaken in the Department of Plant Pathology, Universiti Putra Malaysia under the supervision of Prof. Dr. Madya Kamaruzaman Sijam.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this work has been presented for another degree or diploma at any other institution.

_____	_____	_____
NAME OF STUDENT	SIGNATURE	DATE

Certification

This dissertation titled 'HAZARDS ASSOCIATED WITH ENTERIC AGENTS ON VEGETABLES GROWN IN IRRIGATED FIELDS TREATED WITH MANURE IN PARTS OF KANO AND PLATEAU STATES, NIGERIA' meets the regulations governing the award of Doctorate degree in Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary contribution.

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Abstract

With increased consumption of vegetables and the number of documented outbreaks of human infections associated with it, this study was instituted. The study assessed the hazards of the presence of enteric agents on vegetables grown in irrigated fields treated with manure in parts of Kano and Plateau States, Nigeria. Semi structured questionnaires were administered to farmers in the study area to ascertain their farm management practices. Four hundred and forty (440) samples comprising vegetables and some environmental samples (water, soil and manure) were obtained from vegetable producing regions in Kano and Plateau States, Nigeria, between May, 2010 and March, 2011. All samples were examined using standard procedures to determine physicochemical characteristics, enumeration of faecal coliform and detection of *E.coli* O157: H7, *Salmonella* spp and *Vibrio cholerae* during the dry and wet seasons. Isolated pathogens were characterized conventionally and molecularly, assayed for toxin production, virulence determinants, and mobile genetic elements by Polymerase Chain Reaction (PCR). Representative isolates were further analysed for genetic relatedness and determination of diversity by Deoxyribonucleic Acid (DNA) Fingerprinting. Farm management practices which include use of untreated wastewater and untreated manure were observed to have contributed greatly to preharvest contamination of vegetables in the study areas. Significant difference ($P < 0.05$) was observed in the level of counts of faecal coliform on vegetables, with vegetables irrigated with untreated water having higher counts than those irrigated with treated water. Vegetables obtained from sites which received contamination from livestock waste had higher (7.79 ± 7.53) faecal coliform counts than other point sources of contamination to irrigation water in Kano State while in Plateau State, higher (7.74 ± 7.67) counts were obtained from vegetables that received contamination from domestic waste. Generally, significantly higher

counts ($P<0.05$) were obtained from vegetables in the wet season than in the dry season. Physicochemical parameters of irrigation water exceeded acceptable limits for irrigation of vegetables for human consumption. Faecal coliform counts (FCC) exceeded World Health Organisation guidelines acceptable limits (1000fcc/100ml) for vegetables and irrigation water. Counts were significantly ($P< 0.05$) higher in the wet season than in the dry season in Kano State, while in Plateau there was no observed significant difference in counts of coliform between the two seasons. *Salmonella* spp had the highest prevalence rate of 13.2% in the study with its highest detection rate in vegetables. *E. coli* O157:H7 had a prevalence of 7.3%, while *Vibrio cholerae* had 5.7% prevalence in the study. Seasonal distribution of pathogens by odds ratio showed that *E.coli* O157:H7 was more likely to be detected in the wet season while *Salmonella* spp and *Vibrio cholerae* were more likely to be detected in the dry season. Isolates were assayed for toxin production and their virulence potential was ascertained. *Escherichia coli* O157:H7 produced verocytotoxins (VT), 57.9% of isolates tested produced both VT1 and VT2 while, 61.5% *Vibrio cholerae* isolates produced cholera toxin (*ctx*). Antibiogram of isolates obtained showed multiple antibiotic resistance profiles of pathogens to eight commonly used antibiotics in Nigeria. Polymerase Chain Reaction (PCR) amplified some housekeeping genes (*uid*A), virulence gene markers (*rfb*O_{157}, *inv*A, *stn* and *ctx*A), antibiotic resistance genes and some mobile genetic elements in the pathogens. Enterorepititive Intergenic Concensus DNA Fingerprinting successfully genotyped isolates in the study. Fingerprints revealed genetic similarity among some isolates from vegetables and environmental sources and showed diversity among some. Cluster analysis of *Escherichia coli* O157:H7 fingerprints at a coefficient of similarity of 0.53 grouped strains into 6 different groups; five clusters and one singleton. *Salmonella* strains were grouped into five groups; two clusters and three singletons,

while, *Vibrio cholerae* strains were grouped into six groups at a coefficient of similarity of 0.62. Sequence analysis of pathogens obtained in the study gave an insight on the prevailing strains of each bacterial type studied in the study locations and probably in Nigeria as a whole. These sequences have been deposited into the Genebank and assigned accession number (*Salmonella inv*A gene KF026356) for reference. We recommend continuous monitoring of these vehicles of pathogen transmission from the environment to the foodchain. The need for proper washing and cooking of vegetables before consumption cannot be overemphasized.

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CHAPTER ONE

1.0 INTRODUCTION

Vegetable production, consumption and safety are undergoing rapid changes (Park *et al*., 2013) and production of organic foods has been significantly increased worldwide. Annual increases in production are between 8 and 20% and consumption is between 20 and 30% (Willer and Yussefi, 2004). Fruits and vegetables are important components of a healthy diet (Allen *et al*., 2013). Increased consumption and year-round consumer demand for fresh, minimally processed green vegetables have been observed (Allen *et al*., 2013) as they are important sources of vitamins, fiber and minerals (Marlett *et al*., 2002). Urban vegetable farmers in search of water for irrigation often rely on waste-water for year round production (Nana and Esi, 2010). Other available water sources used are shallow/dugout wells, pipe-borne water and inland valley (Obuobie *et al.,* 2006).

Irrigation is an artificial application of water to the soil to assist the growing of crops in dry areas and during periods of inadequate rainfall. Agricultural irrigation with waste water is a common practise in many parts of the world, although the extent of the practice is a debatable point (Scott *et al*., 2004). Estimate is that worldwide 20 million hectares of irrigated agriculture uses raw treated or partially diluted waste water (Future Harvest, 2001). About two – thirds of the population in the developing world has no hygienic means of disposing excreta, with even a greater number lacking an adequate means of disposing waste water (United Nations Development Programme, UNDP, 2002), hence human sewage and wastewater are disposed into waterbodies like rivers and streams.

Utilisation of waste water for irrigation and waste products as fertilizer have created a convenient means of disposing waste products and adding valuable plant nutrients and organic matter to the soil (Vander Hoek *et al*., 2002) and improving urban food (vegetable) security. The livelihood of farmers and traders are improved as well (Keraita *et al.*, 2008). One of the most economically feasible agricultural uses of reclaimed water is the irrigation of high value horticultural crops and vegetables which typically have high returns per volume of water invested in (Toze, 2006). But this practice has been approached with trepidation, owing primarily to concerns about risks to human health through contamination of food with pathogenic microorganisms (Hamilton *et al.,* 2005; To$_Z$e, 2006). Pathogenic viruses, bacteria, protozoa and helminthes (Doyle and Erickson, 2007) are released from the bodies of infected persons in their excreta and passed on to others through the mouth like through drinking of contaminated water or eating of contaminated vegetables.

There are many potential preharvest sources of pathogens including contaminated manure, manure compost, sewage sludge, irrigation water, runoff water from livestock operations and exposure to waste products from wild and domestic animals, as well as trophic interactions between plants and plant forages like birds, mammals and insects (Doyle and Erickson, 2009). Therefore, contamination of produce can occur in the field by application of raw (or poorly composted) manure, exposure to contaminated water (irrigation or flooding) (Johannessen *et al.,* 2005) or by the use of livestock waste as fertilizer.

Scrutiny of the role of agricultural practices in contamination of fresh vegetables with pathogenic microbes (Sewell and Farber, 2001) has led to the concern about the safety of using animal manure as fertilizer in vegetable production. Bovine manure is a good source of macro and micro nutrients, using it as fertilizer is an important disposal

method (Lung *et al.,* 2001) particularly for organic farmers. However, bovine manure is a well-known source of food- borne pathogenic bacteria (Wells *et al.,* 2001) and using it without prior treatment to destroy pathogens increases the likelihood of contaminating vegetables grown in manure amended soils. There are reports on the persistence of *E. coli* O157 in irrigation waters and its transmission through fresh produce (Renter *et al.,* 2003).

1.1 Statement of the Problem

Fresh fruits and vegetables are important sources of nourishment and provide vital ingredients in healthy and balanced diets, serving as important components of a healthy diet (Allen *et al*., 2013). Vegetables in its broadest sense refers to any kind of plant product and is commonly referred to as the fresh edible portion of a herbaceous plant; roots, stems, leaves or fruits. Vegetables are good sources of vitamins, minerals, flavors, essential oils, flavonoids and other antimicrobial properties (Klansmeyer *et al*., 2004). They are of special nutritional importance being sources of fat and water soluble vitamins (β-carotene, ascorbic acid, riboflavin, thiamine and niacin). Salads particularly are usually consumed without any pre-heat treatment to conserve its vitamins and are acceptable to people of all works of life (Buck and Walcott, 2003).

Food-borne disease outbreaks have been associated with consumption of fresh vegetables and fruits contaminated with human pathogenic bacteria (Sivapalasingam *et al*., 2004). *Klebsiella* has been linked with infection of tomatoes (Viswanathan and Kaur, 2001), *E.coli* infection with lettuce and enterotoxigenic *E.coli* infection with carrots. The increase in documented outbreaks may be related to a change in consumer demand towards a higher consumption of fresh produce (Jacobsen and Bech, 2012) hence, increasesd exposure to foodborne pathogens.

The study of food safety problems is a result of a large number of bacterial pathogens implicated in food borne outbreaks, associated with the consumption of fresh fruits and vegetables (Beuchat, 1998) and the outcome used to safeguard the health of the consumers. The safety of foods has a wide reaching effect on world trade, for some countries, export of fruits and vegetables make up close to half of the total agricultural exports (Brady, 2002). Therefore, unsafe imports may pose a threat to the health of the people consuming them and result in significant economic loss for the exporting country. In Nigeria, most of the vegetables consumed in the south are grown and transported from the Northern parts of the country.

1.2 Justification

An outbreak of food poisoning in the United States occurred from consuming fresh spinach contaminated with *Escherichia coli* O157:H7 which caused at least 183 people to be ill, of which 29 suffered kidney damage from hemolytic uremic syndrome (Centres for Disease Control, CDC, 2006). *Escherichia coli* O157:H7 is the most common bacterial etiologic agent of outbreaks associated with lettuce and other green leafy vegetables (Brandl, 2006). According to the Center for Disease Control and Prevention, 20 outbreaks and 634 cases of illness from *E.coli* O157:H7 were attributed to lettuce alone during 1998 to 2005 (Lynch, 2007) in the United States. Consumption of lettuce has been linked also to several outbreaks of salmonellosis in Australia (Stafford *et al.,* 2002), United States (Sivapalasingam *et al.,* 2004) and Finland (Takkinen *et al.,* 2005).

In Nigeria, in a study of bacterial contamination of ready to eat salad in Calabar, 78% bacterial contamination was reported (Udo *et al.,* 2009). In another study carried out in vegetable markets in Jos, Plateau State, Damen *et al.* (2007)

reported 36% prevalence for intestinal parasites; 64% prevalence in cabbage, 40% prevalence in lettuce 32% for carrot and 24% for green leafy vegetables. In a related study by Okafor *et al*., 2003 a 33% prevalence of pathogenic microorganisms from vegetables and water used for irrigation in Zaria, Kaduna State was reported. Abakpa *et al.,* 2008 reported a 2.9% prevalence of *Escherichia coli* O157:H7 from animal manure in households engaged in livestock farming in Zaria, Kaduna State Nigeria.

Enteric pathogens as human pathogens pose the greatest risk when present on fresh vegetables as a result of the constant high moisture content of vegetables, the nutrients released by the sprouting seeds and warm temperature which is conducive to the growth of human bacterial pathogens enables them to grow in vegetables prior to consumption (Charkowski *et al*., 2002) and cause infection in consumers. An important first step in addressing the safety of agricultural reuse of wastewater is to determine likely hazards associated with consumption of raw (uncooked/undercooked) vegetables grown in irrigation sites. A sound understanding of such hazards not only will be of significant value in managing low-technology reuse schemes but will form the basis of hazard assessments for advanced reuse proposals.

1.3 Aim

This study was aimed to assess the hazards of the presence of enteric agents on vegetables grown in irrigated fields treated with manure in parts of Kano and Plateau States, Nigeria.

1.4 Specific Objectives

1. To determine the factors associated with levels of faecal coliforms in the study areas by questionnaire administration to vegetable farmers.

2. To enumerate faecal coliforms in all the samples for the two seasons (wet and dry) and the physicochemical properties of water (Temperature, pH, BOD, DO and Electrical conductivity) used for irrigation.
3. To isolate and characterize (biochemical and serological) *Escherichia coli* O157:H7, *Salmonella* spp *and Vibrio* spp from samples of vegetables, irrigation water and manure treated soils for the wet and dry seasons.
4. To determine the antibacterial sensitivity of the isolated pathogens.
5. To detect virulent markers of the isolated pathogens and molecular characterization of some of the isolates by polymerase chain reaction.
6. To use molecular techniques to establish relatedness of the pathogens on vegetables and those from environmental samples.

1.5 Research Hypothesis

Null Hypothesis (HO):

There are no hazards associated with vegetables grown in fields treated with organic or inorganic manure in Kano and Plateau States, Nigeria.

Alternate Hypothesis (HA):

There are hazards associated with vegetables grown in fields treated with organic or inorganic manure in Kano and Plateau States, Nigeria.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Organic Production

Population pressure increases, shortened fallow cycles, cropping intensification, inaccessibility and low output prices and concerns about agricultural sustainability and self sufficiency have combined to increase demand for integrated soil fertility management of the agricultural resource base (Baltenweek *et al*., 2002). Following this situation, organic manure in the form of animal manure becomes one of the principal sources of nutrients for soil fertility maintenance and crop production. A survey of 48 sites in Asia, Africa, and Latin America showed that majority of farmers use manure on their crops (Baltenweek *et al*., 2002). In Northern Nigeria, it is reported that the mean manure use of 1850kgha^{-1}, is still insufficient to meet the animal manure of 3 – 5 tons ha^{-1} required to maintain agriculture in the region (Chianu and Tsuji, 2004). An alternative to conventional agriculture is offered by organic farming, which aims to minimize its impact on the environment by crop rotation, pathogen–resistant cultivars, limited amounts of chemical pesticides and organic manure instead of synthetic fertilizers (Schmid *et al*., 2011). However, the beneficial effect of organic agriculture on biodiversity in general and plant- associated microorganisms in particular is still controversial (Ruimy, 2010).

2.2 Enteric Pathogen Contamination of Irrigation Water, Soil, and Vegetables

Irrigation water also called agricultural water refers to water used in the growing environment (field, vineyard or orchard) for agronomic reasons (Center for Food Safety

and Applied Nutrition, CFSAN, 1998). Typical sources of agricultural water include flowing surface waters from rivers, streams, irrigation ditches, open canals, impoundments (such as ponds, reservoirs and lakes), wells and municipal supplies (CFSAN, 1998). In many countries, surface waters are the predominant source for irrigation (Pachepsky *et al*., 2011). Irrigation aids in cultivation of non-indigenous vegetables (Keraita *et al*., 2002) and year round cultivation unaffected by drought (Gemmell and Schmidt, 2010), hence, increasing the farmers profits through multiple cultivation cycles (Raschid-Sally *et al*., 2005). It has the potential to satisfy the fast growing production demands, enabling communities lacking access to potable water have an alternative to using wastewater, as it constitutes the only feasible way of ensuring crop output (Gemmell and Schimdt, 2010). The crop water consumption at global scale can be roughly estimated to 1.8 x 1011m^3 per year, at least half of this amount supplied as irrigation (Forslund *et al*., 2012). The use of wastewater for irrigation has often been propagated as a way to overcome water scarcity (Forslund *et al*., 2012) and it has numerous benefits especially as water shortages are becoming a growing problem (Gemmell and Schmidt, 2010). It can also contribute to reducing the application rate of fertilizer by farmers, as wastewater may contain sufficiently high concentrations of organic and inorganic nutrients (Toze, 2006). Improved fertilization via the use of wastewater aids poorer communities farming at subsistence level improve their nutritional status by potentially increasing food supply and quality (Raschid-Sally *et al*., 2005). Wastewater reuse reduces the water footprint of food production and supports higher crop yields with the simultaneous recycling of nutrients and energy (Hanjra *et al*., 2012). In addition, Haruvy, (1997) stated that wastewater irrigation has the potential to reduce treatment costs as soil and crops can serve as biofilters and

would therefore contribute to reducing the amount of wastewater to be treated in wastewater treatment plants.

On the other hand, both conventional and organic vegetable producers commonly apply animal manure as fertilizer to fields where crops are grown (Natvig *et al.*, 2002). Manure is a valuable source of nutrients for crop production (Harrison *et al*., 2011). Utilization of organic manure such as farmyard and slurry is the most economic and practical option for improving soil quality, while providing as well an additional source of nutrients for growing plants (Harrison *et al*., 2011). Manure incorporation is a routine practice in the production of some crops as fertilizer or soil amendment (Delaquis *et al*., 2007). This incorporation increases water holding capacity, improves aeration and provides beneficial microorganisms (Islam *et al*., 2005). The use of this practice has been increasing (nearly 20% per year) from 2007 to 2012, mainly because of the trend toward organic produce (Laux, 2012).

Unfortunately, given the positive factors associated with organic production involving irrigation and manure application, public health risks remain a concern for both the farming community and consumers (Mara *et al*., 2007). Sources of irrigation water can be generally ranked by the microbial contamination hazard (Leifert *et al*., 2008). In order of increasing risk, these are potable or rain water, groundwater from deep wells, groundwater from shallow wells, surface water and finally raw or inadequately treated wastewater (Pachepsky *et al*., 2011). The production of domestic, industrial and commercial wastewater has increased over the years due to population growth and economic development (Varela and Mania, 2013). As a consequence, the chemical and biological complexity of wastewater increased considerably over the last decades (Varela and Mania, 2013) due to increase in contaminants. These contaminants may contribute to enhance the potential clinical relevance of some

bacteria thriving in wastewater and in the surrounding environment (Abraham, 2011). Human pathogens, organic and inorganic pollutants, found in urban wastewater are a matter of concern for both farmer health and food safety (Toze, 2006). Irrigation water quality is also of international importance because products exposed to microbially contaminated water may put consumers at risk both locally and internationally (Gemmell and Schmidt, 2010). As the international trade of agricultural products is growing, the export of fresh produce that is contaminated could assist in spreading potential pathogens and strains with new virulence characteristics into areas where previously such pathogens were not present or were atleast absent for a number of years (Beuchat, 1998).

Manure and waste waters contain a broad range of pathogenic microorganisms, including *Escherichia coli* O157:H7, *Campylobacter, Cryptosporidium, Salmonella* spp and pathogenic viruses which are released into the environment during organic farming (Hutchison *et al*, 2004). It has been reported that animal manure is a well known source of food borne pathogenic bacteria (Wachtel *et al.,* 2002) and its inappropriate use in vegetable crops especially organic ones contributes a risk to consumer health. Microbial contamination can occur during any of the steps involved in vegetable production, harvesting and packing. However, the steps in the production chain that have the greatest potential for pathogen contamination are soil preparation (use of uncomposted organic manures) and manure from animals grazing locally or near by (Buck and Walcott, 2003). One of the challenges to the development of preharvest interventions is the transmission of *E. coli* O157:H7 between animals and environmental sources, like contaminated water (Shere, 2002). Cattle colonized with *E.coli* O157:H7 are important sources/ animal reservoir of this zoonotic pathogen, which is frequently transmitted to humans through manure- contaminated foods, water

and other environmental source (Karmali *et al*., 2010). Bovine manure and slurry are the main environmental source of these pathogens, with average concentration between 10^3 and 10^4 CFUper g (dry weight) of manure or slurry (Nicholson *et al*, 2005), but the density can be as high as 10^7 cfu gdw^{-1} of manure (Fukushima and Seki, 2004). Spreading of agricultural slurries and manures on land is used by the agricultural sector as a means of nutrient recycling.

Manure has beneficial fertilizer value, however, it frequently contains enteric microorganisms and land spreading can lead to pathogen entry into the food chain (Islam *et al.*, 2004). There are various sources of contamination of soil but evidence suggests that contamination from the spreading of animal slurries and manures on land can be a significant contributor (Unc and Goss, 2004). Soils (and subsoils) often act as zone for mitigating microbial contamination of groundwater associated with the spreading of animal slurries and manures on land, however, in studies where animal waste had been continually applied for several years, enteric bacteria were found in soils and groundwater (Entry *et al.*, 2000). Once pathogens are introduced into fields, soil can become a pathogen reservoir (Erickson, 2010). For example, *S.enterica* persisted for 161 and up to 231 days in soils amended with contaminated composts on which lettuce and parsley were grown (Islam *et al.,* 2004), where as *E. coli* O157:H7 survived in soil samples for 154 to 196 days in onion and carrot fields (Islam *et al.,* 2005). When microorganisms are introduced on or in soil, their movement is mainly determined by the flow of percolating water (Hekman *et al*., 1994). Water flow and the ultimate distribution of bacteria in soil are affected by soil texture, pH, temperature and the structure of the root system in soil (Kemp *et al.*, 1992). Aislabore *et al.* (2001) reported that differences in soil structure between soils resulted in differences in microbial movement with the coarse subsoil structure of poorly drained soils favoring

preferential flow through macro pores and the fine soil structure of well drained soils favoring matrix flow. Some of the pathogenic microorganisms applied to agricultural soils have however been observed to leach through the soil into groundwater which can affect drinking water quality and pose a risk to public health (Jiang, 2005), confirming that soil is not always a sufficient obstruction for protection of ground water (Unc and Gross, 2004). Consequently, characterization of the movement of pathogens through the unsaturated soil and subsoil zone (undose zone) has become critical for assessment of the risk to groundwater posed by agricultural activities (Darnault *et al.*, 2004). Like other bacteria, *E. coli* O157:H7 and *Salmonella* serovar Typhimurium are able to move through the soil profile with water after rainfall or irrigation and can even reach the ground water (Lang and Smith, 2007). In field experiments, 20% of *E. coli* cells applied with contaminated slurry to the field was found in drain water (Vinten *et al*., 2002). Subsequently, these pathogens move into produce in the soil. Previous studies have found enteric bacteria on or inside a wide variety of vegetables (Brandl, 2008) grown in soils amended with animal waste or irrigated with waste water, as internalisations of pathogenic microorganisms have been documented (Bernstein *et al.*, 2007). Cross- contamination of produce from manure or improperly composed manure used as soil amendment results in pathogen contamination during pre harvest of high value cash crops, especially in developing nations (Ibekwe *et al.*, 2007). More specifically, it has been shown that *E. coli* O157:H7 and *Salmonella* serovar Typhimurium may become associated with contaminated manure (Islam *et al*., 2004) and may even be internalized by plants (Klerks *et al*., 2007). Root crops and leafy vegetables have the greatest risk of contamination through manure applied to soil (Natvig *et al.*, 2002), as enteric bacteria can transfer from manure amended soil to leaf and root vegetable (Franz *et al.*, 2005). The presence of plant root is often neglected in

controlled experiments although root exudates may support survival of human pathogens by providing a supply of easily available nutrients (Klerks *et al*., 2007). The common site of entry of conjectured to be penetration at cracks in seed coat (Wachtel *et al.,* 2002) or invasion at lateral root junction in seedling (Cooley *et al.,* 2003, Dong *et al.,* 2003; Warriner *et al.,* 2003). Exudation of nutrients at these entry sites is hypothesized to act as the trigger of mobilization of pathogens to these sites (Cooley *et al.,* 2003, Dong *et al.,* 2003; Jabalasone *et al.,* 2005) although adhesive characteristics and colonization ability (Dong *et al.,* 2003) also act as factor into their proliferation near these sites. Subsequent entry into the tissue is dependent on the type of plant (Solomon *et al.,* 2005). The potential of pathogenic bacteria to exist and survive on plant surfaces is affected by adjacent physicochemical conditions and fluctuations in these environments (Brandl *et al.,* 2004), subsequently leading to greater public health concerns associated with microbiological contamination (Erickson, 2010). Many bacteria that are non pathogenic to plants, hence, do not affect the plants but are pathogenic to man are often on vegetables. Example *Salmonella*, express defense-eliciting molecules, such as flagellin, that trigger recognition when they arrive on leaves (Barak *et al*., 2011).

On ther hand, microorganisms in land applied manure are affected both positively and negatively by factors affecting their ability to survive (Harrison *et al*., 2011). Bacteria constitute the most successful form of life in environmental habitats (Chekabab *et al*., 2013). This is due to their ability to respond to environmental stimuli by phenotypic plasticity (Chekabab *et al*., 2013). The survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium is thought to be better in slurry than in farmyard manure (Semenov *et al.,* 2007) but is also dependent on the way manure or slurry is applied to agricultural fields (Nicholson *et al*., 2005). The survival of the pathogens

may range from several days (turned composed manure) to more than a year (non aerated manure) (Fremaux *et al.*, 2007). This broad difference in survival times is caused by various abiotic factors such as temperature (Semenov *et al*., 2007), presence of oxygen, chemical composition (Franz *et al.*, 2008) as well as by biological factors e.g microbial community composition (Semenov *et al.*, 2007; Franz *et al*., 2008). Other factors which affect the levels and incidence of pathogens present in animal manures and slurries are herd health, age demographics, stress factors, diet, season and manure management and storage (Ogden *et al.*, 2004; Nicholson *et al.*, 2005). Previous studies have identified the key factors which influence the survival of bacteria within animal faeces. These include moisture content, temperature, pH, nutrients, the animal`s diet and the bacterial strain (Sinton *et al*., 2007; Van Kessel *et al*., 2007). The contamination of vegetables grown in soil irrigated with faecal contaminated water depends largely on the survival capabilities of the pathogens in the soil and on plants (Forslund *et al*., 2012). Pathogen survival time in the soil may vary from 4 to 750 days (Jiang *et al*., 2002; Entry *et al.*, 2005) and strongly reflects the organism's ability to respond to adverse environmental conditions. The survival of enteric bacteria in soils is also dependent on a number of other factors (Avery *et al.*, 2004), which include soil factors as porosity, surface area, bulk density and macrospore structure which play an important role in the leaching potential of the introduced bacteria by their influence on absorption and gravitational movement with water (Auty *et al.*, 2005). A principal factor is the moisture content of the soil, particle distribution and organic matter content of the soils (Jamieson *et al.*, 2002). The clay content of soils is particularly important with respect to survival, as clay particles provide a large ecological niche that is protected against predators and greater availability of substances and moisture than sand particles (Lang and Smith, 2007). Survival of enteric pathogen in soil is therefore,

strongly dependent on the soil type. In tropical soils, the growth of fecal bacteria is believed to be limited by the capacity of the indigenous soil microbiota to out compete them for the limited supply of nutrients present in the system, the bacteria can however, proliferate sporadically, when conditions are favorable (Byappanahalli and Fujioka, 2004) and there by maintain themselves as minor populations in such soils. Weather conditions, desiccation, soil type, predatory protozoan populations and the degree of manure incorporation are all likely to have various effects on pathogen survival in manure fertilized soil (Ogden *et al.,* 2001). The preapplication moisture status of the soil, which may be influenced by the season, also has impact on pathogen survival, fate and transport (Chu *et al.,* 2003). Improved soil nutrient status through exudation may yield general bacterial simulation (Lugtenberg *et al.,* 2001) resulting in increased competition for preferred colonization niches between microorganisms. After land application of animal waste, the opportunity for transfer of these organisms from soils to surface and groundwater and ultimately to humans, depends in part on their ability to survive in the soil environment (Entry *et al.,* 2002). Faecal deposition of *E. coli* into soil represents an intermediate step in a host-soil-water cycle, which is one mechanism by which *E. coli* may colonize new hosts (Collins *et al*., 2005). *E. coli* abundance declines over months in soil, but persistent strains can be mobilized in overland or groundwater flow, leading to redeposition in a new soil environment or entry into surface waters and community water supplies (Avery *et al*., 2004, Islam *et al*., 2005; Walsh and Kunapo, 2009).

Microbial pathogens are known to survive for considerable periods of time in groundwater (John and Rose, 2005) which increase the health risk due to utilization of contaminated supplies. Many enteric pathogens can live in groundwater and soil for months (Unc and Goss, 2006), infact, *E. coli* O157:H7 have been reported to survive in

well water up to 65days (Artz and Killham, 2002). Several investigators found that enteric pathogens declined rapidly when transported through dispensed soils indicating that bacterial pollution occurs by transport via water through soil macropores (Jiang *et al.,* 2002; Vinten *et al.,* 2004). Several factors influence the survival of pathogens in soil after waste materials are applied of which soil water content and temperature are the most important factors (Jiang *et al.,* 2002, Saini *et al.,* 2003; Larney *et al.,* 2003). Survival of bacterial pathogens in soil increases in moist warm soil (Entry *et al.,* 2000). Run off from waste- treated agricultural land shows that enteric bacteria increase in spring flows and decrease in the dry period, increase in water after irrigation or after manure is supplied, and rapidly decreases once manure application is halted (Berry and Miller, 2005, Guber *et al.,* 2005; Unc and Goss, 2006). Mutation and horizontal gene transfer as well as short generation times allow bacteria to adapt easily and rapidly to an environment (Varela *et al*., 2013). Genes encoding biodegradation enzymes, antibiotic resistance or defense mechanism are examples of adaptive traits that can spread rapidly (Varela *et al*., 2013). Among the genetic elements that can be transferred between cells, the plasmids, in particular those that can be mobilized and replicated in taxonomically diverse hosts are the most important vectors to spread genetic information within bacterial populations and through distinct environmental compartments (Varela *et al*., 2013). Wastewater bacterial plasmids contain transposons, insertion sequences and integrons, which are involved in gene acquisition and gene rearrangement in cassettes that promote their expressions (Zhang *et al*., 2011) hence pathogens ability to survive and thrive in diverse environments.

Inspite of the advantages of irrigation water and manure, the environmental and public health risks implication of their use cannot be relegated to the background when wastewater reuse is discussed (Hanjra *et al*., 2012). The health risks associated

with the spreading of animal and human wastes containing enteric pathogens have been recognized for a long time (Gerba and Smith, 2005). Though some bacteria promote a series of biochemical and metabolic transformations indispensable to achieve wastewater treatment, some are pathogenic or harbor antibiotic resistance or viulence genes harmful for human health (Varela and Manaia, 2013).

In many less developed countries, raw and untreated sewage liquids are frequently used for the irrigation of crops (Scott *et al.,* 2000). Pollution of surface flow and ground water from such wastewater and animal waste applied to soils has been documented (Gagliardi and Karns, 2002, Tian *et al.,* 2002; Vinten *et al.,* 2004). Urban wastewater can contain high numbers of faecal microorganisms including pathogens like *Salmonella*, *Campylobacter*, *Shigella*, enteric viruses, protozoan parasites and helminth parasites (Girones, 2006). In Nigeria, as in most developing countries, surface waters which constitute an important source of water for drinking, domestic and agricultural purposes are vulnerable to pollution (Shuval, 1990; Umoh *et al.,* 2001, Cisse *et al.,* 2002, Okafor *et al.,* 2003; Agbogu *et al.,* 2006). Even at very low doses, some wastewater pathogens may represent a public health risk (Varela and Manaia, 2013). This low abundance may represent a major limitation for pathogen surveillance in wastewater habitats (Shannon *et al*., 2007). Wastewater used in irrigation has high bacterial density, hence increase in the probability of exchange of genetic material and thus genome rearrangements (Skippington and Ragan, 2011). The disposal of sewage sludge, as an agricultural fertilizer, is another source of potential environmental contamination (Kelessidis and Stasinakis, 2012). In case, disposal/ reuse of sludge or wastewater, there are two important targets of contamination for both environmental and human health (Varela and Manaia, 2013). One of the targets is represented by edible vegetable, which after irrigation with wastewater, may be a vector of undesirable

bacteria or of their genes into the human food chain via soil and water contamination, with the gradual and successive accumulation of chemical and biological pollutants (Varela and Manaia, 2013). Whereas in vegetables the exposure to wastewater pollutants is limited to the cultivation period, in soils and waters, exposure periods will be much longer, leading to the accumulation of those contaminants (Varela and Manaia, 2013). The contamination of soils after short- and long term exposures to wastewater irrigation has also been assessed. Dalkman *et al*. (2012) comparing the short- and long term (0-100 years) effects of wastewater irrigation in soils, reported some non-negligible effects. The investigators reported that, besides the accumulation of some antbiotics which was variable according to the type of soil, also the total microbial biomass and the abundance of antibiotic resistance genes increased.

Many infection outbreaks have been associated with processed vegetables directly or indirectly contaminated with animal manure (CDC, 2002). Reported outbreaks have been linked to contaminated drinking water and irrigation with water contaminated by animal faeces, discharges from sewage treatment facilities or surface runoff (Chalmers *et al.*, 2000, Payment and Riley, 2002; Solomon *et al.*, 2002). It is estimated that 3.2% of deaths globally are attributable to unsafe water caused by poor sanitation and hygiene, a problem that is particularly acute in rural areas in the developing world (Repoila *et al*., 2003). Sivapalasingam *et al.,* (2004) reported that produce associated outbreaks accounted for an increasing proportion of all reported food borne outbreaks with a known food item, rising from 0.7% in the 1970s to 6% in the 1990s. Data from the Centre for Science in the public interest database revealed that produce outbreaks accounted for 13% of foodborne outbreaks between 1900 and 2005 (Deweal and Bhuiya, 2007). The pathogens most frequently linked to vegetable related disease outbreaks include bacteria (*Salmonella, Escherichia coli*), viruses (Norwalk-

like, hepatitis A) and parasites, with *Salmonella* and *E.coli* O157:H7 being the leading causes of produce- related outbreaks (Lynch *et al*., 2009). Udo *et al*. (2009) reported 24.71% prevalence for *E. coli* and 3.53% prevalence of *Salmonella* e*nterica* Var Typhimurium from ready to eat salads in Calabar, South-South, Nigeria. Infact, fresh produce has been implicated in an increasing number of outbreaks of foodborne illness involving *Escherichia coli* O157:H7 and *Salmonella* spp in recent years (Deering *et al*., 2012). Contaminated water was suspected as the source of enteric pathogens during several recent produce related outbreaks (Benjamin *et al*., 2013). Even at very low doses, wastewater pathogens represent public health risk (Varela *et al*., 2013). Farmers working in the agricultural production and processing of fresh produce involving wastewater and wastewater irrigated soil and crops are exposed to potential pathogenic microorganisms present (Gemmell and Schmidt, 2010). Whether people become infected or sick after consuming wastewater irrigated vegetables depends on a number of additional factors such as the minimal infectious dose for a particular microorganism, the level of immunity and simultaneous contamination through other routes (Gemmell and Schmidt, 2010).

2.3 *Escherichia coli*

Escherichia coli or more commonly just *E.coli* is a rod –shaped bacteria described in 1885 by a German pediatrician, Theodor Escherich (Mainil, 2013). They are an important component of the intestinal microflora of humans and warm blooded mammals. *E.coli* is the predominant non pathogenic facultative anaerobic member of the human intestinal microflora (Daniel *et al*., 2013). *Escherichia coli* are prominent members of *enterobacteriacea* and are widely distributed in nature; they are present in the intestinal tract of man, animals, water and in the soil (Smith *et al.,* 2003). *E. coli* is a

well established indicator of faecal contamination in the environment and is currently used as an indicator of faecal contamination in freshwater systems (Ishii and Sadowsky, 2008). *E. coli*, given its ubiquity in faecal material and its relatively short persistence in environmental matrices, is the gold standard for detection of faecal pollution in water and the presence and density of *E. coli* in water is widely used to measure and regulate water quality (Jin *et al*., 2001 ; Page *et al*., 2001). The detection of *E. coli* in water is an implicit indicator of recent faecal contamination and therefore, the risk of co-occurrence of enteric pathogens that can cause illness in susceptible populations (Yates, 2007). Many jurisdictions evaluate and mandate compliance with drinking and recreational water quality standards on the basis of the presence and abundance of *E. coli* (National Research Council and Committee on indicators for water-borne pathogens, 2004). The organism's validity as an indicator of water pollution is dependent, among other factors, on its faecal specificity and its inability to multiply outside the primary host, the gastrointestinal tracts of humans and warm-blooded animals (National Research Council and Committee on indicators for water-borne pathogens, 2004). They are often used as a marker for the presence of recent faecal contamination (Gallup, 2010). The presence of *E. coli* in water indicates the potential presence of enteric pathogens, such as *Salmonella* and *Shigella* (Sher *et al*., 2010). *E.coli* is a model organism for studying many of life`s essential processes (Gallup, 2010). A model organism is a species that has been widely studied, usually because it is easy to maintain and breed in a laboratory setting and has particular experimental advantages (Gallup, 2010). Due to its rapid growth rate, simple nutritional requirements, well established genetics and completed genomic sequence, more is known about *E.coli* than any other living organism, hence it is the most widely used in molecular genetics (Gallup, 2010). *E.coli* has a cell division rate of about once every

30 minutes (Sundararaj *et al*., 2004) enabling adaptation to the environment. This rapid division rate has facilitated a long term evolutionary experiment in laboratories (Gallup, 2010). *E.coli* has been reported to be beneficial in synthesizing vitamin K (Bentley and Meganathan, 1982).

E.coli is a complex organism, some strains are beneficial and others are clearly harmful (Gallup, 2010). In 1893, a Danish veterinarian postulated that the *E.coli* species comprises different strains, some being pathogens, others not (Mainil, 2013). Those strains that cause enteric infections are generally called diarrheaogenic *E. coli* strains and their pathogenesis is associated with a number of virulence attributes which vary according to pathotype (Vidal *et al.,* 2005). While *E. coli* typically harmlessly colonizes the intestinal tract, several *E. coli* clones have evolved the ability to cause a wide spectrum of diseases, ranging from self-limiting to life threatening intestinal and extra-intestinal illnesses (Nataro *et al*., 2011). Studies have revealed enormous diversity in *E. coli* populations in primary and various secondary habitats, these include faeces from various mammalian and avian hosts (Anderson *et al.,* 2006; Hansen *et al.,* 2009), stored diary and swine manure (Lu *et al.,* 2005), home septic systems (Gordon *et al.,* 2002), fresh and marine surface water (Mclellan, 2004, Kon *et al.,*2007; Udenika *et al*., 2009), beach sand (Walk *et al.,* 2007), soils and the freshwater macrophytic green alga Cladophora (Byappanahalli *et al.,*2006). Different strains of *E. coli* differ widely in their abilities to persist or proliferate in manured soil (Topp *et al.,* 2003) or subtropical waters and sediments (Anderson *et al.,* 2005). Environmental isolates of *E. coli* that are distinct from faecal isolates may represent types that have become uniquely adapted for extra intestinal survival and proliferation (Byappanahalli *et al.,* 2006; Ishii *et al.,* 2007). The growth of *E. coli* in soils, sediments and water in tropical and subtropical regions has been widely documented and the organism is considered to have an

established part of the soil biota within these regions (Byappanahalli and Fujioka, 2004; Hartz *et al.,* 2008). The growth and survival of *E. coli* in soils have been examined primarily under laboratory conditions (Franz *et al*., 2008, Habteselassie *et al*., 2008; Vital *et al*., 2008). The integration of *E. coli* as a component of the indigenous microflora in soils of tropical and subtropical regions may be attributable to the nutrient rich nature and warm temperatures of these habitats (Winfield and Groisman, 2003; Ishii and Sadowsky, 2008), combined with the metabolic versatility of the organism and its simple nutritional requirements (Ishii and Sadowsky, 2008). In addition to tropical and subtropical regions, the presence of autochthonous *E. coli* populations in the cooler soils of temperate regions has also been reported (Byappanahalli *et al.*, 2006,Whitman *et al.,* 2006, Ishii *et al.,* 2006; Jiang *et al.,* 2007) with one report on a maritime temperate grassland soil (Breman *et al.*, 2010). The growth of *E. coli* within soils can act as a reservoir for the further contamination of bodies of water (Ishii *et al.,* 2006), compromising the indicator status of *E. coli* within these regions. It has been suggested that *E. coli* can sustain autochthonous populations within soils in temperate regions, where ever favorable conditions exist (Ishii and Sadowsky, 2008). The challenging nature of the soil environment and the disparity of conditions between the primary host and the secondary host raise the question of how these *E. coli* populations survive and compete for niche space among the higher competitive and diverse coexisting populations of the indigenous microflora (Ishii and Sadowsky, 2008). There is some evidence that naturalized *E. coli* populations in soil may have increased environmental fitness, facilitating their residence in soil (Ishii *et al.*, 2006).

Results from several studies have shown that various stresses influence the survival and growth of *E. coli* in soils, including high and low temperature (Topp *et al*., 2003, Ohotomo *et al*., 2004; Ishii *et al*., 2006;), limited moisture (Ohotomo *et al*., 2004,

Byappanahalli and Fujioka, 2004, Byappanahalli *et al*., 2006; Habteselassie *et al*., 2008), variation in soil texture (Topp *et al*., 2003), organic matter content (Berry and Miller, 2005, Ishii *et al*., 2006; Franz *et al*.,2008), solar radiation (Wu *et al*., 2009), competition and predation (Byappanahalli and Fujioka, 2004; van Elsas *et al*., 2007). Topp *et al*. (2003) observed that *E. coli* was capable of growing in soils amended with autoclaved swine manure slurry.

2.3.1 Phylogenetic origin of *Escheichia coli*

Phylogenetic studies have indicated that *E. coli* is composed of four main phylogenetic groups, A, B1, B2 and D, though a fifth group E, has been proposed (Escobar- Paramo *et al.*, 2004). Based on the 22 complete genome sequences of *E. coli* currently available, the *E. coli* pangenome (the complete complement of none core genes distributed within individuals within the species) has been estimated to comprise at least 42, 500 gene families (Snippen *et al.,* 2009) significantly, more than the human genome. Random amplified polymorphic DNA analysis of the intra species distribution of *E. coli* in pregnant women and neonates indicated that there was a correlation between the distribution of phylogenetic groups, random- amplified polymorphic DNA groups and virulence factors (Watt *et al.,* 2003). Based on comparisons of the distribution of *E. coli* phylogenetic groups among humans of different sexes and ages, it has been suggested that *E. coli* genotypes are likely influenced by morphological, physiological and dietary differences (Gordon *et al.,* 2005). In addition, climate has also been proposed to influence the distribution of strains within *E. coli* phylogenetic groups (Escobar- Paramo *et al.,* 2004). There are several reports indicating that there is a potential relationship between *E. coli* phylogenetic groups, age of host and disease caused. Nowrouzian *et al.* (2005) and

Moreno *et al.* (2005) reported that strains belonging to phylogenetic group B_2 persisted among the intestinal microflora of infants and were more likely to cause clinical symptoms. Boyd and Hartl, (1998) also reported that among the *E. coli* in the *E. coli* reference and diarrheogenic *E. coli* collections, strains in phylogenetic group B_2 carry the greatest number of virulence factors, followed by those in group D. Strains harboring a robust extra intestinal virulence factor (VF) repertorie cluster predominantly in groups B2 and D isolates within each phylogenetic group can be further classified as extra intestinal pathogenic *E. coli* (EXPEC) or non-EXPEC depending on whether specific virulence traits are present (Johnson *et al.,* 2003). Strains of groups A and B1 represent most commensal strains and are largely devoid of virulence determinants (Picard *et al.,* 1991).

2.3.2 Pathogencity of *E. coli*

The varieties of *Escherichia coli* that cause diarrhoea are classified into pathogenic groups (pathotypes) according to their virulence determinants (Robins-Browne and Hartland, 2002). *E. coli* is an important cause of disease in humans and agricultural livestock, and various pathotypes that cause specific enteric or extraintestinal disease syndromes are distinguished by the complements of virulence genes they carry (Iyoda and Watanabe, 2005, Aruscavage *et al*., 2008, Friesema *et al*., 2008; Anturez-Lamas *et al*., 2009). The specific nature of these virulence determinants imbues each pathotype with the capacity to cause clinical syndromes with distinctive epidemiologic and pathologic characteristics (Robins – Browne and Hartland, 2002). *Escherichia coli* pathogenicity is a complex mutifactorial mechanism involving a large number of virulence factors which vary according to the pathotype. A single component might not be sufficient to transform an *E. coli* strain into a pathogenic one but could

play a role in combination with other virulence determinant. Pathogeneicity in *E. coli* is largely determined by gene encoding virulence factors (vfs), such as adhesions, toxins and polysaccharide surface coatings (Johnson *et al*, 2001). Escobar- Paramo, (2004) proposed that certain *E. coli* backgrounds appear to be more likely to acquire virulence genes than others. Some important virulence associated properties of *E.coli* that can directly interact with the epithelial cells are adherence factors and exotoxins (Mainil, 2013). Atleast three different general classes of *E.coli* adhesin can be defined: different appendices like fimbriae, fibrillae and curli, the afimbrial family (Afa) and specific outer membrane proteins (Omps) like the intimin (Mainil, 2005). *E.coli* fimbriae were at first described on enterotoxigenic *E.coli* (ETEC) from pigs, calves and then humans (Gyles and Fairbrother, 2010). They were thereafter also described on uropathogenic (UPEC), septicaemic (SEPEC), avian pathogenic (APEC) and necrotoxigenic (NTEC) *E.coli* (Mainil, 2013). Though they received different names at the time of their description, today most are named by the letter F followed by numbers (Orskov and Orskov, 1990): F2 (colonization Factor Antigen (CFA1), F3 (CFA2), F4 (K88 antigen), F5 (K99 antigen), F6 (987 strain pili), F18 (F107, 8818, 2134 pili), F41 (B41 starin fimbriae) of ETEC; F7-F16 (Pap/Prs fimbriae) of UPEC, SEPEC, APEC, NTEC; F1 (type1) causing mannose-sensitive haemaagglutination, produced by almost all strains of *E.coli* and probably involved in the early steps of the colonization of the air sacs by APEC; F17 (FY, Att25, AttIII, Vir adhesion), ETEC, SEPEC, UPEC, APEC and NTEC (Mainil, 2013). The genes coding for the different fimbriae and fibrillae can be plasmid-located especially in ETEC or chromosome- located sometimes on specific pathogenicity islands, like in UPEC (Gyles and Fairbrother, 2010). The *E.coli* best known type 4 fimbriae or bundle- forming pili (bfp) are produced by typical enteropathogenic *E.coli* a subclass of human EPEC (Bardiau *et al*., 2010). Non- bfp

producing EPEC from humans and animals are called atypical EPEC (Hernandes *et al*., 2009). The bfp are encoded by plasmid- located genes and help bacteria to stick together forming micro-colonies at the surface of the host cells, also called "Localised Adherence" (LA) pattern (Kaper *et al*., 2004). Other type 4-like fimbriae, the Longus pilus, are produced by some human ETEC strains that, in addition to promoting intestinal colonization, also play a role in the protection of the bacterial from antimicrobial agents (Clavijo *et al*., 2010). Different specific outer membrane proteins (OMP) of pathogenic *E.coli* strains are involved in bacteria- eukaryotic cell adherence, bacteria and/or biofilm formation (Mainil, 2013), the plasmid- encoded adhesin involved in diffuse adherence (AIDA) (Bardiau *et al*., 2010), the plasmid- encoded shiga toxigenic autoagglutinating adhesin (Saa) of human and bovine VTEC (Bardiau *et al*., 2010) and the chromosome- encoded intimin adhesin (Int) of human and animal EPEC and EHEC (Hernandes *et al*., 2009). The intimin adhesin is a 94KDa OMP encoded by a gene (eae) located on a pathogenicity island named Locus of Enterocyte Effacement (LEE) along with other genes encoding for the formation of the Attaching and Effacing (AE) lesion produced by EPEC and EHEC (Hernandes *et al*., 2009). Different types of intimin adhesins have been described that differ in their amino-acid carboxyl terminal sequence (Mainil, 2013).

E.coli produces different oligopeptide toxins like the heat- stable enterotoxins of enterotoxigenic strains (STap, StaH and STb) and the heat-stable enterotoxin of enteroaggregative strains (EAST1) (Savarino *et al*., 1993). *E.coli* also produces another type of toxin called the AB toxins (Mainil, 2005). The AB toxins are composed of separate subunits, with one copy of the A subunit and one to several copies of the B subunit (Mainil, 2013). Other AB toxins are actually single proteins composed of two domains corresponding to the A and B subunits. The A

subunit/domain is the toxic component and B(s)/domain binds the whole toxins to the receptors on the eukaryotic cell membrane (Mainil, 2013). Examples of AB *E.coli* toxins are heat-labile enterotoxin (LT) of human, porcine and ruminant ETEC and the vero/shiga toxins (Vtx/Stx) of human, porcine and bovine EHEC and VTEC (Mainil, 2013). Another class of toxins found in *E.coli* is the cytolysisns which cause eukaryotic cell death and lysis by forming a pore within the cytoplasmic membrane (Mainil, 2013). An example is the α haemolysin (or HlyA) produced by several pathogenic types of *E.coli* causing extra intestinal (cystitis, nephritis, meiningitis, septicaemia in humans and animals) and intestinal infections such as enteritis and enterotoxaemia in piglets (Mainil, 2013).

2.3.3 *E. coli* classification

Escherichia coli can be divided into five main categories on the basis of distinct epidemiological and clinical features, specific virulence determinants and association with certain serotypes which include enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), entero-aggregative *E. coli* (EAEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), (Yah *et al.*, 2006), diffusely adherent *E. coli* (DHEC) (Kaper *et al.*, 2004) and cytolethal distending toxin producing *E. coli* (CDT - EC) which has also been described as diarrhoeagenic (Clarke, 2001).

Studies of *E. coli* genomes have highlighted the importance of genomic island content in differentiating among various *E. coli* serotypes and pathotypes (Ogura *et al.*, 2007). Comparative genomics have shown that multiple genetic paths of gain and loss of genes can lead to convergent phenotypes (Touchon *et al*., 2009). Its diverse phenotype results principally from a large number of different gene combinations (Clermont *et al*., 2011). Specific sets of these large genetic elements presumably

explain the phenotypic differences among the *E. coli* groups in host adaptation, pathogenesis, virulence, epidemiology and ecology (Karmali *et al.,* 2003).

Enteropathogenic *Escherichia coli* are one of the six pathotypes of diarrhoeagenic *E. coli* (DEC) which promotes attaching- effacing lesions in eukaryotic cells. These lesions are mediated by intimin, an outer membrane adhesive protein encoded by the *eae* (*E. coli* attaching- effacing) gene (Kaper *et al.,* 2004). EPEC is currently subdivided into typical and atypical subgroups. Typical EPEC carry the EPEC adherence factor plasmids (PEAF) that encodes the bundle forming pilus (Bfp) and a complex regulator of various virulence genes (Per) (Kaper *et al.,* 2004). Conversely, atypical EPEC most often expresses the LA- like pattern (with loose bacterial clusters) or adhesive patterns of other DEC pathotypes (Trabulsi *et al.,* 2002). EPEC adheres tightly to epithelial cells and disrupts normal cellular process (Chen and Frankel, 2005) and evidence suggests that atypical EPEC may retard apoptosis of intestinal epithelial cells (Heezko *et al,* 2001) possibly because of the lack of bundle forming pilus (Bfp) (Melo *et al.,* 2005). EPEC virulence is characterized by intimate contact between the bacterium and the apical plasma membrane of the intestinal brush border cells, followed by localized destruction of the intestinal brush border and distortion of the apical enterocyte membrane. The interactions between the bacterium and the host cells result in gross cytoskeletal rearrangements, particularly the formation of an actin-rich, cup – like pedestal at the site of bacterial contact called attaching and effacing (AE) lesion (Clarke *et al.,*2003). The genes associated with attaching and effacing are localized on a 35.6 –kb Pathogenicity Island called the locus of enterocyte effacement (LEE). LEE contains several virulence genes, including the *eae* gene that encodes the adhesin intimin, which is required for the attachment of EPEC to epithelial cells, genes encoding a type III secretion system (TTSS) and *tir*, which encodes the translocated

intimin receptor (Dean *et al.,* 2005). EPEC strains also contain a large plasmid referred to as the EPEC adherence factor (EAF) plasmid though absent in the atypical EPEC (Trabulsi *et al*., 2002). This plasmid encodes a regulator of virulence genes called Per that regulates the expression of a chromosomal regulator, Ler, which then activates the expression of other LEE genes in regulatory cascade (Dean *et al.*, 2005,). The atypical EPEC strains are genetically related to the enterohemorrhagic *E. coli* (EHEC) strains and are both considered emerging pathogens (Trabulsi *et al.,* 2002). While typical EPEC strains have been isolated only from infected humans, atypical strains have been found in several animal hosts, including birds, dogs and cows (Aidar – Ugworich *et al.,* 2007; Ishii *et al.,* 2007). The prevalence of atypical EPEC strains among different animal hosts is largely unknown (Ishii *et al.,* 2007) as is their capacity to survive outside their host. One of the most common causes of infant diarrhoea in developing countries is enteropathogenic *Escherichia coli* (EPEC) (Clarke *et al.,* 2003). It has been identified in children with acute diarrhoeal disease and has also previously been reported in association with prolonged diarrhoea (Afset *et al.,* 2004).

Enterotoxigenic *Escherichia coli* (ETEC) strains colonize the small intestines, produce enterotoxins and are a major cause of diarrhoeal disease in humans and animals (Svnennerholm and Steels, 2004; Walker, 2005). Pathogenesis of ETEC is due to the production of any of several enterotoxins. Its enterotoxin is very similar in size (86KDa), sequence, antigenicity and function to the cholera toxin (CT) (Feng and Weagant, 2011). ETEC may also produce a heat stable toxin (ST) that is low molecular size (44KDa) and resistant to boiling for 30 minutes (Feng and Weagant, 2011). Diarrhoea is often attributed to ETEC carrying the F18 fimbrial adhesion gene (FedA1) and the virulence factors LT, STa and STb (Zhang *et al.,* 2007). ETEC is recognized as the causative agent of traveller's diarrhoea and illness is characterized by watery

diarrhoea with little or no fever (Feng and Weagant, 2011). ETEC infections occur commonly in under-developed countries (Feng and Waegant, 2011). ETEC for adults has been estimated to be at least 10^8 cells; but the young, the elderly and the infirm may be susceptible to lower levels (Feng and Weagant, 2011). Because of its high infectious dose, analysis for ETEC is usually not performed unless high levels of *E. coli* have been found in food (Feng and Weagant, 2011).

Enteroinvasive *E.coli* (EIEC)'s pathogenicity is primarily due to its ability to invade and destroy colonic tissue (Feng and Weagant, 2011). These bacteria are characterized by a large virulence plasmid (220kb) and by their ability to invade epithelial cells and disseminate from cell to cell (Pupo *et al.*, 2000). Unlike typical *E. coli*, EIEC is non-motile, they do not decarboxylase lysine and do not ferment lactose, so they are anaerogenic (Feng and Weagant, 2011). Like *Shigella*, there are no known animal reservoirs (Feng and Weagant, 2011) hence the primary source for EIEC appears to be infected humans (Feng and Weagant, 2011). A number of methodological issues exist that may partially explain why low levels of EIEC are found in other studies. Specifically, microbiological analyses used in other studies often do not distinguish between EIEC and *Shigella* (Rappelli *et al.*, 2005). Differentiating between EIEC and *Shigella* is difficult because of their genetic similarities. The four species of Shigella are often considered to be types of *E. coli* and are most similarly related to EIEC (Pupo *et al.*, 2000). Although the infective dose of *Shigella* is low and in the range of 10 to few hundred cells, volunteer feeding studies showed that atleast 10^6 EIEC organisms are required to cause illness in healthy adults (Feng and Weagant, 2011).

Enteroaggregative *E. coli* (EAEC) is identified by the characteristic aggregative pattern of adherence (AA) in Hela/HEP 2 cells. EAEC attaches in

aggregates to cell surfaces as well as around cells (Kaper *et al.*, 2004; Nataro, 2005). EAEC colonizes the intestinal mucosa, forming a thick biofilm that favours prolonged colonization and induces malnutrition (Kaper *et al.*, 2004; Nataro, 2005). EAEC strains produce an ST like toxin and haemolysin like toxin (Pabst *et al.*, 2003). Like ETEC they also bind to small intestinal cells and are not invasive but differ from ETEC in that they do not adhere uniformly over the surface of the intestinal mucosa but tend to clump in small aggregates. Actually, this pathotype is heterogeneous regarding the presence of putative virulence genes. EAEC has been sub grouped into typical and atypical, which carry or lack AggR (a globular regulator of EAEC virulence) (Nataro, 2005). EAEC is the pathotype responsible for persistent diarrhoeal (>14days) among international travellers who visit developing countries (Adachi *et al.*, 2001).

2.3.4 Verocytotoxigenic *Escherichia coli* (VTEC)

Verotoxins (vt) are the main virulence factors of VTEC and are essential for many of the pathological features and for some of the severe complications of VTEC infection (O`Loughlin *et al*., 2001). Among VTEC factors, verotoxins are considered to be the most critical virulence factor associated with human disease (O`Loughlin, 2001). The genes encoding verotoxins are carried by bacteriophages, in general, vt genes are situated among genes controlled by the late promoter, suggesting that vt production is linked to the induction or progression of the phage lytic cycle (Wagner *et al*., 2001). Thus, vt phages play an important role in both the expression of vt and lateral gene transfer (Muniesa *et al*., 2004). Kohler *et al*. (2000) observed that certain antibacterial agents commonly used as growth promoters in animal husbandry can induce the vt phage lytic cycle and therefore may contribute to the spread of vt and the development of new VTEC pathotypes. Differences in phage induction could be one of the factors

contributing to the variable clinical significance of VTEC strains (Kruger *et al*., 2011). Several studies have shown that VTEC strains with different vt alleles are associated with particular reservoirs (Brett *et al*., 2003) and differ markedly in their association with Hus (Jenkins *et al*., 2003). The amount of vt produced by the strain may also play an important role in the clinical outcome (Ekulund *et al*., 2002, Muniesa *et al*., 2003; Gamage *et al*., 2004). Several variants of the major types of verotoxin genes; vt1 and vt2, have been identified. The vt1 group is more homogenous than the vt2 group, which includes a high number of variants (Scheutz and Strockbine, 2005).

2.3.5 Shiga toxin Producing *Escherichia coli* (STEC)

Shiga toxin (Stx) producing *Escherichia coli* (STEC) organisms also called verocytotoxin producing *E. coli* (VTEC) are one of the most important groups of foodborne pathogens (Beutin, 2004). The excretion of shiga-like toxins (also known as verotoxins) marks an isolate as STEC (or VTEC) (Hlavsa *et al*., 2011). More than 70 different serotypes of Shiga toxin-producing *Escherichia coli* (STEC) that cause disease in humans have been described (Brooks *et al*., 2005). Shiga toxin (Stx) – producing *Escherichia coli* (STEC) O157:H7 is the most prominent STEC serotype, causing hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) in many countries (Karmali *et al*., 2010). Virulence traits of STEC strains are governed by the dynamic exchange and loss of mobile genetic elements such as plasmids, transposons, insertion sequences, integrons, bacteriophages and genomic islands (Kelly *et al*., 2009). These genetic loci characterize the pathogenicity of bacterial species by determining what toxins are produced and how the bacterium attaches to and invades host cells, modulates the host cell cycle and immune responses, survives in stressful environments and produces biofilms. Many virulence loci have been characterized for STEC,

including a number of pathogenicity islands (e.g the locus of enterocyte effacement (LEE), the locus of proteolysis activity (LPA), the high- pathogenicity islands (HPI), the *E. coli* type 111 secretion apparatus (ETT2), the urease gene cluster, the long polar fimbrial operon, O-island 36 (OI-36), OI-57, OI-71,OI-122,OI-154), two key virulence plasmids (pO157 and pO113) and chromosomal lambdiod bacteriophage insertions that carry genes for types of shiga toxin (Coombes *et al*., 2008). Various factors and toxins contribute to the increasing virulence of STEC (Wicham *et al*., 2006). The major virulence factors implicated in STEC infection are potent shiga toxins which are classified into two groups; Stx1 and Stx2 (Gyles, 2007). Shiga toxins differ in toxicity; toxin receptor and amino acid sequence (Lee *et al.,* 2007). Toxins of the stx1 and stx2 families can be divided further into subtypes differing in the composition of their A or B subunit (Scheutz and Strockbine, 2005). Studies on human virulent EHEC have revealed that certain toxin types such as stx2, stx2c and activatable stx2d are significantly with higher virulence of the bacteria for humans, which may cause severe diseases such as hemorrhagic colitis and Hus (Bielaszewska *et al.,* 2006). Besides the production of stx, a number of accessory virulence factors that are probably involved in pathogenesis occur in STEC strains, one of the most prominent accessory pathomechanisms is the formation of attaching and effacing lesions and STEC virulence factors can also be encoded by large plasmids, such as PO157 and PSFO157 (Brunder *et al.,* 2006). Other toxins purported to increase virulence in strains are subtilase (subA) (Khaitan *et al*., 2007; Wolfson, 2009), lymphocyte-activating factor (*lif*A) also known as *efa*l (Klapporth, 2010), cytotoxic necrotizing factor (*cn*f) and heat stable toxin.

2.4 Enterohemorrhagic *Escherichria coli* (EHEC)

EHEC which was first identified as a human pathogen in 1982 is the STEC serotype most often associated with severe forms of disease (Rivero *et al*., 2010). Enterohemorrhagic *Escherichia coli* (EHEC) strains are a subset of Shiga toxin (Stx) - producing *E. coli* (STEC) strains. They are usually isolated from human patients and are responsible for severe clinical symptoms, such as hemorrhagic colitis (HC) and the potentially lethal hemolytic uremic syndrome (HUS) (Karmali *et al*., 2010). Infact, hemolytic- uremic syndrome (HUS), hemorrhagic colitis (HC) and thrombotic thromboticpenic purpura (TTP) are the most worrisome complications of *E. coli* O157:H7 infection (Callaway *et al*., 2009). The major source for human illness is cattle (Callaway *et al*., 2009), but humans may serve as reservoirs for person to person transmission (Picozzi *et al*., 2005).

The pathogenicity of EHEC involves establishment of the organism in the gut, where it has to compete for space and nutrients with other microorganisms of the normal intestinal microflora (Welinder-Olsson and Kaijser, 2005). Enterohemorrhagic *Escherichia coli* (EHEC) pathogenicity is usually linked to a shiga toxin (Makino *et al*., 2003) and virulence factors including, adhesins, toxins, invasions, protein secretion systems, iron uptake systems and several unidentified functions (Hacker and Kaper, 2000). EHEC strains contain a large heterologous virulence plasmid, referred to as the 60-MDa virulence plasmid, which contains a number of different genes associated with disease: *espP*, *katP*, *eptD*, *toxB* and the EHEC hemolysin gene (*ehx*) (Johnson and Nolan, 2009). EHEC has the following serogroups O157:H7, O113:H21, O113:H4, O26:H11, O118:H16 and O146:H21 with *E. coli* O157:H7 predominating (Bettleheim and Beutin, 2003). The most notorious serotype of EHEC, O157:H7 is responsible for numerous reported outbreaks (Hlvasa *et al*., 2011).

Severe damage due to infection with EHEC is attributable to the cytotoxic verotoxin (VT), which damages epithelial cells and endothelial cells leading to bloody diarrhoea and HUS (Kaper *et al.,* 2004). Shiga toxin (stx) producing *Escherichia coli* (STEC) organisms, also called verocytotoxin (VT) producing *E. coli* (VTEC) are one of the most important groups of food borne pathogens (Beutin *et al.,* 2004*;* Ribot *et al.,* 2006). Verotoxigenic *E. coli* is well established as a major virulence factor of EHEC O157:H7 that plays a central role as a toxin in hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) (Tarr *et al.,* 2005) but its role in adherence is controversial (Cornick *et al.,*2007). Infact, production of VT is the single most important factor, which is considered essential for the development of HC and HUS (Melton-Celsa and Brien, 1998). The genetic organization of VT phages is similar to that of lambda and related phages (Herold *et al.,* 2004) and phage genes regulate toxin production through phage replication of toxin released by lysis of the host bacterial cell (Unkmeir and Schmidt, 2000, Johansen *et al.,* 2001; Wagner *et al.,* 2002). Several investigations have reported that VT does not play a role in colonization of the intestine (Best *et al.,* 2003, Sheng *et al.,* 2006; Cornick *et al.,* 2007). However, Robinson *et al.* (2006) reported that VT enhances adherence to epithelial cells and colonization of the mouse intestine by *E.coli* O157:H7. VT was reported to bind to Gb3 on plant cells in the crypt intestinal epithelia of human biopsy specimens (Schuller *et al.,* 2007). This binding could have an inhibitory effect on antibacterial peptide secretion, as has been described by *Shigella* spp, thereby promoting bacterial colonization (Islam *et al.,* 2001; Schuller *et al.,* 2007). Colonization factors may be host specific and/or environmentally regulated (Rashid *et al.,* 2006) resulting in differential activity or expression of virulence genes.

Ruminants, especially bovines are regarded as the principal reservoir of VTEC strains (Naylor *et al*., 2005). Most STEC isolates from healthy cattle do not

carry the *eae* gene (Irino *et al.*, 2007). Although *eae* is considered important for causing attaching and effacing lesions in human intestinal epithelial cells, it may not be essential for STEC pathogenicity, since *eae* negative STEC strains have also been reported to cause severe human infections (Paton *et al.,* 2001). Zheng *et al.*(2008) identified seven STEC isolates carrying activatable Stx_2d among 153 STEC strains isolated from food, cattle and humans and none of them contained the *eae* gene (Zheng *et al.,* 2008). Characterization of *eae*-negative STEC strains have identified *Saa* (STEC autogglutinating adhesin) as a candidate adherence protein (Paton *et al*., 2001), but have also found other putative virulence factors, like subtilase cytotoxin (encoded by sub AB) (Newton *et al.*, 2009), that are also predominant in *eae*-negative STEC strains. In the absence of intimin, other factors such as *saa* and *iha* that correlate with increased adherence have been identified (Tarr, 2000, Paton *et al*., 2001; Jenkins, 2003). The interplay between these various genes is not understood, but each has been described to provide an additional trait that increases virulence in STEC. Finally, comparisons of the genomes of *E. coli* O157:H7 and *E. coli* k-12 have identified numerous islands of genetic material unique to each. The genomic regions specific to *E. coli* O157:H7 have been termed O islands (OIS). Studies of OIS (Jenkins, 2003; Perelle *et al*., 2006) have identified genes associated with increased virulence (Karmali *et al.* , 2003, Wickham, 2006; Coombes, 2008).

Enterohemorrhagic *E. coli* and enteropathogenic *E. coli* strains are able to cause destruction of the microvillous brush border and actin rearrangement beneath the site of bacterial attachment, a type III secretion system and a number of type III effector proteins are involved in this process (Kenny, 2002; Garmendia *et al.,* 2005). They are encoded on a pathogenicity island termed locus of enterocyte effacement (LEE) and detection of the *eae* genes is often used as a maker for the presence of the LEE (Beutin

et al., 2005; Bielaszewska *et al.,* 2005) in addition to the production of toxins and the attaching and effacing lesions, adherence factors may contribute to STEC pathogenesis. In addition to LEE, EHEC are distinguished by the production of stx1 or stx2, the former being identical to the *Shigella* dysenteriae type 1 toxin and the latter only sharing partial homology (Hill, 2013).

STEC strains are genetically heterogenous and although more than 200 STEC serotypes have been described, only a limited number of serotypes have been isolated from human cases (Hussein, 2007). The most important serotypes which can cause severe human disease are O157:H7, O157: Nm and the non- O157 serotypes O26:H11, O111: Nm, O103:H2 and O145: Nm (Brooks *et al.,* 2005).

2.4.1 EHEC (NON O157)

Most outbreaks and sporadic cases of hemorrhagic colitis and HUS have been attributed to the STEC O157 strains (Tarr *et al.,* 2005). However, numerous non- O157 STEC isolates have also been linked to illnesses and outbreaks or disease (Bosilevac and Koohmaraie, 2011). Molecular risk assessment of non-O157 STEC has greatly advanced due to a number of recent studies that found a strong association between the presence of non-LEE effectors (nle) located in STEC O islands with serotypes classified as seropathotype (SPT) A to C and thereby also associated with outbreaks and Hus (Bugarel *et al*., 2010; Imamaovic, 2010). Moreover, EHEC – O157 typically produces Shiga toxin stx1 and/or stx2 encoded by lambdoid bacteriophages (Law, 2000). The frequency of reported EHEC – O157 associated disease varies markedly internationally for example, the incidence of EHEC-O157 (infections/100,000 population annually) was reported as 4.1 in Scotland and 0.08 in Australia (Dresman and Pulz, 2004, Locking *et al.*, 2006; Korean Centres for Disease Control and

Prevention, 2007). In some geographic areas, STEC non O157 strains are commonly isolated from persons with diarrhoea or HUS than O157 strains (Pradel *et al.,* 2000). STEC non-O157 isolates have been shown to be important pathogens, despite severely under reported due to lack of facilities to isolate, identify and characterize them in many laboratories, where facilities do exist, STEC non –O157 isolates are found (Bettelheim, 2007). Although it is infrequently isolated and reported, it is estimated that non-O157 STEC may cause diarrhoea at frequencies similar to those of other important enteric bacterial pathogens, such as *Salmonella* and *Shigella* (Slutsker *et al*., 1997) and also cause infections resulting in HUS and outbreaks (Brooks *et al*., 2005). The proportion of these non O157 STEC serogroups breakdowns are as follows: 22%, O26, 16%, O111, 12% O103, 8%, O121, 7%, O45 and 5%, O145. Other non O157 STEC strains such as O116:H21 have been isolated from animals, the environment (WHO, 1998) and ground beef samples in various countries (Lucchesi *et al*., 2006) but have seldom been isolated from humans (WHO, 1998; Hussein, 2007). Serotype O113:H21 strains have been isolated from foods, animals and the environment in several countries (dos Santos *et al*., 2010). Infact, there are ample data on the prevalence of non-O157 STEC in cattle from fed beef through processing (Arthur *et al*., 2002). Several non-O157 EHEC serogroups are important causes of human disease and the most common of these is O26 (Brook *et al.,* 2005, Karch *et al.,* 2005; Johnson *et al.,* 2006).

Escherichia coli serogroup O26 has members classified as enterohemorrhagic *E. coli* (EHEC) or atypical enteropathogenic (aEPEC). EHEC O26 strains phenotypically resemble other STEC and are pathogenic *E. coli* strains and are not easily detectable by cultural methods (Jenkins *et al*., 2008). Analysis of virulence genes present in *E. coli* O26 strains revealed two groups of pathogens: EPEC O26 strains that were characterized by the presence of the LEE (locus of enterocyte

effacement) pathogenicity island and shiga toxin-producing *E. coli* (STEC) O26 strains that, in addition to LEE, carried phages encoding the genes for shiga toxin1 (Stx1) and/or shiga toxin 2 (Stx2) (Jenkins *et al*., 2008). Hence, EHEC O26 strains produce Shiga toxin (stx1) and Shiga toxin 2 (stx2) either singly or together (Brooks *et al.,* 2005). In addition, they contain the intimin encoding *eae* gene (Bielaszwska *et al.,* 2005), an important characteristic of EHEC (Orth and Wurzer, 2006). EHEC O26 strains constitute the most common non-O157 EHEC group associated with severe diarrhoea and hemolytic uremic syndrome (HUS) in Europe (European Food Safety Authority, 2009). EHEC O26 is also the most common non O157 EHEC serogroup in the United States where between 1983 and 2003 it accounted for 22% of non-O157 EHEC clinical isolates (Brooks *et al.,* 2005). In a study from Montana, half of EHEC O26 isolates originated from patients with bloody diarrhoea (Jelacic *et al.,* 2003).

2.5 *Escherichia coli* O157

Escherichia coli O157 are an important food- borne and water- borne pathogen with a worldwide distribution (Chalmers *et al*., 2000). The first reported outbreak of *E. coli* O157 infection in the developing world occurred in 1982 in Southern Africa (Effler *et al*., 2001). It was followed up with outbreaks in Central African Republic in 1996 and Cameroon in 1997 (Cunin *et al*., 1999). Such outbreaks have been linked to contaminated bovine food products, contaminated drinking water and flood irrigation with water contaminated by animal faeces or surface runoff (Solomon *et al*., 2002). Cattle faeces have been implicated as the major source of contamination (Renter *et al*., 2003). Although not in outbreak proportions, *E. coli* O157:H7 illnesses have also been reported in Southern Nigeria (Olorunshola *et al.,* 2000).

2.5.1 Outbreaks of *Escherichia coli* O157:H7

EHEC infections may be sporadic, in small clusters, or manifest as larger outbreaks (Besser *et al*., 2010). Outbreaks of EHEC infection may result from contamination originating in restaurants, home, kitchens, farms, petting zoos, nursing homes, day care centers, recreational pools/lakes and schools (Davis *et al*., 2005). Transmission is via the feacal-oral route and frequently occurs through ingestion of contaminated food or water; direct with infected animals, humans or objects (Grant *et al*., 2008). *Escherichia coli* O157:H7 is the most common bacterial etiologic agent of outbreaks associated with leafy greens (Brandl, 2006). Outbreaks of *E. coli* O157:H7 infections historically have been associated with consumption of undercooked ground beef; however, outbreaks of food infections associated with consumption of ready-to-eat vegetables have been increasing (Schreck, 2009) since the first reported case in 1982. This highlights the increasing importance of fresh produce as a vehicle of food borne illness. *Escherichia coli* O157:H7 (*Ecoli* O157) infections are increasingly linked to leafy produce with 27 outbreaks of *E.coli* O157 associated disease between 1995 and 2008 (Mandrell, 2009). Infection with *E.coli* O157 is of particular concern due to its potential to cause severe disease, 6 of 11 deaths related to food-borne illness in 2006 were linked to this bacterium, even though only 2% of all reported food-borne illness were attributed to *E.coli* O157 that year (CDC, 2009). Of the documented outbreaks of *E.coli* O157 between 1991 and 2002, more than half (61%) were associated with either lettuce, salad or coleslaw (Nobre *et al.*, 2009). An outbreak in Walkerton, Canada (Clark *et al*., 2003) was related to consumption of faecally contaminated drinking water and resulted in an estimated number of 2,300 disease cases with seven being fatal (Hrude *et al*., 2003). In September, 2006, pre-packed fresh spinach was recalled by the Food and Drug Administration (FDA) in the United States

of America as a result of an outbreak in the California. Another outbreak linked to lettuce was reported in the northern USA (Institute of Food Technologists, IFT, 2007).

2.5.2 Transmission of *Escherichia coli* O157:H7

Ruminant animals, in particular cattle, are identified as a major reservoir of EHEC and STEC strains (Geue *et al*., 2009; O`Reilly *et al*., 2010). Cattle manure is a major reservoir of *Escherichia coli* O157:H7 and it has been demonstrated to be higher in cattle faeces during the warm months (Renter *et al*., 2004) and parallel with the timing of most human illness outbreaks (Farah *et al*., 2003). However, the factors contributing to bovine colonization and shedding of *E. coli* O157:H7 are poorly understood and they are still very few tools available to control these zoonotic bacteria in cattle populations (Lejeune and Wetzel, 2007). Early epidemiological studies in livestock populations showed that individual farms often tended to maintain either a high or low prevalence of *E. coli* O157:H7 over time (Smith, 2001). This relative stability of pathogen prevalence on individual farms has been interpreted to be an indication for a role of stable farm management factors in governing the relative abundance of *E. coli* O157:H7 in cattle populations (McSpadden *et al*., 2011). In addition, studies indicate a peak in the prevalence of the pathogen in cattle, which occurs in summer and is coincident with the seasonal peak in human illnesses (Barkocy-Gallagher, 2003; Cobbold, 2007; Freeman *et al*., 2009). Cooley *et al*. (2007) reported that the incidence of generic *E. coli* and *E. coli* O157:H7 in some locations increased after rain events in winter months and correlated with significant increases in the flow rate of rivers and streams (Cooley *et al*., 2007). *Escherichia coli* O157:H7 may be introduced into crops by flood irrigation with water contaminated with cattle faeces or by contaminated surface runoff (O'Conner, 2002). *E. coli* O157:H7 can be

transmited in lettuce that has been fertilized with manure or irrigated with contaminated water contaminated through the root system and internalized in the leaves, there by resisting traditional external sanitizing treatments (Solomon *et al.,* 2002). A study by Solomon *et al.* (2005), demonstrated the transmission of *E. coli* O157:H7 from manure contaminated soil and irrigation water to lettuce plants using laser scanning confocal microscopy, epifluorescence microscopy and recovery of viable cells from the inner tissues of plants. Ibekwe *et al,* (2004) also described quantification of *E. coli* O157:H7 in rhizosphere and phyllosphere of plants using multiplex real time. In a field study described by Johannessen *et al.*(2004) where the influence of manure on the hygienic quality of lettuce was studied, *E. coli* O157:H7 was unexpectedly isolated from naturally contaminated farm manure and slurry used for fertilizing and also from soil weeks after fertilizing. Johannessen *et al.* (2005) also suggested that the concentration of pathogens played a role in the contamination of vegetables from manure.

Persistence of *E. coli* O157:H7 in the environment is variable and likely dependent upon a variety of factors, including temperature, sunlight, moisture, nutrients and possibly unknown factors that enhance or decrease amplification of *E. coli* O157:H7. Studies indicate that *E. coli* O157 will survive in the field for four to eight weeks (Ibekwe *et al.,* 2004, Johannessen *et al.,* 2005, Nicholson *et al.,* 2005; Mukherjee *et al.,* 2006) and its survival could be improved at lower temperatures in clay soil and in close association with roots (Ibekwe *et al.,* 2004) and in soil amended with cattle feaces (Scott *et al.,* 2006). *Escherichia coli* O157:H7 can survive for extended periods of time in water and in soil at freezing and refrigeration temperatures and under dry and acidic condition (CDC, 2001). The pathogen can also survive for more than 2 months in organic wastes, followed by a significant and gradual decline in bacterial numbers but long term storage cannot be expected to completely, eliminate *E. coli* O157:H7 from

soil (Williams *et al.,* 2005). Longterm survival of *E.coil* O157:H7 in soils has implications and may be a significant factor in its contamination cycle and subsequent transmission to crops, and surrounding water bodies and also to direct infection of humans and animals in contact with the contaminated soil (Maule, 2000) which may even lead to recontamination of produce, surface water and ground water after the initial contamination event. *E. coli* O157:H7 shed from healthy cattle can survive for extended periods of time in the environment; survival times measures in days, weeks and months have been recorded for manure, soil, water and vegetables such as lettuce (Fenlon *et al.,*2000, Jiang *et al.,* 2002; Ogden *et al.,* 2002). The risk factors for carriage and infection of cattle are; age, weaning, shipping, season and feed composition and the bacteria's ability to persist in the farm environment for months (Fairbrother and Nadeau, 2006). Thus, *E.coli* O157:H7 represents an underestimated environmental risk.

Pathogenic *E.coli* O157:H7 from livestock feces is known to survive on grass pasture for atleast 5 months, affording it opportunity to be recycled by animals (Avery *et al*., 2004). Previous studies have revealed that *E. coli* O157:H7 has the ability to persist on leafy vegetables in the field (Islam *et al.,* 2004) and to multiply in the phyllosphere of lettuce plants under warm and wet conditions in the laboratory (Brandl and Amundson, 2008). Islam *et al.* (2004) discovered that the type of vegetable being grown plays a role with regard to the persistence of *E. coli* O157:H7 in soil. Field studies with virulent strain of *E. coli* O157:H7, have shown the survival of the pathogen on lettuce, parsley, carrots and onions surface up to 77 and 177 days (Islam *et al.,* 2004). Gagliardi and Karns (2002) also showed that *E. coli* O157:H7 persisted longer with some cover crops and in the presence of clayey soil. Other studies revealed that *E. coli* O157 multiplied faster on damaged lettuce leaves than on intact leaves with greater growth corresponding to greater leaf damage (Brandl, 2008). Unlike some Gram-

positive bacteria that respond to starvation by producing spores or cysts, *E.coli* O157:H7, a non-sporulating bacterium, respond more by an altered physiological or metabolic state, instead of developing resistant structural modifications (Chekabab *et al*., 2013). When nutrient conditions in aquatic habitats are unfavourable, *E.coli* O157:H7 might reduce cell size, thereby increasing its surface/volume ratio and allowing more efficient uptake of poorly available nutrients (Chekabab *et al*., 2013). This physiological state resulting from an insufficient amount of nutrients is known as the starvation –survival state (Burgess, 1998). In this state, *E.coli* evolves strategies to acclamatise rapidly to surrounding environmental changes (Chekabab *et al*., 2013). The adaptive response begins by activation of enzymes required to catabolise available nutrients (Tao *et al*., 1999). After that *E.coli* may increase its production of toxins or antibiotics, to promote killing or invading of other cells in the environment (Martin, 2004). Finally, the bacterium swithches to a survival state, which enhances its resistance to many stresses and its ability to remain viable during long periods, without nutrients (Siegele and Kolter, 1992).

EHEC are transmitted by the faecal-oral route (Besser *et al*., 2010). They can be spread between animals by direct contact or via water troughs, shared feed, contaminated pastures or other environmental sources (Besser *et al*., 2010). Human to human transmission is known to occur in about 20% of households with an O157:H7 primary patient (Werber *et al*., 2008). The epidemiological analysis of an initially basically foodborne infection becomes even more difficult when the initial pathogen transmission via the food chain is replaced by human- to- human transmission (Welinder-Olsson and Kaijser, 2005). Infected individuals are highly contagious and may be considered a public health hazard (Ahn *et al*., 2009) and one important factor in direct transmission is how long an infected person sheds the pathogen (Welinder-

Olsson and Kaijser, 2005). Approximately 20% of the *E.coli* O157:H7 cases diagonised during an outbreak are the result of secondary transmission and rates of such transmission are particularly high in outbreaks that affect children with a median age of less than six years and those in nurseries (Snedeker *et al*., 2009).

2.5.3 Public health significance of *E.coli* O157:H7 in water, soil and vegetables

E.coli O157: H7 survives at low density in oligotrophic environments such as surface water or groundwater that may be used as a raw water source for drinking (Chekabab *et al*., 2013). The presence of *E.coli* O157:H7 in aquatic environments is the common denominator linking diverse transient habitats and transmission to animals and humans (Chekabab *et al*., 2013). One important transmission route for *E. coli* O157:H7 outbreaks are through drinking water (Gagliardi and Karns, 2000; Lee *et al.,* 2002). Tap water for example, is generally characterized as being oligotrophic and is oxidatively stressful to bacterial cells, and the bacteria can be induced to enter a viable but not culturable (VBNC) state (Oliver, 2005). Microbiological studies demonstrated that *E. coli* O157:H7 and other bacteria may become viable but non culturable (VBNC) when exposed to sublethal stresses such as temperature, available nutrients or pH or exposure to chlorine used in water treatment (Juhna *et al.,* 2007). Un-culturability is a broad sense condition found in about 99% of the environmental bacteria (Vartoukian *et al*., 2010). Under starvation conditions, *E. coli* O157:H7 can also enter into a VBNC state, however, expression of the toxin gene *stx* can still occur in VBNC cells of *E.coli* O157:H7 (Yaron and Mathew, 2002) and strains of this bacterium in the VBNC state can become culturable again in the presence of oxyrase antioxidant, enterobacterial autoinducer, or sodium pyruvate (Asakura *et al.,* 2005), suggesting the potential health concerns regarding the VBNC cells. Therefore, it is particularly important to monitor

the VBNC cells of *E. coli* O157:H7 in drinking water and source water because of the potential of water distribution to transmit this pathogen as there is high level of contamination associated with small- scale and very – small scale drinking water supply (Vinten *et al.*, 2009). *E. coli* O157:H7 can survive bleach treatment of lettuce because bacterial cells are protected in cuticle cracks and on trichomes, possibly in aggregates (Takeuchi and Frank, 2001) hence, indicating that *E. coli* O157:H7 migrated to internal locations in plant tissue and are thus often protected from the action of sanitizing agents by virtue of its accessibility, posing an additional risk in crops consumed in a raw or minimally prepared state (Solomon *et al*., 2002). *E. coli* O157:H7 has been found to be the most acid resistant vegetative pathogen of concern in acidified vegetables (Breidt *et al*., 2007). A survey by Breidt *et al*. (2007) revealed that, depending on the temperature, O157:H7 strains needed 2 to 6 days to achieve a 5-log reduction in the number of bacteria at pH 3.3 in acetic acid solutions (at 25^{o} C and 10^{o}C respectively). Survival of *E. coli* O157:H7 in the environment may play an important role in the persistence and dissemination of the pathogen on farms. It was observed that *E. coli* O157:H7 cell numbers in no till soils (Gagliardi and Karns, 2000) and in soil cores containing rooted grass, declined from 10^{8} to 10^{6} Cfu per gram of soil after 130 days (Maule, 2000). Studies have revealed that *E. coli* O157:H7 inoculated into cattle feces remained detectable at high levels for >50days, where as in cattle slurry the pathogen declined by $>10^{6}$ CFU/Ml in 10days (Maule, 2000); its persistent represents contamination and continuous re-contamination of produce and produce environments. In addition to horizontal transfer of genetic elements to produce, mutation rates of *E. coli* O157 in soil could be increased by the induction of error prone DNA polymerase under starvation conditions, increasing the genotypic and phenotypic diversity for

natural selection to act on, hence, the possibility of new virulent and resistant strains emerging (Ishii and Sadowsky, 2008).

2.5.4 Disease caused by *E.coli* O157:H7

The infectious dose of *E.coli* O157:H7 necessary to cause illness is between 10 and 100 cells (Feng and Weagent, 2011). *E.coli* O157:H7 can produce an asymptomatic infection, but may manifest into more severe symptoms such as a mild diarrhoea or acute bloody diarrhoea termed "hemorrhagic colitis" (Flatamico and Smith, 2006). The organism produces hemorrhagic colitis along with other characteristics, including the presence of necrotic cells in the stools (Martinez, 2012). The production of hemorrhagic uraemic syndrome (HUS) is not well understood but it is known that shiga-toxins are produced in intestine and ultimately transferred into the bloodstream, where it binds the polymorphonuclear leukocytes which can be transported to vital organs, especially the kidney (Karmali, 2004). The toxin can be translocated to the lungs, or brain in extreme cases (Karmali, 2004). Disease caused by this pathogen can be very serious for patients as around 70% need red blood transfusions, 50% need dialysis and around 25% have neurologic involvement, such as stroke, seizure and coma (Noris and Remuzzi, 2005). Finally, in worse cases, patients die as a consequence of the production of the toxins (Martinez, 2012). EHEC strains represent around 70% of the total development of diarrhoeic HUS; and 80% of these HUS cases are produced from *E.coli* O157:H7 in recent years (Boyer and Niaudet, 2011). Some investigators considered that *E.coli* O157:H7 has the strongest association worldwide with HUS (Tarr *et al*., 2005). It is considered one of the primary causes of renal failure in the Western world (Delaquis *et al*., 2007).

2.5.5 Antimicrobial resistance in *Escherichia coli* O157:H7

Antimicrobial resistance is a natural phenomenon (Maal- Bared *et al*., 2013) which bacteria use to protect themselves against competitors. Antimicrobial resistance (AMR) is a global concern for animal and human health (Pruden *et al*., 2006; Skurnik, 2008). In the bacterium of humans and domestic animals, AMR develops primarily as a consequence of exposure to antimicrobials (Sayah *et al*., 2005; Skurnik *et al*., 2006). Antibiotics are extensively used in agriculture to prevent and treat diseases, as well as to promote growth (Martinez, 2009). Consequently, animal waste produced in agricultural settings can contain resistant bacteria, resistance determinants present in gene- transfer units or combinations (Taylor *et al*., 2011). Mobile genetic elements such as plasmids, transposons and the more recently explored integrons, which are able to disseminate antibiotic resistance genes by horizontal or vertical transfer, have been shown to play an important role in the evolution and dissemination of multidrug resistance genes in Gram-negative bacteria (Caratolli, 2001). Integrons are genetic elements that are able to capture gene cassettes from the environment and incorporate them as part of the Tn2 or Tn4 02 transposon family. Colonization or infection by a resistant bacterial strain can occur as a result of consumption of antimicrobials or antimicrobial residues or by direct transfer of resistant bacteria or resistance genes or from consumption of contaminated food and water (Sayah *et al*., 2005; Blanco *et al*., 2009). Recreational water and wastewater have been identified as a potential source of resistant bacteria in the environment (Edge and Hill, 2005; Hamelin, 2006). The transmission of antibiotic resistance through agriculture may have a greater impact on human health than hospital transmission of resistance (Smith *et al*., 2005), as gene transfer and the environmental spread of antibiotic resistance genes might substantially contribute to antibiotic resistance genes in the human microbiome, which exacerbates

the threat to infections with antibiotic-resistant bacteria (Croft *et al*., 2007). Antibiotic resistant genes transferable plasmids are introduced via manure into agro ecosystems and can persist regardless of the viability of the introduced host cell due to horizontal gene transfer (Chee- Sanford, 2009). However, the effects of a single manure application on bacterial resistance levels decreases over time due to diminished selective pressure and eventually the resistance level of untreated soil is restored. Farmers typically apply manure to their fields several times a year (Schluter, 2003) which might lead to an accumulation of resistance genes and antibiotic compounds in soil. There is evidence that antimicrobial use in animals selects for resistance in both pathogenic and commensal organisms (Mathew *et al*., 2001; Docic and Bilkei, 2003) and a commensal organism of interest *Escherichia coli*, may serve as a reservoir of transferable antimicrobial resistance genetic elements (Rajic *et al*., 2005). Beneficial traits for the host, often conferring important competitive advantages under selective pressures, are part of the gene cassettes inserted in integrons and transposons (Varela *et al*., 2013). Such cassettes include genes encoding the adaptive traits, e.g resistance to antibiotics and disinfectants (e.g sulphonomides, streptomycin and quaterinary ammonium compounds) and heavy metals (e.g mercury, chromate). Antimicrobial resistance in *enterobacteriaceae* poses a critical public health threat, especially in the developing countries (Karlowsky *et al*., 2003). Much of the problem has been shown 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).

Antimicrobial resistance (AMR) can potentially contribute to enhanced survival of STEC O157 in cattle, particularly under intensive rearing conditions where antimicrobials are frequently used therapeutically and for disease prevention and

growth promotion (Call *et al*., 2008). Laboratotry- based studies have shown that *E. coli* is capable of transferring resistance to other bacterial species such as *Salmonella* spp., which are dissemninated through the human food chain (Blake *et al*., 2003). Furthermore, the emergence and spread of antimicrobial resistant *E. coli* and other pathogenic bacteria have become serious global public health threats (Ma *et al*., 2007). Water contaminated with effluents from livestock farms, aquaculture, hospitals, municipal wastewater treatment, or more are also source of resistant organisms (Hengge, 2008, Loui *et al*., 2008; Dong and Schellhorn, 2009). Antibiotic resistance and virulence genes identical to those reported in human pathogens are detected worldwide in raw and in treated wastewater (Varela *et al*., 2013). Researchers have speculated that the use of antimicrobials to promote animal performance and well-being has played a role in accelerating the development of antimicrobial- resistant pathogens (Kohler *et al*., 2000; Zhao *et al*., 2001). Several classes of antibiotics, including glycolipids (bambermycin), polypeptides (bacitracin) ionophores (salinomycin) and β-lactams (penicillin) are used in broiler chicken production for growth promotion and prevention of infectious diseases (Butaye *et al*., 2003, Singer and Hofacre, 2006). These antibiotics improve feed conversion and body weight gain presumably by altering the composition and activities of microflora at the same time increasing the dissemination of resistant bacteria in the environment.

Antimicrobial resistance (AMR) in STEC O157 may be relevant clinically, although antimicrobial therapy in STEC- infected patients remains controversial (Ikeda, 2000). AMR in STEC O157 isolates is increasing, with the most common being single-drug or most often multidrug resistance to streptomycin, sulfonamide and tetracycline (AMR-SsuT) (Mora, 2005, Ziebell, 2008; Aslam *et al*., 2010). Antimicrobial drug resistance among extra intestinal *E. coli* is further adding to the cost of treating

infections (Aslam *et al.*, 2009). The use of antimicrobials to treat *E. coli* O157:H7 is a contentious area in the management of infection and is currently contradicted as it may exacerbate the disease (Todd and Dundas, 2001)**.**

2.5.6 Detection of *E. coli* O157:H7

STEC O157 is detected in water samples using membrane filtration followed by selective enrichment in liquid broth and subsequent immune-magnetic separation (IMS), followed by growth on selective culture plates (Standing Committee of Analysts Environment Agency, 2002). Subsequently, presumptive STEC O157 colonies are subcultured and finally characterized using biochemical and immunological tests. Culture based methods may not detect very low numbers of bacteria (Ibekwe *et al*., 2007) although, most models and transport studies demonstrating the risk of *E. coli* O157:H7 in the environment depend on culture techniques (Gagliardi and Karns, 2000). In most studies where culture- based methods are used, enrichment is usually necessary (Gagliardi and Karns, 2002; Johanneson *et al*., 2005). The detection of very low levels of bacterial contamination in soils and faeces requires that the samples be cultured for a few hours in an appropriate enrichment broth (Ibekwe *et al*., 2002). Enrichment dilutes out inhibitory substances and provides conditions conducive to the growth and multiplication of bacterial pathogens to a detectable number (Ibekwe *et al*., 2002). These methods are laborious, time-consuming and not always reliable (Karch and Bielaszewska, 2001). As a result of these limitations, routine applications to demonstrate the presence of STEC *E. coli* O157 in environmental water samples are difficult and ask for the development of simple and more specific methods. Therefore, Polymerase chain reaction (PCR) - based methods have been developed to detect STEC *E. coli* O157 in clinical, food and environmental samples (Fortin *et al*., 2001; Ibekwe *et*

al., 2002). Naturally occurring *E.coli* O157:H7 was quantified in soil, cattle feces, manure, wastewater, rhizosphere and phyllosphere using multiplex real-time PCR (Ibekwe *et al*., 2002, Ibekwe and Grieve, 2003; Ibekwe *et al*., 2004). Although quantification of bacteria by this method is limited due to PCR bias in template- to-product ratios (Huang *et al*., 2004), the detection of a low number of *E. coli* O157:H7 is used for surveillance of pathogen contamination in water or food.

PCR has become an important method for the rapid sensitive and specific detection of bacterial pathogens. Characterisation of EHEC isolates, both O157 and non-O157, by use of PCR provides detailed information about genetic variability (Welinder-Olsson 2002 and Kaijser, 2005). The evaluation of the PCR assays should be based on their rapidity, specificity (ability to target only the desired species), ubiquity (ability to detect all strains of the targeted species; Boissinot and Bergeron, 2002) and analytical detection limit (Maheux *et al*., 2009). PCR is increasingly accepted as the most sensitive means of determining whether a faecal specimen or a food sample contains EHEC (Welinder-Olsson, 2002; Kaijser, 2005). Specific oligonucleotides (primers) are used to amplify short selected sections of DNA specific for the VT genes (Welinder-Olsson, 2002; Kaijser, 2005). However, different laboratories use a variety of systems (Welinder-Olsson *et al*., 2002; Pulz *et al*., 2003).

2.6 *Salmonella*

The genus *Salmonella* is a gram- negative rod shaped bacteria in the family of *Enterobacteriacea* (Salehi *et al*., 2005). The *Salmonella* genus consists of two separate species: *Salmonella bongori* and *Salmonella enterica* and encompasses over 2,500 known serotypes, all of which are considered potential human pathogens (Baggesen *et al*., 2000), however, the majority of human and warm- blooded animal *Salmonella*

infections are caused by relatively few serotypes of *Salmonella enterica* subsp *enterica* (CDC, 2005). The nine most common human *S.enterica* serovars; *Salmonella* serovars Typhimurium, Enteritidis, Newport, Heidelberg, Javiana, Montevideo, Muechen and Saintpaul were responsible for more than 60% of human salmonellosis cases based on the Centers for Disease Control and Prevention`s (CDC) annual summary of 2005 (CDC, 2006; CDC, 2008) and continue to be a major cause of food–borne illness (CDC, 2008, Hanning, 2009; CDC, 2010). Infections due to *Salmonella* serotypes continue to be a major health problem (Lampel *et al*., 2000, Ferretti *et al*., 2001; Malorny *et al.*, 2003). Epidemiology and pathogenic process in salmonellosis are dictated by an array of factors that act in tandem and ultimately manifest in the typical symptoms of salmonellosis (Murugkar *et al*., 2003). *Salmonella enterica* serovar Typhimurium is a common cause of salmonellosis in humans and animals (Ikuo *et al*., 2011). It is the second most reported serovar of *Salmonella* causing human disease and the most prevalent *Salmonella* serovar (CDC, 2007). *S. enterica* epidemics often involve rapid dissemination of the predominant epidemic strains over a large geographic area (Ikuo *et al*., 2011).

Salmonella infections still occur at high frequencies in industrialized nations and developing countries as well (Salehi *et al*., 2005). In the United States, *Salmonella* is the leading cause of bacterial food- borne disease, with approximately 1.4 million human cases each year since 1996 (Voetsch, 2004). *Salmonella enterica* causes severe enteritis in both humans and animals (Sauli, 2005; Murchie *et al*., 2008). It is a major cause of food-borne disease worldwide (Voetsch *et al*., 2004, dejong and Ekdahl, 2006; Galanis *et al.*, 2006). A peculiar epidemiological feature of human salmonellosis is that epidermics are commonly associated with a particular prevalent serovar of *S. enterica* that shows temporal and geographical variation (Bentancor *et al*., 2010). Until the

1980s, *S.enterica* serovar Typhimurium (*S.*Typhimurium) was the serovar most commonly isolated from humans worldwide, but by the late 1980s, *S.enterica* serovar *enteritidis* emerged as the most common cause of salmonellosis in Europe and during the 1990s, it became the most prevalent serovar in many countries worldwide (Heristad *et al*., 2002, Cogan *et al*., 2003; Schroeder *et al*., 2005). *Salmonella* Typhimurium has been a major cause of salmonellosis in humans in Japan until 1989 when *Salmonella enteritidis* suddenly emerged and continued to date (Hamada *et al*., 2003; Asai *et al*., 2007). *Salmonella* enterica serotype Typhi (*S.*Tyhpi) and several non-typhi serotypes of *S.enterica* (non typhoid *Salmonella*) are important causes of childhood bacteremia in African children (Graham, 2002). Although the data is sparse, there seems to be a relatively low burden of pediatric typhoid fever across sub-Saharan Africa (Mweu and Englis, 2008), where non typhoid *Salmonella* has consistently been reported as a leading cause of bacteremia in African children (Morpeth *et al*., 2009). In contrast to industralised countries where non typhoid *Salmonella* usually causes a self- limited gastroenteritis, invasive disease frequently occurs in sub-Saharan Africa with high case fatality rates among hospitalized children (Graham, 2002; Morpeth *et al*., 2009). However, regional data on the demography and prevalence of both (invasive non typhoid *Salmonella*) iNTS and *S.*Typhi for sub-saharan Africa are incomplete (Hotez and Kamath, 2009).

2.6.1 Outbreaks of *Salmonella*

Although, the majority of salmonellosis outbreaks have been linked to the consumption of contaminated foods (Mohle- Boetani *et al*., 2002), *Salmonella* is considered a leading cause of water borne disease outbreaks and is commonly isolated from environmental sources including surface waters (Martinez-Urtaza *et al*., 2004).

Several large regional outbreaks of Salmonellosis have been linked to contaminated water supplies and exposure sources include recreational use, home water use and consumption of produce irrigated with contaminated water (Placha *et al*., 2001). The true incidence of *Salmonella*- associated diseases is difficult to evaluate because of lack of epidemiological surveillance systems especially in developing countries (Akinyemi *et al*., 2012). Typhoid fever is endemic throughout Africa and Asia and persists in the Middle East, a few Southern and Eastern European countries and Central and South America (CDC, 2005). In the Mediterranean African countries, typhoid is considered endemic with medium incidence of 10 to 100 cases per 100, 000 persons (Ghenghensh *et al*., 2009) and in Libya the incidence for the year 2006 was 16 per 100,000 persons per year. While, Egypt remains a country with intermediate incidence of one to 180 per 100,000 cases of enteric fever below nations such as India and Indonesia, which claimed more than 100 cases per 100,000 persons (Afifi *et al*., 2005). In Tunisia, the 2006 Bulletin of the Ministry of Health showed an incidence of about one case per 100,000 per year (Akinyemi *et al*., 2012). In Nigeria, typhoid fever is among the major widespread diseases affecting both young children and adults (Akinyemi *et al*., 2005). In Lagos, Nigeria, out of 85, 187 confirmed cases of *Salmonella* associated diseases, 880 deaths were recorded between 1999 and 2008, giving a case-fatality rate of 1.03% (Akinyemi *et al*., 2012).

2.6.2 Transmission of *Salmonella*

The major food vehicle for *S.enteritidis* is shell eggs, as 80% of the *S.enteritidis* outbreaks and approximately 50,000 to 110,000 cases are egg associated in the United States each year (Patrick, 2004; Braden, 2006). Another common food vehicle for *S.enteritidis* is chicken meat (Kimura *et al*., 2004, Patrick, 2004, Marcus,

2007; Parker *et al.*, 2010). *S.enteritidis* has been isolated from a wide variety of animals, such as rodents, wild birds and insects, which could serve as potential sources (Gast and Beard, 1990). Additional potential sources for *S.enteritidis* include chicken manure, sewage and other moist and organic materials in farm environments (Braden, 2006). *Salmonella* is an environmentally persistent pathogen capable of surviving and proliferating in diverse niches, including many environmental ecosystems, such as food production and processing plants and the intestinal tracts of host animals (Winfield and Groisman, 2003). Multiple vertebrate sources exist for this bacterium, including livestock, wildlife, poultry and companion animals, with water functioning as a documented mode of dissemination in the environment (Mandrell, 2009). Additionally, most *Salmonella* serotypes must be introduced into human populations from exposure to contaminated foods, soil, water or animal contact (Humphrey, 2006; Mermin *et al*., 2006). *Salmonella* is spread by the fecal-oral route of contamination (Levantesi *et al*., 2012). This organism can enter the aquatic environment directly with feces of infected humans or animals or indirectly, via sewage discharge or agricultural land run off (Levantesi *et al*., 2012). *Salmonella* contaminated waters used to irrigate and wash produce crops have been implicated in a large number of food-borne outbreaks demonstrating indirect routes of transmissions (Levantesi *et al*.**,** 2011)**.**

The ubiquitous nature of *Salmonella* and its widespread occurrence in both fresh waters and marine waters suggest that transmission in the aquatic environment from water consumption, contact or the consumption of food treated with or harvested in contaminated water is highly probable (Lechmarchand and Lebaron, 2003, Martinez-Urtaza *et al.,* 2004; Brands *et al*., 2005). Potential routes for pre harvest contamination of fresh produce with human enteric pathogens include exposure to contaminated surface or irrigation water, faecal contamination from livestock or wild animals, raw or

poorly composted manure, farm equipment, human carriers and possibly dust (Gorski *et al*., 2011). Due to the ability of the pathogen to survive for prolonged periods on stainless steel, glass and plastic, the risk of cross contamination through such surfaces has also been recognized (Kusumaningrum *et al*., 2002, de Cesare *et al*., 2003; Humphrey, 2004).

Surveys to measure the incidence and/or amount of *Salmonella* contamination by some of these potential reservoirs have been reported previously (Harley *et al*., 2009; Walderhaug, 2009). In Nigeria, Oguntoke *et al*. (2009) investigated relationships between water- borne diseases, morbidty patterns, drinking water source quality and water handling practices, reporting 39.3% typhoid fever related infections. Abakpa *et al*. (2011) in a study of *Salmonella* in households engaged in livestock farming in some parts of Zaria, Kaduna- Nigeria reported a 2.08% prevalence. The presence of clinically relevant *Salmonella* serotypes in natural waters has been well documented (Catalao *et al*., 2000; Martinez-Urtaza *et al*., 2004) and isolation patterns of serotypes in water follow patterns of infection among humans and local fauna, suggesting a local terrestrial origin (Baudart *et al*., 2000; Olsen *et al*., 2001). Sources may vary between wildlife (Reifsum *et al*., 2002; Chambers and Hulse, 2006) and domesticated animals (Ray *et al.*, 2006) depending on the local land use and waste management facilities. Fresh produce is a recognized vector for *Salmonella* infection and several high- profile outbreaks and recalls have occurred in relevant years (Humphrey, 2004, CDC, 2008; Hanning *et al*., 2009). Consumption of contaminated produce, not animal products, has been the most reported cause of salmonellosis in recent years (CDC, 2009). Other than disease outbreaks caused by sprouted seeds, salmonellosis outbreaks associated with fresh produce have been attributed most frequently to consumption of tomatoes (CDC, 2005; CDC, 2007). Outbreaks resulting

from water and produce contaminated with *Salmonella* contribute to illness (Mohle-Boetani *et al*., 2002) and are consequently now being increasingly investigated as a potentially significant reservoir of *Salmonella* transmission (Schutze *et al*., 1999). However, it has been suggested that the survival capacity of environmental strains may depend on species and pollution sources (Baudart *et al.*, 2000).

Salmonella strains that can persist in the environment for years, withstanding periods of stress and nutrient depletion have been reported (Parker *et al*., 2010). *S.enterica* can survive in soil for extended periods (Jensen *et al*., 2006). It has been shown that *Salmonella enterica* serovar Typhimurium could be isolated from different types of vegetables after sowing seeds in soil amended with artificially inoculated manure or composts (Natvig *et al.*, 2002; Islam *et al.*, 2004). Islam *et al*. (2004) demonstrated in a series of experiments both in a growth chamber and in the field that after compost, *S.enterica* serovar Typhimurium persisted for at least two months on the surface of the vegetables. Low- water- activity medium (Kieboom, 2006) and dry environment (Hiramatsu *et al.*, 2005) were found to enhance *Salmonella* tolerance to sodium hypochlorite and ethanol, respectively. *S.enterica* initiates leaf colonization on grandular trichomes of tomato plants. Grandular trichomes have developed on plants as a physical barrier to small herbivorous insects and chemical deterrents. Exudates from grandular trichomes can entrap or poison insects and act as a source of nutrients for bacteria (Wagner *et al*., 2004), infact plant- associated bacteria often congregate at the base of trichomes to take advantage of an oasis that offers nutrients and moisture (Monier and Lindow, 2004). Hence, *Salmonella*'s ability to survive long on plants. Exposure to a single stressor is commonly associated with the development of cross-tolerance to other stressors (Humphrey, 2004, Boor, 2006; Kultz *et al*., 2006) as bacteria evoke a common stress response when encountering diverse

environmental stresses as exposure to a single stress result in cross- tolerance to heterogeneous stresses (Boor, 2006). Cross-tolerance has been reported in *Salmonella*; carbon starvation induces a protective effect against low pH, hyperosmolarity, heat, polymyxin B and peroxides, while exposure to mild acidic conditions induces tolerance to low pH, heat, NaCl (2.5M), crystal violet and polymyxin B. Exposure to acetic acid was shown to induce protection against high concentrations of NaCl and KCl (Greenarce and Brocklehurst, 2006) as well as against low temperature (4^{o}C) (Xu *et al*., 2008).

The occurrence and persistence of *Salmonella* in ambient waters may be governed in part by extrinsic parameters such as temperature and water chemistry (Mouslim *et al*., 2002) or by seasonal changes in loading from human (Olsen *et al*., 2001) and animal hosts (Smith *et al*., 2002; Srikantiah *et al*., 2004), which may vary spatially and temporarily with disease. The incidence of human salmonellosis peaks in summer months throughout the world (D`Souza *et al.*, 2004, Kovats *et al*., 2005, Fleury *et al*., 2006; Naumova *et al*., 2006). Weather or climate variability may be a factor in this seasonality. In particular, precipitation or storm events may mobilize pathogens from agricultural and urban environments as well as onsite sewage disposal systems and subsequently transport them into the aquatic environment (Lipp *et al*., 2001; Mermin *et al*., 2004). In a study of diversity and seasonality of water borne *Salmonellae* in a rural watershed in the United States, Lipp *et al*. (2009) reported that *Salmonella* abundance and diversity in the environment vary temporally and are strongly influenced by seasonal precipitation and water temperature, they reported a peak in diversity in the summer. Cases of salmonellosis follow typical seasonal patterns with the highest rates noted in summer months (Naumova *et al*., 2006). A study by Pangloli *et al*. (2008) showed a higher prevalence of *Salmonella* in summer months. Similarly,

another report by Mohanty *et al*. (2006) revealed that *Salmonella* occurred more in the dry season than the rainy. Thus, seasonal changes in weather (i.e. temperature and precipitation) along with seasonal changes in carriage rates of *Salmonella* in local human and fauna populations may influence waterborne *Salmonella* densities and ultimately exposures to *Salmonella* from the environment. In an unrelated study, salmonellosis was reported to have occurred more in the winter than summer (Olowe *et al*., 2003). On the other hand, Shahada *et al*. (2008) reported that *Salmonella* prevalence trend did not vary with season when the 12 month time periods were compared in a study from 1998 to 2003 in Southern Japan.

2.6.3 Public health significance of *Salmonella* in water, soil and vegetables

Salmonella spp present on the surface of plants cannot only survive for extended periods (Brandl and Mandrell, 2002) but might overcome the innate immune response of plants (Schikora *et al*., 2008) and actively enter the plant via the stomata (Kroupitski, 2009). Hence, microbes present on fresh produce contaminated by irrigation using microbially polluted water is likely to escape post harvest treatment procedures such as washing and disinfection of vegetables (Gemmell and Schmidt, 2010). Salads are usually consumed without any pre-heat treatment and therefore the possibility of food poisoning exists (Buck and Walcott, 2003). A large part of the outbreaks caused by pathogenic bacteria is related to the consumption of raw produce contaminated with human pathogens such as *Salmonella* spp (Semenov *et al*., 2008). Example, *Salmonella enterica* colonization of tomato plants has caused several multistate and international outbreaks, each involving hundreds of cases (Bidol, 2007). More importantly, studies have found that in certain *Enterobacteriacea*, including *Salmonella*, virulence genes may be colocalizing on transferable resistance plasmids

(Fricke, 2009; Venturini *et al*., 2010), a phenomenon that would lend credence to studies that have shown that antimicrobial- resistant *Salmonella* strains may be more invasive than *Salmonella* strains that are susceptible to antimicrobials (Helmis *et al*., 2004; Varma, 2005; Varma, 2006). The issue of antimicrobial resistance in *Salmonella* poses a significant threat to public health (Wu *et al*., 2013) as several reports have shown that clonal groups of *Salmonella* could be transmitted along the food chain and represents a hazard to human health. The possible role of fecally contaminated waters in the dissemination of multidrug resistant (MDR) *Salmonella*, both typhoid and non-typhoidal, as well as antibiotic resistance genes through horizontal gene transfer is of great intrest (Levantesi *et al*., 2012). Hence, the burden of typhoid fever worldwide is further compounded by the spread of multiple drug resistant *S.*Typhi (Kim, 2010).

Salmonella enterica infections are a significant public health concern worldwide, with an estimated 1.028 million cases, 19,000 hospitalizations and approximately 400 deaths in the United States each year (Scallan, 2011) while the CDC estimates that approximately 40,000 cases of salmonellosis are reported in the United States and 600 persons die each year with acute salmonellosis, though the actual number of infections may be thirty or more times greater (CDC, 2006). In Nigeria, such cases are either not documented or many milder cases are not diagonosed or reported (Olowe *et al.*, 2007). However, cases of systemic disease due to *Salmonella typhimurium* and *Salmonellae* have been reported (Varma *et al*., 2005) else where. Typhoid fever caused by *Salmonella enterica* serotype Typhi (here after referred to as *Salmonella* Typhi) remains a major health problem in the developing world (Crump and Mintz, 2010). It is well known that *Salmonella* infections can lead to the development of a carrier state (Stevens *et al*., 2009) and carriers remain a route of transmission to other uninfected persons.

2.6.4 Pathogenicity of *Salmonella*

The pathogenic ability of *Salmonella* depends on many virulence factors which are either chromosomal or plasmidic (Turkylmaz *et al*., 2009). Virulence genes have previously been used to identify the epidemiology of *Salmonella* among cow and human isolates (Alcaine, 2006). After ingestion, in order to reach their sites of colonization, *Salmonella* must be able to survive the antimicrobial properties of the stomach, including the low pH and the presence of many organic acids (Foley and Lynne, 2007). This is achieved through its virulence genes. *Salmonella* colonises the mucosal cells and peyer`s patches in the host (Bolton *et al.*, 2013). Infection involves adhesion, invasion and replication (Bolton *et al*., 2013). Some factors can be found on plasmids, which are extrachromosomal, circular DNA molecules that typically contain genes that impact selective advantage to the host, such as virulence or antimicrobial resistance (Sheppard *et al*., 2003). The plasmids that are known to carry virulence gene clusters are called virulence plasmids. Some of the most important serovars for human health, including Typhimurium, Enteritidis and Choleraesuis are known to harbor virulence plasmids (Chiu *et al*., 2005, Villa and Carattoli, 2005; Yu *et al*., 2006). Virulence genes, encode products that assist the organism in expressing its virulence in the host cells (Murugkar *et al.*, 2003). Recently, serovar-specific virulence plasmids of various sizes have been found in *Salmonella;* some of these virulence- resistance plasmids, each encoding different resistance genes, have been detected in *Salmonella* isolates collected in various countries (Chu and Chiu, 2006). The acquisition of genomic islands has played a central role in bacterial evolution as a mechanism of diversification and adaptation (Boyd *et al*., 2009). *Salmonella* genomic island 1(SGI1) is a 43-kb mobilizable chromosomal region that is located in *Salmonella enterica* serovars between the genes *thdF* and *yidY* or between thdF and int2 in the retron

sequence of *S.enterica* serovar Typhimurium (Boyd *et al.*, 2000). SGI1 contains an antimicrobial resistance gene cluster (Boyd, 2000; Doublet *et al*., 2005) located on a 13-kb complex class 1 integron designated In104 (Levings *et al*., 2005), which confers pentaresistance (Penta-R) to ampicilin (bla_{PSE-1}), chloramphenicol/florfenicol (floR), streptomycin/spectomycin (aadA2), sulfamethoxazole (sul1) and tetracycline [tet (G)] (Boyd, 2001). The five resistance determinants are characteristic of the pandemic *S.*Typhimurium definite phage type 104(DT104), which was globally most predorminant in causing infections in humans since the 1980s (Threfall, 2002; Doublet *et al*., 2005).

The acquisition of genomic islands has played a central role in bacterial evolution as a mechanism of diversification and adaptation (Boyd *et al*., 2009). Studies have shown that type 111 secretion systems, which are encoded within Pathogenicity Island 1 and 2 (SP1-1 and SP1-2 respectively) are important for the virulence of this organism (Masayuki *et al*., 2012). These secretion systems are involved in invasion of intestinal epithelial cells and *Salmonella* survival in macrophages (Grassl and Finlay, 2008). Among these virulence factors, the chromosomally located gene *inv*A encodes an inner membrane protein and provides the bacteria with the capability to invade epithelial cells (Darwin and Miller, 1999) and this gene has been the target sequence for a number of rapid detection methods for *Salmonella* in foods (Ammari *et al*., 2009). During salmonellosis, the invasion of *Salmonella* into the small intestine is attributed to a number of invasion genes, one of which is *inv*A (Ammari *et al*., 2009). A sequence of the *inv*A gene has been utilized as a target for the detection of *Salmonella* nuclieic acids in soil samples (Chiu and Ou, 1996). This gene is highly conserved in almost all *Salmonella* serotypes (Rahn *et al*., 1992, Galan *et al*., 1992; Boyd *et al*., 1996) and detection of *Salmonella* based on the presence of this gene has been previously reported

(Hoorfar *et al*., 2000; Cortez *et al*., 2006). Rhan *et al*. (1992) reported that the *inv*A primer set was able to discriminate between *Salmonella* and non- *Salmonella* species (Moganedi *et al*., 2007).

Salmonella enterotoxin (*stn*) is a putative virulence factor and causative agent of diarrhoea (Chopra *et al*., 1994; Chopra *et al*., 1999). Infact, it is responsible for the enterotoxicity of *Salmonella* (Masayuki *et al*., 2012) and is a key factor in acute gastroenteritis and diarrhoea (Chopra *et al*., 1999). Intrestingly, it has been shown that the *stn* gene is specifically distributed in *Salmonella* spp (Masayuki *et al*., 2012), irrespective of their serotypes (Lee *et al*., 2009). Hence the *stn* gene is useful for the identification or detection of *Salmonella*. Chopra *et al.* (1999) cloned the *stn* gene and showed it had an enterotoxic activity.

2.6.5 Disease caused by *Salmonella*

This bacterium causes severe clinical manifestations including acute gastroenteritis and typhoid fever (Boyle *et al.*, 2007). Salmonellosis, caused by *Salmonella enterica* is a food borne disease of humans and livestock (Wu *et al*., 2013). **S**almonellosis is complex largely because there are > 2,500 distinct serotypes of *Salmonella* (serovars) with different reservoirs and diverse geographic incidences (Olsen *et al*., 2001). The primary route of *Salmonella* infection in humans and other animal species is the fecal-oral- transmission of the organism (Foley and Lyne, 2007). Salmonellosis is an acute form of enterocolitis characterized by headache, abdominal pain, diarrhoea, occasional vomiting and fever (Bolton *et al*., 2013). The disease has poor prognosis because of co-infection and co-endemicity with malaria (Akinyemi *et al*., 2007). Enteric fevers, typhoid and paratyphoid fever are severe, contagious systemic diseases caused by the infection of the serovars typhi and paratyphi (Levantesi

et al., 2012). Both infections are host adapted and can only infect humans; stools of infected persons are therefore the original source of contamination for these pathogens (Levantesi *et al*., 2012). Water contaminated with feces of human with typhoid disease and carriers is one of the main vehicles of the transmission of the agent of typhoid fever (Levantesi *et al*., 2012). Typhoid fever complications such as septicemia, typhoid psychosis, intestinal perforations, hepatosplenomegally and haemorrhage have been documented (Akinyemi *et al*., 2005). Non typhoid salmonellosis is a diarrhoeal disease also caused by *Salmonella enterica* (Barak *et al*., 2011).

2.6.6 Antibiotic Resistance in *Salmonella*

Multi-drug resistant (MDR) strains of *Salmonella* are now encountered frequently and the rates of multidrug- resistance have increased considerably (CDC, 2006). Even worse, some variants of *Salmonella* have developed multidrug-resistance as an integral part of the genetic material of the organism and are therefore likely to retain their drug resistant genes even when antimicrobial drugs are no longer used, a situation where other resistant strains would typically lose their resistance (CDC, 2006). Class 1 integrons are considered to be the single biggest contributor to MDR in *Salmonella* (where they are integrated into the chromosomal DNA and thus persist even in the absence of selective pressure (Chen *et al*., 2004). The increased level of antimicrobial resistance observed in *Salmonella* strain has become a public health issue (Gorski *et al*., 2011). Extensive research aimed at characterizing antimicrobial resistance phenotypes of *Salmonella enterica* from a variety of food and animal sources has revealed that numerous serotypes may harbor multiple antimicrobial resistance determinants (White, 2001; Zhao, 2007). The development of resistance in *Salmonella* toward antimicrobial agents is attributable to one of multiple mechanisms, including

production of enzymes that inactivate antimicrobial agents through degradation or structural modification, reduction of bacterial cell permeability to antibiotics, activation of antimicrobial efflux pumps and modification of the cellular target for drug (Sefton, 2002). Much of the observed resistance to cephalosporins and penicillins by *Salmonella* is attributable to the acquired ability of the strains to produce β-lactamase enzymes that are able to degrade the chemical structure of the antimicrobial agents (Foley and Lynne, 2007). The production of β-lactamases by *Salmonella* has become an important and common mechanism for β-lactam resistance (Bush, 2003). Among the more worrisome β-lactamases is the AmpC enzyme, which is encoded by bla_{cmy} and has been associated with resistance to a large number of β-lactam antibiotics including ampicillin, ceftiofur and ceftraxone (Aarestrup *et al*., 2004). In other cases, enzymes inactivate antimicrobial agents by modifying their structure. Much of the aminoglycosides resistance in *Salmonella* is associated with modifying enzymes that include the aminoglycoside phosphotransferases, aminoglycoside acetyltransferases and aminoglycoside adenyltransferases, which function by phosphorylation, acetylating and adenylating certain aminoglycosides respectively (Poole, 2005). Numerous outbreaks have been caused by the multidrug resistant *Salmonella* enterica serovar Typhimurium definitive phage type DT104 with resistance to ampicillin, chloramphenicol, streptomycin/spectinomycin, sulphonamides and tetracycline but increasingly other multidrug resistant *Salmonella* serovars have been responsible for infections in humans (Threfall, 2002, Zansky *et al*., 2002; Brown *et al*., 2003). Multidrug resistance in the majority of DT104 strains is due to the presence of five genes aadA2, sul1, floR, tetA(G) and blaP1(variant form bla_{PSE-1} or bla_{CARB-2}) that confer resistance to streptomycin and spectinomycin, sulfonamides, chloramphenicol and forfenicol, tetracyclines and β-lactam antibiotics, respectively (Boyd *et al*., 2001). These isolates

represent a single clone that has emerged as a dominant serovar Typhimurium clone globally and has been isolated from humans, most meat producing animals, exotic birds and various foods, including meat, dairy products and salad ingredients (Threlfall *et al*., 2000; Threlfall, 2002). Severe *Salmonella enterica* infections are commonly treated with fluoroquinolones (e.g ciprofloxacin) (Guerrant *et al*., 2001). Antimicrobial resistance in *Salmonella* is an emerging public health problem, however, β-lactams and fluoroquinolones are two important classes of antibiotics used to treat complicated cases of Salmonellosis (Gonzalez-Sanz *et al*., 2009). Various β-lactamases have been detected worldwide in various serotypes located in plasmids or integrons, facilitating rapid transmission of resistance among serotypes (Yang *et al*., 2010). The spread of bla_{cmy-2} – harboring resistant *Salmonella* through food/produce has important public health implications (Wu *et al*., 2013).

2.6.7 Detection of *Salmonella*

Established conventional methods to detect and identify *Salmonella* are time consuming and include selective enrichment and plating, followed by biochemical tests (Bennasar *et al*., 2000). Although, phenotypic methods play an important role in the identification to the genus level (Turki *et al*., 2012) they are not accurate. Molecular testing has been most successful in areas for which conventional microbiological techniques do not exist, are too slow or are too expensive (Jungkind, 2001). Serotyping is the most commonly used molecular subtyping method for *Salmonella* (Liu *et al*., 2011). Serotyping distinguishes *Salmonella* based on immunological classification of the H and O antigens (Grimont and Weill, 2007) and is routinely used for surveillance of this organism. Historically, *Salmonella* were divided into separate species based on the results of serotyping; for each serotype (serovar), there was a separate species

(Brenner *et al*., 2000). Serotyping uses differences in the polysaccharide portion of lippolysacharide portion of the flagella (H antigen) present on the surface of *Salmonella* to separate strains into distinct serotypes (Voogt *et al*., 2002; CDC, 2006). Serology based on surface antigens is the standard method of identification and classification of *Salmonella* and is a starting point of any meaningful typing investigation (Turki *et al*., 2012). Currently, there are over 2,500 identified *Salmonella* serotypes. Most serotypes share a very high degree of genetic similarity and because of this similarity; the *Salmonellae* are now divided into just 2 species; *Salmonella enterica* and *Salmonella bongori* (Foley and Lyne, 2007). Over 99% of the serotypes are grouped into the species *S. enterica*, which contains all of the major serovars that are pathogenic to humans (Brenner *et al*., 2000; Poppoff *et al*., 2001). Serotyping is performed by mixing a suspension of *Salmonella* with a series of antisera specific for a variety of O and H surface antigens. Following incubation, the mixtures are observed for agglutination and the specific agglutination profile is used to determine the serotype of an isolate (Brenner *et al*., 2000; Voogt *et al.*, 2002). The particular serotypes are designated using the Kauffmann- White scheme used by public health organizations (Popoff *et al*., 2001; CDC, 2006). The host-range and disease can differ considerably between serovars making such classification important (Andrews-Polymenis *et al*., 2009).

Numerous methods have been developed for the detection and quantification of *Salmonella* in different matrices (Fey *et al*., 2004). The introduction of molecular techniques has become an important advance in reducing the time required for detection of *Salmonella* and in detecting active bacteria in environmental samples through their DNA and RNA (Touron *et al*., 2005; Gonzalez- Escalona *et al.*, 2009). PCR offers a more rapid and reliable method for the detection of *Salmonella* in natural environments (Moganedi *et al*., 2007). Polymerase chain Reaction (PCR) is a rapid and

reliable method for detection and identification of food-borne pathogens such as *Salmonella* (Salehi *et al*., 2005). PCR assay has been successfully used to diagonise the presence of bacterial pathogens in aquatic environments, food products and clinical samples (Ellingson *et al*., 2004, Myint *et al*., 2006; Patel *et al*., 2006). The assay combines simplicity with specificity and sensitivity in detecting organisms (Moganedi *et al*., 2007).

2.7 *Vibrio*

Cholera (Greek, chole, bile) is caused by the Gram- negative *Vibrio cholerae* bacterium of the family *Vibrionaceae* (Opajobi *et al.,* 2004). They are short, curved, rod-shaped organisms (Strohl *et al.,* 2001) which display colony variation (Thompson and Swings, 2006). The *Vibrionaceae* are gram–negative *Gammaproteobacteria* that occur in temperate to tropical, coastal and estuarine marine systems (Thomson *et al*., 2004). *Vibrio* spp. occupies a diverse range of ecological niches, including sediments, the water column and in association with organisms either as symbionts (Ruby *et al*., 2005) or pathogens (Heidelberg *et al*., 2000; Makino *et al*., 2003). Overall, the cells are 1micrometer in width and 2 to 3 micrometer in length and motile by atleast one polar flagellum (Thompson and Swings, 2006). *Vibrio cholerae* strains are grouped into two defined biotypes (El Tor and classical) and more than 200 serogroups (Faruque *et al*., 2003). The El Tor and classical biotypes are differentiated based on biochemical properties and phage sensitivity, whereas serogroup differentiation is based on O-antigen structure (Dziejman *et al*., 2005). Pathogenic clinical isolates generally express one or two O antigen (Dziejman *et al*., 2005). Until 1992, only serogroup O1 (out of more than 206 serogroups currently described) was recognized as a cause of cholera (Stine *et al*., 2011). Within the O1 serogroup, is the ability to produce cholera toxin

(CT) and it is an essential determinant of virulence (Haque, 2011). In 1992, a new, non-O1 *V. cholerae* strain (subsequently designated *V.cholerae* O139) appeared in India and rapidly spread across much of Asia (Nair, 1994). *V. cholerae* non-O1 serogroups have been reported to be involved in the emergence of this newer variant *V.cholerae* O139, a serogroup believed to have evolved by horizontal gene transfer from serogroup O1 to a non-O1 serogroup (Singh *et al*., 2001). Extensive studies (Mooi, 1997) demonstrated that the O139 strains was closely related to the O1 El Tor strains of the 7^{th} pandemic, except that the genes responsible for O1- antigen biosynthesis, were deleted and replaced with DNA that encodes the O139 antigen (Stine *et al*., 2011). The O139 strains clearly emerged from a CTX^{+} TCP^{+} seventh pandemic strain through replacement of O139 antigen (Li *et al*., 2002). Since the O antigen is the major protective epitope, its alteration was sufficient to allow O139 strains to move in epidemic form through populations previously immune to cholera caused by O1 (Stine *et al*., 2011). Previous studies report that four serogroups were identified and reported to have been derived from an O1 progenitor by serogroup conversion at the O- antigen region (Blokesch and Schoolnik, 2007). Reports of one serotype displacing another have been on the increase (Opajobi *et al*., 2004). Gomez *et al.* (1995) reported a case in which Inaba strains, which were dominant in Mexico in 1991, were later sub-planted by Ogawa serotype in 1992. In Calcutta, India, *Vibrio cholerae* O139 displaced the predorminant serotype, El-Tor however reappeared in India in 1998 and 1999, almost a decade after its last dominance in the Calcutta episode (Garg *et al.*, 2000). Studies of DNA sequences from single or multiple loci have demonstrated strong clonality of the epidemic O1 and O139 lineages (Farfan *et al*., 2002; Lee *et al*., 2006), while uncovering broader genetic diversity among non- O1/O139 isolates (Kotetishivili *et al*., 2003; O`Shea *et al*., 2004). Genome sequences from several disease- causing

V.cholerae isolates revealed an identical genome ``backbone`` in pandemic strains, with most of the intergenomic diversity generated by illegitimate recombination events (Chun *et al*., 2009). Hence, it has become evident from earlier reports that a new epidemic *V.cholerae* clone could emerge by acquisition and combination of crucial virulence genes from environmental pool through horizontal gene transfer (Faruque *et al*., 2004). Epidemiological surveillance and comparative molecular analysis of isolates have demonstrated clonal diversity among epidemic strains and a clonal emergence of new clones of toxigenic *V.cholerae* (Sack *et al*., 2004). *V. cholerae* O1 was the main causative agent of cholera in Nigeria and much effort was directed towards its control (Isere and Osemwenkhae, 2010).

Most non pathogenic, environmental isolates express other O antigens and are referred to as`` non- O1, non-O139`` (Dziejman *et al*., 2005). However, some non-O1, non-O139 *V.cholerae* are clearly pathogenic and have caused outbreaks or sporadic cases of gastroenteritis and extraintestinal infections in humans (Dalsgaard *et al*., 2001; Ou *et al*., 2003) as they have been isolated from patients with acute secretory diarrhoea worldwide (Singh *et al*., 2001) and reported to be the most frequent causative agents of cholera (Alexander *et al*., 2011).

Studies suggest that the pandemic cluster carries other serogroups as well, including O37, O27, O53, O65 and O75 (Tobin-Dangelo, 2008). These observations are consistent with the hypothesis that pathogenic *V.cholerae* strains are able to easily acquire and/or exchange O-antigen structure and may also provide selective advantages in the environment (Stine *et al*., 2011). During epidemics, bacteriophage may play a cruicial role in controlling the number of *V.cholerae* in the environment (Faruque, 2005) and since the O- antigen may serve as bacteriophage receptors, serogroup conversion also may be beneficial for evading phage predation (Nesper, 2005).

However, strains of *V.cholerae* belonging to a single phage type originated from a single clone (Sarkar *et al*., 2009).

2.7.1 Outbreak of *Vibrio cholerae*

Cholera has unique epidemiologic features; most intriguing is the preposition to cause epidemics with pandemic potential and the ability to remain endemic in all affected areas (Seas and Gottuzzo, 2005). Cholera epidemic was first presented formally in Snow`s seminal work in 1885 (Codeco, 2001). Throughout recorded history, cholera has caused seven pandemics in various areas of the world especially in Asia, the Middle East and Africa (Opajobi *et al*., 2004). Cholera was first mentioned in the India sub- continent in 1817 (Isere and Osemwenkhae, 2010). The disease spread through Asia continent in the 1960s reaching Africa in 1970 and Latin America in 1991 (Codeco, 2001 ; Wearing *et al*, 2005). Since the early 1800s, there have been seven cholera pandemics and 2011 marks not only the 1-year anniversary of the reappearance of cholera in Haiti but also the 50^{th} anniversary of the onset of the current cholera pandemic that began in Indonesia in 1961 (Ryan, 2011). In addition to Haiti, during the past 10years, there have been major cholera epidemics in Zimbabwe, Kenya, Nigeria, Afghanistan, Iraq, Somalia, Angola, Guinea-Bissau, Sudan, South Africa, Malawi, Liberia and Vietnam (Ryan, 2011). Cholera is endemic to 50 countries, affects 2-5 million persons each year and kills 100,000 (Ryan, 2011). *V.cholerae* O1 Ogawa is the major organism of the seventh pandemic (Alam *et al*., 2003). This seventh pandemic, caused by the El Tor biotype of *V. cholerae* O1 and O139, began in Indonesia, spread through Asia and reached Africa in 1971 (Tappero and Tauxe, 2011). In 1991, it appeared unexpectedly in Latin America, causing 1 million reported cases and 9, 170 deaths in the first 3years (PAHO, 2011). The World Health Organization reported in

2006 that 236, 896 cases of cholera occurred in 52 countries, a 79% increase over 2005 (WHO, 2005). *Vibrio cholerae* is a major cause of illness in the developing world (Stine *et al*., 2008). The African continent is the major contributor of cholera in the world, with a proportion of cases that ranged from 93.6% to 99% from 2004 to 2008 (WHO, 2009). This has been exemplified by the cholera outbreak that occurred in Zimbabwe from 2008 to 2009, which spread quickly across the entire country causing 60,055 cases and 2,928 fatalities in 2008 alone (WHO, 2009). In 2008, South Africa reported 3,907 cases and 22 fatalities to WHO (WHO, 2009). According to WHO (2009), as of March 2009, Mpumlanga province recorded the highest number of cholera cases (6, 644). In 2009, 221, 226 cholera cases were reported to the World Health Organisation (WHO) from 45 countries (WHO, 2010). This number includes 4,946 deaths, for a case- fatality rate of 2.24 % (Morris, 2011). Although the disease was reported from all continents, 98% of cases reported during 2009 were from Africa, driven in part by large numbers from the latter part of the 2008-2009 Zimbabwe epidemics (Morris, 2011). This number is however not encompassing of cholera cases because of well-recognised problems with underreporting in the WHO system, particularly because cholera is no longer a notifiable disease and countries can choose whether to report cases (Morris, 2011). A nation wide outbreak of cholera caused by *Vibrio cholerae* O1serotype Ogawa began in Haiti in October 2010 and has since resulted in more than 200,000 illnesses and 4000 deaths (Pan American Health Organisation, PAHO, 2011). Cholera remains a serious public health problem for some developed countries (Pazzani *et al.*, 2006). Additional case of cholera attributed to the neighboring Dominican Republic, in Florida and New Jersey in the United States (Gilmour *et al*., 2011) were reported in these instances, illness was related to travel to Haiti or consumption of contaminated water on the island of Hispaniola (which is

shared by Haiti and Dominican Republic) (Gilmour *et al*., 2011). Except for Haiti, where an epidemic of cholera began in mid-October, 2010 (Piarroux *et al*., 2011), the area experiencing the worst cholera epidemics is sub- Saharan Africa (Bompangue, 2011). During 1995-2005, a total of 632 cholera outbreaks were reported worldwide; 66.0% of the total cases and 87.6% of fatal cases were reported from sub-Saharan Africa (Griffith *et al*., 2006). Cholera outbreaks are common in developing countries where basic infrastructure to provide access to safe water and sanitation is lacking (Jain *et al*., 2011). Cholera outbreaks have occurred in Burundi, Rwanda, Democratic Republic of Congo, Tanzania, Uganda, and Kenya almost every year since 1977-1978, when the disease emerged in these countries (Bompangue *et al*., 2011). In Kenya, 18% prevalence was documented in rural Western Kenya from diarrhoea specimens (Shipiro *et al*., 2001). A 33.8% isolation rate of *Vibrio cholerae* O1 Ogawa was recorded in Karachi, Pakistan from stool samples (Alam *et al*., 2003). In Nigeria, outbreaks of the disease have been occurring with increasing frequency since the first outbreak in modern times in 1972 (Adagbada *et al*., 2012). There was a reported outbreak of cholera in Jos in 1991, in which Ogawa was the predorminant serotype (Opajobi *et al*., 2004). In a related report, a 4.39% isolation rate was reported in 2004 (Opajobi *et al*., 2004). Since then, the disease has remained endemic with occasional outbreaks in some States of the country primarily due to lack of good water supply and poor personal and environmental hygiene (Opajobi *et al*., 2004). Cholera outbreak was reported in Benue State in April 2005, more than 90 people died from this outbreak within two weeks (Vanguard, 2008), in Gwer West and Apa, Local Government Area of Benue State. A source attributed the outbreaks to the fact that many residents in Makurdi depend on the River Benue (which flows through the city) for drinking water. In July 16, 2008, it was reported that cholera claimed six lives leaving 30 others hospitalized in Zaria Local

Government Area (L.G.A) of Kaduna State (Hustin and Luby, 2008). The UN Humanitarian Unit revealed that out of a total of 5600 cholera cases in Nigeria, 340 cholera deaths were reported, of this number, 102 cases were known to have drunk street vended water ((Vanguard, 2008). Most strains causing epidemics in Nigeria have been Ogawa serotype (Adesuyin, 1983; Oyediran, 1991). 1n 2006, a 0.59% isolation rate was reported in Zaria, Kaduna State (Abakpa *et al*., 2011) from households with livestock. A total of 221 226 cases including 4946 deaths were reported to WHO from 45 countries (WHO, 2010). Also in 2010, severe outbreaks which started in the northern part of Nigeria, spreading to other parts of the country claimed 781 lives out of 3,000 recorded cases (Adagbada *et al*., 2012). The lack of infrastructure and economic development has made many parts of Africa susceptible to cholera, a disease associated with a lack of clean water and poor sanitation (Griffith *et al*., 2006). The CDC`s recent FoodNet report suggested a continued increase in *Vibrio* incidences since 2001, pointing to a need for improved prevention measures (CDC, 2010).

2.7.2 Transmission of *Vibrio cholerae*

Both pathogenic and non pathogenic *V.cholerae* resides in the aquatic ecosystems, but non pathogenic strains typically dominate this niche (Singh *et al*., 2001; Faruque *et al*., 2004). Based on a growing understanding of the ecology of *Vibrio cholerae*, the pandemic capacity of pathogenic strains can be understood to result from its characteristic lifecycle within its natural habitat (Sack *et al*., 2004). *V.cholerae* strains associated with epidemic disease can respond to changes in their immediate environment as they move from environmental reservoirs to humans and back (Morris, 2011). Cholera is transmitted through the faecal-oral route after consumption of contaminated water and/or food (Madoroba and Momba, 2010). Epidemic *Vibrio*

strains cause human illness by expression of specific genes that enable *V.cholerae* to exist in the environment long enough to be ingested, overcome host immunity, colonize the intestinal tract and produce cholera toxin in the host (Sharma and Chatruvedi, 2006, Pal *et al*., 2006; Pal *et al*., 2010). The epidemiology and transmission of cholera is greatly influenced by water (Faruque *et al*., 1998). Toxigenic *V.cholerae* is now recognized as an authochthonous member of the microflora in many aquatic environments based on its protracted survival and proliferation without losing the virulence determinants (Sanyal, 2000; Ramamnurthy *et al*., 2000).

In environmental monitoring of cholera, a large number of samples are often required (Wang *et al*., 2010). Each subpopulation is furnished with unique genetic resources so that its ecological niches are diverse (Miller *et al*., 2007). This bacterium attaches to the bodies of zooplankton, crustaceans and aquatic plants in seawater and estuarine water and survives by forming a biofilm on the surface of these organisms (Alam *et al*., 2006) or as a biofilm in the water (Alam *et al*., 2007). No bacterial factors have been correlated with the prevalence of pathogenic strains in the aquatic environment other than resistance to bacteriophages (Faruque *et al*., 2005). *Vibrio* in the environment depends on water temperatures (optimal growth at water temperatures>20^{o}C) and salinity (Martinelli *et al*., 2011). In contrast to most other *Vibrio* species, *Vibrio cholerae* is able to grow in fresh water and is often present in inland rivers and lakes in where it is endemic (Morris, 2011). Studies by Binsztein *et al*. (2004) suggested that in the interim period between cholera epidemics, *V.cholerae* is still present in the environment in a VBNC state. During inter-epidemic period, *V. cholerae* cannot be cultured from the surface water, whereas in epidemic season it can be isolated from the patients' body as well as surface water (Alam *et al*., 2006). In areas with seasonal variations in water temperatures, the microorganism shows clear

seasonality; environmental counts decrease during warmer periods and decline (or become nondetectable) during cold weather (Morris, 2011). Seasonal cycles of infectious diseases have been variously attributed to changes in atmospheric conditions, the prevalence or virulence of the pathogen, or the behavior of the host organism (Dowell, 2001). In a study by Mohanty *et al*. (2004) in North India, *V.cholerae* had peak prevalence from May to June, where a 90.25% resistance to atleast one antibiotic was reported. In Asia, the endemic and seasonal character of cholera largely depends on human exposure to the aquatic reservoir of *Vibrio cholerae* (Constantain and Colwell, 2009). Culturable *V.cholerae* as well as viable but non culturable *V.cholerae* (i.e those that have entered into a dormant stage because of conditions unfavourable for growth or reproduction) attach to zooplankton and phytoplankton, especially in estuarine areas (Alam *et al*., 2007). In these areas, the incidence of cholera is influenced by local factors, such as rainfall and plankton blooms and by global climatic conditions, such as increased sea surface temperatures linked to El Nino Southern oscillation events (Hashizume *et al*., 2010; Ohotomo *et al*., 2010).

2.7.3 Public health significance of *Vibrio cholerae* in water, soil and vegetables

Cholera is a major public health concern in countries where access to safe water and adequate sanitation cannot be guaranteed for all residents (Sithivong *et al.*, 2011). The life threatening diarrhoeal consequences are still major challenges in concern with human health across the world and especially developing country where hygiene and sanitation is not optimal (Yogendra and Mahendra, 2013). Cholera is considered a reemerging disease, in part because infections appearing in novel communities or communities where the disease had been absent for many years and the range of areas of endemicity is expanding (Lipp *et al.*, 2002). This may be due to

changes in the environment that promote favorable conditions for the pathogens and/or its inveterbrate host (Lipp *et al.,* 2002). The differential behavior of this pathogen against external and internal factor not only provides natural adaptation for its survival but also emergence of new potent sub-species (Yogendra and Mahendra, 2013). This bacterium contains a wide variety of strains and biotypes, receiving and transferring genes for toxins, colonization factors, antibiotic resistance, capsular polysaccharides that provide resistance to chlorine and new surface antigen capsule (Heidelberg *et al*., 2000). Hence, the bacterium is able to escape treatment processes. The lateral or horizontal transfer of these virulence genes by phage, pathogenicity islands and other accessory genetic elements provides insights into how bacterial pathogens emerge and evolve to become new strains (Heidelberg *et al*., 2000). The emerging new resistance strains of *Vibrio* are major threats for conventional medications and global spread of pathogen (Yogendra and Mahendra, 2013). The newly emerging strains of *Vibrio* have adapted themselves to survive in changed environmental habitat and in the presence of antibiotics often used for medications (Yogendra and Mahendra, 2013). Antibiotic multidrug resistance is becoming increasingly common among atypical *V.cholerae* strains mostly associated with acquisition of genes and/or modification in the antibiotic target genes (Okeke *et al*., 2007). In Nigeria, for example, it has been reported that recent outbreaks were caused by multidrug resistant atypical El Tor O1 strains, which are reportedly highly virulent (Ceccarelli *et al*., 2011), a common worrisome trend in the current cholera outbreaks around the world (Marin *et al*., 2013). Worldwide, diarrhoeal diseases, caused by cholera is the second leading cause of death for children under the age of 5 and atleast 120,000 deaths are estimated to be caused by cholera each year (Nelson *et al*., 2009). Humans are a reservoir for the disease as are animals

around aquatic environments (Krausis *et al*., 2003), hence, dissemination of *Vibrio* strains could occur through travels and water movements.

2.7.5 Pathogenicity of *Vibrio cholerae*

The pathogenic variants of *V.cholerae* expresses genes encoding virulence associated factors in order to cause diarrhoea, strains require genes for the cholera toxin, the colonization factor TCP (Toxin-coregulated pilus) and the central regulatory protein ToxR (Elhadi *et al*., 2012). They are essential for colonization of the host and enterotoxicity, respectively (Childers and Klose, 2007). These virulence factors are under the control of ToxT, an AraC/Xyls family protein that activates transcription of the genes encoding TCP and CT (Childers and Klose, 2007). ToxR is a transmembrane transcriptional activator that is part of a complex virulence gene regulon that regulates cholera toxin and TCP synthesis and expression of the outer membrane protein Ompu (Huhulescu *et al*., 2007). TOxT is under the control of virulence regulatory cascade known as the ToxR regulon, which responds to environmental stimuli to ensure maximal virulence – factor induction within the human intestine (Childers and Klose, 2007). The most important virulence factor produced by *Vibrio cholerae* is cholera toxin (CT) encoded by *ctx*AB gene (Diallo *et al*., 2007) which is located on the CTX prophage (Sithivong *et al*., 2011). The horizontal acquisition of genes and expression of the transcriptional co-regulated pilus (located on *Vibrio* pathogenicity island) and CTXAB (carried on CTXΦ) determine primary virulence (Sharma and Chaturvedi, 2006; Pal *et al*., 2010). Cholera toxin (CT) is encoded by a transferable filamentous phage (CTΦ) and reports had implied the acquisition of these CT genes under analogous conditions to those in the aquatic environments (Faruque and Nair, 2002). CTxAB operon, which encodes for the A and B subunits of CT, resides in the genomes

of (CTxΦ) (Elhadi *et al*., 2012). All *Vibrio* strains proficient of causing cholera perpetually carry genes for TCP which is an adhesion that is coordinately regulated with CT production (Taylor *et al*., 1987). TCP is the solitary *V.cholerae* pilus that has been demonstrated to have a role in colonization of the human's gut mucuos and of infant mice (Taylor *et al*., 1987), the latter being an experimental model (Elhadi *et al*., 2012).

On the other hand, in the pathogenicity mechanisms of *V.cholerae* non-O1 and non O139, different virulence factors had been suggested to be involved in the disease caused by this pathogen (Morris, 1994). Although some of these strains produce the cholera toxin (CT) or cholera- like toxin, most of them lack the CTΦ phage and toxin coregulated factor (TCP) and even the complete *Vibrio* pathogenicity island (VPI) (Elhadi *et al*., 2012).

2.7.6 Disease of *Vibrio cholerae*

In 1884 during an outbreak in Egypt, Robert Koch noted that the disease was a specific gastrointestinal infection caused by a comma- shaped bacillus, which he isolated and named *Vibrio cholerae* (Potter, 2011). Cholera, a severe watery diarrhoeal disease is caused by toxigenic *Vibrio cholerae* (Lam *et al.,* 2012). It is an acute infection caused most frequently by the colonization and multiplication of *Vibrio cholerae* O1 or O139 within human small intestine (Emch *et al*., 2008). *Vibrio cholerae* causes disease by colonizing, proliferating and secreting toxins in the intestine of the host (Wang *et al*., 2010). Symptoms and signs can range from asymptomatic carriage to severe diarrhoea, vomiting and profound shock (Tappero and Tauxe, 2011). The resulting profused diarrhoea and vomiting can be fatal if left untreated (Wang *et al*., 2010). *Vibrio cholerae* can cause symptoms ranging from asymptomatic to cholera

gravis (Krausis *et al*., 2003). Symptoms include abrupt onset of watery diarrhoea (a grey and cloudy liquid), occasional vomiting and abdominal cramps (Krausis *et al*., 2003). Dehydration ensues with symptoms and signs such as thirst, dry mucous membranes, decreased skin turgor, sunken eyes and hypotension, weak or absent radical pulse, tachycardia, tachyphnea, hoarse voice, oliguria, cramps, renal failure, seizures, sommolence, coma and death (Krausis *et al*., 2003). Death due to dehydration can occur within hours to days in untreated children and the disease is dangerous for pregnant women and their foetus during late pregnancy as abortion, premature labor and fetal death may occur (Fleming and Hunt, 2006).

Cholera is a pandemic diarrhoeal disease that continues to be an important cause of morbidity and mortality worldwide (Stine *et al*., 2011). In animal studies, infectious dose is 1-2 orders of magnitude lower than that for strains grown by using traditional in vitro methods (Morris, 2011). Of particular relevance, it has been shown that *V.cholerae* passed in human rice-water faeces are in a ``hyperinfectious`` state (Nelson *et al*., 2009). The hyper infectious state lasts at least 5 hours after passage of the microorganism from the patients (Morris, 2011). The physiologic basis for this effect is unclear but appears to be associated, at least in part, with changes that include down-regulation of chemotaxis genes (Butler *et al*., 2006). *V.cholerae* as it is passed from the body also up regulates a series of genes that are not required for infection but are needed for survival in the environment (Morris, 2011). Twenty four hours after the microorganism reaches the aquatic environment, these shifts, potentially combined with lytic phage, result in a dramatic decrease in the ability of *V.cholerae* to cause infection (Nelson *et al*., 2008). Cholera epidemics appear in explosive outbreaks that have occurred repeatedly throughout history (Childers and Klose, 2007). The El Tor biotype was responsible for the seventh pandemic of cholera (Cholera- Indonesia, 2008). Since

the early 1990s, the El Tor variant strains, which are biotypes of El Tor but carry the classical type of ctxB, have emerged and prevail in multiple regions where cholera is endemic (Lee *et al*., 2006, Cholera-Indonesia, 2008; Alexander *et al*., 2011).

2.7.7 Antimicrobial resistance in *Vibrio*

The primary treatment for cholera is rehydration with oral or intravenous fluids (WHO, 2011). For severe cases, antimicrobial agents may reduce the volume and duration of diarrhoea (Sack *et al*., 2011). Tetracyclines (e.g doxycycline), fluoroquinolones (ciprofloxacin), macrolides (e.g erythromycin) and trimethoprim/sulfamethoxazole have commonly been used to treat cholera (WHO, 2011). However, *V.cholerae* continues to evolve and resistance to antimicrobial drugs is complicating treatment options in areas with limited resources (Ryan, 2011). Antimicrobial drug resistance can undermine the success of antimicrobial therapy (Sjolound-Karlsson *et al*., 2011). Several reports have documented tetracycline and fluoroquinolone-resistant *V.cholerae* and multidrug resistance is increasing (Kitaoka *et al*., 2011). Antimicrobial drug resistance in *Vibrio* spp can develop through mutation acquisition of resistance genes on mobile genetic elements, such as plasmids, transposons, integrons and integrating congugative elements (ICEs) (Sjoulund-Karlsson *et al*.,2011). ICEs integrate and replicate with the host chromosome and excise themselves and transfer between bacteria by conjugation (Wozniak *et al*., 2009). ICE commonly carries several antimicrobial drug resistances in *V.cholerae* (Burrus *et al*., 2006). The first *V.cholerae* ICE described was in an O139 isolate in Madras, India, in 1992 and was named SXT after the resistance phenotype it conferred (trimethoprim/sulfamethoxazole)(Waldor *et al*., 1996). Many O139 and O1 isolates have since acquired SXT or a closely related ICE (Burrus *et al*., 2006; Wozniak *et al*.,

2009). Drug resistant *V.cholerae* is a global health concern because resulting infections can be more severe and difficult to treat (Sjolund-Karlsson *et al*., 2011). During an outbreak in Guinea-Bissau, case- fatality rates increased from 1% to 5.3% after the outbreak strain acquired multidrug resistance (Dalsgaard *et al.*, 2000). As different populations of bacteria co-exist in aquatic ecosystems, *V.cholerae* may acquire antibiotic resistance plasmids from neighboring microorganisms (Fernandez *et al.*, 2009). Hence, continuous surveillance of antimicrobial resistance in *V.cholerae* needs to be performed in order to detect the emergence of multi-drug resistant strains (Gonzalez- Fraga *et al*., 2007).

2.7.8 Detection of *Vibrio cholerae*

In general, classical and/or molecular-based methods are used for identification of *Vibrio cholerae* (Madoroba and Momba, 2010). Classical methods involve enrichment of samples in alkaline peptone water (APW), followed by inoculation on suitable culture media such as thiosulphate citrate bile salt sucrose (TCBS) agar and aerobic incubation at 37^{o}C (Nandi *et al*., 2010). Due to the general low level of *Vibrio cholerae* in water and to reduce the impact of other bacteria that can interfere with its detection, selective enrichment of bacterial population from water samples, followed by selective isolation and culture in selective medium is required (Wang *et al*., 2010), though very time consuming. Alkaline peptone water is the preferred enrichment medium for *Vibrios*, the high pH of the medium (pH close to 9) and NaCl concentration inhibit many other bacteria and favor *Vibrios* (Gomez- Gill and Roque, 2006). To date, practical typing of *V.cholerae* is mainly serological and requires about 200 antisera (Danin- Poleg *et al*., 2007). There are a number of non-molecular microbiological methods that have been used for detection of *V.cholerae*

(Geol *et al*., 2007) but are laborious and boring (Espineira *et al*., 2010). Since cholera detection requires urgent testing (Mehrabadi *et al*., 2011), applied molecular methods that are confident and rapid are needed (Espineira *et al*., 2010). DNA- based techniques with high resolution are advantageous for diagnosis and epidemiological surveillance of *V.cholerae* (Vandamme *et al*., 1996). These techniques include species-specific PCR targeting the outer membrane protein, which is very sensitive, specific and efficient for *V.cholerae* identification (Nandi *et al*., 2000).

2.8 Multidrug Resistance in Bacteria

Bacteria with resistance to two or more antimicrobial drugs are regarded as multidrug resistant (Gundogdu *et al*., 2011). The intensive use of antibiotics for human, veterinary and agricultural purposes results in their continuous release into the environment (Kummerer, 2009) including effluents from urban wastewater (Rizzo *et al*., 2013). After use, up to 75% of antibiotics are excreted unaltered or as metabolites by humans and animals and end up in the environment (Pei *et al*., 2007). The main concern for the release of antibiotics into the environment is related to the development of antibiotic resistance genes (ARGs) and bacteria (ARB), which reduce the therapeutic potential against human and animal pathogens (Zhang *et al*., 2009). The extensive treatment of humans and animals with antibiotics increases the presence of antibiotic residues in the environment and gives rise to antibiotic resistance in various bacteria by the dissemination of antibiotic resistance genes (ARGs) (Su *et al*., 2012). The acquisition of resistance genes by horizontal transfer is currently thought to play a major role in the development of multi-drug resistant (MDR) strains because a substantial proportion of the resistance genes are located on conjugative plasmids, transposons, insertion sequences and integrons (Chang *et al*., 2011). Integrons are

genetic elements able to acquire exchange and express antibiotic resistance genes embedded within gene cassettes (Rizzo *et al*., 2013). An integron is structurally based on a functional platform composed of 3 elements: a. the int1 gene, encoding an integrase protein b, a specific recombination site 1 attl and c, a promoter (Cambray *et al*., 2010). Integrons are mobile DNA elements with the ability to integrate and express gene cassettes by site- specific recombination (Mazel, 2006). Site- specific recombination is catalysed by an integrase that is encoded within the conserved 5` region of integrons (Gundogu *et al*., 2011). Several classes of integrase have been described with those classes of integrons i.e class 1 integrase (int 1) defines class I integrons (Collins *et al*., 2002). Resistance integrons (RIs) and chromosomal integrons (CIs) are the two major groups of integrons (Rizzo *et al*., 2013). CIs are located on the chromosome of hundreds of bacterial species (Cambray *et al*., 2010), while RIs have been described in a wide range of Gram-negative bacteria. RIs are located on mobile genetic elements such as plasmids and transposons, which promote their dissemination among bacteria (Rizzo *et al*., 2013). The common types of integrons identified are class1, 2 and 3 integrons classified according to the integrase gene (Intl) (Su *et al*., 2012). Most integrons belong to Class 1 and comprise two conserved segment (cs) regions (Su *et al*., 2012). Class 1 and class 2 integrons are the most frequently found and have been detected in human, animal and environmental samples and bacterial isolates (Stokes and Gillings, 2011). Class 1 RIs have been extensively studied and have mainly been described in multidrug- resistant bacteria (Stalder *et al*., 2012). RIs are useful in the tracking of occurrence of resistance genes in the environment and their detection serve as a good indicator of antibiotic resistance acquisition (Rizzo *et al*., 2013). Integrons can transfer among different bacterial species easily because of their association with plasmids (Su *et al*., 2012). They are adapted to transfer and

disseminate antibiotic resistance as a result of their mobility and ability to quickly acquire diverse resistance genes (Caratolli, 2003). Integrons play a role in developing multiple drug resistance amongst microorganisms by transferring various resistance genes simultaneously (Luis-Martinez, 2009).

2.9 Genotypic Characterisation of Bacteria by DNA Fingerprinting

Enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) is a genetic fingerprinting method (Leung *et al*., 2004) with a high level of heterogeneity (Zulikifi *et al*., 2009). ERIC-PCR uses primers corresponding to interspersed repetitive DNA elements present in various locations within the prokaryotic genome to generate highly specific genomic fingerprints (Fu and Li, 2013). These elements are 126bp long, highly conserved at the nucleotide level and include a central core inverted repeat (Zulkifi *et al*., 2009). The repetitive units are highly conserved because rep sites are crucial protein-DNA interaction sites by gene conversion (Fu and Li, 2013). Amplification of the distinct rep sites produces diverse- sized DNA fragments that can be separated by agarose gel electrophoresis, and the resulting banding patterns unique for specific bacterial strain can be compared (Fu and Li, 2013). These elements are 126bp long, highly conserved at the nucleotide level, and include a central core inverted repeat (Zulkifli *et al*., 2009). The position of ERIC elements in enterobacterial genomes varies between different species and has been used as a genetic marker to characterize isolates within a bacterial species (Versalovic *et al*., 1991; Son *et al*., 2002). In ERIC-PCR, a band pattern is obtained by amplification of genomic DNA located between ERIC elements or between ERIC elements and other repetitive DNA sequences (Zulifli *et al*., 2009)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area and Sampling Sites

The study area covered irrigated areas in Kano and Plateau States, Nigeria where major vegetable production is carried out. Sites in Kano include a large vegetable farm at Kwakwachi which was divided into three sites (I-III); Kwakwachi I, Kwakwachi II and Kwakwachi III, site IV at Challawa industrial layout and site V - the Kadawa irrigation station at Tiga dam which served as control, while, in Plateau State, vegetables were collected from site I; Anglo Jos, site II; Rayfield, site III, Zaramaganda, site IV; Barkin Ladi and site 5 at Rukuba which served as control.

3.2 Kano State

Kano State is located in North-Western Nigeria. It occupies a central position in the northern part of Nigeria covering an area extending between latitude $12^{o}40`$ and $10^{o}30`$ and longitude $7^{o}40`$ and $9^{o}\ 40`$. Kano State borders Kastina State to the north-west, Jigawa State to the north-east, Bauchi State to the south-east and Kaduna State to the south-west (Commercial Agricultural Development Project, CADP, 2010). It lies in the tropical wet and dry climate zone. It has two distinct marked seasons; wet (April-October) and dry (November-March) with an average rainfall of 1000mm which lasts between 3 and 5 months and a mean temperature of $26^{o}C$ to $33^{o}C$. Kano State has many rivers, ponds and dams. It is one of the most irrigated States in the country with more than 3 million hectares of cultivable land and two of the most ambitious irrigation projects in West Africa with the Challawa Gorge and Tiga dams, which ensures all year round farming. Kano State is the second most populated State in Nigeria with a

population of about 9,401288 million people (National Population Commission, NPC, 2010). Kano State is historically a commercial and agricultural State, the centre of commerce. It is one of the most developed industrial cities in Nigeria. Tannery and textile are some of the dominating industries and this could be one of the reasons for her high population density (Olarenwaju, 2001). The rivers receive waste from tanneries and textile industries, urban water storm and agricultural runoffs from farming communities along its course. These water bodies serve as source of water for irrigation. Irrigated vegetables grown commonly in Kano include; tomatoes, cabbage, lettuce, carrots, cucumber, spinach, green peas and other leafy vegetables. Livestock farming is predominant in the State. Animal watering is done in the same water/ pond sources used for irrigation of vegetables.

3.3 Jos, Plateau State

Plateau State is within the Northern guinea savannah zone and occupies the North central part of the country on 80^{o} 24`N and longitude $80^{o}$ 32` and 100^{o}38` east. Plateau State got its name from Jos Plateau, a mountainous area in the north of the State with captivating rock formations (Zaccheus Onumba Didiaezue Memorial Libriaries, ZODML, 2013). To its north are Gombe and Bauchi States, while Benue stands at its southern border. It is flanked in the east by Taraba State and in the west by Nassarawa and Kaduna States. Plateau State has a population of about 3,206,531 million (NPC, 2010). Plateau State is characterized by a near temperate climate on the Jos Plateau and a hot humid climate on its lower parts. Generally, weather conditions are warmer during the wet season (April-October) and much colder during the harmattan period (December-February). Plateau State experiences an annual rainfall of 1,300-1,500mm with an average temperature of 21^{o}C in January rising to a peak of 25.5^{o}C in April

(ZODML, 2013). The soil and climatic conditions of the Jos Plateau favour production of exotic crops like vegetables, irish potatoes, apples, grapes, wheat and barley. These crops are grown throughout the year: rainfed during the wet season and irrigated during the dry season. Irrigation water is derived from sources such as; dams, old mine ponds, wells, rivers and tap. These sources are usually highly polluted with domestic, industrial, human and animal wastes (Damen *et al*., 2007). Tin mining, rock quarrying, farming and other commercial activities are common in the area. The natural features of these regions, especially the flood-prone plains, river basins and the development of vast irrigated lands, create conditions that greatly enhance the development of vegetables.

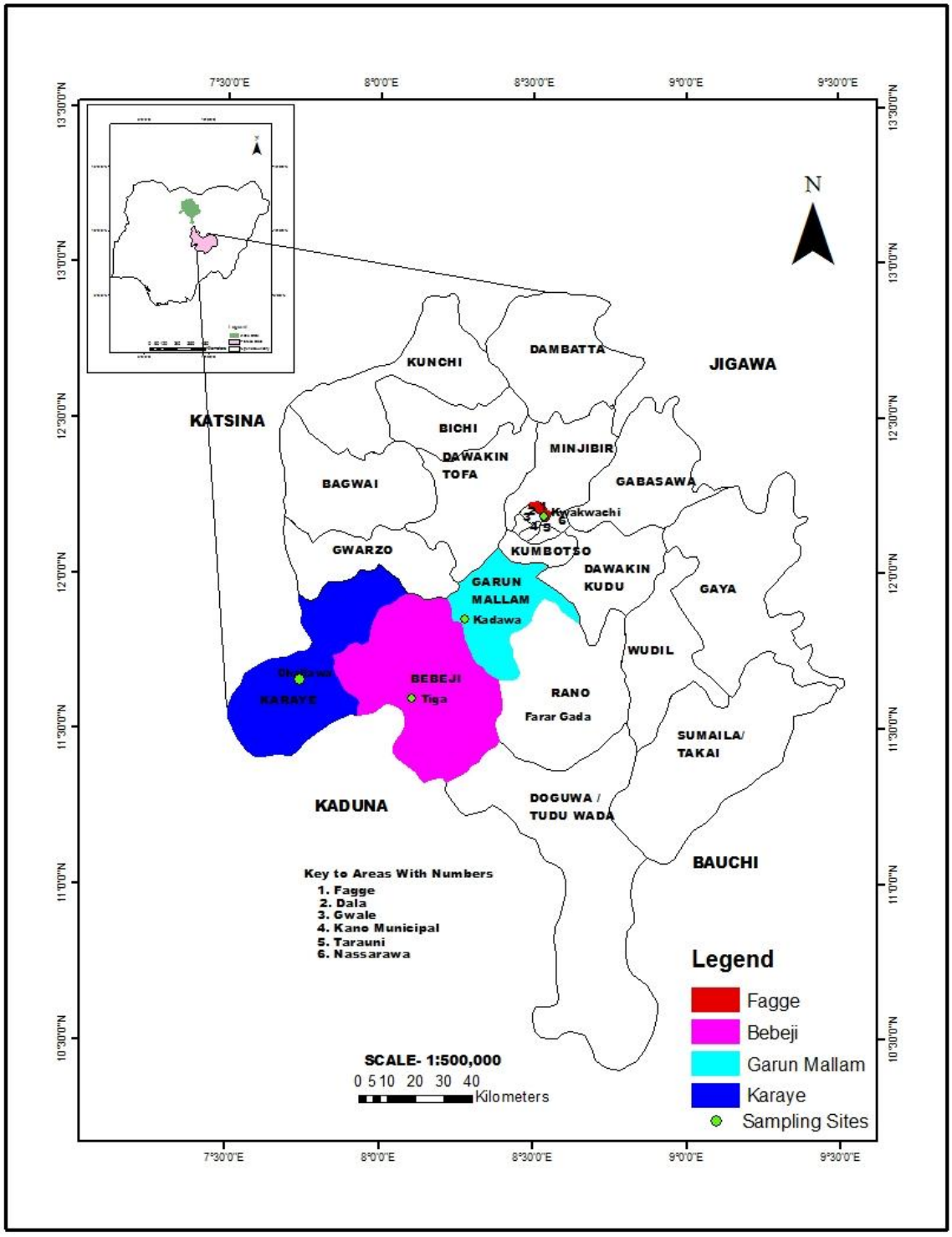
3.4 Sampling Sites Description

Sampling sites were selected after a survey of some irrigation sites in the two States during which copies of questionnaire were administered to farmers. Selection was based on; the availability of vegetables on the farms, the cosent of the farmers, the type of vegetables grown (vegetables commonly consumed raw including; carrots, cabbage, lettuce, cucmber, tomatoes, green pepper and green peas), the source of irrigation water and its point source of contamination. Five designated irrigation sites were selected for the study in Kano State; The Kwakwachi vegetable farm; a very large irrigation field along airport road behind hajj camp in Sabon Gari local Government (where large amount of vegetables are grown all year round and vegetable sellers were found purchasing from the farmers on a wholesale basis for onward retail sales in the market) was divided into sites 1, 2 and 3 based on point source of pollution to water used in irrigation by farmers. Site 1: water source receives waste directly from a large abattoir located close to the site, site 2 had a large refuse dumpsite composed of

domestic waste from inhabitants, and site 3 recieves human faeces, domestic sewage and waste from mechanic workshops from Sabon Gari extension, site 4; a vegetable farm at the Challawa industrial layout, where farmers use a river receiving industrial effluent from industries in the area and site 5 was the Kadawa Ahmadu Bello University irrigation station at the Tiga dam which served as control. In Plateau State also samples were collected from five large vegetable producing areas; site1- a vegetable field behind the Jos International breweries and Anglo Jos where a river receiving wastewater from livestock/abattoir, industrial and municipal waste is used for vegetable production, site 2 - a vegetable farm at Zaramaganda around the Rayfield river, receiving livestock and industrial waste, Site 3 behind the University of Jos permanent site and it receives domestic sewage and livestock waste from the University residence and Yelwa farms adjacent to it, site 4 a vegetable farm at Barkin Ladi receiving domestic sewage from residence around it, and 5, the control site at Rukuba where tap served as water source to grow vegetables (Figure 3.1 and 3. 2).

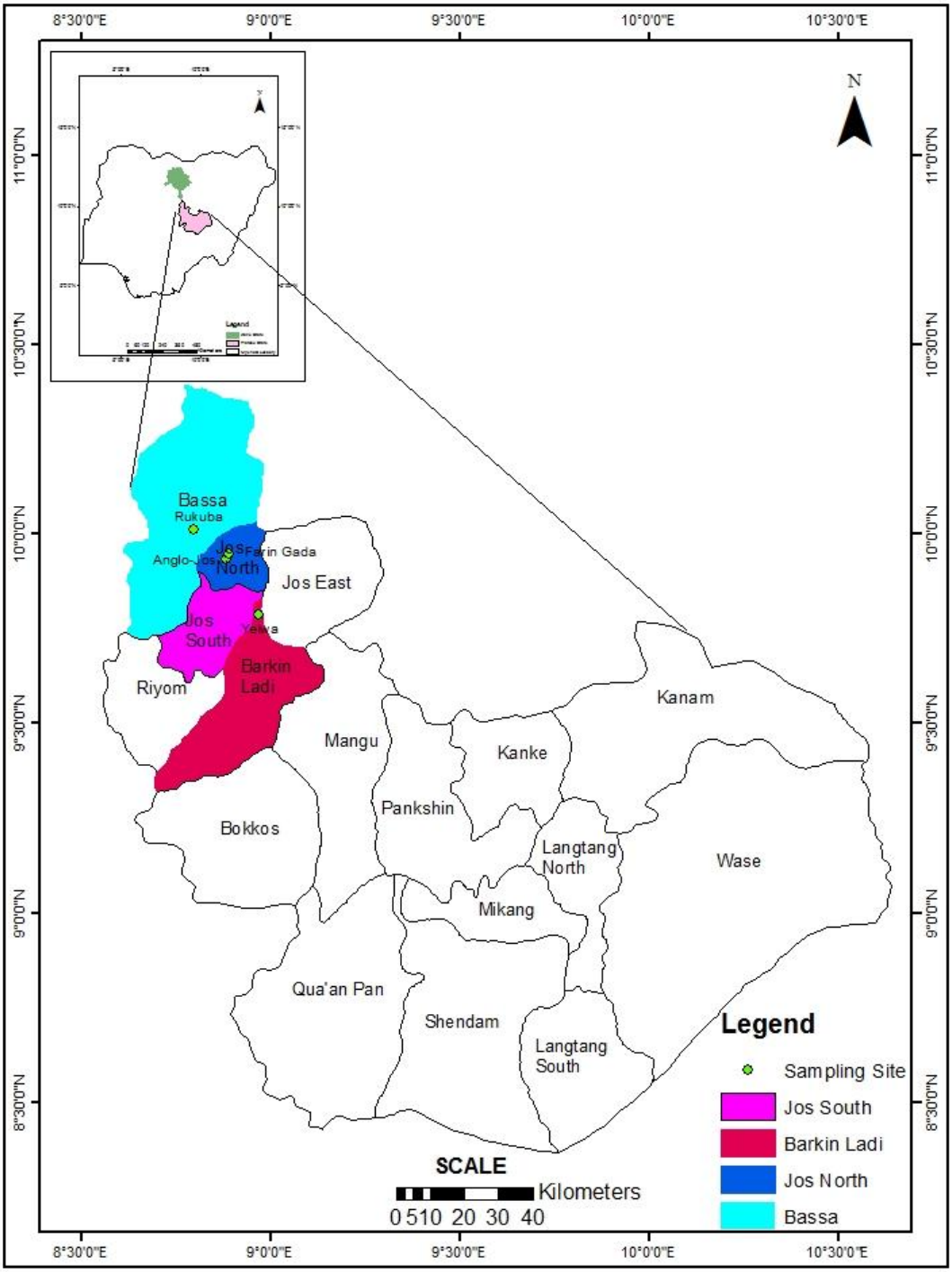
3.5 Questionnaire Administration

A survey of the study area was carried out between March, 2010 and May, 2010. Questionnaire regarding farm management practices was administered at the time of visit to the farms (Appendix I). Information obtained include socio-economic variables such as sex, age, occupation, estimated income from vegetable production and level of formal education. Information on each farm site (source of water for irrigation, treatment to water before use and point source of pollution) as well as on vegetable production; type of fertilizer (organic or inorganic), type of organic manure used (treated or untreated) and stage of fertilizer application was also obtained.



Source: Adapted and modified from the administrative map of Kano State

Figure 3.1 Map of Kano State showing sampling sites location



Source: Adapted and modified from the administrative map of Plateau State

Figure 3.2 Map of Plateau State showing sampling sites location

3.6 Sample Size

From a reported case of bacterial contamination of vegetables in Calabar, Cross river State, Nigeria at 78% prevalence (Udo *et al*., 2009), a sample size was determined using this prevalence rate. Using the equation as described by Niang *et al*. (2006):

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

Where n= sample size

Z= statistics for a level of 95% confidence interval= 1.96

P= Prevalence of 78% (0.78) from Udo *et al*. (2009)

d=precision (allowable error) =5%=0.05

Therefore:

$$n = \frac{1.96^2 \times 0.78\ (1-0.78)}{0.05^2}$$

n= 263.69

A total of 440 samples were collected and analysed during the study, consisting of 230 samples from parts of Kano State (122 in the wet and 108 in the dry season) and 210 samples from parts of Plateau State (122 in the wet and 88 in the dry season).

3.7 Collection of Samples

Samples were collected in the wet (May to October, 2010) and dry (November to March, 2011) seasons.

3.7.1 Water samples

Samples of water were collected from sources where vegetable farmers obtained water for irrigating their vegetables which include; rivers, tap, dam, wells in Kano State and old mine ponds in Plateau State. Water samples were collected according to the procedure recommended by American Public Health Association (APHA, 1992). Water samples were collected in sterile wide mouth, screw capped 250ml bottles, packed on ice during the transportation to the laboratory (Postgraduate Laboratory, Department of Microbiology and the Department of Water Resources, Ahmadu Bello University, Zaria) for analysis. A total of 84 water samples including 57 from Kano State and 27 from Plateau State were collected and analysed during the study.

3.7.2 Manure treated soil samples

Representative samples of manure treated soils were collected aseptically using ethanol-sterilized spatula. A total of 100 gm of sample was collected each at designated site, by taking 20 gm at five different points, 20 gm from the centre and 20 gm each at four different points, on the periphery and mixed together. During the study, 118 manure treated soil samples were collected and analysed; 79 from Kano and 39 from Plateau State. Samples were transported to the laboratory in clean plastic bags.

3.7.3 Vegetable samples

Vegetables available in the study sites during the study period were collected and analysed. Using 90% ethanol sterilized scissors; vegetables were cut into clean unused polythene bags and taken to the laboratory. A total of 238 samples were collected and analysed, from Kano, 94 samples were obtained while 144 vegetable samples were obtained from Plateau State.

All samples were analysed within six hours of collection.

3.8 Physicochemical Analysis of Water Samples

Temperature of each water samples was measured in the field using a thermometer and the pH was determined using a pre- calibrated pH meter (Norylab; LM8). The dissolved oxygen and a five day biological oxygen demand (BOD_5) were determined after incubation in tightly stoppered BOD bottle in the dark at 20^oC. The amount of oxygen consumed was determined. BOD was determined by the Winkler method (APHA, 1992). Each water sample was pipetted into a BOD bottle containing diluent. The dissolved oxygen content was determined and recorded on the first day. The BOD bottle was then incubated in the dark for 5days at 20^oC. At the end of the five days, the final dissolved oxygen content was determined. The difference between the initial DO and the final DO was used to calculate the BOD of the water sample.

Formular: $BOD = \frac{D1-D2}{P}$ (Warren and Hammer, 1993)

Where, BOD= biochemical oxygen demand
D1=Initial diluted seeded wastewatwater dissolved oxygen
D2=Final diluted seeded wastewater dissolved oxygen
P=wastewater decimal fraction

3.9 Determination of Faecal Coliform Count (FCC) from (vegetable, water, manure treated soil) Samples

Vegetable samples were cut in pieces using ethanol sterilized scissors. Twenty –five (25gm) of each vegetable was washed by shaking thoroughly with 225ml of sterile buffered peptone water. Serial dilutions of each vegetable washing was made in sterile peptone water to obtain dilution series ranging from 10^{-1} to 10^{-8}.

Faecal coliform count of water samples was determined using the serial dilution and spread plate method (Ajayi *et al*., 2008). Serial dilution of each water sample was made in sterile peptone water at dilutions 10^{-1} to 10^{-8}. Ten (10gm) of well mixed manure treated soil was placed in 99ml of sterile peptone water and shaken thoroughly. Serial dilution of the suspension was made at dilutions 10^{-1} to 10^{-8}. Aliquot of 0.1ml of 10^{-4}-10^{-6} dilutions were inoculated on Eosine Methylene Blue (EMB) agar plates by spread plate technique. Inoculated EMB agar plates were incubated at 44.5^{o}C for 24h. Characteristic colonies which appeared greenish black with metallic sheen on EMB agar were counted as faecal coliforms. Typical colonies were inoculated into lactose broth in test tubes containing inverted Durham`s tubes and were incubated at 44^{o}C for 24h for confirmatory tests. Gas and acid production confirmed faecal coliform test (Ogunshe *et al*., 2006).

3.10 Isolation of *Escherichia coli* O157 from Water, Vegetable and Manure Treated Soil

E. coli O157 was isolated from samples of water, vegetable and manure treated soil using enrichment in *Escherichia coli* (EC) medium and plating on Sorbitol MacConkey agar (David *et al*., 2003).

Twenty (20ml) millilitres of each water sample was combined with equal volume of sterile E.C medium (Oxoid) in a cotton plugged Erlenmeyer flask and incubated at 37^{o}C for 24h. Twenty five (25gm) of cut vegetables was enriched in 225ml buffered peptone water and incubated at 37^{o}C for 8h (David *et al*., 2003). Five millilitres of the broth was enriched in equal volume of E.C medium and incubated at 37^{o}C for 24h. One (1gm) gram of well mixed manure treated soil was placed in 9ml of *E. coli* medium and incubated at 37^{o}C for 24h for enrichment. After the broth enrichment, the cultures were streaked on sorbitol MaCconkey agar (Oxoid) plates containing cefixime (0.05mg/litre) and potassium tellurite (2.5mg/litre) and incubated at 37^{o}C for 24h (David *et al*., 2003). Three sorbitol negative colonies exihibiting *E. coli* O157 colony characteristics (gray or pale with a darker centre) were selected from each plate as presumptive *E. coli* O157 isolates. Presumptive isolates were inoculated into slants of nutrient agar, incubated at 37^{o}C for 24h and stored in the refridgerator at 4^{o}C as stock cultures for further identification.

3.11 Isolation of *Salmonella* from Water, Vegetable and Manure Treated Soil

Salmonella was isolated from samples using selenite F broth enrichment followed by isolation on *Salmonella –Shigella* agar (Turki *et al*., 2012). Ten millilitres of each water sample was inoculated in ten millilitres of selenite F broth and incubated at 37^{o}C for 24h. Twenty five (25gm) of cut, washed vegetables was pre-enriched in 225ml sterile peptone water and incubated at 37^{o}C for 24h. One millilitre of the broth was transferred into 9ml selenite F broth for selective enrichment (Okafor *et al*., 2003). One (1gm) of well mixed manure treated soil was placed in 9ml of selenite F broth and incubated at 37^{o}C for 24h. After the enrichment, *Salmonella* was detected by plating the broth cultures on *Salmonella-Shigella* (SS) agar and incubation at 37^{o}C for 24h.

Typical *Salmonella* colonies which appeared colourless with black centres were picked and confirmed as Gram negative by Gram-staining. Pure cultures of isolates were made on slants of nutrient agar, incubated at 37^{o}C for 24h and stored in refridgerator at 4^{o}C as stock cultures of presumptive *Salmonella* for further identification.

3.12 Isolation of *Vibrio cholerae* From Water, Vegetable and Manure Treated Soil

Vibrio was isolated from samples using the method of Islam *et al*. (1992). Samples were pre-enriched in alkaline peptone water and incubated at 37^{o}C for 24h. The enriched broth was streaked on thiosulphate citrate bile salt (TCBS) agar and incubated at 37^{o}C for 24h. Typical *Vibrio* colonies which appeared yellow were picked and independently confirmed Gram negative by Gram staining. Pure isolates were stored on nutrient agar slants as stock cultures of presumptive *Vibrio* isolates for further identification.

3.13 Biochemical Characterization By Conventional Method

All the presumptive isolates were subjected to series of biochemical tests. Biochemical identification of isolates was as described by Farmer (1999) as follows:

3.13.1 Motility-Indole-Urease (MIU)

The isolates were each stab-inoculated into the MIU medium and incubated at 37^{o}C for 24h. Growth on the line of inoculation and diffusion of organism in the softer 0.4 % (w/v) agar indicated motility. The formation of a red ring upon the addition of 0.2ml of Kovac`s reagent indicated indole formation and non formation of red ring indicated a negative indole result, while a change in urease agar to pink indicated a positive result.

3.13.2 Methyl red-Voges-Proskaeur (MR-VP) test

The colonies of presumptive isolates were inoculated into five millilitres of phosphate buffered peptone medium (pH 7.5) and incubated at 37^{o}C for 48h. After incubation, one millilitre of broth was transferred into a sterile serological tube and three drops of methyl red indicator was added. A red colouration was considered positive. To one millilitre of the remaining broth culure, 0.6ml of ethanolic solution of 5% α-naphtol and 0.2ml of 40% potassium hydroxide solution were added sequentially. After a vigorous shaking, the tube was placed in a slopping position with its cap loosened. Development of red colour after 30-60minutes was considered positive, while no colour change indicated negative for VP test.

3.13.3 Citrate utilization test

Colonies of presumptive isolates were grown in 0.85% normal saline for 24h. Using a sterile wire loop a loopful of the broth culture was transferred to the surface of sterile Simmon`s citrate agar in bottles. Inoculated bottles were then incubated at 37^{o}C for 24-72h with loose cap. Growth of the organism, with change in colour from green to blue was the evidence of a positive test and no colour change indicated negative.

3.13.4 Kliger`s Iron Agar (KIA) test

Using a sterile straight wire, the isolates were streaked and butt-stabbed into sterile KIA slants. The tubes were loosely capped and incubated at 37^{o}C for 24h. The tubes were then observed for the production of acid butt and gas and for hydrogen sulphide production, indicated by the blackening of butt due to the decomposition of sulphur compounds to release H_2S on KIA. Negative reaction were regarded as presumptive for *E. coli* and *Vibrio* respectively, while acid/alkaline reaction and gas formation with blackening due to H_2S were regarded as presumptive for *Salmonella*.

3.13.5 Oxidase test

Isolates were tested for oxidase production. Smear of test isolates was made on oxidase test strips (Oxoid) and observed for any colour change. Colour change from white to deep violet/purple at the area of the smear with the test organism within 2-3 seconds (maximum 1 minute) was recorded as positive reaction, while smears of test isolates with no colour change indicated negative reaction.

3.14 Biochemical Identification Of Isolates By The Use Of Microbact Gram-Negative System

The microbact- Gram-negative system (Oxoid), a standardized micro-substrate system designed to simulate conventional biochemical substrates for the identification of *Enterobacteriaceae* was further used to confirm conventional biochemical characterization of organisms following manufacturer`s instructions. Organism identification by microbact-Gram–negative system was based on pH change and substrate utilization as established by published reference methodologies. The 24E solid microplate format made up of 24 substrates was used for further biochemical characterization of isolates. From a 24h culture, three isolated colonies were picked and emulsified in a 5.0ml of sterile saline solution and mixed thoroughly to obtain a homogenous bacterial suspension. One hundred (100µl) of the bacterial suspension was dispensed into each well of microbact identification system and incubated at 37^{o}C for 24h. Reactions were evaluated as positive or negative by comparing them with the colour chart. Results were recorded in the report form provided which generated an octal coding system. Each group of three reactions provided a single digit of the code. Using the results obtained, the indices of the positive reactions were circled. The sum of these indices in each group of three reactions formed the code number. The code was

entered into the computer package. The microbact computer aided identification package generated percentage score against the organism's name as share of the probability for that organism as a part of the total probabilities of all choices.

3.15 Serological Identification Of Isolates

All *E. coli* isolates stored on nutrient agar slants as stock culture were identified serologically using *E. coli* O157:H7 latex test kit (Oxoid, Basingstoke, and Hampshire, England). The procedure was according to manufacturer`s instructions. Latex reagents were brought to room temperature, one drop of saline was placed on the circle distant from the latex. Using a loop, a portion of the colony of presumptive *E. coli* O157:H7 on blood agar plates was emulsified in the saline drop on a portion of the circle on the card. The test latex and the resulting smooth suspension were mixed together and spread to cover the reaction area using the loop. The card was rocked in a circular motion observing for agglutination within one minute. Agglutination of the test latex within one minute was recorded as positive for *E. coli* O157:H7 (Oxoid, Basingstoke, and Hampshire, England). Since the Oxoid *E. coli* O157:H7 latex test agglutinates only *E. coli* strains possessing the O157:H7 sero-group antigen.

3.16 Determination of *E. coli* Serogroup

A further determination of the O- antigen was carried out with specific antisera (Denken Seiken, Japan) using the slide agglutination method. Three discrete colonies of the bacterial isolate were suspended in a test tube containing physiological saline heated to 121^{o}C for 15minutes. The heated suspension was centrifuged at 900g (1500rpm) for 20minutes, and the supernatant was discarded. The resulting sediment was suspended in 0.5ml physiological saline and was used as the antigenic suspension. A drop of the antisera and 30µl physiological saline was placed on a clean glass slide

to serve as control. A 10µl antigenic suspension was placed onto the serum and physiological saline on the glass slide. The reagents were mixed together by tilting the glass slide back and forth for 1 minute and the agglutination pattern was observed. Strong agglutination observed within 1 minute in the reaction with each serum was recorded as positive, while delayed or weak agglutination was regarded as negative. ATCC 25922 was used as control strain of *Escherichia coli*.

3.17 Detection of Verotoxin *E. coli* (VTEC) VT1 and VT2

The reverse passive agglutination test method was used for the detection of VT1 and VT2 from *Escherichia coli* O157:H7. In reverse passive latex agglutination (RPLA), the antibody is attached to particles, latex does not play a part in the reaction, hence is passive and reacts with the specific soluble antigen to produce a visible precipitate or lattice structure. Toxin production and extraction was performed using the solid culture method. Isolated organism was inoculated onto Brain Heart infusion agar (Oxoid) slopes and incubated at 37^{o}C for 30minutes for extraction, with occasional shaking. After extraction, the culture was centrifuged at 4,000rpm for 20minutes at 4^{o}C. The filtrate was retained for the verocytotoxin assay. Assay method was according to manufacturer`s (Oxoid) instruction. Toxin controls (TD0964 and TD0965) provided reference as the positive patterns.

3.17.1 Assay method for detection of verotoxin *E. coli* (VTEC) VT1 and VT2

To a row of microtitre plates arranged in three columns each consisting of 8 wells, 25µl of dilluent (TD0966) was dispensed into each well using a micropipette. Twenty five (25µl) of test sample supernatant was added to the first well of each column, a microdiluter was used to pick up 25µl of the resultant suspension and doubling dilutions was carried out down each column, up to and including row 7.25µl

of sample and buffer mix was removed from the 7th well and discarded. With the last row of well left containing dilluent (TD0966) only, 25µl of the test latex VT1 (TD0961) was added to each well in the first column. About 25µl of test latex VT2 (TD0962) to each well in the second column and 25µl of latex control (TD0963) was added to each well in the third column to detect false agglutination reactions. The toxin controls (TD0964 and TD0965) were also assayed in the same manner as test samples. The microtitre plates were incubated in a moisture box in a vibration free surface at room temperature for 24h. Each well in each column was examined for agglutination against a black background after 24h. Agglutination patterns were recorded as negative (−, ±) and positive (+, ++ and +++).

3.18 Serological Identification of *Salmonella*

Salmonella serotyping was done according to Kauffman-White Scheme (Grimont and Weill, 2007) involving the use of *Salmonella* antisera.

Polyvalent *Salmonella* antisera A-G, A-S, surface antigen, phase 1 and phase 2 flagellar H antigens (Denka Seiken, Japan) were used for the serological identification of *Salmonella*.

3.18.1 Principle

Identification was based on the production of agglutination on mixing of *Salmonella* strain with antigens correspondent to the reagent/antiserum. The antigen - antibody reaction occurs to produce agglutination. The reaction was macroscopically observed to determine each serotype.

3.18.2 Procedure

Antigenic suspension of each isolate was made by suspending 3 colonies of pure culture of isolate in 0.5ml physiological saline. A drop of each polyvalent antiserum and 30µl of physiological saline was placed on a clean glass slide and used as control. Ten (10µl) of antigenic suspension was placed on a clean glass slide, serum and physiological saline was placed on the other end of the glass slide. The reagents were mixed by tilting the glass slide back and forth for one minute and the agglutination pattern was observed. Agglutination was observed with light through the slide. Strong agglutination observed within one minute was recorded as positive while delayed or weak agglutination was regarded as negative. To a 2ml of physiological saline, 0.2ml of the antigenic suspension was dispensed and heated to about 121°C for 15minutes. The heated solution was centrifuged at 900g for 20minutes. The supernatant was discarded. The sediment was suspended in 0.2ml physiological saline and was used as heated cell suspension. A drop of the polyvalent antiserum and 30µl physiological saline was placed on a cleaned glass slide and used as control. On another glass slide, 10µl of antigenic suspension was mixed with serum and physiological saline by tilting the glass slide back and forth for one minute and agglutination pattern was observed and results recorded.

3.19 Serological Identification of *Vibrio cholerae*

Serological identification of *Vibrio cholerae* was carried out with the use of *Vibrio cholerae* antisera (Denka Seiken, Japan); Polyvalent antiserum (containing antigenic factor A, B and C), *V.cholerae* serovar Ogawa antiserum (factor B), *V.cholerae* serovar Inaba antiserum (factor C) and the *V.cholerae* O139 ``Bengal`` antiserum.

Vibrio cholerae was serologically classified into O1 and O139 serotypes and the *V.cholerae* O1 strains were further classified into serovar Ogawa and serovar Inaba according to the presence of antigenic factor B. The transitional type from serovar Ogawa to serovar Inaba referred to as serovar Hikojima was also identified.

3.19.1 Method of assay

Bacterial growth (*Vibrio*) was suspended in 0.5ml physiological saline and was used as antigenic suspension. A drop of antiserum and 30µl physiological saline placed on clean test glass slide served as control. To another glass slide containing a drop of serum and 30µl physiological saline, 30µl antigenic suspension was added. The reagents and suspension were added, and mixed by tilting the glass slide back and forth for one minute while observing the agglutination pattern. Agglutination was grossly observed with light through the slide. Strong agglutination observed within one minte was regarded as positive, while delayed/weak agglutination was regarded as negative. Inference was based on the table (Appendix VI) provided by the manufacturer (Denka Seiken, Japan)

3.20 *V. cholerae* Enterotoxin Test

The *V.cholerae* enterotoxin reversed passive latex agglutination (VET-RPLA) test kit was used for toxin assay in the *Vibrio cholerae* isolates.

3.20.1 Principle of assay

Polysterene latex particles are senstised with purified antiserum taken from rabbits immunized with purified *Vibrio cholerae* enterotoxin. These latex particles will agglutinate in the presence of *V.cholerae* enterotoxin (CT). A control reagent is provided which consists of latex particles sensitized with non-immune rabbit globulins.

The test is performed in V-well microtitre plates. Dillutions of the culture filtrate are made in two rows of wells, 0.5ml the latex suspension is added to each well and the contents mixed. If the toxin is present, agglutination occurs which results in the formation of a lattice structure, upon setting, forming a diffuse layer on the base of well. If enterotoxin is absent or at a concentration below the assay detection level, no such lattice structure is formed hence a tight button will be observed.

3.20.2 Assay

Isolates of *Vibrio cholerae* were inoculated in alkaline peptone water adjusted to pH 8.4 and incubated at 37^{o}C for 24h. Two (2ml) mililiters of the broth was centrifuged at 3,000rpm for 20minutes at 4^{o}C. The supernatant obtained served as the test sample. Twenty five microlitre of dilluent was dispensed in two rows of microtitre plates each consisting of eight wells except the first well in each row. To the first and second wells of each row 25µl of test sample was added. Starting at the second well of each row, doubling dilution was carried out along each of the two rows except the last well (leaving the last well containing diluent only). Twenty five (25µl) of sensitized latex was added to each well of the first row, while 25µl of latex control was added to each well of the second row. The contents of each well were mixed together on agitation. The microtitre plates were then placed in a moisture box and left undisturbed on vibration free surface at room temperature for 24h. Agglutination patterns were read as (+), (++) and (+++) and considered positive while non agglutination was considered negative (Oxoid, Basington, England).

3.21 Determination of Antibacterial Drug- Susceptibility

Antibacterial susceptibility testing was performed by the disc diffusion method (Khan *et al.*, 2006). Using commercially available disks (Oxoid) consisting of eight

antibacterial drugs; amoxicillin/clavulanate (30µg), sulfamethoxazole/trimethoprim (25µg), ciprofloxacin (5µg), ceftriaxone (30µg), gentamicin (10µg), tetracycline (30µg), kanamycin (30µg), and cephalothin (30µg).

Three colonies of the test organism were inoculated in Mueller-Hinton (Oxoid) broth and incubated at 37^{o}C until the turbidity approximated 0.5 McFarland`s standard. The broth suspension was spread on the surface of the Mueller-Hinton agar (Oxoid) in the petri dish using a sterile bent glass rod and was allowed to dry for five minutes before the paper discs containing the antibiotics were placed on the agar plates with sterilized forceps. Plates were incubated at 37^{o}C for 24h. The inhibition was measured using a meter rule on the under surface of the petri dish. Test was carried out in duplicates and mean value was recorded. *Escherichia coli* ATCC strain 25922 which is sensitive to all the antibiotics served as control. Isolates were considered susceptible, intermediate and resistant to a particular antibacterial agent on the basis of the diameters of the inhibitory zones that marked the criteria of the manufacturer`s interpretative table, which followed the recommendations of the Clinical and Laboratory Standards Institute guidelines (CLSI, 2010).

3.22 Molecular Characterization of *Escherichia coli* O157:H7

The identities of the *E.coli* O157:H7 isolates were further confirmed through the amplification of the *uid*A, *rfb*O_{157}, Sxt_1 and Sxt_2 gene fragments.

3.22.1 Template DNA preparation using extraction kit

A single colony of pure *E.coli* O157:H7 was inoculated into 10ml of Luria-Bertani (LB) broth medium and incubated at 37^{o}C overnight. One (1ml) of the overnight culture was centrifuged for 2min at 14,000 x g. Pelleted bacterial cells were

resuspended thoroughly in 480 µl of 50mM EDTA, digested in 120 µl10mg/ml lysozyme and incubated at 37^{o}C for 60 minutes before being subjected to DNA extraction using Wizard Genomic DNA Purification Kit (Promega, Madison, USA) by following the manufacturer`s instructions.

3.22.2 DNA amplification and detection

A total of 19 *E.coli* O157:H7 isolated, identified and characterized in the study were further confirmed by detecting the presence of *uid*A (187bp) gene according to Heijen and Medema (2006). The presence of the O157:H7 gene was also further confirmed by detecting the *rfb*O_{157} (420bp) gene according to Visetsripong *et al*. (2007) with some modification (different annealing temperature of 60^{o}C). The primers, PCR preparations and conditions used are presented in Table 3.1. Amplification was performed using thermal cycler (Bio-Rad Thermal cycler, California, USA). In all the PCR assays, a positive control (reaction tube with DNA from *E.coli* ATCC 25922) and a negative control (reaction tube with nuclease-free water) were included.

3.22.3 Genotypic determination of diversity among *E.coli* O157:H7 isolates using DNA fingerprinting assay

From the characterized *E.coli* O157:H7 isolates, 19 representative isolates were analysed using Enterobacterial Repetitive Intergenic Concensus fingerprints (ERIC PCR) to determine their genetic relatedness. The primers, PCR preparations and conditions used are presented in Table 3.1.

3.22.4 Electrophoresis of PCR amplicons

The PCR products (amplicons) were separated by electrophoresis on a 2% (w/v) agarose gel containing 2µl florosafe. Electrophoresis was run at 100v for 30mins

Table 3.1 Primer sequences, PCR preparations and conditions used in molecular characterization and genotyping of *E.coli* O157:H7 in the study

Target	Primer sequence	PCR preparation (25µl)	PCR conditions	Reference (s)
*uid*A	UAL- 1939b 5`-ATGGAATTTCGCCGATTTTGC-3` UAL-2105b 5`-ATTGTTTGCCTCCCTGCTGC-3`	12.5 µl Dream Taq mastermix,8.5 µl nuclease free water, 3 µl template DNA and 0.5 µl of each primer mix.	3mins at 95^{o}C prior to 30cycles of 30secs of 95 ^{o}C, Annealing temperature at 60 ^{o}C for 30secs, extension at 72 ^{o}C for 1min and a final extension of 5mins at 72 ^{o}C.	Heijen and Medema, (2006)
rfbO_{157}	O157PF-8 5`-CGTGATGATGTTGAGTTG-3` O157PR-8 5`-AGATTGGTTGGCATTACTG-3`	12.5 µl Dream Taq mastermix,8.5 µl nuclease free water, 3 µl template DNA and 0.5 µl of each primer mix.	5mins at 94^{o}C prior to 35cycles of 1min at 94 ^{o}C, Annealing temperature at 60 ^{o}C for 90mins, extension at 72 ^{o}C for 1 min and a final extension of 10mins at 72 ^{o}C.	Visetsripong *et al*. (2007)
SXT1	SXTA1- 598 5`-AGTCGTACGGGGATGCAGATAAAAT-3` SXTA1-1015 5`-CCGGACACATAGAAGGAAACTCAT-3`	12.5 µl Dream Taq mastermix, 8.5 µl nuclease free water, 3 µl template DNA and 0.5 µl of each primer mix.	5mins at 94^{o}C prior to 35cycles of 1min at 94 ^{o}C, Annealing temperature at 60 ^{o}C for 90mins, extension at 72 ^{o}C for 1 min and a final extension of 10mins at 72 ^{o}C.	Bellin *et al*. (2001)
SXT2	SXTA2-679 5`-TTCCCGAATGCAAATCAGTC-3` SXTA2-942 5`-CGATACTCCGGAAGCATTG-3`	12.5 µl Dream Taq mastermix, 8.5 µl nuclease free water, 3 µl template DNA and 0.5 µl of each primer mix.	5mins at 94^{o}C prior to 35cycles of 1min at 94 ^{o}C, Annealing temperature at 60 ^{o}C for 90mins, extension at 72 ^{o}C for 1 min and a final extension of 10mins at 72 ^{o}C.	Bellin *et al*. (2001)
ERIC	$ERIC_1$ 5`-ATGTAAGCTCCTGGGGATTCAC-3` $ERIC_2$ 5`-AAGTAAGTGACTGGGGTGAGCG-3`	12.5 µl Dream Taq mastermix, 8.5 µl nuclease free water, 3 µl template DNA and 0.5 µl of each primer mix.	10mins at 95^{o}C prior to 34cycles of 30secs at 94 ^{o}C, Annealing temperature at 50 ^{o}C for 1min, extension at 65 ^{o}C for 1 min and a final extension of 8mins at 65 ^{o}C.	Versalovic *et al*. (1994)

Key: *uid*A=β-glucoronidase gene, *rfb*$O_{157=}$ O157 antigen gene, Sxt1, Sxt2= Shiga toxin 1 and 2 gene, ERIC= Enterobacterial repetitive intergenic concensus.

for gene detection while for DNA fingerprinting, electrophoresis was run at 120v for 45mins and visualized on an ultra violet (UV) transilluminator gel imaging system (Bio-Rad Gel Imaging system, Bio-Rad, California, USA). Bands were photographed and band positions were determined and compared to molecular weight markers (1kb and 100bp DNA ladders, First base, Malaysia).

3.23 Molecular Characterization of *Salmonella enterica*

The identities of the *Salmonella enterica* (10 *S.typhi*, 6 *S.tyhphimurium* and 2 *S.paratyphi*) isolates were selected based on the distribution of their abundance in the study and further confirmed through the amplification of the specific *Salmonella* invasive (*inv*A) gene and the *Salmonella* enterotoxin (*Stn*) gene fragments.

3.23.1 Template DNA preparation using extraction kit

A single colony of pure *Salmonella enterica* was inoculated into 10ml of Luria- Bertani (LB) broth medium and incubated at 37^{o}C overnight. One (1ml) of the overnight culture was centrifuged for 2min at 14,000 x g. Pelleted bacterial cells were resuspended thoroughly in 480 µl of 50mM EDTA, digested in 120 µl of 10mg/ml lysozyme and incubated at 37^{o}C for 60 min before being subjected to DNA extraction using Wizard Genomic DNA Purification Kit (Promega, Madison, USA) by following the manufacturer`s instructions.

3.23.2 DNA amplification and detection

A total of 15 *Salmonella enterica* isolates, identified and characterized in the study were further confirmed by detecting the presence of the *Salmonella* invasion *inv*A (187bp) gene according to Nucera *et al*. (2006). The presence of the *Salmonella* enterotoxin (Stn) gene was also further confirmed (284bp) according to Chopra *et al*.

(1999). The primers, PCR preparations and conditions used are presented in Table 3.2. Amplification was performed using thermal cycler (Bio-Rad Thermal cycler, California, USA). A negative control (reaction tube with nuclease-free water) was included as quality control.

3.23.3 Genotypic determination of diversity among *Salmonella enterica* isolates using DNA fingerprinting assay

From the characterized *Salmonella enterica* isolates, 18 representative isolates were analysed using enterobacterial repetitive intergenic concensus fingerprints (ERIC PCR) to determine their genetic relatedness. The primers, PCR preparations and conditions used are presented in Table 3.2

3.23.4 Electrophoresis of PCR amplicons

The PCR products (amplicons) were separated by electrophoresis on a 2% (w/v) agarose gel containing 2µl florosafe. Electrophoresis was run at 100v for 30min for gene detection while for DNA fingerprinting, electrophoresis was run at 120v for 45min and visualized on an ultra violet (UV) transilluminator gel imaging system (Bio-Rad Gel Imaging system, Bio-Rad, California, USA). Bands were photographed and band positions were determined and compared to molecular weight markers (1kb and 100bp DNA ladders, First base, Malaysia).

3.24 Molecular Characterization of *Vibrio cholerae* O1

The identities of the *Vibrio cholerae* O1 isolates were further confirmed through the amplification of the cholera toxin *ctx*A subunit.

Table 3.2 Primer sequences, PCR preparations and conditions used in molecular characterization and genotyping of *Salmonella enterica* obtained in the study

Target	Primer sequence	PCR preparation (25µl)	PCR conditions	Reference(s)
*inv*A	SAL1 5`-GTGAAATTATCGCCACGTTCGGGCAA-3` SAL2 5`-TCATCGCACCGTCAAAGGAACC-3`	12.5 µl Dream Taq mastermix,8.5 µl nuclease free water, 3 µl template DNA and 0.5 µl of each primer mix.	5mins at 94^{o}C prior to 35cycles of 1min at 94 ^{o}C, Annealing temperature at 55 ^{o}C for 1min, extension at 72 ^{o}C for 1min and a final extension of 10mins at 72 ^{o}C.	Nucera *et al.* (2006)
Stn	Stn P15 5`-TTGTGTCGCTATCACTGGCAACC-3` StnM13- 5`-ATTCGTAACCCGCTCTCGTCC-3`	12.5 µl Dream Taq mastermix,8.5 µl nuclease free water, 3 µl template DNA and 0.5 µl of each primer mix.	5mins at 94^{o}C prior to 35cycles of 1min at 94 ^{o}C, Annealing temperature at 60 ^{o}C for 90mins, extension at 72 ^{o}C for 1 min and a final extension of 10mins at 72 ^{o}C.	Chopra *et al.* (1999)
ERIC	$ERIC_1$ 5`-ATGTAAGCTCCTGGGGATTCAC-3` $ERIC_2$ 5`-AAGTAAGTACTGGGGTGAGCG-3`	12.5 µl Dream Taq mastermix,8.5 µl nuclease free water, 3 µl template DNA and 0.5 µl of each primer mix.	1min at 94^{o}C prior to 1min at 95^{o}C, Annealing temperature at 52 ^{o}C for 1min, extension at 65 ^{o}C for 8 mins and a final extension of 16mins at 65 ^{o}C.	Beyer *et al.*, (1998)

Key: *inv*A=*Salmonella* invasion gene, *Stn*=*Salmonella* enterotoxin gene and ERIC= Enterobacterial repetitive intergenic concensus

3.24.1 Template DNA preparation using extraction kit

A single colony of pure *Vibrio cholerae* O1 was inoculated into 10ml of Luria- Bertani (LB) broth medium and incubated at 37^{o}C overnight. One millilitre of the overnight culture was centrifuged for 2min at 14,000 x g. Pelleted bacterial cells were resuspended thoroughly in 480 µlof 50mM EDTA, digested in 120 µl10mg/ml lysozyme and incubated at 37^{o}C for 60 min before being subjected to DNA extraction using Wizard Genomic DNA Purification Kit (Promega, Madison, USA) by following the manufacturer`s instructions.

3.24.2 DNA amplification and detection

A total of 17 *Vibrio cholerae* O1 isolated, identified and characterized in the study were further confirmed by detecting the presence of the cholera toxin *ctx*A subunit (564bp) gene according to Lipp *et al*. (2003) with some modification (This study of the annealing temperature to 55^{o}C). The primers, PCR preparations and conditions used are presented in Table 3.3. Amplification was performed using thermal cycler (Bio-Rad Thermal cycler, California, USA). A negative control (reaction tube with nuclease-free water) was included as quality control.

3.24.3 Genotypic determination of diversity among *Vibrio cholerae* O1 isolates using DNA fingerprinting assay

From the characterized *Vibrio cholerae* O1 isolates, 17 representative isolates were analysed using Enterobacterial repetitive intergenic concensus fingerprints (ERIC PCR) to determine their genetic relatedness. The primers, PCR preparations and conditions used are presented in Table 3.3.

Table 3.3 Primer sequences, PCR preparations and conditions used in molecular characterization and genotyping of *Vibrio cholerae* O1 obtained in the study

Target	Primer sequence	PCR preparation (25µl)	PCR conditions	Reference (s)
*ctx*A	F 5`-GGGCAGATTCTAGACCTCCTG-3` R 5`-CGATGATCTTGGAGCATTCCCAC-3`	12.5 µl Dream Taq mastermix, 8.5 µl nuclease free water, 3 µl template DNA and 0.5 µl of each primer mix.	2mins at 94^{o}C prior to 30cycles of 1min at 94^{o}C, Annealing temperature at 55^{o}C for 1min, extension at 72 ^{o}C for 1min and a final extension of 10mins at 72 ^{o}C.	This study.
ERIC	ERIC$_1$ 5`-TGTAAGCTCCTGGGGATTCAC-3` ERIC$_2$5`-AGTAAGTGACTGGGGTGAGCG-3`	12.5 µl Dream Taq mastermix,8.5 µl nuclease free water, 3 µl template DNA and 0.5 µl of each primer mix.	10mins at 95^{o}C prior to 34cycles of 30secs at 94 ^{o}C, Annealing temperature at 50 ^{o}C for 1min, extension at 65 ^{o}C for 1 min and a final extension of 8mins at 65 ^{o}C.	Versalovic *et al*. (1994)

Key: *ctx*A= cholera toxin subunitA gene and ERIC= Enterobacterial repetitive intergenic concensus.

3.24.4 Electrophoresis of PCR amplicons

The PCR products (amplicons) were separated by electrophoresis on a 2% (w/v) agarose gel containing 2µl florosafe. Electrophoresis was run at 100v for 30min for gene detection while for DNA fingerprinting, electrophoresis was run at 120v for 45min and visualized on an ultra violet (UV) transilluminator gel imaging system (Bio-Rad Gel Imaging system, Bio-Rad, California, USA). Bands were photographed and band positions were determined and compared to molecular weight markers (1kb and 100bp DNA ladders, First base, Malaysia).

3.25 Detection of Resistance Genes And Mobile Genetic Elements in Pathogens Obtained in The Study

The presence of quinolone resistance- determining region (QRDR) of gyrA (448bp) according to Gomez *et al*. (2004), plasmids conferring multidrug resistance to aminoglycosides (pcT) (428bp) according to Cottell *et al*. (2011), determination of mobile genetic elements; Class 1 integron and sul1 gene (Deekshit *et al*., 2012) were assayed for in the study. The variable region of Class 1 integron was characterized by PCR and sequencing of the amplicons was obtained. The primers, PCR preparations and conditions used are presented in Table 3.4.

3.26 Sequence Analysis

The presence of genetic determinants was confirmed by amplicon sequence analysis. PCR products were purified using PCR clean up kit (First base, Malaysia) according to manufacturer`s instructions. Purified PCR products were sequenced by Sanger method (3730XL, Applied Biosystems). Nucleotide sequences were edited by BioEdit software

Table 3.4 Primer sequences, PCR preparations and conditions used in molecular characterization of antibiotic resistance genes of pathogens obtained in the study

Target	Primer sequence	PCR preparation (25µl)	PCR conditions	Reference (s)
QRDR of *gyr*A	gyrAF 5`-GCGCGTGAGATGACCCGCCGT-3` gyrAR 5`-CTGGCGGTAGAAGAAGGTCAG-3`	12.5 µl Dream Taq mastermix,8.5 µl nuclease free water, 3 µl template DNA and 0.5 µl of each primer mix.	5mins at 94^{o}C prior to 35cycles of 1min at 94 ^{o}C, Annealing temperature at 54 ^{o}C for 1min, extension at 72 ^{o}C for 1min and a final extension of 10mins at 72 ^{o}C.	Gomez *et al*. (2004).
pCT	pCT(008)F 5`-CATTGTATCTATCTTGTGGG-3` Pct(009)R 5`-GCATTCCAGAAGATGACGTT-3`	12.5 µl Dream Taq mastermix,8.5 µl nuclease free water, 3 µl template DNA and 0.5 µl of each primer mix.	5mins at 94^{o}C prior to 35cycles of 1min at 94 ^{o}C, Annealing temperature at 60 ^{o}C for 1min, extension at 72 ^{o}C for 1 min and a final extension of 10mins at 72 ^{o}C.	Cottel *et al*. (2011).
Class one integrone	Int1 F 5`-GCCTTGCTGTTCTTCTACGG-3` Int1 R 5`-GATGCCTGCTTGTTCTACGG-3`	12.5 µl Dream Taq mastermix, 8.5 µl nuclease free water, 3 µl template DNA and 0.5 µl of each primer mix.	30secs at 95^{o}C prior to 35cycles of30secs at 94 ^{o}C, Annealing temperature at 60 ^{o}C for 2mins, extension at 72 ^{o}C for 2 mins and a final extension of 5mins at 72 ^{o}C.	Shahcheraghi *et al*. (2009)
sul1	Sul1 F 5`-TTTCGGCATTCTGAATCTCAC-3` Sul1 R 5` ATGATCTAACCCTCGGTCTC-3`	12.5 µl Dream Taq mastermix, 8.5 µl nuclease free water, 3 µl template DNA and 0.5 µl of each primer mix.	3mins at 95^{o}C prior to 35cycles of 1min at 94 ^{o}C, Annealing temperature at 47 ^{o}C for 90secs extension at 72 ^{o}C for 1 min and a final extension of 10mins at 72 ^{o}C.	Van *et al*. (2008)

Key QRDR=Quinolone resistance determining region, pCT= Multidrug resistant plasmid, sul= Sulfonomide resistance gene.

and compared with known sequences in the Genbank, using BLASTN of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST).

3.27 Statistical Analysis

Numerical data generated in the field/laboratory were entered directly into spreadsheet to generate tables and figures, using Microsoft excel. Significant ($P<0.05$) differences between mean's were determined using analysis of variance (ANOVA) and student's t-test. Differences was established using Duncan's multiple range test. Statistical Package for Social Sciences (SPSS) version 19 and Pearson's correlation were used to determine the relationship between the faecal coliform counts and the physicochemical water quality parameters and the relationship amongst the parameters (Colton *et al*.,1974).

3.28 Cluster Analysis And Calculation of Discriminatory Index

DNA fingerprint positions were defined as presence of DNA band (a score '1') and absence of DNA band (a score '0'). The scores were entered into NTedit to obtain a data matrix and then inserted into NTSYS pc version 2.2 computer software for the construction of dendrogram based on simple matching coefficient and unweighted pair-group arithemetic average clustering (UPGMA).

CHAPTER FOUR

4.0 RESULTS

4.1 Characteristics of Farmers And Sources of Manure

Analysis of responses to questionnaire showed that all farmers visited in Kano State were males, while in Plateau State, 81.2% were male and 18.8% were female farmers (Figure 4.1). Farming and trading of farm products (vegetables) was the basic occupation of those visited. A comparison of the income level generated showed that majority of the farmers earned between ₦100,000 and ₦200,000 per annum (Figure 4.2). Information on the level of education of the farmers revealed that 6.3% had tertiary education, 37.4% had secondary school education/attempted secondary school, 31.3% had primary education, , while 25.0% had no formal education. The sources of water for irrigation by the farmers include well (20%), river (59%), tap (19%) and dam (1%) (Figure 4.3).

Point sources of contamination to irrigation water in the study included; livestock waste, industrial waste, domestic and municipal waste comprising human sewage also. Irrigation water in Kano was observed to be most polluted by livestock (Figure 4.4). In Plateau State, irrigation water was most polluted with domestic waste. Vegetable farmers used organic manure, inorganic fertilizer and some used both oraganic manure and inorganic fertilizer as nutrient to vegetables (Figure 4.5). It was observed that 68% of the farmers used organic manure while 5% used inorganic fertilizers and 10% used both organic and inorganic fertilizers at different stages. Cattle dung, goat/sheep dung and poultry droppings were the various types of organic

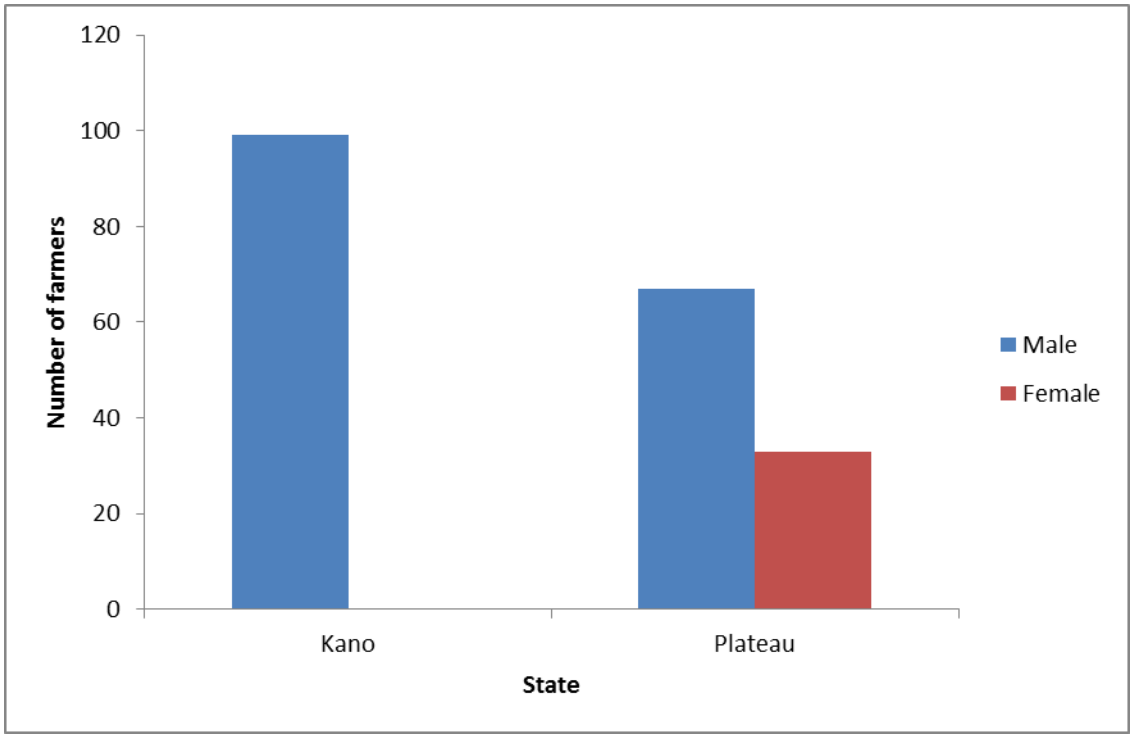


Figure 4.1 Distribution of farmers by gender in the study area

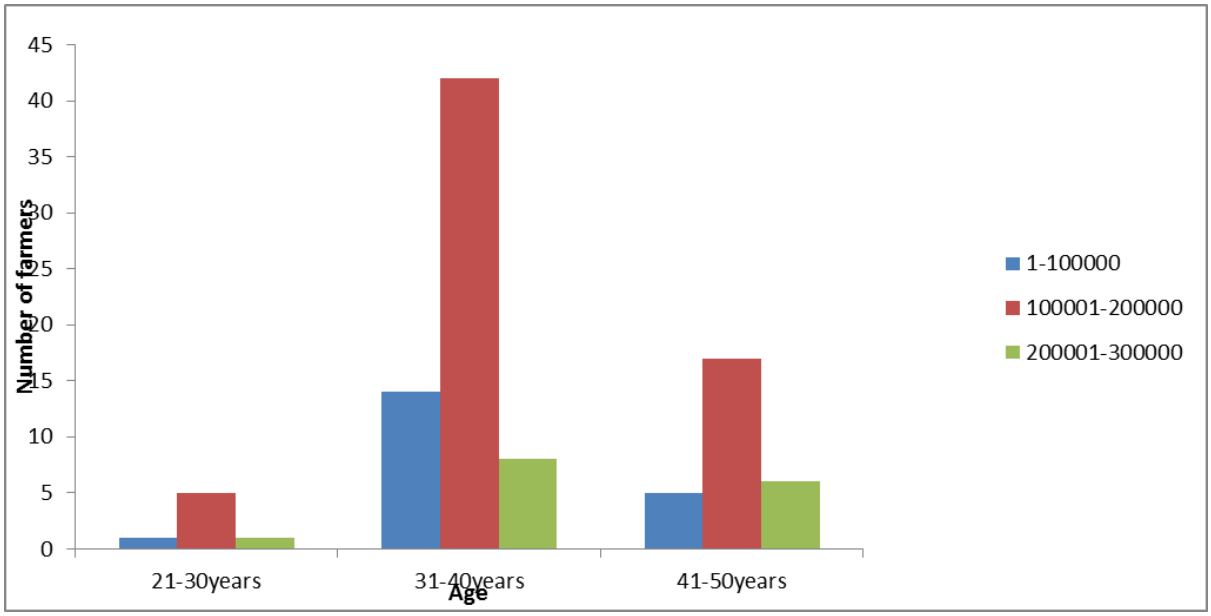


Figure 4.2 Distribution of income level among farmers in the study area

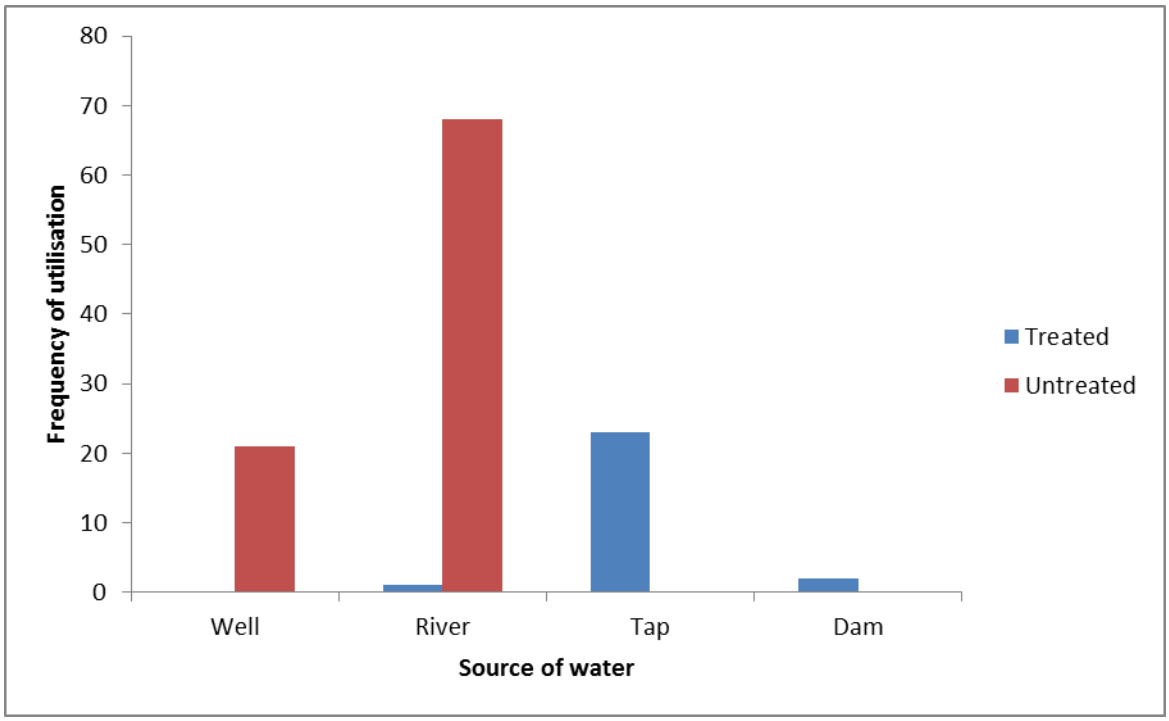


Figure 4.3: Frequency distribution of farmers utilising water source and treatment method in the study area

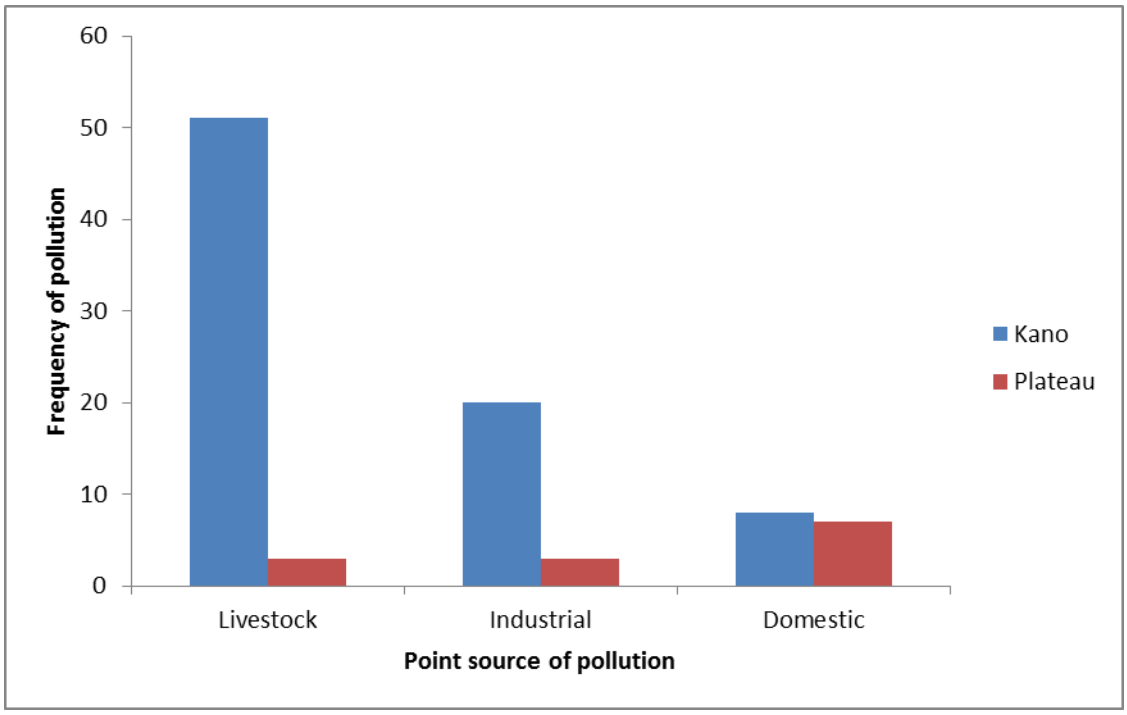


Figure 4.4: Frequency point source of pollution in parts of Kano and Plateau states

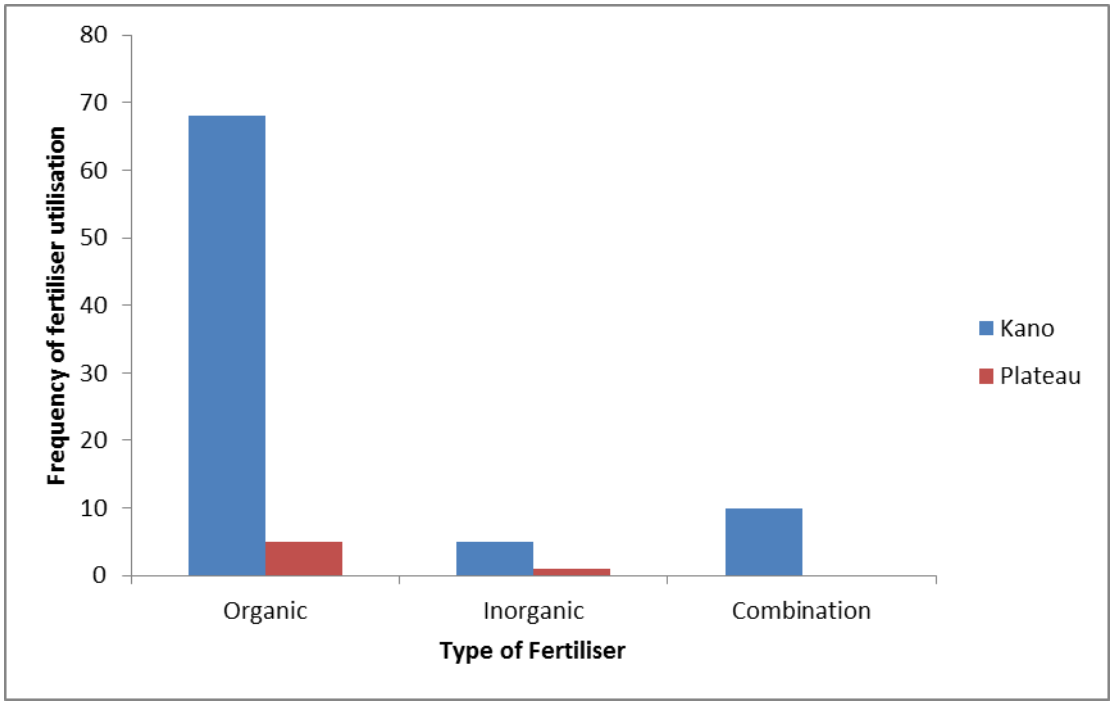


Figure 4.5 The percentage distribution type of fertilisers used in parts of Kano and Plateau States

manure used however, farmers in Kano State used cattle dung more commonly as source of organic manure while, the basic organic manure type used in Plateau State was poultry dropping. Application of manure was done 3-4 weeks after planting by most farmers (Figure 4.6).

On the whole, significant risk factors for faecal contamination of vegetable were point source pollutions to irrigation water sources, use of untreated water for irrigation and use of organic manure for soil replenishment/nutrient supply.

4.2 Faecal Coliform Counts in Samples By Season

Analysis of faecal coliform counts of vegetables from different source of irrigation water in Kano and Plateau State revealed that there was a statistical significant difference ($t=2.237$, $p= 0.047$) in the level of counts on vegetables irrigated with water from well/ponds with a record of higher counts in Kano than that of vegetables irrigated from the same water source type (ponds) in Plateau State (Table 4.1). Vegetables obtained from sites which received contamination from livestock had higher (7.79 ± 7.53) faecal coliform counts than other point source of irrigation water contamination in Kano State while, in Plateau State higher (7.74 ± 7.67) counts were obtained from vegetables that received contamination from domestic waste (Table 4.2).

Significant difference ($t=-3.320$, $P=0.002$) was observed in level of counts of faecal coliform on vegetables irrigated with treated/untreated water, with vegetables irrigated with untreated water having significantly higher counts in Kano and Plateau States ($t= -2.823$, $P=0.007$). However, a comparison of vegetables obtained from varied sites within each State showed no statisticaly significant ($P<0.05$) difference in counts from vegetables irrigated with treated/untreated wastewater, though both recorded higher counts in vegetables.

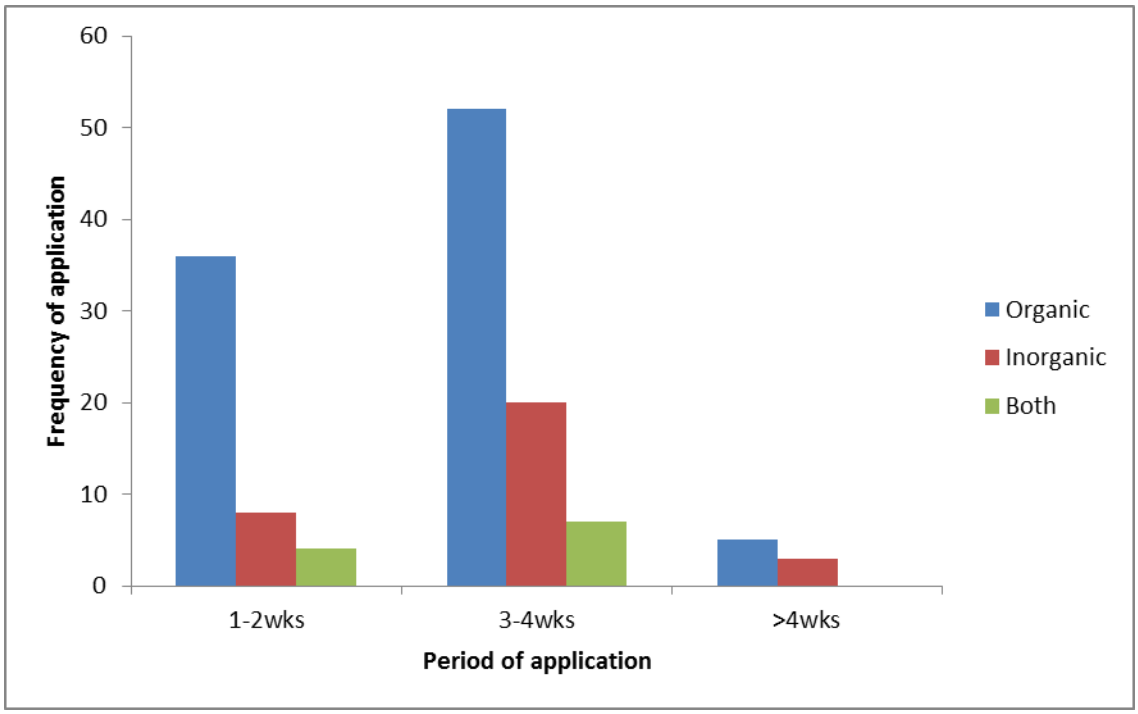


Figure 4.6 Frequency of the period of application of different types of fertilizer used by farmers in the study area

Table 4.1 Effect of source of irrigation water on the mean counts of faecal coliforms on vegetable by State

Parameter	No. of samples analysed	FCC (Kano)[#]	No. of samples analysed	FCC (Plateau)[##]	t-test	p-value
Source of water						
River	74	7.70 ± 7.18	88	7.86 ± 7.40	-0.739	0.463
Well/Ponds	13	7.76 ± 7.38	36	6.46 ± 5.80	2.237	0.047*
Tap	5	5.09 ± 4.90	20	5.85 ± 5.58	-1.484	0.227
Dam	2	5.48 ± 4.81	-	-	-	-
Total	94	7.66 ± 7.06	144	7.66 ± 7.21		

Key: FCC= Feacal coliform counts

[#] F=0.773; p=0.514

*=significant difference exists showing association between counts on vegetables irrigated with water from wells/ponds

[##]F=2.272; p=0.112

Table 4.2 Effect of point source of contamination of irrigation water on the mean counts of faecal coliforms on vegetable by State

Point source pollution	No. of samples analysed	FCC (Kano)[#]	No. of samples analysed	FCC (Plateau)[##]	t-test	p-value
Livestock	20	7.79 ± 7.53	3	7.73 ± 7.45	0.103	0.919
Industrial waste	4	6.46 ± 5.21	11	7.10 ± 6.96	-0.652	0.527
Domestic waste	16	7.45 ± 7.00	5	7.74± 7.67	-0.563	0.610
Total	40	7.61± 7.23	19	7.48 ± 7.11		

[#] F=0.774; p=0.471 [##]F= 1.311; p=0.301

FCC= Mean count (cfu/g) from Kano State
FCC= Mean count (cfu/g) from Plateau State

from untreated irrigation water (Table 4.3). Highest counts were detected from vegetables fertilized with organic manure in both States than those fertilized with inorganic fertilizer (Table 4.4)

Comparison of the level of faecal coliform counts in samples from different point source of contamination in each State of study revealed that in Kano State, vegetables obtained from abattoir wastes contaminated irrigation water sources had significantly (P= 0.045) higher counts than those from other sources. While, in Plateau State, vegetables obtained sewage and industrial wastes contaminated irrigation water sources had higher counts (11.48 ± 11.48). Similarly, sewage and industrial wastes contaminated irrigation water had significantly (P=0.048) higher counts in Plateau State. When all sample types analysed from the different point source pollutions were pooled together, higher counts (12.16 ± 11.67) were obtained from vegetables in Kano State. In Plateau State, irrigation water samples had significantly (P<0.001) higher counts as was also observed in soil/manure. Overall, vegetables obtained from Kano State had significantly (P<0.001) higher counts than those obtained from Plateau State. However, counts observed in soil/manure samples obtained from Plateau State were significantly (P<0.001) higher than those obtained from Kano State (Table 4.5).

Student's t-test revealed that counts of faecal coliform in vegetables obtained from human sewage/ industrial waste polluted site had significantly higher (P= 0.021) counts than all other sites in Kano State in the dry season. In the wet season, vegetables obtained from sites 1, 2, 3 which had abattoir waste, domestic sewage, human sewage and industrial waste had significantly (P<0.005) higher counts than sites 4 which received industrial effluents only and site 5. Generally, significantly higher counts (P=0.043) were obtained from vegetables in the wet season than in the dry season

Table 4.3 Effect of type of irrigation water on faecal coliform counts on vegetables obtained from the two States

Parameter	No of samples analysed	FCC (Kano)[#]	No of samples analysed	FCC (Plateau)[##]	t-test	p-value
Type of irrigation water						
Treated	7	5.53 ± 4.96	20	6.05 ± 5.69	-1.561	0.189
Not treated	77	7.77 ± 7.23	124	7.70 ± 7.24	0.332	0.740

Key: FCC= Feacal coliform counts [#]t= -3.320; p=0.002 [##]t= -2.823; p=0.007

Table 4.4 Effect of type of fertilisers on faecal coliform counts on vegetables obtained from the two States

Fertiliser	No. of samples analysed	FCC (Kano)[#]	No. of samples analysed	FCC (Plateau)[##]	t-test	p-value
Organic	50	7.90 ± 7.45	35	7.98 ± 7.56	-0.323	0.748
Inorganic	14	5.52 ± 5.04	6	5.97± 5.63	2.262	0.299
Organic/Inorganic	26	7.72± 7.21	103	7.87± 7.38	-0.730	0.468
		[#]F = 0.933; p = 0.398		[##]F = 0.513; p = 0.601		

Mean value were separated using Duncan's multiple range test; values with different superscripts in the same column are significantly different.

FCC= Mean count (cfu/g) from Kano State

FCC= Mean count (cfu/g) from PlateauState

Table 4.5 Levels of coliform counts by site contamination of samples by State

Site	Sample type	FCC (Kano)	FCC (Plateau)	t	p-value
I	Vegetable	12.41 ± 12.05[a]	8.06 ± 7.61	2.280	0.040*
	Water	7.77 ± 7.53[b]	12.48 ± 12.48	-2.582	0.020*
	Soil/manure	8.18 ± 7.82[b]	12.84 ± 12.48	-1.195	0.286
		F = 3.455; p = 0.045*	F = 2.265; p = 0.141		
II	Vegetable	12.13 ± 11.87	4.70 ± 4.48	0.566	0.577
	Water	11.88 ± 11.88	7.73 ± 7.45	0.486	0.635
	Soil/manure	7.27 ± 7.17	12.65 ± 12.65	-1.000	0.500
		F = 0.495; p = 0.614	F = 1.429; p = 0.340		
III	Vegetable	11.95 ± 11.95	11.48 ± 11.48[c]	0.824	0.415
	Water	12.29 ± 12.01	12.83 ± 12.35[a]	-2.144	0.048*
	Soil/manure	8.51 ± 8.41	12.56 ± 12.34[b]	-1.633	0.178
		F = 0.450; p = 0.643	F = 13.332; p < 0.001*		
IV	Vegetable	5.54 ± 5.15	6.45 ± 5.80[b]	-3.751	0.001*
	Water	4.97 ± 4.88	5.96 ± 5.95[c]	-0.920	0.525
	Soil/manure	7.41 ± 7.41	7.84 ± 7.78[a]	-0.652	0.552
		F = 1.838; p = 0.252	F = 3.878; p = 0.035*		
V	Vegetable	5.46 ± 5.70	6.13 ± 5.18	-6.704	0.022*
	Water	5.99 ± 5.91	6.69 ± 6.63	-0.707	0.531
	Soil/manure	8.02 ± 7.94	12.83 ± 12.35	-3.000	0.024*
		F = 0.594; p = 0.587	F = 5.000; p = 0.053		
	Total samples				
	Vegetable n=238	12.16 ± 11.67	11.15 ± 11.15[b]	2.649	0.010*
	Water n=84	11.90 ± 11.59	12.38 ± 12.03[a]	-1.414	0.175
	Soil/manure n=118	8.03 ± 7.60	12.54 ± 12.03[a]	-3.289	0.004*
		F = 1.839; p = 0.164	F = 13.305; p < 0.001*		

* Significant difference exists.

N=number tested

Site I (Abattoir liquid waste), site II (Domestic sewage drain), site III (Human sewage/ Industrial waste), site IV (Industrial effluents) siteV (Control: no point source of contamination)

(Table 4.6). In Plateau State, vegetables obtained from abattoir waste contaminated irrigation water had significantly higher (P= 0.002) counts in the wet season than in the dry season. Significantly (P<0.001) higher counts were also obtained from vegetables in the wet season than in the dry (Table 4.7).

The overall results of enumeration of faecal coliform counts in samples showed that higher counts were obtained in the wet than dry season in the study. The effect of physical parameters; water temperature, electrical conductivity (conductance) and three chemical parameters, including hydrogen ion potential (pH), dissolved oxygen (DO) and five-day biochemical oxygen demand (BOD_5) on bacterial level were determined for a total of eighty- four water samples obtained from parts of Kano and Plateau States.

A comparison of mean values of water quality parameters from irrigation water obtained from the different point sources of contamination in Kano State showed that abattoir waste contaminated irrigation water had the highest (8.22 ±7.48) counts of faecal coliform. Higher mean temperature values were obtained from irrigation water obtained from the industrial effluent contaminated water and water obtained from the control site. Irrigation water obtained from human sewage/indusrial waste contaminated source had the highest pH value. Domestic sewage contaminated irrigation water had the highest value of electrical conductivity, while, irrigation water obtained from sites contaminated with industrial effluents and the control site had higher DO and BOD values respectively (Table 4.8). The mean values of water quality parameters in Plateau State showed higher mean temperature values for irrigation water from the control site (Table 4.9).

Table 4.6 Seasonal effects on the mean faecal coliform counts of vegetable samples obtained from Kano State

Site	No of samples analysed	FCC			
		Dry[#]	Wet[##]	t-test	p-value
		Mean count (cfu/g)	Mean count (cfu/g)		
I	63	7.75± 7.50 [b]	12.60± 12.20	-2.580	0.035*
II	72	7.82 ± 7.52 [b]	12.44± 12.08	-2.309	0.040*
III	70	12.65± 12.71 [a]	7.70± 7.50	2.530	0.035*
IV	13	5.54 ± 5.15 [b]	6.32± 6.23	-1.379	0.379
V	20	6.95± 6.80 [b]	5.31 ± 5.03	1.411	0.294
Overall	238	11.61± 11.61	12.29± 11.79	-2.075	0.043*
Key		[#] F = 3.864; p = 0.021*	[##]F= 2.050; p= 0.111		

* = Significant difference exists indicating association between season and feacal coliform counts on vegetables
No=Number
FCC= Faecal coliform counts
Site I (Abattoir liquid waste), site II (Domestic sewage drain), siteIII (Human sewage/ Industrial waste), site IV (Industrial effluents) site V (Control: no point source of contamination)

Table 4.7 Seasonal effects on the mean faecal coliform counts of vegetable samples obtained from Plateau State

Site	No of samples tested	Season		t-test	p-value
		Dry[#]	Wet[##]		
		Mean count (cfu/g)	Mean count (cfu/g)		
I	50	8.09± 8.64	12.78± 12.48	-4.114	0.002*
II	30	6.91± 6.79	7.34 ± 7.34	-0.601	0.580
III	68	11.75± 11.75	6.39 ± 5.92	0.934	0.359
IV	57	6.19± 5.90	6.58± 5.94	-1.874	0.077
V	7	6.21± 6.20	6.48± 5.98	-0.785	0.468
Overall	212	11.35± 11.35	11.70± 11.54	-0.676	0.501

Key [#] F = 0.350; p = 0.842 [##] F= 14.208; p < 0.001*

* = Significant difference exists

No=number

Site I (Abattoir liquid waste), site II (Domestic sewage drain), site III (Human sewage/ Industrial waste), site IV (Industrial effluents) siteV (Control; : no point source of contamination)

Table 4.8 Comparison of mean values of water quality parameters in parts of Kano State

Site (No tested)	Mean ± SEM					
	FCC (Log_{10} cfu)	Temp(^oC)	pH	EC (µs/cm)	DO(mg/l)	BOD(mg/l)
Site 1 (62)	8.22 ± 7.48 [a]	25.80 ± 0.73[b]	6.97 ± 0.77 [ab]	1473.57 ± 222.55	177.71 ± 24.93	140.93 ± 16.02
Site 2 (72)	8.15 ± 7.41 [a]	25.46 ± 0.77[b]	7.07 ± 0.08 [ab]	1884.00 ± 266.73	244.09 ± 23.83	105.00 ± 12.35
Site 3 (60)	8.16 ± 7.45 [a]	25.83 ± 0.75[b]	7.21 ± 0.13 [a]	1325.38 ± 237.17	201.92 ± 24.71	79.69 ± 11.10
Site 4 (13)	5.12 ± 4.67 [b]	30.50 ± 0.89[a]	6.71 ± 0.01 [b]	1145.00 ± 199.20	255.00 ± 14.58	87.00 ± 37.58
Site 5 (22)	7.59 ± 7.43 [b]	28.40 ± 1.95[ab]	6.74 ± 0.03 [b]	649.20 ± 359.59	200.00 ± 14.78	57.90 ± 25.23

For each parameter, mean values with different superscripts are significantly different (P< 0.05)
SEM=Standard Error of mean
FCC= Faecal Coliform Counts, Temp= Temperature, pH= Potential hydrogen, EC=Electrical conductivity, DO= Digested oxygen and BOD= Biological oxygen demand.
Site I (Abattoir liquid waste), site II (Domestic sewage drain), site III (Human sewage/ Industrial waste), site IV (Industrial effluents) siteV (Control; no point source of contamination)

Table 4.9 Comparison of mean values of water quality parameters in parts of Plateau State

Site (No tested)	Mean ± SEM					
	FCC (Log_{10} cfu)	Temp(^oC)	pH	EC (µs/cm)	DO(mg/l)	BOD(mg/l)
Site 1 (50)	7.76 ± 7.29	26.83 ± 0.87[b]	8.12 ± 0.74[a]	625.00 ± 291.45[b]	241.67 ± 16.21	81.67 ± 6.01
Site 2 (28)	8.33 ± 7.36	28.00 ± 2.32[a]	6.40 ± 0.24[c]	2167.50 ± 1104.61[a]	220.00 ± 24.58	115.00 ± 42.77
Site 3 (68)	7.79 ± 7.37	25.80 ± 0.92[c]	6.40 ± 0.19[c]	154.20 ± 67.86[b]	246.00 ± 8.12	91.00 ± 19.13
Site 4 (57)	7.91 ± 7.39	27.14 ± 0.38[b]	6.76 ± 0.11[b]	126.74 ± 36.72[b]	234.55 ± 12.89	84.55 ± 18.16
Site 5 (8)	7.33 ± 8.00	28.25 ± 0.25[a]	6.64 ± 0.75[b]	121.43 ± 23.4[c]	275.00 ± 25.00	56.00 ± 75.00

Means of each parameter for different sites with different superscript are significantly different (P=0.05), while values with the same superscript do not vary significantly using Duncan's multiple range test
Key: FCC=Faecal coliform counts
Temp= Temperature
EC= Electrical conductivity
DO= Digested oxygen
BOD= Biological Oxygen Demand

Site 1(Abattoir liquid waste), site 2 (Domestic sewage drain), site 3 (Human sewage/ Industrial waste), site 4 (Industrial effluents) site5(Control; no point source of contamination)

Effect of seasonal variation on mean values of faecal coliform counts as revealed by Student's t-test showed significantly higher ($t=4.011$, $P<0.001$) counts in wet than dry season in Kano State. Mean temperature values and electrical conductivity were observed to be significantly higher in the wet season (Table 4.10), while, dissolved oxygen was higher in dry than wet season. In Plateau State, higher mean faecal coliform counts in irrigation water were obtained in the dry season than in the wet season. Significantly ($P<0.005$) higher mean values were also obtained for mean temperature, electrical conductivity in this season while dissolved oxygen values were significantly higher in the wet season than in the dry (Table 4.11).

Generally, analysis of the parameters of indicators of pollution of irrigation water for the two States for the seasons showed that higher (8.07 ± 7.14) mean values of faecal coliform counts were obtained in the wet season, when lower mean temperature, neutral pH and higher electrical conductivity values were obtained with resultant lower BOD and DO levels in this season (Table 4.12).

4.3 Frequency of Contaminaton of Samples By Season

A total of 440 samples consisting of vegetables, water and manure treated soil were obtained from the two study locations and examined for the presence of *E. coli* O157:H7, *Salmonella* spp and *Vibrio cholerae*. The distribution of pathogens in 94 vegetable samples analysed from Kano State was: *E.coli* O157:H7 19.1%, *Salmonella* spp 20.2% and *Vibrio cholerae* 2.1%. While, in Plateau State, distribution of pathogens in 144 vegetable samples analysed was *E.coli* O157:H7 2(1.4%), *Salmonella* spp 15(10.4%) and *Vibrio cholerae* 5(8.8%). *Salmonella* was most frequently detected in vegetables obtained from both States.

Table 4.10 Effect of seasonal variation on the mean values of faecal coliform on the mean values of faecal coliform counts and physicochemical parameters of irrigation water in parts of Kano State

Variable	Season		t-value	p-value
	Wet	Dry		
FCC (Log_{10} cfu)	8.25 ± 7.34^{a}	7.86 ± 7.17^{b}	4.011	< 0.001*
Temp	27.19 ± 0.35^{a}	25.26 ± 0.85^{b}	2.259	0.028*
pH	7.02 ± 0.06^{a}	7.00 ± 0.10^{a}	0.181	0.857
EC(μs/cm)	1733.21 ± 139.39^{a}	88.69 ± 183.65^{b}	3.708	0.001*
DO(mg/l)	183.59 ± 17.21^{b}	235.00 ± 10.85^{a}	2.185	0.034*
BOD(mg/l)	70.64 ± 11.63^{a}	105.00 ± 13.39^{a}	1.903	0.064

* = Significant difference exists. Mean values with different superscripts are significantly different ($p \leq 0.05$)

Key: FCC=Faecal coliform counts

Temp= Temperature

pH=Hydrogen ion potential

EC= Electrical conductivity

DO(mg/l)= Digested oxygen

BOD(mg/l)= Biological Oxygen Demand

Table 4.11 Effect of seasonal variations on the mean values of faecal coliform counts and physicochemical parameters of irrigation water in parts of Plateau State

Variable	Season		t-value	p-value
	Wet	Dry		
FCC (Log_{10} cfu)	7.68 ± 7.14^{a}	7.97 ± 7.29^{a}	1.935	0.055
Temperature	23.67 ± 1.41^{b}	29.33 ± 0.61^{a}	3.699	0.001*
pH	9.25 ± 1.56^{a}	6.82 ± 0.16^{a}	1.059	0.299
EC(µs/cm)	153.43 ± 42.94^{b}	1318.89 ± 554.65^{a}	3.077	0.005*
DO(mg/l)	256.84 ± 5.98^{a}	201.11 ± 11.39^{b}	4.333	0.001*
BOD(mg/l)	80.53 ± 13.67^{a}	86.67 ± 16.69^{a}	0.285	0.779

* = Significant difference exists. Mean values with different superscripts are significantly different ($p \leq 0.05$)

Key
FCC=Faecal coliform counts
Temp= Temperature
pH=Hydrogen ion potential
EC= Electrical conductivity
DO(mg/l)= Digested oxygen
BOD(mg/l)= Biological Oxygen Demand

Table 4.12 Seasonal variation of the mean values of faecal coliform counts and Physicochemical parameters of irrigation water in Kano and Plateau States

Variable	Season	Mean ± SEM		
		Both States	t-value	p-value
FCC	wet	8.07 ± 7.14	1.87	0.063
	Dry	7.91 ± 7.08		
Temp	wet	25.80 ± 1.21	0.638	0.526
	Dry	26.41 ± 0.71		
pH	wet	7.92 ± 0.64	1.150	0.254
	Dry	6.94 ± 0.08		
EC(µs/cm)	wet	1094.58 ± 141.96	0.240	0.812
	Dry	1032.07 ± 218.72		
DO(mg/l)	wet	212.58 ± 11.81	0.652	0.516
	Dry	223.70 ± 8.63		
BOD(mg/l)	wet	74.64 ± 8.80	1.774	0.081
	Dry	98.89 ± 10.47		

Key
FCC= Faecal coliform counts
Temp= Temperature
pH= Hydrogen ion potential
EC= Electrical conductivity
DO (mg/l) = Digested oxygen
BOD (mg/l) = Biological Oxygen Demand

Pathogen detection in irrigation water analysed from Kano State showed a prevalence of *E.coli* O157:H7 13 %(7/57), *Salmonella* spp 19.3% (11/57) and 8.8 %(5/57) *Vibrio cholerae* (Table 4.13). While, in Plateau State, distribution of target pathogens in irrigation water was *E.coli* O157:H7 3.7 % (1/27), *Salmonella* spp 11.1% (3/27) and 7.7%(3/27) *Vibrio cholerae*. *Salmonella* spp was also most detected from irrigation water samples from both States. Isolation and characterisation of pathogens from soil/manure analysed in the study showed that in Kano State, of 79 samples analysed, 3.8 % (3/79) *E.coli* O157:H7, 11.4%(9/79) *Salmonella* spp and 11.4%(9/79) *Vibrio cholerae* were detected. In Plateau State however, 2.5 %(1/39) *E.coli* O157:H7, *Salmonella* spp 2.6 %(1/39) and 7.7%(3/39) *Vibrio cholerae* was obtained. The overall distribution of target pathogens in samples analysed showed that *Salmonella* spp was most detected in the study and from vegetable samples (Table 4.13).

When isolation rate in wet season was compared with that of the dry season, *E. coli* O157:H7 was three times more likely to be isolated from vegetables in the wet season than in the dry season (OR=3.022, 95% CI; 1.047, 8.719). It was 2.3 times more likely to be isolated in water used for irrigation in wet than in dry season (OR= 2.308, 95% CI; 0.409, 13.302) in Kano State. Comparing the *Salmonella* spp isolation rate during the wet season with the rate during the dry season in Kano, it was observed that, in general, significantly less *Salmonella* spp was isolated during the wet season (OR= 0.297, 95% CI 0.139, 0.634) particularly from the water used for irrigation (OR= 0.202, 95% CI 0.041, 0.853). In Plateau State also, the isolation rate of *Salmonella* spp in the wet season was less than that in the dry season for vegetable (OR= 0.372, 95% CI 0.120-1.149), water (OR= 0.167, 0.013-2.182) and manure treated soil. Seasonal distribution of *Vibrio* in samples in Kano State revealed that it was two times more likely to be obtained from vegetables in the wet than dry season (OR= 1.556, 95% CI

Table 4.13 Occurrence of enteric pathogens in samples from Kano and Plateau States

State / Sample	No of samples analysed	No. contaminated with enteric agent		
		E. coli O157 No (%)	*Salmonella* species No (%)	*Vibrio cholerae* No (%)
Kano				
Vegetable	94	18(19.1)	19(20.2)	2(2.1)
Water	57	7(12.3)	11(19.3)	5(8.8)
Manure treated soil	79	3(3.8)	9(11.4)	9(11.4)
Total	230	28(12.2)	39(17.0)	16(7.0)
Plateau				
Vegetable	144	2(1.4)	15(10.4)	5(8.8)
Water	27	1(3.7)	3(11.1)	1(3.7)
Manure treated soil	39	1(2.5)	1(2.6)	3(7.7)
Total	210	4(1.9)	19(9.0)	9(4.3)
Grand Total	440	32(7.3)	58(13.2)	25(5.7)

No=Number tested
(%)= Percentage of no. tested

0.094, 25.664). Although there was no statistical significance in its detection rate from water in both seasons, it was observed that isolation rate was higher in the dry season than in the wet (OR=0.183, 95% CI 0.019, 1.756). There was an observed significant difference (OR=0.099 CI 0.019-0.520) with higher detection rate in the dry than wet season in its distribution in manure treated soil samples. The rate of isolation of *Vibrio cholerae* was higher in the dry than wet season for all the samples combined (OR= 0.271, 95%CI 0.085, 0.868) in Kano State. In Plateau State however, there was statistically significant difference in isolation rate in samples between seasons (OR= 1.712, 95% CI 0.506, 5.799) and *Vibrio* was two times more likely to be detected in wet than dry season particularly in manure treated soil. The overall isolation rate was three times higher in wet than dry (OR=2.750, 95% CI=0.402, 18.804) (Table 4.14).

4.3 Distribution of *Vibrio* Serotypes in Samples

Serological classification of *Vibrio* confirmed with *Vibrio cholerae* antiserum (Table 4.15) showed different biotypes of this organism as; three (1.3%) of the Inaba biotype, six (2.6%) of the Ogawa biotype, seven (3.0%) of the Hikojima (O139) biotype, while twelve (5.2%) non O1 *V.cholerae* were found from samples obtained from Kano State. Distribution of these biotypes as obtained from samples analysed were; two *Vibrio cholerae* serogroup O1; both of the Ogawa biotype and one non *V.cholerae* O1 were obtained from vegetables, five (8.8%) *Vibrio cholerae* O1 confirmed from water samples showed two of the Inaba biotype, three Ogawa biotype and five non *V.cholerae* O1. While from manure treated soil samples analysed, nine (6.3%) *Vibrio cholerae* O1 were confirmed, of which one was of the Inaba biotype, one of the Ogawa biotype and seven (O139) Hikojima biotype, while six non *V.cholerae* O1 serogroup were detected. Distribution of these serotypes as obtained from samples

Table 4.14 Seasonal distribution of samples contaminated with *E. coli* O157:H7, *Salmonella* spp and *Vibrio cholerae* in the study

State	*E. coli* O157:H7				*Salmonella* species				*Vibrio cholerae*			
	Dry	Wet			Dry	Wet			Dry	Wet		
Sample	No +ve(%)	No ve(%)	OR	95% CI	No +ve(%)	No - ve(%)	OR	95% CI	No +ve(%)	No - ve(%)	OR	95% CI
Kano												
Vegetable	7(12.3)	11(29.7)	3.022	1.047 – 8.719	14(24.6)	5(13.5)	0.480	0.157-1.469	1(1.8)	1(2.7)	1.556	0.094-25.664
Water	2(7.7)	5(16.1)	2.308	0.409 – 13.032	9(34.6)	3(9.7)	0.202	0.041-0.853	4(15.4)	1(3.2)	0.183	0.019-1.756
Manure treated soil	1(4.0)	2(3.7)	0.923	0.080 – 13.032	4(16.0)	3(5.6)	0.309	0.064-1.501	7(28.0)	2(3.7)	0.099	0.019-0.520
Plateau												
Vegetable	2(3.1)	0(0.0)	–	–	10(15.4)	5(6.3)	0.372	0.120-1.149	3(3.8)	2(3.1)	0.804	0.130-4.964
Water	0(0.0)	1(5.3)	–	–	2(25.0)	1(5.3)	0.167	0.013-2.182	0(0.0)	1(12.5)	–	–
Manure treated soil	0(0.0)	1(4.2)	–	–	0(0.0)	1(4.2)	0.386	0.145-1.023	2(8.3)	3(20.0)	2.750	0.402-18.804

Key
No +ve: Number of samples containing pathogens
-ve:nil
OR= odds ratio
CI= Confidence interval

Table 4.15 Distribution of *Vibrio cholerae* O1, O139 and non O1 *V. cholerae* in samples in the study

Sample Type	No of samples Analysed	No of *Vibrio cholerae* O1		Hikojima O139	Non O1 *V. cholerae*
		Inaba	Ogawa		
Kano					
Vegetables	94	-	2	-	1
Water	57	2	3	-	5
Manure treated soil	79	1	1	7	6
Total	230	3 (1.3%)	6 (2.6%)	7 (3.0%)	12 (5.2%)
Plateau					
Vegetables	144	1	2	2	9
Water	27	-	-	1	-
Manure treated soil	39	2	1	-	-
Total	210	3 (1.4%)	3 (1.4%)	3 (1.4%)	9 (4.3%)

Key
__- = not detected

analysed in Plateau State showed nine confirmed *Vibrio cholerae* O1 isolates of these, three (1.4%) were of the Inaba biotype, three (1.4%) Ogawa and three (1.4%) of the Hikojima biotype, while nine non O1 *V.cholerae* were detected. Distribution of biotypes as found in *Vibrio cholerae* O1 serotypes were; vegetables had one Inaba, two Ogawa, two Hikojima biotypes. Only the Hikojima biotype was found in water, while manure treated soil had two Inaba and one Ogawa biotype respectively.

4.4 Toxin assay in *E.coli* O157:H7 and *Vibrio cholerae*

The confirmed *E. coli* O157:H7 isolates were assayed for toxin production (Table 4.16). Nineteen isolates (twelve from vegetables, three from water and four from manure treated soil) assayed for verocytotoxin (VT1 and VT2) production, four (21.1%) produced VT1, one (5.3%) produced VT2, while eleven (57.9%) produced both VT1 and VT2. Of the four isolates that produced VT1, two (16.7%) were isolates from vegetables, one (33.3%) each from water and manure treated soil (25.0 %) samples. Only one (8.3%) produced VT2. Out of the eleven isolates that produced both VT1 and VT2; eight (66.7%) were isolates from vegetables, two (66.7%) from water samples and one (25.0%) from manure treated soil samples.

Confirmed isolates of *Vibrio cholerae* were assayed for cholera toxin (CT) production. Of the 13 isolates tested (five from vegetables, four from water samples and four from soil/manure samples), eight (61.5%) produced cholera toxin as follows; three were isolates obtained from vegetables, three from water and two from manure treated soil (Table 4.17). Isolates were observed to be potentially pathogenic as they produced toxins.

Table 4.16 Detection of verocytotoxins VT1 and VT2 from *E. coli* O157:H7 isolates

Sample Type	No of isolates assayed	VT1 No (%)	VT2 No (%)	VT1 /VT2 No (%)
Vegetables isolates	12	2(16.7)	1(8.3)	8(66.7)
Water isolates	3	1(33.3)	0(0.0)	2(66.7)
Manure treated soil	4	1(25.0)	0(0.0)	1(25.0)
Total	19	4(21.1)	1(5.3)	11(57.9)

Key: Vt1= verocytotoxin 1 No (%) = Number (percent)
Vt2= verocytotoxin 2

Table 4.17 Detection of cholera toxin (CT) from *Vibrio* isolates

Sample Type	No of isolates assayed	No positive for cholera toxin
Vegetables isolates	5	3
Water isolates	4	3
Manure treated soil	4	2
Total	13	8(61.5%)

4.5 Antibiotic Resistance Patterns of Isolates.

Figure 4.7 shows the results of antibacterial susceptibility patterns of 32 *E. coli* O157:H7 isolated from vegetables and environmental samples (water, soil and manure) using eight commonly used antibiotics in the study area. Of the antibiotics used, ceftriaxone was the most active, but all the isolates were resistant to cephalothin. Out of the 32 isolates obtained, 24(75%) were resistant to tetracycline, with all isolates obtained from soil/manure samples being resistant to tetracycline. Therefore, tetracycline and cephalothin were the least active. Only four (12.5%) isolates were resistant to ceftriaxone, ten (31.3%) showed intermediate susceptibility, while, 18 (56.3%) were sensitive to this drug. Multidrug resistance (MDR) patterns of the 32 isolates tested to antibiotics showed that 30 (93.8%) were resistant to two or more antibiotics viz; nine (28.1%) were resistant to two antibiotics, eight (25%) to three antibiotics, seven (21.9%) to four antibiotics, four (12.5%) to five antibiotics, one (3.1%) to six and one (3.1%) was resistant to all the eight antibiotics used in the study. The remaining two (6.3%) were resistant to only cephalothin. Overall, higher numbers of resistant isolates were identified among the vegetables and soil/manure strains. Three isolates from vegetables were resistant to five antibiotics, while two isolates from manure treated soil were resistant to six and all eight antibiotics tested respectively (Table 4.18).

Figure 4.8 shows the antibacterial susceptibility pattern of *Salmonella* isolates obtained using eight antibiotics. Of all the antibiotics used, gentamicin, ceftriaxone and ciprofloxacin were the most active. All the isolates obtained from the manure treated soil samples were sensitive to gentamicin. Only three (4.5%) and four (6.0%) of the isolates tested, showed resistance to ciprofloxacin and ceftriaxone respectively in the

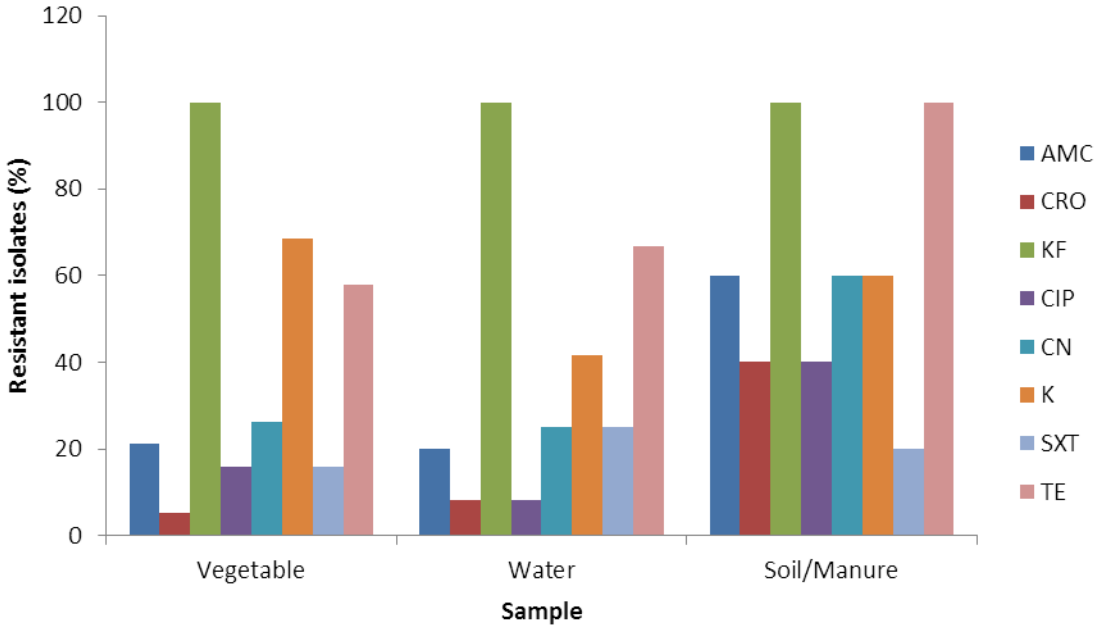


Figure 4.7 Antibiotic susceptibility pattern of *E. coli* O157:H7 isolates from samples of vegetable, water and manure treated soil from both States

Key

AMC=Amoxicillin acid (10µg)

CRO=Ceftriaxone (30µg),

KF=Cephalothin (30µg)

CIP=Ciprofloxacin (5µg)

CN=Kanamycin (30µg)

K=Gentamicin (30µg)

SXT=Sulfamethoxazole25µg

TE=Tetracycline (30µg)

Table 4.18 Multiple antibiotic resistance indexes of *Escherichia* coli O157:H7 isolates obtained from environmental samples from parts of Kano and Plateau States, Nigeria

No of antibiotics resisted	Combination of antibiotics	Vegetable isolates	Water isolates	Manure treated soil isolates	MARI (%)
1R	TE/KF	3	1	1	0.1
2R	AMC, TE/ SXT,TE/KF, TE, KF/ K, KF/ CIP,CRO.	4	3	1	0.3
3R	SXT,CN,TE/CRO,CN,K/ AMC,TE,KF/KF,TE,K.	5	1	-	0.4
4R	AMC,KF,TE,K/AMC, CN,TE,K/ SXT,KF,TE,K/CN,CIP,KF,K/ AMC,CN,KF,K/AMC,SXT,KF, TE.	2	3	2	0.5
5R	CN,CIP,KF,TE,K/AMC,CIP,KF TE,K/CRO,CN,KF,TE,K/AMC, SXT,CN,KF,TE.	3	1	-	0.6
6R	CRO,CN,CIP,KF,TE,K	-	-	1	0.8
8R	AMC,SXT,CRO,CN,CIP,KF,TE,K.	-	-	1	1.0

Key;R=resistance, KF=Cephalothin(30µg), SXT=Sulfamethoxazole(25µg), AMC=Amoxicillin/Clavulanic acid(10µg),Gentamicin=CN(10µg), CIP=Ciprofloxacin(5µg), CRO=Ceftriaxone(10µg), TE=Tetracycline(30µg) and K=Kanamycin(30µg), MARI= multiple antibiotic resistance index, 1R=resistance to 1 antibiotic, 2R= resistance to 2antibiotics, 3R=resistance to 3 antibiotics,4R= resistance to four antibiotics, 5R=resistance to five antibiotics, 6R=resistance to 6antibiotics, 7R=resistance to 7antibiotics, 8R=resistance to 8antibiotics.

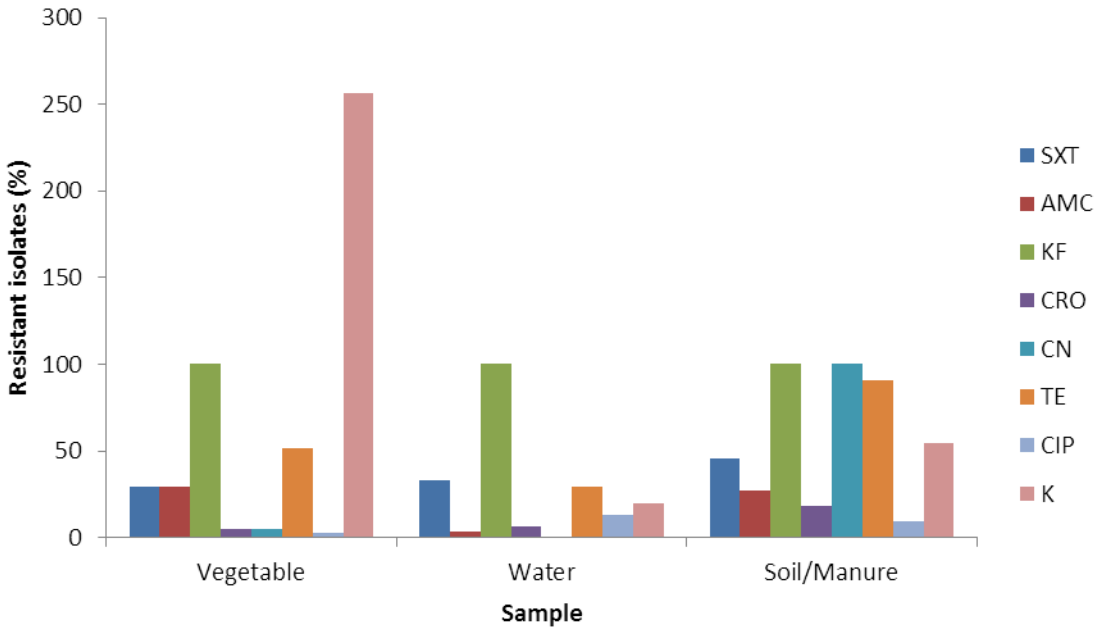


Figure 4.8 Antibiotic susceptibility pattern of *Salmonella* isolates from samples of vegetable, water and manure treated soil from both States

Key

SXT=Sulfamethoxazole25µg

AMC=Amoxicillin acid (10µg)

KF=Cephalothin (30µg)

CRO=Ceftriaxone (30µg),

K=Kanamycin (30µg)

TE=Tetracycline (30µg)

CIP=Ciprofloxacin (5µg)

CN=Gentamicin (10µg)

study. Cephalothin was not active against any of the isolates. Tetracycline and cephalothin were the least active with 42 (64.2%) and 67 (100%) resistance respectively.

The multidrug resistant (MDR) patterns of the 67 *Salmonella* isolates revealed that 55 (82.1%) were resistant to two or more antibiotics. That is, 22 (32.8%) were resistant to three antibiotics, five (7.5%) were resistant to four antibiotics and seven (10.4%) were resistant to five antibiotics. The remaining 12 (17.9%) showed resistance to only one antibiotic, cephalothin. Higher numbers of resistant isolates were identified among isolates from vegetables and manure treated soil, four isolates from vegetables showed resistance to five antibiotics, while three isolates from manure treated soil were resistant to four antibiotics (Table 4.19).

Figure 4.9 shows the antibacterial susceptibility patterns of 41 *Vibrio cholerae* (O1 and non O1) isolates tested in this study using eight antibiotics. Of the eight antibiotics used, the isolates were more sensitive to gentamicin, ciprofloxacin and ceftriaxone and all were resistant to cephalothin. All isolates were sensitive to gentamicin, two (4.9%) were resistant to ceftriaxone while only one (2.4%) was resistant to ciprofloxacin. Multidrug resistance (MDR) patterns of the 41 *Vibrio cholerae* isolates showed that; 30 (73.3%) were resistant to two or more antibiotics, nine (22.0%) were resistant to three antibiotics while, three (7.3%) were resistant to four antibiotics. The remaining 11(26.8%) were resistant to only cephalothin. Isolates from manure treated soil showed resistance to a higher number of antibiotics, one showed resistance to three antibiotics while two isolates showed resistance to four antibiotics (Table 4.20). The study of antibacterial resistance showed that isolates obtained showed multidrug resistance patterns.

Table 4.19 Multiple antibiotic resistance indexes of *Salmonella* isolates obtained from environmental samples from parts of Kano and Plateau States, Nigeria

No of antibiotics resisted	Combination of antibiotics	Isolates from Vegetables	Water	Soil/ manure	MARI (%)
1R	TE/ CN/ KF	3	1	1	0.1
2R	KF,TE/KF,CRO/AMC,KF/KF,K	17	6	1	0.3
3R	SXT,TE,KF/KF,TE,CIP/KF,CRO,CIP ;KF,TE,K/SXT,KF,K/AMC,KF,K/SXT, KF,AMC/AMC,KF,TE	11	6	5	0.4
4R	SXT,KF,TE,K/SXT,AMC,KF,TE/ KF,TE,CIP,K	2	-	3	0.5
5R	SXT,KF,CN,TE,K/SXT,AMC,KF, TE,K/CN,CIP,KF, TE,K/AMC,CIP,KF,TE,K/CRO,CN,KF TE,K/AMC, SXT,CN,KF,TE	7	3	1	0.6
6R	CRO,CN,CIP,KF,TE,K	-	-	1	0.8
8R	AMC,SXT,CRO,CN,CIP,KF,TE,K	-	-	1	1.0

Key;R=resistance, KF=Cephalothin(30µg), SXT=Sulfamethoxazole(25µg), AMC=Amoxicillin/Clavulanic acid(10µg),Gentamicin=CN(10µg), CIP=Ciprofloxacin(5µg), CRO=Ceftriaxone(10µg), TE=Tetracycline(30µg) and K=Kanamycin(30µg), MARI= multiple antibiotic resistance index, 1R=resistance to 1 antibiotic, 2R= resistance to 2antibiotics, 3R=resistance to 3 antibiotics,4R= resistance to four antibiotics, 5R=resistance to five antibiotics, 6R=resistance to 6antibiotics, 7R=resistance to 7antibiotics, 8R=resistance to 8antibiotics.

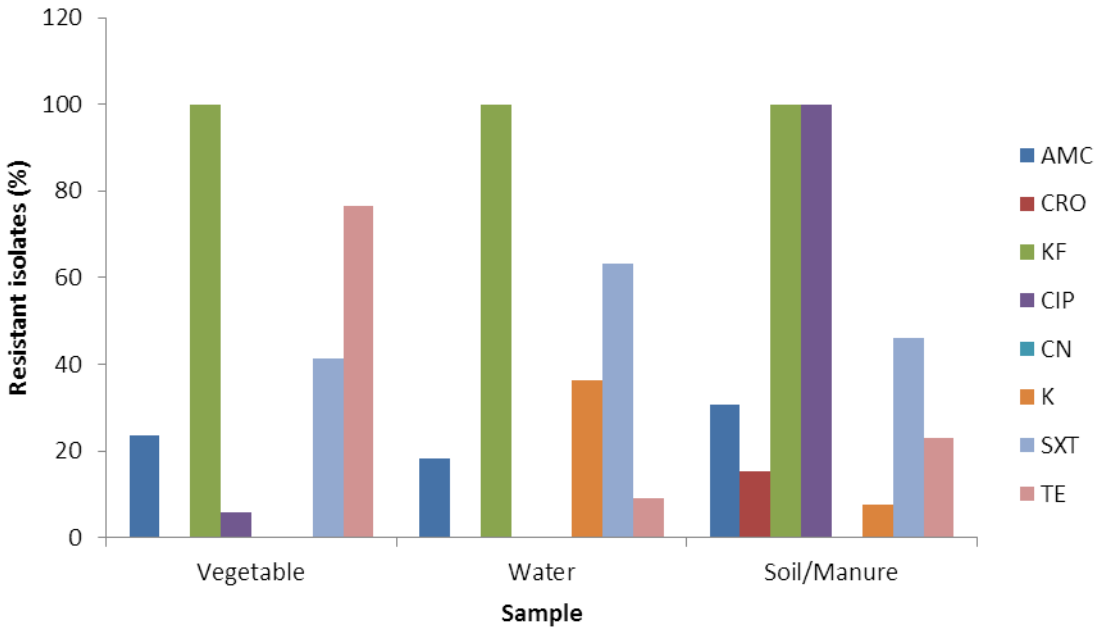


Figure 4.9 Antibiotic susceptibility pattern of *Vibrio cholerae* isolates from samples of vegetable, water and soil/manure

Key

AMC=Amoxicillin acid (10µg)

CRO=Ceftriaxone (30µg),

KF=Cephalothin (30µg)

CIP=Ciprofloxacin (5µg)

K=Kanamycin (30µg)

CN=Gentamicin (10µg)

SXT=Sulfamethoxazole25µg

TE=Tetracycline (30µg)

Table 4.20 Multiple antibiotic resistance indexes of *Vibrio cholerae* isolates obtained from environmental samples from parts of Kano and Plateau States, Nigeria

No of antibiotics resisted	Combination of antibiotics	Isolates vegetables n=41	water n=15	soil/ manure N=11	MARI (%)
1R	TE/CN/KF	3	1	1	0.1
2R	KF,TE/KF,CRO/ AMC,KF/KF,K/	17	6	1	0.3
3R	SXT,TE,KF/KF,TE,CIP/KF,CRO, CIP/KF,TE,K/SXT, KF,K/AMC, KF,K/SXT,KF, AMC/AMC,KF,TE/	11	6	5	0.4
4R	SXT,KF,TE,K/ SXT,AMC,KF,TE/ KF,TE,CIP,K	2	-	3	0.5
5R	SXT,KF,CN,TE,K/SXT,AMC,KF,TE,K/ CN,CIP,KF,TE,K/ AMC,CIP,KF,TE,K/ CRO,CN,KF,TE,K/AMC,SXT,CN,KF,TE	7	3	1	0.6
6R	CRO,CN,CIP,KF,TE,K	-	-	1	0.8
8R	AMC,SXT,CRO,CN, CIP,KF,TE,K	-	-	1	1.0

Key;R=resistance, KF=Cephalothin(30µg), SXT=Sulfamethoxazole25µg, AMC=Amoxicillin acid(10µg),CIP=Ciprofloxacin(5µg), CRO=Ceftriaxone(30µg), TE=Tetracycline (30µg), CN=Kanamycin(30µg), K=Gentamicin(30µg), MARI= Multiple antibiotic resistance index, 1R=Resistance to 1 antibiotic, 2R=Resistance to 2 antibiotics, 3R=Resistance to 3 antibiotics,4R=Resistance to 4 antibiotics, 5R=Resistance to 5 antibiotics, 6R=Resistance to 6 antibiotics, 7R=Resistance to 7 antibiotics, 8R= Resistance to 8 antibiotics.

4.6 Molecular Characterization Of Isolates

Strains of *E. coli* O157:H7 assayed for the *uid*A gene showed amplification of the expected 187 bp (Plate I). The *rfb* $_{O157}$ gene was amplified and showed the expected band size of 420bp (Plate II). However, no amplification was obtained for the shiga toxin genes (Plate III).

Salmonella strains produced an intense band of 187bp for the *inv*A gene (Plate IV) and 267bp for the *stn* gene respectively (Plate V). The *ctx*A subunit of the cholera toxin gene (564bp) was amplified (Plate VI). Multidrug resistance in isolates was further confirmed with primer amplification of the Quinolone resistance determining region, plasmids (pCT) conferring multidrug resistance and resistance to aminoglycosides (Plate VII- IX) with band sizes of 428bp and 484bp respectively. The presence of mobile genetic elements in the isolates was shown in amplification of a variable region of the class one integron (558bp) in *E.coli* O157:H7 (Plate X), *Salmonella enterica* (Plate XI), *Vibrio cholerae* (Plate XII). While amplification of the sulphonamide resistance genes was shown in the amplification of Sul1 gene with an expected band size of 433bp (Plate XIII - XV). However, the negative control was not amplified in the entire assay indicating 100% specificity. Repeat PCR gave reproducible results.

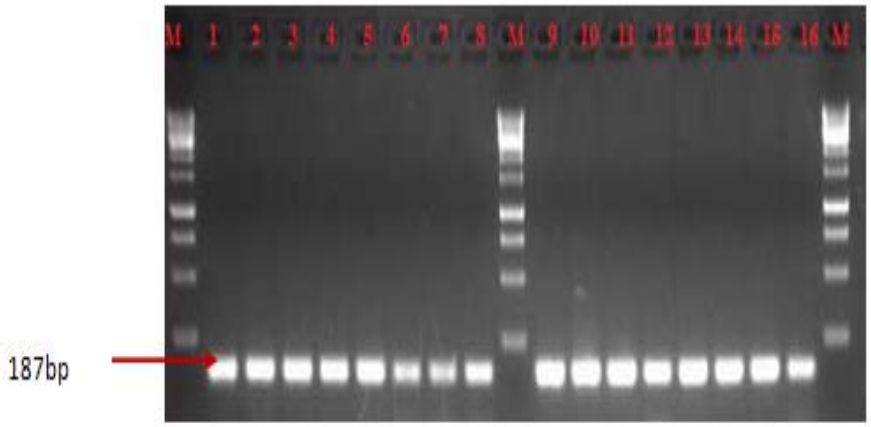


Plate I UAL gene in *E.coli* O157 isolates from the two study locations. M=DNA ladder, 1-8= isolates from Kano State, 9-16= isolates from Plateau State, Nigeria

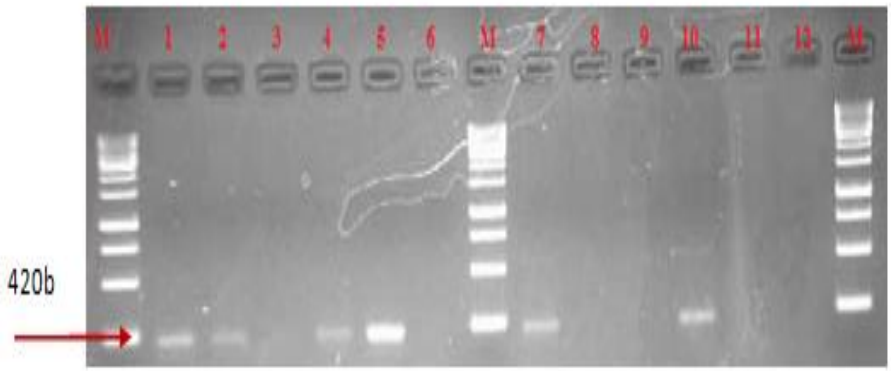


Plate II O157 gene in *E.coli* O157 isolates. M=1kb ladder, Lane 1,2,4,5 (O157) in isolates from Kano State while, 7 and 10 from Plateau State, Lane 12= Negative control

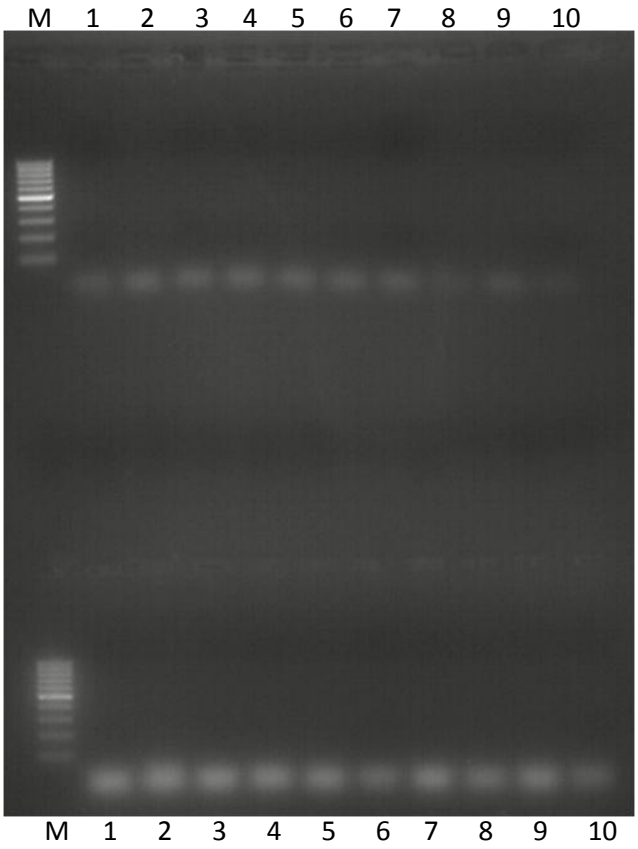


Plate III *E. coli* O157:H7 shiga toxin gene (sxt1: upper Lane 1-7, lower lane 1-7)

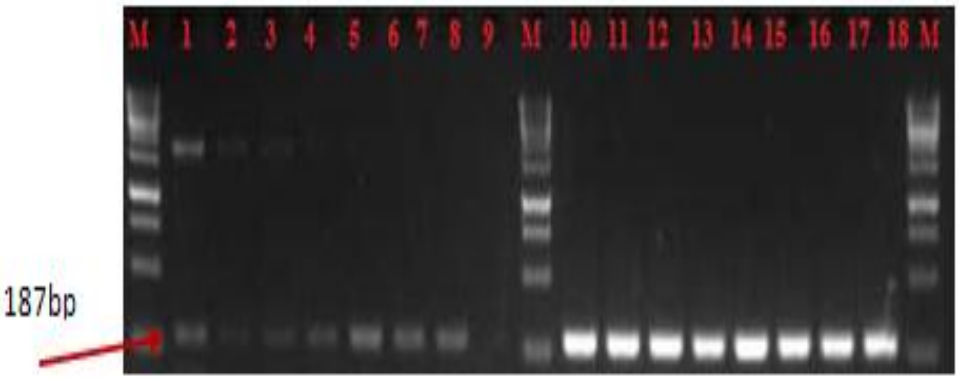


Plate IV *inv*A gene in *Salmonella* isolates. M= 1kb ladder, 1-9= Isolates from Kano State, 10-18= isolates from Plateau State

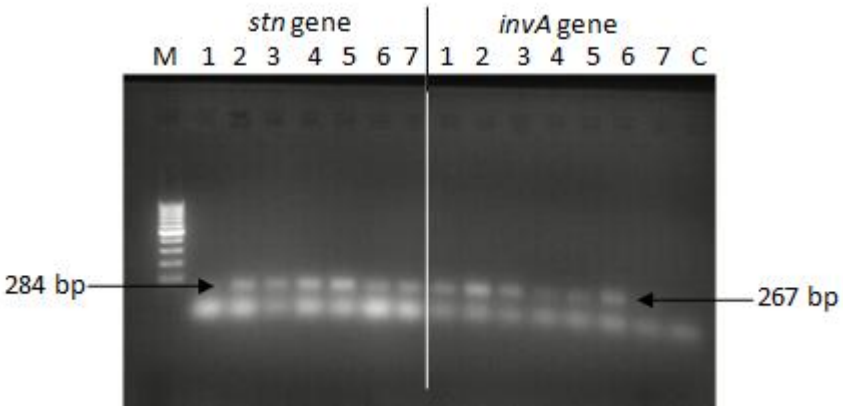


Plate V *Salmonella* enterotoxin (*stn* gene) Lane M= DNA marker, lane1-7 and invasive (*inv*A gene) lane 1-7, Lane C= negative control *E .coli* ATCC 25922

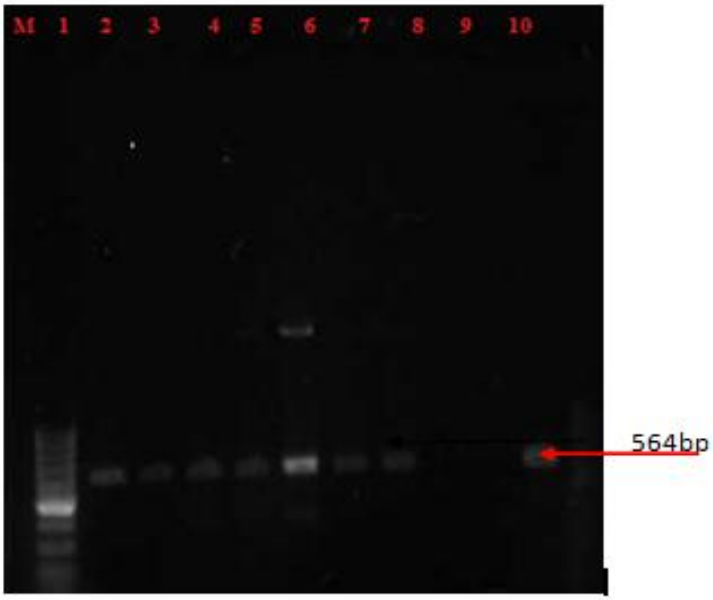


Plate VI Cholera toxin gene (*ctx*A) in *Vibrio* isolates in the study

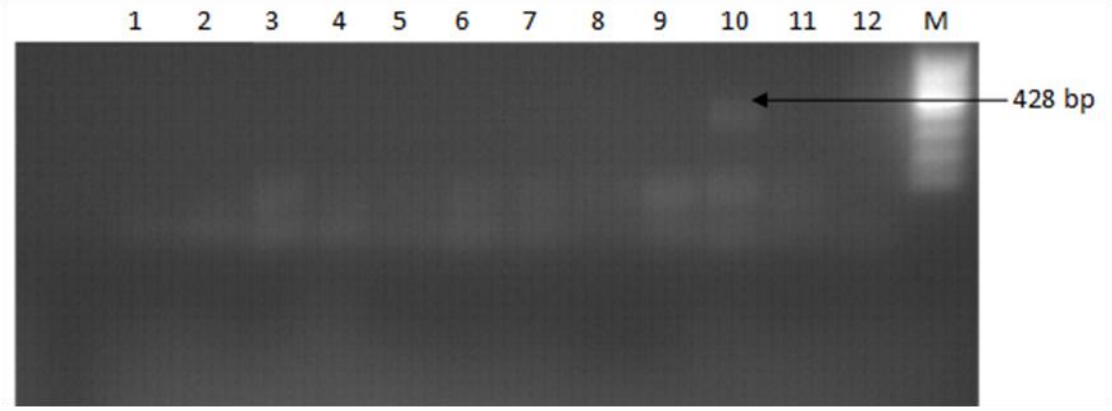


Plate VII *E. coli* O157:H7 pCT (multidrug resistance plasmid): Lane 1-12, Lane M= 100bp Marker lane

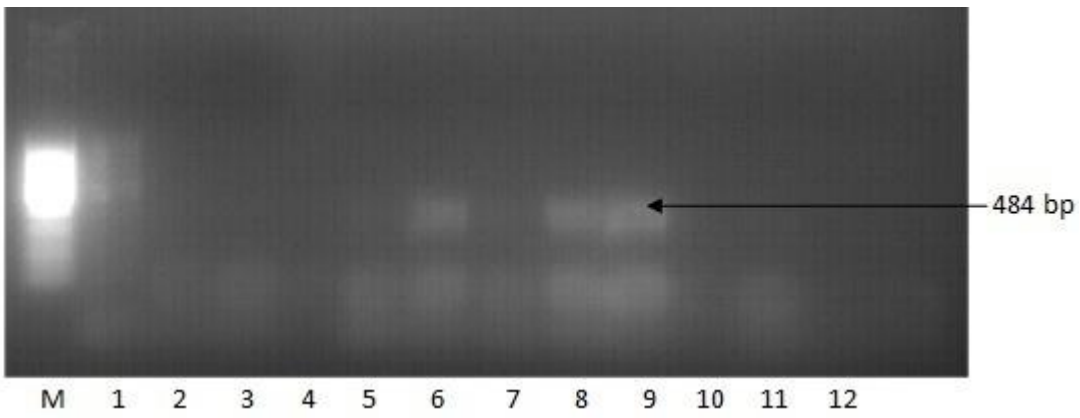


Plate VIII *E. coli* O157:H7 Quinolone resistance determining region (QRDR): Lane 1-12, Lane M= 100bp Marker lane

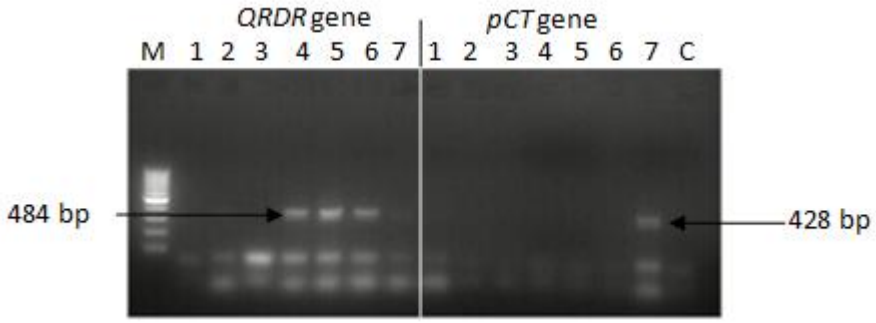


Plate IX Quinolone resistance determining region (QRDR) in *Salmonella*: Lane 1-7, Lane M= 100bp Marker lane, Lane 1-7 (left) = multidrug resistance plasmid, C= control; PCR product- DNA

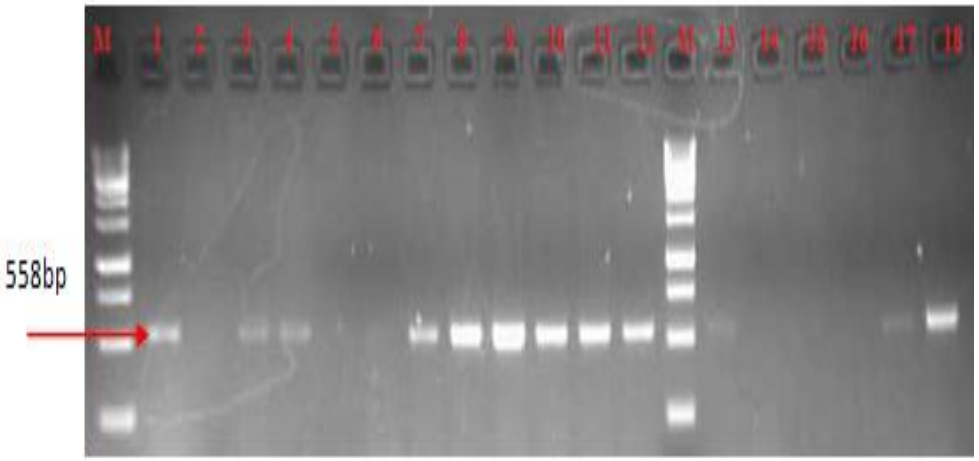


Plate X Class one integron gene in *E.coli* O157 M=1kb, 1- 12= Isolates from Kano State, 13-18= isolates from Plateau State

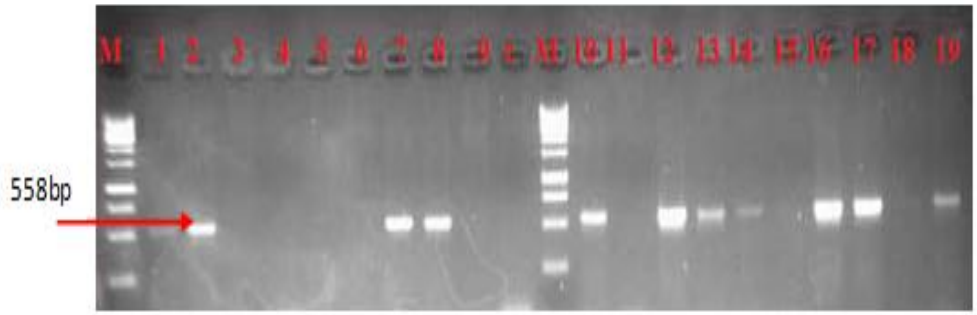


Plate XI Class one integron in *Salmonella* isolates. M= 1kb ladder

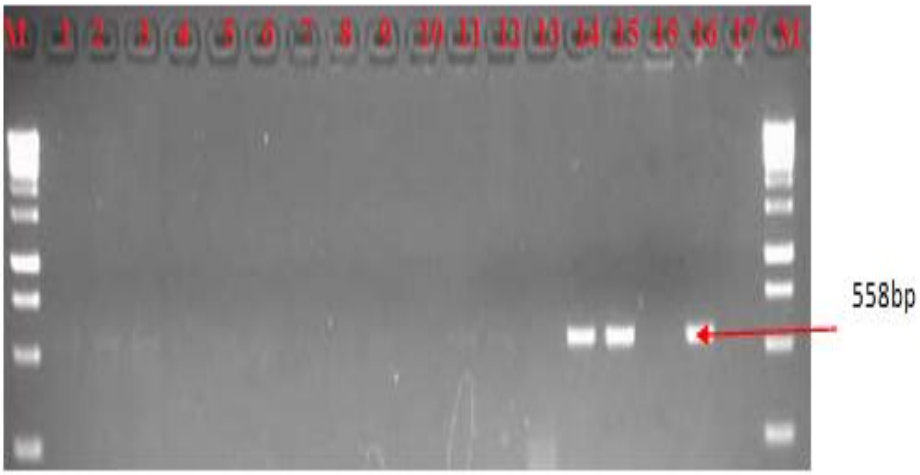


Plate XII Class one integron in *Vibrio cholerae* isolates. M=1kb ladder

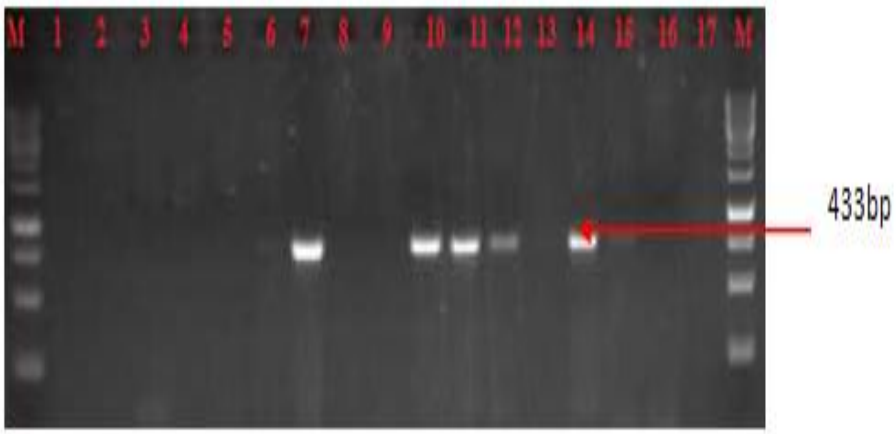


Plate XIII Sulfanomide resistant genes in *E.coli* O157 isolates. M= 1Kb ladder

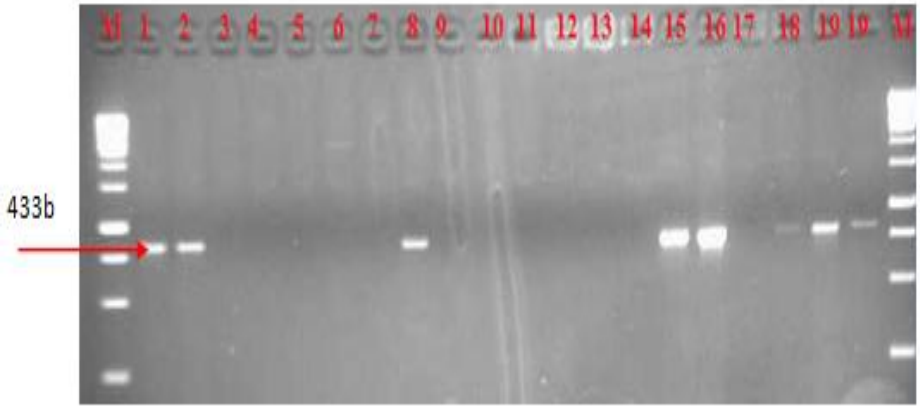


Plate XIV Sulfonamide resistant genes in *Salmonella* isolates, M=1kb ladder

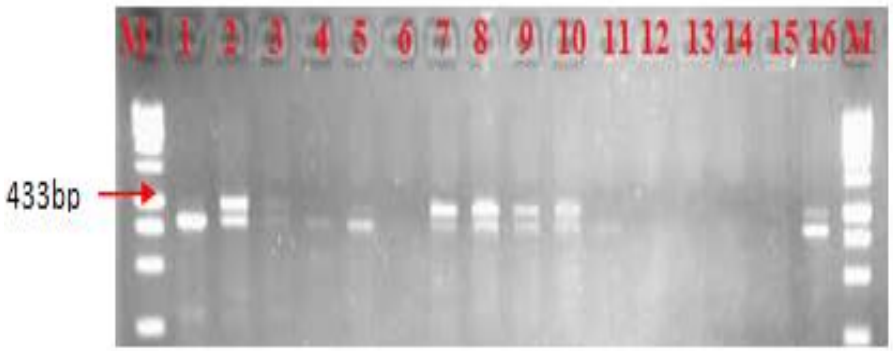


Plate XV Sulfonamide resistant genes in *Vibrio cholerae* isolates. M=1kb ladder

4.7 Genetic Diversity of *E.coli* O157:H7 Isolates

Figure 4.10 shows the results of Enterobacterial repetitive intergenic concensus Deoxyribonucleic acid (ERIC DNA) fingerprints of nineteen representative *E.coli* O157:H7 strains obtained in the study from vegetable and environmental samples.

Fingerprints show 3-9 bands with a molecular weight of 150bp to 1kb. A common intensive band of about 7000bp was obtained in most of the strains. Cluster analysis at a coefficient of similarity of 0.53 grouped the nineteen into 6 different groups, designated 1-6; five clusters and one singleton (Plate XVI). Cluster one comprise isolates (Jva1, Jva2, Jsa6) from vegetables and soil with a similarity coefficient of over 0.58, cluster two comprise strains (Jwa4, Kva7, Kva2, Kva40) obtained from irrigation water and manure with a similarity coefficient of 0.67, while cluster five comprises strains of *E.coli* O157:H7 (Kwa29, Kwa7 and Kma3) obtained from irrigation water and manure with a similarity coefficient of 0.54. However, isolate Kva31 from vegetable exists as a singleton.

4.8 Genetic Diversity of *Salmonella enterica* Isolates

Figure 4.11 shows the results of ERIC DNA fingerprints fifteen representative *Salmonella enterica* strains obtained in the study from vegetable and environmental samples. Fingerprints showed 1-12 bands with a molecular weight of 150bp to 1kb. A common intensive band of about 9000bp was obtained in most of the strains. Cluster analysis at a coefficient of similarity of 0.82 grouped the fifteen strains of *S. enterica* into five different groups, designated 1-5; two clusters and three singletons (Plate XVII). Cluster 2 comprises isolates obtained from 10 samples including vegetables, irrigation water and soil with a similarity coefficient

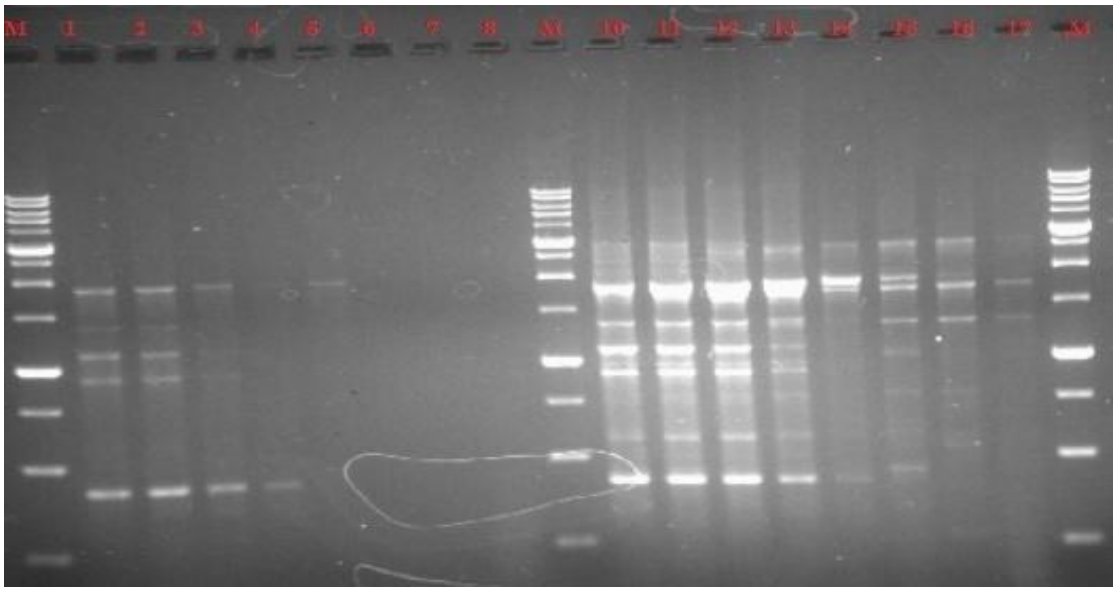


Plate XVI DNA fingerprint of *E.coli* O157:H7 isolates in the study

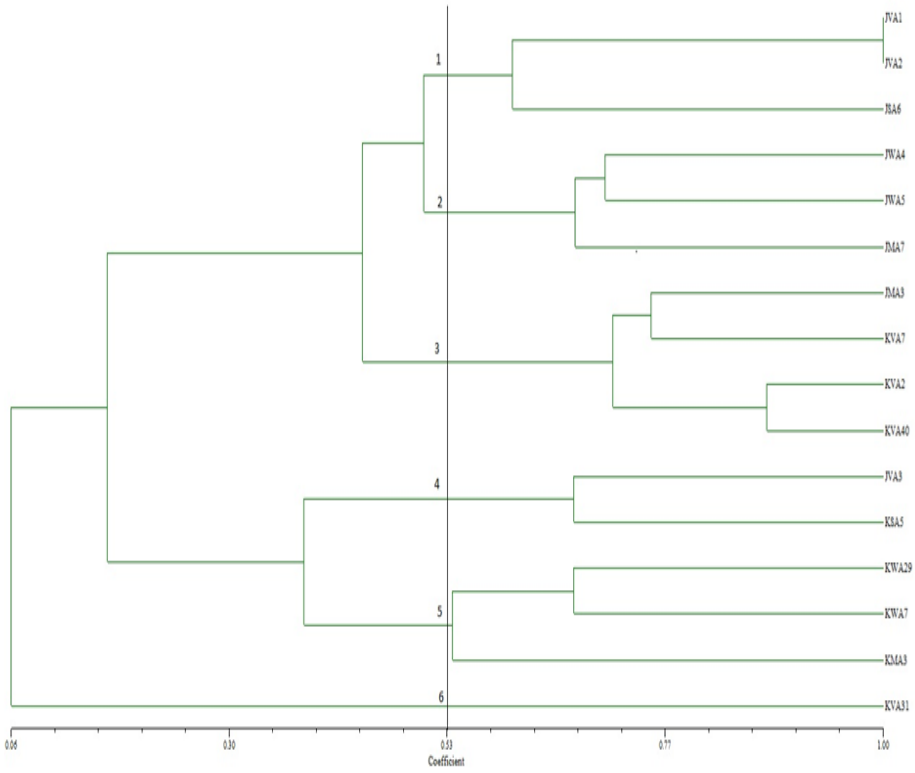


Figure 4.10 Dendrogram showing relatedness of *E.coli* O157:H7 from environmental isolates in the study

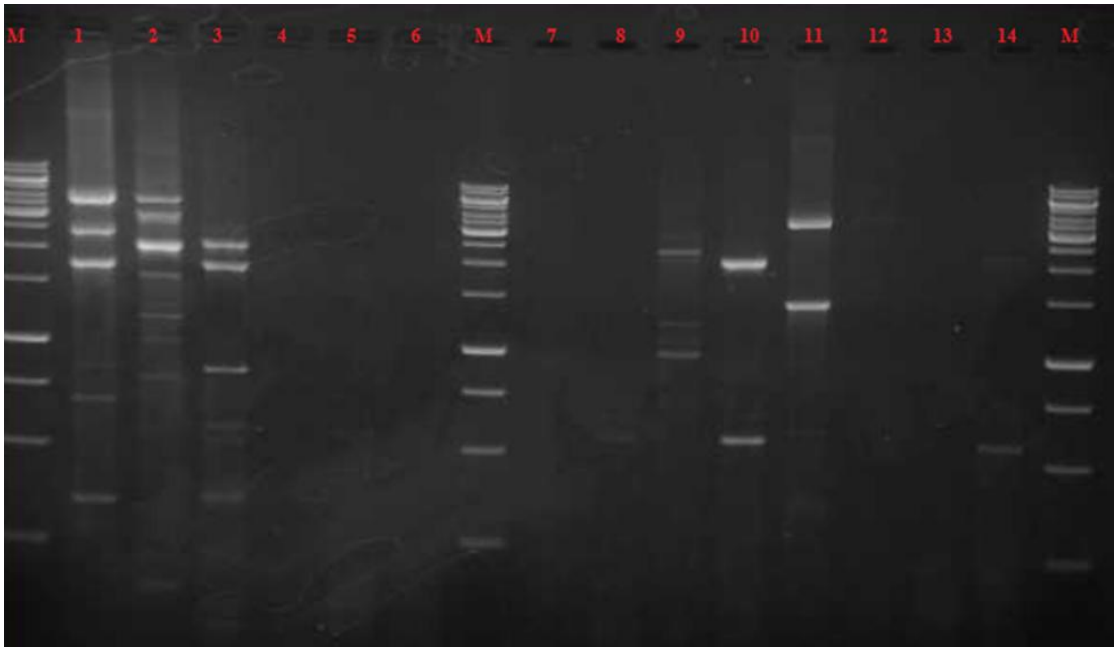


Plate XVII DNA fingerprint of *Salmonella enterica* isolates in the study

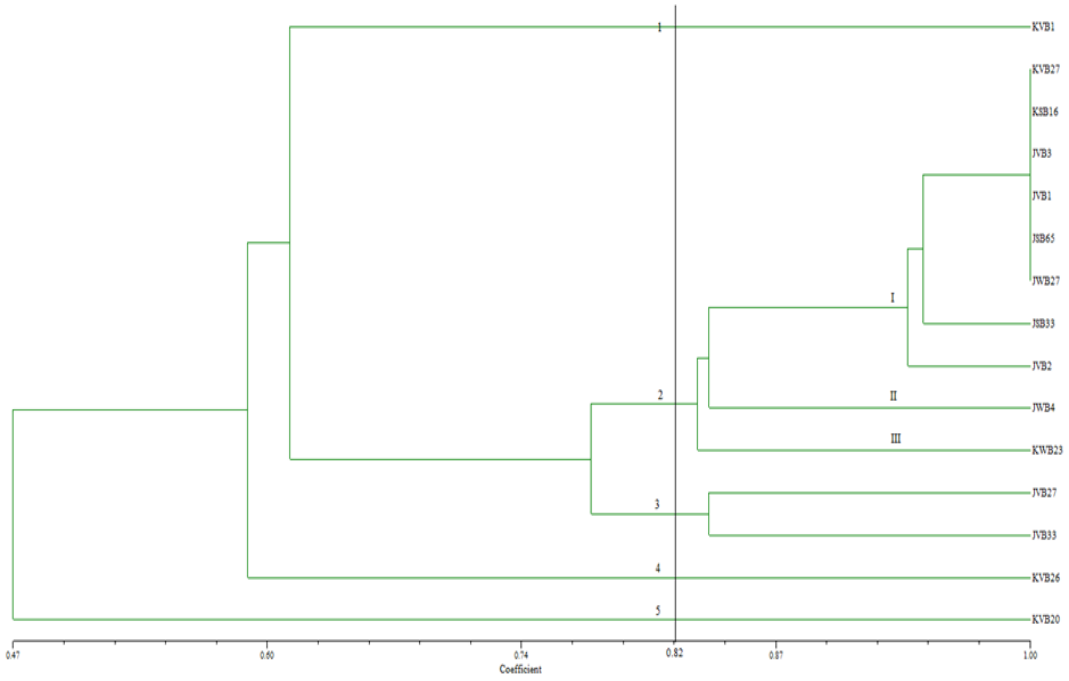


Figure 4.11 Dendrogram showing genetic relatedness among strains of *Salmonella enterica* obtained in the study

of 0.84, cluster 3 comprise strains two different vegetable samples with a similarity coefficient of 0.85, while singletons1, 4 and 5 had an isolate each.

4.9 Genetic Diversity of *Vibrio cholerae* Isolates

Figure 4.12 shows the results of ERIC DNA fingerprints of seventeen representative *Vibrio cholerae* strains obtained in the study from vegetable and environmental samples. Fingerprints showed 5-13 bands with a molecular weight of 150bp to 1kb. Two intensive bands of about 580bp and 8000bp were common in most of the strains. Cluster analysis at a coefficient of 0.62 grouped the seventeen strains of *Vibrio cholerae* into six different groups, designated 1-6; five clusters and one singleton (Plate XVIII). Cluster 1 comprises isolates obtained from irrigation water and vegetable samples at a similarity coefficient of 0.85, cluster 2 comprises isolates obtained from vegetables, irrigation water and soil with a similarity coefficient of 0.72, cluster 3 comprises strains from two different soil samples and vegetable sample at a similarity coefficient of 0.61. Cluster 4 with a similarity coefficient of 0.71 comprises three isolates obtained from three different vegetable samples.While cluster 5 comprises isolates obtained from soil and manure. One isolate existed alone, hence a singleton.

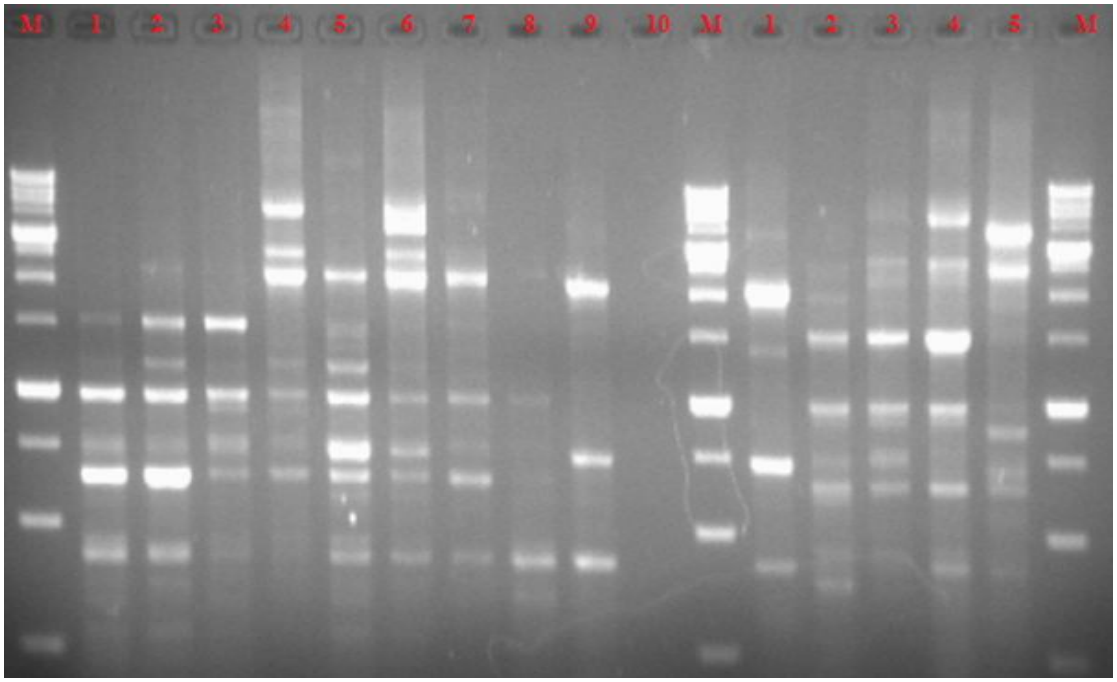


Plate XVIII DNA fingerprints of *Vibrio cholerae* isolates obtained in the study

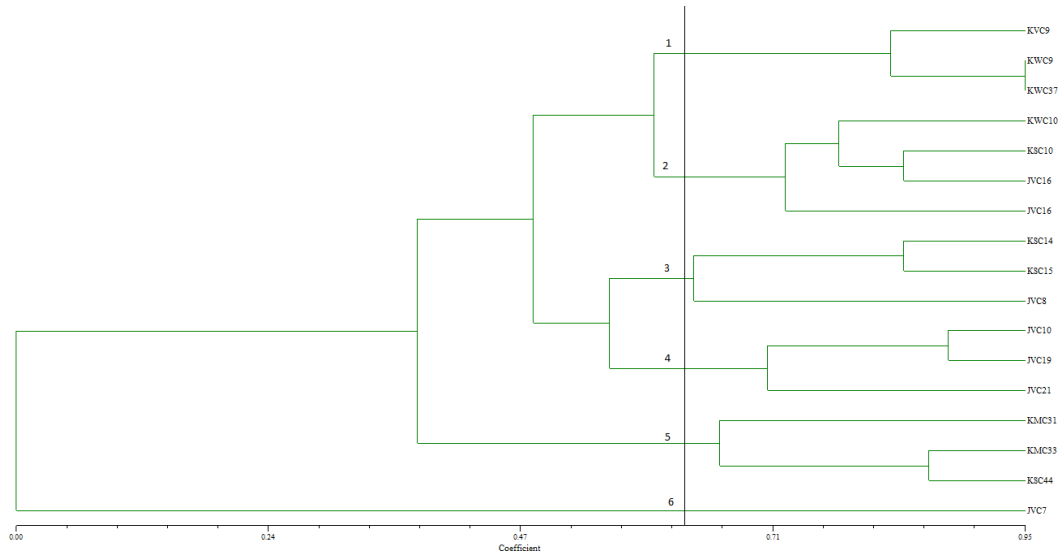


Figure 4.12 Dendrogram showing genetic relatedness of *Vibrio cholerae* strains obtained in the study

CHAPTER FIVE

5.0 DISCUSSION

Majority of the farmers practising irrigation farming in the study areas were males, though few females were noted in Plateau State. This is in line with the report of Drechsel *et al*. (2006) of male dominance in vegetable farming in Ghana. Irrigated farming is labourious and can be attributed to cultural and economic constraints as well as access to land (Zibrilla and Salifu, 2004; Obuobie *et al*., 2006). Almost all the farmers were illiterate, had just primary education, or attempted secondary school. This is similar to the report of Obuobie *et al*. (2006) who reported high illiteracy among open-space urban farmers in Ghana.The illiteracy level among the farmers could be a reason for the ignorance to the risk involved in unhealthy irrigation practices and lack of knowledge of the risk associated with the use of untreated and uncomposted organic manure in vegetable production. The high level of income generated from vegetables is an indication of the high consumption level of vegetables. Erickson *et al*. (2010) stated that increased awareness of the health benefits of eating fresh fruits and vegetables has contributed to greater consumption of produce. Further information obtained from the questionnaires indicated potential preharvest sources of pathogens to include; contaminated manure, manure compost, sewage sludge, irrigation water, runoff water from livestock operations and exposure to waste products from domestic and industrial sources. Generally, irrigation water receives no treatment before use and represents a potential risk factor as irrigation with contaminated water had been reported to lead to pathogen colonization of vegetables (Solomon *et al*., 2003). Farmers applied manure to plants at the seedling stage, this also is a great potential

risk factor as studies have suggested that colonization of plants is most likely when the plant is a seedling and the emerging root is a key area of bacterial attachment (Cooley *et al*., 2003; Dong *et al*., 2003).

The lack of significant difference in counts on vegetables irrigated with water from river in the study is not unassociated with self purification ability of all river water bodies (Okafor *et al*., 2003). The significant count observed in vegetables from well/ponds in the study is not unexpected due to runoff and percolation from surface waters. It has also been observed that standing water provides an environment in which microorganisms can survive and proliferate (Eriksson *et al*., 2002; WHO, 2006). Previous studies have also reported that shallow wells used in urban agriculture in Kumasi, Ghana showed high level of contamination because they were not protected against runoff (Amoah *et al*., 2005). Ndiaye *et al*. (2010) attributed high faecal bacteria in irrigation water to the fact that farmers often walk in them to fill their watering cans, after having walked over their plots treated with animal manure. The higher counts observed in vegetables obtained from Kano State as compared to those from well/ponds in Plateau State could be due to difference in the method of irrigation used in the two States. The method of irrigation used in sites plays an important role in the contamination of vegetables (Ndiaye *et al*., 2011).

In Kano State, farmers were found using irrigation pumps to water vegetables by the sprinkler irrigation method, while in Plateau State, most of the farmers used watering cans to obtain water out of wells/ ponds and applied to vegetables at the base (below the leaves). Previous studies had reported that contamination of vegetables with pathogens originating from wastewater occur

more when overhead irrigation methods such as sprinkler irrigation is used because the edible parts are directly exposed to the applied water (Forslund *et al*., 2012).

The lack of significant difference in counts obtained from vegetables irrigated with treated and untreated water in the study could be attributed to the level and type of treatment applied to the water and possible contamination after treatment such as from dirty watering cans. In a related study, Effler *et al*. (2001) recovered *E. coli* O157:H7 from multiple water sources considered to be treated and untreated water sources; including borehole, standpipe, stream, well and river. The lack of association between faecal coliform counts and fertilizer type used for vegetable production within and across both States could have been due to the presence of other sources of contamination from point and non point sources such as droppings from birds and other wild animals, contamination from foot of people/traders found coming to the farm sites to purchase produce directly from the farmers. Amoah *et al*. (2005) reported other sources of on- farm crop contamination.

Potential routes for preharvest contamination of fresh produce with human enteric pathogens have been reported to include exposure to contaminated surface or irrigation water, faecal contamination from livestock or wild animals, raw or poorly composted manure, farm equipment, human carriers and possibly dust (Haley *et al*., 2009, Wacheck *et al*., 2010; Gorski *et al*., 2011). Other researchers have even reported other non-point source of pollution to include flies. Flies have been reported as potentially effective vectors for dispersion of *E. coli* O157:H7 in the environment (Bach *et al*., 2002). Irrespective of the irrigation water source, mean faecal coliform levels exceeded the recommended standard (WHO, 1989). The WHO (1989) standard recommends less than 1000 faecal coliform

bacteria/ 100ml of water used to irrigate crops that are eaten raw, in addition to a requirement for secondary treatment, filtration and disinfection.

The physicochemical parameters of the irrigation water could have been influenced by the natural and anthrpogenic activities/point source contaminants around the sampling sites of this study. The higher level of faecal coliform counts obtained from abattoir and domestic waste contaminated irrigation water in Kano and Plateau States respectively could have resulted to the high level of BOD observed from irrigation water from these sites. BOD is known to be related to levels of microorganisms in water (Casanova *et al*., 2001). The biodegradable componenets of the wastewater require a large amount of oxygen for oxidation processes of micro-organisms which can cause depletion of DO (Chaudhry *et al*., 2013). The high BOD concentration observed in the irrigation water might be due to the use of chemicals, which are organic or inorganic caused by the inflow of domestic, livestock and industrial waste that contain elevated levels of organic pollutants (Salem *et al*., 2011) as chemical component discharged from sewage and industries contribute to oxygen demand (Morrison *et al*., 2001). However, the low levels of BOD concentrations obtained from water used for irrigation at the control sites reflects some level of treatment application.

Irrigation water obtained from tap in Plateau State served as control. While, at the control site in Kano irrigation water was obtained from the Kadawa dam. Tap water normally undergoes some form of treatment which includes amongst other chemicals, chlorine application. Chlorine has been reported to affect BOD measurements by inhibiting or killing the microorganisms (APHA, 1992). The low BOD values recorded from this water source reflects a lower burden of

organic pollution. Due to the high volume of water, the extent of pollution onward may not appear severe. It could also be attributable to prolonged storage in abstraction pipe and to the dying off or possible transformation of bacterial cells into viable but non-culturable states (Wang and Doyle, 1998). Lesser values of dissolved oxygen might have exterted respiratory stress for the microoganisms as reported by Nnaji *et al*. (2011). Generally, the concentrataion of BOD obtained at all sites were higher than the WHO values of 50mg/L for discharged wastewater (Akin, 2008). The range of values recorded for electrical conductivity was relatively wide.

Electrical conductivity is a reflection of the status of inorganic pollution and is a measure of total dissolved solids and ionized species in water (WHO, 2008). The higher conductance values recorded at some sites relative to other sites were apparently due to the pollutants from point and non point sources. The observed pH in this study fall within the recommended target limits (6.5- 8.5) for agriculture (WHO, 2006) with reference to pH and in lieu of its public health concerns. Similar pH values as observed in this study have been previously reported in literature (El-Shafai *et al*., 2004; Odjajare *et al*., 2011). However, Ogunfowokan *et al. (*2005) reported lower pH values (5.23-6.32) while Akan *et al*. (2008) reported higher pH (8.94-10.34). Though the mean pH values recorded for all irrigation water analysed was within the WHO pH tolerance limit, pH values of 3 to 10.5 have been reported to be favourable both to indicator and pathogenic microorganism's growth (Amaxaka *et al*., 2004). Hence, indicated pH levels seem to support bacterial growth. Changes in temperature may be attributed to the addition of sewage and industrial effluents which in turn might have contributed in enhancing the microbial activities. The reduced temperature observed at some sites may be a

reflection of the recovery of quality of water on gradual improvement due to microbial decomposition activities (Chaudhry *et al*., 2013). Physicochemical parameters have been reported to have a major influence on bacterial population (Ayati, 2013). Wastewaters often have nutrient loads, high number of pathogens is present, increasing the risk of infections occurring from them.

Salmonella, *E. coli* O157:H7 and *Vibrio cholerae* are major food-borne pathogens (Gorski *et al*., 2011) and their implication in documented illness associated with consumption of vegetables has increased in recent years (Ansingkar and Kulkarni, 2010). The prevalence of these organisms in this study raises concerns about microbial safety of fresh produce. The 13.2% prevalence of *Salmonella* spp obtained in this study is worrisome as *Salmonella* is a pathogen of economic and public health importance (Navarro-Gonzalez *et al*., 2012). A similar study reported 14% prevalence from poultry environment in Japan (Shahada *et al*., 2009). It is however lower than that reported from other developing countries from similar samples; a report of study from irrigation water used for lettuce production in Dakar, Senegal gave a 35% contamination of *Salmonella* (Ndiaye *et al*., 2011), in another study, Pangloli *et al*. (2008) reported a 38% *Salmonella* prevalence from animal and farm environment and a 79% contamination was obtained from rural water shed in Bangladesh (Haley *et al*., 2009).

The 7.3% prevalence of *E. coli* O157:H7 obtained in this study raises concern, as this pathogen has been reported to have the ability to colonise the interior of plants and internal plant compartments which makes it difficult to be removed or killed by washing and disinfection (Chekabab *et al*., 2013). The result in this study is higher than previous findings; Chigor *et al*. (2010) reported a 2.1%

prevalence of this organism from water used for irrigation of vegetables in Samaru village Zaria, Nigeria. It is however lower than that reported by Abong` O and Momba (2008) who reported the prevalence of EHEC from drinking water and vegetables as 35.5% and 21.7% respectively. In a related study, Smith *et al*. (2003) reported a prevalence rate of 17% from healthy animals in Lagos, Nigeria.

A 5.7% prevalence of *Vibrio cholerae* was obtained in this study. Reports show that episodes of cholera outbreaks in both States were traced to have been transmitted through water, food and faecal -oral route (Adagbada *et al*., 2012). Studies have shown that about 75% of people infected with this pathogen show no symptoms but shed the pathogen back to the environment hence, infecting other people (Adagbada *et al*., 2012). Its presence in the study and particularly from vegetables which are sometimes consumed raw is therefore of a serious public health concern. The prevalence is however lower than that of previous work in Calabar, Nigeria where 30.6% prevalence was obtained (Ustalo *et al*., 1992) and that of Stine *et al*. (2008) who reported a 20% prevalence from environmental samples in rural Bangladesh. It is however higher than the 0.59% isolation rate reported by Abakpa *et al*. (2011) from Zaria, Kaduna State, Nigeria. Given the low infectious doses of this enteric organisms (Kaper *et al*., 2004), their presence in this study is of public health significance.

Studies have revealed that the prevalence of *E. coli* O157:H7 is seasonally modulated (Westphal *et al*., 2011) and indicate its peak in cattle in summer which coincides with the seasonal peak in human illness (Barkocy- Gallagher, 2003; Freeman *et al*., 2009). Intrestingly, the isolation rate of this organism was higher in the wet season than that obtained in the dry season in this study. The higher

prevalence obtained in the wet season (14.8%) than in the dry season (9.3%) in this study area could be attributable to wash off by rainfall, storm events and grazing animals (Oliver *et al*., 2005; Fremaux *et al*., 2008). In the last decade, increases in *E.coli* O157:H7 outbreaks were associated with environmental contamination in water and through fresh produce (Chekabab *et al*., 2013). In our study, *E. coli* O157:H7 was detected most in vegetables, this could be due to the presence of available nutrients and moisture content in the vegetables. Studies have reported that bacterial motility (Cooley *et al*., 2003) and the ability to use seed/plant exudates as carbon sources (Roberts *et al*., 2000) are related to the extent of bacterial colonization. Internalisation of bacteria in seedling is more likely in hydroponic systems than in soil (Guo *et al*., 2002; Warriner *et al*., 2003) but internalization and transport throughout the plant can still occur in the latter growth medium (Solomon *et al*., 2002). The lower isolation rate of *E. coli* O157:H7 from soil/manure samples in the study area in both seasons could be attributable to the motility of *E. coli* from soil toward new hosts over substantially short time intervals (Oliver *et al.*, 2005; Fremaux *et al*., 2008) and to a more favourable condition, soil pH has also been hypothesized to be an important environmental variable for *E. coli* survival in soil (Fremaux *et al*., 2008). Bergholz *et al*. (2011) reported that *E. coli* genotypes display variation in persistence at different soil pH values. Moreover, the gradient in soil pH should induce variation in the bioavailability of toxic heavy metals in the study site, potentially compounding selective pressures on *E. coli* in soil (Bergholz *et al*., 2011). Johannessen *et al*. (2005) also reported that some organisms native in the soil microflora have antagonistic effects against pathogenic bacteria introduced into soil. This reported antagonistic effect could also have an impact on the presence of this organism in the soil. Though low, the presence of *E.*

coli O157:H7 in cattle manure illustrates the complex, interrelated nature of the environment, livestock production practices, food safety and microbial ecology (Islam *et al*., 2005).

Analysis of irrigation water from the study area also showed high prevalence of this organism. This is not suprising as the point source of pollution, included abattoir waste, human and domestic waste being constantly washed down to the irrigation farmers, which they apply to vegetables particularly as the intestines of ruminants, specifically cattle, have been reported to be the primary reservoir of this pathogen (Muniesa *et al*., 2012). Contamination of fresh produce has previously been observed to be associated with faecal contamination in agricultural irrigation water or runoff (Chekabab *et al*., 2013). The higher level of isolation of *E. coli* O157:H7 found in Kano State as compared to Plateau State may be due to differences in the number of cattle (which are the major carriers) in the two locations (the predorminant livestock in Kano State was observed to be cattle), differences in management practice and environmental conditions. The diversity, frequency distribution and persistence of *E. coli* O157:H7 observed in range cattle environment and confined cattle operations have been reported to vary distinctly (Renter *et al*., 2003). Other factors such as persistent recurring exposure, multiple sources of exposure, or subtype specific survival and propagation mechanisms have been suggested to explain the diversity and persistence of *E. coli* O157:H7 in specific cattle environments (Renter *et al*., 2003).

The significantly higher *Salmonella* spp obtained in the dry season than in the wet season in this study could be due to its persistence, as *Salmonella* is a multi--host pathogen with long environmental persistence (Navarro- Gonzalez *et*

al., 2012). In a related study, Okafor *et al*. (2003) reported a high prevalence of *Salmonella* in the dry season from vegetables and irrigation water in Zaria, Kaduna State, Nigeria. This however is contrary to the reported incidence of human salmonellosis with peaks in summer months throughout the world (D`Souza *et al*., 2004; Fleury *et al*., 2006; Naumova *et al*., 2006). Although, in a report of enteric fever caused by *Salmonella* in North India, the highest cases were in the dry season (Mohanty *et al*., 2006). Another report showed that isolation of *Salmonella* was negatively and significantly associated with temperature, probably because of the effect of solar radiation in the decline of permanence of *Salmonella* bacteria (Martinez- Urtaza and Simental, 2008) and that conversely, human infections prevailed during the warmest months and were negatively correlated with the presence of *Salmonella* in environment. In a study by Haley *et al*. (2009), both rainfall and temperature were positively correlated with *Salmonella* levels in a watershed suggesting that multiple environmental factors like host shedding, enhanced environmental persistence of the pathogen at warm temperatures and loading during storm events played a role in *Salmonella* occurrence.

The higher prevalence of this organism in the dry season despite the harsh environmental conditions in this season shows its persistence at relatively high rates in all seasons (Pangloli *et al*., 2008). Bacteria have been reported to evolve common stress reponse when encountering diverse environmental stresses (Sela *et al*., 2011) and exposure to a single stress may result in cross-tolerance to heterogenous stress (Hecker and Volker, 2001; Vorob`eve, 2003, Kovats *et al*., 2005; Kultz, 2005). Studies have also shown that the capacity of environmental strains of *Salmonella* may depend on species tolerance to multiple stressors (Sela *et al*., 2011). In the dry season, desication is higher and desication stress involves total dependence on

untreated wastewater from domestic sewage and abattoir waste. In Kano State, this must have probably contributed to the higher prevalence obtained in this season. Desication has been documented to increase *Salmonella* tolerance to heat (Hiramatsu *et al*., 2005). *Salmonella* strains that can persist in the environment for years with standing periods of nutrient depletion have been reported (Parker *et al*., 2010). In some reports, however, there was no difference in *Salmonella* prevalence in cold and hot months (Shahada *et al*., 2007).

Water had the highest isolation rate (34.6%) in the dry season. This is not suprising as the highest risk of pathogen movement through the soil profile is immediately after slurry application and precipitation (Semenov *et al*., 2009). In a related study by Haley *et al*. (2009), water yielded the highest percentage of *Salmonella* in samples analysed. Infact the use of wastewater is believed to have contributed to typhoid and cholera epidemics in Senegal during the last decades (Gaye and Niang, 2002). *Salmonella* was detected from soil/manure in the study area in both seasons. This confirms earlier report that soil is considered one of the ancient, biggest and highly diverse reservoirs of pathogens (Rehman *et al*., 2013). *Salmonella* is more persistent in soil compared with other bacterial pathogens (Guan and Holley, 2003). Most importantly, *Salmonella* was isolated from vegetables in both seasons. This shows the persistence and abundance of this organism in the environment. The higher incidence of *Salmonella* in vegetables in the dry season could be due to difference in time and concentration of pathogens from point source of contamination. The time of introduction and the concentration of pathogen might influence the transmission of bacteria (Johannessen *et al*., 2005; Ibekwe *et al*., 2007).

Fresh fruits and vegetables are increasingly recognized as potential sources of diseases (Beuchat, 2002; Ibenyassine *et al*., 2006) and studies have shown that vegetables from fields irrigated with polluted water and amended with manure are contaminated (Al- Laham *et al*., 2003, Amoah *et al*., 2005; Ibenyassine *et al*., 2006). *Salmonella* spp were isolated from all the samples analysed in Kano State. This reflects the ability of *Salmonella* spp to survive well in multiple environments (Gorski *et al*., 2011) and indicates its transportability between two or more environments. Therefore, multiple vertebrate sources exist for this bacterium, including livestock, wildlife, poultry and companion animals with water functioning as a documented mode of dissemination in the environment (Winfield and Groisman, 2003; Mandrell, 2009). Vegetables had the highest isolation rate of 15.4% in Plateau State and its frequency of isolation from samples was higher in the dry season than in the wet season. Studies had shown that periods of dryness on the phylloplane are interrupted by rainfall, dew formation, or crop irrigation, which may be beneficial to bacterial leaf inhabitants by increasing water availability and solubilisation of substrates (Brandl and Amundson, 2008). The low incidence of *Salmonella* spp in soil in both seasons is in line with reports by Barak and Liang (2008) that soil may not be the route of preharvest contamination.

Notably, the prevalence of this organism was higher in Kano State than in Plateau State. This could be due to difference in point source of contamination. The lower incidence of *Salmonella* in Plateau State than in Kano State could also be due to difference in the method of irrigation used. Reducing surface soil contamination through sub surface drip irrigation has been reported to have reduced contamination of produce compared with produce harvested in plots using furrow irrigation (Song *et al*., 2006).

Water had the highest isolation frequency (15.4%) of *Vibrio cholerae* in Kano State. This is expected considering reports that *Vibrio cholerae* occurs naturally in aquatic environments throughout all seasons, even under conditions unfavourable for its growth and multiplication (Zo *et al*., 2009), proliferating when conditions become favourable (Colwell and Spira, 1992). *V.cholerae* was isolated most in the dry season. This is not suprising since cholera outbreaks typically arise in settings where water, sanitation and hygiene infrastructures are inadequate (Guerra *et al*., 2000). Other researchers have also reported cases of higher incidence of *V.cholerae* in the dry season. Ustalo *et al*. (1992) reported seasonal incidence of diarrhoea cases caused by *V.cholerae* with highest cases in the dry season in Calabar. However, Opajobi *et al*. (2004) reported a higher prevalence of *V.cholerae* in the wet season in Plateau State. Similarly, Abakpa *et al*. (2011) reported more *Vibrio cholerae* in the wet season in Zaria, Kaduna State. Other studies rejected the concept of seasonal occurrence of cholera stating that *V.cholerae* occurred all year round in all seasons (Zo *et al*., 2009). Cholera seasonality is reported to vary from place to place (Tappero and Tauxe, 2011). There is acute water shortage in Kano municipality leaving the farmers the only choice of total dependence on wastewater and sludge for watering their vegetables in the dry season. This must have contributed to the seasonality observed.

V.cholerae was detected most in soil/manure samples in both seasons in Plateau State. This further emphasizes the importance of soil/manure as a means of pathogen transport in the environment. This organism also showed marked seasonality in this study area, with a higher prevalence in the wet season, this is in agreement with an earlier report by Opajobi *et al*., (2004) who also reported a higher incidence in the wet season, although contrary to its seasonal incidence in

Kano State. Since *Vibrio cholerae* has the ability to exist in a viable but non culturable state (VBNC), the possibility of high incidence in both seasons as observed elsewhere cannot be ruled out. This organism is known to have community defense mechanisms, mediated by cellular signaling, such as quorum sensing in biofilms, which gives species additional resiliency against changing environmental conditions (Zhu *et al*., 2002). *Vibrio cholerae* had a higher prevalence in Kano State than in Plateau State; this could be due to the difference in temperature of the study areas. In areas with seasonal variations in water temperatures, *Vibrio cholerae* had been observed to show clear seasonality with an increase in environmental counts during warm periods with increase in water temperature (or non detectable) during cold weather (Morris, 2011). The temperature in Kano was higher than that of Plateau State, this could have been a major factor in the higher prevalence in Kano State. Observed cases of cholera outbreak, though grossly under reported are higher in Kano State than in Plateau State.

In Plateau State, the prevalence rate of *V.cholerae* was 5.2%. This prevalence rate is significant and represents a significant risk factor since vegetables are best consumed raw. This agrees with the findings of Opajobi *et al*. (2004), who reported a prevalence rate of 4.4% in Plateau State. The prevalence rate of 1.9% of *E. coli* O157:H7 obtained in Plateau State though low is significant due to its low infective dose (Kaper *et al*., 2004). This however, confirms the absence of incessant gastroenteritis in this study location. The higher prevalence (2.3%) of this organism in the dry season than that obtained in the wet season (1.6%) could be due to the stress in this season as a result of lack of water, cattle are reported to be the

major reservoir of these organism and shed it more frequently when stressed from lack of water (Armstrong *et al*., 1996).

The highest isolation of *E. coli* O157:H7 in Plateau State was from water (5.3%). This could be due to the reported case of high pollution of the old mine ponds used for irrigation in the State with human and animal faeces (Damen *et al*., 2007) as, the inhabitants of such areas, cattle rearers and vegetable farmers defecate there. The inability to detect this organism in vegetables in the wet season could be as a result of low concentration of the pathogen in point source of contamination and the time of application of manure. Solomon *et al*. (2002) reported that manure contaminated with high numbers of *E. coli* O157:H7 could be a source of transmission of this pathogen to the plant tissue. Another study reported that both the time of introduction and the concentration of this pathogen might influence the uptake of bacteria (Johannessen *et al*., 2005; Ibekwe *et al*., 2007). The absence of *E. coli* O157:H7 in vegetables but presence in manure agrees with the result of a field study in which manure naturally contaminated with *E. coli* O157:H7 was used as fertilizer on lettuce and *E. coli* was not isolated from the lettuce (Johannessen *et al*., 2004). Warriner *et al*., (2003) observed that the interaction of *E. coli* with spinach was dependent on the stage of introduction of bacteria, hence its absence could be due to time of manure application.

In the dry season however, *E. coli* O157:H7 was detected in only vegetable samples. This could be as a result of bacterial motility (Cooley *et al*., 2003), ability to utilize carbon sources (Roberts *et al*., 2000) and the constant moisture content of the vegetables as water may be available to the environment only once or twice a day. Once in foods or food ingredients, *E. coli* O157:H7 displays a remarkable ability to survive harsh environmental conditions (Islam *et*

al., 2005). The inability to detect this organism from the soil environment could be a result of decreasing moisture and variation in temperature. Entry *et al*., 2000 found that decreasing soil moisture with increasing soil temperature substantially decreased survival of total and fecal coliform bacteria. Soil moisture seems to be the most important of factors affecting bacterial survival (Entry *et al*., 2000, Jiang *et al*., 2002, Saini *et al*., 2003; Larney *et al*., 2003). The precipitation moisture status of a soil, which may be influenced by season, also impacts pathogen survival, fate and transport (Powelson and Mills, 2001; Chu *et al*., 2003).

The existence of *Vibrio cholerae* O1 (Inaba and Ogawa), the Hikojima strain and even the non O1 *Vibrio cholerae* strains illustrates the increasing diversity of *Vibrio cholerae* in Nigeria as reported elsewhere (Talkington *et al*., 2011) and has great implications, as *Vibrio cholerae* O1 and O139 have pandemic potential (Talkington *et al*., 2011). The El-Tor biotype began the ongoing seventh pandemic, displacing the classical biotype of the fifth and sixth pandemics because of its superior ability to survive in the environment, increased frequency of asymptomatic carriers and more efficient transmissibility (Safa *et al*., 2010). The adaptive immunity of the local population as well as climatic variations will further enhance bacterial evolution; for example, it will not be suprising to observe a gradual switch over time from the Ogawa to the Inaba serotype as population immunity to Ogawa rises, as has been reported during outbreak (Pal *et al*., 2010). The isolation of non O1 *V.cholerae* is also significant since environmental studies suggest that non-O1 *V.cholerae* gastrointestinal infections may also be associated with environmental sources (Udo *et al*., 1994) and it has been linked to outbreaks in various regions of Bangladesh and India (Faruque *et al*., 2013).

Precipitation and storm events have been reported to mobilize pathogens from agricultural and urban environments as well as on-site sewage disposal systems and subsequently transport them into aquatic environment (Lipp *et al*., 2001; Haley *et al*., 2009). This must have greatly contributed to the higher incidence of *E. coli* O157:H7 in the wet season in the study. Cooley *et al*. (2007) reported that the incidence of generic *E. coli* and *E. coli* O157:H7 in some locations increased after rainfall events and correlated with significant increases in the flow rate of rivers and streams. Its detection in soil further emphasizes its persistence in the environment as *E. coli* O157:H7 have been found to persist for a long time in soil (Khandaghi *et al*., 2010).

The production of toxin by *E.coli* isolates assayed represents potential risk to public health as *E.coli* has been reported to have a low infective dose (Chart, 2000). Previous studies have reported that among VTEC factors, verotoxins are considered to be the most critical virulence factor associated with human disease (O`Loughlin and Robins-Browne, 2001). The combination of VT1 and VT2 was highest in the isolates obtained. This is in line with the report of Kruger *et al*. (2011) that a combination of subtypes VT1/VT2 is most frequent in samples. Isolates from vegetables had the highest detection level. This supports the studies of Beutin *et al*. (2007) who found this toxin in food samples, indicating that humans can be exposed to this along the food chain. Isolates that produced VT2 toxin were few. Though few, its production is significant as VT2 positive VTEC strains have been found to be related to higher virulence and are significantly associated with heamorrhagic uremic syndrome (Friedrich, 2002, Beutin *et al*., 2004, Bielaszewska *et al*.,2006; Persson *et al*., 2007). Sablet *et al*. 2008 observed that the expression of VT2 is heterogenous in basal and in induced conditions, depending on the VT2

variant. In this way, some differences reported in basal and in induced VT production between disease associated and bovine associated VTEC bacteria (Ritchie *et al*., 2003) could be explained by the VT subtypes present in the strains studied. Other factors have also been reported to affect toxin production such as growth in low-iron medium and phage induction (Ritchie *et al*., 2003). Polymyxin B was used to induce toxin production in the isolates, the inability of some isolates to produce toxin could have been as a result of some other factors required for toxin production such as amino acids which were lacking in this assay.

Cholera surveillance is in progress in many countries, based primarily on detection of *V.cholerae* O1, O139 and determining the presence of cholera toxin (CT), the principal virulence factor responsible for the most relevant symptoms of cholera (Jain *et al*., 2011) using biological and molecular methods (Rivera *et al*., 2001).Virulence associated factors in *V.cholerae* isolates from environmental sources are of concern (Rivera *et al*., 2001) as toxigenic *Vibrio cholerae* strains possess the potential to cause epidemics and global pandemics of cholera (Jain *et al*., 2011). In this study, the virulence trait of *Vibrio cholerae* CT toxin was detected in strains of *Vibrio cholerae* O1 and O139 obtained. Studies have reported that the bacterium has many serogroups but only toxin producing serogroups O1 and O139 cause epidemic cholera (Sjolund-Karlson *et al*., 2011) and they both have pandemic potential (Talkington *et al*., 2011). Previous reports have stated that the nature of virulence and the infective doses need to be determined for the establishment of guidelines for risk assessment of each species in surface water and food material earmarked for human consumption (Janda *et al*., 1988). The detection of toxin in the *Vibrio* isolates from samples analysed in this study shows that (vegetables) produce and the environment (water and soil/manure) harbor viable and toxigenic

V.cholerae and therefore, represents a potential risk factor for continuous cholera epidemics in Nigeria particularly in the study area.

Acquired resistance to first line antibacterial agents increasingly complicates the management of extraintestinal infections due to *Escherichia coli*, which are a major source of illness, death and increased healthcare costs (Gupta *et al*., 2001; Russo and Johnson, 2003; Pitout *et al*., 2005). Ciprofloxacin and ceftriaxone were the most active antibiotics against the *E. coli* O157:H7 isolates in the study. This is in line with the report of Yah and Eghafona (2007), that ciprofloxacin is effective against diarrhoeagenic *E. coli*. The high level of resistance of these isolates to tetracycline observed agrees with the findings of Chigor *et al*. (2010) who reported 100% resistance of *E. coli* O157 isolates to tetracycline. Sulphamethoxazole and amoxicillin were also active against only few isolates in this study, this reflects the report that the high rates of resistance to trimethoprim/sulphamethoxazole and ampicillin should prompt close review of treatment success rates in settings in which they are commonly used in empiric therapy (Hooton *et al*., 2004).

There is evidence indicating that tetracycline survives longer in the environment than other antibiotics which may be critical in maintaining the level of tetracycline resistance at a high level (Yah and Eghafona, 2007). This could explain the total resistance of *E. coli* isolates obtained from soil/manure to this drug. This is worrisome considering that tetracycline is a first line drug in Nigeria and as in most developing countries, people with gastrointestinal infections readily purchase it across the counter for self medication. The lack of sensitivity of any of the isolates to cephalothin is similar to the findings of Wagner *et al*. (2008). Cephalothin is one of the narrow-spectrum cephalosporins which are beta-lactam

antibacterials that inhibit cellwall development (Wagner *et al.*, 2008) and is widely used in livestock production.It has been reported that 1.6 billion broiler eggs or chicks receive cetiofur (Mellon *et al*., 2001) and *E. coli* isolates resistant to these drugs are found in poultry (Johnson *et al*., 2007) and these could promote resistance effectively.

As humans are continuously exposed to bacteria in the environment, the accumulation of resistance genes in soil due to the spreading of manure is likely to contribute to the threat of antibacterial resistance in the therapy of infectious diseases (Heuer *et al*., 2011). Isolates from vegetables also showed resistance to most of the antibiotics used. Udo *et al.* (2009) also reported 33.33% multiple antibiotic resistance in *E. coli* in salads in Calabar, South-South, Nigeria. This supports the findings of Johnson *et al.* (2007) that one suspected source of drug-resistance *E. coli* in humans is use of antibacterial drugs in agriculture and it presumably selects for drug resistance *E. coli,* which may be transmitted to humans through the food supply (Jones *et al*., 2005; Collingnon and Angulo, 2006). The low level of resistance of these organisms to the quinolones may be because they are relatively new antibiotics and are more expensive than tetracycline, ampicillin and gentamicin (Yah and Eghafona, 2007). Due to their high cost, they may not be commonly purchased and hence less used and presence of reduced residue from it in the environment.

None of the antibiotics used recorded 100% sensitivity from *E. coli* O157:H7 in this study. This supports the report that use of antibacterials to treat *E. coli* O157:H7 is a contentious area in the management of infection and is currently contra-indicated, as it may exarcerbate the disease (Todd and Dundas, 2001). Hence, use of these or any antibacterial for therapeutic and non therapeutic

indications may exert selective pressure and result in the development and maintenance of serotype O157:H7 strains of *E. coli* that are resistant to these and related antibacterials (Byrne *et al*., 2003). *E.coli* carrying several antibiotic resistance genes may act as a donor of those resistance genes to other pathogenic *E.coli* and even other species (Gundogdu *et al*., 2011). Hence, the resistance observed in this organism in the study could be transeferred to other pathogens and represents significant threat to health. Antibacterial therapy may be of value for some forms of diarrhoeagenic *E. coli* (Oberhalm *et al*., 1987), but this resistance pattern is an emerging problem for diarrhoeagenic *E. coli* strains isolated in developing countries (Putnam *et al*., 2004) and for other enterobacteria worldwide (Replogle *et al*., 2000, Bhattacharya and Sur, 2003; Putnam *et al*., 2004). *E. coli* O157:H7 has been reported to be a refactory to developing resistance among pathogenic bacteria (Todd and Dundas, 2001), hence the antibacterial resistance being transferred is of great relevance in pathogen emergence and re-emergence (Oppergaard *et al*., 2001, Poppe *et al*., 2001; Schroeder *et al*., 2002 ; Solomon *et al*., 2002).

The resistance of *Salmonella* isolates to amoxicillin and sulpha methoxazole is similar to reports of *Salmonella* resistance to ampicillin and cotrimoxazole in South east Asia (Wain and Kidgell, 2004), where the incidence of typhoid fever is high and antibacterial agents are widely available without prescription providing the opportunity for the development and selection of resistant strains (Cooke and Wain, 2004) just as in Nigeria. Resistance of some *Salmonella* isolates to ciprofloxacin and ceftriaxone in this study is in line with recent reports that they are among the drugs with an upward trend in resistance (Medalla *et al*., 2011) and is a public health issue, as the extended-spectrum cephalosporin,

ceftrixone is the drug of choice for treatment of pediatric salmonellosis and the fluoroquinolone, ciprofloxacin, which are preferable for treatment of adults (Foyle and Lynn, 2007). Tetracycline and cephalothin were the least active against *Salmonella*. Infact, cephalothin was not active against any of the isolates in the study. It has been suggested that extensive therapeutic use of the veterinary cephalosporin ceftiofur has been a major driving force for the dissemination of this resistance (CDC, 2002 ; Davis, 2007) and there is concern that ceftiofur- resistant *Salmonella* strains may develop cross-resistance to ceftriaxone, because of the structural similarity of these two drugs (Brichta-Harlay *et al*., 2011). Hence, it is likely that environmental selective pressure caused by use of antibacterial drugs has led to the distribution of appropriate resistance genes (Gal-Mor *et al*., 2010). Recent studies have found that in certain *Enterobacteriaceae* including *Salmonella*, virulence genes may be colocalising on transferable resistance plasmids (Fricke, 2009 and Venturini *et al*., 2010). A phenomenon that would lend credence to studies that have shown that antibacterial- resistant *Salmonella* strains may be more invasive than *Salmonella* strains that are susceptible to antibacterials (Helms *et al*., 2004, Varma, 2005; Varma, 2006). Therefore, the multidrug resistance pattern obtained from isolates in this study is of public health significance as salmonellosis is a global foodborne disease of humans and livestock (Wu *et al*., 2013).

The multidrug resistance pattern of the *Vibrio* isolates obtained in this study, confirms earlier report that multidrug resistance is increasing in *Vibrio* as reported elsewhwere (Kitaoka *et al*., 2011). The resistance pattern obtained is similar to that obtained in Haiti, where strains were susceptible to tetracycline and either susceptible or intermediately susceptible to ampicillin/amoxicillin but resistant to other antibiotics such as sulpha methoxazole (Talkington *et al*., 2011).

The low level of resistance gives credence to the fact that tetracycline remains the drug of choice in adults, while treatment with trimethoprim- sulfamethoxazole could still have some effect as alternative drugs for children and pregnant women respectively (WHO, 1992).

Multiple antibiotics resistance (MARI) index is a tool that reveals the spread of bacteria resistance in a given population (Adeshina *et al*., 2012). A MAR index greater than 0.2 implies that, the strains of such bacteria originate from an environment where several antibiotics are used (Adeshina *et al*., 2012). Bacterial isolates in this study showed MAR indices of 0.2 and above reflecting the high level of antibiotics in the environment. The MAR indices obtained could be a result of extensive use of antibiotics by man and in agriculture to prevent, treat as well as to promote growth (Ribeiro *et al*., 2012). Consumption of produce with these bacteria especially as vegetables are considered best eaten raw is worrisome and represents a potential public health risk. Studies have revealed that consumption of raw produce carrying MAR bacteria elevates the resistance gene pool in the human gut micrflora (Schjorring and Krogfeit, 2011). Another study observed that there was continuous mutation selection of bacteria containing MAR genes on mobile genetic elements which continued to spread these MAR genes through green leafy salads, vegetables and fruits (Walia *et al*., 2013). In a previous study, Adeshina *et al*. (2012) reported susceptibility pattern of bacterial isolates obtained from salad sold in restaurants in Zaria, Kaduna State (Nigeria) with MAR indices of 0.2 and above.

The detection of *uid*A gene in *Escherichia coli* O157:H7 in this study is in aggrement with the findings of Tantawiwat *et al*. (2005) and Heijen and Medema (2006) and it supports the detection of this gene in *E.coli* O157:H7 as reported by Ibekwe and Lyon (2008). The most notorious serotype of EHEC, the

O157:H7 is responsible for numerous reported outbreaks (Hlavsa *et al.*, 2011). The amplification of the rfb_{o157} gene in isolates obtained further confirms our serological characterisation of this organism.

The inability to detect the stx gene from the *E. coli* O157:H7 could have been due to the long period of preservation of the isolates as studies revealed that some verotoxin factors are plasmid- encoded and the plasmid could be lost during the many subcultures of strains (Clermont *et al*., 2011). The presence of free *stx* phages interacting with isolates during subculturing (Karmali *et al*., 1985) could also lead to loss of the *sxt* genes upon subcultivation/ subculturing of isolates (Karch *et al*., 1992).

During the process of *Salmonella* infection, invasion genes are required for bacterial entry into host cells (Mezal *et al*., 2013), while *Salmonella* enterotoxin (*stn*) is a putative virulence factor responsible for enterotoxic activity (Chopra *et al*., 1999). Amplification assay for both genes showed postitve for all *Salmonella* species obtained in this study. This gives credence to previous reports that typhoid fever, an infection caused by *Salmonella* is endemic in Nigeria (Adagbada *et al*., 2012). The presence of both genes in the *Salmonella* strains assayed in this study is in aggrement with the reports of Torpdahl *et al*. (2005), Moganedi *et al*. (2007), Ammari *et al*. (2009) and Shanmugasamy *et al*. (2011). The present study supports the ability of these specific primer sets to confirm the isolates as *Salmonella* and indicated that *inv*A and *stn* genes are widely distributed among *Salmonella* strains irrespective of their serovars and source of isolation as reported by Salehi *et al*. (2005). The detection of the *stn* and *inv*A genes confirms that both genes contain sequences unique to *Salmonella* strains, hence both genes are suitable PCR target for detection of *Salmonella* strains from varied sources.

Salmonella isolates obtained from vegetables and environmental samples were found carrying the same virulence genes as have been reported in clinical isolates of *Salmonella.* These virulent genes have been found to play an impotant role in invasion of the intestine during infection (Mezal *et al*., 2013). This confirms that produce and environmental isolates can cause human infections. Similarly, Akinyama *et al*., 2011 reported that *Salmonella* isolates from the environment carried the same virulence genes as clinical isolates.

The amplification of the cholera (CT) *ctx*A gene subunit of an A-B type toxin, a principal virulence factor responsible for cholera symptoms (Jain *et al*., 2011) further emphasizes potential risk to public health and confirms toxigenicity of *Vibrio* strains in the environment studied. The *ctx* genes are located on mobile genetic elements and could be transferred to a virulent strains (Akoachere *et al*., 2013), hence a high potential of spread in the environment and resultant outbreaks of cholera.

Antibiotics are distributed in the environment in low concentrations via agricultural runoff from precipitation, animal and poultry feces used as fertilizer and wastewater effluent (Walia *et al*., 2013). Pathogens in this study were resistant to one to several antibiotics tested against them. Bacterial plasmids are key vectors of horizontal gene transfer, mediating the mobilization of genetic material from bacteria to bacteria (Cottell *et al*., 2011). The detection of multidrug resistance plasmids and the QRDR in these isolates agrees with the findings of Cottell *et al*. (2011). This finding supports the suggestion that pCT persistence and dissemination have been driven by constant β-lactam exposure and that pCT can remain stable within a population. The ability and frequency with which antimicrobial resistance genes disseminate between bacteria in humans, the environment and in animals is

still debated. The role of plasmids in the movement between ecosystems, including the food chain, is also still contested, despite mounting evidence that it occurs (Sheldon, 2010; Hunter *et al*., 2010). Intrestingly, isolates in this study were obtained from vegetables, animal manure and irrigation water. Representative isolates from these sources showed the presence of antibiotic resistance plasmids. Hence, this work supports the hypothesis that pCT is disseminated broadly between bacteria in animal and human ecosystems (Cottell *et al*., 2011).

Antimicrobial resistance remains a serious global health concern (Costa *et al.,* 2013). Isolates in this study were found to be PCR positive for Class 1 intergron and had sulfonamide resistance genes. These elements are able to capture, integrate and express resistance gene cassettes in their variable region (Jain *et al*., 2011). Sulfonamide resistance is associated with treatment failures (Gundogdu *et al*., 2011). The presence of integron and sulfonamide genes in isolates obtained in this study indicates that these mobile genetic elements are common in the study location and is associated with reduced susceptibility to the first-line antimicrobial drugs (Adzitey *et al*., 2013). Co- selection processes have been reported to occur for virulence and resistant genes located in mobile genetic elements or associated with gene capture systems such as integrons (Koczura *et al*., 2012) and integrons are important vectors for the spread of antimicrobial resistance (Farakas *et al*., 2013). The presence of these resistance genes and the observed reduced susceptibility to clinically relevant antibiotics in recovered pathogens demonstrates that fresh produce may serve as a vehicle for the transmission of antimicrobial resistance in the study area and even across national borders.

Molecular typing of bacteria has been widely used in epidemiological studies and for tracing the transmission of food-borne pathogenic bacteria (Chen *et*

al., 2012) and determination of relatedness among strains. In our study, *E.coli* O157:H7 isolates obtained from vegetables were found in the same cluster as isolates obtained from environmental sources; irrigation water and soil/manure. Earlier studies have reported that isolates in the same cluster are genetically related and it indicates possible contamination of pathogens (Adzitey *et al*., 2013). In cluster 1, strains obtained from two different vegetable samples and soil samples were found, hence their genetic relatedness. Isolates in clusters 2, 3, 4 and 5 also comprised strains obtained from different samples hence genetically related at respective coefficients of similarity.

These genetic similarities could possibly indicate that the isolates obtained from vegetables could have resulted from the water used for irrigating the vegetables or the untreated manure used to supply nutrients to the vegetables for maximum yield. Isolate Kva31 clustered as a singleton indicating that these isolate is genetically diverse (Adzitey *et al*., 2013) from other *E.coli* O157:H7 isolates obtained in the study. The diversity observed could be due to other sources of contamination to produce such as wildlife. Based on the result of this study, some isolates from different sources/ samples may have originated from a single source and transmitted through different routes to vegetables or from diverse sources as shown in similar and different banding patterns. Our results are similar to previous studies; Ateba and Mbewe (2013) reported similarity of fingerprints strains of *E.coli* O157:H7 obtained from different sources while, Forslund *et al*. (2012) reported a lack of similarity between *E.coli* strains obtained from soil and water used in irrigated produce.

Fingerprinting pattern of *Salmonella enterica* strains from the different samples also showed marked similarity and close genetic relatedness. The

largest cluster, cluster 2 had isolates from vegetables, soil and irrigation water sources at a similarity coefficient above 0.82, hence highly genetically related. Consistent contamination with irrigation waters has been shown to be routes of crop contamination in produce related *Salmonella* outbreaks (Levantesi *et al*., 2012). This has been shown in the marked genetic similarity observed in our study. Previous studies also reported genetic similarity among *Salmonella* isolates obtained from food, environmental and clinical samples (Mezal *et al*., 2013). The continuous use of untreated wastewater in vegetable production in Nigeria therefore, calls for concern, as *Salmonella* is one of the leading casuses of intestinal illness all over the world as well as the etiologic agent of more severe systemic diseases such as typhoid and paratyphoid fevers (Levantesi *et al*., 2012).

Genotypic analysis of *Vibrio cholerae* isolates also showed close relatedness and diversity among some strains. Several studies have reported that clonal groups could be transmitted along the food chain and represent a hazard to human health (Hauser *et al*., 2012) and contaminated water was suspected as the source of enteric pathogens during several recent produce-related outbreaks (Benjamin *et al*., 2013). The similarity observed between strains obtained from vegetables and those from the environment is alarming and calls for concern as cholera infection, caused by *Vibrio cholerae* is endemic in Nigeria and outbreaks not unusual (Adagbada *et al*., 2012). The high diversity shown among some strains which clustered alone as singletons could be due to other sources of contamination such as wild birds/animals. It could also be from migrant strains as a result of the high possibility of *Vibrio* strains to move from non point sources of contamination leading to the diversity observed and indicative of multiple contamination sources (Zulkifili *et al*., 2009). The relatedness among pathogens obtained from the

environment (irrigation water and soil/manure) and vegetables in this study reflects that the phenomenon of pathogen transmission along the food chain or from the environment to humans does exist in Nigeria.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 SUMMARY

1. Farm management practices which include use of untreated wastewater and untreated manure were observed to have contributed greatly to preharvest contamination of vegetables in both study locations (Kano and Plateau States).

2. The physicochemical parameters and faecal coliform levels of irrigation water evaluated, exceeded acceptable limits for irrigation of crops/vegetables for human consumption and therefore represents a public health risk.

3. The presence of pathogenic bacteria (*E.coli* O157:H7, *Salmonella enterica* and *Vibrio cholerae* in vegetables and environmental samples in both study locations was ascertained. Bacterial isolates obtained were observed to possess housekeeping genes (eg *uid*A), virulence genes (rfb $_{O157}$, *inv*A, *stn*, *ctx*A) and resistance gene (quinolone resistance genes, pct, class one intergrase and sul genes) by Polymerase Chain Reaction.

4. Antibacterial resistance was observed in isolated organisms, indicating the role of the environment/ irrigation water in emerging antibacterial resistance level. The high level of antibiotic resistance observed in this study was most probably due to the widespread and heavy use of numerous antimicrobial agents in human therapy and livestock production.

5. Overall, our data indicated that accumulation of resistance genes in environmental bacterial communities may be the consequences of potential long-term persistence of active pharmaceutical residues in soil.

6. The nucleotide sequence of pathogens obtained in this study gives an insight on the type of prevailing strains in the study locations and possibly in Nigeria as a whole.

7. ERIC-PCR fingerprinting revealed that isolates obtained from vegetables showed identical banding pattern with that obtained from environmental (irrigation water, soil and manure) sources.

6.2 CONCLUSION

This study conclude that management practices which involves the use of untreated wastewater and manure was observed to contribute greatly to preharvest contamination of vegetable with the accumulation of resistance genes in bacteria in the environment. This study reflects the potential risks and hazards associated with production of vegetables by use of organic manure and untreated waste water and therefore the need for prior treatment and continuous monitoring in order to ensure safe consumption of vegetables in Nigeria. Data obtained in this study will be useful in assessing the health risk associated with use of untreated wastewater and/or organic manure in organic production in Nigeria. It further provides a baseline data describing the occurrence and levels of microbiological indicators in produce regions in the study locations and Nigeria as a whole.

6.3 RECOMMENDATIONS

Farmers should be enlightened on the risk associated with farm management practices and relevant precautionary measures such as manure composting to avoid farm to table contamination. Composting is an accepted manure pathogen reduction treatment and compost–generated heat is believed to eliminate pathogenic bacteria. Hence composting should be promoted as it cost little or nothing to practice. Farmers should allow adequate time before using manure as fertilizer. Animal manure should be stored for atleast 120days before use in farming.

Identifying reservoirs and transport of enteric pathogens in this important vegetable production region and other important produce regions in Nigeria is critical to improving food safety and public health, we advocate regular monitoring of these pathogens in the environment.

Faster and quicker identification procedures to identify enteric pathogens in food/food produce are expensive and lacking in Nigeria. More rapid molecular tools which identify pathogens directly in samples should be looked into. Molecular subtyping of pathogens isolated by standard procedures and development of a DNA fingerprinting database of these isolates would enable monitoring of changes in epidemic patterns. Therefore, a sensitive surveillance system based on a molecular subtyping technique such as PFGE or MLVA is needed for early detection of such epidemics. All these molecular techniques, still lacking in Nigeria, calls for concern. Hence, the achievement of this level of technology in Nigeria is strongly recommended.

Further studies are required on these pathogens to determine their allelic variations of integrons and other transposable element in them. As a better understanding of the molecular mechanisms by which antimicrobial resistance emerges and spreads would enable design of intervention strategies to reduce its progression.

Due to worldwide emergence of old and new infectious agents, new strains of pathogens and the fact that the world is now a global village, we advocate the establishment and enforcement of National Microbiological Standards for ready to eat fresh vegetable salads in Nigeria.

The observation of potentially epidermic and endemic biotypes of *Vibrio cholerae* O1 in this study further demonstrates the need for research into the ability of pathogens to persist in epidemiologically complex agricultural settings.

Oral cholera vaccines (ocvs) have been recommended in cholera-endemic settings and preemptively during outbreaks and complex emergencies. Kano State, Nigeria suffers atleast 3 episodes in a year, the use of vaccine is strongly advised.

Introduction of enteropathogenic bacteria via manure into the food chain should be avoided and more precise safety guidelines prepared for defining actions to minimize contamination of plant produce.

The government's policy on the role of the agrarian sector in the economic development of Nigeria should also include measures to reduce risks associated with pre and post harvest contamination of produce.

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APPENIDICES

Appendix I Questionnaire for assessment of risks factors associated with vegetables grown in irrigated fields in Kano and Plateau States, Nigeria

DATA COLLECTION

State/Site ----------

Date of interview---------- (day/month/year)

Location (Kano/ Plateau)
Survey site----------
Name of respondent----------
Age----------
Sex ----------

SOCIO- ECONOMIC VARIABLES

1. Occupation----------
2. Estimated income from vegetables----------
3. Level of formal education----------

INFORMATION ON STUDY AREA

Source of water for irrigation (well, pond, river, tap and dam)
Treatment of water A. Treated B. Untreated
If yes, method of treatment----------
Point source of pollution A. Livestock waste B. Industrial waste C. Domestic or municipal waste.

INFORMATION ON VEGETABLES PRODUCTION

1. Type of fertilizer used A. Organic B. Inorganic
2. If organic: Source----------
3. At what stage did you apply the manure/ fertilizer before harvest?----------

Appendix II Biochemical characterization of presumptive isolates of *Escherichia coli* O157 in the wet season by Microbact

Isolate Code	Oxidase	Motility	Nitrate	Lysine	Ornithine	H_2S	Glucose	Mannitol	Xylose	ONPG	Indole	Urease	VP	Citrate	TDA	Gelatin	Malonite	Inositol	Sorbitol	Rhamnose	Sucrose	Lactose	Arabinose	Adonitol	Raffinose	Salicin	Arginine		Identification no
KVa9	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	+		347600261
KVa8^{D}	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	+		347600261
KVa19	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+		347600061
Kma11	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	+		347600261
KVa14	-	+	+	+	-	-	+	+	+	+	+	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-	+		347600061
KVa29	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+		347600061
KVa3	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	+		347600361
KVa40	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+		347600075
Kwa14	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	+		347600261
KVa13	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	-	+	-	+		347600365
Kma41	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	+		347600261
KVa12	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+		347600161
Jwa38	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	+		347600261
Kma7	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+		347600061
KVa20	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+		347600360
KVa10	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	+		347600261
KVa8$_{b}^{D}$	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	+		347600361
KVa31	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+		347600061
Jsa9	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	+		347600261
KVa6	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+		347600061

Appendix III Biochemical characterization of presumptive isolates of *Escherichia coli* O157 by Microbact (Dry season)

Isolate Code	Oxidase	Motility	Nitrate	Lysine	Ornithine	H_2S	Glucose	Mannitol	Xylose	ONPG	Indole	Urease	VP	Citrate	TDA	Gelatin	Malonite	Inositol	Sorbitol	Rhamnose	Sucrose	Lactose	Arabinose	Adonitol	Raffinose	Salicin	Arginine		Identification no
KVa3	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	+	+	+	-	-	-		347600270
Kwa7	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-		347600260
Kwa4	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-		347600260
Kwa15	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	+	+	-	+	-	-		347600264
KVa8	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-		347600370
KVa7	-	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-		347600264
KVa16	-	+	+	+	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-		367600260
JVa11	-	+	+	+	+	+	+	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-		375310020
Jwa7	-	+	-	+	-	-	+	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-		245600376
KVa13	-	+	-	+	+	-	+	-	+	-	-	-	-	-	-	+	+	-	-	+	-	-	+	-	-	+	+		265006223
KVa11	-	+	+	+	-	-	+	-	+	-	+	-	-	-	+	-	-	+	-	-	-	-	+	-	-	+	+		347600261
JVa3	-	+	-	-	+	-	+	-	+	-	+	+	+	-	+	-	-	-	-	+	-	-	+	-	-	-	-		226350220
JVa8	-	+	+	+	+	-	+	-	+	-	-	+	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-		365101760
JVa10	-	+	-	+	+	+	+	-	+	-	+	+	-	-	-	-	-	-	+	+	+	+	-	-	-	-	+		265300361

Appendix IV Biochemical characterization of presumptive *Salmonella* isolates by Microbact (Rainy/Wet season)

Isolate Code	Oxidase	Motility	Nitrate	Lysine	Ornithine	H_2S	Glucose	Mannitol	Xylose	ONPG	Indole	Urease	VP	Citrate	TDA	Gelatin	Malonite	Inositol	Sorbitol	Rhamnose	Sucrose	Lactose	Arabinose	Adonitol	Raffinose	Salicin	Arginine		Identification no
Jvb 34a	-	+	+	+	+	+	+	+	+	-	-	-	-	+	+	-	-	-	+	+	-	-	+	-	-	-	+		377030621
Jvb 33	-	+	+	+	-	-	+	+	+	+	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	+		347420601
Jsb 65	-	+	+	+	-	-	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	+		347420021
Ksb 1	-	+	+	+	+	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-		375030020
Kwb	-	+	+	+	+	-	+	-	-	-	+	-	-	+	+	-	-	-	-	-	-	-	+	-	-	+	-		364230032
Ksb 5	-	+	+	+	+	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-		375030020
Ksb 4	-	+	+	-	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-		334030020
Kwb 6	-	+	+	+	+	+	+	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-		377030020
Kvb 10	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-		365030020
Kvb 15	-	+	+	+	+	+	+	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-		377030020
Kvb 17	-	+	+	+	+	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-		375030020
Jvb	-	+	+	+	-	+	+	+	+	-	-	-	-	+	-	-	+	-	+	-	-	-	+	-	-	-	+		367022421
Kvb 15b	-	+	+	+	+	+	+	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-		377030020
Jvb 34b	-	+	+	+	+	+	+	+	+	+	-	-	-	+	-	-	-	-	+	+	-	+	+	-	-	-	+		377420661
Jvb 9	-	+	+	+	+	+	+	+	+	+	-	-	-	+	-	-	+	-	+	+	-	-	+	-	-	-	+		377422621
Jwb 4	-	+	+	+	+	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-		375030020

Appendix V Biochemical characterization of presumptive isolates of *Salmonella* spp dry season by Microbact

Isolate Code	Oxidase	Motility	Nitrate	Lysine	Ornithine	H_2S	Glucose	Mannitol	Xylose	ONPG	Indole	Urease	VP	Citrate	TDA	Gelatin	Malonite	Inositol	Sorbitol	Rhamnose	Sucrose	Lactose	Arabinose	Adonitol	Raffinose	Salicin	Arginine		Identification no
Jvb 17	-	+	+	+	+	+	+	+	+	-	-	-	-	-	+	-	-	-	-	+	-	-	+	-	-	-	-		377010220
Jvb 1	-	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-		301000220
Jvb 2	-	+	+	+	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-		367600220
Jwb 7	-	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+		307400021
Jvb 12	-	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+	-		347400222
Jvb 4	-	+	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-		347000362
Jvb 8	-	+	+	-	-	-	+	+	+	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-		307010202
Jvb 9	-	+	+	+	+	-	+	+	+	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-		367010200
Jvb 6	-	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	+		307400321
Jvb 10	-	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	+		307400221
Jvb 3	-	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-		307400320
Jvb 19	-	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	+		317400221
Jvb 15	-	+	+	-	-	-	+	+	+	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	+		307200371
Jvb 1	-	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-		307400660
Kvb 19a	-	+	+	+	-	+	+	+	-	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+		356401777
Jvb 5	-	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	-	+	+	+	+	+	-	-	-		347401370
Kvb 19b	-	+	+	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	+		357400361
Ksb 18	-	+	+	+	+	+	+	-	+	-	-	+	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-		375100320
Kvb 27	-	+	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	+		367000321
Kvb 16	-	+	+	+	+	+	+	+	-	+	-	-	+	-	-	-	-	-	-	+	+	+	+	-	-	-	-		375100361
Kwb 8	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-		377400360
Kwb 20	-	+	+	+	-	+	+	+	+	-	+	+	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+		367300373
Kwb 7	-	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	+		277003521
Kvb 22	-	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	-	+	-	+	-	+		347400725
Kvb 14b	-	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-		377100021

Kwb 21	-	+	+	+	-	+	+	-	+	-	-	+	-	-	-	-	-	-	-	+	-	-	+	-	-	-	+		355100221
Kvb 20	-	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	+	+	-		317000326

Appendix VI Table of inference for agglutination results of *V.cholerae* serotypes

V.cholerae O1 antiserum			*V.cholerae*	
Polyvalent	Serovar Inaba	Serovar Ogawa	O139 antiserum	Judgement
+	+	-	-	*V.cholerae* O1 Serovar Inaba
+	-	+	-	*V.cholerae* O1 Serovar Ogawa
+	+	+	-	*V.cholerae* O1 Serovar Hikojima
-	-	-	+	*V.cholerae* O139

Appendix VII Distribution of income level among age groups of farmers (males) in parts of Kano State

Income (₦)	**Age group (years)**			**Total**
	21-30	**31-40**	**41-50**	**No (%)**
	No (%)	**No (%)**	**No (%)**	
1-100 000	1(14.3)	14(21.9)	5(17.9)	20(20.2)
100 001-200 000	5(71.4)	42(65.6)	17(60.7)	64(64.6)
200 001-300 000	1(14.3)	8(12.5)	6(21.4)	15(15.2)
Total	7(100.0)	64(100.0)	28(100.0)	99(100.0)

$\chi^2 = 1.441$, df = 4, p = 0.837

Appendix VIII Distribution of age group among farmers (male and female) in parts of Plateau State

Age group	Sex		Total
	Male	Female	
	No (%)	No (%)	No (%)
21-30	1(100.0)	0(0.0)	1(6.3)
31-40	4(66.7)	2(33.3)	6(37.5)
41-50	4(100.0)	0(0.0)	4(25.0)
>50	4(80.0)	1(20.0)	5(31.3)
Total	13(81.2)	3(18.8)	16(100)

$\chi^2 = 1.997$, df = 3, p = 0.573

Appendix IX Distribution of income level among farmers in parts of Plateau State

Income level	Sex		Total
	Male	Female	
	No (%)	No (%)	No (%)
1-100 000	9(81.8)	2(18.2)	11(91.7)
100 001-200 000	1(100.0)	0(0.0)	1(8.3)
Total	10(83.3)	2(16.7)	12(100)

$\chi^2 = 0.218$, df = 1 p = 0.640

Appendix X Occupation of farmers

Occupation	Sex		Total
	Male	Female	
	No (%)	No (%)	No (%)
Farming	11(84.6)	2(15.4)	13(81.3)
Trading	1(100.0)	0(0.0)	1(6.3)
Others	1(50.0)	1(50.0)	2(12.5)
Total	13(81.2)	3(18.8)	16(100)

$\chi^2 = 1.609$, df = 2, p = 0.447

Appendix XI Level of education of farmers

Level of education	Sex		Total
	Male	Female	
	No (%)	No (%)	No (%)
No formal education	4(100.0)	0(0.0)	4(25.0)
Primary	3(60.0)	2(40.0)	5(31.3)
Secondary	5(83.3)	1(16.7)	6(37.4)
Tertiary	1(100.0)	0(0.0)	1(6.3)
Total	13(81.3)	3(18.8)	16(100.0)

$\chi^2 = 2.653$, df = 3 p = 0.448

Appendix XII Irrigation water source and treatment of water in parts of Kano State.

Water source	**Treated**	**Untreated**	**Total**
	No (%)	**No (%)**	**No (%)**
Well	0(0.0)	20(100.0)	20(20.2)
River	1(1.7)	58(98.3)	59(59.6)
Tap	19(100.0)	0(0.0)	19(19.2)
Dam	1(100.0)	0(0.0)	1(1.0)
Total	21(21.2)	78(78.8)	99(100.0)

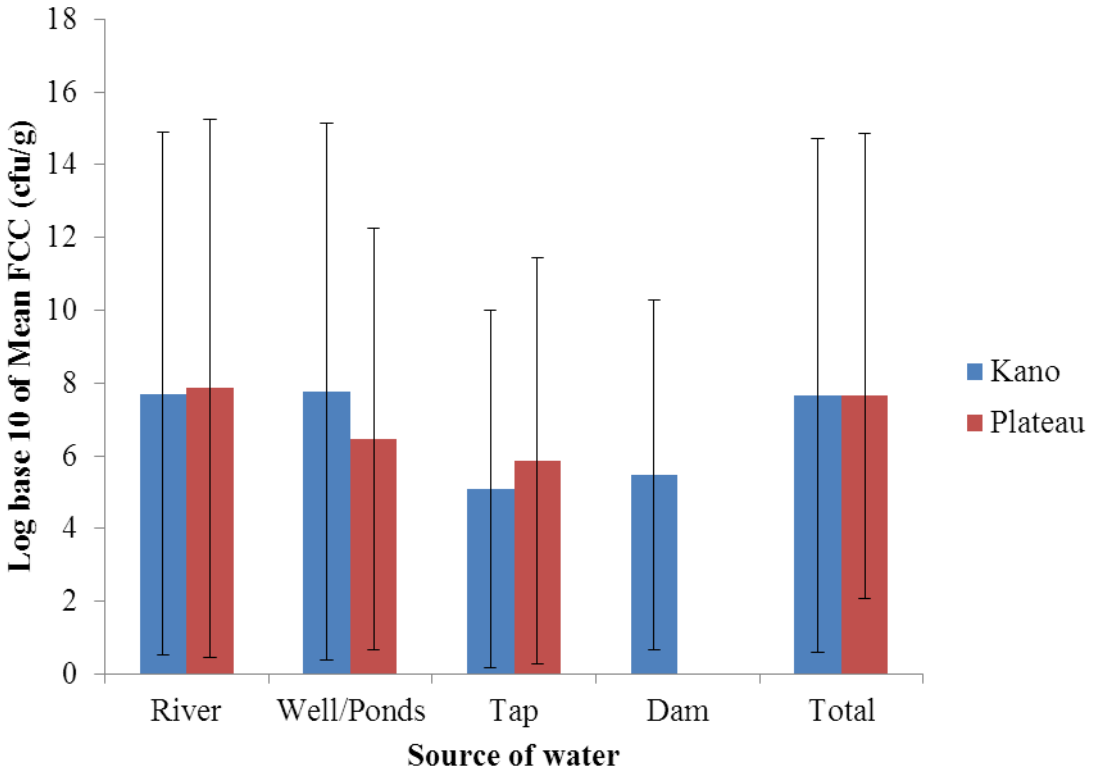
$\chi^2 = 93.118$, df = 3, $p < 0.001$

Appendix XIII Method of treatment

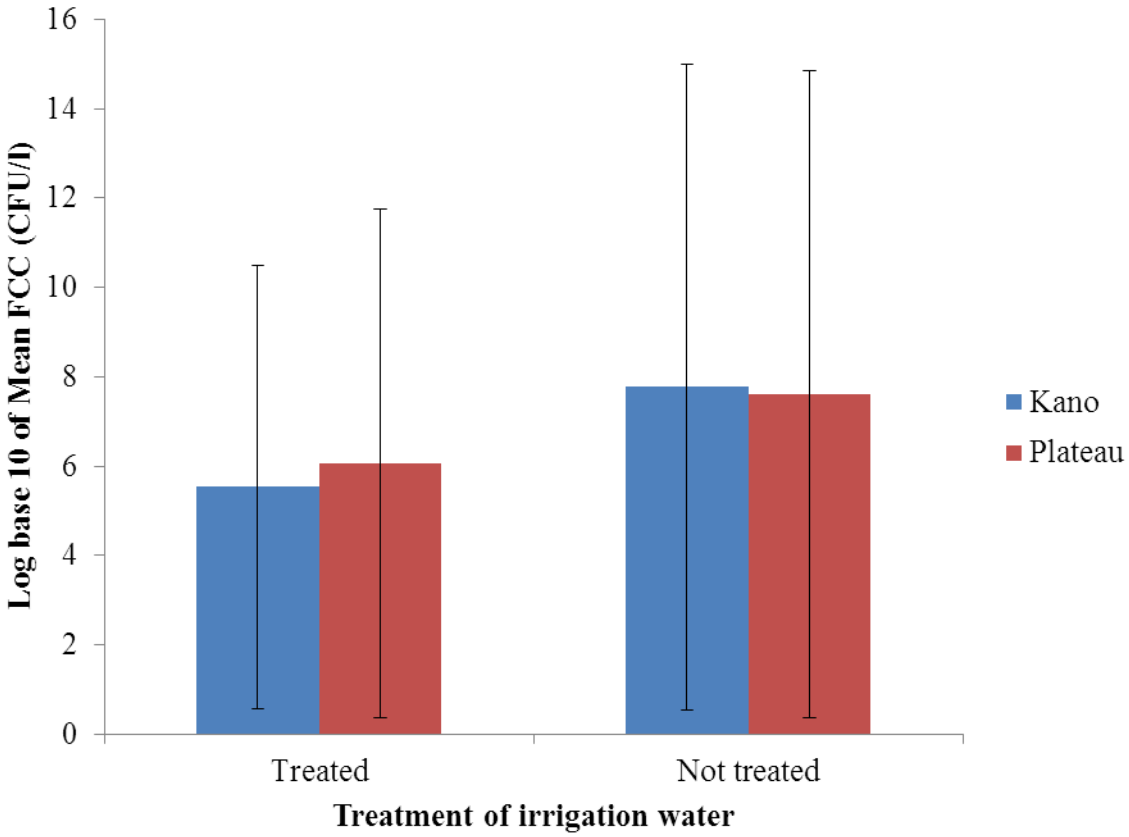
Water source	**No treatment**	**Storage/chemical**	**Total**
	No (%)	**No (%)**	**No (%)**
Well	20(100.0)	0(0.0)	20(20.2)
River	59(100.0)	0(0.0)	59(59.6)
Tap	0(0.0)	19(100.0)	19(19.2)
Dam	0(0.0)	1(100.0)	1(1.0)
Total	79(79.8)	20(20.2)	99(100.0)

$\chi^2 = 99.000$, df = 3, $p < 0.001$

Appendix XIV Effect of source of irrigation water and wastewater on the level of faecal coliforms on vegetable by State



Appendix XV Feacal coliform counts of treated and untreated irrigation water in the study



Kano (t = -3.320, p = 0.002); Plateau (t = -2.823, p = 0.007)

Appendix XVI Point source of pollution to irrigation water used by farmers in parts of Kano State

Watersource	**Livestock waste**	**Industrial waste**	**Domestic and/or industrial waste**	**Nil**	**Total**
	No (%)	**No (%)**	**No (%)**	**No (%)**	**No (%)**
Well	12(60.0)	0(0.0)	8(40.0)	0(0.0)	20(20.2)
River	39(66.1)	20(33.9)	0(0.0)	0(0.0)	59(59.6)
Tap	0(0.0)	0(0.0)	0(0.0)	19(100.0)	19(19.2)
Dam	0(0.0)	0(0.0)	0(0.0)	1(100.0)	1(1.0)
Total	51(51.6)	20(20.2)	8(8.1)	20(20.2)	99(100.0)

$\chi^2 = 137.179$, df = 9, $p < 0.001$

Appendix XVII Point source of pollution to irrigation water used by farmer's in parts of Plateau State

Water source	**Livestockwaste**	**Industrial waste**	**Domesticand/or industrial waste**	**Total**
	No (%)	**No (%)**	**No (%)**	**No (%)**
Well	0(0.0)	0(0.0)	1(100.0)	1(6.3)
River	2(20.0)	2(20.0)	6(60.0)	10(62.5)
Tap	0(0.0)	1(25.0)	3(75.0)	4(25.0)
Dam	1(100.0)	0(0.0)	0(0.0)	1(6.3)
Total	3(18.8)	3(18.8)	10(62.5)	16(100.0)

$\chi^2 = 5.893$, df = 6, p = 0.435

Appendix XVIII Levels of faecal coliform counts on samples from Kano and Plateau State

Site	Sample type	FCC (Kano)	FCC (Plateau)	t	p-value
I	Vegetable	12.41 ± 12.05[a]	8.06 ± 7.61	2.280	0.040*
	Water	7.77 ± 7.53[b]	12.48 ± 12.48	-2.582	0.020*
	Soil/manure	8.18 ± 7.82[b]	12.48 ± 12.48	-1.195	0.286
		F = 3.455; p = 0.045*	F = 2.265; p = 0.141		
II	Vegetable	12.13 ± 11.87	4.70 ± 4.48	0.566	0.577
	Water	11.88 ± 11.88	7.73± 7.45	0.486	0.635
	Soil/manure	7.27 ± 7.17	12.65± 12.65	-1.000	0.500
		F = 0.495; p = 0.614	F = 1.429; p = 0.340		
III	Vegetable	11.95 ± 11.95	11.45± 11.48[c]	0.824	0.415
	Water	12.29 ± 12.01	12.83± 12.35[a]	-2.144	0.048*
	Soil/manure	8.51± 8.41	12.56± 12.34[b]	-1.633	0.178
		F = 0.450; p = 0.643	F = 13.332; p < 0.001*		
IV	Vegetable	5.54 ± 5.15	6.45 ± 5.80[b]	-3.751	0.001*
	Water	4.97 ± 4.88	5.96 ± 5.95[c]	-0.920	0.525
	Soil/manure	7.41 ± 7.41	7.84 ± 7.78[a]	-0.652	0.552
		F = 1.838; p = 0.252	F = 3.878; p = 0.035*		
V	Vegetable	5.46± 5.70	6.13± 5.18	-6.704	0.022*
	Water	5.99 ± 5.91	6.69 ± 6.63	-0.707	0.531
	Soil/manure	8.02 ± 7.94	12.83 ± 12.35	-3.000	0.024*
		F = 0.594; p = 0.587	F = 5.000; p = 0.053		
	Overall				
	Vegetable	12.16 ± 11.67	11.15 ± 11.15[b]	2.649	0.010*
	Water	11.90± 11.59	12.38 ± 12.03[a]	-1.414	0.175
	Soil/manure	8.03 ± 7.60	12.54 ± 12.03[a]	-3.289	0.004*
		F = 1.839; p = 0.164	F = 13.305; p < 0.001*		

* Significant difference exists.

Appendix XIX Mean of faecal coliform counts of samples obtained from Kano and Plateau States

Sample	State	Log_{10} of mean faecal coliform count	t-value	p-value
Water	Kano	8.12	0.721	0.474
	Plateau	8.00		
Vegetable	Kano	8.02	3.609	0.001*
	Plateau	7.42		
Soil/Manure	Kano	8.17	0.971	0.335
	Plateau	8.29		

* = significant difference exists. Mean values with different superscripts are significantly different ($p \leq 0.05$).

Appendix XX Seasonal variation in coliform counts of samples from Kano and Plateau States

State	Sample	Kano			
		Dry	Wet	t-test	p-value
Kano	Water	12.30± 11.96	11.84± 11.68	1.379	0.175
	Vegetable	11.61± 11.61	12.30± 11.80	-2.112	0.039*
	Soil/Manure	8.59 ± 7.99	8.12± 7.71	2.247	0.043
Plateau	Water	12.35± 12.16	12.29± 12.01	0.183	0.856
	Vegetable	11.35± 11.35	11.70± 11.54	-0.676	0.501
	Soil/Manure	12.70 ± 12.20	12.65± 12.06	0.252	0.800

* Significant difference exists.

Appendix XXI Correlation half matrix of water quality parameters for Kano State

	FCC	**Temperature**	**pH**	**EC**	**DO**	**BOD**
FCC	1.000					
Temperature	- 0.016	1.000				
pH	- 0.145	- 0.156	1.000			
EC	- 0.136	- 0.047	0.002	1.000		
DO	- 0.260	- 0.117	- 0.160	- 0.183	1.000	
BOD	- 0.207	- 0.204	- 0.180	- 0.137	0.352*	1.000

* = significant correlation exists between parameters ($p < 0.05$)

Appendix XXII Correlation half matrix of water quality parameters for parts of Plateau State

	FCC	**Temperature**	**pH**	**EC**	**DO**	**BOD**
FCC	1.000					
Temperature	0.034	1.000				
pH	- 0.124	- 0.316	1.000			
EC	- 0.193	0.373	- 0.063	1.000		
DO	- 0.295	- 0.470*	0.199	- 0.465	1.000	
BOD	- 0.306	- 0.209	0.061	- 0.217	0.293	1.000

* = significant correlation exists between parameters ($p < 0.05$)

Key: FCC=Faecal coliform counts

Temp= Temperature

pH=Hydrogen ion potential

EC= Electrical conductivity

DO= Digested oxygen

BOD= Biological Oxygen Demand

Appendix XXIII Correlation matrix of water quality parameters for both parts of Kano and Plateau States

	FCC	Temperature	pH	EC	DO	BOD
FCC	1.000					
Temperature	0.015	1.000				
pH	- 0.116	- 0.763**	1.000			
EC	0.027	0.267*	- 0.156	1.000		
DO	- 0.270*	- 0.219	0.130	- 0.310**	1.000	
BOD	- 0.240*	- 0.190	0.078	- 0.147	0.315**	1.000

* = significant correlation exists between sample parameters ($p < 0.05$)
** = significant correlation exists between sample parameters ($p < 0.01$)

Key

FCC=Faecal coliform counts

Temp= Temperature

pH=Hydrogen ion potential

EC= Electrical conductivity

DO= Digested oxygen

BOD= Biological Oxygen Demand

Appendix XXIV Type of fertiliser used for vegetable production in parts of Kano State

Source of organic manure	Organic	Organic and inorganic	Total
	No (%)	No (%)	No (%)
Cattle dung	57(86.4)	9(13.6)	66(84.6)
Goat/sheep dung	9(100.0)	0(0.0)	9(11.5)
Poultry droppings	2(66.7)	1(33.3)	3(3.8)
Total	68(87.2)	10(12.8)	99(100.0)

Appendix XXV Stage of application of fertiliser/manure to vegetables in Kano State

Stage of application	**Organic**	**Inorganic**	Both	Total
	No (%)	**No (%)**	**No (%)**	No (%)
1-2 weeks	35(81.4)	5(11.6)	3(7.0)	43(43.5)
3-4 weeks	31(57.4)	16(29.6)	7(13.0)	54(54.5)
> 4 weeks	2(100)	0(0.0)	0(0.0)	2(2.0)
Total	68(68.7)	21(21.2)	10(10.1)	99(100.0)

$\chi^2 = 7.442$, df = 4, p = 0.114

Appendix XXVI Stage of application of manure to vegetables in parts of Plateau State

Stage of application	**Organic**	**Inorganic**	Both	Total
	No (%)	**No (%)**	**No (%)**	No (%)
1-2 weeks	1(50.0)	0(0.0)	1(50.0)	2(14.3)
3-4 weeks	21(33.3)	4(66.7)	0(0.0)	64(42.9)
> 4 weeks	3(50.0)	3(50.0)	0(0.0)	6(42.9)
Total	6(42.9)	7(50.0)	1(7.1)	14(100.0)

$\chi^2 = 7.556$, df = 4, p = 0.104

Appendix XXVII Treatment of irrigation water in parts of Plateau State

Water source	**No treatment**	**Storage/chemical**	**Total**
	No (%)	**No (%)**	**No (%)**
Well	0(0.0)	1(100.0)	1(6.3)
River/pond	0(0.0)	10(100.0)	10(62.5)
Tap	0(0.0)	4(100.0)	4(25.0)
Dam	1(100.0)	0(0.0)	1(6.3)
Total	1(6.3)	15(93.8)	16(100.0)

$\chi^2 = 16.000$, df = 3, p = 0.001

Appendix XXVIII Distribution of *Escherichia coli* O157:H7 in all samples studied in the two seasons

Sample	Dry season			Wet season			OR	95% CI
	No +ve (%)	No –ve (%)	Total	No +ve (%)	No –ve (%)	Total		
Vegetable	9	113	122	11	105	116	1.315	0.524 to 3.301
Water	2	32	34	6	44	50	2.182	0.413 to 11.519
Soil/Manure	1	39	40	3	75	78	1.560	0.157 to 15.498
Total	12(6.52)	172	184	20(8.20)	224	244	0.781	0.609 to 2.690

Key

No +ve: Number of samples containing pathogens
No –ve: Number of samples without detectable pathogens
OR= odds ratio
CI= Confidence interval

Appendix XXIX Distribution of *Salmonella* contaminated samples in the study for the two seasons

Sample	Dry season			Wet season			OR	95% CI
	No +ve (%)	No – ve (%)	Total	No +ve (%)	No – ve (%)	Total		
Vegetable	24	98	122	10	106	116	2.596	1.181 to 5.704
Water	11	23	34	4	46	50	5.500	1.577 to 19.179
Soil/Manure	4	36	40	4	74	78	2.056	0.486 to 8.693
Total	39 (21.20)	157	184	18 (7.38)	226	244	3.119	1.721 to 5.652

Key

No +ve: Number of samples containing pathogens
No –ve: Number of samples without detectable pathogens
OR= odds ratio
CI= Confidence interval

Appendix XXX Distribution of *Vibrio cholerae* contaminated samples in the study for two seasons

Sample	Dry season			Wet season			OR	95% CI
	No +ve (%)	No – ve (%)	Total	No +ve (%)	No – ve (%)	Total		
Vegetable	4	132	136	3	99	102	1.000	0.219 to 4.570
Water	4	41	45	2	37	39	1.805	0.312 to 10.434
Soil/Manure	9	40	49	5	64	69	2.880	0.901 to 9.209
Total	17 (7.39)	213	230	10 (4.76)	200	210	1.596	0.714 to 3.569

Key

No +ve: Number of samples containing pathogens
No –ve: Number of samples without detectable pathogens
OR= odds ratio
CI= Confidence interval

Appendix XXXI Distribution of *Salmonella* serotypes in samples obtained from parts of Kano State

Sample type	***S.* Typhi**	***S.* Paratyphi**	***S.*Typhimurium**
Vegetable	10	2	7
Soil/manure	5	3	2
Water	5	3	2
Total	20	8	11

Appendix XXXII Distribution of *Salmonella* serotypes in samples obtained from parts of Plateau State

Sample type	***S.typhi***	***S.paratyphi***	***S.tyhpimurium***
Vegetable	12	1	3
Soil/manure	1	-	2
Water	1	-	2
Total	14	1	7

Appendix XXXIII Distribution of *Vibrio cholerae* O1, O139 and non *V. cholerae* O1 in samples from parts of Kano State

Sample Type	**No of Samples**	**Number of *Vibrio cholerae* O1**		**Hikojima**	**Non *V. cholerae***
		Inaba	**Ogawa**		
Vegetables	94	-	2	-	1
water	57	2	3	-	5
Soil/Manure	79	1	1	7	6
Total	230	3 (1.3%)	6 (2.6%)	7 (3.0%)	12 (5.2%)

Appendix XXXIV Distribution of *Vibrio cholerae* O1, O139 and non O1 *V. cholerae* in samples obtained from parts of Plateau State

Sample type	**No of Samples**	**No of *Vibrio cholerae* O1**		**Hikojima(O139)**	**Non *V.* O1**
		Inaba	**Ogawa**		
Vegetables	144	1	2	2	9
Water	27	-	-	1	-
Soil/Manure	39	2	1	-	-
Total	210	3 (1.4%)	3 (1.4%)	3 (1.4%)	9 (4.3%)

Appendix XXXV Antibiotic susceptibility pattern of *Escherichia coli* O157:H7

	Antimicrobial Agent	Symbol	Disc Content (µg)	Susceptibility Isolates from Vegetables(n=19) R	I	S	Isolates from Water(n=12) R	I	S	Isolates from Soil/manure(n=5) R	I	S	Isolates strongly resistant NO (%)
1	Amoxicillin/ clavulanic acid	AMC	10µg	4(21.1)	13(68.4)	2(10.5)	3(25)	7(58.3)	2(16.7)	3(60.0)	1(20.0)	1(20.0)	10(27.8)
2	Ceftriaxone	CRO	30µg	1(5.3)	9(47.4)	9(47.4)	1(8.3)	3(25.0)	8(66.7)	2(40.0)	2(40.0)	1(20.0)	4(11.1)
3	Cephalothin	KF	30µg	19(100)	-(0)	–(0)	12(100)	–(0)	–(0)	5(100)	–(0)	–(0)	36(100)
4	Ciprofloxacin	CIP	5µg	3(15.8)	4(21.1)	12(63.2)	1(8.3)	–(0)	1(91.7)	2(40.0)	1(20.0)	2(40.0)	6(16.7)
5	Gentamicin	CN	10µg	5(26.3)	–(0)	14(0)	3(25.0)	–(0)	9(75.0)	3(60.0)	–(0)	2(40.0)	11(30.6)
6	Kanamycin	K	30µg	13(68.4)	5(26.3)	1(5.3)	5(41.7)	4(33.3)	3(25.0)	3(60.0)	1(20.0)	1(20.0)	21(58.3)
7	Sulfamethoxazole/ Trimethoprim	SXT	25µg	3(15.8)	4(21.1)	12(63.2)	3(25.0)	–(0)	9(75.0)	1(20.0)	–(0)	4(80.0)	7(19.4)
8	Tetracycline	TE	30µg	11(57.9)	5(26.3)	3(15.8)	8(66.7)	3(25.0)	1(8.3)	5(100)	–(0)	-(0)	24(66.7)

KEY: R=Resistant, I= Intermediate, S= Susceptible.

Appendix XXXVI Antibiotic susceptibility pattern of *Salmonella* isolates

S/N	Antimicrobial Agent	Symbol	Disc Content (µg)	Susceptibility: Isolates from Vegetables(n=41) R I S No%	Isolates from water (n=15) R I S No%	Isolates from Soil/manure (n=11) R I S No%	Isolates strongly Resistant No%
1	Sulphamethoxazole/ Trimethoprim	SXT	25	12(29.3)2(4.9)27(65.9)	5(33.3) 2(13.3) 8(53.3)	5(45.5)1(9.1)5(45.5)	22(32.8)
2	Amoxicillin/Clavulanic acid	AMC	10	12(29.3)11(26.8)18(43.9)	2(3.3)3(7.3)10(90.9)	3(27.3)1(9.1)7(63.6)	17(25.4)
3	Cephalothin	KF	30	-(100) –(0) –(0)	15(100)- (0) –(0)	11(100) –(0) –(0)	26(38.8)
4	Ceftriaxone	CRO	30	2(4.9) 12(29.3) 27(65.9)	1(6.7) 1(6.7) 13(86.7)	-(0) 2(18.2) 9(81.8)	5(7.4)
5	Gentamicin	CN	10	2(4.9) 2(4.9) 37(90.2)	0(0) 1(6.7) 14(93.3)	-(0) –(0) 11(100)	2(3.0)
6	Tetracycline	TE	30	21(51.2) 5(12.2) 15(36.6)	11(29.3) 0(0) 3(20.0)	10(90.9) 1(9.1) 0(0)	42(64.2)
7	Ciprofloxacin	CIP	5	1(2.4) 4(9.8) 36(87.8)	2(13.3)2(13.3)11(73.3)	1(9.1) 0(0) 10(90.9)	4(6.0)
8	Kanamycin	K	30	11(26.8)20(48.8)10(24.4)	3(20.0) 6(40.0)6(40.0)	6(54.5) 3(27.3) 2(18.2)	20(29.9)

KEY: R=Resistant, I= Intermediate, S= Susceptible.

Appendix XXXVII Antibiotic susceptibility pattern of *Vibrio cholerae* isolates

S/N	Antimicrobial Agent	Symbol	Disc Content (µg)	Isolates from Vegetables(n=17) No(%) R	I	S	Isolates from water (n=11) No(%) R	I	S	Isolates from soil/manure (n=13) R	I	S	Isolates Strongly Resistant No(%)
1	Amoxicillin/	AMC	10	4(23.5)	4(23.5)	9(52.9)	2(18.2)	(18.2)	7(63.3)	4(30.8)	2(15.4)	7(53.8)	10(24.4)
2	Ceftriaxone	CRO	30	-(0)	4(23.5)	13(76.5)	-(0)	2(18.2)	9(81.8)	2(15.4)	4(30.8)	7(53.8)	2(4.9)
3	Cephalothin	KF	30	17(100)	–(0)	–(0)	11(100)	–(0)	-(0)	13(100)	–(0)	–(0)	41(100)
4	Ciprofloxacin	CIP	5	1(5.9)	1(5.9)	15(88.2)	-(0)	–(0)	11(100)	-(0)	–(0)	13(100)	1(2.4)
5	Gentamicin	CN	10	-(0)	–(0)	17(100)	-(0)	–(0)	11(100)	-(0)	–(0)	13(100)	0(0)
6	Kanamycin	K	30	0(0)	3(76.5)	14(82.4)	4(36.4)	3(27.3)	4(36.4)	1(7.7)	2(15.4)	10(58.8)	1(12.2)
7	Sulphamethoxazole/ Trimethoprim	SXT	25	7(41.2)	-(0)	10(58.8)	7(63.3)	-(0)	4(36.4)	6(46.2)	0(0)	7(53.8)	20(48.8)
8	Tetracycline	TE	30	3(76.5)	2(11.8	012(70.6)	1(9.1)	0(0)	10(90.9)	3(23.1)	1(7.7)	9(69.2)	7(17.1)

KEY: R=Resistant, I= Intermediate, S= Susceptible.

Appendix XXXVIII Bacteriological media and reagents

1. MacConkey broth: Peptone (20.0), lactose (10.0), bile salts (5.0), sodium chloride (5.0), sodium chloride (5.0), bromocresol purple (0.075), pH 7.4± 0.2
2. Sorbitol MacConkey agar: peptone (20.0), sorbitol (10.0), bile salts (95.0), sodium chloride (5.0), crystal violet (0.001), agar (15.0), pH 7.4±0.2.
3. Nutrient agar: peptone (10.0), sodium chloride (5.0), beef extract (5.0), agar (15.0).
4. Nutrient broth: peptone (10.0), sodium chloride (5.0).
5. Eosine methylene Blue agar: hydrogen phosphate (0.7), potassium dihydrogen, phosphate(1.3), eosine yellow(0.4), methylene blue(0.65), agar(15.0), pH6.8±0.2.
6. Salmonella-Shigella agar: Lab-lemco powder(0.5), peptone(0.5), Lactose(10.0), Bile salts(8.5),sodium citrate(10.0),sodium thiosulphate(8.5), ferric citrate(1.0), Brilliant green(0.00033), neutral red(0.025),agar 15.0,pH 7.0± 0.2.
7. Thiosulphate citrate Bile salt: yeast extract(5.0), Bacteriological peptone(10.0), sodium thiosulphate(10.0),sodium citrate (10.0), Ox bile (8.0), sucrose(20.0), sodium chloride(10.0),ferric citrate(1.0), bromothymol blue(0.04), thymol blue(0.04), agar(15.0), pH 8.6±0.2.
8. Motility –Indole-Urease medium: Tryptone(3.0),potassium dihydrogen phosphate (1.0), sodium chloride(5.0),agar(4.0),phenol red(2.0), urea, pH 6.9-7.3.
9. Kliger`s Iron agar: Lab-lemco powder(3.0),yeast extract(3.0), peptone(20.0),lactose(10.0),sodium thiosulphate(0.3),phenol red(0.05),agar(12).
10. Methyl red Voges Proskaeur : Peptone(5.0), glucose(5.0), phosphate buffer(5.0), pH 7.5±0.2.
11. Kovac`s reagent:Para-dimethylaminobenzaldehyde (5.0), iso-amyl alcohol (75.0ml), concentrated hydrochloric acid(25.0ml).
12. Methyl red reagent: Methyl red (0.1), 95% ethanol(300ml),distilled water(200ml).
13. Voges-Proskaeur reagent: (a) α-naphtol (5.0), absolute ethanol(100ml) and (b) Potassium hydroxide(40.0g), distilled water(100ml).
14. Gram staning reagents
    a. (a) Huckers crystal violet: Mixture of 2 solutions:1 crystal violet(2.0g), 95% ethanol(20.0ml),11 Ammoniumoxalate(0.8g), distilled water(100ml).
    b. (b)Lugol`s iodine Iodine (5.0g), potassium iodide(10.0g), distilled water(100ml).
    c. (c) Counter stain: Safranin (0.25g), 95% ethanol(10.0ml), distilled water(100ml)
15. Agar Mueller Hinton

    Beef infusion 2.0

    Acid casein peptone (H): 17.5

    Corn starch 1.5

Bacteriological agar 17.0

pH 7.4± 0.2 at 25^0C

16. Brain Heart infusion agar

    Nutrient substrate (extracts of brain and heart and peptones) 27.5

    D(+)- Glucose 2.0

    Sodium chloride 5.0

    di-Natriumhydrogen phosphate 2.5

    Agar-agar 15.0

Appendix XXXIX Plates showing point sources of contamination at sampling sites



Plate XXI Sampling site with domestic waste drain



Plate XXII An irrigation pump by a drum for storage



Plate XIX sampling site with domestic dumps



Plate XX sampling site with abattoir waste drain



Plate XXIII Sampling site showing industrial waste drain



Plate XXIV Sampling site showing animals feeding on site to deposit manure



Plate XXV Control site



Plate XXVI Sampling site showing vegetable farm with animals