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ASSESSMENT OF THE QUALITY INDICES AND PREVALENCE OF *Escherichia coli* PATHOTYPES IN SELECTED RIVERS OF OSUN STATE, SOUTHWESTERN NIGERIA

OSUOLALE YINKA TITILAWO

DEPARTMENT OF BIOCHEMISTRY AND MICROBIOLOGY

FACULTY OF SCIENCE AND AGRICULTURE

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BY

OSUOLALE YINKA TITILAWO

A thesis submitted in fulfillment of the requirements for the degree of

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SOUTH AFRICA

Supervisor: **Professor Anthony I. Okoh**

Co-supervisor: **Professor Larry C. Obi**

APRIL 2015

DECLARATION

I, the undersigned, declare that this thesis entitled "Assessment of the quality indices and prevalence of *Escherichia coli* pathotypes in selected rivers of Osun State, Southwestern Nigeria" submitted to the University of Fort Hare for the degree of Doctor of Philosophy in Microbiology in the Faculty of Science and Agriculture, School of Biological and Environmental Sciences, and the work contained herein is my original work with exemption to the citations and that this work has not been submitted to any other University in partial or entirely for the award of any degree.

Name: **Osuolale Yinka Titilawo**

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DECLARATION ON PLAGIARISM

I, **Osuolale Yinka Titilawo**, student number: 201314485 hereby declare that I am fully aware of the University of Fort Hare's policy on plagiarism and I have taken every precaution to comply with the regulations.

Signature…… Date…….

CERTIFICATION

This thesis entitled "Assessment of the quality indices and prevalence of *Escherichia coli* pathotypes in selected rivers of Osun State, Southwestern Nigeria" meets the regulation governing the award of degree of Doctor of Philosophy of the University of Fort Hare and is approved for its contribution to scientific knowledge and literary presentation.

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Prof. A.I. Okoh Date

Head of Department and Major Supervisor

DEDICATION

This thesis is dedicated to my Almighty God, Alpha and Omega, my inspiration, the source of my wisdom, knowledge and understanding, the light and sustenance of my life, the One who has been before and behind me, the One whose finger was clearly seen in this pursuit and the One whose divine agenda for my life has brought this to fulfilment in such a time as this. I humbly sing doxology to say thank you God.

"Praise God from whom all blessings flow

Praise Him all creatures here below

Praise Him above Ye heavenly hosts

Praise Father, Son and Holy Ghost" Amen.

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LIST OF ACRONYMS

AA:	Aggregative Adherence
AAF:	Aggregative Adherence Fimbriae
AAP:	Antiaggregation Protein
AIDS:	Acquired Immune Deficiency Syndrome
AEMREG:	Applied and Environmental Microbiology Research Group
A/E:	Attaching and Effacing
AIEC:	Adherent Invasive *Escherichia coli*
AEPEC:	Atypical Enteropathogenic *Escherichia coli*
AMPs:	Antimicrobial Peptides
AMR:	Antimicrobial Resistance
ANOVA:	Analysis of Variance
APEC:	Avian Pathogenic *Escherichia coli*
APHA:	American Public Health Association
APUA:	Alliance for the Prudent Use of Antibiotics
ARD:	Antimicrobial Resistance Determinant
ARG:	Antimicrobial Resistance Gene
ARI:	Antibiotic Resistance Indexing
ATCC:	American Type Culture Collection
BFP:	Bundle Forming Pilus
CAWMA:	Comprehensive Assessment of Water Management in Agriculture
CCME:	Canadian Council of Ministers of the Environment
CDC:	Centers for Disease Control and Prevention
CDEC:	Cell-Detaching *Escherichia coli*
CEACAM:	Carcinoembryonic-Antigen-related Cell-Adhesion Molecule

CF:	Colonization Factor
CFU:	Colony Forming Unit
CLSI:	Clinical and Laboratory Standards Institute
CFSPH:	Centre for Food Security and Public Health
CNF:	Cytotoxic Necrotizing Factor
CT-SMAC:	Cefixime and Tellurite MacConkey Agar
DA:	Diffuse Aggregative
LLA:	Localized-like Adherence
DAEC:	Diffusely-Adherent *Escherichia coli*
DAEC/DNA:	Diffusely-Adherent *Escherichia coli*/Deoxyribonucleic Acid
DAF:	Decay-Accelerating Factor
DAF/AMPs:	Decay-Accelerating Factor /Antimicrobial Peptides
DALY:	Disability Adjusted Life Years
DEC:	Diarrhoeagenic *Escherichia coli*
DHFR:	Dihydrofolate Reductase
DHPS:	Dihydropteroate Synthase
DNA:	Deoxyribonucleic Acid
DO:	Dissolved Oxygen
EAEC:	Enteroaggregative *Escherichia coli*
EAF:	EPEC Adherence Factor
EAggEC:	Enteroaggregative *Escherichia coli*
EAHEC:	Entero-Aggregative-Haemorrhagic *Escherichia coli*
EAST:	Enteroaggregative Heat-Stable
EC:	Electrical Conductivity
ECOR:	*Escherichia coli* Collection of Reference

EHEC: Enterohaemorrhagic *Escherichia coli*

EIA: Enzyme Immunoassay

EIEC: Enteroinvasive *Escherichia coli*

EMB: Eosin Methylene Blue

ELISA: Enzyme-Linked Immunosorbent Assay

EnPEC: Endometrial Pathogenic *Escherichia coli*

EPA: Environmental Protection Agency

EPEC: Enteropathogenic *Escherichia coli*

ESBL: Extended-Spectrum Beta–Lactamase

ETAG: European Technology Assessment Group

ETEC: Enterotoxigenic *Escherichia coli*

ExPEC: Extra-Intestinal Pathogenic *Escherichia coli*

FAS: Fluorescent Assay

FRN: Federal Republic of Nigeria

GBDs: Global Burden of Diseases

GMRDC: Govan Mbeki Research and Development Centre

GPS: Global Positioning System

HC: Haemorrhagic Colitis

HGT: Horizontal Gene Transfer

HIV: Human Immunodeficiency Virus

HIV/AIDS: Human Immuno-deficiency Virus/Acquired Immune Deficiency Syndrome

HUS: Haemolytic Uremic Syndrome

IBC: Intracellular Bacterial Communities

IBM: International Business Machine

IBS: Irritable Bowel Syndrome

IPS: Inter-Press Service

InPEC: Intestinal Pathogenic *Escherichia coli*

LA: Localized Adherence

LEE: Locus of Enterocyte Effacement

LT: Heat-Labile

MPEC: Mammary Pathogenic *Escherichia coli*

MPN: Most Probable Number

MAR: Multiple Antibiotic Resistance

MARI: Multiple Antibiotic Resistance Indexing

MARP: Multiple Antibiotic Resistance Phenotype

MDGs: Millennium Development Goals

MDR: Multi-Drug Resistance

MRSA: Multi-Drug Resistant *Staphylococcus aureus*

MUG: Methyl Umbelliferyl-β-D-Glucuronide

NARMS: National Antimicrobial Resistance Monitoring System

NBS: National Bureau of Statistics

NHMRC: National Health and Medical Research Council

NIID: National Institute of Infectious Diseases

NMEC: Neonatal Meningitis *Escherichia coli*

NRC: National Research Council

NTEC: Necrotoxigenic *Escherichia coli*

NTU: Nephelometric Turbidity Unit

OMP: Outer Membrane Protein

OSWSP: Osun State Water and Sanitation Projects

PCR: Polymerase Chain Reaction

PCR-RFLP: Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

PER: Plasmid Encoded Regulator

PET: Plasmid-Encoded Toxin

PMN: Polymorph Nuclear Leukocyte

SA-MRC: South African Medical Research Council

RNA: Ribonucleic Acid

SAP: Substance Abuse Professional

SEPEC: Septicemic *Escherichia coli*

SLT: Shiga-like Toxins

SMAC: Sorbitol-MacConkey Agar

SPSS: Statistical Packages for Social Sciences

SPT: Sero-Pathotype

ST: Heat-Stable

STEC: Shiga Toxin–Producing *Escherichia coli*

STP: Sewage Treatment Plant

STX: Shiga Toxin

TC: Total Coliform

TEPEC: Typical Enteropathogenic *Escherichia coli*

TDS: Total Dissolved Solids

TH: Total Hardness

TIR: Translocated Intimin Receptor

TJ: Tight Junction

TMP-SMX: Trimethoprim/Sulfamethoxazole

UK: United Kingdom

UNEP: United Nations Environment Programme

UNDP:	United Nations Development Programme
UPEC:	Uropathogenic *Escherichia coli*
UNICEF:	United Nations Children's Fund
US:	United States
USEPA:	United States Environmental Protection Agency
USFDA:	United States Food and Drug Administration
UTI:	Urinary Tract Infection
VAG:	Virulence-Associated Gene
VG:	Virulence Gene
VTEC:	Verocytotoxigenic *Escherichia coli*
WASH:	Water, Sanitation and Hygiene
WHO:	World Health Organization
WQI:	Water Quality Index
WRC:	Water Research Commission

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GENERAL ABSTRACT

General Abstract

Surface waters are important freshwater sources used for domestic, industrial, agricultural and recreational activities, and the availability of good quality freshwater is indispensable for preventing water-borne diseases and improving quality of life especially in communities that lack pipe-borne water. Water samples were collected from ten rivers at different locations in Osun State, Southwestern Nigeria. A total of 12 physicochemical parameters, counts of total coliforms (TC) and *Escherichia coli* isolates were determined using standard analytical procedures. Confirmed *Escherichia coli* isolates (*n*=300) were assessed for the presence of 10 virulence genes (VGs) associated with *Escherichia coli* strains causing intestinal and extra-intestinal infections. The recovered *Escherichia coli* isolates were elucidated for their antibiogram profiling by disk diffusion method and the resistant isolates were further profiled for their genotypic antimicrobial resistance by polymerase chain reaction technique. The physicochemical qualities ranged as follows: pH (6.9 - 7.6), temperature (26 – 29 ºC), turbidity (2.28 – 9.46 NTU), electrical conductivity (229 – 581 µS/cm), nitrate (0.03 – 0.05 mg/L), nitrite (0.00 – 0.01 mg/L), sulphate (3.33 – 20.33 mg/L), chloride ions (7.83 – 27.33 mg/L), dissolved oxygen (4.23 – 5.57 mg/L), total dissolved solids (56 – 184 mg/L), total hardness (78 – 519 mg/L) and alkalinity (50.67 – 146.67 mg/L). Statistical analysis showed that pH, temperature, electrical conductivities, nitrates, nitrites, chloride, dissolved oxygen, total dissolved solid, total hardness and alkalinity were significantly different ($P < 0.05$), whereas turbidity and sulphate were not significantly different ($P > 0.05$) from each parameter with respect to sampling sites. While the VG *lt* for enterotoxigenic *E. coli* had the highest prevalence of 45%, the enteropathogenic *E. coli* genes *eae* and *bfp* were detected in 6% and 4% of the isolates respectively. The VGs *stx*1 and *stx*2 specific for the enterohemorrhagic *E. coli* pathotypes were equally detected in 7% and 1% of the isolates respectively. Also, the VG *eagg* harboured by

enteroaggregative pathotype and diffusely-adherent *E. coli* VG *daa*E were detected in 2% and 4% of the isolates respectively and enteroinvasive *E. coli* VG *ipa*H was not detected. In addition, the VGs *pap*C for uropathogenic and *ibe*A for neonatal meningitis were frequently detected in 19% and 3% of isolates respectively. While all the isolates tested were susceptible to imipenem, meropenem, amikacin and gatilofloxacin, others were variously susceptible, and resistant as follows; ciprofloxacin (96%), kanamycin (95%), neomycin (92%), streptomycin (84%), chloramphenicol (73%), nalidixic acid (66%), nitrofuratoin (64%), gentamycin (63%), doxycycline (58%), cefepime (57%), tetracycline (49%) and cephalothin (42%). Conversely, all the isolates were resistant to sulphamethoxazole, and high levels of resistance were equally observed against amoxycillin (59%), ampicilin (57%) and cefuroxime (40%). Cefepime, cephalothin, cefuroxime, nalidixic acid, nitrofuratoin, chloramphenicol and tetracycline were not significantly different in their effect against the isolates from all locations ($P > 0.05$), whereas the resistance profile of the isolates against gentamycin, ciprofloxacin, sulphamethoxazole, ampicillin and amoxicillin were significantly different ($P < 0.05$). Amikacin, kanamycin, streptomycin, meropenem, imipenem and gatilofloxacin were statistically excluded from the analysis since all tested isolates showed total susceptibility to these antimicrobials. The multiple antibiotic resistance indexing ranged from 0.50 to 0.80 for all the sampling locations and exceeded the threshold value of 0.2. Prevalence and distributions of the 19 resistance determinants assessed were obtained as follows; [sulfonamides (*sul*I (8%), *sul*II (41%)], [beta-lactams; (*amp*C 22%; $bla_{\text{TEM,}}$ (21%), bla_{Z} (18%),], [tetracyclines *(tet*A (24%), *tet*B (23%), *tet*C (18%), *tet*D (78%), *tet*K (15%), *tet*M, (10%)], [phenicols; (*cat*I (37%), *cat*II (28%), *cml*A1 (19%)] and [aminoglycosides; (*aac*C2 (8%), *aph*A1 (80%), *aph*A2 (80%), *aad*A (79%) and *str*A (38%)]. The Pearson chi square exact test revealed many strong significant associations among

*amp*C, bla_{TEM}, *bla*$_Z$ and *tet*A genes with some determinants screened. In the same vein, a grand total of 366 resistance gene fingerprints were spotted across the sampling locations and among the resistant pathotypes, the modal prevalent gene prints were found among the ETEC strains in 148 (40%), being the predominant pathotype observed, followed by UPEC strains 80 (22%) while the lowest was the least occurring EAEC pathotype 14 (4%). While some physicochemical parameters exceeded prescribed standards for drinking water, some fell within. The total coliforms obtained in all the sampling sites were above the acceptable limits. Findings reveal the presence of diarrhoeagenic and non-diarrhoeagenic *E. coli* in the selected rivers and suggest a potential public health risk as the rivers are important resources for domestic, recreational and livelihood usage by their host communities. The multiple drug resistance indexing signifies isolates and pathotypes of high antimicrobial usage origin. An increase in the antimicrobial resistance signatures towards conventionally used antibiotics as observed in this study necessitates for safe water supply, adequate sanitation facilities and proper surveillance programs towards the monitoring of antimicrobial resistance determinants in water-bodies. Generally, results from this study indicate that the river waters are not suitable for consumption, domestic or recreational use and re-echo the importance of safeguarding the freshwater resources of Southwestern Nigeria.

CHAPTER ONE

GENERAL INTRODUCTION

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CHAPTER ONE

1.1 Introduction and background to the study

Water plays a significant role in the sound health of every human and its essentiality for plant life cannot be underestimated. Good drinking water quality is essential for the well-being of all people. It plays an important role in bodily intake of essential elements by man (Abulude *et al.*, 2007). About 75% of the earth's crust is covered with water and the human body comprises approximately 70% water (Pant, 2004). Therefore, water is the most essential need for life and healthy living of human beings. In Europe and America, much attention has been paid to the challenge of water purity, safety and security (Pant, 2004). The people of developing countries are faced with water-borne diseases than developed countries. Faecal pollution in water systems originates from human and non-human sources and multiple pollution control measures are necessary to meet the requirement of Clean Water Act and its amendments (Simpson *et al.*, 2002).

Rivers are important sources of natural waters for domestic and industrial activities, and the availability of good quality water is indispensable for preventing water-borne diseases and improving quality of life (Anhwange *et al.*, 2012). They are important multi-usage components, such as sources of drinking water, irrigation, fishery and energy production (Hacioglu & Basaran, 2009). They are also self-sustaining ecosystems that, without any human interference and natural disasters, could be able to support both themselves and all life forms included in them indefinitely. They are vital but vulnerable freshwater milieu that are highly significant for the sustenance of all life (Venkatesharaju *et al.*, 2010).

Many parts of the world are experiencing increasing water stress and scarcity. Projections indicate that the population living in water – stressed and water – scarce countries will grow from about 1.2 billion or 18% of the world population in 2007 to 4.0 billion or 44% of the world population by the year 2050 (CAWMA, 2007). According to the World Commission on Water for the 21st Century, more than half of the world's major rivers are so depleted and polluted that they pose danger to human health and surrounding ecosystems (IPS, 1999). The sources of water pollution vary and involve almost every significant human activity. These include mostly the dumping of domestic wastes, sewage, agricultural and industrial effluents into water bodies (Maitera *et al.*, 2010).

The goal of the Safe Drinking Water Act is the development and implementation of prevention and protection strategies to address potential threats to the water supply system identified through the assessment process. This law epitomizes a crusade towards a more preventive approach of avoiding contamination of public water supply systems (Brown & Caldwell, 2001). Each assessment must include a delineation of the watershed and source water assessment areas that drain to the intake location, an inventory of potential pollution and contaminant sources, and a determination of the susceptibility of the drinking water source to contamination. The susceptibility analysis is based on the potential for contaminant to be released into the environment, as well as the risk the contaminant poses to the surface water intake should it be released. In addition, the results of the assessment must be made available to the population served by the public water system. This information may then be useful for developing source water protection strategies as part of comprehensive planning efforts (Brown & Caldwell, 2001).

Safe drinking water or potable water is water that is free of injurious chemicals or microbial contamination (Smith, 1980). It is one of the most important felt needs in public health in the

21st century (Sobsey & Bartram, 2003). At least 884 million people in the world still do not get their drinking-water from improved sources with developing regions being critically hit by the menace. Sub-Saharan Africa accounts for over one third of that number, and is lagging behind in progress towards the realization of millennium development goals (MDGs), with only 60% of the population having access to improved sources of drinking-water (WHO, 2010). Nigeria, being the most populated country in Africa, has an important role to play in the realization of target 10 of the MDGs; to halve by 2015 the proportion of people lacking access to safe drinking water (Bourrigault, 1996).

In Nigeria and many developing countries, diarrhoea caused by bacterial pathogens especially *E. coli* remains one of the major causes of morbidity and mortality in infants and young children (Okeke *et al.*, 2003). Other African nations and South-East Asia show varying degrees of significant contributions of *E. coli* associated diarrhoea and mortalities (Abba *et al*., 2009). More than 80% of child deaths due to diarrhoea occur in these regions and nearly three-quarters of child deaths due to diarrhoea occur in just 15 countries (WHO, 2011) (Table 1.1).

Table 1.1 Rating of Countries in the Global Burden of Disease Estimates

Rank	Country	Total number of annual child deaths due to diarrhoea
1.	India	386,600
2.	**Nigeria**	**151,700**
3.	Democratic Republic of Congo	89,900
4.	Afghanistan	82,100
5.	Ethiopia	73,300
6.	Pakistan	53,300
7.	Bangladesh	50,800
8.	China	40,000
9.	Uganda	29,300
10.	Kenya	27,400
11.	Niger	26,400
12.	Burkina Faso	24,300
13.	United Republic of Tanzania	23,900
14.	Mali	20,900
15.	Angola	19,700

(Source: WHO, 2011).

Throughout history, consumption of drinking water supplies containing enteric pathogenic bacteria has been linked to illnesses in human populations. These illnesses commonly present as gastrointestinal-related symptoms, such as diarrhoea and nausea. Unsafe water is a global public health threat, placing persons at risk for a host of diarrhoeal and other diseases as well as chemical intoxication (Hughes & Koplan, 2005). Unsanitary water has particularly devastating effects on young children in the developing world. Diarrhoeal diseases are major causes of

morbidity and mortality in the developing world, more especially in young children (Kosek *et al.,* 2003). An estimated 1.1 billion persons (one sixth of the world's population) have inadequate access to clean water and 2.6 billion to sanitation (Hughes & Koplan, 2005; WHO, 2005). In developing countries, diarrhoeal diseases are often associated with infant and child mortality (Murray & Lopez, 1996; Sobel *et al.*, 2004). They account for an estimated 4.1% of the total daily global burden of disease and are responsible for the deaths of 1.8 million people every year, 90% of them are children under the age of 5. It was estimated that 88% of this burden is attributable to unsafe water supply, sanitation and hygiene, and is mostly concentrated in children in developing countries (UNICEF, 2011; WHO, 2014).

In recent years, both the anthropogenic influences and agricultural activities have increased exploitations of water resources as well as natural processes such as precipitation inputs, erosion, weathering of crustal materials, degradation of surface waters and rendering the water bodies unsuitable for both primary and secondary use (Najafpour *et al.,* 2008; Agbabire & Obi, 2009). Surface runoff and discharge of sewage into river bodies are the two major means through which various nutrients enter the aquatic ecosystems resulting in water pollution (Kumar *et al.*, 2011). In most cases, waters from these sources become faecally contaminated and devoid of treatment, and surprisingly communities use these waters directly (Momba & Notshe, 2003).

Figure 1.1 Villagers collecting water for domestic activities**.**

(Source: UNICEF, 2011).

Polluted river waters contain a large variety of pathogens including viruses, bacteria and protozoa (Servais *et al.*, 2007). The presence of these bacteria in surface waters also indicates that pathogenic organisms such as *Salmonella* spp., *Shigella* spp. as well as enteric viruses may be present (Ahmed *et al.*, 2006). Indicator bacteria have been used for many years to determine the quality and safety of surface and ground waters (Frenzel & Couvillion, 2002; Ahmed *et al.*, 2010). Non-point sources such as defective septic systems, storm water drainage systems, runoff from animal feedlots and/or point sources such as industrial effluents and municipal waste discharges are also known to be sources of faecal pollution (Ahmed *et al.*, 2010). The most common indicators used today are enterococci, total coliforms and faecal coliforms used as surrogates for human pathogens to assess the health risk and quality of the water (Evanson & Ambrose, 2006).

Much of the current issue with respect to environmental safety is focused on water because of its importance in sustaining the human health and health of the ecosystem. Freshwater is finite resource, essential for agriculture, industry and even human existence, without fresh water of adequate quantity and quality, sustainable development will be practically impossible (Kumar, 1997). Deterioration of freshwater resource has now become a global challenge and is increasing at a faster rate. Discharge of toxic chemicals, over pumping of aquifer and contamination of water bodies with substances that promote algae growth are some of the major causes of water quality deterioration. Direct contamination of surface water with metals in discharges from mining, smelting and industrial manufacturing, is a long-standing phenomenon. Today there is trace contamination not only of surface water but also of groundwater bodies, which are susceptible to leaching from waste dumps, mine tailings and industrial production sites (Mahananda *et al.*, 2005).

Water quality index (WQI) is one of the most effective tools to communicate information on the quality of water to the concerned citizens and policy makers. It, thus, becomes an important parameter for the assessment and management of surface water (Atulegwu & Njoku, 2004). Before water can be described as potable, it has to comply with certain physical, chemical and microbiological standards, which are designed to ensure that the water is palatable and safe for drinking (Tebutt, 1983). Tebutt (1992) listed many physicochemical characteristics to be analysed for different waters. These include pH, temperature, odour, radioactivity, electrical conductivity, total solids, turbidity, chloride, phosphate, nitrate, nitrite, biochemical oxygen demand and dissolved oxygen.

The original source of any drinking water is rich in aquatic microbes, some of which could be hazardous if they enter the human body. The quality of water is described by its physical, chemical and microbiological characteristics (Rajeshwari & Saraswathi, 2009). The annual and seasonal distributions of pH, temperature, turbidity, conductivity, nutrients, etc are investigated so as to understand quality of water dependent season (Chavan *et al.,* 2006). Quality of surface and ground waters is inadequate and is getting deteriorated due to unwise utilization of water resources, dehumanizing manner of organization, industrialization and other developed activities (Elayaraja, 2003). An adequate understanding of the importance of each water quality monitoring parameter will simplify the process of deciding what to test for and will help minimize unnecessary costs (Brown & Caldwell, 2001).

Biological assessments are based on the proposition that the structure and function of aquatic communities provide vital information about the quality of surface waters. Biological monitoring can reveal the cumulative effect of point and non-point sources pollution to streams and rivers. Results can be used to assess the ecological health and integrity of a particular water body (Brown & Caldwell, 2001)**.** Microbiological examination of river water is mandatory for use-related purposes such as drinking water production, irrigation and recreation (Kolarević *et al.,* 2011). Enterococci, faecal and total coliform counts are used as indices for determining the quality of the surface water (Holland *et al.,* 2004). Conformity with microbiological standard is of keen interest because of the capacity of water to spread diseases within a large population. Although the standards vary from place to place, the objective anywhere is to reduce the possibility of spreading water-borne diseases to the barest minimum in addition to being pleasant to drink (Edema *et al*., 2001).

Escherichia coli was first described by Theodor Escherich in 1885 as *Bacterium coli commune*, which he isolated from the faeces of neonates (Todar, 2008). In 1892, Shardinger proposed the use of *E. coli* as an indicator of faecal contamination based on the fact that *E. coli* is found abundantly in human and nearly all warm-blooded animal faeces at a concentration of approximately 10^9 per gram (Edberg *et al.*, 2000) and comprises about 1% of the total biomass in the large intestine (Leclerc *et al.*, 2001). Therefore, this organism is a perfect reliable index of faecal contamination and signifies a risk of water-borne diseases (Szewzyk *et al.*, 2000; Leclerc *et al.*, 2001). This bacterium has been used worldwide to measure the microbial water quality, and the national and international regulations establish the absence of this indicator microorganism in any 100 ml sample of drinking water (WHO, 1996).

Furthermore, since *E. coli* could be easily detected by its ability to ferment glucose (later changed to lactose), it is easier to isolate than known gastrointestinal pathogens. Regrowth of *E. coli* in water distribution systems is not an issue, since the organism rarely grows outside the human or animal gut (Geldreich, 1996). The inability of *E. coli* to grow in water, combined with its short survival time in water environments, suggests that the detection of *E. coli* in a water system is a good indicating of faecal contamination and the possible indicator of the presence of pathogens such as *Salmonella*, *Campylobacter, Giardia Cryptosporidium* or Norovirus (Todar, 1998).

The *E. coli* strains are usually referred to as commensal, intestinal pathogenic or extra intestinal pathogenic strains (Russo & Johnson, 2000). The pathogenic strains have been associated with several diseases including diarrhoea, urinary tract infections and meningitis (Russo & Johnson, 2003). Although identified by culturing on eosin methylene blue (EMB), MacConkey and *E. coli* chromogenic agar but the detection of the 16S rRNA is usually done to confirm the true *E. coli*

isolates. Bej *et al.* (1991) suggested a more sensitive PCR-based technique for the confirmation of *E. coli* isolates from water samples using *uid*A gene.

The pathogenicity of *E.coli* was first demonstrated in 1935 when the strain of *E. coli* was shown to be the causative agent in an outbreak of diarrhoea in infants (Todar, 2008), and ever since then, pathogenic *E. coli* have been classified into numerous categories based on their possession of virulence factors, clinical symptoms and sites of pathogenesis of the host (Nataro & Kaper, 1998; Milon *et al*., 1999; Gyles & Fairbrother, 2004). These pathogenic strains are broadly categorized as either intestinal pathogenic *E. coli* (InPEC) or extraintestinal pathogenic *E. coli* (ExPEC) (Russo & Johnson, 2000; Kaper *et al.,* 2004). Within each of these broad categories are sets of strains called "pathotypes" that share common virulence factors and elicit similar pathogenic outcomes (Marrs *et al.,* 2005).

To date, eight *E. coli* pathovars of two sub-categories have been widely studied. Six pathovars; enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely-adherent *E. coli* (DAEC) are diarrhoeagenic *E. coli* (DEC), and two pathovars; uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) are the most common extra-intestinal pathogenic *E. coli* (ExPEC) pathotypes (Croxen & Finlay, 2010). Other pathovars have been identified, but their mechanisms of pathogenesis are not as well defined. They include necrotoxigenic *E. coli* (NTEC), cell-detaching *E. coli* (CDEC) and adherent invasive *E. coli* (AIEC) (Croxen & Finlay, 2010).

Several pathotypes of diarrhoeagenic *E. coli* give rise to gastroenteritis, but rarely cause disease outside of the intestinal tract. ExPEC, on the other hand, have maintained their impeccable

ability to exist in the gut without consequence, but have the capacity to disseminate and colonize other host niches including the blood, central nervous system, and urinary tract, resulting in disease. DEC strains are food and waterborne pathogens that cause a wide spectrum of symptoms, ranging from mild gastroenteritis to severe diseases such as haemorrhagic colitis, thrombotic thrombocytopenic purpura, and haemolytic uremic syndrome (HUS) (Karmali *et al.*, 1985). Diarrhoeagenic *Escherichia coli* (DEC) is an important agent of childhood diarrhoea which represents a major public health problem in developing countries (Nataro & Kaper, 1998; Mitchell *et al*., 2005; Soltan Dallal., 2006; Akinjogunla *et al*., 2009).

Globally, the occurrence of pathogens in environmental waters is a current concern for public health officials and those in the water resource management area. Contamination of surface waters particularly with faecally-derived bacteria, has long been a water quality issue owing to the potential for disease transmission (Cernat *et al*., 2002). Despite the discovery of penicillin in the 1940s and several other antimicrobials in subsequent years which led to great improvements in the management of infectious diseases, the widespread use of antimicrobial agents in human and veterinary medicine, animal husbandry, aquaculture, agriculture and food technology has led to the inevitable development of resistance, as diseases and disease agents now re-emerging in new forms resistant to antibiotic therapy (Levy & Marshall, 2004; Norrby *et al*., 2005).

Before the abusive antimicrobial use age, only a slight resistance level had been detected among enteric bacterial pathogens. Nowadays, their susceptibility to antimicrobials has changed and resistance patterns have been used as epidemiologic markers (Bechtlufft *et al.*, 2008). Antimicrobial resistance among enteropathogens, including *E. coli* has been reported to be increasing in recent years, sometimes leading to point-break situations where no antibiotic treatment options remain. These situations are of serious concern in developing countries where

enteropathogens are frequently encountered and cause life-threatening infections, especially among children (Pitout & Laupland 2008; Lynch *et al.,* 2013).

Escherichia coli remain one of the major causes of morbidity and mortality in infants and young children especially when these diseases fail to be cured due to development of resistance to commonly prescribed antimicrobial agents (Okeke *et al*., 2003). Generic *E. coli* are frequently used as indicator bacteria to monitor the trends in antimicrobial resistance because they are the prevalent commensal enteric bacteria in humans and animals, can be cultured easily and inexpensively (van Den Bogaard *et al.,* 2000), and they can acquire and preserve resistance determinants from other organisms in the environment and in animal populations (Murray *et al.,* 1997).

Antibiotic resistance in *E. coli* has been globally identified in isolates from environmental, animal and human sources (Heike & Reinhard, 2005).While *E. coli* has diverse phenotypic and genotypic characteristic features, some characteristics are shared among strains exposed to similar environments due to selection pressure (Borgen *et al.,* 2000). The level of selective pressure exerted in a mixed catchment area may be a useful criterion for identifying the host sources of *E. coli* in the watershed. One such tool to aid with examining the selection pressure on *E. coli* is assessing their antimicrobial sensitivities (DeFrancesco *et al.,* 2004; Donaldson *et al.,* 2006).

The health consequences associated with *E. coli* infection have been greatly worsened by the emergence of multidrug-resistant *E. coli*. This mounting phenomenon, which has been deemed to be worse that the methicillin-resistant *Staphylococcus aureus* (MRSA), whose resistance is considered as one of the biggest challenges in the twenty-first century in the field of science and

medicine, already has some established consequences regarding bacteria-host relationships (Santos *et al*., 2007). From the late 1990s, multidrug-resistant Enterobacteriaceae (mostly *Escherichia coli*) that produce extended-spectrum β-lactamases (ESBLs), such as the CTX-M enzymes emerged within the community setting as an important cause of urinary tract infections (UTIs). Recent reports have also described ESBL-producing *E coli* as a cause of bloodstream infections associated with these community-onset UTIs. The shiga toxin–producing *E. coli* (STEC) serotype O104 that was responsible for an outbreak in Germany showed extended spectrum β-lactamase (ESBL) activity (Rubino *et al*., 2011; Struelens *et al.,* 2011).

Excessive and sometimes indiscriminate use of antibiotics in human and veterinary medicines are major promoters for the development and spread of multi-resistant bacteria worldwide (Woodford & Livermore, 2009; Gootz, 2010). Liquid manure of animals and human excretions has led to dissemination of resistant enteric bacteria in the environment (Reinthaler *et al.,* 2003). The emergence of antimicrobial-resistant bacteria presents a major threat to public health because it reduces the effectiveness of antimicrobial treatment, leading to increased morbidity, mortality and healthcare expenditure (Smith & Coast, 2002; Hawkey & Jones, 2003; Collignon *et al.,* 2009). The direct consequences associated with the extensive use of antibiotics in hospitals, swine production areas and fish farms cannot be over-emphasized as antimicrobial resistance determinants (ARDs) can easily be transferred into soils while enriching farmlands with animal manure and processed biosludge from sewage treatment plants (STPs). These ARDs can either leach to groundwater or be carried by runoff and erosion to surface water. Ground and surface waters which are commonly used as sources of drinking water then allow them to go through drinking water treatment facilities and enter into water distribution systems. Also, they can enter into aquatic environments by direct discharge of untreated wastewaters or into STPs

through wastewater collection systems and subsequently into the environments and the genes in surface and ground waters can transfer antibiotic resistance to the bacteria in drinking water or food chain (Chee-Sanford *et al.,* 2001, Schwartz *et al.,* 2003; Yang & Carlson, 2003; Auerbach *et al.,* 2007).

Considering the fact that ARDs are widespread in aquatic environments, there is a need for the development and application of molecular methods to investigate their occurrences, transport, and fate in the environments. So far, the established methods used for detection, typing, and characterization of ARDs include, but not been limited to the following, specific and multiplex polymerase chain reaction (PCR), real-time PCR, DNA sequencing, and hybridization-based techniques including microarray (Zhang *et al.,* 2009).

The spread of resistant genes is made worse when they form part of a mobile gene cassette, which provides for horizontal transfer by several mechanisms including:

(i) mobilization of individual cassettes by the integron-encoded integrase (Hall & Collis, 1995)

(ii) movement when the integron containing the cassette relocates by targeted transposition (Minakhina *et al*., 1999)

(iii) dissemination of larger transposons such as *Tn21* carrying integrons (Liebert *et al*., 1999)

(iv) movement of conjugative plasmids containing integrons among different bacterial species.

Gene cassettes consist of a gene flanked by a recombination site, known as a 59-base element, which is recognized by the integron-encoded site-specific recombinase (*intI*). Gene cassettes are known to exist as free circular molecules (Hall & Collis, 1995) and are transcribed only when captured and inserted into an integron, usually at the *attI* recombination site 104 bp upstream of the *intI1* gene (Hall & Collis, 1995). New cassettes are continually being discovered, and now

over 60 cassettes that confer resistance to a range of antimicrobial agents have been identified (Laraki *et al*., 1999; White *et al*., 2000). Integrons are elements that participate in a powerful site-specific recombination system and play a major role in the spread antibiotic resistance genes. Many antibiotic resistance genes found in Gram-negative bacteria are part of a gene cassette inserted into an integron (Reechia & Hall, 1995). Since many gene cassettes of integron contain antimicrobial resistance genes in Gram-negative bacteria, the horizontal transfer of integron through plasmids and transposons has been found to play an important role in the dissemination of antimicrobial resistance genes and the development of multi-resistance. Few studies have reported the prevalence of integron and gene cassettes in the enteric faecal flora of humans and animals (Hall & Collis, 1995; Leverstein *et al*., 2001).

1.2 Research Justification

Provision of clean and safe drinking water in rural and urban areas is a great challenge for the developing countries of the world since most communities rely on poor traditional sources that often provide unsafe domestic water. Available data from Targets of the State Water and Sanitation Projects (2007-2010) reveals that access to clean water in rural and urban areas are still far from being met. It has been reported that the target is below average, about 48% approximately (Water and Sanitation Projects, 2007–2010). Therefore, microbial water quality is necessary to ensure a sustainable good quality water supply for the entire populace and to prevent diarrhoeal or diarrhoeal–related diseases. The dearth of information on pathotyping, and the combined phenotypic and genotypic characterization of antimicrobial resistance profiles of *Escherichia coli* isolates recovered from surface waterbodies of the state further necessitated the need for this research so as to create awareness on the consequences associated with ingestion of

untreated waters and suggest possible ways of lessening or averting the potential threats in order to safeguard the health of the public.

1.3 Research Hypothesis

The working hypothesis set for this study was that rivers in Osun state, Southwestern Nigeria are potential sources of pathogens and multi-drug resistant *Escherichia coli*.

1.4 Aim and Objectives

The broad aim of this study was to assess the quality indices of the selected rivers and determine, using molecular technique, the prevalence of the *Escherichia coli* pathotypes in selected rivers of Osun state, Southwestern Nigeria.

The specific objectives of the study are as follows:

(a) To determine the physicochemical and bacteriological qualities of selected rivers water in Osun State, Nigeria

(b) To evaluate the prevalence of *Escherichia coli* pathotypes in the selected rivers

(c) To elucidate the antibiogram characteristics of the *E. coli* pathotypes including relevant antimicrobial resistance determinants in the multi-drug resistant *E. coli* pathotypes.

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CHAPTER TWO

LITERATURE REVIEW

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2.1 Water and the planet earth

Water is of vital importance to human health, well-being and existence. However, freshwater is a finite resource; and without it's availability in adequate quantity and quality, sustainable development will be practically impossible (UNEP/WHO, 1996). The total volume of water on Earth is about 1.4 billion cubic kilometers. Of this, about 97.5 % of the total volume is saltwater and only about 2.5% is freshwater. Freshwaters consist of surface water (0.3%), groundwater (30.1%), ice caps and glaciers (68.7%) and others (0.9%) (Figure 2.1). This implies that, of the total freshwater, over 68% is locked up in ice and glaciers, 30% in underground aquifers while the fresh surface waters (water in rivers, streams, lakes, dams, ponds and similar bodies of water) consist only 0.3% of the world's freshwater. From the foregoing, it is clearly evident that the total available freshwater supply for humans and ecosystems is less than 1% of all freshwater resources. Although, a larger percentage of the planet earth is covered by water, yet very little water is accessible.

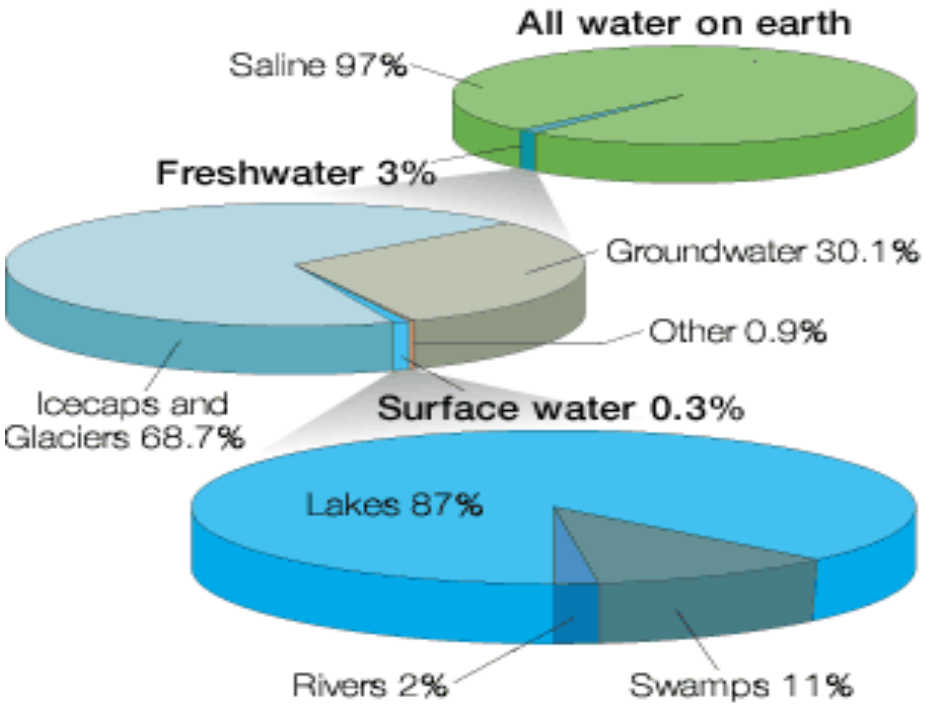

Figure 2.1 Distribution of earth's water.

(Source: http://ga.water.usgs.gov/edu/earthwherewater.html)

2.2 Vulnerability of surface waters

Surface waters serve for drinking, domestic, agricultural, recreational, industrial and other purposes including transportation, hydroelectricity, etc. Universal access to safe drinking water and sanitation has been promoted as an essential step in reducing the avertable diseases (WHO, 1994; 2001). The provision of clean drinking water and discharge of adequately treated wastewater is a fundamental requirement for human life (Akpor & Muchie, 2011). Man need water for industrial development, irrigation to grow food, wash everyday item, sanitations needs, water makes up about 90% of cytoplasm, and is used as hydration to sustain health among others. However, freshwater sources are vulnerable to pollution (Shuval, 1990; Okoh *et al*., 2007; Schwarzenbach *et al*., 2010; Azizullah, *et al*., 2011; Chigor *et al*., 2012), and are

continually being impacted by rapid population growths, land development along river basin, agriculture, urbanization and industrialization that place increased demand on surface waters both as sources of water for different uses and as disposal channels for treated and untreated wastewaters (Solaraj *et al.*, 2010; Suthar *et al.*, 2010). Thus, Samuel Taylor Coleridge's *The Rime of the Ancient Mariner*, comes to mind:

"Water, water, everywhere,
And all the boards did shrink;
Water, water, everywhere,
Nor any drop to drink." (Coleridge, 1798).

In developing nations, the vulnerability of surface water bodies to pollution with decomposable organic materials and pathogenic agents, and the use of raw/treated wastewater for irrigation may constitute serious public health risks (Shuval, 1990; Obi *et al*., 2004; Okoh *et al*., 2007; Igbinosa and Okoh, 2009; Chigor *et al*., 2010a, 2010b; Gemmell & Schmidt, 2012). Pathogens such as bacteria, viruses, fungi, protozoa and helminths render water contaminated and non-potable, and could result in the transmission of water-borne diseases to swimmers, agricultural workers and the consumers of crops irrigated with contaminated waters (Shuval, 1990; Mohanty *et al.*, 2002). Faecal pollution also affects the ecosystem and aquatic life, in addition to causing economic losses from closure of aquatic food harvesting areas, bathing restrictions and diseases (Pruss *et al.*, 2002; Gourmelon *et al*., 2007).

2.3 Surface water pollution and associated risks

There is a broad spectrum of microbial pathogens that have been found in the water environment including bacteria, protozoa, algae, viruses and helminths (WHO, 2011). The types and numbers and distribution of various pathogens in surface waters vary greatly across different water bodies,

regions of the world and times of year (Tani *et al*., 1995). The increase in anthropogenic activities as well as natural processes such as precipitation inputs, erosion, weathering of crustal materials, and degradation of surface waters have rendered most water bodies unsuitable for their multi-purpose usage (Furhan *et al.,* 2004). Many of these water bodies are often impacted by inadequately treated effluents from municipal wastewater plants as receiving water bodies (Fatoki *et al.,* 2003). The major hazard in drinking water supplies is microbial contamination, which is due to agricultural land wash, domestic sewage, industrial effluents, improper storage and handling (Saha *et al*., 2006; WHO, 2006). In addition to anthropogenic activities, meteorological events are also major determinants of physicochemical parameters such as temperature, pH and turbidity of the water (Zamxaka *et al.,* 2004).

Figure 2.2 Dam used for domestic purposes by residents.

(Source: UNICEF/WASH, 2012).

Enteric pathogens are excreted in faeces of infected individuals. It is known that contaminant loads to surface water bodies start off from point or non-point sources (Parveen *et al.,* 2001; Albek, 2003). Point-source pollution originates from discrete sources whose inputs into aquatic systems can often be defined in a spatially explicit manner (Ritter *et al*., 2002). Examples of point-source pollution include industrial effluents (food processing plants), municipal sewage

treatment plants, combined sewage-storm-water overflows and sewage, septic tank leakage (Ritter *et al*., 2002). Non-point-source pollution, in contrast, arises from poorly defined, diffuse sources that typically occur over broad geographical scales. Examples of non-point-source pollution include urban and agricultural runoffs, pathogens, organic matter and storm-water. (Kistemann *et al.,* 2002; Ritter *et al*., 2002).

Surface water pollution also has economic consequences. A serious problem impacting on communities relying on polluted water sources for the production of potable water is the eventual costs of potable water. Treatment costs may become so excessive that water becomes available only to those who can afford it (Hutton *et al.,* 2007). Pollution of surface waters by agricultural runoffs like sediment, nutrients, pesticides, salts and pathogens can impose costs on water users (Water quality impacts of agriculture, 2012). Pesticides are especially difficult to remove from freshwater and thus can be found in municipal or bottled water, even after conventional treatment (Maria, 2003). Eutrophication of surface waters may accelerate algal production, resulting in clogged pipelines, fish kills, which may result in loss of revenue, and reduced recreational opportunities (USEPA, 1998). Sediment is the largest contaminant of surface water by weight and volume (Koltun *et al.,* 1997). Besides increasing the cost of water treatment for municipal and industrial water uses, it can also destroy or degrade aquatic wildlife habitat, reducing diversity and damaging commercial and recreational fisheries. In addition, many toxic materials can be bound to silt and clay particles that are carried into water bodies, including nutrients, pesticides, industrial wastes and metals (Osterkamp *et al.,* 1998).

The resulting effect of water pollution, according to the latest WHO report is that, today, about one billion people lack access to safe drinking water and 2.4 billion to adequate sanitation (WHO/UNICEF, 2010). The report further states that improved sanitation facilities are used by

less than two thirds of the world population, a global picture that however, masks great disparities between regions. Virtually, the entire population of the developed regions uses improved facilities but in developing regions, only about half the population does. The pollution of fresh water resources therefore remains a global concern contributing to high morbidity and mortality rates from waterborne and food-borne diseases (Pruss *et al.*, 2002; WHO, 2009).

2.4 Global burden of diseases

Lack of safe water and poor sanitation are important risk factors for mortality and morbidity, including diarrhoeal diseases, especially in the developing world (Pruss *et al*., 2002; WHO, 2009). A recent report by the WHO/UNICEF Joint Monitoring Programme for Water Supply and Sanitation shows that 884 million people in the world do not still get their drinking-water from improved sources, almost all of them in developing regions. Sub-Saharan Africa accounts for over a third of that number. It further reveals that seven out of ten people without improved sanitation live in rural areas, and that worldwide, 37% of people not using improved source of drinking water live in Sub-Saharan Africa (WHO/UNICEF, 2010).

The contamination of water is a problem of global concern contributing to high morbidity and mortality rates from waterborne and food-borne diseases, such as typhoid fever, cholera and diarrhoeal diseases (Pruss *et al.*, 2002; WHO, 2009). A report from the American Academy of Microbiology on the global burden of gastrointestinal diseases (Payment & Riley, 2002) reveals an estimated 6-60 billion cases of gastrointestinal illness occur annually. The WHO declared that diarrhoeal diseases alone contributes to an estimated 4.1% of the total DALY (disability adjusted life years) global burden of disease and is responsible for the deaths of 1.8 million people every year (Pruss *et al.*, 2002). It was figured that 88% of that burden is attributable to unsafe water supply, sanitation and hygiene, and it is mostly concentrated on children in developing countries.

Annually, diarrhoeal disease affects children in developing countries some 5 billion times, claiming the lives of nearly 1.8 million (UNDP, 2006). A fraction of the world's population (20%) has no access to safe drinking water. This fact, in conjunction with inadequate sanitation, leads to millions of deaths every year (Hunter *et al.*, 2001). Similarly, >2 million persons, mostly children <5 years of age, die of diarrhoeal disease, out of which almost 90% is directly linked to contaminated water, inadequate sanitation and hygiene (WHO, 2003; UNICEF, 2011). These diseases are the major causes of morbidity and mortality in the developing world, more especially in young children, and continue to be a health challenge worldwide, where they are estimated to be responsible for 2.5 million infant deaths per year, with an annual mortality rate of 4.9 per 1,000 children and an incidence of 3.2 episodes per child per year among children under 5 years of age (Avendan *et al.,* 1993; Kosek *et al.,* 2003)**.**

2.5 Surface water quality

The quality of water is affected by natural and anthropogenic factors including, geological structure and mineralogy, rainfall and runoff events, population growth, urbanization and industrialization (Kistermann *et al*., 2002; Solaraj *et al*., 2010; Suthar *et al*., 2010; Alexakis, 2011; Calijuri, *et al*., 2011; Chigor *et al*., 2012). In water quality control monitoring, non-pathogens present in faeces are used to indicate the occurrence of faecal contamination, hence the possibility that pathogens may be present (Byamukama *et al*., 2000; Grabow, 2001; APHA, 2005; Jiang *et al*, 2007; Bosch *et al*, 2008; Abdelzaher *et al*., 2010). Such faecal indicator bacteria (FIB) include faecal coliforms, *Escherichia coli*, enterococci and *Clostridium perfringens* (Abdelzaher *et al*., 2010). However, recent studies have highlighted the shortcomings of using only bacteriological indicators and the necessity of surveillance of source waters for pathogens towards protection of public health (Pourcher *et al*., 2007; Muscillo *et al*.,

2008; Jurzik *et al*., 2010). All human usage of water, either for drinking, irrigation, recreation and industrial processes has some prescribed quality criteria to make it acceptable. This quality requirement can be addressed in terms of physicochemical properties and microbiological examinations of such water (Maitera *et al.*, 2010).

2.5.1 Physicochemical quality

Monitoring physicochemical parameters in water resources to evaluate water quality and identify deficiencies is important for protection of both the environment and public health (Okoh *et al*., 2007). Tebbut (1992) listed many physicochemical characteristics to be analysed for in different waters (river water, drinking water, raw sewage and sewage effluent) including: pH, temperature, odour, electrical conductivity, total dissolved solids, turbidity, chloride, phosphate, nitrate-nitrogen and biochemical oxygen demand. Although some of these parameters may have limited health significance, yet international standards require their determination for the evaluation of surface water quality (Tebbut, 1992; WHO, 2008) and several studies have assayed for these parameters worldwide including in Asia (Solaraj *et al*., 2010; Suthar *et al*., 2010), Europe (Alexakis, 2011; Popa *et al*., 2012), North America (Stewart & Skousen, 2003; Jonathan & Thangadurai, 2011), South America (Debels *et al*., 2005; Calijuri *et al*., 2011) and Africa (Jonnalagadda & Mhere, 2001; Chigor *et al*., 2012).

2.5.2 Microbial water quality

For any indicator organism to be considered reliable, it should:

(i) be present whenever pathogens are present, and in a much greater number,

(ii) be resistant or more resistant to disinfectants and the aqueous environment than the pathogens,

(iii) be easy to grow on a selective artificial medium, and

(iv) have easily identifiable characteristics (Bonde, 1977; Hobson & Poole, 1988).

The most frequently used indicator organisms are faecal coliforms followed by faecal streptococci and *Clostridium perfringens*. Faecal (thermotolerant) coliforms are defined as the group of coliform organisms that are able to ferment lactose at 44-45^{o}C, comprising the genus *Escherichia* and, to a lesser extent, species of *Klebsiella, Enterobacter* and *Citrobacter*. Coliforms are parts of the *Enterobacteriaceae* and refer to Gram-negative, rod-shaped bacteria capable of growth in the presence of bile salts or other surface-active agents with similar growth inhibiting properties and are able to ferment lactose at 35-37 ^{o}C (WHO, 2011).

The basis of using coliforms as indicators of faecal contamination is that the primary natural habitat of these bacteria is the intestine of warm-blooded animals. This assumption is probably true for *Escherichia coli* and large numbers of these bacteria are found in faeces may approach 10^{9}/g. However other coliforms such as *Klebsiella pneumonia* and *Enterobacter aerogenes* can grow in non-animal environments such as the soil, plant surfaces and even industrial effluents (Hobson & Poole, 1988). Furthermore, some reports had that the multiplication of *E. coli* within riverbank or beach soils and showed that soils can be the primary source of *E. coli* in the river between storm events. Such results contest the use of *E. coli* as a suitable indicator of water quality in tidally influenced areas (Solo-Gabriele *et al*., 2000; Desmarais *et al*., 2002; Harwood *et al*., 2005; Abdelzaher *et al*., 2010), that notwithstanding, the total, faecal coliforms and *E. coli* detection in particular, remains the major and most reliable tool in the assessment of the health risks posed by pathogens in waters (Byamukama *et al.,* 2000; APHA, 2005). The concept of using organisms such as *E. coli* as indicators of faecal pollution is a well-established practice in the assessment of drinking-water quality (WHO, 2011). Other indexes of water pollution are viruses such as adenoviruses (Fong *et al*., 2010; Connell *et al*., 2012), and bacteriophages such as

Bacteriodes fragilis, (Savichtcheva & Okabe, 2006), and F-specific RNA coliphages (Skraber *et al*., 2004; Savichtcheva & Okabe, 2006).

Table 2.1 Bacterial pathogens linked to drinking water or recreational water contact.

Organism	Disease	Transmission	Clinical feature
Vibrio cholerae	Cholera	Drinking water	Watery diarrhoea, may be severe
Salmonella spp.	Salmonellosis	Occasional outbreaks with drinking water	Diarrhoea, colicky abdominal pain and fever
Salmonella typhi	Typhoid	Drinking water	Fever, malaise and abdominal pain with high mortality
Shigella spp.	Shigellosis (Bacillary dysentery)	Both drinking and recreational water	Diarrhoea frequently with blood loss
Campylobacter spp.	Campylobacteriosis	Both drinking and recreational water	Diarrhoea frequently with blood loss
Enterotoxigenic *E. coli*		Drinking water	Watery diarrhoea
Enterohaemorrhagic *E. coli*		Drinking water and recreational water contact	Bloody diarrhoea and haemolytic uraemic syndrome in children
Yersinia spp.	Yersiniosis	Drinking water	Fever, diarrhoea and abdominal pain
Francisella tularensis	Tularaemia	Drinking water	Typhoid-like or mucoutaneous with suppurative skin lesions
Helicobacter pylori		Drinking water	Gastritis that can progress to gastric cancer
Mycobacterium spp. (Not *M. tuberculosis*)	Varies	Potable water systems in hospitals, some recreation	Varies, includes respiratory diseases, wound infections, skin disease
Cyanobacteria	Various	Toxins in drinking water or direct contact with surface blooms	Dermatitis, hepatitis, respiratory symptoms, potentially fatal

(Source: Hunter, 2003).

2.6 *Escherichia coli*: Microbiology, laboratory diagnosis and typing

Escherichia coli was first described by Theodor Escherich in 1885 as *Bacterium coli commune*, which he isolated from the faeces of newborns (Todar, 2008). It is a normal microflora of the human intestinal tract and regarded as commensal *E. coli*. The commensal *E. coli* and its host organisms live together for long periods of time with mutual benefits (Kaper *et al.,* 2004). *Escherichia coli*, a member of the *Enterobacteriaceae* family, is a Gram-negative, rod-shaped

(1.1-1.5 × 2-6 μm), motile organism that are oxidase-negative, glucose, lactose and sucrose fermenting, with an optimum growth pH of 6.0-7.0 and temperature of 37 °C but there are certain laboratory strains can multiply at temperatures up to 49 °C (Fotadar *et al.,* 2005). The bacterium can grow in the presence or absence of O_2.

Under anaerobic conditions, it grows by means of fermentation, producing "mixed acids and gas" as end products. It can also grow by means of anaerobic respiration, since it is able to utilize NO_3, NO_2 or fumarate as final electron acceptors for respiratory electron transport processes (Todar, 1998). Cells are positive in the Methyl-Red test, but negative in the Voges-Proskauer assay. Cells do not use citrate, do not produce H_2S or lipase, and do not hydrolyze urea (Holt *et al*., 1994). Physiologically, *E. coli* is versatile and well-adapted to its characteristic habitats. It can grow in media with glucose as the sole organic constituent and can respond to environmental signals such as chemicals, pH, temperature, osmolarity, etc., in a number of very remarkable ways considering it is a unicellular organism. It is the member of the faecal coliform group, unable to breakdown urease (Todar, 1998).

Escherichia coli is one of the United States Environmental Protection Agency (USEPA) recommended indicator organisms for freshwater systems and a sensitive measure of faecal pollution since it is found in almost all warm-blooded animals, including humans (USEPA, 1986; Leclerc *et al.,* 2001). Occurrence of *Escherichia coli* in drinking water is indicative of recent faecal contamination and possible incidence of water-borne diseases that is most serious threat to health (WHO, 2010). However, recovery of indicator bacteria may depend upon level of contamination in a particular water source (Warner *et al.,* 2008).

2.7 Clinical significance of *Escherichia coli*

The acquisition of virulence genes is believed to provide an evolutionary pathway to pathogenicity. As a genetically diverse group, most strains of *Escherichia coli* are harmless commensals of mammals (Hartl & Dykhuizen 1984; Selander *et al.*, 1987), but others are capable of causing either intestinal or extraintestinal disease (Ørskov & Ørskov, 1992). Indeed, PCR analysis has revealed that even commensal *E. coli* isolates possess some of these virulence genes (Beutin *et al*., 2003; Dixit *et al*., 2004). However, mere possession of a single or a few virulence genes does not mean a strain is pathogenic unless that strain possesses the appropriate virulence gene combination to cause disease in a specific host species (Gilmore & Ferretti, 2003). Currently, as to whether isolates that possess one or a few virulence genes represent pathogenic clones that have lost virulence genes or are commensals in the process of acquiring them remain a controversy.

Using a more phylogenetic approach, Clermont *et al.* (2000) described a three-gene combination to differentiate between strains in the ECOR collection that are pathogens (B2 and D) and those members that are mainly commensals (A and B1). Surprisingly, these three genes alone were capable of providing a phylogenetic classification that closely mirrored similar groupings based on a more complex genetic analysis by multilocus enzyme electrophoresis. Furthermore, these relationships were established with a population of assembled clones, primarily of commensal origin, and in the absence of a panel of functionally accredited virulence genes.

While most *E. coli* strains in the gut are commensals, certain strains may carry a combination of VGs which enable them to cause intestinal infections such as diarrhoea or haemolytic colitis, or to cause extra-intestinal infections such as neonatal meningitis, nosocomial septicaemia, haemolytic uraemic syndrome, urinary tract and surgical site infections (Falagas & Gorbach,

1995; Johnson & Stell, 2000). The variety of pathogenic strategies exhibited by *E. coli* strains is attributable to differences in genetic background with each strain carrying unique plasmids or pathogenicity islands (Keskimäki *et al.,* 2001). *E. coli* strains capable of causing diarrhoea, under certain conditions, for example, when the immune system is compromised, or due to environmental exposure, is referred to as diarrhoeagenic *E. coli* (Nataro & Kaper, 1998).

Diarrhoeagenic *E. coli* (DEC) strains possess specific fimbrial antigens that enhance their intestine-colonizing ability and allow adherence to the small mucosa bowel. Once having colonized, the strains use very different pathogenic strategies to cause changes in the arrangement of the bowel's mucosa (Donnenberg, 1999). They are among the bacteria most frequently associated with diarrhoea in children from developing countries (O'Ryan *et al.,* 2005; Thapar & Sanderson, 2004). The specific virulence factors produced by DEC, together with the type of disease they caused have been used to separate them into different pathotypes. They are among the most common etiological agents of diarrhoea and have been differentiated based on their specific virulence factors and phenotypic traits (Vidal *et al.,* 2005; Prescott *et al.,* 2008; CFSPH, 2009).

Six groups of *E. coli* that could potentially cause diarrhoeal diseases are now recognized and are enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (Vidal *et al.,* 2005; Prescott *et al.,* 2008). Although, cell detaching *E. coli* (CDEC) and necrotoxic *E. coli* (NTEC) are other diarrhoeagenic *E. coli* pathotypes have been proposed, however their significance remains uncertain (Russo & Johnson, 2000; Clarke, 2001; Abduch-Fabrega *et al.,* 2002). The two characterized extra intestinal pathogenic *E. coli* (EXPEC) which are non-diarrhoeagenic are uropathogenic *E. coli* (UPEC) and neonatal

meningitis *E. coli* (NMEC), both isolated from humans. Avian pathogenic *E. coli* (APEC) are associated with avian infections and have been isolated from poultry (Wiles *et al.,* 2008; Dubois *et al.*, 2009; Johnson & Stell, 2000; Kaper *et al.,* 2004). Each group has its unique virulence properties and is classified as shown in Figure 2.3.

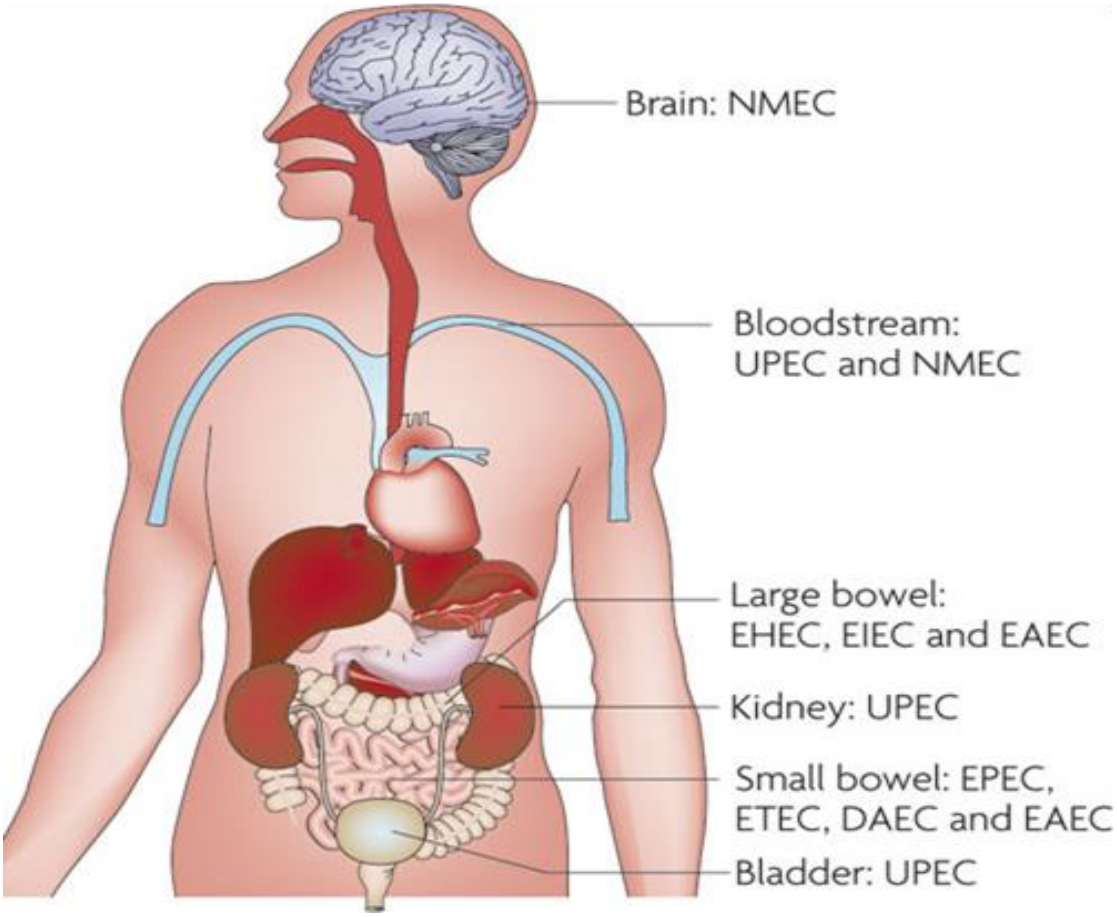

Figure 2.3 Sites of pathogenic *Escherichia coli* colonization.

(Adapted from Croxen & Finlay, 2010).

Pathogenic *Escherichia coli* colonize various sites in the human body. EPEC, ETEC and DAEC colonize the small bowel and cause diarrhoea, whereas EHEC and EIEC cause disease in the large bowel; EAEC can colonize both the small and large bowels. Uropathogenic *E. coli* (UPEC) enters the urinary tract and travels to the bladder to cause cystitis and, if left untreated, can

ascend further into the kidneys to cause pyelonephritis. Septicaemia can occur with both UPEC NMEC, and NMEC crosses the blood–brain barrier into the central nervous system, causing meningitis (Croxen & Finlay, 2010).

2.8 Pathotypes of *Escherichia coli*

2.8.1 Enterotoxigenic *Escherichia coli* (ETEC)

(i) History

ETEC is the most important but under-recognized bacterial cause of diarrhoea or cholera-like disease in all age groups especially in areas with population pressure, poor sanitation and inadequate drinking water (Nataro & Kaper, 2004). Furthermore, of the estimated one billion yearly international travelers, 20-60% of those traveling to low-income countries suffer from travelers' diarrhoea (Hill & Beeching, 2010). Approximately 30-70% of traveler's diarrhoea bacteria are the causative agent, of which ETEC are the most commonly detected (Wiedermann & Kollaritsch, 2006). Often ETEC is the first enteric infection experienced by infants in low resource countries, and in endemic areas almost all children will have had one ETEC diarrhoea episode in their first year of life. One out of every six travelers to endemic areas has been observed to be infected with ETEC (CDC, 2004; Steffen *et al*., 2005).

Development of rabbit ileal loop assay which led to the discovery of cholera toxin was also used for pure cultures of *E. coli* isolated from stools and small bowels of children and adults showing similar symptoms to cholera. Live cultures and culture filtrates of these strains when injected into isolated rabbit ileal loops produced strong cholera-like secretory response leading to the discovery of the heat-labile enterotoxin of *E. coli* and recognition of ETEC pathotype in 1968 (Sack, 2011).

(ii) Pathogenicity

ETEC strains adhere to intestinal epithelial cells via a heterogeneous group of proteinacious surface structures termed colonization factors (CFs) which can be fimbrial, non-fimbrial or fibrillar (Croxen & Finlay, 2010). Enterotoxigenic *Escherichia coli* (ETEC) become anchored to enterocytes of the small bowel through colonization factors (CFs) and an adhesin that is found at the tip of the flagella (EtpA). Tighter adherence is mediated through Tia and TibA. Two toxins, heat-labile enterotoxins (LT) and heat-stable enterotoxins (ST) that cause intestinal epithelial cells to secrete excess fluid through cyclic AMP (cAMP) and cyclic GMP (cGMP)-mediated activation of cystic fibrosis transmembrane conductance regulator (CFTR). Some strains produce only one of the toxins while others produce both (Croxen & Finlay, 2010).

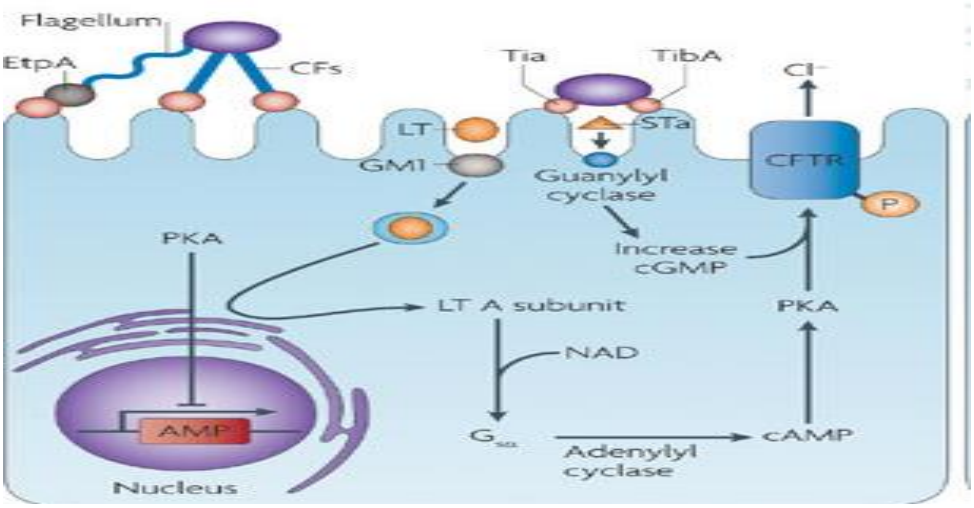

Figure 2.4 Molecular mechanism of ETEC pathogenicity.

(Adapted from Croxen & Finlay, 2010).

(iii) Clinical manifestations

The diarrhoeal disease caused by ETEC that was first recognized consisted of a cholera-like illness in both adults and children in Calcutta. Since then, many studies around the world have

shown that ETEC-induced diarrhoea may range from very mild to very severe. There are, however, short-term, asymptomatic carriers of the organisms (Black, 1993). Diarrhoea produced by ETEC is of the secretory type with a sudden onset of watery stool (without blood or inflammatory cells) and often vomiting, which leads to dehydration from the loss of fluids and electrolytes (sodium, potassium, chloride, and bicarbonate) in the stool (Sack, 1975; Black *et al*., 1981). The loss of fluids progressively results in a dry mouth, rapid pulse, lethargy, decreased skin turgor; decreased blood pressure, muscle cramps, and eventually shock in the most severe forms. The illness is typically abrupt in onset with a short incubation period (14 to 50 h). The diarrhoea is watery, usually without blood, mucus, or pus; fever and vomiting are present in a minority of patients. ETEC diarrhoea may be mild, brief, and self-limiting or may result in severe purging similar to that seen in *Vibrio cholera* infection. Most life-threatening cases of ETEC diarrhoea occur in weanling infants in the developing world (Todar, 1998).

(iv) Epidemiology

A large US nursery outbreak in 1974-75 was attributed to ST-producing ETEC (Ryder *et al*., 1976). Epidemiological studies conducted in different parts of Africa linked strains producing ST alone eliciting diarrhoea, nausea, vomiting and abdominal cramps in adult volunteers, which suggest that ST-producing ETEC are more strongly associated with childhood diarrhoea than are LT-producing strains, even though the latter may be more common overall (Shukry *et al*., 1986; Waiyaki *et al.,* 1986; Okeke, 2000; Steinsland *et al.,* 2002). The same may also be true for travelers' diarrhoea (Shaheen *et al*., 2003). Early studies identified ETEC by screening for toxigenic activity of isolates or with immunologic reagents that agglutinated the heat-stable or heat-labile enterotoxins. ETEC is one of the best documented and predominant causes of

diarrhea in travelers visiting African countries from Europe and North America (Black, 1990; Schults *et al*., 2000; Qadri *et al*., 2005).

From 1996 to 2003, 16 outbreaks of Enterotoxigenic *Escherichia coli* (ETEC) infections in the United States and on cruise ships were confirmed (Beaty *et al*., 2004). The most common symptoms reported by ill passengers were diarrhoea (99%), abdominal cramps (78%), nausea (63%), headache (47%), myalgia (39%), fever (27%), and vomiting (22%). A multifocal outbreak of diarrhoea caused by ETEC, involving 175 Israel soldiers and at least 54 civilians, occurred in the Golan Heights (Huerta *et al*., 2000). Similar reports implicated ETEC in infantile diarrheas in Kivu Province, Zaire, and Lagos, Nigeria (Stintzing, 1982; de Mol *et al*, 1983; Yala *et al.*, 1985).

In countries like Bangladesh and Egypt, the majority of cases of ETEC occur in children less than 2 years of age, and between 15 and 18% of children 3 years and younger experience ETEC-associated diarrhea episodes. ETEC is less prevalent in children 5 years and older, as well as in adults, because of natural immunity that develops following several episodes of the disease (Qadri *et al*., 2005). In Japan, large-scale outbreaks involving more than 500 patients per incident from 1997 to 2000 occurred. Two big outbreaks occurred again in 2010 and 2011 caused by ETEC O148:H28 (NIID, 2012).

(v) Detection and diagnosis

Diagnosis of ETEC is based on the production of LT and/or ST with the rabbit ileal loop and infant mouse physiological assays initially used as gold standards for the identification of these enterotoxins respectively. These tests are difficult to perform and time consuming and for a while efforts were made to use serotyping for this purpose, but soon it became clear that a large number of serotypes could be enterotoxigenic and therefore not applicable (Qadri *et al.*, 2005).

In 1974 it was found that LT produces morphological changes on Y1 adrenal and Chinese hamster ovarian cell lines that were neutralizable by antitoxin (Donta & Smith, 1974; Guerrant *et al.,* 1974). Although, these tissue culture tests were used in preference to the animal models, but these assays were only useful for LT detection and not available in all laboratories making ETEC detection problematic. Enzyme-linked immunosorbent assay (ELISA), passive latex agglutination, immune-precipitation in agar and Biken test were developed subsequently and were found to be specific (Yolken *et al.,* 1977; Honda *et al.,* 1981; Scotland *et al.,* 1989). PCR has revolutionized clinical diagnosis of pathogens and was used in 1994 for detection of ETEC strains (Schultsz *et al.,* 1994) but prior to the advent of PCR methods radioactively and non-radioactively labeled probes were used for detection of enterotoxin genes and the method was shown to be both sensitive and specific (Hill *et al.,* 1983; Echeverria *et al.,* 1984; Qadri *et al.,* 2005).

Different methods have been used for CF detection, including mannose-resistant agglutination of certain species of erythrocytes, serological tests initially using polyclonal sera (Evans *et al.,* 1977; Evans *et al.,* 1978; Ahren *et al.,* 1986) which were subsequently replaced by monoclonal antibodies and eventually molecular methods (Sommerfelt *et al.,* 1996). In 1992 however, both mannose-resistant haemagglutination and polyclonal antisera for CFA/I and CFA/II were used for detection of these antigens among Iranian ETEC isolates and it was concluded that haemagglutination was not specific enough for characterization of these fimbriae (Katouli *et al.,* 1992). Besides determination of the toxins and CFs, serotyping, i.e. determination of O serogroups associated with the cell wall lipopolysaccharides and H serogroups of the flagella, has been applied for identification and characterization of ETEC (Orskov *et al.,* 1978). However,

as shown in studies conducted in different countries, clinical ETEC isolates may belong to a large number of serotypes making this method unsuitable.

(vi) Treatment

Fluoroquinolones (ciprofloxacin, norfloxacin, and ofloxacin) are the most commonly recommended agents, since increasing antimicrobial resistance to traditional agents has been documented in several areas (Du Pont, 1995). Effective antimicrobials that have been used in treatment include doxycycline, trimethoprim-sulfamethoxazole, erythromycin, norfloxacin, ciprofloxacin, ofloxacin, azithromycin, and rifamycin (Ericsson, 2003). The non-absorbable antimicrobial rifaximin (Du Pont *et al.,* 2001) was effective in treating traveler's diarrhoea in adults, using 200 mg two times a day for 3 days. Resistance rates to previously effective drugs such as the tetracyclines, trimethoprim-suphamethoxazole and ampicillin have risen to 30-90% in some parts of the world (Lamikanra *et al.*, 1990; Mikhail *et al*., 1990; Sharp *et al*., 1995; Vila *et al*., 1999; Shaheen *et al*., 2003).

2.8.2 Enteropathogenic *Escherichia coli* (EPEC)

(i) History

Until the 1970s, serotyping was the only means of distinguishing EPEC strains from those of normal flora, since no biochemical, microbiological or animal experiments were available for their differentiation (Levine, 1987). The 12 serogroups originally recognized by the WHO as EPEC or the classical EPEC were; O26, O55¸ O86, O111, O114, O119, O125, O126, O127, O128, O142 and O158 (Hernandes *et al.,* 2009). Current classification of EPEC however, is based on the presence of specific virulence genes, which the use of molecular techniques has shown to be present in serotypes other than classical ones as well (Ochoa *et al.,* 2008). EPEC strains are said to be "moderately-invasive", meaning they are not as invasive as *Shigella,* and

unlike ETEC or EAEC, they cause an inflammatory response (Nataro & Kaper, 2004). Typical EPEC strains also carry a virulence plasmid, which bears genes encoding bundle-forming pili, the plasmid encoded regulator and other putative virulence genes (Todar, 1998).

(ii) Pathogenicity

The distinctive histopathology induced by EPEC is termed attaching and effacing (A/E) lesions and is caused by the intimate attachment of bacteria to the intestinal epithelial cells and effacement of enterocyte microvilli (Chen & Frankel, 2005). Formation of the micro ulcers and exfoliation of the cells at the site of EPEC attachment was first described in experimentally infected pigs (Staley *et al.,* 1969) and subsequently in biopsies from infected infants (Chen & Frankel, 2005). A protein called intimin mediates the bacterial attachment to outer cell membranes and is encoded by *eae* gene which along with all other genetic elements required for this phenomenon are located on the locus of enterocyte effacement (LEE), a large genomic pathogenicity island which was discovered in 1995 (McDaniel *et al.,* 1995). The *eae* is one of the genes currently used for the molecular diagnosis of EPEC. Pathogenesis of these bacteria however is many faceted which has not been fully unraveled as yet and may involve factors other than those directly responsible for A/E lesions as well as more specialized intestinal cells (Cravioto *et al.*, 1979; Nataro & Kaper, 1998). The protein intimin (*eae*A), necessary for the A/E lesion, has been used for the molecular identification of EPEC. The current average prevalence of EPEC in pediatric diarrhoeal episodes in developing countries, using molecular methods, is 5%–10% (Francesca *et al.*, 2011).

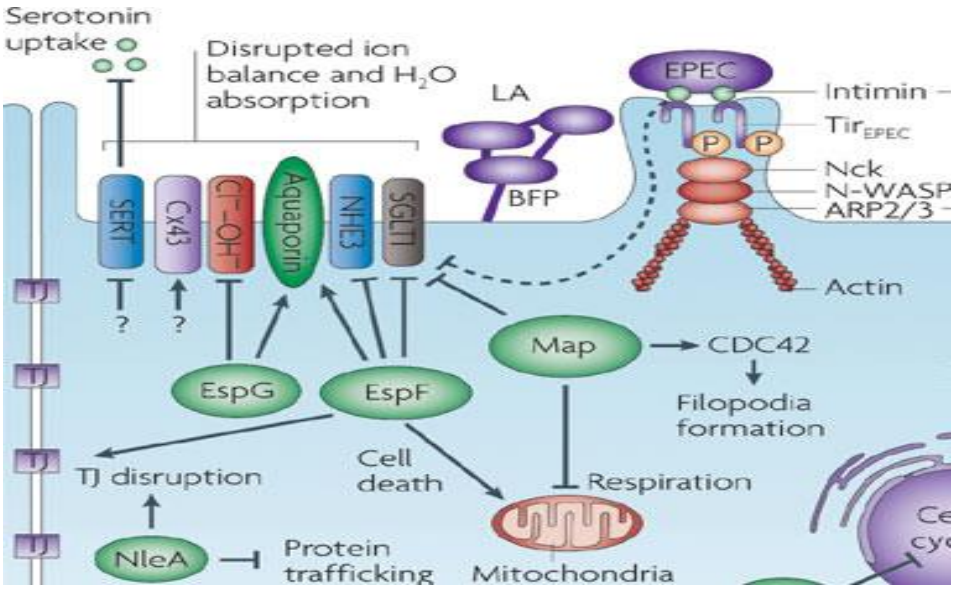

Figure 2.5 Molecular mechanism of EPEC pathogenicity.

(Adapted from Croxen & Finlay, 2010).

(iii) Clinical manifestations

EPEC causes watery diarrhoea that may contain mucus but typically does not have blood in it. Vomiting, fever, malaise and dehydration are equally associated. The symptoms may last a brief period of several days, although instances of long, chronic EPEC disease have also been noted. EPEC induces profuse watery, sometimes bloody diarrhoea. They are a leading cause of infantile diarrhoea in developing countries (Todar, 1998). Faecal leukocytes are observed occasionally, but more sensitive tests for inflammatory diarrhoea such as an anti-lactoferrin latex bead agglutination test are frequently positive with EPEC infection (Miller *et al*., 1994).

(iv) Epidemiology

In Brazil, EPEC has been isolated from stools of over 40% of infants with acute diarrhoea and was associated with a mortality of 7% (Fagundes-Neto & Scaletsky, 2000). Common-source community outbreaks are rare in geographic areas with satisfactory sanitation. However,

sporadic cases are seen in the United States, Canada, and Europe, and outbreaks occur in these areas, but most commonly in close-contact institutions such as hospital nurseries, day-care centers, and nursing homes. EPEC once caused frequent outbreaks of infant diarrhoea in the US and the UK (Robins-Brown, 1987). These community-acquired and nosocomial outbreaks were often explosive, with up to 50% mortality (Robins-Brown *et al*., 1982; Levine & Edelman, 1984).

EPEC strains are no longer as important a cause of diarrhoea in developed countries as they were in the 1940s and 1950s; However, several outbreaks of diarrhoea due to EPEC have been reported in the last two decades in the US, the UK, Finland and other developed countries. These outbreaks frequently occur in day care centers (Paulozi *et al*., 1986; Bower *et al*., 1989) and occasionally occur in paediatric wards (Bower *et al*., 1989). An outbreak due to atypical EPEC was recently reported among adults who ate at a gourmet buffet in Minnesota (Hedberg *et al*., 1994). However, EPEC strains are also associated with sporadic cases of diarrhoea in the United States and other developed countries (Levine & Edelman, 1984; Sherman *et al*., 1989; Bokete *et al*., 1997).

Numerous case-control studies on six continents found EPEC to be more frequently isolated from infants with diarrhoea than from matched healthy controls particularly in the 0- to 6-month age group. Studies in Brazil (Gomes *et al*., 1989), Mexico (Cravioto *et al*., 1988), and South Africa have shown that 30 to 40% of infant diarrhoea can be attributed to EPEC, and in some studies, EPEC infection exceeds rotavirus infection in incidence (Cravioto *et al*., 1988; Gomes *et al*., 1989). EPEC strains are an important cause of disease in all settings: nosocomial outbreaks, outpatient clinics, patients admitted to hospitals, community-based longitudinal studies, and urban and rural settings (Okeke, 2009).

(v) Detection and diagnosis

Originally, HEp-2 cell-adherence assay performed with serologically defined EPEC strains showed that 80% of these strains adhere to HEp-2 cells in vitro (Cravioto *et al.,* 1979). The HEp-2 assay has been modified often since its first description, including such variations as extending the incubation time to 6 h or changing the growth medium during the incubation. However, collaborative studies have shown that the assay performed essentially as first described provides the best ability to differentiate among EPEC, EAEC and DAEC isolates (Nataro & Kaper, 1998). After the introduction of the term "attaching and effacing" actin accumulation under the attached bacteria was demonstrated using *ex vivo* culturing of human intestinal biopsies (Knutton *et al.,* 1987). Staining this electron dense material produced the actin fluorescent assay (FAS) which enabled researchers to detect the ability of a strain to produce A/E lesions in vitro (Knutton *et al.,* 1989). It should however be noted that a negative FAS result may depend on the cell type used and the bacteria should be confirmed as nonpathogenic by alternative methods (Hernandes *et al.,* 2009).

The localized adherence pattern of EPEC strains was shown to be associated with the presence of a 60 MDa plasmid called pMAR2 from which a DNA fragment of 1 kb was isolated which has been used extensively in epidemiological studies (Nataro *et al.,* 1985; Levine *et al.,* 1988; Bouzari *et al.,* 2000). The presence of the *E. coli* adherence factor (EAF) plasmid carrying *bfp* operon, encoding the type IV bundle-forming pilus (BFP), and *per* operons, a transcriptional activator called plasmid encoded regulator (Per) is the basis of typical and atypical classification of EPEC strains (Trabulsi *et al*., 2002). All EPEC strains lack genes encoding Shiga toxin (*stx*) although they share A/E phenotype with some other strains of *E. coli*, therefore, strains that are *eae*+ *bfp*A+ *stx*- are classified as typical EPEC (tEPEC). Production of BFP protein induces the

localized adherence pattern (LA) and most of tEPEC strains belong to classic O:H serotypes (Trabulsi *et al.*, 2002).

On the other hand, atypical EPEC (aEPEC), are of *eae*+*bfp*A-*stx*- genetic background and display localized-like (LLA), diffuse (DA), or aggregative adherence patterns which is associated with the *E. coli* common pilus and other known adhesins (Scaletsky *et al.*, 2010). Most of the over 200 O-serogroups that have been identified among aEPEC strains, do not belong to classical EPEC serogroups and many have been designated non-typeable (Schmidt, 2010). Recently in a study done in Iran, multiplex PCR was used to differentiate between tEPEC, and aEPEC and PCR-RFLP for H typing of conventionally serogrouped isolates (Bouzari *et al.*, 2011) showing the ease and applicability of this method for rapid screening of large number of isolates.

(vi) Treatment

Broad-spectrum antibiotics are recommended in chronic and/or life-threatening cases. Fortunately, EPEC diarrhoea is usually self-limited and rehydration is the most effective treatment (Kandakai-Olukemi *et al.*, 2009).

2.8.3 Enterohaemorrhagic/Shiga toxin-producing *Escherichia coli* (EHEC/ STEC)

(i) History

The term enterohaemorrhagic *E. coli* (EHEC) is applied to those STEC serotypes that have the same clinical, epidemiological and pathogenetic features associated with the prototype strain *E. coli* O157:H7 (Jafari *et al.*, 2012). EHEC bacteria were first discovered in 1977 by the production of cytotoxin, verotoxin (VT), lethal to Vero (African green monkey) cells, which led to these pathogens being called verocytotoxigenic *E. coli* (VTEC) (Konowalchuk *et al.*,1977).

The main virulence factor and the defining feature of this group is a phage-encoded potent cytotoxin the effect of which was shown to be neutralizable by anti-Shiga toxin of *Shigella dysenteriae*. The cell toxicity effect was also demonstrated on vero cells resulting in a parallel nomenclature system of shiga/vero toxin-producing *E.coli* (STEC) and (VTEC) respectively (Nataro & Kaper, 1998; Karmali *et al.*, 2010).

In 1983, an *E. coli* strain serotype O157:H7, was identified in association with outbreaks of a bloody diarrhoea called haemorrhagic colitis (HC) leading to the recognition of EHEC as a new and increasingly important class of enteric pathogens causing intestinal and renal disease (Nataro & Kaper, 1998). The high virulence of STEC strains such as O157:H7 is not only dependent on the virulence factors but partially also on the pathogen's ability to survive environmental stress conditions, such as resistance to low pH levels found in the gastrointestinal tract contributing to its very low infectious dose of 50-100 bacteria or lower (Viazis & Diez-Gonzalez, 2011). Among STEC serotypes, O157:H7 is associated with both outbreaks and sporadic cases of severe disease, but it has been shown that other serotypes may also cause human infections albeit variably (Coombs *et al.,* 2011). This quantitative and qualitative difference in disease association among STEC has given rise to various classification schemes the simplest of which divides STEC into *E. coli* O157 and non-O157. However, in view of the fact that the virulence potential of non-O157 might be genetically determined a seropathotype (SPT) classification has been proposed in which prior association with human epidemics, HUS, and diarrhoea is considered (Coombs *et al.*, 2011).

In this scheme, SPT-A includes O157:H7 and O157:NM, the most commonly isolated serotypes from outbreaks and HUS. SPT-B strains differ from group A in the frequency of isolation from outbreaks and HUS cases, SPT-C strains are only associated with sporadic cases of HUS, SPT-D

are isolated from diarrhoeal cases and have not been encountered in outbreaks or HUS and SPT-E that have never been associated with human disease (Karmali *et al.,* 2003). Furthermore, data collected using different methods of comparative genomic have suggested that several discreet genotypes differing in virulence exist within *E. coli* O157:H7 population and based on these data this serotype has been subdivided into nine clades (Zhang *et al.,* 2007; Manning *et al.,* 2008).

In 1982, the O157:H7 strain of *E. coli* was first recognized during an outbreak of haemorrhagic diarrhoea and colitis in the United States (Nataro & Kaper, 2004). Strain serotype O157:H7 of the enterohaemorrhagic *E. coli* (EHEC) has since then been associated with haemorrhagic diarrhoea, colitis and haemolytic uremic syndrome (HUS). HUS is characterized by low platelet count, haemolytic anemia, and kidney failure. Recently, a new shiga toxin producing *E. coli* strain was identified in Germany causing one of the largest outbreaks of HUS worldwide. The perpetrator belonged to serotype O104:H4 which contained the virulence factors of typical EAEC and a *stx*2 producing prophage, but lacked the LEE pathogenicity island. This discovery has led to the emergence of a new pathotype for which the name Entero-aggregative-haemorrhagic *Escherichia coli* (EAHEC) has been suggested (Brzuszkiewicz *et al.,* 2011; Wu *et al.,* 2011). This event exemplifying the genome plasticity of *E. coli* has highlighted the need for public health surveillance of STEC infections and its important role in devising and implementing control measures.

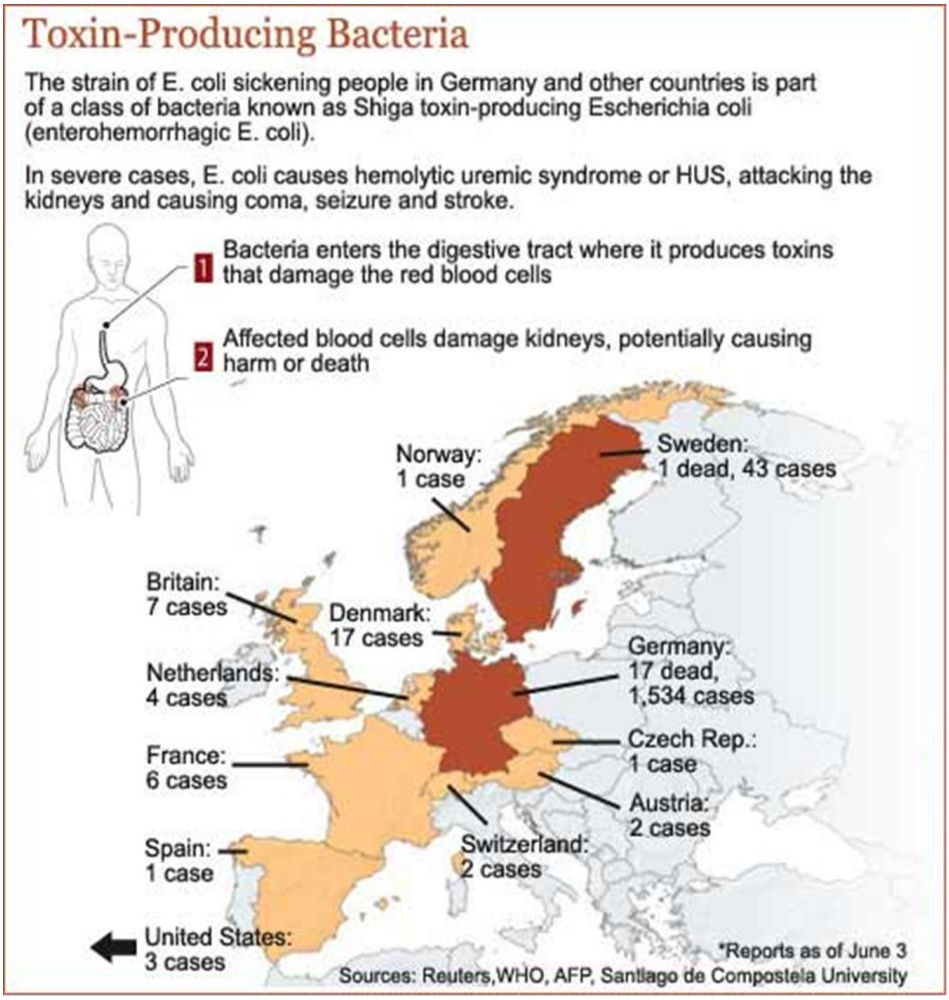

Figure 2.6 Outbreak of diarrhoea in Europe and US.

(Source: WHO, 2011).

(ii) Pathogenicity

Shiga toxin family with related structure and similar biological activity is composed of *stx*1 which is essentially identical to the toxin of *Shigella dysenteriae* differing in a single amino acid and *stx*2 with less than 60% amino acid homology to *stx*1 (Caprioli *et al.*, 2005; Beddoe *et al.*, 2010). Little sequence variation has been reported for *stx*1 (Zhang *et al.*, 2002), but *stx*2 has several subtypes which differ in biological activity and immunological reactivity (Fraser *et al.*, 2004). Shiga toxins similar to the heat-labile enterotoxin of ETEC belong to the AB5 family of

the toxins and consist of a pentameric ring-shaped B subunit that is non-covalently attached to the A subunit. The B subunit interacts with globotriaosylceramides (Gb3s) on the surface of human intestinal mucosa and kidney epithelial cells resulting in the internalization of the toxin where the A subunit is activated causing cell death (Croxen & Finlay, 2010).

Among the *stx*2 variants, *stx*2c has been isolated more frequently from HUS patients but *stx*2e and *stx*2f have been mainly isolated from pigs and birds and rarely from humans (Caprioli *et al.,* 2005). Moreover, a different AB5 toxin has been discovered in this group which differs significantly from other toxins in this group. This subtilase-like toxin (SubAB) was isolated from an HUS outbreak strain in Australia and shows greater cytotoxicity than *stx*2 for a range of cell types including Vero cells (Beddoe *et al.,* 2010). The EHEC genome contains the same locus of enterocyte effacement (LEE) as the EPECs and the intimate attachment of EHEC to host cells occur through interaction between an adhesin called intimin (eaeA), and Tir (translocated intimin receptor).

This intimate attachment induces the characteristic attaching and effacing lesions (A/E), but the initial adherence of EHEC to colonocytes is not well-defined (Caprioli *et al.,* 2005; Johnson & Nolan, 2009; Croxen & Finlay, 2010; Viazis & Diez-Gonzalez, 2011). Sixteen potential fimbria-like operons, which have not been extensively studied have been recognized in STEC strains (Low *et al.,* 2006; Croxen & Finlay, 2010), and recently a pilus involved in adherence and biofilm formation called haemorrhagic coli pilus, a type IV pilus, has also been identified in STECs (Xicohtencatl-Cortes *et al.,* 2009). However, the intimate adherence as in EPECs is through the interactions between Tir and intimin. At least 29 distinct intimin types with heterogeneity in the C-terminal part of the molecule that is involved in binding to Tir in both STEC and EPEC have so far been identified (Gyles, 2007; Mora *et al.,* 2009). The ability of

STEC to produce A/E lesions is sufficient to cause non-bloody diarrhoea but Shiga toxin is essential for the development of bloody diarrhoea, HC, and HUC (Savarino *et al.,* 1996; Nataro & Kaper, 1998).

Another toxin found in many STEC/EHEC isolates is the enteroaggregative heat-stable enterotoxin1 (EAST1) and usually two copies of the *ast*A gene is present in the chromosome (Nataro & Kaper, 1998; Viazis *et al.,* 2011). The significance of this carriage in the pathogenesis of EHEC is unclear, but it has been suggested that some of the non-bloody diarrhoea in person infected with these strains might be due to the production of this toxin (Nataro & Kaper, 1998; Mora *et al.,* 2009). The primary virulence determinants of EHEC strains are chromosomally encoded, but plasmids might play an important role in the pathogenesis of EHEC strains.

Plasmid pO157 is found in 99-100% of O157:H7 serotype isolated from human clinical isolates, most not all *stx*-producing isolates. Presence of this plasmid has been correlated with hemolytic activity and adherence to intestinal epithelial cells, but the overall understanding of the role of plasmids in pathogenesis of STEC/EHEC strains is hindered by the absence of a reliable model of human infection (Johnson & Nolan, 2009; Lim *et al.,* 2010a; 2010b). Shiga toxin-producing *Escherichia coli* (STEC) represent a large, diverse group of bacteria characterized by the production of Shiga toxins (stx). There are two main stx types, designated *stx*1 and *stx*2 and within each are many subtypes. There are hundreds of known STEC serotypes that can produce any of the stx types or combination of subtypes. However, the production of stx alone is deemed to be insufficient to cause severe human illness. Also, some stx subtypes are produced mostly by environmental or animal strains and have not affected humans, so, not all STEC strains appear to be human pathogens (Martin & Beutin, 2011; Hofer *et al.,* 2012).

In contrast, enterohaemorrhagic *E. coli* (EHEC) is a pathogenic subset of STEC strains that carry other virulence factors. Most notable of these is the intimin protein that enables EHEC to attach to epithelial cells. Intimin is encoded by the *eae* gene that resides on a pathogenicity island called locus of enterocyte effacement (LEE). The presence of *eae* and *stx*2 is a reliable predictor that the STEC strain may cause severe illness such as haemorrhagic colitis (HC) or hemolytic-uremic syndrome (HUS) (Ethelberg *et al.,* 2004). The enterohaemorrhagic *E. coli* strain is the major cause of sporadic outbreaks of haemorrhagic colitis (Gerber *et al.*, 2002).

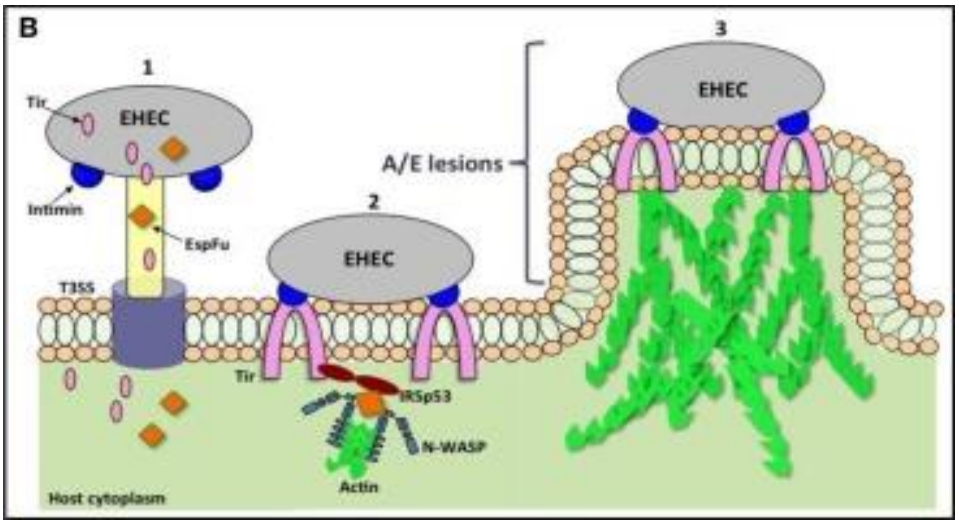

Figure 2.7 Molecular mechanism of EHEC/STEC pathogenicity.

(Adapted from Nguyen and Sperandio, 2012).

(iii) Clinical manifestations

EHEC typically cause an afebrile bloody colitis and, in about 10% of patients, this infection can be followed by HUS (Pickering *et al*., 1994). They are involved in episodes of diarrhoea with complications. Serotype O157:H7 is the prototype of increasing importance and is associated with haemorrhagic colitis, bloody diarrhoea and the haemolytic uremic syndrome (HUS). HUS involves a triad of haemolytic anaemia, thrombocytopenia and renal failure. EHEC cause disease

of the large intestine that may present as simple watery diarrhoea and then progress to bloody stools with ulcerations of the bowel. The incubation period of EHEC diarrhoea is usually 3 to 4 days, although incubation times as long as 5 to 8 days or as short as 1 to 2 days have been described in some outbreaks. The initial complaint is usually non bloody diarrhoea, although this is preceded by abdominal pain and a short-lived fever in many patients. Vomiting occurs in about half of the patients during the period of nobody diarrhoea and/or at other times in the illness. Within 1 or 2 days, the diarrhoea becomes bloody and the patient experiences increased abdominal pain. This stage usually lasts between 4 and 10 days. In severe cases, faecal specimens are described as "all blood and no stool" (WHO, 2011). While the kidney is the organ most commonly affected in HUS, evidence of central nervous system, pancreatic, skeletal and myocardial involvement may also be present (Richardson *et al.*, 1988; Siegler, 1994; Sebbag *et al.*, 1999).

(iv) Epidemiology

Germanii *et al.* (1996) described an EHEC outbreak in Central African Republic. Outbreak of pathogenic *E. coli* have been reported in Canada, in occurred in Canada where an estimated 2300 people became ill and 7 died from exposure to EHEC contaminated drinking water and a recreational water outbreak in 2001 at a beach in Montreal, Quebec, resulted in the hospitalization of 4 children (Bruneau, 2004). A more recent outbreak due to pathogenic *E. coli* occurred during May–June 2011, an outbreak of bloody diarrhoea and haemolytic uremic syndrome occurred in Germany and other parts of Europe (Struelens *et al*., 2011). The shiga toxin–producing *E. coli* (STEC) serotype O104 strain was the cause of this outbreak that caused > 4,000 cases and 50 deaths. This outbreak strain showed an unusual combination of virulence factor of STEC and enteroaggregative *E. coli* (EAggEC) (WHO, 2011). Since the first EHEC

O157:H7 outbreak in the USA in the early 1980s, there has been a dramatic increase in the number of reported infections caused by this O group in the developed countries (Nataro & Kaper, 1998).

Also, outbreaks with hundreds of cases of EHEC infection have been reported from the United Kingdom (Gillespie *et al*., 2005). In United States, 2006: More than 200 people in 26 states were infected with *E coli* O157:H7. Thirty-one cases of HUS with three fatalities occurred (CDC, 2004). In the U.S., the Centers for Disease Control and Prevention (CDC) estimates that EHEC O157:H7 causes approximately 73,000 illnesses, 2,000 hospitalizations, and 50-60 deaths annually. Another recent outbreak due to *E. coli* O157:H7 occurred in Japan, five people were killed and more than 100 were sickened in the Hokkaido area outbreak (Japan Times, 2010). In addition, in Scotland severe, fatal outbreaks with 21 deaths among over 400 individuals infected with the *E. coli* O157:H7 have occurred (Ahmed & Donaghy, 1998). Moreover, an international outbreak caused by EHEC O157 occurred among tourists belonging to five nationalities visiting Spain (Pebody *et al*., 1999). The first documentation of EHEC in Africa was a sporadic case of haemorrhagic uraemic syndrome caused by EHEC O157:H7 and reported from South Africa in 1990 (Browning *et al*., 1990). Three years later, a South African laboratory described one of the largest EHEC outbreaks in the world, which began on a sugar plantation in Swaziland, and resulted in approximately 2,000 deaths (Isaacson *et al*., 1993). An outbreak of O111 EHEC occurred in Nigeria in 1998 (Okeke *et al*., 2003).

Table 2.2 Outbreak of diarrhoea in Europe and US.

Number of cases reported to the WHO as for 21 July 2011[10]			
Country	Deaths	HUS cases	Non-HUS cases
Austria	0	1	4
Canada	0	0	1
Czech Republic	0	0	1
Denmark	0	10	15
France	0	7	10
Germany	48	857	3078
Greece	0	0	1
Luxembourg	0	1	1
Netherlands	0	4	7
Norway	0	0	1
Poland	0	2	1
Spain	0	1	1
Sweden	1	18	35
Switzerland	0	5	0
United Kingdom	0	3	4
United States	1	4	2
Total	**50**	**908**	**3,167**

Enterohaemorrhagic *E. coli* have been associated with several large outbreaks in US, Canada, Europe and Japan (Caprioli *et al.*, 2005; Viazis *et al.*, 2011). However, detection of these bacteria requires an array of different tests and cultures, a combination of molecular and classic methods. Therefore any identification relying on a single method should be considered with caution. Moreover, serotyping for O and H determination especially interpretation of H serology results requires expertise and trained personnel and in view of the fact that so far no epidemics or large outbreaks for this organism have occurred, isolation reports should be assessed more critically.

(v) Detection and diagnosis

Laboratory confirmation of STEC infection can be achieved by isolation and confirmatory tests using culture media, immunoassays, cell toxicity assays and PCR (Gould *et al.,* 2009). Screening of O157 relies on the strain's inability to utilize sorbitol rapidly, leading to the use of sorbitol-MacConkey agar (SMAC) as a differential medium with added cefixime and tellurite (CT-SMAC) although in our setting addition of cefixime has not led to the prevention of other faecal-associated microorganisms. More specific media have also been developed such as Rainbow agar, CHROMagar and O157:H ID agar that are able to recover O157 along with sorbitol-fermenting O157 and non-O157 strains (Gould *et al.,* 2009; Karmali *et al.,* 2010).

The identity of potential STEC isolates should be assessed by serotyping and Shiga toxins detection methods. Cell toxicity assay using Vero and HeLa cell lines for Shiga toxin in stool samples or broth enrichment is a very sensitive method since these cell lines have high concentrations of globotriaosylceramides Gb3 and Gb4 which are the receptors for shiga toxin. Neutralization tests using antibodies against *stx*1 and *stx*2 confirms the results obtained, but this test despite high sensitivity is not routinely used due to its high cost, labor intensity and the expertise required. PCR however offers a fast and reliable method for detection of STEC which similar to immunoassay tests can be used directly with stool samples as well as isolated colonies and depending on the primers used can distinguish between *stx*1 and *stx*2 and detect *eae* and enterohemolysin (*hly*) genes (Jafari *et al.,* 2012).

Use of PCR on DNA extracted from whole stool however, is not recommended because of the low sensitivity (Gould *et al.,* 2009). STECs are the only zoonotic *E. coli* pathotype and more than 380 different OH serotypes have now been isolated from humans with gastrointestinal disease and many of these as well as others have been recovered from animals. However,

majority of human disease appear to be caused by a limited number of serotypes with frequency varying depending on the location and the year. Serogroups O26, O45, O91, O103, O111, O113, O121, O145 are listed as the most commonly encountered non-O157 STEC-associated O antigens (Gould *et al.,* 2009; Karmali *et al.,* 2010; Coombs *et al.,* 2011).

(vi) Treatment

While there is controversy about the use of antibiotics, the use of antimotility (antidiarrhoeal) such as loperamide agents in hemorrhagic colitis also seems to increase the risk for developing HUS is definitely not indicated in the management of disease due to EHEC. Treatment of renal disease due to EHEC is primarily supportive, except for some experimental therapies currently being evaluated in clinical trials. Patients with complications may require intensive care including dialysis, transfusion and/or platelet infusion. Patients who develop irreversible kidney failure may need a renal transplant (Armstrong *et al.,* 2006). Vaccination and pre-vaccination have been shown to protect against *E coli* O157:H7 Shiga toxin 2 in a mouse model. Administration of exogenous SAP to patients who have EHEC and HUS may offer potential benefit, but efficacy in humans must be tested in clinical trials (Armstrong *et al.,* 2006).

2.8.4. Enteroinvasive *Escherichia coli* (EIEC)

(i) History

Bacillary dysentery as opposed to dysentery caused by amoeba was described in 1887 and *Bacillus dysenteriae* as the causal agent was described in 1898 by Shiga during an epidemic of 89,400 cases (Peng *et al.,* 2009). The medical importance of *Shigella* strains led to their separation from *E. coli* and the newly formed genus with its 4 species could be differentiated from *E. coli* on the basis of physiological and biochemical characteristics. However, the discovery of strains which could cause dysentery and were intermediate between *Shigella* and *E.*

coli in biochemical characteristic in 1944 caused the separation of the two genera to be questioned (Van den Beld & Reubsaet, 2012). The ability of these strains which by now were called enteroinvasive *E. coli* (EIEC) to cause diarrhea was demonstrated in volunteer studies in 1971 (Nataro & Kaper, 1998).

It has been shown that EIEC strains and *Shigella* species are biochemically, genetically and pathogenetically very closely related so much so that it has been proposed that they should be classified as one species in genus *Escherichia* (Lan *et al.,* 2004). Like *Shigella* spp, EIEC strains are generally lysine decarboxylase negative, non-motile and 70% are unable to ferment lactose. In fatty acid analysis, *Shigella* and *E. coli* form one cluster with other genera of the family Enterobacteriaceae, therefore it is difficult to distinguish from *Shigella* species (van Der Beld &Reubsaet, 2012).

(ii) Pathogenicity

Acquisition of the invasive plasmid (pINV) encoding the ability to invade host tissues (Harris *et al.,* 1982; Hale *et al.,* 1983) is probably the single most important event that has probably given rise to the evolution of both *Shigella* and EIEC from non-pathogenic *E. coli.* Nearly one third of this large single copy plasmid encodes IS elements and contains a 30 kb region enabling the bacteria to invade intestinal epithelial cells (Parsot, 2005). Many components of type three secretion system (T3SS) such as translocators, transcriptional activators, some effectors and chaperones are coded by this region with the expression of the coded genes being delimited globally by VirB and MxiE (Johnson & Nolan, 2009).

In addition to the genes of pINV many chromosomal genes which are not specific to *Shigella* spp. and are carried on the chromosome are required for pathogenesis (Parsot, 2005). Colonic

mucosa is the infection site of *Shigella* and EIEC where invasion of M cells, macrophages and epithelial cells occur resulting in watery diarrhoea, which in severe cases may be followed by the onset of scanty dysenteric stools containing blood and mucus (Sansonetti *et al.*, 1992). EIEC strains may also produce a 63 kDa toxin designated Sen which contributes to the enterotoxic activity detected in the strains carrying the gene (Nataro *et al.*, 1995).

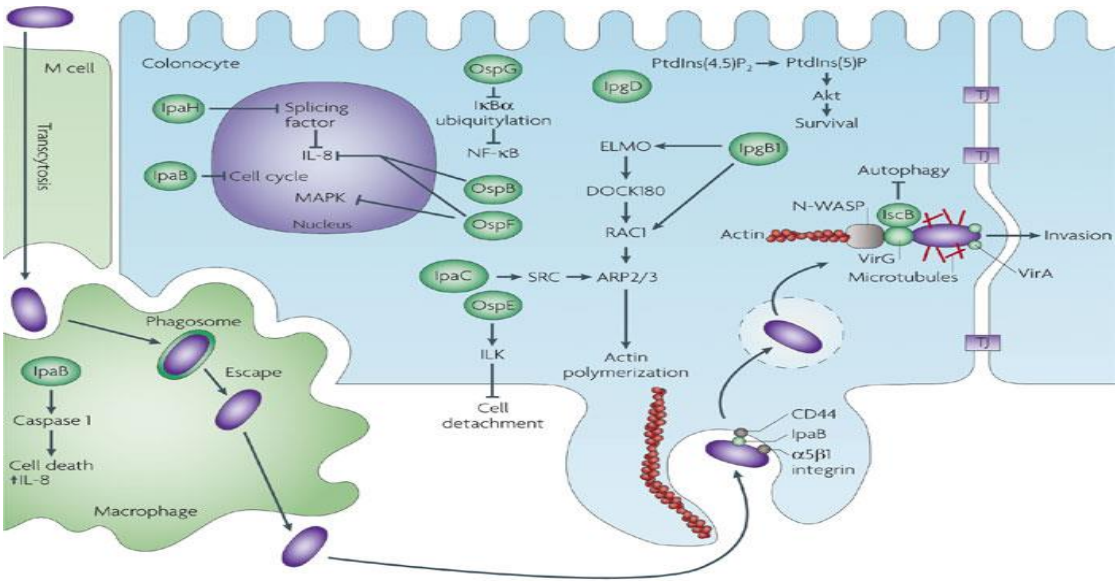

Figure 2. 8 Molecular mechanisms of EIEC pathogenicity.

(Adapted from Croxen & Finlay, 2010).

(iii) Clinical manifestations

The clinical syndrome of EIEC is identical to that of *Shigella* dysentery. It includes a dysentery like diarrhoea with fever (Todar, 1998). EIEC can also cause a broad spectrum of human diseases including an invasive inflammatory colitis. It may also induce a watery diarrhoea syndrome indistinguishable from the secretory diarrhoea seen with ETEC. Only a minority of patients experience the appearance of blood and mucus in the stools (Nataro & Kaper, 1998).

(iv) Epidemiology

No epidemics and recent reports of outbreaks caused by EIEC is found in literature. The available literatures on documented EIEC outbreaks are usually foodborne or waterborne. The data dates back as early as early as 1973 (Tulloch *et al*., 1973). In another recorded EIEC outbreak, Twenty-eight of 37 people developed acute dysentery in from 24 to 48 hours after eating contaminated, imported French Camembert cheese. An invasive strain of *E. coli* (ONT: NM) was isolated from stool specimens from 7 of 10 ill passengers who developed diarrhoea during a 5-day ocean cruise (Snyder *et al*., 1984). A large diarrhoea outbreak due to enteroinvasive *E. coli* (EIEC) serogroup O143 occurred in Houston, Texas (Gordillo *et al*., 1992). In Africa, the epidemiology of EIEC is not thouroughly investigated (Okeke, 2009).

(v) Detection and diagnosis

There are very few biochemical characteristics that differentiate *Shigella* and EIEC from each other and the two most convenient are mucate and acetate tests. EIEC may be positive for either or both, whereas with rare exceptions *Shigella* strains are negative for both (Lan *et al.,* 2004). Salicin fermentation and esculin hydrolysis have also been used to differentiate the two groups (Van den Beld & Reubsaet, 2012). The ability to cause kerato conjunctivitis in guinea pig eyes and to form plaques in HeLa cell monolayers were the standard methods of identification for EIEC isolates. However, molecular methods have replaced these phenotypic assays (Knutton *et al.,* 1989) including amplification of a multicopy gene (4-10 copies) called *ipa*H with copies located on both plasmid and chromosome (Venkatesan *et al.,* 1989; Hartman *et al.,* 1990; Luscher & Altwegg, 1994).

PCR for detection of EIEC is based on the detection of the *ial* and *ipaH* genes (Frankel *et al*., 1990). This assay distinguishes EIEC and *Shigella* from other diarrhoeal pathogens and further

efforts have been made to develop molecular methods to discriminate between the two microorganisms resulting in the development of conventional, multiplex as well as real-time PCR methods for this purpose (Horakova *et al.,* 2008; Pavlovic *et al.,* 2011; Yamazaki & Fukasawa, 2011).

(vi) Treatment

Quinolones are the drugs of choice for the treatment of EIEC (Gassama-Sow *et al*., 2004). In a study conducted by Prado and colleagues, TMP-SMX was found to be effective in the diarrhoea caused by *Shigella* and enteroinvasive *E. coli* in children (Prado *et al*., 1992). In another study, EIEC O164 strain designated " RIMD05091045 " isolated from a travelling patient in Japan, showed multidrug resistance against streptomycin, spectinomycin, co-trimoxazole (trimethoprim/sulfamethoxazole) and ampicillin, and reduced susceptibility to ciprofloxacin (Ahmed & Shimamoto, 2004).

2.8.5. Enteroaggregative *Escherichia coli* (EAEC)

(i) History

EAEC was first described in 1985 and recognized by its distinctive adherence to HEp-2 cells in an aggregative, " stacked brick-like " pattern (Nataro *et al.,* 1998; Pereira *et al*., 2008). This adherence pattern, distinguishable from the adherence patterns manifested by EPEC and DAEC, was first significantly associated with diarrhoea among Chilean children in 1987 (Nataro *et al*., 1987). This pathotype is the most recently identified diarrhoeagenic *E. coli* and is the second most common cause of travelers' diarrhoea after ETEC in both developed and developing countries. EAEC are commonly being recognized as a cause of endemic and epidemic diarrhoea worldwide and recently, has been shown to cause acute diarrhoeal illness in newborns and children in industrialized countries. This organism has also been associated with persistent

diarrhoea. Diarrhoea caused by EAEC is often watery, but it can be accompanied by mucus or blood; (Harrington *et al.,* 2006; Weintraub, 2007; Croxen & Finlay, 2010; Kauer *et al.,* 2010).

The discovery of EAEC as well as diffusely adherent *E. coli* (DAEC) stemmed from the studies showing that EPEC adhere to HEp-2 cells in a distinctive pattern (Cravioto *et al.,* 1979). Examination of a collection of diarrhoeal *E. coli* strains that were not of EPEC serogroups showed that many of these strains also adhered to HEp-2 cells and the phenotype was different from that of EPEC (Scaletsky *et al.,* 1984; Nataro *et al.,* 1985). This pattern of adherence, which had been called ” diffuse ” was subsequently subdivided into aggregative and true diffuse adherence (Nataro *et al.,* 1987). *E. coli* showing aggregative adherence (AA) are auto-agglutinating, but their hallmark is aggregative adhesion, which involves the formation of a stacked-brick pattern on HEp-2 cells. EAEC is also considered as a ‘potential’ bioterrorism agent by the USA National Institutes of Health (Huang & DuPont, 2004). EAEC exert a complex pathogen-host immune interaction where the host inflammatory response to EAEC infection is dependent on the host innate immune system and the EAEC strain (Kaur *et al*., 2010).

(ii) Pathogenicity

Lack of suitable animal models and the heterogeneity of virulence factors caused the paucity of details regarding the EAEC transmission, pathogenicity and epidemiology. However, colonization of intestinal mucosa, mucoid biofilm formation and elaboration of various enterotoxins, cytotoxins and mucosal inflammation are considered the major features of EAEC pathogenesis (Bouzari *et al.,* 1994; Harrington *et al.,* 2006; Weintraub, 2007; Boisen *et al.,* 2009; Croxen & Finlay, 2010; Kauer *et al.,* 2010). Colonization of intestinal mucosa by the EAEC occurs via aggregative adherence fimbriae (AAF) encoded by a 55-65 MDa plasmid named pAA. The first one of which, aggregative adherence fimbriae I (AAF/I), was cloned and

characterized from EAEC prototype strain 17-2 (Nataro e*t al.*, 1992; Harrington *et al.,* 2006). A probe derived from this adhesin did not recognize O42, the second EAEC prototype and subsequently a new fimbria was characterized in this strain called AAF/II (Czeczulin *et al.,* 1997).

Although two other adherence factors (AAF/III and AAF/IV) as well as a non-fimbrial adhesin have been described but some strains are encountered that do not contain any of these known fimbriae despite showing AA phenotype which is indicative of the as yet uncharacterized adhesins (Bernier *et al.,* 2002; Monteiro-Neto *et al.,* 2003; Boisen *et al.,* 2008). Similar to ETEC strains adhesion of EAEC to intestinal tissue is mediated by antigenically heterogenous adhesins and multiple carriage of AAFs by an EAEC strain has been rare (Bouzari *et al.,* 2005; Aslani *et al.,* 2011). A transcriptional activator known as " AggR " encoded by pAAs, regulates the biogenesis of AAFs (Nataro *et al.,* 1994) and is the major EAEC virulence regulator controlling diverse virulence genes encoded by pAAs as well as by chromosomes (Harrington *et al.,* 2006; Kauer *et al.,* 2010). Adherence of EAEC to the mucosa is characterized by the formation of a thick, aggregating mucus layer inside which they survive and this biofilm production has been attributed to the activity of *fis* and *yafK* genes (Sheikh *et al.,* 2001; Huang *et al.,* 2006). However, a secreted 10 kDa protein encoded by pAA and called antiaggregation protein (Aap) or dispersin, facilitates the movement of bacteria across the surface of the cells for subsequent aggregation and adherence (Sheikh *et al.,* 2002; Johnson & Nolan, 2009).

Dispersin is highly immunogenic and is translocated via an ATP binding cassette (ABC) transporter complex (the Aat apparatus) (Nishi *et al.,* 2003). Both these genes have been used for

identification and classification of EAEC isolates, but it has been noted that dispersin gene (*aap*) can be detected in DAEC as well as nonpathogenic *E.coli* (Monteiro *et al.,* 2009).

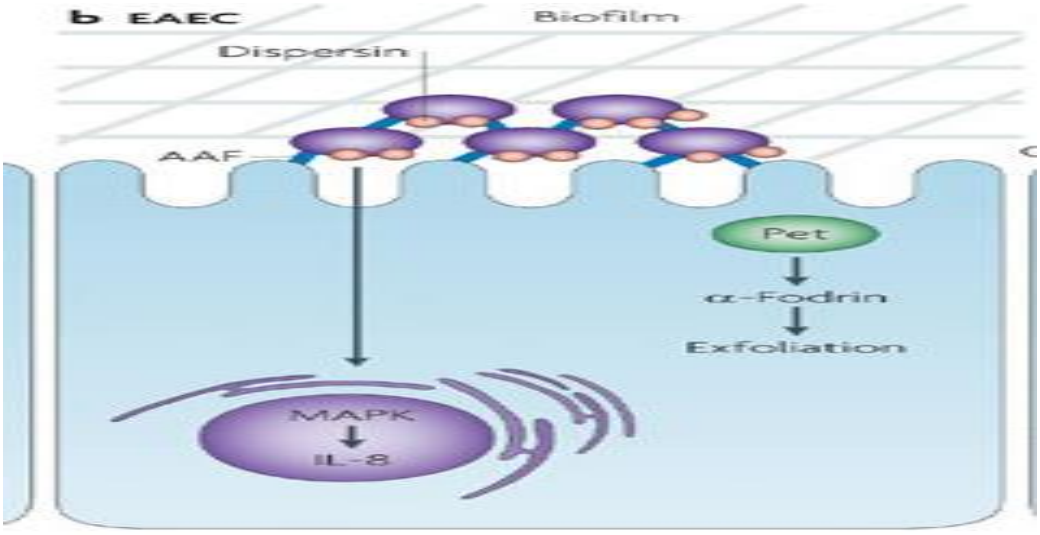

Figure 2. 9 Molecular mechanism of EAEC pathogenicity.

(Adapted from Croxen & Finlay, 2010).

(iii) Clinical manifestations

Not all EAEC infections show in symptomatic illness (Adachi *et al.,* 2002); however, most studies suggest that EAEC infection results in gastrointestinal diseases. The most commonly reported symptoms are watery diarrhoea with or without blood and mucus, abdominal pain, nausea, vomiting, and low-grade fever. EAEC can cause both an acute and a chronic diarrhoea illness. The incubation period of EAEC diarrhoeal illness ranges from 8 to 18 h. Malnourished hosts, especially infants living in developing countries, may be unable to repair mucosal damage and thus may become vulnerable to persistent or chronic diarrhoea. The clinical manifestations of EAEC diarrhoea vary from individual to individual, depending upon the genetic composition

of the host, immune response, heterogeneity of virulence among EAEC strains and the amount of bacteria ingested by the infected host (Jiang *et al*., 2003).

Persistent diarrhoea (lasting more than 14 days) may occur in select populations, including HIV/AIDS patients and malnourished children in developing countries. Risk factors for EAEC include travelers to developing countries, ingestion of contaminated food and water, poor hygiene, host susceptibility, and possibly immunosuppressant HIV infection (Huang *et al*., 2004; Huang & DuPont, 2004). Several studies have suggested that patients infected with EAEC manifest intestinal inflammation (Steiner *et al*., 1998). In a study carried out in Poland, acute episode of diarrhoea by EAEC was showed to be responsible for the development of post infectious irritable bowel syndrome (IBS) (Sobieszanska *et al*., 2007).

(iv) Epidemiology

The populations best studied for infection by EAEC include children, adults and HIV-infected individuals, living in developing and developed regions and travellers to developing countries (Vernacchio *et al*., 2006). Outbreaks of diarrhoeal illness due to EAEC have been widely reported. North American and European travellers to developing regions such Guadalajara, Mexico, Ocho Rios, Jamaica; and Goa, India, frequently experience diarrheal illness caused by EAEC infection (Adachi *et al*., 2001). Travellers are at high risk because of limited exposure to the pathogen at home and, thus, have fragile immunity to the infection (Adachi *et al*., 2001).

In another study, Cobeljic *et al*. (1996) described an outbreak affecting 19 infants in the nursery of a hospital in Serbia. Infants with diarrhoea typically manifested liquid green stools and in 3, mucus was visibly apparent. There was no gross blood and the source of infection was unclear. In reported two outbreaks of severe lethal diarrhoeic malnutrition in Mexico City hospitals

persistent diarrhoea developed in affected infants, and five patients died despite aggressive combative effort. The Autopsy taken revealed that the infants who died illustrated severe necrotic lesions of the ileal mucosa (Eslava, 1993). In 1993, a massive outbreak affected 2697 children in Japan, representing an attack rate of 40 % (Itoh *et al*., 1997).

Between 60-90% of HIV-infected patients in Africa without access to antiretroviral experience bouts of diarrhea and EAEC strains have repeatedly been isolated from HIV-positive patients with diarrhoea (Mwachari *et al*., 1998). In a study carried out in South Africa by Samii *et al.* (2007), among the *E. coli* strains from clinical samples (including stool, sputum and urine) from HIV and AIDS patients, EAEC was the most prevalent with 22.3%. In a Central African Republic study, EAEC was isolated from 12.7% of 110 HIV-positive patients with diarrhoea and none of 73% asymptomatic controls. In Senegal, EAEC were recovered from in 42 (27%) HIV-positive patients with diarrhoea and was only rarely recovered in HIV-negative individuals (Gassama-Sow, 2004). Patients who are infected with HIV frequently develop persistent diarrhoeal disease for which the etiology remains enigmatic (Lambl *et al*., 1999).

(v) Detection and diagnosis

The ability of EAEC to form biofilm has been utilized in an assay which has been suggested as a screening test in both clinical and epidemiological studies (Nataro & Kaper, 1998; Wakimoto *et al.,* 2004; Kauer *et al.,* 2010). Formation of biofilm however, was shown to be method dependent and strongly influenced by culture media, leading to the conclusion that considering the experimental variables the results need to be interpreted cautiously (Naves *et al.,* 2008). The aggregating nature of this pathotype has made serotyping in many cases impractical and the fraction that can be serotyped belong to a wide range of O:H types, making serotyping of little use in EAEC diagnosis (Weintraub, 2007). Bacterial adhesion is followed by the secretion of

various toxins of which the plasmid-encoded toxin (Pet), a serine protease causing cytoskeletal rearrangements and EAST1, an activator of guanylate cyclase, are regulated by AggR.

Within EAEC group, different pathogenicity islands have been identified including *she* pathogenicity island of *Shigella*, containing enterotoxin and mucinase genes, and *Yersinia* high-pathogenicity island, containing the yersinibactin siderophore gene (Schubert *et al.,* 1998; Henderson *et al.,* 1999; Weintraub, 2007). None of these genes however is present in all the EAEC strains and many of them are not specific for this *E. coli* category which makes developing an alternative method to HEp-2 cell adherence assay difficult. In 1990 a diagnostic probe obtained from the aggregative plasmid of strain 17-2 has been reported (Baudry *et al*., 1990). The cryptic 1-kb probe known as " CVD432 " or aggreagative probe (AA) which was later shown to correspond to the site of Aat transporter complex (Harrington *et al.,* 2006) performed variably in different locations (Nataro & Kaper, 1998). This probe was used by Bouzari *et al.* (2001) on a collection of 98 HeLa cell assay-confirmed EAEC isolates of which only 46 (46.9%) reacted with the probe.

A PCR method using primers based on the probe sequence was developed by Schmidt *et al*. (1995) and its sensitivity and specificity was reported as similar to the AA probe (Nataro & Kaper, 1998). A DNA probe from the pAA plasmid of EAEC is specific for EAEC strains, but has variable sensitivity (Scaletsky *et al*., 2002). A problem with using DNA probes and PCR assays to identify EAEC is that EAEC strains are extremely heterogeneous, and this may account for the varying sensitivity of these techniques (Sarantuya *et al*., 2004).

Heterogeneity among EAEC strains in their carriage of putative virulence factors have been well established (Nataro & Kaper, 1998; Harrington *et al.,* 2006; Weintraub, 2007; Johnson & Nolan,

2009; Croxen & Finlay, 2010; Kauer *et al.*, 2010), but in view of the pivotal role played by *aggR* in regulating a large number of virulence factors and its location on pAA, strains positive for this gene are called " typical EAEC " and strains lacking pAA, but showing the characteristic stacked-bricks phenotype in HEp-2 cell adherence assay are considered " atypical EAEC " (Bouzari *et al.*, 2005; Harrington *et al.*, 2006). Typical EAEC has been associated with diarrhoea, but in an extensive genomic analysis of EAEC strains isolated from a case-control study conducted in Mali with children under the age of 5 suffering from moderate to severe diarrhoea presence of *aggR* regulon genes was not correlated with diarrhoea (Boisen *et al.*, 2011). The importance of EAEC in diarrhoeal diseases in various epidemiological and clinical settings and the unusual degree of heterogeneity among EAEC isolates in carrying various putative virulence factors has been well documented. However, data pertaining to the role of individual factors and their contribution in conferring distinct clinical outcomes are required for a true assessment of EAEC as a human pathogen.

(vi) Treatment

A dosage of ciprofloxacin 500 mg twice a day with responses to placebo for 3 days was administered and a significant reduction in diarrhoea was observed among 29 US travellers to Jamaica and Mexico suffering EAEC diarrhoea (Glandt *et al*., 1999). In a similar trial that included US travellers to trial Guatemala, Kenya, Guadalajara and Mexico evaluated the clinical response to rifaximin with responses to placebo among 43 patients with EAEC diarrhoea. Thirty of the patients were treated with rifaximin and 13 with placebo with a significant reduction in the duration of post-treatment diarrhoea compared to placebo (Infante *et al*., 2004). Another attempt was a double blind, placebo-controlled, crossover treatment trial involving 24 HIV infected patients with EAEC diarrhoea, those treated with ciprofloxacin reported 50% fewer bowel

movements and a 42% decrease in other enteric symptoms compared with those who received placebo (Wanke *et al*., 1998). These 3 clinical studies indicate that fluoroquinolones, especially ciprofloxacin and rifaximin may be the antimicrobial treatments of choice for symptomatic EAEC infections.

Likewise, susceptibility patterns of EAEC strains appear to vary by geographical locations. Some studies have reported EAEC to have moderate-to high-level resistance to ampicillin, tetracycline, trimethoprim, sulfamethoxazole and chloramphenicol (Sobieszczanska *et al*., 2003). In most regions of the world, EAEC strains are susceptible to fluoroquinolone, azithromycin, rifaximin, amoxicillin/clavulanic acid, and nalidixic acid (Glandt *et al*., 1999; Infante *et al*., 2004).

2.8.6. Diffusely adherent *Escherichia coli* (DAEC)

(i) History

DAEC is a heterogeneous group that generates a diffuse adherence patterns on HeLa and HEp-2 cells and has been associated with the watery diarrhoea that can become persistent in young children in both developing and developed countries as well as recurring urinary tract infections (Le Bouguénec *et al.,* 2006; Croxen & Finlay, 2010). It has been shown that the relative risk of diarrhoea associated with DAEC increases with age of children from 18 months to 5 years. The intestinal carriage of these strains has also been reported to be widespread in older children and adults. The consequences of this persistence are unknown, but several observations have suggested a potential role in the development of chronic inflammatory intestinal disease (Le Bouguénec *et al.,* 2006).

Two types of adhesins mediating the DA pattern have so far been described dividing the DAEC strains into AIDA-I-dependent group and those that their adhesins is encoded by a family of

related operons, which include both fimbrial and afimbrial adhesins. These groups of proteins are collectively designated Afa-Dr adhesins (Croxen & Finlay, 2010). The first afimbrial adhesin (*afa*) operon belonging to this group was characterized and sequenced in 1984 (Labigne-Roussel *et al.*, 1984), and subsequently another operon in this family as well as the adhesins receptor were described (Nowicki *et al.*, 1987; 1990). AIDA-I is a 100 kDa outer membrane protein which is associated with DA phenotype and was described by Benz *et al*. (Benz & Schmidt, 1989) who also showed that this adhesin was not commonly encountered among DEAC isolates (Benz & Schmidt, 1992; Nataro & Kaper, 1998).

Phenotypic detection of DEAC is based on the mannose-resistant diffuse adhesion of these strains to cultured epithelial HEp-2 or HeLa cells (Scaletsky *et al.*, 1984; Nataro *et al.*, 1987 Nataro & Kaper, 1998). The adhesion assay however, is not specific for Afa/Dr DAEC detection, since other pathogenic *E. coli* including EPEC strains may show this pattern of adhesion (Bouzari *et al.*, 2000; Servin, 2005). Colony hybridization using various probes have also been developed and used in epidemiological studies (Bilge *et al.*, 1989; Stapleton *et al.*, 1991; Foxman *et al.*, 1995), but this technique is laborious and time consuming and not suitable for use on individual strains. Design of PCR methods that allow identification of all known Afa/Dr adhesins has been developed (Le Bouguénec *et al.*, 1992; 2001).

(ii) Pathogenicity

Diffusely adherent *E. coli* (DAEC) forms a diffuse attaching pattern on enterocytes of the small bowel, which is mediated through afimbrial (Afa) and fimbrial adhesins, which are collectively known as Afa–Dr fimbriae. Most Afa–Dr fimbriae bind to complement decay-accelerating factor (DAF); a subset of Afa–Dr fimbriae bind to receptors in the carcinoembryonic-antigen-related cell-adhesion molecule (CEACAM) family. The autotransported toxin Sat has been implicated in

lesions of tight junctions (TJs) in Afa–Dr-expressing DAEC, as well as in increased permeability. Polymorph nuclear leukocyte (PMN) infiltration increases surface localization of DAF.AMP, antimicrobial peptides; $G_{s\alpha}$, stimulatory guanylyl-nucleotide-binding (G) protein α-subunit; MAPK, mitogen-activated protein kinase; PKA, protein kinase A (Fig 2. 3) (Croxen & Finlay, 2010).

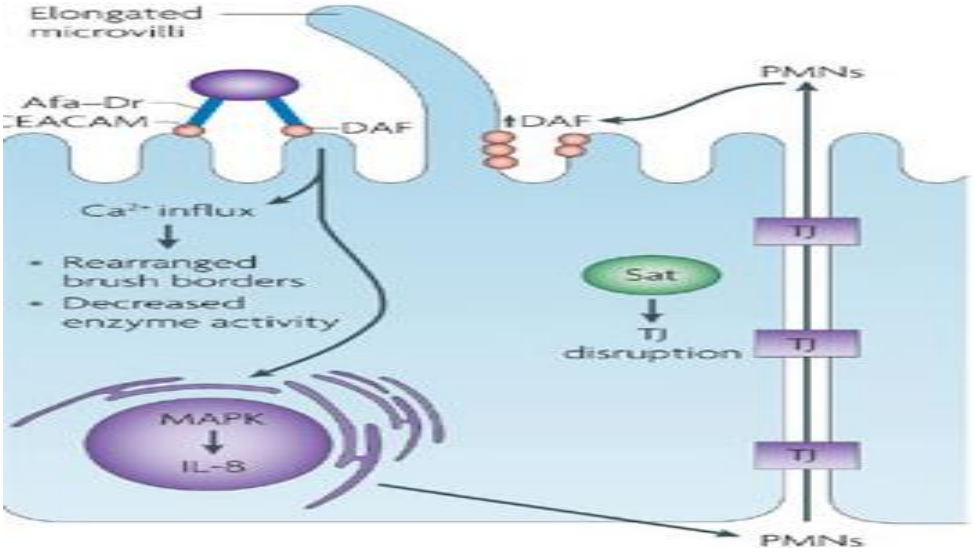

Figure 2.10 Molecular mechanism of DAEC pathogenicity.

(Adapted from Croxen & Finlay, 2010).

(iii) Clinical manifestations

DAEC is associated with watery diarrhoea that can become persistent in young children in both developing and developed countries (Croxen & Finlay, 2010). The intestinal carriage of these strains has also been reported to be widespread in older children and adults. The consequences of this persistence are unknown, but several observations have suggested a potential role in the development of chronic inflammatory intestinal disease (Servin, 2005).

(iv) Epidemiology

The epidemiology of the diffusely adherent *E. coli* (DAEC) is not well understood. Several studies have implicated DAEC strains as agents of diarrhoea, while other studies have not recovered DAEC strains more frequently from diarrhoeal patients than from asymptomatic controls. In the Central African Republic, one study found DAEC in 8.2% of HIV-positive patients with diarrhoea and only 1.4% of symptomless controls (Germani *et al*., 1998). In 49 children with diarrhoea (20.7%) and 40 children without diarrhoea (17.3%), diffusely adherent *E. coli* (DAEC) isolates were detected and were not found to be associated with diarrhoea (Scaletsky *et al.,* 2002).

(v) Detection and diagnosis

DAEC strains are defined by the presence of the DA pattern in the HEp-2 adherence assay. A 700-bp polynucleotide fragment derived from the *daa*C gene has been used as a DAEC DNA probe; *daa*C encodes a molecular usher necessary for expression of the F1845 fimbriae (Bilge *et al*., 1993). Approximately, 75% of DAEC strains from around the world are positive with this F1845 gene probe (Nataro *et al*., 1992). However, due to the genetic relatedness of F1845 to other members of the Dr Family of adhesins, false-positive reactions with the DA probe may occur, albeit with unknown frequency.

Two types of adhesins mediating the DA pattern have so far been described dividing the DAEC strains into AIDA-I-dependent group and those that their adhesins is encoded by a family of related operons, which include both fimbrial and afimbrial adhesins. These groups of proteins are collectively designated Afa-Dr adhesins (Labigne-Roussel, 1984). The first afimbrial adhesin (*afa*) operon belonging to this group was characterized and sequenced in 1984 (Nowick *et al*., 1987), and subsequently another operon in this family as well as the adhesins receptor were

described (Benz and Schmidt, 1989). AIDA-I is a 100 kDa outer membrane protein which is associated with DA phenotype and was described by Benz *et al*. (1992) who also showed that this adhesin was not commonly encountered among DEAC isolates (Nataro & Kaper, 1998). The *afa/dr/daa* operons are genes that arise and are expressed in a variety of genetic backgrounds (Servin, 2005) and the pathogenesis of DAEC seems to be predominantly mediated through Afa/Dr adhesin interactions with host cells. In addition a secreted auto transporter toxin (Sat) has also been implicated in pathogenesis, but nevertheless, the implication of Afa/Dr DAEC strains in diarrhoea remains controversial.

The adhesion assay however, is not specific for Afa/Dr DAEC detection, since other pathogenic *E. coli* including EPEC strains may show this pattern of adhesion. Colony hybridization using various probes have also been developed and used in epidemiological studies (Stapleton *et al*., 1991; Le Bouguenec *et al*., 1992), but this technique is laborious and time consuming and not suitable for use on individual strains. Design of PCR methods that allow identification of all known Afa/Dr adhesins has been achieved (Le Bouguenec *et al*., 2001).

(vi) Treatment

Susceptibilities to ceftazidime, gentamicin, lomefloxacin, ofloxacin and nalidixic acid were observed to all isolates screened by Lopes *et al*. (2005). In a study carried in Brazil, the frequencies of resistance against ampicillin, cephalothin, cotrimoxazole, streptomycin, sulfonamide and tetracycline were each > 50% each, except chloramphenicol that was less frequent (20%).

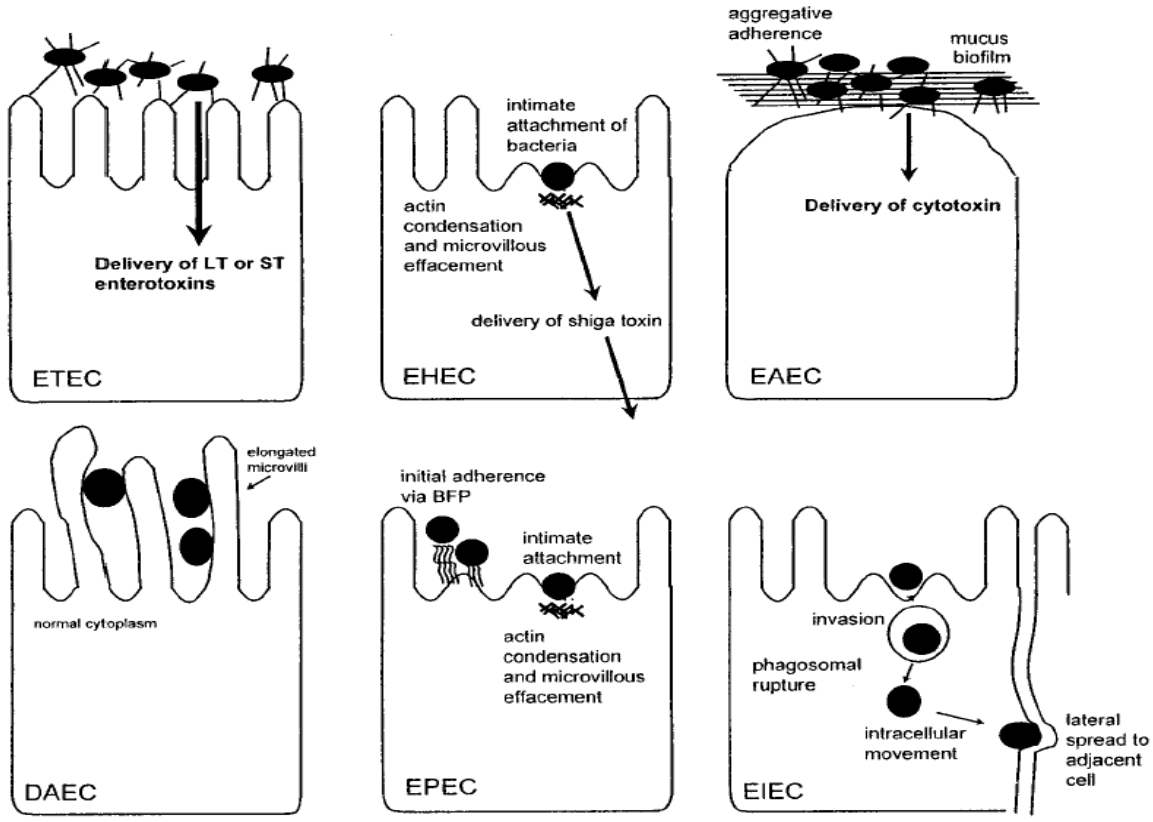

Fig 2.11 Pathogenic schemes of the diarrhoeagenic *E. coli,* each with a unique feature in its interaction with eukaryotic cells.

(Adapted from Nataro & Kaper, 1998).

2.9 Extra Intestinal Pathogenic *Escherichia coli*

Escherichia coli strains isolated from infections outside of the intestinal tract have been grouped as extra intestinal pathogenic *Escherichia coli* (ExPEC) (Russo & Johnson, 2000; Johnson & Russo, 2002, 2005; Smith *et al.,* 2007). Extra intestinal infections caused by *E. coli* can at times supersede disease episodes by other micro-organisms. These infections include pneumonia, bacteremia, meningitis, septicaemia, abscesses, wound infection and urinary tract infections (Diekema *et al.,* 2000; Edmond *et al.,* 2000; Abbott, 2001). A surveillance report on nosocomial

infections in the USA between 1986 to 1989 and 1990 to 1996 indicated that *E. coli* was the most isolated bacterium in urinary tract infections (Diekema *et al.,* 2000).

With the advent of Human Immunodeficiency Virus infection (HIV), *E. coli* has become the major cause of acquired bacteremia (Gilks *et al.,* 2002). Furthermore, ExPEC are the primary gram-negative bacterial pathogens associated with neonatal meningitis and are the second overall cause of the disease after group B Streptococci (Bonacorsi & Bingen, 2005; Furyk *et al.,* 2011). Unlike commensals, they do not establish long-term symbiotic relationships with their host (McCartney *et al.,* 1996; VSN International, 2003).

ExPEC are part of the intestinal microflora of a fraction of the healthy population and normally asymptomatically colonize the gut. Once they get access to niches outside of the gut, they are, however, able to efficiently colonize these niches and cause disease in man, i.e. urinary tract infection (UTI), septicemia or meningitis in newborns, as well as UTI or systemic disease in many animals. Human and animal pathogenic strains share common genetic backgrounds (Johnson *et al.,* 2008b; Clermont *et al.,* 2011).

ExPEC strains are divided into three major pathotypes (Bekal *et al.,* 2003): (i) uropathogenic (UPEC) strains that cause urinary infections, (ii) strains that cause neonatal meningitis (NMEC), and (iii) strains that cause septicemia (Bekal *et al.,* 2003). They possess virulence gene combinations that are distinctive from those found in their counterparts that cause intestinal disease. For instance, UPEC strains are more likely to possess P pili, S pili, afimbrial adhesin, and toxins such as haemolysin and cytotoxic necrotizing factor 1 (Johnson & Stell, 2000, Marrs *et al.,* 2002). In addition, strains which cause systemic infection in poultry, avian pathogenic *E. coli* (APEC), resemble certain human-pathogenic ExPEC variants (Ewers *et al.,* 2007; Moulin-

Schouleur *et al.,* 2007; Johnson *et al.,* 2010; Bélanger *et al.,* 2011; Clermont *et al.,* 2011). Two new animal pathogenic subgroups have been proposed: mammary pathogenic *E. coli* (MPEC) (Shpigel *et al.,* 2008) and endometrial pathogenic *E. coli* (EnPEC) (Sheldon *et al.,* 2010).

Although several important ExPEC virulence factors and their role during pathogenesis have been described (Kaper *et al.,* 2004; Mokady *et al.,* 2005; Smith *et al.,* 2007), many ExPEC cannot be unambiguously distinguished from commensal *E. coli* based on a set of discriminatory virulence factors as it can use multiple virulence factors in a mix-and-match fashion. Nevertheless, ExPEC classification has been proposed on the basis of isolation site and the detection of two virulence-associated genes (VAG) typical of the specific pathotype (Johnson & Russo, 2005), respectively two VAGs for non-host samples like food samples (Johnson *et al.,* 2005). This high level of genetic heterogeneity within the species *E. coli* mirrors the genomic plasticity of this generally clonal group of organisms which results from frequent acquisition and loss of genomic information as well as high recombination rates within the flexible genome (Brzuszkiewicz *et al.,* 2009; Schubert *et al.,* 2009; Touchon *et al.,* 2009; Tenaillon *et al.,* 2010). It has also been shown that the genetic background plays a role in the acquisition and expression of foreign DNA (Escobar-Paramo *et al.*, 2004).

ExPEC strains are implicated in a large number of infections in humans and animals, such as urinary tract infection (UTI), meningitis, diverse intra-abdominal infection, pneumonia, osteomyelitis, and soft-tissue infection; besides, bacteremia can accompany infection at any of these sites. ExPEC, which include avian pathogenic *E. coli* (APEC), uropathogenic *E. coli* (UPEC), septicemic *E. coli* (SEPEC) and neonatal meningitis causing *E. coli* (NMEC), exhibit considerable genome diversity characterized by the possession of various combinations of adhesins, iron-acquisition systems, host defense-avoidance mechanisms, toxins, and others,

which collectively are known as extraintestinal virulence factors (Russo & Johnson, 2000; 2005; Ewers *et al*., 2007). Like strains that cause intestinal infections in humans, ExPEC strains possess virulence genes that have a range of functions, including attachment/invasion, toxin production, iron scavenging, and immune evasion ((Johnson & Stell, 2000; Marrs *et al.*, 2002).

Table 2.3 ExPEC virulence factors.

Functional category	Virulence factor	Reference
Adhesin	Type 1 fimbriae (Fim)	
	P fimbriae (Pap/Prf)	Anfora *et al.*, (2007)
	S/F1C fimbriae (Sfa/Foc)	
	N-acetyl d-glucosamine-specific fimbriae (Gaf)	
	M-agglutinin (Bma)	
	Bifunctional enterobactin receptor/adhesin (Iha)	
	Afimbrial adhesin (Afa)	
	Temperature sensitive hemagglutinin (Tsh)	
Invasin	Invasion of brain endothelium (IbeA)	Ewers *et al.*, (2007)
	Iron acquisition Siderophore receptor IreA	
	Aerobactin (Iuc)	
	Yersiniabactin (Ybt)	
	Salmochelin (Iro)	
	Periplasmic iron binding protein (SitA)	
Toxins	alpha-Hemolysin (HlyA)	Johnson *et al.*, (2003)
	Cytolethal distending toxin IV (CDT 1)	
	Cytotoxic necrotizing factor 1 (CNF-1)	
	Putative hemolysin (HlyF)	
	Colibactin (Clb)	
	Serine protease autotransporters Sat, Pic	
Protectins	Group II capsule incl. K1 capsule	Johnson *et al.*, (2008c)
	Conjugal transfer surface exclusion protein (TraT)	
	Outer membrane protease T (OmpT)	
	Increased serum survival (Iss)	
	Colicin V (Cva)	
Others	d-Serine deaminase (DsdA)	Rodriguez-Siek *et al.*, (2005)
	Maltose and glucose-specific PTS transporter subunit IICB (MalX)	
	Flagella	

2.9.1 Uropathogenic *Escherichia coli*

Uropathogenic *Escherichia coli* (UPEC) is the primary cause of community-acquired UTIs, with an estimated 20% of women over the age of 18 years suffering from an UTI during their lifetime (Foxman, 2010). It is responsible for 70-95% of community-onset UTIs and approximately 50 % of nosocomial UTIs, hence accounting for substantial morbidity, mortality and medical expenses (Nicolle, 2014). Recurrent or relapsing UTIs are especially problematic in many individuals. The primary reservoir of UPEC is believed to be the human intestinal tract and isolates act as opportunistic pathogens that employ diverse repertoire of virulence factors to colonize and infect the urinary tract in an ascending fashion (Foxman, 2010; George & Manges, 2010). However, community-onset clonal outbreaks of UTIs, possibly due to the consumption of food contaminated with UPEC have also been described with some additional evidence that UPEC isolates can also be transmitted via sexual activities (George & Manges, 2010).

UPEC infections account for roughly 80% of all UTIs, causing cystitis in the bladder and acute pyelonephritis in the kidneys. UPEC has the challenge of moving from the intestinal tract to establish an infection in the urinary tract, where it uses peptides and amino acids as the primary carbon source for fitness (Alteri *et al.,* 2009). The ability to ascend the urinary tract from the urethra to the bladder and kidneys reflects exceptional mechanisms for organ tropism, evading innate immunity and avoiding clearance by micturition. Several highly regulated virulence factors contribute to this complex pathogenesis, including multiple pili, secreted toxins [for example Sat and vacuolating autotransporter toxin (vat)], multiple iron acquisition systems and a polysaccharide capsule (Wiles *et al.,* 2008) (Figure 2.12a).

Entry of UPEC into the urinary tract is followed by adhesion to the uroepithelium. This attachment is mediated by fimbrial adhesin H (FimH), which is found at the tip of the phase-variable type 1 pili. FimH binds to the glycosylated uroplakin Ia that coats terminally differentiated superficial facet cells in the bladder (Wiles *et al.*, 2008). Interactions between FimH and uroplakin IIIa were recently found to lead to phosphorylation events that are required to stimulate unknown signalling pathways for invasion and apoptosis (Thumbikat *et al.*, 2009). UPEC invasion is also mediated by FimH binding to α3 and β1 integrins that are clustered with actin at the sites of invasion (Eto *et al.*, 2007) as well as by microtubule destabilization (Dhaka & Mulvey, 2009). These interactions trigger local actin rearrangement by stimulating kinases and Rhofamily GTPases, which results in the envelopment and internalization of the attached bacteria. Once internalized, UPEC can rapidly replicate and form biofilm-like complexes termed intracellular bacterial communities (IBCs) or pods, which serve as transient, protective environments (Anderson *et al.*, 2003). UPEC can leave the IBCs through a fluxing mechanism; motile UPEC leaves the epithelial cells and enters the lumen of the bladder (Justice *et al.*, 2004). Filamentous UPEC has also been observed fluxing out of an infected cell, looping and invading surrounding superficial cells in response to innate immune responses (Justice *et al.*, 2004; 2006).

UTIs that are left untreated can disseminate to the kidney in an ascending progression of disease. Ascension to the kidney is mediated by reciprocal regulation of type 1 pili and motility. Bacteria that express type 1 pili are less flagellated than those that do not, suggesting that when type 1 pili are 'switched off', UPEC can become more motile (Lane *et al.*, 2007). Furthermore, motility was shown to permit the ascension from the bladder to the kidney (Lane *et al.*, 2007). UPEC isolates that are associated with pyelonephritis often express the P fimbriae that adhere to Galα (1–4) Galβ moieties of the globoseries glycolipids that are found on the surface of kidney epithelial

cells1. Similarly to the inverse relationship between type 1 pili and motility, expression of P fimbriae is associated with fewer flagella and repressed motility (Simm *et al.,* 2004). Crosstalk between P fimbriae, type 1 pili and other adhesion clusters prevents co-expression of multiple surface organelles (Lindberg *et al.,* 2008).

2.9.2 Neonatal meningitis *Escherichia coli*

Neonatal meningitis *Escherichia coli* (NMEC), a common inhabitant of the gastrointestinal tract, is the most frequent cause of Gram-negative-associated meningitis in newborns. Fatality rates can approach 40% (Kaper *et al.,* 2004), and survivors are usually burdened with severe neurological attack. The pathogenesis of NMEC is complex, as the bacteria must enter the bloodstream through the intestine and ultimately cross the blood–brain barrier into the central nervous system (Figure 2.12b), which leads to meningeal inflammation and pleocytosis of the cerebrospinal fluid. Initial colonization, after the bacteria have been acquired perinatally from the mother, is followed by transcytosis through enterocytes into the bloodstream. The progression of disease is dependent on high bacteremia (> 103 colony-forming units per ml of blood), so survival in the blood is crucial. Protection from the host immune responses is provided by an antiphagocytic capsule, made up of a homopolymer of polysialic acid, and serum resistance, resulting from manipulation of the classical complement pathway by the bacterial outer-membrane protein A (OmpA) (Wooster *et al.,* 2006).

NMEC has also been shown to interact with immune cells: invasion of macrophages and monocytes prevents apoptosis and chemokine release (Sukumaran *et al.,* 2004), providing a niche for replication before dissemination back into the blood. Maturation of dendritic cells is also inhibited by NMEC (Mittal & Prasadarao, 2008). Recently, a lambdoid phage that encodes O acetyltransferase was discovered, which acetylates the O antigen to provide phase variation

and diversity to the capsule (Deszo *et al.*, 2005) and may therefore hide the bacteria from host defences. The blood–brain barrier is a tight barrier formed by brain microvascular endothelial cells. Attachment of NMEC is mediated by FimH of the type 1 pili binding to CD48 (Khan *et al.*, 2007) and by OmpA binding to its receptor, ECGP96 (Prasadarao, 2002), on the surface of brain microvascular endothelial cells. Invasion occurs through the actions of Ibe proteins, FimH, OmpA and cytotoxic nectrotizing factor 1 (CNF1) (Kim *et al.*, 2008). The receptors for the Ibe proteins are unknown, but the 67 kDa laminin receptor (67LR; also known as RPSA) was shown to be the receptor for CNF1 (Kim *et al.*, 2005). CNF1 is a toxin that deaminates Rho-family GTPases that are involved in myosin rearrangement (Essler *et al.*, 2003).

It is possible that FimH- and OmpA-mediated attachment to brain microvascular endothelial cells may be required before translocation of CNF1 into the host cell can occur. OmpA interaction with its receptor and FimH-mediated increase of intracellular Ca2+ stimulates actin rearrangements (Khan *et al.*, 2007, Maruvada *et al.*, 2008) that, along with the CNF1-stimulated myosin rearrangements, are involved in the invasion of NMEC. The K1 capsule, which is found in approximately 80% of NMEC isolates, also has a role in invasion by preventing lysosomal fusion and thus allowing delivery of live bacteria across the blood–brain barrier (Kim *et al.*, 2003). Collectively, these mechanisms allow NMEC to penetrate the blood–brain barrier and gain access to the central nervous system, where they cause oedema, inflammation and neural damage.

Severe neurological lesions resulting from infection with neonatal meningitis *E. coli* (NMEC) leads to death in 20-40% of infected infants (Bonacorsi & Bingen, 2005). Nosocomial bloodstream infections in hospitals and nursing homes may be caused by ExPEC strains which may also be the cause of respiratory, UTI or bacteremia in long-term hospitalized patients (Ron,

2010). Resistance to antimicrobials has made combating these infections a major problem worldwide (French, 2010).

Antimicrobial therapy remains the cornerstone for treating ExPEC infections. The β-lactam antibiotics, especially the 3rd generation cephalosporins, and the fluroquinolones are the major antimicrobial families used to treat serious community-onset or hospital-acquired infections caused by ExPEC (Pitout, 2012; Shepherd & Pottinger, 2013).

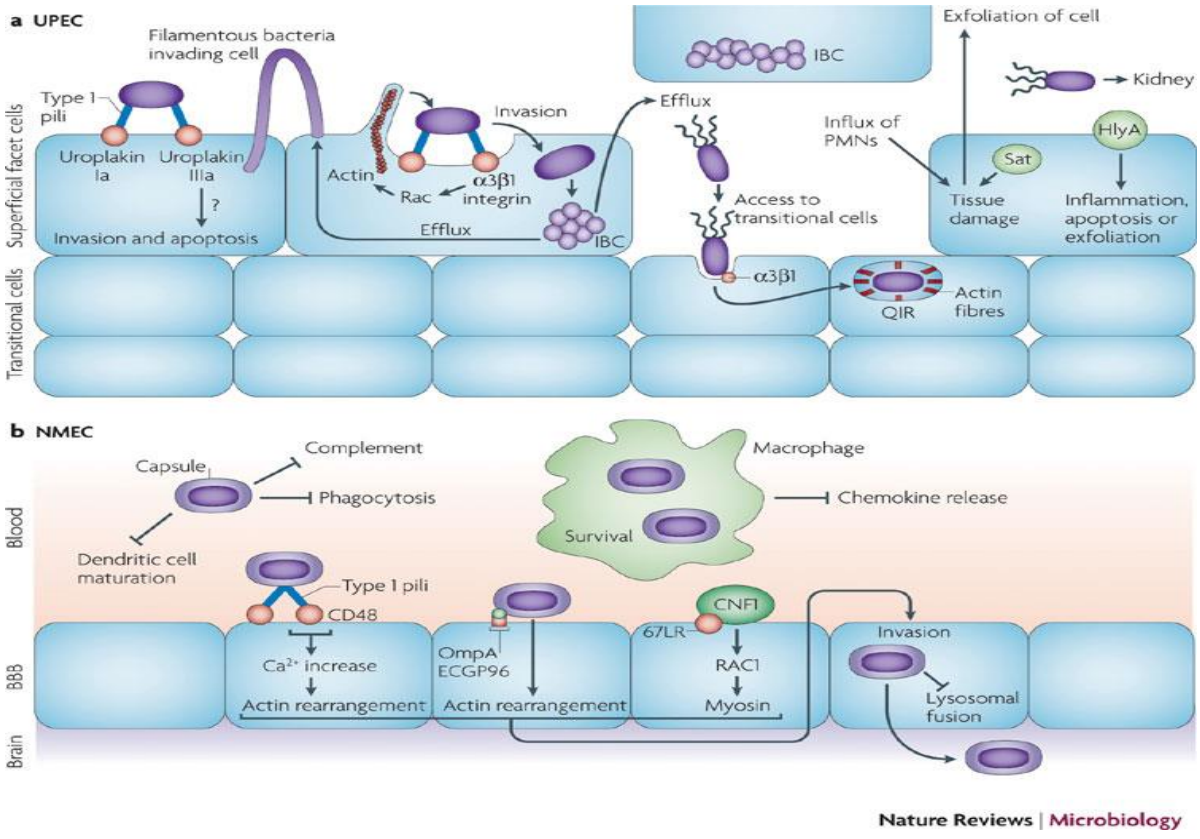

Figure 2.12 Molecular mechanism of ExPEC pathogenicity. (a) UPEC and (b) NMEC **(Adapted from Croxen & Finlay, 2010).**

2.10 *Escherichia coli* and antimicrobial therapy

Antimicrobial agents are widely used to protect the health of humans and animals or to promote growth rate of animals as food additive. The World Health Organization (WHO, 2002) estimated that about 80% of antibiotics consumption takes place in the community for human use and that, at least half of this is based on inadequate indication, mostly viral infections, that normally require symptomatic rather than drug therapy. In industrialized countries, driving forces known to promote antimicrobial resistance basically include the use of antibiotics for growth promotion in animals (Zhang *et al*., 2006; Jacob *et al*., 2008; Bisht *et al*., 2009; Espinal *et al*., 2011; Wagner *et al*., 2011), pesticides in agriculture and frequent prescription of most recent antibiotics (last line) by physicians (Bisht *et al*., 2009). In resource-limited countries, factors like monotherapy, inadequate dosage associated with treatment interruption are most likely responsible for resistance phenotype growth among bacteria (Cohen, 1992; Gold & Moellering, 1996). In these areas, the limited number of antibiotics is increasingly inadequate for infectious disease management (Roger *et al*., 2003; Shetty *et al*., 2009; Andersson & Hughes, 2011).

The discovery of penicillin in the 1940s and several other antibiotics in consequent years led to great improvements in the management of infectious diseases for the most parts in the developed world. Since this discovery, there was a certainty in the medical fraternity that this would lead to the eradication of infectious diseases. Despite this great success, the excessive use of antimicrobials has led to the inevitable development of resistance (Norrby *et al.,* 2005), with diseases and disease agents that were once thought to have been controlled by antibiotics re-emerging in new forms resistant to antibiotic therapies (Levy & Marshall, 2004). This worldwide emergence of multi-drug resistant bacterial strains has rendered the current drugs used for treatment useless, causing treatment failures (Hancock, 2005).

In addition to the wide spread cases of water-borne diseases resulting from the contamination of water sources, concerns have been raised when these diseases fail to be cured due to development of resistance to commonly prescribed antibiotics by the contaminating microorganisms originating from livestock feces and human sewage (Prescott *et al.,* 2000). Now-a-days, antibiotic resistant bacteria species are ubiquitous in the environment and their negative impact has greatly increased (Mathew *et al.,* 2007); improper antibiotic use and lack of awareness are considered as the most important factors for the emergence, selection, and dissemination of antibiotic resistant bacteria species in the environments (Neu, 1992).

The majority of antibiotics are excreted unchanged and find their way into the environments. Thus, concerns about the potential impacts of antibiotic residues in the aquatic ecosystems keep growing in recent years (Sarmah *et al.,* 2006; Wright, 2007; Kemper, 2008). The surfacing of antibiotic resistance usually results from the indiscriminate use of antibiotics as growth-promoters in animal production, for therapy and prophylaxis (Barbosa & Levy, 2000). In many developing countries the unregulated sale and dispensing of antibiotics is very common (Byarugaba, 2004). *E. coli* is also considered as a good indicator of the selective pressure imposed by antimicrobial use in food animals (van den Bogaard & Stobberingh, 2000).

The health consequences associated with *E. coli* infection have been greatly worsened by the emergence of multidrug-resistant *E. coli*. This mounting phenomenon, which has been deemed to be worse that the methicillin–resistant *Staphylococcus aureus* (MRSA), whose restraint is considered as one of the biggest challenges in the twenty-first century in the field of science and medicine, already has some established consequences regarding bacteria-host relationships (Santos *et al.,* 2007). From the late 1990s, multidrug-resistant Enterobacteriaceae (mostly *E. coli*) that produce extended-spectrum β-lactamases (ESBLs), such as the CTX-M enzymes

emerged within the community setting as an important cause of UTIs. Recent reports have also described ESBL-producing *E. coli* as a cause of bloodstream infections associated with these community-onset urinary tract infections (UTIs). The shiga toxin–producing *E. coli* (STEC) serotype O104 that was responsible for an outbreak in Germany showed extended spectrum β-lactamase (ESBL) activity (Struelens *et al.*, 2011).

The emergence of antimicrobial resistant *E. coli* strains has rendered first choice antibiotics as useless. Although some pathotypes show promise for vaccine development, not enough is known about predominant subtypes to assure that vaccines will be effective in the places where they are most needed (Okeke, 2009). The antimicrobial-resistant bacteria presents a major threat to public health because it reduces the effectiveness of antimicrobial treatment, leading to increased morbidity, mortality and healthcare expenditure (Smith & Coast, 2002; Collignon *et al.,* 2009; Hawkey & Jones 2009). The main factor driving this process is the selective pressure of antimicrobial use in human and animal medicine, as well as in aquaculture and agriculture (Smith & Coast, 2002; Collignon *et al.,* 2009; Kummerer *et al.,* 2009). Consequently, this has led to the dissemination of antibiotic-resistant bacteria throughout the environment (Baquero *et al.,* 2008; Martinez, 2009). Residues of human and veterinary drugs are introduced into the environment via a number of pathways, but primarily from discharges of wastewater treatment plants or land application of sewage sludge and animal manure (Kummerer *et al.,* 2004; Kim & Aga, 2007).

2.11 *Escherichia coli* and antimicrobial resistance

Resistance to antimicrobial agents began with their introduction in the 1940s and has increased remarkably in the last three decades (WHO, 1997; Okeke & Edelman, 2001; Chigor *et al.,* 2010). Other reports show that multidrug resistance presents an emerging problem of antibiotic

resistance (Faruque *et al.,* 2003; Fontana *et al.,* 2003). Currently, resistance now spans through all known families of antimicrobial agents (Zhang *et al.,* 2009). Commensal bacteria which are naturally occurring host enteric flora constitute an enormous potential reservoir of resistant genes for pathogenic bacteria (Bartolini *et al.,* 2004; Turnidge, 2004). *E. coli* are used internationally as indicator bacteria for antimicrobial resistance determinance because of their high prevalence in human faeces and also they are significant reservoir of resistance determinants (Catry *et al.,* 2003). The abundance of *E. coli* implicates them as likely candidates for the spread of resistance genes and vectors between the bacterial populations in humans; however, their abundance also makes such spread difficult to trace. The spread of antimicrobial resistance (AMR) into environments where antibiotics are not used remains an hypothesis yet to be thoroughly investigated, although it has been postulated that water could disseminate AMR (WRC, 2001).

A wide range of antimicrobial agents effectively inhibit the growth of *E. coli*. The β-lactams, fluoroquinolones, aminoglycosides, tetracyclines and trimethoprim-sulfamethoxazole are often used to treat community and hospital infections due to *E. coli* (Pitout, 2012). β-lactams disrupt cell wall synthesis by binding to and inhibiting the penicillin-binding proteins essential for transpeptidation and carboxypeptidation reactions in cell wall peptidoglycan synthesis. Fluoroquinolones interfere with DNA supercoiling and promote DNA gyrase-mediated double-stranded DNA. The aminoglycosides bind irreversibly to the 50S subunit of the 70S bacterial ribosomes. Sulfonamides and trimethoprim interfere with bacterial folic acid synthesis by inhibiting tetrahydropteric acid syntheses and dihydrofolate reductase, respectively.

The β-lactam antibiotics, especially the cephalosporins and β-lactamases inhibitor combinations, are major drug classes used to treat community-onset or hospital-acquired infections caused by *E. coli* (Pitout, 2010). Among *E coli,* β-lactamase production remains the most important

contributing factor to β-lactam resistance. β-lactamases are bacterial enzymes that inactivate β-lactam antibiotics by hydrolysis, which results in ineffective compounds (Jacoby, 2009).

Resistance to aminopenicillins (e.g. ampicillin) and early-generation cephalosporins (e.g. cefazolin) among *E. coli* is often mediated by the production of narrow-spectrum β-lactamases such as TEM-1, TEM-2 and to a lesser extent SHV-1 enzyme (Bush & Jacoby, 2010). Most importantly among *E. coli*, is the increasing recognition of isolates producing the so-called "newer b-lactamases" that causes resistance to the expanded-spectrum cephalosporins and/or the carbapenems. These enzymes consist of the plasmid-mediated AmpC β-lactamases (e.g. CMY types), extended-spectrum β-lactamases (e.g. TEM, SHV, CTX-M types), and carbapenemases (KPC types, metallo-β-lactamases (MBLs) and OXA-types) (Bush & Jacoby, 2010, Nordmann *et al.,* 2011). CMY, CTX-M, and NDM types of β-lactamase are mostly responsible for the emerging resistance to the β-lactam antibiotics among *E. coli* (Pitout, 2012). The VIM, IPM, KPC and OXA-48 β-lactamases had been described in various members of the Enterobacteriaceae (especially *Klebsiella* spp.) and are not yet commonly encountered among *E. coli*.

The up regulation of efflux pumps and plasmid-mediated resistance mechanisms (e.g. *qnr* determinants) can reduce fluoroquinolone susceptibilities in *E. coli*, however high level resistance to the fluoroquinolones typically requires 1-2 point mutations within the quinolone resistance determining regions of *gyr*A and *par*C, the chromosomal genes encoding for DNA gyrase and topoisomerase IV respectively (Johnson *et al*., 2013).

Resistance to aminoglycosides may develop because of impaired uptake and aminoglycoside phosphorylation, although enzymatic modification by acetylation of an amino group is

considered the most common mechanism. The genes encoding for enzymatic modification of aminoglycosides are often part of class I integrons (Gillings, 2014). A variant of aminoglycoside acetyltransferase *aac*(*6'*)-*Ib*, named *aac*(*6'*)-*Ib-cr* are very prevalent among antibiotic resistant *E. coli* (Park *et al.,* 2006). *AAC* (*6'*)-*Ib-cr* has the additional ability to acetylate fluoroquinolones with unprotected amino nitrogen on the piperazine ring that includes norfloxacin and ciprofloxacin. Trimethoprim-sulfamethoxazole resistance results from alterations of different substrate enzymes or their overproduction, loss of bacterial drug-binding capacity, and decreased cell permeability.

2.12 Mechanism of antimicrobial resistance

The developmental of resistance to antimicrobials occurs through stable genetic change heritable from generation to generation through specific mechanisms including mutation, transduction, transformation, and or conjugation (Metlay *et al*., 2006). The shedding of the resistant bacteria into the environment by cattle may lead to a widespread dissemination of antibiotic resistant genes to the resident bacteria in the environment (Callaway *et al*., 2004; Mashood, *et al*., 2006).

There are two forms of AMR in bacteria: intrinsic and acquired. Intrinsic or natural resistance is widespread in bacteria and results from evolutionary adaptation of bacteria to the environment (Heinemann, 2001). The inability of the antimicrobial to penetrate a bacterial cell and the lack of a target for the antimicrobial agent to act against, are all natural bacterial adaptations which result in AMR (Mitema *et al.,* 2004). Acquired resistance may be displayed in two forms: by the micro-organisms withstanding relatively high levels of a specific antimicrobial agent. This could be as a result of either single or multiple step mutation in genes responsible for antibiotic uptake or binding sites, or may result from the lateral transfer of resistance traits between bacteria of the same species, of a different species, or acquired from the environment by a process known as

transformation (McManus, 2000; Apley *et al.*, 2003). It may also be by acquisition of mobile extra chromosomal DNA elements such as plasmids, transposons and integrons rather than mutation (Heinemann, 2001).

A plasmid is a circular body of double stranded DNA which is separate from the chromosome and carries genes that encode various traits such as virulence and AMR (Kaye *et al.*, 2000). There are two types of plasmids: conjugative and non-conjugative. Conjugative plasmids transfer resistance via the sex pili whereas non-conjugative plasmids must have direct contact for transfer to occur. In non-conjugative transfer, both the donor bacteria and the recipient bacteria have a copy of the transferred plasmid. Conjugative transfer is an important mechanism in AMR because transfer can occur in a wide range of bacterial species and can spread to unrelated organisms. Moreover, a single plasmid can contain multiple genes conferring resistance to multiple classes of antimicrobials (Akkina & Johnson, 1999). Bacteria can also acquire DNA via transduction and transformation. Transduction occurs when DNA is transferred via bacteriophages, which are viruses that attack bacteria. Bacteriophages are very tightly packaged and do not have room to carry DNA. They also have a narrow host range, and as a result transduction is a less important mechanism for resistance gene transfer (Akkina & Johnson, 1999). Transformation occurs when bacteria pick up free DNA from the environment. While the presence of DNA is common after cell lysis, the compatibility between the free DNA and the intact recipient is narrow (McManus, 2000). Additionally, free DNA in the environment would be highly susceptible to digestion by nuclease. As a result, transduction and transformation are not thought to contribute significantly to the dissemination of AMR (Chopra *et al.*, 2001). A transposon is a genetic element that contains an insertion sequence at each end. The insertion sequence allows the gene to jump to different locations on the chromosomal DNA, from plasmid

to chromosome or from chromosome to plasmid. Movement of a transposon is known as transposition and represents an important facet of AMR transfer because resistance genes can be moved from a non-conjugative plasmid or chromosome to a conjugative plasmid, and hence easily transferred to other bacteria (Emmanuel De *et al.,* 2001).

Integrons are genetic units that include the determinants of the components of a site-specific recombination system called gene cassettes (Hall & Collins, 2001). The essential components of an integron are an *Int* gene which encodes a site-specific recombinase belonging to the intergrase family, an adjacent site *attl,* which is recognized by the intergrase and is the receptor site for the gene cassettes, and a promoter suitably oriented for the expression of the cassette-encoded genes (Hall & Collins, 2001). The integron is composed of a 5' conserved sequence (CS) (the integrase gene) and a 3' CS, which can vary for the four different classes of integrons; classes 1 through 4 (Roe *et al.,* 2003a). The majority of integrons described to date are class 1 integrons and the majority of those are associated with *sul,* a gene commonly found within the 3' conserved sequence (Fluit & Schmitz, 2000; Naas *et al.,* 2001). AMR gene cassettes are not always present in integrons, and the integrase gene *(Int)* can excise gene cassettes as covalently closed supercoiled circular molecules. Integron gene cassettes can be deleted, rearranged and duplicated within the integron (Wiktor *et al.*, 2007). Integron mediated AMR is a major mechanism for transfer of resistance traits within Gram-negative bacteria (Leverstein-van Hall *et al.,* 2002a; O'Brien, 2002; Roe *et al.,* 2003b; Mathai *et al.*, 2004). Integrons have been found to harbor the majority of resistance genes within the mobile resistance elements (transposons and plasmids), which allow for the transfer of resistance between bacteria (Hall & Collins, 2001; White *et al.,* 2001; Levesque *et al.,* 2003; Roe *et al.,* 2003a). Within the integron, more than 60 gene cassettes have been found that confer resistance to a variety of agents (White *et al.,* 2001). Of these, the

most prevalent genes are those coding for aminoglycosides and trimethoprim resistance (Fluit & Schmitz, 2000; White *et al.,* 2001). Integrons also represent an important mechanism for transfer of resistance characteristics from commensal to pathogenic organisms and have been found to harbor multiple resistance genes (Goldstein *et al.,* 2001; Maguire *et al.,* 2001; Zhao *et al.,* 2001).

2.13 Molecular mechanism of antimicrobial resistance

Antimicrobial agents have targets, which are usually functional proteins such as enzymes and ribosomal proteins. The interaction between an antibiotic and target moiety is often quite specific. Alteration of target protein through mutations renders the bacterium resistant to drugs. A single amino acid change in the enzyme alters the sensitivity of the target for β-lactams, macrolides, and folate acid synthesis antagonists. Modification of penicillin binding protein (PBP) can affect the affinities of these molecules for β-lactam antibiotics. Mutations in *gyr*A gene lead to reduced affinity of the subsequent mutated DNA gyrase to quinolones. Mutations in genes coding for dihydropteroic acid synthesis (DHPS) and dihydrofolate reductase (DHFR) reduce affinity for sulfamethazole and trimethoprim respectively (Summers, 2002).

Burns *et al.* (2003) suggested that decreased sensitivity to chloramphenicol was due to low intracellular concentration of the drug. Resistance was observed even in the absence of chloramphenical acetyltransferase. Decreased membrane permeability involves alteration of the outer membrane porins. Emmanuelle De (2001) demonstrated antibiotic resistance in *Enterobacter aerogenes* by inducing structural modification of the major porin. Decreased membrane permeability probably also enhances other mechanisms of antibiotic resistance (Nikaido, 2001; Pratt & Taylor, 2003). Antibiotics can be inactivated by enzymatic cleavage or by chemical modification upon which the drug is rendered ineffective (Pratt & Taylor, 2003). The degrading enzymes can be encoded on genes including transferable ones. Classical examples

of these enzymes are the β-lactamases, acetyltransferases and aminoglycosides modifying enzymes (Rice *et al.*, 2003). Shifting to an alternative pathway is another way by which bacteria develop drug resistance. Organisms are able to utilize substrates from the immediate environment, bypassing the target enzyme. *Enterococcus* strains for example can resist activity of trimethoprim by using available folic acid (Bellaj *et al.*, 2002). Bacteria may fail to metabolize the antibiotic into its active derivatives. This occurs when the drug needs to be converted by the bacterium into an active derivative. *Bacteriodes* are resistant to metranidazole as the bacteria cannot convert the drug to active metabolite (Fluit & Schmitz, 2000).

Table 2.4 Mechanism of action of major antimicrobial families.

Mechanism of action	**Antimicrobial families**
Inhibition of cell wall synthesis	Penicillins; cephalosporins; carbapenems; daptomycin; monobactams; glycopeptides
Inhibition of protein synthesis	Tetracyclines; aminoglycosides; oxazolidonones; streptogramins; ketolides; macrolides; lincosamides
Inhibition of DNA synthesis	Fluoroquinolones
Competitive inhibition of folic acid synthesis	Sulfonamides; trimethoprim
Inhibition of RNA synthesis	Rifampin
Other	Metronidazole

(Source: Levy & Marshall, 2004).

Genetic mechanisms involved in horizontal transfer of ARDs among environmental bacteria may include the following:

(1) conjugative transfer by mobile elements including plasmids, transposons, and integrons on plasmids or transposons

(2) transformation by naked DNA, in the case of naturally competent state of some bacteria, or an environmentally induced competence such as the presence of calcium; and

(3) transduction by bacteriophage. Antibiotic resistance in most environmental bacteria is due to the acquisition of new genes, often associated with the mobile elements.

Bacteria become resistant by any of three routes: inheritance, spontaneous mutations that produce new resistance traits or acquisition of resistance determinants (called R-genes) from other bacteria by conjugation, transduction and transformation, virtually without barriers between species (Schmidt, 2002; Woegerbauer *et al.*, 2002).

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CHAPTER THREE

Physicochemical properties and total coliform distribution of some selected rivers in Osun State, Southwestern Nigeria

This chapter has been submitted for publication in

Environmental Monitoring and Assessment

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Physicochemical properties and total coliform distribution of some selected rivers in Osun State, Southwestern Nigeria

Abstract

Rivers are important freshwater resources for domestic and industrial activities, and the availability of good quality freshwater is indispensable for preventing water-borne diseases and improving quality of life especially in communities that lack pipe-borne water. The physicochemical qualities and coliform distribution of some selected rivers in Osun State, Southwestern Nigeria was evaluated using standard analytical procedures. The physicochemical qualities ranged as follows: pH (6.9 - 7.6), temperature (26 – 29 ºC), turbidity (2.28 – 9.46 NTU), electrical conductivity (229 – 581 μS/cm), nitrate (0.03 – 0.05 mg/L), nitrite (0.00 – 0.01 mg/L), sulphate (3.33 – 20.33 mg/L), chloride ions (7.83 – 27.33 mg/L), dissolved oxygen (4.23 – 5.57 mg/L), total dissolved solids (56 – 184 mg/L), total hardness (78 – 519 mg/L) and alkalinity (50.67 – 146.67 mg/L). Total coliforms were detected in counts ranging from 49 to 136 CFU/100 mL of water sample. One-way ANOVA showed that pH, temperature, electrical conductivities, nitrates, nitrites, chloride, dissolved oxygen, total dissolved solid, total hardness and alkalinity were significantly different ($P < 0.05$), whereas turbidity and sulphate were not significantly different ($P > 0.05$) from each parameter with respect to sampling sites. Generally, results from this study suggest that the waterbodies are not suitable for consumption, domestic or recreational use and re-echo the importance of safeguarding the freshwater resources of Southwestern Nigeria.

Key words: Rivers, Water Quality, Parameters, Coliforms, Pollution, Nigeria.

3.1 Introduction

Rivers are the most important freshwater resources for man. Social, economic and political developments have been largely dependent on the availability and distribution of freshwaters contained in riverine systems and river systems have been considered as arteries of the land supplying life giving water to an abundance of organisms while at the same time supporting modern civilizations (King et al. 2003). They are self-sustaining and vital ecosystems that are capable of supporting plant and animal lives indefinitely if left undisturbed (Venkatesharaju et al. 2010). Water is required by all living things for cellular metabolism. Activities such as domestic, agriculture, industry, transportation and recreation, upon which the continuous existence of man on this planet relies, require good quality water (Awomeso et al. 2010).

Globally, most rural dwellers largely source their water supply from rivers, dams, springs or shallow dug wells (WHO/UNICEF 2006; Aneck-Hahn et al. 2009; Sun et al. 2010), with a concomitant increase in pollution from urban, industrial and agricultural sources rendering the available water resources useless and hazardous to consumer's health (Contaminated surface water 2008). Sources of surface and ground water have become increasingly contaminated due to increased industrial and agricultural activities (Oluduro and Aderiye 2007). The increase in anthropogenic activities as well as natural processes such as precipitation, erosion, weathering of crustal materials and degradation of surface waters have rendered most water bodies unsuitable for their multi-purpose usage (Furhan et al. 2004).

Water intended for human consumption should be safe, wholesome, easily accessible, and adequate in quantity, free from contamination and readily available (CCME/WQI 2005). Although, water has been internationally recognized as a fundamental human right (WHO 2003), owing to its essentiality in human wellbeing and life's sustenance, nonetheless, it has also has

long been suspected of being responsible of many human illnesses. The major hazard in drinking water supplies is microbial contamination, which is due to agricultural land wash, domestic sewage, industrial effluents, improper storage and handling of food (Saha et al. 2006; WHO 2006). Water quality is at present a global issue, especially when considering its implications to humanity in terms of water-borne diseases. The deterioration of water quality as a result of pollution and contamination of ground and surface waters, has led to the destruction of ecosystem balance. Water quality can be regarded as a network of variables (pH, oxygen concentration, temperature, etc) that are linked and co-linked, and any change in these physical and chemical variables can affect aquatic biota in a variety of ways (Annalakshmi and Amsath 2012).

About 1.6 billion children under the age of 5 years die annually due to unsafe drinking water, coupled with a lack of basic sanitation (WHO/UNICEF 2006). In the developing nations alone, almost 5 million deaths annually are due to water-related diseases as water quality challenges are affecting virtually all of the developing world's major rivers (Contaminated surface water 2008). Microbiological examination of river water is obligatory for use-related purposes such as drinking water production, irrigation and recreation (Kolarević et al. 2011). Investigations in hygiene, sanitation and water supplies have proved to control these diseases while universal access to safe drinking water and sanitation has been promoted as an essential step in reducing the preventable diseases (WHO 1994, 2001).

Unfortunately, in many countries around the world, including Nigeria, drinking water supplies have become increasingly compromised by pollution. As a result of inadequate treatment and disposal of waste from human activities, industrial discharges, contaminants such as bacteria, viruses, heavy metals, nitrates and salts have found their ways into water supplies (Sorabjeet and

Luke 2003). Nigeria, despite being the largest African country and the continent's biggest oil exporter, it has been characterized by low levels of access to an improved water source with her coverage rates being amongst the lowest in the world. Access to improved water supply in Nigeria was 47% in 1990, with 79% (27 million) of the urban population of 34 million having access, compared to 30% (19 million) of the 63 million people living in rural areas. By 2008 the percentage of the population with access had increased to 58% (86 million), spread across 75% of the urban population and 42% of the rural population (UNICEF/WHO 2010).

Due to inability of the governments to meet the ever increasing water demand, some communities resort to surface water sources as alternatives sources of drinking water, and Osun State is not exempted from this development. Available data from Targets of the State Water and Sanitation Projects reveal that access to clean water in rural and urban areas is still far from being met. Hence, in this paper we report on the physicochemical and microbiological qualities of some typical rivers in Osun State, Southwestern Nigeria in view of the increasing dependence by the populace on the rivers for their daily water needs.

3.2 Materials and Methods

3.2.1 Description of the study area

Osun State is located in the Southwestern region of Nigeria. It covers an area of approximately 10,456 square kilometers and bounded by Ogun, Kwara, Oyo and Ondo states in the South, North, West and East respectively. Osun State, with co-ordinate $7^{\circ}30'N4^{\circ}30''E$, nicknamed state of the living spring; an inland state with a population of 3, 999,800 people (NBS 2006). The main sources of water supply in the state are by impounding rivers and construction of dams on the major rivers in the state with the state water scheme accounting for about 39% of the total design capacity and actual production level of all the water schemes in the state. Consequently,

the communities have resorted to depending on the surrounding rivers for their daily needs. (Adedeji and Ajibade 2008). Table 3.1 below shows the morphometric details of the sampling sites and their geographic co-ordinates.

Table 3.1 Morphometric details of the sampling sites.

Sampling site	Site name	Description	Coordinates
R1	Erinle-Ede	The *River Erinle* which rises just South of Offa. Its elevation is 269 m and has residential, commercial and industrial areas. The old and new dams on the river supply water to the neighbouring settlements including Osogbo, the state capital.	7°44′44″N; 4°29′22″E
R2	Ido Osun	The *River Ido Osun* has an elevation of 270 m, takes its source from the Erinle river, asides being the closest. Activities sighted along the river courses include animal rearing, fishing, irrigation domestic and recreations.	7°49′01″N; 4° 26′41″E
R3	Osun-Osogbo	The *River Osun* has an elevation of 320 m. Its drainage system rises in Oke-Imesi ridge, about 5 km north of Efon-Alaiye, Ekiti state of Nigeria, and flows north through the Itawure before winding its way Westwards through Osogbo and Ede and southwards to lower course where it empties into Lekki lagoon in Lagos state and the Atlantic Gulf of Guinea. Its global popularity lies in its sacredness as a national and international tourist center and worship shrine. Agricultural activities such as farming and fishing take place along the river courses.	7°46′04″N; 4°34′00″E
R4	Oba-Iwo	The *River Oba* has an elevation of 226 m. It is located in Iwo township, the most populous Local Government in the State and with a density of 245 sq km and a population of 191,348. The town is reliant on the river for its domestic and agricultural activities.	7°38′01″N; 4°11′20″E
R5	Ejigbo	The *River Ejigbo* has an elevation of 354 m. The characteristic heavy annual rainfall in the area greatly supports the development of thick forest and the production of food and cash crops and varieties of fruits. Waters used by women for the processing of palm oils are sourced from the river and the availability of the river has been the strong pillar behind the large-scale fishing and fishing business in the zone. In 2008, Ejigbo was ranked highest producer of life cat fish in Osun State.	7°54′0″N; 4°18′54″E
R6	Ilobu-Okinni	The *River Ilobu* is watered by Ojutu River, Erinle River, Konda River and a few other streams. It is located in a sparsely forested area and bounded in the north by Ifon-Osun, south by Osogbo, east by Oba and west by Erin-Osun. Its elevation above sea level is 301 m. Farming and animal rearing are linked to presence of the river in the zone. Many ugly and undesirable practices such as car washing, swimming and bathing by farmers and bricklayers take place within and around the river.	7°50′06″N; 4°29′14″E

Table 3.1 cont'd Morphometric details of the sampling sites.

Sampling site	Site name	Description	Coordinates
R8	Shasha	The *River Shasha* has an elevation of 208 m, located in Edun Abon in Ife North Local Government Area of the state, a settlement inhabited by the Igbo people whose origin are traced to the Eastern and Southeastern parts of Nigeria. The river is believed to be friendly to all in that, it is harmless, does not discriminate and ever ready to proffer solutions to human problems willingly and freely especially to waiting mothers who only need to collect and drink for conception. Cultivation of food crops, animal rearing and processing of palm oils are some of the activities surrounding the catchment.	7°22′32″N; 4°28′11″E
R9	Ila-Oke Ila	The *River Ila/Oke Ila* has an elevation of 550 m. The availability of the river has greatly enhanced the large scale cultivation of palm tree plantation which in turns support the common traditional profession of the indigenes of the town, *palm-wine tapping*.	8°01′50″N; 4°59′03″E
R10	Inisha-Okuku	The *River Inisha/Okuku* has an elevation of 352 m above sea level. The river is 36 km long and on it was impounded a reservoir with a large capacity designed to supply potable water to the neighbouring communities. Other benefits derived from the rivers are fishing, irrigation and domestic activities.	8°01.32″N; 4°42′11″E

3.2.2 Sample collection and processing

Triplicate water samples were collected from ten different rivers in the state over a period of 3 months (March to May, 2012). The samples were collected in the morning using sterile 5 L plastic containers. Sampling was done by dipping sterile sample bottles at approximately 20-30 cm below the surface of the water after which they were transported in cooler boxes to the laboratory for analysis within 6 h of collection using the recommended procedure (APHA 2005).

3.2.3 Determination of physicochemical parameters

The pH of the samples was determined using Jenway pH meter (3310 model) already standardized by using standard analytical grade buffer solutions of pH 4.0, 7.0 and 10 values before analyses (Skoog et al. 1988). Temperature was determined using calibrated mercury thermometer (Jenway 3015 model) at the sampling sites (EPA 1998). Turbidometer was used to measure turbidity of the water samples while electrical conductivity and total dissolved solids were determined by using HACH conductivity meter (4510 model) following the procedure of

Richard (1954). Dissolved oxygen content of the samples was determined using digital dissolved oxygen meter. Nitrate, nitrite and sulphate contents were determined using calibrated V2000 multi–analyte photometer, while the concentrations of chloride, total hardness and alkalinity were determined using the standard titrimetric methods (APHA 1999).

3.2.4 Enumeration of total coliform populations

The most probable number (MPN) technique was employed for the total coliforms. This was achieved using multiple 5×5 tube regimen with MacConkey broth as the cultivation medium (Usharani et al. 2010). The bottles were incubated and observed at the end of 24 and 48 hours for presumptive and confirmatory tests respectively. The numbers of positive bottles indicated by colour change and gas formation were recorded and counts estimated from the MacCrady interpretative chart. The MPN of the coliform populations was expressed as CFU/100 mL.

3.2.5 Statistical Analysis

Statistical analysis was performed using IBM Statistical Package for Social Sciences [(SPSS) Version 20 software]. Data obtained in this study were subjected to descriptive statistical analysis (95% confidence interval). The one-way analysis of variance (ANOVA) was done to test differences among the parameters measured with respect to sampling locations. Statistical significance was set at P values < 0.05. Correlation between the physico-chemical properties and total coliforms was observed using a 2-tailed Pearson's correlation analysis. Correlations and test of significance were considered statistically significant when P values were < 0.05 and 0.01.

3.3 Results and Discussion

Table 3.2 shows the results of the physicochemical parameters analyzed together with the WHO standards and statistical *P* values while Figure 3.1 shows the mean values obtained for total coliform counts of the river water samples.

Table 3.2 Mean values of physicochemical parameters of river water samples from Osun State, South-Western Nigeria.

Parameter	**WHO limits**	**Sampling sites**										***P* value**
		R1	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**	**R8**	**R9**	**R10**	
pH	6.5 – 8.5	7.1 ± 0.1	7.1 ± 0.1	6.9 ± 0.1	7.4 ± 0.0	7.4 ± 0.0	7.1 ± 0.1	7.3 ± 0.1	7.3 ± 0.1	7.6 ± 0.0	7.2 ± 0.2	0.000
Temperature (^{o}C)	25-30	28 ± 0	29 ± 1	29 ± 0	26 ± 1	26 ± 0	26 ± 1	27 ± 0	27 ± 0	27 ± 0	27 ± 0	0.000
Turbidity (NTU)	5	5.04 ± 0.7	4.64± 0.2	9.46± 0.3	2.28± 0.2	6.55± 7.8	3.30 ± 2.1	2.65± 0.3	4.48± 1.2	2.99± 0.5	3.13 ± 0.6	0.078
EC (µS/cm)	600	256 ± 9	341 ± 4	300 ± 4	581 ± 18	574 ± 6	229 ± 5	325 ± 2	382 ± 2	502 ± 3	425 ± 7	0.000
Nitrate (mg/l)	5	0.03 ± 0.0	0.05± 0.0	0.04± 0.0	0.03± 0.0	0.04± 0.0	0.03± 0.00	0.03± 0.0	0.04± 0.01	0.04± 0.00	0.03 ± 0.0	0.000
Nitrite (mg/l)	≤ 0.1	0.01 ± 0.0	0.01± 0.0	0.01± 0.0	0.01± 0.0	0.01± 0.0	0.01± 0.00	0.01± 0.0	0.01± 0.00	0.01± 0.00	0.01 ± 0.0	0.000
Sulphate (mg/l)	100	16.33± 7.2	15.67± 6.7	16.67± 8.1	12.00± 8.5	20.33± 11.5	21.00± 10.0	19.00± 16.8	11.33± 15.5	3.33± 4.93	18.00± 13.9	0.706
Chloride (mg/l)	250	8.17 ± 0.8	15.50± 0.5	14.50± 1	27.33± 0.3	27.17± 1.3	7.83 ± 0.3	8.83± 0.6	8.33± 0.3	8.83± 0.6	23.67± 16.8	0.000
DO (mg/l)	≥ 5	4.40± 0.17	4.97± 0.23	4.73± 0.15	5.27± 0.23	5.57± 0.55	4.50± 0.10	4.77± 0.58	4.23± 0.06	5.01± 0.95	5.00± 0.10	0.008
TDS (ppm)	1000	64 ± 30	71 ± 19	56 ± 10	184 ± 12	178 ± 1	121 ± 7	67 ± 12	80 ± 4	64 ± 9	80 ± 16	0.000
Total hardness (mg/l)	500	144 ± 17	145 ± 12	97 ± 16	461 ± 24	341 ± 19	79 ± 13	299 ± 18	339 ± 46	519 ± 20	281 ± 16	0.000
Alkalinity (mg/l)	200	50.67± 6.11	52.00± 4.00	57.27± 5.80	90.00± 0.00	84.67± 3.03	46.00± 3.46	62.00± 3.46	100.00 ± 2.00	146.67± 9.02	95.33± 3.06	0.000

The pH measures the intensity of acidity and alkalinity of water. In the present study, the pH obtained in all the sites ranged from 6.9 to 7.6 and fell within the recommended limits of 6.5-8 by WHO (2008) for drinking water. While the lowest pH was obtained at R3, the highest was at R9. The pH regimes in this study showed significant difference in all the ten sampling sites ($P < 0.05$) (Table 3.2). Generally, most of the water samples are slightly basic and this is in agreement with reports from similar studies (Aremu et al. 2011; Edimeh et al. 2011). Changes in pH are brought about by photosynthesis, respiration, temperature exposure to air, disposal of industrial wastes, geology and mineral content of the catchment area, acid mine drainage, agricultural run-off, carbon dioxide concentration in the atmosphere, and accumulation and decomposition of organic detritus in the water producing weak carbonic acids that impact on pH (Sibanda et al. 2014).

Water temperature is an important factor that influences the rate of all biological activities. It can therefore be used as a first step in predicting the effects of man's activities on the aquatic ecosystem (Rivers-Moor et al. 2004). The mean temperature values obtained in this study ranged from 26 – 29 ^{o}C. The maximum surface water temperature was observed at R3 whereas the minimum was at R6. There was a significant difference in the temperature regimes by sampling sites ($P < 0.05$) (Table 3.2). Most of the sites are covered by thick forests whose upper canopies prevent sunlight penetration, cast shadow upon the water surfaces and thereby cooling the waters. Temperature generally drives the chemical reactions in an aquatic system and warmer temperatures (> 25 ^{o}C) may influence the toxicity of some substances like ionized ammonia that may be found dissolved in water (Hargreaves and Tucker 2004). However, in this study, the temperature regimes in all the sampling sites were within the acceptable limit (25 – 30 ^{o}C) for domestic water uses.

Turbidity of water is the expression of optical property in which the light is scattered by the particles present in the water (Verma et al. 2012). It is an indication of the amount of the materials suspended in water (Brown and Caldwell 2001). The mean turbidity values of the river water samples ranged from 2.28 to 9.46 NTU. The highest value was recorded at R3 and the lowest at R4. Sampling sites R1, R3 and R5 turbidities exceeded the WHO acceptable limit of 5 NTU while others fell within acceptable limit. These results did not show any significant difference at the 95 % confidence limit in the sampling locations (Table 3.2). Increase in turbidity is observed when the colour of the water changes from white to light-yellowish, reddish or grayish. Highly turbid water has an altered odour, taste and its visual properties are negatively impacted and where necessary, will significantly increase water treatment costs due to the amount of flocculants needed to clarify the water (Osode and Okoh 2009). Light penetration is also highly affected by turbidity and higher levels of turbidity strongly associate with higher levels of pathogenic microorganisms such as bacteria, viruses and parasites. Thus the occurrence of higher indicator bacterial numbers in any water sample could be linked to the higher turbidity levels (EPA 2009; Momba and Kaleni 2002). Suspended silt and clay, organic matter, and plankton can contribute to turbidity; hence, turbidity in a stream will fluctuate before, during and after storm flow (Igbinosa and Okoh 2009).

Electrical conductivity (EC) estimates the amount of total dissolved salts, or the total amount of dissolved ions in the water and is controlled by, among other factors; the geology of the catchment area which determines the chemistry of the watershed soil and ultimately the water (Chang 2008). The overall conductivity obtained in this study ranged from 229 to 581 µS/cm and fell within the WHO maximum permissible limits (600 µs/cm) for drinking water. A significant difference was observed to EC values in all the sampling sites ($P < 0.05$) (Table 3.2). With

respect to EC, the results obtained in this study suggest that the river waters could be fit for domestic use. The low EC values observed in all the sampling points could indicate the presence of low amount of dissolved inorganic substances in the samples (Murhekar et al. 2011). Previous findings indicate that sewage disposal tends to increase the EC levels of the receiving water body because of the high concentrations of salts and ions in the sewage (Suthar et al. 2010). Sewage disposal and urban surface run-off in the downstream stretch of the river contributes to the increase in conductivity. Similar findings have been observed elsewhere (Igbinosa and Okoh 2009). High EC levels can be disastrous to aquatic life because of increased salinity in the stream and possible smothering of the stream bottom, especially if the stream is deep (DWAF 1996a).

Nitrates indicate the presence of fully oxidized organic matter. The mean nitrate values obtained in the river water samples were lower than the WHO permissible limits for drinking water. Nitrates however were noted in low concentrations within the range of 0.03 and 0.05 mg/L water and fell within the water quality target of 5.0mg/L (WHO 2002). This implies that water samples analysed contained low level of oxidized organic matter. There was a significantly higher difference ($P < 0.05$) of nitrates at all sampling sites (Table 3.2). With regards to the extremely low values obtained for nitrate, the waters could be suitable for sundry purposes. Nitrates themselves are relatively non-toxic and normal individuals have low levels (0.5–2 %) of methaemoglobin in their blood (EPA 2007). When in excess, they may result in eutrophication which leads to loss of diversity in the aquatic biota and overall ecosystem degradation through algal blooms, excessive plant growth, oxygen depletion and reduced sunlight penetration (Odjadjare and Okoh 2010). Levels above permissible limit are considered harmful to aquatic lives (Suthar et al. 2008). Excess levels of nitrates (above 10 mg/L) cause Methemoglobinemia; a blue baby syndrome in which blood loses its ability to carry sufficient oxygen (Burkar and

Kolpin 1993). Although nitrates levels that affect infants do not pose a direct threat to older children and adults, yet they do indicate the possible presence of other more serious residential or agricultural contaminants such as bacteria or pesticides (Robert 2006).

Approximately, nitrite levels complied with the acceptable limit (0.5 mg/L) during the study as the same concentration was recorded. Nitrite concentrations were significantly different at all the sampling sites ($P < 0.05$) (Table 3.2). This indicates that most of the rivers receive very low amount of organic materials. The low concentrations of nitrite obtained in this study depict low anthropogenic activities. However, nitrite easily changes to nitrate as the end product of the oxidation of organic nitrogen and ammonia, and may therefore unable to pose a health risk in the case of people using the raw water since the detected nitrate levels were within the safety guidelines set for water intended for human consumption. Nitrites could be toxic at certain concentrations and cause damage to gills as well as diffusing in to red blood cells resulting in the formation of methhaemoglobin; which lacks the ability to bind with oxygen thereby resulting in stunted oxygen transport. High nitrite level is considered to pose a problem to communities when the receiving water bodies are used for domestic purposes (Fatoki et al. 2003). It could also lead to death due to anoxia during highly active periods (Dallas and Day 1993).

Sulphates are formed due to the decomposition of various sulphur containing substances present in water bodies. The sulphate ions (SO_4^{-2}) occur naturally in most water supplies and hence present in natural water bodies. The mean values obtained for the sampling locations were found between the range of 3.33 and 20.33 mg/l. The results revealed that sulphate concentrations in the river waters did not differ significantly in the sampling sites ($P > 0.05$) (Table 3.2). The concentrations obtained in this study are low compared to the WHO permissible limit of 250 mg/L. This could be an indication that sulphate easily precipitates and settles to the bottom

sediment of the river and are derived from discharge of domestic sewage, surface runoff and agricultural activity and are brought into the rivers (Abdul et al. 2009).

Generally, nutrients are naturally present in the environment and their cycling processes prevent accumulation of very high concentrations of the nutrients. However, human activities have increased environmental nitrate and nitrite concentrations, with agriculture being the major source (Castillo et al. 2000; Ferrier et al. 2001). This includes increased use of nitrogen-containing fertilizers as well as concentrated livestock and poultry farming; the latter two produce millions of tons of nitrate-containing manure each year (EPA 2007). Nitrate and nitrite compounds are highly soluble in water and quite mobile in the environment (Blanchard and Lerch 2000). They have a high potential for entering surface water during rainfall events, as nitrates in applied fertilizers can dissolve in runoff that flows into streams (Brainwood et al. 2004). Sulphur is an important building block of proteins and many other organic compounds. Within water, it occurs mostly as sulphate ion, though non-toxic, but its excess forms sulphuric acid (H_2SO_4) which has a devastating effect upon aquatic ecosystems and also diarrhoea can be caused due to higher amount of sulphate in drinking water. High sulphate levels are capable of causing bad odour (Taylor et al. 1984; Nussey 1998).

Chloride is an important anion used for determining total salinity of water and serves as an indicator of pollution by sewage (Solanki et al. 2007). In this present work, the chloride concentrations ranged from 7.83 to 27.33 mg/L, and were within the allowance of 250mg/L set limit. Chloride concentrations recorded at all sampling locations were significantly different ($P < 0.05$) (Table 3.2). People habituated to higher chloride in water are prone to laxative effects. Low chloride concentration in the water indicates the absence of organic wastes because the greater source of chlorides in freshwater is disposal of sewage and industrial waste. Human body also

releases very high quantity of chlorides through urine and faeces (Julie and Vasantha 2010). The presence of chlorides in natural waters is due to leaching of chloride-containing rocks and soils with which the water comes in contact. Chlorides are the most stable components in water and its concentration is largely unaffected by most natural physico-chemical and biochemical processes. Hence the value of its concentration in water is a useful measure in water sample (Aremu et al. 2011). Dissolved oxygen (DO) is very crucial for the survival of aquatic organisms and used to evaluate the degree of freshness of a river (WHO 1999). It is such a fundamental factor for metabolism of the aerobic aquatic organisms and its distribution affects the solubility of nutrient (Agbabire and Obi 2009). DO levels are important in determining the natural self-purification capacity of a river (Mukherjee et al. 1993).

In this study, the dissolved oxygen (DO) content varied in a range of 4.23 to 5.57 mg/L. (Table 3.2). Sampling sites R4, R5, R9 and R10 concentrations exceeded the acceptable limit which is (≥ 5mg/L) while those of other sites fell within. Measurement of dissolved oxygen is a primary parameter in all pollution studies. The quantity of DO in streams is dependent on the water temperature, the amount of sediment, level of oxygen taken out of the system by respiring and decaying organisms, and the amount of oxygen restored back into the system by plants' photosynthesis, stream flow, and aeration (Brown and Caldwell 2001).The results imply that rivers in Osun state are clean with respect to organic pollution (Bhutiani and Khanna 2007; Kannel et al. 2007). The high temperature, addition of sewage and other waste might be responsible for low values of DO in some sites. Good levels of DO in all the sampling sites of the river may also be indicative of high re-aeration rates and rapid aerobic oxidation of biological substances (Suthar et al. 2010). Depletion of dissolve oxygen in water is due to high temperature and increased microbial activity (Kataria et al. 2006). The threshold limit for DO is 5.0 mg/L in

drinking water and should be greater than 5.0 mg/L in water to be used for agricultural purposes (Cruise and Miller 1994). Although there is no health guideline for DO content in water but low concentration in water supplies may encourage microbial reduction of nitrate to nitrite and sulphate to sulphite and can also cause an increased ferrous iron concentration in solution with subsequent discoloration at the tap point (WHO 2006). Low DO concentrations (below 70% saturation) may result in anaerobic conditions and cause bad odour in water and when combined with the presence of toxic substances, they lead to stress responses in aquatic ecosystems because the toxicity of certain elements, such as zinc, lead and copper (not included in this study) is increased by low concentrations of DO (EPA 1986; Adekunle et al. 2007). Low concentrations of DO, associated with high water temperature, also increase the adverse effects on biota (Enderlein 1996). The water quality criterion for DO, therefore, cannot be taken independently of other water quality determinants.

Another evidence of pollution is high level of total dissolved solid (TDS) which indicates materials carried in suspended form (Amadi et al. 2006) and waters with high TDS are potentially unhealthy (Brown and Caldwell, 2001). TDS levels ranged from 56 to 184 ppm and statistically significant across the sampling sites ($P < 0.05$) (Table 3.2). WHO has suggested a guideline value of 500 ppm of (TDS) for drinking water. TDS values for this study fell within stipulated guidelines (WHO 2006). Low level of TDS shows good quality river waters, while very high value of TDS confirm the observed high conductivity value of the drinking water samples. Increase content of TDS elevates the density of water and such a medium increase in osmo-regulation. As this parameter indicates, these rivers are suitable for domestic and agricultural purposes. This finding strongly agrees with the reports of other researchers (Shrinivasa and Venkateswaralu 2000; Adekunle et al. 2007). Water containing more than 500

ppm of TDS is not considered desirable for drinking water supplies, but in inevitable cases 1500 ppm is also allowed (Shrinivasa and Venkateswaralu 2000). TDS is the sum total of all of the dissolved substances in a given body of water and includes hardness, alkalinity, chlorides, bromides, sulphates, silicates and all manner of organic compounds. Although elevated TDS concentration may not mean that the water is hazardous to health, it however does mean the water may have aesthetic hitches or cause nuisance problems. These problems may be associated with staining, taste, or precipitation. With respect to trace metals, elevated TDS may suggest that toxic metals may be present at an elevated level (Adekunle et al. 2007).

Water hardness is a traditional measure of the capacity of water to react with soap. Hard water requires a considerable amount of soap to form lather. Hardness of water is not a specific constituent but a variable and complex mixture of cations and anions (Angadi et al. 2005). It mainly depends on the amount of calcium or magnesium salts or both. In the present study, the overall mean value for total hardness ranged from 78 to 519 mg/L, and was significantly different by sampling sites ($P < 0.05$). Aside site R9 whose concentrations exceeded the specification limit of 500 mg/L for drinking water, others fell within (Table 3.2) (WHO 2006), although they are sufficient enough to cause water hardness. This further explains the presence of carbonates/bicarbonates which may cause poor lather formation and scales on boilers (Durrance 1986). The high level of calcium, magnesium, aluminum, potassium and sodium may be attributable to natural processes such as weathering of rocks, erosion, human activities like mining, quarrying, farming, calcium laden dust and leaching of rocks (Umeham and Elekwa 2005; Akubugwo et al. 2007; Singh et al. 2008).

Alkalinity in natural water is due to free hydroxyl ion and hydrolysis of salts formed by weak acid and strong bases and also due to salt containing carbonates and bicarbonates or silicate and

phosphate along with hydroxyl ion. Change in carbonates and bicarbonates also depend upon release of CO_2 through respiration (Chaurasia and Pandey 2007). Concentrations of for total alkalinity for the investigated water samples ranged from 50.67 to 146.67 mg/L and were found within the prescribed limit of 200 mg/l (WHO 2006). They also ranged significantly at all the sampling sites ($P < 0.05$) (Table 3.2). The trend indicates the low level of carbonates and bicarbonates in the water and it concurs to what has been reported elsewhere (Abulude et al. 2007; Yusuf 2007). The relatively high alkalinity (> 100 mg/L) observed at sites R7 and R8 might likely be due to the presence of carbonates and bicarbonates in the water samples as they contribute to water hardness (Magit et al. 2002). High alkalinity (above 500 mg/L) is usually associated with high pH, hardness and dissolved solids values in water.

Coliforms are a broad class of bacteria found in the environment, including the human excreta and other warm-blooded animals. Their presence in water suggest the possibility of the presence of pathogens and parasites, with the associated disease symptoms such as diarrhoea, nausea, vomiting, cramps and other gastro intestinal distresses and in severe cases can be fatal (Kilmaren 2011). The result of total coliform (TC) populations in the water samples were found to be generally high and ranged between 49.00 and 136.00 (CFU/100 mL) (Figure 1). The modal TC counts were obtained at site R2 and the least at site R10 (Figure. 3.1). Results of this study indicated that the river water sources were of poor microbiological quality. Although TC counts varied from site to site, however, sites R1, R2 and R7 seemed to be more polluted compared to others. From the figure it was evident that the river waters are highly contaminated, and also denote public health hazards. The maximum permissible value of total coliforms in drinking is 10 per 100 mL (WHO 1993). In the present study, the total coliform counts obtained in all the sampling sites exceeded the set limit. Presence of coliform organisms in water has been regarded

as evidence of faecal contamination. This clearly suggests that the river waters were contaminated by domestic sewage, human and animal excreta, which are objectionable for drinking purposes and also render the waters unsuitable for domestic use.

The total coliform counts obtained for each sampling location are presented in Figure 3.1 below.

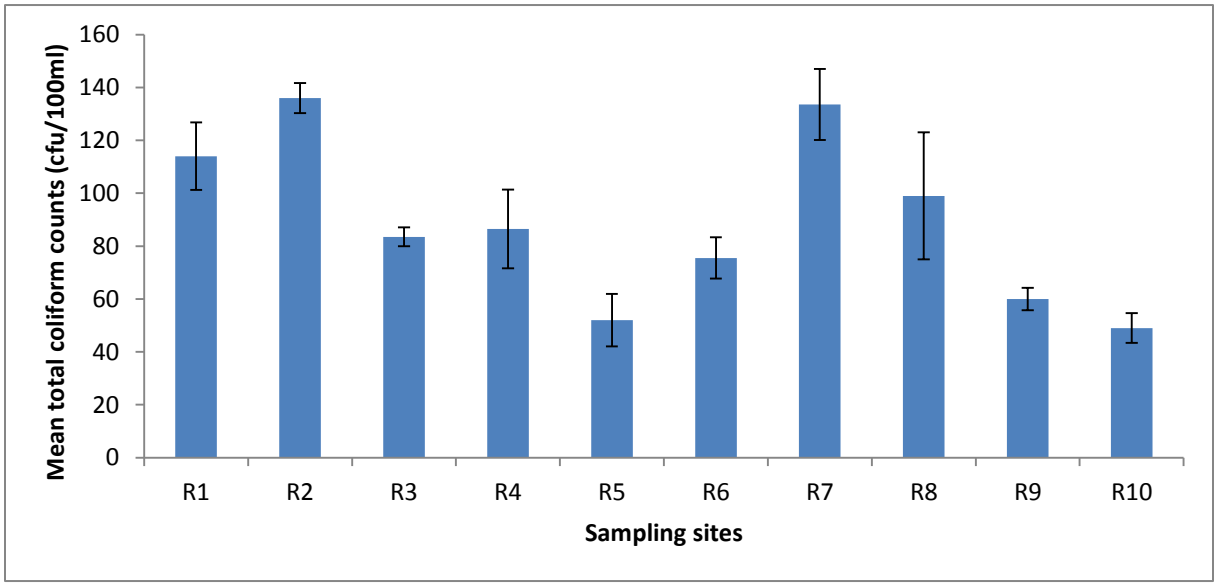

Figure 3.1 Mean values of total coliform counts obtained for each sampling location.

The occurrence of total coliforms in the studied rivers could be attributable to some major factors stated by Fatoki et al. (2001) that inflow of domestic and industrial wastes, anthropogenic activities, discharges from sewage treatment plants and runoff from informal settlements are responsible for microbiological quality of surface water. The ingestion of *E. coli* may result to haemolytic uremic syndrome, a disease similar to dysentery and potentially life-threatening. *E.coli* and *Klebsiella* bacteria may also cause urinary tract infections which primarily affect women, especially pregnant women, due to hormonal changes and physical pressure on the urinary tract (Azalea et al. 2010).

A number of diseases that claim millions of lives annually in developing countries have been attributed to unsafe drinking water and inadequate sanitation measures. The quality of water, whether used for drinking, domestic purposes, food production or recreational purposes has an important impact on health (Zamxaka et al. 2004). The microbiological quality of river systems is majorly influenced by human activities (WHO 1993). Results of the present study suggest pollutions in some of the rivers, even at some points where human activities have not been sternly impacted on the river. Total coliform test is one of the most important biological parameters in drinking water quality. In the present study, the total coliform counts were ranged from 49 to 136 CFU/mL. This result is worrisome, considering the relatively high dependence on the river waters by the populace.

The correlation analysis output for both microbiological and physicochemical data of the selected rivers are performed and presented in Table 3.3. Some significant positive correlations were obvious. DO was positively correlated with TDS and TH at 99 % confidence limit. Likewise, temperature was positively correlated with nitrate and nitrites at the 95 % confidence level. This result disagrees with the findings of Badran (2001) and Manasrah et al. (2006) that nutrient consumption by primary producers increased in favourable temperature condition. Also, nitrates and nitrites were positively correlated with each other, and this is not surprising because nutrients are naturally found in the environmental waters even if they are from different sources.

Similarly, correlation of EC with chloride, DO, TDS, TH and alkalinity was positively significant. This finding strongly concurs with the report of Sunitha et al. (2005) that EC finds higher level correlation significance with many of the water quality parameters like TDS, chlorides, total alkalinity, sulphates, total hardness and magnesium. Other highly significant

positive correlations were observed as follows: pH with EC, TH and alkalinity, chloride to DO and TDS, and TH with alkalinity, all at the 95 % confidence level.

Conversely, temperature was negatively correlated with EC and TDS at 95 % confidence level ($r = – 0.48$) and ($r = – 0.67$) respectively. Some significant negative correlations were equally noted between pH and temperature, temperature and TH, and sulphate and alkalinity at 99 % confidence level. Above all, correlation study and coefficient values help in selecting treatments to minimize contaminants in surface and ground waters (Achuthan et al. 2005).

Table 3.3 Correlation analysis output for microbiological and physicochemical data of selected rivers in Osun State, Nigeria.

	TC	pH	Temperature	Turbidity	EC	Nitrate	Nitrite	Sulphate	Chloride	DO	TDS	TH	Alkalinity
TC	1												
pH	-.021 .913	1											
Temperature	-.092 .628	-.428* .018	1										
Turbidity	-.193 .306	-.294 .115	.309 .097	1									
EC	-.051 .788	.612** .000	-.482** .007	-.128 .501	1								
Nitrate	-.016 .934	-.105 .582	.516** .004	.345 .062	-.023 .903	1							
Nitrite	-.104 .584	-.177 .351	.547** .002	.315 .090	-.013 .944	.930** .000	1						
Sulphate	-.124 .514	-.236 .210	.014 .944	-.010 .957	-.198 .294	-.100 .598	-.111 .558	1					
Chloride	-.168 .376	.012 .949	-.321 .084	.020 .917	.630** .000	-.086 .652	-.006 .977	.265 .157	1				
DO	-.166 .381	.291 .118	-.281 .133	.176 .352	.644** .000	.073 .700	.199 .291	-.075 .694	.556** .001	1			
TDS	.063 .741	.260 .165	-.668** .000	-.103 .589	.595** .001	-.316 .089	-.275 .142	.095 .618	.564** .001	.399* .029	1		
TH	.041 .829	.679** .000	-.416* .022	-.240 .201	.737** .000	-.095 .619	-.148 .436	-.344 .063	.297 .111	.443* .014	.298 .110	1	
Alkalinity	-.128 .499	.636** .000	-.339 .067	-.209 .269	.689** .000	.061 .750	-.022 .907	-.375* .041	.124 .515	.301 .106	.050 .792	.775** .000	1

TC: Total coliforms, EC: Electrical conductivity, DO: Dissolved oxygen, TDS total dissolved solids, TH: Total hardness
* Correlation is significant at 0.01 level (2-tailed).
** Correlation is significant at 0.05 level (2-tailed).

3.4 Conclusion and Recommendations

Water of poor quality can cause disease outbreaks and contribute to background rates of disease manifesting themselves at different times of life. Striving to manage the safety of water will not only support public health, but will also promote socio-economic development and well-being as well. This work has examined the physicochemical properties and total coliform densities of rivers which serve as the sources of water supply to the inhabitants of the state. Although, the results indicated that most of the physicochemical quality parameters of the sampled rivers fell within the acceptable limits for drinking water and, therefore, may be suitable for domestic purposes, nonetheless, those few that exceeded the set limits cannot be overlooked. Similarly, the microbiological quality of the rivers suggest potential health risk if consumed. This emphasizes the need for urgent intervention in the provision of safe water supply and adequate sanitation facilities for the state dwellers. In order to maintain good water quality safe for drinking, the waters obtained from these rivers must be adequately treated by the consumers. The national surveillance agency should set medium-term targets for the continuous improvement of water supplies and safeguarding of freshwater resources.

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Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER FOUR

Occurrence of virulence gene signatures associated with diarrhoeagenic and non-diarrhoeagenic pathovars of *Escherichia coli* isolates from some selected rivers in Southwestern Nigeria

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Occurrence of virulence gene signatures associated with diarrhoeagenic and non-diarrhoeagenic pathovars of *Escherichia coli* isolates from some selected rivers in Southwestern Nigeria

Abstract

Diarrhoeal diseases are attributable to unsafe water stemming from improper sanitation and hygiene and are reportedly responsible for extensive morbidity and mortality particularly among children in developed and developing countries. Water samples from selected rivers in Osun State, Southwestern Nigeria were collected and analysed using standard procedures. Confirmed *Escherichia coli* isolates (*n*=300) were assessed for the presence of 10 virulence genes (VGs) associated with *Escherichia coli* strains causing intestinal and extra-intestinal infections. While the VG *lt* for enterotoxigenic *E. coli* had the highest prevalence of 45%, the enteropathogenic *E. coli* genes *eae* and *bfp* were detected in 6% and 4% of the isolates respectively. The VGs *stx*1 and *stx*2 specific for the enterohaemorrhagic *E. coli* pathotypes were equally detected in 7% and 1% of the isolates respectively. Also, the VG *eagg* harboured by enteroaggregative pathotype and diffusely-adherent *E. coli* VG *daa*E were detected in 2% and 4% of the isolates respectively and enteroinvasive *E. coli* VG *ipa*H was not detected. In addition, the VGs *pap*C for uropathogenic and *ibe*A for neonatal meningitis were frequently detected in 19% and 3% of isolates respectively. These findings reveal the presence of diarrhoeagenic and non-diarrhoeagenic *E. coli* in the selected rivers and a potential public health risk as the rivers are important resources for domestic, recreational and livelihood usage by their host communities.

Keywords: Prevalence, *Escherichia coli* pathovars, Virulence genes, Diarrhoeagenic, Surface water.

4.1 Introduction

Globally, diarrhoeal diseases and other related gastrointestinal illnesses constitute one of the most important causes of illness and death in the world particularly among infants and young children [19, 26, 81], with most of such illnesses contracted through ingestion of polluted waters. Ascertaining the qualities of fresh and marine waters relies heavily on the use of *Escherichia coli* and *Enterococcus* spp. commonly found in mammalian faeces [62, 92]. *Escherichia coli* is the most abundant facultative anaerobe. Most are commensals in the human intestinal microflora, but certain strains have virulence properties that may account for life-threatening infections. The pathogenicity of a particular *E. coli* strain is primarily determined by specific virulence factors which include adhesins, invasins, haemolysins, toxins, effacement factors, cytotoxic necrotic factors and capsules [29, 50], and these have been implicated in human and animal diseases worldwide with the pathogenic strains being categorized into intestinal pathogenic *E. coli* (InPEC) and extra-intestinal pathogenic *E. coli* (ExPEC) on the basis of their virulence factors and clinical symptoms [45, 79]. InPEC can be further classified into enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) [40, 45, 59], and ExPEC into uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC) and avian pathogenic *E. coli* (APEC). Other diarrhoeagenic *E. coli* pathotypes have been proposed, such as cell-detaching *E. coli* (CDEC); however their significance remain uncertain [1, 19].

Common reservoirs of ETEC and EPEC include humans, ruminants, porcine, other domesticated animals such as goats, dogs and cats [23, 53, 59]. EHEC have been isolated from various

ruminants, primarily cattle [73]. The principal reservoir for EIEC, EAEC and DAEC are humans [45, 53]. While UPEC and NMEC are commonly isolated from humans, APEC have been attributed to avian infections from poultry [43, 45]. Enterotoxigenic *E. coli* (ETEC) have been found associated with infantile and traveler's diarrhoea; EPEC with acute infantile diarrhoea; EHEC with sporadic outbreaks of haemorrhagic colitis and hemolytic-uremic syndrome in humans; EAEC with persistent gastroenteritis and diarrhoea in infants and children and is prevalent in developing countries; and EIEC produces shigellosis-like diseases in children and adults, with invasive intestinal infections, watery diarrhoea, and dysentery in humans and animals [45, 59]. DAEC strains have also been associated with diarrhoeal disease in different geographic areas [42]. Uropathogenic *E. coli* (UPEC) enters the urinary tract and travels to the bladder to cause cystitis and, if left untreated, can ascend further into the kidneys to cause pyelonephritis. Septicaemia can occur with both UPEC and neonatal meningitis NMEC, and NMEC can cross the blood–brain barrier into the central nervous system, causing meningitis [20].

Contamination of surface waters with pathogenic strains of *E. coli* has been implicated in increasing number of disease outbreaks and deaths [27, 71]. Disease outbreaks related to exposure to contaminated freshwaters are well documented [2, 17, 71, 85]. Occurrence of pathogenic *E. coli* strains harbouring virulence genes (VGs) in environmental waters could be linked to contamination by storm events, faeces from domestic and wild animals as well as humans, runoffs from agricultural lands, sewage overflows, farm animals, pets and birds [14, 40, 72, 82, 86]. However, only a few studies have investigated the presence of *E. coli* strains carrying VGs in environmental waters [4, 18, 35, 36, 51, 56, 76]. Exposure to recreational waters has been linked to high numbers (21 out of 31) of reported *E. coli* O157:H7 disease outbreaks in

the United States from 1982 to 2002 [77]. Prevalence studies on the various *E. coli* pathotypes are important since it has been shown through various studies that the prevalence of diarrhoeagenic *E. coli* is region-specific [37]. Studies on the prevalence of DEC categories and their importance in diarrhoea have not been carried out extensively in Nigeria [8, 67, 68, 69, 70], with investigations on the southwestern axis being scantily documented on stool samples but not on environmental waters. A controlled study using the traditional culture/serology technique and polymerase chain reaction (PCR) was designed to ascertain the level and spectrum of bacterial pathogens and define the association of various categories of *E. coli* with diarrhoea in Enugu and Onitsha, Southeastern Nigeria [65]. To the best of our knowledge, no investigation on *Escherichia coli* pathotypes distribution has been carried out on the freshwater environments of Nigeria. Hence, in this paper, we report for the first time the prevalence and distribution of diarrhoeagenic *E. coli* pathotypes in surface waters in Osun State, Southwestern Nigeria.

4.2 Materials and Methods

4.2.1 Description of study area and collection of water samples

The ten river water sources used in this study are located in Osun State, Southwestern Nigeria. The state is an inland state with its headquarters located in Osogbo. It is bounded in the north by Kwara state, south by Ogun state, west by Oyo state and east partly by Ekiti and Ondo states. The sampling locations were coded as follows: R1: Erinle-Ede; R2: Ido-Osun; R3: Osun-Osogbo, R4: Oba-Iwo; R5: Ejigbo; R6: Ilobu-Okinni; R7: Asejire-Ikire; R8: Shasha; R9 and Ila-Oke Ila, R10: Inisha-Okuku (Figure 4.1).

Water samples were collected at each site in sterile 2.5 L clean bottles between September 2011 and August 2012 from ten sampling locations, transported to the laboratory on ice and processed

within 6 h of collection. Table 4.1 shows the site code, location, land use and potential sources of faecal pollution of each sampling site.

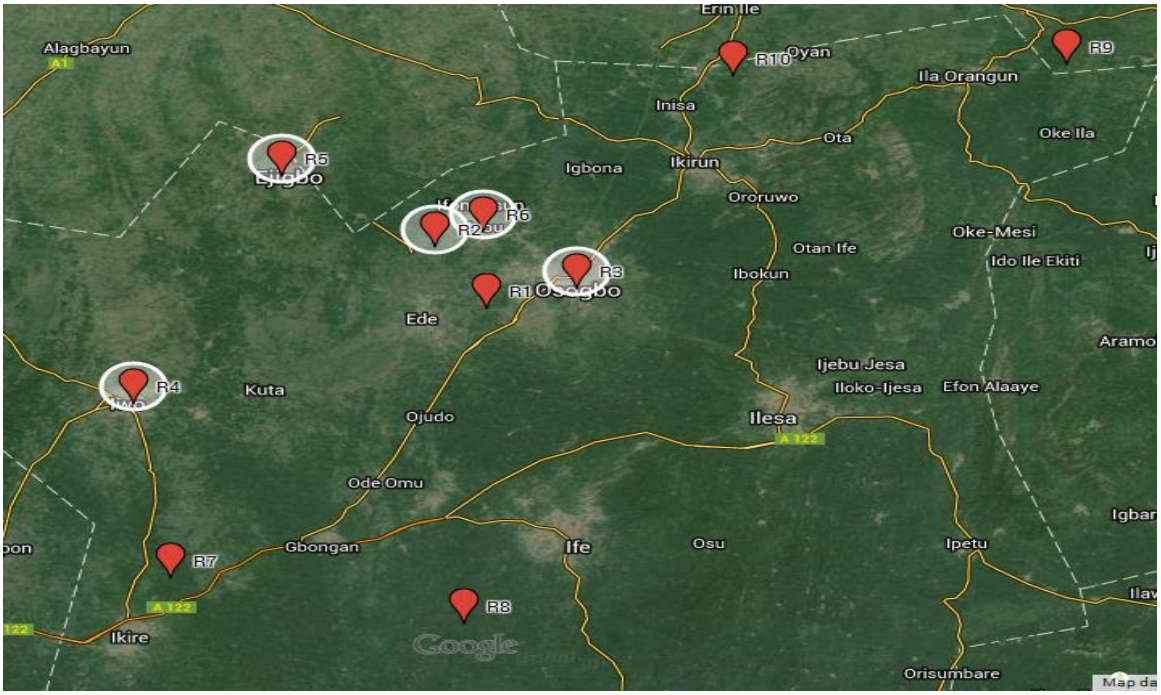

Figure 4.1 Map of Osun State Nigeria showing the locations of the sampling sites

Table 4.1 List of the 10 sampling sites, their location, land use and suspected sources of faecal contamination impacting each site

Site code	Location	Land use	Suspected sources of faecal pollution
R1	Erinle-Ede	Peri-urban	Tourists, coastal birds, cattle, run offs
R2	Ido Osun	Pasture	Fisher men, cattle, wild animals, run offs
R3	Osun-Osogbo	Urban	Farmers, tourists, wild animals, run offs
R4	Oba-Iwo	Peri-urban	Farmers, coastal birds, wild animals, run offs
R5	Ejigbo	Rural	Farmers and wild animals
R6	Ilobu-Okinni	Rural	Dilutions from vehicle washing centers, swimmers, animal inputs, run offs
R7	Asejire-Ikire	Peri-urban	Pollution by a bottling company, fisher men, coastal birds, wild animals
R8	Ishasha	Rural	Fisher men, dilutions from palm oil processing, swimmers, wild animals, run offs
R9	Ila-Oke Ila	Rural	Fisher men, farmers, cattle, wild animals
R10	Otin-Okuku	Pasture	Fisher men, farmers, cattle, wild animals, run offs

4.2.2 Isolation of presumptive of Escherichia coli

The standard membrane filtration method was used for the processing and quantification of presumptive *E. coli* in the water samples [9]. A volume of 100 ml of each water sample was filtered through 0.45 mm pore size nitrocellulose membrane filters (Millipore, Ireland). The filters were placed on eosin methylene blue agar (Oxoid, England), incubated overnight at 44.5°C for characteristic green metallic sheen colonies and thereafter counted. The isolates were further purified on *E. coli* chromogenic agar (Conda Pronadisa, Spain), incubated at 37°C overnight and individual well-isolated typical *E. coli* colonies were selected and transferred onto nutrient agar slants for further studies. A total of 480 presumptive *E. coli* colonies were isolated during the 12-month sampling regime.

4.2.3 PCR confirmation of E. coli and extraction of DNA

The presumptive *E. coli* were confirmed by polymerase chain reaction using the housekeeping 4-methylumbelliferyl-glucuronide (*uid*A) gene marker as previously described [91], and the positive isolates were preserved at -80°C in 20% glycerol. *E. coli* ATCC *25922* (ATCC, USA) was used as positive control. The bacterial genomic DNA was extracted using the boiling method as described elsewhere [28, 44], and the recovered DNA was used as template for amplification reactions.

4.2.4 PCR detection of virulence genes

Using a conventional singleplex PCR, confirmed *E. coli* isolates (*n* =300) were screened for the presence of 10 *E. coli* VGs for a number of adhesion, invasion and toxin determinants to correctly place them under the 8 pathotypes studied. The list of VGs, categorized on the basis of their functional characteristics and association with *Escherichia coli* pathotypes is shown in Table 4.2. The primers used for PCR detection of the VGs and other relevant characteristics are

listed in Table 4.3. For each PCR experiment, appropriate positive and negative controls were included. The PCR amplification was performed using a thermocycler system (Bio-Rad Thermal cycler, USA). Each 25 µl PCR mixture contained 12.5 µl of PCR master mix (Thermo Scientific, (EU) Lithuania), 0.5 µl each of primer (Inqaba Biotech, SA), 5 µl of template DNA and 6.5 µl of PCR grade water. To detect the amplified product, 5 µl of amplicons was visualized by electrophoresis through a 1.8% agarose gel (Merck, SA) at a voltage of 100 for 45 min in 0.5X TBE buffer and stained with ethidium bromide (Sigma-Aldrich, USA) using the gel documentation system (Alliance 4.7, France). Identification of the bands was established by comparison of the band sizes with molecular weight markers of 100-bp (Thermo Scientific, (EU) Lithuania). Samples were considered to be positive for a specific VG when the visible band was the same size as that of the positive control DNA. To minimize PCR contamination, DNA extraction, PCR set up, and gel electrophoresis were performed in isolated rooms. The positive controls were sourced from DSMZ Germany and included: DSM 10819 for NMEC; DSM 4819 for UPEC; DSM 8695 for EPEC; DSM 10973 for ETEC; DSM 10974 for EAEC; and DSM 10975 for EIEC except ATCC 35150 for EHEC from USA. There was no positive control available for DAEC but we went further to optimize the PCR condition of the related gene for possible detection of the expected amplicon band size.

Table 4.2 List of 10 virulence genes screened in this study, categorized based on their functional characteristics and association with *Escherichia coli* pathotypes.

Pathotype	Adhesion gene	Toxin gene	Invasion gene	Function
EPEC	*eae*			Intimin/Attaching and effacing
	bfp			Type IV bundle-forming pili
EAEC	*eagg*			Transcriptional regulator for chromosomal gene/ Enteroaggregative adhesion
EIEC			*ipa*H	Invasion plasmid antigen
ETEC		*lt*		Heat-labile toxin
DAEC	*daa*E			
EHEC		*stx*1		Shiga-toxin 1
		*stx*2		Shiga-toxin 2
UPEC	*pap*C			P fimbriae chaperone
NMEC			*ibe*A	Invasion of brain endothelium

Table 4.3 Primer sequences, expected amplicon sizes and their cycling conditions

Target strain	Target gene	Primer sequence (5'→3')	Amplicon size (bp)	PCR cycling condition	Reference
E. coli	*uid*A	F: AAA ACG GCA AGA AAA AGC AG R: ACG CGT GGT TAA CAG TCT TGC G	147	5 min initial denaturation at 94°C followed by 35 cycles of 95°C for 30 sec, 58°C for 1min, 72°C for 1 min and final extension at 72°C for 8 min	[9]
EPEC	*Eae*	F: TCA ATG CAG TTC CGT TAT CAG TT R: GTA AAG TCC GTT ACC CCA ACC TG	482	15 min initial denaturation at 95°C followed by 35 cycles of 94°C for 45 sec, 55°C for 45 sec, 68°C for 2 min and final extension at 72°C for 5 min	[87]
	Bfp	F: GGA AGT CAA ATT CAT GGG GGT AT R: GGA ATC AGA CGC AGA CTG GTA GT	300	2 min initial denaturation at 94°C followed by 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and final extension at 72°C for 5 min	[87]
ETEC	*Lt*	F: GGC GAC AGA TTA TAC CGT GC G: CGG TCT CTA TAT TCC CTG TT	450	2 min initial denaturation at 94°C followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and final extension at 72°C for 5 min	[54]
EAEC	*Eagg*	F: AGA CTC TGG CGA AAG ACT GTA TC R: ATG GCT GTC TGT AAT AGA TGA GAA C	194	15 min initial denaturation at 95°C followed by 35 cycles of 94°C for 45 sec, 55°C for 45 sec, 68°C for 2 min and final extension at 72°C for 5 min	[48]
EIEC	*ipa*H	F: CTC GGC ACG TTT TAA TAG TCT GG R: GTG GAG AGC TGA AGT TTC TCT GC	933	2 min initial denaturation at 94°C followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and final extension at 72°C for 5 min	[93]
DAEC	*daa*E	F: GAA CGT TGG TTA ATG TGG GGT AA R: TAT TCA CCG GTC GGT TAT CAG T	542	2 min initial denaturation at 94°C followed by 40 cycles of 92°C for 30 sec min, 59°C for 30 sec, 72°C for 30 sec and final extension at 72°C for 5 min	[93]
EHEC	*stx*1	F: CAG TTA ATG TGG TGG CGA AGG R: CAC CAG ACA ATG TAA CCG CTG	384	15 min initial denaturation at 95°C followed by 35 cycles of 94°C for 45 sec, 55°C for 45 sec, 68°C for 2 min and final extension at 72°C for 5 min	[16]
	*stx*2	F: ATC CTA TTC CCG GGA GTT TAC G R GCG TCA TCG TAT ACA CAG GAG C	584	2 min initial denaturation at 94°C followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and final extension at 72°C for 5 min	[16]
NMEC	*ibe*A	F: TGG AAC CCC GCT CGT AAT ATA C R: CTG CCT GTT CAA GCA TTG CA	342	2 min initial denaturation at 94°C followed by 30 cycles of 94°C for 1min, 55°C for 1min, 72°C for 1 min and final extension at 72°C for 5 min	[93]
UPEC	*pap*C	F: GAC GGC TGT ACT GCA GGG TGT GGC G R: ATA TCC TTT CTG CAG GGA TGC AAT A	328	2 min initial denaturation at 94°C followed by 30 cycles of 94°C for 1min, 55°C for 1min, 72°C for 1 min and final extension at 72°C for 5 min	[52]

4.2.5 Statistical analysis

Statistical analysis was performed using IBM Statistical Package for Social Sciences [(SPSS) Version 20 software]. Data on *E. coli* mean counts for each site were recorded in 100 ml per CFU. The one way analysis of variance (ANOVA) was performed to investigate existence of any correlation between *E. coli* counts, the difference in VG distribution and the degree of correlation between the number of *E. coli* isolates and the number of VGs observed with respect to each site. Correlations and test of significance were considered statistically significant when *P* values were < 0.05.

4.3 Results

4.3.1 E. coli confirmation

Of the 480 presumptive *E. coli* isolates recovered from the sampling sites, 410 were confirmed to be *E. coli* out of which 300 isolates made up of 30 from each sampling location were pooled together for further analysis. Figure 4.2 below shows the gel electrophoresis picture of the PCR products of the *uid*A gene amplification.

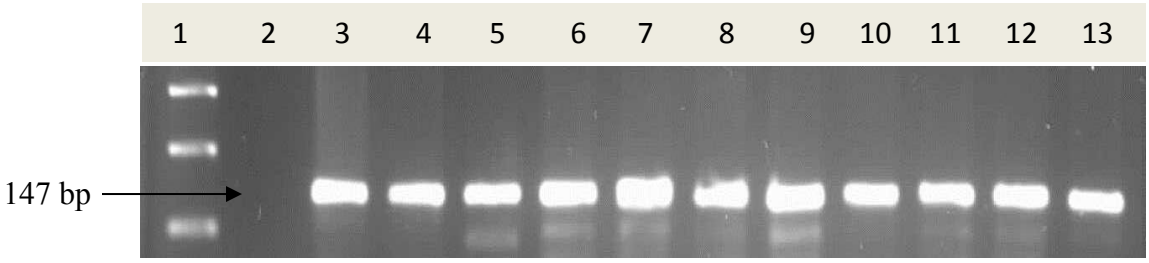

Figure 4.2 PCR products of the amplification of *uid*A gene

Lane 1: molecular weight marker (100 bp); lane 2: negative control; lane 3: positive control (ATCC *25922*); lanes 4-13: positive isolates.

4.3.2 Prevalence of E. coli in the river samples

Confirmed *E. coli* isolates in the river samples at all sites ranged between 34 CFU/100 ml at the R5 site and 76 CFU/100 ml at the R7 site (Figure 4.3).

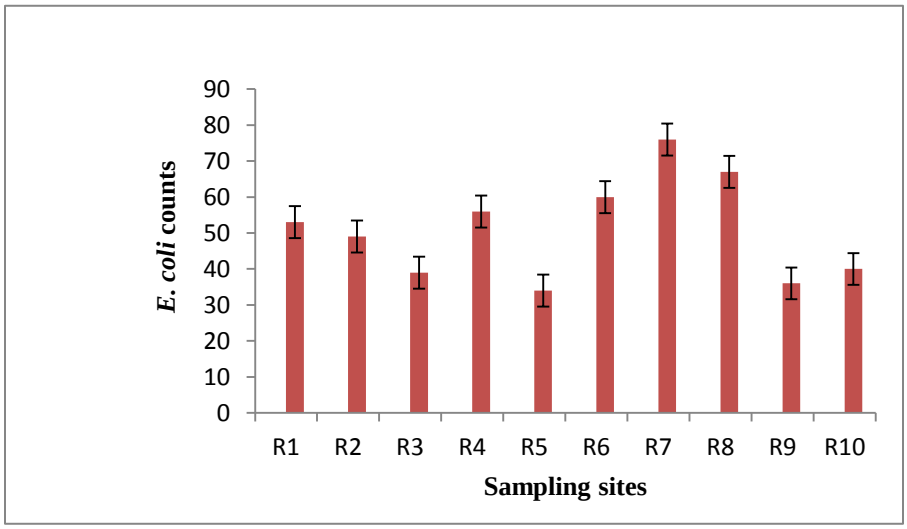

Figure 4.3 Comparative mean counts of *E. coli* at the ten sampling sites

4.3.3 Prevalence of virulence genes (VGs) amongst confirmed E. coli isolates

Among the 300 confirmed *E. coli* isolates assessed for the various VGs, 273 (91%) harboured at least 1 VG while 27 (9%) isolates harboured none. Overall, 91% of the isolates were found to harbour between 1 and 4 VGs (Figure 4.4). The modal occurrence of 1 VG was recorded at site R2 with 50 (33%) of the isolates being positive for 1 VG each. The prevalence of multiple VGs in the *E. coli* isolates was equally higher at R2 than at other sites. The heat-labile toxin, *lt* gene was the most commonly detected gene at site R1 in 24 (80%) of the isolates, followed by the adhesion *pap*C gene, detected in 17 (57%) of the isolates (Figure 4.4). Similarly, the modal occurrence of 2 VGs was at R2 in 12 (10%) isolates. None of the isolates from R6, R7 and R10 sites harboured 2 VGs Also, the modal prevalence of 3 VGs and 4 VGs were at R3 and R2 each in 2 (7%) (Figure 4.4). The invasion plasmid antigen gene, *ipa*H was not observed throughout the study and was therefore omitted from subsequent analysis. The representative gel

electrophoresis profiles of amplified products of the investigated diarrhoeagenic and non-diarrhoeagenic coding genes are shown in Figures 4.5 and 4.6.

Statistical analysis using one-way ANOVA was performed on the pooled data in order to further explore the distribution of the remaining 9 VGs among all the ten sites. There was a significantly higher occurrence ($P < 0.05$) of 1 VG at sites R4, R5, R7 and R8 than at any other sites. While sites R1 and R2 were not significantly different in the prevalence of 2 VGs in their isolates ($P > 0.05$), a significant difference was observed when compared to the other 8 sites tested ($P < 0.05$). Similarly, the differences in the occurrences of 3 VGs among sites R1, R2, R6, R7 and R8 were not significant ($P > 0.05$).

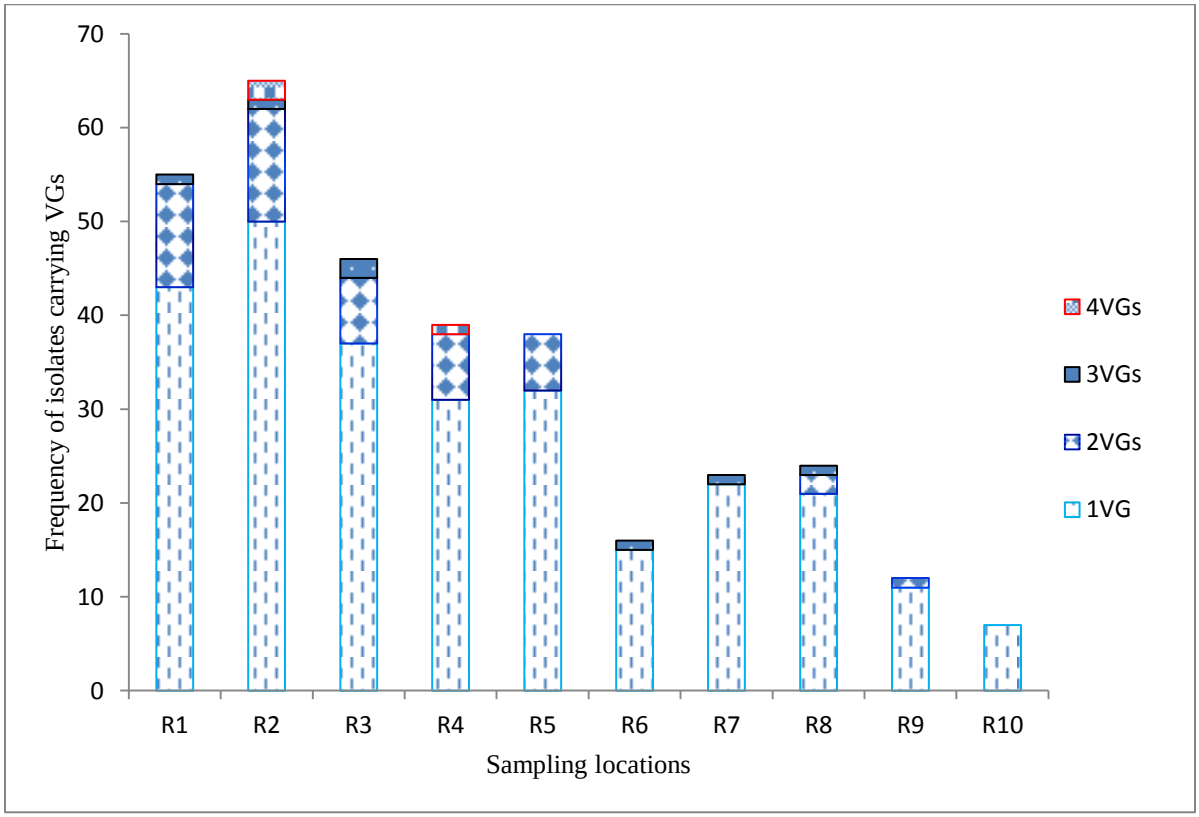

Figure 4.4 Comparative distribution of the virulence genes (VGs) in *E. coli* isolates.

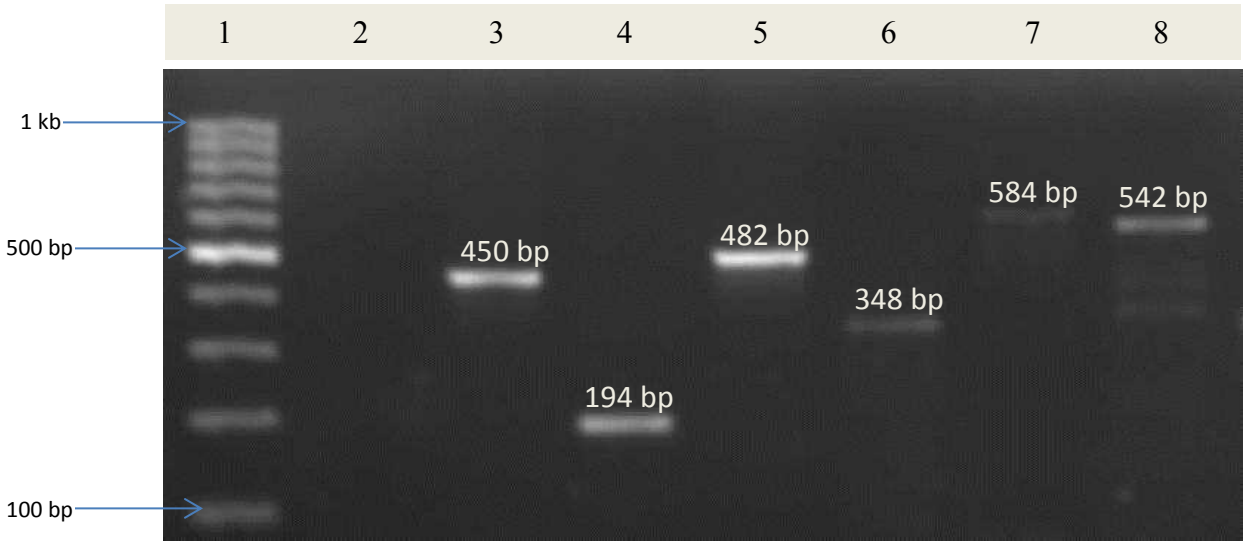

Figure 4.5 A representative gel electrophoresis profile of different virulence genes of the diarrhoeagenic *E. coli* isolate
Lane 1: molecular weight marker (Thermo Scientific 100 bp DNA ladder), lane 2: negative control, lane 3: *lt*, lane 4: *eagg*, lane 5: *eae*, lane 6: *stx*1, lane 7: *stx*2 and lane 8: *daa*E.

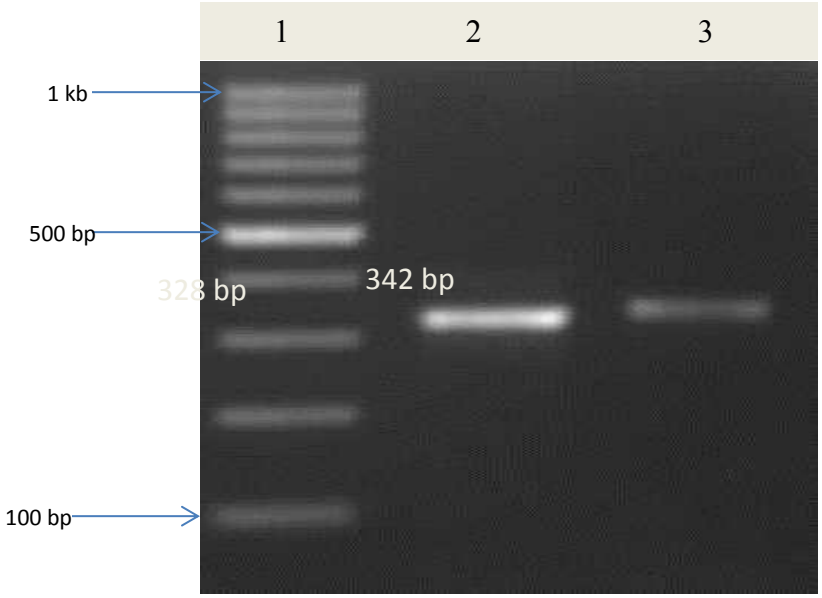

Figure 4.6 A representative gel electrophoresis profile of two virulence genes of the non-diarrhoeagenic *E. coli* isolates.
Lane 1: molecular weight marker (Thermo Scientific 100 bp DNA ladder), lane 2: *pap*C and lane 3: *ibe*A.

4.3.4 Comparative prevalence of E. coli pathotypes

To identify the prevalence of different pathotypes of *E. coli* isolates in all the ten sites, VGs were grouped according to their association with different *E. coli* pathotypes. The percentage site-specific distribution of the *E. coli* pathotypes in the ten sampling locations is shown in Figure 4.7. Overall, isolates belonging to the intestinal ETEC pathotype were the most commonly detected (45%), followed by the extra-intestinal UPEC (19%) and the lowest was EAEC (2%). Approximately, 21%, 18% and 12% of the *E. coli* isolates in sites R3, R5 and R8 could be placed into five main pathotypes with EPEC, ETEC, EHEC, UPEC and NMEC mostly observed. While EAEC was noticed at sites R3 and R5, DAEC was detected at R8 (Figures 4.7c, e and h). Similarly, ETEC pathotype was commonly found associated with UPEC (20%) at sites R2 and R4 each, and 13% at site R3, followed by ETEC/EHEC and ETEC/NMEC (10%) each (Figures 4.7b and d).

In addition, the percentage distribution of pathotypes was uniform at almost all the sampled sites except R6, R7 and R10. Approximately, 3% of *E. coli* isolates could be differentiated into more than three pathotypes and this was observed at sites R1, R2, R3, R6, R7 and R8 in the same proportion (3%) each, with the ETEC/EPEC/UPEC pathotypes commonly found at R2 and R7 (Figures 4.7a, b, c, f, g and h). Conversely, 3% of the isolates positive for multiple VGs could only be grouped into four defined pathotypes as observed at sites R2 and R4 only, with ETEC/EPEC/EHEC/EAEC common to both (Figure 4.7d). Other sampling sites' isolates did not carry multiple VGs sufficient enough to be categorized into four pathotypes.

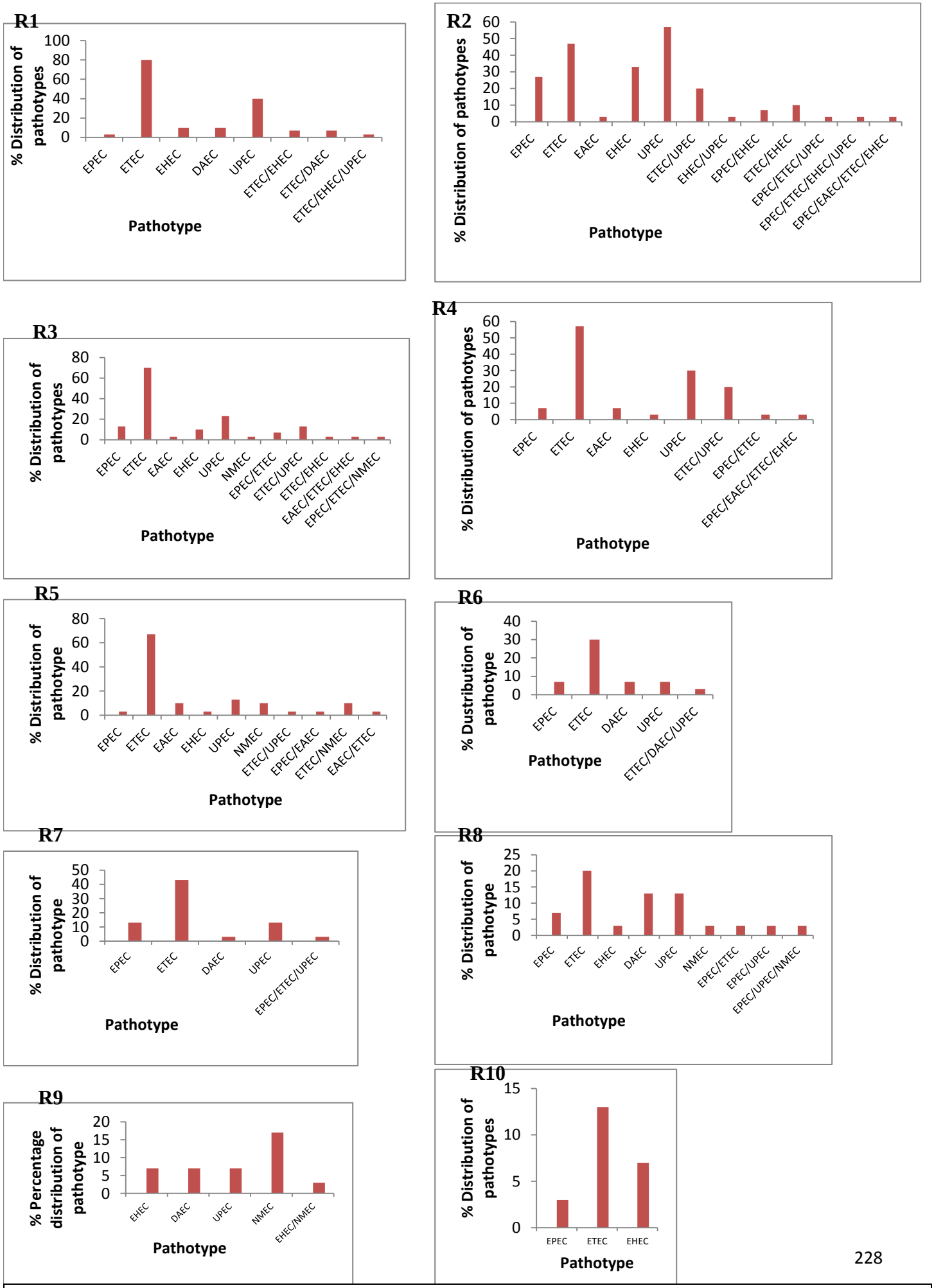

Figure 4.7 Comparative distribution of *E. coli* pathotypes from all ten sites in Osun State, Southwestern Nigeria.

4.3.5 Comparison of E. coli VG profiles from ten sites

VGs were further classified into toxin, adhesion and invasion genes based on functional characteristics of the genes (Table 4.2). This enabled us to identify the prevalence of different virulence genes of *E. coli* with observable differences in each site. A comparative analysis of the distribution of the 9 VGs observed across all the ten sites is presented in Figure 4.8. In general, the highest frequency of VGs in *E. coli* isolates was obtained at site R2 (16%), followed by site R1 (15%) and the lowest at site R10 (2%). Overall, the highest occurrences of VGs were observed at R1 and R3, with 7 VGs each and the lowest at site R10 with 3 VGs (Figures 8a, c and j). The VGs *pap*C and *lt* were the most frequently detected across all the sites, whereas the *bfp*, *eagg*, *stx*2 and *ibe*A genes were infrequently detected (Figure 4.8). Among the toxin genes screened in this study, *lt* was the most prevalent gene at all sites (45%) except at site R9 where it was not detected. Likewise, *pap*C was the most commonly detected adhesion gene observed across all the sampling sites (19%) with *eagg* gene being the least (2%). Conversely, of the two invasion genes assessed, *ibe*A was detected at a very low prevalence (3%) while *ipa*H was not detected in all the isolates (0%).

A comparison between the sites was made (ANOVA) to determine if the sites were similar or different on the basis of occurrence of VGs. Both the sites R1 and R2 were significantly different ($P < 0.05$) in the occurrence of *stx*1 toxin and *eae* adhesion genes compared to other sites, whereas the difference between the occurrence of *stx*2 toxin gene in the *E. coli* isolates from site R2 was found to be statistically non-significant ($P > 0.05$) in relation to those from the other sites except site R1. Similarly, there was a significant difference in the occurrence of *ibe*A invasion gene among the isolates obtained at sites R5 and R9 ($P < 0.05$).

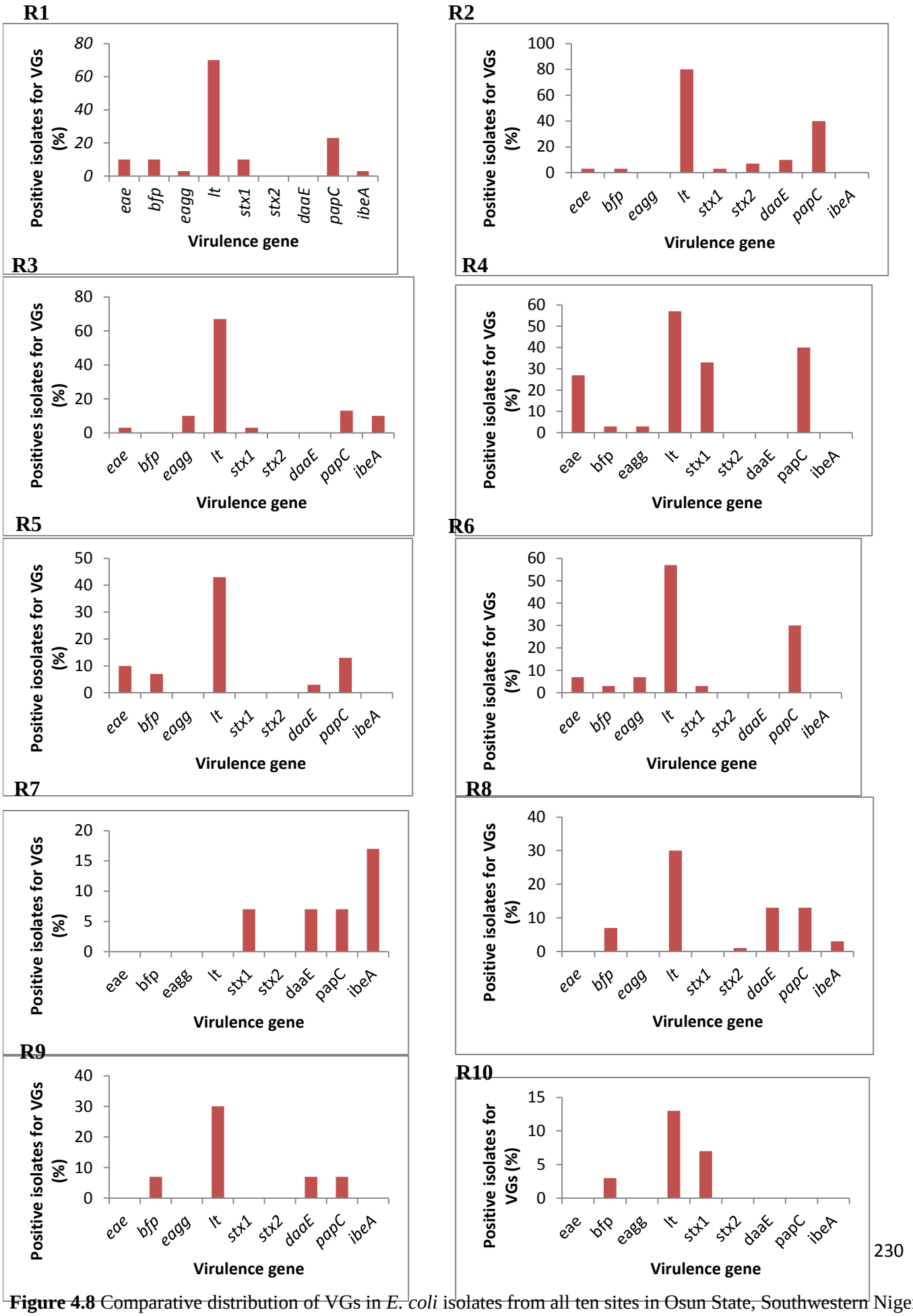

Figure 4.8 Comparative distribution of VGs in *E. coli* isolates from all ten sites in Osun State, Southwestern Nigeria.

4.4 Discussion

The present study investigated the distribution and frequency of defined pathotypes of *E. coli* isolates in river water samples from Osun State, Southwestern Nigeria. Generally, the mean annual counts of the presumptive *E. coli* obtained in all the sampling sites were relatively high. *E. coli* has been used extensively as one of the major faecal indicator bacteria due to the previous notion that it has limited survival ability in the environment though recent studies have suggested that some pedigrees of *E. coli* have adapted and acclimatized within tropical, subtropical and even temperate regions [41, 95], and as such could be the germane reason for *E. coli* to flourish especially within freshwaters. In this study, most of the sites with higher counts of *E. coli* were those located in pasture and peri-urban catchments with multiple sources of faecal pollution such as run-offs and dungs from cattle, horses and wild animals. There is a high likelihood that the isolates were mainly from human and animal excreta because during our sampling periods, human and animal excreta were sighted at the banks of the rivers, livestock were seen drinking water from the rivers, farmers and bricklayer were bathing and used waters from the car washing centers drained into the river. This further implicates both humans and animals as potential sources for the recovered *E. coli* pathovars. A previous study has also reported the presence of high numbers of faecal indicator bacteria originating from defective septic systems and grazing animals in freshwater sites and surface waters of developing countries [4, 5]. Other likely sources include mobilization of *E. coli* persisting in the soil [12], sediments [21] and aquatics [55].

In our study, VGs were detected in the *E. coli* isolates suggesting the presence of pathogenic *E. coli* strains in these waters. Otherwise, this may indicate an incessant input of these bacteria from a common source in the water or a combination of both. Generally, the results illustrate varied

occurrence of diarrhoeagenic and non-diarrhoeagenic *E. coli* pathotypes with (91%) of the isolates grouped under seven main *E. coli* pathotypes.

A large number of the *E. coli* isolates tested positive for toxin genes. The VG *lt* associated with ETEC strains was the most prevalent of all (45%). This finding is worriedsome, considering the fact that it is the most common agent of traveler's diarrhoea with food and water implicated as the modes of transmission [39, 58]. The presence of ST and/or LT enterotoxins which are commonly associated with ETEC strains have been reported by other workers in surface waters [10, 66], and are thought to originate from swine and humans with diarrhoea. ETEC strains are the most frequently isolated bacterial enteric pathogens in children below 5 years of age in developing countries and responsible for approximately 300 million diarrhoea cases and 380 000 deaths annually [88, 96], and their prevalence in surface water sources in developing countries has been documented [46]. The pathotype has been predominant followed by EPEC, EAEC and STEC in developing countries [75].

EHEC causes haemorrhagic colitis and haemolytic uremic syndrome in humans, and the key virulence factors include intimin (*eae* gene) and shiga toxins (*stx*1 and *stx*2 genes) [11]. Though, none of the isolates harboured a combination of the shiga toxin genes, nonetheless the relatively high occurrence of the *stx*1 gene (6%) compared to *stx*2 (1%) in the water *E. coli* isolates suggests the capability of each gene in causing acute diarrhoea in humans. This observation contradicts with the relatively high occurrence of the *stx*2 gene (10%) compared to *stx*1 (6%) in the storm water *E. coli* isolates which suggests that *E. coli* carrying a combination of the EHEC genes, are known to cause more severe diarrhoea in humans [73]. The most prevalent pathotypes of *E. coli* responsible for diarrhoeal diseases include enterohaemorrhagic or shiga toxin producing *E. coli* (EHEC or STEC) and enterotoxigenic *E. coli* (ETEC) [45]. The contamination

of drinking or recreational waters with such *E. coli* pathotypes has been linked to waterborne disease outbreaks and mortality [10, 15].

EPEC has been shown to be a major cause of diarrhoea in young children [50]. The *eae* gene, which codes for intimin protein, was the fourth most prevalent gene in this study (6%). This gene is necessary for intimate attachment to host epithelial cells in both the EHEC and EPEC pathotypes. Our findings tend to strongly disagree with the previous finding of significantly higher prevalence of the *eae* gene (up to 96%) in surface water reported in other studies [57, 85]. Typical EPEC strains carry the LEE pathogenicity island, which encodes for several virulence factors, including intimin (*eae*) and the plasmid-encoded bundle forming pilus (*bfp*), which mediates adhesion to intestinal epithelial cells [36]. Therefore, all the *E. coli* isolates were further screened for the presence of the *eae* and *bfp* genes to determine their association with the EPEC pathotype. In this study, a noticeably low prevalence of the *bfp* gene (4%) was detected, suggesting that prevalence of the EPEC pathotype could be expected in the surface water bodies. In addition, *eae* was also detected in 4% isolates which lacked other typical genes from both EPEC group. This indicates prevalence of this gene in *E. coli* isolated from the freshwater environments. This finding is of great concern, as an atypical EPEC pathotype which lacks the *bfp* gene but carries the *eae* gene has been found to be a major cause of gastroenteritis worldwide [38], in patients suffering from community-acquired gastroenteritis in Melbourne, Australia [78], and from children with diarrhoea in Germany [49]. Approximately 2% of the isolates carried both *eae* and *bfp* genes suggesting the presence of typical EPEC pathotype. The relatively low occurrence of the combination of both atypical EPEC genes in the water *E. coli* isolates is alarming due its possible significance in the cause of severe diarrhoea in humans. However, the

role of atypical EPEC in diarrhoea has not been established assertively [45, 59, 60], and this study did not aim at revealing the diarrhoeagenic role of this pathotype.

In addition to the most prevalent ETEC strains obtained, EPEC and EHEC were the second- and third-more-common diarrhoeagenic pathotypes detected in this study respectively, with each group represented by 10% and 7% isolates respectively. This is a strong indication that the three pathotypes occur widely in the surface water samples. The presence of *E. coli* strains with virulence characteristics similar to ETEC and EHEC [18] and EPEC [51] have been previously reported in the fresh and estuarine waters. Generally, a few EHEC strains identified in this study were in agreement with other studies executed in different parts of the world [32, 61], and a low prevalence of EHEC infection has been observed in developing countries [13, 90].

EAEC is an emerging pathogen associated with diarrhoea. It has been identified in travelers, children in the developing world and human immunodeficiency virus infected patients with diarrhoea [3, 45, 47, 61, 63]. In the present study, among the DEC types, *eagg* gene of EAEC strains was the least frequently isolated adhesion VG with only 7 (2%) strains detected in all the isolates, yet the pathotype has been an important diarrhoeagenic pathogen with its characteristic persistent diarrhoea in children and adults. This finding seems to be inconsistent with the previously reported high prevalence of the EAEC pathotype in fresh and estuarine water samples [57], but tends to align with the earlier observation of a less common DAEC, EAEC and a variety of different EHEC and EPEC pathotypes with the exception of enteroinvasive *E. coli* which was not detected in the 509 samples studied [16, 94]. Several studies have reported that contaminated food and water hygiene are the main vehicles of transmission with EAEC [59]. Similar findings have been described [80] and elsewhere [30, 66]. Our observation on the occurrence of ETEC, EPEC, EAEC and NMEC concurs with the report that ETEC is the most

prevalent pathotype detected, followed by low prevalence of EPEC and NMEC, and absence of EIEC pathotypes in *E coli* isolates of surface water [64].

Uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) are the two other extra-intestinal *E. coli* (ExPEC) pathotypes that have been characterized [24, 97]. The study shows a higher prevalence of VG *pap*C (19%), belonging to UPEC pathotype than the NMEC VG *ibe*A (3%). The presence of *E. coli* strains with virulence characteristics similar to ExPEC have been reported previously in the fresh and estuarine waters [35]. This observation was of interest and may be an indication of a high potential health risk of such waters as the number of ExPEC VG(s) in *E. coli* has been suggested to be proportional to its pathogenic potential [74].

Overall, the detection of most of the VGs tested was relatively low aside *lt* and *pap*C, ranging from 1 to 7%. This finding correlates with the reports of other researchers that the prevalence of *E. coli* isolates harbouring VGs in environmental waters is low ranging from 0.9% to10% [18, 51, 56]. In the light of this, screening of a large number of isolates for possible detection of VGs is advocated. *E. coli* isolates have been concentrated from large volume (1 L) of water samples followed by an enrichment step to increase PCR detection sensitivity [6, 83, 84].

The presence of a single or multiple VGs in an *E. coli* strain does not necessarily indicate that a strain is pathogenic unless that strain has the appropriate combination of VGs to cause disease in the host [15, 31]. The pathogenic *E. coli* strains use a complex multistep mechanism of pathogenesis involving a number of virulence factors depending upon the pathotype, which consists of attachment, host cell surface modification, invasin, a variety of toxins and secretion systems which eventually lead toxins to the target host cells [45]. Thus, VGs are appropriate targets for determining the pathogenic potential of a given *E. coli* isolate [50]. The occurrence of

unusual combinations of VGs in *E. coli* isolates observed in this study could be explained on the basis of horizontal gene transfer between cells, which enables the exchange of genetic material located on mobile elements (transposons, integrons, or plasmids) among related or unrelated bacterial species [22]. Further screening of the *E. coli* isolates with these unusual VG patterns in tissue culture or animal models would be required to demonstrate their pathogenicity.

In this study, we collected water samples from communities with diverse human population densities and land uses to determine if these factors influence the distribution of VGs (Figure 4.8). The results of this study show a relatively low and clear pattern of occurrence of VGs across the sites with a noticeable difference of occurrence of 4 VGs and 3 VGs at sites R9 and R10 respectively. Overall, it was evident that the point and non-point sources of contamination were potentially similar across the sampling sites in their characteristic features. Similarly, all the sampling sites are bordered by farm animals such as ruminants which are known to be potential sources of these VGs [7, 23, 34, 40]. Animals, humans and the environments including water sources serve as natural habitats of virulent strains of *E. coli* [25, 33, 50, 59, 89). Storm runoffs may also increase the prevalence of microbial pathogens, including diarrhoeagenic *E. coli* pathotypes in the surface water bodies due to transport of faecal contamination from land [86].

A better understanding of the prevalence and distribution of *E. coli* pathotypes in water sources used for potable, non-potable or recreation purposes could be an important tool in the development of public health risk mitigation strategies. Pathotyping of *E. coli* isolates may also provide useful information to identify potential sources of pollution, as the principal reservoirs of ETEC and EPEC pathotypes are majorly humans and ruminants, whereas the bovine intestinal tract is the main source of the EHEC pathotype [45, 53]. The lower prevalence of the EHEC pathotype compared to other pathotypes suggests that human faecal contamination of the

waterways is the main source of diarrhoeagenic *E. coli* pathotypes in the surface water as opposed to contamination from animals. This stresses the importance of controlling sources of human faecal pollution such as municipal wastewater sources, sewage leaks and overflows, wastewater treatment plant discharge to reduce potential threats to human health. The results demonstrate that the risk of contracting infection, however, may increase over time if no appropriate preventive and controlling measures are ensured. Since this study aimed at detecting *E. coli* pathotypes carrying VGs, it is logically reasonable to assume that actual distribution of these VGs in surface water could be relatively higher. While the ability of *E. coli* isolates described in this study to cause human diarrhoeal diseases was not established, a high proportion of isolates carrying a full set of VGs have been linked to defined pathotypes. Further screening for other VGs along with serotype testing and other assays may offer further information on pathogenicity of these isolates.

4.5 Conclusion and Recommendations

Confirmation of the presence of *Escherichia coli* in river water in Osun State, Southwestern Nigeria, indicates faecal contamination and the possible presence of other enteric pathogens. The prevalence of virulence markers in *E. coli* isolates from river water sources is indicative of increased risks of mortality, especially among the vulnerable populations, should they contract infections through the use of river water for consumption or other household related purposes. It equally emphasizes the importance of safe water supply, good hygiene and sanitation practices both in rural and urban communities. A need for large sample size has been buttressed and further characterization of these pathogenic strains will improve their detection and possibly the design of protective substances such as vaccines that will constitute a milestone in the control of diarrhoeal infections in this part of the world and elsewhere. Finally, this study has revealed a

number of *E. coli* isolates positive for single and multiple VGs which indicates the presence of potential pathogenic *E. coli* in these waters and it clearly highlights the need to develop a better understanding of public health implications of occurrence of *E. coli* carrying VGs in surface waters used for potable, non-potable and recreational purposes.

4.6 Acknowledgement

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Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER FIVE

Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of faecal contamination of water

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Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of faecal contamination of water

Abstract

We evaluated the antibiogram profile of *Escherichia coli* (*n* = 300) isolated from selected rivers in Osun State, Nigeria. The identities of the *E. coli* isolates were confirmed by polymerase chain reaction (PCR) technique. Susceptibility of the isolates to 20 antibiotics conventionally used in clinical cases was assessed *in vitro* by the standardized agar disc-diffusion method. All the isolates were susceptible to imipenem, meropenem, amikacin and gatilofloxacin. The isolates were variously susceptible to the other antibiotics as follows; ciprofloxacin (96 %), kanamycin (95 %), neomycin (92 %), streptomycin (84 %), chloramphenicol (73 %), nalidixic acid (66 %), nitrofurantoin (64 %), gentamycin (63 %), doxycycline (58 %), cefepime (57 %), tetracycline (49 %) and cephalothin (42 %). The multiple antibiotic resistance indexing ranged from 0.50 to 0.80 for all the sampling locations and exceeded the threshold value of 0.2, suggesting the origin of the isolates to be of high antimicrobial usage. Our findings signify an increase in the incidence of antimicrobial resistance of *E. coli* towards conventionally used antibiotics necessitating proper surveillance programs towards the monitoring of antimicrobial resistance determinants in water-bodies.

Keywords Surface water. *Escherichia coli.* Phenotype. Antibiotic resistance. Multiple antibiotic resistance

5.1 Introduction

Antimicrobial agents play an indispensable role in reducing morbidity and mortality associated with infectious diseases in animals and humans. However, selective pressure exerted by the exaggerated use of antimicrobials has been the main driving force in the emergence and spread of drug-resistance traits among pathogenic and commensal bacteria (Aarestrup et al. 2008). The selection and spread of multi-drug resistance (MDR) organisms can often be traced to complex socioeconomic and behavioural antecedents, and has contributed to the ever-increasing challenge of antibiotic resistance worldwide (Okeke et al. 1999; Mc Manus and Stockwell 2001; Silbergeld et al. 2008; Ghafur 2010). Surveillance data indicate that resistance in *E. coli* is consistently higher for antimicrobials that have been in use for a long time in human and veterinary medicine (USFDA and NARMS 2008). Furthermore, antimicrobials are poorly absorbed in the gut of animals and humans and end up being excreted unchanged in faeces and urine, eventually finding their way into the environment through the disposal of sewage, hospital wastewater and animal wastes (Schlusener and Beste 2006). Antibiotic-resistant bacteria have been noticed in various aquatic habitats, including treated and untreated wastewaters (Anderson and Sobrey 2006; Shehabi et al. 2006), rivers (Cernat et al. 2002), and marine water (Boehm et al. 2003).

Surface waters have emerged as reservoirs of faecal coliforms exhibiting multiple antimicrobial resistance owing to the discharge of municipal sewage and wastes from animals, industries and hospitals (Bruneau et al. 2004; Edge and Hill 2005; Qadri et al. 2005; Hamelin et al. 2006). This is because freshwater milieus are readily accessible for the discharge of wastewater, which may contain antimicrobial residues and are therefore, easily contaminated (Hirsch et al. 1999). Microbial indicators have been used worldwide as a tool to indicate the contamination of water by human wastes and the occurrence of *Escherichia coli* in drinking water is an indication of

faecal contamination and probable incidence of water-borne diseases that are injurious to health (Sivanadham et al. 2012; WHO 2010). Bacterial contamination of surface water, and particularly contamination with faecally derived bacteria, has long been a water quality concern owing to the potential for disease transmission.

The discovery of penicillin in the 1940s and several other antimicrobials in subsequent years led to great improvements in the management of infectious diseases (Norrby et al. 2005). Despite this great achievement, the indiscriminate use of antimicrobials has led to the inevitable development of resistance, as diseases and disease agents are now re-emerging in new forms resistant to antimicrobial therapy (Levy and Marshall 2004), rendering the current drugs used for treatment useless (Hancock 2005).

Antibiotic resistance indexing (ARI) is an excellent tool that enables one to determine the dissemination and prevalence of bacterial resistance in a given population at a specified location (Tandra and Sudha 2014). Isolates from water sources contaminated with antibiotics often give an ARI value >0.2, which is an indication of high-risk source of contamination. However, when antibiotics are seldom or never used, an ARI value <0.2 is observed (Krumperman 1983). A bacterium is termed multiple antibiotic resistant (MAR) if it is found to be resistant to three or more antimicrobials (Manjusha et al. 2005). Antimicrobial resistance increasingly compromises the treatment of many infections that were until recently, controllable, and so remain the most common diseases in Africa. The global challenge of antimicrobial resistance is particularly pressing in developing countries like Nigeria, where the infectious disease burden is high and cost constraints prevent the widespread application of newer and more expensive agents (Okeke et al. 2005).

Globally, about 191 million people rely on surface water as their main source of drinking water with sub-Saharan Africa facing the greatest challenge (WHO 2012). Almost 90 % of child deaths from diarrhoeal diseases are directly linked to contaminated water, inadequate sanitation and hygiene (UNICEF 2013). *E. coli* remain one of the major causes of morbidity and mortality in infants and young children especially when these diseases fail to be cured due to development of resistance to commonly prescribed antimicrobials (Okeke and Sosa 2003). The present study aimed at determining the high-risk contamination of water by indexing the frequency at which multi-drug resistant *E. coli* organisms occurred in selected surface waters from Osun State, South-western Nigeria.

5.2 Materials and methods

5.2.1 Description of study area and sampling sites

Water samples were collected from ten rivers at different locations in Osun State, South-western Nigeria. Table 5.1 shows the description of the sampling sites. The sites were selected after consultation with the State Ministries of Environment, Water Resources, Lands and Housing for proper mapping of the state rivers. The ten rivers were purposefully selected to represent the three senatorial districts of Osun State, in addition to being the most popular and major rivers in the state. The sampling locations were coded as follows: R1: Erinle-Ede; R2: Ido-Osun; R3: Osun-Osogbo, R4: Oba-Iwo; R5: Ejigbo; R6: Ilobu-Okinni; R7: Asejire-Ikire; R8: Shasha; R9 and Ila-Oke Ila, R10: Inisha-Okuku.

Table 5.1 Sampling sites description their location.

Site code	Site name	Activities	GPS coordinates
R1	Erinle-Ede	Fishing, animal rearing, irrigation, tourism and domestic purposes	7°44′44″ N; 4°29′22″ E
R2	Ido Osun	Animal rearing, fishing, irrigation, domestic and recreational purposes	7°49′ 01″ N; 4°26′41″ E
R3	Osun-Osogbo	Farming, fishing, habitation, tourism and worship shrine	7°46′ 04″ N; 4°34′00″ E
R4	Oba-Iwo	Fishing, domestic and recreational purposes	7°38′ 01″ N; 4°11′20″ E
R5	Ejigbo	Farming, fishing and domestic use	7°54′0″ N; 4°18′54″ E
R6	Ilobu-Okinni	Car washing, swimming and domestic use	7°50′06″ N; 4°29′14″ E
R7	Asejire-Ikire	Fishing, irrigation, domestic and industrial activities	7°25′44″ N; 4°13′14″ E
R8	Shasha	Fishing, farming, palm oil processing and domestic purposes.	7°22′32″ N; 4°28′11″ E
R9	Ila-Oke Ila	Farming, irrigation, fishing and domestic activities	8°01′50″ N; 4°59′03″ E
R10	Inisha-Okuku	Fishing, irrigation and domestic activities	8°01′32″ N; 4°42′11″ E

5.2.2 Sampling and isolation of presumptive Escherichia coli

Water samples were aseptically collected monthly over a period of one year from September 2011 to August 2012. All samples were collected in 1.5-L sterile bottles and transported on ice to the laboratory where they were processed within 6 h of collection. The bottles were sterilized by autoclaving and during sampling, were triple rinsed with sample water before being filled. Analysis of water samples was performed according to Standard Methods (APHA 1998). One hundred milliliter (100 ml) aliquots of the water samples were filtered through a 90-mm diameter, 0.45-µm pore-sized membrane filters (Millipore, Ireland). The filters were incubated overnight at 44.5 °C on eosin methylene blue agar (Oxoid, UK). Characteristic metallic-sheen

colonies were selected and purified by streaking on *E. coli* chromogenic agar (Conda Pronadisa, Spain) plates before storing on glycerol for further use.

5.2.3 PCR confirmation of Escherichia coli isolates

All the presumptive *E. coli* isolates were subjected to PCR technique for identification. DNA extraction was done using the boiling method (Torres et al. 2005; Maugeri et al. 2004). *E. coli* isolates were grown on nutrient agar plates at 37 °C for 24 h. The colonies were picked and suspended in 200-µl sterile distilled water, vortexed and boiled at 95 °C for 15 min followed by centrifugation at 15,000 rpm for 10 min. The supernatant was removed and stored at –80 °C for PCR confirmation. The PCR conditions, with some modifications, were in accordance with the protocols described elsewhere (Yokoigawa et al. 1999; Daly et al. 2002; Moyo et al. 2007).

Primers specific for a conserved region situated within the *E. coli* alanine racemase gene were selected [(F: 5´-CTGGAAGAGGCTAGCCTGGACGAG-3´) and (R: 5´-AAAATCGGCACCGGTGGAGCGATC-3´)]. The reaction mixture contained 25 µl of PCR Master Mix (Thermo Scientific, (EU) Lithuania), 0.5 µl each of oligonucleotide primer (Inqaba Biotech, SA), 10 µl of template DNA and 14 µl of nuclease free water to constitute a total reaction volume of 50 µl. PCR amplification was done using the following protocols: initial denaturation at 94 °C for 5 min followed by 35 cycles consisting of 30 s denaturation at 95 °C; primer annealing at 58 °C for 1 min; extension at 72 °C for 1 min and a final extension step for 5 min at 72 °C. The amplicons (5 µl aliquots) were resolved in 1.8% (w/v) agarose gel (Merck, SA) stained with 5 µl ethidium bromide (Sigma-Aldrich, USA) and visualized under the Gel documentation system (Alliance 4.7, France). A 100-bp DNA ladder (Thermo Scientific, (EU) Lithuania) was included on the gel as a molecular size standard. Electrophoresis was carried out at 100 V for 1 h. *E. coli* ATCC *25922* was used as a reference strain (ATCC, USA).

5.2.4 Antibiotic susceptibility testing

Antimicrobial susceptibility tests were performed on the *E. coli* isolates by the disc-diffusion method, as previously described (Kirby-Bauer et al. 1966). To prepare the inocula for antibiotic susceptibility testing, 4-5 lone colonies of an 18-h old culture were picked up with a sterile loop and transferred to a micro-centrifuge tube containing 2 ml of 0.85 % physiological sterile saline and gently vortexed to mix. The turbidity of the resulting solution was adjusted to 0.5 McFarland standard solution (using 0.5 ml of 0.048 M $BaCl_2$ added to 99.5 ml of 0.18 M H_2SO_4 as a standard for comparison) which is approximately equal to 1×10^8 cells/ml (McFarland 1907). The turbidity of the bacterial suspension was adjusted by either adding inocula or sterile saline solution as required (Panda et al. 2012). One hundred microliter of the suspension was then spread plated onto Mueller Hinton agar plates and left to dry for 10 min prior to placing antibiotic discs on them.

A panel of twenty antibiotic discs (Mast Diagnostics, UK) impregnated with amikacin (30 µg), streptomycin (300 µg), kanamycin (30 µg), neomycin (10 µg) gentamycin (10 µg), cefepime (30 µg), cephalothin (30 µg), cefuroxine (30 µg), meropenem (10 µg), imipenem (10 µg), ciprofloxacin (5 µg), gatilofloxacin (5 µg), nalidixic acid (30 µg), sulphamethoxazole (25 µg); nitrofurantoin (200 µg), chloramphenicol (30 µg), tetracycline (30 µg), doxycycline (30 µg), amoxycillin (25 µg) and ampicillin (25 µg), were placed on the Mueller Hinton agar plates and incubated at 37 °C for 24 h. After incubation, the inhibition zone diameters were measured and classified as resistant (R), intermediate (I) and susceptible (S) to antibiotics according to the zone diameter interpretation standard recommended by the Clinical and Laboratory Standards Institute (CLSI 2010). Multiple antibiotic-resistant phenotypes (MARPs) for each sampling location were then generated for isolates that showed resistance to three or more antimicrobials following the

method of Wose et al. (2010). The resistance pattern, number of antimicrobials to which the isolates were resistant, frequencies and percentages were obtained from the result of antimicrobial susceptibility testing.

The antibiotic resistance index (ARI) for each sampling site was also determined using the formula described by Tandra and Sudha (2014). It was mathematically expressed as:

$ARI = A / N(Y),$

A being the total number of resistant determinants recorded, N being the number of isolates and Y being the total number of antibiotics tested.

The multiple antibiotic resistance index (MARI) for each sampled location was equally derived using the mathematical expression of Blasco et al. (2008) which was given as:

$MAR_{index} = a / b,$

where 'a' represented the number of antibiotics to which the isolate was resistant and 'b' the total number of antibiotics against which an individual isolate was tested.

5.2.5 Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences [(SPSS) IBM version 20 software]. One way analysis of variance (ANOVA) was done to determine the variation in resistance among the isolates with respect to different locations. Correlations and test of significance were considered statistically significant when *P* values were >0.05.

5.3 Results

Table 5.2 shows mean annual presumptive and confirmed *E. coli* counts obtained at each of the sampling sites while Figure 5.1 shows the gel electrophoresis picture of *E. coli* confirmation by PCR amplification of the *uid*A gene.

Table 5.2 Mean annual counts of the presumptive and confirmed *E. coli* isolates.

Site code	**Site name**	**Average annual presumptive *E. coli* count (CFU/100ml)**	**Number of confirmed *E. coli* isolates**
R1	Erinle-Ede	62	53
R2	Ido-Osun	56	49
R3	Osun-Osogbo	44	39
R4	Oba-Iwo	61	56
R5	Ejigbo	38	34
R6	Ilobu-Okinni	77	60
R7	Asejire-Ikire	83	76
R8	Shasha	72	67
R9	Ila-Oke Ila	41	36
R10	Inisha-Okuku	46	40

The lowest and highest counts were recorded in R7 and R9 with 83 and 41 CFU/ml respectively. A total of 300 confirmed *E. coli* isolates comprising 30 isolates from each study site, was pooled together and subjected to antibiogram analysis.

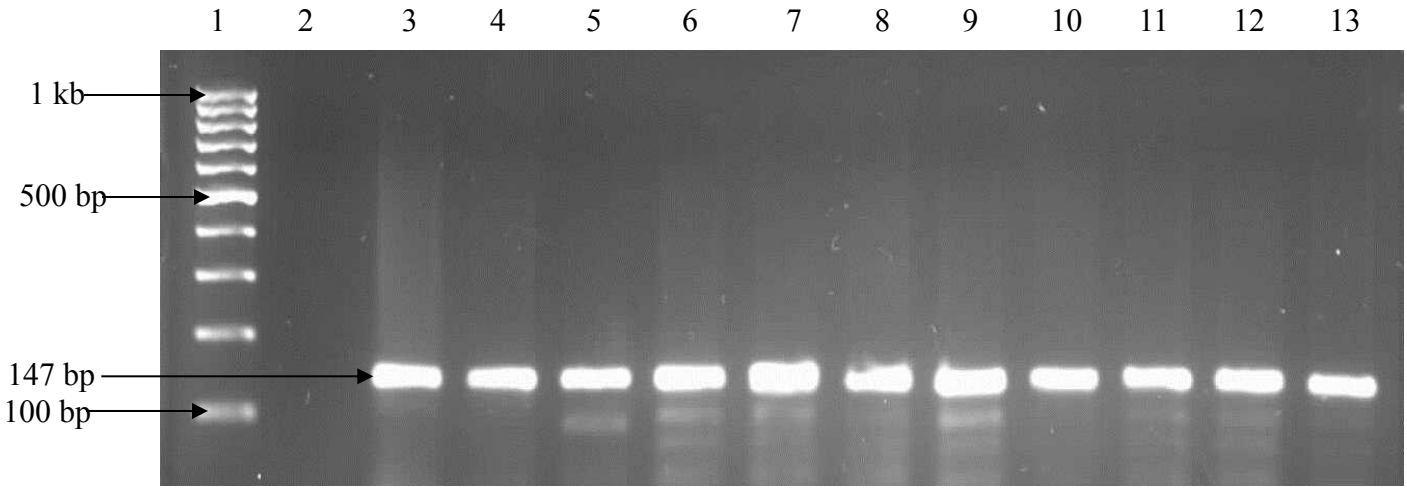

Figure 5.1 PCR confirmation of *E. coli* isolates from surface water.

Lane 1: molecular weight marker (100 bp); lane 2: negative control; lane 3: positive control (ATCC *25922*); lanes 4-13: positive isolates.

All the *E. coli* isolates were susceptible to all members of imipenem, meropenem, amikacin and gatilofloxacin antimicrobials. Similarly, high sensitivities were detected against ciprofloxacin (96 %), kanamycin (95 %), neomycin (92 %), streptomycin (84 %) and chloramphenicol (73 %). Varied susceptibilities were recorded for other antibiotics as follows: nalidixic acid (66 %), nitrofurantoin (64 %), gentamycin (63 %) and cefepime (57 %). Susceptibilities observed against other antibiotics tested were below average as shown in Figure 5.2.

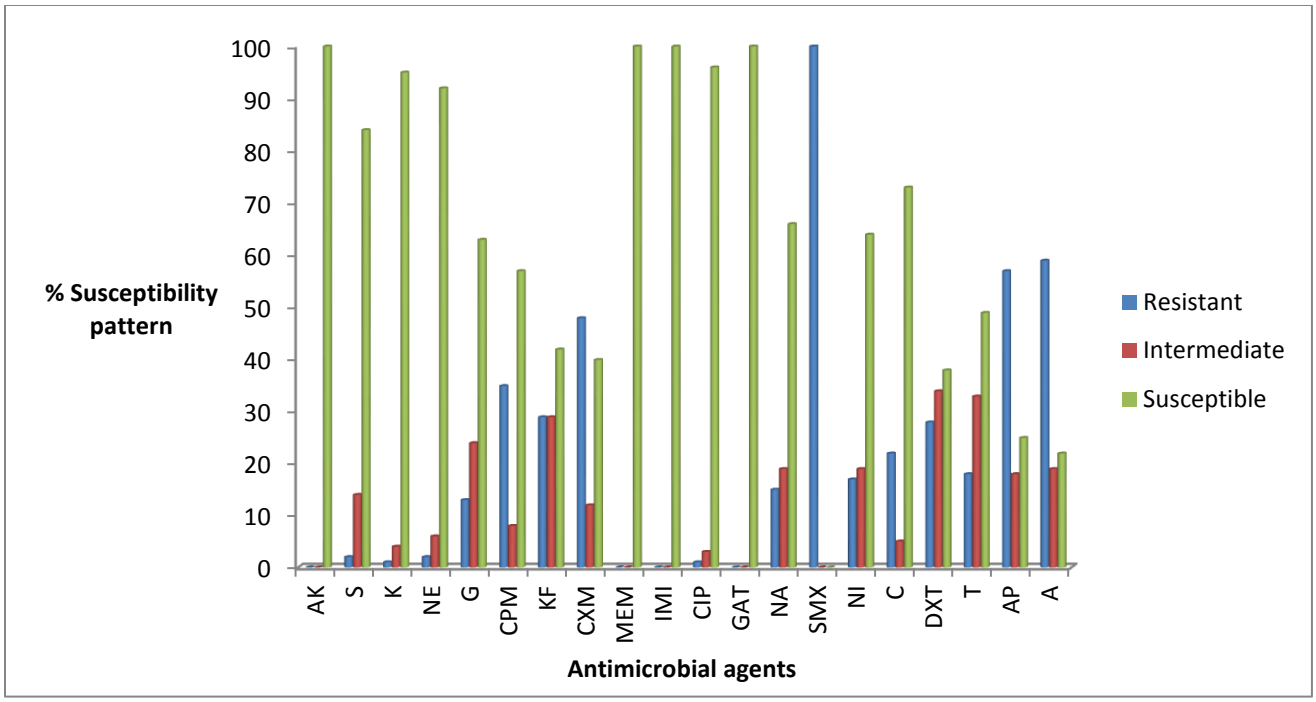

Figure 5.2 Susceptibility pattern of *Escherichia coli* isolates against selected antimicrobials across different classes.

AK Amikacin, *S* Streptomycin, *K* Kanamycin, *NE* Neomycin, *G* Gentamycin, *CPM* Cefepime, *KF* Cephalotin, *CXM* Cefuroxin, *MEM* Meropenenm, *IMI* Imipenem, *CIP* Ciprofloxacin, *GAT* Gatilofloxacin, *NA* Nalidixic acid, *SMX* Suphamethoxazole, *NI* Nitrofurantoin, *C* Chloramphenicol, *DXT* Doxycycline, *T* Tetracycline, *AP* Ampicilllin, *A* Amoxycilin.

All the isolates were resistant to sulphamethoxazole. High levels of resistance were equally observed against amoxycillin (59 %) and ampicilin (57 %) while the resistance to cefuroxime at 40 % was slightly below average. The lowest susceptibilities were observed against doxycycline (34 %) and tetracycline (33 %). Others varied as follows: cephalotin (29 %), gentamycin (24 %), nalidixic acid and nitrofurantoin (19 %), amoxycillin (19 %), ampicillin (18 %) and streptomycin (14 %) (Figure 5.2).

Compilation of the MAR phenotypes indicated that about 75 % of *E. coli* isolates in this study exhibited resistance to three or more antimicrobial agents. The frequency of MARPs ranged from

24.8 to 2.2 % for MARPs 5 and 9 respectively. When this was expressed in terms of prevalence, 4, 5, 10, 16, 20, 20, and 25 % of the isolates showed multiple antibiotic resistance to nine, seven, eight, three, four, six and five antimicrobials respectively (Table 5.3). The highest prevalence of MARPs across all the sampled sites was recorded in R7 at 70 % and lowest at R9 with 4.4 % (Figure 5.3).

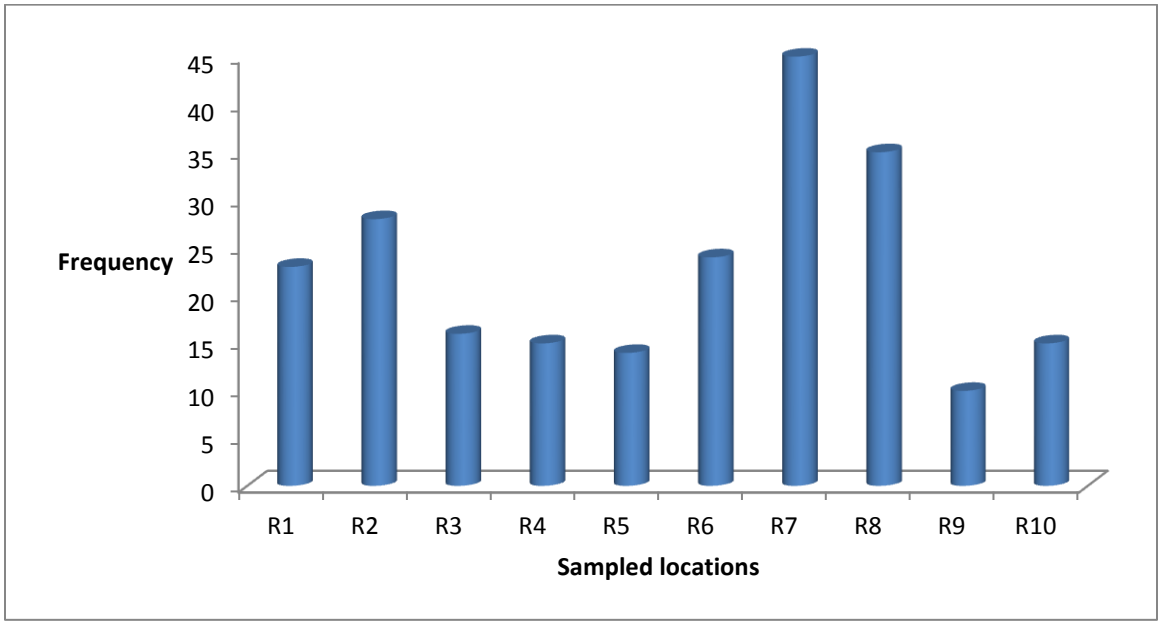

Figure 5.3 Frequency distribution of multiple antibiotic resistance phenotypes of *E. coli* isolates

The predominant multiple antibiotic resistant phenotypes (MARPs) of the *E. coli* isolates, indicating the numbers of antimicrobials, resistance patterns and frequencies obtained in all the sampling sites are presented in Table 5.3.

Table 5.3 Patterns of multiple antibiotic resistance phenotypes (MARPs) of *E. coli* isolates.

Sampling site R1 (N=30)			**Sampling site R2 (N=30)**		
№ of antimicrobials	**Resistance pattern**	**№ observed**	**№ of antimicrobials**	**Resistance pattern**	**№ observed**
3	SMX-T-CIP	1	4	SMX-C-G-AP	1
	SXM-A-DXT	2		SMX-C-A-AP	2
	SMX-CXM-G	2		SMX-DXT-A-AP	2
	SMX-A-AP	2	5	SMX-C-S-G-NA	1
4	SMX-CPM-A-AP	1		SMX-G-CXM-CPM-NA	1
	SMX-G-T-DXT	1		SMX-A-CXM-KF-AP	1
	SMX-C-G-AP	1		SMX-G-DXT-A-AP	1
	SMX-C-A-AP	1		SMX-DXT-T-A-AP	2
	SMX-DXT-A-AP	1	6	SMX-NI-C-DXT-T-A	1
5	SMX-G-NA-A-AP	1		SMX-G-CXM-DXT-A-AP	1
	SMX-C-G-CXM-CPM	1		SMX-T-CXM-KF-A-AP	2
	SMX-C-KF-A-AP	1		SMX-G-DXT-T-A-AP	2
	SMX-G-DXT-A-AP	1	7	SMX-CXM-KF-DXT-T-A-AP	1
	SMX-DXT-T-A-AP	1	8	SMX-C-G-NI-NA-KF-A-AP	1
6	SMX-C-KF-G-A-AP	1	9	SMX-G-K-KF-DXT-T-CIP-A-AP	1
	SMX-C-G-NA-A-AP	1	**Sampling Site R3 (N=30)**		
	SMX-G-DXT-T-A-AP	1	3	SMX-C-NA	1
7	SMX-CXM-KF-DXT-T-A-AP	1		SMX-G-CPM	1
8	SMX-C- G-NI-CXM-KF-A-AP	1		SMX-A-AP	1
9	SMX-S-T-CXM-KF-DXT-NA-A-AP	1	4	SMX-G-CPM-NA	1
Sampling site R2 (N=30)				SMX-G-A-AP	1
3	SMX-T-AP	1		SMX-C-A-AP	2
	SMX-CXM-T	1	5	SMX-CXM-DXT-CIP-A	1
	SMX-C-KF	2		SMX-G-K-A-AP	1
	SMX-A-AP	2		SMX-DXT-T-A-AP	3
4	SMX-CXM-G-A	1	6	SMX-A-G-DXT-T-AP	1
	SMX-KF-A-AP	1		SMX-G-T-CPM-DXT-AP	1

Sampling site R3 (N=30)		
№ of antimicrobials	**Resistance pattern**	**№ observed**
7	SMX-C-DXT-T-NA-A-AP	1
8	SMX-C-S-G-NE-CXM-NA-A	1
Sampling site R4 (N=30)		
3	SMX-T-CIP	1
	SMX-A-AP	2
4	SMX-CXM-DXT-AP	1
	SMX-G-A-AP	1
	SMX-G-CXM-CPM	1
	SMX-DXT-A-AP	1
5	SMX-G-DXT-A-AP	1
	SMX-CPM-NA-AP	1
	SMX-C-DXT-A-AP	1
	SMX-DXT-T-A-AP	1
6	SMX-CXM-CPM-NA-A-AP	1
	SMX-KF-DXT-NA-A-AP	1
7	SMX-C-DXT-T-NA-A-AP	1
8	SMX-C-S-CXM-CPM-DXT-A-AP	1
Sampling site R5 (N=30)		
3	SMX-CXM-AP	1`
	SMX-A-AP	1
4	SMX-G-A-K	1
	SMX-G-A-AP	3
	SMX-G-CXM-CPM	1
5	SMX-NI-CXM-CPM-KF	1
	SMX-T-KF-A-AP	1
	SMX-C-DXT-A-AP	1
6	SMX-C-G-KF-A-AP	1
	SMX-G-KF-T-A-AP	1
7	SMX-G-NE-CXM-NA-A-AP	1
8	SMX-G-KF-DXT-T-NA-A-AP	1

Sampling site R6 (N=30)		
№ of antimicrobials	**Resistance pattern**	**№ observed**
3	SMX-DXT-NA	1
	SMX-CPM-A	2
	SMX-A-AP	2
4	SMX-G-DXT-T	1
	SMX-CXM-CPM-AP	1
	SMX-G-K-A	1
	SMX-G-CXM-CPM	1
	SMX-DXT-A-AP	1
5	SMX-T-CPM-DXT-AP	1
	SMX-CXM-KF-NA-A	1
	SMX-G-CXM-A-AP	1
	SMX-KF-DXT-A-AP	3
	SMX-DXT-T-A-AP	2
6	SMX-G-KF-DXT-T-A-AP	1
	SMX-G-KF-DXT-NA-AP	1
	SMX-G-DXT-T-A-AP	1
7	SXM-NI-CXM-KF-G-A-AP	1
8	SMX-C-G-DXT-T-NA-A-AP	1
9	SMX-G-NI-CXM-KF-DXT-T-A-AP	1
Sampling site R7 (N=30)		
3	SMX-G-A	1
	SMX-A-NI	1
	SMX-DXT-AP	1
	SMX-K-A	2
	SMX-G-CPM	2
	SMX-A-AP	4
4	SMX-C-CXM-CPM	1
	SMX-C-NA-A	1
	SMX-KF-DXT-AP	1
	SMX-G-K-A	1
	SMX-G-CXM-CPM	1

Sampling site R7 (N=30)		
№ of antimicrobials	**Resistance pattern**	**№ observed**
	SMX-DXT-A-AP	2
5	SMX-CXM-CPM-DXT-AP	1
	SMX-CXM-CPM-DXT-A	1
	SMX-T-CXM-KF-A	1
	SMX-CXM-CPM-A-AP	1
	SMX-G-T-A-AP	2
	SMX-C-T-A-AP	3
	SMX-DXT-T-A-AP	2
6	SMX-G-CPM-DXT-T-A-AP	1
	SMX-C-KF-DXT-A-AP	1
	SMX-A-NI-CXM-KF-G	2
	SMX-G-NI-CPM-KF-AP	2
	SMX-G-DXT-T-A-AP	3
7	SXM-NI-CXM-KF-G-A-AP	1
8	SMX-NI-CXM-KF-G-T-A-AP	1
	SMX-G-NI-CXM-CPM-KF-A-AP	2
9	SMX-CXM-CPM-KF-DXT-NA-A-AP	1
Sampling site R8 (N=30)		
3	SMX-NI-AP	1
	SMX-KF-AP	1
	SMX-CXM-KF	1
	SMX-G-A	2
	SMX-G-CPM	2
	SMX-A-AP	2
4	SMX-CXM-CPM-A	1
	SMX-C-DXT-A	1
	SMX-CXM-CPM-AP	1
	SMX-G-K-A	1
	SMX-T-A-AP	1

Sampling site R8 (N=30)		
№ of antimicrobials	**Resistance pattern**	**№ observed**
	SMX-DXT-A-AP	1
5	SMX-CPM-DXT-A-AP	1
	SMX-CPM-KF-A-AP	1
	SMX-G-CXM-CPM-CIP	1
	SMX-G-T-A-AP	2
	SMX-C-T-A-AP	3
	SMX-DXT-T-A-AP	2
6	SMX-C-CXM-DXT-A-AP	1
	SMX-G-KF-DXT-A-AP	1
	SMX-G-CXM-KF-A-AP	2
7	SXM-NI-CXM-KF-G-A-AP	1
8	SMX-G-KF-DXT-NA-A-AP	1
	SMX-C-G-NI-KF-DXT-A-AP	3
9	SMX-NE-CXM-CPM-KF-DXT-NA-A-AP	1
Sampling site R9 (N=30)		
3	SMX-G-CPM	1
	SMX-A-AP	1
4	SMX-NA-A-AP	1
	SMX-T-A-AP	1
5	SMX-C-CXM-CPM-A	1
	SMX-CPM-DXT-NA-AP	1
6	SMX-CPM-KF-DXT-A-AP	1
	SMX-T-CXM-KF-A-AP	1
7	SMX-C-S-T-CXM-KF-AP	1
8	SMX-C-G-K-DXT-T-A-AP	1
Sampling site R10 (N=30)		
3	SMX-DXT-KF	1
	SMX-A-AP	1
	SMX-CXM-G	2

Sampling site R10 (N=30)		
№ of antimicrobials	**Resistance pattern**	**№ observed**
4	SMX-G-DXT-NA	1
	SMX-CXM-A-AP	2
	SMX-T-A-AP	1
5	SMX-G-DXT-A-AP	1
	SMX-CXM-KF-DXT-A	1
6	SMX-C-T-CXM-A-AP	1
	SMX-C-CXM-NA-A-AP	1
	SMX-G-DXT-T-A-AP	1
7	SMX-NI-CXM-KF-DXT-A-AP	1
8	SMX-G-KF-K-DXT-T-A-AP	1

SMX Suphamethoxazole, *T* Tetracycline, *CIP* Ciprofloxacin, *A* Amoxycilin, *DXT* Doxycycline, *CXM* Cefuroxin, *G* Gentamycin, *AP* Ampicilllin, *CPM* Cefepime, *C* Chloramphenicol, *NA* Nalidixic acid, *KF* Cephalotin, *NI* Nitrofurantoin, *S* Streptomycin, *K* Kanamycin, *NE* Neomycin.

Table 5.4 presents the ARI and MARI values of all the sampling stations. In general, the MAR indices in all the river samples were found to be higher than the 0.2 threshold value, revealing imprudent use and greater exposure to antibiotics in humans, aquaculture, poultry and livestock which may pose high ecological risk to the waters. The MAR index actually ranged from 0.5 to 0.8. The modal MAR index for the tested isolates was 0.8 at R7 which is approximately four times the 0.2 limit while the lowest was at R9 with 0.5. This implies that the burden of antibiotics being discharged into site R7 is greater than others.

Table 5.4 Predominant antibiotic resistance patterns of *E. coli* isolates from different sampling locations

	Antimicrobial agents																					
Sampling locations	AK	S	K	NE	G	CPM	KF	CXM	MEM	IMI	CIP	GAT	NA	SMX	NI	C	DXT	T	AP	A	Total	ARI	MARI
R1	0	0	1	0	5	21	8	13	0	0	0	0	2	30	4	5	9	5	17	12	132	0.022	0.65
R2	0	1	1	0	3	19	13	18	0	0	0	0	9	30	7	3	18	10	21	19	172	0.029	0.70
R3	0	0	0	2	2	5	3	7	0	0	0	0	0	30	3	12	8	1	16	11	100	0.017	0.60
R4	0	1	0	1	3	10	9	13	0	0	0	0	6	30	1	8	12	5	20	21	140	0.023	0.70
R5	0	0	0	0	3	2	3	9	0	0	0	0	0	30	2	11	3	1	12	11	87	0.015	0.55
R6	0	1	0	0	4	12	11	19	0	0	1	0	12	30	7	4	7	6	22	24	160	0.026	0.70
R7	0	2	2	1	8	25	21	24	0	0	1	0	10	30	10	7	16	11	24	25	217	0.036	0.80
R8	0	1	0	1	5	5	14	19	0	0	1	0	5	30	9	10	8	6	21	23	158	0.026	0.75
R9	0	0	0	1	3	0	2	5	0	0	0	0	0	30	2	1	0	1	7	12	64	0.011	0.50
R10	0	0	0	0	3	6	4	17	0	0	0	0	0	30	5	4	2	7	12	19	109	0.018	0.55
Total	0	6	4	6	39	105	88	144	0	0	3	0	44	300	50	65	83	53	172	177	1326		

AK Amikacin, *S* Streptomycin, *K* Kanamycin, *NE* Neomycin, *G* Gentamycin, *CPM* Cefepime, *KF* Cephalotin, *CXM* Cefuroxin, *MEM* Meropenenm, *IMI* Imipenem, *CIP* Ciprofloxacin, *GAT* Gatilofloxacin, *NA* Nalidixic acid, *SMX* Suphamethoxazole, *NI* Nitrofurantoin, *C* Chloramphenicol, *DXT* Doxycycline, *T* Tetracycline, *AP* Ampicilllin, *A* Amoxycilin.

Generally, the one-way ANOVA showed that cefepime, cephalothin, cefuroxime, nalidixic acid, nitrofurantoin, chloramphenicol and tetracycline were not significantly different in their effect against the isolates from all locations ($P>0.05$), whereas the resistance profile of the isolates against gentamycin, ciprofloxacin, sulphamethoxazole, ampicillin and amoxicillin were significantly different ($P<0.05$). Amikacin, kanamycin, streptomycin, meropenem, imipenem and gatilofloxacin were statistically excluded from the analysis since all tested isolates showed total susceptibility to these antimicrobials.

A multiple comparisons analysis was done to determine the resistance patterns of isolates to antimicrobials with respect to isolate sources, i.e., R1–R10. We observed that the differences in resistance patterns of the isolates to ciprofloxacin, cefuroxime, gentamycin, nitrofurantoin, chloramphenicol, doxycycline, tetracycline, ampicillin and amoxicillin were not statistically significant in relation to sampling sites R1, R2, R4, R5, R7 and R8. Similarly, isolates from all locations (R1–R10) did not significantly differ in their resistance profile to sulphamethoxazole as they were all resistant to it. The homogeneous subsets analysis indicated over 60 % resistance to the antimicrobials by isolates from over 70 % of the locations; hence the widespread of antimicrobial resistance in the surface water samples.

5.4 Discussion

The presumptive *E. coli* isolates were confirmed by PCR technique by targeting the *uid*A gene. The *uid*A gene has been used in PCR assays for *E. coli* as a control amplicon due to the shared ability to cleave the colisure indicator 4-methylumbelliferyl-β-D-glucuronide (MUG) among different strains (Bej et al. 1991). Antibiogram results also show that *E. coli* isolates revealed resistance to more than one antimicrobial, similar to reports by other researchers (Reinthaler et al. 2003; Noble et al. 2003; Lin et al. 2004). The finding on the susceptibility pattern of the

bacterial isolates to imipenem and meropenem (cephems), amikacin, kanamycin, neomycin and streptomycin (aminoglycosides) and gatilofloxacin and ciprofloxacin (fluoroquinolones) is consistent with that of other studies which reported high susceptibility to imipenem and low resistance to the quinolones and aminoglycosides respectively (Osundiya et al. 2013; Olayinka et al. 2009). Results from this study indicate that gatilofloxacin and ciprofloxacin (fluoroquinolones) and amikacin, kanamycin and streptomycin (aminoglycosides) are the drugs of choice for *E. coli* infections, since none or few of the isolates were resistant to them. This shows the effectiveness of the fluoroquinolones and aminoglycosides, and is in agreement with the finding which suggested the use of these classes of antimicrobial agents against a range of pathogens (Scheld 2003).

The antibiotic sensitivity patterns of the *E. coli* isolates obtained in this research reveals that a larger percentage of the *E. coli* was resistant to one or more of the antimicrobial agents tested. The isolates' resistance to ampicillin and amoxycillin, sulphamethoxazole, and chloramphenicol treatments has been described elsewhere (Manikandan et al. 2011). Although, sampling site R7 is a moderately populated place in the state, the availability of the river has formed the basis for industrial location (e.g. Bottling Company) and a large scale fishing business at sites R4, R7 and R10, as a means of livelihood for the inhabitants of the area. Fishing is common in most of the sampling sites, hence the high possibility of influence of domestic and industrial wastes into the environmental waters and the likelihood of antimicrobials being discharged into the waters is feasible as evidenced by the present findings. Both the presence of human settlements and small-scale industries are among the key factors that may directly or indirectly contribute to the increasing resistance of bacteria in the rivers, a finding that mirrors all the sampling sites except R3, possibly because it is a sacred site. The fact that some of these *E. coli* isolates showed high

levels of resistance to some of the antimicrobials used is an indication that the antibiotics may have been abused or overly used for the treatment of bacterial infections hence, the possibility of building resistance against the antimicrobials (Ramesh et al. 2010). Resistance to antimicrobial agents is most common in areas with high usage of antibiotics such as hospitals (Sternbuerg 1999). Patterns of multi-drug resistance have also been observed in *E. coli* isolated from patients with urinary tract infections (Sevanan et al. 2011). The detection of multi-drug *E. coli* resistance in this study was neither shocking nor surprising as some of the rivers act as sinks for hospital wastes in upstream areas. The distribution and occurrence of *E. coli* having reduced susceptibility to multiple antimicrobial agents in surface water and other environmental media has equally been reported (Boerlin et al. 2005; Sayah et al. 2005).

The development of antimicrobial resistance by the bacteria to these drugs poses a major challenge in both human and animal medicine because these drugs are commonly used in animal and human therapeutic practices. Uncontrolled usage of antibiotics in treatment of animals and their incorporation in animal feeds has been suspected to account significantly to the increase in antimicrobial resistance in pathogenic bacterial isolates (WHO 2000; Galland et al. 2001). High *E. coli* resistance to tetracyclines and penicillins has been observed by many researchers (Al-Haj et al. 2007; Shitandi and Sternesjö 2001; O'Brien 1987). Animal rearing coupled with slaughtering of animals are a common practice around sampling locations R1, R2 and R8. Since these animals or poultry have often been treated with antibiotics during their breeding, the waste generated contains fairly high levels of antimicrobials. The high level of resistance to tetracyclines obtained in this study may be as a result of it being the most commonly available antimicrobial agent used as a growth promoter and routine chemoprophylaxis among livestock in Nigeria (Olatoye 2010). Penicillin and tetracycline are known to be extensively used in

developing countries to treat respiratory infections, diarrhoea, mastitis and other infectious diseases in beef and dairy cattle (Hart and Ariuki 1998; Okeke et al. 1995).

It is bothersome bearing in mind that tetracycline is a first line drug in Nigeria, and as in most developing countries, people with gastrointestinal infections readily procure it across the counter for self-prescription and medication (Chigor et al. 2010). Resistance was found to be relatively low against streptomycin. This probably may be because of less exposure to the antibiotic due to the discouraged use of the antibiotic and the fact that it is usually administered intravenously thereby restricting indiscriminate use (Cheesbrough 2000). Results of a similar study revealed that more than 90 % of *E. coli* isolates were multi-drug resistant to three or more commonly used antibiotics (Olowe et al. 2008). All the MDR isolates were resistant to sulphamethoxazole and most to ampicillin, amoxycillin, cephalotin, cefuroxime and cefepime. Findings from water samples of Southwest Nigeria also showed that most of the *E. coli* isolates were resistant to various antimicrobial agents ranging from two to seven kinds including cotrimoxazole, tetracycline and amoxicillin (Lateef et al. 2003). The study around Jimma, Ethiopia, showed that almost all *E. coli* isolates from environmental sources were found to be multi-drug resistant to the commonly used antimicrobials including amoxicillin, tetracycline, and cotrimoxazole (Wolde-Tenssay 2002). Also, 96.7 % of *E. coli* isolated from drinking water samples from Hyderabad, India, were found to be resistant to two to six antibiotics (Atif et al. 2010). Another similar report also indicated that bacterial isolates from household drinking water demonstrated multiple drug resistance to antibiotics (Obi et al. 2004).

Resistance patterns may demonstrate multiple resistances to many antimicrobial agents phenotypically and genotypically and could present therapeutic consequences. Multiple resistances capable of regional dissemination can emerge as a result of antimicrobial selection

pressure in either livestock or humans. This increase in multidrug resistance is quite alarming if coupled with the fact that such isolates harbour plasmids on which these genes may be located, because they are highly transferable (Aarestrup 1995; Levin et al. 1997).

The increased concentration of multi-drug resistant bacteria in the aquatic environment creates selective pressures on natural bacterial strains (Alpay-Karoglu et al. 2007). Basically, the influence of the various anthropogenic and other related activities within the sampling sites might be a strong indication for a high-risk source contamination of water as evidenced by the widespread antimicrobial resistance in the environment since their MARI values were by far greater than the threshold value of 0.2. Based on the comparison of MAR indices for *E. coli* isolates from a variety of sources, a suggestion was made by (Krumperman 1998) to use a MAR index of 0.2 for differentiating between low- and high-risk contamination, although he acknowledged that this value was arbitrary. Occurrence of antibiotic-resistant bacteria in a given environment may be an indication that an area is contaminated with antimicrobials (Gunaseelan and Ruban 2011). For example, Al-Bahry et al. (2009) suggested that the main reasons causing marine environmental contamination were improper and unnecessary use of antimicrobial drugs by human and animals.

Two major intrinsic mechanisms were reported to confer bacterial resistance to multiple antimicrobial drug classes: mutations in outer membrane porins resulting in reduced permeability to antimicrobials and over expression of multidrug efflux pumps, which tend to pump out antibiotics before they have the opportunity of acting on their target. Multiple antibiotic-resistant bacterial strains may also arise due to dissimilar mechanisms accumulating sequentially in an organism (Navon-Venezia et al. 2005). The observation on high MAR indices indicates that isolates originated from high risks source(s) of contamination where antibiotics are frequently

used and high levels of antibiotics usage and resistance have been found to be associated with animal husbandry (Paul et al. 1997; Bohm et al. 2004).

5.5 Conclusion

Confirmation of the presence of *E. coli* in river water samples in Osun State, Southwestern Nigeria, indicates faecal contamination and the possible presence of other enteric pathogens. Multiple antibiotic-resistant phenotypes and indices evaluated suggest increased presence of antibiotic-resistant *E. coli* in the surface waters tested and the prevalence can lead to serious health risk for communities that depend on the waters for sundry purposes. Resistant bacterial strains will continue to emerge unless indiscriminate use of drugs is curtailed by public awareness programs coupled with enforcement of legislation that limits the prescription and dispensing of antimicrobials to only qualified professionals. Our findings indicate a high incidence of antimicrobial resistance of *E. coli* towards the conventionally used antibiotics. There is, therefore, a need for good surveillance programs to monitor antimicrobial resistance patterns in water bodies.

Acknowledgements

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Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER SIX

Antimicrobial resistance determinants of *Escherichia coli* isolates recovered from some rivers in Osun State, South-Western Nigeria: implications for public health

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Antimicrobial resistance determinants of *Escherichia coli* isolates recovered from some rivers in Osun State, South-Western Nigeria: implications for public health

Abstract

The inevitable development of resistance has sunk the great success achieved in the discovery of antimicrobial agents and dashed the hope of man in the recovery from infections and illnesses, as diseases and disease agents that were once thought to be controlled by antimicrobials are now re-emerging in new leagues resistance to therapy. A total of 300 PCR confirmed *Escherichia coli* isolates recovered from different river sources in Osun State, Nigeria were evaluated for their antibiogram profiling by the disc diffusion method and the resistant isolates were further profiled for their genotypic antimicrobial resistance determinants by polymerase chain reaction assays. Among the 20 antimicrobials selected from 10 families, resistance among sulfonamides, β-lactams and tetracyclines were found to be most frequent than phenicols and aminoglycosides with a noticeable increase in the number of multi-drug resistance ranging from three to nine antimicrobials. A total of 19 resistance determinants were assessed with their prevalence and distributions obtained as follows; [sulfonamides: *sul*I (8%) and *sul*II (41%)], [β-lactams: *amp*C 22%; $bla_{TEM,}$ (21%) and bla_{Z} (18%),], [tetracyclines: *tet*A (24%), *tet*B (23%), *tet*C (18%), *tet*D (78%), *tet*K (15%) and *tet*M, (10%)], [phenicols: *cat*I (37%), *cat*II (28%) and *cml*A1 (19%)] and [aminoglycosides: *aac*C2 (8%), *aph*A1 (80%), *aph*A2 (80%), *aad*A (79%) and *str*A (38%)]. The Pearson chi-square exact test revealed many strong significant associations among *amp*C, bla_{TEM}, bla_{Z} and *tet*A genes with some determinants screened. The findings signify high increase in the prevalence of multi-drug resistant *E. coli* isolates and resistance determinants indicating increased public health risks associated with the ingestion of waters from untreated sources.

Hence, a necessity for safe water supply, provision of proper sanitation facilities and good surveillance programmes to monitor antimicrobial resistance patterns in water bodies.

Keywords: Rivers, Prevalence, *Escherichia coli*, Multi-drug resistance, Genetic marker, Nigeria.

6.1 Introduction

Antimicrobial agents are widely used in human and veterinary medicine to control bacterial infections. Their usage for livestock can be for therapeutic, prophylactic, metaphylactic or growth promotion purposes. About 90% of the antimicrobials used in animal husbandry are for growth promotion and prophylaxis (Sarmah et al., 2006; Pitout and Laupland, 2008). With the majority of them being excreted unchanged into the environment, concerns about their potential impact on the aquatic environments keep increasing over time (Sarmah et al., 2006; Wright, 2007; Kemper, 2008).

Antimicrobial resistance has an important impact on health policies and involves an increasing number of bacterial species and resistance mechanisms. It has been observed that the highest prevalence of antimicrobial-resistant bacteria occur in those countries where they are extensively used for prevention and treatment of microbial infections in humans as well as animals (Kummerer, 2004; Junco-Diaz et al., 2006). Although antimicrobial therapy is a significant tool for the treatment of these infections, its widespread resistance has become a cause of great concern in veterinary medicine (Monroe and Polk, 2000; Teshager et al., 2000; Schoevaerdts et al., 2011). Indeed, a close association and direct correlation exist between the use of antimicrobial agents for the treatment of infections in animals and the levels of resistance observed (Schwarz and Chaslus-Dancla, 2001; Mellon et al., 2001; Bibbal et al., 2009). Antimicrobial resistance among pathogens has become an emerging threat to human and veterinary medicine due to the excessive and indiscriminate use for treatment, prophylaxis or

growth promotion, with increased multidrug resistant *Escherichia coli* now becoming a major public health issue both in the developed and developing countries (Ajamaluddin et al., 2000; Okeke et al., 2005; Chandran et al., 2008).

Contamination of water sources with faecal indicator bacteria like *E. coli* is a serious challenge due to its ability to transmit diseases. The risk associated with these bacteria further increases with a corresponding increase in their resistance to antimicrobial therapy (Da Silva and Mendonca, 2012). These bacteria could be transmitted from environment to human via direct or indirect route (Iversen et al., 2004; Kim et al., 2005; Rodríguez et al. 2006). *E. coli*, has been the foremost indicator of faecal contamination and possible incidence of water-borne diseases that are most injurious to health and a significant reservoir of genes coding for antimicrobial resistance and therefore is a useful indicator for resistance in bacterial communities (Bucknell et al., 1997; WHO, 2010). However, recovery of indicator bacteria may depend upon the extent of contamination in a particular water source (Warner et al., 2008). *E. coli* has been reported to acquire and transfer virulence and antimicrobial resistance genes to enteric pathogenic and normal flora bacteria in the environment through horizontal transfer of resistance (R) plasmids, transposons and integrons (Platt et al., 1986; Pang et al., 1994; Schwarz and Chaslus-Dancla, 2001; Nordmann and Poirel, 2005; Leverstein-van Hall et al., 2002; Pruden et al., 2006; Tenover, 2006; Ozgumus et al., 2007), although the resistance they carry may not be a problem as such but the transfer of resistance factors to zoonotic pathogens inhabiting the gut has stern implications for animal and human health (Ranjana Sharma et al., 2008).

In surface water, it is difficult to find an area where antibiotics cannot be detected, except for the pristine spot in the mountains before the rivers or streams go through urban or agricultural areas (Yang and Carlson, 2003). Apart from chemical pollution caused by antibiotics themselves,

their use may also accelerate the development of antimicrobial resistance determinants and bacteria, which further shade health risks to humans and animals (Kemper, 2008).

Antimicrobial resistance determinants (ARDs) from hospital wastewaters, animal husbandry and aquaculture areas directly exposed to the environment are eventually carried to nearby streams, rivers, lakes, or other aquatic bodies or even leach downward through the soil during rainfall. Several reports have indicated that bacteria harbouring ARDs can be released from sewage treatment plants (STPs) into surface waters (Tennstedt et al., 2005; Chen et al., 2007; Auerbach et al., 2007) and the genes in surface and ground waters can transfer antibiotic resistance to the bacteria in drinking water or the food chain (Chee-Sanford et al., 2001).

Scores of ARDs have been found in the isolates or microbial communities in the natural waters, which were not or slightly polluted (Jacobs and Chenia, 2007; Mohapatra et al., 2008; Rahman et al., 2008). Several types of aminoglycoside resistance genes have been detected in the microorganisms isolated from surface water, including *aac* (Lee et al., 1998), *aad* (Park et al., 2003; Mukherjee and Chakraborty, 2006), *aph* (Poppe et al., 2006) and *str* (Mohapatra et al., 2008). Sulfonamide resistance genes including *sulI, II, III* and *A* were detected in the microorganisms of river water and sediments (Lin and Biyela, 2005; Pei et al., 2006; Poppe et al., 2006; Mohapatra et al., 2008). The presence of ARGs, such as *tet*, *van* and *sul* has been reported in wastewater, surface water and sediments (Schwartz et al., 2003; Pei et al., 2006; Ram et al., 2007). Similarly, beta lactams *ampC* and *bla* genes have been detected in surface water biofilms and estuarine water (Schwartz et al., 2003, Henriques et al., 2006).

Globally, antimicrobial resistant bacteria and their associated determinants have become an important environmental contamination issue which is currently receiving an increased attention (Kummerer, 2004; Pruden et al., 2006; Sapkota et al., 2007). Thus, adequate

information on the prevalence of antimicrobial resistance in pathogens would serve as basis for proper selection of optimal treatment when necessary (Okeke et al., 2005). Several waterborne outbreaks attributed to multidrug resistant *E. coli* have been reported worldwide and high prevalence of multi-drug resistance indicates a serious need for antibiotic surveillance and planning of effective interventions to reduce multidrug resistance in such pathogens (Olayinka et al., 2004). The monitoring and surveillance of both antibiotic usage and multiple antibiotic resistances especially in nosocomial infections is necessary to setting up of effective containment programs and audit of such programmme (Mthembu, 2008; Kamat et al., 2008). The spread of ARDs into environments where antibiotics are not used remains a postulation yet to be thoroughly investigated; although it has been hypothesized that water could disseminate AMR (WRC, 2001). Modern food animal production which depends on the use of large amounts of antibiotics for disease control and agricultural purposes, particularly for growth enhancement provides favourable conditions for selection, spread and persistence of antimicrobial-resistant bacteria capable of causing infections in animals and humans through food chains (ETAG, 2005; Mathew et al., 2007). As a result, when water bodies are contaminated with faecal materials containing antibiotic resistant bacteria, they could serve as a source and/or pool of antibiotic resistant genetic elements that could possibly be transferred to other bacterial species and make the water quality unpleasant to health (Biyela and Bezuidenhout, 2004) and also create a favourable condition for the spread of antibiotic resistant genetic elements from place to place (APUA, 1999).

Unfortunately, in Nigeria and particularly Osun State, previous studies have only reported high levels of microbial contamination and phenotypic antimicrobial susceptibility profiling of water sources used by the population (Lateef et al., 2003; Olowe et al., 2008). To the

best of our knowledge, there is dearth of information regarding the genotypic characteristics of antibiotic resistance determinants of *E. coli* isolates recovered from aquatic environments in South Western Nigeria and it is in the light of this that the study aimed at investigating the prevalence and distribution of antimicrobial resistance determinants of *E.coli* isolates from some selected rivers of Osun State, South Western Nigeria, as the first of its kind in the region.

6.2 Materials and methods

6.2.1 Description of study area and sampling sites

Water samples were collected from ten rivers at different locations in Osun State, South-Western Nigeria. The sites were selected after due consultation with the State Ministries of Environment, Water Resources, Lands and Housing for proper mapping of the state rivers. Table 1 shows the description of the sampling sites. For convenience, the sampling locations were coded as follows: R1: Erinle-Ede; R2: Ido-Osun; R3: Osun-Osogbo, R4: Oba-Iwo; R5: Ejigbo; R6: Ilobu-Okinni; R7: Asejire-Ikire; R8: Shasha; R9 and Ila-Oke Ila, R10: Inisha-Okuku and illustrated on the map found below (Figure 6.1).

Table 6.1 Sampling sites description and their location.

Site code	Site name	Activities	GPS coordinates
R1	Erinle-Ede	Fishing, animal rearing, irrigation, tourism and domestic purposes	7°44′44″ N; 4°29′22″ E
R2	Ido Osun	Animal rearing, fishing, irrigation domestic and recreational purposes	7°49′ 01″ N; 4° 26′41″ E
R3	Osun-Osogbo	Farming, fishing, habitation, tourism and worship shrine	7°46′ 04″ N; 4°34′00″ E
R4	Oba-Iwo	Fishing, domestic and recreational purposes	7°38′ 01″ N; 4°11′20″ E
R5	Ejigbo	Farming, fishing and domestic use	7°54′0″ N; 4°18′54″ E
R6	Ilobu-Okinni	Car washing, swimming and domestic use	7°50′06″ N; 4°29′14″ E
R7	Asejire-Ikire	Fishing, irrigation, domestic and industrial activities	7°25′44″ N; 4°13′14″ E
R8	Shasha	Fishing, farming, palm oil processing and domestic purposes	7°22′32″ N; 4°28′11″ E
R9	Ila-Oke Ila	Farming, irrigation, fishing and domestic activities	8°01′50″ N; 4°59′03″ E
R10	Inisha-Okuku	Fishing, irrigation and domestic activities	8°01′.32″ N; 4°42′11″ E

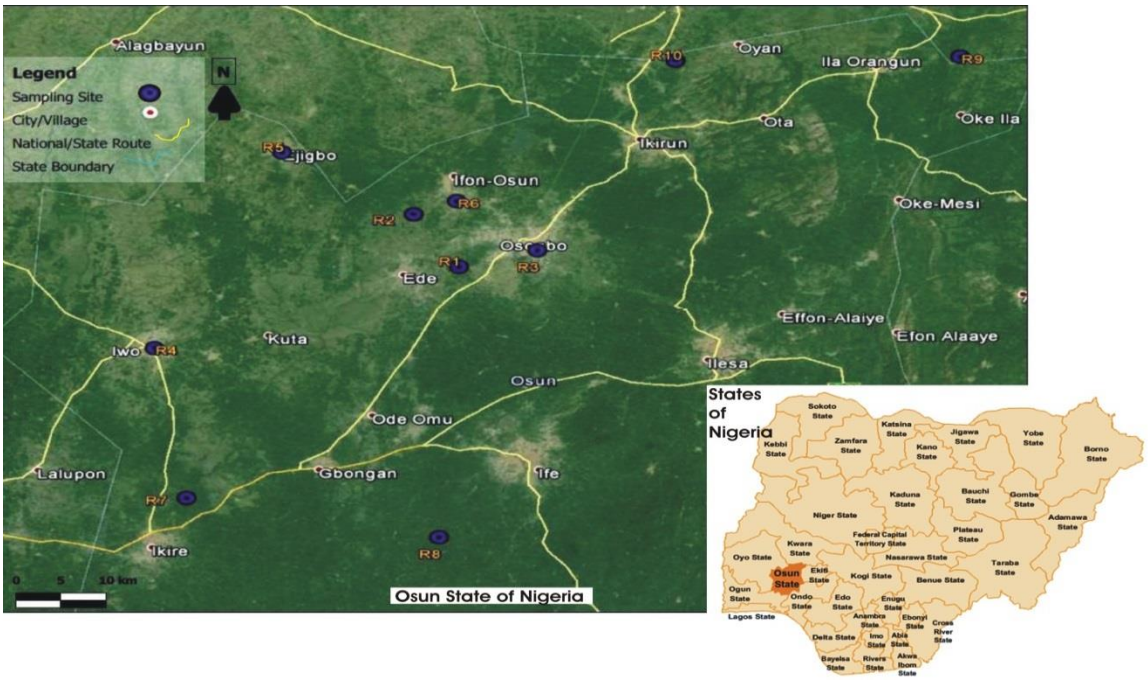

Figure 6.1 Map of Osun State, Nigeria showing the sampling locations.

6.2.2 Sampling and isolation of presumptive E. coli

Water samples were aseptically collected monthly over a period of one year from September 2011 to August 2012. All samples were collected in 1.5 L sterile bottles and transported on ice to the laboratory where they were processed within 6 h of collection. The bottles were sterilized by autoclaving and during sampling, were triple-rinsed with sample water before being filled. Analysis of water samples was performed according to Standard Methods (APHA, 1998). One hundred millilitre (100 ml) aliquots of the water samples were filtered through a 90 mm diameter, 0.45 μm pore-sized membrane filters (Millipore, Ireland). The filters were incubated overnight at 44.5 °C on eosin methylene blue agar (Oxoid, UK). Characteristic metallic-sheen colonies were selected and purified by streaking on *E. coli* chromogenic agar (Conda Pronadisa, Spain) plates before storing on glycerol for further use.

6.2.3 PCR confirmation of E. coli isolates and DNA isolation

Genomic DNA was extracted from the isolates using the boiling method as described by Gueimonde et al., (2004) and Naas et al., (2007). Briefly, a few colonies of each bacterial strain was suspended in 200 µl of distilled water and heated at 100^{o}C for 10 min followed by centrifugation at 13 500 rpm for 10 min and the recovered supernatant was frozen at –20^{o}C until use.

6.2.4 Antibiotic susceptibility testing

Antibiotic resistance of *E. coli* isolates was determined by the Kirby Bauer disc diffusion assay method using the standard procedure of the Clinical and Laboratory Standards Institute (CLSI, 2010). The isolates were screened for susceptibility to a panel of twenty antibiotic discs belonging to ten classes (Mast Diagnostics, UK), impregnated with aminoglycosides: amikacin

(30 µg), streptomycin (300 µg), kanamycin (30 µg), neomycin (10 µg) gentamycin (10 µg); cephems: cefepime (30 µg), cephalothin (30 µg), cefuroxime (30 µg); carbapenems meropenem (10 µg), imipenem (10 µg); fluoroquinolones: ciprofloxacin (5 µg), gatilofloxacin (5 µg); quinolones: nalidixic acid (30 µg), sulfonamides: sulphamethoxazole (25 µg); nitrofuratoins: nitrofuratoin (200 µg); phenicols: chloramphenicol (30 µg); tetracyclines: tetracycline (30 µg), doxycycline (30 µg) and beta-lactamases: amoxycillin (25 µg) and ampicillin (25 µg). The inoculum for antibiotic susceptibility pattern testing was suspended in stroke physiological saline solution (0.85% NaCl) by dispensing a single colony picked up with a sterile cotton swab (Copan, Italy). The turbidity of the resulting solution was adjusted to 0.5 McFarland standard (using 0.5 ml of 0.048 M $BaCl_2$ added to 99.5 ml of 0.18 M H_2SO_4 as a standard for comparison) which is approximately equal to 1×10^8cells/ml (McFarland, 1907). One hundred microlitre of solution was spread plated onto Mueller Hinton agar plates. The antibiotic discs were placed 30 mm apart on the inoculated plates by using a disc dispensing apparatus. Fifteen minutes after the discs were applied; the plates were inverted and incubated at 37°C for 24 h. The inhibition zone diameters were measured to the nearest millimetre and recorded. Each isolate was categorized as susceptible (S), intermediate (I) and resistant (R) to antibiotics according to the zone diameter interpretation standard recommended by the Clinical and Laboratory Standards Institute (CLSI, 2010) to establish the antibiogram profiles of the isolates.

The frequency of antibiotic-resistant isolates was calculated by the equation: $A/B \times 100\%$, where A is the number of isolate resistant to an antibiotic and B is the total number of isolates from the sample.

6.2.5 PCR detection of antimicrobial resistance genes

The phenotypic antimicrobial resistant *E. coli* isolates obtained from the ten sampling sites was analysed for the presence of relevant resistance genes using simplex PCR assays. Sulfonamide resistance genes (*sulI* and *sulII*), β-lactamases-encoding genes (*ampC, bla_{TEM}* and *bla_{Z}*), tetracycline resistance genes (*tetA, tetB, tetC, tetD, tetK* and *tetM*), chloramphenicol resistance genes (*catI, catII* and *cmlA1),* and aminoglycoside resistance genes *(aacC2, apHAI, apHAII, aadA* and *strA)* were the selected target genes. For the PCR amplification of the target genes, the reaction mixture contained 12.5 µl of PCR Master Mix (Thermo Scientific, (EU) Lithuania), 0.5 µl each of oligonucleotide primer (Inqaba Biotech, SA), 5 µl of template DNA and 6.5 µl of nuclease free water to constitute a total reaction volume of 25 µl. The sequences of primers, PCR conditions and amplicon sizes are provided in Table 2. All PCR assays were performed on a thermocycler (Bio-Rad Mycycler, USA) and each run included a negative control. Amplicons of each sample (5 µl) was mixed with 2 µl loading dye and resolved on 1.5% agarose gels (Merck, SA) containing 5 µl ethidium bromide (Sigma-Aldrich, USA). A 100-bp marker (Thermo Scientific, (EU) Lithuania) was also included for DNA band size estimation purposes. All gels were run in 0.5 X TBE buffer at 100 V for 45 min, and visualized by UV trans-illumination (Alliance 4.7, France).

Table 6.2 PCR primers used for antimicrobial resistance gene amplification.

Antimicrobial family	Primer	PCR primer sequence (5 ′–3 ′)	Amplicon size (bp)	PCR cycling condition	Reference
Sulfonamides	*sul*1	F: TTCGGCATTCTGAATCTCAC R: ATGATCTAACCCTCGGTCTC	822	Initial denaturation at 94°C for 5 min, followed by 1 min of denaturation at 94°C, 1 min of annealing at 55°C, 5 min of extension at 72°C for a total of 35 cycles and 5min of final extension at 72°C.	Maynard et al. 2004
	*sul*11	F: CGGCATCGTCAACATAACC R: GTGTGCGGATGAAGTCAG	625	Initial denaturation for 5 min at 94°C, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1.5 min and final extension at 72°C for 5 min.	Falbo et al. 1999
Beta-lactams	*amp*C	F: TTCTATCAAMACTGGCARCC R: CCYTTTTATGTACCCAYGA	550	94°C for 4 min followed by 30 cycles: denaturation at 94°C for 45s, annealing for 45s and extension at 72°C for 45s and final extension for 7 min at 72°C.	Velusamy et al. 2007
	bla_{TEM}	F: TTTCGTGTCGCCCTTATTCC R: CCGGCTCCAGATTTATCAGC	690	94°C for 5 min followed by 30 cycles of denaturation (94°C for 30 s), annealing (60°C for 30 s), extension (72°C for 90 s) and final incubation at 72°C for 5 min.	Jannine et al. 2010
	bla_{Z}	F: ACT TCA ACA CCT GCT GCT TTC R: TGA CCA CTT TTA TCA GCA ACC	490	94°C for 5 min followed by 30 cycles of denaturation (94°C for 30 s), annealing (60°C for 30 s), extension (72°C for 90 s) and final incubation at 72°C for 5 min.	Baddour et al. 2007
Tetracyclines	*tet*A	F: GCTACATCCTGCTTGCCTTC R: CATAGATCGCCGTGAAGAGG	201	5 min initial denaturation at 94°C followed by 35 cycles of 94°C for 1min, 55°C for 1min and 72°C for 1.5 min and final incubation at 72°C for 5 min.	Ng et al. 2001
	*tet*B	F: TTGGTTAGGGGCAAGTTTTG R: GTAATGGGCCAATAACACCG	359	5 min initial denature at 94°C followed by 35 cycles of 94°C for 1min, 55°C for 1min, 72°C for 1.5 min and final incubation at 72°C for 5 min.	Ng et al. 2001
	*tet*C	F: CTTGAGAGCCTTCAACCCAG R: ATGGTCGTCATCTACCTGCC	418	5 min initial denaturation at 94°C followed by 35 cycles of 94°C for 1min, 55°C for 1min, 72°C for 1.5 min and final incubation at 72°C for 5 min.	Ng et al. 2001
	*tet*D	F: AAACCATTACGGCATTCTGC R: GACCGGATACACCATCCATC	300	5 min initial denaturation at 94°C followed by 35 cycles of 94°C for 1min, 55°C for 1min, 72°C for 1.5 min and final incubation at 72°C for 5 min.	Ng et al. 2001
	*tet*K	F: GTA GCG ACA ATA GGT AAT AGT R: GTA GTG ACA ATA AAC CTC CTA	460	5 min initial denaturation at 94°C followed by 35 cycles of 94°C for 1min, 55°C for 1min and72°C for 1.5 min and final incubation at 72°C for 5 min.	Strommenger et al. 2003
	*tet*M	F: AGT GGA GCG ATT ACA GAA R: CAT ATG TCC TGG CGT GTC TA	158	5 min initial denaturation at 94°C followed by 35 cycles of 94°C for 1min, 55°C for 1min and72°C for 1.5 min and final incubation at 72°C for 5 min.	Strommenger et al. 2003
Phenicols	*cml*A1	F: CACCAATCATGACCAAG R: GGCATCACTCGGCATGGACATG	115	94°C for 5 min followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for1.5 min and final incubation at 72°C for 5 min.	Post and Hall 2009
	*cat*I	F: AGTTGCTCAATGTACCTATAACC R:TTGTAATTCATTAAGCATTCTGCC	320	5 min at 94°C, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1.5 min and final incubation at 72°C for 5 min.	Maynard et al. 2004
	*cat*II	F: ACACTTTGCCCTTTATCGTC R: TGAAAGCCATCACATACTGC	543	5 min at 94°C, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1.5 min and final incubation at 72°C for 5 min.	Maynard et al. 2004
Aminoglycosides	*str*A	F: CTTGGTGATAACGGCAATTC R: CCAATCGCAGATAGAAGGC	348	94°C for 4 min of initial denaturation, followed by 30 cycles of denaturation at 94°C for 45s, annealing for 45s at 50°C, extension at 72°C for 45s and final extension for 7	Velusamy et al. 2007

Antimicrobial family	Primer	PCR primer sequence (5′–3′)	Amplicon size (bp)	PCR cycling condition	Reference
				min at 72°C.	
	*aad*A	F: GTGGATGGCGGCCTGAAGCC R: AATGCCCAGTCGGCAGCG	525	94°C for 4 min followed by 30 cycles of 94°C for 45s, 50°C for 45 s and extension at 72°C for 45s and final extension for 7 min at 72°C.	Velusamy et al. 2007
	aac(3)-IIa (*aac*C2)[a]	F: CGGAAGGCAATAACGGAG R: TCGAACAGGTAGCACTGAG	428	5 min at 94°C, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1.5 min and final incubation at 72°C for 5 min.	Maynard et al. 2004
	aph(3)-Ia (*aph*A1)[a]	F: ATGGGCTCGCGATAATGTC R: CTCACCGAGGCAGTTCCAT	600	5 min at 94°C, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1.5 min and final incubation at 72°C for 5 min.	Maynard et al. 2004
	aph(3)-IIa (*aph*A2)[a]	F: GAACAAGATGGATTGCACGC R: GCTCTTCAGCAATATCACGG	510	5 min at 94°C, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1.5 min and final incubation at 72°C for 5 min.	Maynard et al. 2004

6.2.6 Determination of association between the resistance genes and statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences [(SPSS) IBM version 20 software]. Comparisons of the associations between resistance genes detected in the *E. coli* isolates were performed separately by using Pearson's chi-square exact test. The statistical significance was set at $P<0.05$. An association between two genes can either be positive, indicating that the genes are found together, or negative, indicating that the genes are not found together.

6.3 Results

6.3.1 PCR identification and prevalence of E. coli isolates from the ten sampling sites

In general, counts of confirmed *E. coli* isolates in the river water samples at all sites ranged between 34 CFU/100 ml at the R5 site and 76 CFU/100 ml at the R7 site. The counts of the confirmed *E. coli* obtained during the study period are depicted in Figure 6.2 while the representative gel electrophoresis picture of *E. coli* confirmation by PCR amplification of the *uidA* gene is also shown in Figure 6.3.

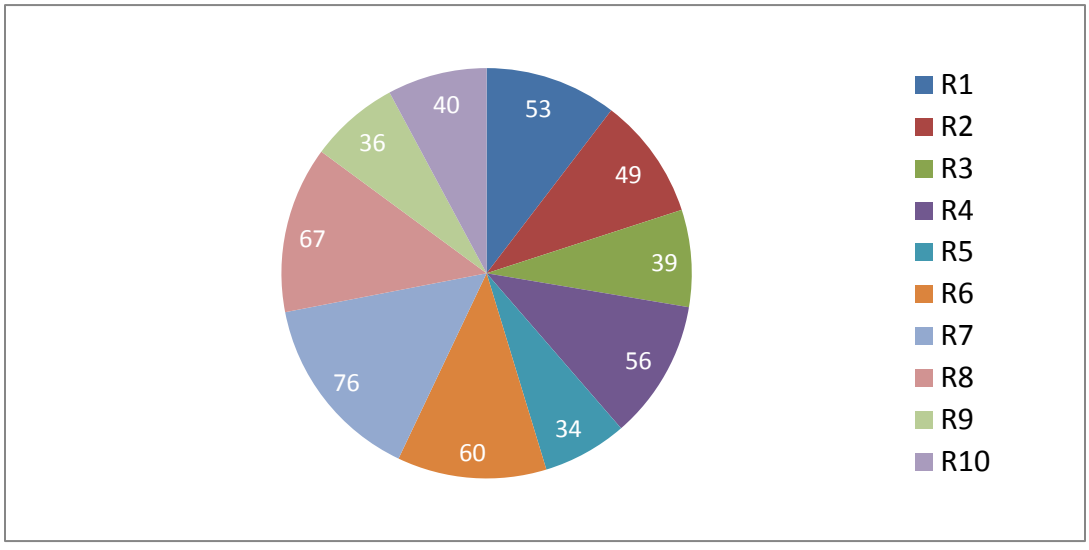

Figure 6.2 Comparative mean counts of *E. coli* at the ten sampling sites

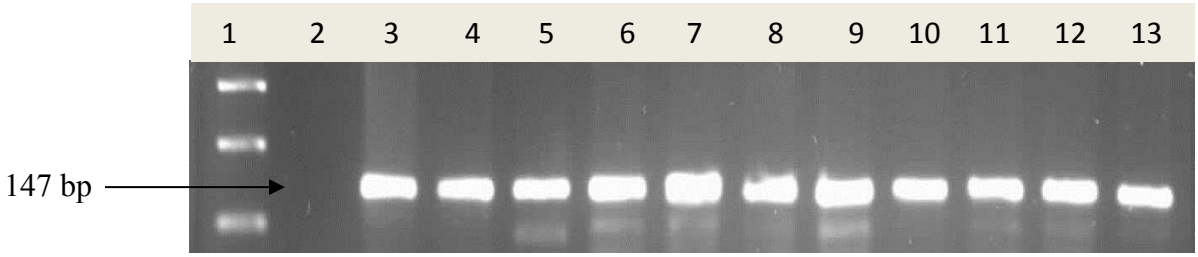

Figure 6.3 PCR confirmation of *E. coli* isolates from surface water.

Lane 1: molecular weight marker (100 bp); lane 2: negative control;
lane 3: positive control (ATCC *25922*); lanes 4–13: positive isolates.

6.3.2 Antimicrobial resistance and susceptibility phenotype characteristics

A total of 300 confirmed *E. coli* isolates comprising 30 isolates from each site, were pooled together and profiled for their probable phenotypic resistance to 20 different antimicrobials selected across 10 antimicrobial families. Surprisingly, all the isolates were resistant to sulphamethoxazole (100%) with high levels of resistance equally observed against amoxycillin (59%) and ampicillin (57%). Varied resistances for other antibiotics were recorded as follows; cefuroxime (48%), cefepime (35%), cephalotin (29%), tetracycline (28%), chloramphenicol (22%), doxycycline (18%), nitrofurantoin (17%), streptomycin (16%), nalidixic acid (15%) and gentamycin (13%) (Table 6.3).

Conversely, all the *E. coli* isolates were susceptible to all members of amikacin, meropenem, imipenem and gatilofloxacin and so therefore excluded from the subsequent analyses. Similarly, high sensitivities were detected against ciprofloxacin (96%), kanamycin (95%), neomycin (92%), streptomycin (84%) and chloramphenicol (73%) (Table 6.3).

Table 6.3 Antibiogram profile of *E. coli* isolates from river water sources in Osun State, Nigeria.

Antimicrobial family	Antimicrobial agent	Disc code	Potency (µg)	No of antimicrobial resistant isolates (%)										Total
				R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	
Sulfonamides	Sulphamethoxazole	SMX	25	30	30	30	30	30	30	30	30	30	30	300 (100)
Aminoglycosides	Amikacin	AK	30	0	0	0	0	0	0	0	0	0	0	0 (0)
	Gentamycin	G	10	5	3	2	3	3	4	8	5	3	3	39 (13)
	Kanamycin	K	30	1	2	1	0	0	5	3	3	0	0	15 (5)
	Neomycin	NE	10	1	1	0	0	0	3	4	7	3	6	25 (8)
	Streptomycin	S	300	0	3	2	3	2	8	5	12	5	7	47 (16)
β-lactams	Ampicillin	AP	25	7	12	16	12	17	22	24	21	21	20	172 (57)
	Amoxycillin	A	25	19	25	23	21	11	19	24	11	12	12	177 (59)
Cephems	Cefepime	CPM	30	21	19	5	10	2	12	25	5	0	6	105 (35)
	Cephalotin	KF	30	8	13	3	9	3	11	21	14	2	4	88 (29)
	Cefuroxine	CXM	30	13	18	7	13	9	19	24	19	5	17	144 (48)
Carbapenems	Meropenem	MEM	10	0	0	0	0	0	0	0	0	0	0	0 (0)
	Imipenem	IMI	10	0	0	0	0	0	0	0	0	0	0	0 (0)
Fluoroquinolones	Ciprofloxacin	CIP	5	0	0	0	0	0	1	1	1	0	0	3 (1)
	Gatilofloxacin	GAT	5	0	0	0	0	0	0	0	0	0	0	0 (0)
Quinolones	Nalidixic acid	NA	30	2	9	0	6	0	12	10	5	0	0	44 (15)
Nitrofuratoins	Nitrofurantoin	NI	200	4	7	3	1	2	7	10	9	2	5	50 (17)
Phenicols	Chloramphenicol	C	30	11	8	4	3	5	4	7	10	5	8	65 (22)
Tetracyclines	Tetracycline	T	30	9	17	15	7	8	7	8	2	4	6	83 (28)
	Doxycycline	DXT	30	5	10	1	5	1	6	11	6	1	7	53 (18)

[a]As determined by the disc diffusion.

6.3.3 Distributions of antimicrobial resistance genes among E. coli isolates

The choice of the resistance genes assessed was based on their high frequency of occurrence in phenotypically resistant *E. coli* isolates (Bush et al., 1995; Huovinen et al., 1995; Miller et al., 1997; Murray and Shaw, 1997; Sandvang and Aarestrup, 2000; White et al., 2000; Chopra and Roberts, 2001; Maynard et al., 2003). Therefore, 19 genes coding for resistance to antimicrobials in five families (sulfonamides, beta-lactams, tetracyclines, phenicols and aminoglycosides) were screened for possible detection, prevalence and distribution of resistance determinants among *E. coli* isolates from river water samples (Table 6.4). Likewise, the pattern and frequency of dual and multiple antimicrobial resistance genes in the isolates were compiled and presented in Table 6.5.

(i) Sulfonamides

Eight percent of the 300 sulfonamide-resistant isolates possessed the *sul*I gene, whereas 41% harboured *sul*II (Table 6.4). More than half of the isolates (53%) did not harbour either of the 2 genes and only 2% carried *sul*I–*sul*II (Table 6.5).

(ii) β-lactams

While the PCR amplification undertaken shows that 22% of the 172–ampicillin-resistant isolates were *amp*C positive, 21% and 18% of the 177–amoxycillin-resistant isolates harboured bla_{TEM}–bla_{Z} respectively (Table 6.4). Nine percent of the amoxycillin-resistant isolates possessed dual bla_{TEM}–bla_{Z} resistance genes (Table 6.5). Thirty seven percent of the amoxycillin resistant isolates and 38% of the ampicillin-resistant isolates were found negative to the β-lactam resistance genes examined.

(iii) Tetracyclines

The 83 *E. coli* isolates resistant to tetracycline treatment were screened for possible detection of tetracycline resistance genes; *tet*A, *tet*B, *tet*C, *tet*D, *tet*K and *tet*M. Of the six genes targeted, *tet*D was the predominant allele detected. Overall the frequency of detection of the tet alleles was *tet*A–*tet*B–*tet*C–*tet*D–*tet*K–*tet*M, being spotted in 24%, 23%, 18%, 78%, 15% and 12% of the total tetracycline resistant isolates, respectively (Table 6.4). Patterns of dual and multiple tetracycline-resistance genes were equally observed. Dual *tet*A–*tet*D had the highest occurrence (5%), followed by multiple *tet*A–*tet*D–*tet*M (3%) while those with multiple 4 genes were all uniform in their occurrences (1%) each, with *tet*D commonly associated with all. None of the isolates harboured up to 5 and 6 tetracycline resistance genes (Table 6.5).

(iv) Phenicols

Among the 65 chloramphenicol-resistant isolates screened, the most frequent chloramphenicol resistance gene was *cat*I (37%). *cat*II and *cml*A1 genes were frequently detected in 28% and 19% of isolates respectively (Table 6.4), whereas 59% of the isolates were negative to *cat*I, *cat*II and *cml*A1. Dual patterns *cat*I–*cat*II, *cat*I–*cml*A1 and *cat*II–*cml*A1 were detected in 12%, 2% and 9% respectively and none of the isolates carried all the three genes tested (Table 6.5).

(v) Aminoglycosides

Of the five resistance genes encoding for aminoglycosides examined in this study, only 8% gentamycin-resistance gene, *aac*C2 was detected among the 39 gentamycin-resistant isolates (Table 6.4), with 92% showing no observable genotypic feature. The *aph*A1 and *aph*A2 genes encode a kanamycin and neomycin resistance phenotypes, and these genes were found respectively, in 33% and 47% of the 15 kanamycin-resistant isolates, and 28% and 52% of the 25

neomycin-resistant isolates (Table 6.4). Dual *aph*A1–*aph*A2 was carried in 20% each of the kanamycin and neomycin-resistant *E. coli* isolates (Table 6.5). Similarly, streptomycin-resistance genes *aad*A and *str*A were detected in 79% and 38% among the 47 streptomycin-resistant isolates respectively (Table 6.4). Only one isolate (2%) was found positive to both *aad*A and *str*A genes (Table 6.5).

Table 6.4 Prevalence and distributions of antimicrobial resistance determinants among *E. coli* isolates.

Antimicrobial family	Antimicrobial agent	Antimicrobial resistance gene	No (%) of positive isolates by origin										Total
			R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	
Sulfonamides	Sulphamethoxazole (n = 300)	*sul*I	5	4	10	3	1	0	0	0	0	0	23 (8)
		*sul*II	6	7	6	9	16	16	15	19	23	6	123 (41)
Beta-lactams	Ampicillin (n = 172)	*amp*C	0	2	1	1	1	6	5	12	4	6	38 (22)
	Amoxycillin (n = 177)	bla_{TEM}	2	12	14	1	0	1	1	2	2	2	37 (21)
		bla_{Z}	9	6	9	4	4	0	0	0	0	0	32 (18)
Tetracyclines	Tetracycline (n = 83)	*tet*A	3	3	3	0	1	4	2	0	3	1	20 (24)
		*tet*B	0	0	2	4	2	1	1	2	4	3	19 (23)
		*tet*C	0	2	1	5	6	0	0	0	1	0	15 (18)
		*tet*D	7	15	14	6	3	7	7	0	0	6	65 (78)
		*tet*K	4	5	0	0	1	0	1	1	0	0	12 (15)
		*tet*M	2	6	0	1	0	1	0	0	0	0	10 (12)
Phenicols	Chloramphenicol (n = 65)	*cat*I	3	3	2	2	0	2	2	2	2	6	24 (37)
		*cat*II	4	0	3	1	2	1	0	0	2	5	18 (28)
		*cml*A1	4	1	2	0	1	0	1	0	0	3	12 (19)
Aminoglycosides	Gentamycin (n = 39)	*aac(3)–IIa* (*aac*C2)[a]	0	0	0	0	0	1	0	0	0	2	3 (8)
	Kanamycin (n = 15)	*aph(3)–Ia* (*aph*A1)[a]	0	1	0	0	0	2	2	0	0	0	5 (33)
		aph(3)–IIa (*aphA2*)[a]	0	0	0	0	1	3	1	2	0	0	7 (47)
	Neomycin (n = 25)	*aph(3)–Ia (aph*A1)[a]	0	0	0	0	0	0	1	3	1	2	7 (28)
		*aph(3)–IIa (aph*A2)[a]	0	0	0	0	0	2	2	3	2	4	13 (52)
	Streptomycin (n = 47)	*aad*A	0	2	0	1	1	7	6	10	4	6	37 (79)
		*str*A	0	0	2	0	1	5	1	5	2	2	18 (38)
Total			**49**	**69**	**69**	**38**	**41**	**59**	**48**	**61**	**50**	**54**	**538**

Table 6.5 Patterns of dual and multiple resistance determinants tested on the antimicrobial-resistant *E. coli* isolates.

Antimicrobial family	Antimicrobial agent	Antimicrobial resistance determinants pattern	Total (%)
Sulfonamides	Sulphamethoxazole	*sul*I–*sul*II	5 (2)
Beta-lactams	Amoxycillin	bla_{TEM}–bla_{Z}	15 (9)
Tetracyclines	Tetracycline	*tet*A–*tet*B	2 (2)
		*tet*A–*tet*D	5 (5)
		*tet*A–*tet*K	1 (1)
		*tet*B–*tet*C	3 (3)
		*tet*C–*tet*K	1 (1)
		*tet*B–*tet*D	3 (3)
		*tet*D–*tet*K	2 (2)
		*tet*D–*tet*M	3 (3)
		*tet*A–*tet*B–*tet*D	2 (2)
		*tet*A–*tet*C–*tet*D	1 (1)
		*tet*A–*tet*D–*tet*M	3 (3)
		*tet*C–*tet*K–*tet*M	1 (1)
		*tet*C–*tet*D–*tet*K	1 (1)
		*tet*B–*tet*C–*tet*D	1 (1)
		*tet*A–*tet*B–*tet*C–*tet*D	1 (1)
		*tet*A–*tet*C–*tet*D–*tet*K	1 (1)
		*tet*A–*tet*D–*tet*K–*tet*M	1 (1)
		*tet*B–*tet*C–*tet*D–*tet*M	1 (1)
		*tet*C–*tet*D–*tet*K–*tet*M	1 (1)
Phenicols	Chloramphenicol	*cat*I–*cat*II	8 (12)
		*cat*I–*cml*A1	1 (2)
		*cat*II–*cml*A1	6 (9)
Aminoglycosides	Kanamycin	*aph*(3)*–Ia* (*aph*A1)[a]–*aph(3)–IIa* (*aph*A2)[a]	3 (20)
	Neomycin	*aph*(3)*–Ia (aph*A1)[a]*–aph(3)-IIa (aph*A2)[a]	5 (20)
	Streptomycin	*aad*A–*str*A	1 (2)

[a]Alternative nomenclatures are presented in parentheses

Generally, of the sum total of 538 resistance gene fingerprints obtained across the sampling sites, the highest prevalence was recorded at sites R2 and R3 with the overall total of 69 prints each while the lowest was at R4 with 38 (Figure 6.4). Likewise, the prevalence of dual and multiple antimicrobial resistance genes across the sampling sites ranged between 2 and 4 (Table 6.5) with *tet* gene combinations most occurring, due to their high number assessed in the study. The representative gel electrophoresis profiles of amplified products of the investigated antimicrobial resistance encoding genes are shown in Figures 6.5a and b.

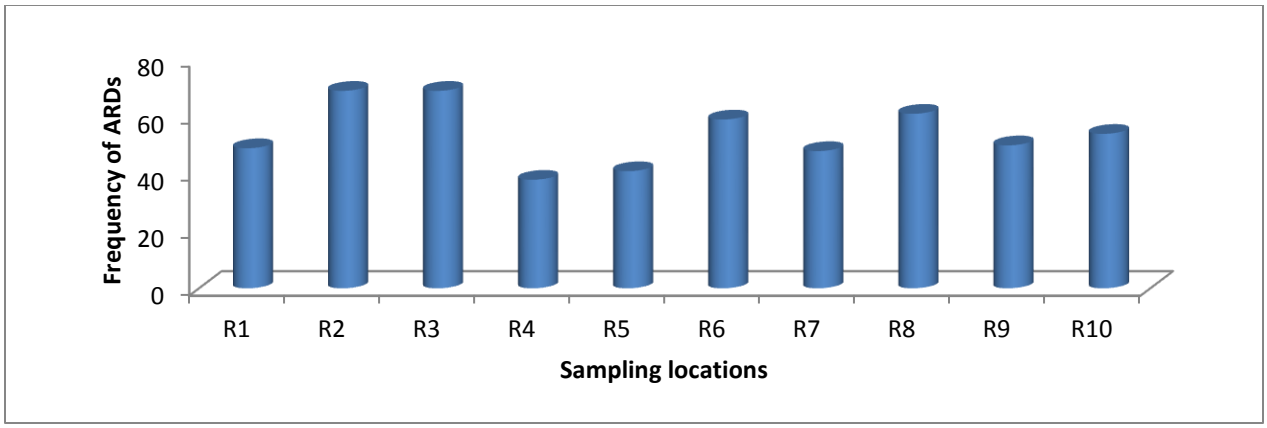

Figure 6.4 Frequency distribution of antimicrobial resistance determinants among *E. coli* isolates from the ten sampling sites.

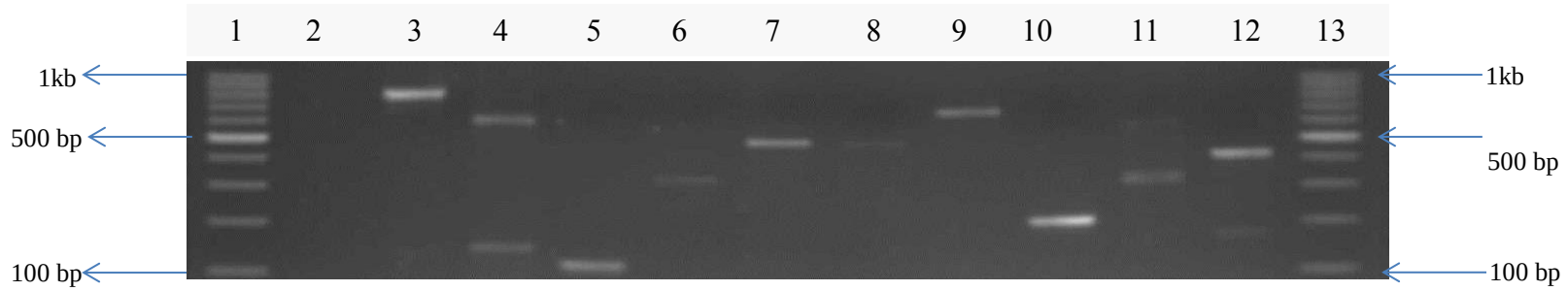

Figure 6.5a A representative gel electrophoresis profile of various antimicrobial virulence genes of *E. coli* isolates.

Lanes 1 and 13: molecular weight marker (Thermo Scientific 100 bp DNA ladder), lane 2: negative control, lane 3: *sul*I (822 bp), lane 4: *sul*II (625 bp), lane 5: *cml*A1 (115 bp), lane 6: *cat*1 (320 bp), lane 7: *cat*11 (543 bp), lane 8: bla_{Z} (490 bp), lane 9: bla_{TEM} (690 bp), lane 10: *tet*A (201 bp), lane 11: *tet*B (359 bp) and lane 12: *tet*C (418 bp).

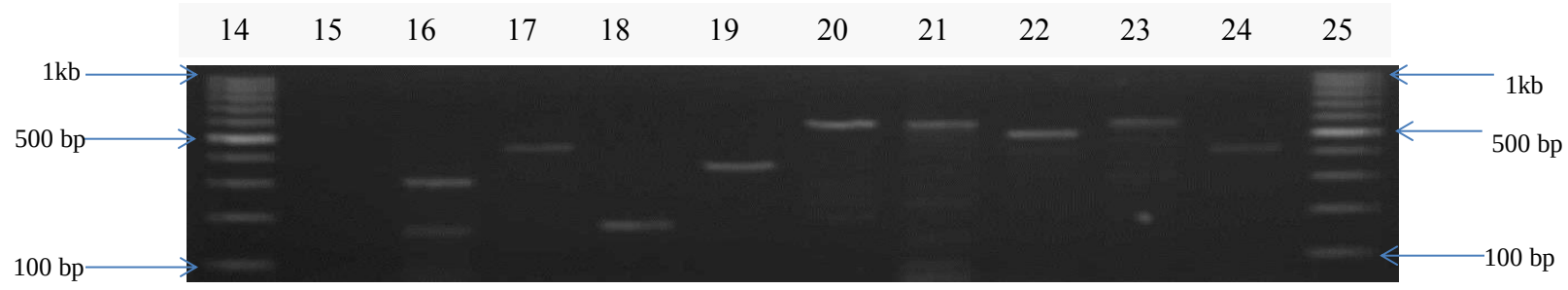

Figure 6.5b. A representative gel electrophoresis profile of different antimicrobial resistance genes of *E. coli* isolates.

Lanes 14 and 25: molecular weight marker (Thermo Scientific 100 bp DNA ladder), lane 15: negative control, lane 16: *tet*D (300 bp), lane 17: *tet*K (460 bp), lane 18: *tet*M (158 bp), lane 19: *str*A (348 bp), lane 20: *amp*C (550 bp), lane 21: *aad*A (525 bp), lane 22: *aph*A2 (510 bp), lane 23: *aph*A1 (600 bp) and lane 24: *aac*C2 (428 bp).

6.3.4 Association between resistance genes

In order to determine whether possible associations exist between the resistance genes detected among our isolates and whether the co-appearance of some resistance genes could be confirmed statistically, an analysis of association was done by using Pearson's chi-square exact test. Significant associations ($P < 0.05$) with respect to the occurrence of individual resistance genes among the whole collection of *E. coli* isolates were detected (Table 6.6).

Many positive associations were obvious, for example, the association of the *sul*I and $bla_{TEM,}$ bla_{Z} and *tet*C, as well as *tet*A and *tet*B genes. The *sul*I gene was positively associated with bla_{TEM}, bla_{Z} genes and also, although less strongly, with the *sul*II and *amp*C genes (Table 6.6). The *amp*C gene showed a very strong association with the *cml*AI, *aac*C2, *aph*A1, *aph*A2, *aad*A and *str*A genes. The same trend was observed for bla_{Z} gene with *tet*C, *tet*D, *tet*K, *tet*M, *cat*II, *cml*A1 genes and less strongly associated with *tet*A gene (Table 6.6). None of the genes was negatively associated with one and another.

Table 6.6 Association between various antimicrobial resistance genes among *E. coli* isolates

	Significance of association for the following antimicrobial resistance genes[a]																
Genetic marker	*sul*I	*sul*II	*amp*C	bla_{TEM}	bla_{Z}	*tet*A	*tet*B	*tet*C	*tet*D	*tet*K	*tet*M	*cat*I	*cat*II	*cml*A1	*aac*C2	*aph*A1	*aph*A2	*aad*A
*sul*II	+																	
*amp*C	+	–																
bla_{TEM}	+++	–	–															
bla_{Z}	++	–	–	+++														
*tet*A	–	–	–	+++	+													
*tet*B	–	++	+	–	–	+++												
*tet*C	–	+++	–	–	+++	+++	+++											
*tet*D	–	+	–	+++	+++	+++	+	–										
*tet*K	–	–	–	+	+++	++	–	+++	+++									
*tet*M	–	–	–	+++	+++	+++	–	+++	+++	+++								
*cat*I	–	–	–	–	–	–	–	–	–	–	–							
*cat*II	–	–	–	++	+++	–	–	–	–	–	–	+++						
*cml*A1	–	–	–	+	+++	–	–	–	–	–	–	–	+++					
*aac*C2	–	–	+++	–	–	–	–	–	–	–	–	–	–	–				
*aph*A1	–	–	+++	–	–	+	–	–	–	–	–	–	–	–	+++			
*aph*A2	–	–	+++	–	–	+	+++	–	–	–	–	–	–	–	++	+++		
*aad*A	–	+	+++	–	–	–	++	+	–	–	–	–	–	–	+++	+++	+++	
*str*A	–	–	+++	–	–	+	–	–	–	–	–	–	–	–	–	+++	+++	+++

[a]Only antimicrobial resistance genes that exhibited an association with another gene at the $P<0.05$ level are shown. The levels of significance of the association (as assessed by the chi-square exact test) were as follows: –, $P > 0.05$; +, $0.05 \geq P \geq 0.01$; ++, $0.01 \geq P \geq 0.001$; and +++, $0.001 \geq P$.

6.4 Discussion

Contaminated drinking water is a major source of gastrointestinal infections and responsible for many waterborne related diseases, with *E. coli* being used extensively as one of the major faecal indicator bacteria. In this study, the overall mean annual counts of both the presumptive and confirmed *E. coli* obtained in all the sampling sites studied were relatively high and this finding is comparable to previous results (Warner et al., 2008; Pant, 2011).

The occurrence of antimicrobial resistant bacteria in surface waters is a major public health concern as they could be transmitted to humans through ingestion of contaminated water which then contributes to the spread and persistence of antimicrobial resistance bacteria in general population and environment with its associated potential health threats (Tao et al., 2010). The present investigation revealed a wide presence of phenotypic antimicrobial resistant *E. coli* in the river waters of Osun State, South Western Nigeria. The high level of resistance to sulphamethoxazole, amoxycillin, ampicillin, cefuroxime and tetracycline observed in all the sampling sites could be attributable to influence of domestic, industrial and hospital wastes, surface runoff and various anthropogenic activities in the catchments (Table 6.1). The presence of antibiotic-resistant bacteria in freshwater sources has been documented on the basis of the phenotypic antimicrobial resistance testing (Young, 1993; Ronald, 2002; Reinthaler et al., 2003; Seino et al., 2004).

Sulfonamides act by their competing ability to inhibit the synthesis of folic acid, β-lactams inhibit the last step involved in the bacterial cell wall synthesis while tetracyclines inhibit protein synthesis (Levy and Marshall, 2004). The extremely low toxicity of the antimicrobial agents in these classes has resulted in their overuse in the medical world, hence the observed increased

resistance. The relatively high level of resistance to antimicrobial agents in the environment depicts a true reflection of their indiscriminate and excessive usage (Lateef, 2004).

Chloramphenicol inhibits translation during protein synthesis and causes aplastic anaemia in a small percentage of patients, and its use is very minimal in non-life-threatening situations. The observed low *E. coli* resistance to chloramphenicol in our findings could be attributed to the ban placed on its usage. The resistance of sulphamethoxazole, ampicillin, amoxycillin and chloramphenicol treatments by microbial isolates has been described elsewhere (Manikandan et al., 2011). Aminoglycosides are protein synthesis inhibitors binding to bacterial ribosomes to prevent the initiation of protein synthesis. Their usage has been restricted because persistent use has been found to cause kidney damage and injury to the auditory nerves leading to deafness (Goni-Urriza et al., 2000). The reduced use of this class of antibiotics may justify its low resistance profiles against our isolates except streptomycin (Table 6.3). The excessive use of streptomycin in the treatment of tuberculosis has been found solely behind its high resistance compared to other aminoglycosides (Goni-Urriza et al., 2000).

With *E. coli* being the most widely investigated bacteria, the resistance to at least two classes of antimicrobial agents has been frequently detected in the environment (Young, 1993; Baum and Marre, 2005). The current study revealed multi-drug resistance to numbers of antimicrobials ranging from three to nine. The antibiotic resistance patterns of the *E. coli* isolates across the selected sites include no-drug resistance (4), single-drug (36), two-drug (25), three-drug (19), four-drug (21), five-drug (24), six-drug (20), seven-drug (6), eight-drug (13) and nine-drug resistance (5) (results not shown). The antimicrobial resistance patterns of the isolates obtained in this study corroborate results from previous reports (Wasfy et al., 2000; Obi et al., 2004b). *E. coli* with multiple antimicrobial resistances in surface water and other environmental samples

have been reported (Carattoli, 2002; Olaniran et al., 2009; Martinez, 2012). Multi-drug resistances have also been detected in strains of Enterobacteriaceae isolated from the Venda Region rivers in South Africa (Obi et al., 2004), Aksu river in Turkey (Toroglu et al., 2005), rivers in Malaysia (ALHaj et al., 2007), River Nile in Egypt (Ezzat, 2008), Durban rivers in South Africa (Olaniran et al., 2009), Pearl Rivers in South China (Tao et al., 2010) and Almendares River in Cuba (Graham et al., 2011). Industrial and human activities also contribute to the multi-drug resistance patterns and since rivers are the primary reservoirs of industrial effluents, exposure to environmental pollutants and changes in nutrient composition can lead to selective pressure that favours antimicrobial resistance in certain organisms (Furuya and Lowy, 2006; Ram et al., 2008).

Polymerase chain reaction assays have been widely used in both pure cultures and mixed environmental samples for detection of certain antimicrobial resistance genes encoding resistances to aminoglycosides (Mohapatra et al., 2008; Taviani et al., 2008), chloramphenicols (Dang et al., 2008), β-lactams (Taviani et al., 2008), macrolides (Chen et al., 2007; Patterson et al., 2007), penicillins (Srinivasan et al., 2005), sulfonamides (Agersø and Petersen, 2007), tetracyclines (Jacobs and Chenia, 2007), trimethoprims (Moura et al., 2007) and vancomycin (Caplin et al., 2008).

The present work investigated characterization of antimicrobial resistance determinants in *E. coli* isolates from some selected rivers. Although *sul*II (41%) was more frequently detected than *sul*I (8%) among our isolates (Table 6.4), but the detection of both genes suggests the misuse of this drug in the geo-political region. Similar higher *sul*II than *sul*I detection has been reported as an abundant sulfonamide resistance gene in the aquatic environment and it is in agreement with those for two other studies demonstrating similar antimicrobial gene occurrences in the

environment (Sayah et al., 2005; Hammelin et al., 2006). Similarly, *sul*I and *sul*II have been detected in bacterial isolates from water or sediments of aquaculture areas (Akinbowale et al., 2007a; Agersø and Petersen, 2007), faecal, slurry of dairy farms (Srinivasan et al., 2005) and even from the river or seawater without evidence of pollution (Lin and Biyela, 2005; Hu et al., 2008; Mohapatra et al., 2008).

Beta lactams are the most widely used antibiotics. They are characterized by a low toxicity and used to treat a broad range of infections. Nevertheless, the associated severe threat posed by their resistances cannot be overemphasized (Livermore, 1996). In the present study, β-lactams *amp*C bla_{TEM} and bla_{Z} genes were all observed in their respective resistant isolates. Ampicillin resistance gene *amp*C was the highly prevalent gene and amoxycillin resistance gene bla_{TEM} was detected at a higher frequency than bla_{Z} (Table 6.4). Our ample result for β-lactams resistance genes *amp*C and bla_{TEM} agrees with another study in the aquatic environment (Hamelin et al., 2006). Ampicillin-resistance gene detected in the river water isolates might likely be due to the presence of natural populations of β-lactam resistant bacteria with a long term exposure of bacteria to trace levels of antibiotics known to increase antibiotic resistance, as low levels of β-lactam drug resistance are known to be inherently present in some gram-negative environment isolates (Esiobu et al., 2002). The environmental compartments may further serve as reservoirs for β-lactam resistance genes. *amp*C gene encoding β-lactams has been detected in the microbial isolates from wastewater, surface water and even from drinking water films (Schwartz et al., 2003). A variety of *bla* genes have been identified in bacteria isolates from faecal slurry and lagoon water of dairy farms (Srinivasan et al., 2005), water or sediments of aquaculture areas (Dalsgaard et al., 2000; Jacobs and Chenia, 2007), STPs (Szczepanowski et al., 2004; Volkmann

et al., 2004; Antunes et al., 2006; Taviani et al., 2008) and surface water (Schwartz et al., 2003; Poppe et al., 2006 Alpay-Karaoglu et al., 2007).

Tetracycline-resistant bacteria have been widely distributed in the environments with the introduction of tetracycline. About 38 different tetracycline (*tet*) resistance genes have been characterized to date, out of which over 22 have been detected in bacterial isolates from aquatic environments (Dancer et al., 1997; Roberts, 2005; Thompson et al., 2007). The present investigation screened and detected six tetracycline resistance genes *tet*A, *tet*B, *tet*C, *tet*D, *tet*K and *tet*M in the tetracycline resistant *E coli* isolates from the studied rivers at varying frequencies (Table 6.4). The wide distribution of these genes among the isolates could be attributed to sewage inflow, indiscriminate wastewater disposal and animal manures which are typical characteristic features sighted at most of the sampling sites (Table 6.1). The finding shows that about 28% of the tetracycline resistant *E. coli* isolates from the river water samples carried at least one different *tet* gene, a result found to be in a strong conformity with previous reports (Schmidt et al., 2001; Bryan et al., 2004). Tao et al (2010) also detected *tets* A, B, C and D genes in Enterobacteriaceae isolated from the Pearl rivers in South China. The *tet* genes including *tet*A (Agersø and Sandvang, 2005; Srinivasan et al., 2005), *tet*B (Agersø and Sandvang, 2005; Dang et al., 2007), *tet*C (Agersø and Sandvang, 2005; Akinbowale et al., 2007b) and *tet*D (Schmidt et al., 2001) have been previously reported in bacterial isolates from aquatic environment. Twenty *tet* genes have equally been detected in aquatic microbial communities around the world with *tets* A, B, C, D, E and *tets* M, O, S, Q, W more frequently detected (Zhang et al., 2009). The efflux genes of *tets* A, B, C, D and E frequently appeared in various environmental compartments including activated sludge of STPs (Guillaume et al., 2000), fish farming ponds (Schmidt et al., 2001; Dang et al., 2007), surface water (Poppe et al., 2006) and swine lagoon (Macauley et al.,

2007). Recently, the tetracycline resistance genes including *tets* M, O, S, Q and W, coding for ribosomal protection proteins, have also been detected in microbial communities of sewage treatment systems (Auerbach et al., 2007), hospital or animal production wastewaters (Kim et al., 2007; Nonaka et al., 2007) and even in natural water environments (Mackie et al., 2006).

Chloramphenicol is an important antibiotic in the treatment of central nervous system infections and some epidemic diseases in humans and none food producing animals (Chinabut et al., 2005). Of the chloramphenicol resistance genes known to date, several types of *cat* and *cml* genes have been reported to be of environmental origin (Heuer et al., 2004; Dang et al., 2008). In the present investigation, *cat*I, *cat*II and *cml*A1 were tested on the chloramphenicol resistant *E. coli* isolates and the results obtained were quite low (Table 6.4). This could likely be due to the fact that the use of this antibiotic has been restricted, hence it's vulnerability to excessive and indiscriminate use is drastically reduced. Resistance to chloramphenicol was closely associated with the presence of the *catI* gene (Bischoff et al., 2002).

Our findings on dual chloramphenicol resistance (Table 5), strongly agree with the report of Yoo et al. (2003) that none of the isolates used in their study carried more than two different types of *cat* genes, not even the use of multiplex PCR with four sense primers and an antisense primer to discover and characterize four different types of *cat* genes carried in microorganisms. In addition, Schwarz et al. (2004) stated that chloramphenicol-resistant bacteria could still be found in the environments despite the restriction placed on it. This is because chloramphenicol resistance genes could be transferred between aquatic microorganisms without a high particular selective pressure (Yoo et al., 2003).

The genes of *aac*C1, C2, C3, and C4, encoding aminoglycoside-3-N-acetyltransferase, were often detected in microbial communities especially isolates of STP origin (Heuer et al., 2002; Tennstedt et al., 2003, 2005) and the two adenylyltransferase genes, *aad*A1 and *aad*A2, were frequently reported worldwide in the isolates from aquaculture areas (Dalsgaard et al., 2000), river water (Park et al., 2003), STPs (Szczepanowski et al., 2004; da Silva et al., 2007) and surface urban water (Taviani et al., 2008). In this study, a relatively low resistance and cross resistance were observed between the occurrence of streptomycin resistance gene *aad*A and *str*A, gentamycin resistance gene *aac*C2, and *aph*A1 and *aph*A2 encoding kanamycin and neomycin resistance genes occurred at varying frequencies among the aminoglycoside resistant isolates (Table 6.4). These findings could likely be linked to the cross-resistance caused by most of the aminoglycoside resistance genes (Werckenthin et al., 2002). Previous uses of antibiotics in earlier outbreaks may be partly responsible for the extensive increase in antibiotic resistance (Okoh and Igbinosa, 2010).

The current study did not aim at investigating the exact mechanisms of resistance. Nevertheless, previous molecular studies have reported strong statistical associations between different resistance genes in *E. coli* isolates (Smith et al., 2002; Boerlin et al., 2005; Travis et al., 2006). In our study, some strong associations between certain antimicrobial resistance determinants were apparent, although few were less strong. For example, some strong associations were seen in *tet*A gene with *tet*B, *tet*C, *tet*D, *tet*K and *tet*M genes and also with *aph*AI, *aph*A2 and *str*A genes although less strong. Similarly, bla_{TEM} gene was strongly associated with *tet*A gene (Table 6.6). These findings indicate that the resistance to a given antimicrobial was likely caused by a single gene and in some cases two to three genes. The mechanism involved in the transfer of antimicrobial resistance genes between aquatic microorganisms could either be cross-resistance,

co-resistance or multidrug transport (Alekshun and Levy, 2000; Alonso et al., 2001; Courvalin and Trieu-Cuot, 2001; Schwartz et al., 2004; Poole, 2005).

During this study, the highly prevalent antimicrobial resistance genes were *sul*II, ampC and $bla_{TEM,}$ *tet*A, *tet*B and tetD, *cat*I and *cat*II, and *aph*A1, *aph*A2, *aad*A and *str*A among the *E. coli* isolates (Table 6.4). These findings are consistent with other studies demonstrating similar antimicrobial gene occurrences in the environment (Hamelin et al., 2006; Sayah et al., 2005). Urban sewage, waste waters from poultry and dairy farms, hospital wastes, animal and human remains contribute antimicrobial agent residues to the rivers. Hence, the close contact of the human population with the surface waters will enrich the environmental genetic pool of *E. coli* isolates that may serve as reservoirs of antimicrobial resistance genes.

Antibiotic resistant phenotypes can emerge from many different genetic determinants, and each determinant may represent unique epidemiological features (Gow et al., 2008; Lanz et al., 2003). For example, *bla* genes often coexist with other antimicrobial resistance determinants and can also be associated with mobile genetic elements, increasing the possibility of multidrug resistance and environmental dissemination (Tennstedt et al., 2003; Weldhagen, 2004; Schlüter et al., 2007). The plasmids containing *bla* obtained from a wastewater treatment plant are frequently associated with transposons and integrons and often simultaneously carry other resistance determinants including *aad* or *aac* encoding aminoglycoside nucleotidyltransferase (or acetyltransferase), *cml* encoding chloramphenicol efflux protein, and *cat* encoding chloramphenicol acetyltransferase (Tennstedt et al., 2003).

The presence of *tet* genes in different members of Enterobacteriaceae family occurs due to transfer of drug resistance genes between bacteria through plasmids, transposons and integrons

(Pang et al., 1994; Nordmann and Poirel, 2005; Pruden et al., 2006). Tetracycline resistance genes are located on mobile genetic elements, and can be transmissible between bacteria (Roberts, 2005). Osun River is protected locally as a sacred place for worship, shrine and internationally recognized as a world heritage tourist centre in the state, yet the river water has still been polluted by antimicrobial resistance genes due to some agricultural and practices along its courses. Tetracycline resistance genes have been detected in a protected Liuxi River in China by both point and non-point sources along its course with the development of agriculture and industry in the river, despite its protection for drinking purposes (Liu et al., 2004; Zhang et al., 2004; Song et al., 2007).

The prevalence of antibiotics or traces thereof in characteristic environments can challenge the populace flow and the physiology of natural microbial populations (Carattoli, 2002). However, several reports indicate that the resistance genes currently present in human or animal associated microbiota are found in environments without antibiotic pollution (Martinez, 2012; D'Costa et al., 2011; Somme et al., 2009). This strongly supports the fact that resistant genes can persist and spread in the environment via horizontal gene transfer (HGT), thus, increasing the chances of pathogens acquiring resistance. With resistant *E. coli* types present in the environment, the chance of lateral gene transfer events, like the acquisition of by a pathogen, is higher (Lopez-Cerero et al 2011).

Resistance genes are associated with mobile DNA such as plasmids, transposons, and integrons, which facilitate resistance gene distribution (Jacoby, 1994; Tenover and Rasheed, 1998). Of the three mechanisms known to contribute to horizontal gene transfer (conjugation, transformation, and transduction), the conjugal transfer of plasmids between microorganisms plays a pivotal role in the evolution of antibiotic resistance and virulence among bacteria including the emergence of

novel variants of *E. coli* (Lopez-Cerero et al., 2011; Van Meervenne et al., 2012; Chouchani et al., 2012; Tenaillon et al., 2010]. Whether plasmids (conjugal or otherwise) contributed to the antibiotic resistance among our *E. coli* isolates is unknown. As a result of extensive use of human and veterinary antibiotics, hospital wastewater and livestock manure are considered as the major sources of environmental ARDs.

In conclusion, the *E. coli* isolates recovered from the rivers showed phenotypic and genotypic expressions to be quite different as genotypes do not always correspond with the phenotypic expression of individual isolates. The *E. coli* isolates were analysed for antimicrobial susceptibility and the presence of resistance genes and none of our findings showed total agreement of phenotypic resistance and the presence of genes encoding the phenotype. Often, more than one gene was associated with a given phenotypic resistance. A slightly different distribution of resistance genes and multigene resistance was observed across all the ten sampling sites, thus suggesting that relative resistance gene frequencies could vary within a population and geographical origins. Therefore, this study reinforces the necessity of using genotypic resistance analyses in future epidemiological studies.

To the best of our knowledge, this is the first study that describes the occurrence of antimicrobial resistance determinants known to confer resistances to common classes of antibiotics in *E. coli* isolates from river water sources in Osun state, South Western Nigeria. The presence of multiple antimicrobial resistant *E. coli* in all the river water sources could pose a serious health threat to consumers if ingested without treatment. This underscores the importance of safe water supply and provision of proper sanitation facilities for the inhabitants of the catchments and entire state. The *E. coli* isolates showed a high level of resistance to antimicrobial agents and multidrug resistance was extremely common. This is of great concern and demands for caution in the

indiscriminate and inappropriate use of antimicrobial agents, and related compounds on animals and humans. It also appears that drug resistant *E. coli* are widely distributed in most of the rivers sampled and so sewage inflow, animal dung, wastewater disposal along with antibiotic use must be carefully regulated and monitored to control the distribution of drug resistant bacteria and genes in environment. These findings have implications for the choice of antibiotics for empiric management of infections, continuous surveillance of antimicrobial susceptibility patterns and effective hospital infection control. A continuous surveillance of *E. coli* isolates from surface waters used for recreational or domestic purposes and the development of adequate prevention strategies to diminish the spread of multi-resistant bacteria and/or the mobile resistance elements are needed for public health purposes.

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Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER SEVEN

Prevalence of multidrug resistant *Escherichia coli* pathotypes in some selected surface waters in Southwestern Nigeria

This chapter has been submitted for publication in

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Prevalence of multidrug resistant *Escherichia coli* pathotypes in some selected surface waters in Southwestern Nigeria

Abstract

Despite advances in knowledge, understanding and management that have ensued in recent years, diarrhoeal disease is still the most prevalent and important public health threat in developing and developed countries as well as leading cause of morbidity and mortality in the paediatric populations. Water samples from selected rivers in Osun State, Nigeria were collected and analyzed using standard procedures. While the polymerase chain reaction (PCR) confirmed *Escherichia coli* (*n*=300) were assessed for 10 virulence genes, the disc diffusion method was used to determine the antibiogram profiling of the pathotypes and the resistant pathotypes were further profiled for their genotypic antimicrobial resistance determinants using PCR assay. The results indicated that virulence gene *lt* coding for ETEC pathotype had the highest detected (46%), followed by *pap*C for UPEC (17%) and the least being *ibe*A for EAEC (2 %). Antimicrobial resistance profiling showed all the pathotypes to be resistant against sulphamethoxazole and high levels of resistance were observed against ampicillin (63%), amoxycillin (55%), gentamycin (41%), cefuroxin (39%), chloramphenicol (28%) and cefepime (26%) among all *E. coli* strains, with high susceptibilities observed against neomycin, streptomycin and kanamycin. A total of 13 resistance determinants were assessed for all resistant strains, and their overall prevalence and distributions were obtained as follows: [sulfonamides: *sul*I (10%) and *sul*II (26%)], [β-lactams: *amp*C (7%), $bla_{\text{TEM,}}$ (13%) and bla_{Z} (15%)], [tetracyclines: *tet*A (3%), *tet*B (1%), *tet*C (4%) and *tet*D (10%)], [phenicols: *cat*I (4%), *cat*II

(3%) and *cmI*A1 (2%)] and [aminoglycoside (gentamycin): *aacC*2 (2%)]. One way analysis of variance revealed the difference between the prevalence of EPEC, EAEC, ETEC, EHEC, DAEC and NMEC strains to be statistically non-significant ($P > 0.05$) in almost all the sites whereas a significant difference in the prevalence of UPEC was noticed at site R1 only ($P < 0.05$). The presence of pathogenic and multi-drug resistant *E. coli* strains in the catchment could poses a serious threat to human health and therefore necessitates for safe water supply, good hygiene and sanitation practices to safeguard public health and monitor surface waters for forecasting and management of water-borne outbreaks.

Keywords: Surface water, Prevalence, *Escherichia coli* pathotypes, multi-drug resistance, Gene marker.

7.1 Introduction

Globally, diarrhoeal diseases remain a major health challenge, especially in developing countries and are estimated to be responsible for 2.5 million infantile deaths per year, with an annual mortality rate of 4.9 per 1,000 children and an incidence of 3.2 episodes per child per year among children under 5 years of age [62]. In developing countries, infection could be due to consumption of contaminated ground and surface waters [35, 128, 133], with *E. coli* being reported as the predominant bacterial agent of diarrhoeal diseases in most countries of the world [2, 3].

Escherichia coli is a natural inhabitant of the gut of humans, birds and other warm-blooded animals and is widely accepted as an indicator of faecal contamination of water. It is a robust bacterium which is genetically highly adaptable to environmental stresses, and has been shown to survive and multiply in the environment [127, 130]. Although most strains are commensals, yet, the pathogenic strains can contain various virulence properties for a variety of infections,

and interact and share their genomic contents (e.g. virulence an antibiotic resistance genes), with native microbiota in other places or hosts (such as in the human gut) [54, 111, 114].

There are six major categories of diarrhoeagenic *E. coli* diagnosed responsible for gastroenteritis in humans and are regarded as intestinal pathogenic *Escherichia coli* (InPEC). These include enterotoxigenic *E. coli* (ETEC) that produces a toxin resulting in traveler's and infantile diarrhoea and is the main cause of haemolytic-uraemic syndrome associated with food-borne infections, enteroinvasive *E. coli* (EIEC) that penetrates the epithelial cells lining the intestinal mucosa and produces shigellosis-like diseases in children and adults, enteropathogenic *E. coli* (EPEC) that causes acute infantile diarrhoea, enterohaemorrhagic *E. coli* (EHEC) that causes diarrhoea as a result of haemolysin production and enteroaggregative *E. coli* (EAEC) that causes prolonged diarrhoea and persistent gastroenteritis, DAEC strains have also been associated with diarrhoeal disease in different geographic areas, and each of them has different symptoms and mechanisms of infection [17, 50, 54, 67].

The extraintestinal pathogenic *E. coli* (ExPEC) group is composed of uropathogenic *E. coli* (UPEC), which is the main causes of UTIs, meningitis associated *E. coli* (MNEC), sepsis-associated *E. coli* (SEPEC) and the avian pathogenic *E. coli* (APEC), which is associated with respiratory infections, pericarditis, and septicaemia in poultry [54].

The global widespread use of antibiotics remains popular among both healthcare providers and patients for their low toxicity, high efficiency and relatively low cost. However, overuse and misuse has promoted the emergence of antibiotic-resistant strains at an alarming rate [7, 11, 106, 123]. The relationship between antibiotic use and resistance is complex; a major driving factor for antibiotic resistance is antibiotic use/abuse both within human and veterinary medicine. A

survey carried out in Nigeria reported that, 53% of respondents took incomplete regimen of antibiotics; a significant proportion of which were self-prescribed for unspecified ailments [138, 139]. Antimicrobial resistance among enteropathogens, including *E. coli* has been reported to be increasing in recent years [92], and compromises the outcome of many infections that were, until recently, treatable and remain the most common diseases in Africa [86]. The global increase of antimicrobial resistance is particularly pressing in developing countries, where the infectious disease burden is high and cost constraints prevent the widespread application of newer, more expensive agents [86].

Multiple studies from different parts of Nigeria have observed progressive trends in the prevalence of resistance among enteric organisms and have shown increasing prevalence in the last fifteen years [85, 86, 88]. However, none of the studies examined multidrug resistant pathotypes in freshwater environments, particularly in the state. Therefore, this study for the very first time, aimed at assessing the prevalence of pathogenic *E. coli* and antimicrobial resistance profiles of *E. coli* pathotypes from selected rivers of Osun State, Southwestern Nigeria.

7.2 Materials and Methods

7.2.1 Mapping and determination of geographic coordinates of the sampling locations

A hand-held global positioning system (GPS) receiver (Garmin etrex 0630p, US) was used to obtain co-ordinates and elevation of the study locations. The data obtained were incorporated into the existing GIS-RS Software for proper mapping of the sampling locations. The sampling locations were coded as follows: R1: Erinle-Ede; R2: Ido-Osun; R3: Osun-Osogbo, R4: Oba-Iwo; R5: Ejigbo; R6: Ilobu-Okinni; R7: Asejire-Ikire; R8: Shasha; R9 and Ila-Oke Ila, R10: Inisha-Okuku (Figure 7.1).

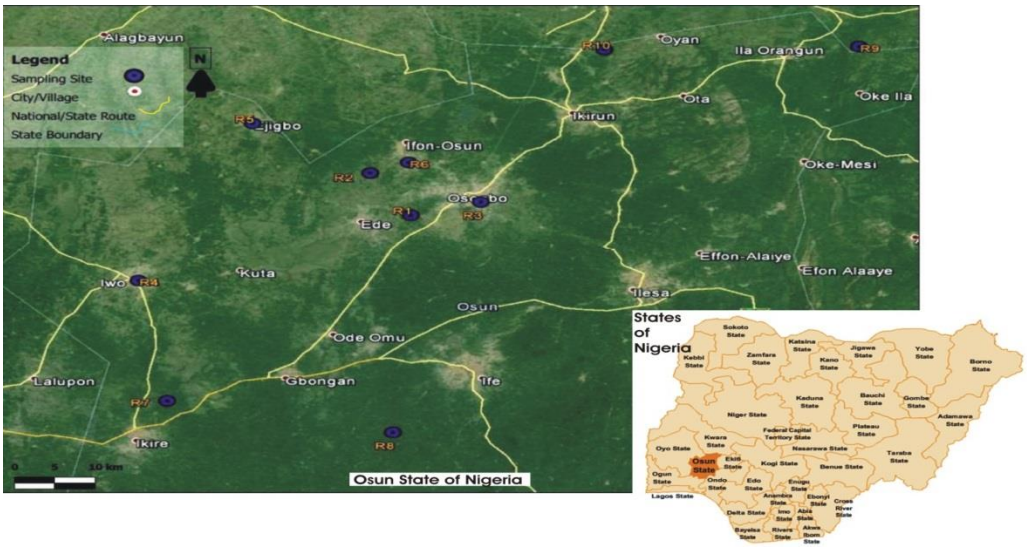

Figure 7.1 Map of Osun State, Nigeria showing the sampling locations.

7.2.2 Resuscitation of E. coli isolates and DNA extraction

Polymerase chain reaction confirmed *E. coli* isolates stored at -80 °C in Luria Bertani (LB) broth (Biolab, Merck SA) containing 20% glycerol recovered from frozen stocks, plated on LB agar (Biolab, Merck SA) and sub-cultured before DNA extraction. DNA extraction of all confirmed *E. coli* isolates was performed using the boiling method as previously described [18]. Briefly, this involved inoculating a single colony into 2 ml LB broth and incubated at 37 °C with gentle shaking (100 rpm) overnight. The culture was then pelleted using centrifugation at 13,000 rpm, the supernatant was removed, and the pellet re-suspended in 200 µl of sterile distilled water, followed by heat lysis at 100 °C for 15 min. After centrifugation, the supernatant which is the DNA was carefully transferred into a sterile 1.5 ml Eppendorf tube and stored at –20 °C for further analysis.

7.2.3 PCR detection of virulence genes

Using a conventional simplex PCR, confirmed *E. coli* isolates (*n* =300) were screened for the presence of 10 VGs associated with diarrhoeagenic and non-diarrhoeagenic *E. coli* strains, in order to correctly differentiate them into the 8 pathotypes studied. The primers used for PCR detection of the VGs and other relevant characteristics are listed in Table 7.1. For each PCR experiment, appropriate positive and negative controls were included. The PCR amplification was performed using a thermocycler system (Bio-Rad Thermal cycler, USA). Each 25 µl PCR mixture contained 12.5 µl of PCR master mix (Thermo Scientific, (EU) Lithuania), 0.5 µl each of primer (Inqaba Biotech, SA), 5 µl of template DNA and 6.5 µl of PCR grade water. To detect the amplified product, 5 µl of amplicons was visualized by electrophoresis through a 1.8% agarose gel (Merck, SA) at a voltage of 100 for 45 min in 0.5X TBE buffer and stained with ethidium bromide (Sigma-Aldrich, USA) using the gel documentation system (Alliance 4.7, France). Identification of the bands was established by comparison of the band sizes with molecular weight markers of 100-bp (Thermo Scientific, (EU) Lithuania). Samples were considered positive for a specific VG when the visible band was the same size as that of the positive control DNA. To minimize PCR contamination, DNA extraction, PCR set up, and gel electrophoresis were performed in isolated rooms. The positive controls were sourced from DSMZ Germany and included: DSM 8695 for EPEC; DSM 10973 for ETEC; DSM 10974 for EAEC; and DSM 10975 for EIEC except ATCC 35150 for EHEC from USA. There was no positive control available for DAEC but we went further to optimize the PCR condition of the related gene for possible detection of the expected amplicon band size.

Table 7.1 Primer sequences, expected amplicon sizes and their cycling conditions.

Target strain	Target gene	Primer sequence (5'→3')	Amplicon size (bp)	PCR cycling condition	Reference
EPEC	*Eae*	F: TCA ATG CAG TTC CGT TAT CAG TT R: GTA AAG TCC GTT ACC CCA ACC TG	482	15 min initial denaturation at 95°C followed by 35 cycles of 94°C for 45 sec, 55°C for 45 sec, 68°C for 2 min and final extension at 72°C for 5 min	[116]
	Bfp	F: GGA AGT CAA ATT CAT GGG GGT AT R: GGA ATC AGA CGC AGA CTG GTA GT	300	2 min initial denaturation at 94°C followed by 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and final extension at 72°C for 5 min	[116]
ETEC	*Lt*	F: GGC GAC AGA TTA TAC CGT GC G: CGG TCT CTA TAT TCC CTG TT	450	2 min initial denaturation at 94°C followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and final extension at 72°C for 5 min	[67]
EAEC	*Eagg*	F: AGA CTC TGG CGA AAG ACT GTA TC R: ATG GCT GTC TGT AAT AGA TGA GAA C	194	15 min initial denaturation at 95°C followed by 35 cycles of 94°C for 45 sec, 55°C for 45 sec, 68°C for 2 min and final extension at 72°C for 5 min	[61]
EIEC	*ipa*H	F: CTC GGC ACG TTT TAA TAG TCT GG R: GTG GAG AGC TGA AGT TTC TCT GC	933	2 min initial denaturation at 94°C followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and final extension at 72°C for 5 min	[132]
DAEC	*daa*E	F: GAA CGT TGG TTA ATG TGG GGT AA R: TAT TCA CCG GTC GGT TAT CAG T	542	2 min initial denaturation at 94°C followed by 40 cycles of 92°C for 30 sec min, 59°C for 30 sec, 72°C for 30 sec and final extension at 72°C for 5 min	[132]
EHEC	*stx*1	F: CAG TTA ATG TGG TGG CGA AGG R: CAC CAG ACA ATG TAA CCG CTG	384	15 min initial denaturation at 95°C followed by 35 cycles of 94°C for 45 sec, 55°C for 45 sec, 68°C for 2 min and final extension at 72°C for 5 min	[16]
	*stx*2	F: ATC CTA TTC CCG GGA GTT TAC G R GCG TCA TCG TAT ACA CAG GAG C	584	2 min initial denaturation at 94°C followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and final extension at 72°C for 5 min	[16]
NMEC	*ibe*A	F: TGG AAC CCC GCT CGT AAT ATA C R: CTG CCT GTT CAA GCA TTG CA	342	2 min initial denaturation at 94°C followed by 30 cycles of 94°C for 1min, 55°C for 1min, 72°C for 1 min and final extension at 72°C for 5 min	[132]
UPEC	*pap*C	F: GAC GGC TGT ACT GCA GGG TGT GGC G R: ATA TCC TTT CTG CAG GGA TGC AAT A	328	2 min initial denaturation at 94°C followed by 30 cycles of 94°C for 1min, 55°C for 1min, 72°C for 1 min and final extension at 72°C for 5 min	[68]

7.2.4 Antimicrobial susceptibility testing

Antimicrobial susceptibility of *E. coli* strains was determined by the Kirby Bauer disc diffusion assay method using the standard procedure of the Clinical and Laboratory Standards Institute [21]. The pathotypes were screened for susceptibility to a panel of sixteen antibiotic discs

belonging to nine families (Mast Diagnostics, UK), impregnated with aminoglycosides, streptomycin (300 µg), kanamycin (30 µg), neomycin (10 µg) gentamycin (10 µg); cephems: cefepime (30 µg), cephalothin (30 µg), cefuroxine (30 µg); fluoroquinolones: ciprofloxacin (5 µg); quinolones: nalidixic acid (30 µg), sulfonamides: sulphamethoxazole (25 µg); nitrofurantoins: nitrofurantoin (200 µg); phenicols: chloramphenicol (30 µg); tetracyclines: tetracycline (30 µg), doxycycline (30 µg) and β-lactamases: amoxycillin (25 µg) and ampicillin (25 µg). The inoculum for antibiotic susceptibility pattern testing was suspended in stroke physiological saline solution (0.85 % NaCl) by dispensing a single colony picked up with a sterile cotton swab (Copan, Italy). The turbidity of the resulting solution was adjusted to 0.5 McFarland standard One hundred microliter of solution was spread plated onto Mueller Hinton agar plates. The antibiotic discs were placed 30 mm apart on the inoculated plates by using a disc dispensing apparatus. Fifteen minutes after the discs were applied; the plates were inverted and incubated at 37 °C for 24 h. The inhibition zone diameters were measured to the nearest millimeter and recorded. Each isolate was categorized as susceptible (S), intermediate (I) and resistant (R) to antibiotics according to the zone diameter interpretative chart recommended by the Clinical and Laboratory Standards Institute [21].

7.2.5 Multiple Antibiotic Resistance Phenotyping and Indexing (MARP and MARI)

Multiple antibiotic resistant (MAR) phenotypes were generated for strains that showed resistance to three or more antibiotics. MAR index was calculated as previously described by Blasco et al. [14] and is mathematically expressed as:

$$MARI = a/b$$

where a = number of antibiotics to which the isolate was resistant;

b = total number of antibiotics against which individual isolate was tested.

7.2.6 PCR detection of antimicrobial resistance genes

The phenotypic antimicrobial resistant *E. coli* pathotypes obtained from the ten sampling sites were analyzed for the presence of relevant resistance genes using simplex PCR assays. Sulfonamides resistance genes *sul*I and *sul*II, β-lactamases-encoding genes *amp*C, bla_{TEM} and bla_{Z}, tetracycline resistance genes *tet*A, *tet*B, *tet*C and *tet*D, chloramphenicol resistance genes *cat*I, *cat*II and *cml*A1 and aminoglycoside resistance gene *aacC*2, were the selected target genes. For the PCR amplification of the target genes, the reaction mixture contained 12.5 µl of PCR Master Mix (Thermo Scientific, (EU) Lithuania), 0.5 µl each of oligonucleotide primer (Inqaba Biotech, SA), 5 µl of template DNA and 6.5 µl of nuclease free water to constitute a total reaction volume of 25 µl. The sequences of primers, PCR conditions and amplicon sizes are provided in Table 7.2. All PCR assays were performed on a thermocycler (Bio-Rad Mycycler, USA) and each run included a negative control. Amplicons of each sample (5 µl) was mixed with 2 µl loading dye and resolved on 1.5% agarose gels (Merck, SA) containing 5 µl ethidium bromide (Sigma-Aldrich, USA). A 100-bp marker (Thermo Scientific, (EU) Lithuania) was also included for DNA band size estimation purposes. All gels were run in 0.5X TBE buffer at 100 V for 45 min, and visualized by UV trans-illumination (Alliance 4.7, France).

Table 7.2 PCR primers used for antimicrobial resistance gene amplification.

Antimicrobial family	Primer	PCR primer sequence (5′–3′)	Amplicon size (bp)	PCR cycling condition	Reference
Sulfonamides	*sul*1	F: TTCGGCATTCTGAATCTCAC R: ATGATCTAACCCTCGGTCTC	822	Initial denaturation at 94°C for 5 min, followed by 1 min of denaturation at 94°C, 1 min of annealing at 55°C, 5 min of extension at 72°C for a total of 35 cycles and 5min of final extension at 72°C.	[77]
	*sul*11	F: CGGCATCGTCAACATAACC R: GTGTGCGGATGAAGTCAG	625	Initial denaturation for 5 min at 94°C, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1.5 min and final extension at 72°C for 5 min.	[32]
Beta-lactams	*amp*C	F: TTCTATCAAMACTGGCARCC R: CCYTTTTATGTACCCAYGA	550	94°C for 4 min followed by 30 cycles: denaturation at 94°C for 45s, annealing for 45s and extension at 72°C for 45s and final extension for 7 min at 72°C.	[131]
	bla_{TEM}	F: TTTCGTGTCGCCCTTATTCC R: CCGGCTCCAGATTTATCAGC	690	94°C for 5 min followed by 30 cycles of denaturation (94°C for 30 s), annealing (60°C for 30 s), extension (72°C for 90 s) and final incubation at 72°C for 5 min.	[51]
	bla_{Z}	F: ACT TCA ACA CCT GCT GCT TTC R: TGA CCA CTT TTA TCA GCA ACC	490	94°C for 5 min followed by 30 cycles of denaturation (94°C for 30 s), annealing (60°C for 30 s), extension (72°C for 90 s) and final incubation at 72°C for 5 min.	[6]
Tetracyclines	*tet*A	F: GCTACATCCTGCTTGCCTTC R: CATAGATCGCCGTGAAGAGG	201	5 min initial denaturation at 94°C followed by 35 cycles of 94°C for 1min, 55°C for 1min and 72°C for 1·5 min and final incubation at 72°C for 5 min.	[82]
	*tet*B	F: TTGGTTAGGGGCAAGTTTTG R: GTAATGGGCCAATAACACCG	359	5 min initial denature at 94°C followed by 35 cycles of 94°C for 1min, 55°C for 1min, 72°C for 1·5 min and final incubation at 72°C for 5 min.	[82]
	*tet*C	F: CTTGAGAGCCTTCAACCCAG R: ATGGTCGTCATCTACCTGCC	418	5 min initial denaturation at 94°C followed by 35 cycles of 94°C for 1min, 55°C for 1min, 72°C for 1.5 min and final incubation at 72°C for 5 min.	[82]
	*tet*D	F: AAACCATTACGGCATTCTGC R: GACCGGATACACCATCCATC	300	5 min initial denaturation at 94°C followed by 35 cycles of 94°C for 1min, 55°C for 1min, 72°C for 1·5 min and final incubation at 72°C for 5 min.	[82]
Phenicols	*cml*A1	F: CACCAATCATGACCAAG R: GGCATCACTCGGCATGGACATG	115	94°C for 5 min followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for1.5 min and final incubation at 72°C for 5 min.	[93]
	*cat*I	F: AGTTGCTCAATGTACCTATAACC R:TTGTAATTCATTAAGCATTCTGCC	320	5 min at 94°C, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1.5 min and final incubation at 72°C for 5 min.	[77]
	*cat*II	F: ACACTTTGCCCTTTATCGTC R: TGAAAGCCATCACATACTGC	543	5 min at 94°C, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1.5 min and final incubation at 72°C for 5 min.	[77]
Aminoglycoside	*aacC2*	F: CGGAAGGCAATAACGGAG R: TCGAACAGGTAGCACTGAG	428	5 min at 94°C, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1.5 min and final incubation at 72°C for 5 min.	[77]

7.2.7 Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences [(SPSS) IBM version 20 software]. The one way analysis of variance (ANOVA) was performed to investigate the difference in the prevalence of pathotypes observed with respect to each site. Test of significance was considered statistically significant when *P* values were < 0.05**.**

7.3 Results

7.3.1 PCR detection of virulence genes encoding pathotypes

Overall, of the 300 confirmed *E. coli* isolates assessed for the 10 VGs, the heat-labile toxin, *lt* gene was the most commonly detected gene in 134 (45%), followed by the adhesion *pap*C gene in 17 (56%) of the isolates, both encoding ETEC and UPEC pathotypes respectively. While the *eae* and *bfp* coding for typical and atypical EPEC were both detected in 24 (8%), *stx*1 and *stx*2 were equally detected in approximately 23 (8%) for EHEC. Other VGs were variously detected as follows; *daa*E 12 (4%), *ibe*A 10 (3%) and *eagg* 7 (2%) for DAEC, NMEC and EAEC pathotypes respectively (Table 7.3). Throughout the study, the invasion plasmid antigen gene, *ipa*H coding for EIEC was not detected and therefore omitted from subsequent analysis. The representative gel electrophoresis profiles of amplified products of the investigated virulence genes are shown in Figure 7.2. A comparison between the sites was made (ANOVA) to determine if the sites were similar or different on the basis of prevalence of pathotypes. The difference between the prevalence of EPEC, EAEC, ETEC, EHEC, DAEC and NMEC strains were found to be statistically non-significant ($P > 0.05$) in almost all the sites whereas a significant difference in the prevalence of UPEC was noticed at site R1 ($P < 0.05$).

Table 7.3 Prevalence of *E. coli* pathotypes in selected rivers of Southwestern Nigeria.

	Sampling locations										Total
***E. coli* strains**	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**	**R8**	**R9**	**R10**	
EPEC	1 (3)	8 (27)	4 (13)	2 (7)	1 (3)	1 (3)	4 (13)	2 (7)	0 (0)	1 (3)	24 (8)
EAEC	0 (0)	1 (3)	1 (3)	2 (7)	2 (7)	0 (0)	0 (0)	1 (3)	0 (0)	0 (0)	7 (2)
EIEC	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
ETEC	24 (80)	17 (57)	21 (70)	17 (57)	20 (67)	9 (30)	13 (43)	9 (30)	0 (0)	4 (13)	134 (45)
EHEC	3 (10)	10 (33)	3 (10)	1 (3)	1 (3)	0 (0)	0 (0)	1 (0)	2 (7)	2 (7)	23 (8)
DAEC	3 (10)	0 (0)	0 (0)	0 (0)	0 (0)	2 (7)	1 (3)	4 (13)	2 (7)	0 (0)	12 (4)
UPEC	12 (40)	12 (40)	7 (23)	9 (30)	4 (13)	2 (7)	4 (13)	4 (13)	2 (7)	0 (0)	56 (19)
NMEC	0 (0)	0 (0)	1 (3)	0 (0)	3 (10)	0 (0)	0 (0)	1 (3)	5 (17)	0 (0)	10 (3)

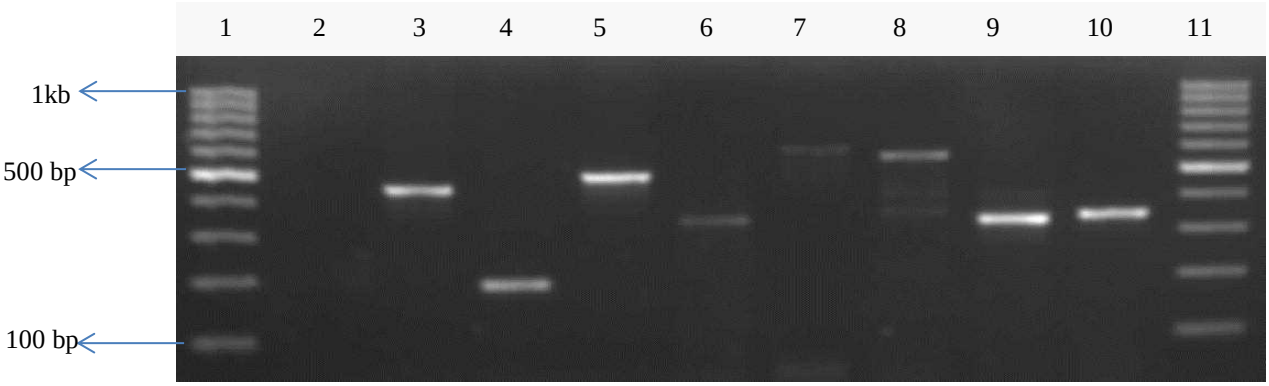

Figure 7.2 A representative gel electrophoresis profile of different virulence genes of isolated *E. coli*.

Lanes 1 and 11: molecular weight marker (Thermo Scientific 100 bp DNA ladder), lane 2: negative control, lane 3: *lt* (450 bp), lane 4: *eagg* (194 bp), lane 5: *eae* (482 bp), lane 6: *stx*1 (348 bp), lane 7: *stx*2 (584 bp), lane 8: *daa*E (542 bp), lane 9: *pap*C (328 bp) and lane 10: *ibe*A (342 bp).

7.3.2 Antibiogram profiles of E. coli pathotypes

Antimicrobial resistance profiling was conducted *in-vivo* on the *E. coli* pathotypes. Overall, all the tested pathotypes showed complete resistance to sulphamethoxazole (100%) and high levels of resistance were observed against ampicillin (63%), amoxycillin (55%), gentamycin (41%), cefuroxin (39%), chloramphenicol (28%) and cefepime (26%) among ETEC strains. Similarly, a high level of resistance was observed against ampicillin (70%), amoxycillin (59%), cefuroxin

(39%), gentamycin (34%), chloramphenicol (27%), cefepime (23%) and tetracycline (21%) among UPEC pathotype (Table 7.4). Although, ETEC and UPEC pathotypes were the predominant pathotypes observed in this study, nonetheless others were variously resistant to one or more antimicrobial agents at low frequencies. Aside gentamycin, other members of aminoglycosides family; neomycin, streptomycin and kanamycin proved effective as most strains were susceptible to them as follows: EPEC, EAEC, UPEC and NMEC strains to kanamycin (100%), EHEC, DAEC and NMEC to streptomycin (100%), and EAEC, DAEC and UPEC to neomycin (100%). Likewise, high susceptibilities were recorded among ETEC strains for neomycin, streptomycin and kanamycin in 99%, 98% and 97% respectively (Table 7.4).

Table 7.4 Antibiogram profile of *E. coli* pathotypes from river water sources in Osun State, Southwestern Nigeria

	Antimicrobial agent																MARI
***E. coli* strain**	**SMX**	**T**	**C**	**A**	**AP**	**G**	**DXT**	**CPM**	**CXM**	**KF**	**NI**	**NA**	**S**	**K**	**NE**	**CIP**	
EPEC (n=24)	24 (100)	7 (29)	4 (17)	12 (50)	13 (54)	8 (33)	8 (33)	10 (42)	10 (42)	7 (29)	4 (17)	5 (21)	1 (4)	0 (0)	2 (8)	3 (13)	0.9
EAEC (n=7)	7 (100)	1 (14)	2 (29)	3 (43)	6 (86)	3 (43)	1 (14)	2 (29)	5 (71)	4 (57)	0 (0)	2 (29)	1 (14)	0 (0)	0 (0)	0 (0)	0.8
ETEC(n=134)	134 (100)	37 (28)	26 (19)	73 (55)	84 (63)	55 (41)	44 (33)	35 (26)	52 (39)	31 (23)	17 (13)	10 (8)	3 (2)	4 (3)	1 (1)	6 (5)	1.0
EHEC (23)	23 (100)	9 (39)	9 (39)	15 (65)	17 (74)	11 (49)	12 (52)	7 (30)	9 (39)	7 (30)	5 (22)	2 (9)	0 (0)	2 (9)	1 (4)	1 (4)	0.9
DAEC (n=12)	12 (100)	4 (33)	1 (8)	7 (58)	8 (67)	3 (25)	4 (33)	4 (33)	4 (33)	3 (25)	1 (8)	0 (0)	0 (0)	1 (8)	0 (0)	0 (0)	0.8
UPEC (n=56)	56 (100)	12 (21)	15 (27)	33 (59)	39 (70)	19 (34)	17 (30)	13 (23)	22 (39)	14 (25)	7 (13)	4 (7)	3 (5)	0 (0)	0 (0)	2 (4)	0.9
NMEC (n=10)	10 (100)	2 (20)	1 (10)	6 (60)	6 (60)	4 (40)	2 (20)	2 (20)	3 (0)	1 (10)	2 (20)	1 (10)	0 (0)	(0)	1 (10)	1 (10)	0.8

SMX Suphamethoxazole, *T* Tetracycline, *C* Chloramphenicol, *A* Amoxycilin, *AP* Ampicilllin, *G* Gentamycin, *DXT* Doxycycline, *CPM* Cefepime, *CXM* Cefuroxin, *KF* Cephalotin, *NI* Nitrofurantoin, *NA* Nalidixic acid, *S* Streptomycin, *K* Kanamycin, *NE* Neomycin, *CIP* Ciprofloxacin.

7.3.3 Multiple Antibiotic Resistance Phenotyping and Indexing (MARP and MARI)

MAR phenotypes were compiled and the result indicated a high degree of MARPs ranging from three to nine antimicrobials with ETEC as the predominant pathotype followed by UPEC. The modal MARP for the *E. coli* pathotypes was MARP 4 (24%) and the least was MARP 9 (7%) (Table 7.5). Overall, the prevalences showed multiple antibiotic resistance to nine, eight, three, seven, six, five and four antimicrobials in 7%, 10%, 11%, 13%, 16%, 20%, and 24% respectively in all *E. coli* strains (Figure 7.3). In the same vein, MAR indices were calculated and obtained approximately as 0.9, 0.8, 1.0, 0.9, 0.8, 0.9 and 0.8 for EPEC, EAEC, ETEC, EHEC, DAEC, UPEC and NMEC strains respectively. The modal MAR index for the tested strains was 1.0 for ETEC pathotype.

Table 7.5 Multiple antimicrobial resistance patterns (MARPs) of *E. coli* pathotypes from selected rivers of Southwestern Nigeria.

E. coli strains	№ of antimicrobials	Resistance patterns	Sampling locations									
EPEC			**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**	**R8**	**R9**	**R10**
	3	SMX/T/CIP	1	–	–	–	–	–	–	–	–	–
		SMX/DXT/T	–	–	1	–	–	–	–	–	–	–
		SMX/NA/CPM	–	–	–	–	–	–	–	1	–	–
	4	SMX/CXM/G/CPM	–	1	–	–	–	–	–	–	–	–
		SMX/CXM/KF/AP	–	–	–	1	–	–	–	–	–	–
		SMX/C/A/NA	–	–	–	–	–	–	1	–	–	–
		SMX/CXM/CPM/AP	–	–	–	–	–	–	1	–	–	–
	5	SMX/A/DXT/T/AP	–	1	–	–	–	–	–	–	–	–
		SMX/CXM/G/CPM/AP	–	1	–	–	–	–	–	–	–	–
		SMX/CXM/G/CPM/CIP	–	1	–	–	–	–	–	–	–	–
		SMX/A/NE/CPM/AP	–	–	–	–	–	–	–	1	–	–
	6	SMX/A/G/DXT/T/AP	–	2	–	–	–	–	–	–	–	–
	7	SMX/A/CXM/DXT/T/NA/KF	–	–	1	–	–	–	–	–	–	–
		SMX/A/CXM/CPM/KF/CIP/AP	–	–	–	–	1	–	–	–	–	–
	8	SMX/C/A/NI/CXM/G/KF/AP	–	–	1	–	–	–	–	–	–	–
		SMX/A/G/DXT/NA/CPM/KF/AP	–	–	–	–	–	1	–	–	–	–
	9	SMX/C/A/NI/CXM/S/DXT/KF/AP	–	1	–	–	–	–	–	–	–	–
		SMX/A/NI/NE/CXM/DXT/NA/CPM/AP	–	–	–	–	–	–	1	–	–	–
		SMX/C/A/NI/CXM/G/T/KF/AP	–	–	–	–	–	–	–	–	–	1

E. coli strains	№ of antimicrobials	Resistance patterns	Sampling locations									
EAEC	3	SMX/NA/AP	–	–	1	–	–	–	–	–	–	–
	4	SMX/CXM/KF/AP	–	–	–	1	–	–	–	–	–	–
		SMX/G/NA/CPM	–	–	–	–	1	–	–	1	–	–
	5	SMX/C/A/T/AP	–	–	–	–	1	–	–	–	–	–
	8	SMX/A/CXM/G/NA/CPM/KF/AP	–	–	–	–	1	–	–	–	–	–
	9	SMX/C/A/N/CXM/S/DXT/KF/AP	–	1	–	–	–	–	–	–	–	–
ETEC	3	SMX/T/CIP/	1	–	–	–	–	–	–	–	–	–
		SMX/S/DXT	1	–	–	–	–	–	–	–	–	–
		SMX/A/AP	3	–	1	–	–	–	1	1	–	–
		SMX/CXM/T	–	1	–	–	–	–	–	–	–	–
		SMX/NA/AP	–	–	–	–	–	–	–	–	–	–
		SMX/DXT/T	–	–	1	–	–	–	–	–	–	–
		SMX/G/CPM	–	–	–	1	–	–	–	–	–	–
		SMX/CXM/AP	–	–	–	–	–	–	1	–	–	–
		SMX/NA/CPM	–	–	–	–	–	–	–	1	–	–
	4	SMX/C/A/AP	4	–	–	–	–	–	–	–	–	–
		SMX/G/DXT/T	1	–	–	–	–	–	–	–	–	–
		SMX/C/G/AP	1	–	–	–	–	–	–	–	–	–
		SMX/A/DXT/AP	–	1	–	2	–	–	–	–	–	–
		SMX/T/KF/AP	–	1	–	–	–	–	–	–	–	–
		SMX/A/CXM/G	–	1	–	–	–	–	–	–	–	–
		SMX/CXM/G/CPM	–	1	–	–	–	–	–	–	–	–
		SMX/A/DXT/KF	–	–	–	1	–	–	–	–	–	–
		SMX/C/G/NA	–	–	–	–	1	–	–	–	–	–

E. coli strains	№ of antimicrobials	Resistance patterns	Sampling locations									
			R1	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**	**R8**	**R9**	**R10**
		SMX/G/NA/CPM	–	–	–	–	1	–	–	–	–	–
		SMX/CXM/G/CPM	–	–	–	–	2	–	–	–	–	–
		CPM/CXM/DXT/CPM	–	–	–	–	1	–	–	–	–	–
		SMX/A/T/AP	–	–	–	–	–	–	1	–	–	–
		SMX/A/CXM/CPM	–	–	–	–	–	–	1	–	–	–
		SMX/DXT/CPM/AP	–	–	–	–	–	–	1	–	–	–
		SMX/CXM/CPM/AP	–	–	–	–	–	–	–	1	–	–
		SMX/A/CXM/AP	–	–	–	–	–	–	–	–	–	1
	5	SMX/C/A/T/AP	1	–	–	–	–	–	–	–	–	–
		SMX/A/T/CPM/AP	1	–	–	–	–	–	–	–	–	–
		SMX/A/CXM/CPM/A	–	1	–	–	–	–	–	–	–	–
		SMX/CXM/G/CPM/AP	–	1	–	–	–	–	–	–	–	–
		SMX/CXM/G/CPM/CIP	–	1	–	–	–	–	–	–	–	–
		SMX/C/A/DXT/AP	–	1	–	–	–	–	–	–	–	–
		SMX/A/DXT/T/AP	–	–	1	2	–	–	1	–	–	–
		SMX/A/G/DXT/AP	–	–	–	1	–	–	–	–	–	–
		SMX/C/G/KF/AP	–	–	–	1	–	–	–	–	–	–
		SMX/CXM/DXT/KF/AP	–	–	–	1	–	–	–	–	–	–
		SMX/C/A/T/AP	–	–	–	–	1	–	–	–	–	–
		SMX/C/S/G/NA	–	–	–	–	1	–	–	–	–	–
		SMX/A/DXT/KF/AP	–	–	–	–	1	–	–	–	–	–
		SMX/A/CXM/G/CPM	–	–	–	–	1	–	–	–	–	–
		SMX/G/DXT/T/CPM	–	–	–	–	1	–	–	–	–	_

E. coli strains	№ of antimicrobials	Resistance patterns	Sampling locations									
			R1	R2	R3	R4	R5	R6	R7	R8	R9	R10
		SMX/CXM/DXT/NA/AP	–	–	–	–	–	1	–	–	_	–
		SMX/CXM/NA/CPM/AP	–	–	–	–	–	1	–	–	–	–
		SMX/A/G/T/AP	–	–	–	–	–	–	–	1	–	–
		SMX/A/CXM/KF/AP	–	–	–	–	–	–	–	1	–	–
		SMX/A/CXM/CPM/AP	–	–	–	–	–	–	–	–	–	1
	6	SMX/C/A/DXT/T/AP	1	–	–	–	–	–	–	–	–	–
		SMX/C/A/CXM/KF/AP	–	1	–	–	–	–	–	–	–	–
		SMX/A/G/DXT/T/AP	–	1	–	–	1	–	–	–	–	–
		SMX/A/DXT/T/CIP/AP	–	1	–	–	–	–	–	–	–	–
		SMX/A/CXM/G/CPM/CIP	–	1	–	–	–	–	–	–	–	–
		SMX/NI/CXM/G/KF/AP	–	–	1	–	–	–	–	–	–	–
		SMX/A/NI/CXM/G/KF	–	–	1	–	–	–	–	–	–	–
		SMX/A/CXM/G/KF/AP	–	–	1	–	–	–	–	–	–	–
		SMX/A/CXM/T/KF/AP	–	–	1	–	–	–	–	–	–	–
		SMX/A/CXM/DXT/KF/AP	–	–	1	–	–	–	–	–	–	–
		SMX/C/CXM/G/CPM/AP	–	–	–	–	1	–	–	–	–	–
		SMX/A/NE/CPM/KF/AP	–	–	–	–	1	–	–	–	–	–
		SMX/CXM/G/DXT/CPM/K	–	–	–	–	–	1	–	–	–	–
		SMX/A/G/DXT/T/AP	–	–	–	–	–	1	–	1	–	–
		SMX/G/DXT/T/CPM/AP	–	–	–	–	–	1	–	–	–	–
		SMX/A/NI/CXM/CPM/KF	–	–	–	–	–	–	1	–	–	–
		SMX/C/A/CXM/CPM/AP	–	–	–	–	–	–	–	–	–	1
	7	SMX/A/NI/CXM/G/KF/AP	1	–	1	–	–	–	–	–	–	–

E. coli strains	№ of antimicrobials	Resistance patterns	Sampling locations									
			R1	R2	R3	R4	R5	R6	R7	R8	R9	R10
		SMX/A/CXM/G/DXT/KF/AP	–	–	1	–	–	–	–	–	–	–
		SMX/A/CXM/DXT/T/NA/KF	–	–	1	–	–	–	–	–	–	–
		SMX/C/A/CXM/G/KF/AP	–	–	1	–	–	–	–	–	–	–
		SMX/C/A/G/DXT/T/AP	–	–	–	–	1	–	–	–	–	–
		SMX/A/G//DXT/T/K/AP	–	–	–	–	–	1	–	–	–	–
		SMX/G/DXT/NA/CPM/KF/AP	–	–	–	–	–	–	1	–	–	–
		SMX/A/CXM/DXT/NA/CPM/AP	–	–	–	–	–	–	1	–	–	–
		SMX/C/A/CXM/NA/CPM/AP	–	–	–	–	–	–	–	1	–	–
		SMX/A/CXM/G/CPM/KF/AP	–	–	–	–	–	–	–	–	–	1
	8	SMX/C/A/NI/CXM/G/KF/AP	2	–	–	–	–	–	–	–	–	–
		SMX/A/NI/CXM/G/DXT/KF/AP	1	–	–	–	–	–	–	–	–	–
		SMX/A/NI/CXM/G/T/KF/AP	1	–	–	–	–	–	–	–	–	–
		SMX/C/A/NI/DXT/T/KF/CIP	–	1	–	–	–	–	–	–	–	–
		SMX/A/NI/CXM/G/DXT/KF/AP	–	–	1	–	–	–	–	–	–	–
		SMX/C/A/G/DXT/T/NA/AP	–	–	1	–	–	–	–	–	–	–
		SMX/A/G/DXT/T/CIP/K/AP	–	–	–	–	–	1	–	–	–	–
	9	SMX/A/NI/CXM/G/DXT/T/KF/AP	1	–	–	–	–	–	–	–	–	–
		SMX/C/A/N/CXM/S/DXT/KF/AP	–	1	–	–	–	–	–	–	–	–
		SMX/C/NI/CXM/S/DXT/T/CPM/AP	–	1	–	–	–	–	–	–	–	–
		SMX/C/A/NI/CXM/G/CPM/KF/AP	–	–	1	–	–	–	–	–	–	–
		SMX/A/NI/CXM/G/DXT/T/KF/AP	–	–	1	–	–	–	–	–	–	–
		SMX/A/NI/CXM/G/T/CPM/KF/AP	–	–	–	–	1	–	–	–	–	–
		SMX/A/CXM/G/DXT/T/CPM/K/AP	–	–	–	–	–	1	–	–	–	–

E. coli strains	№ of antimicrobials	Resistance patterns	Sampling locations									
			R1	R2	R3	R4	R5	R6	R7	R8	R9	R10
EHEC	4	SMX/G/DXT/T	1	–	–	–	–	–	–	–	–	–
		SMX/CXM/G/CPM	–	1	–	–	1	–	–	–	–	–
		SMX/A/CPM/AP	–	–	–	–	–	–	–	1	–	–
		SMX/A/DXT/AP	–	–	–	–	–	–	–	–	1	–
	5	SMX/C/A/T/AP	1	–	–	–	–	–	–	–	–	–
		SMX/CXM/G/CPM/CIP	–	1	–	–	–	–	–	–	–	–
		SMX/C/A/DXT/AP	–	1	–	–	–	–	–	–	–	–
	6	SMX/A/G/DXT/T/AP	–	1	–	–	–	–	–	1	–	–
	7	SMX/A/NI/CXM/G/KF/AP	1	–	1	–	–	–	–	–	–	–
		SMX/C/A/CXM/DXT/KF/AP	–	1	–	–	–	–	–	–	–	–
		SMX/NI/CXM/T/CPM/KF/AP	–	–	–	–	–	–	–	–	–	1
	8	SMX/A/CXM/G/CPM/KF/K/AP	–	1	–	–	–	–	–	–	–	–
		SMX/C/A/N/DXT/T/KF/CIP	–	1	–	–	–	–	–	–	–	–
		SMX/C/A/G/DXT/T/NA/AP	–	–	2	–	–	–	–	–	–	–
		SMX/C/A/G/DXT/T/K/AP	–	–	–	–	–	–	–	–	1	–
	9	SMX/C/A/N/NE/S/DXT/CPM/AP	–	1	–	–	–	–	–	–	–	–
		SMX/A/N/CXM/G/DXT/CPM/KF/AP	–	–	–	–	–	–	–	–	–	1
DAEC	3	SMX/A/AP	1	–	–	–	–	–	–	–	–	–
	4	SMX/CXM/KF/AP	–	–	–	–	–	–	–	1	–	–
		SMX/A/CPM/AP	–	–	–	–	–	–	–	1	–	–
	5	SMX/A/DXT/T/AP	1	–	–	–	–	–	–	–	–	–
		SMX/A/G/T/AP	–	–	–	–	–	–	–	–	1	–
	7	SMX/C/G/DXT/T/K/AP	–	–	–	–	–	1	–	–	–	–

E. coli strains	№ of antimicrobials	Resistance patterns	Sampling locations									
			R1	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**	**R8**	**R9**	**R10**
		SMX/A/CXM/G/T/CPM/AP	–	–	–	–	–	–	–	1	–	–
		SMX/A/CXM/DXT/CPM/KF/AP	–	–	–	–	–	–	–	1	–	–
	8	SMX/A/NI/CXM/DXT/CPM/KF/A	–	–	–	–	–	1	–	–	–	–
UPEC	3	SMX/A/AP	2	–	–	–	–	–	–	–	–	–
		SMX/C/AP	1	–	–	–	–	–	–	–	–	–
		SMX/S/CPM	–	–	–	1	–	–	–	–	–	–
		SMX/G/AP	–	–	–	–	–	–	–	1	–	–
	4	SMX/C/A/AP	1	–	–	1	–	–	–	–	–	–
		SMX/A/DXT/AP	–	1	–	2	–	–	–	–	–	–
		SMX/CXM/G/CPM	–	1	–	–	1	–	–	–	–	–
		SMX/A/CXM/KF	–	–	1	–	–	–	–	–	–	–
		SMX/C/G/AP	–	–	–	1	–	–	–	–	–	–
		SMX/CXM/G/KF	–	–	–	1	–	–	–	–	–	–
		SMX/C/CXM/CPM	–	–	–	–	1	–	–	–	–	–
		SMX/A/G/P	–	–	–	–	–	1	–	–	–	–
		SMX/CXM/CPM/AP	–	–	–	–	–	–	–	1	–	–
		SMX/A/CPM/AP	–	–	–	–	–	–	–	–	1	–
	5	SMX/C/A/T/AP	1	–	–	–	–	–	–	–	–	–
		SMX/A/CXM/CPM/AP	–	1	–	–	–	–	–	1	–	–
		SMX/A/DXT/T/AP	–	1	–	–	–	–	–	–	–	–
		SMX/C/A/DXT/AP	–	1	–	–	–	–	–	–	–	–
		SMX/CXM/G/CPM/AP	–	1	–	–	–	–	–	–	–	–
		SMX/CXM/G/CPM/CIP	–	1	–	–	–	–	–	–	–	–

E. coli strains	№ of antimicrobials	Resistance patterns	Sampling locations									
			R1	**R2**	**R3**	**R4**	**R5**	**R6**	**R7**	**R8**	**R9**	**R10**
		SMX/C/A/G/AP	–	–	–	–	–	1	–	–	–	–
	6	SMX/A/NI/CXM/G/KF	1	–	–	–	–	–	–	–	–	–
		SMX/C/A/DXT/T/AP	–	2	–	–	1	–	–	–	–	–
		SMX/A/S/DXT/T/AP	–	1	–	–	–	–	–	–	–	–
		SMX/A/DXT/T/CIP/AP	–	1	–	–	–	–	–	–	–	–
		SMX/A/CXM/G/KF/AP	–	–	1	–	–	–	–	–	–	–
		SMX/A/CXM/DXT/KF/AP	–	–	1	–	1	–	–	–	–	–
		SMX/C/A/G/NA/AP	–	–	–	1	–	–	–	–	–	–
	7	SMX/C/A/NI/CXM/G/KF/AP	1	–	–	–	–	–	–	–	–	–
		SMX/CXM/DXT/T/KF/CIP/AP	–	1	–	–	–	–	–	–	–	–
		SMX/NI/CXM/G/DXT/KF/AP	1	–	–	–	–	–	–	–	–	–
		SMX/NI/CXM/G/CPM/KF/AP	–	–	1	–	–	–	–	–	–	–
		SMX/C/A/CXM/NA/CPM/AP	–	–	–	–	–	–	–	1	–	–
	8	SMX/A/N/CXM/G/T/KF/AP	1	–	–	–	–	–	–	–	–	–
		SMX/C/A/NI/S/NA/KF/AP	–	–	–	1	–	–	–	–	–	–
		SMX/A/CXM/DXT/NA/CPM/KF/AP	–	–	–	–	–	–	1	–	–	–
		SMX/NI/CXM/G/DXT/NA/CPM/AP	–	–	–	–	–	–	–	–	1	–
	9	SMX/A/NI/CXM/G/DXT/T/KF/AP	1	–	–	–	–	–	–	–	–	–
NMEC	3	SMX/CXM/G	–	–	1	–	–	–	–	–	–	–
	4	SMX/A/DXT/AP	–	–	–	–	–	–	–	–	1	–
		SMX/A/CXM/AP	–	–	–	–	–	–	–	–	1	–
	5	SMX/A/G/T/AP	–	–	–	–	1	–	–	–	–	–
		SMX/NE/NA/CPM/AP	–	–	–	–	–	–	–	1	–	–
	6	SMX/A/NI/CXM/G/CPM	–	–	–	–	–	–	–	–	1	–
	7	SMX/C/A/G/DXT/T/AP	–	–	–	–	1	–	–	–	–	–
		SMX/A/N/CXM/CPM/KF/AP	–	–	–	–	–	–	–	–	1	–

SMX Suphamethoxazole, *T* Tetracycline, *C* Chloramphenicol, *A* Amoxycilin, *AP* Ampicilllin, *G* Gentamycin, *DXT* Doxycycline, *CPM* Cefepime, *CXM* Cefuroxin, *KF* Cephalotin, *NI* Nitrofurantoin, *NA* Nalidixic acid, *S* Streptomycin, *K* Kanamycin, *NE* Neomycin, *CIP* Ciprofloxacin

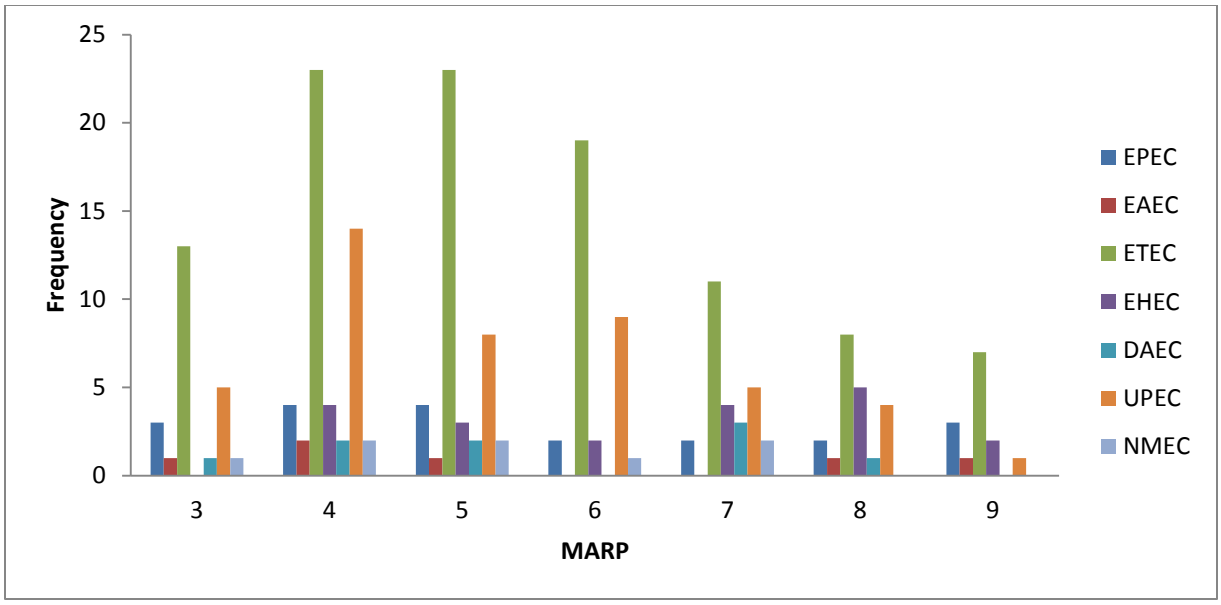

Figure 7.3 Frequency distribution of multiple antibiotic resistance phenotypes of *E. coli* pathotypes

7.3.4 Distributions of antimicrobial resistance determinants among E. coli resistant pathotypes

(i) *EPEC*

Of the 19 sulphamethoxazole-resistant EPEC strains, 10 (52%) harboured *sul*II. While 4 (21%) strains were *sul*I positive and 5 (26%) strains possessed none of the sulfonamide resistance genes (Table 6). Twenty three percent of the 13 ampicillin-resistant strains were *amp*C positive. The genes bla_{TEM} and bla_{Z} were frequently detected in 8 (67%) and 6 (50%) of 12 strains resistant to amoxycillin respectively. Of the 7 tetracycline-resistant strains, the detection of tet alleles was spotted in 2 (29%), 1 (14%), 1 (14%) and 3 (43%) for *tets* A, B, C and D respectively. Interestingly, all the 4 chloramphenicol-resistant EPEC strains were positive for *cat*1 and only 1 (25%) was found to harbour *cat*II. Similarly, of the 8 gentamycin-resistant strains, only 1 (13%) possessed *aacC*2 while the remaining 7 (87%) did not (Table 7.6).

(ii) *EAEC*

Seven EAEC strains were found to be resistant to sulphamethoxazole therapy, out of which 1 (14%) and 2 (28%) were found to harbour *sul*I and *sul*II respectively. Also, 2 (33%) of the 6 ampicillin-resistant strains were *amp*C positive. The gene bla_{TEM} was frequently detected in 2 (67%) higher than bla_{Z} 1 (33%) of the 3 amoxycillin-resistant strains. The only tetracycline-resistant strain was tested positive for *tet*D. The gene *cat*II harboured 1 (50%) of the 2 chloramphenicol-resistant strains while the 2 gentamycin-resistant strains were negative for *aacC*2 (Table 7.6).

(iii) *ETEC*

A high frequency of ETEC resistance was observed against sulphamethoxazole therapy. Of the 106 strains, 14 (13%) possessed *sul*I, whereas 40 (38%) had *sul*II. Eight (10%) of the 82 ampicillin-resistant strains were *amp*C positive. The genes bla_{TEM} and bla_{Z} were frequently detected in 13 (18%) and 20 (28%) of 71 amoxycillin-resistant strains respectively. Of the four tetracycline-resistance genes targeted, *tetD* was the predominant allele detected. Overall the detection of the tet alleles was *tetA* > *tetB* < *tetC* < *tetD* > *tetK*, being spotted in 6 (19%), 3 (9%), 6 (19%), 20 (63%) respectively (Table 7.6). Surprisingly, each of *cmlA*I, *cat*I and *cat*II were equally detected in 5 (16%) of 27 chloramphenicol-resistant strains. Only 3 (7%) of 46 gentamycin-resistant strains were *aacC*2 positive (Table 7.6).

(iv) *EHEC*

While the PCR amplification performed shows that 3 (14%) and 9 (41%) of 22 sulphamethoxazole-resistant EHEC strains were *sul*I and *sul*II positive respectively, 5 (30%) of 17 ampicillin-resistant strains harboured *amp*C (Table 7.6). Six (40%), each of bla_{TEM} and bla_{Z} genes was detected in 6 (40%) among the 15 amoxycillin-resistant strains. Only *tet*D was found

positive in 6 (67%) of 9 tetracycline-resistant strains and other tet alleles were tested negative. Two (22%) and 1(11%) of *cat*I and *cat*II genes were observed among the 9 chloramphenicol-resistant strains respectively, whereas they were negative for *cml*AI gene. Only one gentamycin-resistant strain harboured *aacC*2 while others were tested negative (Table 7.6).

(v) *DAEC*

The prevalence of *sul*II 8 (89%) was higher than *sul*I 3 (33%) among the 9 sulphamethoxazole-resistant DAEC strains. While 3 (38%) of 8 ampicillin-resistant strains were positive for *amp*C, bla_{TEM} and bla_{Z} were equally detected in 4 (57%) and 3 (43%) of 7 amoxycillin-resistant strains respectively. The *tet*A and *tet*D genes were detected in 1 (25%) and 2 (50%) of 4 tetracycline-resistant strains respectively, whereas no single strain carrying both *tet*B and *tet*C was detected. Neither the single chloramphenicol nor 3 gentamycin-resistant strains was found to harbour their associated and respective *cml*AI, *cat*I and *cat*II, and *aacC*2 resistance genes (Table 7.6).

(vi) *UPEC*

Screening for sulphamethoxazole-resistance genes in the resistant strains reveals the presence of *sul*I and *sul*II in 11 (23%) and 18 (38%) of 47 strains respectively. While the gene *amp*C was detected in 2 (5%) strains of 39 ampicillin-resistant strains, bla_{TEM} and bla_{Z} were consequently detected in 11 (34%) and 14 (44%) of 32 amoxycillin-resistant strains respectively. One (10%) *tet*B, and 6 (60%), each of *tets* C and D were detected among the 10 tetracycline-resistant strains respectively, and none of the strains was found to harbour *tet*A. Three (20%) each of 15 chloramphenicol-resistant strains harboured *cml*AI and *cat*II, with 4 (27%) were *cat*I positive. Of the 16 gentamycin-resistant strains, just one strain was noticed to possess *aacC*2 (Table 7.6).

(vii) *NMEC*

Among the 8 sulphamethoxazole-resistant strains, *sul*I and *sul*II resistance determinants were detected in 2 (25%) and 7 (88%) respectively, whilst in 6 ampicillin-resistant strains, 2 (33%) were tested positive for *amp*C. Two (33%), each of bla_{TEM} and bla_{Z} were detected among the 6 amoxycillin-resistant pathotypes. Interestingly, all the 2 chloramphenicol-resistant strains were found to harbour *tet*C only while *tets* A, B and D were negative. Neither the lone chloramphenicol nor 3 gentamycin-resistant strains was found to possess their associated *cml*AI, *cat*I and *cat*II, and *aacC*2 resistance genes respectively (Table 7.6).

Overall, of the grand total 366 resistance gene fingerprints spotted across the sampling locations and among the resistant pathotypes, the modal prevalent gene prints were found among the ETEC strains in 148 (40%), being the predominant pathotype observed in this study, followed by UPEC strains 80 (22%) while the lowest was the least occurring EAEC pathotype 14 (4%) (Table 7.6). The representative gel electrophoresis profiles of amplified products of the investigated antimicrobial resistance encoding genes are shown in Figure 7.4.

Table 7.6 Summary of antimicrobial resistance determinants among *E. coli* pathotypes across the sampling sites**.**

Antimicrobial agent / № of resistant pathotype (RP)	Resistance determinant	*E. coli* pathotype							Total
		EPEC	EAEC	ETEC	EHEC	DAEC	UPEC	NMEC	
Sulphamethoxazole	*sul*I	4	1	14	3	3	11	2	**38**
	*sul*II	10	2	40	9	8	18	7	**94**
№ of RP		19	7	106	22	9	47	8	
Ampicillin	*amp*C	3	2	8	5	3	2	2	**25**
№ of RP		13	6	82	17	8	39	6	
Amoxycillin	bla_{TEM}	8	4	13	6	4	11	2	**48**
	bla_{Z}	6	3	20	6	3	14	2	**54**
№ of RP		12	3	71	15	7	32	6	
Tetracycline	*tet*A	2	–-	6	–	1	–	–	**9**
	*tet*B	1	–	3	–	–	1	–	**5**
	*tet*C	1	–	6	–	–	6	2	**15**
	*tet*D	3	1	20	6	2	6	–	**38**
№ of RP		7	1	32	9	4	10	2	
Chloramphenicol	*cml*A1	–	–	5	–	–	3	–	**8**
	*cat*I	4	–	5	2	–	4	–	**15**
	*cat*II	1	1	5	1	–	3	–	**11**
№ of RP		4	2	27	9	1	15	1	
Gentamycin	*aacC2*	1	–	3	1	–	1	–	**6**
№ of RP		8	2	46	13	3	16	3	
Total		**44**	**14**	**148**	**39**	**24**	**80**	**17**	**366**

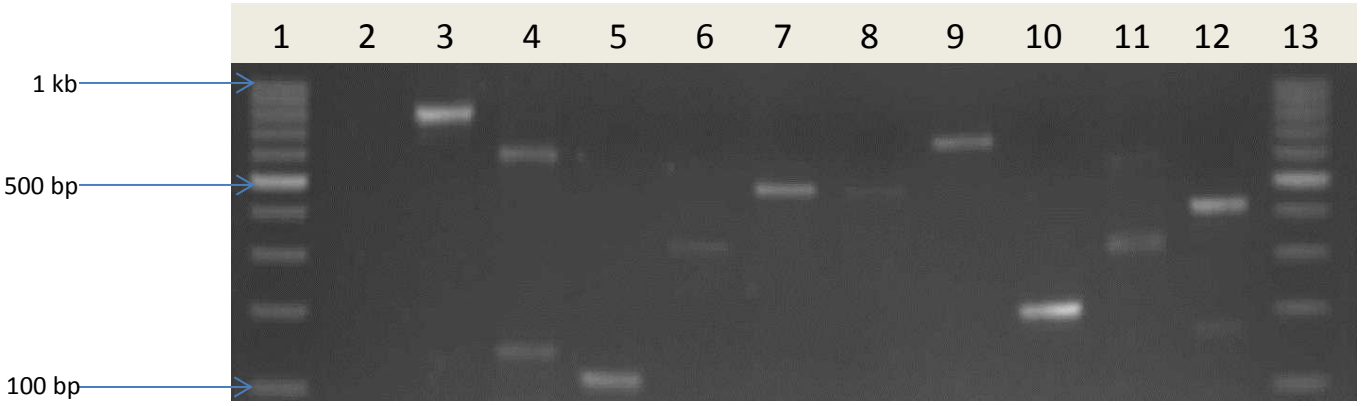

Figure 7.4 A representative gel electrophoresis profile of various antimicrobial genes of *E. coli* pathotypes.

Lanes 1 and 13: molecular weight marker (Thermo Scientific 100 bp DNA ladder), lane 2: negative control, lane 3: *sul*I (822 bp), lane 4: *sul*II (625 bp), lane 5: *cml*A1 (115 bp), lane 6: *cat*1 (320 bp), lane 7: *cat*11 (543 bp), lane 8: bla_{Z} (490 bp), lane 9: bla_{TEM} (690 bp), lane 10: *tet*A (201 bp), lane 11: *tet*B (359 bp), lane 12: *tet*C (418 bp).

7.4 Discussion

Globally, 41.1 billion people drink from unsafe water sources and a vast majority of diarrhoeal diseases are linked to unsafe water, inadequate sanitation and hygiene. Outbreaks of waterborne diarrhoeal diseases as a result of consumption of contaminated drinking water remains a serious health threat worldwide, despite the fact that drinking water is one of the most closely monitored and strictly regulated resource [135].

The current investigation resuscitated previously confirmed *E. coli* isolates and assessed them for the presence of intestinal and extra-intestinal pathogenic virulence genes. However, result on *E. coli* counts exceeded the WHO set limit of zero *E coli* in 100ml water sample [136]. The presence of *E. coli* in water is widely used as a microbiological indicator of faecal pollution and water quality [134]. Highest recovery of *E. coli* in this study is an indication that the waters have been subjected to faecal contamination as a result of poor sanitation, improper sewage disposal systems, surface runoff and seepage from contaminated ground and waste waters [81]. Major factors affecting the microbial quality of surface and underground waters are improper sewage disposal, surface runoff, seepage from nearby sewage or septic tank [33].

E. coli is used as a specific indicator organism of the level of faecal pollution from warm-blooded animals [102, 104]. The presence of pathogenic *E. coli* in environmental waters poses a potential risk for infections in humans and animals especially since water is used for irrigation, drinking and recreational purposes [39, 59, 65]. In this study, intestinal and extraintestinal *E. coli* (InPEC and ExPEC) were variously distributed across the sampling locations. Overall, the results show that ETEC 134 (45%) was the predominant pathotype demonstrating the highest percentage of defined pathotype observed at all sampling sites other than site R9 (Table 7.3). Similarly, UPEC was found to occur at every sampled river location except site R10 (Table 7.3).

It has been noted earlier that leakages from sewage lines, human and animal excreta flowing into open drains are potential sources of contamination of pathogenic *E. coli* in defective drinking water distribution systems [97]. Water sources such as wells and rivers serve as natural habitats for pathogenic *E. coli* strains that possess virulence factors that could cause gastrointestinal diseases [1, 64, 84].

Pathogenic *Escherichia coli* that colonize the epithelial lining of the small intestine primarily cause gastrointestinal illness in infants and travelers, with ETEC being implicated in the menace. ETEC induces watery diarrhoea worldwide in humans, affecting mainly children and travelers [129]. The LT gene commonly present in strains associated with human illness has been frequently observed in ETEC strains recovered from surface waters of India and other South Asian countries contaminated by faecal wastes of human origin [10, 99]. Hence, the occurrence of ETEC is used extensively in water resources and serves as an important health concern because large populace of developing countries depend on surface waters for drinking and other domestic usage [61]. The detection of other pathotypes was relatively low and this seems not to concur with the reports on EPEC EAEC and EIEC prevalence in fresh and estuarine waters [76]. Also, our findings on ETEC and EAEC partly agrees and disagrees with previous studies conducted in Mexico [30], since in clinical settings, EAEC and ETEC are the most prevalent pathotypes in Mexico [30, 31].

Resistance to antibiotics such as sulfamethoxazole, ampicillin, chloramphenicol is found in DEC isolated from children with diarrhoea in developing countries [26, 83]. The current investigation assessed the pathotypes for their antibiogram profiling. Antibiotic resistance analysis reveals that, all the strains exhibited total resistance to sulphamethoxazole and displays a significant high resistance to ampicillin, amoxycillin, gentamycin, cefuroxin, tetracycliine and chloramphenicol

(Table 7.4), and thus can be referred to as multidrug resistant (MDR) strains. This conclusion is based on the accepted definition of MDR which refers to the co-resistance that a strain can have to three or more classes of antimicrobials [27], and consistent with other reports that revealed high level of these antibiotics in water isolates [5, 20, 48]. This finding equally aligns with previous observations that multiple antibiotic resistances are common for EPEC [126]. Other studies reported multidrug resistance profiles for *E. coli* strains; EPEC and ETEC [89], EAEC and EPEC [39].

Some recent studies reported the prevalence of multi antimicrobial-resistant *Escherichia coli* isolates positive for virulence determinants for ETEC in surface waters that are being used as raw water to supply drinking water [10, 22, 98, 112]. Tetracycline has sequentially been replaced by trimethoprim-sulfamethoxazole and, more recently, quinolones, because of the emergence and spread of resistant strains. *Escherichia coli* is the most frequently isolated etiological agent of urinary tract infections (UTIs), and trimethoprim-sulfamethoxazole (TMP-SMZ) is one of the primary antibiotics empirically prescribed for the treatment of community acquired UTIs [54]. In the United States, there has been a notable increase in the isolation of uropathogenic *E. coli* strains resistant to TMP-SMZ [55].

Similarly, high resistance levels to ampicillin, trimethoprim/sulfamethoxazole, chloramphenicol, gentamicin and tetracycline have been detected in *E. coli* strains from drinking water sources in Jordan [110]. The presence of antibiotic resistant *E. coli* was also observed in other studies from human and animal faecal sources, wastewater treatment plants and surface waters [47, 79, 103, 121]. Multi-drug resistant *E. coli* strains have also been isolated from surface and ground waters in KwaZulu-Natal and North-West Provinces of South Africa [87, 91, 137]. The rate of resistance against β-lactams and cephalosporins obtained in this study is quite high compared to

that reported elsewhere [80, 107]. Furthermore, several intestinal pathogens with multi-drug resistance were isolated from the sampled rivers. Thus, the occurrence of pathogenic *E. coli* with multiple antimicrobial resistances in the these rivers represents a great concern due to possible transfer of resistant genes and may increase the possibility of infections with a higher cost of treatment.

The current study reveals multi-drug resistance to numbers of antimicrobials ranging from three to nine. Overall, the multiple antibiotic resistance patterns of all the *E. coli* pathotypes include three-drug (24), four-drug (51), five-drug (43), six-drug (33), seven-drug (27), eight-drug (31) and nine-drug resistance (14). The differences in resistance patterns may have been due to exposure to different agents. The differences in antimicrobial agent resistance patterns can be used to differentiate sources of faecal contamination in water with analytical tools such as discriminant functional analysis [36, 37, 38, 41, 56].

The MARI values calculated for each pathotype reflects high exposure to antibiotics since their values are greater than the threshold value of 0.2 and suggests contamination of high risk origin. It also indicates that the burden of antibiotics being discharged into the rivers is high, coupled with other factors such as heavy populations, various small and large-scale industrial activities. These factors may directly or indirectly contribute to the increasing resistance of bacteria in the rivers. MAR indices are used to assess the relative prevalence of resistant *E. coli* stains in the environment [63]. He suggested a MAR index of 0.2 to differentiate between low and high-risk contamination, although he acknowledged that the value is arbitrary.

The present investigation selected a total of 13 resistance determinants on the basis of their prevalences and distributions in their resistances to the tested antimicrobials. Only those with

high resistances were chosen for genotypic characterization. Sulfonamides act as competitive inhibitors of the enzyme dihydropteroate synthase in the folic acid pathway of bacterial and some eukaryotic cells. *sul*I and *sul*II encode alternative sulfonamide-resistant dihydropteroate synthase commonly occur (often at roughly the same frequency) in sulphamethoxazole-resistant strains [46]. Our findings show that both *sul*I and *sul*II frequencies were relatively high (Table 6). A similar pattern was also found in sulfonamide resistant *E. coli* strains from a natural river basin [45]. In addition, some strains harboured neither *sul*I nor *sul*II, as drug-resistant bacteria can occur by other mechanism of modification of the antibiotic target (mutations in the chromosomal DHPS gene).

The *sul*II was obviously detected with higher frequency than *sul*I among all the pathotypes (Table 6). The detection of *sul*I and *sul*II in our study corroborates with other reports elsewhere [52]. There are four *sul* genes coding for sulfonamide resistance identified up to now, with *sul*I and *sul*II being commonly reported in various aquatic environments such as wastewater, dairy lagoons, and river water in different regions [45, 72, 94]. This observation is consistent with previous studies [13, 28, 34], that showed that the *sul*I was typically associated with class1 integrons, while *sul*II was mostly found on small non-conjugative plasmids [113], or large transmissible multi-resistant plasmids [29], which may lead to *sul*II widespread detection in the environment.

Of the 4 tetracycline resistance genes assessed, *tet*D was the dominant gene detected among the *E. coli* strains over *tets* A, B and C. Most of the detectable *tets* have been found to be widespread in aquatic environments, since previous studies have revealed the occurrence of *tet*B, *tet*C and *tet*D in wastewater lagoons [90], and rivers impacted by anthropogenic influence [119, 124], *tet*B, *tet*C and *tet*M in surface water [118]. This finding also differs from those observed in other

regions *tet*C, *tet*H, *tet*O and *tet*W in the Poudre River, U.S. (94, 120], and *tet*A, *tet*B and *tet*M in the Wenyu River, China [45]. The difference in *tet* gene detection may be caused by the differential tetracycline antibiotic use patterns and host bacterial species in these regions.

Chloramphenicol is a broad spectrum antibiotic that is effective against both gram-positive and gram-negative bacteria [115]. Our *E. coli* strains show the relatively high frequency of the *cat*I, followed by *cat*II and *cml*AI. This observation tends to contradict the finding of Yoo et al. [140], who obtained a higher result for *cat*II than *cat*I. Nevertheless, the detection of these genes calls for concern, moreso that the antibiotic has been banned. Schwarz et al. [109] stated that chloramphenicol-resistant bacteria could still exist in the environments although the drug has no longer been used. This is because chloramphenicol resistance genes could be transferred between aquatic microorganisms without a high particular selective pressure [140]. Meanwhile, the mechanism involved could be either the cross-resistance caused by cross-selection, or co-resistance caused by co-selection [4, 24, 109].

Beta-lactam antibiotics are the most commonly prescribed antibacterial agents, and the production of β-lactamases is the most common and important resistance mechanism of bacteria against these agents. In the present study, bla_{z} detection was higher than bla_{TEM} and *amp*C among their respective *E. coli* strains. This result is in not in accordance with the reports of other researchers [43, 70]. The high level of resistance to β-lactam antibiotics in these *E. coli* strains may be attributable to the discharge of high concentrations of antibiotics and resistance genes in natural ecosystems [8, 57, 75]. It is also noteworthy that the presence of multiple drug resistance genes in bacteria strains from aquatics has been reported previously [19, 72]. *amp*C β-lactamases are cephalosporinases encoded on the chromosomes of many of the Enterobacteriaceae and a few other organisms, where they mediate resistance to cephalothin,

cefazolin, cefoxitin, most penicillins, and β-lactamase inhibitor-β-lactam combinations [49]. *amp*C gene encoding β-lactams has been detected in the microbial isolates from wastewater, surface water and even from drinking water films [108].

Freshwater environments such as rivers, lake, ponds and streams are considered ideal reservoirs for antibiotic resistance dissemination, since antimicrobials and antimicrobial resistant bacteria are often directly released in the environment [73, 101, 141]. A study on Mhlathuze river in South Africa revealed the presence of antibiotic resistant bacteria and genes and inferred that the river can act as reservoir as well as a medium for the spread of bacterial antibiotic resistance genes [12]. Previous studies have correlated an increased incidence of antibiotic resistance among culturable bacteria in surface water [41, 100] and groundwater [78].

The emergence and spread of antibiotic resistance in bacteria is a major public health issue [69]. Anthropogenic-driven selective pressures may be contributing to the persistence and dissemination of genes and antimicrobial resistant bacteria usually relevant in clinical environments [122]. Resistance genes may be horizontally or vertically transferred between bacterial communities in the environment [12]. Multiple antibiotic resistance (MAR) in bacteria is most commonly associated with the presence of plasmids which contain one or more resistance genes, each encoding a single antibiotic resistance phenotype [25].

The emergence of antimicrobial resistant bacteria increases in environments where antimicrobials are indiscriminately used by the public [40]. In Nigeria and other developing countries, acquired bacterial resistance to antimicrobial agents is a common phenomenon and the complex socio-economic and behavioural factors associated with this it include indiscriminate and excessive use of antibiotics among others. It has been reported that bacteria can obtain

resistance by horizontal gene transfer of mobile genetic elements and that gross usage of antibiotic influences the selection of existing resistance mechanisms [117], and under the selective pressure of the antibiotics used in aquatic environment. Results showed that all isolates in this study are resistant to more than 1 antibiotic (multiple drug resistances) which could be due to their long term exposure to pollutants in the rivers. Recent studies have also identified antibiotics themselves in surface waters [9, 15, 23, 60], and the role of these antibiotics in the development, transfer, and maintenance of resistance is largely unknown. In a limited number of studies workers have identified antibiotic-resistant bacteria in the aquatic environment.

Multiple bacterial resistances to drugs, up to six different resistance patterns, had earlier been reported. The relatively high resistance of bacterial pathogens to antibiotics in this study agrees with the [42, 95, 96]. Resistance to multiple antibiotics can lead to occurrence of newly emerging resistant bacteria which may be transmitted to consumers causing infections that are difficult to treat. The observed high frequency of bacterial resistance may not only result in the therapeutic failure in the river fauna population, but also endanger the health of the people who are at risk of infection with pathogens from these animals coupled with the possibility of plasmid transfer of resistance to human pathogenic bacteria [105].

Resistance is common where antibiotics are heavily used, and additionally antibiotic resistant bacteria are present in wastewater, surface water, ground water, sediments and soils, and increasingly in aquatic environments [8, 58, 66, 75, 142]. Resistance can also be acquired through horizontal gene transfer via uptake of resistance determinants via conjugation, transduction and transformation [125].

7.5 Conclusion and Recommendations

Conclusively, this present study is the first documented evidence aimed at investigating multi-drug resistance among *E. coli* strains in the region. The detection of *E. coli* strains in the river water samples clearly indicates that the waters are unfit for domestic and recreational purposes. The study underscores the widespread distribution and prevalence of multidrug resistant *E. coli* strains known to be indicators of water contamination. Isolation of MDR *E. coli* from the river water samples in this study further sends alarms to scientists for urgent warfare against the menace. High prevalence of multidrug resistance demands serious need for broad-based, local antimicrobial resistance surveillance and planning of effective interventions to reduce multidrug resistance in the environments. Monitoring programmes for antibiotic usage should be formulated and implemented both at the national and state levels as none is currently in place. The persistence and proliferation risk of antibiotic resistance genes equally necessitate a dire need for further research in order to better understand their routes and mechanisms which will in turn mitigate the public health risks.

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Conflicts of Interest

All authors hereby declare that there was no conflict of interest whatsoever throughout the period of this research. We all have agreed and approved this submission.

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CHAPTER EIGHT

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

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GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

8.1 General Discussion

Surface waters serve such important purposes as industrial, agricultural and recreational purposes. However the vulnerability of such water resources to pollution presents serious public health and ecological problems of global concerns (Shuval, 1990; Pruss *et al*., 2002; WHO, 2009). The river water can be affected by point and non-point pollution sources along the river with the development of industry and agriculture in the river catchment (Liu *et al.,* 2004; Zhang *et al.,* 2004; Song *et al.,* 2007). Environmental discharge of untreated sewage and animal wastes can also lead to contamination of surface and ground waters (Reinthaler *et al.,* 2003; Sapkota *et al.,* 2007). Monitoring physicochemical parameters in water resources to evaluate water quality and identify impairments is very important for protection of both the environment and public health (Tebbut, 1992; Okoh *et al*., 2007; WHO, 2008). Also, microbial quality of aquatic environments is assessed by the use of bacterial indicators (Abdelzaher *et al*., 2010; WHO, 2011).

The present study investigated physicochemical properties and total coliform distribution of some selected rivers in Osun State, Southwestern Nigeria. From our findings, it was evident that the river water is highly contaminated, and also denotes the potential public health hazards. The physicochemical qualities ranged as follows: pH (6.9 - 7.6), temperature (26 – 29 ºC), turbidity (2.28 – 9.46 NTU), electrical conductivity (229 – 581 µS/cm), nitrate (0.03 – 0.05 mg/L), nitrite (0.00 – 0.01 mg/L), sulphate (3.33 – 20.33 mg/L), chloride ions (7.83 – 27.33 mg/L), dissolved oxygen (4.23 – 5.57 mg/L), total dissolved solids (56 – 184 mg/L), total hardness (78 – 519 mg/L) and alkalinity (50.67 – 146.67 mg/L). Total coliforms were detected in counts ranging

from 49 to 136 CFU/100 mL of water sample. One-way ANOVA showed that pH, temperature, electrical conductivities, nitrates, nitrites, chloride, dissolved oxygen, total dissolved solid, total hardness and alkalinity were significantly different ($P < 0.05$), whereas turbidity and sulphate were not significantly different ($P > 0.05$) from each parameter with respect to sampling sites.

The correlation analysis for both microbiological and physicochemical data of the selected rivers was performed. Some significant positive correlations were obvious. DO was positively correlated with TDS and TH at 99 % confidence limit. Likewise, temperature was positively correlated with nitrate and nitrites at the 95 % confidence level. This result disagrees with the findings of Badran (2001) and Manasrah *et al.* (2006) that nutrient consumption by primary producers increased in favourable temperature condition. Also, nitrates and nitrites were positively correlated with each other, and this is not surprising because nutrients are naturally found in the environmental waters even if they are from different sources.

Similarly, correlation of EC with chloride, DO, TDS, TH and alkalinity was positively significant. This finding strongly concurs with the report of Sunitha *et al.* (2005) that EC finds higher level correlation significance with many of the water quality parameters like TDS, chlorides, total alkalinity, sulphates, total hardness and magnesium. Other highly significant positive correlations were observed as follows: pH with EC, TH and alkalinity, chloride with DO and TDS, and TH with alkalinity, all at the 95 % confidence level. Conversely, temperature was negatively correlated with EC and TDS at 95 % confidence level ($r = – 0.48$) and ($r = – 0.67$) respectively. Some significant negative correlations were equally noted between pH and temperature, temperature and TH, and sulphate and alkalinity at 99 % confidence level. Above all, correlation study and coefficient values help in selecting treatments to minimize contaminants in surface and ground waters (Achuthan *et al.* 2005).

Total coliform bacteria are widespread in nature. All members of the total coliform group can occur in human faeces, but some can also be present in animal manure, soil and submerged wood and in other places outside the human body (Shilklomanov, 2000). Thus, the usefulness of total coliforms as an indicator of faecal contamination depends on the extent to which the bacteria species found are both faecal and human in origin (USEPA, 2012). While total coliforms are no longer recommended as an indicator for recreational waters, they are still the standard test for drinking water because their presence indicates contamination of a water supply by an outside source (USEPA, 2012).

Coliforms are a broad class of bacteria found in the environment, including the human excreta and other warm-blooded animals. Their presence in water suggest the possibility of the presence of pathogens and parasites, with the associated disease symptoms such as diarrhoea, nausea, vomiting, cramps and other gastro intestinal distresses and in severe cases can be fatal (Kilmaren, 2011). The result of total coliform (TC) populations in the water samples were found to be generally high and ranged between 49.00 and 136.00 (CFU/100 mL). The modal TC counts were obtained at site R2 and the least at site R10. Results of this study indicated that the river water sources were of poor microbiological quality.

Although TC counts varied from site to site, however, sites R1, R2 and R7 seemed to be more polluted compared to others. From the figure it was evident that the river waters are highly contaminated, and also denote public health hazards. The maximum permissible value of total coliforms in drinking is 10 per 100 mL (WHO, 1993). In the present study, the total coliform counts obtained in all the sampling sites exceeded the set limit. Presence of coliform organisms in water has been regarded as evidence of faecal contamination. This clearly suggests that the river waters were contaminated by domestic sewage, human and animal excreta, which are

objectionable for drinking purposes and also render the waters unsuitable for domestic use. Generally, results from this study suggest that the river waters are not suitable for consumption, domestic or recreational use and re-echo the importance of safeguarding the freshwater resources of Southwestern Nigeria.

Escherichia coli can be used as indicators of aquatic ecosystem dynamics and determination of their occurrence may help to assess the water quality. The present study evaluated the distribution and frequency of *E. coli* isolates in river water samples from Osun State, South-western Nigeria. Generally, the mean annual counts of the presumptive *E. coli* obtained in all the sampling sites were relatively high. Confirmed *E. coli* isolates in the river samples at all sites ranged between 34 CFU/100 mL at the R5 site and 76 CFU/100 mL at the R7 site. Thus, the occurrence of higher indicator bacterial numbers in the samples could present a microbiological hazard to consumers of the raw river waters.

In this study, most of the sites with higher counts of *E. coli* were those located in pasture and peri-urban catchments with multiple sources of faecal pollution such as run-offs and dungs from cattle, horses and wild animals. There is a high likelihood that the isolates were mainly from human and animal excreta because during our sampling periods, human and animal excreta were sighted at the banks of the rivers, livestock were seen drinking water from the rivers, farmers and bricklayer were bathing and used waters from the car washing centers drained into the river. This further implicates both humans and animals as potential sources for the recovered *E. coli* pathovars. *E. coli* has been used extensively as one of the major faecal indicator bacteria due to the previous notion that it has limited survival ability in the environment though recent studies have suggested that some pedigrees of *E. coli* have adapted and acclimatized within tropical, subtropical and even temperate regions (Walk *et al.,* 2007; Ishii & Sadowsky 2008).

In developing countries, *E. coli* have been reported to be the leading cause of diarrhoeal diseases in addition to pathogens such as *Salmonella, Shigella, Yersinia, Vibrio, Campylobacter* species, *Entamoeba histolytica* and *Giardia lamblia* (Pratt & Taylor, 2003). Although most strains are harmless, several others are known to produce toxins while others possess O and H antigens that can induce diarrhoeal diseases and other extra intestinal infections (Kelly *et al.,* 2002). Over the years, strains of *E. coli* causing diarrhoea worldwide, mostly in infants have been categorized depending on their mechanisms of producing gastroenteritis. These groups are enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC) and diffusely adhering *E. coli* (DAEC) (Levin *et al.,* 2000; Patterson, 2002; Berge *et al.,* 2003; Pratt & Taylor, 2003).

The occurrence of virulence gene signatures associated with diarrhoeagenic and non-diarrhoeagenic pathovars of *Escherichia coli* isolates from some selected rivers was determined. Generally, the results illustrate varied occurrence of diarrhoeagenic and non-diarrhoeagenic *E. coli* pathotypes with (91%) of the isolates grouped under seven main *E. coli* pathotypes. In our study, VGs were detected in the *E. coli* isolates suggesting the presence of pathogenic *E. coli* strains in these waters. A large number of the *E. coli* isolates tested positive for toxin genes. The VG *lt* associated with ETEC strains was the most prevalent of all (45%). This finding is a serious concern, considering the fact that it is the most common agent of traveler's diarrhoea with food and water implicated as the modes of transmission (Huang *et al.,* 2006; Mohamed *et al.,* 2007). The presence of ST and/or LT enterotoxins which are commonly associated with ETEC strains have been reported by other workers in surface waters (Obi *et al.,* 2004; Begum *et al.,* 2005) and are thought to originate from swine and humans with diarrhoea. Though, none of the isolates harboured a combination of the shiga toxin genes, nonetheless the relatively high occurrence of

the *stx*1 gene (6%) compared to *stx*2 (1%) in the water *E. coli* isolates suggests the capability of each gene in causing acute diarrhoea in humans. This observation contradicts with the relatively high occurrence of the *stx*2 gene (10%) compared to *stx*1 (6%) in the storm water *E. coli* isolates which suggests that *E. coli* carrying a combination of the EHEC genes, are known to cause more severe diarrhoea in humans (Paton & Paton 1998). The most prevalent pathotypes of *E. coli* responsible for diarrhoeal diseases include enterohaemorrhagic or shiga toxin producing *E. coli* (EHEC or STEC) and enterotoxigenic *E. coli* (ETEC) (Kaper *et al.,* 2004). The contamination of drinking or recreational waters with such *E. coli* pathotypes has been linked to waterborne disease outbreaks and mortality (Begum *et al.,* 2005; Bruneau *et al.,* 2004).

The *eae* gene, which codes for intimin protein, was the fourth most prevalent gene in this study (6%). This gene is necessary for intimate attachment to host epithelial cells in both the EHEC and EPEC pathotypes. Our findings tend to strongly disagree with the previous finding of significantly higher prevalence of the *eae* gene (up to 96%) in surface water reported in other studies (Shelton *et al.,* 2006; Masters *et al.,* 2011). In this study, a noticeably low prevalence of the *bfp* gene (4%) was detected, suggesting that prevalence of the EPEC pathotype could be expected in the surface water bodies. In addition, *eae* was also detected in 4% isolates which lacked other typical genes from both EPEC group. This indicates prevalence of this gene in *E. coli* isolated from the freshwater environments. This finding is of great concern, as an atypical EPEC pathotype which lacks the *bfp* gene but carries the *eae* gene has been found to be a major cause of gastroenteritis worldwide (Hernandes *et al.,* 2009) in patients suffering from community-acquired gastroenteritis in Melbourne, Australia (Robins-Browne *et al.,* 2004), and from children with diarrhoea in Germany (Kozub-Witkowski *et al.,* 2008).

Approximately 2% of the isolates carried both *eae* and *bfp* genes suggesting the presence of typical EPEC pathotype. The relatively low occurrence of the combination of both atypical EPEC genes in the water *E. coli* isolates is alarming due its possible significance in the cause of severe diarrhoea in humans. However, the role of atypical EPEC in diarrhoea has not been established assertively (Nataro & Kaper 1998; Kaper *et al.,* 2004; Nataro *et al.,* 2006) and this study did not aim at revealing the diarrhoeagenic role of this pathotype. Generally, a few EHEC strains identified in this study were in agreement with other studies executed in different parts of the world (Gomes *et al.,* 1991; Nguyen *et al.,* 2005) and a low prevalence of EHEC infection has been observed in developing countries (Brown *et al.,* 1989; Strockbine *et al.,* 1992).

In the present study, among the DEC types, *eagg* gene of EAEC strains was the least frequently isolated adhesion VG with only 7 (2%) strains detected in all the isolates, yet the pathotype has been an important diarrhoeagenic pathogen with its characteristic persistent diarrhoea in children and adults. This finding seems to be inconsistent with the previously reported high prevalence of the EAEC pathotype in fresh and estuarine water samples (Masters *et al.,* 2011) but tends to align with the earlier observation of a less common DAEC, EAEC and a variety of different EHEC and EPEC pathotypes with the exception of enteroinvasive *E. coli* which was not detected in the 509 samples studied (Cebula *et al.,* 1995; Vidal *et al.,* 2004).

Uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) are the two other extra-intestinal *E. coli* (ExPEC) pathotypes that have been characterized (Wiles *et al.,* 2008; Dubois *et al.,* 2009). The study shows a higher prevalence of VG *pap*C (19%), belonging to UPEC pathotype than the NMEC VG *ibe*A (3%). The presence of *E. coli* strains with virulence characteristics similar to ExPEC have been reported previously in the fresh and estuarine waters (Hamelin *et al.,* 2006). This observation was of interest and may be an indication of a high

potential health risk of such waters as the number of ExPEC VG(s) in *E. coli* has been suggested to be proportional to its pathogenic potential (Picard *et al.,* 1999). Pathogenic strains are divided into intestinal pathogenic *E. coli* (InPEC) causing diarrhoea and extra-intestinal *E. coli* (ExPEC) causing a variety of infections in both humans and animals including urinary tract infections (UTIs), bacteremia, meningitis and septicemia (Kaper *et al.* 2004).

Overall, the detection of most of the VGs tested was relatively low aside *lt* and *pap*C, ranging from 1 to 7%. This finding correlates with the reports of (Martin *et al.*, 1992; Lauber *et al.,* 2003; Chern *et al.,* 2004) that the prevalence of *E. coli* isolates harbouring VGs in environmental waters is low ranging from 0.9% to10%. The presence of a single or multiple VGs in an *E. coli* strain does not necessarily indicate that a strain is pathogenic unless that strain has the appropriate combination of VGs to cause disease in the host (Gilmore & Ferretti 2003; Bruneau *et al.,* 2004).

In the present investigation, we collected water samples from communities with diverse human population densities and land uses to determine if these factors influence the distribution of VGs. The results of this study show a relatively low and clear pattern of occurrence of VGs across the sites with a noticeable difference of occurrence of 4 VGs and 3 VGs at sites R9 and R10 respectively. Overall, it was evident that the point and non-point sources of contamination were potentially similar across the sampling sites in their characteristic features. Similarly, all the sampling sites are bordered by farm animals such as ruminants which are known to be potential sources of these VGs (Djordjevic *et al.*, 2004; Ahmed *et al.,* 2007; Gyles 2007; Ishii *et al.,* 2007).

Rise of antimicrobial resistance in *E. coli* to multiple antibiotics is a major concern both in developed and developing countries (Ajamaluddin *et al.,* 2000; Chandran *et al.,* 2008). Contamination of water sources with faecal bacteria like *E. coli* is a serious problem due to its ability to transmit diseases. The risk associated with these bacteria further increases if they are antibiotic resistant (Da Silva *et al.,* 2012).

The study further employed the multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of faecal contamination of water**.** Our findings revealed all the *E. coli* isolates were susceptible to all members of imipenem, meropenem, amikacin and gatilofloxacin antimicrobials. Similarly, high sensitivities were detected against ciprofloxacin (96 %), kanamycin (95 %), neomycin (92 %), streptomycin (84 %) and chloramphenicol (73 %). Varied susceptibilities were recorded for other antibiotics as follows: nalidixic acid (66%), nitrofuratoin (64 %), gentamycin (63 %) and cefepime (57 %). Susceptibilities observed against other antibiotics tested were below average of 50%.

All the isolates were resistant to sulphamethoxazole. High levels of resistance were equally observed against amoxycillin (59 %) and ampicilin (57 %) while the resistance to cefuroxime at 40% was slightly below average. The lowest susceptibilities were observed against doxycycline (34 %) and tetracycline (33 %). Others varied as follows; cephalotin (29 %), gentamycin (24 %), nalidixic acid and nitrofuratoin (19 %), amoxycillin (19 %), ampicillin (18 %) and streptomycin (14 %).

Compilation of the MAR phenotypes indicate that about 75 % of *E. coli* isolates in this study exhibited resistance to three or more antimicrobial agents. The frequency of MARPs ranged from 24.8% to 2.2% for MARPs 5 and 9 respectively. When this was expressed in terms of

prevalence, 4%, 5%, 10%, 16%, 20%, 20%, and 25% of the isolates showed multiple antibiotic resistance to nine, seven, eight, three, four, six and five antimicrobials respectively. The highest prevalence of MARPs across all the sampled sites was recorded at R7 in 70% and lowest at R9 in 4.4%. The current study revealed multi-drug resistance to numbers of antimicrobials ranging from three to nine. The antibiotic resistance patterns of the *E. coli* isolates across the selected sites include no-drug resistance (4), single-drug (36), two-drug (25), three-drug (19), four-drug (21), five-drug (24), six-drug (20), seven-drug (6), eight-drug (13) and nine-drug resistance (5).

Overall, the MAR indices in all the river samples were found to be higher than the 0.2 threshold value, revealing imprudent use and greater exposure to antibiotics in humans, aquaculture, poultry and livestock which may pose high ecological risk to the waters. The MAR index actually ranged from 0.5 to 0.8. The modal MAR index for the tested isolates was 0.8 at R7 which is approximately four times the 0.2 limit while the lowest was at R9 with 0.5. This implies that the burden of antibiotics being discharged into site R7 is greater than others.

Generally, the one-way ANOVA showed that cefepime, cephalothin, cefuroxime, nalidixic acid, nitrofuratoin, chloramphenicol and tetracycline were not significantly different in their effect against the isolates from all locations ($P > 0.05$), whereas the resistance profile of the isolates against gentamycin, ciprofloxacin, sulphamethoxazole, ampicillin and amoxicillin were significantly different ($P < 0.05$). Amikacin, kanamycin, streptomycin, meropenem, imipenem and gatilofloxacin were statistically excluded from the analysis since all tested isolates showed total susceptibility to these antimicrobials.

Previous studies also suggest that sewage pollution contribute to the dissemination of antibiotic resistant bacteria in the environment (Reinthaler *et al.,* 2003; Martins da Costa *et al.,* 2006).

Indiscriminate and inappropriate use of antibiotics, and related compounds, for various purposes also causes significant antibiotic contamination of the natural environment (Mellon *et al.,* 2001; McEwen & Fedorka-Cray, 2002) and the development and proliferation of antimicrobial resistant pathogens (Martinez & Baquero 2002). In addition to the problem of the detection of pathogens in water samples in population is the wide antibiotics resistance commonly demonstrated by these pathogens (Engberg *et al.,* 2001; Ash *et al.,* 2002).

Multiple antimicrobial resistances may partly result from the spread of genetic elements including plasmids, transposons, and integrons that may confer resistance to numerous antimicrobials (Obi *et al.,* 2004b). It should be noted that susceptibility of bacteria to antibiotics is not static and resistance may be due to antibiotic abuse, antibiotic overuse or may be chromosomally or plasmid mediated (Obi *et al.,* 1998). Several pathogens have been shown to demonstrate a significant increase in resistance to some specific antibiotics over a short period of time (Coker & Adefeso, 1994; Hoge *et al.,* 1998), either as a result of selective pressure, antibiotic abuse by humans or over use in animals (White *et al.,* 2000).

The drug resistance patterns observed suggest that most of the isolates had multiple drug resistance. Presence of multiple drug resistance (MDR) in enteric bacteria isolates from aquatic environment has been reported previously (Akinbowale *et al.,* 2006; Olaniran *et al.,* 2009; Abdo *et al.* 2010; Emmanuel *et al.* 2011; Florea, 2011). Resistant bacteria have been isolated from a variety of sources, including domestic sewage, drinking water, rivers, and lakes (Kasper *et al.,* 1990; McKeon 1995; Mulamattathil *et al.,* 2000). Studies conducted in other countries demonstrated the presence of MDR pathogenic bacteria in water sources including rivers, ponds and lakes (Hu *et al.,* 2008; Ram *et al.,* 2008). Multiple antimicrobial drug resistance profiles have been used to identify and differentiate *E. coli* strains from different animal (Krumperman *et*

al., 1983) and water (Wiggins 1996; Kasper *et al.,* 1990; Parveen *et al.,* 1997; Hagedorn *et al.,* 1999; Wiggins *et al.,* 1999; Harwood *et al.,* 2000; Graves *et al.,* 2002; Guan *et al.,* 2002) sources. This type of testing is simple, cost-effective and suitable for surveillance (Troy *et al.,* 2002).

In a study of 16 United States Rivers, antibiotic-resistant bacteria were found to be widespread, and the resistance included to chemically modified and synthesized antibiotics (Ash *et al.,* 2002). Several rivers in the United States have therefore been indicated as reservoirs of antibiotic resistant bacteria (Ash *et al.,* 2002). Antibiotic resistance among pathogens is emerging as a threat to human and veterinary medicine. This is due to the extensive and indiscriminate use of antibiotics for treatment, prophylaxis or growth promotion.

Antimicrobial resistance genes (ARGs) can enter into aquatic environments either by direct discharging of untreated wastewater or into sewage treatment plants through wastewater collection systems and subsequently into the environments with effluents and discharged sludge (Auerbach *et al.,* 2007). ARGs can be transferred into soils by amending farm land with animal manure and processed biosludge from sewage treatment plants (STPs) and then can leach to groundwater or be carried by runoff and erosion to surface water (Yang & Carlson, 2003). Surface water and shallow groundwater are commonly used as source of drinking water; thus, ARGs can go through drinking water treatment facilities and enter into water distribution systems (Schwartz *et al.,* 2003).

E. coli, being commensal in the human and animal gut has been a sensitive indicator of distinct therapeutic and non-therapeutic uses of antimicrobial drugs (Stelling *et al.,* 2005), and has been reported to transfer the antibiotic resistant genes to enteric pathogenic and normal flora bacteria

(Platt *et al.,* 1986; Ozgumus *et al.,* 2007). The problem in increasing infectious disease is the acquisition and transfer of antibiotic resistance and virulence factor genes by the bacterium through horizontal transfer of the resistance (R) plasmids, transposons and integrons (Leverstein-van Hall *et al.,* 2002; Tenover *et al.,* 2006).

Sulfonamides are the first antibiotic developed for large scale introduction into clinical use, which target dihydropteroate synthase (DHPS). Eight percent of the 300 sulfonamide-resistant isolates possessed the *sul*I gene, whereas 41% harboured *sul*II. More than half of the isolates (53%) did not harbour either of the 2 genes and only 2% carried *sul*I and *sul*II. Four kinds of sul genes (*sul*I, II, III, and A) have been found in the bacteria of environmental origin (Lin & Biyela 2005; Pei *et al.,* 2006; Agersø & Petersen 2007; Akinbowale *et al.,* 2007a; Cernat *et al.,* 2007; Hu *et al.,* 2008; Mohapatra *et al.,* 2008).

Over 22 tet genes have been found in bacterial isolates from water environments such as natural water, drinking water, sediments, wastewater from hospitals, animal production and aquaculture, untreated sewage, effluent water and activated sludge of sewage treatment plant (Szczepanowski *et al*., 2004; Agersø & Sandvang 2005; Srinivasan *et al.,* 2005; Tennstedt *et al.,* 2005; Poppe *et al.,* 2006; Rodríguez *et al.,* 2006; Cernat *et al.,* 2007; Dang *et al.,* 2007; Macauley *et al.,* 2007; Hu *et al.,* 2008). The 83 *E. coli* isolates resistant to tetracycline treatment were screened for possible detection of tetracycline resistance genes; *tet*A, *tet*B, *tet*C, *tet*D, *tet*K and *tet*M. Of the six genes targeted, *tet*D was the predominant allele detected. Overall the frequency of detection of the tet alleles was *tet*A > *tet*B > *tet*C < *tet*D > *tet*K > *tet*M, being spotted in 24%, 23%, 18%, 78%, 15% and 12% of the total tetracycline resistant isolates, respectively. Patterns of dual and multiple tetracycline-resistance genes were equally observed. Dual *tet*A-*tet*D had the highest occurrence (5 %), followed by multiple *tet*A-*tet*D-*tet*M (3 %) while those with multiple 4 genes

were all uniform in their occurrences (1 %) each, with *tet*D commonly associated with all. None of the isolates harboured up to 5 and 6 tetracycline resistance genes.

Different from tetracycline resistance mechanisms mentioned above, the most major mechanism of aminoglycoside resistance is direct deactivation of this type of antibiotics by enzymatic modification (Shakil *et al.,* 2008). More than 50 modification enzymes have been found so far (Vakulenko & Mobashery, 2003; Ramón-García *et al.,* 2006). These enzymes are divided into three groups based upon their biochemical actions on the aminoglycoside substrates, including acetyltransferases, phosphotransferases, and nucleotidyltransferases (adenylyltransferases), encoded by three types of genes, namely, aac, aph, and ant (aad), respectively.

Of the five resistance genes encoding for aminoglycosides examined in this study, only 8% gentamycin-resistance gene, *aac*C2 was detected among the 39 gentamycin-resistant isolates, with 92% showing no observable genotypic feature. The *aph*A1 and *aph*A2 genes encode a kanamycin and neomycin resistance phenotypes, and these genes were found respectively, in 33% and 47% of the 15 kanamycin-resistant isolates, and 28% and 52% of the 25 neomycin-resistant isolates. Dual *aph*A1-*aph*A2 was carried in 20% each of the kanamycin and neomycin-resistant *E. coli* isolates. Similarly, streptomycin-resistance genes *aad*A and *str*A were detected in 79% and 38% among the 47 streptomycin-resistant isolates respectively. Only one isolate (2%) was found positive to both *aad*A and *str*A genes. Different aminoglycoside-modifying enzymes have been reported in a broad range of bacteria isolated from patients or clinical environments (Filipova *et al.,* 2006; Kelmani Chandrakanth *et al.,* 2008). ARGs encoding resistances to other antibiotics in aminoglycoside group, for example, phosphotransferase genes encoding resistance to neomycin (nptII) and streptothricin (strAB), have also been detected in the river water of Canada (Zhu, 2007) and Ganges river of India (Mohapatra *et al.,* 2008).

The mechanisms responsible for resistance to chloramphenicol and florfenicol include chloramphenicol acetyltransferases (encoded by cat genes), specific exporters (encoded by cml genes) and multidrug transporters (Schwarz *et al.,* 2004). Among the 65 chloramphenicol-resistant isolates screened, the most frequent chloramphenicol resistance gene was *cat*I (37%). *cat*II and *cml*A1 genes were frequently detected in 28% and 19% of isolates respectively, whereas 59% of the isolates were negative to *cat*I, *cat*II and *cml*A1. Dual patterns *cat*I-*cat*II, *cat*I-*cml*A1 and *catI*I-*cml*A1 were detected in 12%, 2% and 9% respectively and none of the isolates carried all the three genes tested.

The mechanisms of β-lactam resistance include inaccessibility of the antibiotics to their target enzymes, modifications of target enzymes, and/or direct deactivation of the antibiotics by β-lactamases (Walsh, 2000; Li *et al.,* 2007). In Gram-negative bacteria, the primary resistance mechanism is enzymatic inactivation through the cleavage of the β-lactam ring by β-lactamases. More than 400 different β-lactamases encoded by hundreds of ARGs (bla) have been identified, and the enzymes are divided into four molecular classes, A‑D, mediating resistances to a broad range of β-lactams including penicillins and cephalosporins (Li *et al.,* 2007). While the PCR amplification undertaken shows that 22% of the 172–ampicillin-resistant isolates were *amp*C positive, 21% and 18% of the 177 amoxycillin-resistant isolates harboured bla_{TEM} and bla_{Z} respectively. Nine percent of the amoxycillin-resistant isolates possessed dual bla_{TEM}-bla_{Z} resistance genes. Thirty seven percent of the amoxycillin resistant isolates and 38% of the ampicillin-resistant isolates were found negative to the β-lactam resistance genes examined.

This worldwide emergence of multi-drug resistant bacterial strains has rendered the current drugs used for treatment useless, causing treatment failures (Hancock, 2005). Resistance to

antimicrobials is as a result of three main strategies namely enzymatic inactivation of the drug (Davies, 1994), modification of target sites (Spratt, 1994) and extrusion by efflux (Nikaido, 1994). They are prevalent in different water bodies and their spread pathways in various aquatic environments are usually complicated (Zhang *et al.,* 2009).

Generally, of the sum total of 538 resistance gene fingerprints obtained across the sampling sites among the resistant isolates, the highest prevalence was recorded at sites R2 and R3 with the overall total of 69 prints each while the lowest was at R4 with 38. Likewise, the prevalence of dual and multiple antimicrobial resistance genes across the sampling sites ranged between 2 and 4, with *tet* gene combinations most occurring, due to their high number assessed in the study. Some physicochemical factors can influence the dissemination of ARGs in aquatic environments. The first factor contributing to the horizontal transfer of ARGs is the selective pressure from ever-increasing production and consumption of antibiotics for treatment of disease and growth promotion. High selective pressure facilitates the acquisition of ARGs, which may actually increase the fitness of certain bacteria and allow the rapid emergence and dissemination on a worldwide scale (Enne *et al.,* 2004; Luo *et al.,* 2005). In addition, the presence of antibiotics at low sub-inhibitory concentrations can accelerate horizontal transfer and dissemination of environmental ARGs (Kümmerer, 2004). It was found that keeping antibiotic concentration at a sub-inhibitory level in the mating medium significantly enhances conjugal transfer mediated by plasmid or transposon in the environments (Ohlsen *et al.,* 2003; Hecht *et al.,* 2007).

In order to determine whether possible associations exist between the resistance genes detected among our isolates and whether the co-appearance of some resistance genes could be confirmed statistically, an analysis of association was done by using Pearson's chi-square exact test. Significant associations ($P < 0.05$) with respect to the occurrence of individual resistance genes

among the whole collection of *E. coli* isolates were detected. Many positive associations were obvious, for example, the association of the *sul*I and $bla_{TEM,}$ bla_{Z} and *tet*C, as well as *tet*A and *tet*B genes. The *sul*I gene was positively associated with bla_{TEM}, bla_{Z} genes and also, although less strongly, with the *sulI*I and *amp*C genes. The *amp*C gene showed a very strong association with the *cml*AI, *aac*C2, *apH*A1, *apH*A2, *aad*A and *str*A genes. The same trend was observed for bla_{Z} *gene* with *tet*C, *tet*D, *tet*K, *tet*M, *cat*II, *cml*A1 genes and less strongly associated with *tet*A gene. None of the genes was negatively associated with one and another.

8.2 Conclusion

In Nigeria today, inadequate water for domestic, irrigation and others purposes in rural and urban centers is one of the most challenging issues. The rise of the inflow of waste is clearly due to the rapid growth of residential and commercial activities in the study area. Due to the discharge of sewage, domestic wastes and human activities, the physicochemical and bacteriological qualities of the river waters exceeded the permissible limit of WHO drinking water standards and in turns make the waters unfit for human consumption. Confirmation of the presence of *Escherichia coli* in river water samples indicates faecal contamination and the possible presence of other enteric pathogens. A better understanding of the prevalence and distribution of *E. coli* pathotypes in water sources used for potable, non-potable or recreation purposes could be an important tool in the development of public health risk mitigation strategies. Pathotyping of *E. coli* isolates may also provide useful information to identify potential sources of pollution. The prevalence of virulence markers in *E. coli* isolates from river water sources is indicative of increased risks of mortality, especially among the vulnerable populations, should they contract infections through the use of river water for consumption or other household related purposes. Multiple antibiotic resistant phenotypes and indices of *E. coli* observed in the surface waters suggest serious health

risk for communities that depend on them for sundry purposes. The *Escherichia coli* isolates recovered from the rivers showed in some cases differences in antibiotics phenotypic and genotypic expressions. Often, more than one gene was associated with a given phenotypic resistance. A slightly different distribution of resistance genes and multigene resistance observed across the sampling sites suggests that relative resistance gene frequencies could vary within a population and geographical origins. Therefore, this study reinforces the necessity of using genotypic resistance analysis in future epidemiological studies.

8.3 Recommendations

Based on the findings of the study, the following recommendations could be deduced:

(i) A better access to safe and water supply, adequate sanitation facilities and better hygiene practices is very essential to health, food security and any sustainable development both in rural and urban communities. The state government should strive towards safety of water as this will not only support public health, but will also promote socio-economic development and individual well-being as well.

(ii) The state and national surveillance agencies should be set up and shouldered with a responsibility of setting up short, medium and long-term targets for the continuous improvement of water supplies and safeguarding of freshwater resources. The targets could be directed towards meeting up with the millennium development goals (MDGs) on water quality.

(iii) The presence of multiple antimicrobial resistant *E. coli* in all the river water sources calls for caution in the indiscriminate and inappropriate use of antimicrobial agents, and related compounds on animals and humans. There is need for government to legislate

and implement policies geared towards controlling sewage inflow, animal dung and wastewater disposal along with antibiotic use.

(iv) Our findings indicate a high incidence of antimicrobial resistance of *E. coli* towards the conventionally used antibiotics. There is a need for good surveillance programs to monitor antimicrobial resistance patterns in water bodies. Therefore, together with water quality assessments, studies that provide valuable information about antimicrobial resistant microbes from environmental sources like water, food, soil and other ecological niches should be encouraged to avoid disease complications.

(v) The wide distribution of multidrug resistance genes in *E. coli* and other indicator bacteria in the environments has implications for the choice of antibiotics and underscores the importance of empiric management of infections, continuous regulation and monitoring of antimicrobial susceptibility patterns, and effective hospital infection control by the government. A continuous surveillance of *E. coli* isolates from surface waters used for recreational or domestic purposes and the development of adequate prevention strategies to diminish the spread of multi-resistant bacteria and/or the mobile resistance elements is equally advocated for public health purposes.

(vi) Some concise efforts have to be made by the government to reduce the possible spread of antimicrobial resistance genes in the environments because resistant bacterial strains will continue to emerge unless indiscriminate and imprudent use of drugs is curtailed by public awareness programs coupled with enforcement of legislation that limits the prescription and dispensing of antimicrobials to only qualified professionals. Researches on transfer of environmental antimicrobial resistance genes and health risk assessment on the genes need to be properly carried out in order to provide more scientific information

for relevant and appropriate authorities to make up regulatory standards and guidelines to control environmental dissemination of these " pollutants ".

8.4 Potentials for future development of the study

(i) Mechanism of action of virulence and antimicrobial resistance genes needs to be elucidated. This will help to understand the physiological or anatomical change at the cellular and molecular level, resulting from the exposure of a living organism to a substance or treatment. It is also important in classifying chemicals as it represents an intermediate level of complexity in between molecular mechanisms and physiological outcomes, especially when the exact molecular target has not yet been elucidated.

(ii) Sequencing and phylogenetic analysis of the isolates and antibiotic resistance markers. Evolutionary knowledge on the structure of identification and classification of isolates will help further to understand the epidemiology of infectious diseases and their relatedness.

(iii) Detection of integrons and gene cassettes. Cassettes are important genetic elements and play an important role in the dissemination and spread of antimicrobial resistance genes in a clinical setting. This will help to understand the transference of resistance determinants among the antimicrobial resistant isolates in the environment.

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