MOLECULAR CHARACTERIZATION OF ANTIBIOTIC RESISTANCE PLASMIDS IN SOME EXTENDED SPECTRUM β-LACTAMASE PRODUCING GRAM NEGATIVE BACTERIAL ISOLATES RESISTANT TO METHANO LIC EXTRACT OF *CARICA PAPAYA*

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NIGERIA

MARCH, 2014

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A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES AHMADU BELLO UNIVERSITY, ZARIA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF A DOCTOR OF PHILOSOPHY DEGREE IN MICROBIOLOGY

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MARCH, 2014

DECLARATION

I declare that the work in this dissertation entitled "**MOLECULAR CHARACTERIZATION OF ANTIBIOTIC RESISTANCE PLASMIDS IN SOME EXTENDED SPECTRUM β-LACTAMASE PRODUCING GRAM NEGATIVE BACTERIAL ISOLATES RESISTANT TO METHANOLIC EXTRACT OF *CARICA PAPAYA*"** was performed by me in the laboratories of the Department of Microbiology, Ahmadu Bello University, Zaria, under the supervision of Professors O.S. Olonitola, J.B. Ameh and C.M.Z. Whong.

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

______________________ __________ ___________

Name of Student Signature Date

CERTIFICATION

This dissertation entitled **"MOLECULAR CHARACTERIZATION OF ANTIBIOTIC RESISTANCE PLASMIDS IN SOME EXTENDED SPECTRUM β-LACTAMASE PRODUCING GRAM NEGATIVE BACTERIAL ISOLATES RESISTANT TO METHANOLIC EXTRACT OF *CARICA PAPAYA*"** by Omolara ADENAIKE meets the regulations governing the award of the degree of Doctor of Philosophy of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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ACKNOWLEDGEMENTS

I give all the glory to God Almighty, the Giver of life and every good thing, for the privilege of this additional degree, may His name be praised forever more. Amen.

I'm greatly indebted to my Supervisors, Professors O.S. Olonitola, J.B. Ameh and C. M. Z. Whong, for all I enjoyed in the course of the supervision of this work, their concern for the progress of the work, the search for needed resources and their belief in my person that I could bring forth a worthwhile research. May the Lord bless you abundantly in Jesus name. Amen.

I appreciate the concern and generosity of Prof. C.A. Okuofu, Dept. of Water Resources and Environmental Engineering, for giving me a number of media and reagents for the first set of analysis, and his concern for the completion of the work; also by extension the hand of fellowship accorded me by Messers Alika and Yahaya of the same Department. The entire staffs of the Dept. of Microbiology are instrumental to the success of this work. I want to appreciate the efforts of Mr. Ayo Odewumi, Prof. E.D. Jatau, Dr. I.O. Abdullahi, Prof. A.A. Ahmad, Dr. (Mrs.) H.I. Inabo, Dr. (Mrs.) Maryam Aminu-Muktar and the fatherly support of Dr. S.E. Yakubu. Thanks to Mrs. T. E. Addai and Mr. Alexander Shaibu for permitting access to their offices within a period of time in order to use the laboratory facilities therein. To Dr. E.E. Ella, Messers Adamu Shittu and Shuaibu Garba, I owe you a million thanks. May God bless you.

I wish to appreciate the Head, and technical staff of the Department of Pharmacognosy and drug development, Faculty of Pharmaceutical Sciences, A.B.U. Zaria; for providing bench space and technical assistance for the plant extraction and phytochemical screening aspect of this work.

Also, I'm grateful to the Management and Staff of DNALABS Nigeria, Kaduna. They are really a team of research support group and wonderful people to work with. Their tremendous support and consideration contributed immensely to success of the molecular aspect of this work.

I want to appreciate my loving Parents, Deacon and Mrs. D. O. Ogunwole, and my maternal Aunt, Mrs. Adejoke Ajibola, for their prayers, moral and financial support to see to the success of this work. Also, the concerns and support of my late Brother and his Sister in-laws, Mr. John Adewale Adenaike and Mrs Adebimpe Adeborogun, are well appreciated. The tremendous support of my siblings and their spouses cannot be overemphasized. So, to Elder and Mrs.

Kehinde Olugbeminiyi (Sogbesan), Prof. and Mrs. J. O. Ogunwole, Pastor and Mrs. Oladele Olabode and lastly, Mr. and Mrs. Emmanuel Ibisagba, I say God bless you. Our cord of love will ever remain binding.

I'm grateful for the support and encouragement received from Prof. and Mrs. J. F. Iyun, Engr. and Dr. (Mrs.) James Babatunde, Dr. and Mrs James Sambo, Mr. and Mrs Barnabas Jatau, Mrs. Janet Sangowawa and Dr. (Mrs.) Olubunmi Negedu-Momoh. Also, I appreciate every assistance from friends, fellow postgraduate students and co-workers in the laboratory, to mention a few are Evelyn Fatokun, Tarfena Amapu, Grace Abakpa, Sakina Bello, Mrs Mulika Agboola Abdul-rahman, Dr. (Mrs.) Grace Gberikon, Mrs Juliana Mohammed Christopher (Mama Jerry), Theresa Tafida, Blessing Obasi, Mrs Helen Ibukun Ikilama, Sister Esther Femi Ojo, Mrs. Nike Oladokun, Messers Hacinth Dapiya, Barnabas Olukotun, Oluwaseye Adedirin, Julius Okojukwu, Jacob Koduah, Engr. James Shiraki and Dr. (Mrs.) Mercy Bassi. Others worthy of note are the contributions and concerns of Bro. Johnson David, Dr. Asabe Dzikwi, Mary Okpe, Mrs Juliana Gambo, Mr. Samuel Oyebode, Dr. Nura Sani Mohammed, Pst. Dr. Joseph Okopi and Mr. Joseph Orabuike (Uncle Joe).

Finally, I'm indebted to my husband, Dr. Emmanuel Adeoye Adenaike, for his cooperation, moral and financial support to enhance the completion of this work. I sincerely appreciate his endurance during those long hours of loneliness while I was away in the laboratory. Together, we shall soar high. Thanks so much.

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ABBREVIATIONS

Acronyms	Meanings
AGP	Antimicrobial growth promoters
AmpC	Class C β-lactamase
bla	Beta-lactamase
CMY	Cephamycin resistance
CTX-M	Cefotaximase
DNA	Deoxyribonucleic acid
ESBLs	Extended spectrum β-lactamases
FOX	Cefoxitin resistance
GES	Gulana extended-spectrum β-lactamase
IMP	Imipenemase
KPC	*Klebsiella pneumonia* carbapenemase
MDR	Multidrug resistance
MOX	Moxalactam resistance
NAFDAC	National Agency for Food and Drug Administration Control
NDDIC	National Digestive Diseases Information Clearinghouse
NDM	New Delhi metallo-β-lactamase
OXA	Oxacillinase
PABA	Para-aminobenzoic acid

PBP	Penicillin binding protein
SHV	Sulphydryl variable
TEM	Temoniera
VEB	Vetnam extended spectrum β-lactamases
VIM	Verona integron-encoded-metallo- β-lactamase
WHO	World Health Organization

ABSTRACT

Some samples of ready-to-eat foods and drinks ('zoborodo', 'kunun zaki', smoked fish and 'suya') sold within the environs of Ahmadu Bello University, Zaria, Nigeria were assessed for the presence of antibiotic resistant E. coli and Klebsiella spp. The bacteria isolated were characterized using Microgen Gram negative identification kit and tested for their susceptibility to prepared concentrations of methanolic extracts of the leaves, stem-bark and root of Carica papaya using impregnated paper discs. Phytochemical screening revealed presence of more active constituents in the leaf extract than in the extracts of the root and stem-bark. All the organisms were found to be resistant to the Carica papaya methanolic extracts. Determination of β-lactamase production using nitrocefin-containing beta-lactamase identification sticks was carried out to test sensitivity of the rapid test. The test was found to produce false negatives and so had 12.9% sensitivity but 100% specificity when compared with disc diffusion test. Antibiogram of the test organisms to nine antibiotics showed 75% broad spectrum resistance (i.e. resistance to ampicillin or cephalothin), 35% ESBLs production (i.e. resistance to cefpodoxime or cefotaxime). Student t-test shows higher significant difference between the numbers of E. coli and Klebsiella spp. Resistant to the antibiotic tested in the study. Pearson's correlation showed significant association between ESBLs production and multidrug resistance in the entire sample populations. A high percentage of the bacteria had Multiple Antibiotic Resistance (MAR) index greater than 0.2 which shows that the isolates were obtained from high risk environment. Duncan multiple range test (DMRT) showed that the mean of antibiotic resistance from isolates obtained from 'suya' was significantly higher than those obtained from 'zoborodo' or smoked fish. For the molecular studies, TEM and SHV β-lactamase genes were assayed for among 12 isolates. TEM had a frequency of 66.7% while SHV had 8.3%. No isolate was found to harbour both TEM and SHV genes together. Sequence analysis results showed that the bla_{TEM} *in Sye10 (isolate 8) had 86% homology with β-lactamase TEM-1gene. The entire* bla_{TEM} *genes were not sequenced, so it cannot be stated categorically that the entire TEM genes present were TEM-1. Sequence analysis of* bla_{SHV} *in Syk2 (isolate 10) could not confirm the subtype. The reason for this is not yet known but must be because the primers only amplified a portion of the* bla_{SHV} *open reading frame and not the entire portion. Sperman's correlation showed moderate correlation between the presence of any of the two genes and resistance to third generation cephalosporins. Therefore, there is no significant correlation (*$r_s = 0.258$*) between the presence of any of the two genes and resistance to third generation cephalosporins (*$p>0.05$*).*

CHAPTER 1

INTRODUCTION

1.1 General Background

Medicinal plants, since time immemorial have been used in virtually all cultures as sources of medicine. The widespread use of herbal remedies and healthcare preparations, obtained from commonly used traditional herbs and medicinal plants has been traced to the occurrence of natural products with medicinal properties (Hoareau and DaSilva, 1999). Herbal medicine, in several developing countries, using local traditions and beliefs, is still mainstay of healthcare. As defined by World Health Organization (WHO), health is a state of complete physical, mental and social well being and most merely the absence of disease or infirmity (Hoareau 90rely chiefly on traditional medicines for their primary health care needs (Weignenand *et al.,* 2004; Murugesan *et al*., 2011; Velanganni *et al*., 2011). Some plant products have been historically used as therapeutics in folk medicine to treat diseases caused by pathogens (Sanchez *et al*., 2010). Medicinal plants would be the best source to obtain a variety of drugs and therefore such plants should be investigated to understand better about their properties, safety and efficacy. They are the major sources of obtaining antimicrobial drugs (Velanganni *et al*., 2011). Bioactive compounds from a variety of natural sources have been used for the treatment of a number of human diseases. Selection of plant species to be studied could be based on ethno-medicinal information, chemotaxonomic relationship and the use of the plants in traditional medicine (Salihu and Garba, 2008).

Africa is a rich source of medicinal plants yet not without its problems. One of the major problems associated with the use of traditional medical remedies is the lack of standardization of dosage. Hence, the call of its formulation into modern and appropriate dosage forms to remedy this problem. This will guarantee the quality, safety and efficacy of the medicament (Olowosulu and Ishaku, 2005). In Nigeria, a large proportion of the people depend on traditional medicine for drug therapy. Over 60% of the rural dwellers depend on traditional medicine for the treatment of their ailments. It is therefore pertinent to study Nigerian plants due to fear of their extinction through bush burning, tree felling and agricultural requirement (Ayandele and Adebiyi, 2007; Salihu and Garba, 2008). Several reports have been published on the scientifically confirmed antimicrobial activity of some natural products derived from plants (Savoia *et al*., 2004). A large number of plant species still need to be analyzed for their antimicrobial activity against diverse bacteria, it is therefore critical to develop simple systems for rapid antimicrobial screening (Sanchez *et al*., 2010). Despite the increasing use of medicinal plants, their future, seemingly, is being threatened by complacency concerning their conservation. They are continuously under the threat of extinction as a result of growth exploitation, environment-unfriendly, harvest techniques, loss of growth habitats and unmonitored trade of medicinal plants (Hoareau and DaSilva, 1999).

The pawpaw tree (*Carica papaya*) is a small tree, native to tropical America, but cultivated in tropical areas throughout the world. It has a non-woody and hollow trunk, which produces large, deeply lobed leaves, which are eaten as vegetable in some geographical areas but more importantly, as antipyretic, for diuresis, antisyphilitic, abortifacient and antidiabetes. It is also used to promote healing, as an antidote for venoms and rabies. Studies on its leaf extracts have

been found to exhibit antimicrobial action against disease-causing microbes such as *Salmonella typhimurium* and opportunistic organisms such as *Escherichia coli* (Ojekale *et al*., 2006).

Food has a long association with the transmission of disease. Despite our increased knowledge, food borne disease is perhaps the most widespread health problem in the contemporary world and an important cause of reduced economic productivity. The various ways in which foods can transmit illness clearly indicates that biological contaminants are the major cause (Adams and Moss, 1999). When pathogens are discharged in faeces and urine, the hands of infected persons may become contaminated by the materials which are easily transferred to foods. These, when ingested find their way into the alimentary canal. Diseases such as typhoid and paratyphoid, bacillary dysentery, food poisoning, are transmitted through the ingestion of contaminated food or water (Okuofu, 2002).

Antimicrobial agents are one of the most useful groups of therapeutic agents available today. In fact, they constitute the only group of therapeutic agent which has had a measurable effect on overall mortality rate in the population. Unfortunately, the emergence of resistance against many agents in almost all human pathogens is now widespread and a cause of great concern for future therapeutic effectiveness (Berg *et al*., 2004). Recent studies have demonstrated that antibiotic resistant bacteria occur in the community. In the past, these organisms were confined to nosocomial (hospital) settings, but in recent years, community associated antibiotic resistant bacteria are being identified in different parts of the world (Olonitola *et al.,* 2006). Antimicrobial drug failure may occur for many reasons, e.g., reduced adherence to drug therapy, suboptimal dosing, diagnostic and laboratory error, ineffective infection control, counterfeit or altered drugs,

and resistance (innate or acquired). Although much attention is focused on the resistance patterns of eubacteria, resistance is being found for virtually all microbial agents including mycobacteria, viruses, parasites and fungi (MacPherson *et al.,* 2009). The use, over-use, and misuse of antibiotics has led to an alarming increase in the frequency of human pathogens that do not respond to antibiotic therapy, underscoring the need for new antibiotics and a better understanding of the origins of antibiotic resistance (Donato *et al*., 2010). In the developing countries, individuals may purchase antibiotics in pharmacies, stores and even marked stalls without laboratory susceptibility studies, without being dispensed by pharmacists and without a prescription. Thus, there is a widespread and uncontrolled use of antibiotics and patients often do not take a full course of treatment particularly if they are unable to afford it, coupled with the poor qualities and potencies of many drugs locally manufactured (Olonitola *et al.,* 2006).

The β-lactam antibiotics are a family of antimicrobial agents consisting of four major groups: the penicillins, cephalosporins, carbapenems and monobactams; in each case the molecules include a four-membered nitrogen-containing ring, the β-lactam ring. β-lactam antibiotics act by disrupting synthesis of the cell envelope in growing cells. In many of these antibiotics the β-lactam ring is susceptible to cleavage by certain bacterial enzymes (β-lactamases); such cleavage destroys the antibiotic and organisms which produce the enzymes generally show at least some degree of resistance to particular β-lactam antibiotics(s) (Singleton, 1997; Samaha-Kfoury and Araj, 2003). In recent years, the problem of gradually increasing resistance to antibiotics has threathened the entire world. Production of β-lactamases, which hydrolyses and inactivates β-lactam antibiotics, has been one of the resistance mechanisms of bacterial species, mainly in the

family Enterobacteriaceae (Bali *et al.*, 2010). Many of the second and third generation penicillins and cephalosporins were specifically designed to resist the hydrolytic action of major β-lactamases. However, new β-lactamases emerged against each of the new classes of β-lactams that were introduced and caused resistance. The latest in the arsenal of these enzymes has been the evolution of extended spectrum β-lactamases (ESBLs). These enzymes are commonly produced by many members of Enterobacteriaceae, especially *E. coli* and *Klebsiella pnemoniae* and efficiently hydrolyze oxyimino-cephalosporins conferring resistance to third generation cephalosporins such as cefotaxime, ceftazidime, ceftriaxone and to monobactams such as aztreonam (Kumar *et al*., 2006).

Human activity strongly affects acquired resistance. Emergence of drug resistance in environments that enable sharing of drug-resistance genes between organisms has been documented. Human activities that contribute to ecological niche pressures, such as antimicrobial drug use and manufacturing or biological waste disposal into the environment can support the development of resistance (MacPherson *et al*., 2009). Microbial identification and typing systems, antibiograms and new technologies for identifying genetic clones and 'fingerprints' of microbes are better at defining the origin and patterns of spread of multidrug resistant (MDR) organisms. Local monitoring of susceptibility patterns combined with knowledge of emerging drug resistance, regionally or internationally, is already recognized as a component of some resistant infections. Growing population mobility makes local monitoring an increasing important component of routine surveillance for antimicrobial resistance (MacPherson *et al*., 2009).

1.2 Statement of Research Problem

The development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from other sources including plants (Balaraju *et al*., 2008a). The prevalence of extended spectrum β-lactamases (ESBLs) among members of *Enterobacteriaceae* constitutes a serious threat to current β-lactam therapy leading to treatment failure and consequent escalation of cost of treatment (Kumar *et al.,* 2006). ESBLs can be difficult to detect because of inoculum effects and substrate specificity, hence their detection is a major challenge, organisms possessing genes for inducible β-lactamases show false susceptibility if tested in the un-induced state (Chaudhary and Aggrawai, 2004).

1.3 Justification for the Study

Multidrug resistance by various bacteria against the most commonly prescribed antibiotics is a cause for concern among medical practitioners, pharmaceutical industries, research institutions and the general populace (Mbuh *et al., 2*008).

Also, most patients from the tropics and particularly from Africa are from low socioeconomic groups who can ill-afford imported and expensive medicines, hence the need for renewable, affordable and readily available local alternatives cannot be overemphasized (Okeniyi *et al.,* 2007). This has resulted in many people, both in urban and rural areas, seeking for succor in plants in search for cure of their infections (Mbuh *et al.,* 2008).

More has been focused on hospitals as the primary reservoir and place of transmission of many antimicrobial-resistant organisms, there is need to shift interest to the role of non-hospital community, such as foods as a significant reservoir of resistant pathogens (Hunter *et al*., 2008).

Updated knowledge of the prevailing causative bacteria and their susceptibility patterns are important for the proper selection and use of antimicrobial drugs and for the development of an appropriate prescribing policy (Ahmed *et al.,* 2000).

Extended-spectrum β-lactamases (ESBLs) had been the largest source of resistance to broad spectrum oxyimino-cephalosporins among the *Enterobacteriaceae* (Olonitola *et al.,* 2007).
Some ESBLs may fail to reach a level to be detectable by disk diffusion tests but result in treatment failure in the infected patient (Cornejo-Juarez *et al*., 2012).

Plasmid-encoded β-lactamase genes are therefore characterized using molecular techniques such as polymerase chain reaction (PCR) with primer sets specific for β-lactamase and DNA sequencing and because of the implications for treating such infections, particularly in developing countries, the spread of ESBL producing *Enterobacteriaceae* merits close surveillance (Frank *et al.,* 2006).

Therefore, there is a need to use molecular detection methods that will enable the identification and monitoring of the emergence of ESBL types (Xu *et al*., 2005).

1.4 Aim of Study

The aim of this study is to determine the antibiotic resistance pattern of *Escherichia coli* and *Klebsiella* species that are resistant to methanolic extracts of *Carica papaya* and characterize some of the plasmids responsible for the drug resistance.

1.5 Specific Objectives

1. To isolate and characterize *Escherichia coli* and *Klebsiella* sp. from some 'ready- to- eat' food items sold in A.B.U. Zaria main campus and its environs.
2. To prepare the leaf, stem-bark and root extracts of *Carica papaya* and determine the phytochemical properties of the extracts.
3. To determine the antibacterial properties of the extracts against the isolates.
4. To determine antibiotic susceptibility pattern of the isolates as well as confirm ESBL production from isolates identified as potential β-lactamase producers.
5. To isolate plasmid DNA of the isolates and amplify the β-lactamase genes (TEM and SHV) from the plasmid DNAs by polymerase chain reaction using specific primers.
6. To characterize the β-lactamase genes by agarose gel electrophoresis and validate the amplified genes by DNA sequencing.

1.6 Research Questions

This work tends to answer the following questions:

i. Will *Carica papaya* extracts be effective against the isolates?

ii. Will *E. coli* and *Klebsiella* spp. isolated from 'ready-to-eat' food items be found to produce β-lactamase enzymes?

iii. Will ESBL production be detected among the resistant isolates?

iv. Is the resistance mediated by the possession of plasmids in these isolates?

CHAPTER 2

LITERATURE REVIEW

2.1 Medicinal Plants

Over the years, plants have provided human beings with a source of essentials of life such as food, medicine and raw materials for clothing and shelters (Akiniyi and Efiom, 2005). The use of medicinal plants all over the world predates the introduction of antibiotics and other modern drugs into Africa (Akinyemi *et al*., 2005). They are used locally in the treatment of infections caused by fungi, bacteria, viruses and other parasites (Ayandele and Adebiyi, 2007). Currently, plant products are considered to be important alternative sources of new antimicrobial drugs against antibiotic-resistant microorganisms (Sanchez *et al*., 2010). According to Kuete *et al*. (2011), infectious diseases are the first cause of death worldwide with more than 50% of the death appearing in tropical countries. In the developing countries, treatment of such diseases is complicated not only because of the occurrence of resistant microorganisms to the commonly

used antibiotics, but also because of the low income of the population, which drastically reduce their accessibilities to appropriate drugs. It is reported that about 80% of the world population is dependent (wholly or partially) on plant-based drugs. Scientific experiments on the antimicrobial properties of plant components were first documented in the late 19th century. Naturally occurring antimicrobials can be derived from plants, animal tissues, or microorganisms. The short comings of the drugs available today propel the discovery of new pharmacotherapeutic agents in herbal medicine (Kuete *et al*., 2011).

Industrial interest in exploiting plants for medicinal purpose is exclusively found in China and Japan. Some African countries have also made advances in the area of the use of plants for the production of new drugs (Olukemi and Kandakai-Olukemi, 2004). The industrial uses of medicinal plants are many; these range from traditional medicines, herbal teas and health foods such as nutriceuticals to galenicals, phytopharmaceuticals and industrially produced pharmaceuticals. Furthermore, they constitute a source of valuable foreign exchange for most developing countries, as they are a ready source of drugs such as quinine and reserpine. The world market for plant-derived chemicals, pharmaceuticals, fragrances, flavours and colour ingredients alone exceeds several billion dollars per year (Hoareau and DaSilva, 1999).

In the last few decades, medicinal plants have been the subject for every intense pharmacological study. This has been brought about by the acknowledgement of their value as potential sources of new compounds of therapeutic value and as sources of lead compounds in drug development (Balaraju *et al*., 2008a). There is already increased shifting of interest from the use of synthetic

drug to the use of plant-derived drugs. It is believed that many of these phytomedicines have fewer side effects compared with their synthetic alternatives (Olowosulu and Ishaku, 2005). The increasing resistance to most synthetically derived antimicrobial agents is of utmost concern. Microbial infections pose a health problem throughout the world with the alarming increase in the rates of infection by antibiotic resistance in human pathogens against commonly used antibiotics. This has necessitated a search for new antimicrobial substances from other sources including plants (Balaraju *et al*., 2008b). Currently, plant products are considered to be important alternative sources of new antimicrobial drugs against antibiotic-resistant microorganisms (Sanchez *et al*., 2010).

Medicinal plants represent a rich source from which antimicrobial agents may be obtained. Plants are used medicinally in different countries and have been found to be sources of many potent and powerful drugs (Chaudhary and Khanam, 2008). Among the diseases that have been successfully managed traditionally include malaria, epilepsy, infantile convulsion, diarrhea dysentery, gonorrhea, flatulence, tonsillitis, sterility, asthma, scabies, eye aches, mental illness, worm infections, and several other bacterial and fungal infections. Curative uses of these plants include the administration of the roots, barks, stems, leaves, and seeds to the use of extract from a whole plant (Ogbulie *et al.,* 2004; Oyewale *et al*., 2006).

Medicinal plants constitute an effective source of both traditional and modern medicines. Herbal medicine has been shown to have genuine utility and about 80% of rural population depends on it as primary health care. Over the years, the World Health Organization (WHO) advocated that

countries should interact with traditional medicine with a view to identifying and exploiting aspects that provide safe and effective remedies for ailments of both microbial and non-microbial origins (Akinyemi *et al.,* 2005). Evaluation of plant products for pharmacological and medicinal effects is of interest as they contain many bioactive substances which have therapeutic potential and because phytotherapy is cheap and locally available (Balaraju *et al*., 2008b).

In Nigeria today, the prevalence of infectious diseases and the fact that the average citizen cannot afford the cost of modern chemotherapy makes the assay of plants important and more so herbal medicine can be found in the remotest parts of the country where medical doctors are absent (Olukemi and Kandakai-Olukemi, 2004; Oyewale *et al*., 2006). The World Health Organization has recommended the evaluation of the effectiveness of plants in conditions where we lack safe modern drugs (Balaraju *et al*., 2008b). Therefore, the integration of traditional and modern medicine is now to be regarded as supplemental to each other as opposed to being competitive (Satheesh and Pari, 2003).

According to Hoareau and DaSilva (1999), scientific validation of the antimicrobial properties of plants has been extensively reported. In the pharmaceutical industry, medicinal plants are an integral component of research development. Such researcher focuses on the isolation and direct use of active medicinal constituents, or on the development of semi- synthetic drugs, or still again on the active screenings of natural products to yield synthetic pharmacologically-active compounds. (Hoareau and DaSilva, 1999). However, little information is available about the mechanisms of action of antimicrobial compounds in bacteria. Several proposed mechanisms

include membrane damage, changes in intracellular pH, membrane potential, and ATP synthesis (Sanchez *et al*., 2010).

In Germany, over 1,500 plant species encountered in some 200 families and 800 genera have been processed into medicinal products. In South Africa likewise, some 500 species are commercialized trade products. Today, Bulgaria, Germany and Poland are recognized as major exporters of plant-based medicinal products (Hoareau and DaSilva, 1999). The development and commercialization of medicinal plants based bioindustries in the developing countries is dependent upon the availability of facilities and information concerning upstream and downstream bioprocessing, extraction, purification and marketing of the industrial potential of medicinal plants. Absence of such infrastructure compounded by lack of governmental interest and financial support restricts the evolution of traditional herbal extracts into authenticated market products. Furthermore, the absence of modernized socio-economic and public health care systems reinforces reliance of rural and lower-income urban populations on the use of traditional medicinal herbs and plants as complementary aid to routine pharmaceutical market products (Hoareau and DaSilva, 1999).

2.1.1 Major groups of bioactive components in medicinal plants

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives (Cowan, 1999). Phenols and phenol derivatives called phenolics disrupt cell membranes, denature proteins and inactivate enzymes. They are used to disinfect surfaces and to destroy discarded cultures because their action is not impaired by organic materials. Amphyl, which contains amylphenol, destroys vegetative forms of bacteria

and fungi, and inactivates viruses. It can be used on skin, medical instruments, dishes and furniture. When used on surfaces, it retains its antimicrobial action for several days (Black, 2005). Phenols are compounds possessing one or more aromatic rings with one or more hydroxyl groups. They are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants, with more than 8,000 phenolic structures currently known, ranging from simple molecules such as phenolic acids to highly polymerized substances such as tannins. It is known that phenolics are the most important compounds affecting flavour and colour difference among white, pink and red wines; they react with oxygen and are critical to the preservation, maturation and aging of the wine (Dai and Mumper, 2010).

The active principles of many drugs found in plants are secondary metabolites (Chaudhary and Khanam, 2008). About 12,000 secondary metabolites from plants have been isolated, a number estimated to be less than 10% of the total secondary metabolites found in plants. In many cases, these substances serve as plant defense mechanism against predation by microorganisms, insects, and herbivores (Salihu and Garba, 2008). Some, such as terpenoids, give plants their odors; others (quinines and tannins) are responsible for plant pigment. Many compounds are responsible for plant flavor (e.g., the terpenoid capsaicin from Chili peppers), and some of the same herbs and spices used by humans to season food yield useful medicinal compounds (Cowan, 1999).

2.1.1.1 *Simple phenols and phenolic acids*

Some of the simplest bioactive photochemical consist of a single substituted phenolic ring. Cinnamic and caffeic acids are common representatives of a wide group of phenylpropane-

derived compounds which are in the highest oxidation state (Cowan, 1999). The common herbs tarragon and thyme both contain caffeic acid, which is effective against viruses, bacteria and fungi (Cowan, 1999).

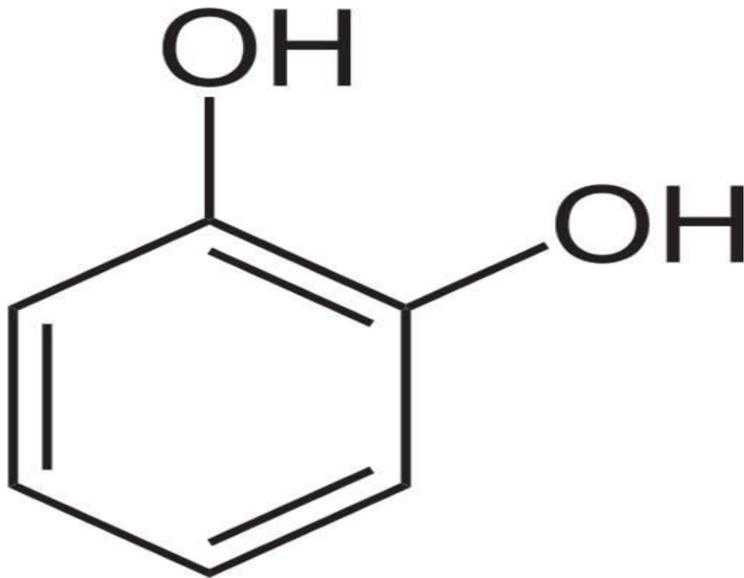


Fig. 2.1 Chemical structure of catechol (Helmenstine, 2013)

Catechol and pyrogallol both are hydroxylated phenols, shown to be toxic to microorganisms. Catechol has two-OH groups, and pyrogallol has three. The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity. In addition, some authors have found that more highly oxidized phenols are more inhibitory.

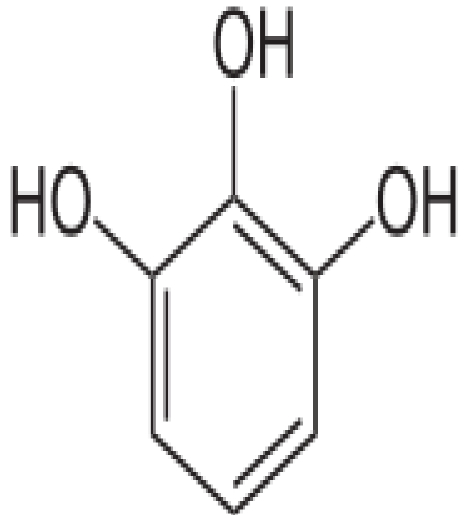


Fig. 2.2 Chemical structure of pyrogallol (Hardie *et al*., 2007)

The mechanisms thought to be responsible for phenol toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more non-specific interactions with proteins (Cowan, 1999). Phenolic compounds possessing a C_3 side chain at a lower level of oxidation and containing no oxygen are classified as essential oils and often cited as antimicrobial as well. Eugenol is a well characterized representative found in clove oil. Eugenol is considered bacteriostatic against both fungi and bacteria (Cowan, 1999).

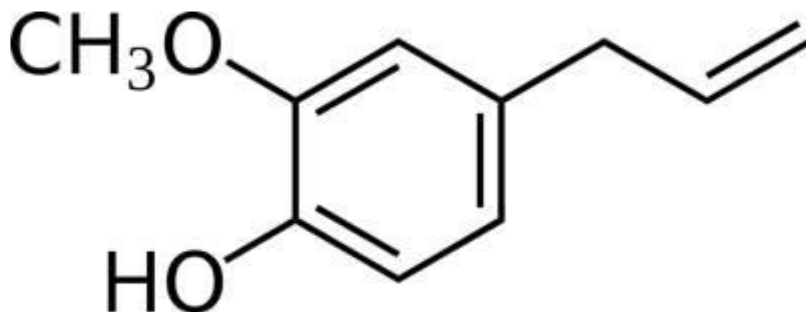


Fig. 2.3 Chemical structure of Eugenol (Helmenstine, 2013)

2.1.1.2 *Quinones*

Quinones are aromatic rings with two ketone substitutions. They are ubiquitous in nature and are characteristically highly reactive (Cowan, 1999). These compounds, being colored, are responsible for the browning reaction in cut or injured fruits and vegetables and are an intermediate in the melanin synthesis pathway in human skin. Their presence in henna gives that material its dyeing properties. The switch between diphenol (or hydroquinone) and diketone (or quinone) occurs easily through oxidation and reduction reaction. Vitamin K is a complex naptithoquinone. Its antihemorrhagic activity may be related to its ease of oxidation in body tissues (Cowan, 1999).

Fig. 2.4 Structures of quinones (Khullar, 2010)

According to Cowan (1999), in addition to providing a source of stable free radicals, quinones are known to complex irreversibly with nucleophilic amino acids in protein often leading to inactivation of the protein and loss of function. For that reason, the potential range of quinone

antimicrobial effect is great. Probable targets in the microbial cell are surface-exposed adhesions, cell wall polypeptides, and membrane bound enzymes. Quinones may also render substrates unavailable to the microorganism. However, as with all plant-derived antimicrobials, the possible toxic effects of quinones need to be thoroughly examined (Cowan, 1999). Anthraquinone from *Cassia italica*, a Pakistani tree has been described to be bacteriostatic for *Bacillus anthracis*, *Corynebacterium pseudodiphthericum* and *Pseudomonas aeruginosa* and bactericidal for *Pseudomonas pseudomalliae*. Hypercin, another anthraquinone from St. John's wort (*Hypericum perforatum*), has received much attention that it had general antimicrobial properties (Cowan, 1999).

2.1.1.3 *Flavonoids*

Flavonoids are the most abundant polyphenols in our diets. The basic flavonoid structure is the flavan nucleus, containing 15 carbon atoms arranged in three rings (C6-C3-C6), which are labelled as A, B and C. Flavonoids are themselves divided into six subgroups: flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins, according to the oxidation state of the central C ring. Their structural variation in each subgroup is partly due to the degree and pattern of hydroxylation, methoxylation, prenylation, or glycosylation (Dai and Mumper, 2010).

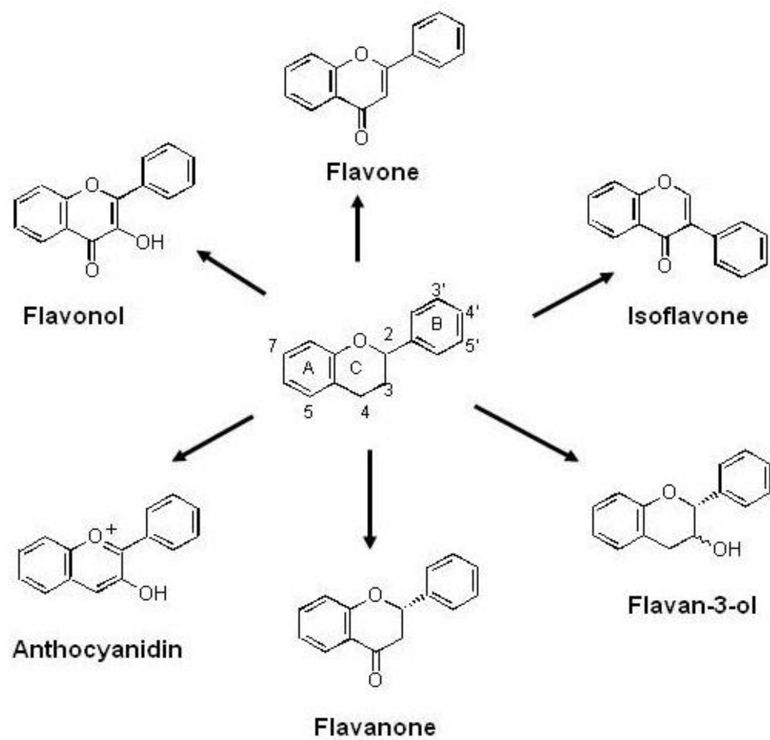


Fig. 2.5 Chemical structures of flavonoids (Ghasemzadeh and Ghasemzadeh, 2011)

Flavones are phenolic structures containing one carbonyl group (as opposed to the two carbonyls in quinones). From the report of Cowan (1999), the addition of a 3-hydroxyl group yields a flavonol. Flavonoids are also hydroxylated phenolic substances but occur as a C_6 –C_3 unit linked to an aromatic ring. Since they are known to be synthesized by plants in response to microbial infection, it should not be surprising that they have been found in vitro to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with bacterial cell walls, as described for quinones. More lipophilic flavonoids may also disrupt microbial membranes (Cowan, 1999). Catechin, the most reduced form of the C_3 unit in Flavonoid compounds, deserve special mention. These flavonoids have been extensively researched due to their occurrence in oolong green teas. It was observed some time ago that these teas exerted antimicrobial activity and that they contain a mixture of catechin compounds. These compounds inhibited *Vibrio cholerae* 01 in vitro, *Streptococcus mutans*, *Shigella* and other bacteria and microorganisms (Cowan, 1999).

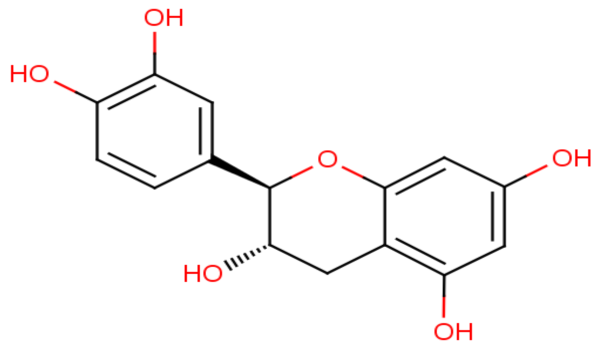


Fig. 2.6 Chemical structure of catechin (Maoela *et al*., 2009)

The catechins inactivated cholera toxin in *Vibrio* and inhibited isolated bacterial glucosyltransferases in *Streptococcus mutans*, possibly due to complexing activities described for quinones. This latter activity was borne out in *in vivo* tests of conventional rats. When the rats were fed on a diet containing 0.1% tea catechins, fissure caries (caused by *Streptococcus mutans*) was reduced by 40% (Cowan, 1999). According to Khullah (2010), flavonoid compounds exhibit inhibitory effects against multiple viruses. Numerous studies have documented the effectiveness of flavonoids such as swertifrancheside, glycyrrhizin (from licorice), and chrysin against HIV (Khullah, 2010). More than one study has found that flavone derivatives are inhibitory to respiratory syncytial virus (RSV). The average western daily diet contain approximately one gram of mixed flavonoids; pharmacologically active concentrations are not likely to be harmful to human host (Cowan, 1999). An isoflavone, found in a West Africa legume, alpinumisoflavone, prevents schistosomal infection when applied topically (Cowan, 1999). Phloretin, found in certain serovars of apples, may have activity against a variety of microorganisms. Galangin (3,5,7-trihydroxyflavone), derived from the perennial herb *Helichrysum aureonitens*, seems to be a particularly useful compound, since it has shown

activity against a wide range of gram-positive bacteria as well as fungi and viruses, in particular HSV-1 and coxsackie B virus type 1 (Cowan, 1999).

Delineation of the possible mechanism of action of flavones and flavonoids is hampered by conflicting findings. Sharafati-Chaleshtori *et al*. (2010) reported that flavonoids lacking hydroxyl groups on their β-rings are more active against microorganisms than are those with the – OH groups; this finding supports the idea that their microbial target is the membrane (Sharafati-Chaleshtori *et al*., 2010). Lipophilic compounds would be more disruptive of this structure. However, several authors have also found the opposite effect; i.e. the more the hydroxylation, the greater the antimicrobial activity. This latter finding reflects the similar result for simple phenolics. It is safe to say that there is no clear predictability for the degree of hydroxylation and toxicity of microorganisms (Cowan, 1999).

2.1.1.4 *Tannins*

Tannins are another major group of polyphenols in our diets and usually subdivided into two groups: Hydrolysable tannins and condensed tannins (Dai and Mumper, 2010). Tannins consist mainly of gallic acid residues that are linked to glucose *via* glycosidic bonds (Legesse and Emire, 2012). Tannin is a general descriptive name for a group of polymeric phenolic substance capable of tanning leather or precipitating gelation from solution, a property known as astringency. Their molecular weights range from 500 to 3,000 unit and are found in almost every plant part: bark, wood, leaves, fruits and roots (Samy and Gopalakrishnakone, 2010). Hydrolysable tannins are based on gallic acid, usually as multiple esters with D-glucose; while the more numerous condensed tannins (often called proanthocyanidins) are derived from flavonoid monomers.

Tannins may be formed by condensations of flavan derivatives which have been transported to woody tissues of plants. Alternatively, tannins may be formed by polymerization of quinone units. This group of compounds has received a great deal of attention in recent years, since it was suggested that the consumption of tannin-containing beverages, especially green teas and red wines can cure or prevent a variety of illness (Cowan, 1999). They are known to possess general antimicrobial and antioxidant activities (Sermakkani and Thangapandian, 2010).

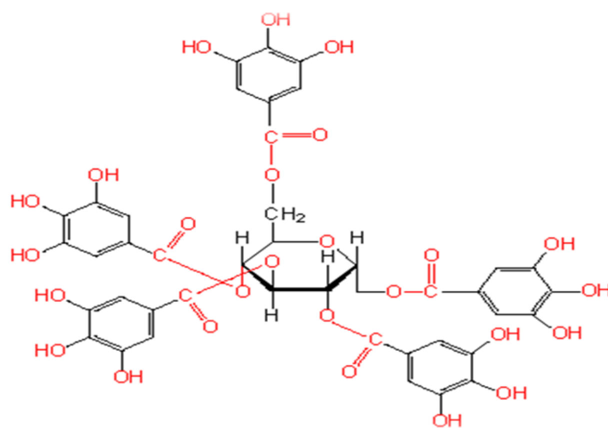


Fig. 2.7 General structure of tannin (Gunduz *et al*., 2011)

Mode of antimicrobial action of tannins, as described for quinones, may be related to their ability to inactivate microbial adhesives, enzymes, cell envelope transport proteins, etc they also complex with polysaccharides. The antimicrobial significance of this particular activity has not been explored. There is also evidence for direct inactivation of microorganisms: low tannin concentrations modify the morphology of germ tubes of *Crinipellis perniciosa*. Tannins in plants inhibit insect growth and disrupt digestive events in ruminant animals (Cowan, 1999). According to previous studies, tannins can be toxic to filamentous fungi, yeast and bacteria and condensed tannins have been determined to bind cell walls of ruminant bacteria, preventing growth and

protease activity (Cowan, 1999). Despite their wide distribution, the health effects of dietary polyphenols have come to the attention of nutritionists only in recent years. Researchers and food manufacturers have become more interested in polyphenols due to their potent antioxidant properties, their abundance in the diet, and their credible effects in the prevention of various oxidative stress associated diseases. The preventive effects of these plant metabolites in terms of cardiovascular, neurodegenerative diseases and cancer are deduced from epidemiologic data as well as *in vitro* and *in vivo* results in respective nutritional recommendations (Dai and Mumper, 2010). Furthermore, polyphenols were found to modulate the activity of a wide range of enzymes and cell receptors. In this way, in addition to having antioxidant properties, polyphenols have several other specific biological actions in preventing and or treating diseases (Dai and Mumper, 2010).

2.1.1.5 *Terpenoids and essential oils*

Among the antimicrobial extracts of plants, essential oils receive particular attention because of the ease of extraction (Karou *et al*., 2007). The fragrance of plants is carried in the so called *Quinta essentia*, or essential oil fractions. These oils are secondary metabolites that are highly enriched in compounds based on an isoprene structures. They are called terpenes, their general chemical structure is $C_{10}H_{16}$, and they occur as diterpenes, triterpenes and tetraterpenes (C_{20}, C_{30} and C_{40}), as well as hemiterpenes (C_5) and sesquiterpenes (C_{15}). When the compounds contain additional elements, usually oxygen, they are termed terpenoids (Cowan, 1999). Terpenoids are synthesized from acetate unit and as such they share their origins with fatty acids. They differ from fatty acids in that they contain extensive branching and are cyclized. Examples of common terpenoids are menthol and camphor (monoterpenes), also farnesol and artemisin (sesquiterpenoids) (Cowan, 1999). Menthol is a flavour additive widely used in consumer and

medicinal products.It can be natural or synthetic and has a minty taste and aroma, and may have cooling, analgesic or irritating properties. Menthol is an active ingredient in certain medicinal products, such as cough drops and when used in medicinal products, it is regulated as a drug (Menthol, 2013).

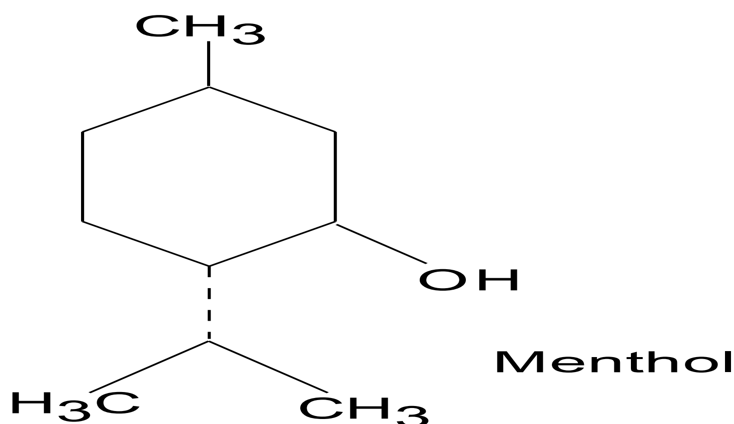


Fig. 2.8 Chemical structure of Menthol (Menthol, 2013)

It is a naturally occurring chemical chiefly derived from the peppermint plant (*Mentha piperita*) or the corn mint (*Mentha arvensis*) but it can also be synthetically produced. Menthol increases blood flow at the site of application, which may also contribute to local aanalgesia. Menthol's other attributes include antibacterial and antifungal properties and the ability to enhance topical drugs and chemicals (Menthol, 2013). Artemisin and its derivative ά – arteether, also known by the name ginghaosu, find current use as antimalarials. In 1985, the steering committee of the scientific working group of the World Health Organization decided to develop the latter drug as a treatment for cerebral malaria (Cowan, 1999).

Food scientists have found the terpenoids present in essential oils of plants to be useful in the control of *Listeria monocytogenes* (Jamine *et al*. 2007). Oil of basil, a commercially available

herbal, was found to be as effective as 125 ppm chlorine in disinfecting lettuce leaves. A terpenoid constituent, capsaicin was found to be bactericidal to *Helicobacter pylori.* Another hot-tasting diterpene aframodial, from a Cameroonian spice, is a broad-spectrum antifungal (Cowan, 1999). Neem seed (*Azadirachta indicia)* oil extract has been found to show both antibacterial and antifungal effects on food spoilage isolates due to the presence of phenolic compounds and essential oils. The seed oil disrupt cell membrane synthesis in little concentrations hence its application in medical, agricultural and household products (Idise, 2007).

2.1.1.6 *Alkaloids*

Alkaloids are a group of nitrogen-containing bases. Most of them are drugs, only a few (like caffeine) are derived from purines or pyrimidines, while the large majority is produced from amino acids. The amino acid, tyrosine is the starting product of a large family of alkaloids. The first important intermediate is dopamine which is the starting product of the biosynthesis of berberine, papaverine and morphine too (Sengbusch, 2008).

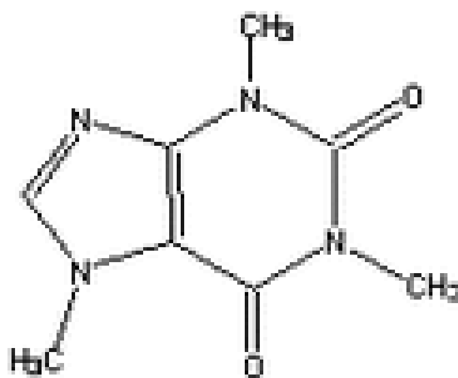


Fig. 2.9 Chemical structure of Caffeine (Helmenstine, 2013)

Alkaloids are heterocyclic nitrogen compounds. The first medically useful example of an alkaloid was morphine isolated in 1805 from the opium poppy *Papaver somniferum*; the name

morphine comes from the Greek Morpheus, god of dreams. Codeine and heroin are both derivatives of morphine (Cowan, 1999).

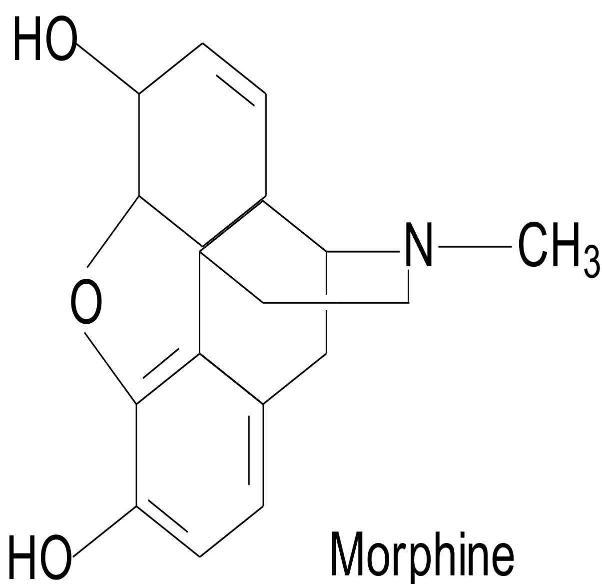


Fig. 2.10 Structure of Morphine
www.sciencebase.com/images/structure_of_morphine

Diterpeniod alkaloids, commonly isolated from the plant of the Ranunculaceae, or butter cup family, are commonly found to have antimicrobial properties. Solamargine, a glycoalkaloid from the berries of *Solanum khasianum*, and other alkaloids may be useful in HIV infections associated with AIDS (Cowan, 1999). While alkaloids have been found to have microbiocidal effects (including against *Giardia* and *Entamoeba* species), the major anti-diarrhoeal effect is probably due to their effects on transit time in the small intestine (Cowan, 1999). Furthermore, Berberine is an important representative of the alkaloid group. It is potentially effective against trypanosomes and plasmodia. The mechanism of action of alkaloids such as berberine and harmane is attributed to their ability to intercalate with DNA (Cowan, 1999).

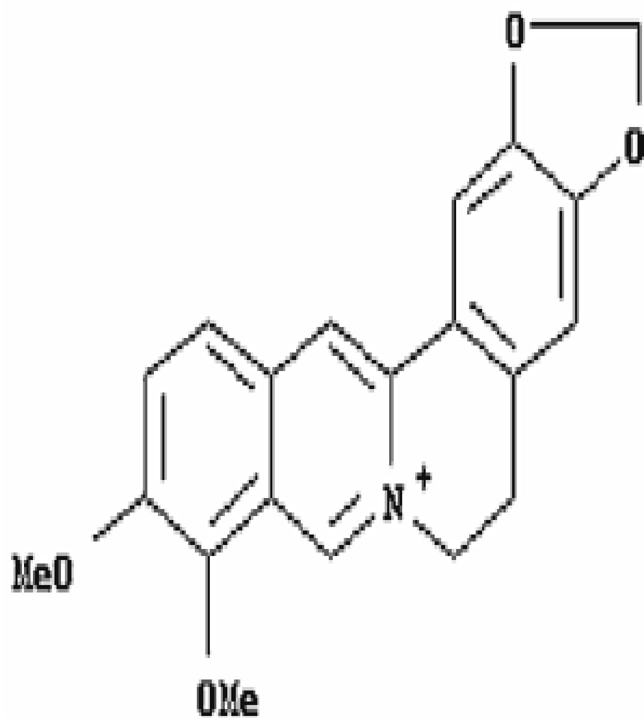


Fig. 2.11 Chemical structure of berberine (Singh *et al*., 2010)

Berberine is an isoquinoline alkaloid with a bright yellow color that is easily seen in most of the herb materials that contain any significant amount of this compound. Berberine was isolated and used as an herbal drug in China 50 years ago (the drug forms are usually the hydrochloride or sulfate; the chloride, as used in the dye, may have the strongest antiseptic action). It has since become an ingredient in several Western herbal products, particularly for treatment of intestinal infections (Dharmananda, 2005). Tests of the antiseptic action of berberine against bacteria, yeasts, viruses, and amoebas have shown a range of activity levels from apparent potent action to mild suppression. Inhibition of *Giardia* and of *Candida* have been areas of considerable interest and initial positive research results have led to development of several herb products for those applications (Dharmananda, 2005).

The chewing stick is widely used in African countries as an oral hygiene aid (in place of a toothbrush). Chewing sticks come from different species of plants, and within one stick the chemically active component may be heterogeneous. Crude extracts of one species used for this purpose, *Serindeia werneckei*, inhibited the periodontal pathogens *Porphyromonas gingivalis* and *Bacteroides melaninogenicus in vitro*. The active component of the Nigerian chewing stick (*Fagara zanthoxyloides*) was found to consist of various alkaloids. Whether these compounds long utilized in developing countries, might find use in the Western world is not yet known (Cowan, 1999).

2.1.1.7 *Saponins*

Saponins are natural detergents found in many plants. They have detergent or surfactant properties by reason that they contain both water-soluble and fat-soluble components. Certain desert plants are especially rich in saponins (Vaclavkova and Beckova, 2008). According to Hassan *et al*. (2010), they are glycoside compounds whose chemical structures are composed of a fat soluble nucleus (aglycone) that is either a triterpenoid (C-30) or neutral or alkaloid steroid (C-27) attached to one or more side chains of water soluble sugars (glycone) through ester linkages to the aglycone nucleus at different carbon site. Variability of saponin aglycone side chains in terms of number, chemical composition, and specific point of attachment to the steroid or triterpenoid nucleus is critical to saponins biological effects. Hassan *et al*. (2010) reported that the mode of action of antibacterial activity of saponins against both gram-negative and gram-positive bacteria is not yet clear. Some researchers noted that the aglycone part of the saponin is the antibacterial determination suggesting that the sugar moiety is not important for the antimicrobial efficacy while another study reported that saponins hydrolysed by bacterial enzymes to its corresponding aglycone resulted in decreased antibacterial activity (Hassan *et al*.,

2010). The antifungal and antibacterial properties of saponins are important in cosmestic applications, in addition to their emollient effects (Aghel *et al*., 2006).

2.1.1.8 *Steroids*

Plant steroids are known to be important for their cardiotonic activities, possess insecticidal and antimicrobial properties. They are also in nutrition, herbal medicine and cosmetics (Sermakkani and Thangapandian, 2010).

2.1.2 Solvent extraction of medicinal plants

The extraction of bioactive compounds from plant materials is the first step in the utilization of phytochemicals in the preparation of dietary supplements or nutraceuticals, food ingredients, pharmaceutical, and cosmetic products (Dai and Mumper, 2010). Initial screenings of plants for possible antimicrobial activities typically begin by using crude aqueous or alcohol extraction can be followed by various organic extraction methods. Since nearly all of the identified components from plants active against microorganisms are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol extraction. In fact, many studies avoid the use of aqueous fractionation altogether. The exceptional water-soluble compounds, such as polysaccharides (e.g., starch) and polypeptides, including fabatin and various lectins, are commonly more effective as inhibitors of pathogen usually virus adsorption and would not be identified in the screening techniques commonly used. Occasionally tannins and terpenoids will be found in the aqueous phase, but they are more often obtained by treatment with less polar solvents (Cowan, 2009). Solvent extractions are the most commonly used procedures to prepare extracts from plant materials due to their ease of use, efficiency, and wide applicability (Dai and Mumper, 2010). Scientists generally avoid using water extraction, which is the method used by

traditional healers in most cases, because of the complexity and difficulty involved in developing a suitable workup procedure with aqueous extracts. Organic solvent extractions are therefore used as a good alternative in evaluating the antimicrobial activities of plants. To this end, alcohol or aqueous alcohol, in any case, is a good all-purpose solvent for preliminary extractions in a screening program. Particularly, methyl or ethyl alcohol has the ability to extract a broad spectrum of chemical substances (Rasoanaivo *et al*., 2004).

In the single-solvent extraction procedure, the plant material is subjected to extraction exhaustively, by repeated maceration with alcohol or aqueous alcohol at room temperature. The alcohol fraction in the combined extracts is evaporated off under reduced pressure at a temperature not exceeding 45^{0}C, and the residual water extract is freeze dried or evaporated to dryness by azeotropic methods by repeatedly adding 95% ethanol to the residual water until this water is completely removed. As a general rule in some laboratories, approximately 25 g of dried plant material is used for extraction in the primary screening (Rasoanaivo *et al*., 2004). Solvents, such as methanol, ethanol, acetone, ethyl acetate, and their combinations have been used for the extraction of phenolics from plant materials, often with different proportions of water. Selecting the right solvent affects the amount and rate of polyphenols extracted. In particular, methanol has been generally found to be more efficient in extraction of lower molecular weight polyphenols while the higher molecular weight flavanols are better extracted with aqueous acetone. Ethanol is another good solvent for polyphenol extraction and is safe for human consumption (Dai and Mumper, 2010).

Extraction with alcohol in a soxhlet apparatus has been reported for various parts of medicinal plants, but some scientists avoid the used of this technique because extracts are continuously boiled with the solvent for several hours, which may alter labile constituents (Rasoanaivo *et al*., 2004). Successive extractions with solvents in increasing order of polarity are also a useful practice followed in several laboratories. In this procedure, plant material is defatted with petroleum ether, cyclohexane or heptane, the use of heptane being avoided because of its toxicity and flammability. The residual powdered plant is then extracted, preferably, with ethyl acetate because of its lower toxicity compared to chlorinated hydrocarbon solvents or alternatively with dichloromethane or chloroform. Thereafter, the residue is extracted with methanol or ethanol and finally with water. The procedure is based on the old Roman principle of solubility: *similia similibus solvuntur* (the similar dissolves the similar). Scientifically speaking, nonpolar solvents dissolve selectively nonpolar compounds; polar solvents dissolve preferably polar compounds. A reasonable alternative is to shorten the procedure by using only one nonpolar solvent (ethylacetate) and one polar solvent (methanol or water) (Rasoanaivo *et al*., 2004).

2.1.3 *Carica papaya*

Carica Papaya L., more commonly known as the papaya, belongs to the Caricaceae. Its classification is as follows: Division: Magnoliophyta, class: Magnoliopside, subclass: Dilleniidae, Order: violales and as previously mentioned, Family: Caricaceae. It was first described by the Spanish chronicler Oviedo in 1526, from the Caribbean coast of Panama and Colombia (Dawson, 2007). *Carica papaya* is a fruit also called papaya, papaw, pawpaw, and mamao or tree melon. It is found in virtually every tropical and subtropical country and soon after it was grown throughout the tropics, its distribution was being aided by the abundance of its seeds (Dawson, 2007; Okeniyi *et al*., 2007).

The papaya seed is viable for up to three years under cool, dry conditions and it is a herbaceous, dicotyledonous plant that may produce fruits for more than twenty years. The plant usually has a single trunk with several well developed branches. The melon- like fruit varies in size and shape, and hangs from short, thick peduncles at the leaf axil. Its flowers are mostly dioecious and resemble each other until they start to develop sexual organs. The species is polygamous and can be classified into three sex types: male staminate, hermaphroditic (bisexual) and female pistillate. In addition, some plants can produce more than one kind of flowers. The fruits which are orange-yellow when ripen, are popular breakfast staple that also used in jellies, preserves, fruit juices and as a beverage in certain Latin countries. In addition, the leaves and roots of the plants are also used in variety of dishes. The bark can also be used for rope making and the leaves as a soap substitute, being an excellent stain remover (Dawson, 2007). In Java, even the flowers are eaten (Dawson, 2007).

Papain obtained from pawpaw is used to treat commercial beer, to degumm natural silk, and in the production of chewing gums. Cosmetically, papain is used in shampoos and in a number of face-lifting operations (Dawson, 2007). Dawson (2007) also stated that papaya can be used as diuretic (the roots and leaves), antihelmintic (the leaves and seeds) and to treat bious conditions (the fruit). Parts of the plant are also used to combat dyspepsia and other digestive disorders and a liquid portion has been used to reduce enlarged tonsils (Dawson, 2007). In addition, pawpaw juice is used against warts, cancers, tumors, corns and skin defects while the root is said to help tumors of the uterus (Dawson, 2007). In Africa, a root infusion of papaya is also used for syphilis

and the leaf is smoked to relieve asthma attacks. The Javanese believes that eating papaya prevents rheumatism and in Cuba the latex is used for psoriasis, ringworm and the removal of cancerous growth (Dawson, 2007). Thus, the successful use of *Carica papaya* in ethno-medicine offers cheap, natural, harmless, readily available monotherapy and preventive strategy against several diseases, especially in tropical communities. Further and large-scale intervention studies to compare *C. papaya* with standard antimicrobial preparations are desirous (Okeniyi *et al.*, 2007).

2.2 Food Safety

Food safety and security all over the world have no substitute. This essential commodity of life is continuously threatened by spoilage through adverse changes caused by the presence of enzymes, oxygen, light, loss of moisture, or most importantly, the action of microorganisms and their enzymes (Shide and Whong, 2003). Food safety can be defined as assurance that food will not cause harm to the consumer when it is prepared and \ or eaten according to its intended use (NAFDAC, 2004). For food producers and manufacturers the guiding principle in hygiene is the exclusion or elimination of pathogens or the reduction of contamination to safe ('acceptable') levels (Singleton, 1997). Microbiological limits of the set guidelines for ready-to-eats foods are organized under three components (Microbiological Guidelines, 2007).

The Standard Plate Count (SPC), also referred to as the aerobic plate count or the total viable count, is one of the most common tests applied to indicate the microbiological quality of food. The significance of SPCs, however, varies markedly according to the type of food product and

the processing it has received. When SPC testing is applied on a regular basis it can be a useful means of observing trends by comparing SPC results over time (Guidelines, 2001). It is generally believed that high SPCs in foods indicate greater risks of pathogen being present in consumable products, poor implementation of sanitation procedures or problems in process controls to which a test food item has been subjected. It is generally used for descriptive evaluation of microorganisms on nonselective media under mesophilic and aerobic conditions (Avanza, 2005). It is useful for indicating the sanitary quality of food. Generally, it does not relate to food safety hazards, but is taken as a food quality parameter (Microbiological Guidelines, 2009).

Indicator organisms refer to the selected surrogate markers employed to reflect the hygienic quality of food. *E. coli* is commonly used as surrogate indicator. The native habitat for *E. coli* is the enteric tract of humans and animals. Its presence in food generally indicates direct or indirect fecal contamination. Substantial number of *E. coli* in food suggests a general lack of cleanliness in handling and improper storage. The presence of *E. coli* in foods does not connote directly the presence of a pathogen, but implies a certain risk that it may be present (Microbiological Guidelines, 2009). The presence of *E. coli* in ready-to-eat foods is undesirable because it indicates poor hygienic conditions which have led to contamination or inadequate heat treatment. Ideally *E. coli* should not be detected and as such a level of <3 per gram (the limit of the Most Probable Number test) has been given as the satisfactory criteria for this organism. Levels exceeding 100 per gram are unacceptable and indicate a level of contamination, which may have introduced pathogens or that pathogens, if present in the food prior to processing, may have survived (Guidelines, 2001).

Specific pathogens refer to bacteria that may cause food poisoning. Mechanisms involved may be toxins produced in food or intestinal infection. Nine specific bacterial pathogens included in this set of guidelines are *Campylobacter* spp*.*, *E.coli* O157, *Listeria monocytogenes, Salmonella spp., Vibrio cholerae, Vibrio parahaemolyticus, Staphylococcus aureus, Clostridium perfringes* and *Bacillus cereus.* The symptoms of food poisoning caused by these pathogens vary from nausea to vomiting (e.g. caused by *S. aureus*), through diarrhoea and dehydration (*Salmonella* spp. and *Campylobacter* spp.) to paralysis and death in the rare cases of botulism. The infectious doses vary from less than ten (10) to more than a million (10^6) organisms (Microbiological Guidelines, 2007).

2.3 Microbial Food Borne Diseases

Food borne diseases continue to be a common and serious threat to public health over the world and are a major cause of morbidity (Blackburn and McClure, 2004). They are caused by eating food or drinking beverages and/or water contaminated with bacteria, parasites or viruses (NDDIC, 2007). Both industrialized and developing countries suffer large numbers of illnesses and the incidence on a global basis appears to be increasing (Blackburn and McClure, 2004). Most food borne illnesses are mild, and associated with gastrointestinal symptoms such as diarrhoea and vomiting. Sometimes a food borne disease is much more serious and is life threatening, particularly in children in developing countries, and infection can also be followed by chronic sequalae or disability. In many countries, where information on food borne diseases are documented the total number of cases has been increasing over the past 20-30 years

(Blackburn and McClure, 2004). In recent years, the epidemiology of food borne diseases has been changing as new pathogens have emerged. "Emerging diseases" are described as those that have increased in prevalence in recent decades or are likely to do so in the near future, so it is not necessary for an emerging pathogen to be evolved. Food borne diseases that are regarded as emerging include illnesses caused by enterohaemorrhagic *Escherichia coli* [(EHEC) particularly serovar O157:H7], *Campylobacter jejuni, Salmonella typhimurium* Definitive Type (DT) 104 (Blackburn and McClure, 2004). In some cases, diseases have been associated with food vehicles only relatively recently. Examples of these pathogens include *Listeria monocytogenes, Cryptosporidium parvum* and *Cyclospora cayetanensis*. Many of these food borne pathogens have a non-human animal reservoir, and are termed zoonoses, but they do not necessarily cause diseases in the animal. Previously, animal or carcass inspection was used as a method of preventing zoonotic diseases being transferred through food, but this can no longer be relied upon (Blackburn and McClure, 2004).

2.3.1 Indicators of bacterial food pathogens

All pathogenic microorganisms indicated in food borne diseases are considered enteric pathogens, except *Staphylococcus aureus, Bacillus cereus, Clostridium botulinum* (except in the case of infant botulism), *Cl. perfringens,* and toxicogenic molds. This means they can survive and multiply or establish in the gastrointestinal (GI) tract of humans, food animals and birds. A food contaminated directly or indirectly with faecal materials from these sources may theoretically contain one or more pathogens and can thus be potentially hazardous to consumers. To implement regulatory requirements and ensure consumer safety, it is necessary to know that a food is either free of some enteric pathogens, such as *Salmonella* serovars and *Escherichia coli*

0157:H7, or contains low levels of some other enteric pathogens, such as *Yersinia enterolitica* and *Vibro parahaemolyticus* (Ray, 2004).

Food samples are examined for the number (or level) of groups or a series of bacteria that are of faecal or enteric origin, usually present in higher density than pathogens, but usually considered to be non-pathogenic. Their presence is viewed as resulting from direct or indirect contamination of a food with faecal materials and indicates the possible presence of enteric pathogens in the food. These bacterial groups of species are termed indicators of enteric pathogens. Although, *Staphylococcus aureus*, *Costridium botulinum, Cl. perfringens* and *Bacillus cereus* can be present in faecal matters of humans and food animals, they, along with toxicogenic molds are not considered classical enteric pathogens. Their presence in a food is not normally considered to be because of faecal contamination, and the indicators of enteric pathogens are not very effective for the purpose (Ray, 2004).

2.3.1.1 *Coliforms*

The term 'coliform' does not have taxonomic value; rather, it represents a group of species from several genera namely, *Escherichia*, *Enterobacter*, *Klebsiella* and *Citrobacter* (Ray, 2004). They are all Gram negative non-spore forming rod-shaped bacteria, aerobic and facultative anaerobic organisms that ferment lactose in 24-48 hr at 35°C (Okpokwasili, 2007). Some species grow at higher temperature (44.5^{o}C), whereas others can grow at 4-5°C. All are able to grow in foods except in those that are at pH$\leq$0.92. All are sensitive to low-heat treatment and are killed by pasteurization (Ray, 2004). Coliforms were historically used as indicator microorganisms to serve as a measure of faecal contamination, and thus potentially of the presence of enteric

pathogens in foods (Cakir *et al.,* 2002). Although with some disadvantages, coliforms are probably the most useful and most extensively used indicators (Ray, 2004).

2.3.1.2 *Faecal coliforms*

Faecal coliforms also constitute a group of bacteria and include those coliforms whose specificity as faecal contaminants is much higher than that of other coliforms. This group includes mostly *E.coli,* along with some *Klebsiella* and *Enterobacter* spp. Non-faecal coliforms are eliminated by using a high incubation temperature (44.5 ± 0.2 or $45.0 \pm 0.2^{o}C$) for 24 hr in selective broth containing lactose. Lactose fermentation with the production of gas is considered a presumptive test (Ray, 2004). Faecal coliforms are therefore defined as bacteria which in the presence of bile salts or other equivalent selective agents, can grow and produce acid and gas from lactose when incubated at 44-45.5^{o}C (Harrigan and McCance, 1976). In heated and ready-to-eat products, presence of faecal coliforms especially above a certain level is viewed cautiously for possible faecal contamination and presence of enteric pathogens. A food can be accepted or rejected based on the numbers present. This group is extensively used as an indicator in foods of marine origin (shellfish) and wastewater (Ray, 2004).

2.3.1.3 *Escherichia coli*

In contrast to either coliforms or faecal coliforms, *E.coli* has a taxonomic basis. It includes only the *Escherichia* spp. of the coliform and faecal coliform groups. *E.coli* strains conform to the general characteristics described for coliform groups. Following a great deal of the work on the phenotypic characteristics of the bacteria, by the 1960s, the genus *Escherichia* was described as: Gram negative, non-sporing rods; often motile, with peritrichate flagella. It is easy to cultivate on ordinary laboratory media, aerobic and facultative anaerobic. According to Donnenberg and

Nataro (2000) all species ferment glucose with the formation of acid or and gas, both aerobically and anaerobically. All reduce nitrate to nitrite and are oxidase negative but catalase positive. Typically, they are intestinal parasites of humans and animals, though some species may occur in other parts of the body, on plants and in the soil (Donnenberg and Nataro, 2000).

Biochemically, they are differentiated from other coliforms by the indole production from tryptone, methyl red reduction due to acid production (red colouration), Voges Proskauer reaction (production of acetyl-methyl carbinol from glucose) and citrate utilization as a sole carbon-source (IMViC) reaction patterns. *E. coli* Type 1 and Type 2 give IMViC reaction patterns respectively, of ++-- and -+--. The -+-- reaction pattern of *E.coli* type 2 could also be due to slow or low production of indole from tryptone (or peptone) (Ray, 2004). By the mid-1940s, a serogrouping scheme was developed that allowed *E.coli* to be divided into more than 170 different serogroups based on their somatic (O) antigen. In addition, over 50 flagella (H) antigens and approximately 100 capsular (K) antigens are now also recognized and these are used to further subdivide *E. coli* into serotypes. Serogroupings and serotyping, together with other information such as biotype, phage type and enterotoxin production, now facilitates distinction between those strains able to cause infectious diseases in humans and animals. Some correlation has been established between the *E.coli* serogroup and virulence (Bell and Kyriakides, 2004). *E.coli* has been found associated with diarrhoea (particularly in children), haemorrhagic colitis, dysentery, bladder and kidney infections, surgical wound infection, septicaemia, haemolytic uraemic syndrome, pneumonia and meningitis; some of these conditions result in death (Bell and Kyriakides, 2004). *E.coli* may be the most versatile of human pathogens (Donnenberg and Nataro, 2000). Strains that share virulence features with more than one group

have been identified and will likely be recognized with increasing frequency. The species is not static, but is constantly evolving in leaps and bounds by the acquisition of new genetic determinants. Organized by pathotype, six categories of diarrhoeagenic *E.coli* and two *E.coli* that cause extraintestinal infections are identified (Donnenberg and Nataro, 2000).

E. coli, particularly serotype O157:H7 has become an important food borne pathogen responsible for gastroenteritis epidermics in North America, Europe, Asia and Africa. The most frequently implicated foods have been under cooked contaminated ground beef, raw milk, unpasteurised cider and apple juice, bean sprouts or fresh leafy vegetables such as lettuce and spinach (Hosein *et al*., 2008). The increased consumption of Ready-to-eat foods coupled with the associated risk of disease to which consumers may be exposed, is a matter of great concern. It is difficult for one to attest to the hygiene of the processors or to the sanitary conditions at points of preparation (Oranusi and Olorunfemi, 2011).

2.3.1.4 *Non-Escherichia coli coliforms*

Coliform groups include species from genera *Escherichia*, *Klebsiella*, *Enterobacter* and *Citrobacter,* all belonging to the family *Enterobacteriaceae* and thus sharing some common characteristics. Previously, *E.coli* strains (both pathogenic and nonpathogenic) were thought to mainly inhabit the intestinal tract of humans and warm blooded animals and birds, and most species in the other three genera were thought to be mainly of non- intestinal origin. However, other studies have shown that species and strains of *Klebsiella*, *Enterobacter* and *Citrobacter* (together referred to as non *E. coli* coliforms) can colonize the human gut and produce potent enterotoxins. In several acute and chronic cases of diarrhoea, they were isolated from stools and

the intestinal tract. Some isolates of *Enterobacter cloacae*, *Klebsiella pneumonia* and *Citrobacter* spp. were found to produce enterotoxins similar to heat- labile or heat-stable toxins of enterotoxigenic *E.coli* strains probably due to the intergeneric transfer of plasmids encoding these phenotypes (Ray, 2004). Non *E.coli* coliforms are normally present in raw food materials as well as in some pasteurized foods because of post-heat contamination. They can grow in many foods if the growth parameters are not limiting. Some strains can grow at refrigerated temperature. Temperature abuse during storage can also facilitate their rapid growth in a food. The significance of their presence in a food may need to be reevaluated (Ray, 2004).

2.4 Ready-to-eat Foods

A ready-to-eat food is food in a form that is edible without washing, cooking or additional preparation by the food establishment or the consumer, and is consumed in the ordinary state (Microbiological Examination, 2010). Furthermore, ready-to-eat is defined as the status of the food being ready for immediate consumption at the point of sale. It could be raw or cooked, hot or chilled, and can be consumed without further heat-treatment including re-heating (Microbiology Guidelines, 2007). It can also be defined as food that

is ordinarily consumed in the same state as that in which it is sold or distributed and does not include nuts in the shell, raw fruits and vegetables that are intended for hulling, peeling or washing by the consumer (Guidelines, 2001).

The presence of enteric bacteria in ready-to-eat foods provides undeniable evidence of the poor microbiological quality of the foods in different countries (Owoseni and Onilude, 2011). Contamination of food by enteric pathogens can occur from the farm if human sewage is used to fertilize the soils or if sewage water is used to irrigate the crops. Such risks are further increased if the food is mishandled during processing and preparations where pathogens could multiply exponentially under favourable conditions (Nyenje *et al*., 2012). It is mandatory that foods must be free from contaminants as much as possible. The presence of *E.coli*, *S. aureus* and *B.cereus* demonstrates a potential health risk as these organisms are pathogenic and have been implicated in food borne diseases. Foodborne illness can be prevented by good hygiene practices (Oranusi *et al*., 2013). The category ready-to-eat can be considered as high risk foods because they do not require any heating or process prior to consumption. In addition, food workers may transmit pathogens to food from a contaminated surface, from another food, or from hands contaminated with organisms from their gastrointestinal tract. Therefore, hand contact with ready-to-eat foods represents a potentially important mechanism by which pathogens may enter the food supply (Jacob, 2010). Inadequate refrigeration and/or sanitation that prepared ready-to-eat foods experience might create conditions under which any existing bacteria may flourish, especially if lack of proper handling practices occurs. Small corner markets in high poverty areas may be very

limited in their resources available to train employees in safe food handling and guarantee the safest food supply to consumers (Jacob, 2010). The use of sensitive, quatitative methods for the detection of food borne pathogens during food processing could be used to determine points in the food production process where contamination occurs and where controls could be introduced to reduce or eliminate enterobacteria from readt-to-eat food products, thereby reducing risk to the consumer (Owoseni and Onilude, 2011).

2.4.1 Processed meat ('suya')

Processed meat products are defined as those in which the properties of fresh meat must have been modified by the use of one or more procedures such as grinding, addition of seasoning agents, alteration of color or heat treatment (Abdullahi *et al.*, 2005). Processed meat in Nigeria includes 'Tsire' or 'Suya', 'Kilishi' and 'Balangwu'.

'Suya' is a popular Nigerian traditional processed ready-to-eat smoked meat product. It is served or sold in public places, along streets, in club houses, restaurants, picnics and homes. It is prepared from boneless meat of animals such as mutton, beef or goat. The meat is trimmed from associated connective tissues, nerves and vessels. The meat is artfully sliced into very thin continuous sheets which are then cut into pieces. The pieces of meat are staked on sticks, spiced with groundnut powder/flour, salt, vegetable oil and flavorings such as monosodium glutamate or others. The sticks are then arranged round the fire place for the meat to roast, the duration of roasting depend on such factors as fire intensity and pressure from consumers. The traditional smoking of 'suya' is usually done by wood smoke (Inyang *et al*., 2005). The prepared 'suya' when being sold are usually packaged in newspapers and sometimes in cellophane or nylon bags

(Uzeh *et al*., 2006). Much of ready-to-eat foods, including processed meat, have been associated with gastroenteritis of *E. coli* origin in many countries. Direct and indirect contamination of these foods with fecal materials, along with improper storage temperature and inadequate heat treatment, were involved in these incidences (Ray, 2004). Most of the stages of 'suya' preparation, materials used and the surrounding environment can serve as sources of contaminants to the meat product (Uzeh *et al*., 2006).

2.4.2 Smoked fish

Fish is endowed with one of the cheapest sources of protein (Whong *et al*., 2003). It competes favourably with those of eggs, milk and meat in its amino acid composition. In fact, it often has higher levels of essential lysine and methionine, both of which are lacking in tuber-based or cereal based diet. This makes fish protein particularly valuable in many countries where the staple diet consists of starchy foods like cassava, yam, rice, sorghum and millet. The lack of livestock and fresh meat products in many parts of Nigeria makes fish protein all the more essential (Daniel *et al*., 2006). Fish is however highly perishable if not properly processed and preserved because of its high protein and fat content (Whong *et al*., 2003). It has been reported that 40% of the total fish catch in Nigeria is lost annually due to improper storage and inadequate preservation infrastructure. Hence, fish storage stability remains a serious factor affecting fish supply in Nigeria (Adebayo *et al*., 2003).

Traditional fish smoking is one method of preserving fish against spoilage. It involves gutting, cutting and then smoke-drying. Smoking remains an important means of preservation in most developing countries, as it requires low capital investment. Common methods of fish smoking involve the use of conical or rectangular mud or drum kilns, and pot oven. (Adebayo *et al*.,

2003). First of all, fish are smoked to obtain a product of high sensory virtues not containing microorganisms (Sobota *et al*., 2006). Smoke contributes a pleasant and agreeable flavor to fish and its chemical constituents, which include formic acid, acetic acid, propionic acid, phenol, cresol, isobutyl alcohol, formaldehyde and acetaldehyde, confirm it as a preservative. These organic compounds act as bacteriostatic, bactericidal and antioxidant chemicals in fish (Adebayo *et al*., 2003; Whong *et al*., 2003).

In spite of its acceptability, there is need to determine the shelf life of smoked fish and the nutritional change during storage. Nutritional and microbiological qualities are important considerations for all forms of food processing. When fish are smoked, the duration of storage poses a great challenge to product quality. Information on the storage period, within which the fish will still retain its nutritional qualities, is invaluable to the processors and consumers of the smoke fish products (Adebayo *et al.,* 2003). Smoked dried fish are liable to water absorption and during raining season such a situation is inevitable. Also, smoked fish containing moisture content greater than 10% will be liable to microbial spoilage, shorter shelf-life, off-odor and flavor. Such fish when consumed directly may result in food poisoning (Whong *et al*., 2003). Most critical are the hygienic conditions for handling fish after smoking (Novotny *et al*., 2004).

2.4.3 'Zoborodo'

'Zoborodo' drink is extracted from the dry calyces of Roselle plant (*Hibiscus sabdariffa*), a member of the family Malvaceae. It is usually prepared as a sorrel drink. The product has today become popular, with much acceptance in Nigeria and commonly referred to as 'zobo' (Abdullahi and Elegbe, 2001; Ayo *et al*., 2004). 'Zobo' drink is prepared by boiling the dry

calyces of *Hibiscus sabdariffa* in water for about 10-15 min from which the pigment or flavour embedded is extracted. After extraction the filtrate may be taken hot as tea or allowed to cool and packaged in plastic sachet containers then taken as a refreshing drink when chilled. The sharp sour taste of the raw extract is usually sweetened with sugar cane or granulated sugar, pineapple, orange or other fruits depending on choice. The sweetness of ‘zobo’ drink does not last long due spoilage by microbial activities. There is increase in the demand for ‘zobo’ drinks due to its low prices, nutritional and medicinal properties (Nwachukwu *et al.,* 2007). Economically, zobo is cheap and has been shown to be good source of natural carbohydrates, protein and vitamin C which constitute a major reason for consuming the soft drink by the increasing population (Braide *et al*., 2012). The greatest limitation for large-scale production of ‘zobo’ drinks is the rapid deterioration of the drink. Its shell-life is approximately twenty-four hours following production if not refrigerated. Microorganisms associated with the dried calyx and the processing for the production of ‘zobo’ drinks and other factors may contribute to its spoilage (Nwachukwu *et al.,* 2007). ‘Zoborodo’ is normally packed in sachet form or bottle and presented to the consuming public at refrigeration temperature. Production and sale of ‘zoborodo’ is still at the local level. The low sanitary practice during production and sales accounts for the poor quality and a cause for concern (Ayo *et al*., 2004). The production process of ‘zobo’ however, is neither standardized nor mechanized. This allows proliferation of the associated micro-organisms which potentiate spoilage and the short shelf-life associated with this sorrel beverage. Some of these organisms have been found to pose serious health risks to consumers as they are associated with food spoilage and intoxication (Braide *et al*., 2012).

2.4.4 ‘Kunun zaki’

‘Kunun zaki’ is one of the indigenous non-alcoholic beverages prepared from guinea corn (*Sorghum bicolor*), millet (*Pennisetum typhoides*), maize (*Zea mays)*, rice (*Oryza sativa*) and wheat (*Triticum aestivum*).The fermented cereal beverage is widely consumed in most parts of Northern Nigeria and beyond the Savanna region of Nigeria. It is taken at anytime of the day by both adults and children, as breakfast drink, food complement, refreshing drink for visitors , appetizer, and is commonly served in social gatherings (Umoh *et al*., 2004). ‘Kunun zaki’ is spiced with ginger, cloves, red and black pepper, sweetened with sugar, packed for sale in polythene bags, bottles, and as bulk package in large containers and distributed under ambient temperature or cold in refrigerator where available (Umoh *et al*., 2004). Like other local beverages, the traditional method of production and sales of ‘kunun zaki’ exposes it to high level of contamination by pathogenic organisms. Factors such as handling, spicing and the use of untreated water have been reported to be contributing to the unwholesomeness of the drink (Ayo *et al*., 2004).

2.5 Antibiotics

In the middle of the 20th century, the discovery of antibiotics dramatically changed tools available to cure infectious disease (Donato *et al*., 2010). An antibiotic [Greek *anti*, against, and *bios*, life], is a chemical substance produced by a microorganism that is able to kill or inhibit the growth or activity of other microorganisms (Black, 2005). They were originally discovered as secretions of fungi or soil bacteria (Antibiotics, 2010). In the 1940s, Selman Waksman, the discoverer of streptomycin, defined an antibiotic as “a chemical substance produced by microorganisms which have the capacity to inhibit the growth of bacteria and even destroy bacteria and other microorganisms in dilute solution”. In contrast, agents synthesized in the

laboratory are called synthetic drugs. Some antimicrobial agents are synthesized by chemically modifying a substance from a microorganism. Antimicrobial agents made partly by laboratory synthesis and partly by microorganisms are called semi synthetic drugs (Black, 2005). Antibiotics are selectively toxic to the microbes but not to the host (Thakur, 2006). The range of different microbes against which an antimicrobial agent act is called spectrum of activity. Those agents that are effective against a great number of microorganisms from a wide range of taxonomic groups, including, both Gram-positive and Gram-negative bacteria, are said to have a broad spectrum of activity. (Black, 2005). The mechanism of action of most antibacterial drugs were worked out after the discovery that the molecules had effects on bacterial growth, either showing growth dramatically (bacteriostatic) or killing (bactericidal). Molecules of clear therapeutic utility and potential were then examined for the molecular basis of their antibacterial properties, their selectivity, and their associated toxicity (Walsh, 2003). Five different modes of action of antimicrobials are identified: Inhibition of cell wall synthesis, disruption of cell membrane function, Inhibitors of protein synthesis, Inhibitors of nucleic acid synthesis and action as antimetabolites (Black, 2005).

2.5.1 Antibiotics that inhibit cell wall synthesis

The most celebrated of the antibiotics that kill bacteria by blocking the crucial transpeptidations that lead to mechanically strong peptidoglycan through the covalent cross-links of peptide strands are the β-lactam antibiotics (Walsh, 2003). These antibiotics such as penicillins and cephalosporins contain a chemical structure called β-lactam ring, which attaches to the enzymes that cross- link peptidoglycans. By interfering with the cross-linking of tetrapeptidases, these antibiotics prevent cell wall synthesis. Fungi and Archaea, whose cell walls lack peptidoglycan,

are unaffected by these antibiotics (Black, 2005). β-lactams account for approximately two-thirds, by weight of all antibiotics administered to humans (Lachmayr *et al*., 2009).

2.5.2 Antibiotics that disrupt cell membrane function

Five polymyxins designated A, B, C, D, and E, have been obtained from the soil bacterium *Bacillus polymyxa* (Black 2005). They have limited spectra of antimicrobial activity and significant toxicity, with a unique fatty acid component that contribute to the detergent activity (Talaro and Talaro, 2002; Yao and Moellering, 2007). Polymyxins are peptides which are active against many Gram negative bacteria (Singleton, 1997). Acting like detergents or surfactants, members of this group of antibiotics interact with the phospholipids of the bacterial cell membrane, thereby increasing cell permeability and disrupting osmotic integrity. This process results in leakage of intracellular constituents, leading to cell death (Yao and Moellering, 2007). Polymyxins B and E are the most common clinically. They are usually applied topically, often with bacitracin, to treat skin infections caused by Gram negative bacteria such as *Pseudomonas.* Used internally, polymyxins can cause numbness in the extremities, serious kidney damage, and respiratory arrest. They are administered by injection when the patient is hospitalized and kidney function can be monitored (Black 2005).

2.5.3 Antibiotics that inhibit protein synthesis

In all cells, protein synthesis requires not only the information stored in DNA, plus several kinds of RNA, but also ribosomes. Differences between bacterial (70S) and animal (80S) ribosomes allow antimicrobial agents to attack bacterial cells – that is, with selective toxicity. Aminoglycoside antibiotics, such as streptomycin, derived their name from the amino acids and

glycosidic bonds they contain. They act on the 30S portion of bacterial ribosomes by interfering with the accurate reading (translation) of the mRNA message-that is, the incorporation of the correct amino acids. Chloramphenicol and Erthromycin act on the 50S portion of bacteria ribosomes, inhibiting the formation of the growing peptide. Because animal cell ribosomes consist of 60S and 40S subunits, these antibiotics have little effect on host cells. Mitochondria, however, which have 70S ribosomes, can be affected by such drugs (Black, 2005). Other aminoglycosides, such as neomycin, kanamycin, amikacin, gentamicin, toramycin and Netilmicin, also have special uses and display varying degrees of toxicity to the kidneys and inner ear. At lower, less toxic doses, aminoglycosides tend to be bacteriostatic. They are usually administered intramuscularly or intravenously because they are poorly absorbed when given orally (Black, 2005). An important property of aminoglycosides is their ability to act synergistically with other drugs- an aminoglycoside and another drug together often control an infection better than either could alone. For example, gentamicin and penicillin or ampicillins are effective against penicillin-resistant streptococci. In other synergistic actions, gentamicin or tobramycin work with carbenicillin or ticarcillin to control *Pseudomonas* infections, especially in burn patients, and aminoglycosides work with cephalosporins to control *Klebsiella* infections. Other antibacterial agents that affect protein synthesis are the tetracyclines, chloramphenicol, macrolides and lincosamides (Black, 2005).

2.5.4 Antibiotics that inhibit nucleic acid synthesis

From among the rifamycins produced by *Streptomyces mediterranei*, only the semi synthetic rifampin is currently used. It blocks RNA transcription. Although, it is bactericidal and has a wide spectrum of activity, it is approved in the United States only for treating tuberculosis and eliminating meningococci from the nasopharynx of carriers. It is unusual among antibiotics in its

ability to interact with other drugs, and possibilities of such interactions should be considered before the drug is given (Black, 2005).

The Quinolones are a new group of synthetic bactericidal analogs of *nalidixic acid.* They are effective against many Gram-positive and Gram-negative bacteria. Quinolones' mode of action is to inhibit bacterial DNA synthesis by blocking DNA gyrase, the enzyme that unwinds the DNA double helix preparation to its replication. *Norfloxacin, Ciprofloxacin* (Cipro), and *enoxacin* are examples of this group of antibiotics. They are especially effective in the treatment of traveler's diarrhea and in urinary tract infections caused by multiple resistant organisms (Black, 2005). A recent advancement has produced a hybrid class of antibiotics. One of these, a quinolone-cephalosporin combination, is currently being tested. When the β-lactamase acts on the cephalosporin component, the quinolone is released from the hybrid molecule and is available to kill the cephalosporin-resistant organisms. The use of such a dual-acting synergistic antibiotic may also prevent or delay development of antibiotic resistance organisms (Black, 2005).

2.5.5 Antibiotics that act as antimetabolites

The sulfonamides or sulfa drugs are a large group of entirely synthetic, bacteriostatic agents. They act by blocking the synthesis of folic acid, which is needed to make the nitrogenous bases of DNA (Black, 2005). In practice, sulfa drugs are very similar to the structural metabolic compound PABA (para-aminobenzoic acid) acquired by the bacteria to synthesize the co-enzyme tetrahydrofolic acid, which participates in the synthesis of purines and certain amino acids. A sulfonamide molecule has high affinity for the PABA site on the enzyme and can

successfully compete in a ‘chemical race’ with PABA to occupy those sites. This ultimately causes an inadequate supply of tetrahydrofolic acid for purine production, which invariably halts nucleic acid synthesis and prevents bacterial cells from multiplying (Talaro and Talaro, 2002). Sulfonamides have been characterized as broad-spectrum antibiotics with a bacteriostatic mode of action based on inhibition of folic acid metabolism (Olliver *et al*., 2010). Sulfonamides have now been largely replaced by antibiotics because antibiotics are more specific in their actions and less toxic than sulfonamides. When sulfonamides first came into use in the 1930s, they frequently lead to kidney damage. Newer forms of these drugs usually do not damage kidneys, but they do occasionally produce nausea and skin rashes. Certain sulfonamides are still used to suppress intestinal micro flora prior to colon surgery. They are also used to treat some kinds of meningitis because they enter cerebrospinal fluid more easily than do antibiotics. *Cotrimoxazole* (Septra), a combination of sulfamethoxazole and trimethoprim, is used to treat urinary tract infections and a few other infections. Cotrimoxazole is the primary drug of choice to control *Pneumocystis* pneumonia, a common fungal complication to AIDS patients. Unfortunately, both drugs are toxic to bone marrow and may cause nausea and skin rashes (Black, 2005). Isoniazid is an antimetabolite for two vitamins-nicotinamide (niacin) and pyridoxal (vitamin B_6). It binds to and inactivates the enzyme that converts the vitamins to useful molecules. This bacteriostatic, synthetic agent has little effect on most bacteria and is effective against the mycobacterium that causes tuberculosis: because of isoniazid-resistant organisms; isoniazid usually is given with other two or three agents such as rifampin or ethambutol. Isoniazid kills the rapidly dividing bacilli; the other agents kill slow or dormant bacilli. Dietary supplements of nicotinamide and pyridoxal also should be given along with isoniazid (Black, 2005).

2.6 Beta-Lactam Antibiotics

Beta-lactam antibiotics are among the safest and most frequently prescribed antimicrobial drugs in the world (Aminzadeh *et al*., 2008). They got their name from the characteristic ring structure- the β-lactam ring. They work by interfering with the synthesis of the bacterial cell wall, a structure that is not found in eukaryotes. The walls of bacteria are made of a complex polymeric material called peptidoglycan. The beta-lactam antibiotics bind to and inhibit enzymes needed for the synthesis of the peptidoglycan wall. While they have little effect on resting bacteria, they are lethal to dividing bacteria as defective walls cannot protect the organism from bursting in hypotonic surroundings (Antibiotics, 2010). β-lactams account for approximately two-thirds, by weight of all antibiotics administered to humans (Lachmayr *et al*., 2009).

2.6.1 Penicillins

The penicillins are the oldest class of antibiotics (Bayarski, 2006). They are a group of natural and semi synthetic antibiotics containing the chemical nucleus 6-aminopenicillanic acid, which consists of a β-lactam ring fused to a thiazolidine ring. The naturally occurring compounds are produced by a number of *Penicillum* spp. The penicillins differ from one another in the substitution at position 6, where changes in the side chain may modify the pharmacokinetic and antibacterial properties of the drug (Yao and Moellering, 2007). The semi-synthetic products are those that have been chemically modified in the laboratory (and pharmaceutical facility) to improve the efficacy of the natural product, reduce its side effects, circumvent developing resistance by the target bacteria, and expand the range of bacteria that can be treated with it (Antibiotics, 2010). The natural penicillins are based on the original penicillin-G structure (Bayarski, 2006). Penicillin G is very effective against penicillin-susceptible *Staphylococcus*

aureus, Streptococcus pneumoniae, S. pyogenes, viridans group Streptococcus, *S. bovis, Neisseria gonorrhoeae, Neisseria meningitides, Pasteurella multocida,* anaerobic cocci, *Clostridium* spp., *Fusobacterium* spp., *Prevotella* spp. and *Porphyromonas* spp. However, the occurrence of penicillin-resistant pneumococci has recently been increasing worldwide. Penicillin V has a spectrum of activity similar to that of penicillin G except that it is less active against *Neisseria gonorrhoeae* (Yao and Moellering, 2007). The aminopenicillins are penicillinase-resistant. Methicillin is the prototype. They are primarily effective against penicillinase-producing Staphylococci. They are also active against *Streptococcus pneumoniae* and *S. pyogenes,* but not active against enterococci, members of the family Enterobacteriaceae, *Pseudomonas* spp., or members of the *Bacteroides fragilis* group. Ampicillin and Amoxicillin have spectra of activity similar to that of penicillin G, but they are more active against enterococci and *Listeria monocytogenes*. Although, they are also more active against *Haemophilus influenzae* and *Haemophilus parainfluenzae,* up to 35% of *H. influenzae* isolates are resistant, usually because of β-lactamase production. Ampicillin is more effective against Salmonellae. Both of these agents are degraded by β-lactamase and are inactive against many Enterobacteriaceae and *Pseudomonas* spp. (Yao and Moellering, 2007). The carboxypenicillins and ureidopenicillins have increased activity against gram-negative bacteria that are resistant to ampicillin. Although, these drugs are susceptible to Staphylococcal penicillase, they are more stable against hydrolysis by the β-lactamases of *Enterobacteriaceae* and *Pseudomonas aeruginosa*. Carbenicillin and ticarcillin are relatively active against streptococci as well as against *Haemophilus* spp., *Neisseria* spp., and a variety of anaerobes.

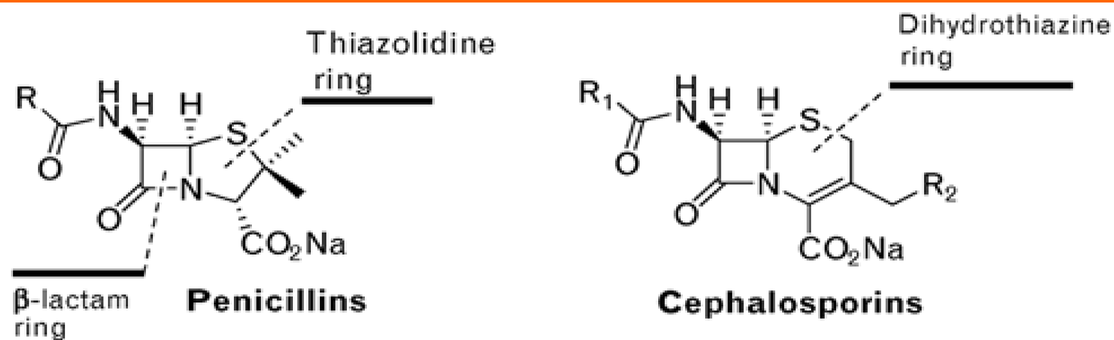


Fig. 2.12 Chemical structures of Penicillins and Cephalosporins

They inhibit Enterobacteriaceae but are inactive against *Klebsiella* spp. Although, carboxypenicillins such as carbenicillin are not particularly active against the enterococci, they may act synergistically with aminoglycosides against these organisms (Yao and Moellering, 2007). The ureidopenicillins such as piperacillin have greater *in vitro* activity against streptococci and enterococci than do the carboxypenicillins, and they inhibit more than 75% of *Klebsiella* spp. They have excellent activity against many Enterobacteriaceae of the *Bacillus fragilis* group. These agents also act synergistically with aminoglycosides against *Pseudomonas aeruginosa* (Yao and Moellering, 2007).

2.6.2 Cephalosporins

Acremonium chrysogenum (formerly named *Cephalosporium acremonium*) is the industrial producer of the pharmaceutical relevant β-lactam antibiotic, cephalosporin C. *Acremonium chrysogenum* was isolated from seawater close to a sewage outfall area at Cagliari (Sardinia, Italy) in 1945 by Giuseppe Brotzu and was found to produce, among other antibiotics, a β-lactam compound designated cephalosporin C, which is structurally related to penicillin. Today, cephalosporin derivatives are widely used in the treatment of infectious diseases and are one of

the world's major biotechnological products, with a total world market of about $10 billion (Poggeler *et al*., 2008*).* Cephalosporins contain a 7-aminocephalosporanic acid nucleus, which consists of a β-lactam ring fused to a dihydrothiazine ring. Various substitutions at positions 3 and 7 alter their antibacterial activities and pharmacokinetic properties. The addition of a methoxy group at position 7 of the β-lactam ring results in a new group of compounds called cephamycins, which are highly resistant to a variety of β-lactamases (Yao and Moellering, 2007). Cephalosporins have a mechanism of action identical to that of the penicillins, in that they interfere with synthesis of the bacteria cell wall and so are bactericidal. However, the basic chemical structure of the two differs in other respects, resulting in some difference in the spectrum of antibacterial activity (Bayarski, 2006). They are classified by a well-accepted but somewhat arbitrary scheme of grouping by generations based on general features of their antibacterial activity (Yao and Moellering, 2007).

The first-generation (narrow spectrum) drugs have good activity against gram positive organisms and relatively modest activity against gram-negative organisms. They are active against penicillin-susceptible and resistant *S. aureus* as well as *Streptococcus pnemoniae*, S. pyogenes, and other aerobic and anaerobic streptococcus. Methicillin-resistant *S. aureus* (MRSA), *Staphylococcus epidermidis,* and enterococci are resistant. Some *Enterobacteriaceae*, including many strains of *E. coli, Klebsiella* spp., and *Proteus mirabilis,* are susceptible. *Pseudomonas* spp. (including *P. aeruginosa*), many *Proteus* spp., and *Serratia* and *Enterobacter* spp. are resistant. These agents are active against penicillin-susceptible anaerobes except members of the *Bacillus fragilis* group. They have only modest activity against *H. influenzae* (Yao and Moellering, 2007).

The second-generation (expanded-spectrum) cephalosporins are stable against certain β-lactamases found in gram-negative bacteria and, as a result, have increased activity against gram-negative organisms. They are also more active than narrow-spectrum drugs against *E. coli, Klebsiella* spp. and *Proteus* spp. Their activity also extends to cover some *Enterobacter* and *Serratia* strains, and they have good activity against *Haemophilus* spp., *Neisseria* spp., and many anaerobes (Yao and Moellering, 2007).

Third-generation (broad spectrum) cephalosporins are generally less active than the narrow-spectrum agents against gram-positive cocci, but they are much more active against the *Enterobacteriaceae* and *P. aeruginosa*. Their potent broad spectra of activity against gram-negative organisms are due to their stability against β-lactamases and their ability to pass through the outer cell envelopes of gram-negative rods. Cefotaxime inhibits more than 90% of strains of *Enterobacteriaceae,* including those resistant to aminoglycosides (Yao and Moellering, 2007). They have the advantage of convenient dosing schedules, but they are expensive (Bayarski, 2006).

Table 2.1: Groups and examples of β-lactam antimicrobial agents

β lactam groups	**Examples of Antimicrobial Agents**
PENICILLINS	Penicillin G, Penicillin
	Penicillinase resistant penicillins : *methicillin*, *nafcillin*, *oxacillin*, *cloxacillin*
	Aminopenicillins: *ampicillin*, *amoxicillin*
	Carboxypenicillins: *Carbenicillin*, *ticarcillin*

	Ureidopenicillins:*Meiocillin*, *piperacillin*
CEPHALOSPORINS	First generation :*cefazolin*, *cephalothin*, *cephalexin* Second generation: *cefuroxime*, *cefaclor*, *cefamandole*, *cefamycins (cefotetan*,*cefoxitin*) Third generation: *cefotaxime*, *ceftriaxone*, *cefpodoxime*, *ceftizoxime*, *cefoperazone*, *ceftazidime* Fourth generation :*cefepime*, *cefpirome*
CARBAPENEMS	*imipenem, meropenem,ertapenem*
MONOBACTAMS	*Aztreonam*

(Samaha-Kfoury and Araj, 2003)

The fourth generation cephalosporins are extended spectrum agents with similar activity against gram-positive organisms as first generation extended spectrum. They also have a greater resistance to β-lactamases than the third generation cephalosporins. Many fourth generation cephalosporins can cross blood brain barrier and are effective in meningitis (Bayarski, 2006).

2.6.3 Monobactams

Unlike other β-lactams, the monobactams contains a nucleus with no fused ring attached. Thus, there is less probability of cross-sensitivity reactions (Beta-lactam Antibiotic, 2013). Aztreonam is the only monobactam antibiotic currently in clinical use. The monobactams are β-lactams with various side chains affixed to a monocyclic nucleus. Aztreonam binds primarily to penicillin binding proteins (PBP 3) of gram-negative aerobes, including *Pseudomonas aeruginosa*, thereby disrupting bacteria cell wall synthesis. It is not hydrolyzed by most commonly occurring plasmid-mediated and chromosomally mediated β-lactamases, and it does not induce the

production of these enzymes. The antibacterial activity of aztreonam is limited to aerobic gram-negative rods, inhibiting most Enterobacteriaceae, *Neisseria* spp. and *Haemophilus* spp. Bacterial tolerance and inoculum effect are generally not seen with this agent. Aztreonam is not active against gram-positive bacteria or anaerobes (Yao and Moellering, 2007).

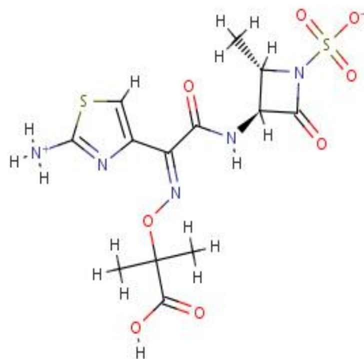
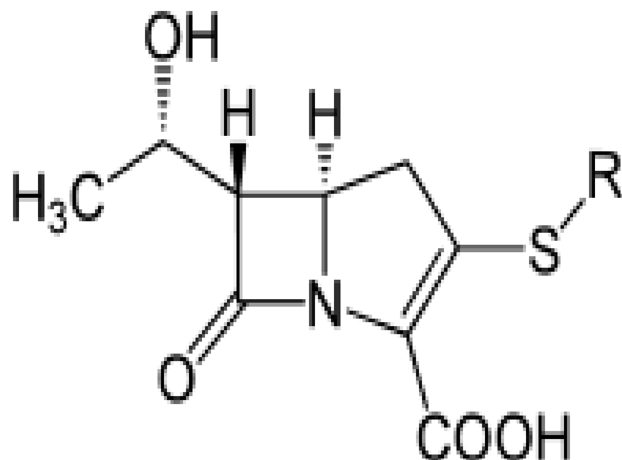


Fig. 2.13 Chemical structure of aztreonam

2.6.4 Carbapenems

The carbapenems are structurally very similar to the penicillins, but the sulfur atom in position 1 of the structure has been replaced with a carbon atom, and hence the name of the group, the carbapenems (Carbapenem: Structure, 2010). They are a unique class of β-lactam agents with the widest spectrum of antibacterial activity of the currently available antibiotics. Structurally, they differ from β-lactams in having a hydroxyethyl side chain in trans configuration at position 6 and lacking a sulphur or oxygen atom in the bicyclic nucleus. The unique stereochemistry of the hydroxyethyl side chain confers stability against β-lactamases.Carbapenem antibiotics have an important niche in that they retain activity against the chromosomal cephalosporinases and extended-spectrum beta-lactamases found in many gram-negative pathogens (Quale and Spelman, 2011).



www.chemicalbook.com/chemicalProductProperty

Fig. 2.14 Carbapenem: Structure

Imipenem (N-formimidoyl thienamycin), a semi synthetic derivative of thienamycin produced by *Stretomyces* spp., meropenem and ertapenem are the carbapenems currently available for clinical use. Carbapenems bind to PBP 1 and PBP 2 of gram-negative and gram-positive bacteria, causing cell elongation and lysis. More than 90% of Enterobacteriaceae, including those resistant to other β-lactams and aminoglycosides are susceptible to carbanepems. They are highly active against clinical isolates of extended spectrum β-lactamase-producing *Klebsiella pneumonia* and *E.coli* (Yao and Moellering, 2007). They are the most potent β-lactams against anaerobes, with activities comparable to those of clindamycin and metronidazole. Yao and Moellering (2007) stated that they are excellent *in vitro* activities against aerobic gram-positive species but methicillin-resistant Staphylococci are usually resistant to all carbapenems. The drug may show *in vitro* antagonism when combined with broad-spectrum cephalosporins or extended-spectrum penicillins as a result of its ability to induce class 1 β-lactamase production (Yao and Moellering, 2007). The emergence of carbapenem-hydrolyzing beta-lactamases has threathened the clinical

utility of this antibiotic class and brings us a step closer to the challenge of ‘extreme drug resistance’ in gram-negative bacilli (Quale and Spelman, 2011).

2.7 Susceptibility Test Methods

Antimicrobial susceptibility testing methods are *in vitro* procedures used to determine antimicrobial resistance in individual bacterial isolates. Antibiotic sensitivity testing aims to determine the susceptibility of an isolate to a range of potential therapeutic agents. There are several antimicrobial susceptibility testing methods available today, and each one has its respective advantages and disadvantages. They all have one and the same goal, which is to provide a reliable prediction of whether an infection caused by a bacterial isolate will respond therapeutically to a particular antibiotic treatment. These data may be utilized as guidelines for chemotherapy, or at the population level as indicators of emergence and spread of resistance based on passive or active surveillance (Microbiology module, 2013). The choice of methodology to be used in individual laboratories may be based on factors such as relative ease of performance, cost, flexibility in selection of drugs for testing, use of automated or semi automated devices to facilitate testing, and the perceived accuracy of the methodology (Jorgensen and Turnidge, 2007). Here is an overview of commonly used susceptibility testing methods.

2.7.1 Disk diffusion method

In the disk diffusion method or Kirby-Bauer method, a standard quantity of the causative organism is uniformly spread over an agar plate. Then, the several filter paper disks impregnated with specific concentrations of selected chemotherapeutic agents are placed on the agar surface.

The disk diffusion susceptibility method is simple and practical and has been well standardized. The test is performed by applying a bacterial inoculum of approximately 1–2 x 10^8 cfu/ml to the surface of a large (150 mm diameter) Mueller-Hinton agar plate. Plates are incubated for 16–24 h at 35^0C prior to determination of results (Jorgensen and Ferraro, 2009). During incubation, each chemotherapeutic agent diffuses out from the disk in all directions. Agents with lower molecular weights diffuse faster than those with higher molecular weights. Clear areas, called zones of inhibition, appear on the agar around disks where the agents inhibit the organism. The size of a zone diameter of inhibition is not necessarily a measure of the degree of inhibition because of differences in the diffusion rates of chemotherapy. An agent of large molecular size might be a powerful inhibitor even though it might diffuse only a small distance and produce a small zone of inhibition. Standard measurements of zone diameters for particular media, quantity of organisms, and drug concentrations have been established and correlated to zone diameters (Black, 2005). The diameter of the zones of complete inhibition is measured (as judged by the unaided eye), including the diameter of the disk. The Petri-dish is held a few inches above a black, non reflecting background illuminated with reflected light. The zone margin is considered the area showing no obvious, visible growth that can be detected with the unaided eye (CLSI, 2008). The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The zone diameters of each drug are interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards or NCCLS) for the disks. The results of the disk diffusion test are "qualitative," in that a category of susceptibility (i.e., susceptible, intermediate, or resistant) is derived from the test (Jorgensen and Ferraro, 2009).

Fig. 2.15 Agar plate of disk diffusion test showing different sizes of zones of inhibition

The three interpretative categories are defined as follows. **Susceptible** indicates that an infection caused by the tested microorganism may be appropriately treated with the usually recommended dose of antibiotic. **Intermediate** indicates that the isolate may be inhibited by attainable concentrations of certain drugs (e.g., the beta-lactams) if higher dosages can be used safely or if the infection involves a body site indicating that the drug is physiologically concentrated (e.g., the urinary tract). The intermediate category also serves as a buffer zone that prevents slight technical artifacts from causing major interpretive discrepancies. **Resistant** isolates are not inhibited by the concentration of antimicrobial agent normally achievable with the recommended dose and / or yield results that fall within a range indicating that specific resistance mechanisms are likely to be present (Jorgensen and Turnidge, 2007). The advantages of the disk method are the test simplicity that does not require any special equipment, the provision of categorical results easily interpreted by all clinicians, and flexibility in selection of disks for testing. It is the least costly of all susceptibility methods. The disadvantages of the disk test are the lack of mechanization or automation of the test. Although not all fastidious or slow growing bacteria can be accurately tested by this method, the disk test has been standardized for testing several organisms such as streptococci, *Haemophilus influenzae,* and *N. meningitides* through use of

specialized media, incubation conditions, and specific zone size interpretive criteria (Jorgensen and Ferraro, 2009).

2.7.2 Dilution method (broth and agar dilution method)

One of the earliest antimicrobial susceptibility testing methods was the macro broth or tube-dilution method. This procedure involved preparing two-fold dilutions of antibiotics (e.g., 1, 2, 4, 8, and 16 mg/ml) in a liquid growth medium dispensed in test tubes. The antibiotic containing tubes were inoculated with a standardized bacterial suspension of 1–5 x10^5 cfu/ml. Following overnight incubation at 35^0C, the tubes were examined for visible bacterial growth as evidenced by turbidity. The lowest concentration of antibiotic that prevented growth represented the minimal inhibitory concentration (MIC). The advantage of this technique was the generation of a quantitative result (i.e., the MIC) (Jorgensen and Ferraro, 2009). Finding an inhibitory agent by the dilution method does no more to prove that it will kill the infectious organism in the patient than finding one by the disk diffusion method. However, the dilution method allows a second test to distinguish between bactericidal agents, which kill microorganisms, and bacteriostatic agents, which merely inhibit their growth. Samples from tubes that show no growth but that might contain inhibited organisms can be used to inoculate broth that contains no chemotherapeutic agent. In this test, the lowest concentration of the chemotherapeutic agent that yields no growth following this second inoculation, or subculturing, is the **minimum bactericidal concentration** (MBC). Thus, both an effective chemotherapeutic agent and an appropriate concentration to control an infection can be determined. That concentration should be maintained at the sites of infection because it is the minimum concentration that will cure the disease (Black, 2005). The principal disadvantages of the macro dilution method were the tedious, manual task of preparing the antibiotic solutions for each test, the possibility of errors in

preparation of the antibiotic solutions, and the relatively large amount of reagents and space required for each test (Jorgensen and Ferraro, 2009).

The miniaturization and mechanization of the test by use of small, disposable, plastic "micro dilution" trays has made broth dilution testing practical and popular. (Jorgensen and Ferraro, 2009).

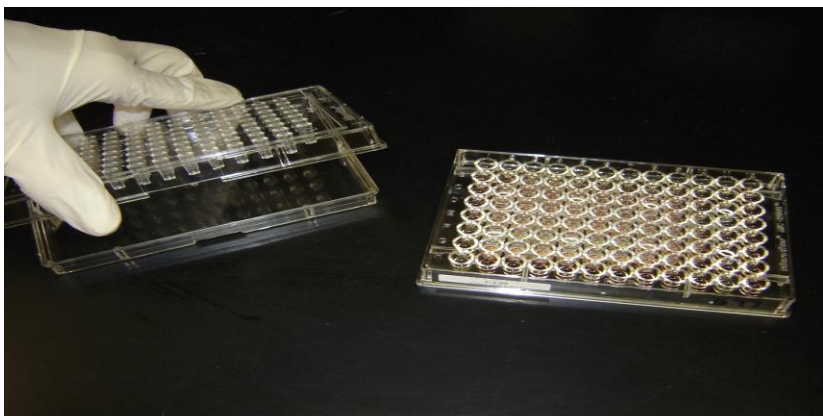


Fig. 2.16 A broth micro dilution susceptibility panel and disposable tray inoculators

Standard trays contain 96 wells, each containing a volume of 0.1 ml that allows approximately 12 antibiotics to be tested in a range of 8 two-fold dilutions in a single tray Micro dilution panels are typically prepared using dispensing instruments that aliquot precise volume of pre-weighed and diluted antibiotics in broth into the individual wells of trays from large volume vessels. Hundreds of identical trays can be prepared from a single master set of dilutions in a relatively brief period (Jorgensen and Ferraro, 2009).

Few clinical microbiology laboratories prepare their own panels; instead frozen or dried micro dilution panels are purchased from one of several commercial suppliers. Inoculation of panels with the standard 5 x10^5 cfu/ml is accomplished using a disposable device that transfers 0.01 to 0.05 ml of standardized bacterial suspension into each well of the micro dilution tray or by use of a mechanized dispenser. Following incubation, MICs are determined using a manual or automated viewing device for inspection of each of the panel wells for growth. The advantages of the micro dilution procedure include the generation of MICs, the reproducibility and convenience of having pre-prepared panels, and the economy of reagents and space that occurs due to the miniaturization of the test. There is also assistance in generating computerized reports if an automated panel reader is used. The main disadvantage of the micro dilution method is some inflexibility of drug selections available in standard commercial panels' growth. The advantages of the micro dilution procedure include the generation of MICs, the reproducibility and convenience of having pre-prepared panels, and the economy of reagents and space that occurs due to the miniaturization of the test. There is also assistance in generating computerized reports if an automated panel reader is used. The main disadvantage of the micro dilution method is some inflexibility of drug selections available in standard commercial panels (Jorgensen and Ferraro, 2009).

A procedure similar to broth dilution is agar dilution. Agar dilution method follows the principle of establishing the lowest concentration of the serially diluted antibiotic concentration at which bacterial growth is still inhibited (Microbiology module, 2013).

2.7.3 Antimicrobial gradient method

The antimicrobial gradient diffusion method uses the principle of establishment of an antimicrobial concentration gradient in an agar medium as a means of determining susceptibility (Jorgensen and Ferraro, 2009). It is a newer version of the diffusion test, called the **E-(epsilometer) test** uses a plastic strip containing a gradient of concentration of antibiotic. Printed on the strips are concentration values which allow the laboratory technician to directly read off the minimum concentration needed to inhibit growth (Black, 2005). This method provides for a convenient quantitative test of antibiotic resistance of a clinical isolate. However, a separate strip is needed for each antibiotic, and therefore this method can be expensive (Microbiology module, 2013).

Fig. 2:17 The E-test gradient diffusion method

As many as 5 or 6 strips may be placed in a radial fashion on the surface of an appropriate 150-mm agar plate that has been inoculated with a standardized organism suspension like that used for a disk diffusion test. After overnight incubation, the tests are read by viewing the strips from

the top of the plate. The MIC is determined by the intersection of the lower part of the ellipse shaped growth inhibition area with the test strip. The gradient diffusion method has intrinsic flexibility by being able to test the drugs the laboratory chooses. This method is best suited to situations in which an MIC for only 1 or 2 drugs is needed or when a fastidious organism requiring enriched medium or special incubation atmosphere is to be tested (e.g., penicillin and ceftriaxone with pneumococci). Generally, E-test results have correlated well with MICs generated by broth or agar dilution methods. However, there are some systematic biases toward higher or lower MICs determined by the E-test when testing certain organism-antimicrobial agent combinations. This can represent a potential shortcoming when standard MIC interpretive criteria derived from broth dilution testing are applied to E-test MICs that may not be identical (Jorgensen and Ferraro, 2009).

2.7.4 Automated methods

Automated methods are now available to identify pathogenic organisms and to determine which antimicrobial agents will effectively combat them (Black, 2005). These commercial systems have been developed to provide conveniently prepared and formatted micro-dilution panels as well as instrumentation and automated reading of plates. These methods are intended to reduce technical errors and lengthy preparation times. Most automated antimicrobial susceptibility testing systems provide automated inoculation, reading and interpretation. These systems have the advantage of being rapid (some results can be generated within hours) and convenient, but one major limitation for most laboratories is the cost entailed in initial purchase, operation and maintenance of the machinery (Microbiology module, 2013). Machines vary in their degree of automation and the speed with which results become available. Some require technicians to perform some steps; others provide results in 3 to 6 hours and most provide them overnight,

although slow-growing organisms may require 48 hours (Black, 2005). Some examples of these include: Vitek System (bioMerieux, France), Walk-Away System (Dade International, Sacramento, Calif.), Sensititre ARIS (Trek Diagnostic Systems, East Grinstead, UK), Avantage Test System (Abbott Laboratories, Irving, Texas), Micronaut (Merlin, Bornheim-Hesel, Germany), Phoenix (BD Biosciences, Maryland) and many more (Microbiology module, 2013).

2.7.5 Mechanism-specific tests

Resistance may also be established through tests that directly detect the presence of a particular resistance mechanism. For example, beta lactamase detection can be accomplished using an assay such as the chromogenic cephalosporinase test (Cefinase disk by BD Microbiology Systems, Cockeysville, MD and BBL DrySlide Nitrocefin, Becton Dickinson, Sparks, MD) and detection for chloramphenicol modifying enzyme chloramphenicol acetyltransferase (CAT) may utilize commercial colorimetric assays such as a CAT reagent kit (Remel, Lenexa, Kansas) (Microbiology module, 2013).

2.7.6 Genotypic methods

Since resistance traits are genetically encoded, we can sometimes test for the specific genes that confer antibiotic resistance. However, although nucleic acid-based detections systems are generally rapid and sensitive, it is important to remember that the presence of a resistance gene does not necessarily equate to treatment failure, because resistance is also dependent on the mode and level of expression of these genes. Some of the most common molecular techniques utilized for antimicrobial resistance detection are as follows:

2.7.6.1 *Polymerase chain reaction (PCR)*

Polymerase chain reaction (PCR) is one of the most commonly used molecular techniques for detecting certain DNA sequences of interest. This involves several cycles of denaturation of sample DNA, annealing of specific primers to the target sequence (if present), and the extension of this sequence as facilitated by a thermo stable polymerase leading to replication of a duplicate DNA sequence, in an exponential manner, to a point which will be visibly detectable by gel electrophoresis with the aid of

a DNA-intercalating chemical which fluoresces under UV light. (Microbiology module, 2013).

2.7.6.2 *DNA hybridization*

This is based on the fact that the DNA pyrimidines (cytosine and thymidine) specifically pair up with purines (guanine and adenine; or uracil for RNA). Therefore, a labeled probe with a known specific sequence can pair up with opened or denatured DNA from the test sample, as long as their sequences complement each other. If this "hybridization" occurs, the probe labels this with a detectable radioactive isotope, antigenic substrate, enzyme or chemiluminescent compound. Whereas if no target sequence is present or the isolate does not have the specific gene of interest, no attachment of probes will occur, and therefore no signals will be detected (Microbiology module, 2013).

2.7.6.3 *Modifications of PCR and DNA hybridization*

With the above basic principles, several modifications have been introduced which further improve the sensitivity and specificity of these standard procedures. Examples of

such development were the use of 5′-fluorescence-labeled oligonucleotides, the development of molecular beacons, development of DNA arrays and DNA chips, among many others. (Microbiology module, 2013).

2.8 Antimicrobial Resistance

Antimicrobial resistance is a broad term with many meanings. It describes the response of a multitude of microbes to a variety of agents by many different mechanisms. Broadly, it refers to the 'temporary or permanent ability of an organism and its progeny to remain viable and / or multiply under conditions that would destroy or inhibit other members of the strain'. Resistance to antibiotics is generally genetically based and progeny of antibiotic-resistant bacteria are also resistant. It can also result from spontaneous mutation in the absence of antibiotic use (McEntire and Montville, 2007). Intrinsic characteristics or chromosomal resistance to antibacterial substances depends upon mutants to emerge. Plasmid-mediated resistance (R-factor or acquired resistance) is more complex (Evermann, 2000).

An increasing health problem is the appearance and spread of antimicrobial resistance (Rijavec *et al.,* 2007). The use, over-use, and misuse of antibiotics have led to an alarming increase in the frequency of human pathogens that do not respond to antibiotic therapy, underscoring the need for new antibiotics and a better understanding of the origins of antibiotic resistance (Donato *et al*., 2010). The use of antibiotics in the treatment of clinical enteric infection has been heavily

compromised by emerging multidrug-resistant microbes (Rayamajhi *et al*., 2010). Antibiotic resistance has been classified by the WHO as one of the three major public health threats of the 21st century (Lachmayr *et al.,* 2009). It poses a significant, serious and increasing threat to modern health care. Patients infected with or colonized by resistant pathogens are reported to suffer greater mortality, require longer hospital stay, and cost the health service substantially more than patients with antimicrobial-sensitive infections (Hunter *et al*., 2008).

Bacterial antibiotic-resistance can be either intrinsic or acquired. Intrinsic resistance is a natural resistance present in all strains of a bacterial species, while acquired resistance is often identifiable as a resistance found in only a certain number of members of a particular species. Although intrinsic resistance is generally accepted to be non-transferable, acquired resistance may be more easily transferred to other bacterial species, particularly if the resistance trait is located on a plasmid in the carrier strain (Rosander *et al.*, 2008). Identification of the main route of the evolution of antibiotic resistance and its transmission to humans is essential for effective mitigation (Manuzon *et al.,* 2007). While there may be several contributing causes for the increase in antimicrobial resistance in pathogenic bacteria, most researchers focus on increased use of antimicrobial agents (Wagner *et al*., 2008). The use of antibiotics in treatments or as food supplements for farm animals leaves behind drug-resistant microbes in milk, eggs and meat that could encourage the development of resistant traits and transfer to other bacteria, making consumers more vulnerable to the resistant varieties (Denwe, 2006). There is evidence that antimicrobial use in animals select for resistance in both pathogenic and commensal organisms (Wagner *et al*., 2008). A commensal organism of interest, *Escherichia coli,* may serve as reservoir of transferable antimicrobial resistance genetic elements. Laboratory based studies have

shown that *E .coli* is capable of transferring resistance to other bacterial species, such as *Salmonella* spp*.,* which are disseminated through the human food chain. This mechanism of transfer has been shown to occur within and between many different bacterial genera and has been proposed to be a major cause behind the rapid spread of resistance genes during the last five decades (Wagner *et al*., 2008). In developed countries, the main reservoirs for antimicrobial drug resistance in enteric bacteria have been attributed to farm animals such as cattle, sheep, pigs and poultry. Contact with these animals or consumption of food products from them has been the main route of dissemination of resistance into the human populations. Therefore, transmission of drug resistant bacteria from farms into the community and subsequently to patients in hospital may occur through food. This demonstrates how resistant bacteria arising from indiscriminate use of antibiotics in animals may impact on human health (Ombui *et al*., 2000).

The ‘prudent use of antimicrobials’ has become a major objective of the human and veterinary medical care establishment. The possibility of resistant organisms passing from animals to humans has been recognized by the World Organization of Animal Health and the World Health Organization (Wagner *et al*., 2008). Consequently, the role of antimicrobial administration and the extent to which it affects the development of resistance in animals are receiving much attention (Sharma *et al.,* 2008). In North America, antibiotics are widely used in beef cattle production as prophylactics or antimicrobial growth promoters (AGP). Used in this manner, antibiotics are generally administered in the diet either at times of high disease risk or on a continuous basis to improve feed efficiency. Employment of AGP in this manner may increase the prevalence of commensal antimicrobial-resistant bacteria (Alexander *et al*., 2009).

A major public health concern is that use of third-generation cephalosporins, such as ceftiofur, in food animals is leading to resistance to other extended-spectrum cephalosporins, such as ceftriaxone and cephamycins, a group of antimicrobial agents used to treat a wide variety of human infections (Dutil *et al*., 2010). Antimicrobial usage for livestock can be for therapeutic, prophylactic, metaphylactic, or growth promotion purposes. Reportedly, 90% of the antimicrobials used in animal agriculture are for growth promotion and prophylaxis and this widespread use is suggested to be an important contributor to the emergence, selection and dissemination of antimicrobial-resistant bacteria, as indicated in recent studies (Sharma *et al.,* 2008). Antibiotic resistant bacteria, including *Escherichia coli*, are frequently isolated from commensal gut flora of food animals, and although the resistance they carry may not be a problem *per se*, the transfer of resistant elements to zoonotic pathogens inhabiting the gut has serious implications for animal and human health (Sharma *et al.,* 2008). Antimicrobial drug resistance phenotype is commonly described in terms of the resistance characteristics of the organism. These characteristics are constitutionally based intrinsic characteristics of the organism or resistance factors acquired through induced genetic expression or gene transfer between organisms (MacPherson *et al*., 2009).

2.8.1 Beta lactamases

The ability of bacteria to produce enzymes that destroy the β-lactam antibiotics began even before penicillin was developed. The first β-lactamase was identified in an isolate of *Escherichia coli* in 1940 (Turner, 2005). Beta lactamases are enzymes which inactivate (susceptible) β-lactam antibiotics by hydrolyzing the β- lactam ring (Singleton, 1997). Enzymatic inactivation of

antibiotics occurs with several of the natural product antibiotic classes but has not yet been observed as a major route of resistance development for some classes of synthetic antibacterials: the sulfamethoxazone-trimethoprim combination, the fluoroquinolones, or the oxazolidinones. The most widespread mode of clinical resistance development to β-lactam antibiotics is the expression of β-lactamases that hydrolyze the antibiotic (Walsh, 2003). Many of the 2nd and 3rd generation penicillins and cephalosporins were specifically designed to resist the hydrolytic action of major β-lactamases. However, new β-lactamases emerged against each of new classes of β-lactams that were introduced and caused resistance. In fact, since β-lactam antibiotics came into clinical use, β-lactamases have evolved with them (Okesola and Makanjuola, 2009). Although β-lactamases are estimated to have existed for the past 2 billion years, their evolution and spread have been highly correlated to the anthropogenic development and prolificacy of β-lactam antibiotics during the past 60 years (Lachmayr *et al*., 2009).

Two systems are commonly used to classify beta-lactamases: the Ambler scheme and the Bush-Medeiros-Jacoby system. They are summarized in the Table 2.2. Both systems are used interchangeably in literature: ESBLs belong to group 2be in the Bush-Medeiros-Jacoby system and to class A in the Ambler system (Perez *et al.,* 2007). β-lactamase enzymes destroy the β-lactam ring by two major mechanisms of action. Firstly, most common β-lactamases have a serine based mechanism of action. They are divided into three classes (A, C and D) on the basis of the amino acid sequences. They contain an active site consisting of a narrow longitudinal groove, with a cavity on its floor (the oxyanion pocket), which is loosely constructed in order to have conformational flexibility in terms of substrate binding. These enzymes are active against many penicillins, cephalosporins and monobactams. Secondly, a less commonly encountered

group of β-lactamases is the metallo β-lactamases, or class B β-lactamases (Samaha-Kfoury and Araj, 2003). The class B lactamases are zinc enzymes, containing a binuclear zinc cluster in the active site. Unlike the class A, C and D lactamases, which do lactam ring opening via covalent acyl enzyme intermediate , the class B lactamases use zinc to activate a water molecule and catalyze its direct addition to the β-lactam ring (Walsh, 2003).

Table 2.2 Classification of beta-lactamases.

Bush-Jacoby-Medeiros system	**Major subgroups**	**Ambler System**	**Main attributes**
Group 1	–	C	Usually chromosomal; Resistance to

Bush-Jacoby-Medeiros system	Major subgroups	Ambler System	Main attributes
cephalosporinases		(cephalosporinases)	all β-lactams except carbapenems; Not inhibited by clavulanate
Group 2 penicillinases	2a	A (serine β-lactamases)	Staphylococcal penicillinases
(clavulanic acid susceptible)	2b	A	Broad-spectrum – TEM-1, TEM-2, SHV-1
	2be	A	Extended-spectrum – TEM-3–160, SHV-2–101
	2br	A	Inhibitor resistant TEM (IRT)
	2c	A	Carbenicillin-hydrolyzing
	2e	A	Cephalosporinases inhibited by clavulanate
	2f	A	Carbapenemases inhibited by clavulanate
	2d	D(oxacillin-hydrolyzing)	Cloxacillin-hydrolyzing (OXA)
Group 3 metallo-β-Lactamase	3a	B (metalloenzymes)	Zinc-dependent Carbapenemases
	3b	B	
	3c	B	
Group 4		Not classified	Miscellaneous enzymes, most not yet sequenced

(Perez *et al.,* 2007).

The metallo β-lactamases of type B are thought to be the major sub-class of hydrolases that destroy the carbapenem antibiotics such as imipenem (thienamycin) and meropenem. Many bacteria that produce the type D metallo hydrolases also produce a type A, C or D lactamase; for example, a clinical isolate of *Serratia marcescens* carries a type A and a type B bla gene on a plasmid (Walsh, 2003). Resistant bacteria may transfer resistance genes to other bacteria and become important in the spread of antibiotic resistance. Indiscriminate use of antimicrobial agents and antibiotic sale behaviour (for example, sale of antibiotics without prescription, sale of

under dose and substituting brands) enhances the development of drug resistance (Ombui *et al*., 2000).

2.8.2 Extended spectrum β- lactamases

The persistent exposure of bacterial strains to a multitude of β-lactams has induced a dynamic and continuous production and mutation of β-lactamases in bacteria, expanding their activity even against the third and fourth generation cephalosporins such as ceftazidime, cefotaxime and cefepime and against aztreonam. Thus, these new β- lactamases are called extended spectrum β-lactamases (Samaha-Kfoury and Araj, 2003). ESBLs, first isolated in 1983 in Germany, spread rapidly to the rest of Europe, US and Asia and are now found all over the world. Being plasmid mediated, they are easily transmitted among members of Enterobacteriaceae thus facilitating the dissemination of resistance not only to β-lactams but to other commonly used antibiotics (Kumar *et al*., 2006). Extended spectrum β-lactamases (ESBLs) are a group of enzymes that break down antibiotics belonging to the penicillin and cephalosporin groups and render them ineffective. ESBLs have traditionally been defined as transmissible beta-lactamases that can be inhibited by clavulanic acid, tazobactam or sulbactam, and which are encoded by genes that can be exchanged between bacteria (Strama, 2007). They are enzymes mediating resistance to most beta-lactams used in human and veterinary medicine, including expanded spectrum-cephalosporins but excluding carbapenems and cephamycins (Valat *et al.,* 2012). ESBLs production is often plasmid-mediated. They share highly conserved amino acid sequence with penicillin binding proteins (PBPs). They are known to attack amide bonds in the β-lactam ring of penicillins and cephalosporins (Jeong *et al*., 2004; Olonitola *et al.,* 2007). The presence of ESBL has been associated with increased mortality, longer duration of hospitalization and increased hospital cost (Cornejo-Juarez *et al*., 2012). Many clinical laboratories are not fully aware of the

importance of ESBLs and how to detect them. Laboratories may also lack the resources to curb the spread of these resistance mechanisms. This lack of understanding or resources is responsible for a continuing failure to respond appropriately to prevent the rapid worldwide dissemination of pathogens possessing these β-lactamases (Sharma *et al*., 2010).

ESBL has traditionally been defined as transmissible beta- lactamases that can be inhibited by clavulanic acid, tazobactam or sulbactam, and which are encoded by genes that can be exchanged between bacteria (Strama, 2007). Extended spectrum β-lactamases (ESBLs) are enzymes conferring broad resistance to penicillins, aztreonam and cephalosporins (with the exception of cephamycins). They generally result from point mutations in the genes of broad-spectrum β-lactamase such as TEM or SHV (Deschamps *et al*., 2009). There are four classes of β-lactamases based on the primary sequence. Classes A, C, and D involve a serine residue in the active site. Class B enzymes are less abundant and require a catalytic zinc for activity (Petrosino and Palzkill, 1996).

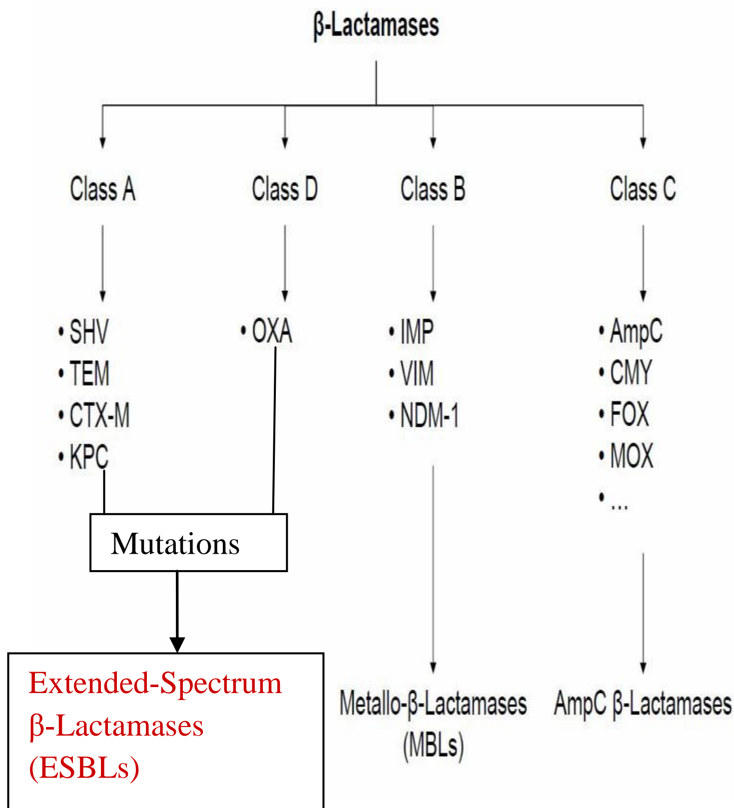


Fig. 2: 19 Overview of classification and types of β-lactamases
(Petrosino and Palkill, 1996).

They are usually located on plasmids that often carry genes responsible for resistance to other antimicrobial agents making it extremely difficult to treat infections caused by bacteria that produce ESBL enzymes (Iroha *et al*., 2008). The production of extended-spectrum β-lactamases (ESBLs) is a major mechanism of resistance to third- generation cephalosporins, and plasmid-encoded ESBLs are mostly of the TEM, SHV or CTX-M types. Over the last decade, CTX-M enzymes have replaced TEM and SHV mutants as the most prevalent ESBLs worldwide, with *E. coli* as a major host. They have been reported in both hospital and community settings. They have also been detected in pets and farm animals, products of the food chain and sewage (Deschamps *et al*., 2009). ESBLs belong to group 2be in the Bush-Medeiros-Jacoby system and

to class A in the Ambler system (Perez *et al*., 2007). Resistance to extended-spectrum β-lactam antibiotics is mainly caused by extended-spectrum β-lactamases (ESBLs) such as bla_{TEM}, bla_{SHV} and bla_{CTX-M} (Cullik *et al*., 2010).

2.8.2.1 *TEM-type extended spectrum β- lactamases*

The first plasmid-mediated β-lactamase in gram-negative bacteria, TEM-1, was described in 1965. This occurred in a strain of *E. coli* isolated from culture of blood from a patient in Greece (the designation "TEM" came from the patient's name, Temoniera), because this β-lactamase was plasmid borne, it soon spread to other members of the Enterobacteriaceae family, *Haemophilus influenzae, Neisseria gonorrhoeae,* and *Pseudomonas aeruginosa* (Turner *et al*., 2005). Up to 90% of ampicillin resistance in *E.coli* is due to the production of TEM-1 (Lim *et al*., 2009). This enzyme is able to hydrolyze penicillins and early cephalosporins such as cephalothin and cephaloridine. TEM-type β-lactamases are most often found in *E. coli* and *K. pneumonia* and also in other species of gram-negative bacteria with increasing frequency (Bradford, 2001).

2.8.2 .2 *SHV-type extended spectrum β- lactamases*

The SHV enzymes, named after the 'sulfhydryl variable' active site, are commonly associated with *Klebsiella pneumonia* (Samaha-Kfoury and Araj, 2003). The native SHV-1 β-lactamase, found primarily in *K. pneumoniae*, is a plasmid or chromosomally encoded-enzyme that confers resistance to penicillins and first-generation cephalosporins. Specific mutations within the bla_{SHV-1} to extended-spectrum cephalosporins and monobactam. Fewer ESBL variants have been described (Rupp and Fey, 2003). The first extended-spectrum SHV enzyme was described in 1983 in clinical isolates of *K. pneumonia*, *K. ozaenae* and *Serratia marcescens*. Because of its

similarity to SHV-1 the new enzyme was named SHV-2. A single amino acid substitution alters the spectrum of activity of the SHV-1 β-lactamase to encompass extended spectrum cephalosporins. Glycine at position 238 in SHV-1 is replaced by serine in SHV-2 (Heritage *et al*., 1999).

2.8.3 β-lactamase inhibitors

Further attempt to combat resistance due hydrolytic lactamases has led to the screening for inhibitors and inactivators of lactamase activity and then combine these molecules with a β-lactam. This approach has had success (Walsh, 2003). The inhibitors include clavulanic acid, tazobactam and sulbactam (Beta-lactam Antibiotic, 2010). The first is the natural product clavulanate, an enol- β-lactam from *Streptomyces clavuligerus*, the second class is represented by penicillin sulfone and a substituted congener taxobactam (Walsh, 2003). Although, they exhibit negligible antimicrobial activity, and they contain the β-lactam ring. Their sole purpose is to prevent the inactivation of β-lactam antibiotics by binding to the β-lactamases (Beta-lactam Antibiotic, 2010). They are not potent enough as β-lactam antibiotics to be used on their own, as such; they are co-administered with β-lactam antibiotics as follows:

Clavulanate + Amoxicillin→ Augmentin

Clavulanate + Ticarcillin → Timentin

Sulbactam + Ampicillin → Unasyn

Tazobactam + Piperacillin → Zocin

The combination of amoxicillin and clavulanate, known as Augmentin, for the augmenting powers that clavulanate confers to amoxicillin, has been the most widely used form of penicillin

in recent years (Walsh, 2003). In general, sulbactam, clavulanate and tazobactam are all potent inhibitors of staphylococcal penicillinase; chromosomal beta-lactamases produced by *Bacteroides* species, *Proteus vulgaris*, *Haemophilus influenza*, *Neisseria gonorrhoeae*, and type IV enzymes of *Klebsiella* species. Although sulbactam possesses activity against TEM-1 and TEM-2 beta-lactamases, it does not have reliable activity against SHV-1 beta-lactamases. Clavulanate and tazobactam are potent inhibitors of both TEM and SHV-1 beta-lactamases. *P. aeruginosa* and some Enterobacteriaceae produce an inducible, extremely potent, broad spectrum enzyme (type 1 beta lactamase). Tazobactam is the only currently available beta lactamase inhibitor with activity against type 1 beta-lactamases; however, some enzymes are not inhibited by tazobactam (Rotschafer and Ostergard, 1995).

2.8.4 Methods of detecting ESBLs

Identifying organisms that are ESBL producers is a major challenge for the clinical microbiology laboratory. It however, appears that there is a difference in the ability of various susceptibility-testing methods used for detecting cephalosporin resistance in an ESBL –producing strain (Bradford, 2001). The Clinical Laboratory Institute Standard (CLSI) has developed broth microdilution and disk diffusion screening tests using selected antimicrobial agents. These selected antimicrobial agents are known as indicator cephalosporins for screening ESBL production in bacteria. They are cefpodoxime, ceftazidime, aztreonam, cefotaxime and ceftriaxone. The use of more than one of the five antimicrobial agents suggested for screening will improve the sensitivity of detection. Cefpodoxime and ceftazidime show the highest sensitivity for ESBL detection (Rawat and Nair, 2010). A number of investigators have suggested that either dilution tests or disk diffusion susceptibility tests performed with

cefpodoxime detected more ESBLs than other cephalosporins such as ceftazidime, cefotaxime and ceftriaxone (Bradford, 2001).

Cefpodoxime and ceftazidime have therefore been proposed as indicators of ESBL production as compared to cefotaxime and ceftriaxone. Hence, an institution where only cefotaxime and ceftriaxone are used in the routine sensitivity testing panel may have difficulty in detecting ESBLs (Chaudhary and Aggarwai, 2004). Ideally, all Enterobacteriaceae isolates should be tested with *both* ceftazidime and cefotaxime as this achieves the best sensitivity and specificity in ESBL detection. If only a single cephalosporin can be accommodated in the testing scheme, then the best choice is cefpodoxime, which has good sensitivity for detection of ESBL producers but poorer specificity than testing both cefotaxime and ceftazidime (ESBLs, 2013). Also, these enzymes can be induced by certain antibiotics, amino acids or body fluids. Organisms possessing genes for inducible β-lactamase show false susceptibility if tested in the uninduced state (Chaudhary and Aggarwai, 2004).

Generally, an isolate is suspected to be an ESBL producer when it shows *in vitro* susceptibility to the second generation cephalosporins (cefoxitin, cefotan) but resistant to the third generation cephalosporins and to aztreonam. Moreover, one should suspect these strains when treatment with these agents for Gram negative infections fails despite reported *in vitro* susceptibility. Once an ESBL producing strain is detected, the laboratory is expected to report it as 'resistance' to all penicillins, cephalosporins, and aztreonam, even if they test as susceptible. Other antimicrobial agents can be reported as they are tested (Samaha-Kfoury and Araj, 2003). Several tests have been developed to confirm the presence of ESBLs (Chaudhary and Aggarwai, 2004).

2.8.4.1 Double disc synergy test (DDST)

In this test, discs of third generation cephalosporins and augmentin are kept 30 mm apart from center to centre on inoculated Mueller-Hinton Agar (MHA). A clear extension of the edge of the inhibition zone of cephalosporin towards augmentin disc is interpreted as positive for ESBL production (Chaudhary and Aggarwai, 2004). ESBLs are inhibited by β-lactamase inhibitors like clavulanic acid, sulbactam and tazobactam and the property of specific inhibition is utilized in this test for the detection and confirmation of ESBLs (Kumar *et al.,* 2006).

2.8.4.2 Three dimensional test

This test provides the advantage of simultaneous determination of antibiotic susceptibility and β-lactamase substrate profile. Inoculum produced in this method contains between $10^9$and 10^{10} cfu/ml of cells that actively produced β-lactamase. Two types of inocula are prepared, one disc diffusion test inoculum (optical density equal to that of 0.5 McFarland standard) and a three dimensional inoculum (contain between 10^9 and10^{10} CFU of cells). Plate is inoculated by disc diffusion procedure. Circular silt is cut on the agar 4mm inside the position at which the antibiotic discs are placed. Conventional (two dimensional) disc diffusion susceptibility test results are measured according to the recommendations of Clinical Laboratory Standard Institute (CLSI). Distortion or discontinuity in the circular inhibition zone is positive for ESBL production (Chaudhary and Aggarwal, 2004).

2.8.4.3 Inhibitor potentiated disc diffusion test

Cephalosporin disc is placed on clavulanate containing and without clavulanate containing Mueller Hinton Agar (MHA) plates. More than 10 mm increase in the zone of inhibition on the

clavulanate containing MHA plates indicates ESBL production (Chaudhary and Aggarwal, 2004).

2.8.4.4 Disc approximation test

Cefoxitin (inducer) disc is placed at a distance of 2.5cm from cephalosporin disc. Production of inducible β-lactamase is indicated by flattening of the zone of inhibition of the cephalosporin disc towards inducer disc by $\geq$1mm (Chaudhary and Aggarwal, 2004).

2.7.4.5 MIC reduction test

An 8 fold reduction in the MIC of cephalosporin in the presence of clavulanic acid indicates production of ESBL (Chaudhary and Aggarwal, 2004).

2.8.4.6 Vitex ESBL test

Four wells containing cards are inoculated. A pre-determined reduction in growth of cephalosporin well containing clavulanic acid: when compared with the level of growth in well with cephalosporin alone indicates presence of ESBL (Chaudhary and Aggarwal, 2004).

2.8.4.7 E-test

The E-test ESBL strip carries two gradients, on the one end, ceftazidime and on the opposite end ceftazidime plus clavulanic acid. MIC is interpreted as the point of intersection of the inhibition ellipse with the E-test strip edge: Ratio of ceftazidime MIC and ceftazidime-clavulanic acid MIC equal to or greater than 8 indicates the presence of ESBL (Chaudhary and Aggarwal, 2004).

2.8.5 Treatment of ESBLs

Of all the available β-lactams, carbapenems are the drug of choice for treating ESBL producing bacteria (Samaha-Kfoury and Araj, 2003). They are most effective and reliable as they are highly resistant to the hydrolytic activity of all ESBL enzymes, due to the trans-6- hydroxyethyl group. Meropenem is the most active with MICs generally lower than those of imipenem (0.03-0.12μg/ml vs 0.06-0.5μg/ml). Several new carbapenems, ertapenem and faropenem are being studied in the various phases of clinical trials. Alternately, fluoroquinolones and aminoglycosides may be used if they show *in vitro* activity. Although, clinical data for their use are absent, a β-lactam- β-lactamase inhibitor combination such as amoxillin-clavulanate or piperacillin-tazobactam may also be a further option to consider. All these agents should be used with caution, however, as their susceptibility varies among ESBL producers. Cefamycins, such as cefoxitin and cefotetan, although active *in vitro*, are not recommended for treating infections caused by enzyme producing bacteria or otherwise used with caution, because of the relative ease with which these strains decrease the expression of outer membrane proteins, rendering them resistant (Samaha-Kfoury and Araj, 2003; Chaudhary and Aggarwal, 2004).

2.8.6 Prevention and Control

Proper infection control practices and barriers are essential to prevent spreading and outbreaks of ESBL producing bacteria. Essential infection control practices should include hand washing by hospital personnels, increased barrier precautions, and isolation of patients colonized or infected with ESBL producers. Other practices that have minimized the spread of such organisms include clinical and bacteriological surveillance of patients admitted to intensive care units and antibiotic recycling, as well as policies of restriction, especially on the empirical use of broad spectrum antimicrobial agents (Samaha-Kfoury and Araj, 2003). Several studies have shown that by limiting the use of these agents alone or in combination with infection control measures, the

frequency of ESBL isolates can be reduced substantially. Educational programs for medical staff to increase awareness also should be developed (Chaudhary and Aggarwal, 2004).

2.9 Plasmids and Antibiotic Resistance

Plasmids are usually extrachromosomal, circular, double stranded DNA molecules found in diverse bacteria. They replicate autonomously from bacterial chromosome. (Carattoli, 2011). Plasmids range in size from a few hundred to several hundred thousand base pairs and are present in most bacterial species.

Plasmids allow the movement of genetic material, including antimicrobial resistance genes, between bacterial species and genera. They frequently mediate resistance to multiple antimicrobials and can result in the acquisition by a pathogen of resistance to all or most clinically relevant antimicrobials (Sherley *et al.*, 2004). Plasmids may contain 20-500 genes that can carry resistance to a number of different antibacterial substances, as well as specific virulence factors (Evermann, 2000).

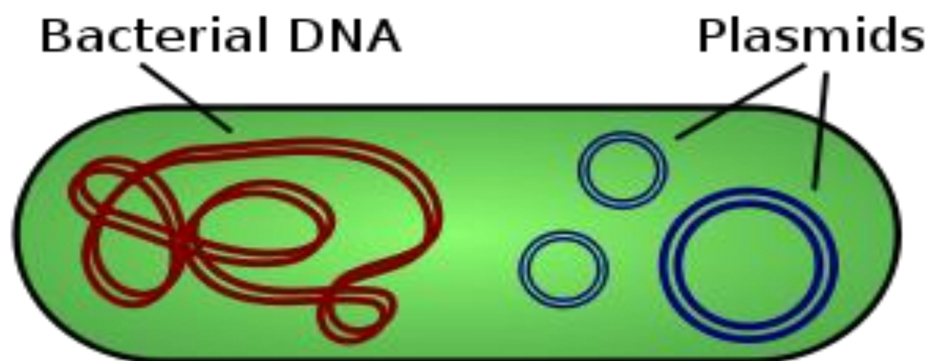


Fig. 2:19 Bacterial genomic and plasmid DNA

The most notorious property of plasmids lies in their ability to disseminate antibiotic resistance genes (Dale and von Schantz, 2003). Plasmids are a major cause of spread of bacterial resistance, as they can be transferred between Gram negative bacteria by conjugation and Gram positive bacteria by bacterial viruses called transducing phages. This transferability is responsible for many outbreaks of resistance, especially when appropriate infection control measures are breached in hospital settings (Samaha-Kfoury and Araj, 2003). Plasmid-associated resistance genes have been discovered for a majority of the known antimicrobials, including the quinolones and fluoroquinolones, and it is not uncommon for a single plasmid to simultaneously mediate resistance to five or six antimicrobials (Sherley *et al*., 2004).

Antibiotic resistance is not the limit of the ability of plasmids, nor the reason for their existence. Apart from resistance to antibiotics, accessory functional region of plasmids may also bear resistance genes to heavy metals, disinfectants, production of colicins (bacteriocins), degradation of various compounds, and virulence functions (Carattoli *et al*., 2005). Interest tends to focus on antibiotic resistance because of its importance in medical Microbiology, and because of the ease with which resistance genes can be isolated and studied. However, many naturally occurring plasmids code for other properties or even for none at all. Plasmids exist because they can replicate within bacteria, and sometimes spread from one bacterium to another. They are a form of DNA parasite. Any advantage they confer on the host bacterium is a bonus that helps the plasmid to survive (Dale and von Schantz, 2003).

Extended-spectrum β-lactamases (ESBLs) genes are located on plasmids that can be easily transferred between and within bacterial species. Some ESBL genes are mutant derivatives of established plasmid-mediated β-lactamases (e.g., $bla_{TEM/SHV}$), and others are mobilized from the

environment (bla_{CTX-M}) (Overdevest *et al*., 2011). They have an extended substrate profile which allows the hydrolysis of all cephalosporins, penicillins, and aztreonam. These enzymes are most commonly produced by *Klebsiella* sp. and *Escherichia coli* (Sharma *et al*., 2010).

CHAPTER 3

MATERIALS AND METHODS

3.1 Study Area

The Study Area is Ahmadu Bello University, main campus, Samaru, Zaria. Kaduna state, Nigeria. Ahmadu Bello University was founded in 1962 from 3 previously independent institutions; the Nigerian college of Arts, Science and Technology, Institute of Administration in Tudun Wada area of Zaria, and the Regional Research Station of the Ministry of Agriculture now the Institute for Agricultural Research. The University is situated in Samaru, Zaria in the Sabon Gari Local Government area of Kaduna State. It is located on latitude 11° 15'N to 11°3'N of the equator and longitude 7° 30'E to 7°45'E of Greenwich Meridian (Abbas and Arigbede, 2012).

3.2 Media, Reagents and Steriliations

The brands and formulation of media used in this study as well as composition of reagents are stated in Appendices V and VI of this write up. The temperature conditions for incubations and sterilizations are stated in the methodology.

3.3 Determination of Sample Size

The sample size was determined using the equation $n = \frac{Z^2 P (1-P)}{d^2}$ (Bland, 1999)

Where

n = sample size

Z= 95% confidence interval = 1.96

P= prevalence rate =16.8 (Mansouri and Ramazanzadeh, 2009)

d= precision allowable error = 5% = 0.05

$$n = \frac{(1.96)^2 \; 0.17 \, (0.83)}{(0.05)^2} = \frac{3.84 \times 0.1411}{0.0025} = \frac{0.5418}{0.0025} = 216.72$$

$n \approx 217$

This was rounded off to 300 samples

3.4 Collection of Food Samples

A total of three hundred (300) samples of ‘ready- to eat’ food and drinks items (75 samples from each item namely, ‘zoborodo’, ‘kunun zaki’, processed meat [‘suya’] and smoked fish) were purchased from sellers in stores and markets within A.B.U. main campus and its environs. The food items were immediately transported to the laboratory for microbiological analyses.

3.5 Enrichment and Serial Dilution

Upon arrival at the laboratory, 25 ml/25 g of each food sample was homogenized in 225 ml of buffered peptone water. A disinfected blender (using 70% ethanol for disinfection) was used to obtain the homogenate in the case of the solid food samples. The homogenate was pre-enriched by incubating for 18-24 hr at 37°C before serial dilution. Ten fold serial dilutions were prepared at the end of the enrichment using 1% buffered peptone water as diluent (Valentine-Bon *et al*., 2008).

3.6 Isolation of Test Organisms

Eosin Methylene Blue (EMB) agar was used for the differential and selective culturing of the faecal coliforms at 44.5^{o}C within 18-24 hr (Ajayi *et al.,* 2008). Colonies which appear pinkish-red on MacConkey agar plates were considered lactose fermenters (Chapin and Lauderable, 2007). While colonies with greenish-black appearance with metallic sheen on and those with dark purple appearance with no sheen were considered as presumptive *E. coli* and *Klebsiella* sp. respectively. Representatives of bacterial colony types with the above features were picked and sub-cultured on sterile EMB plates by streaking and incubated at 44.5^{o}C for 24 hr. The isolates were purified by repeated streaking on plates until pure culture was obtained before storing on nutrient agar slants at 4°C as working and stock cultures (Ogunshe *et al.,* 2006).

3.7 Characterization of the Isolates

The presumptive isolates were further subjected to a series of biochemical tests, which include carbohydrate fermentation test, indole test, methyl red and Voges Proskaur tests, citrate utilization test, hydrogen sulphide production and motility tests (Farasat *et al*., 2012).

3.7.1 Carbohydrate utilization test

The purple base broth was used for carbohydrate (lactose) fermentation test. Precisely, 35 g/L of DEV Lactose Peptone Broth (Merck, Germany) was dissolved in distilled water by heating gently and dispensed 10 ml of the broth in test-tubes. Inverted Durham tubes were afterward inserted into the base of the test-tubes to trap any gas produced. Sterilization was done by autoclaving at 121°C for 15mins. After cooling, the sterile tubes were inoculated with a loop-full each of 18-24 hr old organism growing in test tubes containing nutrient broth . These were labelled and incubated at 37°C for 24 hr. An uninoculated fermentation tube was used as the control. Colour change of broth from purple to yellow indicated acid production while formation of bubble in Durham tube indicates gas production.

3.7.2 Sulphur Indole Motility (SIM) test

Sulphur Indole Motility (SIM) medium was used for three (3) tests in one.

3.7.2.1 *Production of hydrogen sulphide (H_2S)*

This test was used to determine metabolism of certain sulphur- containing amino acids to produce hydrogen sulphide. Organisms which formed a lot of sulphide form visible amounts of black ferrous sulphide in SIM medium. The SIM medium was stab-inoculated with a suspension of 18-24 hr old culture on loop straight into the medium and incubate at 35^{0}C for 24-48 hr. Medium turned black in hydrogen sulphide positive test.

3.7.2.2 *Indole test*

This test detected the ability of an organism to produce indole from the amino acid tryptophan. The organism was grown for 48 hr in the SIM medium and then Kovàc’s indole reagent was added at the surface of the tube. The closed container was gently shaken. In the positive test,

indole (present in the culture) dissolved in the reagent which then became pink or red, and formed a layer at the surface of the medium (Singleton, 1997). In the negative test, no change will occurred when Kovàc's reagent was added.

3.7.2.3 *Motility test*

The test showed the way an organism grows on solid media. The SIM medium was stab-inoculated with culture on straight wire into the medium and incubated at 35^{0}C for 18-24 hr. The motile organisms spread throughout the medium from the stab while the motility negative bacteria only grew in the stabbed region of the medium (Singleton, 1997).

3.7.3 Citrate utilization test

This test detected the ability of an organism to use citrate as the sole source of carbon. Precisely, 24.28 g/L of Simmon's citrate agar was prepared according to manufacturer's instruction. A saline suspension of the test organism was made from growth on a solid medium. Using a straight wire, Simmon's citrate agar was stabbed-inoculated with the suspension and incubated at 30-35°C for five days. Bacteria with citrate permease uptook citric acid, causing alkaline end products that changed pH indicator to blue., hence slant changed to blue; for the citrate utilization negative organism slant, remained green (Singleton, 1997).

3.7.4 Methyl red (MR) test

The MR test detected the ability of an organism, growing in a phosphate-buffered glucose-peptone medium, to produce sufficient acid (from the metabolism of glucose) to reduce pH of the medium from 7.5 to about 4.4 or below. The glucose medium was inoculated with and then incubated for at least 48 hours at 37°C, following which the pH of the culture was tested xby

adding a few drops of methyl red (yellow at pH 6.2, red at pH 4.4); with a MR-positive organism the culture became red (Singleton, 1997).

3.7.5 Voges Proskauer (VP) test

The test detected the ability of an organism to form acetoin (acetyl-methylcarbinol). A phosphate-buffered glucose-peptone medium was inoculated with the test strain and incubated at 37°C for 2 days. Some 0.6 ml of 5% α-napthol, and 0.2 ml of 40% potassium hydroxide solution, were added sequentially to 1 ml of culture; the tube was then shaken vigorously, placed in a sloping position (for maximum exposure of the culture to air), and examined after 30 and 60 minutes. Acetoin (where present) was apparently oxidized to diacetyl (CH_3.CO.CO CH_3) which, under test conditions, gave a red colouration (a positive VP test). [Singleton, 1997]. The VP test was used to aid in the differentiation between genera (such as *E. coli* from the *Klebsiella and Enterobacter* groups) and other species of the Enterobacteriaceae family (Chapin and Lauderable, 2007).

3.7.6 Identification of the isolates

Isolates giving atypical responses to any of the above named tests were examined further using Microgen™Gram negative Identification A system. The data obtained by the Microgen GN-ID A microwell strip was designed to generate a 4 digit octal code which was used to interpret the result by the Microgen Identification System Software (Appendices I &II).

3.8 Collection of Plant Materials

Healthy, disease-free leaves, bark and roots of *Carica papaya* were collected within A.B.U. Zaria main campus. Authentication of the plant was carried out at the Herbarium, by a Taxonomist, Mr. U.S. Gallah of the Department of Biological Sciences, Ahmadu Bello University, Zaria. The voucher specimen number was 285.

3.9 Pretreatment of plant parts

The leaves were cleaned with cloth; roots were washed under running tap water and then spread out along with the stem-bark in the laboratory to air-dry away from sunlight. After proper drying has been ensured, the plant materials were pulverized by mechanical grinding using mortal and pestle. These were then weighed, packed in nylon bags and labelled.

3.10 Preparation of Plant Extracts

Plant extraction was carried out using the Soxhlet method and maceration with separating funnels.

3.10.1 Extraction using the Soxhlet method

The Soxhlet apparatus was used for the extraction of the dry stem bark. The coarse plant material (stem bark, 568 g) was placed in a porous container made of cotton, called timble. Methanol was added to the distilling pot as well as the timble to soak the plant material and the condensed methanol from the pot to extract continuously. The methanol vapour generated by gently heating the reservoir condensed and it was allowed to drip back onto the timble. The liquid condensate that dripped onto the sample performed the extraction which then passed through the container and back into the reservoir. The cycle was repeated continuously and sustained as long as needed until a clear solvent was obtained from the timble. As the extraction of the stem extract

progressed the bioactive components were concentrated in the reservoir. This was transferred onto an evaporatory dish and concentrated *in vacuo* at about 45-50^{0}C to obtain 99.88 g chocolate brown mass of the stem bark residue which is equivalent to 17.58% ($^w/_w$) and coded Methanol extract stem bark (MESB) [Beginners, 2013].

3.10.2 Extraction by maceration using separating funnels

The coarse leaves (300 g) and roots (468 g) were separately extracted by maceration using separating funnels. The separating funnels were suspended in iron rings (retort stand). The stoppers were removed and made sure the stop clocks were closed. The plant materials were added to about half the volume of the funnels and 300 ml methanol-water was added as the solvent in the ratio 70:30, then the stoppers placed on the tops. These were allowed to stand for 72 hr; forming two separate layers (see Appendix 4, plate 3). The stoppers were then removed, and the liquid extracts were drained into clean containers. This was followed by washing the funnels with about 150 ml each of the methanol: water mixture. The liquid extracts obtained were poured in evaporatory dishes and concentrated in avacuo at about 45-50^{0}C. The open vessels were left to evaporate the last traces of the solvent to obtain 58.66 g and 54.44 g of the leaf and root residue respectively (Beginners, 2013). The leaf extract gave a black mass of 19.55% while root bark extract was 11.63%.

3.11 Phytochemical Screening

3.11.1 Detection of Carbohydrates

3.11.1.1 Molisch's *test*

Two to three drops of Molisch reagent were added to 2 g of extract in a test tube and a small quantity of concentrated tetra-oxo-sulphate (iv) acid (H_2SO_4) was allowed to run down the side of the test tube. The presence of a lower, purple to violet colour at the interface indicated the presence of carbohydrates (Trease and Evans, 1983).

3.11.1.2 *Fehlings test for reducing sugar*

The residue was redissolved in 5 ml of water in the water bath. To 2 ml of the solution in the test tube, 1ml each of Fehling's solutions A and B were added. The mixture was shaken and heated in a water bath for 10 min. A brick-red precipitate indicated presence of reducing sugar (Onwukaeme *et al*., 2007).

3.11.2 Detection of Glycosides

About 5 ml of conc. H_2SO_4 was added to the extract and boiled for 15 min. This was then cooled and neutralized with 20% KOH and was divided into two portions. Another part of the extract was dissolved in distilled water, this was used as a control; no acid hydrolysis.

3.11.2.1 *Fehling's solution test*

Five millilitres each of Fehling's solutions A and B were added to the first portion and boiled for three minutes. A brick-red precipitate indicated the glycone portion as a result of hydrolysis of glycoside.

3.11.2.2 *Ferric chloride test*

Two to three drops of ferric chloride solution were added to the second portion. Green to black precipitate indicated phenolic aglycone as a result of hydrolysis of glycosides (Trease and Evans, 1983).

3.11.2.3 *Test for cardiac glycosides (Kella Killiani's test)*

About 2 g of extract was dissolved in 10 ml glacier acetic acid containing traces of ferric chloride. The test tube was held at an angle of 45 degree, 1 ml of conc. H_2SO_4 was added down the side. Purple ring colour at the interface indicated cardiac glycosides (Trease and Evans, 1983).

3.11.2.4 *Kadde's test*

About 1 ml of 2% 3,5-dinitrobenzoic acid in 5 ml 95% alcohol was added to the 2 g of the extract. The solution was made alkaline with 2 ml 5% sodium hydroxide; appearance of purple-blue colour indicated the presence of cardenolides in the ring (Trease and Evans, 1983).

3.11.3 Detection of Saponins (Frothing test)

To about 0.5 g of the coarse powder in a test tube, 5 ml of distilled water was added and vigorously shaken for about 30 sec. A persistent froth that last for at least 15 min indicated presence of saponins (Ibrahim *et al*., 2006).

3.11.4 Detection of Flavonoids (Sodium hydroxide test)

Two to three drops of aqueous NaOH were added to 5 ml of extract, a yellow colouration showed the presence of flavonoid (Trease and Evans, 1983).

3.11.5 Detection of Tannins (Ferric chloride test)

About 0.5 g of extract was dissolved in 10 ml of distilled water and filtered. Two to three drops of ferric chloride solution were added to the filtrate. Formation of a blue-black precipitate indicated hydrolysable tannins while green precipitate indicated the presence of condensed tannin (Trease and Evans, 1983).

3.11.6 Detection of Alkaloids

3.11.6.1 *Mayer's test*

Two to three drops of Mayer's reagent were added to the extract in a test tube. A cream precipitate indicated presence of alkaloids.

3.11.6.2 *Dragendoff's test*

Two to three drops of Dragendoff's reagent were added to the extract in a test tube. A rose red precipitate indicated presence of alkaloids.

3.11.6.3 *Wagner's test*

Two to three drops of Wagner's reagent were added to the extract in a test tube. A whitish precipitate indicated presence of alkaloids.

3.11.7 Detection of Resins

A portion of 2 g of the extract was dissolved in 10 ml of acetic anhydride. One drop of conc. H_2SO_4 was added. Appearance of purple colour, which rapidly changed to violet, indicated presence of resins (Ibrahim *et al*., 2006).

3.11.8 Detection of Anthraquinone derivatives

3.11.8.1 *Test for free anthraquinones (Borntrager's test)*

An amount of 5 g of the extract was shaken with 10 ml of benezene and filtered. Five millilitres (5 ml) of 10% of ammonia solution was added to the filtrate and stirred. The production of a pink-red or violet colour indicated the presence of free anthraquinones.

3.11.8.2 *Test for combined anthracene (Modified Borntrager's test)*

Two gram of the extract was boiled with 5 ml of 10% hydrochloric acid for 3 mins. This was to hydrolyse the glycosides to yield aglycones which were soluble in hot water only. The solution was filtered hot; the filterate was cooled and extracted with 5 ml of benzene. The benezene layer was filtered off and shaken gently with half its volume of 10% ammonia solution. A rose-pink or a cherry red colour indicated combined antracene (Trease and Evans, 1983).

3.11.9 Detection of Steroids and Triterpenes

3.11.9.1 *Salkowsk's test*

An amount of 0.5 g of the extract was dissolved in 2 ml of chloroform and 3-4 drops of conc. H_2SO_4 were added to form a lower layer. A reddish-brown colour indicated the presence of a steroidal ring (Trease and Evans, 1983).

3.11.9.2 *Lieberman-Burchard's test*

Five millilitres of acetic anhydride was added to 2 g of the extract. About 1ml of conc. H_2SO_4 was added down the side of the tube; the colour change was observed immediately and later

found to retain the colour. A red, pink or purple colour indicated the presence of triterpenes while blue or blue-green indicated steroids (Trease and Evans, 1983).

3.12 Preparation of different concentrations of the extracts

Stock solutions were prepared according to the method of Rahman *et al*. (2011) with slight modifications. The plant extracts were prepared at the concentration of 1gml^{-1}(1000ugml^{-1}) in dimethylsulphoxide (DMSO) and used as stock solution. From the stock solutions, double fold dilutions were prepared to obtain the following concentrations (500, 250 and 125µg ml^{-1}). Briefly, 10 g of the extract was dissolved in 10 ml of DMSO to obtain 1 gml^{-1} or 1000µg ml^{-1} which served as the stock solution. Four tubes were obtained and labelled 1, 2, 3, and 4. An amount of 10 ml of the stock solution was dissolved in 10 ml of DMSO in tube 1 to obtain 500µg ml^{-1}. 10ml of the solution from tube 1 was further dissolved in 10 ml of DMSO in tube 2 to obtain 250µg ml^{-1}. In the same manner, solution in tube 3 was prepared to obtain 125µg ml^{-1}. Disc impregnated with only DMSO in tube 4 without the extract was used as negative control. Standard drug used as positive control were ampicillin disc (10µg) and later amikacin as second positive controls.

3.13 Screening of Plant Extracts for Antibacterial Activity

This was performed using paper disc diffusion assay method as described by Elayaraja *et al*., (2008). Paper discs of uniform size (8mm in diameter) were prepared using Whatmann No. 1 filter paper. The paper discs were sterilized in hot air oven at 160^{0}C for one hour. The discs were

then impregnated with 0.1 ml of 500, 250 and 125μg ml^{-1} concentrations of the plant extracts. The solvent dimethylsulphoxide (DMSO) was used as negative control. According to Bauer *et al*. (1966) with a little modification, few pure colonies (3 to 10) of the 18-24 hr test organisms were picked from nutrient agar plates and introduced onto Mueller-Hinton broth and incubated at 37^0C for 2-5 hrs to produce a bacterial suspension of moderate cloudiness. This suspension was diluted (where necessary) with physiological saline to match 0.5 McFaland standard (0.5ml of one percent $BaCl_2$ to 99.5ml of one percent H_2SO_4) [equivalent to 3.0 x 10^8 bacterial density]. An aliquot of 0.1ml broth suspension was used to streak the large petridishes of sterile Mueller-Hinton agar and allowed to dry for about 3-5 minutes before the extract paper discs were placed on the agar plates with sterile forceps, gently pressed down to ensure contact. Plates were incubated within 30 minutes at 35^0C for 18-24 hr. The zone diameters of inhibition were measured using a transparent metre rule on the undersurface of the petridishes. The tests were carried out in duplicates and average values were recorded.

3.14 Determination of Beta-Lactamase Production using nitrocefin sticks

All characterized isolates were tested for β-lactamase production using nitrocefin-containing identification sticks (Oxoid Ltd., Basingstoke, Hamphire, England) [AppendixVII, plate VI]. The container with the sticks was removed from freezer and allowed to attain room temperature (26-28^0C). The technique according to the manufacturer's manual was carried out thus: An 18-24 hr representative colony was selected from nutrient agar medium. A stick was removed from the container, and holding the coloured end, touched the colony with the nitrocefin impregnated end of the stick, the stick was rotated to pick off a small mass of cells from a 24 hr culture of test bacteria and kept up to 24 hr in an incubator at 37^oC. A change in the colour of the stick indicated β-lactamase production (Oncel *et al*., 2004). The reaction required moisture, so the tip

of the stick was placed in the moisture condensate on the lid before picking up the organism. Where condensate was not available in the inverted plate, a drop of distilled water was added to the lid and used to moisten the tip of the stick.

3.15 Antibiotic Susceptibility Testing

Susceptibility of the isolates to some β-lactams and commonly used antibiotics was determined using the disc- diffusion method as recommended by Clinical Laboratory Institute Standards (CLSI, 2008). The bacterial isolates were grown for 18 to 24 h on nutrient agar. They were suspended in 2 ml sterile normal saline and turbidity adjusted to match McFarland Opacity Standard No0.5 (equivalent to 3.0 x 10^8 bacterial density). Bacterial suspensions of 0.1 ml were dispensed on the surface of the Mueller-Hinton agar plate and spread evenly using a sterile spreader. The inoculum was allowed to dry for 5 min and antibiotic discs were dispensed on the surface of the media and incubated aerobically at 37^oC for 18 h. Results were classified as susceptible, intermediate or resistant, according to the approved clinical breakpoints (CLSI, 2008). A standard strain *E.coli* ATCC 25922, obtained from National Institute for Pharmaceutical Research, Idu, Abuja was used as quality control. The following antimicrobial agents (single discs, Oxoid Ltd., Basingstoke, Hamphire, England) were tested Ampicillin (10µg), Cephalothin (30µg), Cefpodoxime (10µg), Ceftriaxone (30µg), Ciprofloxacin (5µg), Trimethoprim/Sulphamethoxazole (25µg), Tetracycline (30µg) Amikacin (30µg) and amoxicillin-clavulanic acid (25µg) [CLIS, 2008].

3.15.1 Detection of ESBLs Producing Bacteria

This was carried out in two stages.

Screening test- Measurement of diameter of zone of inhibition for cefpodoxime and ceftriaxone taken in the susceptibility test was used. Organisms were considered potential ESBL-producer if zone of inhibition measured less than 22mm and less than 25mm for cefpodoxime and ceftriaxone discs respectively (CLIS, 2008).

Confirmatory test- The double disc synergy test (DDST) was performed. Discs of cefpodoxime and ceftriaxone alone were placed at a distance of 25 mm to amoxicillin-clavulanic acid on a Mueller Hinton agar plate earlier inoculated with a bacterial suspension of 0.5 McFarland turbidity standards and incubated overnight at 35-37^{0}C for 18 hr. Organisms were confirmed ESBLs producers if synergy between cefpodoxime and ceftriaxone and amoxicillin associated with clavulanic acid was detected i.e zones of inhibitions of 5 mm or greater obtained when compared with discs without clavulante (Tande *et al*., 2009. Tawfik *et al*., 2012).

3.16 Identification of Multidrug Resistance (MDR) Strains

The number of antibiotic each bacterium was resistant to in the disc diffusion test was noted for identification of multidrugresistant strains. Multidrug resistance (MDR) was taken as resistant to four or more antibiotics tested (Ezekiel *et al*., 2011).

3.17 Calculation of Multiple Antibiotic Resistance (MAR) Index

Multiple antibiotic resistance (MAR) index is a measure of the extent of antimicrobial agent resistance for the isolates in the group studied. It was calculated as a/b where a represents the number of antibiotics to which the isolates were resistant and ‘b’ represents the total number of antibiotics to which the isolate was exposed (Apun *et al*., 2008).

3.18 Genotypic detection of β-lactamase genes

3.18.1 Plasmid DNA extraction

DNA extraction was carried out with Zyppy™ Plasmid Miniprep Kit (Inqaba Biotech, South Africa) using the Manufacturer's protocol.

- To a 1.5 ml microcentrifuge tube, 600 µl of bacterial culture grown overnight in LB medium[1] was added
- Followed by the addition of 100 µl of lysis buffer (blue), this was mixed thoroughly by inverting the tube 4-6 times.
- Within 2 minutes of mixing with the lysis buffer, 350 µl of cold neutralization buffer[2] (yellow), was added and mixed thoroughly.

  The sample turned yellow when the neutralization was completed and a yellowish precipitate formed. Sample was inverted additional 2-3 times to ensure complete neutralization.
- It was centrifuged at 13,500 x *g* for 3 minutes.
- Approximately, 900 µl of the supernatant was transferred into the spin column.

  Disturbance of the cell debris pellet was avoided by gently transferring the supernatant.
- The column was placed into a collection tube and centrifuged at 13,500 x g for 15 seconds.
- The flow-through was discarded and the column was placed back into the same collection tube.
- This was followed by addition of 200 µl of Endo-Wash buffer[3] to the column and centrifuge at 13,500 x g for 15 seconds.
- After this, 400 µl of Zyppy™ wash buffer[3] was added to the column and centrifuged at 13,500 x g for 30 seconds.
- The column was transferred into a clean 1.5 ml microcentrifuge tube followed by addition of 30 µl Zyppy™ elution buffer (10 mM Tris-HCl, pH 8.5 and 0.1 mM EDTA) directly to the column matrix and allowed to stand for one minute at room temperature.
- Centrifugation was carried out at 13,500 x g for 15 seconds to elute the plasmid DNA.

[1] LB medium- Luria Betani medium (Yeast extract 5g, Tryptone 10g and NaCl 5g)/L.
[2] RNase A added to Neutralization buffer
[3] Ethanol added to wash buffer

3.18.2 Primer design

The oligonucleotide primer sequences (Bioneer Inc., USA) used for the PCR assays were obtained from Bali *et* al., (2010), and are as shown in Table 3.1.

Table 3.1: Oligonucleotide primers used for detection of β-lactamase genes.

Primers	**Melting Temperature (^oC)**	**Nucleotide Sequences (5'-3')**	**References (GenBank number)**	**Expected Amplicon Size (bp)**
SHV-F	50.6	CGCCTGTGTATTATCTCCCT	EF125011	293
SHV-R	50.2	CGAGTAGTCCACCAGATCCT		
TEM-F	56.2	TTTCGTGTCGCCCTTATTCC	AB282997	403
TEM-R	50.1	ATCGTTGTCAGAAGTAAGTTGG		

Key: F-Forward

R-Reversed

3.18.3 PCR amplification of β-lactamase gene

To amplify the sequences of TEM and SHV β-lactamase genes, PCR was carried out with the primer sets as described by Chang *et al*. (2001) and Bali *et al*. (2010) with slight modifications. Reactions were performed in a GeneAmp PCR system 2400 (Perkin-Elmer) in 20 µl reaction mixtures containing 10 µl Premix with non-interfering dye (consisting of Taq DNA polymerase, dNTPs, $MgCl_2$, reaction buffer, PCR stabilizer and enchancer at optimer concentrations). Each oligonucleotide primer concentration (i.e. forward and reversed) added was 0.5µl, while template concentration added was 1.0µl. The PCR conditions used were 35 cycles of amplification at a denaturation temperature of 94^0C for 3 mins (first cycle only), subsequently 94^0C for 45s, an annealing temperature of 51^0C for 30s, and an extension temperature 72^0C for one min. This step was followed by a final extension at 72^0C for one min.

3.18.4 Agarose gel electrophoresis

Ten microliters (10µl) of PCR products were loaded into wells of 1.0% agarose gel containing ethidium bromide. A molecular size marker, O'GeneRuler^{TM} 100bp DNA ladder (Fermentas) or at other times Perfect 1,000bp DNA ladder- SibGene was run on both sides with PCR products**.** Electrophoresis was carried out in Tris Acetate EDTA buffer (1x contains; Tris 40mM, Acetic Acid 20Mm, EDTA 1Mm, pH 8.0; BIOLAND SCIENTIFIC LLC) containing ethidium bromide (20 ml of 50 X TAE and 4.0 µl of 10 µg/ml ethidium bromide per litre) at 90 V for 40min. Plasmids were viewed on a U/V transilluminator and photographs taken using a gel documenting machine (Gel doc 2000; BIO-RAD). Plasmid sizes were assessed and estimated from the molecular sizes of the DNA ladder against their migration distance (Ombui *et al*., 2000).

3.18.5 Purification of PCR products

One strand each of the bands obtained for the genes were purified for sequencing. Protocol for PCR purification using AccuPrep PCR purification kit (Bioneer Inc. USA) was carried out as follows:

1. DNA fragments were excised from the agarose gel, and the gel slice was weighed in a clean 1.5 ml micro-centrifuge tube.
2. Three volumes of buffer $_{(1)}$ (gel binding buffer) were added to one volume gel slice.
3. Incubation was carried out at 60^{0}C for 10 min while the tubes were vortexed every 2-3min. during incubation for dissolution.
4. Further incubation and vortexing were repeated until complete dissolution was ensured by the appearance of a yellow colour.
5. The mixtures were transferred into the DNA binding column tubes and centrifuge for 1min. at 13,000rpm.

6. The flow-through is poured off and the DNA binding filter column re-assembled with the 2.0 ml collection tube.
7. To the DNA binding column tubes, 500 µl of buffer $_{(2)}$ was added and centrifuged for one min. at 13,000 rpm. *This step removes salts and soluble impurities in the DNA binding column tube. The loss of DNA in this step is negligible.*
8. The flow-through was poured off and the DNA filter column was re-assembled with the 2.0 ml collection tube.
9. Steps 7 and 8 were repeated.
10. Drying was carried out by additional centrifugation at 13,000rpm for one min to remove the residual ethanol. The DNA binding filter column was transferred to the , new 1.5ml micro-centrifuge tube.
11. Thirty microlitres (30µl) of buffer $_{(3)}$ was added to the centre of the DNA binding filter column, and allowed to stand for at least one min, at room temperature for elution.
12. Fragment DNA were eluted by centrifugation at 13,000rpm for 1min.

3.18.6 Plasmid DNA sequencing

Sequencing of the purified PCR products were performed with the Dye Terminator Cycle Sequencing (DTCS) Quick Start kit using the sequencer CEQ 2000 XL DNA Analysis System (BECKMAN COULTER, U.S.A.). Sequence alignment and analysis were performed online using the Basic Local Alignment Search Tool (BLAST) program of the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov) (Kolar *et al*., 2000).

Procedure for sequencing reactions were as follows;

1. Preparation of the DNA sequencing reaction

   Sequencing reactions were prepared in 0.2 ml thin-wall tubes and all the reagents were added in the order listed below.

   dH_2O 8.0µl

DNA template	2.0 µl
Primer	2.0 µl
DTCS Quick start Master Mix	8.0 µl

2. Thermal cycling programme

   Initial temperature 96^{0}C for 20sec;

   Annealing temperature 50^{0}C for 20sec;

   Extension temperature 60^{0}C for 4min; for 30 cycles followed by holding at 4^{0}C.

3. Ethanol Precipitation

   Precipitation in individual tubes was carried out as follows:

   a. A labelled, sterile 0.5ml microfuge tube was prepared for each sample.
   b. Fresh Stop Solution/Glycogen mixture was prepared as follows (per sequencing reaction); 2 µl of 3 M Sodium Acetate (pH5.2), 2 µl of 100 mM Na_2-EDTA (pH8.0) and 1µl of 20 mg/ml of glycogen (supplied with the kit). To each of the labelled tubes, 5 µl of the Stop Solution/glycogen was added.
   c. The sequencing reaction was transferred to the appropriately labelled 0.5 ml microfuge tubes and mixed thoroughly.
   d. To the above, 60 µl cold 95% (v/v) ethanol from -20^{0}C freezer was added and mixed thoroughly. Centrifugation was carried out immediately at 14,000rpm at 4^{0}C for 15 minutes. Supernatant was carefully removed using micropipettes. *The pellets were visible at this stage*.
   e. Pellets were rinsed twice with 200 µl 70% (v/v) ethanol from -20^{0}C freezer. Centrifugation was carried out immediately at 14,000rpm at 4^{0}C for 4min. Supernatant was carefully removed after centrifugation with a micropipette.
   f. This was followed by vacuum drying for 10 min.
   g. Samples were resuspended in 40 µl of the Sample Loading Solution (Provided in the kit) and allowed to stand for 10 min.

4. Sample preparation for loading into the instrument:

a. Resuspended samples were transferred to the appropriate wells of the sample plate.
b. Each of the resuspended samples was overlaid with one drop of light mineral oil.
c. Sample plate was loaded into the instrument and the desired method started.

3.19 Statistical Analysis

Data obtained were subjected to statistical analysis using different tools. Student t-test was used to compare if there were significant differences between the number of *E.coli* and *Klebsiella* spp. resistant to each antibiotic used in this study (Tables 4.8 and 4.10). One way analysis of variance was used to determine whether there significant differences in the response to the tested antibiotics by the bacteria isolated from the different food samples. Duncan multiple range test was used to separate the means. (Table 4.12). Pearson's correlation was used to establish significance in association between multidrug resistance and ESBLs production (Appendix VII). Finally, Spearman's rank correlation coefficient was used to identify whether there is a relationship between the presence of either TEM or SHV genes and resistance to third generation cephalosporins. All statistical analysis was performed at 95% confidence interval and p values less than 0.05 were considered significant (Bland, 1999).

CHAPTER 4

RESULTS

A total of 53 bacterial isolates identified as *E.coli* and *Klebsiella* spp. were obtained from the ready-to-eat food and drink items. Characterization and identification of the isolates using Microgen Identification kit is stated in Appendix II. *E.coli* were 49 (92%) while Klebsiella spp. obtained were 4 (8%). Table 1 shows their distribution in the food samples. This showed that *E.coli* was widely distributed in the food samples. *Klebsiella* spp. was not isolated from 'kunun-

zaki' and smoked fish but was isolated from 'zoborodo' and processed meat 'suya'. Three of the *Klebsiella* spp. were *K. oxytoca* while one was *K. ozaenae* (Fig. 4.1). Unfortunately, the only *E.coli* isolated from 'kunun-zaki' was lost during subculture as it became unculturable after characterization; hence fifty two (52) bacterial isolates were used in this study.

Table 4.1: Distribution of bacterial isolates from food samples

Type of Food sample	**No. of food sample analysed**	**No. of *E. coli* Isolates**	**No. of *Klebsiella spp.* isolates**	**Total no. of isolates**
'Kununzaki'	75	1	0	**1**
'Zoborodo'	75	11	2	**13**
Smoked fish	75	19	0	**19**

'Suya'	75	18	2	**20**
TOTAL	**300**	**49**	**4**	**53**

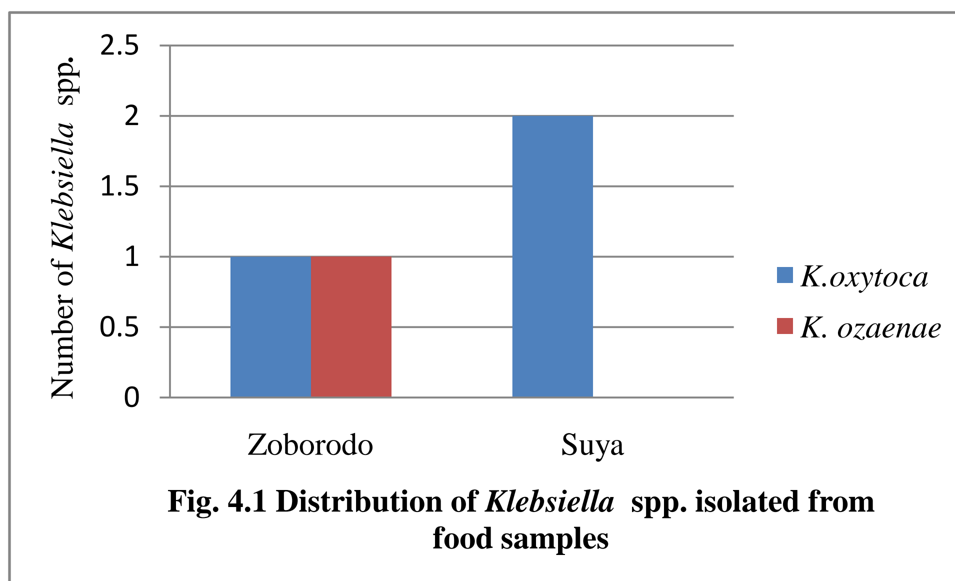


Fig. 4.1 Distribution of *Klebsiella* spp. isolated from food samples

The results of the phytochemical analyses of the stem-bark, leaf and root extracts of *Carica papaya* are shown in Table 4.2. The leaf extract contained more of the active components than the root or stem-bark. Tannins, flavonoids, glycosides, steroids and triterpenes, resins, carbohydrates were present in the leaf extract while only carbohydrates, saponins and steroids

were present in the root extract. The stem-bark contains carbohydrates, glycosides, saponins, flavonoids, alkaloids, steroids and triterpenes.

Table 4.2: Phytochemical Constituents of the Leaf, Root and Stem bark of *Carica papaya* extracts

Phytochemical constituents	**Test**	**Inferences**		
		Leaves	Root	Stem-bark

Carbohydrates	**Molisch's test**	+	+	+
Reducing sugar	**Fehling's test**	+	-	-
Glycosides				
General tests	**Fehling's test**	+	-	-
	$FeCl_3$ test	+	-	+
Cardiac glycosides	Kella-Killiani test	+	-	+
	Kadde test	+	-	-
Anthraquinone derivatives				
Free antraquinones				
Combined anthracene	Borntrager's test	-	-	-
	Modified Borntrager's test	+	-	-
Resins				
	Acetic anhydride test	+	-	-
Saponins				
	Frothing test	-	+	+
Flavonoids				
	Sodium hydroxide test	+	-	+
Tannins				
Hydrolysable tannins				
	Ferric chloride test	+	-	-
Alkaloids				
	Mayer's test	-	-	+
	Dragendoff's test	-	-	+
	Wagner's test	-	-	+
Steroids and triterpenes				
Steroids				
Steroids and triterpenes	Salkowsk's test	+	+	+
	Lieberman-Burchard's test	**+(T)**	-	**+(S)**

Key: + = Present; - = Absent; T = Triterpene; S=Steroid

The methanolic extracts showed no antibacterial activity in all the concentrations prepared (500µg, 250µg, 125µg,) for the *E.coli* reference strain ATCC 25922 and the bacterial strains tested which were *E.coli* and *Klebsiella* spp. (Tables 4.3, 4.4 and 4.5). There was growth up to the edge of the filter paper disks and so the 8 mm obtained in all the measurements was the size of the Whatmann filter paper used. Ampicillin disc alone was used as positive control in the sensitivity test against the bacteria isolated from smoked fish (Table 4.3). However, some strains were resistant to it. The zones of inhibitions of 6 mm were obtained from plates where bacteria grew to the edge of the ampicillin disc, and so what was left to measure was the diameter of the disc which was 6 mm. The DMSO remained the negative control.

In Table 4.4, the antibiotic disc amikacin was added to the regimen of positive controls and was found to be better as positive control than the ampicillin discs because all the bacterial strains were susceptible to amikacin.

In Table 4.5 amikacin was also used along with ampicillin as positive controls. Several of the bacterial strains were resistant to ampicillin with growth to the edge of the disc but all were susceptible to amikacin.

Table 4.3: Zone diameters of inhibition (mm) of plant extracts against isolates from smoked fish

Isolate Code	Conc. of root-bark extract (µg/ml)			Positive controls		Conc. Of stem-bark extract (µg/ml)			Positive controls		Conc. Of leaf extract (µg/ml)			Positive Controls		Nega-tive control
	500	250	125	AMP	AK	500	250	125	AMP	AK	500	250	125	AMP	AK	DMSO
SFe1	8	8	8	19	ND	8	8	8	19	ND	8	8	8	20	ND	8
SFe2	8	8	8	6	ND	8	8	8	6	ND	8	8	8	6	ND	8
SFe3	8	8	8	7	ND	8	8	8	18	ND	8	8	8	16	ND	8
SFe4	8	8	8	19	ND	8	8	8	20	ND	8	8	8	20	ND	8
SFe5	8	8	8	18	ND	8	8	8	16	ND	8	8	8	16	ND	8
SFe6	8	8	8	16	ND	8	8	8	16	ND	8	8	8	16	ND	8
SFe7	8	8	8	18	ND	8	8	8	16	ND	8	8	8	18	ND	8
SFe8	8	8	8	19	ND	8	8	8	17	ND	8	8	8	16	ND	8
SFe9	8	8	8	17	ND	8	8	8	20	ND	8	8	8	16	ND	8
SFe10	8	8	8	20	ND	8	8	8	16	ND	8	8	8	18	ND	8
SFe11	8	8	8	18	ND	8	8	8	6	ND	8	8	8	6	ND	8
SFe12	8	8	8	10	ND	8	8	8	6	ND	8	8	8	6	ND	8
SFe13	8	8	8	6	ND	8	8	8	16	ND	8	8	8	16	ND	8
SFe14	8	8	8	20	ND	8	8	8	16	ND	8	8	8	18	ND	8
SFe15	8	8	8	20	ND	8	8	8	6	ND	8	8	8	6	ND	8
SFe16	8	8	8	19	ND	8	8	8	18	ND	8	8	8	18	ND	8
SFe17	8	8	8	20	ND	8	8	8	18	ND	8	8	8	18	ND	8
SFe18	8	8	8	22	ND	8	8	8	20	ND	8	8	8	18	ND	8
SFe19	8	8	8	16	ND	8	8	8	18	ND	8	8	8	16	ND	8

Key: AMP-Ampicillin; AK- Amikacin; DMSO-Dimethylsulphoxide; ND-Not determined
SFe-*E.coli* isolated from smoked fish
Zone diameterof paper disc is 8mm
Zone diameterof antibiotic disc is 6mm

Table 4.4: Zone diameters of inhibition (mm) of plant extracts against isolates from processed meat 'suya'

Isolate Code	Conc. of root-bark extract (µg/ml)			Positive controls		Conc. Of stem-bark extract (µg/ml)			Positive controls		Conc. of leaf extract (µg/ml)			Positive Controls		Negative control
	500	250	125	AMP	AK	500	250	125	AMP	AK	500	250	125	AMP	AK	DMSO
SYe1	8	8	8	6	23	8	8	8	6	23	8	8	8	6	24	8
SYe2	8	8	8	7	22	8	8	8	6	22	8	8	8	6	23	8
SYe3	8	8	8	8	18	8	8	8	13	18	8	8	8	13	19	8
SYe4	8	8	8	20	21	8	8	8	16	21	8	8	8	16	22	8
SYe5	8	8	8	21	21	8	8	8	18	21	8	8	8	18	22	8
SYe6	8	8	8	6	21	8	8	8	6	21	8	8	8	6	22	8
SYe7	8	8	8	6	25	8	8	8	6	25	8	8	8	6	26	8
SYe8	8	8	8	6	21	8	8	8	6	21	8	8	8	6	22	8
SYe9	8	8	8	6	22	8	8	8	6	22	8	8	8	6	23	8
SYe10	8	8	8	6	21	8	8	8	6	21	8	8	8	6	22	8
SYe11	8	8	8	20	25	8	8	8	18	25	8	8	8	18	26	8
SYe12	8	8	8	21	21	8	8	8	18	21	8	8	8	18	22	8
SYe13	8	8	8	15	20	8	8	8	12	20	8	8	8	12	21	8
SYe14	8	8	8	6	20	8	8	8	6	20	8	8	8	6	21	8
SYe15	8	8	8	21	22	8	8	8	21	22	8	8	8	21	23	8
SYe16	8	8	8	6	22	8	8	8	6	22	8	8	8	6	23	8
SYe17	8	8	8	6	20	8	8	8	6	20	8	8	8	6	21	8
SYe18	8	8	8	19	21	8	8	8	16	21	8	8	8	16	22	8
SYk1	8	8	8	6	22	8	8	8	6	22	8	8	8	6	23	8
SYk2	8	8	8	7	20	8	8	8	6	20	8	8	8	6	21	8

Key: AMP-Ampicillin; AK- Amikacin; DMSO-Dimethylsulphoxide
SYe- *E.coli* isolated from processed meat 'suya'
SYk-*Klebsiella* spp. isolated from processed meat 'suya'
Zone diameterof paper disc is 8mm
Zone diameterof antibiotic disc is 6mm

Table 4.5: Zone diameters of inhibition (mm) of plant extracts against isolates from 'zoborodo' drink

Isolate Code	Conc. of root-bark extract (µg/ml)			Positive controls		Conc. Of stem-bark extract (µg/ml)			Positive controls		Conc. of leaf extract (µg/ml)			Positive Controls		Negative control
	500	**250**	**125**	**AMP**	**AK**	**500**	**250**	**125**	**AMP**	**AK**	**500**	**250**	**125**	**AMP**	**AK**	**DMSO**
Ze1	8	8	8	18	26	8	8	8	20	26	8	8	8	16	25	8
Ze2	8	8	8	17	22	8	8	8	16	22	8	8	8	17	21	8
Ze3	8	8	8	18	25	8	8	8	16	24	8	8	8	16	24	8
Ze4	8	8	8	17	21	8	8	8	15	22	8	8	8	16	21	8
Ze5	8	8	8	6	24	8	8	8	6	24	8	8	8	6	24	8
Ze6	8	8	8	6	23	8	8	8	6	24	8	8	8	6	23	8
Ze7	8	8	8	6	24	8	8	8	6	24	8	8	8	6	24	8
Ze8	8	8	8	6	25	8	8	8	6	24	8	8	8	6	24	8
Ze9	8	8	8	6	20	8	8	8	6	21	8	8	8	6	21	8
Ze10	8	8	8	20	22	8	8	8	18	23	8	8	8	18	22	8
Ze11	8	8	8	6	27	8	8	8	6	26	8	8	8	6	25	8
Zk1	8	8	8	6	27	8	8	8	6	25	8	8	8	6	25	8
Zk2	8	8	8	6	25	8	8	8	6	24	8	8	8	6	25	8

Key: AMP-Ampicillin; AK- Amikacin; DMSO-Dimethylsulphoxide
Ze- *E.coli* isolated from 'zoborodo' drink
Zk-*Klebsiella* spp. isolated from 'zoborodo' drink
Zone diameterof paper disc is 8mm
Zone diameterof antibiotic disc is 6mm

Antibiotic sensitivity of *E.coli* isolated from smoked fish is shown on Table 4.6. The best activity was found in ciprofloxacin, amikacin and amoxicillin-clavulanic acid with 100% activity against all the isolates. This is followed by susceptibility to sulphamethoxazole-trimethoprim (84%). Lowest activity was found in cephalothin and tetracycline with 53% and 42% resistance respectively. Minimal resistance was observedin response to the third generation cephalosporins i.e cefpodoxime and ceftriaxone having 26% and 21% resistance respectively. Resistance to ampicillin was found in 7 (37%) of the isolates.

Table 4.6: Antibiotic sensitivity of *E.coli* isolated from smoked fish (n=19)

Antibiotics class/structural group	Abbreviation	Disc content µg	Number (%) resistant organisms	Number (%) Susceptible organisms
AMPICILLIN (β-lactam-amino-penicillin)	AMP	10	7 (37)	12 (63)
CEPHALOTHIN (β-lactam-1st generation Cephalosporin)	KF	30	10 (53)	9(47)
CEFPODOXIME* (β-lactam-3rd generation Cephalosporin)	CPD	10	5 (26)	14 (74)
CEFTRIAXONE* (β-lactam-3rd generation Cephalosporin)	CRO	30	4 (21)	15 (79)
CIPROFLOXACIN (Fluoroquinone)	CIP	5	0 (0)	19 (100)
SULPHAMETHOXAZOLE/ TRIMETOPRIM (Sulphonamide)	SXT	25	3 (16)	16 (84)

TETRACYCLINE (Tetracyclines)	TE	30	8 (42)	11 (58)
AMIKACIN (Aminoglycosides)	AK	30	0 (0)	19 (100)
AMOXICILLIN-CLAVULANIC ACID (β-lactam-β-lactamase inhibitor)	AMC	25	0 (0)	19 (100)

*One of the five indicator cephalosporins for detection of ESBLs production

The pattern of resistance is shown in Table 4.7. Ten resistance phenotypes were obtained with single antibiotic resistance types (KF and AMP resistance) and eight multiple resistance types with varying combinations of 2, 3, 4 and 6 antibiotics. Highest frequency (4) was found in combinations with three antibiotics. Broad spectrum resistance (i.e. resistance to ampicillin or cephalothin) was identified in 11 (58%) of the isolates (Fig. 4.2) while production of ESBLs was detected in 5(26%) of the isolates (Fig. 4.3). Multidrug resistance (MDR) is regarded as resistance to four or more antibiotics. Three (16%) of the *E. coli* isolates exhibited multidrug resistance and two of the three MDR strains were ESBLs producers. Pearson correlation showed highly significant correlation between ESBLs production and multidrug resistance (Appendix VIII). Among the *E. coli* isolates from smoked fish, 10 (53%) of the tested bacteria had MAR index greater than 0.2 (Fig. 4.4).

Table 4.7: Resistance pattern of *E. coli* isolated from smoked fish (n=19)

Single antibiotic resistance		Multiple antibiotic resistance		
Number of Isolates (%) in the Category	Resistance Phenotype	Number of Antibiotic Combinations	Number of Isolates (%) with the Pattern	Resistance Phenotype
1 (11)	KF	2	1 (5)	KF, TE
			1 (5)	AMP, KF
1 (5)	AMP		1 (5)	CPD, TE
		3	1 (5)	KF, CPD, CRO
			2 (11)	AMP, KF, TE
			1 (5)	CPD, CRO, TE
		4	1 (5)	AMP, KF, SXT, TE
		6	2 (11)	AMP, KF, CPD, CRO, SXT, TE

Key: AMP-Ampicillin; KF- Cephalothin; CPD- Cefpodoxime; CRO-Ceftriaxone; CIP-Ciprofloxacin; SXT- Sulphamethoxazole-trimethoprim (Co-trimethoprim); TE-Tetracycline; AK-Amikacin

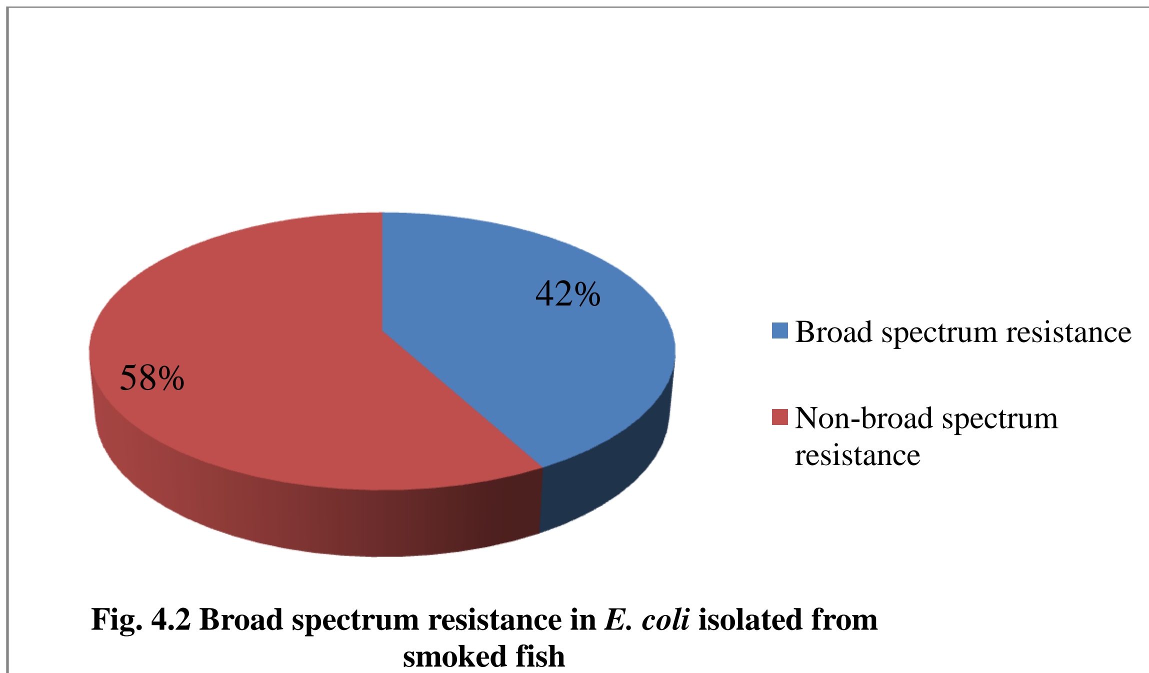


Fig. 4.2 Broad spectrum resistance in *E. coli* isolated from smoked fish

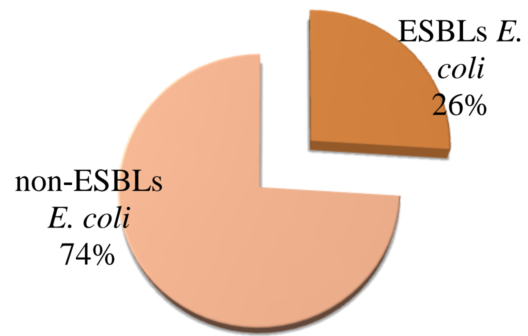


Fig. 4.3 Percentage ESBLs producing *E.coli* from smoked fish

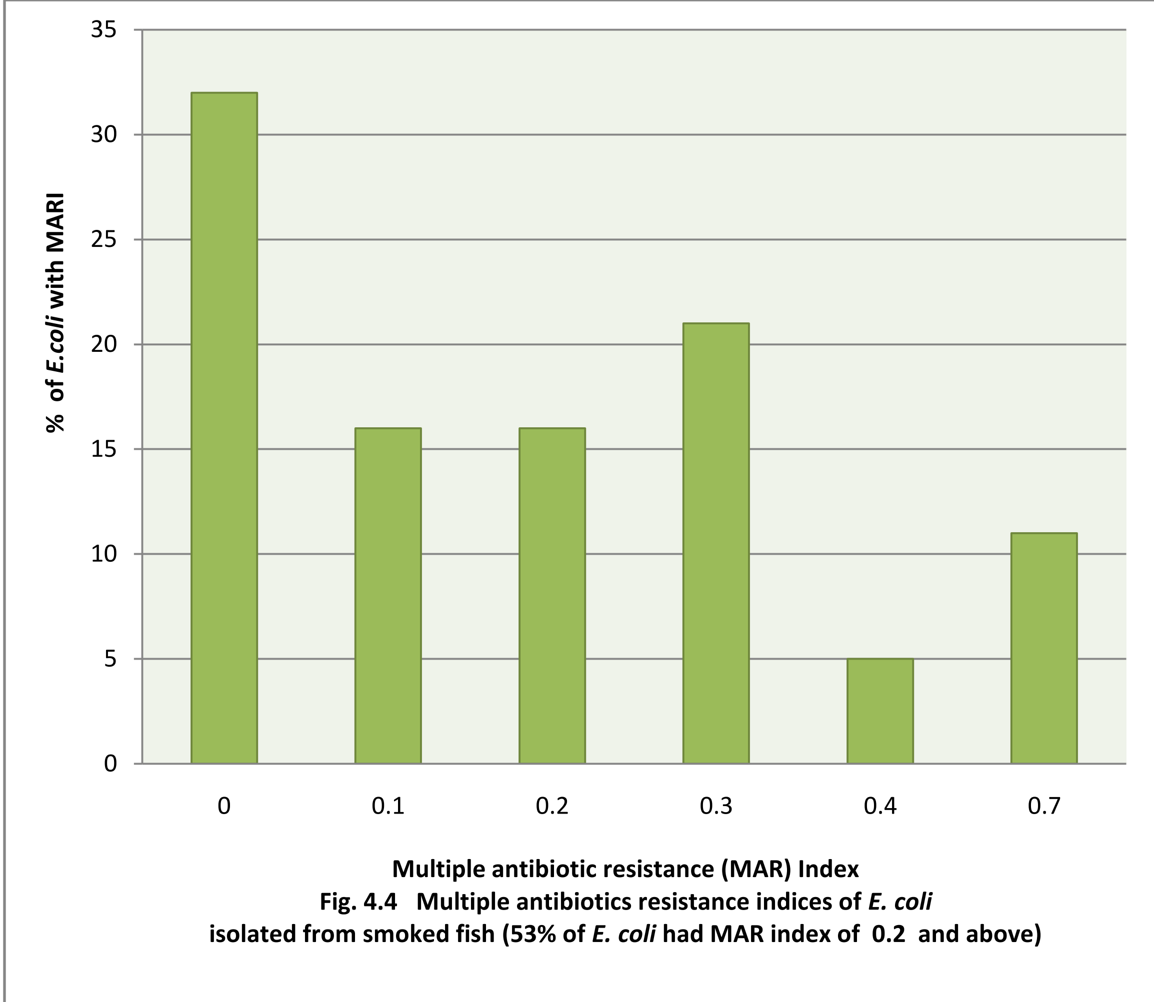


Fig. 4.4 Multiple antibiotics resistance indices of *E. coli* isolated from smoked fish (53% of *E. coli* had MAR index of 0.2 and above)

Antibiotic resistance of *E.coli* and *Klebsiella* spp. isolated from processed meat 'suya' is shown in Table 4.8. The best activity was found in ciprofloxacin and amikacin with no resistant isolate, which is 100% sensitivity. Two (11%) of the isolates were resistant to amoxicillin-clavulanic acid. Lowest activity was recorded in cephalothin and ampicillin with 17(85%) and 15(75%) of the resistant isolates respectively. The two *Klebsiella spp*. were both resistant to ampicillin, cephalothin and tetracycline. Student t-test was used to compare mean of resistance of *E. coli* and *Klebsiella* spp. within the group and found that significant differences exist in resistance to ampicillin, cephalothin, cefpodoxime and tetracycline ($p<0.05$). In all the cases the mean antibiotic resistance in *Klebsiella* spp. were found to be higher than those of *E.coli* strains.

Table 4.8: Antibiotic resistance of *E.coli* and *Klebsiella* spp. isolated from processed meat 'suya' (n=20)

Bacteria isolates	Number of isolates	Antibiotics tested Number (%) of resistant bacteria to the antimicrobial agents								
		AMP	KF	**CPD**	**CRO**	**CIP**	**SXT**	**TE**	**AK**	**AMC**
E. coli	18	13 (72)	15 (83)	8 (44)	3 (17)	0 (0)	9 (50)	11 (61)	0 (0)	2 (11)
***Klebsiella* spp.**	2	2 (100)	2 (100)	1 (50)	0 (0)	0 (0)	1 (50)	2 (100)	0 (0)	0 (0)
Total	**20**	**15 (75)**	**17 (85)**	**9 (45)**	**3 (15)**	**0 (0)**	**10(50)**	**13 (65)**	**0 (0)**	**2 (10)**
t-value		**7.778**	**9.192**	**6.965**	**3.000**		**0.000**	**6.363**		**2.000**
p-value		**0.008***	**0.005***	**0.045***	**0.102**		**0.05**	**0.011***		**0.147**

Student t-test * =Significant at the 0.05 level (2-tailed)

Key: AMP-Ampicillin; KF- Cephalothin; CPD- Cefpodoxime; CRO-Ceftriaxone; CIP-Ciprofloxacin; SXT- Sulphamethoxazole-trimethoprim (Co-trimethoprim); TE-Tetracycline; AK-Amikacin; AMC- Amoxicillin-clavulanic acid (Augmentin)

The resistance pattern of *E.coli* and *Klebsiella* spp. isolated from 'suya' is shown in Table 4.9. Twelve resistance phenotypes were obtained with a single antibiotic resistance type (KF resistance) and eleven (11) multiple resistance types with varying combinations of 2, 3, 4, 5, 6 and 7 antibiotics. Highest frequency of five (5) was found in combinations of five antibiotics. Broad spectrum resistance (i.e. resistance to ampicillin or cephalothin) was identified in 15 (83%) of *E. coli* strains and one out of two (50%) of the *Klebsiella* spp. (Fig. 4.5) while production of ESBLs was detected in 8 (44%) of the *E. coli* strains and one out of two (50%) of the *Klebsiella* spp.. (Fig.4.6). Multidrug resistance (MDR) that is, resistance to four or more antibiotics was exhibited by eleven (55%) of the entire isolates obtained from 'suya', eight of the MDR strains were ESBLs producers. Pearson correlation showed significant correlation ($P<0.05$) between ESBLs production and multidrug resistance in *E. coli* and *Klebsiella* spp. isolated from 'suya' (AppendixVII). Two (11%) *E. coli* strains were susceptible to all the antibiotics and constitute the population with multiple antibiotic resistance index of 0.0 (Fig.4.7). A high antibiotic resistance is observed in this category of organisms which had 67% *E.coli* strains and 100% *Klebsiella* spp. with MAR index greater than 0.2.

Table 4.9: Resistance pattern of *E. coli* and *Klebsiella* spp. isolated from 'suya'

Single antibiotic Resistance		**Multiple antibiotic resistance**		
Number of Isolates (%) in the Category	Resistance Phenotype	Number of Antibiotic Combinations	Number of Isolates (%) with the Pattern	Resistance Phenotype

2(10)	KF	2	1 (5)	SXT, TE
			1 (5)	AMP, KF
		3	1 (5)	AMP, KF, CPD
			1 (5)	AMP, KF, SXT
			1 (5)	AMP, KF, TE
		4	3 (15)	AMP, KF, SXT, TE
			1 (5)	AMP, KF, CPD, TE
		5	2 (10)	AMP, KF, CPD, CRO, TE
			3 (15)	AMP, KF, CPD, SXT, TE
		6	1 (5)	AMP, KF, CRO, SXT, TE, AMC
		7	1 (5)	AMP, KF, CPD, CRO, SXT, TE, AMC

Key: AMP-Ampicillin; KF- Cephalothin; CPD- Cefpodoxime; CRO-Ceftriaxone; CIP-Ciprofloxacin; SXT- Sulphamethoxazole-trimethoprim (Co-trimethoprim); TE-Tetracycline; AK-Amikacin; AMC- Amoxycillin-clavulanic acid (Augmentin)

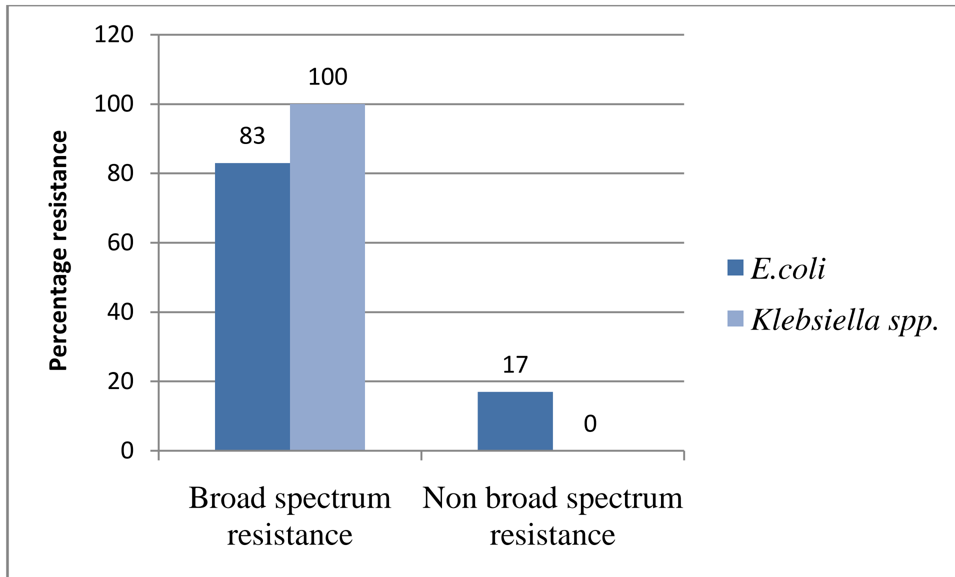


Fig. 4.5 Broad spectrum resistance in *E. coli* and *Klebsiella* spp. isolated from 'suya'

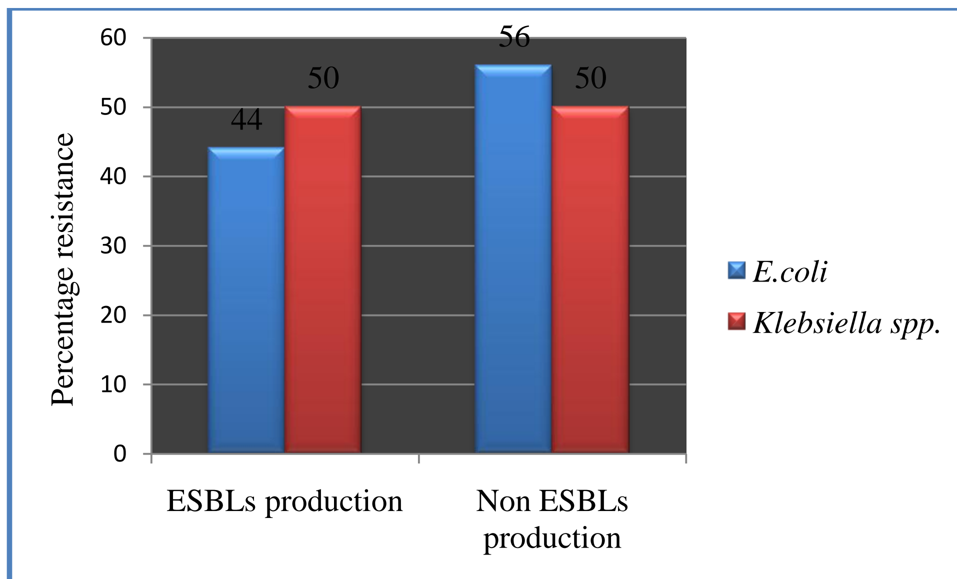


Fig. 4.6 ESBLs production among *E. coli* and *Klebsiella* spp. isolated from 'suya'

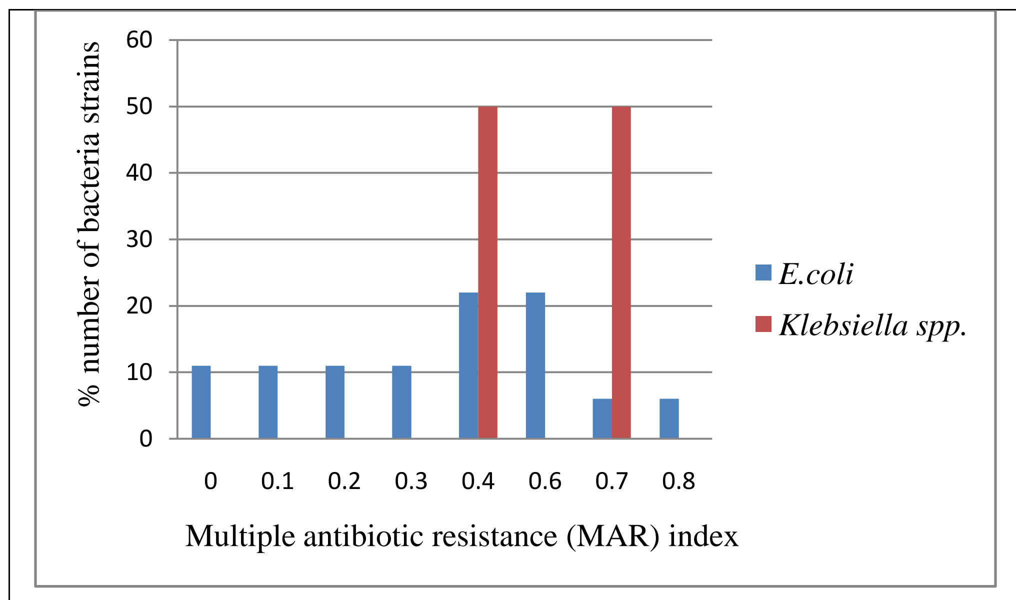


Fig. 4.7 MAR indices of *E.coli* and *Klebsiella* spp. isolated from 'suya'

(About 67% *E.coli* strains and 100% *Klebsiella* spp. had MAR index greater than 0.2)

Antibiotic resistance of *E.coli* and *Klebsiella* spp. isolated from ‘zoborodo’ is shown in Table 4.10. The best activity was found in ciprofloxacillin and amikacin with no resistant isolate that is 100% subceptibility. Lowest activity was recorded in cephalothin with 92% resistance, followed by ampicillin and tetracycline with 9(69%) and 7(54%) respectively. The two *Klebsiella spp*. were both resistant to cefpodoxime but sensitive to ceftriaxone. Student t-test was used to compare mean of resistance of *E. coli* and *Klebsiella* spp. within the group and found that significant differences exist in resistance to ampicillin, cephalothin, sulphamethoxazole-trimethoprim and tetracycline ($p<0.05$).

Table 4.10: Antibiotic resistance of *E.coli* and *Klebsiella* spp. isolated from 'zoborodo' (n=13)

Bacteria isolates	Number of isolates	Antibiotics tested Number (%) of resistant bacteria to the antimicrobial agents								
		AMP	**KF**	**CPD**	**CRO**	**CIP**	**SXT**	**TE**	**AK**	**AMC**
E. coli	11	7 (64)	10 (91)	2 (18)	1 (9)	0 (0)	1 (9)	6 (55)	0 (0)	0 (0)
***Klebsiella* spp.**	2	2 (100)	2 (100)	2(100)	0 (0)	0 (0)	1 (50)	1 (50)	0 (0)	2(100)
Total	**13**	**9 (69)**	**12 (92)**	**4 (31)**	**1 (8)**	**0 (0)**	**2(15)**	**7 (54)**	**0 (0)**	**2 (15)**
t-value		**3.536**	**5.657**	**0.500**	**0.032**		**7.960**	**6.364**		**2.00**
p-value		**0.035***	**0.015***	**2.920**	**6.314**		**0.040***	**0.012***		**6.314**

Student t-test * =Significant at the 0.05 level (2-tailed)
Key: AMP-Ampicillin; KF- Cephalothin; CPD- Cefpodoxime; CRO-Ceftriaxone; CIP-Ciprofloxacin; SXT- Sulphamethoxazole-trimethoprim (Co-trimethoprim); TE-Tetracycline; AK-Amikacin; AMC- Amoxicillin-clavulanic acid (Augmentin)

The pattern of resistance of *E. coli* and *Klebsiella* spp. isolated from 'zoborodo' drink is shown in Table 4.11. Seven resistance phenotypes were obtained with a single antibiotic resistance type (KF resistance) and six (6) multiple resistance types with varying combinations of 2, 3, 4, 5 and 6 antibiotics. Highest frequency of four (4) was found in combinations of three antibiotics. Broad spectrum resistance (i.e. resistance to ampicillin or cephalothin) was identified in 10 (91%) of the *E. coli* strains and in the two *Klebsiella* spp. (100%) (Fig. 4.8) while production of ESBLs was detected in two (18%) of *E. coli* strains and in the two (100%) *Klebsiella* spp. (Fig.4.9). Multidrug resistance (MDR) that is, resistance to four or more antibiotics was exhibited by four (31%) of the entire isolates and all were ESBLs producers. Pearson correlation showed complete correlation (P= 0.00) between ESBLs production and multidrug resistance in *E. coli* and *Klebsiella* spp. isolated from zoborodo' (Appendix VIII). One (9%) *E. coli* strain was susceptible to all the antibiotics and had multiple antibiotic resistance index of 0.0 (Fig. 4.10). A high antibiotic resistance was also observed in this category of bacteria with 54% *E. coli* and 100% *Klebsiella* spp. having multiple antibiotic resistance (MAR) index greater than 0.2.

Table 4.11: Resistance pattern of *E. coli* and *Klebsiella* spp. isolated from 'zoborodo' drink

Single antibiotic Resistance		**Multiple antibiotic resistance**		
Number of Isolates (%) in the Category	Resistance Phenotype	Number of Antibiotic Combinations	Number of Isolates (%) with the Pattern	Resistance Phenotype
3 (23)	KF	2	1 (8)	AMP, KF
		3	4(31)	AMP, KF, TE
		4	1 (8)	AMP, KF, CPD, AMC
		5	1 (8)	AMP, KF, CPD, CRO, TE
				AMP, KF, CPD, SXT, TE
			1 (8)	AMP, KF, CPD, SXT, TE, AMC
		6	1 (8)	

Key: AMP-Ampicillin; KF- Cephalothin; CPD- Cefpodoxime; CRO-Ceftriaxone; CIP-Ciprofloxacin; SXT- Sulphamethoxazole-trimethoprim (Co-trimethoprim); TE-Tetracycline; AK-Amikacin; AMC- Amoxycillin-clavulanic acid (Augmentin)

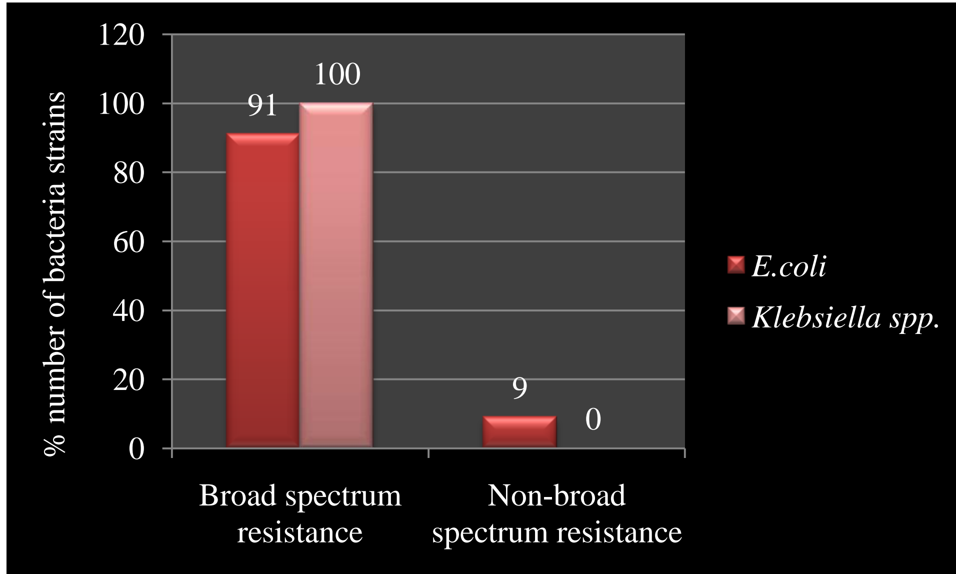


Fig. 4.8 Broad spectrum resistance in *E.coli* and *Klebsiella* spp. isolated from zoborodo drink

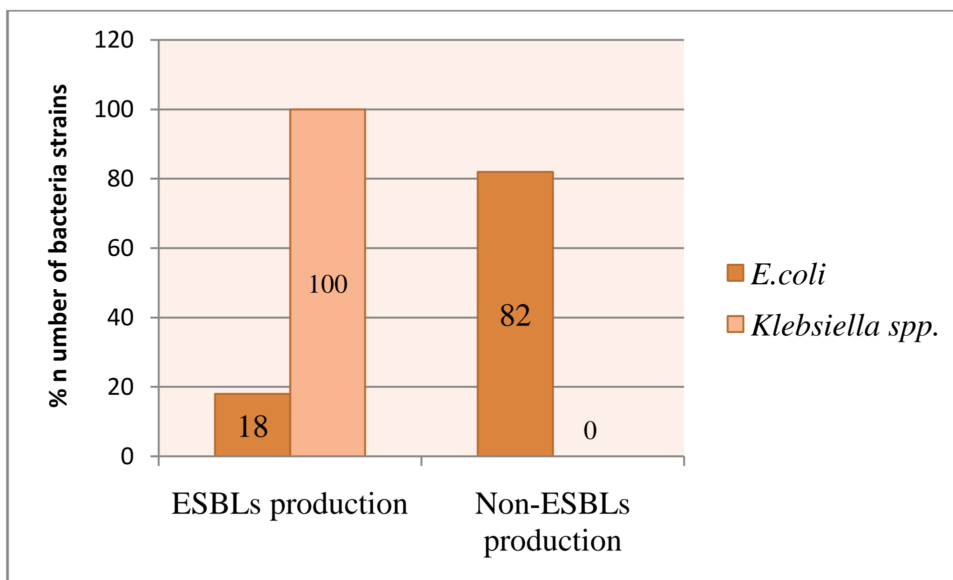


Fig. 4.9 ESBLs producing *E.coli* and *Klebsiella* spp. isolated from zoborodo drink

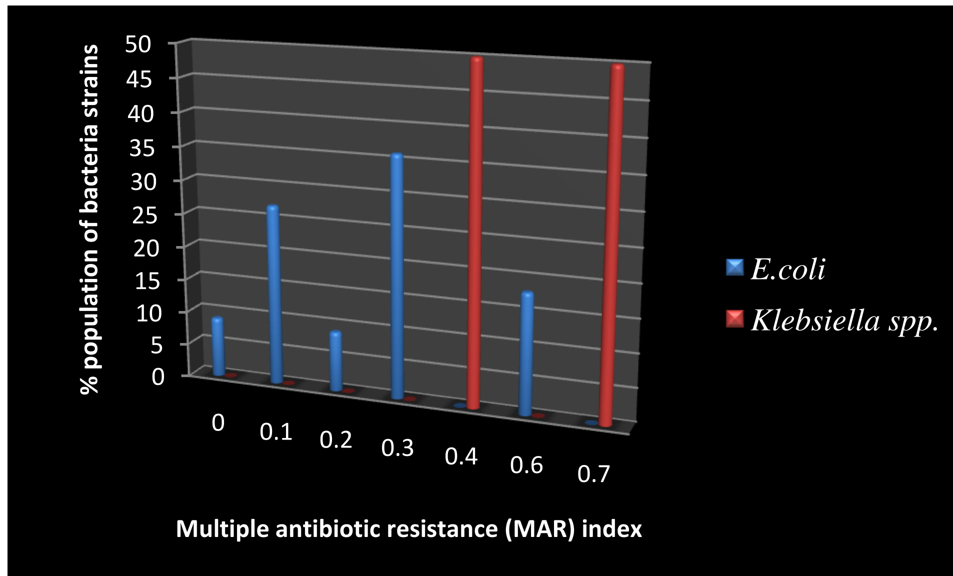


Fig. 4.10 MAR indices of *E.coli* and *Klebsiella* spp. isolated from zoborodo drink.
(About 54% *E.coli* strains and 100% *Klebsiella* spp. had MAR index greater than 0.2)

Analysis of the pooled antibiotic resistance of all the isolates from the ready-to-eat food items is shown in Table 4.12. As in the different food samples, all the isolates were sensitive to ciprofloxacin and amikacin. Four (15%) isolates were resistant to amoxicillin-clavulanic acid. Cephalothin and ampicillin had the lowest activity with 75% and 60% resistance, followed by tetracycline having 54% resistance. Prevalence of ESBLs resistance obtained for all the isolates was 35%. Analysis of variance was used to compare the means. Significance difference was found to exist in the resistances to ampicillin, cephalothin, sulphamethoxazole-trimethoprim and tetracycline. Duncan Multiple Range Test was used to separate the means. Values with different superscripts are significantly different ($p<0.05$). Antibiotic resistance exhibited by isolates from 'suya' were found to be higher than those of smoked fish or 'zoborodo'.

Table 4.12: Analysis of the pooled number (%) of antibiotic resistance of all the bacteria isolated from the food samples; n=52

Sources of bacteria isolates	No. of isolates	**Antibiotics tested** Number (%) of resistant bacteria to the antimicrobial agents								
		AMP	**KF**	**CPD**	**CRO**	**CIP**	**SXT**	**TE**	**AK**	**AMC**
Suya	20	15 (75)[a]	17 (85)[a]	9 (45)	3 (15)	0 (0)	10(50)[a]	13 (65)[a]	0 (0)	2 (10)
Smoked fish	19	7 (37)[b]	10(53)[b]	5(26)	4 (21)	0 (0)	3 (16)[b]	8 (42)[b]	0 (0)	0(0)
Zoborodo	13	9 (69)[b]	12 (92)[ab]	4 (31)	1 (8)	0 (0)	2(15)[b]	7 (54)[b]	0 (0)	2 (15)
Total	**52**	**31(60)**	**39(75)**	**18(35)**	**8(15)**	**0 (0)**	**15(29)**	**28(54)**	**0 (0)**	**4(8)**
F-value		**17.33**	**16.33**	**7.00**	**3.48**		**19.00**	**10.33**		**2.00**
p-value		**0.023***	**0.024***	**0.074**	**0.165**		**0.020***	**0.045***		**0.281**

Key: AMP-Ampicillin; KF- Cephalothin; CPD- Cefpodoxime; CRO-Ceftriaxone; CIP-Ciprofloxacin; SXT- Sulphamethoxazole-trimethoprim (Co-trimethoprim); TE-Tetracycline; AK-Amikacin; AMC- Amoxycillin-clavulanic acid (Augmentin)

*=Significant differences exist between the isolates from the different food samples
Values with different superscripts within the group are significantly different ($p<0.05$)
Means were separated using Duncan Multiple Range Test (DMRT)

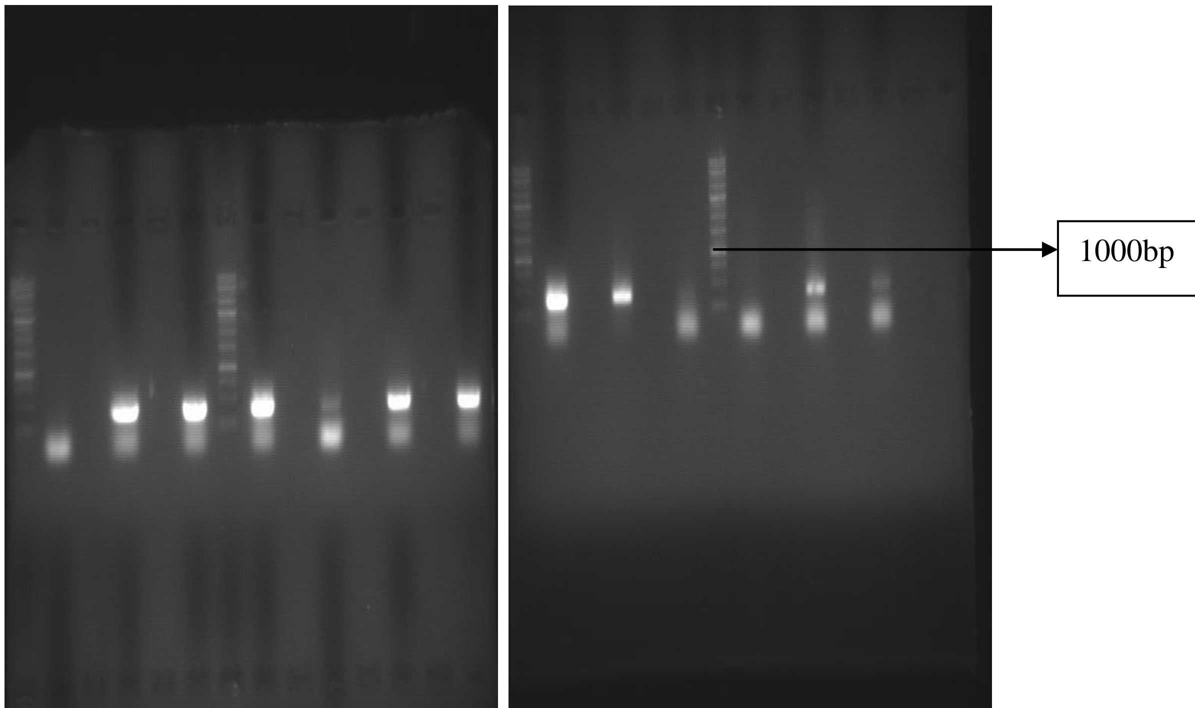
Sensitivity of nitrocefin sticks was calculated in comparism to the disc diffusion test shown in Table 4.13. The significance is that rapid β-lactamase tests can yield clinically relevant information earlier than a MIC test or disc diffusion. In this test, the nitrocefin sticks detected β-lactamase in only four (4) isolates while ampicillin resistance (due β-lactamase production) was detected in 31 isolated using disc diffusion test. Sensitivity of the new and rapid test was therefore calculated to be $\frac{4}{31} \times 100 = 12.9\%$

Table 4.13: Sensitivity of nitrocefin sticks using disc diffusion test (DDT) as the gold standard

Source of isolates	Number of organisms	Ampicillin Resistance Using DDT	β-lactamase detection using nitrocefin sticks	Calculated sensitivity
Smoked fish	19	7	0	**12.9%**
Suya	20	15	1	
Zoborodo	13	9	3	
Total	**52**	**31**	**4**	

For the molecular studies, a total of 12 isolates were used to assay for the presence of TEM and SHV genes. TEM gene was detected in 8 (66.7%) bacterial isolates (Plate I) while the SHV gene was harboured by only one (8.3%) isolate. It should be noted that there were two bands on the SHV gel picture but that of lane 5 does not correspond to the expected base pair size and so it was regarded as a contaminant (Plate II). No isolate was found to harbour both TEM and SHV genes together. SHV gene was detected in one bacterium, *Klebsiella oxytoca* only, while TEM genes were detected in only *E.coli* strains all through. One isolate SFe 16, that was susceptible to all the antibiotics in the disc diffusion test (DDT) was found to harbour a TEM gene. The faint band underneath the bands were said to be primer dimers and this was verified when running SHV amplicons on gel. Two controls were prepared; one was tagged C-plus (C^{+}), which contained the premix, set of SHV primers, but no DNA template while the other control, C-minus (C^{-}), contained the premix only, no primers, no DNA template. Gel picture showed that the faint band did not appear in the lane C^{-} but present in all the lanes with primers. They are the primer dimers.

M 1 2 3 M 4 5 6 7 M 8 9 10 M 11 12 C



(a) (b)

Plate I: Gel electrophoresis of TEM amplicons
M-1000bp DNA Marker C-Negative Control

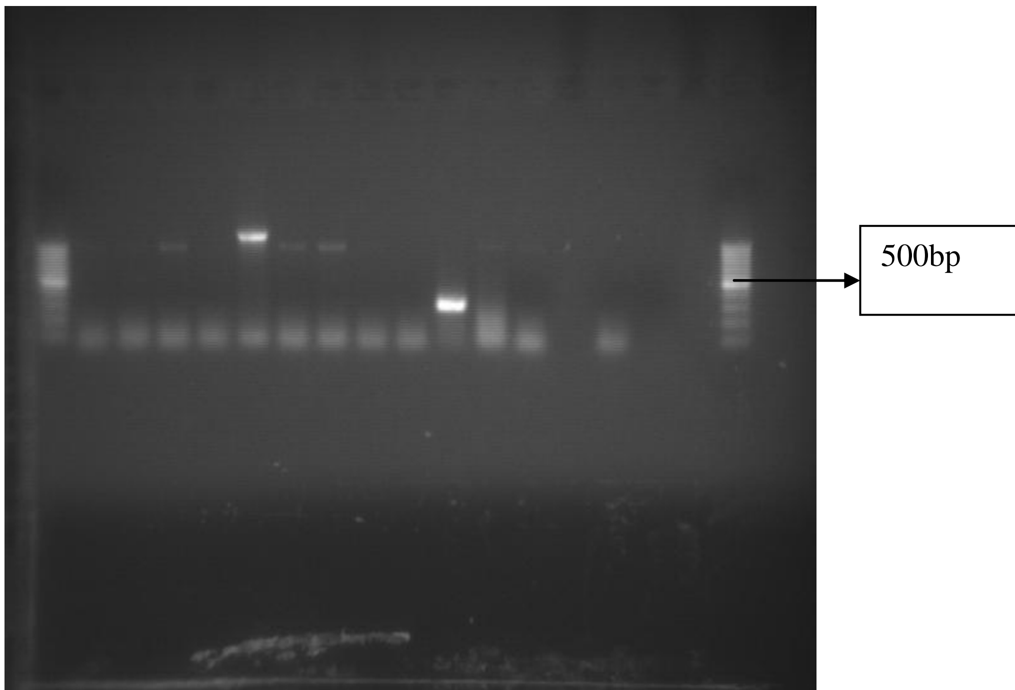

M 1 2 3 4 5 6 7 8 9 10 11 12 C+ C- M
500bp

Plate II: Gel electrophoresis of SHV amplicon
M-100bp DNA Marker
C^+-Negative control (plus primer)
C^--Negative control (no primer)

Table 4.14 shows the antibiotic resistance profile of the isolates as obtained by phenotypic test using disc diffusion test. Eleven (92%) of the isolates were resistant to Ampicillin and Cephalothin which is broad spectrum resistance while 10 (83%) of them were resistant to Cefpodoxime (CPD) and Ceftriaxone (CRO) or both which indicated extended spectrum resistance. Fig. 4.11 shows the distribution of both genes in the bacterial isolates.

Table 4.14: Antibiotic resistance profiles of the isolates used for molecular studies

S/no	Isolate code	Identification of isolate	Source of isolate	No. of antibiotics the isolate is resistant to	Antibiotic resistant phenotype	β-lactamase genes identified
1	SFe15	*E.coli*	Smoked fish	3	AMP, KF, TE	Nil
2	SFe16	*E.coli*	Smoked fish	-	Nil	TEM
3	Ze11	*E.coli*	Zoborodo	*5	AMP, KF, CPD, CRO, TE	TEM
4	Ze7	*E.coli*	Zoborodo	3	AMP, KF, CPD, SXT, TE	TEM

5	Zk2	*Klebsiella ozaenae*	Zoborodo	3	AMP, KF, CPD	Nil
6	Sye1	*E.coli*	Suya	* 6	AMP, KF, CPD, SXT, TE, AMC	TEM
7	Sye8	*E.coli*	Suya	* 5	AMP, KF, CPD, CRO, TE	TEM
8	Sye10	*E.coli*	Suya	* 5	AMP, KF, CPD, SXT, TE,	TEM
9	Sye17	*E.coli*	Suya	*7	AMP, KF, CPD, CRO, SXT, TE, AMC	TEM
10	Syk2	*Klebsiella oxytoca*	Suya	*5	AMP, KF, CPD, SXT, TE	SHV
11	Sye6	*E.coli*	Suya	*5	AMP, KF, CPD, SXT, TE	Nil
12	Sye9	*E.coli*	Suya	3	AMP, KF, CPD	TEM

Key: Sye -*E.coli* from suya; SFe- *E.coli* from smoked fish; Ze- *E.coli* from zoborodo; Zk- *Klebsiella* sp. from zoborodo; *Multidrug resistance **;** AMP-Ampicillin; KF- Cephalothin; CPD- Cefpodoxime; CRO-Ceftriaxone; CIP-Ciprofloxacin; SXT- Sulphamethoxazole-trimethoprim (Co-trimethoprim); TE-Tetracycline; AK-Amikacin; AMC-Amoxicillin-clavulanic acid (Augmentin)

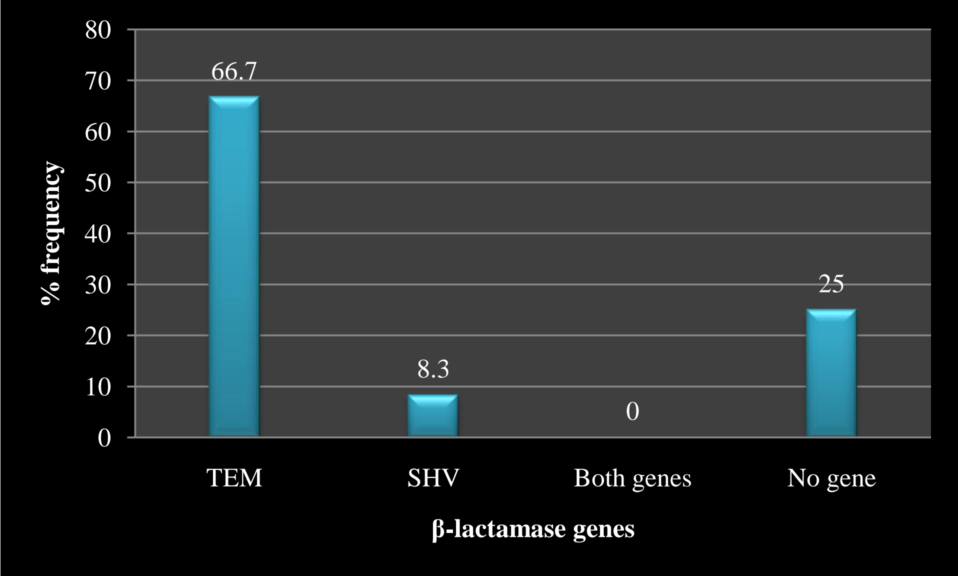


Fig. 4.11 Distribution of TEM and SHV genes in the tested bacterial strains

Result of nucleotide sequence in Sye10 (isolate 8) showed 86% homology with β-lactamase TEM-1gene but the sub-type of SHV gene could not be confirmed by sequencing possibly because the primer could not amplify the entire open reading frame of the bla_{SHV} (Table 4.15).

Table 4.15: Sequence analysis of TEM gene

Sample code	Nucleotide blasted	Number of Base pairs blasted	Sequence identity via blast	Query coverage (%)	Maximum Identity (%)	Accession Number
Sye10 (Isolate 8)	GGGACTTTTGTGGCCTTCCTGTT TGTTTGGCTCA**N**CCCAGAAACG ACTGGGGTGGAAAGTAGAAAG AATTGCTGAAGAATCAGTTGGG TTGCACGAGTGGGTTACATTCG AACTGGATCTCAACAGCGGTAA GATCTGAGAGTTTTCGCCCGAA GAACGTTTTCCATTGATGAGCA CTTTAAGTCTGCTATGTGGTGC GTATATCCGTGTTGACGCCGGG CAGAGCACTCGGTCGCGCATAC CTATTCTCGA**N**GACTGGGTGAG TACTCACAGTACAGAAAGCTTC TACGATGCTGACGTAGAAATAT GCATGCGCCTACATATATACCG CGCACTACTTGCGAAGA	347	*Escherichia coli* Plasmid pECDF 16 Extended spectrum 2 (TEM-1) gene, complete cds	87	86	JX976326.1

Sperman's correlation shows moderate correlation ($r_s = 0.258$) between the presence of any of the two genes and resistance to third generation cephalosporins ($p = 0.418$). So, the correlation is insignificant (AppendixVII).

CHAPTER 5

DISCUSSION

From the results of this study, there was no antibacterial activity of *Carica papaya* extract against the organisms by the paper disk diffusion test. This is similar to the result obtained by Nweze and Eze (2009) where there was no antibacterial activity against *E.coli* both for the type culture and the clinical isolate by a medicinal plant; *Ocimium gratissimum* leaf extract. The explanation was that some of the secondary metabolites were absent and that when present, probablyare in low concentrations or a slight alteration in the rate of diffusion of the test agent. According to Agbagwa and Okolo (2012), plant extracts are usually more active against Gram positive bacteria than Gram negative bacteria. Cheruiyot *et al*. (2009) found out that *Vernonia amygdalina* showed no activity against *E.coli* and *Pseudomonas aeruginosa* thus indicating its narrow spectrum of activity. These Scientists also stated that *Lantana camara* showed activity against *Staphylococcus aureus* but was inactive against *E.coli* probably due to the cell wall structure. In agreement with other Researchers, they stated that these observations were likely to be due to the differences in the cell wall structure between Gram-negative outer membranes acting as a barrier to many environmental substances, including antibiotics. Karou *et al*. (2007) explained further that Gram positive bacteria are often found to be more susceptible to plant extracts than the Gram-negative ones because Gram-positive bacteria have only an outer

peptidoglycan layer which is not an effective barrier while the Gram-negatives have an outer phospholipid membrane that makes the cell wall impermeable to lipophilic solutes, while the porines constitute a selective barrier to hydrophilic solutes. The test bacteria used in this study were all Gram-negative organisms. The cell walls of *E.coli* and *Klebsiella* spp. used in this study possibly inhibited the root, stem-bark and leaf *extracts* of *Carica papaya.*

The choice of the antibiotics regimen was useful in the determination of the spectra of resistance since they represent the penicillin and cephalosporin structural subclasses as well as members of other families of commonly used antibiotics for infections caused by the test bacteria. Broad-spectrum resistance was taken as resistance to ampicillin or cephalothin. In this study, most of the bacteria were resistant to cephalothin and had a pooled resistance of 75%. Cephalothin, a prototype of first of generation cephalosporins, is a narrow spectrum drug, which has it's best activity against Gram-positive pathogens except methicillin-resistant *S. aureus* (MRSA), and is active against some Gram-negative organisms, such as *E.coli* and *Klebsiella* strains (Walsh, 2003). Ampicillin resistance followed with 31 resistant organisms (60%) as shown in Table 4.12. This shows that almost all the organisms that are resistant to ampicillin are equally resistant to cephalothin while the organisms that were resistant to just one antibiotic were resistant to either ampicillin or cephalothin. Ampicillin-resistance is increasingly common and at an alarming rate. Up to 90% of ampicillin resistance in *E.coli* is due to the production of TEM-1 (Lim *et al*., 2009). This enzyme is able to hydrolyse penicillins and early cephalosporins such as cephalothin and cephaloridine. The frequency of Tem-genes in the bacteria used for the molecular aspect of this work was 66.7%. TEM-type β-lactamases are most often found in *E. coli* and *K. Pneumoniae,* also in other species of gram-negative bacteria with increasing frequency

(Bradford, 2001). Anguzu and Olila (2007) obtained as low as 9.4% sensitivity to ampicillin among Gram-negative organisms

.

Twenty eight (54%) of the isolates were resistant to tetracycline while only 15(29%) were resistant to the action of co-trimoxazole. Tetracycline resistance is already emerging in clinical isolates in many communities (Hassan *et al*., 2011).

ESBL was detected if organism was resistant to one of the indicator cephalosporins (cefpodoxime and ceftriaxone) (Aminzadeh *et al*., 2008). ESBLs are able to hydrolyse 3rd generation cephalosporins and monobactams (Bali *et al*., 2010). For this study, cefpodoxime and ceftriaxone discs were used. The result reveals that ceppodoxime detected more ESBL than ceftriaxone (Table 4.12). Best activity was found in ciprofloxacin (Fluoroquinolone) and amikacin (Aminoglycoside) with 100% sensitivity to all the isolates. Ciprofloxacin is a broad spectrum fluoroquinolone which has been found to possess excellent activity *in vivo* against *Enterobacteriaceae* (Yao and Moellering, Jr. 2007). Several studies have established that susceptibility to ciprofloxacin or other fluoroquinolones are quite high among ESBL producing *Enterobacteriaceae* (Hassan *et al*., 2011). The Aminoglycosides are bactericidal agents that inhibit bacterial protein synthesis and have been found to be particularly potent against the *Enterobacteriaceae* among other aerobic Gram-negative rods (Yao and Moellering, Jr. 2007). Aminazadeh *et al*. (2008) obtained 93.5% susceptibility of *E.coli* isolates to amikacin. Several studies have shown that treating infection caused by ESBL with cephalosporins often do not yield good therapeutic result and suggested that fluoroquinolones and aminoglycosides could be alternative choices (Iroha *et al*., 2008). Very high susceptibility to amoxicillin-clavulanic acid

was also observed in the study. The combination of amoxicillin and clavulanate, known as Augmentin, for the augmentin powers that clavulanate confers to amoxicillin, has been the most widely used form of penicillin in recent years (Walsh, 2003). Clavulanic acid is a naturally occurring, weak antimicrobial agent found initially in cultures of *Streptomyces clavuligerus* (Yao and Moellering, Jr., 2007). On its own it is a poor substrate for PBP and so is not considered an antibiotic. Its utility derives from its 'suicide substrate' properties with β-lactamases (Walsh, 2003). It inhibits β-lactamases from Staphylococci and many Gram-negative bacteria, forming an irreversible acyl enzyme complex with the β-lactamase, leading to loss of activity of the enzyme. This synergistic effect of clavulanate and various penicillins and cephalosporins has yielded much success in the battle against resistance due to β-lactamase production. Plasmid-mediated TEM β-lactamases present in strains of *K. pneumoniae* and *E.coli* especially are inactivated by this drug (Yao and Moellering, 2007). This result agrees with Edelstein *et al*. (2003) which states that β-lactam- β-lactamase inhibitor combinations, carbapenems, aminoglycosides and fluoroquinones are considered to be potentially active drugs against ESBL-producing organisms.

Multidrug resistance (MDR) was taken as resistance to four or more antibiotics tested (Ezekiel *et al*., 2011). Infections caused by ESBL-producers often exhibit a multidrug-resistance phenotype, leaving only a few reliable therapeutic options (Fam and El-Damarawy, 2008). ESBLs production is increasingly an important cause of transferable multidrug resistance in Gram-negative bacteria throughout the World (Bali *et al*., 2010). In this study, Pearson correlation showed significant correlation between ESBLs production and multi-drug resistance ($p<0.005$) (AppendixVII).

Multiple antibiotic resistance (MAR) index is a measure of the extent of antimicrobial agent resistance for the isolates in the group studied. (Apun *et al*., 2008). It gives an indirect suggestion of the probable source of the organism (Olayinka *et al*., 2004). MAR index values greater than 0.2 indicate that the isolates were recovered from samples originating from high-risk sources (Apun *et al*., 2008). Most probably, there are no strict rules concerning antibiotic prescriptions and usage in such areas.

Analysis of pooled antibiotic resistance of the isolates showed significally higher mean values of antibiotic resistance of isolates from 'suya' than from smoked fish or 'zoborodo'(Table 4:12). The main reason still remains in the fact that 'suya' is of animal origin while the other samples were not. The use of antibiotics in food animals selects for bacteria resistant to antibiotics used in humans. Resistance can be selected in food animals, and resistant bacteria can contaminate animal-derived food (Philips *et al*., 2004). The dissemination of *E.coli* in food production units may equally occur via faecal cross-contamination between groups of animals (or individuals), and the contamination of food derived from animals may occur during processing in the abattoir (Horton *et al*., 2011). Antibaoterial resistant bacteria have been identified along production path of 'suya'. Amosun *et al*. (2012) confirmed that on-farm and slaughter cattle are important sources of antibacterial resistant *E.coli* transmissible to humans through beef.

In developed countries, the main reservoirs for antimicrobial drug resistance in enteric bacteria have been attributed to farm animals such as cattle, sheep, pigs and poultry (Ombui *et al*., 2000). Contact with these animals or consumption of food products from them has been the main route

of dissemination of resistance into the human populations. Therefore, transmission of drug resistant bacteria from farms into the community and subsequently to patients in hospitals may occur through food. This demonstrates how resistant bacteria arising from indiscriminate use of antibiotics in animals may impact on human health (Ombui *et al*., 2000). Of particular concern are antimicrobial growth promoters that are used in both human and veterinary medical applications (e.g. Tetracycline) or that share a common antibiotic family with antibiotic essential for treatment of bacterial diseases in humans (Alexander *et al*., 2008) . Drug resistance in animals is caused mainly by the large amount of antimicrobial drugs used in food production. In addition to their presence in farm animals, ESBL genes have been found in retail meat (Overdevest *et al*., 2011).

The sensitivity of the nitrocefin sticks was calculated as 12.9%. Different β-lactamase enzymes have been found to exhibit differences in substrate specificities, this leads to partial colour reactions and an increase in false negatives for the colour based test. For this study, another factor could be storage conditions during shipping and handling. The sticks were to be stored freezed at -10°C but this temperature was not achieved at all during the study. The implication is that as good as these sticks may be, being a cold chain test kit reduces its sensitivity rate when used in under-developed countries where power supply is inconsistent.

Among the most clinically and economically important antibiotic resistance genes are those encoding the β-lactamases (bla genes) producing high level resistance to β-lactam antibiotics, the most widely used antibiotics in clinical and veterinary practice (Brusetti *et al*., 2008). The

description of antibiotic resistant bacteria in non-clinical environments such as farm animals, fish farms, sewage, drinking water, polluted rivers, and food items has mostly been on phenotypic investigations of the antibiotic resistant bacteria. There is need to detect the specific antibiotic resistance genes by molecular methods and their pattern of resistance can provide useful information and aid in rational antibiotic therapy. This study also provides an assessment of the presence of TEM and SHV genes in some of the bacterial isolates. Emphasis has been on clinical and environmental samples, there is a need to assess Ready-to-Eat foods as reservoirs of ESBL-encoding bacteria.

This study presents genotypic identification of TEM and SHV genes among 12 isolates that were mostly resistant to broad spectrum cephalosporins from the phenotypic test (Disc diffusion test). TEM gene had higher frequency of 66.7% compared to SHV gene with 8.3%. A fact similar to previous studies (Lal *et al*., 2007; Zaniani *et al*., 2012). No isolate was found to harbour both TEM and SHV genes together. This was also the case in the result of Zhang *et al*. (2010) where none of the ESBLs producing bacteria had both TEM and SHV kind. Three (25%) isolates of which two were categorized as ESBL producers based on double disk synergy test (DDST) did not contain any of the genes. They must have contained other genes that were not tested for in this study. Further studies are required for finding the other genes in ESBLs producing *E. coli* and *Klebsiella* sp. from these foods.

It should also be noted that the isolates may have more than one bla_{TEM} or bla_{SHV} gene present, and amplification and sequencing only detected a single genotype. This is probable because, if multiple bla_{TEM} and bla_{SHV} genes are present, the predominant one will preferentially amplify and

produce sequence (Paterson *et al*., 2003). Recently, 167 TEM β-lactamases that are commonly found in the *Enterobacteriaceae* family have been identified (Yazdi *et al*., 2011). The most prevalent ESBL types have evolved through point mutations of key amino acid substitutions in the parent TEM and SHV enzymes (Al-Jasser, 2006). Over 100 variations and point mutations in TEM gene had been reported during DNA sequencing. These mutations are mostly responsible for resistance to beta lactams in these isolates (Jain and Mondal, 2008).

It was also noted that while SHV gene was detected in *Klebsiella oxytoca* only, TEM genes were detected in only *E.coli* all through. This is in agreement with the observations of Podbielski *et al***.** (1991) which stated that *Klebsiella* strains sometimes contain the plasmid-encoded β-lactamases of the SHV-type and less often of the TEM-type. One isolate SFe 16, that was susceptible to all the antibiotics in the DDT was found to harbour a TEM gene. This discrepancy in the DDT and PCR could be due to inoculum effect and substrate specificity which may render the enzyme in an un-induced state at the time of testing with DDT. This creates a major challenge in laboratory routine susceptibility tests. The degree of resistance against third-generation cephalosporins can be highly variable among different ESBL enzymes. While some ESBL producing strains have overt resistance to broad spectrum β-lactam antibiotics, many will not be phenotypically resistant (Jain and Mondal, 2008).

Sequencing results showed that the bla_{TEM} in Sye10 (isolate 8) had 86% homology with β-lactamase TEM-1gene. Higher identity would probably have been obtained but for two nucleotides (designated as "N"), which were not specified by the sequencer (Table 4.15). Although, the entire bla_{TEM} genes were not sequenced and so we cannot categorically say that the entire TEM genes present are TEM-1. However, TEM-1 is the most commonly encountered β -

lactamase in Gram-negative bacteria (Al-Jasser, 2006). Up to 90% of ampicillin resistance in *E.coli* is due to the production of TEM-1 (Lim *et al*., 2009). This enzyme is able to hydrolyse penicillins and early cephalosporins such as cephalothin and cephaloridine (Bradford, 2001). That is not suprising because diverse point *mutations* in the $bla_{\text{TEM-1}}$ gene have contributed to the emergence of TEM-type extended-spectrum β-lactamases (ESBLs), resulting in simultaneous resistance to penicillins and broad-spectrum cephalosporins (Balsalobre *et al*., 2010)

Sequence analysis of bla_{SHV} in Syk2 (isolate 10) could not confirm the subtype. The reason for this is not yet known but must be similar to the case of Lim *et al.* (2009), who could not confirm specific SHV- subtypes in their study because their primers only amplified a portion of the bla_{SHV} reading frame. Kolar *et al*. (2010) also had a similar experience with bla_{TEM} gene. The SHV-type of ESBL may be found in clinical isolates more frequently than any other type of ESBLs. Unlike the TEM-type β-lactamases, there were relatively few derivatives of SHV-1. The majority of SHV variants possessing an ESBL phenotype were characterized by the substitution of a serine for glycine at position 238. Some had a substitution of lysine for glutamate at position 240. The serine residue at position 238 is critical for efficient hydrolysis of ceftazidime while lysine residue is critical for the efficient hydrolysis of cefotaxime. More than 50 SHV varieties have been described worldwide. SHV-type of ESBLs has been detected in a wide range of *Enterobacteriaceae* (Al-Jasser, 2006). A total of 40 types of SHV-type ESBL enzymes are already reported (Jain and Mondal, 2008).

The fact that these genes also confer resistance to other commonly used antibiotics such as Tetracycline and Co-trimethoprim is a well known fact and this was evident in the organisms

used for this study as 7 (58.3%) of the organisms exhibited multidrug resistance. However, Sperman's correlation showed moderate correlation between the presence of any of the two genes and resistance to third generation cephalosporins ($r_s = 0.258$). There is no significant correlation between the presence of any of the two genes and resistance to third generation cephalosporins ($p>0.05$) (Appendix VIII).

CHAPTER 6

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 SUMMARY

This study presents to us the fact that the antibiotic resistance bacteria are no longer confined to the hospitals and with nosocomial infections but are ever present in our environment, and successfully gain access into the food chain through various means. The use of plant-based systems continues to play an essential role in health care (Karou *et al*., 2007). This however, is increasingly facing resistance by certain groups of microorganisms especially Gram-negative bacteria. Although Gram-negative rods have several layers of the peptidoglycan, they are overlaid with an outer membrane composed mainly of lipopolysaccharides. The outer

membrane is an important permeability barrier which provides protection against various antibacterial materials (Shimamura *et al*., 2007). Ready-to-eat foods could be raw or cooked, hot or chilled and can be consumed without any further heat treatment (Clarence *et al*., 2009). As consumption of ready-to- eat foods increases, these categories of food have become potent reservoirs for antimicrobial resistant genes. Concerns persist regarding the potential negative impacts of antimicrobial use in livestock and, in particular the potential for the emergence of antimicrobial resistance in human and animal pathogens. The introduction into clinical practice of the oxyimino-cephalosporins for treatment of serious infections due to Gram-negative bacteria was soon followed by emergence of the so-called extended spectrum β-lactamases (ESBLs) .Third generation cephalosporins have important applications to both human and veterinary medicine due to their broad spectrum, generally bactericidal effects (Singer *et al*., 2008). Over reliance on third generation cephalosporins to treat Gram negative infections is one prime factresponsible for increased resistance to this class of antibiotics (Kumar *et al*., 2006). These enzymes are mostly plasmid-encoded derivatives of TEM-1, TEM-2 and SHV-1. They confer resistance to third generation cephalosporins as well as to monobactams, in addition to broad-spectrum penicillins and narrow-spectrum cephalosporins (Haeggman *et al*., 2004). The ESBLs constitute a serious potential hazard for clinicians attempting to treat patients who are infected with bacteria that express these resistance determinants. They also provide the opportunity for continuing basic scientific research into the evolution and dissemination of resistance determinants that threaten the continued use of a valuable family of antimicrobial agent (Heritage *et al*., 1999).

6.2 CONCLUSION

The result from this study indicate that most of the ESBL-encoding genes especially bla_{TEM} are carried on plasmids which are transmissible, suggesting that the spread of ESBLs and other antibiotic resistance determinants are most likely to be plasmid mediated. This is in agreement with the conclusion of other Workers (Lim *et al*., 2009). Therefore, the widespread uses of antibiotics, coupled with the transmissibility of resistance determinant mediated by plasmids, transposons, and gene cassettes in integrons are factors that contribute to the increase in antibiotic resistance in bacteria pathogens.

Determination of ESBL type in ESBL-producing bacteria could provide useful information for management and control of antibiotic resistance spread in several regions. To achieve this, molecular techniques, such as PCR and further characterization with sequencing are indispensable since the phenotypic method cannot efficiently differentiate ESBL type.

6.3 RECOMMENDATIONS

i. Fast Detection and Identification of Reservoirs

The incorporation of fast and adequate tests for detection of ESBLs as a routine in all Microbiology laboratories remained a main issue that needs to be addressed. This can be obtained by using molecular-biological methods of gene analysis. A staggeringly diverse group of species maintain a large capacity for carrying and mobilizing resistance genes. These bacteria constitute a largely ignored “reservoir” of resistance genes and provide multiple complex pathways by which resistance genes propagated in animals can directly, or more likely indirectly,

make their way over time into human pathogens via food, water, and sludge and manure applied as fertilizer (Marshall and Levy, 2011).

ii. Reduced and Appropriate Consumption of Antimicrobials both for Human and Animals

It is now generally accepted that the main risk factor for the increase in antibiotic resistance in pathogenic bacteria is the increased use of antibiotics. This has inevitably led to the emergence and dissemination of resistant bacteria and genes. This situation applies to antibiotic usage both in animals and in humans. In both populations antibiotics are used for therapy and prophylaxis of infectious diseases (van den Bogaard and Stobberingh, 2000).

iii. Strict Adherence to Standard Hygiene Practices

Available data suggest that food can contribute to the dissemination of resistant *Enterobacteriaceae* in the community. To ensure that ready-to-eat food is microbiologically safe, both the manipulators and the food need to be continually monitored. The commercial manufacture of ready-to-eat foods consists of a small number of the operations, but this critical process can lead to the introduction of the microorganisms or the proliferation of those already present. Possible sources of the microbial contamination have been identified as a) unhygienic handling; b) raw material; c) inadequate cleaning of the machines used to cut the food, knives, contact surfaces, clothes and manipulators hands, and d) airborne contamination (de Sousa, 2008).

6.4 CHALLENGES

Carrying out this research work was not without several constrain; they include the following:

1. Insufficient fund

2. Non availability of several needed equipment in the research laboratory
3. Scarcity of some media and reagents
4. Inconsistency of power supply

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APPENDICES

APPENDIX I: Microgen GN A substrate reference table

Well	Reaction	Description	Positive	Negative
1.	Lysine	Lysine decarboxylase-Bromothymol blue changes to green/blue indicating the production	Green/Blue	Yellow

		of the amine cadaverine.		
2.	Ornithine	Ornithine decarboxylase- Bromothymol blue changes to blue indicating the production of the amine putrescin.	Blue	Yellow/Green
3.	H_2S	H_2S production-Thiosulphate is reduced to H_2S that reacts with ferric salts producing a black precipitate.	Brown/black	Straw
4.	Glucose	Fermentation- Bromothymol blue changes from blue to yellow as a result of acid produced from the carbohydrate fermentation.	Yellow	Blue/green
5.	Mannitol			
6.	Xylose			
7.	ONPG	Hydrolysis-ONPGHydrolysis by B-galactosidase results in the production of yellow ortho-nitrophenol	Yellow	Colourless
8.	Indole	Indole is produced from tryptophan and gives a pink/red complex when Kovac's reagent is added.	Pink/red	Colourless
9.	Urease	Hydrolysis of urea results in the formation of ammonia leading to an increase in pH which turns phenol red from yellow to pink/red.	V.Deep Pink	Straw to pale salmon pink colour
10.	VP	Acetoin production from glucose is detected by the formation of a pink /red complex after the addition of alpha naphthol and creatine in the presence of KOH.	Deep Pink/Red	Colourless to Pale Pink
11.	Citrate	Utilization of citrate (only carbon source) leading to a colour change in bromothymol blue from green to blue.	Blue	Yellow/Pale Green
12.	TDA	Indolepyruvic acid is produced from tryptophan by tryptophan deaminase giving a cherry red colour when ferric ions are added. Indole positive isolates may give a brown colour- this is a negative result.	Cherry red	Straw colour

Key: H_2S-Hydrogen sulphide; ONPG-Ortho-nitrophenol- galactosidase; TDA-Tryptophan deaminase acid

APPENDIX II: MICROGEN GN A IDENTIFICATION RESULTS

APPENDIX IIa: Microgen GN A Identification result (Suya Isolates)

Code	Lys	Orn	H_2S	Glu	Man	Xyl	ONPG	Ind	Ure	VP	Cit	TDA	Octal code	Identi-fication
Sye1	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
Sye2	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
Sye3	+	-	-	+	+	+	+	+	-	-	-	-	4760	*E.coli*
Sye4	+	-	-	+	+	+	+	+	-	-	-	-	4760	*E.coli*
Sye5	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
Sye6	+	-	-	+	+	+	+	+	-	-	-	-	4760	*E.coli*
Sye7	+	-	-	+	+	+	+	+	-	-	-	-	4760	*E.coli*
Sye8	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
Sye9	+	-	-	+	+	+	+	+	-	-	-	-	4760	*E.coli*
Sye10	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
Sye11	+	-	-	+	+	+	+	+	-	-	-	-	4760	*E.coli*
Sye12	+	-	-	+	+	+	+	+	-	-	-	-	4760	*E.coli*
Sye13	+	-	-	+	+	+	+	+	-	-	-	-	4760	*E.coli*
Sye14	+	-	-	+	+	+	+	+	-	-	-	-	4760	*E.coli*
Sye15	+	-	-	+	+	+	+	+	-	-	-	-	4760	*E.coli*
Sye16	+	+	-	+	+	+	+	+	+	-	-	-	6770	*E.coli*
Sye17	+	-	-	+	+	+	+	+	-	-	-	-	4760	*E.coli*
Sye18	+	-	-	+	+	+	+	+	-	-	-	-	4760	*E.coli*
Syk1	+	-	-	+	+	+	+	+	-	-	+	-	4762	*Klebsiella Oxytoca*
Syk2	+	-	-	+	+	+	+	+	-	+	-	-	4764	*Klebsiella oxytoca*

APPENDIX II b: Microgen GN A Identification result (Smoked fish Isolates)

Code	Lys	Orn	H_2S	Glu	Man	Xyl	ONPG	Ind	Ure	VP	Cit	TDA	Octal Code	Identification
SFe1	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
SFe2	+	-	-	+	+	+	+	+	-	-	-	-	4760	*E.coli*
SFe3	+	-	-	+	+	+	+	+	-	-	-	-	4760	*E.coli*
SFe4	+	-	-	+	+	+	+	+	-	-	-	-	4760	*E.coli*
SFe5	-	-	-	+	+	+	+	+	-	-	-	-	0760	*E.coli*-inactive
SFe6	-	+	-	+	+	+	+	+	-	-	-	-	2760	*E.coli*
SFe7	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
SFe8	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
SFe9	-	-	-	+	+	+	+	+	-	-	-	-	0760	*E.coli*-inactive
SFe10	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
SFe11	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
SFe12	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
SFe13	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
SFe14	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
SFe15	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
SFe16	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
SFe17	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
SFe18	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
SFe19	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*

Key: Lys-Lysine; Orn-Ornithine; H_2S-Hydrogen sulphide; Glu-Glucose; Man-Mannitol; Xyl-Xylose; ONPG-Ortho-nitrophenol- galactosidase; Ind-Indole; Ure-Urease; VP-Voges Proskauer; Cit-Citrate; TDA-Tryptophan deaminase acid.

APPENDIX II c: Microgen GN A Identification result (Zoborodo Isolates)

Code	Lys	Orn	H_2S	Glu	Man	Xyl	ONPG	Ind	Ure	VP	Cit	TDA	Octal code	Identification
Ze1	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
Ze2	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
Ze3	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
Ze4	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
Ze5	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
Ze6	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
Ze7	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
Ze8	+	+	-	+	+	+	+	+	+	-	-	-	6770	*E.coli*
Ze9	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
Ze10	+	+	-	+	+	+	+	+	-	-	+	-	6762	*E.coli*
Ze11	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*
Zk1	+	+	-	+	-	+	+	+	+	-	+	+	4573	*Klebsiella Oxytoca*
Zk2	+	-	-	+	+	+	+	-	-	-	+	-	2742	*Klebsiella ozaenae*

Key: Lys-Lysine; Orn-Ornithine; H_2S-Hydrogen sulphide; Glu-Glucose; Man-Mannitol; Xyl-Xylose; ONPG-Ortho-nitrophenol- galactosidase; Ind-Indole; Ure-Urease; VP-Voges Proskauer; Cit-Citrate; TDA-Tryptophan deaminase acid.

APPENDIX IId: Microgen GN A Identification result (Kunun zaki Isolate)

Code	Lys	Orn	H_2S	Glu	Man	Xyl	ONPG	Ind	Ure	VP	Cit	TDA	Octal code	Identification
Ke1	+	+	-	+	+	+	+	+	-	-	-	-	6760	*E.coli*

Key: Lys-Lysine; Orn-Ornithine; H_2S-Hydrogen sulphide; Glu-Glucose; Man-Mannitol; Xyl-Xylose; ONPG-Ortho-nitrophenol- galactosidase; Ind-Indole; Ure-Urease; VP-Voges Proskauer; Cit-Citrate; TDA-Tryptophan deaminase acid.

APPENDIX III: Zone Diameter Interpretative Standard Breakpoints (for *Enterobacteriaceae*) of antibiotics used in this study.

Antimicrobial Agents	Disk potency (µg)	Zone diameter (mm)			Testing conditions
		R≤	I	S ≥	
Ampicillin	10	13	14-16	17	**Medium** Mueller-Hinton agar
Amoxicillin-clavulanic acid	20/10	13	14-17	18	
Cephalothin	30	14	15-17	18	**Inoculum** Growth method or direct colony suspension equivalent to a 0.5 McFarland Standard
Cefpodoxime	10	17	18-20	21	
Ceftriaxone	30	13	14-20	21	
Ciprofloxacin	5	15	16-20	21	
Sulphamethoxazole/Trimethoprim	25	10	11-15	16	**Incubation** 35 ± 2^0C, ambient air; 16-18hr
Tetracycline	30	11	12-14	15	
Amikacin	30	14	15-16	17	

Key: R= Resistant I=Intermediate S=Sensitive

Source. CLSI, 2008

APPENDIX IV: Screening and Confirmatory Tests for ESBLs in *Klebsiella pneumoniae*, *K. oxytoca*, *Escherichia coli*

Test	**Initial Screening Test**	**Phenotypic Confirmatory Test**
Test Method	Disk diffusion	Disk diffusion
Medium	Mueller Hinton Agar	Mueller Hinton Agar
Antimicrobial Concentration	Cefpodoxime 10 µg or Ceftazidime 30 µg or Aztreonam 30 µg or Cefotaxime 30 µg or Ceftriaxone 30µg (The use of more than one antimicrobial agent for screening improves the sensitivity of detection)	Ceftazidime 30 µg Ceftazidime-clavulanic 30/10 µg and Cefotaxime 30µg Cefotaxime-clavulanic acid 30/10 µg (Confimatory testing requires use of both cefotaxime and ceftazidime alone and in combination with clavulanic acid)
Inoculum	Standard disk diffusion recommendations	Standard disk diffusion recommendations
Incubation conditions	35 ± 2^{0}C; ambient air	35 ± 2^{0}C; ambient air
Incubation length	16-18hr	16-18hr
	Cefpodoxime zone $\leq$ 17mm	

Result	Ceftazzidime zone ≤ 22mm Aztreonam zone ≤ 27mm Cefotaxime zone ≤ 27mm Ceftriaxone zone ≤ 25mm Zones above may indicate ESBL production	A ≤ 5mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid vs its zone when tested alone-ESBL (e.g. ceftazidime zone-16; ceftazidime-clavulanic acid zone-21)
Reporting		For all confirmed ESBL-producing strains, the test interpretation should be reported as resistant for all penicillins, cephalosporins and aztreonam.

Source: CLSI, 2008

APPENDIX V: Media Formulation

1. DEV Lactose peptone broth **(Merck, Germany)**

g/L	
Peptone from casein	10.0
Soyameal	3.0
Lactose	10.0
Sodium chloride	5.0
Bromocresol purple	0.02

2. Eosin methylene blue (EMB) agar **(Biomark, India)**

g/L	
Peptic digest of animal tissue	10.0
Dipotassium phosphate	2.0
Lactose	5.0
Sucrose	5.0

Eosin-Y	0.4
Metyhlene blue	0.065
Agar	13.5
pH 7.2 ±0.2	

3. MacConkey agar (Antec, U.K)

g/L	
Peptone	20.0
Agar	12.0
Lactose	10.0
Neutral red	0.05
Bile salts	5.0
Sodium chloride	5.0
pH 7.4 ± 0.2	

4. Methyl Red Voges Proskauer Broth (Scharlau, Spain)

g/L	
Peptone	7.0
Dextrose	5.0
Potassium phosphate	5.0
pH 7.0 ± 0.2	

5. Mueller-Hinton-Agar (Merck, Germany)

g/L	
Infusion from meat	2.0
Casein hydrolysate	17.5
Starch	1.5
Agar-Agar	13.0

pH 7.4 ± 0.2

5. Nutrient broth (agar) (Oxoid, England)

g/L	
Lab- Lemco powder	1.0
Yeast extract	2.0
Peptone	5.0
Sodium chloride	5.0
(Bacteriological agar)	15.0

pH 7.4 ± 0.2

7. Peptone water phosphate buffered (Scharlau, Spain)

g/L	
Peptone	10.0
Sodium chloride	5.0
Disodium phosphate	3.5
Potassium phosphate	1.5

pH 7.2 ± 0.2

8. Plate count agar (Biotec, U.K.)

g/L	
Tryptone	5.0
Yeast extract	2.5
Glucose	1.0
Agar No2	12.0

pH 7.0 ± 0.2

9. SIM medium (Oxoid, England)

g/L	
Tryptone	20.0
Peptone	6.0
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.2
Agar	3.5

pH 7.3 ± 0.2

10. Simmon’s citrate agar (Biomark, India)

g/L	
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bromothymol blue	0.08
Agar	15.0

pH 6.8 ± 0.2

11. Tryptone Soya broth (Oxoid, England)

g/L	
Pancreatic digest of casein	17.0
Enzymatic*digest of soya bean	3.0
Sodium chloride	5.0
Di-potassium hydrogen phosphate	2.5
Glucose	2.5

*(Contains papain)

pH 7.3 ± 0.2

APPENDIX VI: Composition of reagents

1. Kovàc's Indole Reagent

Para-dimethylaminobenzaldehyde	2g
Isoamyl alcohol (3-methyl-1-butanol)	30ml
Hydrochloric acid, concentrated	10ml

2. Methyl red solution

Methyl red (pH indicator)	0.05g
Absolute ethanol	28ml
Distilled water	22ml

3. 5%-alpha-napthol

Alpha-napthol	5g
Absolute ethanol	100ml

4. 40% Potassium hydroxide

Potassium hydroxide	40g
Distilled water	100ml

.

APPENDIX VII: List of Plates

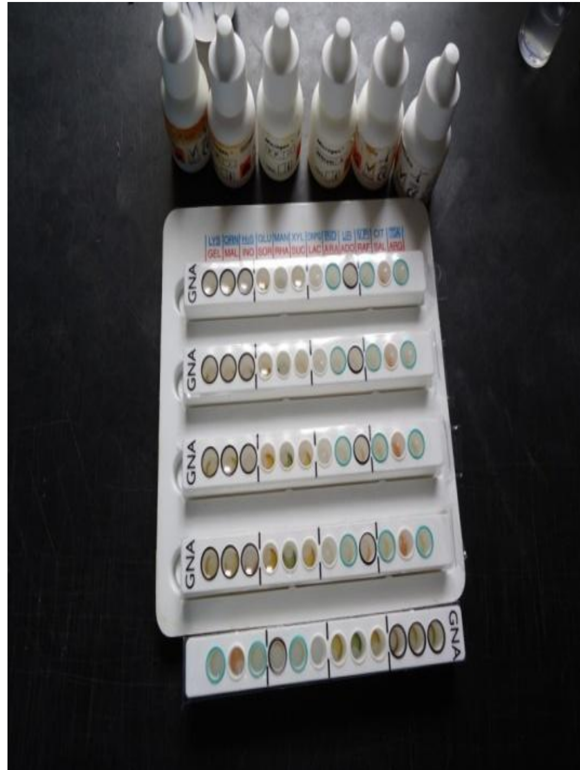

Plate III: Carica papaya tree **Plate IV: Microgen Identification kit**

Plate V: Soxhlet extraction **Plate VI: Maceration with separating funnels**

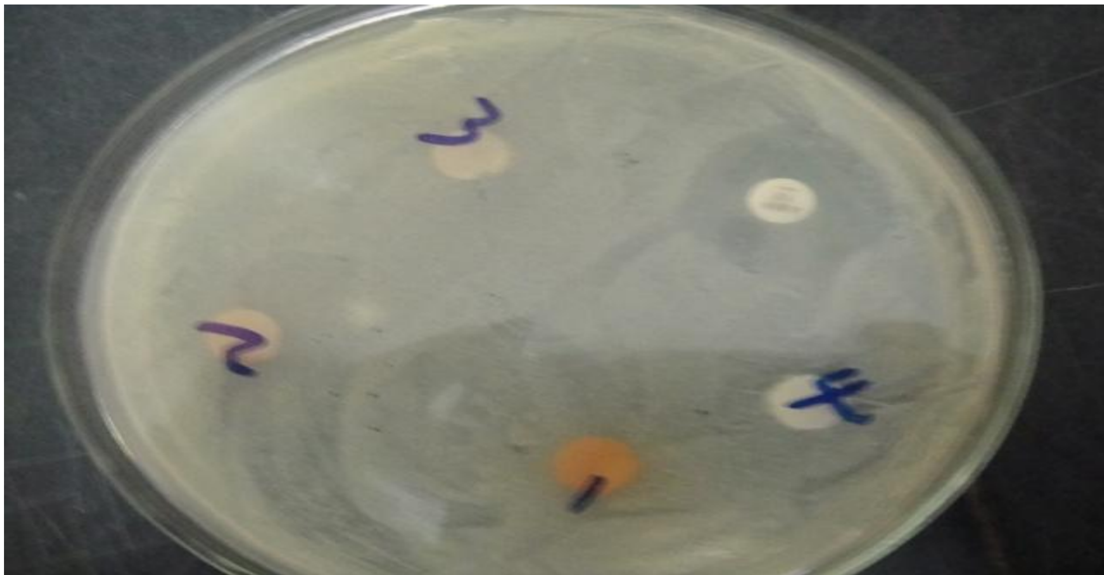

Plate VII: Susceptibility to plant extracts

1=500μg/ml; 2=250μg/ml; 3=125μg/ml; 4=DMSO (Negative control);
Ampicillin disc (Positive control)

Plate VIII: Nitrocefin containing β-lactamase Identification sticks

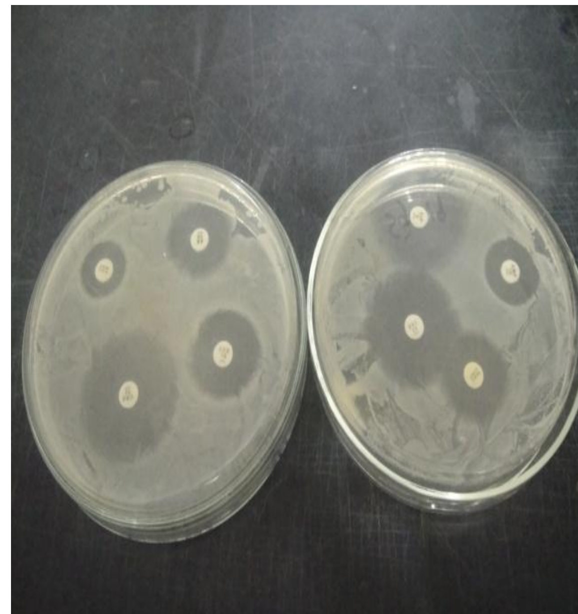

Plate IX: Antibiotic susceptibility testing

APPENDIX VIII: Statistical Analysis

Correlation between ESBLs production and Multidrug resistance (MDR) in *E. coli* isolated from smoked fish

	MDR Strains	Non MDR strains	Total
4ESBLs detected	2	3	**5 (26%)**
ESBLs not detected	1	13	**14 (74%)**
Total	**3 (16%)**	**16 (84%)**	**19**

P=0.000 **. Correlation is significant at the 0.01 level (2-tailed).

Correlation between ESBLs production and Multidrug resistance (MDR) in *E. coli* and *Klebsiella* spp. isolated from 'suya'

	MDR Strains	Non MDR strains	Total
ESBLs detected	8	1	**9(45%)**
ESBLs not detected	3	8	**11 (55%)**
Total	**11 (55%)**	**9 (45%)**	**20**

P=0.038 * Correlation is significant at the 0.05 level (2-tailed)

Correlation between ESBLs production and Multidrug resistance (MDR) in *E. coli* and *Klebsiella* spp, isolated from 'zoborodo' drink

	MDR Strains	Non MDR strains	Total
ESBLs detected	4	0	**4 (31%)**
ESBLs not detected	0	9	**9 (69%)**
Total	**4 (31%)**	**9 (69%)**	**13**

P=0.000 **. Correlation is significant at the 0.01 level (2-tailed).

Nonparametric Correlations

Correlations

			Resistance	Gene
Spearman's rho	Resistance	Correlation Coefficient	1.000	.258
		Sig. (2-tailed)	.	.418
		N	12	12
	Gene	Correlation Coefficient	.258	1.000
		Sig. (2-tailed)	.418	.
		N	12	12

Crosstabs

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
Gene * Resistance	12	100.0%	0	.0%	12	100.0%

Gene * ResistanceCrosstabulation

Count

		Resistance		
		ESBLs Resistance Presence	ESBLs Resistance Absent	Total
Gene	TEM/SHV Genes present	8	1	9
	TEM/SHV Absent	2	1	3
Total		10	2	12

Symmetric Measures

		Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
Interval by Interval	Pearson's R	.258	.324	.845	.418[c]
Ordinal by Ordinal	Spearman Correlation	.258	.324	.845	.418[c]
N of Valid Cases		12			

a. Not assuming the null hypothesis.

b. Using the asymptotic standard error assuming the null hypothesis.

c. Based on normal approximation.