DETECTION OF VIRULENCE AND RESISTANCE GENES IN BACTERIAL ISOLATES FROM SEAFOOD OBTAINED FROM NEMBE AND OX-BOW LAKE, BAYELSA STATE, NIGERIA

BY

JUSTICE-ALUCHO, CHINWE HAPPINESS (B.Sc , FUTO)

20174079398

A THESIS SUBMITTED TO

POST GRADUATE SCHOOL,

FEDERAL UNIVERSITY OF TECHNOLOGY,

OWERRI, IMO STATE

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

AWARD OF MASTER OF SCIENCE (M.Sc.) DEGREE IN FOOD AND INDUSTRIAL MICROBIOLOGY,

SEPTEMBER, 2019

CERTIFICATION

This is to certify that the research work "**DETECTION OF VIRULENCE AND RESISTANCE GENES IN BACTERIAL ISOLATES FROM SEAFOOD SAMPLES OBTAINED FROM NEMBE AND OX-BOW LAKE IN BAYELSA STATE, NIGERIA**" was carried out by **JUSTICE-ALUCHO, CHINWE HAPPINESS** (20174079398) in partial fulfillment of the requirements for the award of Master of Science (M.Sc) in Food and Industrial Microbiology in the Department of Microbiology, Federal University of Technology, Owerri.

……………… 02/11/2021
PROF. W. BRAIDE Date
(Supervisor)

……………… ………………
DR (MRS) E.E MIKE-ANOSIKE Date
(Co-Supervisor)

……………… 02/11/2021
PROF. C.E NWANYANWU Date
HOD, Microbiology

……………… ………………
PROF. C.S. ALISI Date
Dean, School of Science

……………… ………………
PROF. C.C. EZE Date
Dean, Post-Graduate School

……………… 28th Sept. 2021
PROF. E. NWACHUKWU Date
External Examiner

DEDICATION

This Thesis work is dedicated to Almighty God who has always been my help in time of need and to all who have contributed to my academic success

ACKNOWLEDGEMENTS

My gratitude goes to God Almighty for His grace to be able to complete this work. I also want to acknowledge my able supervisors, Prof.Wesley Braide and Dr Mrs E.E Mike-Anosike. I appreciate most especially my Husband, Engineer Justice Alucho for his moral and financial support.

I also appreciate my mentor Professor Tatfeng, my lecturers, my colleagues, Mr Adeleye A. Samuel, Microbilogy Deparment as a whole and all who were committed to ensuring the success of this work through moral and financial support.

I also acknowledge my friends, Mike Miriam, Mrs Oluchi Nwangwu, Mr Emmanuel and Mr Okeke Azubuike Francklin.

TABLE OF CONTENTS

CHAPTER **PAGE**

Title Page i

Certification ii

Dedication iii

Acknowledgements iv

Table of Contents v

List of Tables xi

List of Figures xiii

Appendices xiv

Abstract xv

CHAPTER ONE

1.0 Introduction 1

1.1 Background Information 1

1.2 Statement of the Problem 5

1.3 Aim of Study 5

1.4 Objectives 6

1.5 Justification 6

1.6 Significance of Study 7

CHAPTER TWO

2.1 Review of Literature 8

2.2 Seafood 11

2.2.1 Periwinkle 11

2.2.2 Classification of Periwinkle 12

2.2.3 Crab 12

2.2.4 Classification of crab 13
2.2.5 Shrimps 13
2.2.6 Classification of shrimps 14
2.2.7 Oyster 14
2.2.8 Classification of Oyster 15
2.3 Staphylococcus Mediated Infections 15
2.4 Klebsiella Mediated Infections 16
2.5 Vibrio Mediated Infections 17
2.6 Resistance Mechanisms of Vibrio species 18
2.7 Resistance Mechanisms of Coagulase Negative Staphylococcus Species 19
2.8 Resistance Mechanisms of *Klebsiella* Species 20
2.9 Extensive Drug Resistance/Multi-Drug Resistance in Microrganisms 21
2.10 Molecular Identification of Microbiota in Seafood 22
2.10.1 Polymerase Chain Reaction 22
2.10.2 Agarose Gel Electrophoresis 23
2.10.3 Sanger Sequencing Method 25
2.10.4 Next-Generation Sequencing—second Generation 25
2.10.5 454 Pyrosequencing 26
2.10.6 Illumina/Solexa Genome Analyser 27

CHAPTER THREE

3.0 Materials and Method 29
3.1 Materials 29
3.1.1 Samples used and location 29
3.1.2 Sample size 29
3.2 Methodology 29

3.2.1 Sample Collection 29

3.2.2 Sample Preparation 29

3.2.3 Inoculation 30

3.2.4 Microscopy and Biochemical Characterization 30

3.2.5 Pathogenicity Test 30

3.2.6 Antimicrobial Susceptibility Analysis 30

3.2.7 Molecular Characterization 31

3.2.7.1 DNA Extraction (Boiling Method) 31

3.2.7.2. DNA Quantification 31

3.2.7.3 16SrRNA Amplification 32

3.2.7.4. Sequencing 32

3.2.7.5. Phylogenetic Analysis 33

3.2.8. Amplification of Resistance Genes in Isolates 33

3.2.8.1 Amplification of *shv* Genes 33

3.2.8.2 Amplification of *ctx-M* Genes 34

3.2.8.3 Amplification of *acrAB* Genes 34

3.2.8.4 Amplification of *mecA* Genes 35

3.2.8.5 Amplification of *sxt* Genes 35

3.2.9 Amplification of Virulence Genes in Isolates 36

3.2.9.1 Amplification of *tdh* Genes 36

3.2.10 Statistical Analysis 37

CHAPTER FOUR

4.0 Results and Discussion 38

4.1 Results 38

4.1.1 Heterotrophic Bacterial Isolates from Seafood obtained from Nembe and Ox-bow Lake 38

4.1.2 Evolutionary Relations of Bacterial Isolates from Seafood Samples 39

4.1.3 Percentage Occurrence and Distribution of organisms Isolated from Seafood Samples in Nembe (n=80) 41

4.1.4 Percentage Occurrence and Distribution of Isolates in the Seafood Samples from Ox-Bow Lake. (n=120) 43

4.1.5 Comparative analysis of the Mean of Total Colony Count (Log_{10}cfu/g) of Isolates from Seafood obtained from Nembe and Ox-Bow Lake 45

4.1.6 Pathogenicity of the Bacterial isolates 47

4.1.7 Antimicrobial Resistance Profiles of Isolates from Seafood obtained from Nembe (n=80). 49

4.1.8 Antimicrobial Resistance Profile of Isolates from Seafoood obtained from Ox-Bow Lake (n=120). 51

4.1.9 Frequency (%) of Resistant Isolates from Seafood Samples obtained from Nembe (n=80) 53

4.1.10 Frequency (%) of Resistant Isolates from Seafood Samples obtained from Ox-Bow Lake (n=120) 55

4.1.11 Multi-Drug Resistance Patterns of Isolates from Seafood obtained from Nembe 57

4.1.12 Multi-Drug Resistance Pattern of Isolates from Seafood obtained from Ox-bow-Lake 63

4.1.13 Amplified Resistant and Virulent Genes 69

4.1.13.1 The *acrAB* Gene 69

4.1.13.2 The *ctx-M* Gene 71

4.1.13.3 The *shv* Gene 73

4.1.13.4 The *tdh* Gene 75

4.1.13.5 The *mecA* Gene 77

4.2 Discussion 79

CHAPTER FIVE 84

5.0 Conclusion and Recommendation 84

5.1 Conclusion 84

5.2 Recommendation 85

References 86

Appendices 95

LIST OF TABLES

TABLE **TITLES** **PAGE**

4.1: Total Colony Count (Log$_{10}$CFU/g) of Isolates from Seafoods obtained from Nembe and Ox-Bow Lake 46

4.2: Virulence Factors present in the Bacterial isolates from Nembe and Ox-bow 48

4.3: Frequency (%) of Resistant Isolates from Seafood from Nembe (n=80) 54

4.4: Frequency (%) Of Resistant Isolates obtained from Seafood gotten from Ox-Bow (n=120) 56

4.5: Multi-Drug Resistance Pattern of *Staphylococcus gallinarum (*n=13*)* from Seafood Samples from Nembe 58

4.6: Multi-Drug Resistance Pattern of *Klebsiella aerogenes (*n=10) from Seafood Samples in Nembe 59

4.7: Multi-Drug Resistance Patterns of *Klebsiella quasipneumoniae* (n=17) from Seafood Samples in Nembe 60

4.8: Multi-Drug Resistance Patterns of *Vibrio rotiferanus* (n=9) from Seafood Samples in Nembe 61

4.9: Multi-Drug Resistance Patterns of *Vibrio parahaemoyticus* (n=31) from Seafood Samples in Nembe. 62

4.10: Multi-Drug Resistance Patterns of *S. gallinarum* (n=27) from Seafood Samples in Ox-Bow Lake 64

4.11: Multi-Drug Resistance Patterns of *K. aerogenes* (n=10) from Seafood Samples in Ox-Bow Lake 65

4.12: Multi-Drug Resistance Patterns of *K. quasipneumoniae* (n=18) from Seafood Samples in Ox-Bow Lake 66

4.13: Multi-Drug Resistance Patterns of *V.rotiferianus* (n=17) from Seafood Samples in Ox-Bow Lake 67

4.14: Multi-Drug Resistance Patterns of *V.parahaemolyticus* (n=48) from Seafood Samples in Ox-Bow Lake 68

LIST OF FIGURES

FIGURE **TITLES** **PAGE**

4.1: Evolutionary Distances between the Bacterial Isolates 40

4.2: Percentage distribution of isolates in the seafood samples from Nembe 42

4.3: Percentage distribution of isolates in the seafood samples from Ox-bow Lake 44

4.4: Antimicrobial Resistant Profile of Isolates from Nembe (n=80) 50

4.5: Antimicrobial Resistant Profile of Isolates from Ox-bow (n=120) 52

4.6: Agarose gel electrophoresis of the *acrAB* genes 70

4.8: Agarose gel electrophoresis of the *ctx-m* genes 72

4.9: Agarose gel electrophoresis of the *shv* genes 74

4.10: Agarose gel electrophoresis of the *tdh* genes 76

4.11: Agarose gel electrophoresis of the *mecA* gene 78

APPENDICES **PAGE**

Appendix 1: Media Used 95

Appendix 2: Antibiotics Used 97

Appendix 3: Media Preparation 101

Appendix 4: Microscopy 102

Appendix 5: Biochemical Test 102

Appendix 6: Total colony count, colonial characteristics and biochemical reaction of cultures isolated from periwinkle in Nembe 104

Appendix 7: Total colony count, colonial characteristics and biochemical reaction of cultures isolated from periwinkle in Ox-bow Lake 105

Appendix 8: Total colony count, colonial characteristics and biochemical reaction of cultures isolated from crab Nembe 106

Appendix 9: Total colony count, colonial characteristics and biochemical reaction of cultures isolated from crab from Ox-bow Lake 107

Appendix 10: Total colony count, colonial characteristics and biochemical reaction of cultures isolated from oyster from Nembe 108

Appendix 11: Total colony count, colonial characteristics and biochemical reaction of cultures obtained from oyster from Ox-bow Lake 109

Appendix 12: Total colony count, colonial characteristics and biochemical reaction of cultures obtained from shrimp from Nembe 110

Appendix 13: Total colony count, colonial characteristics and biochemical reaction of cultures obtained from shrimp from Ox-bow Lake 111

Appendix 14: Percentage Distribution and Occurrence of Bacterial Isolates in Seafood Isolated from Nembe (n=80) 112

Appendix 15: Percentage Distribution and Occurrence of Bacterial Isolates in Seafood Isolated From Ox-Bow Lake (n=120) 113

Appendix 16: Analysis of Variation in the Colony Counts of Bacterial Isolates from Seafood Samples from Nembe (Single Factor**)** 114

Appendix 17: Analysis of variation in the colony counts of bacterial isolates from seafood samples from Ox-bow Lake (single factor) 115

Appendix 18: Analysis of variation in the colony counts of bacterial isolates from seafood samples from Nembe and oxbow samples (two-ways without replication) 116

Appendix 19: Antimicrobial Resistance Profiles of Bacterial Isolates obtained from Periwinkle in Nembe 117

Appendix 20: Antimicrobial Resistance Profiles of Bacterial Isolates obtained from Periwinkle in Ox-Bow Lake. (According to CLSI 2013) 118

Appendix 21: Antimicrobial Resistance Profiles of Bacterial Isolates Obtained from Crab in Nembe. (According to CLSI 2013) 119

Appendix 22: Antimicrobial Resistance Profiles of Bacterial Isolates obtained from Crab in Ox-Bow Lake. (According to CLSI 2013) 120

Appendix 23: Antimicrobial Resistance Profiles of Bacterial Isolates obtained from Oyster in Nembe. (According to CLSI 2013) 121

Appendix 24: Antimicrobial Resistance Profiles of Bacterial Isolates obtained from Oyster in Ox-Bow Lake. (According to CLSI 2013) 122

Appendix 25: Antimicrobial Resistance Profiles of Bacterial Isolates obtained from Shrimps in Nembe. (According to CLSI 2013). 123

Appendix 26: Antimicrobial Resistance Profiles of Bacterial Isolates obtained from Shrimps in Ox-Bow Lake. (According to CLSI 2013). 124

Appendix 27: Antimicrobial Resistance Profile of Isolates from Nembe (n=80) 125

Appendix 28: Antimicrobial Resistance Profile of Isolates from Ox-bow lake (n=120) 126

FIGURES IN APPENDIX **PAGE**

A: Luria betani (lb) 96

B: Antibiotic discs 98

C:1000 Nano Spectrophotometer, Electrophoretic tank and UVP chamber 99

D: Discrete colonies growing on TCBS 100

E: Zones of Inhibition on Meuller Hinton Agar 101

ABSTRACT

Microorganisms have developed different mechanisms of evading the actions of antimicrobial agents, consequently, reducing the effectiveness of these antimicrobial agents. Antibiotics abuse and their use in animal husbandry have increased the acquisition and transfer of antibiotic resistant genes from and within the environment. This study is aimed at detecting resistance and virulence genes in bacterial isolates from seafood. A total of 200 fresh seafood samples (crab, shrimp, oyster and periwinkle) were collected randomly from Nembe River and Ox-bow Lake. Isolates were obtained using the conventional microbiological methods after an initial enrichment treatment in peptone for six (6) hours. The pure cultures were screened by gram staining and biochemical test for preliminary identification and representative isolates were characterized for 16SrRNA using Polymerase Chain Reaction and Sequencing. The isolates were further subjected to pathogenicity test to identify the presence of hemolysin and coagulase as well as antimicrobial susceptibility testing using standard oxoid commercial antibiotics. The 16srRNA sequence of the isolates produced an exact match during the megablast search to *Staphylococcus gallinarum, Vibrio rotiferianus, Vibrio parahaemolyticus, Klebsiella aerogenes* and *Klebsiella quasipneumoniae.* All isolates showed resistance to ceftriazone, ceftazidime, ampicillin and penicillin. The multi-drug resistant isolates were analyzed for the presence of the resistance genes *ctx-M*, *shv*, *mec A* and *acrAB* as well as virulence genes; *tdh* and *sxt. acrAB* genes were detected in *Klebsiella aerogenes* and *Klebsiella quasipneumoniae*, the *ctx-M* was detected only in *Klebsiella quasipneumoniae,* the *shv* was detected in *Klebsiella quasipneumoniae, Klebsiella aerogenes* and *Vibrio rotiferianus.* The *tdh* genes were detected in *Vibrio parahaemolyticus* only. *Staphylococcus gallinarum* was positive for *mecA* genes. The presence of these Extended Spectrum β-Lactamases and other resistance genes explains why the isolates were resistant to the third generation cephalosporins as well as the β-lactam antibiotics. The presence of virulence gene, *tdh* in *Vibrio* sp codes for the virulence in these organisms therefore rendering the food unsafe for consumption. Adequate handling as well as proper cooking of seafood before consumption should be highly recommended so as to reduce the incidence of food-borne infections as well as antibiotic resistance.

KEY WORDS: SEA FOODS, MOLECULAR CHARACTERIZATION, ANTIBIOTICS VIRULENCE GENES, RESISTANCE GENES, BACTERIAL ISOLATES, MULTI-DRUG RESISTANCE,

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND INFORMATION

The quest for better food quality has led to an exponential increase in the consumption of seafood because of the nutritional composition. Increased consumption of these foods without proper handling and preparation has led to an increase in the incidence of food borne infection and food poisoning thus posing high public health risk. There is therefore a need to ensure food safety at all levels by eliminating every source of contamination in these sea foods. Bacteria of the genus *Vibrio, Klebsiella* and *Staphylococcus* have been implicated in seafood. *Vibrio* species inhabit the estuarine/coastal regions and have been implicated in raw or undercooked seafood. They are widely distributed worldwide in seawater and are found commonly in association with resident aquatic organisms (Arungiri *et al.,* 2016). Common examples include *Vibrio paraheamolyticus, Vibrio cholerae, Vibrio harveyi, Vibrio rotifernus* and *Vibrio vulnificus.* These species are found in sea foods such as shell fish, bivalves mollusks, oysters, shrimps and other benthic animals and have been implicated in a number of gastro enteric diseases. Consumption of uncooked or undercooked sea food contaminated with *Vibrio* secies pose a considerable public health threat as these infections can become sporadic or even pandemic. *Vibrio* species are gram negative, rod shaped halophilic bacteria. Some *Vibrio* species are motile with the aid of horizontally unsheathed flagella while others are non-motile. They are obligate anaerobes and are non-endospore formers. They are positive to oxidase test with the exception of *V.metchnikovii* (Farmer *et al.,* 2003).*Vibrio* species with the exception of *V. cholerae* and *V. mimicus* are halophilic in

nature and as such require medium that is rich in sodium chloride for growth and thus explains the reason why they survive in marine and estuarine waters (Sridhar *et al.*, 2006).
The *Vibrio* pathogenic species produce various virulence factors including enterotoxin, haemolysin, cytotoxin, protease, lipase, phospholipase, siderophore, adhesive factor and haemagglutinins (Zhang and Austin, 2005).The pathogenicity of *V.parahaemolyticus* is associated with the production of thermostable direct hemolysis and TDH-related hemolysin encoded by the *tdh* and *trh* genes respectively and these are responsible for the hemolysis of human red blood cells. It also possesses a thermolabile hemolysin encoded by the *tlh* genes (Zhang and Austin, 2005).

These bacteria have been recognized as the main agents causing food borne diseases in many countries including Asian countries, the United States, France, Mexico, Peru and Chile (Cabanillas-Beltran *et al.,* 2006). Due to the increase in the consumption of sea foods worldwide there has been a drastic increase in food borne infection which in turn has led to the increase in gastro intestinal diseases in humans (Cabanillas-Beltran *et al.,* 2006). Sporadic cases of cholera have been reported in Nigeria as well as gastroenteritis caused by *V. parahaemolyticus* due to the consumption of raw and undercooked seafood (Cabanillas - Beltran *et al.,* 2006).

Staphylococcus species are usually associated with the skin and mucous membrane of animals and humans. They are gram positive, mesophilic, oxidase negative cocci organisms that have been implicated in several clinical and environmental samples. The diseases caused by *Staphylococcus* species range from acute septicemia to osteomyelitis in poultry (Xu *et al*., 2018). Example of *Staphylococcus* species include; *Staphylococcus pyogenes, Staphylococcus aureus, Staphylococcus epidemidis, Staphylococcus equorum,* *Staphylococcus felis*, *Staphylococcus gallinarum* etc. *Staphylococcus gallinarum* are usually isolated from chicken skin but have also been isolated from seafood. *Staphylococcus* species possess virulence factors such as enterotoxin, coagulase, hemolysins, toxins etc. Food poisoning arising from ingesting food infected by *Staphylococcal* enterotoxins is usually self-limiting (Kérouanton *et al*., 2007; Le-Loir *et al.*, 2003). *Staphylococcus* species pose a high food risk due to the presence of staphylococcal enterotoxins (SEs) produced by enterotoxigenic strains which are highly heat stable and resistant to enzymatic actions like proteolysis (Omoe *et al*., 2005). Other species of *Staphylococcus* have evolved different methods through which they evade the actions of certain antibiotics such as methicillin, penicillin and oxacillin. Example is the Methicillin-resistant *Staphylococcus aureus* (MRSA) (Zhang *et al.,* 2005). The gene that codes for this resistance is the *mecA* gene located on the *mec A operons* of Staphylococci cassette chromosome (*SCCmec*, 21 to 67 kb size) (Bronner *et al.,* 2004). The *mec A* also codes for penicillin binding proteins (PBP2α) responsible for the resistance of these organisms to β-lactams such as methicillin and oxacillin (Plata *et al.,* 2009). The resistance is acquired through the horizontal transfer of the *mec A* genes from *S. epidermidis* and other coagulase negative staphylococci (CoNS) to *S. aureus* (Xu *et al*., 2018).

Klebsiella species have been implicated in both human and animal samples where they play roles as pathogens widely distributed in the environment (Brisse *et al*., 2009; Shon *et al*., 2013). They are gram negative rod shaped oxidase negative bacteria that have been implicated in so many clinical infections such as pneumonia, wound infection, meningitis, urinary tract infections, nosocomial infections, etc (Holt *et al.*, 2015). The pathogenicity factors of *Klebsiella* are capsules or lipopolysaccharide and are considered potential candidate for vaccination that will serve as infection immunological control measures (Holt *et al.*, 2015). Different species of *Klebsiella* are *Klebsiella pneumoniae, Klesiella aerogenes, Klebsiella quasipneumoniae, Klebsiella varicola* etc (Brisse & Verhoef, 2001). *K. pneumonia* is responsible for most of the hospital acquired infections (Celinska, 2012). The phylogenetic groups are subdivided into KpI, KpII-A, KpII-B and KpIII (Brisse *et al*., 2004; Fevre *et al*., 2005). They are divided into three different taxonomic subspecies which include; *K. pneumoniae* subsp. *pneumoniae, K. pneumoniae* subsp. *rhinoscleromatis* and *K. pneumoniae* subsp. *ozaenae* belonging to KpI phylogenetic groups (Brisse & Verhoef, 2001; Brisse *et al.*, 2009; Grimont and Grimont, 2005). *Klebsiella variicola, Klebsiella singaporensis* and *Klebsiella michiganensis* are the three novel species of the genus *Klebsiella* that have been described in the last decade however, *K. variicola* and *K. quasipneumoniae* are sister species of *K. pneumoniae*. *K. variicola* has also been isolated from plant tissues (Martnez *et al*., 2004; Rosenblueth *et al*., 2004). Despite being rarely documented, *K. quasipneumoniae* appears to be capable of undergoing homologous recombination with other *Klebsiella* spp., allowing it to exchange both chromosomal and plasmid-borne antibiotic resistance genes (Holt *et al.,* 2015). *K. quasipneumoniae* was initially known as a commensal intestinal colonizer. However, recent genomics driven studies have documented it as an etiologic agent in a number of clinical *Klebsiella*-related infection cases (Long *et al.*, 2017).

The people of Nembe and Ox-bow live around the river banks and are known majorly for fishing as an occupation. There is constant discharge of sewage matter into the water bodies where these seafoods are harvested from and thus poses a high risk to the community who consume these foods undercooked or even raw. This study is aimed at screening seafood samples obtained from Bayelsa for the presence of virulence and resistance genes obtained from *Vibrio* species, *Klebsiella* species and *Staphylococcus* species. This knowledge will help sensitize people on the risks associated with consuming raw and undercooked seafood and the need to subject them to heat treatment before consumption. Additionally, knowledge of the virulence factors of these organisms will provide new insights into pathogenic strategies of the bacteria as virulence factors are promising candidates for vaccination efforts that may serve as immunological infection control measures (Holt *et al.*, 2015).

1.2 STATEMENT OF THE PROBLEM

The demand for better food quality in terms of its nutritional properties has led to increase in the consumption of seafood. However, Seafood harbor microorganisms that if consumed without proper handling or preparation can lead to food borne illnesses. Also, treatments of these infections have been a challenge lately due to antibiotic resistance caused by antibiotic abuse, mutation or transfer of resistance genes in the environment. There are lots of reports on the food-borne pathogens associated with seafood. However, there is little or no information on the antibiotic resistance profile and multidrug resistance of the bacterial species isolated from this locations. Also, there is paucity of information on the virulence and resistance genes associated with the bacterial species isolated from seafood obtained from these locations. It is therefore imperative to isolate and identify pathogens present in the seafood's as well as determine the resistance and virulence factors present in the pathogen.

1.3 AIM OF STUDY

The aim of this study is to detect the virulence and resistance genes responsible for the pathogenicity and resistance of some bacteria species isolated from seafood samples collected from Nembe and Ox-bow lakes in Bayelsa State.

1.4 OBJECTIVES

The specific objectives of this study are:

1. To isolate and identify some bacteria species implicated in some seafood such as periwinkle, oysters, crabs and shrimps.
2. To identify the antibiotic resistance profile of the bacterial species isolated.
3. To ascertain the multidrug resistant patterns of the isolates
4. To identify the phylogenetic relationship between the isolates.
5. To identify the virulence genes associated with the species isolated
6. To identify antibiotic resistant genes associated with the species isolated from the seafood.

1.5 JUSTIFICATION

Antibiotic resistance has become an issue of global concern and as such scientists, researchers, pharmaceutical industries and health institutions are working tirelessly to tackle this problem. Mutation, antibiotic abuse and horizontal gene transfer are some of the factors responsible for antibiotic resistance. Also, microorganisms have continued to develop different strategies to overcome the effects of antimicrobial agents and this is largely due to lack of innovation in the production of novel antibiotics. This study will identify the genetic factors responsible for the virulence and resistance of some bacterial species implicated in in selected seafood. Identifying and reporting resistance and virulence genes in these isolates can give an insight into new antibiotic combinations that can be used to overcome the cases of antibiotic resistance and curb future emergence of potentially pathogenic bacteria.

Also, this present study will not only identify the pathogens present in seafood, but also their antibiotic resistant patterns as well as the genetic factors responsible for their resistance. This study will bring to the knowledge of the public the need to heat treat seafood before consumption as well as sensitize people on the risk associated with comsuming undercooked and uncooked seafoods. The identification and reporting of these resistance and virulence genes in these isolates can give an insight into new antibiotic combinations that can be used to overcome the cases of antibiotic resistance.

1.6 SIGNIFICANCE OF STUDY

The isolation and molecular characterization of these organisms implicated in sea food will help to determine the incidence of these organisms in such food and also help to reduce the diseases associated with consumption of these sea foods, thus, ensuring food safety. This will also help enlighten people on the risks associated with eating raw sea foods and also reduce the cases of antibiotic resistance.

CHAPTER TWO

2.1 REVIEW OF LITERATURE

Vibrio parahaemolyticus is the causative agent of acute gastroenteritis. It was first isolated in 1950, during a large outbreak of gastroenteritis that occurred in Japan (Farmer *et al.,* 2005). It is characterized by profuse watery diarrhea, cramps, vomiting, and nausea. *V. parahaemolyticus* food poisoning is caused by the consumption of raw oyster, clams and shellfish (Nasreldin, 2013). The pandemic have been recorded in America and Europe. It has also been recorded in Japan, south East Asia, India and Africa where raw fish is implicated as a vehicle (Jay *et al.,* 2005). *V. parahaemolyticus* is usually resident in coastal waters but recently it has been isolated from fresh water fish (Jay *et al.,* 2005). *V. parahaemolyticus* is halophilic, mesophilic and is unable to ferment sucrose making it easier to be isolated in temperate regions. It is associated with crustaceans, shrimps, crab and molluscan shell fish and free swimming fish at a concentration of 10^3 cells per gram (Papovic *et al.,* 2010).

V. cholerae is the causative agent of cholera. It is characterized by profuse watery diarrhea with flakes, mucus, dehydration and even death (Jay *et al.,* 2005). It was first discovered in 1817 and since then about seven pandemics have been recorded (Jay *et al.,* 2005). They are found in highly saline waters. Studies have shown that the *V. cholerae* remains viable for 14days in refrigerated raw vegetables and at room temperatures for 28 days. On dry cereals like maize, rice and biscuits, it survives for 1- 5 days at 4^0C (Jay *et al.,* 2005). It also survives in cooked foods at room temperature for 14-24 days (Shicongo-Nambabi *et al.,* 2012). The *V. cholerae* strains have more than 150 O serotypes belonging to O1 and O139 serotype. They are considered virulent because they contain the cholera toxin gene (ctx), the toxincoregulated pilus gene (tcp) and other virulent genes. *V. cholerae* causes diarrheal disease and could lead to other diseases such as bacteremia, invasive soft tissue infection, cholecystitis and peritonitis (Farmachidi *et al.*, 2003).

V. vulnificus infection case in human was first recorded in USA in the year 1964 and in Taiwan in the year 1987 (Harwood *et al.*, 2004). It is an opportunistic pathogen in the elderly, immunocompromised persons and people with impaired functions or other disease like liver cirrhosis, diabetes and on those who take steroids (Farmachidi *et al.*, 2003). *V. vulnificus* causes three important diseases which are septicemia, necrotizing wound infection and gastroenteritis with a mortality rate of about 50% occurring one to two days after onset of the symptoms (Cazoria *et al.*, 2011). The organism is found to be associated with seafood such as shell fish, oyster, mussels and clams. It is usually found in environments with high salt content (Cazoria *et al.*, 2011).

The incidence of Vibrio species has shown great correlation between the ingestion of these sea foods and the food borne infections (Arungiri *et al*., 2016).

A lot of studies have been done on Vibrio species associated with sea foods. A study carried out by Chigozie and Ogunbanwo (2015) on the prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated from ninety sea foods in Lagos Nigeria showed that shrimps and crabs were the main sources of *V. parahaemolyticus* in Lagos Nigeria and that the organism was resistant to ampicillin.

Another study carried out by Arungiri *et al*., (2016) on the incidence of *Vibrio* species associated with blue crabs (Callinectes sapidus) collected from Galveston Bay Texas showed that the hemolymph of most infected crabs contained very high number of *Vibrio cholerae*, *V.vulnificus* and *V. parahaemolyticus*.

According to a study carried out by Arunagiri *et al*., (2016) on the study of Vibrio species and its occurrence frequencies in seafood samples, V.cholerae and V. parahaemolyticus had the highest percentage occurrence in seafood and as such posed a very high public health hazard. The quality of marine fish is dependent on the microbial load and the microbial load depends on the physiochemical parameters of the water known as the extrinsic factors. It also depends on the intrinsic factors of the fish itself, food processing, transportation and storage (Shikongo-Nambabi *et al*., 2012).

Ahmed *et al*., (2012) worked on the occurrence and characteristics of methicillin-resistant *Staphylococcus aureus* and methicillin-resistant coagulase-negative staphylococci from Japanese retail ready-to-eat raw fish with the aim of determining the prevalence, molecular genetic characteristics, antibiotics resistance and virulence factors of bacteria. The bacteria; methicillin-susceptible *Staphylococcus aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant coagulase-negative *staphylococcus* (MR-CoNS) were isolated from 200 samples of retail ready-to-eat raw fish (sashimi) collected from the Japanese prefecture of Hiroshima. The SCC*mec* typing was carried out and it revealed the presence of a type IV SCC*mec* cassette in *S. warneri* isolates, a type II SCC*mec* cassette in *S. haemolyticus* isolates and a cassette in methicillin-resistant *S. aureus* (MRSA) isolates that could not be typed. The Molecular typing carried out on two MRSA isolates by *spa* sequencing and multilocus sequence typing (MLST) identified t1767 and ST8, respectively. Also, antibiotic resistance genes coding for resistance to aminoglycosides, tetracyclines, β-lactams, macrolides, lincosamides and streptogramin B (MLS_B) antibiotics were detected as well (Ahmed *et al.,* 2012). A study by Kim *et al*., (2017) revealed the presence of *Bacillus cereus, Escherichia coli, S. aureus*, *V. parahaemolyticus and V.*

vulnificus with samples from the fishery market showing a higher detection rate especially in *V. parahaemolyticus* (21·6%) and *V. vulnificus* (1·7%) which indicates the need to improve microbiological safety of raw ready-to-eat seafood products in fishery market (Kim *et al.*, 2017).

2.2 SEAFOODS

Benthic organisms otherwise called benthos are community of organisms that live in, on or near the sea bed called the benthic zone. Seafood such as fishes, shrimps, bivalves (oysters), sea stars, crabs, periwinkle, clams, sea cucumbers, brittle stars, sea anemones, etc serve as important sources of food due to their nutritional properties (Ryan, 2007). Studies have shown that in recent years, consumption of seafood has been on the increase because of their health benefits and nurtitional importance. Seafood is rich in proteins, vitamins, minerals, 2-aminoethane sulfonic acid and n-3 polyunsaturated fatty acids which are not usually present in other terrestrial animals (Mohiuddin, 2019).

2.2.1 PERIWINKLE

The *Littorina littorea is a* common periwinkle that belongs to the specie of edible sea snails and marine gastropods that possesses gills and operculum characterized by a broad ovate, thick and sharply pointed shell with six to seven whorls with fine thread and wrinkles (Chapman *et al.*, 2007). The width of the shell ranges from 10 to 12 mm at maturity, with an average length of 16–38 mm.Shell height can reach up to 30 mm, 43 mm or 52 mm. The length is measured from the end of the aperture to the apex. The height is measured by placing the shell with the aperture flat on a surface and measuring vertically (Chapman *et al.*, 2007).

2.2.2 CLASSIFICATION OF PERIWINKLE (*Littorina littorea*)

Kingdom	Animalia
Phylum	Mollusca
Class	Gastropoda
Superfamily	Littorinoidae
Family	Littorinidae
Genus	Littorina
Species	Littorea

(Chapman *et al.*, 2007).

2.2.3 CRABS

Crabs (Corystes cassivelaunus) along with others such as shrimps lobsters and crayfish belong to the subphylum crustacean and infraorder Bachurya characterized by having eight long legs, two pairs of claws and chitinous exoskeleton. While some crabs live in fresh water others live on salt water and land. Crab serve as a good source of protein and so is usually eaten as food. Crabs are omnivorous in nature and they primarily feed on Algea, worms, crustaceans, fungi, bacteria and detritus (Mohiuddin, 2019).

2.2.4 CLASSIFICATION OF CRAB (Corystes cassivelaunus)

Kingdom:	Animalia
Phylum:	Arthropoda
Subphylu	Crustacea
Class:	Malacostrac
Order:	Decapoda
Suborder:	Pleocyemat
Infraorder:	**Brachyura**

Sections and subsections

- Dromiacea
- Raninoida
- Cyclodorippoida
- Eubrachyura
  - Heterotremata
  - Thoracotremata

(Mohiuddin, 2019)

2.2.5 SHRIMPS

Shrimps known as Black Tiger Shrimps scientifically known as *Panaeus monodon* are decapod crustaceans with elongated bodies and have swimming mode of locomotion. Shrimps are very nutritious, fairly low in calories and possess high protein content as well as healthy fats and variety of vitamins and minerals (Maheswamdu, 2016).

2.2.6 CLASSIFICATION OF BLACK TIGER SHRIMP (*Panaeus monodon*)

Kingdom	Animalia
Phylum	Arthropoda
Sub phylum	Crustecea
Class	Malacostraca
Order	Decapoda
Sub order	Dendrobranchiata
Family	Penaeidae
Genus	Panaeus
Species	Monodon

(Maheswamdu, 2016).

2.2.7 OYSTERS

Oysters (*Crassostrea grigas)* are molluscan shellfish called bivalves. They are an excellent source of zinc, iron, calcium, and selenium, as well as vitamin A and vitamin B_{12}. Oysters are low in calories but are rich in protein and vitamins such as zinc and vitamin B_{12} (Maheswamdu, 2016).

2.2.8 CLASSIFICATION OF OYSTER (*Crassostrea grigas)*

Kingdom	Animalia
Phylum	Mollusca
Sub phylum	Bivalva
Class	Lamellibranchia
Order	ostreioda
Sub order	pteriomorphia
Family	ostreidea
Genus	crassostrea
Species	grigas

(Maheswamdu, 2016).

2.3 STAPHYLOCOCCUS MEDIATED INFECTIONS

Coagulase negative *Staphylococcus* are staphylococcal species other than *Staphylococcus aureus* that are usually non-pathogenic unless when opportunistic and are usually implicated in clinical, environmental as well as seafood samples (Becker *et al*., 2014). Some factor such as clinical procedural changes have made Coagulase Negative *Staphylococcus* species more of nosocomial rather than commensals (Becker *et al*., 2014). *Staphylococcus* species are generally implicated in pyogenic infections, acute urethritis, endocarditis and food borne intoxication (Heilmann, 2011). They also colonize the skin and mucous membranes of humans and animals and are less frequently involved in clinically manifested infections (Heilmann, 2011). Example of Coagulase Negative *Staphylococcus* species isolated from human clinical specimens comprise *S. jettensis*, *S. massiliensis*, *S. petrasii* (including *S. petrasii* subsp. *petrasii* and *S. petrasii* subsp. *croceilyticus*), *S. gallinarum* and *S. pettenkoferi*. Staphylococci have the capacity to colonize and infect human and animal hosts.

They possess a species and strain-specific weapon of diverse strategies to enable them carry out adherence, aggression, invasion, persistence, and/or evasion of both innate and adaptive immunity (Heilmann, 2011). However, *S. aureus*, has a defined clearly virulence mechanisms unlike CoNS which on the other hand is involved in biofilm formation as in the case of *S. epidermidis* (Becker *et al*., 2014). In general, CoNS isolates lack the virulence determinants responsible for aggression. Nevertheless, factors involved in colonization may successfully support the bacterium-host interaction, a phenomenon that may be based, at least partly, on the multifunctional character of various staphylococcal virulence factors known to exhibit redundant and overlapping functions (Becker *et al*., 2014).

2.4 KLEBSIELLA MEDIATED INFECTIONS

Klebsiella pneumoniae was initially classified into three genetically closely-related phylogroups, and more recently, they have been classified into three distinct species: *K. pneumoniae* [KPI], *K. quasipneumoniae* [KpII],and *K. variicola* [KpIII] (Holt *et al.*, 2015). These enterobacteria have been considered to be one of the most prevalent nosocomial enterobacterial pathogens responsible for infections that range from mild urinary infections to severe bacteremia and pneumonia characterized by high mortality and morbidity rates (Long *et al*., 2017). Traditional microbiological assay and Biochemical testing have not shown accurate differentiation between *Klebsiella* spp leading to false reporting of clinically isolated *K. quasipneumoniae* to be *K. pneumoniae* (Long *et al*., 2017). *K. quasipneumoniae* was initially known as a commensal intestinal colonizer but has now been implicated in seafood samples. However, recent genomics driven studies have documented it as an etiologic agent in a number of clinical *Klebsiella*-related infection cases and with minimal positive yield in the effectiveness of antibiotic treatment of the infections caused by them.

This is clearly due to antibiotic resistance increasing emergence of multidrug resistance among *K.pneumoniae* nosocomial isolates as this has limited the therapeutic options for treatment of these infections (Bradford, 2001).

2.5 VIBRIO MEDIATED INFECTIONS

Vibrio species causes a number of infections ranging from human to animals which are usually more distributed during the warmer months (Adams & Moss, 2008). Sources of infections range from contaminated water to consumption of uncooked or undercooked contaminated sea food (Adams & Moss, 2008). Infections caused by *Vibrio* species can be divided into two: cholera caused by consuming food or water contaminated with *Vibrio cholerae* characterized by diarrhea and non-cholera infections called vibriosis (Adams & Moss, 2008). This causes septicaemia and mild gastroenteritis. *Vibrio* species responsible for these are the *V. parahaemolyticus* and *V. vulnificus* (Adams &Moss, 2008). Several *Vibrio* bacteria species can cause serious diseases in humans or animals. Twelve *Vibrio* species have been recognized as potential foodborne disease agents in humans, of which, *V. parahaemolyticus* is the most common (Adams &Moss, 2008). Although strains of *V. parahaemolyticus* are environmental, many strains are pathogenic to humans. Virulent strains of *V. parahaemolyticus* can cause wound infections, septicemia, or more commonly acute gastroenteritis which is acquired through the consumption of raw or undercooked seafood, especially shellfish (Letchumanan *et al.,* 2014). Outbreaks of *V. parahaemolyticus* have been reported in many countries such as the USA, France, and New Zealand because of the increase in seafood consumption and the global warming, which may be resulting in a higher prevalence of *Vibrio* species and increase the risk of *Vibrio*-borne infections (Nair, 2007;

Cruz *et al.,* 2015). Recognized infections from *Vibrio* species are increasing. Most researchers predict that climate change will increase cases (Burge *et al.,* 2014; Letchumanan *et al.,* 2014).

2.6 RESISTANCE MECHANISMS OF VIBRIO SPECIES

Vibrio species exhibit several antibiotic resistance mechanisms which are usually not restricted to the following: exporting drugs through efflux pumps, chromosomal mutations, or developing genetic resistance via the exchange of conjugative plasmids, conjugative transposons, integrons, or self-transmissible chromosomally integrating SXT elements (Kitaoka *et al.,* 2011; Burrus *et al,* 2006). Some antibiotic-resistant *Vibrio* species in sub-Saharan Africa, as well as their resistance mechanisms include multi-drug efflux pumps to establish resistance against antimicrobial agents and other toxic compounds by a mechanism that prevents the accumulation of drugs inside the bacterial cells. *V. cholerae* contains more than forty different antimicrobial resistant encoding genes that can confer resistance to as much as nine different classes of antimicrobial drugs (Verma *et al.,* 2019). Antimicrobial resistant mechanism in *V. cholerae* is usually acquisition of Antimicrobial Resistant Genes through Horizontal Gene Transfer (HGT) and physically linked with Mobile Genetic Elements (MGEs) (Wozniak & Waldor, 2010). Antimicrobial Resistance Genes have been shown to be distributed in the genome environment by MGEs. The processes mediating this distribution include Transduction, Tranformation, conjugation and fusion of outer membrane vesicles (Das, 2020). *V. cholerae* has shown its ability in using multidrug efflux pumps to export a wide range of antibiotics, detergents, and dyes that are chemically and structurally unrelated (Das, 2020). Collectively, multi-drug efflux pumps are not employed only for drug resistance, but have also been implicated in the expression of important virulence genes in *Vibrio* pathogens (Das, 2020).

2.7 RESISTANCE MECHANISMS OF COAGULASE NEGATIVE STAPHYLOCOCCUS SPECIES

Coagulase Negative Staphylococci have in recent times developed novel methods of evading antibiotic actions particularly for penicillin, oxacillin, ciprofloxacin, clindamycin, erythromycin, and gentamicin and this could be as a result of mutation or acquisition of resistant genes from other related species through genetic elements (García-Vázquez*et al.*, 2013). Acquired resistance is caused by mobile genetic elements which can be found in CoNS of both animal and human origins (García-Vázquez*et al.*, 2013). They possess a reservoir of genetic elements leading to resistance not only to the β-lactam antibiotics but also to other antibiotic classes (García-Vázquez*et al.*, 2013). This is of the utmost importance for human and veterinary medicine, because these genetic elements are mobile by nature and thus, they may be transfered into the medically most significant staphylococcal species, *S. aureus*, leading to problems related to the emergence of MRSA as seen after the introduction of the penicillinase-stable penicillins (Gill *et al.*, 2005). An example is the resistance of Coagulase Negative *Staphylococcus* to tetracyclines which is based primarily on the acquisition of mobile *tet* and *otr* genes. This leads to ribosomal protection through dissociation of tetracyclines from their ribosomal binding sites and also drug efflux through active transportation of the agents out of the bacterial cell (Kriegeskorte *et al.*, 2012).

The resistance of Coagulase Negative *Staphylococcus* to glycopeptid is by cell wall alterations, resulting in reorganization and thickening as well as reduction in autolytic activity (Howden *et al.,* 2010). Cell wall thickening has been reported for glycopeptide-resistant CoNS (*S. epidermidis* and *S. haemolyticus*) (Howden *et al.,* 2010).

Methicillin resistance is mediated by the *mec* genes harbored by a staphylococcal Chromosomal Cassette *mec* mobile genetic element inserted into the chromosome (Howden *et al.,* 2010). This cassette is composed of three major elements: the *mec* gene complex, the *ccr* gene complex, and the joining ("junkyard" or J) regions. The *mec* gene complex comprises the *mecA* gene itself, its regulatory genes; *mecI* (a repressor) and *mecR1* (a sensor inducer), as well as an insertion sequence, IS*431mec* (Gill *et al.,* 2005). The cassette chromosome recombinase genes *ccrAB* and *ccrC* encode site-specific integrases catalyzing the integration or excision of the entire SCC*mec* into or from the *orfX* locus at its 3′ end in the staphylococcal genome (Howden *et al.,* 2010).

2.8 RESISTANCE MECHANISMS OF *KLEBSIELLA* SPECIES

Studies on *Klebsiella* resistance pattern and mechanisms have shown that there is an increased percentage of *Klebsiella* strains that are resistant to antimicrobials of the cephalosporins and flouroquinolone groups (Wang *et al.,* 2004). Resistance to Fluoroquinolone is associated with mutations in the quinolone resistance-determining region of the *gyrA* and or *parC* gene coding for the target proteins DNA gyrase and topoisomerase IV, respectively (Hooper**,** 2000). Plasmid-mediated resistance to quinolones has also been described (Wang *et al.,* 2004), and its frequency seems to be increasing in recent years (Rodríguez-Martínez *et al.,* 2003). Besides topoisomerase mutations and plasmids, altered permeability (usually because of porin loss) and energy-dependent efflux have also been shown to contribute to the fluoroquinolone resistance phenotype in *K.*

pneumoniae (Martínez-Martínez *et al.,* 2002). One of the efflux systems involved in this resistance phenotype is the *acrAB* multidrug efflux system that in *K. pneumoniae* is encoded by the *acrAB* operon. In this operon, *acrR* encodes the *acrAB* repressor, while *acrA* and *acrB* encode a periplasmic lipoprotein of 40 kDa, anchored to the inner membrane, that bridges the outer and inner membranes and an integral membrane protein of 113.5 kDa with 12 membrane-spanning α-helices, located in the cytoplasmic membrane, respectively (Domenech-Sanchez *et al.,* 2001). The *acrB* connects with TolC, an outer membrane protein that belongs to a family of envelope proteins found in all Gram-negative bacteria and that is essential for the expulsion of a plethora of compounds (Eswaran *et al.,* 2003). Most enterobacteriacea are known to possess Extended Spetrum B-Lactamases such as SHV and CTX-M which are responsible for the hydrolytic activity against cefotaxime and ceftazidime leading to resistance in these cephalosporins (Olusegun *et al.,* 2006). The first CTX-M-type b-lactamases were identified as plasmid-encoded enzymes in clinical isolates from the Enterobacteriaceae (Martinez *et al.,* 2004).

2.9 EXTENSIVE DRUG RESISTANCE/MULTI-DRUG RESISTANCE IN MICRORGANISMS

The spread of multidrug resistant genes in *V. cholerae* is mainly facilitated by horizontal gene transfer through self-transmissible mobile genetic elements, autonomously replicating plasmids or Integrative Genetic Mobile Elements such as SXT genetic mobile elements (Das, 2020). The SXT genetic mobile element ICE conferring resistance to sulfamethoxazole-trimethoprim was first documented in *V. cholerae* O139 or a closely related ICE in Madras, India, owing to its ability to harbor resistance to trimethoprim, sulfamethoxazole, and streptomycin (Das, 2020). Also, Multi Drug Resistance *V. cholerae* isolates of O1 serotype that are resistant to tetracycline, streptomycin and chloramphenicol have been identified (Das, 2020). SXT elements also carry resistant genes that code for resistance to sulphametazole-trimethoprim, ampicillin, streptomycin and furazolidine (Das, 2020). The relationship between self-transmissible elements and multidrug resistance has been well documented in *Vibrio* species (Das, 2020).A recent study in Cameroun revealed that *Vibrio cholerae* O1 of environmental origin harbours heterogeneous multidrug resistance towards Amoxicillin (AML), Ampicillin (AMP), Tetracycline (TE), Chloramphenicol (C), Doxycycline (DXT), and Cotrimoxazole (SXT) (Akoachere *et al.,* 2013).The frequent usage of antibiotics as part of the *Vibrio* infection treatment regimen has resulted in the development of multidrug resistance in *V. cholerae* and seafood pathogens such as pathogenic *Vibrio* species (Sudha *et al.,* 2014).

2.10 MOLECULAR IDENTIFICATION OF MICROBIOTA IN SEAFOODS

2.10.1 POLYMERASE CHAIN REACTION

Molecular identification of microorganisms has in recent times proven to be the most reliable method of identifying isolates from clinical and environmental samples. PCR is the most applied molecular biology technique for the identification of a particular microorganism where a specific genetic segment of interest in the isolate is amplified (multiplied) through the action of the enzyme Taq polymerase, polymerized deoxyribonucleotides and primer (Oliveira *et al.,* 2016). This technique is so sensitive that a single DNA molecule can serve as a template for the exponential amplification of a specific gene so as to attain a sufficient concentration used for further analysis such as cloning and sequencing (Oliveira *et al.,* 2016). There are different types of PCR which include conventional PCR, multiplex PCR, reverse transcriptase polymerase chain reaction (RT-PCR), Random Amplified Polymorphic DNA (RAPD) and Quantitative Polymerase Chain Reaction (qPCR) (Oliveira *et al.*, 2016).

2.10.2 AGAROSE GEL ELECTROPHORESIS

The amplified genetic material is further identified either by agarose Gel Electrophoresis (AGE), Denaturing Gradient Gel Electrophoresis (DGGE), Temporal-Temperature Gradient Electrophoresis (TTGE) and Pulse Field Gel Electrophoresis (PFGE). The genetic material already amplified using conventional PCR now called amplicons are visualized through gel electrophoresis (Xu *et al.*, 2017).

Xu *et al.* (2017) used multiplex PCR for the simultaneous detection of *Vibrio cholerae, V. parahaemolyticus, V. vulnificus* and *V. alginolyticus* in seafood samples, which was proven to be a fast and reliable tool for the detection of these pathogenic species. Kou *et al.* (2008) used RT-PCR multiplex to simultaneously detect artificial and natural Norovirus and Rotavirus infections in oysters and described the technique as a quick, specific and sensitive method and can be applied for virus detection in the seafood product before being marketed. Vongkamjan *et al.* (2017), after isolating *Listeria monocytogenes* from seafood and environment samples by cultivation-dependent means, confirmed the isolation by conventional PCR, followed by multiplex PCR techniques for the detection of serotypes, and RAPD for the characterization of the isolates, detecting 3 serotypes and 11 RAPD profiles. After serotype identification through multiplex PCR, it was possible to verify that the majority of the serotypes identified were the same ones detected in cases of human listeriosis. The RAPD technique confirmed the existence of genetic diversity among *L. monocytogenes* isolates. The researchers also concluded that this technique was adequate for this purpose due to the cost, time and prior knowledge required to perform the analysis (Vongkamjan *et al.*, 2017).

Taminiau *et al.* (2014) developed qPCR protocols to identify and quantify six seafood-borne pathogens and arrived at the conclusion that the protocols displayed good selectivity, specificity and sensitivity in the detection and quantification of the evaluated pathogens. They also evaluated the microbiota profile of air-conditioned and modified atmosphere using qPCR. They evaluated the limit of quantification of the elaborated protocols for artificially contaminated seafood, oysters gills stored at 4°C by PCR-DGGE techniques regarding the 16S rRNA V3 gene region. Through the DGGE profile, they verified a great bacterial diversity in the gills of both groups of oysters.

Mac *et al.* (2012) evaluated the bacterial deterioration of raw salmon conditioned under vacuum and under a modified atmosphere, using culture independent and dependent methods. In this case, some bacteria were only identified by one method, while others were identified by both. The authors state that the cultivation-dependent technique displayed greater precision in the identification of bacteria genera and species. However, PCR allowed for the confirmation of species of the dominant bacterial species during 3, 7 and 10 days of storage in samples packed in vacuum and modified atmosphere, previously identified by the traditional methodology and verified through electrophoretic profiles generated by TTGE (Mac *et al.,* 2012).

Hara-Kudo *et al.* (2012) compared *V.parahaemolyticus* characteristics isolated from patients during the 1997–2001 outbreaks and seafood contamination cases from 2007 to 2009 in Japan. The authors first carried out an epidemiological study regarding the outbreak cases and subsequently analysed the seafood samples. The culture-dependent methodology was applied to isolate *Vibrio* sp. strains from seafood, while biochemical analyses were performed to identify *V.parahaemolyticus* strains. The authors then verified whether the strains indicative of *V. parahaemolyticus* contained the *tdh* virulence gene and which serotype they belonged to, through PCR. Finally, they used PFGE to verify the similarity between strains isolated from patients in the outbreaks and samples collected from seafood. Of the 842 seafood samples, 717 were *V. parahaemolyticus* positive and 18 contained the *tdh* virulence gene (Rodriguez *et al.,* 2018).

2.10.3 SANGER SEQUENCING METHOD

Sequencing has emerged as a reliable way of elucidating the composition and genetic potential of microorganisms in seafood samples (Foster *et al.,* 2012). This technique characterizes the organism to the specie level (Foster *et al.,* 2012). The different sequencing types are first-generation sequencing, and next-generation sequencing (NGS). To this end, researchers began using Sanger sequencing technologies known as first-generation sequencing followed by next-generation sequencing (NGS) (Siqueira *et al.,* 2012). The first generation sequencing could not sequence high number of samples at a time so the next generation sequencing (NGS) which has the ability to generate a greater number of readings in a single reaction, allowing for the massive detection of the genetic material in a fast and economically viable way was identified **(**Roeselers *et al.,* 2011; Star *et al.,* 2013; Wong *et al.,* 2013; Ghanbari *et al.,*2015).

2.10.4 NEXT-GENERATION SEQUENCING—SECOND GENERATION

Next generation sequencing makes use of Five technologies namely 454 pyrosequencing (Roche Applied Science, Basel, Switzerland), Illumina/Solexa Genome Analyzer (Illumina, San Diego, CA), Sequencing by Oligonucleotide Ligation and Detection (SOLiD) (Applied

Biosystems, Foster City, CA) and HeliScope Single Molecule Sequencer (Helicos BioSciences, Cambridge, MA) (Ghanbari *et al.,* 2015). The 454 pyrosequencing, Illumina/Solexa Genome Analyzer and Sequencing by Oligonucleotide Ligation and Detection (SOLiD) clone products are usually amplified by PCR while the platform HeliScope Single Molecule Sequencer do not require prior amplification (Siqueira *et al.*, 2012).

These novel technologies were developed with the intention of improving sequencing process, increasing the length of final sequencing data, correcting errors detected from the previous platform and reducing timeframe of the process (Ghanbari *et al.*, 2015). The effects of DNA extraction and foreground choice are common problems in other molecular culture-independent methods (Kuczynski *et al.*, 2012). However, with respect to NGS specifically, short readings made by certain platforms show comparable difficulties when assembling and mapping reference sequences (Lui 2012), while longer readings, such as those produced by 454 pyrosequencing are prone to quality error **(**Siqueira *et al.*, 2012).

2.10.5 454 PYROSEQUENCING

The 454 platform marked the beginning of NGS and belongs to the second generation of genomic sequencing technologies. The principle of the method is synthesis sequencing, while the library preparation is by PCR emulsion and the chemical process is named pyrosequencing. With each addition of a nucleotide by DNA polymerase, the formation of phosphodiester bonding occurs in the single strand of amplified DNA, releasing a pyrophosphate molecule, which after a cascade of chemical reactions will generate a luminous intensity for each added nucleotide. This light is captured by the equipment, generating peaks in the plotted graph, with peak heights proportional to the amount of added nucleotides and corresponding to the specific type of embedded dNTP (Metzker 2010; Liu *et al.,* 2012; Siqueira *et al.,* 2012; Ghanbari *et al.,* 2015).A total of 454 models exist, such as 454 FLX Titanium and 454 FLX +. While the first has a read length of 450–600 bp, 10 h of sequencing, data output per run of 450 Mb and output read of 1 million, the second performs readings of 700–100 bp in 23 h with 700 Mb of data output per run and a 1 million output read (Ghanbari *et al.,* 2015)**.** In seafood, numerous studies have been carried out using such pioneering technology for sequencing (Star *et al.,* 2013). It was used to elucidate the composition of the gut microbiota of Atlantic Cod caught in a single location (Star *et al.,* 2013). It was also used to verify the effects of regulation of intestinal absorption and fatty acid metabolism in the Zebrafish (Semova *et al.,* 2012). It has also been used to characterize gastrointestinal bacteria of Atlantic salmon (*Salmo salar*) (Zarkasi *et al.,*2014) as well as identify risk markers for foodborne diseases in swimming crab (*Portunus trituberculatus*) microbiota in South Korea (Kim *et al.,* 2017).

2.10.6 ILLUMINA/SOLEXA GENOME ANALYSER

This platform also works with the synthesis sequencing principle, with library preparation by bridge PCR and chemical processing named reversible terminators (Siqueira *et al.,* 2012). Following the amplification process, sequencing takes place by the addition of nucleotides labelled with fluorophores and terminators which ensure that only one base will be incorporated at a time. The fact that the terminator is reversible enables the polymerization to continue even after the detection of the fluorophores. The fluorescence is recorded by the equipment, allowing for the detection of the added nucleotide (Siqueira *et al.,* 2012). Different models based on Illumina sequencing are available, such as Illumina GAIIx, Illumina HiSeq1000, Illumina HiSeq1500, Illumina HiSeq2000, Illumina HiSeq2500, Illumina MiSeq and Illumina NextSeq500. Comparing the HiSeq2000 platform with MiSeq, it is possible to verify that there is not only an increase in length (from 2 9 125 bp to 2 9 300 bp), 6-day reduction in 26 h of sequencing, but also a decrease in the data output per run, from 900–1000 Gb to 100–125 Gb, and output read from 250 million to 44–50 million (Ghanbari *et al.*,2015).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 SAMPLES USED AND LOCATION

The samples used in this study were Periwinkle, Crab, Oyster and Shrimps. A total of 200 samples were analyzed with 80 samples bought from Nembe river side market and 120 samples bought from Ox-bow lake market.

3.1.2 SAMPLE SIZE

Sample size was determined through the approach based on the Precision Rate and Confidence level using the formular $n=Z^2pq/d^2$ (Kothari, 2004). Using a single proportion formula, the sample size is calculated thus:

$n = Z^2pq/d^2$
Where: n = desired sample size, when population is more than 10,000
Z = Critical value at 90% or 1.64 confidence interval
p = Prevalence or population with the desired attribute pegged at 25% i.e 0.25
$q = 1 – p$
d = degree of accuracy usually set at 0.05
Hence, $Z = 1.64$, $p = 0.25$, $q = 0.75$, $d = 0.05$
$n = 1.64^2 \times 0.25 \times 0.75/0.052$ $n = 0.5043/0.0025$ $n = 201.72$ $n \approx 200$

3.2 METHODOLOGY

3.2.1 SAMPLE COLLECTION

A total of 200 seafood samples made up of Periwinkle, Crab, Oyster and Shrimps. Twenty each of the seafood samples were bought from Nembe water side market while thirty each of the four different seafood samples stated above were bought from Ox-bow lake market women in Bayelsa state making a total of 200 samples in all.

3.2.2 SAMPLE PREPARATION

Each of the samples collected were macerated using a clean blender to increase surface area to volume ratio and to obtain a homogenous state of the sample. The periwinkle samples were carefully removed from their husks using a sterile needle. All the samples were placed in peptone for six (6) hours for the enrichment treatment purpose. The samples were serially diluted (10 folds dilution) using peptone water and an aliquot (0.1ml) of the diluted sample was inoculated onto the already prepared sterile, Nutrient Agar, EMB Agar, Mannitol salt Agar and Thiosulphate Citrate Bile salt Sucrose (TCBS) agar plates.

3.2.3 INOCULATION

An aliquot (0.1ml) was aseptically collected from the required dilution factor (10^4) and inoculated onto an already prepared plate using a sterilized pipette. An L-shaped spreader was used to evenly distribute the inoculum round the surface of the agar plate so as to obtain discrete colonies after incubation. The agar plates were allowed to dry and were incubated at 35^0C for 24 hours. The results of the colonies grown were recorded.

3.2.4 MICROSCOPY AND BIOCHEMICAL CHARACTERIZATION

Isolates were identified microscopically by Gram staining. The biochemical analysis for each organism was adopted from Cheesbrough, (2006). The result for each inoculum was recorded. The biochemical tests carried out were; catalase test, oxidase test, nitrate test, indole test, and sugar fermentation test.

3.2.5 PATHOGENICITY TEST

The isolates were subjected to pathogenicity test to determine the presence of the virulence factors Haemolysin and Coagulase. The test for haemolysis was done by preparing a blood agar and inoculating it with the test isolates. The coagulase test was carried out using human plasma and the methods used were adopted from Cheesbrough, (2006).

3.2.6 ANTIMICROBIAL SUSCEPTIBILITY ANALYSIS

The antibiogram pattern of the isolated organisms were tested using 10 different conventional antibiotics from varying groups which include; Erythromycin, Ciprofloxacin, Tetracyclin, Ceftriaxone, Trimethoprim\Sulphonamide, ceftazidime, Ampicillin, Penicillin, Streptomycin, Erythromycin and Gentamycin.

The antimicrobial susceptibility testing was carried out using Kirby Bauer Disc method (Disk Diffusion Method).

The cells were collected from agar slant and thereafter sub cultured into a nutrient broth which was incubated for 24hours. This was done so as to obtain fresh 24hr cultures necessary for the antibiotic susceptibility test.

The cultures were standardized using 0.5Macfalands standards so as to obtain cells equivalent to $1.5x10^8$ CFU/ml.

Thereafter 0.1ml ($1.5x10^8$ CFU/ml) of the standardized inoculum was inoculated into a Mueller Hinton agar using the spread plate method.

Antibiotics impregnated disc were placed gently and aseptically on the Mueller Hinton agar some millimeters apart. The plates were incubated at 35^0C for 48 hours.

The diameter of the “zone of inhibition” was measured using a meter rule and compared to the standards on the Clinical and Laboratory Standard Institute (CLSI) table 2013.

3.2.7 MOLECULAR CHARACTERIZATION

3.2.7.1. DNA Extraction (Boiling method)

Overnight broth culture of the bacterial isolate (5ml) was inoculated into Luria Bertani (LB) and was spun at 14000rpm for 3 min. The cells were re-suspended in 500µl of normal saline and heated at 95^0C for 20 min. The heated bactererial suspension was cooled on ice and spun for 3 min at 14000rpm. The supernatant containing the DNA was transferred to a 1.5ml microcentrifuge tube and stored at -20^oC for other down-stream reactions (Tamura *et al.*, 2013).

3.2.7.2. DNA quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was launched by double clicking on the Nanodrop icon. The equipment was initialized with 2µl of sterile distilled water and blanked using normal saline. Two microlitre of the extracted DNA was loaded onto the lower pedestal; the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the “measure” button (Tamura *et al.,* 2013).

3.2.7.3 16S rRNA Amplification

The 16srRNA region of the rRNA gene of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.5µM and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extention, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light transilluminator (Carter *et al.,* 2010).

3.2.7.4. Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10µl, the components included 0.25 µl BigDye® terminator v1.1/v3.1, 2.25µl of 5 x BigDye sequencing buffer, 10µM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing conditions were as follows; 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min (Tamura *et al.,* 2013).

3.2.7.5. Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using MAFFT. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou and Nei, 1987, (Tamura *et al.,* 2013). The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method (Tamura *et al.,* 2013).

3.2.8. AMPLIFICATION OF RESISTANCE GENES IN ISOLATES

3.2.8.1 Amplification of *shv* Genes

shv genes from the isolates were amplified using the *shv* F: 5' CGCCTGTGTATTATCTCCCT-3' and *shv* R: 5'-CGAGTAGTCCACCAGATCCT-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master Mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 56ºC for 40 seconds; extension, 72ºC for 50 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 25 minutes and visualized on a UV (Carter *et al.,* 2010).

3.2.8.2 Amplification of *ctx-M* Genes

ctx-M genes from the isolates were amplified using the *ctx-M*F:5'-CGCTTTGCGATGTGCAG-3' and *ctx-M* R: 5'-ACCGCGATATCGTTGGT-3'primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 25 minutes and visualized on a UV transilluminator (Carter *et al.*, 2010).

3.2.8.3 Amplification of *acrAB* Genes

acrAB genes from the isolates were amplified using the *acrAB*F: 5'-ATCAGCGGCCGGATTGGTAAA-3'and *acrAB*R:5'- CGGGTTCGGGAAAATAGCGCG-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 60ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 25 minutes and visualized on a UV transilluminator (Carter *et al.*, 2010).

3.2.8.4 Amplification of *mecA* Genes

mecA genes from the isolates were amplified using the ***mecAF:*** **TGGCTATCGTGTCACAATCG**

mecAR: **CTGGAACTTGTTGAGCAGAG** primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 58ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 25 minutes and visualized on a UV transilluminator (Carter *et al.*, 2010).

3.2.8.5 Amplification of *sxt* Genes

The *sxtl* genes of the isolates were amplified using the *sxt1* F: AGCGATGCAGCTATTAATAA and

sxt1 R: GAAGAGTCCGTGGGATTACG primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; anealing, 53ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a blue light transilluminator (Carter *et al.,* 2010).

3.2.9 AMPLIFICATION OF VIRULENCE GENES IN ISOLATES

3.2.9.1 Amplification of *tdh* Genes

tdh genes from the isolates were amplified using the TdhF: 5'-CTGTCCCTTTTCCTGCCCCCG-3'and TdhR:5'-AGCCAGACACCGCTGCCATTG-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 50ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 25 minutes and visualized on a UV transilluminator (Carter *et al.*, 2010).

3.2.10 STATISTICAL ANALYSIS

The data generated from the study were subjected to statistical analysis.

The means and standard deviation were calculated using the Microsoft Excel 2007.

The ANOVA was used to compare the means of the colony counts using the Microsoft Excel 2007

CHAPTER FOUR

4.0. RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 Heterotrophic Bacterial Isolates from Seafood obtained from Nembe and Ox-bow Lake.

The colonial characteristics as well as the biochemical characteristics of the pure isolates obtained from periwinkle, crab, oysters and shrimps were presumptive of *Staphylococcus, Vibrio* and *Klebsiella* sp. Some of the pure isolates obtained were yellow, gram positive, round (cocci) and oxidase negative. However, the isolates were all coagulase negative indicating that they are not *Staphylococcus aureus*. All other isolates were gram negative, sucrose/glucose fermenters, citrate positive as well as catalase positive. Some of the gram negative pure isolates were yellow flat colonies while others were green round colonies growing on the Thio sulphate Citrate Bile-Salt Sucrose agar. The gram negative pure isolates were oxidase positive, sucrose/glucose fermenters, citrate positive as well as catalase positive which is indicative of the presence of *Vibrio* species.

Other gram negative pure isolates were oxidase negative as well as indole negative which is characteristic of *Klebsiella* species.

4.1.2: Evolutionary Relations of Bacterial Isolates from Seafood Samples

The obtained 16SrRNA sequence from the isolates from the seafood samples produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotides (nr/nt) database. The 16SrRNA of the isolates showed a percentage similarity to other species at 100%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16SrRNA of the isolates within the *Vibrio*, *Klebsiella* and *Staphylococcus* sp and revealed a closely relatedness to *Vibrio rotiferianus*, *Vibrio paraheamolyticus*, *Klebsiella aerogenes*, *Klebsiella quasipneumonia* and *Staphylococcus gallinarum* respectively a shown in Figure 4.6.

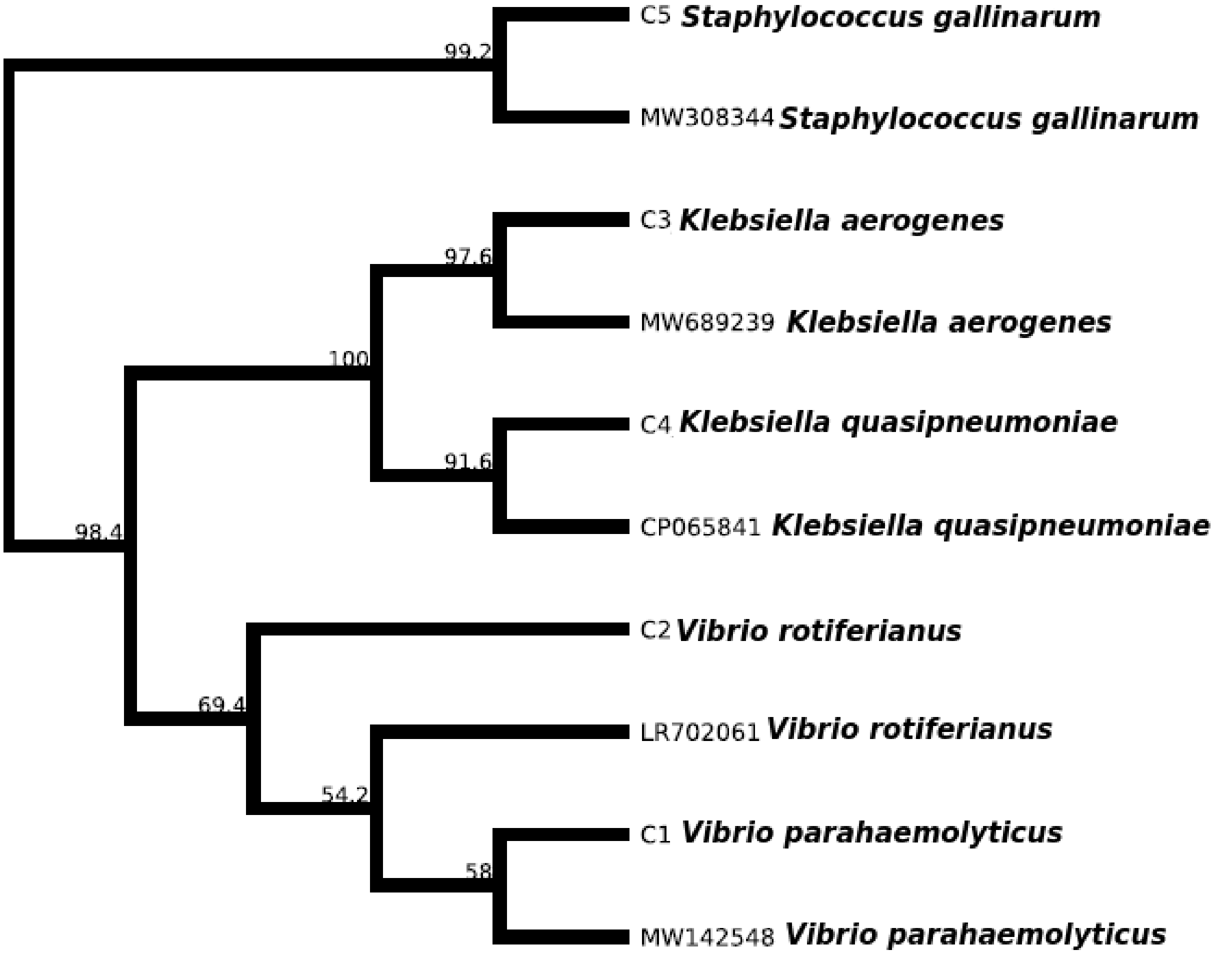


Figure: 4.1 Evolutionary Distances between the Bacterial Isolates

4.1.3 Percentage Occurrence and Distribution of Organisms Isolated from Seafood Samples in Nembe (n=80)

The percentage occurrence of the bacterial isolates from the seafood samples from Nembe were obtained by taking the percentage of the colony counts from each seafood sample (periwinkle, crab, oyster and shrimps). This was also done to determine the percentage distribution of the different bacterial isolates in the different seafood samples. The total number of pure isolates obatained from sea food samples in Nembe were eighty (80). *Staphylococcus gallinarum* made up 13(16.25%) of the isolates, *Vibrio rotiferianus* made up 9(11.25%) of the total isolates, *Vibrio paahaemolyticus* made up 31(38.75%) of the isolates, *Klebsiella aerogenes* made up 10(12.5%) of the isolates while *Klebsiella quasipneumoniae* made up 17(21.25%) of the total isolates. The percentage distribution of *Staphylococcus gallinarum,* V*ibrio rotiferianus, Vibrio parahaemolyticus, Klebsiella aerogenes,* and *Klebsiella quasipneumoniae* in Periwinkle was 20%, 5%, 25%, 15% and 35% reapectively. Periwinkle haboured the highest percentage of *Klebsiella quasipneumoniae* (35%) compared to the other bacterial isolates. Crab had 15%, 15%, 45%, 10% and 15% of the bacterial isolates respectively. Crab haboured more *Vibrio parahaemolyticus* (45%) than other organisms. Oyster had 10%, 10%, 35%, 20% and 25% of the bacterial isolates respevtively. Shrimps had 20%, 15%, 50%, 5% and 10% of the bacterial species stated respectively. *Vibrio* species had the highest occurrence in all the seafoods except for Periwinkle as shown in Figure 4.2.

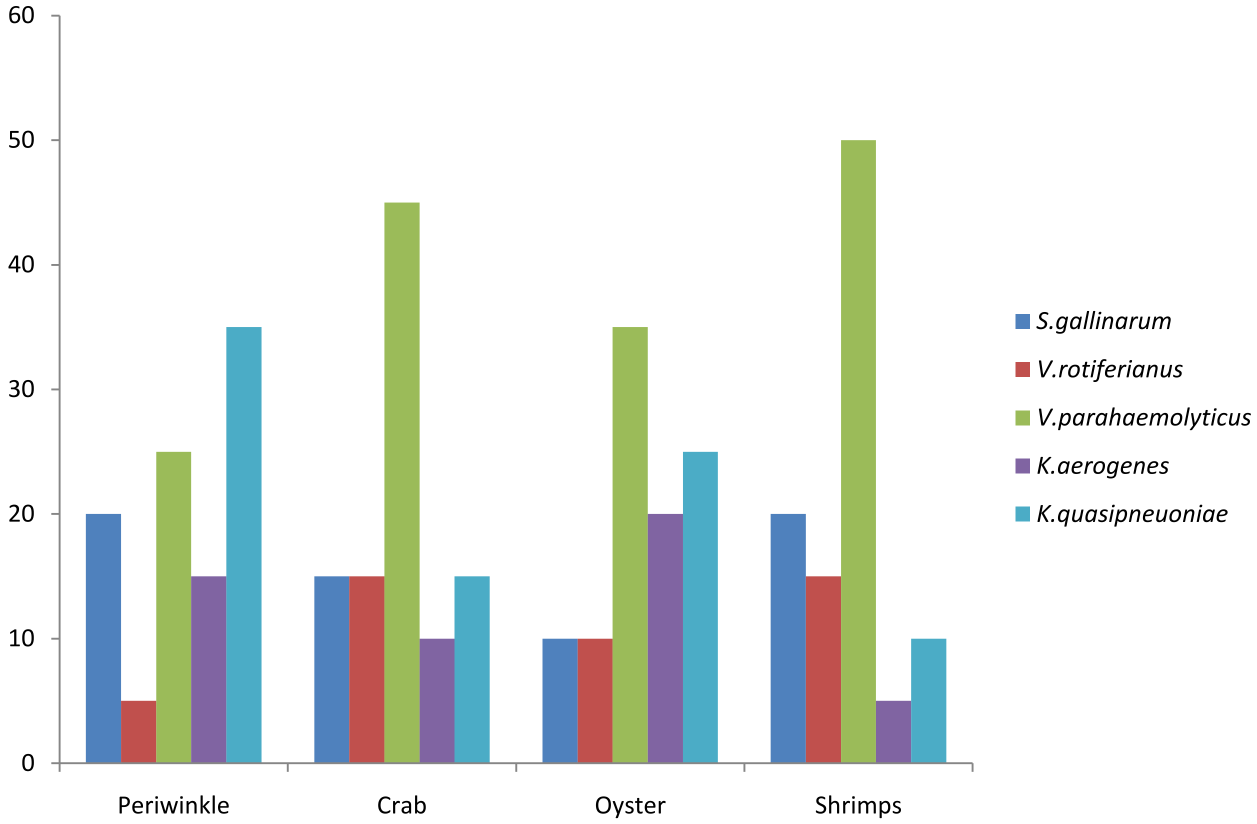


Figure 4.2: Percentage distribution of isolates in the seafood samples from Nembe

4.1.4 Percentage Occurrence and Distribution of Isolates in the Seafood Samples (n=120) obtained from Ox-Bow Lake.

The percentage occurrence of the bacterial isolates from the seafood samples from Ox-bow were obtained by taking the percentage of the colony counts from each seafood sample (periwinkle, crab, oyster and shrimps). This was also done to determine the percentage distribution of the different bacterial isolates in the different seafood samples. The total number of pure isolates obatained from the seafood samples in Ox-bow Lake were one hundred and twenty (120). *Staphylococcus gallinarum* made up 27(22.5%) of the isolates, V*ibrio rotiferianus* was 17(14.2%), *Vibrio parahaemolyticus* was 48(40%), *Klebsiella aerogenes* made up 10(8.3%) and *Klebsiella quasipneumoniae* made up 18(15%) of the isolates obtained from the seafood samples. The percentage distributions of *Staphylococcus gallinarum,* V*ibrio rotiferianus, Vibrio parahaemolyticus, Klebsiella aerogenes,* and *Klebsiella quasipneumoniae* in Periwinkle were 30%, 16.7%, 33.3%, 10% and 10% respectively. Periwinkle haboured the highest percentage of *Vibrio parahaemolyticus* (33.3%) compared to the other bacterial isolates. Crab had 20%, 16.7%, 40%, 0% and 23.3% of all the isolates respectively. Crab haboured more *Vibrio parahaemolyticus* than other organisms but had no *Kebsiella aerogenes* present. Oyster had 16.7% 10%, 43.3%, 13.3% and 16.7% for the bacterial isolates respectively. Shrimps had 23.3%, 13.3%, 43.3, 10% and 10% of all the bacterial species isolated respectively. *Vibrio* species had the highest occurrence in all the seafood samples with the highest occurrence in shrimps as shown in Figure 4.3.

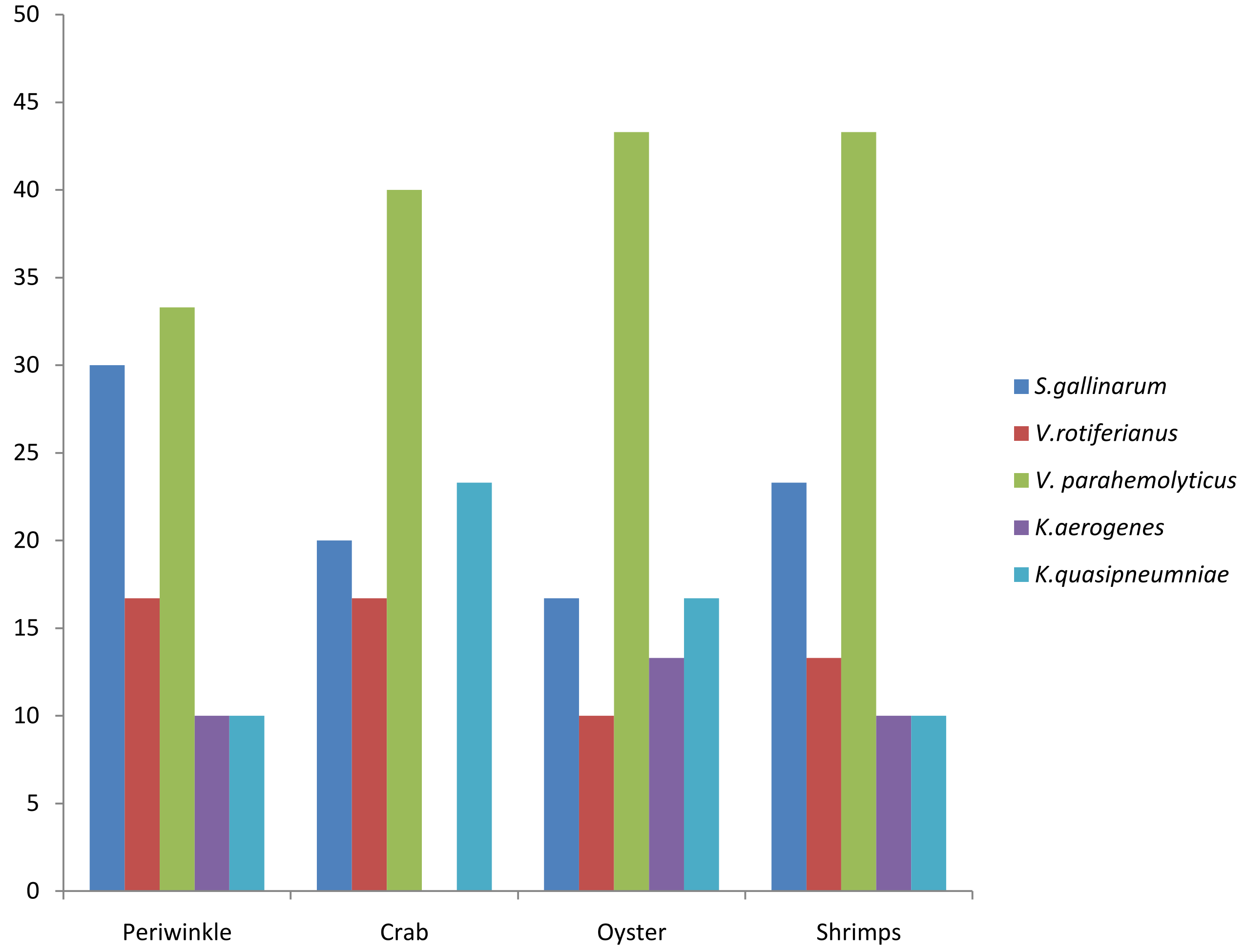


Figure 4.3: Percentage distribution of isolates in the seafood samples from Ox-bow Lake

4.1.5 Comparative Analysis of the Mean of Total Colony Count (LOG_{10}CFU/g) of Isolates from Seafoods obtained from Nembe and Ox-Bow Lake

A comparative analysis on the LOG_{10} of the mean of the colony count of the isolates from the different seafood samples from Nembe and Ox-bow shows that there was no significant difference in the colony counts obtained from the seafoods from Nembe and Ox-bow lake as shown in table 4.5. In as much as there were differences in the colony counts obtained from periwinkle from both locations (6.43 and 6.54), crab from both locations (6.52 and 6.51) and shrimps from the two different locations (6.51 and 6.41), the ANOVA shows that there is statistically no significant difference in the colony count at 5% level of confidence as $P>0.05$ as shown in Table 4.1.

Table 4.1: Total Colony Count (LOG_{10}CFU/g) of isolates from Seafoods obtained from Nembe and Ox-Bow Lake

Samples	**NEMBE LAKE**	**OX-BOW LAKE**
PERIWINKLE	6.43±0.45	6.54±0.18
OYSTER	6.41±0.31	6.41±0.36
CRAB	6.52±0.28	6.51±0.23
SHRIMP	6.51±0.22	6.42±0.33

4.1.6 Pathogenicity of the Bacterial Isolates

The bacterial isolates were tested for the presence of the virulence factors; Hemolysin and Coagulase. The *Vibrio parahaemolyticus* isolated from the seafood samples obtained from Nembe and Ox-bow Lake were positive for the hemolysis test. A positive test was detected by characteristic zones of hemolytic reaction on the blood agar. All the other isolates were negative for this test. The *Vibrio parahaemolyticus* isolated lysed the red blood cells present in the blood producing a complete hemolysis known as β-hemolysis. This indicates that the organism possesses the hemolysin virulence factor responsible for the hemolysis of human red blood cells. All the isolates were negative for the Coagulase test as shown in Table 4.2

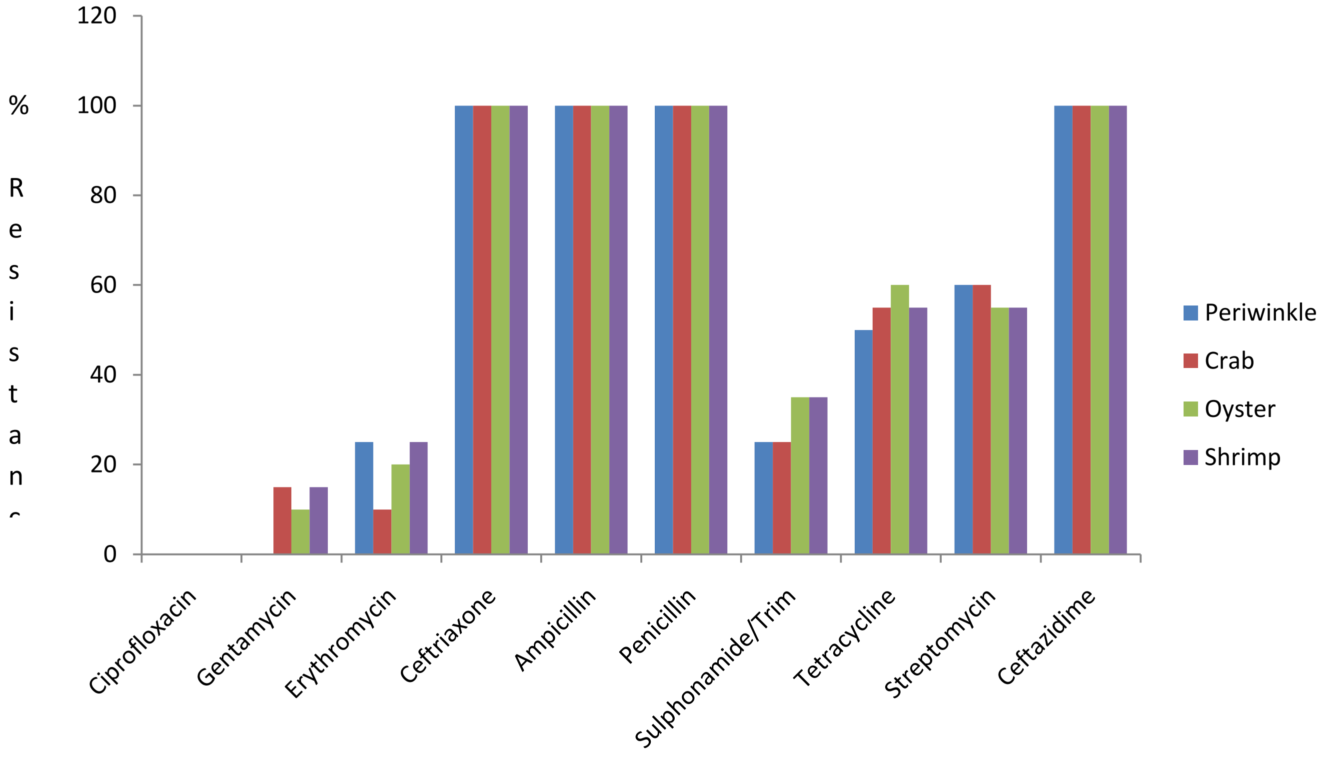
Table 4.2: Virulence factors present in the Bacterial Isolates from Nembe and Ox-bow Lake

Virulence Factor	Bacterial	Isolates			
	S.gallinarum	*V.rotiferianus*	*V. parahaemolyticus*	*K.aerogenes*	*K. quasipneumoniae*
Hemolysin	-	-	+	-	-
Coagulase	-	-	-	-	-

+ = Present, - = Absent

4.1.7 Antimicrobial Resistance Profiles of Isolates from Seafood obtained from Nembe (n=80).

The antimicrobial susceptibility test carried out on the isolates gotten from seafoods obtained from Nembe showed that all the isolates were resistant to Penicillin, Ampicillin, Ceftriaxone and Ceftazidime. However, all the isolates (100%) were susceptible to Ciprofloxacin(C) i.e none of the isolates from the different seafood samples was resistant to Ciprofloxacin. Also, no isolate from periwinkle was resistant to gentamycin but 15%, 10% and 15% of the isolates from crab, oyster and shrimp respectively were resistant to gentamycin. 25% of the isolates from periwinkle and shrimps were resistant to Erythromycin while 10% and 20% of isolates from crab and oysters respectively were resistant to it. 25% each of the isolates from periwinkle and crab were resistant to Sulphonamide/Trimethoprim while 35% each of the isolates from oysters and shrimps were resistant to it. 55% each of the isolates from crab and shrimps were resistant to tetracycline while 50% and 60% of the isolates from periwinkle and oyster respectively were resistant to it. Again, 55% of the isolates from oyster and shrimp were resistant to streptomycin while 60% of the isolates from periwinkle and crab were resistant to it as shown in Figure 4.4



Antibiotics

Figure 4.4: Antimicrobial Resistance Profile of Isolates from the different seafood from Nembe (n=80)

4.1.8 Antimicrobial Resistance Profile of Isolates from Seafoood obtained from Ox-Bow Lake (n=120).

The antimicrobial susceptibility test carried out on the isolates gotten from seafoods obtained from Ox-bow Lake showed that all the isolates were resistant to Penicillin, Ampicillin, Ceftriaxone and Ceftazidime. However, all the isolates (100%) were susceptible to Ciprofloxacin(C) i.e none of the isolates from the the different seafood samples was resistant to Ciprofloxacin. Also, no isolate from crab and shrimp was resistant to gentamycin but 6.7%, and 10% of the isolates from periwinkle and oyster respectively were resistant to gentamycin. 6.7% and 20% of the isolates from periwinkle and crab respectively were resistant to Erythromycin while 10% of isolates each from shrimp and oysters were resistant to it. 26.7% of the isolates from periwinkle, 33.3% from crab, 23.3% from oysters and 13.3% of the isolates from shrimps were resistant to Sulphonamide/Trimethoprim. Furthermore, 53.3% each of the isolates from periwinkle and crab were resistant to tetracycline while isolates from oysters and shrimps showed close resistance at 33.3% and 30% respectively. Again, 63.3% of the isolates from periwinkle and 50% of the islates from oyster were resistant to streptomycin while 30% of the isolates from crab were resistant to it and 23.3% from shrimps as shown in figure 4.5.

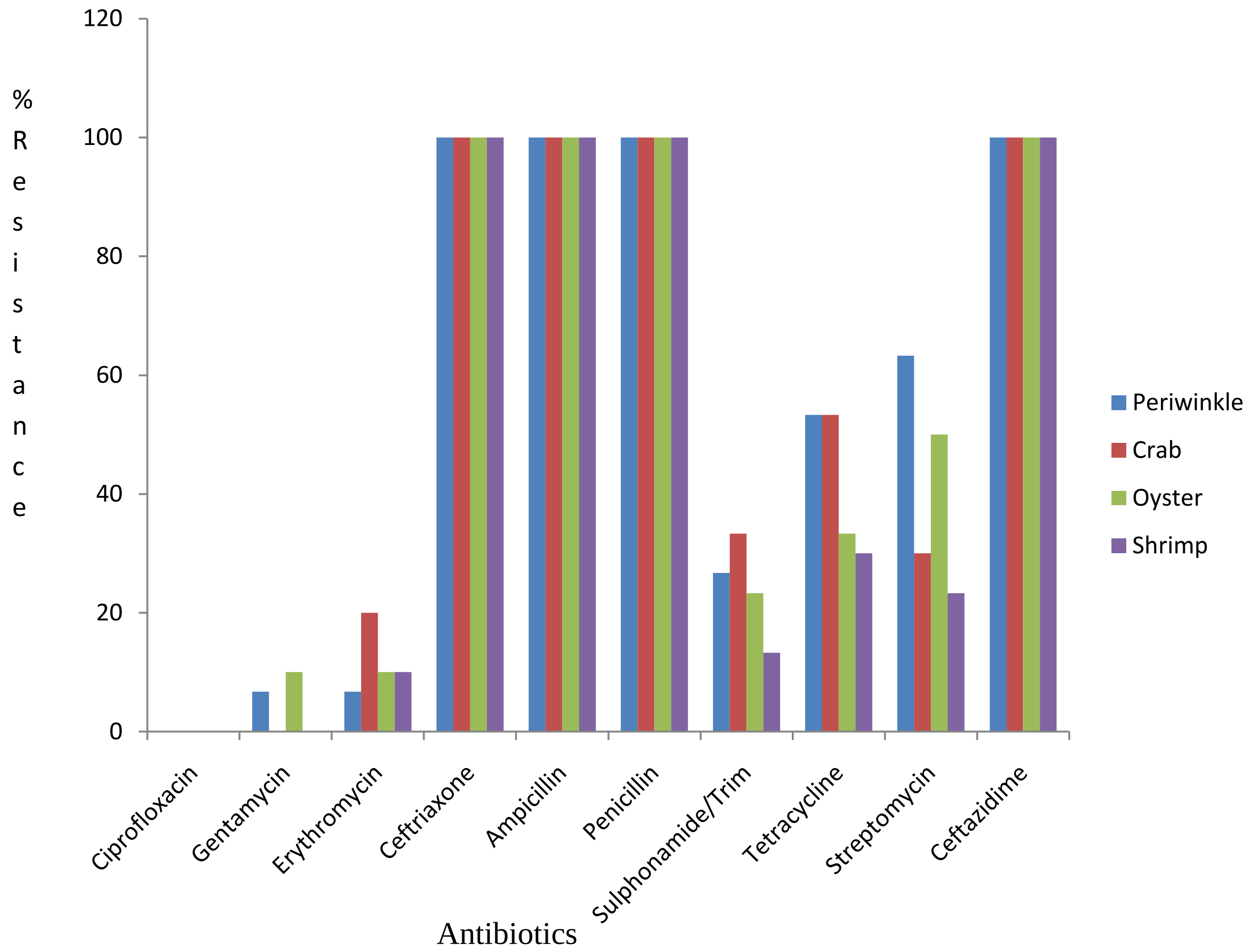


Figure 4.5: Antimicrobial Resistance Profile of Isolates from the different seafood from Ox-bow lake (n=120)

4.1.9 Frequency (%) of Resistant Isolates from Seafood Samples obtained From Nembe

The frequency of the resistance of the bacterial isolates obtained from seafood samples from Nembe clearly showed that the frequency of resistance in the bacterial isolates was higher than the frequency of susceptibility of the isolates to the antibiotics. Clearly, the isolates were resistant to 60% (6) of the antibiotics which shows that a higher resistant frequency is being recorded. Streptomycin and tetracycline both have close resistant frequencies viz a viz 46 and 43. The four antibiotics with the highest resistant frequencies are Ceftriazone, Ampicillin, Penicillin and Ceftazidime as shown in the table 4.3.

Table 4.3: Frequency (%) of Resistant Isolates from Seafood obtained from Nembe (n=80)

Antibiotics	Isolates					Total Resistant Isolates (n=80)
	S.gallinarum (n=13)	*K. aerogenes (n=10)*	*K. quasipneumoniae (n=17)*	*V.rotiferanus (n=9)*	*V.parahaemolyticus (n=31)*	
CIP	0	0	0	0	0	0
CN	0	0	2(11.8)	0	0	2(2.5)
E	2(15.4)	6(60)	1 (5.9)	2(22.2)	5(16.1)	16(2)
CRO	13(100)	10(100)	17(100)	9(100)	31(100)	80(100)
AMP	13(100)	10(100)	17(100)	9(100)	31(100)	80(100)
P	13(100)	10(100)	17(100)	9(100)	31(100)	80(100)
SXT	1(7.7)	5(50)	6(35.3)	3(33.3)	8(25.8)	23(28.8)
TE	3(23.1)	6(60)	15(88.2)	5(55.6)	14(45.2)	43(53.8)
S	2(15.4)	7(70)	15(88.2)	2(22.2)	20(64.5)	46(57.5)
CAZ	13(100)	10(100)	17(100)	9(100)	31(100)	80(100)

Key:CIP=Ciprofloxacin, CN=Gentamycin, E=Erythromycin, CRO= Ceftriaxone, AMP=Ampicillin, P=Penicillin, SXT= Sulphonamide/Trimethoprime, T=Tetracycline, S=Streptomycin, CAZ=Ceftazidime

4.1.10 Frequency (%) of Resistant Isolates from Seafood Samples obtained from Ox-Bow Lake (n=120)

The frequency and percentage of the resistance of the bacterial isolates to the different antibiotics was recorded. Unlike the frequencies recorded from Nembe, the frequencies as recoded from isolates of the samples from ox-bow Lake clearly shows that the frequency of susceptibility of the bacterial isolates from ox-bow Lake is higher than the frequency of resistance of the isolates to the antibiotics. Moreso, the frequency of resistance was only higher in 4(40%) antibiotics. The four antibiotics with the highest resistant frequencies are Ceftriazone, Ampicillin, Penicillin and Ceftazidime as shown in table 4.4.

Table 4.4: Frequency (%) of Resistant Isolates obtained from Seafood from Ox-Bow (n=120)

Antibio ics			Isolates			Total Resistant Isolates (n=120)
	S. gallinarum (n=27)	*K. aerogenes (n=10)*	*K. quasipneumoniae (n=18)*	*V.rotiferanus (n=17)*	*V.parah emolyticus (n=48)*	
CIP	0	0	0	0	0	0
CN	2(7.4)	0	0	0	1(2.1)	3(2.5)
E	1(3.7)	0	2 (11.1)	3(17.7)	7(14.6)	13(10.8)
CRO	27(100)	10(100)	18(100)	17(100)	48(100)	120(100)
AMP	27(100)	10(100)	18(100)	17(100)	48(100)	120(100)
P	27(100)	10(100)	18(100)	17(100)	48(100)	120(100)
SXT	10(37)	0	7(38.9)	6(35.3)	5(10.4)	28(23.3)
T	8(29.6)	6(60)	10(55.6)	3(17.6)	22(45.8)	49(40.8)
S	10(37)	5(50)	7(38.9)	5(29.4)	23(48)	50(41.7)
CAZ	27(100)	10(100)	18(100)	17(100)	48(100)	120(100)

Key: CIP=Ciprofloxacin, CN=Gentamycin, E=Erythromycin, CRO= Ceftriaxone, AMP=Ampicillin, P=Penicillin, SXT= Sulphonamide/Trimethoprime, T=Tetracycline, S=Streptomycin, CAZ=Ceftazidime

4.1.11 MULTI-DRUG RESISTANCE PATTERNS OF ISOLATES FROM SEAFOOD IN NEMBE.

The multidrug resistant pattern of the bacterial isolates from Nembe were analysed and recorded on the terms that isolates resistant to more than one antibiotic are multi-drug resistant. *Staphylococcus gallinarum* isolated was resistant to six antibiotics at different frequencies and different patterns and as such is multi-drug resistant as recorded below. The highest frequency (7) was recorded in its resistance to Ceftriazone, Ampicillin, Penicillin and Ceftazidime indicating that seven of the *S. gallinarum* isolates were resistant to Ceftriazone, Ampicillin, Penicillin and Ceftazidime at the same time as shown in table 4.5.

Klebsiella aerogenes isolated was multi drug resistant. It was resistant to eight (8) antibiotics at a frequency of five which indicates that five of the *K. aerogenes* isolated were all resistant to the eight antibiotics. The highest frequency (5) was recorded in its resistance to Erythromycin, Ceftriazone, Ampicillin, Penicillin, Sulphonamide/Trimehoprim, Tetracycline, Streptomycin and Ceftazidime as shown in table 4.6.

Klebsiella quasipneumonia isolated was multi drug resistant. It was resistant to Nine (9) antibiotics at a frequency of one which indicates that one of the *K. quasipneumoniae* isolated was resistant to these nine antibiotics. The highest frequency (9) was recorded in its resistance to Ceftriazone, Ampicillin, Penicillin, Tetracycline and Ceftazidime as hown in table 4.7.

Vibrio rotiferanus isolated was multi drug resistant. It was resistant to eight (8) antibiotics at a frequency of two which indicates that two of the *V. rotiferanus* isolated were resistant to these eight antibiotics. The highest frequency (5) was recorded in its resistance to Ceftriazone, Ampicillin, Penicillin and Ceftazidime as shown in table 4.8.

Vibrio parahaemolyticus isolated was multi drug resistant. It was resistant to eight (8) antibiotics at a frequency of five which indicates that two of the *V. parahaemolyticus* isolates were resistant to these eight antibiotics. The highest frequency (11) was recorded in its resistance to Ceftriazone, Ampicillin, Penicillin and Ceftazidime as shown in table 4.9.

Table 4.5: Multi-Drug Resistance Pattern of *Staphylococcus gallinarum (*n=13*)* from Seafood Samples from Nembe

ANTIBIOTICS	FREQUENCY
CRO+AMP+P+SXT+T+CAZ	1
E+CRO+AMP+P+S+CAZ	1
CRO+AMP+P+T+CAZ	2
E+CRO+AMP+P+CAZ	1
CRO+AMP+P+S+CAZ	1
CRO+AMP+P+CAZ	7

Key:E=Erythromycin, CRO= Ceftriaxone, AMP=Ampicillin, P=Penicillin, SXT= Sulphonamide/Trimethoprime, T=Tetracycline, S=Streptomycin, CAZ=Ceftazidime

Table 4.6: Multi-Drug Resistance Pattern of *Klebsiella aerogenes (*n=10) from Seafood Samples in Nembe

ANTIBIOTICS	FREQUENCY
E+CRO+AMP+P+SXT+T+S+CAZ	5
E+CRO+AMP+P+T+S+CAZ	1
CRO+AMP+P+S+CAZ	1
CRO+AMP+P+CAZ	3

Key:E=Erythromycin, CRO= Ceftriaxone, AMP=Ampicillin, P=Penicillin, SXT= Sulphonamide/Trimethoprime, T=Tetracycline, S=Streptomycin, CAZ=Ceftazidime

Table 4.7: Multi-Drug Resistance Patterns of *Klebsiella quasipneumoniae* (n=17) from Seafood Samples in Nembe

ANTIBIOTICS	FREQUENCY
CN+E+CRO+AMP+P+SXT+T+S+CAZ	1
CN+CRO+AMP+P+SXT+T+S+CAZ	1
CRO+AMP+P+SXT+T+S+CAZ	4
CRO+AMP+P+T+CAZ	9
CRO+AMP+P+CAZ	2

Key:E=Erythromycin, CRO= Ceftriaxone, AMP=Ampicillin, P=Penicillin, SXT= Sulphonamide/Trimethoprime, T=Tetracycline, S=Streptomycin, CAZ=Ceftazidime

Table 4.8: Multi-Drug Resistance Patterns of *Vibrio rotiferanus* (n=9) from Seafood Samples in Nembe

ANTIBIOTICS	FREQUENCY
E+CRO+AMP+P+SXT+T+S+CAZ	2
CRO+AMP+P+SXT+T+CAZ	1
CRO+AMP+P+T+CAZ	2
CRO+AMP+P+CAZ	5

Key:E=Erythromycin, CRO= Ceftriaxone, AMP=Ampicillin, P=Penicillin, SXT= Sulphonamide/Trimethoprime, T=Tetracycline, S=Streptomycin, CAZ=Ceftazidime

Table 4.9: Multi-Drug Resistance Patterns of *Vibrio parahaemoyticus* (n=31) from Seafood Samples in Nembe.

ANTIBIOTICS	FREQUENCY
E+CRO+AMP+P+SXT+T+S+CAZ	5
CRO+AMP+P+SXT+T+S+CAZ	3
CRO+AMP+P+T+S+CAZ	6
CRO+AMP+P+S+CAZ	6
CRO+AMP+P+CAZ	11

Key:E=Erythromycin, CRO= Ceftriaxone, AMP=Ampicillin, P=Penicillin, SXT= Sulphonamide/Trimethoprime, T=Tetracycline, S=Streptomycin, CAZ=Ceftazidime

4.1.12 MULTI-DRUG RESISTANCE PATTERN OF ISOLATES FROM SEAFOOD OBTAINED FROM OXBOW-LAKE

The multidrug resistant pattern of the bacterial isolates from Ox-bow Lake were analysed and recorded on the terms that isolates resistant to more than one antibiotic are multi-drug resistant. *Staphylococcus gallinarum* isolated was resistant to nine antibiotics at different frequencies and different patterns and as such is multi-drug resistant as recorded below. The highest frequency (17) was recorded in its resistance to Ceftriazone, Ampicillin, Penicillin and Ceftazidime indicating that seventeen of the *S. gallinarum* isolates were resistant to Ceftriazone, Ampicillin, Penicillin and Ceftazidime at the same time as shown in table 4.10.

K. aerogenes isolated were multi drug resistant. The isolates were resistant to six (6) antibiotics at a frequency of five which indicates that five of the *K. aerogenes* isolated were all resistant to these same six antibiotics. The highest frequency (5) was recorded in their resistance to Ceftriazone, Ampicillin, Penicillin, Tetracycline, Streptomycin and Ceftazidime as shown in table 4.11.

K. quasipneumonia isolated was multi drug resistant. It was resistant to eight (8) antibiotics at a frequency of two which indicates that two of the *K. quasipneumoniae* isolated were resistant to these eight antibiotics. The highest frequency (8) was recorded in its resistance to Ceftriazone, Ampicillin, Penicillin and Ceftazidime as shown in table 4.12.

V. rotiferanus isolated was multi drug resistant. It was resistant to eight (8) antibiotics at a frequency of three which indicates that three of the *V. rotiferanus* isolated were resistant to eight antibiotics. The highest frequency (11) was recorded in its resistance to Ceftriazone, Ampicillin, Penicillin and Ceftazidime as shown in table 4.13.

V. parahaemolyticus isolated was multi drug resistant. It was resistant to Nine (9) antibiotics at a frequency of one which indicates that only one of the *V. parahaemolyticus* isolates was resistant to the nine antibiotics. The highest frequency (25) was recorded in its resistance to Ceftriazone, Ampicillin, Penicillin and Ceftazidime as shown in table 4.14.

Table 4.10: Multi-Drug Resistance Patterns of *S. gallinarum* (n=27) from Seafood Samples in Ox-Bow Lake

ANTIBIOTICS	FREQUENCY
CN+E+CRO+AMP+P+SXT+T+S+CAZ	1
CN+CRO+AMP+P+SXT+T+S+CAZ	1
CRO+AMP+P+SXT+T+S+CAZ	6
CRO+AMP+P+SXT+S+CAZ	2
CRO+AMP+P+CAZ	17

Key:E=Erythromycin, CRO= Ceftriaxone, AMP=Ampicillin, P=Penicillin, SXT= Sulphonamide/Trimethoprime, T=Tetracycline, S=Streptomycin, CAZ=Ceftazidime

Table 4.11: Multi-Drug Resistance Patterns of *K. aerogenes* (n=10) from Seafood Samples in Ox-Bow Lake

ANTIBIOTICS	FREQUENC
CRO+AMP+P+T+S+CAZ	5
CRO+AMP+P+T+CAZ	1
CRO+AMP+P+CAZ	4

Key:CRO= Ceftriaxone, AMP=Ampicillin, P=Penicillin, T=Tetracycline, S=Streptomycin, CAZ=Ceftazidime

Table 4.12: Multi-Drug Resistance Patterns of *K. quasipneumoniae* (n=18) from Seafood Samples in Ox-Bow Lake

ANTIBIOTICS	FREQUENCY
E+CRO+AMP+P+SXT+T+S+CAZ	2
CRO+AMP+P+SXT+T+S+CAZ	5
CRO+AMP+P+T+CAZ	3
CRO+AMP+P+CAZ	8

Key:E=Erythromycin, CRO= Ceftriaxone, AMP=Ampicillin, P=Penicillin, SXT= Sulphonamide/Trimethoprime, T=Tetracycline, S=Streptomycin, CAZ=Ceftazidime

Table 4.13: Multi-Drug Resistance Patterns of *V.rotiferianus* (n=17) from Seafood Samples in Ox-Bow Lake

ANTIBIOTICS	FREQUENCY
E+CRO+AMP+P+SXT+T+S+CAZ	3
CRO+AMP+P+SXT+S+CAZ	2
CRO+AMP+P+SXT+CAZ	1
CRO+AMP+P+CAZ	11

Key:E=Erythromycin, CRO= Ceftriaxone, AMP=Ampicillin, P=Penicillin, SXT= Sulphonamide/Trimethoprime, T=Tetracycline, S=Streptomycin, CAZ=Ceftazidime

Table 4.14: Multi-Drug Resistance Patterns of *V.parahaemolyticus* (n=48) from Seafood Samples in Ox-Bow Lake

ANTIBIOTICS	FREQUENCY
CN+E+CRO+AMP+P+SXT+T+S+CAZ	1
E+CRO+AMP+P+SXT+T+S+CAZ	4
E+CRO+AMP+P+T+S+CAZ	2
CRO+AMP+P+SXT+S+T+CAZ	15
CRO+AMP+P+S+CAZ	1
CRO+AMP+P+CAZ	25

Key: CN=Gentamycin, E=Erythromycin, CRO= Ceftriaxone, AMP=Ampicillin, P=Penicillin, SXT= Sulphonamide/Trimethoprime, T=Tetracycline, S=Streptomycin, CAZ=Ceftazidime

4.1.13 AMPLIFIED RESISTANCE AND VIRULENCE GENES

The DNA samples isolated from the bacterial isolates were analyzed for the presence of the following genes; *acrAB, ctx-M, shv, tdh, sxt, and mecA.* The primers for each gene were used to amplify the specific gene regions using PCR and they were resolved by Agarose Gel Electrophoresis and the bands were detected as shown in the figures below.

4.1.13.1 The *acrAB* Gene

The *acrAB* genes were detected in the *Klebsiella* species ie *Klebsiella aerognes* and *Klebsiella quasipneumoniae* but absent in the other bacterial isolates as shown in figure 4.6. These genes are responsible for the multidrug resistance of *Klebsiella* species isolated to tetracycline, penicillin, ampicillin and erythromycin used in this study.

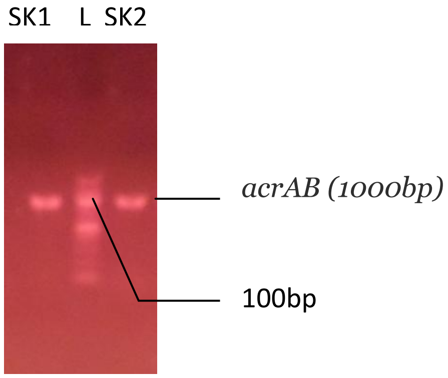


Figure 4.6: Agarose gel electrophoresis of the *acrAB* genes. Lanes SK1 and SK2 represent the *acrAB* genes at 1000bp while lane L represents the 100bp molecular ladder.

4.1.13.2 The *ctx-M* Gene

The *ctx-M* genes were resolved at 500bp on the Agarose Gel Electrophoresis. The *ctx-M* genes were identified in *Klebsiella quasipneumoniae* only while absent in other bacterial isolates as shown in figure 4.7. The *ctx-M* gene is responsible for the resistance of the *Klebsiella* sp to the third generation cephalosporins which include; ceftriaxone and ceftazidime. It is also responsible for the resistance of the bacterial isolates to β-Lactam antibiotics used in this study. The β-Lactam used in this study were penicillin and ampicillin.

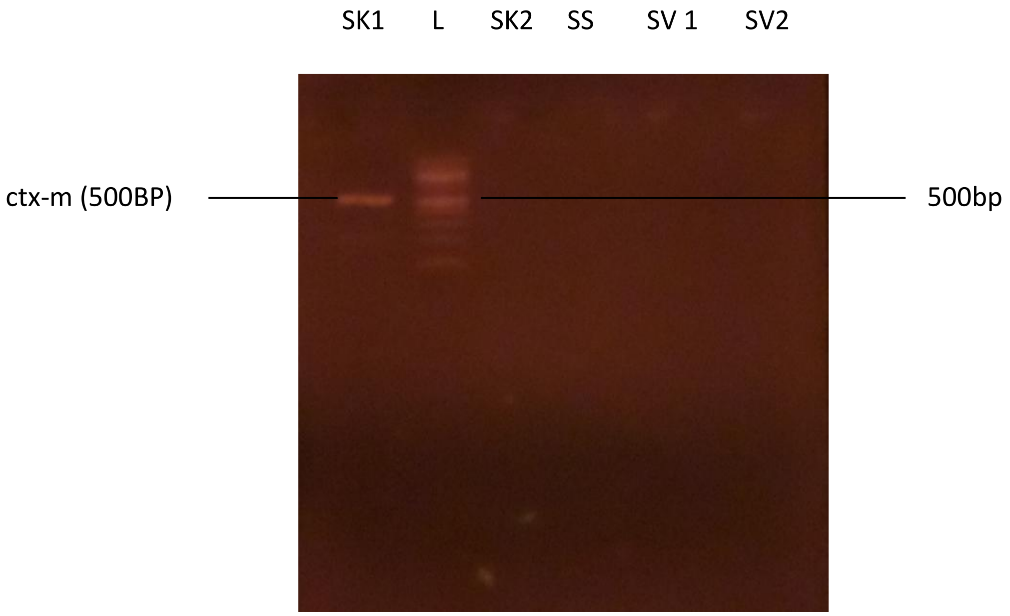


Figure 4.7: Agarose gel electrophoresis of the *ctx-m* genes. Lanes SK1 represents the *ctx-M* gene at 500bp while lane L represents the 100bp molecular ladder.

4.1.13.3 The *shv* Gene

The *shv* genes were resolved at 200bp on the Agarose Gel Electrophoresis. The presence of the bands shows that the gene was present in *Klebsiella aerognes, Klebsiella quasipneumoniae* and *Vibrio rotiferianus* as shown in figure 4.8. This gene is responsible for the resistance of these bacterial isolates to cephalosporins i.e ceftriaxone and ceftazidime used in this study as well as their resistance to β-lactam antibiotics such as ampicillin and penicillin.

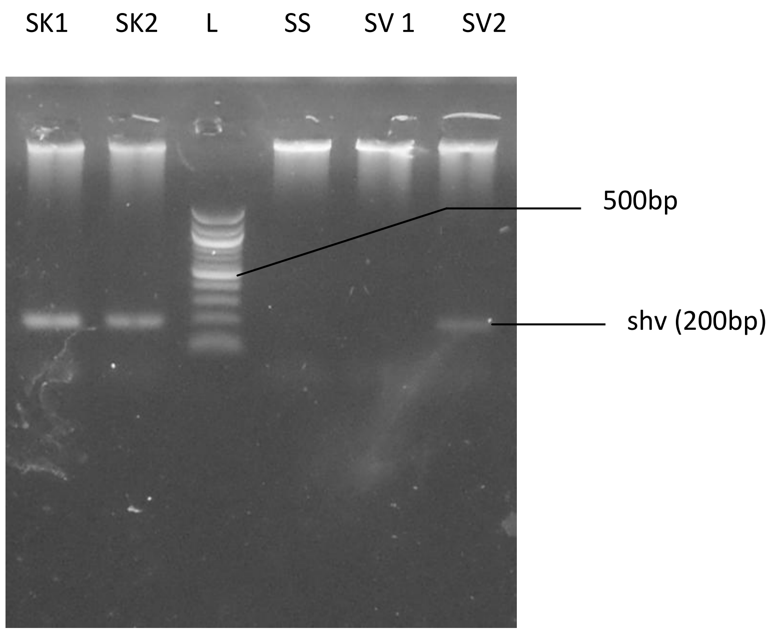


Figure 4.8: Agarose gel electrophoresis of the *shv* genes. Lanes SK1, SK2 and SV2 represent the *shv* genes at 200bp while lane L represents the 100bp molecular ladder.

4.1.13.4 The *tdh* Gene

The Thermo Stable Direct Hemolysin (*tdh*) genes were resolved at 281bp on the Agarose Gel Electrophoresis. The presence of the bands shows that the gene was present in *Vibrio parahaemolyticus.* The virulence gene *tdh* was detected in *Vibrio parahaemolyticus* only as shown in figure 4.9 and is responsible for the pathogenicity of *Vibrio parahaemolyticus*.

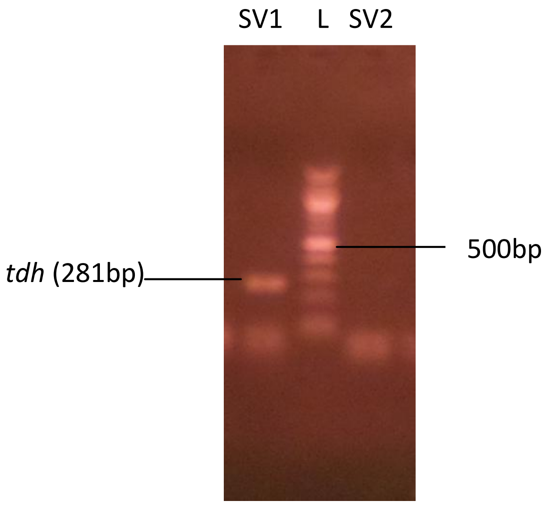


Figure 4.9: Agarose gel electrophoresis of the *tdh* genes. Lanes SV1 and SV2 represents the *tdh* genes at 281bp while lane L represents the 100bp molecular ladder.

4.1.13.5 The *mecA* Gene

The *mecA* genes were resolved at 550bp on the Agarose Gel Electrophoresis. The presence of the bands shows that the gene was present in *Staphylococcus gallinarum.* The *mecA* gene was detected in *Staphylococcus gallinarum* only as shown in figure 4.10 and this explains why the organism was resistance to the β-lactam antibiotics used in this study.

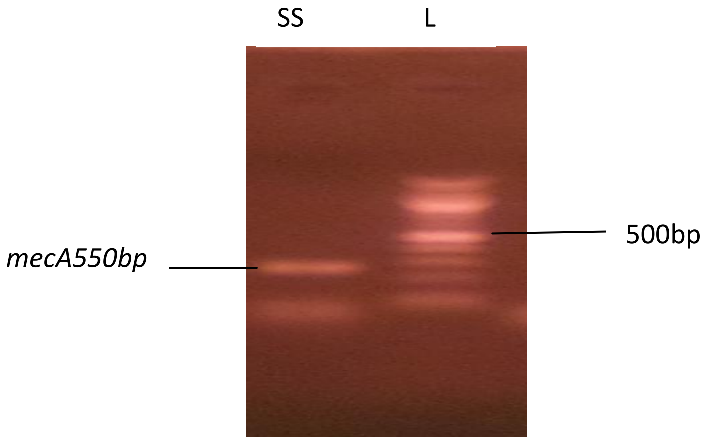


Figure 4.10: Agarose gel electrophoresis of the *mecA* gene. Lanes SS represents the *mecA* genes at 520bp while lane L represents the 100bp molecular ladder.

4.2 DISCUSSION

Seafood samples harbour a consortium of microorganisms ranging from pathogenic microorganisms to normal commensals. The community of microorganisms found in a particular habitat is dependent on a number of factors such as composition, weather, pollution, location, time, etc (Farmer *et al.*, 2005).

Results obtained from this study showed that microorganisms of the genera *Staphyococcus, Vibrio,* and *Klebsiella* were isolated and this coroborate reports that seafood harbour diverse groups of organisms. Thio- sulphate citrate bile salt sucrose agar (TCBS), Eosine Methylene Blue Agar, Nutrient Agar and Mannittol Salt Agar were used for the isolation of these organisms. Kriem *et al.,* (2015), in comparing the performance of two plating media Chromagar and TCBS proved that the former was more selective for *Vibrios* than the later. Also, the TCBS does not give a fast, accurate and reliable result and could allow for growth of other species (Kriem *et al.,* 2015). The growth of *Staphylococcus* and *Klebsiella* clearly shows that TCBS can indeed allow the growth of other microorganisms other than *Vibrios.* Out of the 200 pure cultures isolated, *Vibrio* species constitute about 52.5% of the bacteria isolated while *Klebsiella* and *Staphylococcus* constitute 27.5% and 20% respectively. *Staphylococcus* species are not usually associated with marine environments but could be seen probably as commensals in the sea-foods or the aquatic environment. The dominance of *Vibrio* species is evident of their normal commensals in seafood (Cabanillas-Beltrán *et al.*, 2006*)*. The presence of the *Klebsiella* genera in the seafood could perhaps be caused by the kind of activities carried out by the people living around the environment such as sewage and effluent discharged as well as other domestic activities. The MEAN±SD results obtained for the total colony count at n=80 for samples obtained from Nembe and n=120 for samples obtained from Ox-bow, P value obtained from the analysis was greater than 0.05 ($p>0.05$) meaning that there is statistically no significant difference between the colony count of isolates obtained from the different seafood samples in both locations. The Anova analysis carried out shows statistically there is no significant difference between the isolates in the samples from the same loation and between the two locations at $p>0.05$.

According to medscape and center for disease control (CDC), Cephalosporins, Tetracycline, Aminoglycosides, B-lactams and Trimethoprim, are first line treatment for infections caused by *Vibrio, Staphylococcus* and *Klebsiella* (Ahmed *et al.,* 2012).

All the isolates were susceptible to ciprofloxacin which is a broad spectrum antibiotic. Ten of the isolates which represent 5% of the whole isolates were resistant to gentamycin. All the isolates (100%) were resistant to ceftriaxone, penicillin, ampicillin and ceftazidime. This could be attributed to the constant abuse of cephalosporin leading to their resistance by microorganisms.

In as much as Erythromycin is a broad spectrum antibiotic that can kill or inhibit a wide range of bacterial isolates, 10% of the isolates from the seafood samples were resistant to it. None of the isolates were susceptible to ceftazidime, ampicillin, ceftriaxone, and penicillin. *Staphylococcus* species were 100% sensitive to ciprofloxacin but 1%, 1.5%, 5.5%, 5.5%, and 6% resistant to gentamycin, erythromycin, sulphanamide/trimethoprim, tetracycline and streptomycin respectively. *Staphylococcal* isolates were 100 % resistant to ampicillin, penicillin, ceftazidime and ceftriazone and this can be attributed to the presence of rhe *mecA* gene in the bacterial isolate. This is also similar to the results obtained by Tibra *et al.,* (2010) which showed that *Staphylococcus gallinarum* was sensitive to ciprofloxacin, gatifloxacin, moxifloxacin, ofloxacin, cefazolin, vancomycin, amikacin and gentamicin and resistant to oxacillin and ceftazidime.

The 100% resistance of the isolates to ceftriazone and ceftazidime could be attributed to the presence of *ctm-x* and *shv* genes in the isolates. The *Vibrio* isolates were all resistant to ampicillin, penicillin, ceftazidime and ceftriazone.

Test for association between antimicrobial susceptibility of isolates and seafood done using Chi-square showed that the susceptibility or resistance of isolates on Erythromycin, Sulphonamide/Trimethoprim, tetracycline and streptomycin are not associated with type of seafood ($p>0.05$), hence the seafood cannot be said to be responsible for the susceptibility or resistance of the isolates to the above mentioned antimicrobials. The isolates from seafood samples obtained from Ox-bow Lake were all resistant to Ceftazidime, Penicillin, ceftriaxone and Ampicillin as well. However, all the Isolates from Ox-bow seafood samples were susceptible to Ciprofloxacin. Isolates from Crab and shrimp gotten from Ox-bow Lake showed more susceptiblity to Streptomycin than isolates from Periwinkle and Oysters. Isolates from Periwinkle gotten from Ox-bow Lake were more resistance than other isolates from other seafood sample.

The characterization of the isolates was done by PCR using 16srRNA as a target gene and all isolates possess the 16srRNA at 1500bp. The sequenced isolates all had revolutionary relations with *Staphylococcus gallinarum, Klebsiella quasipneumoniae, Klebsiella aerogenes, Vibrio parahaemolyticus and Vibrio rotiferianus.*The report of Kriem *et al.,* (2015) confirmed that the phenotypic identification of bacterial isolates using TCBS is not reliable in identifying organisms making molecular analysis the most reliable method of characterizing isolates to species level.

The molecular analysis shows that the isolates possess *SHV, CTM-X, mecA, TDL, acrAB* resistance and virulence genes. None of the isolates were susceptible to ceftazidime, ampicillin, ceftriaxone, and penicillin and this prompted the need for the detection of ESBLs especially bla_{shv} *and* $bla_{ctm\text{-}x}$in the isolates. Also resistance of *Staphylococcu*s to ampicillin and penicillin could be seen as a result of the presence of *mecA* genes in the *Staphylococcus* species.

The molecular analysis also identified the presence of *shv, ctx-m* genes in *klebsiella quasipneumoniae* and *Klebsiella aerogenes*. Hala *et al.* (2019) reported that *Klebsiella quasipneumoniae* harbours blaKPC-2 and shows that *Klebsiella quasipneumoniae* isolates were resistant to ampicillin, ciprofloxacin, ceftazidime, ceftriaxone, sulphonamide/trimethoprim, gentamycin and tetracycline.

Also, *tdh* genes were also detected in *Vibrio parahaemolyticus* and *mecA* genes were detected in *Staphylococcus gallinarum. acrAB* genes were also identified in *Klebsiella quasipneumoniae* and *Klebsiella aerogenes* and this is related to the results obtained from a study on *Klebsiella variicola* and *Klebsiella quasipneumoniae* by Martínez-Romero *et al.,* (2018).

The results recorded indicates that seafoods are a reservoir for multidrug resistant *Klebsiella* and *Vibrio* species and potential health risks posed by such strains should not be underestimated. The results of this study suggest that, in addition to contributing to the multidrug resistance phenotype, the *AcrAB* efflux pump may represent a novel virulence factor required for *K. quasipneumoniae* to resist innate immune defense mechanisms of the lung, thus facilitating the onset of pneumonia (Martínez-Romero *et al.,* 2018).

The amplification for *sxt* genes in the *Vibrio* species showed absence of *sxt* genes in the isolates. None of the isolates showed significant resistance to sulphonamide/trimethoprime antibiotic and this explains the absence of the *stx* genes in the isolates.

CHAPTER 5

5.0 CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

The detection of *ctx-M, shv, mecA* and *acrAB* genes in *Staphylococcus gallinarum, Klebsiella aerogenes Klebsiella quasipneumoniae, Vibrio rotifernus* and *Vibrio parahaemolyticus* isolated from seafoods in Nembe, Bayelsa State portends a probable risk of public health. The detection of these virulence and antibiotic resistance genes which are plasmid mediated shows the possibility of gene transfer between and within related and unrelated species and this will continue to hamper the effectiveness of antibiotic treatment.

5.2 RECOMMENDATION

People should be sensitized on the dangers associated with consuming undercooked or raw seafood. There should also be an intensive and continuous monitoring of potentially pathogenic *Staphylococcus, Klebsiella and Vibrio* species in seafoods and the human health risk associated with eating seafood should be evaluated periodically. Health professionals should be directed, in cases of gastroenteritis associated with seafood consumption, to request specific culture for the isolation and characterization of these isolates as well as molecular characterization of the isolates. Such measures could provide relevant information to Health Surveillance services. Pharmaceutical and health institutions should adopt this study as this might be a key to developing vaccines necessary to curb the health risk associated with seafood consumption.

REFERENCES

Adams, M. R. & Moss, M. O. (2008). Bacterial agents of food borne illness In biology (3rd Edn), (pp182-269). United Kingdom: RSC, Cambridge.

Ahmed, M.H., Wataru, W., Tomoko, F. & Tadashi, S. (2012). Occurrence and characteristics of methicillin-resistant and -susceptible *Staphylococcus aureus* and methicillin-resistant coagulase-negative staphylococci from Japanese retail ready-to-eat raw fish. *International Journal of Food Microbiology. 156(*3), 286-289

Akoachere, J.F.,T.K., Masalla, T.N.& Njom, H.A.(2013). Multi-drug resistant toxigenic Vibrio cholerae O1 is persistent in water sources in New Bell-Douala Cameroon. *BMC Infectious Diseases, 13*,366.

Arungiri, K., Sivakumar, T. & Murugan, T. (2016). Study of *Vibrio* species and its occurrence in seafood samples. *Asian Journal of Phamaceutical and Clinical Research, 9*(1), 229-231.

Becker, K., Heilmann, C. & Peters, G. (2014). Coagulase-Negative *Staphylococci Clinical Microbiology Reviews*, 27(*4*), 870 –926.

Bradford, P. A. (2001). Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clinical Microbiology Review*, *14*,933-951.

Brisse, S., Fevre, C., Passet, V., Issenhuth-Jeanjean, S., Tournebize, R., Diancourt, L. & Grimont, P. (2009). Virulent clones of *Klebsiella* pneumoniae: identification and evolutionary scenario based on genomic and phenotypic characterization. *PLoS ONE*, 4, 4982.

Brisse, S. & Verhoef, J. (2001). Phylogenetic diversity of *Klebsiella* pneumoniae and *Klebsiella* oxytoca clinical isolates revealed by randomly amplified polymorphic DNA, gyrA and parC genes sequencing and automated ribotyping. *International Journal for Systemic Evolutionary Microbiology*, *51*, 915–924.

Brisse, S., van Himbergen, T., Kusters, K. & Verhoef, J. (2004). Development of a rapid identification method for *Klebsiella* pneumoniae phylogenetic groups and analysis of 420 clinical isolates. *Clinical Microbiology and Infection*, *10*, 942–945.

Bronner, S., Monteil, H., & Prevost, G. (2004). Regulation of virulence determinants in *Staphylococcus aureus*: Complexity and applications. *FEMS Microbiology Reviews, 28*(2), 183–200.

Burge, C. A., Mark Eakin, C., Friedman C. S., Froelich, B., Hershberger, P. K., Hofmann E. E., Petes, L. E., Prager, K. C., Weil, E., Willis, B. L., Ford, S. E. and Harvell, C. D. (2014). Climate change influences on marine infectious diseases: implications for management and society. *Annual Review of Marine Science 6*, 249–277.

Burrus, V., Quezada-Calvillo, R., Marrero, J. & Waldo, M.K. (2006). SXT-related integrating conjugative element in New World *Vibrio cholerae*. *Applied Environmental Microbiology, 72*, 3054–3057.

Cabanillas-Beltrán, H., Lausás-Magaña, E., Romero, R., Espinoza, A., García-Gasca, A. & Nishibuchi, M. (2006). Outbreak of gastroenteritis caused by the pandemic *Vibrio parahaemolyticus* O3:K6 in Mexico. *FEMS Microbiology Letters*. *265*,76-80.

Carter, I., Catriona H., Theo, P. S., Todd M. P., Ian, D. K., Gerald, B.H., Glenys, R. C., & Philip, M. G. (2010). PCR for clinical microbiology.*Springer,* 11-47

Cazoria, C., Guigon A., Noel, M., Quilici, M.L. & Laccasin, F. (2011). Fatal *Vibrio vulnificus* infections associated with Eating Raw Oyster. *New Celodonia. Emergence of infectious Diseass*, *17*,136-137.

Celinska, E. (2012). *Klebsiella* spp as a 1, 3-propanediol producer: the metabolic engineering approach. *Critical Reviews in Biotechnology, 32*, 274– 288.

Chapman, J. W., Carlton, J. T., Bellinger, M. R. & Blakeslee, A. M. H. (2007). "Premature refutation of a human-mediated marine species introduction: the case history of the marine snail *Littorina littorea* in the northwestern Atlantic". *Biological Invasions* **9**,737-750.

Chigozie, O., Ogunbanwo, S. T. & Faith, Y. (2015). Prevalence and antimicrobial susceptibility of *Vibrio parahamolyticus* isolated from seafood in Lagos Lagoon Nigeria. *Cogent Food and Agriculture, 1*(1):1-10

Cruz, C. D., Hedderley D. and Fletcher, G. C. (2015). Long-term study of *Vibrio parahaemolyticus* prevalence and distribution in New Zealand shellfish. *Applied Environmental Microbiology 81*(**7**), 2320-2327.

Das, B., Jyoti, V., Pawan, K., Amit, G. & Thandavarayan, R. (2020). Antibiotic resistance in *Vibrio cholerae*: Understanding the ecology of resistance genes and mechanisms. *Vaccine, 38,* 83-92

Domenech-Sanchez, A., Alberti, S., Martinez-Martinez, L., Pascual, A., Garcia, I. & Benedi, V. J. (2001). Antimicrobial Agents Chemotherapy.*Abstract of 41st international conference*

Eswaran, J., Hughes, C. & Koronakis, V. (2003). Locking TolC entrance helices to prevent protein translocation by the bacterial type I export apparatus. *Journal of Molecular Biology, 21,*309-315.

Farmer, J.J., Janda, J.M. & Birkhead, K. (2003). "*Vibrio*".In Murray, P.R., (eds) *Manual of Clinical Microbiology* (pp. 706–718).Washington, DC, USA: ASM Press.

Farmer, J.J., Janda, M., Brenner, F.W., Cameron, D.N., Birkhead, K. M., Genus, I. and Vibrio, P. (2005). 411AL. In: Brenner, D.J., Krieg, N.R., Staley, J.T. editors. Bergey's manual of systematic Bacteriology. The Proteobacteria. Part B. The Gamma proteobacteria. 2nd ed. New York: Springer *2*,494-546.

Farmerchidi, J.P., Sobesky, R., Boussoughant, Y., Quilici, M. L & Coffin, B. (2003). Septicemia and liver abscesses secondary to non-01/non-0139 *Vibrio cholerae* colitis. *European Journal of Gastroenterol Hepatol, 15*, 699-700

Fevre, C., Passet, V., Weill, F. X., Grimont, P. A. & Brisse, S. (2005). Variants of the *Klebsiella* pneumoniae OKP chromosomal beta-lactamase are divided into two main groups, OKP-A and OKPB. *Antimicrobial Agents and Chemotherapy* 49, 5149–5152

Foster, J.A., Bunge, J., Gilbert, J.A. & Moore, J.H. (2012). Measuring the microbiome: perspectives on advances in DNA-based techniques for exploring microbial life. *Brief Bioinform*, *13*, 420–429.

GarcíaVázquez, E., FernándezRufete, A., HernándezTorres, A., Canteras, M., Ruiz , J. & Gómez. J. (2013). When is coagulase negative *Staphylococcus* bacteraemia clinical. *Scandinavian Journal of Infectious Diseases, 45*,664-671.

Ghanbari, M., Kneifel, W. & Domig, K.J. (2015). A new view of the fish gut microbiome: advances from next generation sequencing. *Aquaculture, 448,* 464–475.

Gill, S. R., Fouts, D. E., Archer, G. L., Mongodin, E. F., DeBoy, R. T., Ravel, J. & Dodson, R. J. (2005). Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *Journal of Bacteriology*, *187*(7), 2426–2438.

Grimont, P. A. D. & Grimont, F. (2005). Genus *Klebsiella*., D. J. Brenner, N. R. Krieg & J. T. Staley (Eds). In Bergey's Manual of Systematic Bacteriology (pp.685–693). New York: Springer.

Hala, S., Antony, C.P., Alshehri, M., Althaqafi, A. O., Alsaedi, A., Mufti, A.A., Kaaki M., Alhaj-Hussein, B.T., Zowawi H,M., Al-Amri, A. & Pain, A. (2019). First report of *Klebsiella quasipneumoniae* harboring blaKPC-2 in Saudi Arabia. *Antimicrobial Resistance and Infection Control* , *8*:203

Hara-Kudo, Y., Saito, S., Ohtsuka, K., Yamasaki, S., Yahiro, S., Nishio, T., Iwade, Y. & Otomo, Y. (2012). Characteristics of a sharp decrease in *Vibrio parahaemolyticu*s infections and seafood contamination in Japan. *International Journal of Food Microbiology, 157,* 95–101.

Harwood, V.J., Gandhi, J.P. & Wright, A.C. (2004). Methods for Isolation and Confirmation of *V. vulnificus* from oysters and environmental sources. *Review of Journal of Microbiological Methods*, 59,301-316

Heilmann, C. (2011). Adhesion mechanisms of staphylococci. *Advances in Experimental Medical Biology, 715*,105–123.

Holt, K.E., Wertheim, H., Zadoks, R.N., Baker, S., Whitehouse, C.A. & Dance, D. (2015). Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in *Klebsiella pneumoniae*, an urgent threat to public health. *Proceedings of National Academy of Science*, *112*(27), 3574–3581.

Hooper, D. C. (2000).Mechanisms of action and resistance of older and newer fluoroquinolones. Clinic*al Infectious Disease, 31*, 24-28.

Howden, B.P., Davies, J.K., Johnson, P.D., Stinear, T.P & Grayson, M.L. (2010). Reduced vancomycin susceptibility in *Staphylococcus aureus*, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: resistance mechanisms, laboratory detection, and clinical implications. *Clininical Microbiology Review, 23*, 99–139.

Jay, J.M., Loessner M.J & Golden, D. A. (2005). Foodborne gastroenteritis caused by *Vibrio, Yersinia and Campylobacter* species. *Modern food microbiology. Springer science,* 657-664.

Kérouanton, A., Hennekinne, J.A., Letertre, C., Petit, L., Chesneau, O., Brisabois, & De Buyser, M.L. (2007). Characterization of Staphylococcus aureus strains associated with food poisoning outbreaks in France. *International Journal of Food Microbiology, 115*, 369- 10 375.

Kim, H.W., Hong, Y.J., Jo, J I., Ha, S. D., Kim, S. H., Lee, H.J. & Rhee, M.S. (2017) Raw ready-to-eat seafood safety: microbiological quality of the various seafood species available in fishery, hyper and online markets. *Letter of Applied Microbiology 64*(1)27-34.

Kitaoka, M., Miyata, S .T., Unterweger, D. & Pukatzki**, S.** (2011).Antibiotic resistance mechanisms of *Vibrio cholerae. Medical Microbiology, 60*, 397-407.

Kou, X., Wu, Q., Wang, D. & Zhang, J. (2008). Simultaneous detection of Norovirus and Notavirus in oysters by multiplex RT–PCR *Food Control, 19*,722–726.

Kriegeskorte, A., Ballhausen, B., Idelevich, E.A., Köck, R., Friedrich, A.W., Karc, H., Peters, G. & Becker, K. (2012). Human MRSA isolates with novel genetic homolog Germany. *Emergence of Infectious Diseases*, *18*, 1016–1018.

Kriem, M.R., Banni, B., El Bouchtaoui, H., Hamama, A., El Marrakchi, A., Chaouqy, N., Robert-pilot, A. & Quilici, M.L (2015). Prevalence of *Vibrio* species in raw shrimps (*Paraperaus longirotris)* and performance of a chromogenic medium for the isolation of *vibrio* strains. *Letters in Applied Microbiology 61*, 224-230

Kuczynski, J., Lauber, C.L., Walters, W.A., Parfrey, L.W., Clemente, J.C., Gevers, D. & Knight, R. (2012). Experimental and analytical tools for studying the human microbiome. *National Review Genet, 13*, 47–58.

Le-Loir, Y., Baron, F. & Gautier, M. (2003). *Staphylococcus aureus* and food poisoning. *Genetics and Molecular Research, 2* (1), 63-76.

Letchumanan, V., Chan, K. G. & Lee, L. H. (2014). *Vibrio parahaemolyticus*: a review on the pathogenesis, prevalence, and advance molecular identification techniques. *Frontiers in Microbiology, 5*,705.

Liu, L., Li, Y., Li, S., Hu, N., He, Y., Pong, R., Lin, D. & Lu, L. (2012). Comparison of next-generation sequencingsystems. *Journal of Biomedical Biotechnology, 251364*, 1–11.

Long, S.W., Linson, S.E., Saavedra, M.O., Cantu, C., Davis, J.J. and Brettin, T. (2017). Wholegenome sequencing of human clinical *Klebsiella* pneumoniae isolates reveals misidentification and misunderstandings of *Klebsiella* pneumoniae, *Klebsiella* variicola, and *Klebsiella quasipneumoniae* . *Msphere*, *2*(4), 290–317

Mac, E, S., Cornet, J., Chevalier, F., Cardinal, M., Pilet, M.F., Dousset, X. & Joffraud, J.J. (2012). Characterisation of the spoilage microbiota in raw salmon (Salmo salar) steaks stored under vacuum or modified atmosphere packaging combining conventional methods and PCR–TTGE. *Food Microbiology, 30*, 164–172.

Maheswamdu, G. (2016). Experimental culture of Black Tiger Shrimp *penaeus monodon* Fabricus, 1798 in open sea floating cage. *Indian journal of fisheries. 63*(2), 46459-46506.

Mohiuddin, A .K. (2019). Nutritional value and associated potential risks of seafood consumption. *Biomedical journal of science and seafood. 19*(4) 14456-14459.

Martı´nez, J., Martı´nez, L., Rosenblueth, M., Silva, J. & Martınez-Romero, E. (2004). How are gene sequence analyses modifying bacterial taxonomy? The case of *Klebsiella*. *International Journal of Microbiology*, 7, 261–268.

Martínez-Martínez, L., Pascual, A., Conejo, M. C., García, I., Joyanes, P., Doménech-Sánchez, A. & Benedí. V. J. (2002). Energy-dependent accumulation of norfloxacin and porin expression in clinical isolates of *Klebsiella pneumoniae* and relationship to extended-spectrum beta-lactamase production. *Antimicrobial Agents Chemotherapy 46*, 3926-3932

Martínez-Romero, E., Rodríguez-Medina, N., Beltrán-Rojel, M., Toribio Jiménez, J. and Garza-Ramos, U. (2018). *Klebsiella variicola* and *Klebsiella quasipneumoniae* with capacity to adapt to clinical and plant settings. *Salud Publication Mexico*, *60*, 29-40.

Metzker, M.L. (2010). Sequencing technologies – the next generation. *National Review Genet*, 11, 31–46.

Miyoshi, S. (2013) Extracellular proteolytic enzymes produced by human pathogenic Vibrio species. *Frontiers of Microbiology, 4*,339-345.

Nair, G. B., Ramamurthy, T., Bhattacharya, S. K., Dutta, B., Takeda, Y. and Sack, D. A. (2007). Global dissemination of *Vibrio parahaemolyticus* serotype O3:K6 and its serovariants, *Clinical Microbiology Reviews 20*, 39- 48.

Nasreldin, E. (2013). Occurence of Potentially Human Pathogenic *Vibrio* species in coastal water of Eastern Province of Saudi Arabia. *Research journal of Microbiology 8*,1-12.

Oliveira, M.A., Silva, S. G., & Pereira, I. S. (2016) Amplificacado de DNA por PCR PCR em tempo real. *InT ecnicas basicas em biologia molecular (2nd edn)*. ed. Souza, M. T., Brigido, M.M. &Maranhao, A.Q. (pp. 145-168). Brasilia: *Editora Universidade de Brasilia*.

Olusegun O. S., Anne, M. Q., Kayode K. O. , Bolanle A. A. & Marilyn C. R. (2006). CTX-M-15 extended-spectrum b-lactamase from Nigerian *Klebsiella pneumonia*. *Journal of Antimicrobial Chemotherapy, 57*,24–30

Omoe, K., Dong-Liang, H., Takahashi-Omoe, H., Nakane, A. & Shinagawa, K. (2005). Comprehensive analysis of classical and newly described staphylococcal superantigenic toxin genes in *Staphylococcus aureus* isolates. *FEMS Microbiology Letters, 246*, 191-198.

Papovic, T., Skukan, A. B., Dzidara, P., Coz-Rakovac, R., Strunjak-Perovic, I., Kozacinski, L., Jadan, M. & Briek-Gorski, D. (2010) Microbiological quality of marketed fresh and frozen seafood caught off the Adriatic coast of Croatia. *Vetinary Medcin-Czech, 55*, 233-241.

Plata, K., Rosato, A. E., & Wegrzyn, G. (2009). *Staphylococcus aureus* as an infectious agent: Overview of biochemistry and molecular genetics of its pathogenicity. *Acta Biochimica Polonica*, *56*(4), 597–612.

Rodríguez-Martínez, J. M., Pascual, A., García, I. & Martínez-Martínez, L.(2003). Detection of the plasmid-mediated quinolone resistance determinant qnr among clinical isolates of *Klebsiella pneumoniae* producing AmpC-type beta-lactamase. *Journal of Antimicrobial Chemotherapy, 52,*703-706.

Rodriguez, P.A., Ferrari, R. G. & Conte-junior, C. A. (2018). Application of molecular tools to elucidate the microbiota of seafood. *Journal of Applied Microbiology, 124,* 1347-1365.

Roeselers, G., Mittge, E.K., Stephens, W.Z., Parichy, D.M., Cavanaugh, C.M., Guillemin, K. & Rawls, J.F. (2011). Evidence for a core gut microbiota in the Zebrafish. *International Society of Microbial Ecology Journal, 5*, 1595–1608.

Rosenblueth, M., Martınez, L., Silva, J. & Martınez-Romero, E. (2004). *Klebsiella variicola*, a novel species with clinical and plant associated isolates. *Systematic and Applied Microbiology*, 27, 27–35.

Ryan, P. (2007) Benthic communitues. *Te Ara*. The encyclopedia of new Zealand.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution 4,*406-425.

Semova, I., Carten, J.D., Stombaugh, J., Mackey, L.C., Knight,R., Farber, S.A. & Rawls, J.F. (2012). Microbiota regulateintestinal absorption and metabolism of fatty acids in the Zebrafish. *Cell Host Microbe, 12,*277–288.

Shicongo- Nambabi, M.N.M., Petrus,N.P & Schneider, M.B. (2012). The role, isolation and identification of *Vibrio* species on the quality and safety of seafood. *Biotechnology and Molecular Biology Review*, *7*(2), 16-30

Siqueira, J.F. Jr., Fouad, A.F. & Rocas, I.N. (2012). Pyrosequencing as a tool for better understanding of human microbiomes. *Journal of Oral Microbiology, 4,*107-143.

Shon, A. S., Bajwa, R. P. & Russo, T. A. (2013). Hypervirulent (hypermucoviscous) *Klebsiella* pneumoniae: a new and dangerous breed. *Virulence 4*, 107–118.

Sridhar, M., Sridhar, N., Robertson, P.A.W. & Austin, B. (2006**)** Role of gut probionts in enhancing growth and disease resistance in rainbow trout (Oncorhynchus mykiss, Walbaum) fingerlings. *Asian Fisheries Science, 19,*1–13

Star, B., Haverkamp, T., Jentoft, S. & Jakobsen, K. (2013). Next generation sequencing shows high variation of the intestinal microbial species composition in Atlantic cod caught at a single location. *BMC Microbiology, 13,* 248.

Sudha, S., Mridula, C., Silvester, R. & Hatha, A.A.M. (2014). Prevalence and antibiotic resistance of pathogenic Vibrios in shellfishes from Cochin market. *Indian Journal of Marine Science, 43,* 815–824.

Taminiau, B., Korsak, N., Lemaire, C., Delcenserie, V. & Daube, G. (2014). Validation of real-time PCR for detection of six major pathogens in seafood products. *Food Control, 44,* 130–137.

Tamura, K., Peterson, D., Peterson, N., Strecher, G. & Kumar, S. (2013). Molecular Evolutionary Genetic Analysis Version 6.0. *Molecular Biology and Evolution*. *30,* (12):2725-2729

Tibra, N.K., Jalali, S., Reddy, A.K., Narayanan, R. & Agarwal,R. (2010).Traumatic endophthalmitis caused by *Staphylococcus gallinarum. Journal of Medical Microbiology, 59,* 365–366

Verma, J., Bag, S., Saha, B., Kumar, P., & Ghosh, T.S. M. (2019). Genomic plasticity associated with antimicrobial resistance in *Vibrio cholerae*. *Proceedings of the National Academy of Science 13,* 6226–6231.

Vongkamjan, K., Benjakul, S., Vu, H.T.K. & Vuddhakul, V. (2017). Longitudinal monitoring of *Listeria monocytogenes* and Listeria phages in seafood processing environments in Thailand. *Food Microbiology 66,* 11–19.

Wang, M., Sahm, D. F. Jacoby, G. A. & Hooper, D. C. (2004). Emerging plasmid-mediated quinolone resistance associated with the qnr gene in *Klebsiella pneumoniae* clinical isolates in the United States. *Antimicrobial Agents Chemotherapy 48,* 1295-1299.

Wong, S., Waldrop, T., Summerfelt, S., Davidson, J., Barrows, F., Kenney, P.B., Welch, T. & Wiens, G.D. (2013). Aquacultured rainbow trout (*Oncorhynchus mykiss*) possess a large core intestinal microbiota that is resistant to variation in diet and rearing density. *Applied Environmental Microbiology*, *79*, 4974–4984.

Wozniak, R.A. & Waldor, M.K. (2010). Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. *National Review of Microbiology, 8,* 552–563.

Xu, F., Gonzalez-Escalona, N., Haendiges, J., Myers, R.A., Ferguson, J., Stiles,T., Moore, M. (2017). Sequence type 631 Vibrioparahaemolyticus, an emerging foodborne pathogen in North America. *Journal of Clinical Microbiology, 55,* 645–648.

Xu, Z., Shah, H. N., Misra, R., Chen, J., Zhang,W., Liu, Y. & Mkrtchyan, H. V. (2018). The prevalence, antibiotic resistance and mecA characterization of coagulase negative *staphylococci* recovered from non-healthcare settings in London, UK. *Antimicrobial Resistance & Infection Control*, *7*(1), 73.

Zarkasi, K.Z., Abell, G.C., Taylor, R.S., Neuman, C., Hatje, E., Tamplin, M.L., Katouli, M. &Bowman, J.P. (2014). Pyrosequencing-based characterization of gastrointestinalbacteria of Atlantic salmon (Salmo salar L.) within a commercial mariculture system. *Journal of Applied Microbiology*, 117,18–27.

Zhang, X.H. & Austin, B. (2005). Haemolysins in Vibrio species. *Journal of Applied Microbiology*, *98*(5),1011–1019.

Zhang, K., McClure, J. A., Elsayed, S., Louie, T., & Conly, J. M. (2005). Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome mec types I to V in methicillin-resistant *Staphylococcus aureus. Journal of Clinical Microbiology*, *43*(10), 5026–5033.

APPENDICES

Appendix 1: Media Used

- Thio sulphate-citrate- Bile –Salt- Sucrose Agar
- Nutrient broth
- EMB Agar
- Mannitol salt Agar
- Mueller Hinton Agar
- Blood Agar
- Luria Betani (LB)

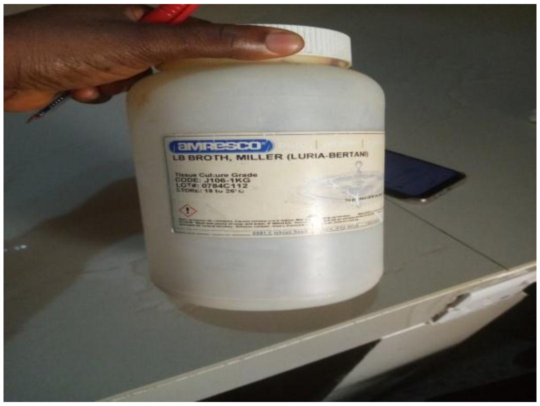


Figure A: Luria Betani (LB)

Appendix 2: Antibiotics Used

- Erythromycin (E) 15ug
- Streptomycin (S) 15ug
- Ciprofloxacin (CIP) 5ug
- Tetracycline (T) 30ug
- Ceftriazone (CRO) 30ug
- Trimethroprim/sulphonamide (SXT) 25ug
- Gentamycin (CN) 30ug
- Penicillin (P) 15ug
- Ampicillin (AMP) 15ug
- Ceftazidime (CAZ) 30ug

Figure B:Antibiotic discs

Figure C:1000 Nano Spectrophotometer, Electrophoretic tank and UVP chamber

ORGANISMS GROWING ON TCBS AGAR

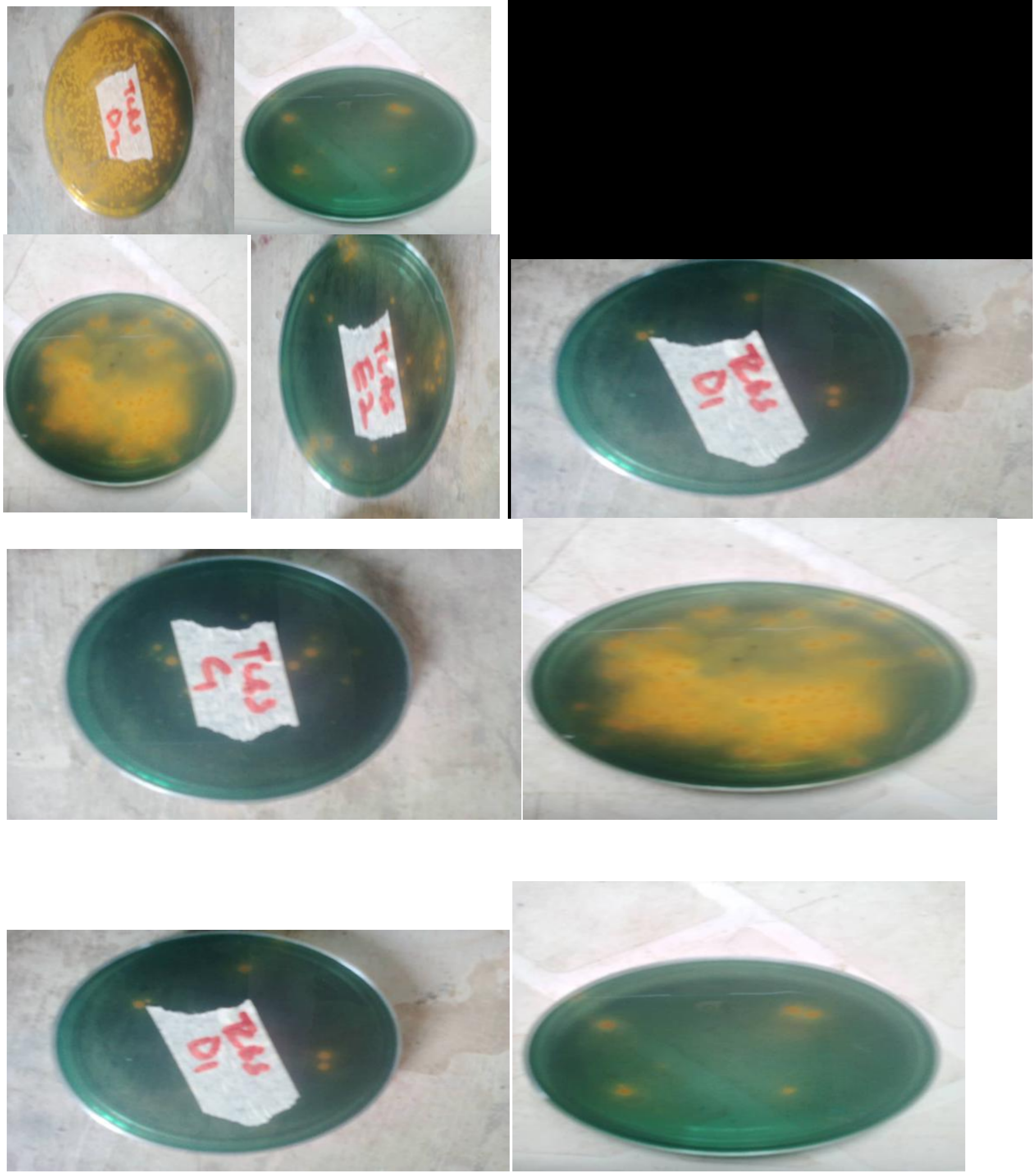


Figure D: Discrete colonies growing on TCBS

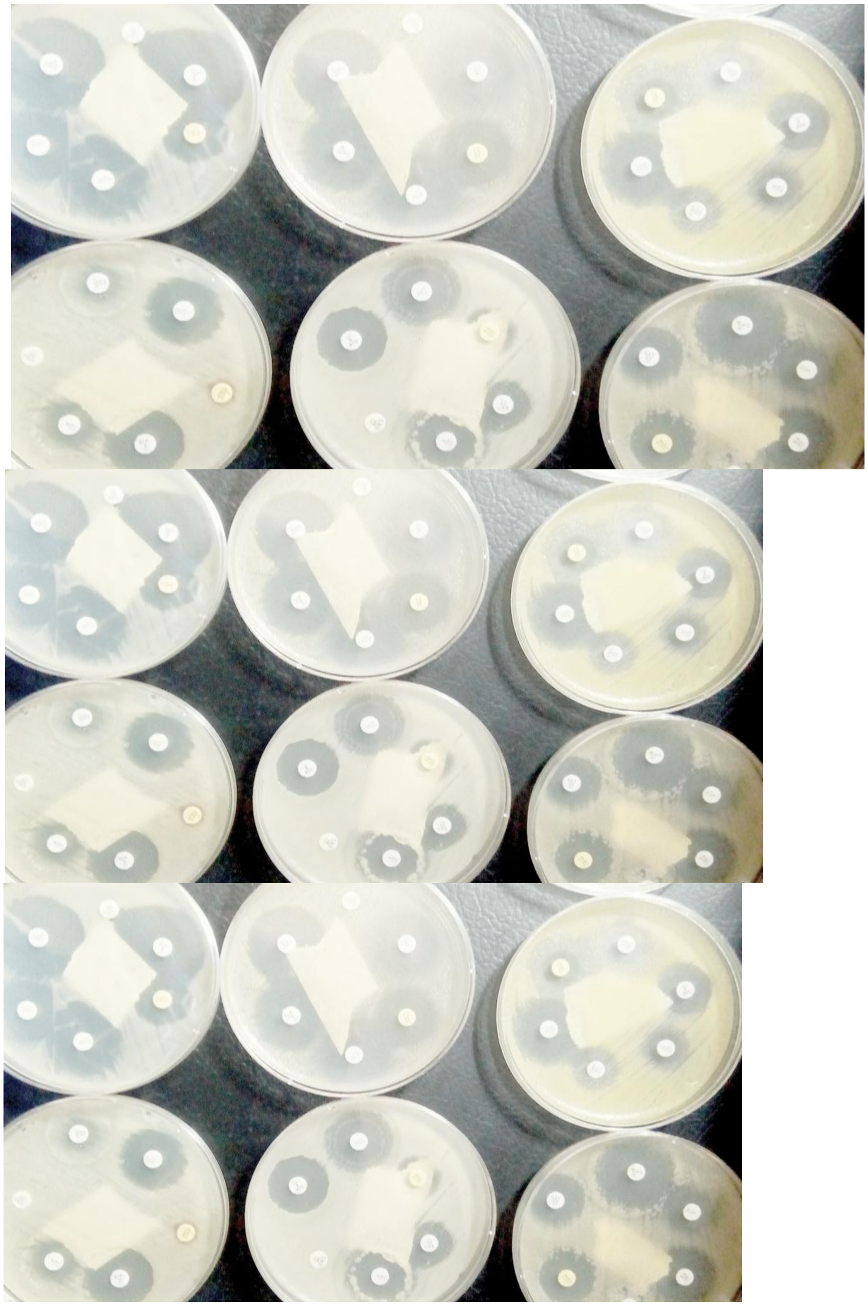


Figure E: Zones of Inhibition on Meuller Hinton Agar

Appendix 3: Media Preparation

The Thiosulphate-Citrate –Bile- Salt-Sucrose (TCBS) agar was prepared according to manufacturer’s guideline. For the 200 samples, 200(20ml) plates were needed so 352.4g of the TCBS powder was dissolved in 4 liters of distilled water.

It was gradually heated while stirring the solution to ensure homogenous mixture of the agar.

The molten agar was poured into 200 Petri dishes and allowed to cool

The Petri dishes were surface dried using the oven heat to reduce contamination and also to remove oxygen bubbles to avoid false positive results.

The blood agar was prepared according to the manufacturer’s guideline. The agar was sterilized in the autoclave at 121^0C for 15 minutes. The sterile blood was added to the already cooled agar and mixed gently while the agar was still molten.

Appendix 4: Microscopy

Isolates were identified microscopically by gram staining method.

The inoculums were collected aseptically from each plate and plated on a glass slide to make a smear which was heat fixed to ensure that the organism will remain intact on the slide. The slide was flooded with crystal violet the primary dye and allowed to stay for 60 seconds. The slide was flooded with iodine so as to increase the binding affinity of the crystal violet to the cells and it was washed out after 60 seconds it was washed out. It was further flooded with a decolourizer acetone. This is done to make the cell membrane lipids accessible to the effect secondary dye. The counter stain was then safranin red was then used to flood the slide after which it was washed with water to remove unfixed dyes on the smear. The slide was observed under the microscope to determine the morphology of the cells.

Appendix 5: Biochemical Test

Catalase Test

This test was carried out to detect the ability of microorganisms to produce the enzyme catalase. A drop of hydrogen peroxide was dropped on a side. An inoculom of the test isolates was collected and place on the slide and mixed with the hydrogen peroxide to make a smear on the slide. The mixture was observed for gas or bubble production.

Oxidase Test:

This test was done to determine the presence of cytochrome oxidase in the isolates. A piece of filter paper was placed on a petri dish and was flooded with oxidase reagent. An inoculom of the test isolates was picked and smeared onto the moistened filter paper. It was allowed to react for 10 minutes and observed it for the production of violet/purple color.

Indole Test

This test was carried out to determine the ability of the isolates to convert typhtophan into indole in other words to detect the presence of tryphtophanase enzyme in the isolates. This test is used to differentiate between gram negative rods.The isolates were incubated in broth containing 0.03% tryptophan, 0.1% peptone, and 0.5% depotasium phosphate for 24 hours. Six drops of kovacs reagent was added to the isolates and observed for the production of violet ring.

Sugar Fermentation Test

This test is carried out to determine the potential of the isolates to utilize sugar and produce acid and gas. The test isolate was placed in the broth containing glucose and sucrose and was incubated. A yellow color and production of gas in the Durham tube indicates a positive result.

Coagulase Test

This test was done to differentiate between *Staphylococcus aureus* from other Staphylococcal species. This test detects the ability of the isolate to produce enzyme coagulase. Rabbit serum was placed on a clean glass slide and saline was added to form a smooth milky emulsion. The mixture was observed for coagulation of the serum within 10 seconds.

Appendix 6: Total Colony Count, Colonial Characteristics And Biochemical Reaction Of Cultures Isolated From Periwinkle In Nembe.

sample code	Colony (CFU/Ml)	Colony morp	Grm	Biochemical Rnx							Probable Orgs
				Oxi	**Ind**	**Glu**	**Suc**	**Cit**	**Coa**	**Cat**	
PN1	7.2x106	Green rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
PN2	$6.6\text{x}10^6$	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus* sp
PN3	$4.2\text{X}10^6$	yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
PN4	$2\text{x}10^6$	Green rod	-	-	-	-	+	+	-	+	*Klebsiella* sp
PN5	$5.8\text{x}10^6$	Yellow cocci	+	-	-	-	+	+	-	+	*Staphylococcus* sp
PN6	$6.3\text{x}10^6$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
PN7	$3.4\text{x}10^6$	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
PN8	$5.9\text{x}10^6$	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus* sp
PN9	$2\text{x}10^5$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
PN10	$4\text{x}10^6$	Green rod	-	-	-	-	+	+	-	+	*Klebsiella* sp
PN11	$9.0\text{x}10^6$	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
PN12	$2.0\text{x}10^6$	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
PN13	$3.2\text{x}10^6$	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
PN14	$1.5\text{x}10^6$	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
PN15	$1.1\text{x}10^6$	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
PN16	$2.4\text{x}10^6$	Green rod	-	-	-	-	+	+	-	+	*Klebsiella* sp
PN17	$3.5\text{x}10^6$	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
PN18	$7\text{x}10^5$	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus* sp
PN19	$4\text{x}10^5$	yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
PN20	$6.4\text{x}10^6$	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp

PN=Periwinkle from nembe, -=negative, +=positive, Oxi=Oxidase, Ind=Indole, Glu=Glucose, Suc=Sucrose, Cit=Citrate, Coa=Coagulase , Cat=Catalase

Appendix 7: Total Colony Count, Colonial Characteristics and Biochemical Reaction of Cultures Isolated from Periwinkle in Oxbow Lake.

Sample	Colony No(CFU/Ml)	Colonial Morph	Gram Rxn	Biochemical Rnx							Probable Orgs
				Oxi	Ind	Glu	Suc	Cit	Coa	Cat	
PO1	2.2×10^6	Green rod	-	-	-	+	+	+	-	+	*Vibrio* sp
PO2	3.6×10^6	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus* sp
PO3	$6.2X10^6$	yellow rod	-	+	+	-	-	+	-	+	*Klebsiella* sp
PO4	2×10^6	Green rod	-	-	-	-	-	+	-	+	*Klebsiella* sp
PO5	5.8×10^6	Yellow cocci	+	-	-	-	+	+	-	+	*Staphylococcus* sp
PO6	6.3×10^6	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
PO7	3.4×10^6	Yellow rod	-	-	-	+	+	+	-	+	*Vibrio* sp
PO8	5.9×10^6	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus* sp
PO9	2.1×10^6	Cream rod	-	+	+	+	+	+	-	+	*Vibrio* sp
PO10	3.2×10^6	Yellow cocci	+	-	-	-	+	+	-	+	*Staphylococcus* sp
PO11	2.9×10^6	Black rod	-	-	-	+	+	+	-	+	*Vibrio* sp
PO12	5.0×10^6	Yellow rod	-	-	-	+	+	+	-	+	*Vibrio* sp
PO13	3.2×10^6	Green rod	-	+	+	-	-	+	-	+	*Klebsiella* sp
PO14	1.5×10^6	Cream rod	-	+	+	-	-	+	-	+	*Klebsiella* sp
PO15	4.1×10^6	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
PO16	2.7×10^6	Green rod	-	-	-	-	+	+	-	+	*Vibrio* sp
PO17	2.5×10^6	Yellow rod	-	-	-	+	+	+	-	+	*Vibrio* sp
PO18	7×10^6	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus* sp
PO19	4.3×10^6	yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
PO20	4.0×10^6	Green rod	-	-	-	+	+	+	-	+	*Vibrio* sp
PO21	4.0×10^6	Green rod	-	-	-	+	+	+	-	+	*Vibrio* sp
PO22	1.9×10^6	Green rod	-	+	+	+	+	+	-	+	*Vibrio* sp
PO23	4.2×10^6	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
PO24	2.9×10^6	Green rod	-	+	+	+	+	+	-	+	*Vibrio* sp
PO25	5.0×10^6	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
PO26	3.2×10^6	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
PO27	2.4×10^6	Cream cocci	+	-	-	+	+	+	-	+	*Staphylococcus* sp
PO28	3.5×10^6	Cream cocci	+	-	-	+	+	+	-	+	*Staphylococcus* sp
PO29	7×10^5	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus* sp
PO30	2.0×10^6	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus* sp

PO=Periwinkle from ox-bow, -=negative, +=positive, Oxi=Oxidase, Ind=Indole, Glu=Glucose, Suc=Sucrose, Cit=Citrate, Coa=Coagulase , Cat=Catalase

Appendix 8: Total Colony Count, Colonial Characteristics and Biochemical Reaction of Cultures Isolated from Crab Nembe.

Sample Code	**Colony Cfu/ML)**	**Colonial Morph**	**Gram Rxn**	**Biochemical Rnx**							**Probable Orgs**
				Oxi	**Ind**	**Glu**	**Suc**	**Cit**	**Coa**	**Ca**	
CN1	$9.0x10^6$	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
CN2	$2.0x10^6$	Green rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
CN3	$3.2x10^6$	yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
CN4	$4.0x10^6$	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
CN5	$4.0x10^6$	Green rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
CN6	$5.5x10^6$	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
CN7	$3.0x10^6$	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
CN8	$2.0x10^6$	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus* sp
CN9	$2.2x10^6$	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
CN10	$4.0x10^6$	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
CN11	$5.0x10^6$	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
CN12	$3.9x10^6$	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
CN13	$2.9x10^6$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
CN14	$3.0x10^6$	Yellow round	+	-	-	+	+	+	-	+	*Staphylococcus* sp
CN15	$3.2x10^6$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
CN16	$2.4x10^6$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
CN17	$3.5x10^6$	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus* sp
CN18	$7x10^5$	Green round	-	+	+	-	-	+	-	+	*Vibrio* sp
CN19	$4x10^5$	yellow round	-	+	+	+	+	+	-	+	*Vibrio* sp
CN20	$6.4x10^6$	Yellow round	-	+	+	+	+	+	-	+	*Vibrio* sp

C=Crab from Nembe, -=negative, +=positive, Oxi=Oxidase, Ind=Indole, Glu=Glucose, Suc=Sucrose, Cit=Citrate, Coa=Coagulase , Cat=Catalase

Appendix 9: Total Colony Count, Colonial Characteristics and Biochemical Reaction of Cultures Isolated from Crab from Ox-Bow.

Sample Code	Colony No (CFU/ML)	Colonial Morph	Gram Rxn	Biochemical Rnx							Probable Orgs
				Oxi	Ind	Glu	Suc	Cit	Coa	Ca	
CO1	$9.0x10^{6}$	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
CO2	$4.0x10^{6}$	Green rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
CO3	$1.2x10^{6}$	yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
CO4	$3.9x10^{6}$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
CO5	$3.0x10^{6}$	Yellow round	+	-	-	+	+	+	-	+	*Staphylococcus* sp
CO6	$3.2x10^{6}$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
CO7	$2.8x10^{6}$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
CO8	$3.5x10^{6}$	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
CO9	$2.2x10^{6}$	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
CO10	$4.0x10^{6}$	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
CO11	$5.0x10^{6}$	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
CO12	$3.9x10^{6}$	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
CO13	$2.9x10^{6}$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
CO14	$3.0x10^{6}$	Yellow round	+	-	-	+	+	+	-	+	*Staphylococcus* sp
CO15	$3.2x10^{6}$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
CO16	$2.4x10^{6}$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
CO17	$3.5x10^{6}$	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus* sp
CO18	$7x10^{6}$	Green round	-	+	+	-	-	+	-	+	*Vibrio* sp
CO19	$3x10^{6}$	yellow round	-	+	+	+	+	+	-	+	*Vibrio* sp
CO20	$2x10^{6}$	Yellow round	+	-	-	+	+	+	-	+	*Staphylococcus* sp
CO21	$8x10^{5}$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
CO22	$2.2x10^{6}$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
CO23	$6.0x10^{6}$	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
CO24	$2.0x10^{6}$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
CO25	$2x10^{6}$	Green rod	-	-	-	-	+	+	-	+	*Klebsiella* sp
CO26	$5.8x10^{6}$	Yellow cocci	+	-	-	-	+	+	-	+	*Staphylococcus* sp
CO27	$6.3x10^{6}$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
CO28	$3.4x10^{6}$	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
CO29	$2.5x10^{6}$	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
CO30	$7x10^{6}$	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus* sp

CO=Crab from Ox-bow, -=negative, +=positive, Oxi=Oxidase, Ind=Indole, Glu=Glucose, Suc=Sucrose, Cit=Citrate, Coa=Coagulase , Cat=Catalase

Appendix 10: Total Colony Count, Colonial Characteristics and Biochemical Reaction of Cultures Isolated from Oyster from Nembe.

Sample Code	Colony No (CFU/ML)	Colonial Morph	Gram	Biochemical Rnx							Probable Orgs
				Oxi	Ind	Glu	Suc	Cit	Coa	Cat	
ON1	$7.9x10^6$	Green rod	-	+	-	+	+	+	-	+	*Vibrio* sp
ON2	$6.3x10^6$	Green rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
ON3	$3.2x10^6$	yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
ON4	$3.6x10^6$	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
ON5	$4.0x10^6$	Green rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
ON6	$5.5x10^6$	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
ON7	$3.0x10^6$	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus* sp
ON8	$2.0x10^6$	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
ON9	$2.2x10^6$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
ON10	$4.0x10^6$	Green rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
ON11	$1.9x10^6$	Green rod	-	+	+	+	+	+	-	+	*Klebsiella* sp
ON12	$4.2x10^6$	Yellow rod	-	+	+	+	+	+	-	+	*Klebsiella* sp
ON13	$2.9x10^6$	yellow rod	-	+	+	+	+	+	-	+	*Klebsiella* sp
ON14	$5.0x10^6$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
ON15	$3.2x10^6$	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
ON16	$2.4x10^6$	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
ON17	$3.5x10^6$	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus* sp
ON18	$7x10^5$	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
ON19	$4x10^5$	Yellow rod	-	+	+	+	+	+	-	+	*Klebsiella* sp
ON20	$1.4x10^6$	Yellow rod	-	+	+	+	+	+	-	+	*Klebsiella* sp

ON=Oyster from Nembe, -=negative, +=positive, Oxi=Oxidase, Ind=Indole, Glu=Glucose, Suc=Sucrose, Cit=Citrate, Coa=Coagulase , Cat=Catalase

Appendix 11: Total Colony Count, Colonial Characteristics and Biochemical Reaction of Cultures Obtained from Oyster from Ox-Bow.

Sample Code	Colony No (CFU/Ml)	Colonial Morph	Gram			Biochemical Rnx					Probable Orgs
				Oxi	Ind	Glu	Suc	Cit	Coa	Cat	
OO1	5.0x10^{6}	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
OO2	3.9x10^{6}	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
OO3	2.9x10^{6}	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
OO4	3.0x10^{6}	Yellow round	+	-	-	+	+	+	-	+	*Staphylococcus* sp
OO5	3.2x10^{6}	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
OO6	2.4x10^{6}	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
OO7	3.0x10^{6}	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus* sp
OO8	2.0x10^{6}	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
OO9	2.4x10^{6}	Yellow rod	-	+	+	+	+	+	-	+	*Klebsiella* sp
OO10	3.5x10^{6}	Yellow rod	-	+	+	+	+	+	-	+	*Klebsiella* sp
OO11	4x10^{5}	Yellow round	-	+	+	+	+	+	-	+	*Vibrio* sp
OO12	8x10^{5}	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
OO13	3.4x10^{6}	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
OO14	5.0x10^{6}	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
OO15	3.2x10^{6}	Green rod	-	+	+	-	-	+	-	+	*Klebsiella* sp
OO16	2.4x10^{6}	Green rod	-	+	+	-	-	+	-	+	*Klebsiella* sp
OO17	3.5x10^{6}	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus* sp
OO18	7x10^{5}	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
OO19	4x10^{5}	Yellow rod	-	+	+	+	+	+	-	+	*Klebsiella* sp
OO20	2.0x10^{6}	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
OO21	3.9x10^{6}	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
OO22	2.9x10^{6}	Green rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
OO23	3.0x10^{6}	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus* sp
OO24	4.2x10^{6}	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus* sp
OO25	2x10^{5}	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
OO26	4x10^{6}	Green rod	-	-	-	-	+	+	-	+	*Klebsiella* sp
OO27	9.0x10^{6}	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
OO28	2.2x10^{6}	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
OO29	6.0x10^{6}	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
OO30	2.0x10^{6}	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp

OO=Oyster from ox-bow, -=negative, +=positive, Oxi=Oxidase, Ind=Indole, Glu=Glucose, Suc=Sucrose, Cit=Citrate, Coa=Coagulase , Cat=Catalase

Appendix 12: Total Colony Count, Colonial Characteristics and Biochemical Reaction of Cultures obtained from Shrimp from Nembe.

Sample Code	Colony No (CFU/ML)	Colonial Morph	Gram Rxn	Biochemical Rnx							Probable Orgs
				Oxi	Ind	Glu	Suc	Cit	Coa	Cat	
SN1	$8.3x10^{6}$	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
SN2	$6.4x10^{6}$	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
SN3	$3.2x10^{6}$	yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
SN4	$2.8x10^{6}$	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
SN5	$2.9x10^{6}$	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
SN6	$4.5x10^{6}$	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus sp*
SN7	$3.7x10^{6}$	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
SN8	$2.0x10^{6}$	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus sp*
SN9	$2.2x10^{6}$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
SN10	$6.0x10^{6}$	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
SN11	$2.0x10^{6}$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
SN12	$3.9x10^{6}$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
SN13	$2.9x10^{6}$	Green rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
SN14	$3.0x10^{6}$	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus sp*
SN15	$4.2x10^{6}$	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus sp*
SN16	$2.4x10^{6}$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
SN17	$3.5x10^{6}$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
SN18	$4x10^{5}$	Yellow round	-	+	+	+	+	+	-	+	*Vibrio* sp
SN19	$8x10^{5}$	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
SN20	$3.4x10^{6}$	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp

SN=Shrimp from Nembe, -=negative, +=positive, Oxi=Oxidase, Ind=Indole, Glu=Glucose, Suc=Sucrose, Cit=Citrate, Coa=Coagulase , Cat=Catalase

Appendix 13: Total Colony Count, Colonial Characteristics and Biochemical Reaction of Cultures obtained from Shrimp from Ox-Bow.

Sample Code	**Colony No (CFU/ML)**	**Colonial Morph**	**Gram Rxn**	**Biochemical Rnx**							**Probable Orgs**
				Oxi	**Ind**	**Glu**	**Suc**	**Cit**	**Coa**	**Cat**	
SO1	4.3×10^6	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
SO2	2.4×10^6	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
SO3	1.2×10^6	yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
SO4	5.8×10^6	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
SO5	3.9×10^6	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
SO6	1.5×10^6	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus sp*
SO7	2.7×10^6	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
SO8	7.0×10^6	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus sp*
SO9	2.7×10^6	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
SO10	4.0×10^6	Green rod	-	+	+	-	-	+	-	+	*Vibrio* sp
SO11	3.0×10^6	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
SO12	1.9×10^6	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
SO13	5.9×10^6	Green rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
SO14	3.0×10^6	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus sp*
SO15	6.2×10^6	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus sp*
SO16	2.4×10^6	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
SO17	4.5×10^6	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
SO18	4×10^5	Yellow round	-	+	+	+	+	+	-	+	*Vibrio* sp
SO19	6×10^5	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
SO20	3.0×10^6	Yellow round	+	-	-	+	+	+	-	+	*Staphylococcus sp*
SO21	2.2×10^6	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
SO22	2.4×10^6	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
SO23	3.5×10^6	Yellow cocci	+	-	-	+	+	+	-	+	*Staphylococcus sp*
SO24	1×10^6	Green round	-	+	+	-	-	+	-	+	*Vibrio* sp
SO25	4×10^5	yellow round	-	+	+	+	+	+	-	+	*Vibrio* sp
S026	5.8×10^6	Yellow cocci	+	-	-	-	+	+	-	+	*Staphylococcus sp*
SO27	6.3×10^6	Yellow rod	-	+	+	+	+	+	-	+	*Vibrio* sp
SO28	3.4×10^6	Yellow rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
SO29	4.0×10^6	Green rod	-	-	-	+	+	+	-	+	*Klebsiella* sp
SO30	1.9×10^6	Green rod	-	+	+	+	+	+	-	+	*Klebsiella* sp

SN=Shrimp from Nembe, -=negative, +=positive, Oxi=Oxidase, Ind=Indole, Glu=Glucose, Suc=Sucrose, Cit=Citrate, Coa=Coagulase , Cat=Catalase

Appendix 14: Percentage Distribution and Occurrence of Bacterial Isolates in Seafood Isolated from Nembe (n=80).

Bacterial Isolates/Seafoods	Periwinkle(20)	Crab(20)	Oyster(20)	Shrimp(20)
S.gallinarum	20	15	10	20
V. rotiferanus	5	15	10	15
V.parahemolyticus	25	45	35	50
K. aerogene	15	10	20	5
K. quasipneumoniae	35	15	25	10

Appendix 15: Percentage Distribution and Occurrence of Bacterial Isolates in Seafood Isolated from Ox-Bow Lake (n=120).

Bacterial Isolates/Seafoods	Periwinkle(30)	Crab(30)	Oyster(30)	Shrimp(30)
S.gallinarum	30	20	16.7	23.3
V. rotiferanus	16.7	16.7	10	13.3
V.parahemolyticus	33.3	40	43.3	43.3
K. aerogene	10	-	13.3	10
K. quasipneumoniae	10	23.3	16.7	10

Appendix 16: Analysis of Variation in the Colony Counts of Bacterial Isolates from Seafood Samples from Nembe (Single Factor)

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.124647	3	0.041549	0.39718	0.755398	2.724944
Within Groups	7.950343	76	0.10461			
Total	8.074989	79				

Appendix 17: Analysis of Variation in the Colony Counts of Bacterial Isolates from Seafood Samples from Ox-Bow Lake (Single Factor)

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.429608	3	0.143203	1.743	0.162073	2.682809
Within Groups	9.53042	116	0.082159			
Total	9.960029	119				

Appendix 18: Analysis of Variation in the Colony Counts of Bacterial Isolates from Seafood Samples from Nembe and Oxbow Samples (Two-Ways Without Replication)

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.011737	3	0.003912	1.15783	0.453477	9.276628
Columns	1.25E-05	1	1.25E-05	0.003699	0.955327	10.12796
Error	0.010138	3	0.003379			
Total	0.021887	7				

Appendix 19: Antimicrobial Resistance Profiles of Bacterial Isolates Obtained from Periwinkle in Nembe. (According to CLSI 2013)

				ANTIBIOTICS(µg)					
CODE	**CIP**	**CN**	**E**	**CRO**	**AMP**	**P**	**SXT**	**TE**	**S**	**CAZ**
PN1	S	S	S	R	R	R	S	R	R	R
PN2	S	S	S	R	R	R	S	S	S	R
PN3	S	S	S	R	R	R	S	S	R	R
PN4	S	S	R	R	R	R	R	R	R	R
PN5	S	S	S	R	R	R	S	S	S	R
PN6	S	S	S	R	R	R	S	S	S	R
PN7	S	S	S	R	R	R	S	R	R	R
PN8	S	S	S	R	R	R	S	S	S	R
PN9	S	S	S	R	R	R	S	R	R	R
PN10	S	S	S	R	R	R	S	R	R	R
PN11	S	S	R	R	R	R	R	R	R	R
PN12	S	S	R	R	R	R	R	R	R	R
PN13	S	S	S	R	R	R	S	R	R	R
PN14	S	S	S	R	R	R	S	R	R	R
PN15	S	S	S	R	R	R	R	S	S	R
PN16	S	S	R	R	R	R	R	S	S	R
PN17	S	S	R	R	R	R	S	S	R	R
PN18	S	S	S	R	R	R	S	S	S	R
PN19	S	S	S	R	R	R	S	R	R	R
PN20	S	S	S	R	R	R	S	S	S	R

PN=PERIWINKLE FROM NEMBE, CIP=CIPROFLOXACIN, CN=GENTAMYCIN, CRO=CEFTRIAXONE, AMP=AMPICILLIN, P=PENICILLIN, SXT=SULPHONAMIDE/TRIMETHOPRIM, T=TETRACYCLINE, S=STEPTOMYCIN, CAZ=CEFTAZIDIME, S=SUSCEPTIBLE, R=RESISTANT

Appendix 20: Antimicrobial Resistance Profiles of Bacterial Isolates obtained from Periwinkle in Ox-Bow Lake. (According to CLSI 2013)

	ANTIBIOTICS(μg)									
CODE	**CIP**	**CN**	**E**	**CRO**	**AMP**	**P**	**SXT**	**TE**	**S**	**CAZ**
PO1	S	S	S	R	R	R	S	R	R	R
PO2	S	S	S	R	R	R	S	R	S	R
PO3	S	S	S	R	R	R	S	R	S	R
PO4	S	S	S	R	R	R	S	R	R	R
PO5	S	S	S	R	R	R	R	S	S	R
PO6	S	S	S	R	R	R	S	S	S	R
PO7	S	S	S	R	R	R	S	S	R	R
PO8	S	S	S	R	R	R	S	S	S	R
PO9	S	S	S	R	R	R	S	S	R	R
PO10	S	S	R	R	R	R	S	R	R	R
PO11	S	S	S	R	R	R	S	S	R	R
PO12	S	S	S	R	R	R	S	R	R	R
PO13	S	S	S	R	R	R	S	R	R	R
PO14	S	S	S	R	R	R	S	R	R	R
PO15	S	S	S	R	R	R	S	S	S	R
PO16	S	S	S	R	R	R	S	S	S	R
PO17	S	S	S	R	R	R	R	S	R	R
PO18	S	S	S	R	R	R	S	S	S	R
PO19	S	S	S	R	R	R	S	R	R	R
PO20	S	S	S	R	R	R	S	R	R	R
PO21	S	R	S	R	R	R	S	S	S	R
PO22	S	S	S	R	R	R	S	R	S	R
PO23	S	S	S	R	R	R	S	S	S	R
PO24	S	S	R	R	R	R	S	S	R	R
PO25	S	S	S	R	R	R	R	R	R	R
PO26	S	S	S	R	R	R	R	S	R	R
PO27	S	S	S	R	R	R	R	R	R	R
PO28	S	S	S	R	R	R	R	R	R	R
PO29	S	S	S	R	R	R	R	R	R	R
PO30	S	R	S	R	R	R	R	R	R	R

PN=PERIWINKLE FROM NEMBE, CIP=CIPROFLOXACIN, CN=GENTAMYCIN, CRO=CEFTRIAXONE, AMP=AMPICILLIN, P=PENICILLIN, SXT=SULPHONAMIDE/TRIMETHOPRIM, TE=TETRACYCLINE, S=STEPTOMYCIN, CAZ=CEFTAZIDIME, mm=MILLIMETER

Appendix 21: Antimicrobial Resistance Profiles of Bacterial Isolates obtained from Crab in Nembe. (According To CLSI 2013)

	ANTIBIOTICS(μg)									
CODE	**CIP**	**CN**	**E**	**CRO**	**AMP**	**P**	**SXT**	**TE**	**S**	**CAZ**
CN1	S	S	S	R	R	R	S	R	R	R
CN2	S	S	S	R	R	R	S	R	R	R
CN3	S	S	S	R	R	R	S	R	S	R
CN4	S	S	S	R	R	R	R	S	R	R
CN5	S	S	S	R	R	R	S	R	R	S
CN6	S	R	S	R	R	R	S	S	S	R
CN7	S	S	S	R	R	R	S	R	R	R
CN8	S	S	S	R	R	R	S	S	S	R
CN9	S	S	S	R	R	R	S	R	R	R
CN10	S	S	S	R	R	R	S	R	R	R
CN11	S	S	S	R	R	R	R	R	R	R
CN12	S	S	S	R	R	R	R	R	R	R
CN13	S	S	S	R	R	R	S	R	R	R
CN14	S	S	S	R	R	R	S	S	S	R
CN15	S	R	S	R	R	R	R	S	S	R
CN16	S	S	R	R	R	R	R	S	S	R
CN17	S	S	R	R	R	R	S	S	R	R
CN18	S	S	S	R	R	R	S	S	S	R
CN19	S	R	S	R	R	R	S	R	R	R
CN20	S	S	S	R	R	R	S	S	S	R

CN=CRAB FROM NEMBE, CIP=CIPROFLOXACIN, CN=GENTAMYCIN, CRO=CEFTRIAXONE, AMP=AMPICILLIN, P=PENICILLIN, SXT=SULPHONAMIDE/TRIMETHOPRIM, T=TETRACYCLINE, S=STEPTOMYCIN, CAZ=CEFTAZIDIME, S=SUSCEPTIBLE, R=RESISTANT

Appendix 22: Antimicrobial Resistance Profiles of Bacterial Isolates obtained from Crab in Ox-Bow Lake. (According To CLSI 2013)

				ANTIBIOTICS(µg)						
CODE	**CIP**	**CN**	**E**	**CRO**	**AMP**	**P**	**SXT**	**TE**	**S**	**CAZ**
CO1	S	S	S	R	R	R	S	R	R	R
CO2	S	S	S	R	R	R	S	R	S	R
CO3	S	S	R	R	R	R	S	R	S	R
CO4	S	S	S	R	R	R	S	R	R	R
CO5	S	S	S	R	R	R	R	S	S	R
CO6	S	S	S	R	R	R	S	S	S	R
CO7	S	S	S	R	R	R	S	S	R	R
CO8	S	S	R	R	R	R	S	S	S	R
CO9	S	S	S	R	R	R	R	S	S	R
CO10	S	S	R	R	R	R	S	R	S	R
CO11	S	S	S	R	R	R	S	S	S	R
CO12	S	S	S	R	R	R	S	R	S	R
CO13	S	S	S	R	R	R	S	R	S	R
CO14	S	S	S	R	R	R	S	R	R	R
CO15	S	S	S	R	R	R	S	S	S	R
CO16	S	S	R	R	R	R	S	S	S	R
CO17	S	S	S	R	R	R	R	S	R	R
CO18	S	S	S	R	R	R	S	S	S	R
CO19	S	S	S	R	R	R	S	R	R	R
CO20	S	S	S	R	R	R	S	R	R	R
CO21	S	S	S	R	R	R	S	S	S	R
CO22	S	S	S	R	R	R	R	R	S	R
CO23	S	S	S	R	R	R	S	S	S	R
CO24	S	S	R	R	R	R	S	S	R	R
CO25	S	S	S	R	R	R	R	R	S	R
CO26	S	S	S	R	R	R	R	S	S	R
CO27	S	S	S	R	R	R	R	R	S	R
CO28	S	S	S	R	R	R	R	R	S	R
CO29	S	S	S	R	R	R	R	R	S	R
CO30	S	S	R	R	R	R	R	R	R	R

PN=PERIWINKLE FROM NEMBE, CIP=CIPROFLOXACIN, CN=GENTAMYCIN, CRO=CEFTRIAXONE, AMP=AMPICILLIN, P=PENICILLIN, SXT=SULPHONAMIDE/TRIMETHOPRIM, T=TETRACYCLINE, S=STEPTOMYCIN, CAZ=CEFTAZIDIME, mm=MILLIMETER

Appendix 23: Antimicrobial Resistance Profiles of Bacterial Isolates obtained from Oyster in Nembe. (According to CLSI 2013)

	ANTIBIOTICS(µg)									
CODE	**CIP**	**CN**	**E**	**CRO**	**AMP**	**P**	**SXT**	**TE**	**S**	**CAZ**
ON1	S	S	S	R	R	R	S	R	R	R
ON2	S	S	S	R	R	R	S	R	R	R
ON3	S	S	S	R	R	R	S	R	S	R
ON4	S	S	S	R	R	R	R	S	R	R
ON5	S	S	S	R	R	R	S	R	R	S
ON6	S	R	S	R	R	R	S	S	S	R
ON7	S	S	S	R	R	R	S	R	S	R
ON8	S	S	S	R	R	R	S	S	S	R
ON9	S	S	S	R	R	R	S	R	R	R
ON10	S	S	R	R	R	R	R	R	R	R
ON11	S	S	S	R	R	R	R	R	R	R
ON12	S	S	S	R	R	R	R	R	R	R
ON13	S	S	S	R	R	R	S	R	R	R
ON14	S	S	S	R	R	R	S	S	S	R
ON15	S	R	S	R	R	R	R	S	S	R
ON16	S	S	R	R	R	R	R	S	S	R
ON17	S	S	R	R	R	R	S	S	S	R
ON18	S	S	S	R	R	R	S	R	R	R
ON19	S	S	R	R	R	R	S	R	R	R
ON20	S	S	S	R	R	R	R	S	S	R

ON=OYSTER FROM NEMBE, CIP=CIPROFLOXACIN, CN=GENTAMYCIN, CRO=CEFTRIAXONE, AMP=AMPICILLIN, P=PENICILLIN, SXT=SULPHONAMIDE/TRIMETHOPRIM, T=TETRACYCLINE, S=STEPTOMYCIN, CAZ=CEFTAZIDIME, S=SUSCEPTIBLE, R=RESISTANT

Appendix 24: Antimicrobial Resistance Profiles of Bacterial Isolates obtained from Oyster in Ox-Bow Lake. (According to CLSI 2013).

	ANTIBIOTICS(μg)									
CODE	**CIP**	**CN**	**E**	**CRO**	**AMP**	**P**	**SXT**	**TE**	**S**	**CAZ**
OO1	S	S	S	R	R	R	S	S	S	R
OO2	S	S	R	R	R	R	S	R	R	R
OO3	S	S	S	R	R	R	S	S	R	R
OO4	S	S	S	R	R	R	S	S	R	R
OO5	S	S	S	R	R	R	S	S	S	R
OO6	S	S	S	R	R	R	R	S	R	R
OO7	S	S	S	R	R	R	S	S	S	R
OO8	S	S	S	R	R	R	S	S	S	R
OO9	S	S	S	R	R	R	S	S	R	R
OO10	S	S	S	R	R	R	S	S	R	R
OO11	S	S	S	R	R	R	R	R	R	R
OO12	S	S	S	R	R	R	R	R	R	R
OO13	S	S	S	R	R	R	S	R	R	R
OO14	S	S	R	R	R	R	S	S	S	R
OO15	S	S	S	R	R	R	R	S	S	R
OO16	S	S	S	R	R	R	R	S	S	R
OO17	S	S	S	R	R	R	S	S	S	R
OO18	S	S	R	R	R	R	S	S	R	R
OO19	S	S	S	R	R	R	S	R	R	R
OO20	S	S	S	R	R	R	S	S	S	R
OO21	S	S	S	R	R	R	R	S	R	R
OO22	S	S	S	R	R	R	S	R	R	R
OO23	S	S	S	R	R	R	S	S	S	R
OO24	S	S	S	R	R	R	S	S	S	R
OO25	S	S	S	R	R	R	S	R	S	R
OO26	S	S	S	R	R	R	R	S	S	R
OO27	S	S	S	R	R	R	S	R	R	R
OO28	S	S	S	R	R	R	S	S	S	R
OO29	S	S	S	R	R	R	S	R	S	R
OO30	S	S	S	R	R	R	S	R	R	R

OO=OYSTER FROM OX-BOW, CIP=CIPROFLOXACIN, CN=GENTAMYCIN, CRO=CEFTRIAXONE, AMP=AMPICILLIN, P=PENICILLIN, SXT=SULPHONAMIDE/TRIMETHOPRIM, TE=TETRACYCLINE, S=STEPTOMYCIN, CAZ=CEFTAZIDIME, mm=MILLIMETER

Appendix 25: Antimicrobial Resistance Profiles of Bacterial Isolates obtained from Shrimps in Nembe. (According to CLSI 2013).

				ANTIBIOTICS(μg)						
CODE	**CIP**	**CN**	**E**	**CRO**	**AMP**	**P**	**SXT**	**TE**	**S**	**CAZ**
SN1	S	R	S	R	R	R	S	R	R	R
SN2	S	S	S	R	R	R	S	R	R	R
SN3	S	R	S	R	R	R	S	R	S	R
SN4	S	S	R	R	R	R	R	S	R	R
SN5	S	S	S	R	R	R	S	R	R	S
SN6	S	S	S	R	R	R	S	S	S	R
SN7	S	S	S	R	R	R	S	S	S	R
SN8	S	S	S	R	R	R	S	R	S	R
SN9	S	S	S	R	R	R	S	R	R	R
SN10	S	S	R	R	R	R	R	S	R	R
SN11	S	S	S	R	R	R	R	S	R	R
SN12	S	S	S	R	R	R	R	R	R	R
SN13	S	S	S	R	R	R	S	R	R	R
SN14	S	S	S	R	R	R	S	S	S	R
SN15	S	S	S	R	R	R	R	R	S	R
SN16	S	S	R	R	R	R	R	S	S	R
SN17	S	S	R	R	R	R	S	S	S	R
SN18	S	R	S	R	R	R	S	R	R	R
SN19	S	S	R	R	R	R	S	R	R	R
SN20	S	S	S	R	R	R	R	S	S	R

SN=SHRIMPS FROM NEMBE, CIP=CIPROFLOXACIN, CN=GENTAMYCIN, CRO=CEFTRIAXONE, AMP=AMPICILLIN, P=PENICILLIN, SXT=SULPHONAMIDE/TRIMETHOPRIM, T=TETRACYCLINE, S=STEPTOMYCIN, CAZ=CEFTAZIDIME, S=SUSCEPTIBLE, R=RESISTANT

Appendix 26: Antimicrobial Resistance Profiles of Bacterial Isolates obtained from Shrimps in Ox-Bow Lake. (According to CLSI 2013).

	ANTIBIOTICS(μg)									
CODE	**CIP**	**CN**	**E**	**CRO**	**AMP**	**P**	**SXT**	**TE**	**S**	**CAZ**
SO1	S	S	S	R	R	R	S	S	S	R
SO2	S	S	S	R	R	R	S	S	R	R
SO3	S	S	S	R	R	R	S	S	S	R
SO4	S	S	S	R	R	R	S	S	S	R
SO5	S	S	S	R	R	R	S	S	S	R
SO6	S	S	S	R	R	R	R	S	S	R
SO7	S	S	S	R	R	R	S	S	S	R
SO8	S	S	S	R	R	R	R	S	S	R
SO9	S	S	S	R	R	R	S	S	S	R
SO10	S	S	R	R	R	R	S	S	S	R
SO11	S	S	S	R	R	R	S	R	S	R
SO12	S	S	S	R	R	R	S	R	S	R
SO13	S	S	S	R	R	R	S	R	R	R
SO14	S	S	S	R	R	R	S	S	S	R
SO15	S	S	S	R	R	R	S	S	S	R
SO16	S	S	S	R	R	R	S	S	S	R
SO17	S	S	R	R	R	R	R	S	S	R
SO18	S	S	R	R	R	R	S	S	S	R
SO19	S	S	S	R	R	R	S	R	R	R
SO20	S	S	S	R	R	R	S	S	S	R
SO21	S	S	S	R	R	R	S	S	R	R
SO22	S	S	S	R	R	R	S	R	R	R
SO23	S	S	S	R	R	R	S	S	S	R
SO24	S	S	S	R	R	R	S	S	S	R
SO25	S	S	S	R	R	R	S	R	S	R
SO26	S	S	S	R	R	R	S	S	S	R
SO27	S	S	S	R	R	R	S	R	R	R
SO28	S	S	S	R	R	R	R	S	S	R
SO29	S	S	S	R	R	R	S	R	S	R
SO30	S	S	S	R	R	R	S	R	R	R

SO=OYSTER FROM OX-BOW, CIP=CIPROFLOXACIN, CN=GENTAMYCIN, CRO=CEFTRIAXONE, AMP=AMPICILLIN, P=PENICILLIN, SXT=SULPHONAMIDE/TRIMETHOPRIM, TE=TETRACYCLINE, S=STEPTOMYCIN, CAZ=CEFTAZIDIME, mm=MILLIMETER

Appendix 27: Antimicrobial Resistance Profile of Isolates from Nembe (n=80)

Antibiotics	No(%) of Resistant isolates from the seafood samples			
	Periwinkle (n=20)	Crab (n=20)	Oyster (n=20)	Shrimps (n=20)
Ciprofloxacin	0(0)	0(0)	0(0)	0(0)
Gentamycin	0(0)	3(15)	2(10)	3(15)
Erythromycin	5(25)	2(10)	4(20)	5(25)
Ceftriaxone	20(100)	20(100)	20(100)	20(100)
Ampicillin	20(100)	20(100)	20(100)	20(100)
Penicillin	20(100)	20(100)	20(100)	20(100)
Sulphonamide/Trim	5(25)	5(25)	7(35)	7(35)
Tetracycline	10(50)	11(55)	12(60)	11(55)
Streptomycin	12(60)	12(60)	11(55)	11(55)
Ceftazidime	20(100)	20(100)	20(100)	20(100)

Appendix 28: Antimicrobial Resistance Profile of Isolates from Ox-bow (n=120)

Antibiotics	No(%) of Resistant isolates from the seafood samples			
	Periwinkle (n=30)	Crab (n=30)	Oyster (n=30)	Shrimps (n=30)
Ciprofloxacin	0(0)	0(0)	0(0)	0(0)
Gentamycin	2(6.7)	0(0)	2(10)	0(0)
Erythromycin	2(6.7)	6(20)	3(10)	3(10)
Ceftriaxone	30(100)	30(100)	30(100)	30(100)
Ampicillin	30(100)	30(100)	30(100)	30(100)
Penicillin	30(100)	30(100)	30(100)	30(100)
Sulphonamide/Trim	8(26.7)	10(33.3)	7(23.3)	4(13.3)
Tetracycline	16(53.3)	16(53.3)	10(33.3)	9(30)
Streptomycin	19(63.3)	9(30)	15(50)	7(23.3)
Ceftazidime	30(100)	30(100)	30(100)	30(100)