UNIVERSITY OF CAPE COAST

ISOLATION AND CHARACTERIZATION OF ANTIBIOTIC RESISTANT BACTERIA FROM SELECTED FISH FARMS IN THE CENTRAL AND WESTERN REGIONS OF GHANA

ROSEMARY AGBEKO

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BY

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Thesis submitted to the Department of Molecular Biology and Biotechnology of the School of Biological Sciences, College of Agriculture and Natural Sciences, University of Cape Coast, in partial fulfillment of the requirements for the award of Master of Philosophy degree in Molecular Biology and Biotechnology

SEPTEMBER, 2020

DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original research and that no part of it has been presented for another degree in this University or elsewhere.

Candidate's Signature: ..... Date: .....

Name: .....

Supervisors' Declaration

We hereby declare that the preparation and presentation of this thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

Principal Supervisor's Signature: ..... Date: .....

Name: .....

Co-Supervisor's Signature: ..... Date: .....

Name: .....

ABSTRACT

The study sought to investigate antibiotic resistant bacteria from fish farms in the Central and Western regions of Ghana. Management practices and antibiotic use at the fish farms were obtained through questionnaire. Bacterial loads of catfish (*Clarias gariepinus*), tilapia (*Oreochromis niloticus*), and pond water samples recovered on MacConkey Agar and Mannitol Salt Agar were determined. Bacterial isolates were identified using biochemical assays. Antibiotic resistance profile and resistant genes of isolates were determined using disc diffusion method and Polymerase Chain Reaction technique respectively. The study revealed that none of the selected farms made use of antibiotics for prevention and treatment of diseases and no major disease outbreak had ever been recorded. Bacterial loads of pond water and fish samples exceeded the acceptable level of ≤100 *E. coli* and <10 coliforms per mL for wastewater recommended for use in fish farming and 5×10^5CFU/g for fresh fish regarded as wholesome. Total of 145 bacterial isolates consisting of 99 (68.30%) coliforms and 46 (31.70%) Gram-positive bacteria were obtained. All isolates showed resistance to at least an antibiotic except *Edwardsiella tarda*. Both coliform and Gram-positive bacteria were highly resistant to beta-lactams with corresponding high detection of *TEM* gene compared to other classes of antibiotics. This study has demonstrated that antibiotic resistant bacteria are present at the fish farms and might have been introduced into the fish farms from the environment where antibiotics are regularly used. Education on risks associated with the use of antibiotics and its impact on bacteria in the environment needs to be intensified.

KEY WORDS

Antibiotic resistance genes

Coliforms

Catfish

Fish farming

Gram-positive bacteria

Tilapia

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DEDICATION

To my dear husband, Lawrence Kusi and my beloved mother Emelia Adomako.

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LIST OF ABBREVIATIONS

AMP	Ampicillin
AOUN.FD	Agriculture Organization of the United Nations. Fisheries Department
bp	Base pair
bla	beta –lactamase
CHL	Chloramphenicol
CIA	Critically important antimicrobials
CLSI	Clinical Laboratory Standard Institute
COT	Cotrimoxazole
CSIR-IACUC	Council for Scientific and Industrial Research-Institutional Animal Care and Use Committee
CRX	Cefuroxime
CTR	Ceftriaxone
CTX	Cefotaxime
CTX-M	cefotaximase variant
CYP450	Cytochrome P450 enzymes
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ERY	Erythromycin
FAO	Food and Agriculture Organization
FLU	Flucoxacillin
GEN	Gentamicin
LB	Luria Bertani
MSA	Mannitol Salt Agar

NaCl	Sodium chloride
NAP	National Action plan
PBPs	Penicillin binding proteins
PCR	Polymerase Chain Reaction
PEN	Penicillin
RNA	Ribonucleic acid
rRNA	ribosomal RNA
TE	Tris-EDTA
TEM	Temienora
tRNA	Transfer RNA
TET	Tetracycline
TSI	Triple Sugar Iron
WHO	World Health Organization
WRIARDEC	Water Research Institute and Development Centre

CHAPTER ONE

INTRODUCTION

Background to the Study

As an important subsector of aquaculture, fish farming has recently grown rapidly across the world (Golub & Varma, 2014; Okocha, Olatoye & Adedeji, 2018) . Half of the total fish produce used as food worldwide is obtained from fish farming and exports from developing countries have been reported to increase for the past two decades (Assefa & Abunna, 2018). The mass production of cultured fish was intensified with support from government of many countries to supplement the deficit of fish harvest obtained from capture fisheries (Risius, Janssen & Hamm, 2017).

Recently, harvest of fishes as a source of protein from naturally occurring marine and freshwater environments have not been able to meet the increasing demand. There is an increasing pressure on harvest of fish from these wild sources, which has translated into depletion of the breeding population. Almost 50% of ocean fisheries known are absolutely overfished (MacLennan, 1995). The situation in Ghana is not different and this has compelled the government to introduce closed season within the country's marine enclave as a way of managing overfishing.

Fish has maintained an important animal protein source in Ghana over the years with 75% of yearly production consumed locally (Onumah *et al*., 2020). The preference of fish is due to the source of high-quality protein. It is also rich in micronutrients such as vitamins A, B and D, calcium, iron, iodine and omega-3 (FAO, 2016) that reduce the risk of non-communicable disease

such as cardiovascular diseases (Tilman & Clark, 2014), Alzheimer's disease and as well improve vision (Connor & Connor, 2007).

In Ghana, fish farming started about 60 years ago, and it is still at the developmental stage. The country has great potential for brackish water and freshwater suitable for fish farming usually carried out in cage system and other culture-based fisheries in ponds; dug out, concrete or tanks. This form of farming is mostly carried out in the central and southern belts. Two main fish types are produced on large scale in the country, Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarias gariepinus*) (Takyi, Nunoo, Ziddah, & Oddoye, 2012).

In as much as the interest in this sector of farming is gradually increasing in the country, outbreak of infectious diseases is a significant challenge to fish farming management practice leading to year round economic losses (Pridgeon & Klesius, 2012: Takyi *et al*., 2012). The occurrence of infectious diseases comes about due to low resistance of fish stock to stress, presence of pathogens, and unfavorable water environment (Folke & Kautsky, 1992). When the pathogenic microbes number increases due to external factors and the fish immune system is unable to withstand them the fish becomes susceptible and disease results (Naylor & Burke, 2005). In Ghana, most disease outbreaks at fish farms have been attributed to be of bacterial origin with recorded recurrent annual losses. For instance, bacteriological finding on tilapia sampled from some selected farms on the Volta Lake revealed the presence of *Streptococcus agalatiae, Aeromonas* sp. *Staphylococcus* sp. *Citrobacter freundii*, *Vibrio* sp. *and F. columnare* (Takyi *et al*., 2012). In the year 2018, Ghana reported high mortality with more than

50% loss of production of tilapia in intensive cage culture systems across Lake Volta. Polymerase Chain Reaction sampling and gene sequencing at two farms in separate areas of the lake recorded a significant role of tilapia Lake Virus with coinfection by *Streptococcus agalactiae* in the mortalities experienced at the sites (Verner-Jeffreys *et al*., 2018).

Antibiotics have been one key option used to salvage the spread and treatment of infectious diseases in cultured fish farms. Antibiotics mostly used in fish farms include sulphamethoxazole, chloramphenicol, tetracycline, flucloxacillin, gentamicin, ciprofloxacin and ampicillin which are usually added to their feed or by direct application into pond water. Other benefits of the use of antibiotics are to disinfect fish eggs, improve water quality and to promote growth (Agoba, Adu, Agyare & Boamah, 2017; Ringø, Olsen, Jensen, Romero, & Lauzon, 2014). However, estimation of the right quantity of antibiotics applied in fish farming is difficult to assess due to unavailable consumption data. The continuous and indiscriminate use of these antibiotics has resulted in requiring higher dose for effective control that has put selective pressure on resistant bacteria (Agoba *et al*., 2017).

Statement of the Problem

Antibiotic use in aquaculture has made a significant contribution to the global increase in antibiotic resistance (Donkor *et al*., 2018; Okocha *et al*., 2018). The emergence and spread of antimicrobial resistant bacteria continue to increase despite substantial interventions (Abadi, Rizvanov, Haertlé, & Blatt, 2019). Water environments of cultured fish are well known reservoirs and routes of transmission of antibiotic resistance (Karkman, Do, Walsh, & Virta, 2018; Suzuki, Pruden, Virta, & Zhang, 2017). Lists of major antibiotic

resistant coliforms and Gram-positive bacterial pathogens such as *Staphylococcus aureus* have been reported by the World Health Organization (WHO) to be threat to human health (Shrivastava, Shrivastava, & Ramasamy, 2018). These resistant bacterial pathogens can be transmitted to humans through consumption of contaminated food (Smith, 2008) or through transfer of their resistant genes into fish bacterial pathogens or human pathogens (Watts, Schreier, Lanska, & Hale, 2017). These resistant bacteria cause infections that are associated with long hospital stay, rise in death rates and high hospital costs (Dadgostar, 2019).

In Ghana, studies on the presence of antibiotic resistant bacterial pathogens in some fish farms in the Ashanti and Greater Accra Regions have been reported (Agoba *et al*., 2017; Apenteng, *et al*., 2017). The increasing rate of antibiotic resistance in Ghanaian fish farms is worrisome (Agoba *et al*., 2017) and there is the need to know the current status of antibiotics use in fish farms in other regions of the country. Aside these, there is the need to also investigate the resistance pattern, presence of resistance genes of these pathogens with the use of scientific techniques such as polymerase chain reaction (PCR). The study aims to determine the presence of antibiotic resistant bacteria in certain fish farming areas and to study certain management practices of fish farmers that contribute to spread of antibiotic resistance.

Research Questions

1. Are antibiotics currently used for prevention and treatment of diseases at fish farms in Western and Central Regions of Ghana?
2. Are good sanitary practices carried out at the fish farms?

3. Are bacterial isolates from the fish farms resistant to antibiotics?
4. Which genes are involved in antibiotic resistance in fish farm bacteria?

Main Objective:

The main objective of this research is to isolate and characterize antibiotic resistant bacteria from selected fish farms in Western and Central Regions of Ghana.

Specific Objectives:

The specific objectives were to:

1. document farming practices including antibiotic usage at the selected fish farms in the Central and Western regions of Ghana,
2. isolate and determine the bacterial loads of fish and water samples,
3. identify the bacterial isolates obtained from fish and water samples,
4. evaluate antibiotic susceptibility pattern of the bacterial isolates, and
5. determine specific antibiotic resistant genes of the bacterial isolates.

Significance of the Study

The usefulness of antibiotics in the prevention and treatment of infectious diseases in food-producing animals is being hindered by the global spread of antibiotic resistance. This presents threat to health following the use of antibiotics whose class and structure are similar to those used in treating infections in humans and thus promotes cross resistance and co-resistance (Marshall & Levy, 2011). Some last-resort antibiotics used in treating human diseases are extensively used in animals without any replacement. Classes of antibiotics which include penicillins, macrolides, aminoglycosides, quinolones, tetracyclines, and sulfonamides reported to be critically important antimicrobials (CIA) used in human medicine are also commonly used in

aquaculture (WHO, 2017). Of the fifty-one antibiotic types reported to be used by some major aquaculture producing countries, 39 of them are on the WHO list. Thirty-seven (37) out of the total list are considered as either highly or critically important in human medicine (Done, Venkatesan, & Halden, 2015).

For instance, polymyxins, a last resort antibiotic used in treating infections caused by multi-drug resistant *Klebsiella* in humans, is a good indicator of the size and scope of the problem. Recently, nosocomial infection in humans with carbapenemase-producing enterobacteria (*Klebsiella* sp and *E. coli*.) as well as multidrug-resistant *Acinetobacter* species and *Pseudomonas*, have prompted the re-introduction of systemic colistin, as a last resort treatment drug (Santos & Ramos, 2018).

In order to reinforce the need for appropriate use of antibiotics in fish farms in Ghana this requires an investigation on the present state of the use of antibiotics. The information that will be gathered could help to develop measures to reduce their use as these antibiotics are also used for treating diseases in humans. It will also serve as a knowledge resource to ensure proper precautions in preventing infectious diseases of bacterial origin by instituting appropriate monitoring measures at the farms. It will also inform policymakers to focus on improving routines of management and regulate the use of antibiotics by instituting strict guidelines and monitoring systems.

CHAPTER TWO

LITERATURE REVIEW

Fish Farming in Ghana

Fish farming has been in existence for more than six decades in Ghana. The then Department of Fisheries was set up to assist implementation and development of culture-based fishery programme initiated by the colonial administration to boost livelihood of communities through generation of income with the intent of reducing poverty and to improve nutrition (Aeschliman, 2005; Agriculture Organization of the United Nations. Fisheries Department [AOUN.FD], 2000). Subsequently, the government of Ghana adopted a policy in which some irrigation schemes in the country were developed into fish ponds for culturing purposes predominantly in the northern part of the country (Kassam, 2014).

Further, the government undertook the interest to promote fish culture nationwide. The sector yielded positive impact in the northern part of the country and was thus extended to other regions of the country. As a result, a number of fish ponds were constructed (Hiheglo, 2008). However, the boost in this sector was short-lived as some fish ponds constructed were deserted and those that were in operation were less productive (Cobbina & Eiriksdottir, 2010). Failure of this programme was attributed to lack of extension services and support from the government (Cobbina & Eiriksdottir, 2010; Doku, Chen, Alhassan, Abdullateef, & Rahman, 2018).

Despite earlier failures, aquaculture production in Ghana in recent years is growing at an increasing rate, with a rise from less than 11,000 tons in 2010 to over 57,000 tons in 2017 contributing remarkably to the economy of

the country. The growth observed in this sector is due to increased production from private commercial operators (Amenyogbe *et al*., 2018).

Fish Farming Systems and Practices in Ghana

Generally, fish farming in the country consists of both small-scale subsistence farming and commercial farming practiced mainly on freshwater environment. The commonest form of fish farming is small-scale subsistence farming using a semi-intensive production method. Extensive culture is also practiced, but it is most commonly associated with dams and small reservoirs. Commercial fish farmers, on the other hand, practice intensive culture systems in cages of floating pens, mostly on rivers and lakes, despite being a minority, they account for 75% of aquaculture output (Amenyogbe *et al*., 2018).

Currently, pond culture system is widely practiced across the country especially in the southern part of the country. In this culture type tilapia (*Oreochromis niloticous*) and African catfish (*Clarias gariepinus*) are the most common (Amenyogbe *et al*., 2018). Tilapia and catfish fingerlings are often obtained from production ponds of colleague farmers, reservoirs and rivers. Also a few fingerling producers such as Aquaculture Research, Water Research Institute and Development Centre (WRIARDEC) at Akosombo now produce and supply good quality fingerlings to fish farmers. Commercial fish farmers mostly produce their own fingerlings in concrete tanks for pond stocking (Antwi-Asare & Abbey, 2011). Depending on the kind of system practiced, some farmers engage in polyculture practice, while others engage in monoculture practice.

Common Fishes Cultured in Ghana

Nile tilapia (Oreochromis niloticus)

Nile tilapia, *Oreochromis niloticus* is a tropical fish type that thrives well in freshwater and belong to the family, Cichlidae. It is one of the first fish species cultured in Ghana. They are surface feeding omnivores that feed on both phytoplankton and zooplankton. It has wide range of ecological adaptations and rapid reproductive rate that makes it suitable for commercial aquaculture production. In Ghana, tilapia accounts for 80% of aquaculture production (Duodu, 2020).

Catfish (Clarias gariepinus)

Catfish, *Clarias gariepinus* is a fish type native to Africa that dwells well in freshwaters. It has specialised breathing organ and can tolerate harsh environmental conditions. It is a scavenger that eats insects, plankton and other fishes. It grows fast with high rate of production. *C. gariepinus* has mostly been used in earthen pond culture to control over-breeding in mixed-sex tilapia. It is the second commercially cultured fish species in Ghana next to tilapia (Duodu, 2020).

Fish Feed

The success of fish farming highly depends on feed that provides enough nutrients for fish to attain marketable size within a short time (Frimpong & Adwani, 2015). The total productivity of fish farming is highly dependent on the choice of feed. This is because growth performance of fish is influenced by the development of nutritionally balanced diet. In Ghana, farmers mostly rely on natural productivity of ponds usually enhanced by pond fertilization to achieve their production. Agricultural by-products of

brans of cereals such as maize and wheat are occasionally used (Aggrey-Fynn, 2001).

Recently, commercially formulated feeds are widely used and accounts for about 60 to 80% of operational cost (Anani *et al.*, 2017). These commercial feeds are produced in the country with a few more imported to cater for the deficit of locally produced ones. The feeds are normally extruded into pellets of varying sizes. The compositions of commercially formulated feeds differ and are prepared to suit particular fish type under culture and with varying formulations for a specific growth stage. For instance, the feed for fingerlings normally comes in powdered form whiles that of broodstock and growers is in the form of pellet (Awity, 2005). Most farmers prefer commercially formulated feed due to its ability to float, high nutritional value and high palatability (Aheto, Acheampong, & Odoi, 2019). Commercially formulated feed has high content of protein ranging between 30 to 40%, followed by lipid between 4.5 to 10%. Other feed components include carbohydrate and premixes or additives consisting of vitamins, minerals and preservatives.

Bacterial Infection in Fish

Fish disease is a major challenge that hampers the sustainability of fish farming (Subasinghe & Phillips, 2002). A number of pathogens have been identified as the primary cause of disease in aquaculture. Although in Ghana, pathogens like parasites, viruses, bacteria and fungi exist in many water bodies, disease of bacterial origin is the most prevalent (Amenyogbe *et al.*, 2018). The observable yearly bacterial disease outbreaks recorded in fish culture in Ghana is partly attributed to favorable weather conditions that

support the survival of pathogenic bacteria. Other factors which include temperature, seasonal variation in pH and dissolved oxygen play important role in the multiplication of these pathogens (Leung & Bates, 2013).

Different genera of both Gram-positive and negative bacteria cause infections in cultured fish (Ampofo, 2000; Brooker, Shinn, & Bron, 2007; Meyer, 1991). Disease-causing bacteria are present in the fish's environment as well as in or on the fish itself. Fish normally become prone to bacterial infections after they have been exposed to stress such as poor nutrition, poor water quality and temperature extremes. Stress suppresses the immune system of fish and increases their susceptibility to disease. Transmission of bacteria in fish occurs through oral, skin abrasions, gill damage, and latent carriers. Frequently observed symptoms of bacterial diseased cultured fish include ulcers, hemorrhages, fin and tail rot, "mouth fungus", "saddle back lesions", exophthalmia, ascites and color changes. The fish may also feed poorly and appear inactive (Chen *et al*., 1994).

Type of Bacteria under Study

Coliforms

Coliform bacteria are Gram-negative, non-spore-forming facultative anaerobes that ferment lactose. Coliform bacteria are indicator organisms whose presence in foods and water indicates possible presence of enteric pathogens signifying poor sanitary conditions (Robinson, 2014). Coliform bacteria belong to the large family of enterobactericeae which can adapt in the aquatic environment, in soil and on vegetation. They are common pathogens that can be found in the tissues of apparently healthy fish (Thillai Sekar *et al*., 2008; Zheng *et al*., 2004). They act as either primary pathogens or cause

opportunistic infections, especially when fish are stressed. Some coliforms such as *Escherichia coli, Enterobacter spp., Shigella spp., and Klebsiella pneumonia* have been frequently isolated from cultured fish which are linked to most species related to human infections (Agoba, 2016). Even though, the survival of enteric organisms in fish ponds that are well-managed is minimal, large numbers of these pathogens capable of causing disease can be present in harvested fish products (World Health Organization [WHO] & Joint FAO/NACA/WHO [JFNW], 1999).

***Staphylococcus* species**

Staphylococci are Gram-positive bacteria of the Staphylococcaceae family belonging to the order Bacillales. The genus *Staphylococcus* comprises of several species mostly found on the mucous membrane and skin of animals and humans, but also thrives well in the environment. *Staphylococcus* spp. are threat on human health due to their ability to cause zoonotic infection. Some of the species are also important food borne opportunistic bacteria in fishes. Species such as *S. warneri, S. epidermidis, S. haemolyticus, S. pasteuri, S. hominis, S. aureus, S. and xylosus* have been isolated from fish (Agoba, 2016; Huicab-Pech,, Castañeda-Chavez, & Lango-Reynoso, 2017; Lauková *et al*., 2013).

Of all the species, *S. aureus* is considered the most dangerous. It has been frequently isolated from fish and attributed to facilitate fish spoilage with associated food poisoning when contaminated fish products are consumed. The food poisoning ability is through the production of enterotoxin in foods (Agoba, 2016; Palilu & Budiarso, 2017) that leads to minor skin and soft

tissue infections to potentially fatal septicemia. Species like *S. xylosus* has also been cited to cause infections in fish (Oh *et al*., 2019; Padilla *et al*., 2001).

Isolation and Characterization of Bacteria

Microorganisms, specifically, bacteria normally live in mixed population. Identification of a bacterial strain requires isolation and characterization procedures under axenic conditions. The main purpose of bacterial identification is to equate the properties of pure cultures with those of a well-characterized taxon. Generally, bacterial identification can be based on phenotypic and genotypic techniques. Phenotypic techniques provide information that depicts specific metabolic activities enabling survival, growth, and development of the bacterium of interest (Emerson, Agulto, Liu & Liu, 2008). Genotypic techniques, on the contrary, require profiling an organism's genetic material, Deoxyribonucleic acid (DNA) independent of the physiological state of the bacterium and are not influenced by the composition of the growth medium or by the organism's phase of growth. Sequence-based method under genotypic techniques requiring analysis of 16S rRNA gene has proved effective in establishing broader phylogenetic relationships among bacteria at the genus, family, order, and phylum levels (Vandamme *et al*. 1996).

The technique of isolating microbes in the laboratory was first developed in the 19th century which referred to the separation of a strain from a natural, mixed population of living microbes, as present in the environment, for example, in water or soil flora, or from living beings with skin flora, oral flora or gut flora, in order to identify the microbe of interest. In addition to using sterile technique for isolation, differential and selective media are

mostly used to assist growth of a specific type of bacteria. A selective medium allows growth of specific microbes while inhibiting the growth of others by virtue of some distinguishing nutritional or environmental factors. Differential medium highlights bacterial property to allow visual differentiation of one organism from another.

Following the isolation procedure is characterization of pure cultures that aids in obtaining a complete collection of data describing the properties of a specific strain using different biochemical assays (Trüper & Krämer, 1981).

Another conventional technique of identifying bacteria is the use of Gram stain reaction. This type of reaction provides firsthand information on the type of bacteria based on the cell wall structure. Based on differences in the thickness of a peptidoglycan layer in the cell membrane, bacteria could be classified as Gram positive or Gram negative through a series of staining and decolorization procedures (Smith & Hussey, 2005).

The use of Antibiotics in Fish Farming

The discovery of antibiotics has played an important role with great impact in modern medicine (Högberg, Heddini, & Cars, 2010). Antibiotics are substances produced by microorganisms or synthesized artificially that has the ability to kill or inhibit growth of other microbes. Antibiotic use in animal medicine have been documented since 1940s (Lozano, Díaz, Muñoz, & Riquelme, 2018). Approximately 80% of animals that produce food have received antibiotic treatments and its application in fish farming is no exception. Factors which include genetic, immunological, poor sanitary practices, physical and chemical properties of the water environment account for the necessary use of antibiotics in fish farming (Darwish *et al.*, 2013).

Antibiotics are often used for prophylactic and therapeutic purposes in fish farming as feed additives (Li, Shi, Gao, Liu, & Cai, 2012; Rahman, Akanda, Rahman, & Chowdhury, 2009). Apart from its application in fish feed, application by bath and parenteral administration have been reported (Inglis, 2000; Yanong, 2003; Rodgers, 2009). Other purposes of antibiotic use in fish farming include promotion of fish growth and disinfection of fish eggs (Aly, & Albutti, 2014). Different classes of antibiotics, tetracyclines, β-lactams, aminoglycosides, macrolides and sulphonamides are employed in fish farming (Agoba, 2016).

Countries like Norway and United States have approved the use of oxytetracycline, florfenicol, and Sulfadimethoxine/ormetoprim but administered on prescription under strict guidelines to prevent development and spread of antibiotic resistance (Romero *et al*., 2012). Some African countries like Egypt and Kenya have also reported the use of tetracyclines, sulphonamides and aminoglycosides for veterinary purposes (Mitema, Kikuvi, Wegener, & Stohr, 2001). A study in Ghana has indicated residues of chloramphenicol, β-lactams, sulphonamides, macrolides, aminoglycosides, tetracyclines, and quinolones in fish product at some markets in the nation's capital (Donkor *et al*., 2018). Also, antibiotics use in fish farming has been confirmed in some districts in Ashanti region (Agoba, 2016).

Mode of Action of Antibiotics to Fish Bacterial Pathogens

Each class of antibiotics have different chemical structure and hence elicit their function differently (Figure 1). Generally, antibiotics could be bactericidal or bacteriostatic. Antibiotics that are bactericidal are able to kill bacteria whiles antibiotics that exhibit bacteriostatic effect prevent bacteria

from growing or multiplying other than killing it. Five different mode of action of antibiotics have been elucidated. These include cell membrane modification, inhibition of cell wall synthesis, protein synthesis, nucleic acid synthesis, and antimetabolite activity.

Inhibition of cell wall synthesis

Bacterial cell wall is composed of a rigid layer of peptidoglycan which protects the cell against osmotic pressure. Antibiotics that target cell wall synthesis block formation of the peptidoglycan units by inhibiting penicillin binding proteins (PBPs) activity or transpeptidase which are required for completion of cell wall synthesis (Browne *et al*., 2020). Examples of such antibiotics include penicillins, carbapenems, cephalosporins and glycopeptide.

Inhibition of nucleic acids

The metabolic pathways for nucleic acids synthesis are very essential for the survival of bacteria. Antibiotics disrupt nuclei acid synthesis by blocking DNA replication or halting protein synthesis. Quinolones group of antibiotics normally function to inhibit the activity of helicase enzyme. This action of quinolones eventually cut short DNA replication and repair in bacteria. Alternatively, antibiotics that target synthesis of DNA also inhibit the activities of topoisomerase II and topoisomerase IV which adversely affects RNA polymerase and subsequently prevents RNA synthesis (Chen, Malik, Snyder, & Drlica, 1996).

Inhibition of protein synthesis

Proteins play significant role in metabolic processes of living organisms, therefore, interference in synthesis of protein in a bacterial cell would eventually inhibit its growth or kill it completely. Antibiotics that

prevent synthesis of protein can be categorised into two subclasses: the 50S inhibitors and 30S inhibitors. Examples of 50S ribosome inhibitors include chloramphenicol, clindamycin, erythromycin, lincomycin, and linezolid (Browne *et al*., 2020). Generally, 50S ribosome inhibitors function by preventing initiation phase of protein translation or the elongation phase of protein synthesis. Oxazolidinones are examples of antibiotic that block protein translation initiation whiles macrolides (eg. streptogramin and lincosamide) interfere with synthesis of protein by preventing the elongation phase of mRNA translation (Browne *et al*., 2020). On the other hand, inhibitors of 30S ribosome mainly function by preventing aminoacyl-tRNAs from accessing the ribosome. Tetracycline, spectinomycin and streptomycin are examples of antibiotics that function in this manner (Li *et al*., 2012).

Alteration of cell membrane

Antibiotics that target bacterial cell membrane bind to the lipid moiety of the lipopolysaccharide (Falagas, Kastoris, Kapaskelis, & Karageorgopoulos, 2010). The bacterial cell losses its membrane selective permeability resulting in loss of cytoplasmic content. The cellular ion gradient becomes distorted, causing cellular damage and, eventually, death in the bacteria. Examples include polymyxin B, Valinomycin and Daphtomycin. Polymycin B possesses hydrophilic and lipophilic groups that interfere with phosphatidylethanolamine of the membrane whiles valinomycin interferes with membrane potential of cells which contributes to oxidative phosphorylation by forming pores in the cell membrane. Daphtomycin depolarizes membrane potential, causing potassium ions to leak from the cytoplasm into the extracellular matrix.

Antimetabolite activity

Antibiotics such as sulphonamides and trimethoprim function by mimicking a substrate required for cellular metabolism in bacteria. Bacterial enzymes have high affinity to these structural analogs compared to the normal substrate. Sulphonamides, in particular, mimic tetrahydrofolate which is needed for the production of folic acid. Folic acid is required for amino acids and nucleic acid metabolism. Sulphonamides ultimately interfere amino acids and nucleic acids production (Browne *et al*., 2020; Etebu & Arikekpar, 2016).

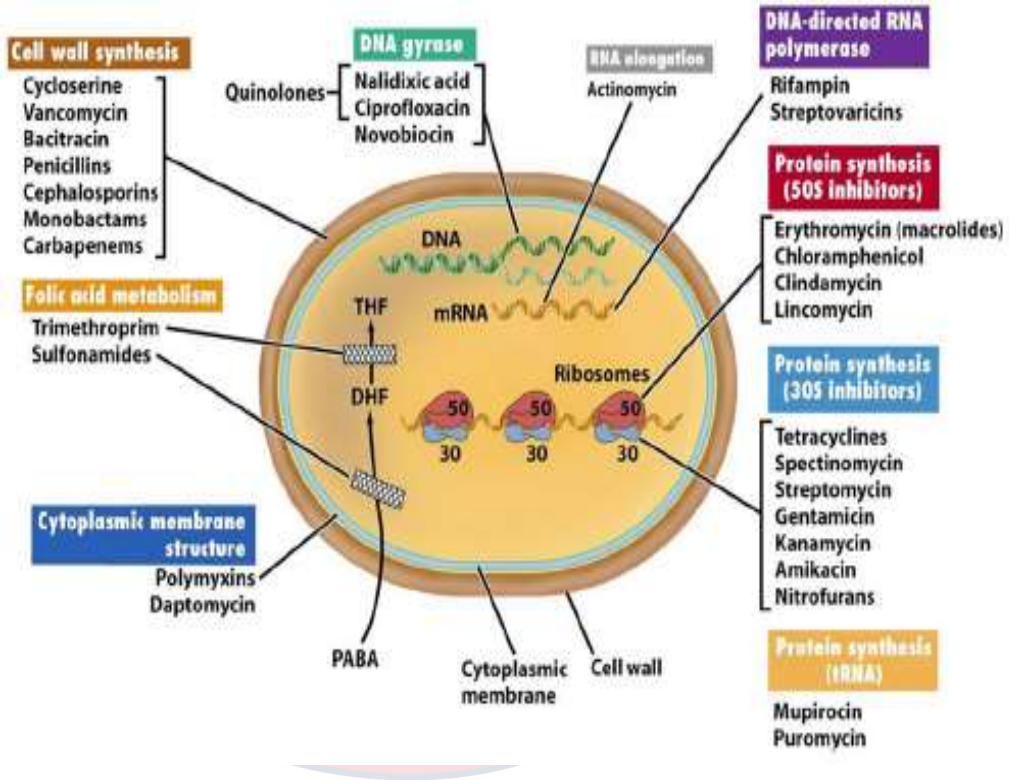

Figure 1: Antibiotic target sites (Madigan, Martinko, Dunlap, & Clark, 2006)

Metabolism of Antibiotics in Fish

Assessment of the effect of antibiotics on cultured fish depends on the kind of antibiotic and the concentration administered to a particular fish species type. Metabolism of antibiotics is similar to that of mammals. Fish use cytochrome P450 enzymes (CYP450) to metabolize drugs. The activity of the

enzyme is a critical determining factor of the detoxification abilities of organisms (Burkina, Zlabek & Zamaratskaia, 2015). Fish carry out most of the Phase I reactions; oxidation, reduction and hydrolysis similar to that of mammals. Phase II reactions which involve conjugate formation and subsequent excretion are also carried out by fish for a variety of compounds. The detoxification of all drugs and chemicals in fish is carried out by the liver. Temperature optimum for many of these reactions in fish is close to the temperature of the natural environment of the fish. As a result, the rate of metabolism is about one tenth of that of mammals.

Bacterial Resistance to Antibiotics

Recently, resistance of bacterial pathogens to antibiotics has become prevalent and of importance to public health. Antibiotic resistance refers to the ability of microorganisms to resist antimicrobial treatments that was designed to kill it (Oliveira *et al*., 2017). Antibiotics use in fish farming has been cited to be contributing factor to the spread of resistant bacteria. Antibiotics use in aquaculture may affect broad variety of bacteria leaving resistant mutants in the selective antibiotic environment.

Bacterial resistance to antibiotics may be inherent or acquired. Inherent resistance is when the antibacterial agent is unable to reach its target in the bacteria or has low affinity between the cells target and the antibacterial agent or there is absence of target site in the cell. In such cases, all strains of the bacterial species become naturally immune to all members belonging to the class of that antibacterial agent (Dixon, 2000).

Acquired resistance on the other hand arises by means of mutation of DNA of the bacteria or through external resistant-conferring DNA (Barker,

1999). Resistant genes may be located on the bacterial chromosome, plasmid or transposons and could be transferred to the progeny of the parent bacterium during replication. The resistant genome can also be transferred to bacteria of the same species or even different species through the process of conjugation, transduction and transformation (Hackman, 2015; Romero *et al*., 2012).

Mechanism of bacterial resistance to antibiotic

Bacteria can use different mechanisms to survive the effect of an antibiotic. One of these mechanisms is to render the antibiotic inactive through physical removal from the cellular membrane through efflux pumps. Membrane proteins that transport antibiotics from the cell reduces intracellular concentrations and can be specific to antibiotics but most of them are capable of pumping different types of unrelated antibiotics resulting in multidrug resistant organisms. This mechanism is well recognized for tetracycline and is encoded by a wide range of related genes distributed in enteric bacteria. Some enteric bacteria isolated from aquaculture setting have been reported to express *tetA*, *tet S* and *tet M* resistant genes to tetracycline (Hedayatianfard, Akhlaghi, & Sharifiyazdi, 2014). Another mechanism is to alter the target site so that the bacterium is not recognized by the antibiotic. An antibiotic that enters the cell is unable to inhibit the target's activity due to structural changes in the target molecule. Spontaneous mutation of a bacterial gene on the chromosome is the most common cause of target site changes (Kapoor, Saigal, & Elongavan, 2017). Another resistant mechanism is by enzymatic inactivation of the antibiotic (Hackman, 2015). The bacterium retains its sensitive target, however; the antibiotic is unable to reach the target.

CHAPTER THREE

MATERIALS AND METHOD

Area of Study

The Central and Western Regions of Ghana were considered for the study. Both regions can be found in the southern part of the country. With a total population of 2,605,492, and land area of 9,908 km^2, the Central region is composed of twenty-two districts (Ghana Statistical Service, 2020). The region is bordered to the north by the Ashanti Region, to the north-east by the Eastern Region, to the south-east by the Greater Accra Region, and to the west by the Western Region. The Gulf of Guinea borders it on the south. The Central region is known for its tourist sites which include forts, castles and beaches located along the region's coastal line.

The Western Region on the other hand covers 10% of Ghana's total land area of approximately 24,000 Km^2. It shares border with the Central Region to the east, Western-north to the north, Cote D'Ivoire to the west and to the south with Gulf of Guinea. The region currently has a population of 2,214,660 (Ghana Statistical Service, 2020).

Both regions contribute to the country's economy due to abundance of industrial minerals, tourism and fishing. The indigenes of the coastal belts rely heavily on fishing for a living (Finegold, Gordon, Mills, Curtis, & Pulis, 2010). In-land fish farming is currently promoted as well to supplement harvest from the sea to close the gap between supply and demand for fish (Amenyogbe *et al.*, 2018). Samples for this study were obtained from Mempaesem, Ansapetu, Okyereko, Assin Fosu and Assin Fosu-Dompim

townships all in the Central region and Dompim No. 1, Daboase and old Daboase in the Western region of Ghana (Figure 2).

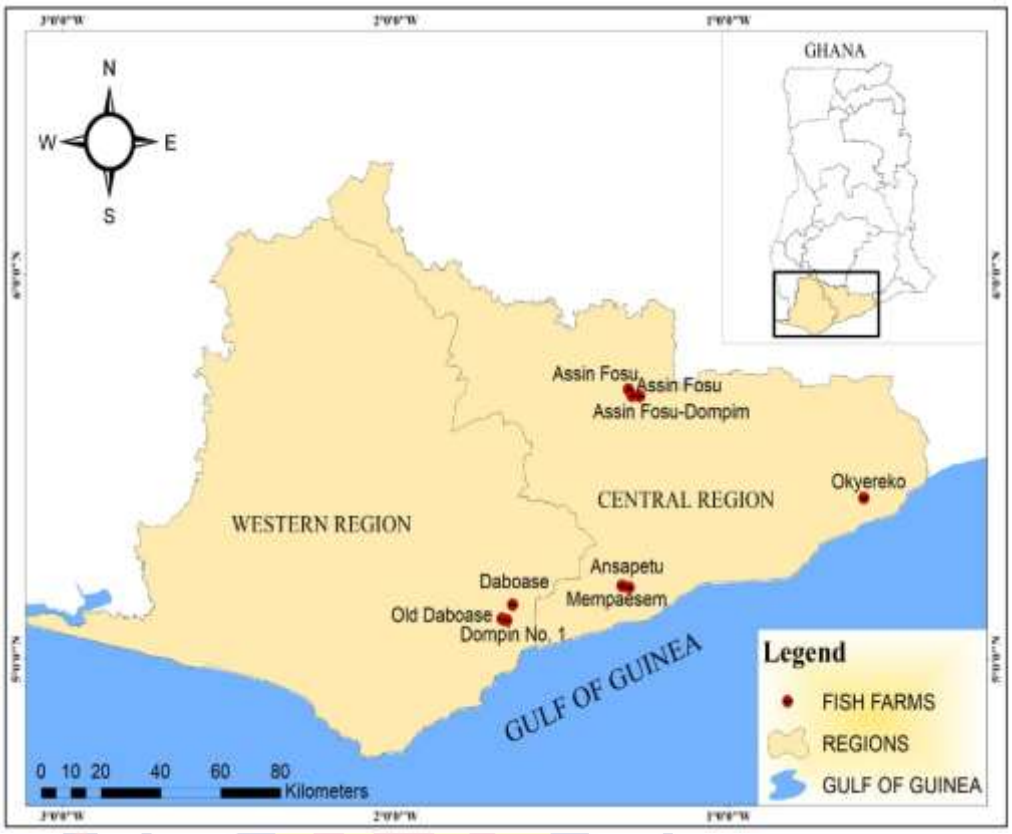

Figure 2: Geographic location of fish farms where samples were obtained for the study

Ethical Clearance

The protocol of the entire research was reviewed and approved by the Council for Scientific and Industrial Research-Institutional Animal Care and Use Committee (CSIR-IACUC) before starting the study (APPENDIX A). Consent was sought from fish farmers or farm managers before administering questionnaire (APPENDIX B) following collection of samples at each farm.

Experimental Design and Sampling Method

The research was divided into five phases which included questionnaire administration, sample collection and processing, bacteria isolation and identification, antibiotic susceptibility testing of bacterial isolates

and DNA extraction and screening for antibiotic resistant genes in bacterial isolates.

The Questionnaire was developed to gain insights into fish farming practices at the farms. The structured questionnaire addressed issues in relation to the use of antibiotics at the farms, types of antibiotics used, sources of antibiotics and methods of administering antibiotics, history of disease outbreaks, and how pond waste was disposed. Information on type of feed used and other uses of antibiotics at the farms were also solicited.

Purposive and snowball sampling methods were adopted to obtain samples from the various fish farms visited. Farmers that engaged in aquaculture were specifically recruited in this study. Some other farms were identified and included in the study based on information from fish farmers. Farms that did not consent to the research were excluded. All fish farms visited were designated with alphabet letters. The location and photographic records of some farm sites visited are shown on Table 1 and Figure 3 respectively.

Table 1: Location of fish farms and type of samples collected

Region	Farm	Location	Sample collected		
			Water	Tilapia	Catfish
Central Region	A	Mempaesem	✓	×	✓
	B	Ansapetu	✓	×	✓
	C	Okyereko	✓	✓	×
	D	Assin Fosu	✓	✓	×
	E	Assin Fosu	✓	✓	✓
	F	Assin Fosu-Dompim	✓	×	✓
Western Region	G	Dompim No. 1	✓	×	✓
	H	Daboase	✓	×	✓
	I	Old-Daboase	✓	✓	✓

✓ = sample collected; × = sample not collected, type of fish was not cultured on those farms

x1/20

x1/20

Figure 3: Study sites in the Western Region of Ghana
A: Farm G at Dompim No.1with concrete pond

B: Farm H at Daboase with earthen pond

Sterilization Procedure

Petri dishes, conical flasks, test-tubes and other glassware were washed with soap, rinsed with running tap water, and finally with distilled water to completely remove all contaminants. They were air dried, wrapped in brown paper and autoclaved for 15 minutes at121 °C. Polyethylene bags for collection of fish sample were wrapped with aluminium foil, pipette tips and containers were all autoclaved for 15 minutes at 121 °C and then oven-dried at 60 °C to evaporate water condensing on the various items. Forceps, inoculation loops and needles were heated in the spirit lamp flame until they turned red-hot and air-cooled before and after use. The working bench surface of the laminar flow cabinet was cleaned and disinfected with 70% ethanol before and after use.

Preparation of culture media

MacConkey Agar (without salt)

MacConkey agar powder (Lab M Ltd. Topley House, UK) weighing 48.5g was dissolved in one litre of distilled water. The resultant mixture was swirled and heated using an electronic hot plate to evenly dissolve agar in the medium. The warm mixture was evenly distributed into 4 conical flasks which were corked with cotton wool plug and autoclaved for 15 minutes at 121 ℃.

Mannitol Salt Agar

Mannitol Salt Agar powder (Oxoid Ltd. England) weighing 111 g was dissolved in a litre of distilled water. The mixture was swirled and heated on an electronic hot plate to evenly dissolve agar in the medium. The mixture was evenly distributed into 4 conical flasks which were corked with a cotton wool plugs and autoclaved for 15 minutes at 121℃.

Nutrient Agar

Nutrient agar powder (Oxoid Ltd., England) weighing 28 g was dissolved in a litre of distilled water. The mixture was swirled and heated on an electronic hot plate. Approximately 1 mL of the warm mixture was dispensed into 1.5 mL-Eppendorf tubes which were autoclaved for 15 minutes at 121℃.

Muller Hinton Agar

Muller Hinton Agar powder (Lab M Ltd. Topley House UK) weighing 36g was dissolved in a litre of distilled water. The mixture was swirled and heated using an electronic hot plate to evenly dissolve agar in the medium. The warm mixture was distributed in 4 conical flasks, corked with cotton wool plugs and autoclaved at 121 ℃ for 15 minutes.

Simmon's Citrate Agar

Simmon's Citrate Agar powder (Lab M Ltd. Topley House UK) weighing 24.28 g was dissolved in a litre of distilled water. The mixture was swirled and heated using an electronic hot plate. Approximately 5 mL of the warm mixture was distributed into screw cap test tubes and autoclaved for 15 minutes at 121 ℃. The media were immediately put in slant position after autoclaving till it solidified to be used for analysis.

Christensen's Urea Agar

One litre of Christensen's Urea agar medium was prepared in two parts, thus, basal medium and the urea concentrate. The basal medium was prepared by dissolving 1.0 g of peptone powder, 5.0 g of sodium chloride (NaCl), 2.0 g of potassium dihydrogen phosphate, 15.0 g of agar powder, 1.0 g of glucose and 0.012 g of phenol red in a 900 mL of distilled water. The

resultant mixture was swirled, heated on an electronic hot plate and autoclaved at 121 ℃ for 15 minutes.

The urea concentrate was also prepared by dissolving 20 g of urea in 100 mL of sterile distilled water. The urea concentrate was thoroughly mixed with the basal medium and 5 mL of the medium subsequently distributed into sterile crew cap test tubes. The media were immediately put in slant position till it solidified to be used for analysis.

Triple Sugar Iron Agar (TSI)

Triple Sugar Iron Agar (TSI) powder (Liofilchem s.r.l. Bacteriology Products, Italy) weighing 64.5 g was dissolved in one litre of distilled water in a conical flask. The mixture was swirled and heated on an electric hot plate. A volume of 5 mL of the warm mixture was distributed into screw cap test tubes and autoclaved for 15 minutes at 121℃. After the sterilization process, the media were immediately put in slant position till it solidified to be used for the analysis.

Peptone water

Peptone water powder (Oxoid Ltd., England) weighing 15g was completely dissolved in one litre of distilled water. A volume of 5 mL was dispensed into test tubes with screw caps and autoclaved for 15 minutes at 121℃.

Luria Bertani broth

Luria Bertani (LB) powder (Sigma Aldrich Logistik GmbH, Germany) weighing 15 g was dissolved in one litre of distilled water. A volume of 5 mL was distributed into screw cap test tubes and autoclaved for 15 minutes at 121°C.

Nutrient Gelatin medium

Nutrient Broth powder (Oxoid Ltd., UK) weighing 8 g and 120 g of gelatin were dissolved in one litre of distilled water. The mixture was heated on hot plate and 5 mL-aliquots were dispensed into screw cap test tubes and autoclaved for 15 minutes at 121℃.

Normal saline

Approximately, 8.5 g of sodium chloride (NaCl) was dissolved in a litre mL of distilled water. Approximately, 9 mL of the solution were distributed into screw cap test tubes and autoclaved for 15 minutes at 121 ℃.

Sample Collection

At each farm, water samples were collected at three different points from the pond. At each spot; the inflow, the middle and the outflow area of the pond, 100 mL of water was collected into sterile bottles at 50 cm deep below the water surface. Each sample was well labelled and kept on ice.

Cast net was used to collect fish samples from farms that had dug-out pond with assistance of fish farmers. An average of 5 live fish samples were randomly selected from the catch at each farm. However, scoop net was used for samples obtained from concrete ponds. Fish samples obtained were placed in a well labelled sterile polyethylene bags and transported to the laboratory on ice. In cases where farm location was not far, the fish samples were brought alive in a sterile container filled with pond water to the laboratory for analysis.

Sample processing, bacterial isolation and storage of isolates

Water samples

A representative sample of pond water from each farm was prepared by making a composite from samples obtained at the three spots from the

pond. Each of the water sample sets was thoroughly mixed. A volume of 3 mL from each bottle was pipetted into a sterile test tube to obtain a 9 mL-composite water sample (Huys, 2003). The composite water sample was thoroughly mixed and 1 mL was serially diluted in 9 mL of sterile normal saline solution to the 5th tenfold-dilution factor.

Pour plate method was adopted in the process of inoculation. About 1 mL of the diluted suspension was dispensed into sterile petri dish. Molten MacConkey agar or Mannitol Salt Agar was allowed to cool to about 48 ℃, and then poured onto the inoculum in the petri dish. It was then swirled gently to attain even distribution of the inoculum into the medium. The inoculated MacConkey agar and Mannitol Salt Agar (MSA) plates were then incubated at 35 ℃ for 16 -24 h.

Fish sample

Ethanol (70%) was used to disinfect the surface of every fish sample before dissecting the gut (Banu, Islam & Chowdhury, 2001). The peritoneal cavity was aseptically dissected from the pyloric valve to the anus. About 0.5 g of the intestinal sample was transferred into test tube containing 4.5 mL sterile normal saline solution. It was vortexed and 3 mL of the intestinal samples from all three sampling spots were pooled into a sterile test tube to obtain a composite intestinal sample for every cultured fish type at each farm. The resulting 9 mL-composite intestinal samples were diluted up the 6th tenfold-dilution factor (Huys, 2003).

A volume of 1mL of the diluted suspension was pipetted into sterile petri dish. Molten MacConkey agar or MSA media which was allowed to cool to about 48 ℃ was then poured onto the inoculum in the petri dish. It was then

swirled gently for even dissemination of the inoculum into the medium. The inoculated MacConkey agar and Mannitol Salt Agar (MSA) plates were then incubated at 35 ℃ for 16-24 h (Cheesbrough, 2005; Huys, 2003).

Determination of Bacterial Load

The colony forming unit (CFU) of bacteria on culture plates were determined after the incubation period using the colony counter (Stuart Scientific, UK). The CFU of culture plates within the range of 30 to 300 were estimated and records were taken for samples from which they were obtained. The original cell density (OCD) for total bacterial load per gram (g) or per millilitre (mL) of the sample was determined using the formula: $OCD = CFU/(D \times V)$ where:

OCD = Original cell density

CFU = Colony-forming unit counted on the agar plate

D = Dilution factor of inoculum plated

V = Volume of inoculum plated

Mean total bacterial load of each sample type was calculated as the average value of the total bacterial load obtained from triplicates (Leboffe & Pierce, 2019).

Storage of bacterial isolates

About 3 to 4 colonies were randomly picked from colonies that grew on each MacConkey agar and Mannitol Salt agar plates and individually stored in Eppendorf tubes containing sterile nutrient agar. These were then incubated at 35 ℃ for 16-24 h and subsequently stored at 4 ℃ for identification and further analysis.

Biochemical Tests used in Identifying the Isolates

Each of the stored bacterial isolates was identified based on different biochemical tests. Prior to identification, isolates were sub-cultured on MacConkey and Mannitol salt agar for coliforms and presumptive *Staphylococcus species* respectively at 35 °C for 24 h. About 2-3 discrete colonies were picked with inoculation loop and suspended in a 5mL sterile normal saline and vortexed to obtain bacterial suspension.

Major tests included in this study for identification of coliform isolates grown on MacConkey Agar were Triple Sugar Iron (TSI) agar tests, citrate, indole and urea tests. Additional tests which include catalase, gelatinase, oxidase and coagulase tests were carried out on isolates that grew on Mannitol salt Agar. The principle behind each test and the procedure has been elaborated below.

Indole test

Indole test determines the ability of an organism to produce the enzyme, tryptophanase to hydrolyse tryptophan to form indole. Indole subsequently forms a complex with 4 p-dimethylamino benzaldehyde in Kovac's reagent to produce red coloured compound.

A loop full of bacterial suspension of the isolate was inoculated into 5 mL of sterile peptone water in screw cap test tube. After an incubation period of 24 h at 35 ºC, 2 drops of Kovac's reagent were added and observed immediately. A pink to red ring colouration formed on the top of the inoculated peptone water indicated a positive test. Production of light brownish ring indicated a negative indole test as shown in Figure 5 (WHO, 2003; Agoba, 2016).

Citrate test

Citrate test determines the ability of bacteria to break down citrate as its source of carbon and energy. A sterile inoculating needle was used to inoculate the bacteria isolate from the bacterial suspension onto the Simmon's citrate medium slant prepared. The caps of the tubes were tightened and tubes incubated at 35 ºC for 24 h. Formation of blue colour indicated citrate positive and no change in green colour of the medium indicated citrate negative as shown in Figure 5 (Harley, 2004).

Triple Sugar Iron (TSI) test

Triple Sugar Iron (TSI) tests for the ability of bacteria to ferment glucose, lactose and sucrose and produce hydrogen sulphide (H_2S). Using an inoculating needle, the bacterial suspension of the respective isolate was inoculated on TSI agar slant. The tubes were sealed and incubated for 24 h at 35 ºC.

The tubes were observed for colour change; a yellow colouration formed was an indication of acid production due to glucose, lactose or sucrose fermentation whereas a red colouration indicated alkaline production due to non-fermentation of the sugars; glucose, lactose or sucrose on butt and slant. The tubes were also observed for gas production indicated by cracks in both slant and butt as shown in Figure 5. Hydrogen sulphide gas (H_2S) production was observed as black precipitate at the medium (Forbes *et al*., 2007).

Urea test

The urea test determines the ability of organisms to hydrolyse urea to produce ammonia and carbon dioxide. With an aid of an inoculating needle, the bacterial suspension was inoculated by stabbing the butt and streaking on

the entire Christensen's urea agar slant surface. The cultures were observed after 24 h incubation at 35 ℃. Formation of magenta colour indicated positive test whereas no colour change of the medium indicated negative test (Figure 5).

Catalase test

Catalase test determines the ability of bacteria to breakdown hydrogen peroxide (H_2O_2) to produce oxygen and water.

A drop of 3% hydrogen peroxide solution was added to a colony of bacteria of the respective isolate on a slide. The formation of bubbles within 10 seconds indicated positive test whiles no bubble formation was observed for negative test (Figure 6). Standard *Staphylococcus aureus* strain (ATCC 662813) was used as positive control.

Coagulase test

Coagulase test is used to differentiate *Staphylococcus aureus* from other bacterial species based on their ability to produce the enzyme coagulase by converting soluble fibrinogen in plasma to insoluble fibrin.

One in ten diluted rabbit plasma was prepared in sterile physiological saline. About 2-3 colony of bacterial isolate grown on MSA was inoculated in 0.5 mL of diluted rabbit plasma, vortexed and incubated at 35 ºC. The suspension was observed every 30 minutes for the first four hours and after 24 h for coagulum formation. The formation of coagulum in the test tube indicated a coagulase positive test. Absence of clot formation indicated negative results (Figure 6). Standard *Staphylococcus aureus* strain (ATCC 662813) was used as positive control and an uninoculated test as negative control (Rakotovao-Ravahatra *et al*., 2019).

Oxidase test

Oxidase test identifies bacteria that produce cytochrome c oxidase enzyme to be utilised for aerobic respiration. Based on manufacturer's instruction, a few drops of oxidase test reagent (Becton, Dickinson-USA) containing 1% tetramethyl-p-phenylenediamine dihydrochloride were added to a strip of sterile filter paper. A 24 h old colony culture of each isolate was picked and streaked on the oxidase reagent-saturated filter paper and observe for colour change within 30 seconds. Positive reactions turned the bacteria violet or purple while negative reactions either remain colourless or turned light pink (Figure 6). Standard strain of *Pseudomonas aeruginosa* (ATCC 27853) was used as positive control.

Gelatinase test

Gelatinase test helps to differentiate organisms based on their ability to break down gelatin into smaller polypeptides, peptides and amino acids. About 3 isolated colonies of 24 h old culture of each isolate was picked with a sterile needle and inoculated by stabbing 4 to 5 times into the gelatin nutrient medium and incubated at 35 °C for 48 h. They were removed from the incubator and refrigerated for at least 30 minutes. Partial or complete liquefaction of the inoculated tube at 4°C for at least 30 minutes indicated positive test. Negative test cultures showed no liquefaction after exposure to cold temperature. An un-inoculated tube and *Pseudomonas aeruginosa* (ATCC 27853) were used as negative and positive controls respectively (Ekpenyong, Asitok, Odey, & Antai, 2016) (Figure 6).

Gram staining

Bacterial colonies on MacConkey and MSA media were examined microscopically to determine the morphology of cells. A loopful of isolate was heat-fixed on a clean glass slide and flooded gently with ammonium oxalate crystal violet solution for 1 minute. After rinsing the slide with running tap water, Gram's iodine was applied for 1 minute before being washed again. Absolute ethanol (95%) was used to decolorize the smear and then washed with water and stained with the contrast stain, safranin for 1 minute 30 seconds. Gram's iodine was applied for 1 minute after rinsing the slide with running tap water before it was washed again (Brown, 2012). The slides were allowed to dry at room temperature and visualized using light microscope (Olympus $CX_{43,}$ UK) Photographic images were obtained as shown on Figure 7.

Antimicrobial Susceptibility Testing

The disk diffusion method described by Kirby-Bauer was used to determine the antibiotic susceptibility pattern of each isolate (Clinical and Laboratory Standards Institute [CLSI], 2018). Antibiotics commonly used in managing diseases in fish farms were selected for both Gram positive and negative bacterial isolates. Bacterial suspensions of all isolates were prepared, adjusted to the 0.5 McFarland's standard and used to inoculate Mueller-Hinton Agar plates in order to create bacterial lawns. On the surface of the inoculated plates antibiotic disks were dispensed with sterile forceps and incubated for 16 to 24 hours at 35 ℃. Following the incubation period, growth inhibition zones around each disc were measured using a meter rule. Results were interpreted

as susceptible, intermediate or resistant based on CLSI, guidelines (CLSI, 2018). Details of the antibiotics set used are shown in Table 2.

Table 2: Antibiotic disc for sensitivity testing of Gram positive and Gram negative isolates

Antibiotic (Abtek Biologicals Limited)	Dose	Gram Positive	Gram Negative
Tetracycline (TET)	10 µg	✓	✓
Erythromycin (ERY)	5 µg	✓	×
Ceftriaxone (CTR)	30 µg	×	✓
Flucoxacillin (FLX)	5 µg	✓	×
Ampicillin (AMP)	10 µg	✓	✓
Cefotaxime (CTX)	30 µg	×	✓
Penicillin (PEN)	1.5 i.u	✓	×
Cefuroxime (CRX)	30 µg	✓	✓
Cotrimoxazole (COT)	25 µg	✓	✓
Gentamicin (GEN)	10 µg	✓	✓
Chloramphenicol (CHL)	10 µg	×	✓

✓ = antibiotic used; × = antibiotic not used

Extraction of DNA

DNA extraction from each bacterial isolate was conducted using a *Maglisto*™ 5M DNA Extraction kit (Bioneer Corporation, USA) following the instructions of the manufacturer. Each kit comprised of a Proteinase K, Rnase A, NanoBead solution and washing buffers. All bacterial isolates were sub-cultured on the respective media used in isolation. About 2 to 3 discrete colonies were picked and grown in 5 mL Luria-Bertani broth. A volume of 2

mL overnight culture of bacterial cells (approximately 1 x 10^6 cfu/mL) was dispensed into sterile 3mL-Eppendorf tubes and centrifuged at 5,000 rpm for 10 minutes. The supernatant was then decanted and the cell pellets re-suspended in 200 µL phosphate buffered saline (1X PBS). To lyse the cells and degrade RNA, 20 µL of proteinase K and 10 µL of Rnase were added and incubated for 2 minutes at room temperature. Subsequently, 200 µL of protein precipitation solution was added to the RNase-treated cell lysate and the contents thoroughly mixed by vortexing. Absolute ethanol, 400 µL was added to the mixture and vortex mixed thoroughly followed by addition of 100 µL Magnetic Nanobead Solution. The mixture was vortexed and the tube placed in *MagListo*™-2 Magnetic separation rack. It was gently inverted 3 to 4 times with the magnet plate attached to allow DNA to bind to the NanoBeads. The supernatant was removed without removing the tube from the rack. The magnetic plate was detached from the *MagListo*™ stand and 700 µL of WM1 buffer added and vortex mixed thoroughly. The tube was placed back in the *MagListo*™ stand with the magnetic plate attached and inverted 3 to 4 times gently for the beads to bind to the magnet. The supernatant was removed without removing the tube from the rack. A volume of 700 µL of W2 buffer was added, vortex mixed and supernatant removed with the magnetic plate still attached to the rack. Without removing the tube from the rack, 700 µL of WE buffer was added to the opposite side of the bead and the tubes gently inverted twice. The supernatant was discarded completely by blotting. Exactly 100 µL of elution (EA) buffer was added, vortex mixed and incubated at 60 °C for 1 minute. The magnetic plate was attached to the rack and the supernatant

containing the DNA was carefully transferred into a sterile Eppendorf tube. The DNA samples were kept at -20 ℃ to be used for further analysis.

Determination of the Integrity, Purity and Concentration of DNA Samples

The integrity of each DNA sample was tested by loading 5 µL of the DNA on 1% agarose gel. The loaded gel was then electrophoresed in 1X TBE buffer for 45 minutes at 90 V. An Ultraviolet Transilluminator (UVP Products, United Kingdom) was used to visualise the gel and photographs were taken with a digital camera. The purity and concentration of the DNA samples were determined and recorded using T70 UV/VIS spectrometer (PG Instrument Ltd., U.K). The absorbance readings for each DNA sample were taken at 260 nm (A_{260}) and at 280 nm (A_{280}) and the ratio A_{260}/ A_{280} calculated to determine the purity. A ratio within the range of 1.8 to 2.0 indicated that the UV absorption was due to nucleic acids, implying that the DNA sample was pure. Proteins and/or other UV absorbers (contaminants) were detected if the ratio was less than 1.8.

The concentrations of the DNA samples were also determined based on the formula:

DNA concentration (µg/mL) = $A_{260} \times D \times 50 \frac{\mu g}{mL} / 1000$

Where, A_{260} represents the measured absorbance at 260 nm and D is the dilution factor.

The concentrations obtained aided in standardising DNA samples before carrying out PCR assays. Highly concentrated DNA samples were diluted with sterile Tris-EDTA (TE) buffer. The DNA extraction procedure was repeated for samples that had low concentrations.

Confirmation of DNA Samples as Bacterial DNA

DNA samples were confirmed to be from bacterial source using the 16S rRNA primers 27F (5'-GAGTTTGATCCTGGCTCAG-3') and 1492R ('5-GGTTACCTTGTTACGACTT-3') (Bioneer Cooperation, South Korea) based on the presence of a product size of about 1500 bp after the amplification process (Lane, 1991).

Determination of the Presence of Resistant Genes

Primer sequences were selected based on different classes of antibiotics used in aquaculture. Multiplex PCR assay procedure was employed in the amplification process. The primers were grouped into five categories for PCR amplification process with each set consisting of three primer sets with the exception of group one which consisted of two primer sets. The primer grouping was based on the differences of their product size and annealing temperature (Table 3**)**. Approximately, 1 µL of forward and reverse (1 pmol each) of each primer were added to the Accupower Multiplex PCR premix (Bioneer Corporation, South Korea) and the reaction volume made up to 20 µL by adding 5 µL of DNA template and 9 µL of sterile molecular biology grade water. The amplification procedure was carried out using the 96-well plate of T100^{TM} thermal cycler (Biorad Laboratories, USA). The amplification programme constituted an initial denaturation for 5 minutes at 95 °C; 35 cycles at 95 °C for 30 seconds, annealing at 55 °C, extension at 72 °C for 1 minute; and a final extension at 72 °C for 5 min. In each well of 2% (w/v) ethidium bromide-stained agarose gel, 5 µL of each PCR product was loaded and a 100 bp DNA ladder (Bioneer Corporation. Korea) was used as a molecular weight marker. The PCR products loaded in the agarose gel were

subjected to electrophoresis at 90 V for 60 minutes. Each gel was examined with an ultraviolet transilluminator (UVP products, UK), photograph were taken using a digital camera. Records were taken for genes amplified for each isolate.

Statistical Analysis

Data recorded were entered into Microsoft Excel and then transferred to SPSS software version 22. Data obtained from questionnaire, antibiotic resistance profile and amplified DNA fragments were interpreted using descriptive statistics. Analysis of variance (ANOVA) was used to compare the mean of bacterial loads of pond water, catfish and tilapia. Independent t-test was used to compare the means of bacterial loads of earthen and concrete pond. Confidence interval was set at 95% and probability value, $p \leq 0.05$ was considered statistically significant.

Table 3: Primer groupings used in PCR analysis

Group of primer sets	Primer	Primer sequence (5' – 3')	Amplicon size (bp)	References
1	cmIA	**F** GGCCTCGCTCTTACGTCATC **R** GCGACACCAATACCCACTAGC	662	Ma *et al*., 2007
	Cat 1	**F** AACCAGACCGTTCAGCTGGAT **R** CCTGCCACTCATCGCAGTAC	549	Zhao *et al*., 2001
2	TEM	**F** GAGTATTCAACATTTCCGTGTCGC **R** TACCAATGCTTAATCAGTGAGGC	865	Zhang *et al*., 2011
	qnr S	**F** ACGACATTCGTCAACTGCAA **R** TAAATTGGCACCCTGTAGGC	417	Robicsek, 2006
	gyrA	**F** CGACCTTGCGAGAGAAAT **R** GTTCCATCAGCCCTTCAA	626	Hossain, 2017
3	^{bla}EBC	**F** TCGGTAAAGCCGATGTTGCGG **R** CTTCCACTGCGGCTGCCAGTT	302	Perez-Perez, 2002
	Sul 3	**F** CAGATAAGGCAATTGAGCATGCTCTGC **R** GATTTCCGTGACACTGCAATCATT	569	Arabi *et al*., 2015
	Sul 1	**F** CGGCGTGGGCTACCTGAACG **R** GCCGATCGCGTGAAGTTCCG	432	Arabi *et al*., 2015

Table 3: Cont'D

4	*bla*TEM-1	**F** CCAATGCTTAATCAGTGAGG **R** ATGAGTATTCAACATTTCCG	858	Domínguez-Pérez *et al*, 2018
	qnrB	**F** GATCGTGAAAGCCAGAAAGG **R** ACGATGCCTGGTAGTTGTCC	469	Wang *et al*., 2008
	tet B	**F** CAGTGCTGTTGTTGTCATTAA **R** GCTTGGAATACTGAGTGTTAA	571	Ma *et al*., 2007
5	mecA	**F** AAAATCGATGGTAAAGGTTGGC **R** AGTTCTGCAGTACCGGATTTG	533	Azimian, 2012
	tet A	**F** TTGGCATTCTGCATTCACTC **R** GTATAGCTTGCCGGAAGTCG	494	Ma *et al*., 2007
	CTX-M	**F** ACGCTGTTGTTAGGAAGTG **R** TTGAGGCTGGGTGAAGT	857	Seyedjavadi, 2016

CHAPTER FOUR

RESULTS

Survey at the Farms- Analysis of Questionnaire

A total of nine (9) fish farms were visited and farm owners were observed to engage in fish farming activities either as part time or full-time job.

All the fish farms made use of commercially formulated feed to feed their fish. Different brands of the fish feed were patronized by the farmers which included Aller aqua, Copens, Raanan and Enam papa. Three of the farms had their own hatchery that also supplied fingerlings to other fish farmers depending on the fish type cultured. None of the farms had witnessed disease outbreak and according to farmers antibiotics are not used in any way at all the culturing stages. However, few mortality occasionally occurred due to overcrowding or change in water quality (Table 4).

The pond types at the fish farms were either dug out or concrete whiles some farms had both types of pond (Figure 3). Water sources for rearing of fish were from tap water, boreholes or nearby streams. At some farms, pond water was changed every three months or during restocking whiles others depended on change of water quality. Also, monoculture and polyculture types of farming were observed to be practiced (Table 4).

Table 4: Management practices adopted at the fish farms

Framing practices	Response	Number of farms
Source of pond water	Bore hole	2
	Tap water	2
	Streams	7
Use of antibiotics	None	9
Incidence of disease outbreak	None	9
Frequency of water change	1-3 months	3
	Change of water quality	4
	During re-stocking	6
Methods of disposing waste water from pond	Outlet into stream	8
	Outlet into drains and pond	1
Culturing type	Monoculture	6
	Polyculture	3
Kind of fish feed patronized	Raanan	6
	Aller aqua	2
	Copens, Enam papa and Raanan	1
Presence of Hatchery		3

Bacterial Loads of Fish and Water Samples

All fish farms visited were active in production. Water and fish samples were obtained from 6 farms in the Central Region and 3 farms in the Western Region between the hours of 09:00 to 14:00 GMT. Tilapia

(*Oreochromis niloticus*) and Catfish (*Clarias gariepinus*) were the main types of fish species sampled. The most commonly sampled fish was catfish from seven (7) fish farms whiles both fish species type were obtained from two (2) farms. At each study site, the actual fish holding systems from which both water and fish samples were obtained composed of five earthen ponds and four concrete tanks. After sample processing and subsequent plating of composite suspension on respective media for coliform and *Staphylococcus* sp. isolation (Figure 4), the mean bacterial loads were recorded as shown on Table 5.

Generally, coliform loads of water samples were higher than that of fish samples. Farm I recorded the highest total coliform load of (167.500±13.500) $x10^4$CFU/mL for water sample, followed by farm B, with record of (154.000±2.301) x 10^4 CFU/mL. Farm A recorded the least coliform load of (2.470±0.013) x 10^4 CFU/mL followed by farm C (8.000±2.028) x 10^4 CFU/mL. Farms D, E, F, G and H recorded (25.300±8.145), (44.600±8.511), (137.000±7.172), (28.300±2.028) and (87.500±3.500) $x10^4$ CFU/mL respectively.

With regard to fish samples, the highest total coliform load was recorded for farm I (58.000±4.360) $x10^4$ CFU/g for catfish whiles that of tilapia was (43.700±4.000) x 10^4 CFU/g for farm E. Farm C and H recorded the least values of (0.323±0.040) x 10^4 CFU/g and (0.181±0.0143) x 10^4 CFU/g for tilapia and catfish respectively, with farm H recording the overall least total coliform load for catfish (Table 5).

Similarly, the mean bacterial loads of water samples cultured on MSA media were relatively higher than that of fish samples. Farm D recorded the

highest mean bacterial load of (15.000±5.292) x10^4 CFU/mL followed by Farm E (11.8±3.667) x10^4 CFU/mL and Farm F recording the least (0.103±0.006) x10^4 CFU/mL for water samples. However, for fish samples, farm D and A recorded the least bacterial loads of (0.153±0.008) x10^4 CFU/g and (0.032±0.007) x10^4 CFU/g for tilapia and catfish respectively. The overall least total bacterial load was recorded for Farm A and the highest bacterial load for fish samples was recorded for Farm E, (6.8±0.608) x 10^4 CFU/g and (1.3) x 10^4 CFU/g for tilapia and catfish respectively (Table 5).

Comparably, coliform and Gram positive bacterial loads of water samples from earthen ponds were relatively higher than that obtained from concrete tanks.

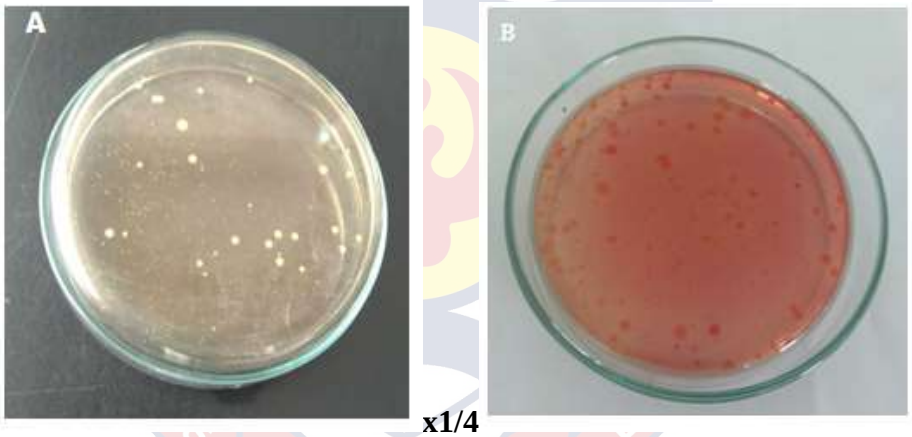

Figure 4: Mixed cultures of bacteria after 16-24 hours of incubation at 35 °C
A: Presumptive *Staphylococcus aureus* growing on Mannitol salt agar

B: Coliforms growing on MacConkey Agar

Table 5: Total coliform loads and Gram-positive bacterial loads of fish and water samples from the various farms

Farm	Type of fish holding facility	Mean total coliform load			Mean bacterial load of Gram-positive isolates		
		Water ($x10^4$ cfu/mL)	Tilapia ($x\ 10^4$ cfu/g)	Catfish ($x\ 10^4$ cfu/g)	Water ($x10^4$ cfu/mL)	Tilapia ($x\ 10^4$ cfu/g)	Catfish ($x\ 10^4$ cfu/g)
A	Concrete tank	2.470±0.013	_	0.936±0.023	0.147±0.010	_	0.032±0.007
B	Earthen pond	154.000±2.301	_	2.080±0.118	0.287±0.043	_	0.106±0.009
C	Concrete tank	8.000±2.028	0.323±0.040	_	0.340±0.066	0.194±0.007	_
D	Earthen pond	25.300±8.145	0.584±0.034	_	15.000±5.292	0.153±0.008	_
E	Earthen pond	44.600±8.511	43.700±4.000	29.100±6.100	11.8±3.667	6.8±0.608	14.3±4.706
F	Concrete tank	137.000±7.172	_	31.300±2.019	0.103±0.006	_	0.700±0.252
G	Concrete tank	28.300±2.028	_	0.310±0.005	0.950±0.035	_	0.176±0.060
H	Earthen pond	87.500±3.500	_	0.181±0.0143	0.870±0.070	_	0.072±0.120
I	Earthen pond	167.500±13.500	2.200±0.208	58.000±4.360	2.360±0.220	0.80±0.093	0.64±.1200

(−) = type of fish not available at the farm

Identification and Distribution of Bacterial Isolates

From water and fish samples, a total of 145 bacterial isolates were obtained on both MacConkey and MSA media. Based on biochemical assays and Gram staining results (Figure 5, 6 and 7), 22 different types of bacteria were identified. Ninety-nine coliforms were recovered from MacConkey Agar representing 68.30% and 46 (31.70%) Gram- positive bacterial isolates recovered from Mannitol Salt Agar. *Citrobacter freundii*, was the most predominant with a total of 38 (26.20%) isolates among coliforms followed by *Klebsiella pneumoniae* representing 22 (15.17%) across all the farms. The least represented with one isolate each were *Serratia marcescens, Escherichia coli, Edwardsiella tarda, Citrobacter diversus, Shigella sonnei, Athrobacter* sp., *Staphylococcus saprophyticus*, *Staphylococcus capitis* and S*taphylococcus intermedius*. Among Gram positive bacteria, *Staphylococcus aureus* was the most predominant with a total of 25 (17.24%) isolates. Farm H had the most recovered number of isolates (33) and the least recovered number of 10 isolates each for Farms C and F (Table 6).

The presence of these pathogens identified, especially, coliforms indicates poor sanitary conditions at the farms and the potential risk to the health of fish, fish farmers and consumers.

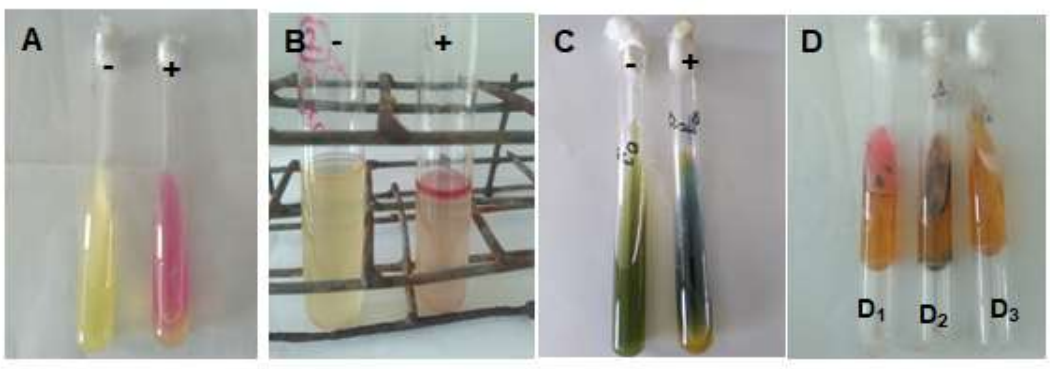

Figure 5: Biochemical tests using Christensen's urea agar (A), Peptone water

+ Kovac's reagent (B), Simmon's citrate agar (C) and Triple Sugar Iron agar (D). For Triple Sugar Iron agar test, D_1: red slope + yellow butt + air bubbles = glucose fermentation only + gas production; D_2: yellow slope + yellow butt + black staining + air bubbles = glucose, lactose and sucrose fermentation + gas and H2S production; D_3: yellow slope + yellow butt + air bubbles = glucose, lactose and sucrose fermentation + gas.

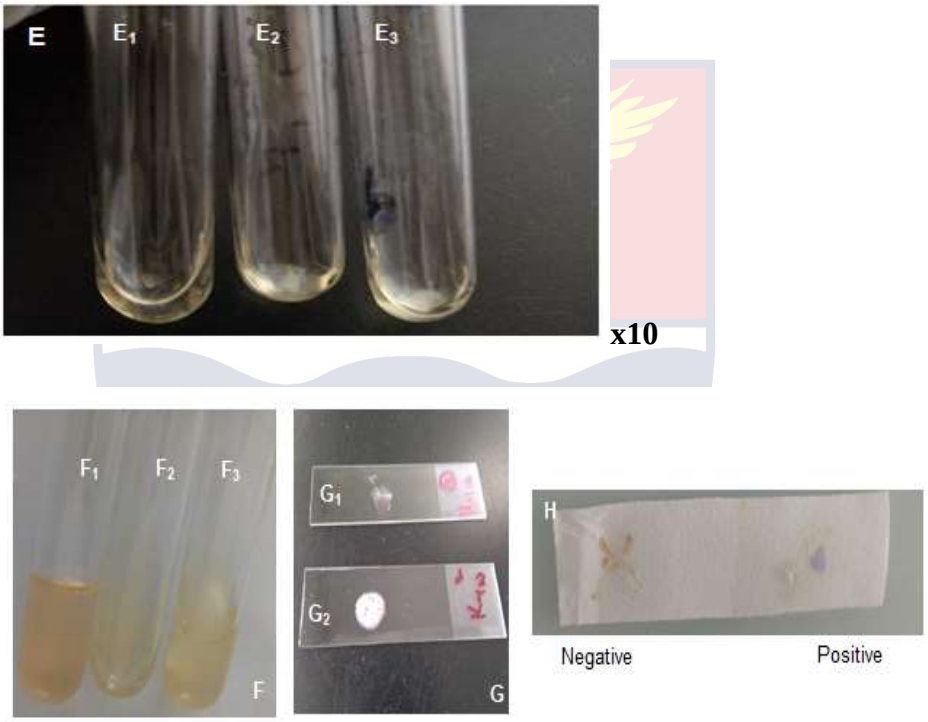

Figure 6: Additional biochemical tests Coagulase test (E) -E_1= Negative control, E_2= Positive control, E_3= Test (Positive); Gelatinase test (F) - F_1= Negative control, F_2=Positive control, F_3 =Positive test; Catalase test (G) – Negative test (G_1), Positive test (G_2); Oxidase test (H).

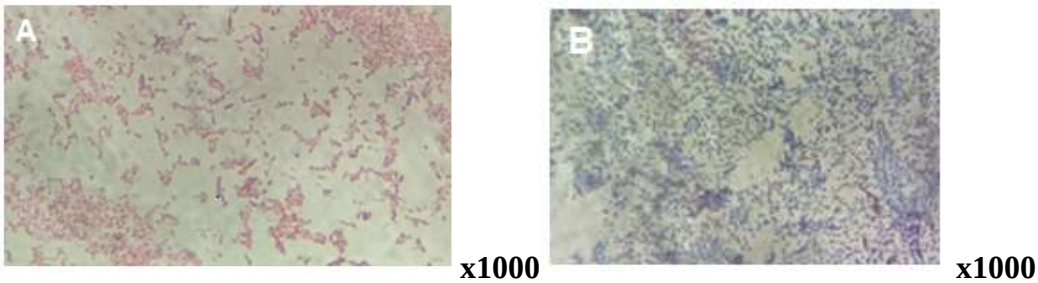

Figure 7: Gram staining of bacterial isolates
A: Gram-negative rods of coliform
B: Gram-positive cocci of presumptive *Staphylococcus* sp.

Table 6: Distribution of bacterial isolates from various fish farms

Type of bacteria	Bacterial species	A	B	C	D	E	F	G	H	I	Total (%)
Coliforms	*Serratia marcescens*	0	0	1	0	0	0	0	0	0	**1 (0.69)**
	Edwardsiella tarda	0	0	0	0	0	0	0	1	0	**1 (0.69)**
	Citrobacter freundii	1	6	3	1	4	1	4	9	9	**38(26.20)**
	Klebsiella oxytoca	4	0	0	2	1	0	2	0	2	**11(7.58)**
	Klebsiella pneumoniae	3	2	0	1	2	4	2	4	4	**22(15.17)**
	Salmonella sp.	0	0	0	1	1	0	0	3	0	**5 (3.40)**
	Escherichia coli	1	0	0	0	0	0	0	0	0	**1 (0.69)**
	Proteus mirabilis	1	0	0	0	0	0	0	0	1	**2 (1.37)**
	Citrobacter diversus	0	0	0	0	0	1	0	0	0	**1 (0.69)**
	Salmonella paratyphi 'A'	0	0	0	1	0	0	0	0	1	**2 (1.40)**
	Shigella sonnei	0	0	0	0	1	0	0	0	0	**1 (0.69)**
	Enterobacter aerogenes	0	0	1	3	1	2	2	2	0	**11(7.58)**
	Salmonella cholereausius	0	0	0	0	0	0	0	1	2	**3 (2.10)**
Gram Positive Bacteria	*Staphylococcus aureus*	3	4	2	2	1	1	2	6	4	**25 (17.24)**
	Streptococcus sp.	0	2	2	1	1	0	0	0	0	**6 (4.10)**
	Cellobioscoccus sp.	0	0	0	1	0	0	0	3	0	**4 (2.80)**
	Micrococcus sp.	0	0	0	0	0	1	0	3	1	**5 (3.40)**
	Athrobacter sp.	0	0	0	0	0	0	0	0	1	**1 (0.69)**
	Staphylococcus xylosus	0	0	1	0	1	0	0	0	0	**2 (2.10)**
	Staphylococcus saprophyticus	0	0	0	0	0	0	0	0	1	**1 (0.69)**
	Staphylococcus capitis	0	0	0	0	1	0	0	0	0	**1 (0.69)**
	Staphylococcus intermedius	0	0	0	0	0	0	0	1	0	**1 (0.69)**
	Total	**13**	**14**	**10**	**13**	**14**	**10**	**12**	**33**	**26**	**145**

Antibiotic Susceptibility Profile of Coliforms and Gram-positive Isolates from the various Fish Farms

The sensitivity of isolates to antibiotics was determined (Figure 8) and the results interpreted. The antibiotic resistance profile of coliforms and Gram-positive bacteria is shown on Table 7 and 8 respectively. All coliform bacteria, with the exception of *Edwardsiella tarda,* showed resistance to at least one of the antibiotics. Apart from *Salmonella cholereausius* that showed resistance to only ampicillin, most of the bacteria showed multiple resistance to the 8 antibiotics. In general, coliform bacteria were highly resistant (94.9%) to ampicillin followed by cefuroxime (80.8%). Cotrimoxazole, chloramphenicol, ceftriaxone, cefotaxime and tetracycline recorded 32.3%, 40.4%, 33.3%, 54.5% and 45.5% resistance respectively. The least was gentamicin (5.1%) (Table 7).

Similarly, all Gram-positive bacterial isolates showed resistance to more than one antibiotic. The percentage resistance of the isolates to flucoxacillin and penicillin was 100.0% and ampicillin recording 97.6%. The isolates recorded 13.0%, 47.8%, 56.5% and 17.4% resistances to cotrimoxazole, cefuroxime, erythromycin, and tetracycline respectively. None of the Gram positive isolate was resistant to gentamicin (0.0 %) (Table 8).

Most of the isolates showed multiple antibiotic resistance indicating exposure of the bacterial isolates to antibiotics, particularly beta-lactam class of antibiotics.

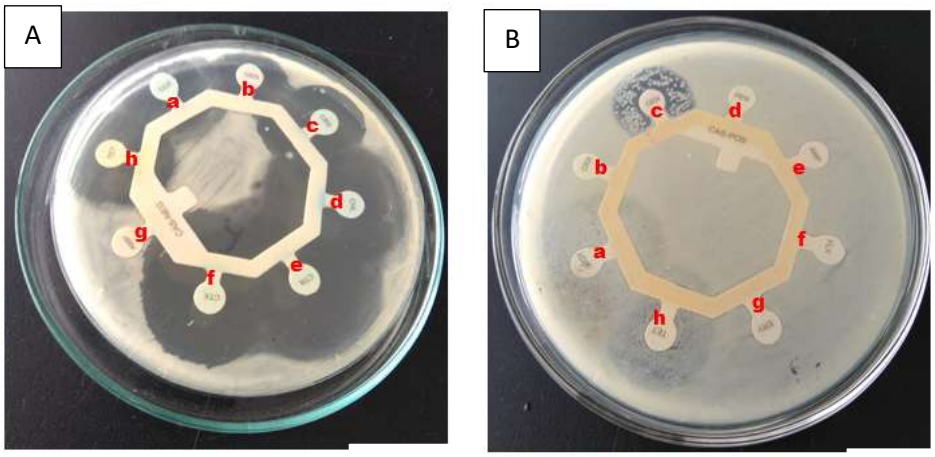

Figure 8: Mueller-Hinton Agar culture plates with antibiotics after 16-24 h incubation at 35 °C

A: (a) Cotrimoxazole (25µg), (b) Gentamycin (10µg),

(c) Cefuroxime (30µg), (d) Chloramphenicol (30µg),

(e) Ceftriaxone (30µg), (f) Cefotaxime (30µg),

(g), Ampicillin (10µg), (h) Tetracycline (30µg),

B: (a) Cotrimoxazole (25µg), (b) Cefuroxime (30µg),

(c) Gentamycin (10µg), (d) Penicillin (1.5 i.u),

(e) Ampicillin (10µg), (f) Flucoxacillin (5µg),

(g) Erythromycin (5µg), (h) Tetracycline (30µg)

Table 7: Antibiotic resistance profile of coliforms (Percentage in brackets)

Bacterial isolate	Number of isolates	Antibiotic resistance profile							
		COT	GEN	CRX	CHL	CTR	CTX	AMP	TET
Serratia marcescens	1	1(100.0)	0(0.0)	1(100.0)	1(100.0)	0(0.0)	0(0.0)	1(100.0)	1(100.0)
Citrobacter freundii	38	8(21.1)	2(5.3)	31(81.6)	14(36.8)	17(44.7)	29(76.3)	38(100.0)	17(44.7)
Klebsiella oxytoca	11	8(72.7)	1(9.1)	10(90.9)	6(54.5)	4(36.4)	4(36.4)	11(100.0)	7(63.6)
Escherichia coli	1	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(100.0)	0(0.0)
Enterobacter aerogenes	11	4(36.4)	0(0.0)	9(81.8)	4(36.4)	5(45.5)	6(54.5)	11(100.0)	5(45.5)
Klebsiella pneumoniae	22	7(31.8)	2(9.1)	22(100.0)	11(50.0)	4(18.2)	13(59.1)	22(100.0)	10(45.5)
Edwardsiella tarda	1	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
Salmonella sp.	5	3(60.0)	0(0.0)	1(20.0)	4(80.0)	0(0.0)	0(0.0)	2(40.0)	3(60.0)
Proteus mirabilis	2	0(0.0)	0(0.0)	2(100.0)	0(0.0)	1(50.0)	0(0.0)	2(100.0)	0(0.0)
Citrobacter diversus	1	1(100.0)	0(0.0)	1(100.0)	0(0.0)	0(0.0)	1(100.0)	1(100.0)	1(100.0)
Salmonella paratyphi 'A'	2	0(0.0)	0(0.0)	2(100.0)	0(0.0)	1(50.0)	0(0.0)	2(100.0)	0(0.0)
Shigella sonnei	1	0(0.0)	0(0.0)	1(100.0)	0(0.0)	1(100.0)	1(100.0)	1(100.0)	1(100.0)
Salmonella cholereausius	3	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	3(100.0)	0(0.0)
Total	**99**	**32(32.3)**	**5(5.1)**	**80(80.8)**	**40(40.4)**	**33(33.3)**	**54(54.5)**	**94(94.9)**	**45(45.5)**

COT= Co-trimoxazole, GEN = Gentamicin, CRX = Cefuroxime, CHL = Chloramphenicol, CTR = Ceftriaxone, CTX= Cefotaxime, AMP = Ampicillin, TET = Tetracycline

Table 8: Antibiotic resistance profile of Gram-positive bacterial isolates (Percentage in brackets)

Bacterial isolate	Number of isolates	Antibiotic resistance profile							
		COT	GEN	CRX	FLX	ERY	PEN	AMP	TET
Staphylococcus aureus	**25**	1(4.0)	0(0.0)	11(44.0)	25(100.0)	14(56.0)	25(100.0)	25(100.0)	4(16.0)
Streptococcus sp.	**6**	1(16.7)	0(0.0)	3(50.0)	6(100.0)	2(33.3)	6(100.0)	5(83.3)	1(16.6)
Cellobioscoccus sp.	**4**	2(50.0)	0(0.0)	3(75.0)	4(100.0)	3(75.0)	4(100.0)	4(100.0)	1(25.5)
Micrococcus sp.	**5**	0(0.0)	0(0.0)	2(40.0)	5(100.0)	3(60.0)	5(100.0)	5(100.0)	0(0.0)
Athrobacter sp.	**1**	0(0.0)	0(0.0)	0(0.0)	1(100.0)	0(0.0)	1(100.0)	1(100.0)	0(0.0)
Staphylococcus xylosus	**2**	1(100.0)	0(0.0)	1(50.0)	2(100.0)	2(100.0)	2(100.0)	2(100.0)	1(50.0)
Staphylococcus saprophyticus	**1**	1(100.0)	0(0.0)	1(100.0)	1(100.0)	1(100.0)	1(100.0)	1(100.0)	0(0.0)
Staphylococcus capitis	**1**	0(0.0)	0(0.0)	0(0.0)	1(100.0)	0(0.0)	1(100.0)	1(100.0)	0(0.0)
Staphylococcus intermedius	**1**	0(0.0)	0(0.0)	1(100.0)	1(100.0)	1(100.0)	1(100.0)	1(100.0)	1(100.0)
Total	**46**	**6(13.0)**	**0(0.0)**	**22(47.8)**	**46(100)**	**26(56.5)**	**46(100)**	**45(97.8)**	**8(17.4)**

COT= Co-trimoxazole, GEN = Gentamicin, CRX = Cefuroxime, CHL = Chloramphenicol, CTR = Ceftriaxone, CTX= Cefotaxime, AMP = Ampicillin, TET = Tetracycline

Analysis of DNA Samples from Bacterial Isolates

A total of 145 DNA samples were obtained from all the isolates of which 99 were from coliforms and 46 from Gram positive isolates. The DNA samples integrity was checked on 1.5% ethidium bromide-stained agarose gel (Figure 9). All DNA samples were confirmed to be bacterial DNA with the presence of 16S rRNA as shown in Figure 10.

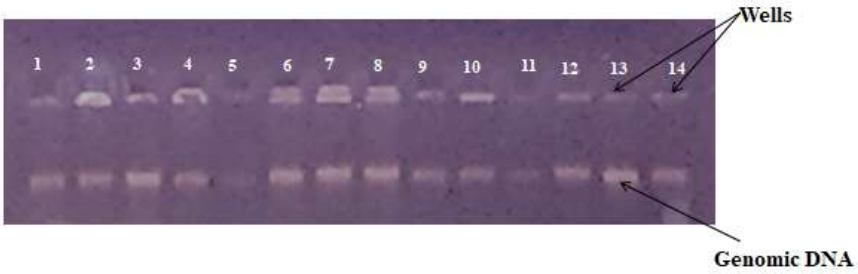

Figure 9: Agarose gel (1.5%) stained with ethidium bromide showing genomic DNA samples from coliforms isolates

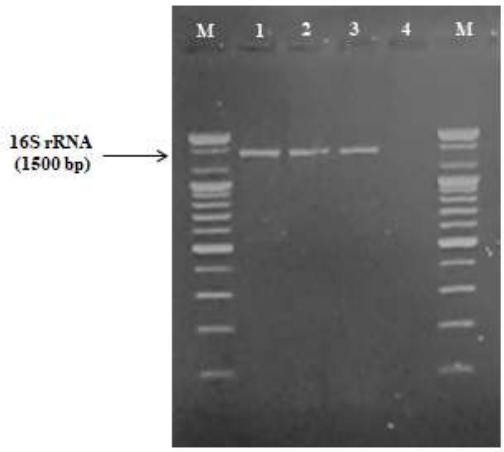

Figure 10: PCR products on 2.0% ethidium bromide-stained agarose gel showing confirmation of DNA samples as bacterial DNA using 16S rDNA primers. Lane 1, 2, and 3, presence of 16S rRNA gene; Lane M, 100 bp molecular ladder; Lane 4, Negative control (molecular grade water)

Detection of Antibiotic Resistance Genes using PCR

The PCR products after the amplification process were separated on 2% ethidium bromide-stained agarose gel for detection of resistant genes as shown in Figures 11, 12, and 13. The percentage detection of a particular gene among coliforms and Gram positive bacteria was recorded. The frequencies of genes detected for coliform and Gram-positive bacteria varied for each gene type. Both coliform and Gram-positive bacteria mostly harboured *TEM* gene, each respectively recording highest percentage detection of 54 (54.5%) and 34 (73.9%). *cmIA*, *qnr S*, *tet B*, and *CTX-M* each recorded a score of 1 (2.2%) for Gram-positive bacteria only whiles none of the coliform isolates harboured these genes. None of the isolates (coliform and Gram-positive isolates) harboured *tet A* gene (Figure 14).

High detection of *TEM* gene among the isolates confirms phenotypic expression of resistance to antibiotics belonging to beta-lactams class.

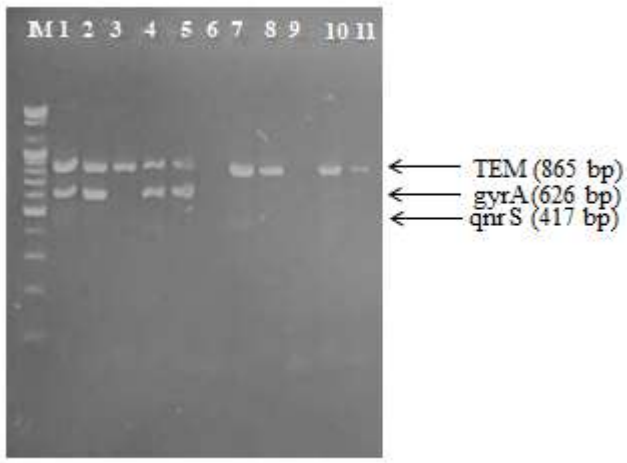

Figure 11: Ethidium bromide-stained 2.0% agarose gel showing presence or absence of *TEM*, *qnrS* and *gyrA* genes using multiplex PCR. Lane M, 100 bp molecular ladder; lanes 1, 2, 3, 4, 5, 7, 8, 10 and 11, presence of *TEM* gene; Lane 6 and 9, absence of *TEM* gene; lanes 1, 2, 4 and 5, presence of *gyrA* gene; lanes 3, 6, 7, 8, 9, 10, and 11, absence of *gyrA* gene; lanes 1 to 11 absence of *qnrS* gene.

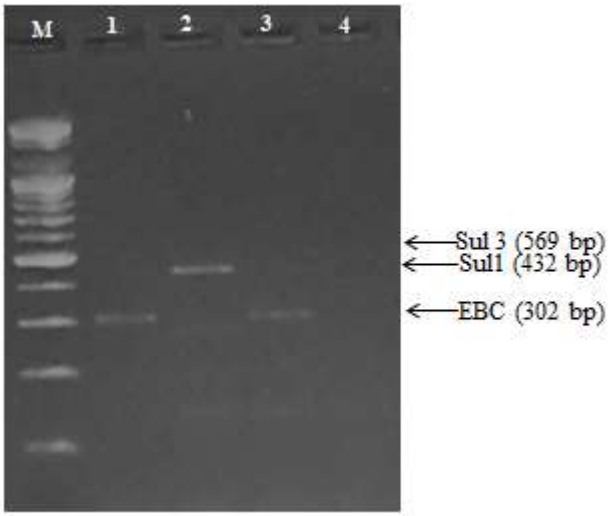

Figure 12: Ethidium bromide-stained 2.0% agarose gel showing presence or absence of *EBC*, *Sul 3* and *Sul 1* genes using multiplex PCR. Lane M, 100 bp molecular ladder; lane 2 and 1, 3, 4 showing presence and absence of *Sul* 1 gene respectively; lanes 1 and 3, presence of *EBC* gene and lanes 2 and 4 showing absence of *EBC* gene; lane 1to 4, showing absence of *Sul* 3 gene

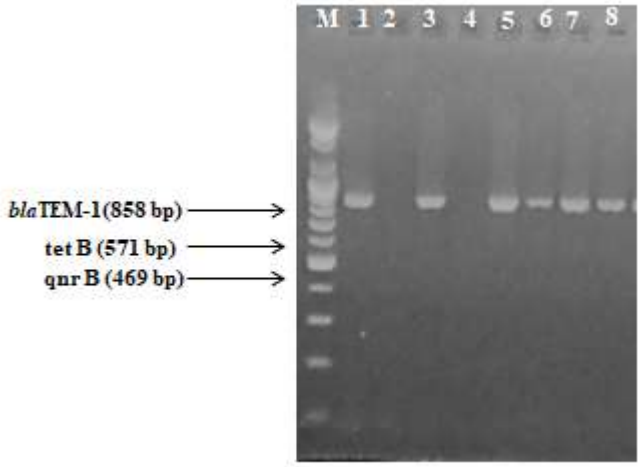

Figure 13: Ethidium bromide-stained 2.0% agarose gel showing presence or absence of ^{bla}*TEM*-1, *qnr B* and *tet B* genes using multiplex PCR. Lane M, 100 bp molecular ladder; lanes 1 and 3, 5, 6, 7 and 8 showing presence of ^{bla}*TEM-1* gene and lane 2 and 4 the absence of ^{bla}*TEM-1* gene; Lanes 1 to 8 showing absence of both *qnr B* and *tet B* genes.

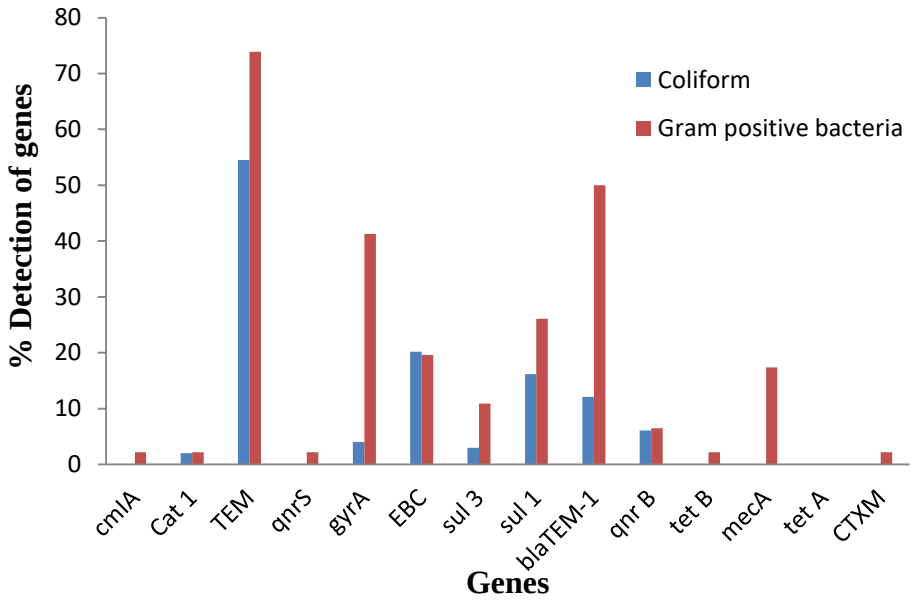

Figure 14: Percentage distribution of resistance genes detected among coliform and Gram positive bacteria

CHAPTER FIVE

DISCUSSION

Antibiotic resistance has become a global issue and the urgency to curb it focuses on all areas that require the use of antibiotics. Fish farming has been reported to contribute significantly to antibiotic resistance in bacteria due to its use for prophylactic and therapeutic purposes. The present study sought to investigate the current use of antibiotics and fish farm management practices at some selected fish farms of the Central and Western regions of Ghana. It also aimed at isolating and characterizing antibiotic resistant bacterial pathogens from fish and pond water samples from the fish farms as well as specific genes conferring resistance to antibiotics in order to ascertain necessary interventions to curb the spread of antibiotic resistance.

Information obtained from fish farmers suggested that none of the farmers directly used antibiotics at their farm and had not witnessed any major disease outbreak (Table 4). This could be that fingerlings that were used to stock the ponds at the various farms may be of good quality that could have resisted infection by pathogens. Patronage of commercially formulated feed at the farms is also advantageous since they are of good quality and farmers can directly monitor the feeding behaviour of their fish stock. This could also account for no record of disease outbreak at the farms since provision of proper fish nutrition promotes growth and also enhance overall health status of fish stock (Craig, Helfrich, Kuhn, & Schwarz, 2017). However, according to Anani *et al*., (2017) most of these commercially formulated feeds are expensive due to high manufacturing costs. Therefore, increased production of feeds from locally available farm-made ingredients that are of quality and rich

in proteins should be encouraged in the country to reduce high cost of fish feed.

Also, according to farmers a few mortalities recorded were due to overcrowding or poor water quality. High stocking density and poor water quality are important factors that cause stress to fish and subsequently weaken their immune system (Ojonugwa, & Solomon, 2017). In the present study, stocking density and parameters that determine quality of pond water such as pH, temperature and dissolved oxygen were not investigated. Hence, few deaths recorded at the farms may be those that could not withstand these stresses. The recommended stocking densities and routine check of water quality should be encouraged at the farms to limit stress to fish under culture.

Fish survive solely in water environment, therefore, different genera of bacterial pathogens in the gastrointestinal tract (GIT) of fish represent the diversity and abundance of microorganisms of pond water (Banu, Islam, & Chowdhury, 2001; Cahill, 1990). However, low levels of bacterial loads in fish sample compared to water samples (Table 5) could be due to the action of beneficial microbes found on the mucosal surface of the gastrointestinal tract (GIT) of the fish. These beneficial bacteria colonize the GIT and therefore block pathogens from attaching to the mucosal surface and also secrete extracellular enzymes that kill the microbial pathogens (Banerjee & Ray, 2017).

Generally, bacterial loads of pond water samples recorded in this study exceeded the acceptable level of ≤100 *E. coli* and <10 coliforms per mL for wastewater recommended for use in fish farming (WHO, 1989). Similarly, fish samples also had levels of bacterial loads exceeding the acceptable limit

for fresh fish regarded as wholesome, 5×10^5CFU/g at 37°C (Surendran, Thampuran, Nambiar, & Lalitha, 2006) (Table 5). High levels of bacterial loads of the samples could suggest possible contamination of pond water used in culturing fish at the farms from its source, thus, streams, borehole and tap water. The contamination may also be due to poor sanitary practices at the farms. Presence of these bacterial pathogens in addition to stress to fish could hamper proper growth and ultimately decrease productivity through infection transmission (Benchalgo, 2014). This could subsequently result in acute disease outbreak at the farms. Even though fish catch from these farms may be processed before being consumed, however, contamination may result from improperly cooked food that could put health of consumers at risk (Alikunhi, Batang, AlJahdali, Aziz, & Al-Suwailem, 2017). Moreover, fish farmers who are constantly exposed to these bacterial pathogens, especially, through handling may be infected by these bacterial pathogens. Hence, effective health protection measure such as proper personal hygiene must be ensured in order to reduce contamination.

Furthermore, the type of holding facility at the farm had influence on the bacterial loads of the samples (Table 5). The relatively higher levels of bacterial load of samples obtained from earthen pond as compared to that of concrete pond is that, earthen ponds mimic the fish's natural habitat with characteristic of the presence of soil beneath the water. The sediment therefore harbour wide range of microorganisms unlike concrete tanks that are more or less close system and not easily accessed by bacteria from the surrounding soils and runoff water. Also, concrete ponds are fully discharged and refilled

with fresh water. Hence, the level of microbial pollution may be reduced unlike earthen ponds.

The level of microbial contaminants recorded at the farms could be reduced by treating pond water using ozone and ultraviolet light that will sterilize it before stocking the ponds with fingerlings. Furthermore, the use of lime could also be used during pond preparation prior stocking. Subsequently, microbial contamination of potentially dangerous pathogens will be reduced that will eventually lead to production of high-value fish product (Boyd & Massaut, 1999).

Each of the bacterial isolates characterized in this study has the ability to cause infections to fish under culture or in human consumers (Table 6). Among coliforms, species like *Escherichia coli* has been linked to food-borne infections whiles *Citrobacter freundii* is generally regarded to cause opportunistic infections in humans. *Serratia marcescens* has been isolated from frozen fish and associated with fish deterioration (de Pádua *et al*., 2014). *Edwardsiella tarda* has also been pronounced to be the causative agent of edwardsiellosis with huge mortalities in both cultured and wild fish (Mohanty & Sahoo, 2007). *Edwardsiella tarda* causes haemorrhagic septicaemia in fish and has been associated with gastrointestinal infections in humans through consumption of raw fish (Greenlees, Machado, Bell, & Sundlof, 1998; Michael & Abbott, 1993). *Salmonella* species also cause food borne illness worldwide. Consumption of fish infected with *Salmonella* spp. by humans comes with major cases of gastroenteritis (Bibi *et al*., 2015). *Klebsiella* and *proteus* species have been reported to be most important histamine-producing bacteria (HPB) isolated from fish. They can cause mild illness in humans,

however, severe cardiac and respiratory complications may arise in individual with pre-existing conditions (Lehane & Olley, 2000). *Shigella sonnei* has been implicated in foodborne outbreaks causing bacillary dysentery with symptoms of acute bloody diarrhea associated with abdominal cramps and fever (Long, Prober, & Fischer, 2017).

Among Gram-positive isolates, *Staphylococcus aureus* was the most predominant and has been isolated from pond water and fish samples in other studies (Agoba, 2016; Saharan, Verma, & Singh, 2020; Singh, & Kulshreshtha, 1994). It is the most occurring Gram-positive bacteria isolated from skin lesions of fish species studied including tilapia and catfish (Ibrahim, Nimir., El-Sanousi, & Shuaib, 2016). Streptococcal diseases of fish are not common but cause significant mortalities in fish when it occurs. Some aquatic *Streptococcus* species may cause disease in immune compromised humans. *Staphylococcus intermedius, Staphylococcus xylosis, Staphylococcus saprophyticus*, *Athrobacter* sp., and *Micrococcus* sp., have all been isolated from fish samples and implicated in human diseases except *Cellosbioscoccus* sp (Oh *et al*., 2019; Sudheesh, Al-Ghabshi, Al-Mazrooei, & Al-Habsi, 2012).

Isolation of these bacterial pathogens from the samples with coliforms being predominant among the isolates indicates poor sanitary conditions at the farms (Table 6). Coliform bacteria are normally present in the environment and the faeces of warm-blooded animals and humans. They are important indicator organisms in an environmental sample to assess quality of water prior to or in place of culturing other organisms. Their presence in a water and fish samples indicates the presence of potentially harmful pathogenic bacteria (Pepper& Gerba, 2015). Hence, presence of coliforms in both water and fish

samples from the farms may suggest faecal contaminations. On the other hand, *Staphylococcus aureus* being predominant among Gram positive bacteria (Table 6) identified is one of the major bacterial agents causing food borne diseases in humans worldwide (European Food Safety Authority [EFSA], 2010; Le-Loir *et al*., 2003). It has been identified as the causative agent in many food poisoning outbreaks and is probably responsible for even more cases in individuals and family groups in most countries of the world (Bennett and Lancette, 1998). This poses risk to the health of consumers when insufficiently processed or raw fish and fish products from these farms are consumed. It becomes worrisome, most importantly in era of high demand for tilapia and catfish in Ghana. Proper hygienic measures such as personal hygiene and the use of clean fish farm equipment should be used to reduce the prevalence of these pathogens. Also some beneficial bacteria, such as *Lactobacillus* and *Carnobacterium* species which have been investigated and recommended for use in fish farming could be explored to control growth and abundance of these bacterial pathogens (Martínez, Ibáñez, Monroy Hermosillo & Ramírez Saad, 2012).

Furthermore, findings of the current study indicate that antibiotic resistance is widespread among different genera of Gram positive and negative bacterial isolates from pond water and fish samples (Table 7 & 8), although none of the farms directly used antibiotics. Even though, *Edwarsiella tarda* was not resistant to any of the antibiotics whiles *Salmonella cholereausius* was resistant to only ampicillin, phenotypic expression of multiple antibiotic resistance by most of the bacterial isolates suggest possible exposure of these bacteria to antibiotics. According to Manyahi *et al*., (2017), antibiotics are

normally released into the environment through feed for stockbreeding and in fish farming, human and veterinary medicine and pharmaceutical wastewaters.

Antibiotics are mostly used as an additive in animal feed (Agoba *et al.*, 2017; Van, Yidana, Smooker, & Coloe, 2020) however commercially formulated feed patronised by fish farmers may presumed not to contain antibiotics as information of feed component were not provided on the feed label. However, study by Agoba *et al*., (2017) at some fish farms in Ashanti region identified presence of multidrug resistant bacteria of which some fish farmers mixed fish feed with antibiotics such as tetracycline and chloramphenicol. Though the present study did not determine antibiotic residues in fish feed patronised by farmers, there has been report on ban of antibiotics use in animal feed for prophylactic purposes (Food and Drug Administration [FDA], 2012) based on recommendation by World Health Organization on measures to reduce antimicrobial resistance (WHO, FAO &NACA, 1999; WHO, 2017). According to guidelines by WHO, antibiotics can be used for treatment of infections in animals but not for the promotion of growth, increased performance, and improved feed efficiency.

On the contrary, in Ghana, antibiotics are easily obtained over the counter; hence abuse and improper disposal of these antibiotics are common. Therefore, multiple resistance of both coliforms and Gram positive bacteria to antibiotics, especially to beta-lactams (ampicillin, penicillin and flucoxacillin) and cephalosporins (cefuroxime, Cefotaxime, ceftriaxone) drugs (Table 8 & 9) indicate that these resistant bacteria may have emerged and found in the environment as these drugs are commonly used in the country. According to a study by Tagoe & Attah, 2010, cefuroxime, penicillin, cotrimoxazole etc. that

belong to beta-lactam class are frequently prescribed over the years and are easily obtained over the counter in the country. Hence, most bacteria have become resistant to beta-lactams as they are able to produce beta-lactamase to render the drugs inactive. A study by Azanu, Styrishave, Darko, Weisser, & Abaidoo, (2018) and Borquaye *et al*., (2019) have confirmed the presence of substantial amount of antibiotics in waste water and landfill sites in the country. Therefore, influx of antibiotics even in low concentrations into aquatic environments might have contributed to the widespread of antibiotic resistant bacteria.

Antibiotic resistance by bacteria develops through mutation of their DNA. Currently, phenotypic determination of antibiotic resistance is confirmed with molecular assays to determine specific genes that confer resistance to aid in decision making on measures to curb spread of resistant traits. Polymerase Chain reaction technique is used to genotype bacteria in relation to antibiotic resistance by the use of molecular markers (Abraham *et al*., 2018).

In this study, varying percentage detection of resistant genes was recorded for all the isolates (Figure 14). Genes that confer resistance to different classes of antibiotics commonly used in fish farming and of importance in human medicine was determined by multiplex PCR reaction assay. Among genes that confer resistance to beta-lactams ^{bla}*TEM,* ^{bla}*TEM-1,* ^{bla}*mecA,* ^{bla}*CTX-M* and ^{bla}*EBC* , high detection of *TEM* gene was recorded among coliforms (54.5%) and Gram-positive bacteria (73.9%). This confirms phenotypic expression of resistance of the isolates to antibiotics belonging to beta-lactam class (Table 8 & 9). *CTX-M* gene was detected in 1 (0.7%) isolate

whiles *mecA* gene *was* detected in 8 (5.5%) isolates of Gram-positive bacteria. *CTX-M* result runs contrary to other studies by Hackman, (2015) and Richter, Du Plessis, Duvenage, & Korsten, (2019) who recorded high detection of *CTX-M* gene in enterobacteria. *CTX-M* has been cited to be the most frequent beta-lactamase (Hackman, 2015), hence, least percentage detection of *CTX-M* could imply the resistance could be conferred by different gene variant. ^{bla}*EBC* genes was also detected in 20 (20.2%) and 9 (19.6%) among coliforms and Gram positive bacteria respectively.

An increasing number of beta-lactam variants have been discovered that differs in sequences of amino acid and their catalytic activity against β-lactam antibiotics (Bush, & Jacoby, 2010). Generally, Gram-negative bacteria produce β-lactamases, enzymes that inactive antibiotics. β-lactamases confer resistance to β-lactams by splitting the four-membered ring of the antibiotics with the release of an inactive product (Toth *et al*., 2016). Gram-positive bacteria on the other hand also use target modification mechanism to confer resistance to beta-lactams. This mechanism ensures structural changes to specific enzyme targets of the β-lactam antibiotics which renders the enzyme inactive to β-lactam (Ogawara, 2015). This could account for detection of diverse variant genes of both coliforms and Gram positive bacteria to most beta-lactams drugs. Similarly, a study conducted by Ishida *et al*., (2010), in Egypt at some fish farms showed that, Gram negative bacteria isolated at some fish farms showed phenotypic resistance to beta-lactams with corresponding detection of *blaTEM-1*, *blaTEM-104* , *blaCTX-M-15*, *blaCTX-M-15* and *blaSHV-89* resistant genes.

Genes that are resistant to tetracycline, *tet A* and *tet B*, recorded low percentage detection of 0(0.0%) and 1(0.7%) in coliforms and Gram positive bacteria respectively. Even though, 45.5% phenotypic resistance to tetracycline was expressed among coliforms (Table 7), no detection of resistant genes in coliforms could suggest the resistance may be due to other resistant gene variants such *tet C, O, W* other than *tet A* and *B*. Similarly, chloramphenicol resistant genes, *cmIA* and *Cat1* recorded 1(0.7%) and 3(2.1%) respectively. The results also confirm that the phenotypic resistant profile recorded is not attributed to these genes.

*Sul1*and *Sul3* resistant genes determined for cotrimoxazole are mostly found on the chromosome other than on plasmids (Hoa, Nonaka, Viet, & Suzuki, 2008). *Sul1* gene was relatively highly detected recording 16 (16.2%) for coliforms and 12 (26.1%) for Gram-positive isolates with overall detection of 28 (19.3%). *Sul3* rather recorded overall percentage detection of 8 (5.5%) with coliforms representing 3 (3.0%) and Gram- positive 5 (10.9%). A study by Manyani *et al*., (2017) on determination of *Sul* (1, 2 and 3) genes among Gram-positive bacteria was higher (98.4%) than that recorded in this study. However, high detection rate recorded for *Sul* 1 gene is confirmed by Dominguez *et al*., (2019) with none of the isolates producing *Sul 3* gene among bacterial strains that was studied. Cotrimoxazole consist of sulfamethoxazole and trimethoprim. Hence resistant genes that were not detected could be attributed to trimethoprim resistant genes as the genes only detected sulphonamide component of the drug.

Even though antibiotics belonging to the class of quinolones were not used in the susceptibility testing, *qnrS, qnrB* and *gyrA* genes were determined.

Quinolones such as ciprofloxacin, oxonilic acid and nalidixic acid are not commonly used in fish farming especially in Ghana (Apenteng *et al*., 2017). However, high detection rate was recorded for *gryA* genes 23 (15.9%) as compared to *qnrS* 1 (0.7%) and *qnrB* 9 (6.2%). *qnr S* and *qnr B* are mostly found in plasmids and this may account for low detection observed in the genomic DNA of the isolates used in the present study. Multiple antibiotic resistance among the isolates and detection of genes that confer resistance suggests widespread of resistance in aquatic environments indicating that fish farms may be a potential source for the dissemination of antimicrobial resistance genes.

Antibiotic resistance is spreading globally, limiting the effectiveness of antibiotics use in the prevention and treatment of infectious diseases. This poses a threat to health following the use of antibiotics whose class and structure are similar to those used in treating infections in humans (Marshall and Levy, 2011). This is a major concern to public health as treatment of diseases caused by these pathogens to fish and subsequently to human consumers would be difficult. Recently some last-resort antibiotics are continuously used without any replacement. First hand measure to prevent widespread of antibiotic resistance ensures reduction in indiscriminate use of antibiotics. Previously, some European countries like Norway and Canada (Heuer *et al*., 2009) have adopted restriction guidelines on the use of antibiotics by prescription only. Similarly, in 2017 Ghana instituted a five-year National Action Plan (NAP) on antimicrobial resistance with objectives focusing on regular surveillance and optimizing the administration of antimicrobial drugs in human medicine, plant production, and animal health

including aquaculture. Even though NAP is at the phase of implementation, per the results from this study on the prevalence of antibiotic resistance, immediate measures are required to curb this menace. Programs that will prompt and educate the citizenry on usefulness of antibiotics and dangers associated with antibiotic resistance must therefore be intensified.

CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Summary

The study investigated the use of antibiotics at some selected fish farms in Central and Western regions of Ghana. It also aimed to isolate and characterize antibiotic resistant bacterial pathogens and specific genes that confer resistance to antibiotics at the various fish farms.

Questionnaire was developed and administered to fish farmers to obtain first-hand information on management practices and the use of antibiotics at the farms. Fish (Catfish and Tilapia) and pond water samples were subsequently obtained at the farms. Bacterial isolates were recovered on MacConkey Agar and Mannitol Salt Agar and the loads determined to know the level of contamination of the samples. Biochemical tests including Gram staining were performed to identify the isolates. Sensitivity test was performed on the identified isolates and the antibiotic resistant profile determined. The presence of resistant genes was determined using primers of tetracycline (tet A and tet B), quinolones (qnrS, qnrB and gyrA), Cotrimoxazole (Sul 1 and Sul 3), beta-lactms such as penicillin, ampicillin and flucoxacillin (^{bla}TEM, ^{bla}TEM-1, ^{bla}CTX-M and mecA), chloramphenicol (Cat 1 and cmIA), cefuroxime and ceftriaxone (^{bla}EBC) by multiplex assay.

Information obtained on antibiotic use at the fish farms indicates that none of the farms made use of antibiotics and no incidence of any major disease outbreak had been recorded. Generally, bacterial loads of pond water samples recorded in this study exceeded the acceptable level of ≤100 *E. coli* and <10 coliforms per mL for wastewater recommended for use in fish

farming (WHO, 1989). Similarly, fish samples also had levels of bacterial loads exceeding the acceptable limit for fresh fish regarded as wholesome, 5×10^5CFU/g at 37°C.

A total of 145 bacterial isolates were obtained of which 99 (68.30%) of them were identified to be coliforms and 46 (31.70%) were Gram-positive bacteria. Apart from *Edwardsiella tarda* and *Salmonella cholereausius*, all other bacterial isolates showed multiple resistance to antibiotics with highest resistance recorded for beta-lactams compared to other classes of antibiotics. Similarly, high detection of resistant genes for beta-lactams, especially, *TEM* gene was recorded among coliform 54 (54.5%) and Gram-positive bacteria 34 (73.9%) compared to other classes of antibiotics.

Conclusions

The study has revealed that none of the farms used antibiotics and no record of disease outbreak observed. A few deaths occasionally recorded were due to stress to fish as a result of overcrowding and poor water quality.

The bacterial loads of water samples in this study far exceeded the acceptable level of ≤100 *E. coli* and <10 coliforms per mL for wastewater recommended for use in fish farming. With regard to fish samples, bacterial loads exceeding the acceptable limit for fresh fish, 5×10^5CFU/g were recorded. Contamination of fish and pond water samples with different genera of bacteria, especially, coliforms indicate poor sanitary conditions at the farms.

A total of 145 bacterial isolates belonging to 22 different genera consisting of 99 coliforms and 46 Gram-positive bacteria were obtained. With the exception of *Edwardsiella tarda*, all isolates showed resistance to at least

an antibiotic used. The isolates were highly resistant to beta-lactam class of antibiotics and were confirmed by corresponding high detection of *TEM* genes that confer resistance to beta-lactams. The presence of antibiotic resistant bacterial isolates at the fish farms indicate possible introduction of these pathogens from the environment where antibiotics are regularly used.

Recommendations

1 The study concentrated on only two regions. It would be more informative to investigate the spread of antibiotic resistance in aquaculture settings in other regions of the country.

2 There is the need to further investigate the presence of antibiotic residues at the fish farms as well as fish feed to confirm none use of antibiotics as indicated by the farmers.

3 An integrated approach involving all stakeholders should be put in place to properly regulate the use of antibiotics that will limit abuse and ultimately minimize the emergence of antibiotic resistance.

REFERENCES

Abadi, A. T. B., Rizvanov, A. A., Haertlé, T., & Blatt, N. L. (2019). World Health Organization report: current crisis of antibiotic resistance. *BioNanoScience*, *9*(4), 778-788.

Abraham, T. J., Mallick, P. K., & Paul, P. (2018). African catfish Clarias gariepinus farming practices in North and South 24 Parganas districts of West Bengal, India. *Journal of Fisheries*, *6*(1), 579-586.

Aeschliman, C. (2005). Results of the 2003–2004 Baseline Survey of Ghanaian Fish Farmers. *FAO Regional Office for Africa Accra, Ghana*.

Aggrey-Fynn, E. (2001). The contribution of the fisheries sector to Ghana's economy. *A paper presented on behalf of the FAO as an input into sustainable fisheries livelihoods study. available at: firms. fao. org/fi/website/FIRetrieveAction. do*.

Agoba, E. E. (2016). *The use of antibiotics and resistance patterns of bacterial isolates from selected fish farms in the Ashanti Region of Ghana* (Doctoral dissertation). Retrieved from http:ir.knust.edu.gh/handle/123456789/9432

Agoba, E. E., Adu, F., Agyare, C., & Boamah, V. E. (2017). Antibiotic use and practices in selected fish farms in the Ashanti region of Ghana. *Journal of Infectious Diseases and Treatment*, *3*(02), 2472-1093.

Agriculture Organization of the United Nations. Fisheries Department. (2000). *The State of World Fisheries and Aquaculture, 2000* (Vol. 3). Food & Agriculture Organization.

Aheto, D. W., Acheampong, E., & Odoi, J. O. (2019). Are small-scale freshwater aquaculture farms in coastal areas of Ghana economically profitable?. *Aquaculture International*, *27*(3), 785-805.

Alikunhi, N. M., Batang, Z. B., AlJahdali, H. A., Aziz, M. A., & Al-Suwailem, A. M. (2017). Culture-dependent bacteria in commercial fishes: Qualitative assessment and molecular identification using 16S rRNA gene sequencing. *Saudi Journal of Biological Sciences*, *24*(6), 1105-1116.

Aly, S. M., & Albutti, A. (2014). Antimicrobials use in aquaculture and their public health impact. *Journal of Aquaculture Research & Development*, *5*(4), 1-6.

Amenyogbe, E., Chen, G., Wang, Z., Lin, M., Lu, X., Atujona, D., & Abarike, E. D. (2018). A review of Ghana's aquaculture industry. *Journal of Aquaculture Research & Development*, *9*(545), 1-6.

Ampofo, J. A. (2000). *Studies On the Occurrence and Diversity of Bacteria In Fish Culture Systems In Ghana With Special Reference to Species Pathogenic to Fish and Humans* (Doctoral dissertation, University of Ghana).

Anani, F. A., Nunoo, F. K. E., Steiner-Asiedu, M., Agbo, N. W., & Nortey, T. N. N. (2017). Production and use of farm-made fish feeds by small-scale pond fish farmers in Ghana. *Journal of Energy and Natural Resource Management (JENRM)*, *4*(1), 9-14.

Antwi-Asare, T. O., & Abbey, E. N. (2011). Fishery value chain analysis—Ghana. *Food and Agriculture Organization, Fisheries*. 3-34

Apenteng, J. A., Osei-Asare, C., Oppong, E. E., Amihere, I., & Hafiz, M. Y. (2017). Antibiotic sensitivity patterns of microbial isolates from fish ponds: a study in the Greater Accra Region of Ghana. *African Journal of Pharmacy and Pharmacology*, *11*(28), 314-320.

Arabi, H., Pakzad, I., Nasrollahi, A., Hosainzadegan, H., Jalilian, F. A., Taherikalani, M., ... & Sefidan, A. M. (2015). Sulfonamide resistance genes (sul) M in extended Spectrum Beta lactamase (ESBL) and non-ESBL producing Escherichia Coli isolated from Iranian hospitals. *Jundishapur journal of microbiology*, *8*(7), 1-6.

Assefa, A., & Abunna, F. (2018). Maintenance of fish health in aquaculture: review of epidemiological approaches for prevention and control of infectious disease of fish. *Veterinary medicine international*, *2018*.

Awity, L. (2005). National Aquaculture Sector Overview-Ghana. National Aquaculture Sector Overview Fact Sheets. *FAO Fisheries and Aquaculture Department [online]. Rome. Accessed March*, *20*, 2010.

Azanu, D., Styrishave, B., Darko, G., Weisser, J. J., & Abaidoo, R. C. (2018). Occurrence and risk assessment of antibiotics in water and lettuce in Ghana. *Science of the Total Environment*, *622*, 293-305.

Azimian, A., Havaei, S. A., Fazeli, H., Naderi, M., Ghazvini, K., Samiee, S. M., ... & Peerayeh, S. N. (2012). Genetic characterization of a vancomycin-resistant Staphylococcus aureus isolate from the respiratory tract of a patient in a university hospital in northeastern Iran. *Journal of clinical microbiology*, *50*(11), 3581-3585.

Banerjee, G., & Ray, A. K. (2017). Bacterial symbiosis in the fish gut and its role in health and metabolism. *Symbiosis*, *72*(1), 1-11.

Banu, A. N. H., Islam, M. A., & Chowdhury, M. B. R. (2001). Bacterial load in pond water and different organs of a Indian major carp Cirrhinus mrigala Ham. *Bangladesh Journal of Fisheries Research*, *5*(1), 53-58.

Barker, K. F. (1999). Antibiotic resistance: a current perspective. *British journal of clinical pharmacology*, *48(2): 109–124*.

Benchalgo, N. (2014). Biomarkers study in rainbow trout exposed to industrially contaminated groundwater. *Journal of Xenobiotics*, *4*(1), 1-7.

Bennett, R. W., & Lancette, G. A. (1998). Staphylococcus aureus, p. 12.01–12.05. *FDA bacteriological analytical manual, 8th ed., rev. A. AOAC International, Gaithersburg, Md*.

Bibi, F., Qaisrani, S. N., Ahmad, A. N., Akhtar, M., Khan, B. N., & Ali, Z. (2015). Occurrence of Salmonella in freshwater fishes: A review. *Journal of Animal and Plant Sciences*, *25*(3), 303-310.

Borquaye, L. S., Ekuadzi, E., Darko, G., Ahor, H. S., Nsiah, S. T., Lartey, J. A., ... & Woode, E. (2019). Occurrence of antibiotics and antibiotic-resistant bacteria in landfill sites in Kumasi, Ghana. *Journal of Chemistry*, *2019*.

Boyd, C. E., & Massaut, L. (1999). Risks associated with the use of chemicals in pond aquaculture. *Aquacultural engineering*, *20*(2), 113-132.

Brooker, A. J., Shinn, A. P., & Bron, J. E. (2007). A review of the biology of the parasitic copepod Lernaeocera branchialis (L., 1767)(Copepoda: Pennellidae). *Advances in Parasitology*, *65*, 297-341.

Brown, A. E. (2012). *Benson's Microbiological applications: Laboratory manual in General Microbiology*, McGraw-Hill Companies.

Browne, K., Chakraborty, S., Chen, R., Willcox, M. D., Black, D. S., Walsh, W. R., & Kumar, N. (2020). A new era of antibiotics: The clinical potential of antimicrobial peptides. *International Journal of Molecular Sciences*, *21*(19), 7047.

Burkina, V., Zlabek, V., & Zamaratskaia, G. (2015). Effects of pharmaceuticals present in aquatic environment on Phase I metabolism in fish. *Environmental toxicology and pharmacology*, *40*(2), 430-444.

Bush, K., & Jacoby, G. A. (2010). Updated functional classification of β-lactamases. *Antimicrobial agents and chemotherapy*, *54*(3), 969-976.

Cahill, M. M. (1990). Bacterial flora of fishes: a review. *Microbial ecology*, *19*(1), 21-41.

Cheesbrough, M. (2005). *District laboratory practice in tropical countries, part 2*. Cambridge university press.

Chen, C. R., Malik, M., Snyder, M., & Drlica, K. (1996). DNA gyrase and topoisomerase IV on the bacterial chromosome: quinolone-induced DNA cleavage. *Journal of molecular biology*, *258*(4), 627-637.

Chen, S. C., Tung, M. C., Chen, S. P., Tsai, J. F., Wang, P. C., Chen, R. S., ... & Adams, A. (1994). Systematic granulomas caused by a rickettsia-like organism in Nile tilapia, Oreochronuis niloticus (L.), from southern Taiwan. *Journal of Fish Diseases*, *17*(6), 591-599.

Clinical and Laboratory Standards Institute (CLSI). (2018). *Performance Standards for Antimicrobial Susceptibility Testing*. (28th ed.). Clinical and Laboratory Standard Institute

Cobbina, R., & Eiriksdottir, K. (2010). Aquaculture in Ghana: economic perspectives of Ghanaian aquaculture for policy development. *Final project). United Nations University-fisheries training programme, 47*.

Connor, W. E., & Connor, S. L. (2007). The importance of fish and docosahexaenoic acid in Alzheimer disease. *The American Journal of Clinical Nutrition, 85*(4), 929–930.

Craig, S., Helfrich, L. A., Kuhn, D., & Schwarz, M. H. (2017). *Understanding fish nutrition, feeds, and feeding.*Virginia Coporative Extension: Virginia, USA. Retrieved from http: www.ext.vt.edu

Dadgostar, P. (2019). Antimicrobial resistance: implications and costs. *Infection and drug resistance*, *12*, 3903-3910.

Darwish, W. S., Eldaly, E. A., El-Abbasy, M. T., Ikenaka, Y., Nakayama, S., & Ishizuka, M. (2013). Antibiotic residues in food: the African scenario. *Japanese Journal of Veterinary Research*, *61*(Supplement), S13-S22.

de Pádua, S. B., Marques, D. P., FA, S., Pilarski, F., Martins, M. L., & Ishikawa, M. M. (2014). Isolation, characterization and pathology of Citrobacter freundii infection in Native Brazilian Catfish Pseudoplatystoma. *Embrapa Agropecuária Oeste-Artigo em periódico indexado (ALICE)*.

Dixon, B. (2000). Antibiotics as growth promoters: risks and alternatives. *American Society of Microbiology news*, *66(5):*264-265.

Doku, B. N. A., Chen, S., Alhassan, E. H., Abdullateef, Y., & Rahman, M. M. (2018). Fisheries resources of Ghana: present status and future direction. *Fisheries*, *3*(4).

Domínguez, M., Miranda, C. D., Fuentes, O., de la Fuente, M., Godoy, F. A., Bello-Toledo, H., & González-Rocha, G. (2019). Occurrence of transferable integrons and sul and dfr genes among sulfonamide-and/or trimethoprim-resistant bacteria isolated from Chilean salmonid farms. *Frontiers in microbiology*, *10*, 748.

Domínguez-Pérez, R. A., De la Torre-Luna, R., Ahumada-Cantillano, M., Vázquez-Garcidueñas, M. S., Pérez-Serrano, R. M., Martínez-Martínez, R. E., & Guillén-Nepita, A. L. (2018). Detection of the antimicrobial resistance genes blaTEM-1, cfxA, tetQ, tetM, tetW and ermC in endodontic infections of a Mexican population. *Journal of global antimicrobial resistance*, *15*, 20-24.

Done, H. Y., Venkatesan, A. K., & Halden, R. U. (2015). Does the recent growth of aquaculture create antibiotic resistance threats different from those associated with land animal production in agriculture?. *Journal of the American Association of Pharmaceutical Scientist*, *17*(3), 513-524.

Donkor, E. S., Anim-Baidoo, I., Fei, E., Amponsah, C., Olu-Taiwo, M., Nana-Adjei, D., ... & Forson, A. O. (2018). Occurrence of antibiotic residues and antibiotic-resistant bacteria in Nile tilapia sold in Accra, Ghana: public health implications. *Journal of Food Research*, *7*(6), 129-137.

Duodu, C. P., Boateng, D. A., & Edziyie, R. E. (2020). Effect of pond fertilization on productivity of tilapia pond culture in Ghana. *Journal of Fisheries and Coastal Management*, *2*(1), 56-64.

Ekpenyong, M., Asitok, A., Odey, A., & Antai, S. (2016). Production and activity kinetics of gelatinase by Serratia sp. SLO3. *Nigerian Journal of Biopesticides*, *1*, 70-82.

Emerson, D., Agulto, L., Liu, H., & Liu, L. (2008). Identifying and characterizing bacteria in an era of genomics and proteomics. *BioScience*, *58*(10), 925-936.

Etebu, E., & Arikekpar, I. (2016). Antibiotics: Classification and mechanisms of action with emphasis on molecular perspectives. *International Journal of Applied Microbiology and Biotechnology Research*, *4*(2016), 90-101.

European Food Safety Authority (EFSA), European Centre for Disease Prevention and Control, 2010. The Community Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in the European Union in 2 008. *EFSA J.*, 8(1): 1496.

Falagas, M. E., Kastoris, A. C., Kapaskelis, A. M., & Karageorgopoulos, D. E. (2010). Fosfomycin for the treatment of multidrug-resistant, including extended-spectrum β-lactamase producing, Enterobacteriaceae infections: a systematic review. *The Lancet infectious diseases*, *10*(1), 43-50.

FAO, I. (2016). The state of world fisheries and aquaculture 2016. *Contributing to food security and nutrition for all*, 200.

Finegold, C., Gordon, A., Mills, D., Curtis, L., & Pulis, A. (2010). Western region fisheries sector review. *World Fish Center. USAID Integrated Coastal and Fisheries Governance Initiative for the Western Region, Ghana*.

Fisheries, F. A. O. (2016). Aquaculture. Fishery and aquaculture country profiles—the republic of Ghana.

Folke, C., & Kautsky, N. (1992). Aquaculture with its environment: prospects for sustainability. *Ocean & coastal management*, *17*(1), 5-24.

Food and Drug Administration, U. S., Department of Health and Human Services, Center for Veterinary Medicine. (2012). *Guidance for Industry: The Judicious Use of Medically Important Antimicrobial Drugs in Food-Producing Animals*. Retrieved from: http://www.fda.gov/downloads/animalveterinary/guidancecomplianceenforcement/guidanceforindustry/ucm216936.pdf

Forbes, B. A., Sahm, D. F., & Weissfeld, A. S. (2007). *Diagnostic microbiology* (pp. 288-302). St Louis: Mosby.

Frimpong, S. K., & Adwani, A. (2015). The challenges and prospects of fish farming in Ghana: A Project Management perspective. *International Journal of ICT and Management*, *3*(2), 29-34.

Ghana Statistical Service (2020). *Ghana's Population By Region*. Accra, Ghana: Author.

Golub, S., & Varma, A. (2014). Fishing exports and economic development of least developed countries: Bangladesh, Cambodia, Comoros, Sierra Leone and Uganda. *UNCTAD. Swarthmore College.*

Greenlees, K. J., Machado, J., Bell, T., & Sundlof, S. F. (1998). Food borne microbial pathogens of cultured aquatic species. *Veterinary Clinics of North America: Food Animal Practice*, *14*(1), 101-112.

Hackman, H. K. (2015). *Phenotypic and Molecular Characterization of Extended-Spectrum Beta-Lactamases in Klebsiella Pneumoniae and Escherichia Coli Isolates in Accra, Ghana* (Doctoral dissertation, University of Ghana). Retrieved from http://ugspace.ug.edu.gh/handle/123456789/7485

Harley, J.P.(2004). Laboratory exercises in microbiology. McGraw-Hill Science Engineering.

Hedayatianfard, K., Akhlaghi, M., & Sharifiyazdi, H. (2014). Detection of tetracycline resistance genes in bacteria isolated from fish farms using polymerase chain reaction. In *Veterinary Research Forum* (Vol. 5, No. 4, p. 269). Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

Heuer, O. E., Kruse, H., Grave, K., Collignon, P., Karunasagar, I., & Angulo, F. J. (2009). Human health consequences of use of antimicrobial agents in aquaculture. *Clinical Infectious Diseases*, *49*(8), 1248-1253.

Hiheglo, P. K. (2008). *Aquaculture in Ghana: Prospects, challenges, antidotes and future perspectives* (Master's thesis, Universitetet i Tromsø). Retrieved from https://munin.uit.no/handle/10037/1431

Hoa, P. T. P., Nonaka, L., Viet, P. H., & Suzuki, S. (2008). Detection of the sul1, sul2, and sul3 genes in sulfonamide-resistant bacteria from wastewater and shrimp ponds of north Vietnam. *Science of the Total Environment*, *405*(1-3), 377-384.

Högberg, L. D., Heddini, A., & Cars, O. (2010). The global need for effective antibiotics: challenges and recent advances. *Trends in pharmacological sciences*, *31*(11), 509-515.

Hossain, A., Nakamichi, S., Habibullah-Al-Mamun, M., Tani, K., Masunaga, S., & Matsuda, H. (2017). Occurrence, distribution, ecological and resistance risks of antibiotics in surface water of finfish and shellfish aquaculture in Bangladesh. *Chemosphere*, *188*, 329-336.

Huicab-Pech, Z. G., Castañeda-Chavez, M. R., & Lango-Reynoso, F. (2017). Pathogenic bacteria in Oreochromis niloticus var. Stirling tilapia culture. *Fisheries and Aquaculture Journal*, *8*(2), 1-6.

Huys, G. (2003). Standard Operating Procedure: Sampling and Sample Processing Procedures for the Isolation of Aquaculture-Associated Bacteria, *Springer,* Berlin, Germany, pp. 1–15.

Ibrahim, W. H., Nimir, A. H. H., El-Sanousi, S. M., & Shuaib, Y. A. (2016). Aerobic bacteria and fungi from skin lesions of fish in Khartoum state. *Journal of Advanced Veterinary and Animal Research*, *3*(4), 375-385.

Inglis, V. (2000). Antibacterial chemotherapy in aquaculture: review of practice, associated risks and need for action. In *Use of Chemicals in Aquaculture in Asia: Proceedings of the Meeting on the Use of Chemicals in Aquaculture in Asia 20-22 May 1996, Tigbauan, Iloilo, Philippines* (pp. 7-22). Aquaculture Department, Southeast Asian Fisheries Development Center.

Ishida, Y., Ahmed, A.M., Mahfouz, N.B., Kimura, T., El-Khodery, S.A., Moawad, A.A., and Shimamoto, T. (2010) Molecular analysis of antimicrobial resistance in Gram-negative bacteria isolated from fish farms in Egypt. *Journal of Veterinary Medical Science*76: 727– 734.

Kapoor, G., Saigal, S., & Elongavan, A. (2017). Action and resistance mechanisms of antibiotics: A guide for clinicians. *Journal of anaesthesiology, clinical pharmacology*, *33*(3), 300-305.

Karkman, A., Do, T. T., Walsh, F., & Virta, M. P. (2018). Antibiotic-resistance genes in waste water. *Trends in microbiology*, *26*(3), 220-228.

Kassam, L. (2014). *Aquaculture and food security, poverty alleviation and nutrition in Ghana: Case study prepared for the Aquaculture for Food Security, Poverty Alleviation and Nutrition project*. WorldFish.

Lane, D. J. (1991). 16S/23S rRNA sequencing. *Nucleic acid techniques in bacterial systematics*, 115-175.

Lauková, A., Kandričáková, A., Strompfová, V., Chacornac, J. P., Léroy, S., & Žitňan, R. (2013). Staphylococcal species detected in free-living trouts of east Slovakian water sources and their relation to antimicrobials. *Bulletin of the Veterinary Institute in Pulawy*, *57*(2), 167-171.

Le Loir, Y., Baron, F., & Gautier, M. (2003). [i] Staphylococcus aureus [/i] and food poisoning. *Genetics and molecular research: GMR*, *2*(1), 63-76.

Leboffe, M. J., & Pierce, B. E. (2019). *Microbiology: Laboratory Theory and Application, Essentials*. Morton Publishing Company.

Lehane, L., & Olley, J. (2000). Histamine fish poisoning revisited. *International journal of food microbiology*, *58*(1-2), 1-37.

Leung, T. L., & Bates, A. E. (2013). More rapid and severe disease outbreaks for aquaculture at the tropics: implications for food security. *Journal of applied ecology*, *50*(1), 215-222.

Li, D., Liu, S., Li, D., & Liu, S. (2019). Water quality monitoring in aquaculture. *Water quality monitoring and management: basis, technology and case studies. Academic Press, Cambridge*.

Li, W., Shi, Y., Gao, L., Liu, J., & Cai, Y. (2012). Occurrence of antibiotics in water, sediments, aquatic plants, and animals from Baiyangdian Lake in North China. *Chemosphere*, *89*(11), 1307-1315.

Liu, X., Steele, J. C., & Meng, X. Z. (2017). Usage, residue, and human health risk of antibiotics in Chinese aquaculture: a review. *Environmental Pollution*, *223*, 161-169.

Long, S. S., Prober, C. G., & Fischer, M. (2017). *Principles and practice of pediatric infectious diseases E-Book*. Elsevier Health Sciences.

Lozano, I., Díaz, N. F., Muñoz, S., & Riquelme, C. (2018). Antibiotics in Chilean aquaculture: a review. *Antibiotic use in animals*, *3*, 25-44.

Ma, M., Wang, H., Yu, Y., Zhang, D., & Liu, S. (2007). Detection of antimicrobial resistance genes of pathogenic Salmonella from swine with DNA microarray. *Journal of Veterinary Diagnostic Investigation*, *19*(2), 161-167.

MacLennan, D. N. (1995). Technology in the capture fisheries. In International Conference on the Sustainable Contribution of Fisheries to Food Security, Kyoto (Japan), 4-9 Dec 1995.

Madigan, M. T., Martinko, J. M., Dunlap, P. V., & Clark, D. P. (2006). Brock biology of microorganisms. 11. *internat. Ed. Englewood Cliiffs: Prentice Hall*.

Manyahi, J., Tellevik, M. G., Ndugulile, F., Moyo, S. J., Langeland, N., & Blomberg, B. (2017). Molecular characterization of cotrimoxazole resistance genes and their associated integrons in clinical isolates of Gram-negative bacteria from Tanzania. *Microbial Drug Resistance*, *23*(1), 37-43.

Marshall, B. M., & Levy, S. B. (2011). Food animals and antimicrobials: impacts on human health. *Clinical microbiology reviews*, *24*(4), 718-733.

Martínez Cruz, P., Ibáñez, A. L., Monroy Hermosillo, O. A., & Ramírez Saad, H. C. (2012). Use of probiotics in aquaculture. *International Scholarly Research Notices*, *2012*.

Meyer, F. P. (1991). Aquaculture disease and health management. *Journal of animal science*, *69*(10), 4201-4208.

Michael, J., & Abbott, S. L. (1993). Infections associated with the genus Edwardsiella: the role of Edwardsiella tarda in human disease. *Clinical Infectious Diseases*, *17*(4), 742-748.

Mitema, E. S., Kikuvi, G. M., Wegener, H. C., & Stohr, K. (2001). An assessment of antimicrobial consumption in food producing animals in Kenya. *Journal of veterinary pharmacology and therapeutics*, *24*(6), 385-390.

Mohanty, B. R., & Sahoo, P. K. (2007). Edwardsiellosis in fish: a brief review. *Journal of biosciences*, *32*(3), 1331-1344.

Naylor, R., & Burke, M. (2005). Aquaculture and ocean resources: raising tigers of the sea. *Annual Review of Environment and Resources*, *30*: 185-218

Ogawara, H. (2015). Penicillin-binding proteins in Actinobacteria. *The Journal of antibiotics*, *68*(4), 223-245.

Oh, W. T., Jun, J. W., Giri, S. S., Yun, S., Kim, H. J., Kim, S. G., ... & Park, S. C. (2019). *Staphylococcus xylosus* infection in rainbow trout (Oncorhynchus mykiss) as a primary pathogenic cause of eye protrusion and mortality. *Microorganisms*, *7*(9), 330-341.

Ojonugwa, E. B., & Solomon, R. J. (2017). Effects of over stocking on the growth rate of Clarias gariepinus. *Journal of Animal Science and Veterinary Medicine*, *2*, 84-95.

Okocha, R. C., Olatoye, I. O., & Adedeji, O. B. (2018). Food safety impacts of antimicrobial use and their residues in aquaculture. *Public health reviews*, *39*(1), 1-22.

Oliveira, R. V., Oliveira, M. C., & Pelli, A. (2017). Disease infection by Enterobacteriaceae family in fishes: a review. *Journal of Microbiology & Experimentation*, *4*(5), 00128.

Onumah, E. E., Quaye, E. A., Ahwireng, A. K., & Campion, B. B. (2020). Fish Consumption Behaviour and Perception of Food Security of Low-Income Households in Urban Areas of Ghana. *Sustainability*, *12*(19), 1-16.

Padilla, D., Acosta, F., Rodriguéz, F., Góméz, V., Déniz, S., & Real, F. (2001). Mortality caused by *Staphylococcus xylosus* in red porgy (Pagrus pagrus) fish. *Journal of Applied Animal Research*, *19*(2), 203-208.

Palilu, P. T., & Budiarso, T. Y. (2017). *Isolation and identification of Staphylococcus sp. in powdered infant milk*. In *AIP Conference Proceedings* (Vol. 1844, No. 1, p. 020016). AIP Publishing LLC.

Pepper, I. L., & Gerba, C. P. (2015). Cultural methods. In *Environmental Microbiology* (pp. 195-212). Academic Press.

Pérez-Pérez, F. J., & Hanson, N. D. (2002). Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. *Journal of clinical microbiology*, *40*(6), 2153-2162.

Pridgeon, J. W., & Klesius, P. H. (2012). Major bacterial diseases in aquaculture and their vaccine development. *Anim. Sci. Rev*, *7*, 1-16.

Rahman, T., Akanda, M. M. R., Rahman, M. M., & Chowdhury, M. B. R. (2009). Evaluation of the efficacies of selected antibiotics and medicinal plants on common bacterial fish pathogens. *Journal of the Bangladesh Agricultural University*, *7*(1), 163-168.

Rakotovao-Ravahatra, Z. D., Randriatsarafara, F. M., Milasoanjara, R. N., Ranaivosoa, M. K., Rakotovao, A. L., & Rasamindrakotroka, A. (2019). Assessment of the Coagulase Test in the Identification of Staphylococcus Aureus Strains. *Journal of Biotechnology and Biomedicine*, *2*(3), 105-111.

Richter, L., Du Plessis, E. M., Duvenage, S., & Korsten, L. (2019). Occurrence, identification, and antimicrobial resistance profiles of extended-spectrum and AmpC β-lactamase-producing Enterobacteriaceae from fresh vegetables retailed in Gauteng Province, South Africa. *Foodborne pathogens and disease*, *16*(6), 421-427.

Ringø, E., Olsen, R. E., Jensen, I., Romero, J., & Lauzon, H. L. (2014). Application of vaccines and dietary supplements in aquaculture: possibilities and challenges. *Reviews in Fish Biology and Fisheries*, *24*(4), 1005-1032.

Risius, A., Janssen, M., & Hamm, U. (2017). Consumer preferences for sustainable aquaculture products: Evidence from in-depth interviews, think aloud protocols and choice experiments. *Appetite*, *113*, 246-254.

Robicsek, A., Strahilevitz, J., Sahm, D. F., Jacoby, G. A., & Hooper, D. C. (2006). qnr prevalence in ceftazidime-resistant Enterobacteriaceae isolates from the United States. *Antimicrobial agents and chemotherapy*, *50*(8), 2872-2874.

Robinson, R. K. (2014). *Encyclopedia of food microbiology*. Academic press.

Rodgers, C. J., & Furones, M. D. (2009). Antimicrobial agents in aquaculture: practice, needs and issues. *Options Méditerranéennes*, *86*, 41-59.

Romero, J., Feijoó, C. G., & Navarrete, P. (2012). Antibiotics in aquaculture–use, abuse and alternatives. *Health and environment in aquaculture*, *159*.

Saharan, V. V., Verma, P., & Singh, A. P. (2020). High prevalence of antimicrobial resistance in Escherichia coli, Salmonella spp. and

Staphylococcus aureus isolated from fish samples in India. *Aquaculture Research*, *51*(3), 1200-1210.

Santos, L., & Ramos, F. (2018). Antimicrobial resistance in aquaculture: current knowledge and alternatives to tackle the problem. *International Journal of Antimicrobial Agents*, *52*(2), 135-143.

Seyedjavadi, S. S., Goudarzi, M., & Sabzehali, F. (2016). Relation between blaTEM, blaSHV and blaCTX-M genes and acute urinary tract infections. *Journal of Acute Disease*, *5*(1), 71-76.

Shrivastava, S. R., Shrivastava, P. S., & Ramasamy, J. (2018). World health organization releases global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. *Journal of Medical Society*, *32*(1), 76-77.

Singh, B., & Kulshreshtha, S. B. (1994). A study on prevalence of Staphylococcus aureus in fish and fish products and their public health significance. *Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases*, *14*, 25-28.

Smith, A. C., & Hussey, M. A. (2005). Gram stain protocols. *American Society for Microbiology*, *1*, 14.

Smith, P. (2008). Antimicrobial resistance in aquaculture. *Revue scientifique et technique (International Office of Epizootics)*, *27*(1), 243-264.

Subasinghe, R. P., & Phillips, M. J. (2002). Aquatic Animal Health Management: Opportunities and Challenges for Rural, Small-scale Aquaculture, and Enhanced-fisheries Development: Workshop Introductory Remarks. *FAO fisheries technical paper*, 1-5.

Sudheesh, P. S., Al-Ghabshi, A., Al-Mazrooei, N., & Al-Habsi, S. (2012). Comparative pathogenomics of bacteria causing infectious diseases in fish. *International journal of evolutionary biology*, *2012*.

Surendran, P. K., Thampuran, N., Nambiar, V. N., & Lalitha, K. V. (2006). Laboratory manual on microbiological examination of seafood. *CIFT, Cochin,*.

Suzuki, S., Pruden, A., Virta, M., & Zhang, T. (2017). Antibiotic resistance in aquatic systems. *Frontiers in microbiology*, *8*, 14.

Tagoe, D., & Attah, C. (2010). A Study of Antibiotic Use and Abuse in Ghana: a case study of the Cape Coast Metropolis. *The Internet Journal of Health*, *11*(2), 1-5.

Takyi, R., Nunoo, F. K. E., Ziddah, P., & Oddoye, J. (2012). Occurrence of bacterial infection in two commonly cultured fish species on two fish farms in southern Ghana. *World Journal of Biological Research*, *5*(2), 81-92.

Thillai Sekar, V., Santiago, T. C., Vijayan, K. K., Alavandi, S. V., Stalin Raj, V., Rajan, J. J. S., ... & Kalaimani, N. (2008). Involvement of Enterobacter cloacae in the mortality of the fish, Mugil cephalus. *Letters in applied microbiology*, *46*(6), 667-672.

Tilman, D., & Clark, M. (2014). Global diets link environmental sustainability and human health. *Nature*, *515*(7528), 518-522.

Toth, M., Antunes, N. T., Stewart, N. K., Frase, H., Bhattacharya, M., Smith, C. A., & Vakulenko, S. B. (2016). Class D β-lactamases do exist in Gram-positive bacteria. *Nature chemical biology*, *12*(1), 9-14.

Trüper, H. G., & Krämer, J. (1981). Principles of characterization and identification of prokaryotes. In *The prokaryotes* (pp. 176-193). Springer, Berlin, Heidelberg.

Van, T. T. H., Yidana, Z., Smooker, P. M., & Coloe, P. J. (2020). Antibiotic use in food animals worldwide, with a focus on Africa: Pluses and minuses. *Journal of global antimicrobial resistance, 20*, 170-177.

Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K., & Swings, J. (1996). Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiological reviews*, *60*(2), 407-438.

Verner-Jeffreys, D. W., Wallis, T. J., Cejas, I. C., Ryder, D., Haydon, D. J., Domazoro, J. F., ... & Feist, S. W. (2018). Streptococcus agalactiae Multilocus sequence type 261 is associated with mortalities in the emerging Ghanaian tilapia industry. *Journal of fish diseases*, *41*(1), 175-179.

Wang, A., Yang, Y., Lu, Q., Wang, Y., Chen, Y., Deng, L., ... & Shen, X. (2008). Presence of qnr gene in Escherichia coli and Klebsiella pneumoniae resistant to ciprofloxacin isolated from pediatric patients in China. *BMC Infectious Diseases*, *8*(1), 1-6.

Watts, J. E., Schreier, H. J., Lanska, L., & Hale, M. S. (2017). The rising tide of antimicrobial resistance in aquaculture: sources, sinks and solutions. *Marine drugs*, *15*(6), 158-174.

WHO Scientific Group on Health Aspects of Use of Treated Wastewater for Agriculture and Aquaculture. (1989). *Health guidelines for the use of wastewater in agriculture and aquaculture: report of a WHO Scientific group*. World Health Organization.

World Health Organization, & Joint FAO/NACA/WHO Study Group on Food Safety Issues Associated with Products from Aquaculture. (1999). *Food safety issues associated with products from aquaculture: report of a joint FAO/NACA/WHO study group* (Vol. 883). World Health Organization.

World Health Organization. (2003). *Manual for the laboratory identification and antimicrobial susceptibility testing of bacterial pathogens of public health importance in the developing world: Haemophilus influenzae, Neisseria meningitidis, Streptococcus pneumoniae, Neisseria gonorrhoea, Salmonella serotype Typhi, Shigella, and Vibrio cholerae* (No. WHO/CDS/CSR/RMD/2003.6). World Health Organization.

World Health Organization. (2017). WHO Advisory Group on Integrated Surveillance of antimicrobial resistance (AGISAR). *Critically important antimicrobials for human medicine: ranking of antimicrobial agents for risk management of antimicrobial resistance due to non-human use, 5th rev. Geneva: World Health Organization*.

Yanong, R. P. (2003b). *Use of antibiotics in ornamental fish aquaculture*. University of Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, EDIS. Retrievedd from http://edis.ifas.ufl.edu

Zhang, C., Zhang, X., Shen, Y., & Wang, J. (2011). Study on the resistance of extended spectrum β-lactamases. *Journal of Animal and Veterinary Advances*, *10*(8), 1032-1036.

Zhao, S., White, D. G., Ge, B., Ayers, S., Friedman, S., English, L., ... & Meng, J. (2001). Identification and characterization of integron-mediated antibiotic resistance among Shiga toxin-producing Escherichia coli isolates. *Applied and Environmental Microbiology*, *67*(4), 1558-1564.

Zheng, D., Mai, K., Liu, S., Cao, L., Liufu, Z., Xu, W., ... & Zhang, W. (2004). Effect of temperature and salinity on virulence of Edwardsiella tarda to Japanese flounder, Paralichthys olivaceus (Temminck et Schlegel). *Aquaculture Research*, *35*(5), 494-500.

APPENDICES

APPENDIX A

ETHICAL CLEARANCE

COUNCIL FOR SCIENTIFIC AND INDUSTRIAL RESEARCH
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Date: 18TH AUGUST, 2020

ETHICAL CLEARANCE

RPN 002/CSIR-IACUC/2020

The Council for Scientific and Industrial Research (CSIR) Institutional Animal Care and Use Committee (IACUC) has reviewed and approved your protocol.

TITLE OF PROTOCOL	:	**PHENOTYPIC AND MOLECULAR CHARACTERIZATION ANTIBIOTIC RESISTANT BACTERIA ISOLATES FROM SOME SELECTED FISH FARMS IN GHANA**
PRINCIPAL INVESTIGATOR	:	**ROSEMARY AGBEKO**
SPONSOR	:	**SELF SPONSORED**

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to the IACUC for review and approval prior to implementation.

Please report all serious adverse events related to this study to CSIR-IACUC within seven days verbally and fourteen days in writing.

This certificate is valid till **5TH AUGUST, 2021**.

Prof. K. G. ANING
(CSIR-IACUC, Chairman)

Cc: Prof. Victor K. Agyeman
(Director General, CSIR)

APPENDIX B

QUESTIONNAIRE

UNIVERSITY OF CAPE COAST

COLLEGE OF AGRICULTURE AND NATURAL SCIENCES

SCHOOL OF BIOLOGICAL SCIENCES

DEPARTMENT OF MOLECULAR BIOLOGY AND BIOTECHNOLOGY

QUESTIONNAIRE

This study is being conducted to assess the use of antibiotics in aquaculture practices in Ghana. This farm/institution has been chosen to collect the primary data to obtain the most credible source of information for this research. Your candid opinion is welcome. Thank you in advance for your priceless input.

Instructions: Please write or tick [√] where appropriate

Name of farm: ……

Location……

……

Demographic Information of Respondents

1. Name……

2. Sex a. Male [ ] b. Female [ ]

3. Age …….

4. Email Address (optional): …….

5. Level of education of respondent……

6. How long has the farm been operating?

[a] <2 years [b] 2-5 years [c] 5-10 years [d] >10years

7. How many ponds do you have? ……..……

8. Which type of fish do you culture? ……

9. Which antibiotics do you use in farming?

[a] Tetracycline [b] Chloramphenicol [c] Amoxicillin [d] None

[e] Others…….

10. Where do you obtain the antibiotics?

[a] Pharmacy [b]chemical sellers [c]veterinary shops [d] other……

11. What do you use antibiotics for?

[a] Disease prevention [b] disease treatment [c] growth promotion [d] Other……

12. What other purposes do you use antibiotics for around the ponds?

……

13. How do you administer the antibiotics?

……

14. At what stage of fish growth do you use the antibiotics?

……

15. What is the source of water for the pond?

[a] River [b] Stream [c] Pipe borne water [d] Other……

16. How often do you change the water in the pond?

[a] 1-3 months [b]4-6 months [c]7-12months [d]>12 months

17. How do you discard water from the pond?

……

18. What disease do you most commonly find in the fish?

……

19. How do you identify diseased fish?

……

20. How do you treat diseased fish?

……

21. How many types of fishes do you deal with within a particular pond?

.....

22. Do the fishes suffer similar diseases?

[a]YES [b] NO

23. Has there ever been an outbreak of a dangerous disease on the farm?

[a] YES [b] NO

If YES, What disease was it? ……

24. What feed do you give to the fish?

[a] Formulated feed from shops [b] Self-manufactured feed [c] rice bran [d]others

25. Do you add manure to the ponds?

[a] YES [b] NO

26. How do you dispose of waste from around the pond?

……

APPENDIX C

TEST OF DIFFERENCE IN COLIFORM LOADS OF WATER, TILAPIA AND CATFISH SAMPLES

DESCRIPTIVE STATISTIC

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean Lower Bound	95% Confidence Interval for Mean Upper Bound	Minimum	Maximum
Tilapia	4	11.7017500	21.35	10.67	-22.27	45.67	.32300	43.70
Catfish	7	17.4152857	22.63	8.55	-3.51	38.34	.18100	58.00
Water	9	72.7411111	65.27	21.76	22.57	122.91	2.47000	167.500
Total	20	41.1692000	53.76	12.02	16.01	66.33	.18100	167.50

Test of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
Total coliform load	Based on Mean	10.457	2	17	.001
	Based on Median	3.294	2	17	.062
	Based on Median and with adjusted df	3.294	2	12.022	.072
	Based on trimmed mean	10.050	2	17	.001

Robust Tests of Equality of Means

Total coliform load

	Statistic[a]	df1	df2	Sig.
Welch	3.075	2	9.703	.092
Brown-Forsythe	5.392	2	12.343	.021

a. Asymptotically F distributed.

APPENDIX C CONTINUED

Test of difference in coliform load of pond type

Group Statistic

	Pond type	N	Mean	Std. Deviation	Std. Error Mean
total coliform load	Concrete pond	4	31.56750	38.906780	19.453390
	Earthen pond	5	105.68000	65.820187	29.435682

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	T	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Total coliform load	Equal variances assumed	4.278	.093	-1.314	5	.246	-65.204000	49.635168	-192.795261	62.387261
	Equal variances not assumed			-2.119	4.581	.093	-65.204000	30.776862	-146.546599	16.138599

APPENDIX C CONTINUED

Test of difference in Gram-positive bacteria load for water, tilapia and catfish samples

Descriptive Statistic

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean Lower Bound	95% Confidence Interval for Mean Upper Bound	Minimum	Maximum
Tilapia	4	2.4867500	4.22	2.10	-4.23	9.20	.153	8.80
Catfish	7	2.2894286	5.30	2.00	-2.62	7.19	.032	14.30
Water	9	3.5396667	5.69	1.89	-.83	7.91	.103	15.00
Total	20	2.8915000	5.07	1.13	.52	5.26	.032	15.00

Test of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
Gram-positive bacteria	Based on Mean	.259	2	17	.775
	Based on Median	.097	2	17	.908
	Based on Median and with adjusted df	.097	2	16.469	.908
	Based on trimmed mean	.218	2	17	.806

ANOVA

Gram-positive bacteria

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.974	2	3.487	.123	.885
Within Groups	481.089	17	28.299		
Total	488.063	19			

APPENDIX C CONTINUED

Test of difference in Gram-positive bacterial loads of pond type

Group Statistics

	Pond type	N	Mean	Std. Deviation	Std. Error Mean
Gram-positive bacteria	Concrete pond	8	.3302500	.32457302	.11475389
	Earthen pond	12	4.5990000	6.02916160	1.74046904

APPENDIX D

BIOCHEMICAL TEST RESULTS FOR IDENTIFICATION OF ISOLATES (COLIFORMS)

Isolate	Cit	Urea	Ind	TSI			H_2S	Identity
				slant	butt	gas		
A_{2w}^{1}	+	-	-	R	Y	+	+	*Citrobacter freundii*
A_{2w}^{2}	+	-	-	R	Y	+	-	*Enterobaccter aerogenes*
D_{2w}^{2}	+	-	+	Y	Y	+	-	*Klebsiella oxytoca*
D_{2w}^{1}	+	-	+	Y	Y	+	+	*Klebsiella oxytoca*
D_{4w}^{1}	+	-	-	Y	Y	+	-	*Enterobaccter aerogenes*
D_{2w}^{1a}	+	+	+	Y	Y	+	+	*Citrobacter freundii*
G_{2w}^{2}	+	-	+	Y	Y	+	-	*Klebsiella oxytoca*
G_{2w}^{1}	+	+	+	Y	Y	+	-	*Klebsiella oxytoca*
G_{5w}^{1}	-	-	+	Y	Y	+	-	*E. coli*
G_{1w}^{1}	+	-	+	Y	Y	+	-	*Klebsiella oxytoca*
G_{1w}^{2}	+	-	+	Y	Y	+	+	*Citrobacter freundii*
J_{5w}^{1}	+	+	+	Y	Y	+	+	*Citrobacter freundii*
G_{2w}^{3}	+	-	+	Y	Y	+	-	*Klebsiella oxytoca*
A_{1f}^{2}	+	-	-	Y	Y	-	-	*Serratia marcescens*
A_{1f}^{3}	-	+	+	R	Y	+	+	*Citrobacter freundii*
D_{2f}^{3}	+	-	-	Y	Y	+	-	*Enterobaccter aerogenes*

A_{1f}^{1}	+	+	-	Y	Y	+	+	*Citrobacter freundii*
J_2f^2	+	+	-	Y	Y	+	+	*Citrobacter freundii*
J_{5w}^{2}	+	+	-	Y	Y	+	+	*Citrobacter freundii*
J_2f^3	-	+	-	Y	Y	+	-	*Klebsiella pneumonia*
G_{1f}^{1}	+	+	-	Y	Y	+	-	*Klebsiella pneumonia*
D_{2f}^{4}	+	+	-	Y	Y	+	-	*Klebsiella pneumonia*
G_{1f}^{3}	+	+	-	Y	Y	+	-	*Klebsiella pneumonia*
D_{2f}^{2}	+	+	-	Y	Y	+	+	*Citrobacter freundii*
J_5f^2	+	+	-	Y	Y	+	-	*Klebsiella pneumonia*
J_1f^1	-	+	-	Y	Y	+	+	*Citrobacter freundii*
J_5f^1	+	+	-	Y	Y	+	+	*Citrobacter freundii*
K_{1w}^{1}	+	-	-	Y	Y	+	+	*Citrobacter freundii*
H_{3f}^{3}	+	+	-	Y	Y	+	+	*Citrobacter freundii*
K_{0w}^{1}	+	-	+	R	Y	+	-	*Citrobacter diversus*
H_{3f}^{2}	+	-	-	Y	Y	+	-	*Enterobacter areogenes*
H_{ow}^{1}	-	-	-	R	Y	+	-	*Salmonella paratyphityphi 'A'*

Isolate	Cit	Urea	Ind	TSI				Identity
				slant	butt	gas	H_2S	
H_{3f}^{1}	+	+	-	Y	Y	+	-	*Enterobacter areogenes*
H_{1w}^{1}	+	-	-	R	Y	+	+	*Salmonella sp.*
K_{ow}^{2}	-	-	-	Y	Y	+	-	*Enterobacter areogenes*
K_{4w}^{1}	+	-	-	Y	Y	+	-	*Enterobacter areogenes*
K_{1w}^{3}	+	+	-	Y	Y	+	-	*Klebsiella pneumonia*
K_{1w}^{2}	+	+	-	Y	Y	+	-	*Klebsiella pneumonia*
H_{1f}^{2}	+	+	+	Y	Y	+	-	*Klebsiella oxytoca*
H_{ow}^{2}	+	-	-	Y	Y	+	-	*Enterobacter areogenes*
H_{ow}^{2i}	+	-	+	Y	Y	+	-	*Klebsiella pneumonia*
D_{2w}^{3}	+	+	-	Y	Y	+	+	*Citrobacter freundii*
K_{3w}^{1}	+	+	-	Y	Y	+	-	*Klebsiella pneumonia*
G_{1f}^{3}	+	+	-	Y	Y	+	-	*Klebsiella pneumonia*
D_{2f}^{4}	+	+	-	R	Y	+	-	*Citrobacter freundii*
Tt_{ow}^{2}	+	+	-	Y	Y	+	+	*Citrobacter freundii*
T_{ow}^{1}	+	+	-	Y	Y	+	-	*Klebsiella pneumonia*
T_{5w}^{1}	+	+	-	Y	Y	+	-	*Klebsiella pneumonia*
H_{1f}^{1}	+	+	-	Y	Y	+	-	*Klebsiella pneumonia*
K_{3w}^{3}	+	+	-	Y	Y	+	-	*Klebsiella pneumonia*
T_{3w}^{1}	+	+	+	Y	Y	+	+	*Citrobacter freundii*
T_{5w}^{3}	+	+	+	Y	Y	+	-	*Klebsiella oxytoca*
D_{2f}^{1}	+	+	-	Y	Y	+	-	*Klebsiella pneumoniae*

J_{2f}^{1}	-	-	-	Y	Y	+	+	*Citrobacter freundii*
T_{5w}^{2}	+	-	-	R	Y	+	+	*Salmonella sp.*
G_{5w}^{2}	+	+	-	R	Y	+	+	*Proteus mirabilis*
T_{3w}^{2}	+	+	-	Y	Y	+	+	*Citrobacter freundii*
Tt_{w}^{1}	+	-	-	Y	Y	+	-	*Enterobacter areogenes*
Tt_{ow}^{2}	+		-	Y	Y	+	+	*Citrobacter freundii*
T_{3f}^{2}	-	-	-	R	R	-	-	*Shigella sonnei*
$UT_{f\,1}^{1}$	+	+	-	Y	Y	+	+	*Citrobacter freundii*
$UT_{f\,1}^{3}$	+	+	-	Y	Y	+	-	*Klebsiella pneumoniae*
$UT_{f\,1}^{4}$	+	+	-	R	Y	+	+	*Proteus mirabilis*
$UT_{f\,2}^{1}$	+	-	-	R	Y	+	-	*Salmonella chloreausius*
$UT_{f\,1}^{2}$	+	+	-	Y	Y	+	+	*Citrobacter freundii*
UT_{w6}^{1}	-	+	-	Y	Y	+	-	*Klebsiella pneumoniae*
UT_{w6}^{2}	+	+	-	R	Y	+	-	*Citrobacter freundii*

Isolate	Cit	Urea	Ind	TSI				Identity
				slant	butt	gas	H_2S	
UT_{w4}^{1}	+	+	-	R	Y	+	-	*Citrobacter freundii*
UT_{f3}^{1}	+	+	-	Y	Y	+	-	*Klebsiella pneumoniae*
UC_{fo}^{1}	+	+	+	Y	Y	+	-	*Klebsiella oxytoca*
UC_{f4}^{2}	+	+	-	R	Y	+	-	*Citrobacter freundii*
UT_{f2}^{2}	+	-	-	R	Y	+	-	*Salmonella chloreausius*
UC_{f0}^{2}	+	+	-	Y	Y	+	-	*Klebsiella pneumoniae*
UC_{f4}^{1}	-	-	-	R	Y	+	-	*Salmonella paratyphi 'A'*
UC_{w4}^{1}	+	+	+	Y	Y	+	+	*Citrobacter freundii*
AKf_{0}^{4}	+	+	+	Y	Y	+	+	*Citrobacter freundii*
AKf_{0}^{2}	+	+	+	Y	Y	+	-	*Klebsiella oxytoca*
AKf_{2}^{2}	+	-	-	Y	Y	+	-	*Enterobacter aerogenes*
AKf_{1}^{2}	+	+	-	R	Y	+	-	*Citrobacter freundii*
AKw_{7}^{1}	+	-	+	R	Y	+	+	*Edwardsiella tarda*
AKw_{6}^{1}	+	-	-	Y	Y	+	-	*Enterobacter aerogenes*
AKw_{4}^{3}	+	+	-	Y	Y	+	-	*Klebsiella pneumoniae*
AKw_{4}^{1}	+	+	+	Y	Y	+	+	*Citrobacter freundii*
AKw_{7}^{4}	+	-	-	R	Y	+	+	*Citrobacter freundii*
AKf_{1}^{1}	+	-	-	R	Y	+	+	*Salmonella sp.*
AKw_{6}^{2}	+	-	-	R	Y	+	-	*Enterobacter aerogenes*

AKw_4^2	+		-	R	Y	+	-	*Salmonella sp.*
AKf_0^3	+	-	+	Y	Y	+	+	*Citrobacter freundii*
AKw_4^{1a}	+	+	-	Y	Y	+	-	*Klebsiella pneumoniae*
AKw_4^4	+	+	-	Y	Y	+	-	*Klebsiella pneumoniae*
AKw_7^5	+	-	-	Y	Y	+	+	*Citrobacter freundii*
AKw_5^2	+	-	+	Y	Y	+	+	*Citrobacter freundii*
AKw_7^3	+	-	+	Y	Y	+	+	*Citrobacter freundii*
AKw_6^3	+	+	-	Y	Y	+	-	*Citrobacter freundii*
AKw_5^1	+	+	-	Y	Y	+	+	*Citrobacter freundii*
AKw_7^2	+	-	-	Y	Y	+	+	*Citrobacter freundii*
AKf_2^1	+	+	-	Y	Y	+	+	*Citrobacter freundii*
AKw_7^3	+		-	R	Y	+	+	*Salmonella sp.*
AKw_4^2	+	-	-	R	Y	+	+	*Salmonella choleraesuis*

APPENDIX D CONTINUED

Gram-positive bacterial isolates

Isolate	Cit	Urea	Ind	TSI				Cat	Coag	Oxi	Gel	Man	Col	Identity
				Slant	Butt	Gas	H_2S							
AK_{f01}	-	-	-	**Y**	**Y**	+	-	+	+	-	-	+	**W**	*S. aureaus*
AK_{w3}^{2}	+	+	-	**Y**	**Y**	+	-	-	+	-	+	+	**W**	*S. intermedius*
AK_{w6}^{3}	-	-	-	**Y**	**Y**	+	-	+	+	-	-	+	**W**	*S. aureaus*
AK_{w6}^{1}	-	-	-	**Y**	**Y**	+	+	+	-	+	+	+	**G**	*Micrococcus sp.*
UC_{w2}	+	+	-	**Y**	**Y**	+	-	-	-	-	-	+	**G**	*S. saprophyticus*
UT_{w2}^{2}	-	-	-	**R**	**R**	-	-	+	-	-	+	+	**W**	*Athrobacter sp.*
UT_{f2}^{2}	+	+	-	**Y**	**Y**	+	-	+	+	+	+	-	**P**	*Micrococcus sp.*
UT_{f2}^{1}	-	-	-	**Y**	**Y**	+	-	+	+	-	-	+	**W**	*S. aureaus*
UC_{f2}	+	+	-	**Y**	**Y**	+	-	+	+	-	+	+	**W**	*S. aureaus*
UT_{w2}^{1}	-	-	-	**R**	**Y**	-	-	+	+	-	-	+	**W**	*S. aureaus*
UT_{w4}	-	-	-	**R**	**Y**	-	-	+	+	-	-	+	**G**	*S. aureaus*
A_{1f}^{4}	+	-	-	**R**	**R**	-	-	+	+	-	+	+	**W**	*S. aureaus*

A_{1f}^{2}	+	+	-	**Y**	**Y**	+	-		-	-	+	+	**S**	*Streptococcus sp.*
D_{2f}^{5}	-	-	-	**R**	**R**	-	-		+	-	-	+	**W**	*S. aureaus*
G_{1f}^{4}	-	-	-	**R**	**R**	-	-		+	-	-	+	**W**	*S. aureaus*
J_{1f}	-	-	-	**Y**	**Y**	-	-		+	-	+	+	**W**	*S. aureaus*
J_{2f}^{4}	+	+	-	**R**	**Y**	+	+		-	-	-	+	**G**	*Streptococcus sp.*
J_{2f}^{5}	-	-	-	**Y**	**Y**	+	-		+	-	-	+	**W**	*S. aureaus*
A_{1f}^{5}	-	+	-	**R**	**R**	-	-		-	-	-	+	**W**	*S. xylosis*
H_{0w}^{2}	-	+	-	**Y**	**Y**	-	-	+	+	-	-	+	**W**	*S. aureaus*
K_{ow}^{1}	-	+	-	**Y**	**Y**	-	-	+	+	-	-	+	**W**	*S. aureaus*
J_{2f}^{5}	-	-	-	**Y**	**Y**	-	-	+	+	-	+	+	**G**	*S. aureaus*
A_{1f}^{2}	-	-	-	**R**	**Y**	+	-	-	-	-	+	+	**G**	*Streptococcus sp.*
H_{0w}^{3}	-	-	-	**Y**	**Y**	-	-	+	-	+	+	+	**W**	*S. aureaus*
G_{1f}^{4}	-	+	-	**Y**	**Y**	+	-	+	+	-	-	+	**W**	*S. aureaus*
D_{2f}^{1}	-	-	-	**Y**	**Y**	+	-	+	+	-	+	+	**W**	*S. aureaus*
T_{0w}^{2}	-	-	-	**R**	**Y**	+	-	+	-	+	+	+	**G**	*S. aureaus*
A_{1f}^{5}	-	+	+	**Y**	**Y**	+	+	-	-	-	-	+	**G**	*S. xylosus.*
T_{3f}^{1}	-	-	-	**Y**	**Y**	+	-	+	+	-	+	+	**W**	*S. aureaus*
H_{0w}^{1}	-	-	-	**Y**	**Y**	-	-	+	+	-	+	+	**W**	*S. aureaus*

H_{1f}^{1}	-	-	-	**Y**	**Y**	-	-	+	+	-	-	+	**W**	*Streptococcus sp.*
K_{0w}^{2}	-	-	-	**Y**	**Y**	-	-	+	-	-	-	-	**W**	*Micrococcus sp.*
Tt_{3f}^{1}	-	-	-	**Y**	**Y**	-	-	-	+	-	+	+	**W**	*Streptococcus sp.*
J_{1f}	-	-	-	**Y**	**Y**	-	-	+	+	-	+	+	**W**	*S. aureaus*
A_{1f}^{4}	-	+	-	**Y**	**Y**	+	+	-	+	-	+	+	**W**	*S. aureus*
T_{0w}^{1}	-	-	-	**Y**	**Y**	-	-	+	-	-	+	+	**W**	*S. aureaus*
AK_{f0}^{3}	-	-	-	**Y**	**Y**	-	-	+	+	-	+	+	**W**	*S. aureaus*
AK_{f1}^{1}	-	-	-	**Y**	**Y**	-	-	+	+	-	+	+	**W**	*S. aureaus*
AK_{f0}^{2}	+	+	-	**R**	**Y**	+	-	+	+	-	+	+	**W**	*S. aureaus*
AK_{f1}^{2}	-	+	-	**Y**	**Y**	-	-	+	+	+	-	+	**W**	*Cellobioscoccus sp.*
AK_{w3}^{1}	+	+	-	**Y**	**Y**	+	-	+	+	+	+	+	**G**	*Micrococcus sp.*
AK_{w0}^{1}	-	-	-	**Y**	**Y**	+	-	+	+	+	-	+	**G**	*Cellobioscoccus sp..*
AK_{w6}^{2}	-	-	-	**Y**	**Y**	+	-	+	-	+	+	+	**G**	*Cellobioscoccus sp.*
AK_{w0}^{2}	-	-	-	**Y**	**Y**	+	-	+	+	+	-	+	**G**	*S. aureus*
AK_{f2}^{1}	-	-	-	**Y**	**Y**	+	-	+	+	+	-	+	**G**	*Mocrococcus sp.*

APPENDIX E

Antibiotic susceptibility profile of coliforms

		MEAN DIAMETER(CM)															
Identity	**Isolate**	**COT**		**GEN**		**CRX**		**CHL**		**CTR**		**CTX**		**AMP**		**TET**	
Citrobacter freundii	A_{2w}^{1}	2.8	S	2.0	S	0.0	R	2.2	S	2.8	S	2.0	S	0.0	R	2.0	S
Enterobaccter aerogenes	A_{2w}^{2}	0.0	R	1.9	S	0.0	R	3.7	S	4.3	S	3.5	S	0.0	R	2.5	S
Klebsiella oxytoca	D_{2w}^{2}	0.0	R	2.1	S	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R
Klebsiella oxytoca	D_{2w}^{1}	0.0	R	2.0	S	0.0	R	0.0	R	2.5	S	2.6	S	0.0	R	2.0	S
Enterobaccter aerogenes	D_{4w}^{1}	2.7	S	2.1	S	0.0	R	2.7	S	3.0	S	3.1	S	0.0	R	0.0	R
Citrobacter freundii	D_{2w}^{1a}	0.0	R	2.1	S	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R
Klebsiella oxytoca	G_{2w}^{2}	0.0	R	1.6	S	0.0	R	0.0	R	1.0	R	0.0	R	0.0	R	2.3	S
Klebsiella oxytoca	G_{2w}^{1}	0.0	R	2.3	S	0.0	R	2.9	S	3.4	S	2.9	S	0.0	R	1.3	R
E. coli	G_{5w}^{1}	2.30	S	2.3	S	2.5	R	1.5	S	2.5	S	2.5	S	0.0	R	3.0	R
Klebsiella oxytoca	G_{1w}^{1}	2.1	S	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R

Citrobacter freundii	G_{1w}^{2}	1.1	R	0.0	R	1.2	R	2.7	S	0.0	R	0.0	R	0.0	R	0.0	R
Citrobacter freundii	J_{5w}^{1}	2.7	S	2.1	S	0.0	R	2.4	S	3.0	S	2.1	S	0.0	R	2.1	S
Klebsiella oxytoca	G_{2w}^{3}	0.0	R	1.9	S	0.0	S	0.0	R	0.0	S	3.2	S	0.0	R	0.0	R
Serratia marcescens	A_{1f}^{2}	0.0	R	1.8	S	0.0	R	0.0	S	3.3	S	3.0	S	0.0	R	0.0	R
Citrobacter freundii	A_{1f}^{3}	0.0	S	1.5	S	0.0	R	0.0	R	1.2	R	0.0	R	0.0	R	0.0	R
Enterobaccter aerogenes	D_{2f}^{3}	0.0	R	2.0	S	0.0	R	1.0	R	3.2	S	2.8	S	0.0	R	1.4	R
Citrobacter freundii	A_{1f}^{1}	0.0	R	1.7	S	0.0	R	0.0	R	1.3	R	0.0	R	0.0	R	0.0	R
Citrobacter freundii	$J_{2}f^{2}$	0.0	R	1.9	S	0.0	R	0.0	R	0.0	R	0.0	0	0.0	R	0.0	R
Citrobacter freundii	J_{5w}^{2}	2.0	S	2.0	S	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R
Klebsiella pneumoniae	$J_{2}f^{3}$	2.4	S	1.9	S	0.0	R	0.0	R	1.2	R	0.0	0	0.0	R	0.0	R
Klebsiella pneumoniae	G_{1f}^{1}	3.0	S	2.0	S	0.0	R	2.7	S	3.2	S	2.9	S	0.0	R	2.4	S
Klebsiella pneumoniae	D_{2f}^{4}	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R	0.0	0	0.0	R	0.0	R
Klebsiella pneumoniae	G_{1f}^{3}	0.0	R	2.3	S	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R	1.9	S
Citrobacter freundii	D_{2f}^{2}	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R	0.0	0	0.0	R	0.0	R

Klebsiella pneumoniae	J_5f^2	2.8	S	2.2	S	0.0	R	2.3	S	3.1	S	2.7	S	0.0	R	0.0	R
Citrobacter freundii	J_1f^1	0.0	R	2.0	S	0.0	R	0.0	R	0.0	R	0.0	0	0.0	R	0.0	R
Citrobacter freundii	J_5f^1	2.5	S	1.5	S	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R
Citrobacter freundii	K_{1w}^1	2.6	S	1.9	S	0.0	R	2.2	S	2.8	S	2.9	0	0.0	R	1.5	S
Citrobacter freundii	H_{3f}^3	1.9	S	1.8	S	0.0	R	2.2	S	0.0	R	1.2	R	0.0	R	1.1	R
Citrobacter diversus	K_{0w}^1	0.0	R	2.0	S	0.0	R	2.6	S	2.1	S	0.0	R	0.0	R	0.0	R
Enterobacter areogenes	H_{3f}^2	2.6	S	2.0	S	0.0	R	2.7	S	1.2	R	0.0	R	0.0	R	1.1	R
Salmonella paratyphityphi 'A'	H_{ow}^1	1.8	S	1.7	S	1.3	R	3.4	S	1.2	R	2.5	S	0.0	R	2.5	S
Enterobacter areogenes	H_{3f}^1	1.6	S	1.9	S	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R	1.0	R
Salmonella sp.	H_{1w}^1	3.2	S	1.5	S	0.0	R	2.4	S	3.2	S	2.9	S	0.0	R	0.0	R
Enterobacter areogenes	K_{ow}^2	2.4	S	2.0	S	0.0	R	3.5	S	1.6	R	0.0	R	0.0	R	2.5	R
Enterobacter areogenes	K_{4w}^1	2.0	S	1.7	S	0.0	R	2.9	S	2.9	S	2.2	R	0.0	R	2.1	R
Klebsiella pneumoniae	K_{1w}^3	0.0	R	2.0	S	0.0	R	2.8	S	2.6	S	2.8	S	0.0	R	0.0	R
Klebsiella pneumoniae	K_{1w}^2	0.0	R	2.0	S	0.0	R	0.0	R	2.7	S	2.9	S	0.0	R	0.0	R
Klebsiella oxytoca	H_{1f}^2	0.0	R	1.6	S	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R

Enterobacter areogenes	H_{ow}^{2}	0.0	R	1.9	S	0.0	R	0.0	R	3.0	S	2.6	S	0.0	R	0.0	R
Klebsiella pneumoniae	H_{ow}^{2i}	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R
Citrobacter freundii	D_{2w}^{3}	1.9	S	1.9	S	0.0	R	1.3	S	0.0	R	0.0	R	0.0	R	0.0	R
Klebsiella pneumoniae	K_{3w}^{1}	2.5	S	2.0	S	0.0	R	0.0	R	1.0	R	0.0	R	0.0	R	0.0	R
Klebsiella pneumoniae	G_{1f}^{3}	2.5	S	2.0	S	0.0	R	2.4	S	3.0	S	2.9	S	0.0	R	2.0	S
Citrobacter freundii	D_{2f}^{4}	2.0	S	2.5	S	0.0	R	0.0	R	2.1	S	1.2	R	0.0	R	0.0	R
Citrobacter freundii	Tt_{ow}^{2}	2.2	S	2.4	S	0.0	R	2.9	S	2.6	S	1.3	R	0.0	R	2.1	S
Klebsiella pneumoniae	T_{ow}^{1}	0.0	R	1.9	S	0.0	R	0.0	R	3.0	S	3.0	S	0.0	R	0.0	R
Klebsiella pneumoniae	T_{5w}^{1}	2.0	S	2.1	S	0.0	R	2.8	S	1.8	R	0.0	R	0.0	R	0.0	R
Klebsiella pneumoniae	H_{1f}^{1}	1.7	S	2.0	S	0.0	R	1.9	R	1.1	R	0.0	R	0.0	R	0.0	R
Klebsiella pneumoniae	K_{3w}^{3}	2.4	S	2.1	S	0.0	R	2.9	S	2.5	S	1.3	R	0.0	R	1.9	S
Citrobacter freundii	T_{3w}^{1}	0.0	R	1.9	S	2.1	S	0.0	R	3.0	S	2.7	S	0.0	R	0.0	R
Klebsiella oxytoca	T_{5w}^{3}	2.1	S	1.6	S	0.0	R	2.2	S	3.0	S	2.6	S	0.0	R	1.0	R
Klebsiella pneumoniae	D_{2f}^{1}	0.0	R	2.0	S	0.0	R	0.0	R	2.5	S	1.2	R	0.0	R	1.7	S
Citrobacter freundii	J_{2f}^{1}	2.6	S	2.1	S	O.O	R	0.0	R	1.8	R	0.0	R	0.0	R	0.0	R

Salmonella sp.	T_{5w}^{2}	0.0	R	1.7	S	1.7	S	0.0	R	3.5	S	3.3	S	0.0	R	0.0	R
Proteus mirabilis	G_{5w}^{2}	3.0	S	2.0	S	0.0	R	1.5	S	2.7	S	2.5	S	0.0	R	1.5	S
Citrobacter freundii	T_{3w}^{2}	2.1	S	1.7	S	0.0	R	1.2	R	2.8	S	2.8	S	0.0	R	1.7	S
Enterobacter areogenes	Tt_{w}^{1}	0.0	R	2.4	S	1.1	R	2.3	S	1.8	R	0.0	R	0.0	R	0.0	R
Citrobacter freundii	Tt_{ow}^{2}	2.2	S	2.4	S	0.0	R	2.9	S	2.6	S	1.3	R	0.0	R	2.1	S
Shigella sonnei	T_{3f}^{2}	2.5	S	2.5	S	0.0	R	2.5	R	0.0	R	0.0	R	0.0	R	0.0	R
Citrobacter freundii	UT_{f1}^{1}	2.3	S	1.9	S	0.0	R	1.7	S	1.2	R	0.0	R	0.0	R	1.9	S
Klebsiella pneumoniae	UT_{f1}^{3}	2.4	S	2.1	S	0.9	R	2.1	S	3.0	S	2.3	S	0.0	R	2.0	S
Proteus mirabilis	UT_{f1}^{4}	2.2	S	1.9	S	0.0	R	1.8	S	0.0	R	0.0	R	0.0	R	2.0	R
Salmonella chloreausius	UT_{f2}^{1}	3.0	S	2.0	S	2.4	S	3.1	S	3.3	S	3.3	S	0.0	R	2.4	S
Citrobacter freundii	UT_{f1}^{2}	2.8	S	1.8	S	1.0	R	2.8	S	2.7	S	2.7	S	0.0	R	2.3	S
Klebsiella pneumoniae	UT_{w6}^{1}	2.4	S	1.0	R	0.0	R	1.8	S	2.0	S	1.0	R	0.0	R	1.9	S
Citrobacter freundii	UT_{w6}^{2}	2.2	S	2.0	S	0.0	R	0.9	R	1.0	R	0.0	R	0.0	R	2.0	S
Citrobacter freundii	UT_{w4}^{1}	2.1	S	2.0	S	0.0	R	0.0	R	1.9	R	0.0	R	0.0	R	2.0	S
Klebsiella pneumoniae	UT_{f3}^{1}	2.6	S	1.7	S	1.0	R	0.0	R	2.6	S	2.8	S	0.0	R	2.0	S

Klebsiella oxytoca	UC_{fo}^{1}	2.2	S	1.9	S	0.0	R	0.0	R	3.2	S	2.4	S	0.0	R	0.0	R
Citrobacter freundii	UC_{f4}^{2}	2.1	S	1.9	S	0.0	R	1.2	R	2.5	S	2.0	R	0.0	R	1.2	R
Salmonella chloreausius	UT_{f2}^{2}	3.4	S	2.0	S	2.4	S	3.5	S	3.6	S	3.4	S	0.0	R	2.9	S
Klebsiella pneumoniae	UC_{f0}^{2}	2.1	S	2.0	S	0.0	R	2.5	S	2.8	S	2.1	R	0.0	R	1.2	R
Salmonella paratyphi 'A'	UC_{f4}^{1}	3.1	S	1.8	S	0.0	R	2.0	S	2.8	S	2.4	S	0.0	R	18	S
Citrobacter freundii	UC_{w4}^{1}	3.0	S	2.1	S	0.0	R	3.0	S	2.9	S	2.5	S	0.0	R	2.0	S
Citrobacter freundii	AKf_{0}^{4}	2.5	S	2.2	S	0.0	R	1.8	S	3.2	S	0.0	R	0.0	R	1.0	R
Klebsiella oxytoca	AKf_{0}^{2}	2.6	S	1.8	S	0.0	R	2.5	S	3.3	S	2.9	S	0.0	R	2.3	S
Enterobacter aerogenes	AKf_{2}^{2}	2.4	S	1.9	S	2.9	S	3.5	S	3.8	S	3.4	S	0.0	R	2.8	S
Citrobacter freundii	AKf_{1}^{2}	2.5	S	2.1	S	0.0	R	1.9	S	2.5	S	3.0	S	0.0	R	2.0	R
Edwardsiella tarda	AKw_{7}^{1}	2.6	S	1.8	S	2.5	S	2.0	S	2.8	S	2.9	S	2.9	S	2.2	S
Enterobacter aerogenes	AKw_{6}^{1}	0.0	S	2.2	S	0.0	R	0.0	R	3.2	S	3.0	S	0.0	R	1.0	R
Klebsiella pneumoniae	AKw_{4}^{3}	2.6	S	1.8	S	0.0	R	0.0	R	3.3	S	0.0	R	0.0	R	2.3	S
Citrobacter freundii	AKw_{4}^{1}	2.4	S	1.9	S	0.0	R	0.0	R	3.8	S	2.2	R	0.0	R	2.8	S
Citrobacter freundii	AKw_{7}^{4}	2.5	S	2.1	S	0.0	R	0.0	R	2.5	S	3.0	S	0.0	R	2.0	R

Salmonella sp.	AKf_1^1	2.6	S	1.8	S	2.5	S	0.0	R	2.8	S	2.9	S	0.0	S	2.2	S
Enterobacter aerogenes	AKw_6^2	2.5	S	2.2	S	0.0	R	1.8	S	0.9	R	2.2	R	0.0	R	1.0	R
Salmonella sp.	AKw_4^2	0.0	R	1.7	S	2.6	S	1.2	R	0.0	R	2.8	S	0.0	R	1.0	R
Citrobacter freundii	AKf_0^3	2.3	S	2.4	S	1.1	R	1.0	R	1.8	R	0.0	R	0.0	R	0.0	R
Klebsiella pneumoniae	AKw_4^{1a}	2.2	S	2.4	S	0.0	R	0.0	R	2.6	S	2.8	S	0.0	R	2.1	S
Klebsiella pneumoniae	AKw_4^4	2.5	S	2.5	S	0.0	R	2.5	S	0.0	R	2.9	S	0.0	R	0.0	R
Citrobacter freundii	AKw_7^5	2.3	S	1.9	S	0.0	R	0.0	R	3.0	S	0.0	R	0.0	R	2.3	R
Citrobacter freundii	AKw_5^2	2.4	S	2.4	S	1.1	R	2.3	S	2.7	S	0.0	R	0.0	R	2.3	S
Citrobacter freundii	AKw_7^3	2.2	S	2.4	S	0.0	R	0.0	R	0.0	R	1.3	R	0.0	R	2.1	S
Citrobacter freundii	AKw_6^3	2.5	S	2.5	S	0.0	R	2.5	S	2.9	S	0.0	R	0.0	R	2.2	S
Citrobacter freundii	AKw_5^1	2.3	S	1.9	S	0.0	R	0.0	R	2.9	S	2.5	S	0.0	R	1.9	S
Citrobacter freundii	AKw_7^2	2.4	S	2.1	S	0.9	R	0.0	R	3.0	S	0.0	R	0.0	R	2.0	S
Citrobacter freundii	AKf_2^1	2.2	S	1.9	S	0.0	R	1.8	S	2.8	S	0.0	R	0.0	R	2.0	S
Salmonella sp.	AKw_7^3	0.0	R	2.4	S	3.0	S	0.0	R	3.0	S	2.9	S	0.0	R	1.8	S

Salmonella choleraesuis	AKw_4^2	2.2	S	2.4	S	2.9	S	2.9	S	2.6	S	2.8	S	0.0	R	2.1	S

APPENDIX E CONTINUED

Antibiotic susceptibility profile of Gram-positive bacterial isolates

		MEAN DIAMETER(CM)															
Identity	**Isolate**	COT		CRX		GEN		PEN		AMP		FLX		ERY		TET	
S. aureaus	AK_{f01}	3.2	S	0.0	R	1.9	S	0.0	R	0.0	R	0.0	R	0.0	R	2.5	S
S. intermedius	AK_{w3}^2	2.4	S	0.0	R	2.1	S	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R
S. aureaus	AK_{w6}^3	2.9	S	0.0	R	2.3	S	0.0	R	0.0	R	0.0	R	0.0	R	2.3	S
Micrococcus sp.	AK_{w6}^1	3.2	S	3.5	S	2.4	S	1.8	R	2.8	R	1.9	R	2.3	S	2.9	S
S. saprophyticus	UC_{w2}	3.1	S	0.0	R	2.0	S	0.0	R	0.0	R	0.0	R	0.0	R	2.0	S
Athrobacter sp.	UT_{w2}^2	2.6	S	2.8	S	2.5	S	0.0	R	2.0	R	0.0	R	2.1	S	2.5	S
Micrococcus sp.	UT_{f2}^2	2.4	S	0.0	R	2.0	S	0.0	R	0.0	R	0.0	R	0.0	R	2.1	S
S. aureaus	UT_{f2}^1	2.9	S	0.0	R	2.3	S	0.0	R	0.0	R	0.0	R	0.0	R	2.1	S

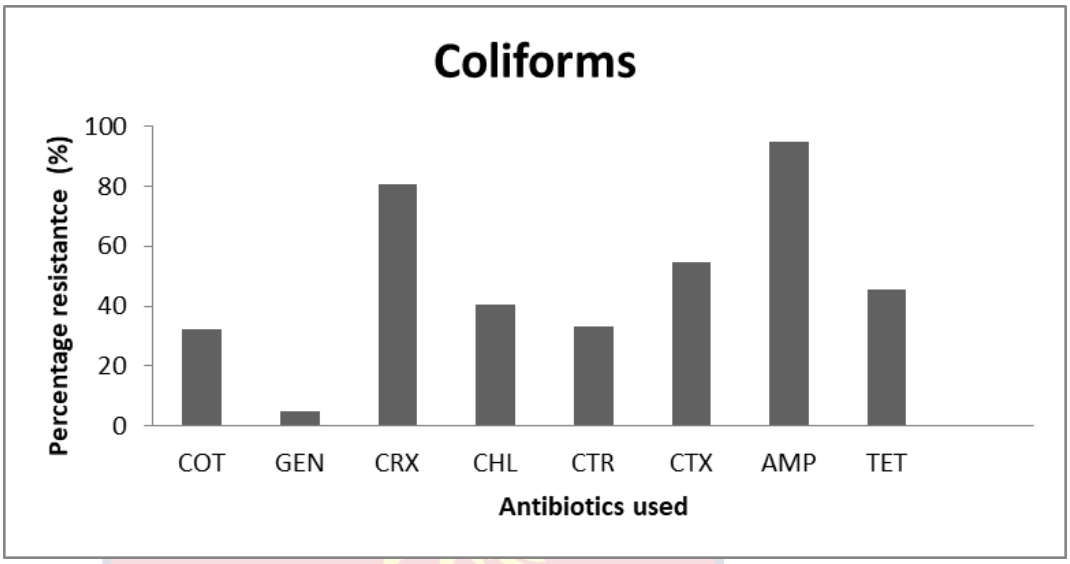
S. aureaus	UC_{f2}	2.7	S	0.0	R	2.3	S	0.0	R	0.0	R	0.0	R	0.0	R	2.1	S
S. aureaus	UT_{w2}^{1}	3.0	S	2.8	S	2.3	S	0.0	R	1.9	R	0.0	R	2.5	S	2.5	S
S. aureaus	UT_{w4}	2.9	S	2.7	S	2.7	S	0	R	2.0	R	0.0	R	2.7	S	2.8	S
S. aureaus	A_{1f}^{4}	3.0	S	2.5	S	3.8	S	2.5	R	2.5	R	1.3	R	1.0	R	3.0	S
Streptococcus sp.	A_{1f}^{2}	3.5	S	0.0	R	1.8	S	0.0	R	0.0	R	0.0	R	1.0	R	3.5	S
S. aureaus	D_{2f}^{5}	3.5	S	2.6	S	2.8	S	0.0	R	2.0	R	0.0	R	3.1	S	3.2	S
S. aureaus	G_{1f}^{4}	3.7	S	3.2	S	2.7	S	0.0	R	2.3	R	0.0	R	3.2	S	3.7	S
S. aureaus	J_{1f}	3.7	S	3.8	S	2.3	S	0.0	R	2.4	R	0.0	R	2.9	S	3.6	S
Streptococcus sp.	J_{2f}^{4}	0.0	R	0.0	R	2.4	R	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R
S. aureaus	J_{2f}^{5}	3.7	S	2.9	S	2.7	S	0.0	R	2.1	R	0.0	R	2.8	S	3.5	S
S. xylosis	A_{1f}^{5}	4.6	S	3.8	S	3.1	S	0.0	R	0.0	R	0.0	R	0.0	R	4.3	S
S. aureaus	H_{0w}^{2}	3.2	S	2.8	S	2.6	S	0.0	R	2.0	R	0.0	0	0.0	R	2.7	S
S. aureaus	K_{ow}^{1}	3.1	S	3.2	S	2.9	S	0.0	R	1.9	R	0.0	R	0.0	R	2.9	S
S. aureaus	J_{2f}^{5}	3.1	S	0.0	R	2.6	S	0.0	R	0.0	R	0.0	R	0.0	R	3.0	S
Streptococcus sp.	A_{1f}^{2}	3.5	S	3.8	S	3.0	S	2.1	R	2.8	R	2.5	S	2.7	S	3.0	S

S. aureaus	H_{0w}^{3}	3.4	S	2.8	S	2.8	S	0.0	R	0.0	R	0.0	R	2.8	R	3.2	S
S. aureaus	G_{1f}^{4}	4.0	S	3.1	S	3.0	S	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R
S. aureaus	D_{2f}^{1}	3.2	S	0.0	R	2.2	S	0.0	R	1.0	R	0.0	0	0.0	R	3.1	S
S. aureaus	T_{0w}^{2}	3.3	S	2.9	S	2.2	S	0.0	R	2.2	R	0.0	R	2.9	S	1.1	R
S. xylosus.	A_{1f}^{5}	0.0	R	0.0	R	2.1	S	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R
S. aureaus	T_{3f}^{1}	3.5	S	3.2	S	2.9	S	0.0	R	2.3	R	0.0	R	3.0	S	1.9	R
S. aureaus	H_{0w}^{1}	3.9	S	3.2	S	3.8	S	0.0	R	2.3	R	0.0	R	3.2	S	3.6	S
Streptococcus sp.	H_{1f}^{1}	3.4	S	3.1	S	3.0	S	0.0	R	1.9	R	1.8	R	3.0	S	2.1	S
Micrococcus sp.	K_{0w}^{2}	3.1	S	2.6	S	2.9	S	0.0	R	0.0	R	1.1	R	0.0	R	1.5	S
Streptococcus sp.	Tt_{3f}^{1}	4.1	S	4.1	S	3.2	S	1.5	R	2.9	S	0.0	R	2.7	S	3.4	S
S. aureaus	J_{1f}	3.0	S	0.0	R	2.6	S	0.0	R	1.5	R	0.0	R	0.0	R	0.0	R
S. aureus	A_{1f}^{4}	3.0	S	0.0	R	3.0	S	1.5	R	2.7	R	2.3	R	3.1	S	3.5	S
S. aureaus	T_{0w}^{1}	3.2	S	2.8	S	2.7	S	0.0	R	0.0	R	0.0	R	2.1	S	3.0	S
S. aureaus	AK_{f0}^{3}	2.5	S	0.0	R	2.5	S	0.0	R	0.0	R	0.0	R	0.0	R	2.3	R
S. aureaus	AK_{f1}^{1}	3.1	S	3.3	S	2.3	S	1.5	R	2.7	R	1.8	R	2.9	S	3.1	S

S. aureaus	AK_{f0}^{2}	2.3	S	0.0	R	2.0	S	0.0	R	0.0	R	0.0	R	0.0	R	2.1	S
Cellobioscoccus sp.	AK_{f1}^{2}	0.0	R	0.0	R	2.1	S	0.0	R	0.0	R	0.0	R	0.0	R	2.4	S
Micrococcus sp.	AK_{w3}^{1}	2.9	S	3.5	S	2.6	S	0.0	R	0.0	R	0.0	S	2.3	S	2.9	S
Cellobioscoccus sp..	AK_{w0}^{1}	2.9	S	0.0	R	2.2	S	0.0	R	0.0	R	0.0	R	0.0	R	2.1	S
Cellobioscoccus sp.	AK_{w6}^{2}	0.0	R	0.0	R	2.3	S	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R
S. aureus	AK_{w0}^{2}	0.0	R	0.0	R	2.2	S	0.0	R	0.0	R	0.0	R	0.0	R	0.0	R
Mocrococcus sp.	AK_{f2}^{1}	3.0	S	0.0	R	2.2	S	0.0	R	0.0	R	0.0	R	0.0	R	2.2	S

APPENDIX F

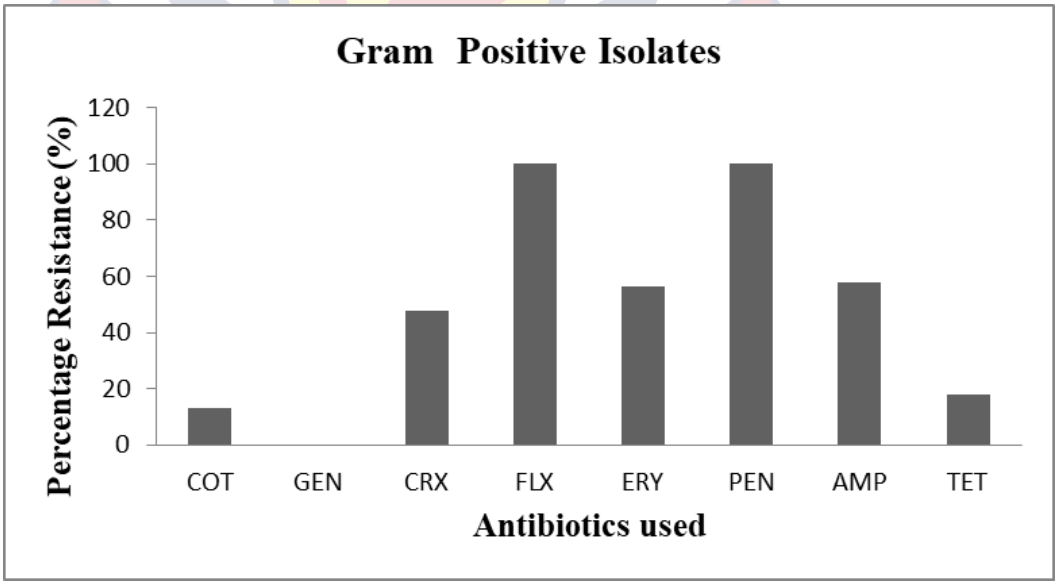
ANTIBIOTIC RESISTANT PROFILE OF COLIFORMS



Percentage resistance of coliform isolates to antibiotics

COT = Co-trimoxazole, GEN = Gentamicin, CRX = Cefuroxime, CHL = Chloramphenicol, CTR = Ceftriaxone, CTX= Cefotaxime, AMP = Ampicillin, TET = Tetracycline

Antibiotic resistant profile of Gram-positive bacterial isolates



Percentage resistance of Gram positive bacteria isolates to antibiotics

COT = Cotrimoxazole, GEN = Gentamicin, CRX = Cefuroxime, FLX= flucoxacillin, ERY= erythromycin, PEN = penicillin, AMP = Ampicillin, TET = Tetracycline

APPENDIX G

DISTRIBUTION OF RESISTANT GENES DETECTED FOR COLIFORM AND GRAM POSITIVE ISOLATES (PERCENTAGE IN BRACKET)

Gene	Coliform (n=99)	Gram Positive isolates (n=46)	Total (n=145)
cmIA	_(0.0)	1 (2.2)	1 (0.7)
Cat 1	2 (2.0)	1 (2.2)	3 (2.1)
TEM	54 (54.5)	34 (73.9)	88 (60.7)
qnrS	_ (0.0)	1 (2.2)	1 (0.7)
gyrA	4 (4.0)	19 (41.3)	23 (15.9)
EBC	20 (20.2)	9 (19.6)	29 (20)
Sul 3	3 (3.0)	5 (10.9)	8 (5.5)
Sul 1	16 (16.2)	12 (26.1)	28 (19.3)
blaTEM-1	12 (12.1)	23 (50.0)	35 (24.1)
qnrB	6 (6.1)	3 (6.5)	9 (6.2)
tetB	_(0.0)	1 (2,2)	1 (0.7)
mecA	_(0.0)	8 (17.4)	8 (5.5)
tetA	_(0.0)	_(0.0)	0 (0.0)
CTXM	_(0.0)	1 (2.2)	1 (0.7)

(_) = Absence of gene