PREVALENCE AND CHARACTERIZATION OF *Staphylococcus aureus* IN FRESH INDIAN MACKEREL (*Rastrelliger kanagurta)* IN UNGUJA ISLAND

FATMA SULEIMAN ALI

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ABSTRACT

A cross-sectional study design was conducted to assess the prevalence and characterization of *Staphylococcus aureus* in fresh Indian mackerel fish (IMF) in Unguja Island. A total of 400 fish samples of IMF were collected from three landing sites Malindi, Mangapwani and Mkokotoni. From each fish two samples were collected namely fish skin swab and fish tissue (muscle). The primary culture were obtained from Mannitol salt agar, Nutrient and Blood agar followed by Gram staining, Catalase, Coagulase (slide and tube) tests. The DNA from the representative isolates was extracted using boiling method. DNA fragments were amplified from isolated DNA based on 16S rRNA, *nuc*, *mecA*, *pvl*, *spa* and enterotoxins genes depending on specific primers. The PCR products were detected by agarose gel electrophoresis. The results indicated that growth of bacteria from fish skin swabs were 359 (89.75%) and fish tissue were 102 (25.5%). Based on biochemical tests, 27 isolates (6.75%) were confirmed to be *Staphylococcus* bacteria. Of the 27 isolates, seven (1.75%) were confirmed *S. aureus* based on PCR results. All twenty seven isolates confirmed to be positive in16S rRNA gene, two isolates demonstrated *mecA* gene and one had enterotoxins SEB and SEC. The SEA, *spa* and *pvl* genes were not detected. In conclusion, the prevalence of *S. aureus* was rather low (1.75%), but this has an implication in public health to community using IMF. This will lead to incidences of food borne diseases caused by *S. aureus*. Therefore, data presented here are useful for risk assessment and management of pathogenic *S. aureus* in IMF.

DECLARATION

I, **Fatma Suleiman Ali,** do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work and that it has neither been submitted nor concurrently submitted in any other institution.

________________________ ________________

Fatma Suleiman Ali Date

(MSc. Health of Aquatic Resources)

The declaration is hereby confirmed by;

________________________ ________________

Dr. Lupindu A. M. Date

(Supervisor)

________________________ ________________

Prof. Mdegela R. H. Date

(Supervisor)

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DEDICATION

This work is dedicated to: My parents Mr. Suleiman Ali Humoud and Mrs. Salma Ali Salim for taking me to school.

My young sister and brothers Nasra, Sharifa, Wahdat, Ali and Said for supporting me.

My husband Mohammed Ali Ameir

My kids: Maliha and Mahad. I always love you.

TABLE OF CONTENTS

ABSTRACT .... **ii**

DECLARATION .... **iii**

COPY RIGHT .... **iv**

ACKNOWLEDGEMENTS .... **v**

DEDICATION .... **vi**

TABLE OF CONTENTS .... **vii**

LIST OF TABLES .... **xi**

LIST OF FIGURES .... **xii**

LIST OF ABBREVIATIONS, SYMBOLS AND ACRONYMS .... **xiii**

CHAPTER ONE **1**

1.0 INTRODUCTION **1**

1.2 Statement of the problem and justification of the study 3

1.2.1 Problem statement 3

1.2.2 Justification 4

1.3 Objectives 5

1.3.1 Overall objective 5

1.3.2 Specific objectives 5

1.3.3 Hypothesis 5

CHAPTER TWO **6**

2.0 LITERATURE REVIEW **6**

2.1 Background Information/Introduction 6

2.1.1 *Staphylococcus aureus* 6

2.1.2 Growth and survival characteristics of *S. aureus*. 7

2.1.3 Pathogenicity of *S. aureus* 7

2.1.4 *Staphylococcus aureus* and food poisoning 7

2.1.5 Virulence and virulence factors of *S. aureus* 9

2.1.6 Enzymes produced by *S. aureus* 10

2.1.7 Diagnosis of *S. aureus* 11

2.1.8 Control of *S. aureus* 12

CHAPTER THREE 14

3.0 MATERIALS AND METHODS 14

3.1 Study Area 14

3.2 Study Design and Sample Collection 15

3.3 Microbiological Analysis 16

3.3.1 Media preparation 16

3.3.1.1 Stuart transport media 16

3.3.1.2 Peptone water 16

3.3.1.3 Mannitol salt agar 17

3.3.1.4 Nutrient agar 17

3.3.1.5 Blood agar 17

3.3.1.6 Rabbit plasma preparation 17

3.4 Isolation and Identification of *S. aureus* 18

3.4.1 Isolation of *S. aureus* 18

3.4.2 Identification of *S. aureus* by Gram stain 18

3.5 Biochemical Tests 19

3.5.1 Coagulase slide test 19

3.5.2 Coagulase tube test 19

3.5.3 Catalase test 20

3.6 Molecular Analysis 20

3.6.1 DNA extraction 20

3.6.2 Preparation of Agarose gel 21

3.6.3 Loading of PCR products in agarose gel and electrophoresis 21

3.6.4 Molecular identification of *S. aureus* 21

3.6.5 PCR amplification 22

3.6.5.1 16S rRNA gene and PVL gene 23

3.6.5.2 *nuc* genes 23

3.6.5.3 Enterotoxins 24

3.6.5.4 *spa* gene 24

3.6.5.5 16S rRNA gene and *mec*A gene 25

3.7 Data Analysis 25

CHAPTER FOUR 26

4.0 RESULTS 26

4.1 Isolation 26

4.1.1 Bacterial contamination on fish samples 26

4.1.2 Morphological appearance of bacterial colony 27

4.3 Biochemical Tests 28

4.3.1 Coagulase slide and tube test 28

4.3.2 Catalase Test 28

4.4 Molecular Analysis of Suspected *Staphylococcus aureus* 29

4.4.1 Monoplex PCR for *nuc* gene 29

4.4.2 Multiplex PCR for *mec*A gene 30

4.4.3 Enterotoxins (SEA, SEB and SEC) 31

4.4.4 PCR for detecting *spa* and *pvl gene* 32

CHAPTER FIVE 33

5.0 DISCUSSION 33

CHAPTER SIX 40

6.0 CONCLUSION AND RECOMMENDATIONS 40

6.1 CONCLUSION 40

6.2 Recommandations 40

REFERENCES 41

LIST OF TABLES

Table 1: Primer sequences used for Staphylococcus aureus 22

Table 2: Bacterial growth from fish skin (swabs) samples 26

Table 3: Bacterial growth from fish tissue (muscles) samples 27

LIST OF FIGURES

Figure 1: Map of Unguja Island indicating landing sites used in the study 14

Figure 2: Gram positive coccus isolated from the samples................................ 27

Figure 3: From left coagulase slide (A) test and coagulase tube (B) test and arrows were used to show the positive area on the slide and positive tube 28

Figure 4: Microscopic slide show the positive Catalase test of bacteria isolate 29

Figure 5: *nuc* gene detected in the suspected *S. aureus* positive samples. MK are samples tested from Mkokotoni, -VE Co represents negative control while +VE Co is for positive control.. 30

Figure 6: Samples MK 40 and MK 54 having *mec*A bands. MK are samples tested from Mkokotoni, -VE Co represents negative control while +VE is for positive control of *mecA* and 16S is for positive control of 16S rRNA gene 31

Figure 7: Sample MK 39 showing enterotoxin bands. MKs' are samples tested from Mkokotoni and -VE Co represents negative control 32

LIST OF ABBREVIATIONS, SYMBOLS AND ACRONYMS

% Percentage

& and

/ and/or

+VE Positive

< less than

> greater than

µl micro-litres

bp base pair

CFU Colony Forming Unit

DNA Deoxyribose Nucleic Acid

e.g example

EDTA Ethylenediaminetetra acetic acid

et al., and others

FAO Food and Agriculture Organisation

FDA Food and Drug Administration

g gram

hrs hours

IMF Indian Mackerel Fish

IMS Institute of Marine Science –Zanzibar

JMHLW Japanese Ministry of Health, Labour and Welfare

lbs pounds

*mec*A Methicillin Resistant Gene Class A

MK Mkokotoni

ML	Malindi
Mls	millilitres
MN	Mangapwani
MRSA	Mecithillin Resistant Strain
MSSA	Mecithillin Susceptible Strain
NNISS	National Nosocomial Surveillance System
nuc	Nuclease
OHCEA	One Heath Central and Eastern Africa
^{o}C	Degree centigrade
P	Probability
PCR	Polymerase Chain Reaction
pH	Power of Hydrogen
Pvl	Panton-valentine Leukocidin
r	Row
RNA	Ribose Nucleic Acid
rpm	revolution per minute
SAS	Statistical Analysis System
SE	Staphylococcus enterotoxins
sec	second
Spa	Staphylococcus Protein A
TRAHESA	Training and Research in Aquatic and Environmental health in
Eastern	and Southern Africa
TSST	Toxic Shock Syndrome Toxin
USA	United States of America

V	voltage
–VE	Negative
Vol	volume
X^2	Chi-square

CHAPTER ONE

1.0 INTRODUCTION

Fish is a vital source of food for people and contributes about 60% of the world's supply of protein. It is man's most important source of high quality protein, providing approximately 16% of the animal protein consumed by the world's population (FAO, 1997). Sixty percent of developing countries derive 30% of their annual protein from fish (Abisoye *et al.,* 2011). In Africa, fish supplies 17% of protein and it is one of the cheapest sources of protein (Claucas and Ward, 1996). In Tanzania, the per capita fish consumption is 8.0 kilogram and about 30% of animal protein consumption is from fish (National Economic Survey, 2009).

The advantage of fish as food is as a result of its easy digestibility and high nutritional value. However, fish are susceptible to wide variety of bacterial pathogens, most of which are capable of causing disease and are considered to be saprophytic in nature (Lipp and Rose, 1997). Unlike other animal products, quality of fish is often more difficult to control due to variations of fishes in species, sex, age, habitants and action of autolytic as well as hydrolytic enzymes of microorganisms on the fish muscles (Venugopal, 2002).

Food safety is a matter of concern in both developed and developing countries. Poor sanitation in developing countries increases susceptibility to food borne diseases. In Africa, a number of foods including meat and fish have been reported to have high bacteria contamination e.g. *Escherichia coli, Staphylococcus aureus* (Caroline and

Nadine, 2005). Possible sources of bacteria are fish skin, shells, tissues and processing equipments used for each operation performed until the final product is eaten (Prince Antwi–Agyei and Maalekuu, 2014). Food borne diseases are diseases resulting from ingestion of bacteria, toxins and cells produced by microorganisms in food (Clarence *et al.,* 2009). Food borne diseases such as diarrhoea, cholera, gastro intestinal tract infections and related death cases have been reported worldwide (Adak *et al*., 2005). The symptoms of food borne diseases are vomiting, diarrhea, abdominal pain, nausea and usually start within four hours after consumption of infected food (FDA, 1998). The intensity of the signs and symptoms vary with the rate of contamination of ingested food and susceptibility of the individuals to the bacteria and their toxins (Clarence *et al*., 2009).

Staphylococcus aureus is the leading cause of nosocomial infections as reported by National Nosocomial Infections Surveillance System (NNISS, 1999). The bacteria is responsible for a wide range of human diseases, including endocarditis, food poisoning, toxic shock syndrome, septicemia, skin and soft tissue infections and bone infections as well as bovine mastitis (Costa *et al.,* 2013). The incidence of *S. aureus* differs in different fish species (Bujjammna and Padmavathi, 2015). Apart from food, these bacteria are ubiquitously found in human body as well as in soil, water and air (Alzbeta *et al.,* 2009; Pinchuk *et al.,* 2010). Furthermore, *S. aureus* enters the food chain as a result of poor hygiene conditions during processing and storage of food stuff (Diana *et al.,* 2012). The microbial association with fish compromises safety and the quality for human consumption, particularly critical when the microorganisms are opportunistic and /or pathogenic in nature (Mhango *et*

al., 2010). The risks of contracting food borne diseases by fish users may be high, but also fish industry is one of the most important protein and economic sources for the rapid growing coastal population of Tanzania (National Economic Survey, 2009). The hygienic situation during fishing, handling, processing and preservation techniques is still questionable in Tanzanian fish industry. Due to this problem then this study aimed at investigating the fishing chain status and fish products contaminated by *S. aureus* along the coast of Zanzibar.

1.2 Statement of the problem and justification of the study

1.2.1 Problem statement

Poor hygienic practices during handling, processing and preservation of seafood are very common in developing countries including Tanzania. Consequently, such practices pose threats to public health and socio-economic services (Vigano *et al*., 2007; Karimuribo *et al.,* 2015). Fish become contaminated due to its adverse environment including sewage, contaminated water, harvesting area as well as from contamination by workers, utensils, equipments and unhygienic handling (Haifaa, 2013). It was observed that in Pemba over 30% of all ready to eat sea foods were contaminated with thermo tolerant coliforms of which *E. coli* was the most dominant among bacteria species (Vigano *et al*., 2007). In the same study it was also observed that there were additional 15% of the samples heavily contaminated with *Vibrio alginolyticus*. Lack of basic infrastructures, poor knowledge of adherence to hygiene practices have been cited to be the principal courses of outbreaks of food borne illnesses including fish in many parts of Africa (Kibret and Abera, 2012). Studies on bacterial contamination in various food materials in Tanzania have been concentrated

on just measuring extent of bacterial loads without identifying the actual pathogenic strains (Harris *et al.,* 2013; Simforiana *et al.*, 2015; Nonga *et al.,* 2015). Such shortfall could lead to improper measures of control and may even be a cause for bacterial resistance to food treatment methods. The information on bacteria pathogens' contamination status along the fishing chain in Unguja Island is limited. There is inadequacy of studies in Unguja Island describing the levels of contamination and identifying the bacteria including *Staphylococcus* strain on fish.

1.2.2 Justification

This proposed study intended to determine the prevalence and characterization of common bacteria afflicting *Rastrelliger kanagurta* focusing on *S. aureus.* Studying *S. aureus* is important due to its occurrence in sea foods and meat products. In addition, most studies reporting on *Staphylococcus* did not give the actual strains observed thereby giving both producer and consumers wrong messages. Furthermore, this study used *R. kanagurta* which is a leading fish species in terms of consumption and catch in Unguja Island. Other fish species are anchovies, sardines and emperors (Sheikh and Nassir, 2013). Therefore, this study will be used to set guidelines on appropriate measures for handling the *R. kanagurta* that will minimize the load of pathogenic *S. aureus* in sea food and reduce public health problems to consumers.

1.3 Objectives

1.3.1 Overall objective

To estimate the prevalence and characterize *S. aureus* obtained from fresh Indian mackerel fish (*R. kanagurta)* from fish landing sites in Unguja Island.

1.3.2 Specific objectives

i. To determine the prevalence of *S. aureus* contamination in fresh landed *R. kanagurta* in Unguja Island.

ii. To identify virulent strain of *S. aureus* from fresh landed *R. kanagurta* in Unguja Island.

1.3.3 Hypothesis

Ho: Fish (*Rastrelliger kanagurta*) from fish landing sites in Unguja Island are contaminated by pathogenic virulent *Staphylococcus aureus.*

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Background Information/Introduction

2.1.1 Staphylococcus aureus

This is a Gram-positive coccal bacterium belonging to member of the Firmicutes bacteria. It is non-spore forming spherical bacterium that belongs to the genus *Staphylococcus*. The genus comprises more than 40 species that differ in their potential to endanger human and animal health, ranging from non-pathogenic food grade members to dangerous pathogens causing severe infections and being resistant to the treatment by most of commonly applied antibiotics (Gotz *et al.,* 2006). Many staphylococci are found on humans, mammals, or birds where they are located on the skin, skin glands, or mucous membranes. They are either found to coexist indigenously as commensals or to be transiently present as colonizers of their hosts. The transitory presence of some staphylococcal cohabitants often hampers the identification of their natural host range (Gotz *et al.,* 2006). A part from being a normal flora to human being and some animals, they also play major role on health importance by causing varieties of clinical infectious diseases from minor skin disease such as pimples, impetigo, boils, cellulitis, folliculitis, carbuncles, scalded skin syndrome, abscesses to life threaten diseases such as pneumonia, meningitis, osteomyelitis, endocarditis and toxic shock syndrome (Julianna and Mariusz, 2012). The highly pathogenic *Staphycoccus aureus* strains developed an immune variety of mechanisms that enable them to specifically interact with host factors and escape the hosts defense mechanisms, to enhance their fitness in the competition for rare

substrates, to detach nutrition's by disintegration of host tissue and to cope with antibacterial compounds like antibiotics (Guinane *et al*., 2010).

2.1.2 Growth and survival characteristics of *S. aureus.*

The bacteria can grow in substrates with a low water activity of 0.86 over a wide temperature range of 7 to 48°C and at pH values ranging from 4.2 to 9.3 (Norman *et al*., 2005). It is a thermo tolerant bacterium that survives at a temperature as high as 50°C, high salt concentration in marine environments as well as in dry environments. Although it is poor competitor, but has an ability to grow under osmotic and pH stress and thus can lead to thrive in a wide variety of foods (Stewart, 2003). It was also observed to survive in vacuum that stored in room temperature up to 21°C on packaged ready to eat food (Ingham *et al.,* 2007).

2.1.3 Pathogenicity of *S. aureus*

The *S.aureus* repertoire of surface protein allows interactions with virtually every structural component of the host's extracellular matrix and with many plasma proteins. Correspondingly, *S. aureus* cells are able to adhere to fibril forming collagens of types I, II and III, laminun, elastin, fibronectin, vitronectin, fibrinogen, von wile brand factor and thrombospondin (Foster and Hook, 1998).

2.1.4 *Staphylococcus aureus* and food poisoning

The food poisoning as a result of staphylococcal intoxication is due to ingestion of food containing pre-formed enterotoxin of *S. aureus* (Argudin *et al.,* 2010). Consumption of this staphylococcus enterotoxin may cause disease in human.

Staphylococcus enterotoxins are classified into several categories including enterotoxin A, D, E. H. B. G. that have also been associated with staphylococcal food poisoning (Pinchuk *et al.,* 2010). Food can be contaminated through different ways but the most common known is through contact with food workers carried bacteria. Several studies have investigated the potential paths of transmission of this dangerous strain by human carriers or environment, such as transport and packaging, contaminated hands and contact with infected respiratory secretions from workers with seas food products (Atyah *et al*., 2010). In some parts of the world, more than 50% of food poisoning is caused by *Staphylococcus* Enterotoxin A (SEA). In Great Britain and America, *Staphylococcus* Enterotoxin A (SEA) and *Staphylococcus* Enterotoxin B (SEB) are the cause of more than 69% of all food poisonings (Adams and Moss, 2002). In Japan, main causes of staphylococcal food poisoning are processed foods composed mainly of rice, such as rice balls which are made by hands (Oda, 1998). Actually, several cases of staphylococcal food poisoning due to consumption of fish were reported between 2002 and 2008, including two cases caused by consumption of cooked eel, two cases caused by consumption sashimi and three cases caused by consumption of flaked fish as reported by Japanese Ministry of Health, Labor and Welfare (JMHLW, 2008). In Zimbabwe, human infections caused by *S. aureus* transmitted from fish are quite common and depend on the season, patients contact with fish and related environment, dietary habits and the immune system status of individual (Novotny *et al.,* 2009). In Iran, food poisoning outbreaks by *S. aureus* have increased during recent years. This might be due to changes in the environment, the development of the food service industry and communal feeding (Arfatahery *et al.,* 2015).

It has been revealed that for *S. aureus* to produce a certain detectible amount of entero-toxins it needs the cellular population of 5-6 long CFU/g, either during stationary stage of its growth or at the end of the exponential phase (Castillejo–Rodriguez *et al*., 2002; Fujikawa and Morozumi, 2006). Staphylococcal food poisoning is usually self- limiting and resolves within 24 to 48 hours after onset (Lawrynowicz–Paciorek *et al.,* 2007).

2.1.5 Virulence and virulence factors of *S. aureus*

Staphylococcus aureus represents by far the most versatile species of the genus. It is reported to have different virulence factors and additional supportive gene products that increase the capability to survive within the living host. This make the *S. aureus* the leading pathogen which is the most threatening microorganisms regarding hospitality and community acquired infections (Rosenstein and Gotz, 2013). As a widespread colonizer of human skin and mucous membranes (Lowy, 1998), *S. aureus* exhibits two life styles, as a tolerated commensal on one hand and as a dangerous pathogen on the other hand. Its abilities as human pathogen are based on a comprehensive collection of various virulence factors and supportive fitness factors that play roles during the various steps of the infection process. These include adhesion to host tissue, forming multilayered and encapsulated biofilms, evasion of the hosts immune system, and coping with limited supply of nutrients like iron compounds (Guggenberger *et al*., 2012). Correspondingly, the staphylococcal virulence factors may be sub divided into adhesions or soluble factors that mediate the attachment host cells or extracellular matrix proteins, Exo-enzymes that are involved in the destruction of host tissues. Toxins that directly exert detrimental

effects to the host and a heterogeneous group comprising iron uptake systems, immune system evasion mechanisms and other factors that enhance the fitness to survive in the host (Roche *et al*., 2003). The staphylococcal pathogenic potential is completed by a variety of genes that are mediating resistance to antibiotics and other antibacterial agents. Its opulent arsenal of factors involved in the course of infection makes *S. aureus* the outstanding, pathogen within the genus and thus represents the benchmark to which the other pathogenic species have to be compared (Heilbronner *et al.,* 2011; Tse *et al*., 2010).

The ability of *S. aureus* to cause various infections and intoxication, results from the production of different extracellular and surface virulence factors with adhesive properties to a range of molecules (Costa *et al.,* 2013). The extracellular products include especially toxins with super antigenic properties, namely enterotoxins A –E, G –K, M –O and Q (SEA –SEQ genes), exfoliate toxins A and B (eta, etb), toxic shock syndrome toxin (TSST –1) as well as for example, Panton valentine Leukocidin (PVL) (Bohach *et al.,* 1990). The cytotoxin that causes leukocyte destruction and tissue necrosis, its presence is associated with the increased virulence of certain strains of *S. aureus* where the latter is a virulence gene which is covalently anchored to the peptidoglycan of *S. aureus*.

2.1.6 Enzymes produced by *S. aureus*

Staphylococcus aureus produces various enzymes such as coagulase which clots plasma and coats the bacterial cell, probably to prevent phagocytosis, Hyaluronidase (also known as spreading factor) and breaks down hyaluronic acid and helps in spreading it (Ben Zakour *et al*., 2011). *S. aureus* also produces deoxyribonuclease,

which breaks down the DNA, lipase to digest lipids, staphylokinase to dissolve fibrin and aid in spread, and beta –lactamase for drug resistance (Cenci –Goga *et al*., 2003). Depending on the strain, *S. aureus* is capable of secreting several exotoxins, which can be categorized into two groups that include staphylococcal enterotoxins (SE) and toxic shock syndrome toxin (TSST). Many of these toxins are associated with specific diseases (Dinges, 2000). Their toxins are dangerous in terms of food safety where by their biological activity remains unchanged even after thermal processing of food.

2.1.7 Diagnosis of *S. aureus*

Staphylococcus aureus can be diagnosed using macro-morphology whereby colony characteristic are observed and the media used is selective containing 0.1% potassium tellurite then incubated at 37°C for 24 –48 hours (Arfatahery *et al*., 2015). Micro-morphologically, *S. aureus* presents as gram positive cocci where samples are swabbed onto microscopic glass slide. This is then stained with Gram stain or dyes like Crystal violet and basic fuchsine space and viewed under the microscope. *S. aureus* is gram positive and stains blue or purple and appears as small round cocci or short chains and most commonly as grape –like clusters (Arfatahery *et al*., 2015). Based on biochemical test identification, sample placed onto a culture media, that provides sources of nutrition, carbon, energy and nitrogen for the bacteria to grow and commonly used is Mannitol salt agar (Smeltzer *et al.*, 2009). It is a selective medium with 7-9% salt or sodium chloride that allows *S. aureus* to grow selectively (Tenover *et al.,* 1994). Other various tests can be used to identify *S. aureus*, including production of protein A cell –bound clumping factor, extracellular coagulase and heat - stable nuclease and catalase test (Marti *et al*., 2010).

Free (extracellular) coagulase clots plasma in the absence of calcium. Slide agglutination test involves clumping factor (bound coagulase) in that it is cell –bound and requires only fibrinogen. Commercial latex agglutination tests for *S. aureus* detected protein A and/ or clumping factor which detect various surface antigens (Sloot *et al*., 1992). Deoxyribonuclease (DNase) plates can be used to screen isolates but as various amounts of DNase are produced by CoNs, positives should be confirmed with an additional test. Heat – stable nuclease tests can be used to identify *S. aureus,* although some rare coagulase – negative species can be positive and *S. aureus* is catalase positive (Derek *et al.,* 2005).

2.1.8 Control of *S. aureus*

Staphylococcus aureus is a much feared nosocomial pathogen. For infection caused by this species, the drugs of choice are beta – lactam antibiotics (in particular methilicilin and oxacillin). However, increasing resistance to these drugs (Katakweba *et al.,* 2012) has recently raised the concerns of both microbiologists and clinicians, especially in the case of methicillin – resistance strains (MRSA). Methicillin resistance is characterized by the presence of the *mec*A gene coding for modified transpeptidases (Penicillin – binding proteins 2a) with very low affinity to beta-lactam antibiotics (Chambers, 1997).

Proper hand washing and disinfection has been recognized as one of the most effective measure to control the spread of pathogens, especially when considering employed workers (Montville *et al.,* 2001). A part from personal hygiene of workers, unclean, insufficiently or in adequately cleaned processing equipments have been identified as a source of bacterial contamination in processed fish (Reij *et al.,* 2003).

In order to avoid microbial contaminations, the whole process should be under hygienic control.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was conducted in Unguja Island –Zanzibar, Tanzania. Unguja Island is located between latitudes 5°40' and 6°30' South and longitude 39° East. Samples were collected at Malindi, Mkokotoni and Mangapwani landing sites. These are very famous landing sites in Unguja for collecting fish species that is why they were used in this study (Figure 1).

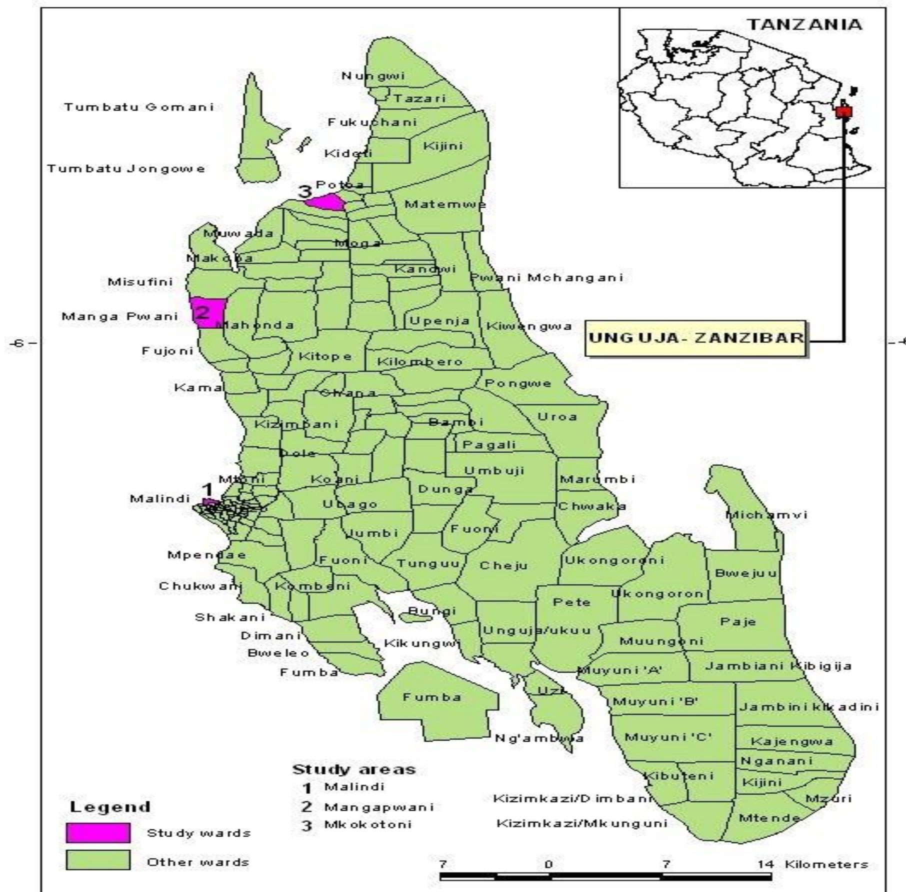

Figure 1: Map of Unguja Island indicating landing sites used in the study

3.2 Study Design and Sample Collection

A cross-sectional study design was used in which a total of 400 fresh fish (Indian Mackerel) were collected from three fish landing sites within Unguja Island (ranging fish size was 13cm to 25cm). From each fish, one fish skin and fish tissue (muscles) was collected making a total of 800 samples. The sample size was determined by Simple Random Sampling formula by Thrusfield (2005) as follows.

$N = Z^2 P (1-P) / \Sigma^2$.

N is sample size, Z is constant (1.96), P is prevalence and Σ is error margin (0.05). Due to the absence of prevalence study on *S. aureus* on sea food in Zanzibar, an assumed prevalence of 50 percent was used in the calculation, which gave us a sample size, N=384, however, total number of samples collected from all three landing sites were 400 samples.

In Malindi landing site one hundred (100) fish samples were collected from randomly selected twenty five (25) boats among eighty seven (87) boats that are used in fishing. In Mkokotoni landing site two separate days were spent for collecting 150 fish samples. In the first day, 100 fish samples were collected from eleven boats among twenty nine boats and in day two 50 fish samples were collected from eleven among twenty three boats. The boats used to collect samples for the second day were different from those used in the first day. In Mangapwani landing site also two separate days were spent for collecting 150 fish samples. In the first day 100 fish samples were collected from sixteen boats among fifty one boats and day two 50 fish samples were collected from twelve among fifty six boats. Each fish sample was

placed in sterilized plastic zip bag then were kept in cool box with ice pack and transferred to the analytical laboratory, Zanzibar. In the laboratory, swabs from fish skin were collected then kept in tubes containing Stuart transport media and stored in a fridge around 4°C while fish samples were stored in a freezer around -5°C to minimize change in quality and control growth of microorganism until used and the tissue (muscles) were collected by cutting one gram of fish muscles using sterilized scalpel blade (Arfatahery *et al*., 2015).

3.3 Microbiological Analysis

3.3.1 Media preparation

3.3.1.1 Stuart transport media

The Stuart Transport Medium (HIMEDIA®, HiMedia Laboratories Pvt. Ltd India Batch number M306-500G) was prepared according to manufacturer instructions where 14.1g was suspended on 1000mls of distilled water and heated to boil to dissolve the medium completely then sterilized by autoclaving at 15lbs pressure121^{o}C for 15 minutes. The medium then was cooled to about 20°C before use. Two milliliters of medium were poured in each tube under laminar flow.

3.3.1.2 Peptone water

The peptone water (HIMEDIA®, HiMedia Laboratories Pvt, Ltd, INDIA Batch number M028-500G) was prepared according to manufacturer instructions where 15.0 grams were suspend in 1000 mls of distilled water and mixed well then sterilized by autoclaving at 121°C for 15 minutes. The peptone water then was cooled to about 20°C before use.

3.3.1.3 Mannitol salt agar

Mannitol Salt Agar (OXOIDHAMPSHIRE, ENGLAND Batch number CM0085) was prepared according to manufacturer instructions by suspending 111g in 1000 mls of distilled water and boil to dissolve completely and was sterilized by autoclaving at 121°C for 15 minutes then cooled to about 20°C prior to pouring in sterile Petri-dishes.

3.3.1.4 Nutrient agar

The Nutrient Agar (TECHNO PHARMCHEM, BAHADURGARH, HARYANA INDIA CM0148) was prepared according to manufacturer instructions by adding 28g of the powdered medium into 1000 mls of distilled water. It was then mixed well and heated to boiling to dissolve the medium completely. It was sterilized by autoclaving at 15 lbs pressure for 15 minutes at 121°C then poured in petri-dishes at 50°C.

3.3.1.5 Blood agar

The blood agar (HIMEDIA®, HiMedia Laboratories Pvt, Ltd, INDIA Batch number M834-500G) of 40.0 grams were suspend in 1000 mls of distilled water and mixed well then sterilized by autoclaving at 121°C for 15 minutes. The blood agar then it was allowed to cool but not to solidify up to 45 °C, then 5% (vol) sterile blood was added and mixed gently before being poured into sterile petri-dishes.

3.3.1.6 Rabbit plasma preparation

The blood from rabbit was placed in vacutainer tube containing heparine anticoagulant and centrifuged to get plasma. The normal saline of 5 mls was added in 1 ml of rabbit plasma to make a solution of 6 mls. Two mls were used in coagulase

tube test while 1 to 2 drops used in coagulase slide test. The remaining rabbit plasma was stored in fridge around 4°C for future use.

3.4 Isolation and Identification of *S. aureus*

3.4.1 Isolation of *S. aureus*

The collected samples were grown in Manitol salt agar and Nutrient agar for primary isolation of bacteria. The blood agar was also used for identification of bacterial morphology. A loop of each swab sample of skin from transport media was put on media plate and was streaked properly with a sterile inoculating wire loop. In the case of fish tissue (muscle), one gram of each fish sample were cut using sterile scalpel blade then ground and homogenized into peptone water before inoculated into Mannitol salt agar and incubated at 37°C for 24hrs. Then, all plates that had suspected positive isolates like morphological appearance, medium size and yellow in color colony, were collected and further sub cultured on nutrient agar. The blood agar was then used for identification of suspected bacteria isolates basing on morphology, those appeared medium colony, yellowish and haemolytic were suspected as *S. aureus*. The colonies were selected and stored in nutrient agar and refrigerated at 4℃ awaiting further analysis.

3.4.2 Identification of *S. aureus* by Gram stain

Isolates of suspected *S. aureus* cultured in Mannitol salt agar which is selective media for *Staphylococcus* species and then sub-cultured in Nutrient Agar plate. The colonies appeared yellowish in color and had medium size.

Gram staining method was used for further identification based on cellular morphology and staining effect. One drop of normal saline was emulsified with

colony organism in microscopic slide and thereafter dried and fixed by heat. Crystal violet was poured on sample slide for one minute then Lugos iodine poured and left for about a minute. After washing by running water, sample slide were decolorized by acetone. After wash in neutral red was poured and left for about three minutes. After washing, slides were dried, smeared with oil immersion and finally examined under a light microscope at 100x magnification for the morphology and if were gram positive or negative.

3.5 Biochemical Tests

Different biochemical tests were performed to confirm the suspected isolates. These included slide and tube coagulase test, as well as catalase test (Shakeri *et al.,* 2010).

3.5.1 Coagulase slide test

The organisms were grown on nutrient agar and cultured overnight to obtain pure isolates. One drop of rabbit plasma was spread onto the slide labeled with sample number. The one drop of rabbit plasma was emulsified with the colony (test organisms) using a wire loop then the slide was rocked gently for about ten minutes. Macroscopic clumping was observed in positive suspects while for negatives there were no clumping observed. The positive colony was further tested with coagulase tube for more confirmation.

3.5.2 Coagulase tube test

Rabbit plasma was also used in coagulase tube test. The dilution of one rabbit plasma in five milliliters of saline (1:5) was used in coagulase tube test. One ml of rabbit plasma was placed in small tubes, and several isolate colonies of organisms were

emulsified in sterile conditions, then the tube was shaken and incubated in 37°C for four hours (4 hrs). Examination was done in 1, 2, 3 and 4 hrs for clot formation. The formation of clots under the tubes indicated a positive result while where plasma remained wholly liquid indicated negative results. Negative tubes were left in incubator for overnight and re-examined.

3.5.3 Catalase test

One drop of hydrogen peroxide 3% was used in catalase test. The drop was placed on microscopic slide and the bacteria colony from nutrient agar plate was added. The formation of foam (bubbles) indicated positive results.

3.6 Molecular Analysis

Conventional Polymerase Chain Reaction (PCR) was used to identify Pathogenic *S. aureus* and their enterotoxins from bacteria isolate using specific primers. This was done to those coagulase positive isolates, catalase positive isolates, catalase positive isolates and coccus with grape like appearance in Gram Stain isolates.

3.6.1 DNA extraction

The DNA extraction was by boiling (Sila *et al.,* 2009) where *S. aureus* bacteria colony was placed in Eppendorf tube containing 200µl of distilled water. The Eppendorf tube containing samples then placed in water bath at 100°C for 15 minutes. The tubes were then centrifuged for maximum speed of 1500 rpm for 5minutes. The DNA was then extracted from the supernatant using micropipette.

3.6.2 Preparation of Agarose gel

Agarose gel was prepared by mixing 1.5g of agarose powder in 100 mls of 1X (Tris-Boric-EDTA buffer to obtain a 1.5% concentration of gel. The mixture was completely dissolved by boiling on a hot plate, 3µl of GelRed (Biotium Products-USA) was added into the 100 mls of the molten agarose and mixed gently. The agarose was poured in the horizontal electrophoresis casting equipment/tray in the presence of a comb and left to solidify.

3.6.3 Loading of PCR products in agarose gel and electrophoresis

A volume of 8µl of the PCR products was mixed thoroughly with 2µl of blue 6X loading dye (Thermo Fisher Scientific) on a laboratory paraphilm. The PCR products were loaded in the wells of the agarose gel. The gel electrophoresis was carried out at a constant voltage of 110 V for 60 minutes and examined under ulta-violet light.

3.6.4 Molecular identification of *S. aureus*

The molecular identification of *S. aureus* was performed by PCR using primer sets (Table 1) that amplified 16S rRNAfor *Staphylococcus* genus*, nuc* to confirm *S. aureus* bacteria*, mec*A genes, PVL genes and Protein A (*spa*) gene for pathogenic genes and *Staphylococcus* enterotoxins using specific primes SEA, SEB and SEC.

Table 1: Primer sequences used for *Staphylococcus aureus*

Target gene	Primer sequence (5'-3')	Bp	Reference
SEA (GSEAR-1)	GGTTATCAATGTGCGGGTGG	102	Mehrotra *et al.,* 2000
(GSEAR-2)	CGGCACTTTTTTCTCTTCGG		
SEB (GSEBR-1)	GTATGGTGGTGTAACTGAGC	164	
(GSEBR-2)	CCAAATAGTGACGAGTTAGG		
SEC (GSECR-1)	AGATGAAGTAGTTGATGTGTATGG	451	
(GSECR-2)	CACACTTTTAGAATCAACCG		
PVL-1	GTAGAAATGACTGAACGTCCGATAA	443	Sila *et al.,* 2009
PVL-2	CCAATTCCACATTGTTTCGGTCTA		
16S rRNA-1	GTACCAGCAGCCGCGGTAA		
16S rRNA-2	AGACCCGGGAACGTATTCAC	886	Chikkala *et al.,* 2012
16S rRNA-1	GTACCAGCAGCCGCGGTAA	886	Chikkala *et al.,* 2012
16S rRNA-2	AGACCCGGGAACGTATTCAC		
mecA-1	ATCATTAGGTAAAATGTCTGGACATGATCCA	293	
	GCATCAAGTGTATTGGATAGCAAAAGC		
mecA-2			
spa typing-1	ATGTGGTGGCGTAACACCTG	1150-1500	Shakeri *et al.,* 2010
spa typing-1	CGCTGCACCTAACGCTAATG		
nuc-1	GCGATTGATGGTGATACGGTT	270	Chikkala *et al.,* 2012
nuc-2	AGCCAAGCCTTGACGAACTAAAGC		

3.6.5 PCR amplification

The first targeted gene was 16S ribosomal RNA gene that has been used for detection, identification and taxonomic classification of bacteria. Every bacterial 16S rRNA consists of approximately 1500 nucleotides and includes several highly conserved regions. The second is the *nuc*gene that considered as a baseline and golden standard of identification of *S. aureus* genes. Another targeted gene is Mecithillin-resistance Strains (MRSA) *mec*A, which is a gene that produces a ‘penicillin Binding Protein 2a’ (PBP2a) a modified transpeptidase which lower

affinity to penicillin. The *pvl* gene (Panton-Valentine leukocidin) and the *spa* gene (Staphylococcal protein A) were also targeted in this study. Ninety percent (90%) of protein A is found in cell wall and remaining is free in the cytoplasm of bacteria. The last targeted gene in this study was Staphylococcal enterotoxins (SEA, SEB and SEC) which belong to a group of pyrogenic and exotoxins sharing common phylogenic relationships, structure, function and sequence homology.

3.6.5.1 16S rRNA gene and PVL gene

Staphylococcus aureus 16S rRNA and pathogenic genes PVL gene were assessed as described by Sila *et al.* (2009). PCR was modified in 16S rRNA gene by using PVL gene condition. The PCR was performed in total volume of 25µl containing 2 X ready mix PCR of 12.5µl, PCR primer for forward and reverse 1µl for each, 6.5µl of RNase free water and 4µof DNA template. The DNA Thermocycler was programmed for initial denaturation at 94°C for 5 min, 40 cycles for amplification, denaturation at 94°C for 10 sec, annealing at 56°C for 20 sec and extension at 72°C for 40 sec and final extension was performed at 72°C for 5 min. After DNA amplification, PCR products were analysed using 1.5% agarose gel at 110 voltages and visualized under ultra violet rays.

3.6.5.2 *nuc* genes

The *S. aureus nuc* genes were determined as described by Chikkala *et al.* (2012). The PCR was performed in total volume of 25µl containing 2X ready mix PCR of 12.5µl, PCR primer for forward and reverse 1µl for each, 6.5µl of RNase free water 4µof DNA template. The DNA Thermocycler was programmed for initial denaturation at 94°C for 5 min, 30 cycles for amplification, denaturation at 94°C for 30sec,

annealing at 45°C for 1min, and extension at 72°C for 45sec and final extension was performed at 72°C for 10 min. After DNA amplification, PCR products were analysed using 1.5% agarose gel at 110 voltages and visualized under ultra-violent trans-illuminator

3.6.5.3 Enterotoxins

The *S. aureus* enterotoxins genes of SEA, SEB and SEC were tested as described by Mehrotra *et al*. (2000). The PCR was performed in total volume of 25µl containing 2 X ready mix PCR of 12.5µl, PCR primer for forward and reverse 1µl for each, 6.5µl of RNase free water, 1µl of each primer and 4µof DNA template. The DNA Thermocycler was programmed for initial denaturation at 94°C for 4 min, 35 cycles for amplification, denaturation at 94°C for 2min, annealing at 57°C for 2min, and extension at 72°C for 1min and final extension was performed at 72°C for 7 min. After DNA amplification, PCR products were analysed using 1.5% agarose gel at 110 voltages visualized under ultra violet rays.

3.6.5.4 *spa* gene

Staphylococcus aureus spa gene was carried out as described by Shakeri *et al.* (2010). The PCR was performed in total volume of 25µl containing 2 X ready mix PCR of 12.5µl, PCR primer for forward and reverse 1µl for each, 6.5µl of RNase free water and 4µof DNA template. The DNA Thermocycler was programmed for initial denaturation at 94°C for 4 min, 35 cycles for amplification, denaturation at 94°C for 1min, annealing at 56°C for 1min, and extension at 72°C for 3min and final extension was performed at 72°C for 5 min. After DNA amplification, PCR products were analysed using 1.5% agarose gel at 110 voltages and visualized under ultra violet rays.

3.6.5.5 16S rRNA gene and *mec*A gene

The *S. aureus* 16S rRNA and pathogenic genes of *mec*A were amplified as described by Chikkala *et al.* (2012). The PCR was performed in total volume of 25µl containing 2 X ready mix PCR of 12.5µl, PCR primer for forward and reverse 1µl for each, 6.5µl of RNase free water, 1µl of each primer and 4µof DNA template. The DNA Thermocycler (Gradient PCR.TAKARA, Japan) was programmed for initial denaturation at 94°C for 5 min, 40 cycles for amplification, denaturation at 94°C for 30s, annealing at 55°C for 40s, and extension at 72°C for 50s and final extension was performed at 72°C for 10 min. After DNA amplification, PCR products were analyzed using 1.5% agarose gel at 110 voltages and visualized under ultra violet trans-illuminator.

3.7 Data Analysis

In this study, all data were dichotomous, were entered in Microsoft Excel sheet and analyzed by using SAS (2009) Software basing on growth frequency. The 95% was taken as a significance level of the results. The Chi-square(x^2) and Probability tests were applied to test the association and difference between isolates from different sites.

CHAPTER FOUR

4.0 RESULTS

4.1 Isolation

4.1.1 Bacterial contamination on fish samples

In Mkokotoni landing site out of 150 fish swab samples 132 (88%) had growth of bacteria, in Malindi, out of 100 fish swab samples, 80 (80%) had growth while in Mangapwani out of 150 swab samples 147 (98%) bacteria growth making a total of 359 (89.75%) of fish swab samples with bacteria growth (Table 2). Out of all samples with growth of bacteria only 27samples were confirmed to be *Staphylococcus* species. For the fish tissue (muscle) samples, 30 (20%) grow bacteria, from 150 samples collected at Mkokotoni landing site. In Malindi only 18 (18%) bacteria growth out of 100 samples and in Mangapwani were 54 (36%) from 150 samples. All three sites had a total of 102 (25.5%) samples that had growth of bacteria (Table 3). The *S. aureus* was not detected in all fish tissue samples after carrying morphological and biochemical tests.

Table 2: Bacterial growth from fish skin (swabs) samples (N=400)

Landing site	Samples collected (N)	Bacteria growth (n)	% growth ($n/N\times100$)	χ^2	P>r
Mkokotoni	150	132	88.00		
Malindi	100	80	80.00	21.9308	<.0001
Mangapwani	150	147	98.00		

Chi Square and P-value in which P-value shows that there is significance difference between three different landing sites and bacteria growth

Table 3: Bacterial growth from fish tissue (muscles) samples (N=400)

Landing site	Samples collected (N)	Bacteria growth (n)	% growth (n/N×100)	χ^2	P>r
Mkokotoni	150	30	20.00		
Malindi	100	18	18.00	14.0545	0.0009
Mangapwani	150	54	36.00		

Chi Square and P-value in which P-value shows that there is significance difference between three different landing sites bacteria bacteria growth

4.1.2 Morphological appearance of bacterial colony

Isolates of suspected *S. aureus* were cultured in Blood agar that was used to see the colony morphology in which its colony was surrounded by zones of clear beta hemolysis.

A total of 27 samples (6.75%) were found to be the typical Staphylococcal colonies. After staining the samples with Gram stain, the isolates appeared purple in color indicating gram positive cocci with grape like shape (Fig. 2).

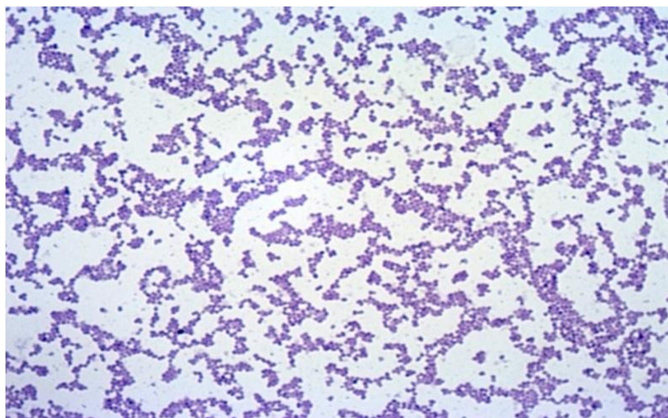

Figure 2: Gram positive coccus isolated from the samples

4.3 Biochemical Tests

4.3.1 Coagulase slide and tube test

All tested isolates (27) confirmed to be positive *S. aureus* in coagulase slide and tube test.

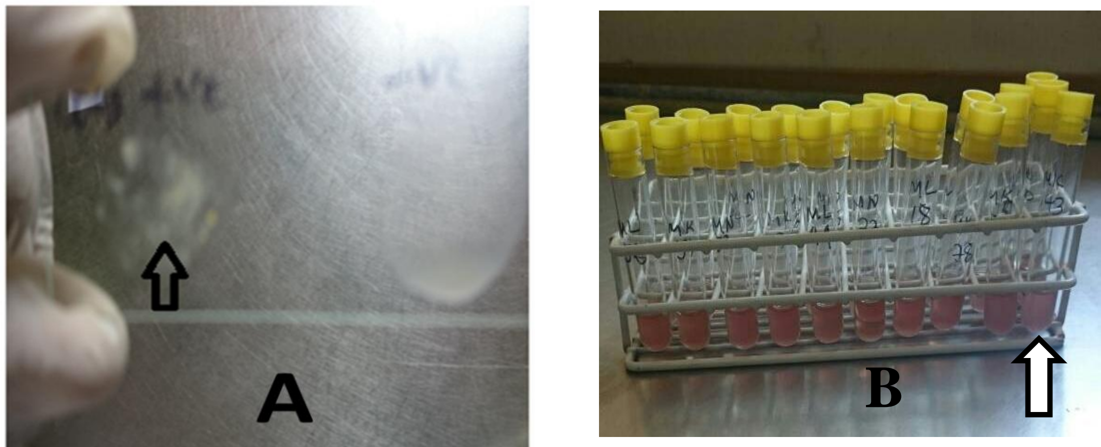

Figure 3: From left coagulase slide (A) test and coagulase tube (B) test and arrows were used to show the positive area on the slide and positive tube

4.3.2 Catalase Test

The suspected positive *Staphylococcus* isolates (27) were all confirmed to be Catalase positive in Catalase test (Fig. 4).

Figure 4: Microscopic slide show the positive Catalase test of bacteria isolate

4.4 Molecular Analysis of Suspected *Staphylococcus aureus*

Conventional Polymerase Chain Reaction (PCR) was used to identify pathogenic *S. aureus*. Confirmation of *S. aureus* on targeted genes for 16S rRNA was carried out. Out of 27 samples that were positive Staphylococcus species in Gram stain, catalase and coagulase tests, only 7 samples were confirmed by PCR to be *S. aureus*. Other tests included *mec*A, PVL, *nuc*, *spa* gene and Enterotoxins (SEA, SEB and SEC).

4.4.1 Monoplex PCR for *nuc gene*

All seven suspected *S. aureus* samples were positive to the *nuc* gene. The gene was detected between 200bp and 300bp at 270bp (Fig. 5). All positive samples were obtained from Mkokotoni (MK) land site.

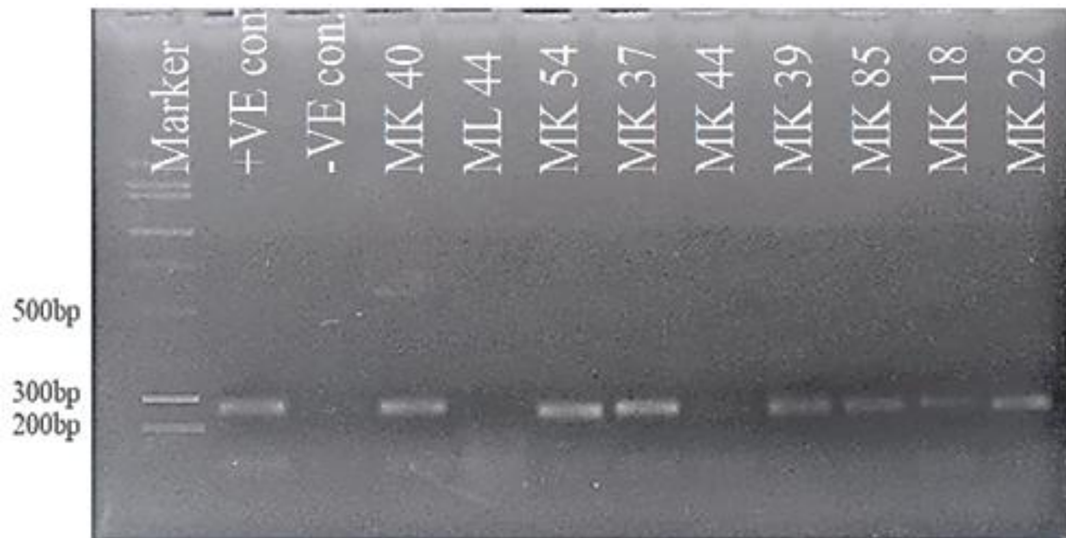

Figure 5: *nuc* gene detected in the suspected *S. aureus* positive samples. MK are samples tested from Mkokotoni, -VE Co represents negative control while +VE Co is for positive control

From the molecular analysis results seven out of 400 skin swab samples were confirmed to be *S. aureus* by showing a band for 16S rRNA and nuc genes. This makes prevalence of 1.75% (95% CI: 0.0007 –0.036).

4.4.2 Multiplex PCR for *mec*A gene

All 7 samples were run for *mec*A gene. Out of seven samples two samples (MK 40 and MK 54) had *mec*A genes. Both positive control and 16S had the same bands (double) as those of two positive samples (Fig 6). The amplification of *mec*A was detected at 280bp.

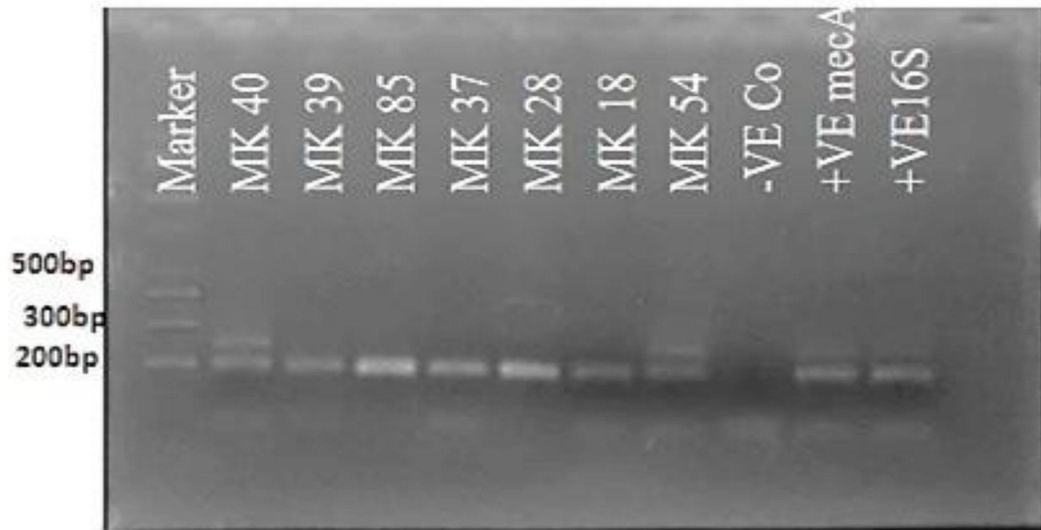

Figure 6: Samples MK 40 and MK 54 is having *mec*A bands. MK are samples tested from Mkokotoni, -VE Co represents negative control while +VE is for positive control of *mecA* and 16S is for positive control of 16S rRNA gene

Out of seven confirmed *S. aureus* isolates, two had pathogenic *mec*A gene.

4.4.3 Enterotoxins (SEA, SEB and SEC)

Multiplex PCR was conducted to all seven *S. aureus* isolates. Three enterotoxins genes namely SEA 102bp, SEB 164bp and SEC 451bp were tested from the samples. To note only sample MK 39 was positive and amplified two bands of enterotoxin B and C (SEB and SEC) (Fig. 7).

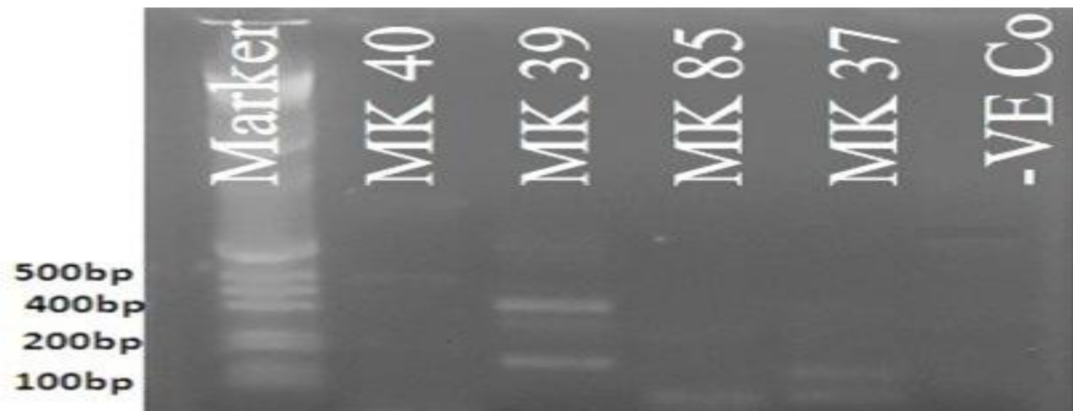

Figure 7: Sample MK 39 showing enterotoxin bands. MKs' are samples tested from Mkokotoni and -VE Co represents negative control

One *S. aureus* isolate has enterotoxins, hence posing a threat to public health.

4.4.4 PCR for detecting *spa* and PVL gene

Detection of *S. aureus* by using Monoplex PCR for *spa* gene to confirm *S. aureus* pathogenic gene at 1150-1500 bp using primer pairs targeting spa-1 and spa-2. There was no any positive isolates with amplicons in *spa g*ene. On the other hand PVL was also tested in all 7 samples and all samples were negative.

CHAPTER FIVE

5.0 DISCUSSION

Various factors pose risks to sea food safety and they range from contamination within the environment where it is caught up to the point of consumption (Mohammed *et al.,* 2003; Rhee and Woo, 2010). According to Ng *et al.,* 2000, potentially pathogenic bacteria present in foods can reach high numbers without necessary producing noticeable alterations in features, odor or taste. Among contaminated sea food is Indian mackerel fish (IMF) *R. kanagurta* which was used in this study. Findings from the primary culture demonstrated a high growth of bacteria in *R. kanagurta* in all three studied landing sites namely Mangapwani 147 (98%), Mkokotoni 132 (88%) and Malindi 80 (80%). The Gram stain, Catalase and biochemical tests revealed that 27 isolates confirmed to be *Staphylococcus* bacteria. After PCR tests to the isolates, only 7 (1.75%) samples were confirmed to be *S. aureus.* Although in formula used to estimate the sample size the prevalence was estimated at 50% but the actual percentage was 1.75%.

Despite of very high contamination with other bacteria in this study, *S. aureus* were very low compared with other studies, that had prevalence from 17% - 61.67% in India and Japan (Shimizu *et al.,* 2000; Simon and Sanjeev, 2007; Rhee and Woo, 2010). Low contamination of Indian mackerel fish in this study might be due to the different sources of contamination including pollution of the landing sites with human waste products and tools (knives, plastic bags, plastic buckets and fishing nets) used to unpack the fish from the boats and containers used in freezing systems.

Furthermore, long distances from trapping sites to the shore, improper storage during transportation, in appropriate accumulation and packaging of frozen fish are the other areas for the high contamination of frozen fish compared with fresh fish (Reij *et al.,* 2003). In the present study, through my personal observation, the presence of *S. aureus* was also attributed by contamination of the fish samples by raw sewage that is discharged directly into sewage disposal system to the sea, the situation that was found in all three landing sites (Sujatha *et al.,* 2010). In the another study conducted by Sujatha *et al.* (2010), reported isolation of *S. aureus* from the gills, intestines, muscles and skin of *Megalapsiscoedyla* and muscle of *Pricanthushamrur* from Royapuram waters in Indian Ocean although this species of fish were not included in my study. This was also attributed by the heavy loads of sewage disposal into the seas that could act as a suitable environment for the growth and survival of the human pathogens.

Furthermore, presence of *S. aureus* found in Indian mackerel fish from the landing sites are more likely to be attributed by multiplier effect as the fish are poorly handled and stored until they are consumed. They are disposed directly on the dirty ground, dirty containers and flies get free access to the fish. Contamination of IMF from fish market at landing sites was evident from my personal observations. Also the contamination increased during the course of distribution process (Montville, 2002).

The process of distribution, selling and moving IMF from one area/container to another contributed the transmission of *S. aureus* and other *Staphylococcus* species

from contaminated surfaces of fish and their products (Reij *et al*., 2003; Gutierrez *et al*., 2012). Hygienic practices like washing of containers, hands, knives and distribution of fish in the grounds at Mkokotoni, Malindi and Mangapwani landing sites are not improved which may have resulted in the higher incidence of bacterial contamination in the present study.

Transfer of micro-organisms by personnel involved in fish industry particularly from their hands is so vital importance (Chen *et al.,* 2001; Montville *et al.,* 2001; Bloomfield, 2003). During handling and preparation, bacteria transferred from contaminated hands of workers to fish and subsequently to other surfaces (Montville, 2002). Poor hygiene, particularly deficient or absence of hand washing and washing detergents has been identified as the causative mode of transmission of bacteria (Reij *et al.,* 2003). This problem has been observed in all three landing sites, thus why bacteria contamination were recorded.

Proper hand washing and disinfection has been recognized as one of the most effective measure to control the spread of pathogens, especially when considering employed workers (Montville *et al.,* 2001). Apart from personal hygiene of workers, unclean, insufficiently or in adequately cleaning of processing equipments has been identified as a source of bacteria contamination in the processed fish (Reij *et al.,* 2003). In order to avoid bacteria contaminations, the whole process should be under hygienic control.

In the present study, Gram staining technique was used for identification of bacteria on cellular morphology and staining effect. Based on positive results from coagulase

slide, catalase and coagulase tube test, out of 400 samples from skin swabs, only 27 isolates (6.75%) were *Staphylococcus* species showing Gram positive cocci for all three landing sites. These results agree with that of Arfatahery *et al*. (2015) showing that *Staphylococcus* species viewed under microscope presents as gram positive cocci. The dominance of coagulase and catalase positive cocci in isolates conformed to the other studies (Ezzeldeen *et al.,* 2011; Vazquezsanchez *et al*., 2012) as both studies reported higher rates of *Staphylococcus* species found in sardine 86.7% and 90% respectively. Of note, the observation of coagulase activity in this study was not exclusive for *S. aureus* since various other bacteria species have been described as coagulase positive. Based on the positivity of isolates that were not *S. aureus* to the catalase and coagulase, other methods of detecting *S. aureus* were carried out in this study (Ezzeldeen *et al*., 2011).

The PCR technology uses oligonucleotide primers targeted to species specific parts of the gene encoding the 16SrRNA and the genes encoding coagulase and protein. A comparable PCR based systems for identification of *S. aureus* isolates from various origins have been used by several researchers (Brakstad *et al*., 1992; Martineau *et al.,* 1998; Straub *et al.,* 1999). All these target genes allowed a rapid identification of the bacteria species with high specific part sensitivity and specificity. As was reported by Straub *et al.* (1999), the amplification of the gene encoding an *S. aureus* specific part of the 16S rRNA revealed an amplicon with a size of 200bp for *S. aureus* isolates investigated.

In the present study *S. aureus* was identified and classified by using PCR with specific primers on a diagnostic protocol to detect the different genes (Mehrotra *et*

al., 2000). It has been found that the *nuc* gene of all isolates were equal to 270bp (Figure 5). The *nuc* gene was used to detect isolates of *S. aureus* in IMF and the present findings indicated that the *nuc* gene was equal 270bp (Daniel *et al.,* 1994; Kipp *et al.,* 2003; Chikkala *et al.,* 2012). The *nuc* gene is a baseline in identification and classification of *S. aureus* and some reports indicated that *nuc* gene was encoded to enzyme the thermonuclease and the length fragment of *nuc* gene was equal to 270bp (Brakstad *et al.,* 1992).

The presence of *mec*A gene in *S. aureus* strains was tested. In this study *mec*Aamplicons were identified in some strains. The presence of *mec*A in species of *Staphylococcus* other than *S. aureus* was previously demonstrated (Carneiro *et al.,* 2004). Recent studies pointed out that the *mec*A gene is present in mobile Staphylococcal chromosomal cassette *mec*A (Jansen *et al.,* 2006; Katayama *et al.,* 2003) and some of the enterotoxin genes, along with other virulence factors are part of mobile pathogenesity Islands (Novick, 2003; Novick and Subedi, 2007). A recent study indicated that high mobility of the *mec*A gene may be more prevalent than the movement of enterotoxins genes (Witte *et al.,* 2000).

In this study the PCR product appeared with a size 280bp fragment which is close to the *mec*A (Fig. 6) which is close to 293bp reported by Chikkala *et al.* (2012). Also in the same figure the product of 16S rRNA appeared with a size of 200bp fragment. The findings from this study correspond to the study carried out by Saruta *et al.* (1995) who obtained 273bp fragments of 16S rRNA gene. The variation of bp in *mec*A gene and 16S rRNA in this study when compared with other studies could be due to *S. aureus* of different strains.

In this study, the selected isolates were found negative for PVL genes. This could indicate that PVL is not normally found in the isolates of landing site environment. However, these isolates are not necessarily representative of landing sites environment in general. Absence of PVL in *S. aureus* from landing site environment may indicate limited role of antileucocytic activity outside the host. The absence of PVL in this study could also be contributed by the sample of *S. aureus* isolates being 7 out of 400 samples examined. Reports from various countries showed that there is increasing prevalence of PVL gene among isolates (Eckhardth *et al.,* 2003; Linde *et al.,* 2005). Souza *et al.* (2010) reported prevalence of 64% PVL positive isolates in *S. aureus*. A lower prevalence of PVL has been also reported from other parts of world (5% in France, 4.9% in UK; 8.1% in Saudi Arabia and 14.3% in Bangladesh) (Lina *et al*., 1999; Holmes *et al*., 2005; Afroz *et al*., 2008).

It is common for *S. aureus* to produce one or more toxins simultaneously; therefore appropriate measures are required to detect these toxins. Formerly, SEs has been divided into five major serological types (SEA, SEB, SEC, SED and SEE), on the basis of their antigenic properties (Su and Wong, 2000). But in the last few years, nine new SEs types have been identified (SEG to SEO) (Letertre *et al*., 2003). Based on this study, two types of enterotoxins were detected namely SEB and SEC (14.29%) that possessed enterotoxins genes. This finding is in agreement with findings reported by Igarashi (2000) in Japan. Staphylococcal food poisoning is mainly caused by SEA and SEC, therefore it is notable that IMF caught at Mkokotoni landing site were contaminated with SEB and SEC enterotoxins (Figure 7). Despite the fact that SEA was not detected in this study, SEA is the most

common enterotoxin recovered from food poisoning outbreaks (Balaban and Rasooly, 2000) and it is known that 59% of staphylococcal food poisonings out breaks are caused by SEA to SEE (Bergdoll, 2000). In this study, the toxin genotypes of *S. aureus* strains isolated from IMF found to be positive for SEC at 451bp (Mehrotra *et al.,* 2000). These results are in accordance with previous finding that many healthy individuals are carriers of toxin producing strains of *S. aureus* (Chance, 2001).

Occurrence of enterotoxigenic Staphylococci has been reported previously in India (Sanjeev *et al*., 1985; Sanjeev *et al*., 1986; Sanjeev and Surrendran, 1994). Also Sneha (2004), reported that 41.1% of the isolates produced enterotoxins. Sindhu and Surrendran (2006) reported enterotoxin SEB was dominant toxin (52.63%) followed by SEC (47.36%). Sneha (2004) reported that 50% of the isolates produced SEC followed by SEB (16.7%). Rajalakshmi and Rajyalakshmi (1982) reported that majority of the *S. aureus* isolates recovered from cases of bacterial food poisoning in India were found to produce SEC. All tested samples for *S. aureus* showed negative results in *spa* gene. This may show that the gene is not present in the IMF in the study areas. So this is in accordance with the study carried out by Shakeri *et al.* (2010). But also the small sample size of isolated *S. aureus* could also be a contributory factor.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

This work revealed the presence of potentially pathogenic bacteria *S. aureus* in Indian mackerel fish. The presence of *S. aureus* was at low prevalence and was only detected at Mkokotoni landing site. Among the epidemiological factors were unhygienic conditions in handling and distribution of IMF and sewage disposal directly into the sea. The presence of *S. aureus* is the source of health risk for consumers of raw seafood and that improperly cooked fish and fish products. The fish act as a reservoir of human pathogens and the presence of highly pathogenic agents such as *S. aureus* is a potential health risk or hazard to human beings and may cause diseases to susceptible individuals especially the immune compromised consumers.

6.2 Recommandations

This study focused only on *S. aureus* bacteria but further studies should be carried out to identify other bacteria that were isolated and were not identified as target species was *S. aureus*. There could be other species of bacteria that cause health problems to the community.

Further studies of different fish species and their bacterial loads should be carried out as reported in other studies especially during handling, storage and up to the point of consumption for the protection and maintenance of health by keeping food borne disease to a minimum.

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