# INCIDENCE OF MULTIDRUG RESISTANT *STAPHYLOCOCCUS AUREUS* AND ENTEROBACTERIACEAE FROM GROUNDNUT CAKES (*KULI-KULI*) AND ROASTED GROUNDNUTS OBTAINED FROM ZARIA METROPOLIS

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**JULY, 2017**

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**P13PHPM8003**

**A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER DEGREE IN PHARMACEUTICAL MICROBIOLOGY**

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**JULY, 2017**

## DECLARATION

I declare that the work reported in this dissertation entitled "Incidence of Multidrug Resistant *Staphylococcus aureus* and Enterobacteriaceae from Groundnut Cakes (*kuli-kuli*) and Roasted Groundnuts Obtained from Zaria Metropolis." was carried out by me in the Department of Pharmaceutics and Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, under the supervision of Prof. J. A. Onaolapo and Dr. (Mrs) R. O. Bolaji.

The information derived from the literature reviewed has been duly acknowledged in the text and a list of references provided. No part of this dissertation has been presented in any previous application for another degree or diploma at any University.

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## CERTIFICATION

This dissertation entitled "Incidence of Multidrug Resistant *Staphylococcus aureus* and Enterobacteriaceae from Groundnut Cakes (*kuli-kuli*) and Roasted Groundnut Obtained from Zaria Metropolis." by Mojisola Omotayo TENDE meets the regulations governing the award of the Degree Master of Science of Ahmadu Bello University, Zaria, Nigeria and is approved for its contribution to knowledge and literary presentation.

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## DEDICATION

This work is dedicated to God Almighty for His Amazing Grace; I am one of the products of His Grace. I stand in awe of Him and I bless His Name alone.

## ACKNOWLEDGEMENT

I appreciate the Almighty God for seeing me through this work successfully.

I sincerely appreciate the fatherly contributions of Prof. J.A. Onaolapo under whose supervision this work was carried out. His advice, encouragement and constructive criticisms are highly appreciated. My profound gratitude goes to Dr. R. O. Bolaji for her valuable contributions to this work.

I also sincerely appreciate all academic and non-academic staff of the Department of Pharmaceutics and Pharmaceutical Microbiology, for their various contributions to the success of this work, especially the H.O.D, Prof. B. O. Olayinka and Prof. G. O Adeshina.

Also to be acknowledged is the support and contributions of the Pastors and members of The Father's Delight International Ministry worldwide.

I highly appreciate the understanding and prayers of my dear families: Tendes, Wuraolas, Oyeyipos, and Aroyewuns especially my loving Husband, Precious as I call him, for his encouragement and understanding in ensuring that this program was a success.

Not to be forgotten are my postgraduate colleagues, who have in one way or the other contributed to the success of this work, especially Dr Igwe James and my dear friend (Ezeh Peace).

May God Almighty in His infinite mercy and favour bless you all in Jesus name, Amen.

## ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a bacterium responsible for several difficult-to-treat infections in humans. Previous studies reported the identification of MRSA in food demonstrating that food can represent a source of *Staphylococcus aureus* which may carry the *mecA* gene. The aim of this study is to determine the incidence of multidrug resistant *Staphylococcus aureus* and Enterobacteriaceae from groundnut cakes (Kuli-Kuli) and roasted groundnut obtained from Zaria metropolis. A total of 294 samples were obtained from different batches of roasted groundnut, kuli-kuli, and swabs from the hands of the vendors. Bacteriological analysis of roasted groundnut and kuli-kuli samples were carried out by growth on specific culture media. Nutrient Agar plates were also opened around the environment where the kuli-kuli and groundnut were sold.

A total of 152 bacterial isolates were recovered from the 106 samples with significant bacteria growth. Using the Microgen kits for identification, it was found that the isolates consisted mainly of Enterobacteriaceae and *Staphylococci*. 82% (125/152) of the isolates were Staphylococci. The Staphylococci were *Staphylococcus aureus* (40.6%), *Staphylococcus xylosus* (23.2%), *Staphylococcus intermedius* and *Staphylococcus hyicus* (8.7%), *Staphylococcus chromogenes* (7.2%), *Staphylococcus schleiferi* (5.8%), *Staphylococcus haemolyticus* and *Staphylococcus epidermidis* (2.9%).

Full identification of isolates showed that the most prevalent Enterobacteriaceae were *Citrobacter freundii* (22.2%), *Klebsiella oxytoca* (11.1%), *Salmonella arizonae* (7.4%), *Salmonella species* (7.4%), *Acinetobacter lwoffi* (7.4%), *Proteus vulgaris* (7.4%), *Serratia marcescens and Acinetobacter haemolyticus* (7.4%). The other Enterobacteriaceae isolated include *Escherichia coli inactive* (3.7%), *Providencia stuartii* (3.7%), *Proteus mirabilis*

(3.7%), *Enterobacter agglomerans* (3.7%), *Klebsiella pneumoniae* (3.7%) and *Providencia alcalifaciens* (3.7%).

Antibiotic resistance profile of the bacteria isolates showed that most of the Enterobacteriaceae isolates were resistant to the inhibitory activities of Cefixime (96%) and Amoxicillin/Clavulanic acid (74%). Gentamicin, Imipenem and Quinolones showed high activities with 0%, 0% and 4% resistance respectively. *Staphylococcus aureus* isolates also showed high resistance to Cefixime (82%). High percentages, (88.9%) of Enterobacteriaceae, and (50.0%) of *Staphylococcus aureus* were observed to have MAR index of 0.3 and above and 78% of Enterobacteriaceae and 61% of *Staphylococcus aureus* were MDR, indicating that the isolates might have been pre-exposed to the antibiotics used in this study. Six (6) of the *Staphylococcus aureus* isolates were found to be MRSA phenotypically and molecular characterization of the isolates confirmed them to be carriers of *mecA* gene, while 33.3% of them carried the *vanA* gene but, none of the MRSA expressed the *blaZ* gene.

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# CHAPTER ONE

## 1.0 Introduction

Groundnut with the botanical name *Arachis hypogaea L*. occupies an important position worldwide, especially in the developing countries. In Nigeria, it is known as “epa”,”gyeda” and “apapa” or “ahuekele” in the Yoruba, Hausa and Igbo languages respectively. The major groundnut producing countries are India, China and the United States. It was introduced in to Nigeria in the 16th century and it has been estimated that about 1.4 million hectare of cultivated land is devoted to groundnut in Nigeria (Salunkhe *et al*., 1985; Taru *et al*., 2008).

In Nigeria, groundnut is usually consumed as boiled or roasted nuts. Groundnut is dense in nutrients. It is very high in energy due to its high fat and protein content. The carbohydrate content of groundnut is relatively low, being under 30% of the whole nut. The nut has relatively high content of fiber. It is an industrial crop whose major utilization is a source of oil (Elegbede, 1998).

Groundnut cake, referred to as kuli-kuli in Hausa language is a popular snack of all age groups (especially school-age children and the middle age) in West Africa particularly in northern Nigeria. "Kuli-kuli" is the residue obtained after the extraction of oil and it is high in protein and used as supplement in feed and food. The groundnut cake is usually fried in oil and is used as a delicious snacks or food supplement. It is also used as a major ingredient in the formulation of poultry feeds (Adebesin *et al.,* 2001).

In Nigeria, roasted groundnuts and kuli-kuli are usually hawked in open trays or wide bowls and packed in polythene bags when sold to the consumers.

Gram-negative and Gram positive organisms have been isolated from groundnut and its

products. Some of these organisms include *Escherichia coli, Staphylococcus aureus, Yesinia enterocolitca, Salmonella sp, Proteus spp, Bacillus spp, Serratia spp, Shigella spp, Klebsiella spp, Micrococcus sp, Yeasts* and moulds (Ezekiel *et al*., 2011; Odu and Okonko, 2012).

Some of these organisms have been used to assess the microbiological safety and sanitation conditions during processing and keeping quality of peanut butter product (Consumer report, 2009).

Oyenuga (1968) assayed the nutrient content of kuli-kuli and reported it to contain carbohydrate 28.3%, fat 10.2%, crude protein 61% and ash content of 5.50%.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a strain of *Staphylococcus aureus* that has developed, through the process of natural selection, resistance to beta-lactam antibiotics, which include the penicillins and the cephalosporins. This resistance makes MRSA infection more difficult to treat with standard types of antibiotics and thus more dangerous (McDougal *et al*., 2003).

Healthy individuals may carry MRSA asymptomatically for periods ranging from a few weeks to many years and patients with compromised immune systems are at a significantly greater risk of symptomatic secondary infection.

The initial presentation of MRSA is small red bumps that resemble pimples, spider bites, or boils; they may be accompanied by fever and, occasionally, rashes. Within a few days, the bumps become larger and more painful; they eventually open into deep, pus-filled boils. Some of the populations at risk of MRSA infection include: people who are frequently in crowded places, especially with shared equipment and skin-to-skin contact (CDC, 2013), people with weak immune systems, diabetics (Lipsky *et al*., 2010), Intravenous drug users

(Otter and French, 2011; Golding *et al*., 2012), the elderly (Elias *et al*., 2013), People staying or working in a health care facility for an extended period of time (CDC, 2013).

A defining characteristic of MRSA is its ability to thrive in the presence of penicillin-like antibiotics, which normally prevent bacterial growth by inhibiting synthesis of cell wall material. This has been shown to be due to a resistance gene, *mecA*, which stops β-lactam antibiotics from inactivating the enzymes (transpeptidases) critical for cell wall synthesis. The gene is responsible for resistance to methicillin and other β-lactam antibiotics. After acquisition of *mecA* (either by horizontal gene transfer or plasmid-mediated), the gene must be integrated and localized in the *S. aureus* chromosome (Lowy, 2003). The *mecA* gene encodes penicillin-binding protein 2a (PBP2a), which differs from other penicillin-binding proteins as its active site does not bind methicillin or other β-lactam antibiotics (Lowy, 2003). As such, PBP2a can continue to catalyze the transpeptidation reaction required for peptidoglycan cross-linking, enabling cell wall synthesis in the presence of β-lactam antibiotics. As a consequence of the inability of PBP2a to interact with β-lactam moieties, acquisition of *mecA* confers resistance to all β-lactam antibiotics in addition to methicillin (Lowy, 2003; Sahebnasagh *et al*., 2011).

*mecA* is under the control of two regulatory genes, *mecI* and *mecR1*. *MecI* is usually bound to the *mecA* promoter and functions as a repressor (Jensen and Lyon, 2009). In the presence of a β-lactam antibiotic, *MecR1* initiates a signal transduction cascade that leads to transcriptional activation of *mecA* (Jensen and Lyon, 2003). This is achieved by *MecR1*-mediated cleavage of *MecI*, which alleviates *MecI* repression (Jensen and Lyon, 2003). *mecA* is further controlled by two co-repressors, *BlaI* and *BlaR1*. *BlaI* and *BlaR1* are homologous to *mecI* and *mecR1*, respectively, and normally function as regulators of *blaZ*, which is responsible for penicillin resistance (Berger-Bachi, 1999; Lowy, 2003). The DNA

sequences bound by *MecI* and *BlaI* are identical (Lowy, 2003) therefore; *BlaI* can also bind the *mecA* operator to repress transcription of *mecA* (Berger-Bachi, 1999).

Vancomycin has been an effective treatment for staphylococcal infection and the first clinical isolate of *Staphylococcus aureus* that showed resistance to Vancomycin was reported in 1997 (Chang *et al.,* 2003). Due to the widespread occurrence of *mecA*-encoded methicillin resistance in *Stapylococcus aureus* (MRSA), treatment of staphylococcal infections is shifted to glycopeptides antibiotics like vancomycin and teicoplanin. The selective pressure of glycopetide has eventually led to the emergence of staphylococci with increased resistance (Icgen, 2016). MRSA showing reduced susceptibility to vancomycin has been reported to harbor *vanA* gene (Rossi *et al*., 2014; Icgen, 2016).

### 1.1 Statement of Research Problem

Groundnut-based foods have been associated with food illness in which initial contamination is traceable to food handlers (Sokari, 1991). Numerous epidemiological reports and studies have implicated foods of ready to eat origin as the major vehicles associated with illness caused by food- borne pathogens. Person to person transmission has also been described (Sokari, 1991). The enterobacteria are a large group of related bacteria that are capable of food and water contamination through faecal sources. Many of the strains and species are known to be enterotoxigenic and contribute a major quota to the many diarrheal illnesses experienced by man (Talaro and Talaro, 2002). In a study carried out by Ezekiel *et al*. (2011), 49 kuli-kuli samples obtained from markets in nine districts within Nigeria were subjected to microbial and proximate analyses in order to ascertain the quality of this food material and all the samples had bacterial contamination at varying levels ranging from $4.2\times10^6$ to $1.0\times10^7$ cfu/g. Some other studies have recorded incidence

of bacterial contamination of groundnut and kuli-kuli (Adetunji and Olaoye, 2011; Euloge *et al.,* 2012; Odu and Okonko, 2012).

Roasted groundnut and kuli-kuli are popular snacks in Nigeria and are often eaten with meals such as "Gari", "Akamu" and sometimes put into salad. They are produced locally and local processing and packaging methods are commonly adopted. This predisposes the groundnut cake to microbiological contamination especially during hawking of the product, which is often exposed or packaged in hand knotted thin polythene bags. Vital information such as the name and address of producers, nutritional content, and recommendations for storage and expiration date for human consumption are also not indicated, despite the fact that mycotoxicoses have been implicated in human and animal pathology (Bacha *et al*., 1988). The situation maybe worsened by consumers' reluctance to discard fairly moldy food samples such as groundnut cake due to the cakes' irresistible taste and flavor.

*Staphylococcus aureus* which is one of the several organisms that have been isolated from groundnut and kuli-kuli samples can cause many type of diseases. The development of antibiotic resistance poses an even greater threat to public health. *Staphylococcus aureus* is known to possess the adaptive capability to promptly respond to antibiotics making it resistant and increasingly difficult to treat. Methicillin-resistant strains of *Staphylococcus aureus* (MRSA) are a major concern with regard to these species. The identification of MRSA in food reported by previous studies shows that food can represent a source of *Staphylococcus aureus* which may carry the *mecA* gene (Rizek *et al*., 2011).

Healthy individuals may carry MRSA asymptomatically for periods ranging from a few weeks to many years. Patients with compromised immune systems are at a significantly greater risk of symptomatic secondary infection. The burden of MRSA is significant. In the United States, there were an estimated 463,017 (95% confidence interval: 441,595,

484,439) MRSA-related hospitalisations, or a rate of 11.74 (95% confidence interval: 11.20, 12.28) per 1,000 hospitalisations, in 2009 (Klein *et al*., 2013). Many of these infections are less serious, but the Centers for Disease Control and Prevention (CDC) estimates that there are 80,461 invasive MRSA infections and 11,285 deaths due to MRSA annually (CDC, 2013) Worldwide, an estimated 2 billion people carry some form of *S. aureus*; of these, up to 53 million (2.7% of carriers) are thought to carry MRSA.

## 1.2 Justification of the Study

Roasted groundnuts and kuli-kuli are popularly consumed as snacks especially among school age children of low income earners, whose immunity are sometimes compromised because of their inadequate nutritional intake. Ingestion of these snacks if contaminated, can lead to infection. Staphylococcal food poisoning represents one of the most prevalent food intoxication worldwide. Oral intake of staphylococcal enterotoxins from food can result in emesis and diarrhea and can be fatal in children and the elderly (Baumgartner *et al*., 2014) hence it is important to investigate the bacterial load of locally sold roasted groundnuts and kuli-kuli.

Most local sellers and hawkers of roasted groundnuts and kuli-kuli do not observe proper hygiene. *Staphylococcus aureus* being a normal floral of the skin can be introduced during the processing, packaging and distribution of the snacks since most of the hawkers use their bare hands in processing and packaging. Reports have shown that the organism has been isolated from food samples (Baumgartner *et al*., 2014).

It has been popularly reported that there has been increase in the incidence of MRSA infection which is due to the misuse of antibiotics and natural selection (Awad *et al*., 2007; David *et al.,* 2012). *MecA* which has been implicated in methicillin resistance is being more

frequently isolated from infections associated with the ingestion of contaminated food substances (Ndahi *et* al., 2014).

In Nigeria, roasted groundnuts and kuli-kuli are produced traditionally on a small scale by local sellers and hawkers and as a result little or no attention has been given to the microbiological quality and safety of traditionally processed Nigerian roasted groundnuts and kuli-kuli. Also there are few studies that have been done on them especially in this part of the country, so no information on the public health implication of the consumption of contaminated roasted groundnut and kuli-kuli.

## 1.3 Aim and Objectives

### 1.3.1 Research aim

The aim of this research work is to determine the incidence of Methicilin-Resistant *Staphylococcus aureus* from kuli-kuli and roasted groundnuts obtained from Zaria metropolis.

### 1.3.2 Research objectives

The specific objectives of this research work are:

i) To isolate and identify Staphylococcus and Enterobacteriaceae species from kuli-kuli, roasted groundnuts, and hand swab samples of their vendors collected from markets in Zaria metropolis.

ii) To determine the susceptibility of the isolates to commonly prescribed antibiotics by disc agar diffusion technique (DAD)

iii) To phenotypically test for the presence of Methicillin-resistance in *Staphylococcus aureus* isolates using cefoxitin discs.

iv) To molecularly test for the presence of *mec*A genes in suspected isolates using PCR.

v) To test for the presence of *blaZ* and *vanA* genes in suspected isolates.

## 1.4 Hypothesis

### 1.4.1 Null hypothesis

- There is no occurrence of multidrug-resistant *Staphylococcus aureus* and Enterobacteriaceae in isolates from kuli-kuli and roasted groundnuts obtained from Zaria metropolis

### 1.4.2 Alternate hypothesis

- There is an occurrence of multidrug-resistant *Staphylococcus aureus* and Enterobacteriaceae in isolates from kuli-kuli and roasted groundnuts obtained from Zaria metropolis

## 1.5 Inclusion and Exclusion Criteria

### 1.5.1 Inclusion criteria

- Local sellers of groundnut and kuli-kuli in the five selected markets
- Local sellers who will give their consent to be included in the study

### 1.5.2 Exclusion criteria

- Local sellers who will not give their consent to be included in the study.
- Samples will only be taken from markets in Zaria (markets outside Zaria are excluded)

# CHAPTER TWO

## 2.0 Literature Review

### 2.1 Groundnut and Groundnut Products

Groundnut (*Arachis hypogaea* L.) is an annual herb belonging to the Papilionaceae division of the family *Leguminoseae* whose pods grow and mature under the soil surface. It originated from Latin America and in the 16$^{th}$ century, the Portuguese from Brazil introduced it to West Africa (Adinya *et al*, 2010). Groundnut is an herbaceous plant of which there are many varieties worldwide, some of which are Boro light, Boro red, Ela, Mokwa, Guta and Campala and it is commonly known as peanut, okpa (Ibo), gya'da (Hausa) and epa (Yoruba)(Ayoola and Adeyeye, 2010).

Groundnut is cultivated around the world, especially in the tropics. Nigeria is the largest groundnut producing country in West Africa accounting for 51% of the production in the region, accounting for 10% and 39% of the World and Africa's total production respectively (Ndjeunga *et al*., 2013). It is estimated that Nigeria's cultivated area under groundnut cultivation is about 1.0 to 2.5 million hectares annually and yield in the range of 500 – 3000 kg/ha while the seed yield in Northern Nigeria is about 3000 Kg/ha (Ibrahim, 2010). Before diverting the economy into an oil dependent one, the growth of groundnut was a major contributor to the gross domestic product of Nigeria. It is however the main export product of states in northern Nigeria (Taru *et al.*, 2010). It is cultivated for its kernels, the oil and hay for livestock (Ibrahim, 2010).

Atasie *et al.* (2009) reported that groundnut contained carbohydrate, fat, crude protein, crude fibre, ash and moisture content of 1.81, 47, 38.61, 3.70, 3.08 and 5.80% respectively. Groundnut plays a very important role in nutrition across the world because it is a rich source of vegetable protein and edible oil, especially in developing countries where access to animal protein is expensive. Groundnut is the 13$^{th}$ most important food crop of the world. It is the world's 4th most important source of edible oil and 3rd most important source of vegetable protein (Girei *et al.*, 2013).The defatted seed can be used to prepare food, peanut butter, soup, oil and snacks, the seed is also fried and eaten directly. Groundnut and groundnut products are sold commercially and hawked all over Nigeria.

Some important bioactive compounds known for anti-inflammatory activities such as procyanidins and catechins are present in groundnut skin (Lewis *et al*., 2013). Groundnuts contain a number of nutrients beneficial to the heart such as flavonoids, phytosterols, folates, alpha-tocopherol, fiber, arginine, niacin, copper, magnesium, potassium, monounsaturated fatty acid (MUFA) and polyunsaturated fatty acids (PUFA). The amino acid profile of groundnut is complementary to that of cereals, such that combining them in meals raises the nutritional effectiveness of both. Groundnuts are rich in zinc and iron (Ndjeunga *et al.*, 2013). Groundnuts along with other legumes are considered a part of meat and meat alternative group in the Food Guide Pyramid. Groundnut consists of mainly of two globulins namely arachin (93% of defatted seed protein) and co-arachin (Kochlar and Dhanesh, 2013). Groundnut is consumed in the roasted or boiled form, and also as groundnut cake ('kuli-kuli') (Adjou *et al.*, 2012).

Fig 2.1a, 2.1b and 2.1c show groundnuts in shells, roasted groundnuts with skins and peeled roasted groundnuts respectively.



Fig 2.1a: Groundnuts with shell

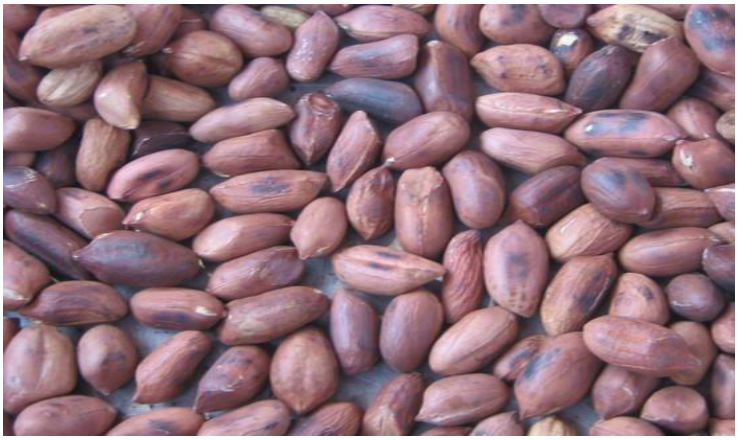


Fig 2.1b: Roasted Groundnuts



Fig 2.1c: Peeled Roasted Groundnuts
**Source: Lawrence (2016)**

### 2.1.1 Kuli-kuli

Kuli-kuli is a traditional snack made from groundnut residue after oil extraction by drying or deep frying. It makes part of the most important diet in some rural populations. It is crunchy, delicious and sometimes pepper spiced. It is sometimes referred to as defatted groundnut flour. It is high in dietary protein and could be a source of protein for many populaces in Nigeria (Osuolale and Olayiwoola, 2014). Kuli-kuli is usually at its best within 14 days after production; therefore its nutritive value is maximized during this period. It is used as supplement in food and in feed and is sometimes grounded into flour and used in soup making. It has been reported to be a popular snack in Nigeria and often eaten with meals such as Garri, Akamu and sometimes put into salad (Ezekiel *et al.*, 2011). Kuli-kuli is also used as a supplement in fish feed because it is rich in crude protein (Davies and Ezenwa, 2010). An example is shown in fig.2.2.




Fig 2.2: Kuli-kuli

**Source:** afrofusionbrands.com/category/blog (2016).

#### *2.1.1.1 Nutritional value*

Groundnut is a good source of vitamins B, E and K. It is the richest plant source of thiamine (B1) and also rich in niacin (Davies and Ezenwa, 2010).

Groundnut cake (kuli-kuli) has been reported to have a moisture content of about 8.43%, ash content of 5.43%; crude fiber, crude protein and fat were 7.40, 39.93 and 17.53% respectively and carbohydrate 21.29% of whole cake (Osuolale and Olayiwoola, 2014). Ezekiel *et al.* (2011) reported that the kuli-kuli products from the northern regions of Nigeria have significantly higher protein content than those produced from other regions indicating that the kuli-kuli samples produced in the northern parts are of a higher nutritional quality and may have been from higher quality groundnut. Groundnut cake protein is however said to be lacking in methionine and lysine and also has a limited amount of threonine and tryptophan but the quality of the amino acid is improved in diets when supplemented with tryptophan, methionine and lysine (Eyo and Olatunde, 1998).

#### *2.1.1.2 Processing and packaging*

The first stage of processing groundnut into kuli-kuli is harvesting the pods from the farm by plowing and manually stacking mature groundnut plants. Drying of the harvested plants to between 7 to 10 percent but not more than 12 percent of its original weight reduces the risk of aflatoxin production (Nautiyal, 2002). These groundnuts are then further cleaned, stored and processed for various uses. The shelled groundnuts are then cleaned to separate it from foreign materials. The cleaned groundnuts are then crushed ensuring minimal damage to the kernels, and the kernels separated from the shells. Roasting is then done and this imparts the typical flavor to groundnuts which can be achieved by dry roasting or oil roasting. During roasting, amino acids and carbohydrates react to produce tetrahydrofuran

derivatives. The oil in the groundnut kernels is then expelled manually by solvent or hydraulic methods or with machines and the defatted flour obtained after oil extraction from seeds is molded and fried into groundnut cake (Dhanesh and Kochbar, 2015). Local packaging in nylon bags is used when sold to consumers (Oko *et al.,* 2015). The processing of groundnut into various products such as kuli-kuli is mostly done by women in Nigeria, either for commercial purposes or home consumption (Ibrahim *et al*., 2005) thereby providing a source of employment and income to many women in the rural areas of northern Nigeria.

Production and marketing of groundnut and its derivative is a profitable business in Nigeria with attractive net return on investment (Adinya, 2009; Taru *et al*., 2010). Although there is limited information on the profitability of groundnut, it is also important to note that there is sparse detail about groundnut cake (kuli-kuli) market structure. It is however commonly hawked in markets, streets and waysides, but with little efficiency.

The flow chart for the processing of kuli-kuli is shown in Fig 2.3

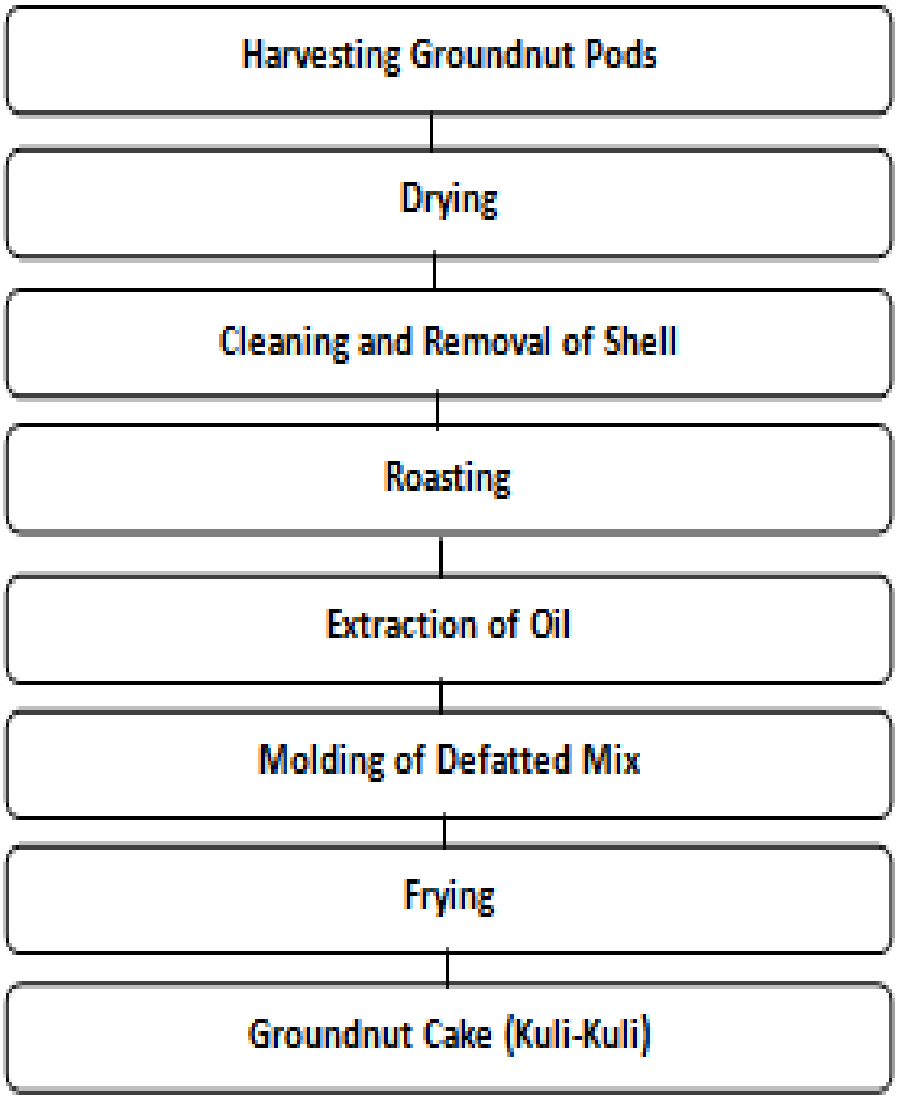



Fig 2.3: Processing of Kuli-kuli

Fig 2.4a shows kuli-kuli as sold in the markets while Fig 2.4b shows groundnuts as sold in some of the markets and shops.



**Fig 2.4a: Kuli-kuli sold in the market**




**Fig2.4b: Groundnuts sold in the market**

**Source: Rafael (2012)**

### *2.1.1.3 Associated health risk*

In microbial analysis of food, the number and type of microbes present in the food material under examination reflect quality of the food and extent of associated risk posed to the consumers (Lund *et al*., 2000) and is a concern to public health. The number and type of fungi recovered from the Kuli-kuli samples is also of immense public health importance since some of the species are notable toxin producers while the others are mere saprophytes; inciting deterioration of the food material in their bid to adapt and survive in the microenvironment. The presence of *Rhizopus, Penicillium* species, *Fusarium* species and *Aspergillus* species such as *A. niger* and *A. flavus,* and in the kuli-kuli samples exposes the consumers to high toxicological risk since majority of the strains of these fungal species are toxigenic (Makun *et al.,* 2010; Ezekiel *et al.,* 2011). These are probably introduced into the product by poor handling, low sanitary standards, poor hygiene, post production exposures as well as bad transportation methods. Contamination of groundnut and its products such as kuli-kuli reduces its quality and also lead to an increased mycotoxin production (Sultan and Magan, 2010).

The occurrences of enterobacteria such as *E. coli, Salmonella*, *Shigella*, *Klebsiella* and *Proteus* which have been implicated in human diseases were found in kuli-kuli samples obtained in markets across Nigeria, including Kaduna state. Also *Shigella*, *E. coli* and *Salmonella* were implicated for their ability to cause food intoxications and poisoning while some strains produce enterotoxins (Ezekiel *et al.,* 2011). These bacteria are usually conveyed into food, drink or water by vectors or faecally-contaminated handlers who maintain a low level of hygiene (Lund *et al.,* 2000; Nzeako *et al*., 2002). Oko *et al*. (2015) also isolated *Pseudomonas aeruginosa, Streptococcus feacalis, Klebsiella oxytoca*, *Bacillus cereus* and *Staphylococcus aureus* in kuli-kuli samples obtained in Zaria, Kaduna State.

The presence of these microbes in a product consumed by many customers of different economic and social class is therefore a public health concern.

## 2.2 Methicillin Resistant *Staphylococcus Aureus* (MRSA)

Staphylococci are a universal pathogens implicated in many diseases found in man and its colonies are responsible for a lot of nosocomial infections, especially those associated with long hospital stays which can sometimes be very fatal (DeLeo *et al.*, 2010; Nwankwo *et al.*, 2010; de Kraker *et al.*, 2011). *S. aureus* is a non-motile Gram-positive, coagulase-positive coccus of the family *Staphylococcaceae*. Staphylococcal species occur worldwide as commensal colonizers of the skin of animals and humans. Staphylococci are resistant to dehydration and are stable for months in the environment. They produce harmful toxin and majority of this specie show resistance to antibiotics due to a consistent genetic variation, thereby posing a great harm to the human race (Monecke *et al*., 2011; Sanchini *et al*., 2011). MRSA infection can be healthcare associated/acquired, community associated/acquired or livestock associated (Stefani *et al.,* 2012). *S. aureus* colonies are found in the gastrointestinal tract, vagina, skin and the nares. They are additionally found on mucous membranes of the upper respiratory tract and lower urogenital tract, and transiently in the digestive tract or other body surfaces which is asymptomatic most times (Kluytmans, 2009). Methicillin-resistant *Staphylococcus aureus* was discovered in 1961 in the United Kingdom. It made its first major appearance in the United States in 1981 among intravenous drug users, now it is found in many regions worldwide (Stefani *et al*., 2012; Target Health Global, 2017).

### 2.2.1 Prevalence of MRSA

In healthy people, MRSA carriage is associated with a minor risk of developing an infection. However, when the integrity of the skin is broken, the risk of infection dramatically increases (Kluytmans, 2009). In 2005, it was said that 25–35% of healthy human individuals carry *S. aureus* on the skin or mucous membranes (Wertheim *et al.,* 2005). This means that as much as two billion individuals may currently carry *S. aureus* worldwide, and conservative estimates based on Dutch and US prevalence data predict that between 2 to 53 million people carry MRSA (Grundmann *et al.,* 2010).In general, its prevalence have been severally reported in healthy populations; 17.3% in nasal cavity of Turkish children (Soysal *et al.,* 2006) , 36% in nares of Japanese adults (Uemura *et al.,* 2004), 32.4% in nasal cavity of adults in USA (Moellering, 1998) and 27.5% and 40% cases were reportedin Nigeria (Onanuga and Temedie, 2011). Specifically, a prevalence of 28.6% was seen in Kano state, Nigeria (Nwankwo *et al.,* 2010), however, prevalence rate of about 69% of MRSA isolates was obtained in a study among healthy women in Zaria, Nigeria (Onanuga *et al*., 2005).

### 2.2.2 Mechanism of Resistance of MRSA to Antibiotics

An important feature of *S. aureus* is the ability to acquire resistance to antimicrobials. Today, only about 10% of *S. aureus* are susceptible to penicillin when compared to 95% susceptibility seen in 1994 (CDC, 2016). When Penicillin binds Penicillin binding Proteins (PBPs) which are normally present at the cell membrane of *S. aureus*, it stops the process of cell membrane synthesis, resulting in lysis of bacterial cell. However, PBP2a (a variant of PBPs) produced by MRSA has a reduced affinity for β-lactam antibiotics, this leaves the PBP2a free hence, causing no harm to the integrity of the cell membrane and consequently

leaving the microorganism intact (Pinho *et al.,* 2001). Broad spectrum antibiotics that are used in the hospital have continuously shown a decline in their effectiveness against this specie of microorganism. This antibiotic resistance may be acquired via plasmid mediated (*mecA*) gene that is not native to *S. aureus*. This is responsible for the production of a modified penicillin-binding protein (PBP2a) which has minimal affinity for β-lactams (Pinho *et al.,* 2001; Stefani *et al.,* 2012).This is often transferred to the chromosome as mobile genetic elements. Methicillin resistant strains usually possess more than four genes encoding different resistant mechanism. This makes MRSA a problematic pathogen in human medicine and more recently in veterinary medicine.

It had been observed that the indiscriminate use of antibiotics with and without prescriptions in the developing countries such as Nigeria where regulatory policies are not adhered to has rendered most of the commonly used antibiotics ineffective in the treatment of *Staphylococcus aureus* infections (Onwubiko and Sadiq, 2011). Indiscriminate availability of antimicrobials to drug users with or without prescription, high levels of self-medication, often associated with failure to comply with treatment and inadequacy of dosage has led to an increased level of antibiotic drug abuse. Inadequate awareness has also contributed to this negative effect (Olowe *et al.,* 2007).

### 2.2.3 Associated infections

*S. aureus* is the primary cause of lower respiratory tract infections and surgical site infections and the second leading cause of nosocomial bacteremia, pneumonia, and cardiovascular infections (Klein *et al.,* 2007). S*taphylococcus aureus* is also an important cause of skin and soft-tissue infections (SSTIs), endovascular infections, septic arthritis, endocarditis, osteomyelitis, foreign-body infections, and sepsis. It causes endocarditis,

osteomyelitis, urinary tract infections, and meningitis and even boils (Talaro and Talaro, 2002; Umaru *et al.,* 2011).

Food poisoning is sometimes caused by the contamination of food products with *S. aureus.* A form of inflammation of the stomach and intestines that is manifested clinically as vomiting, with or without diarrhea results from the intake of staphylococcal enterotoxins from food inoculated with *S. aureus*. It is usually a self-limiting condition that is typically resolved within 24 to 48 hours of onset.

### 2.2.4 Isolation of MRSA from food products

Risk assessment in foodstuffs relies on classic microbial detection and quantification on a selective medium (meat, meat and milk products) occurred during production, processing and at retail point and can be a potential threat to humans who handle the foods as well as those who consume raw or undercooked food (Van Loo *et al.,* 2007; Umaru, 2011). A low-degree contamination by *S. aureus* is tolerated in most foodstuffs while it is found more frequently in retail meat. MRSA isolation from animals was first seen in 1972, after its detection in raw and pasteurized milk from cows (Kluytmans, 2009; Umaru, 2013). Since then, MRSA has been isolated from several animal species, including fowl, chicken, calves, pigs, sheep, horses, pigs and dogs (Kluytmans, 2009; Otaru *et al.,* 2011). Studies in Japan and Switzerland showed a prevalence of *S. aureus* in meat products of 65% and 23% respectively. A survey traced the contamination of meat products back to slaughter houses (Kitai *et al.,* 2005; Kluytmans, 2009).

The isolates in food are usually detected via standard bacteriological methods which includes biochemical tests [sugar fermentation (mannitol, lactose, sucrose, glucose), coagulase and catalase], Gram staining characteristics and colony morphology, (Bauer *et*

*al*., 1996; Cheesbrough, 2000; Onwubiko and Sadiq, 2011). Molecular methods such as polymerase chain reaction (PCR) are also used in the detection of MRSA. Rapid latex agglutination test which detects the PBP2a protein is another well-known laboratory test used to detect MRSA.

### 2.2.5 Treatment of infections caused by MRSA

Vancomycin and teicoplanin are glycopeptides antibiotics used to treat MRSA infections**.** Teicoplanin is a similar anti-microbial to Vancomycin with related activity spectrum but, has a longer half-life (Rybak *et al*., 1991). In more severe cases of infection that is resistant to glycopeptides such as vancomycin, tetracycline, daptomycin, dalfopristin, quinupristin and Linezolid are used. MRSA infection can be treated with oral agents including clindamycin, doxycycline or minocyline, cotrimoxazole (trimethoprim - sulphamethoxazole), pristinamycin, rifampicin + fluoroquinolone, rifampicin-fusidic acid and lineziod (Birmingham *et al*., 2003). MRSA can be eliminated from mucosal membrane colonization by using mupirocin (Furtalo *et al*., 2006). Platensimycin is another antibiotic that has been used successfully against MRSA (Wang *et al*., 2007). Onwubiko and Sadiq (2011) in their study discovered that flouroquinolones are effective in the control of MRSA. Sulfonamides, rifampin, quinupristin/dalfopristin, mupirocin, linezolid, ciprofloxacin and chloramphenicol were effective on all MRSA strains isolated from poultry in Belgium (Furtalo *et al*., 2006, Umaru *et al.*, 2011).

Plant and plant products play a vital role in providing immediate health care of many folks in the developing country. Extracts of plants in Nigeria such as *Mormodica basalmina, Boscia senegalensis, Pavetta crassipes,* and *Acacia albida* have shown antibacterial activity against MRSA and associated diseases (Aliyu *et al.*, 2008). In a different study, allicin, a

compound in garlic and small quantities of silver carbonate was found to successfully treat MRSA at University of East London and University of York (Cutler and Wilson, 2004).

### 2.2.6 Prevention and control of MRSA

Proper hand hygiene is an important step towards the management of the spread of MRSA between animals and humans. Frequent hand washing with soap/detergent and proper disinfection of hard surfaces and equipment between patients is important (AVMA, 2009).Hand sanitizers should be provided in all consulting rooms and as well as sensitization to remind staff of hospitals of the need for frequent hand sanitization (Umaru *et al.*, 2011). Protective gears such as masks, disposable aprons, gloves and uniforms should be worn to prevent unnecessary exposure to body fluids or contaminated tissues, especially when dressing infected wounds (AVMA, 2009).Eye protection is needed if splashing or volatiles are expected (AVMA,2009). The clinic environment should be kept sterile to the best possible extent. It is also necessary to avoid too much close contact and overcrowding to curtail the persistence and spread of MRSA (Umaru *et al*., 2011).

# CHAPTER THREE

## 3.0 Materials and Methods

### 3.1 Materials

#### 3.1.1 Equipment

Autoclave (Portable 230V and 1850W Adelphi MFG CO. Ltd, England), incubator (National appliance Co. Ltd, Oregon, USA: model 1630, 240V and 2340W), hot air oven (Baird and Tatlock (London) Ltd, Chad Well Health Essex, England), refrigerator (Haier Thermocool: Model No. HRF-688-FF/A), PCR thermocycler (Techne TC-312), gel electrophoresis machine (SMax Fill Scie-plas. Model HU10 serial no 5237), comb, laminar air flow (PCR-8 recirculating laminar flow pre station Labcaire product 220/240v) and microscope (Wild Heerbrugg M11, made in Switzerland), bunsen-burner, spectrophotometer (Eppendorf Biophotometer 8,5mm, Lichtstrahihohe), UV illuminator (Vilberb Lourmat TFX-35-M serial no N$^{o}$V02 8104), centrifuge (Eppendorf centrifuge 5417R), micropipette, Electronic weighing balance (QT 600), Votexing mixer (Touch plate Super Mixer, CAT No 1291 Lab-line instrument inc USA).

#### 3.1.2 Glassware

Petri-dishes, beakers, bijou bottles, universal bottles, glass slides, test tubes, measuring cylinder, Eppendorf tube, magnetic stirrer (W and T Avery Ltd., England).

#### 3.1.3 Reagents and chemicals

Methyl red, Barritts solution, α-napthol, Acridine orange, TAE (Tris base, acetic acid and Ethylenediaminetetraacetic acid) buffer, potassium hydroxide, sodium hydroxide, Ethidium bromide, Glycerol, Glacia acetic acid, loading dye, Dettol® and Jik® (Reckit Benckiser

Ltd., Nigeria), Neutralizing solution (Fermentas, UK), ethanol (BDH Chemical Ltd., England), wash solution (Fermentas, UK), Elution buffer (Fermentas, UK), Microgen™ Staph ID kit (Microgen Bioproducts Ltd,UK), Microgen™ GNA ID kit (Microgen Bioproducts Ltd,UK), Zymo DNA extraction kit (Zymo Research Corps, U.S.A).

### 3.1.4 Culture media

MacConkey agar (Oxoid Ltd., England), Nutrient agar (Oxoid Ltd., England), Nutrient broth (Fluka Spain), Mannitol Salt Agar, Blood Agar, Mueller Hilton agar (Oxoid Ltd., England), Yeast extract (Biotech Laboratory Ltd., UK), Peptone water (Fluka Spain) and Lauria-Bertani Broth (Oxoid Ltd., England).

### 3.1.5 Antibiotic discs

The antibiotic discs used were obtained from Oxoid Ltd. They are some of the most commonly prescribed and those recommended for treatment of *Staphylococcus aureus* infections.They include; Ofloxacin (5µg), Vancomycin (30µg), Clindamycin (2µg), Gentamicin (30µg), Imipenem (10µg), Erythromycin (15µg), Mupirocin (200µg), Ciprofloxacin (5µg), Amoxicillin/Clavulanic Acid (20/10µg), Ampicillin (10µg), Cefoxitin (30µg), Quinupristin/Dalfopristin (15µg) and Cefixime (5µg).

## 3.2 Methods

### 3.2.1 Study population

Samples for analysis were obtained from five (5) markets in Zaria metropolis and the study population was seventy-five (75) local sellers of roasted groundnuts and kuli-kuli in the five selected markets - 15 sellers from each market (Samaru, Wusasa/Dan magaji, Sabongari, Zaria city and Tudun-Wada).

### 3.2.2 Sample size

A total of 294 samples were randomly obtained from roasted groundnuts, kuli-kuli and swabs from the hands of the sellers (Ezekiel *et al.*, 2011; Euloge *et al.*, 2012). Nutrient Agar plates were opened around the environment where the kuli-kuli and groundnuts are being sold to sample the microorganisms in the environment. Two hundred and twenty five (225) samples of kuli-kuli and groundnut were collected from the five selected markets, 54 hand swabs were collected and 15 different areas of the markets (where the food products are being sold) were sampled.

### 3.2.3 Sample collection and transportation

Traditionally processed and packaged (in nylon bags) samples of groundnut and kuli-kuli used for this study were purchased from five local markets (Samaru, Wusasa/Dan magaji, Sabongari, Zaria city and Tudun-Wada) in Zaria metropolis, Kaduna State, Nigeria. Samples were collected from 15 different sellers from each selected market area. The hands of the hawkers were swabbed using a sterile swab stick moistened with sterile normal saline and these were labeled appropriately with sample number, date and time of

collection. The environment where the kuli-kuli and roasted groundnuts are sold was sampled using settle plate technique as described by Cheesbrough (2000). Plates of nutrient agar were opened for 5 minutes at the site where samples were obtained; these plates were then closed and transported to the laboratory for incubation. Samples which were collected and transported in previously alcohol-sterilized food flasks were analyzed within two hours of arrival to the laboratory.

### 3.2.4 Bacteriological analysis of roasted groundnut and Kuli-kuli samples

Each sample was subjected to bacteriological analysis to determine the total bacterial load in consumable roasted groundnuts and Kuli-kuli as described by Ezekiel *et al*. (2011). One gram of each sample was suspended in 9ml of sterile peptone water and serially diluted. Aliquots were spread-plated on Nutrient agar, Mannitol Salt agar and MacConkey agar. The nutrient agar plates were used for the Total bacterial count (TBC) in colony forming units per gram (cfu/g), while the MacConkey plates were for initial isolation of Enterobacteriaceae. Each distinct colony on MacConkey plate was picked and sub cultured on Nutrient agar plates, incubated at 37$^{o}$C for 24 hours and then stored on Nutrient agar slants. The isolates were subjected to biochemical characterization and identification using identification kits and then sub-cultured onto Nutrient agar slants. The swabs collected were cut off into sterile nutrient broth and incubated at 37$^{o}$C for 24 hours, after which broth culture was inoculated into plates of Nutrient agar, Mannitol Salt agar and MacConkey agar. Plates were incubated at 37$^{o}$C for 24 hours after which isolates were sub-cultured onto Nutrient agar slants. The plates used for environment sampling were also incubated and isolates were identified and sub-cultured onto nutrient agar slants for further analysis.

### 3.2.5 Purification and preliminary identification of Staphylococcal isolates

#### *3.2.5.1 Gram-staining*

Gram-staining of isolates was carried out as described by Cheesbrough (2000). A smear of each isolate was made on a slide. The smear was heat-fixed and stained with crystal violet. This was allowed to stand for 10-60 seconds; the stain was poured off and rinsed gently with water. The culture was fixed with lugol's iodine solution, decolorized with ethanol and then counterstained with neutral red. The stained slide was examined under an oil-immersion lens (x600 or more) and the isolates that appear as violet cocci predominantly clusters were selected for further identification procedures.

#### *3.2.5.2 Biochemical tests*

**Catalase test:**

The ability to produce the enzyme catalase by the organisms was demonstrated by the addition of about 1ml of a 3% hydrogen peroxide solution on a 24 hour nutrient agar slope culture of the isolate. This was carried out for all the isolates suspected to be Staphylococci and evolution of gas was noted.

**Coagulase test:**

The method described by Cheesbrough (2000) was used to differentiate *Staphylococcus aureus* which produce the enzyme coagulase from other coagulase negative Staphylococci. To detect coagulase, a drop of physiological saline was placed on each end of a slide. A colony of the test organisms was emulsified in each drop to make two separate thick suspensions. A drop of plasma was then added to one of the suspensions and mixed gently and the ability or inability to form a clumping within 10 seconds was noted.

#### *3.2.5.3 Specie identification by Microgen$^{TM}$ Staph ID*

Isolates that are positive for coagulase and catalase tests were used. A single colony of isolates from an 18-24 hours culture was emulsified into sterile normal saline and mixed thoroughly. Using a sterile micro pipette, 3-4 drops (approximately 100µl) of the bacterial suspension were added to each well of the strip(s). After inoculation, mineral oil was added into appropriate wells and test strips were incubated at 35-37$^{o}$C for 18-24 hours. After 24 hours of incubation, appropriate reagents were added and results were taken based on color changes compared to the provided color chart.

### 3.2.6 Antibiotic susceptibility test

Antibiotic susceptibility of the isolates that were confirmed to be *Staphylococcus aureus* and the enterobacteriaceae was determined by the modified Kirby-Bauer disc agar diffusion method described by Cheesbrough (2000) and CLSI (2014). A suspension of overnight growth of each isolate on Nutrient agar plate was standardized by comparing the turbidity with 0.5 McFarland standards corresponding to approximately 1.5 x $10^{8}$cfu/ml. Suspension of the isolates was inoculated on Mueller Hinton agar plate using a sterile swab. The swab was streaked evenly over the surface of the medium to ensure confluent growth. The surface of the agar was allowed to dry and the antibiotic discs were placed on the surface of the agar using a sterile forcep. Thirty minutes was allowed for prediffusion of the discs, the plates were inverted and incubated at 35$^{o}$C for 16–18 hours. A plate of Mueller Hinton agar was also incubated alongside but, without being inoculated in order to confirm the sterility of the medium. After overnight incubation, plates were examined and the diameter of each zone of growth inhibition around the discs was measured in millimeter (mm), using a ruler on the underside of the plate. Using the Interpretative Chart (CLSI, 2014), the zone sizes of

each antimicrobial were interpreted and the organisms were reported as 'Resistant', 'Intermediate/Moderately susceptible 'or 'Susceptible'. Multidrug resistance (MDR) was defined as resistance to one or more agents in three or more different classes of antibiotics (Magiorakos *et al.*, 2012).

### 3.2.7. Test for Beta-lactamase production (Penicillin Zone Edge Test)

Different tests can be performed to evaluate beta-lactamase production in Staphylococci, these include: Penicillin Zone Edge Test, Nitrocefin Disks Test and Clover Leaf Test (Robles *et al*., 2014). The test for Beta-lactamase production among resistant isolates in this study was carried out using the Penicillin zone edge test as described by CLSI (2012). Suspensions of *Staphylococcus aureus* isolates with penicillin zone of ≥ 29mm were standardized by comparing the turbidity with 0.5 McFarland standards. Suspensions of the isolates were inoculated on Mueller Hinton Agar plates and 10 U Penicillin discs were placed on the inoculated Mueller Hinton Agar plates. The plates were incubated at $35^{o}$C for 16-18 hours and zones of growth inhibition with sharp zone edge were interpreted as B-lactamase positive while zones with fuzzy zone edge were interpreted as B-lactamase negative.

### 3.2.8 Detection of Methicillin resistance *Staphylococcus aureus* (MRSA)

Cefoxitin antibiotics discs were used to phenotypically detect the presence of MRSA in the isolates. A 0.5 McFarland standard suspension of the isolates were made and a lawn culture was done on Mueller Hinton Agar plate. Test discs were placed on the inoculated agar plates and incubated at $37^{o}$C for 18 hours and zone diameter was measured and reported as resistant or susceptible according to CLSI (2014).

### 3.2.9 Molecular analysis

The six (6) isolates that were confirmed to be *Staphylococcus aureus,* β-lactamase positive and resistant to cefoxitin were used for molecular analysis.

#### *3.2.9.1 Extraction of DNA*

DNA extraction was carried out using Zymo DNA extraction kit. Bacterial lysate preparation was done according to the manufacturer's instructions: an aliquot of 1.5ml of overnight Lauria-Bertani (LB) broth *Staphylococcus aureus* culture was placed in a micro centrifuge tube and centrifuged for 30 seconds to pellet the cells. The supernatant was carefully poured off and 200 µl of resuspension buffer (containing RNAse) was added to the cell pellets. This was then incubated at room temperature for 5mins. 250µl of lysis solution was added to the cell suspension, the tube capped and the content mixed gently by inverting the tube several times. 350µl of binding solution was added and immediately mixed by inverting the tube several times and then centrifuged for 10mins to clarify the lysate. An insoluble pellicle was collected on the bottom of the centrifuge tube. The lysate was transferred into spin column and centrifuged for 1 minute. The spin column (DNA bound to the column) was assembled with a fresh 1.7 ml elution tube included in the kit. 50µl of elution buffer was added to the center of the column and allowed to stand at room temperature for 1 minute then the column centrifuged for 2 minutes.

#### *3.2.9.2 Separation of plasmid DNA by Agarose gel electrophoresis*

This was done by the resolution of the DNA using agarose gel electrophoresis and comparing their weight to a molecular weight marker. Agarose gels were prepared and

electrophoresis carried out (Sambrook *et al*., 1989). One percent (1% w/v) agarose (Sigma) was dissolved in 1× TAE (Tris base, acetic acid and Ethylenediaminetetraacetic acid) buffer by bringing to boil in a microwave oven. The gel was allowed to cool to about 40˚C before adding a drop (1 µg/ml) of green nucleic acid gel stain which was used to replace the mutagenic ethidium bromide (EB). The gel was poured into a gel mold containing a well comb and allowed to polymerize at room temperature. Isolated DNA samples were mixed with 5 µl gel loading buffer and 20 µl of the sample was then loaded on to the wells of the gel. Electrophoresis was carried out at 100 mV for 45 minutes to allow easy separation of sample based on molecular weight. DNA bands were visualized and documented using an electrophoresis gel documentation system.

***3.2.9.3 Polymerase chain reaction (PCR) method for detection of mecA and blaZ genes.***

The *mecA, blaZ* and *vanA* genes were amplified using PCR. Gene detection was carried out using multiplex PCR technique as described by Cattoir *et al*. (2007). Rapid DNA preparation was performed by a boiling technique that includes a heating step at $100^{o}$C of a single colony in a total volume of 100 ml of distilled water followed by a centrifugation step of the cell suspension. Total DNA was subjected to multiplex PCR in a 50 ml reaction mixture containing 1 X PCR buffer, KCl, MgCl2, deoxynucleotide triphosphate, primers and Taq polymerase. Amplification was carried out with the thermal cycling profile in Tables 2 below. Amplification products were identified by their sizes after electrophoresis in a 2% agarose gel and staining with ethidium bromide.

**The run conditions for *mecA*, *vanA* and *blaZ* primers:**

Table 3.0 shows the run conditions for the genes isolated.

**Table 3.0: Multiplex PCR run conditions for the *mecA*, *vanA* and *blaZ* primers (Duran *et al.*, 2012)**

| STEP | TEMPERATURE ($^0$C) | TIME | NUMBER OF CYCLES |
|---|---|---|---|
| Initial lysing of cells | 95 | 5 min | 1 |
| Denaturation | 94 | 30 sec | 35 |
| Annealing | 60 | 1 min | 1 |
| Extension | 72 | 45 sec | 1 |
| Final extension | 72 | 5 mins | 1 |
| Final hold | 4 | 10 mins | 0 |

**Primers for the Isolated Genes:**

Table 3.1 shows the sequence and amplicon size of each of the primers used.

**Table 3.1: Primers for multiplex PCR (Duran *et al.*, 2012)**

| Gene | Oligonucleotide sequence (5' – 3') | Expected amplicon size (bp) |
|---|---|---|
| *MecA* | 5' CCTAGTAAAGCTCCGGA 3'<br>5' CTAGTCCATTCGGTCCA 3' | 314 |
| *BlaZ* | 5' ACTTCAACACCTGCTGCTTTC3'<br>5' TGACCACTTTTATCAGCAAC 3' | 173 |
| *VanA* | 5' GGGAAAACGACAATTGC 3'<br>5' GTACAATGCGGCCGTTA 3' | 732 |

# CHAPTER FOUR

## 4.0 Results

### 4.1 Sample Collection

A total of 294 samples consisting of kuli-kuli (75), roasted groundnut (75), unpeeled roasted groundnut (75), hands of the sellers (54) and the environments (15) were collected from five markets. The breakdown is shown in Table 4.1.

### 4.2 Isolation of Bacteria from Samples by Market

A total of 152 isolates were obtained from the five markets sampled in the study. The highest number of bacteria was isolated from Samaru market (27.63%) as shown in Table 4.2.

Table 4.2 shows the breakdown of bacteria isolates from the samples by the markets.

### 4.3 Gram Staining of Isolates

A total of 125(82%) out of the 152 isolates were Gram positive cocci while 27 (18%) were Gram negative rods.

### 4.4 Distribution of Enterobacteriaceae in Samples

Full identification of Enterobacteriaceae isolates using Microgen$^{TM}$GNA-ID kit showed that the most prevalent organisms were *Citrobacter freundii* (22.2%) and *Klebsiella oxytoca* (11.1%).

A full breakdown of all the organisms isolated is shown in Table 4.3. Table 4.4 represents the distribution of the Enterobacteriaceae isolates by sources, while Table 4.5 shows the distribution of the Enterobacteriaceae isolates by markets.

**Table 4.1: Sample collection**

| S/N | Samples Evaluated | Samples Collected | Plates with Colonies > 25* | No of Diff Isolates (%) |
|---|---|---|---|---|
| 1 | Kuli-kuli | 75 | 19 | 24 (15.8) |
| 2 | G/nut | 75 | 9 | 9(5.9) |
| 3 | AG/nut | 75 | 11 | 11(7.2) |
| 4 | Envi. | 15 | 14 | 25(16.5) |
| 5 | Hands Swab | 54 | 53 | 83(54.6) |
| | Total | 294 | 106 | 152(100) |

**Keys:** G/nut- Roasted Groundnuts, AG/nut- Unpeeled roasted groundnuts, Envi- Environment, No- Number, Diff- Different

* According to U.S FDA, plates with colonies less than 25 should be discarded (Maturin and Peeler, 2001).

**Table 4.2: Isolation of Bacteria Isolates from the Samples by the Markets**

| Market | Number of Isolates | | | | | Total (%) |
|---|---|---|---|---|---|---|
| | Kuli-kuli | Roasted G/nuts | Roasted G/nut with unpeeled skin | Skin swabs | Environments | |
| Samaru | 11 | 2 | 1 | 21 | 7 | 42(27.6) |
| Sabon-gari | 4 | 1 | 3 | 15 | 4 | 27(17.8) |
| Tudun-wada | 2 | 5 | 2 | 16 | 4 | 29(19.1) |
| Danmagaji/Wusasa | 5 | 0 | 2 | 16 | 5 | 28(18.4) |
| Zaria city | 2 | 1 | 3 | 15 | 5 | 26(17.1) |
| Total | 24 | 9 | 11 | 83 | 25 | 152 (100) |

**Keys**: G/nut-Groundnut

**Table 4.3: Distribution of Enterobacteriaceae**

| S/N. | ISOLATES | NUMBER | PERCENTAGE |
|---|---|---|---|
| 1. | *Citrobacter freundii* | 6 | 22.2 |
| 2. | *Escherichia coli inactive* | 1 | 3.7 |
| 3. | *Salmonella arizonae* | 2 | 7.4 |
| 4. | *Klebsiella oxytoca* | 3 | 11.1 |
| 5. | *Salmonella* species | 2 | 7.4 |
| 6. | *Providencia stuartii* | 1 | 3.7 |
| 7. | *Acinetobacter lwoffii* | 2 | 7.4 |
| 8. | *Proteus vulgaris* | 2 | 7.4 |
| 9. | *Proteus mirabilis* | 1 | 3.7 |
| 10. | *Serratia marcescens* | 2 | 7.4 |
| 11. | *Acinetobacter haemolyticus* | 2 | 7.4 |
| 12. | *Enterobacter agglomerans* | 1 | 3.7 |
| 13. | *Klebsiella pneumonia* | 1 | 3.7 |
| 14. | *Providencia alcalifaciens* | 1 | 3.7 |
| | Total | 27 | |

**Table 4.4 Distribution of Enterobacteriaceae isolates by sources**

| S/N | ORGANISM | NUMBER OF ISOLATES | | | | |
|---|---|---|---|---|---|---|
| | | KULI-KULI | G/NUT | AG/NUT | HAND SWAB | ENVIRON |
| 1 | *Citrobacter fruendii* | 1 | 0 | 0 | 5 | 0 |
| 2 | *Klebsiella oxytoca* | 0 | 0 | 0 | 3 | 0 |
| 3 | *Salmonella arizonae* | 0 | 0 | 0 | 2 | 0 |
| 4 | *Salmonella spp* | 0 | 0 | 0 | 2 | 0 |
| 5 | *Acinetobacter lwoffi* | 1 | 0 | 1 | 0 | 0 |
| 6 | *Proteus vulgaris* | 0 | 0 | 0 | 2 | 0 |
| 7 | *Serratia marcescens* | 0 | 0 | 0 | 2 | 0 |
| 8 | *Acinetobacter hemolyticus* | 0 | 0 | 1 | 1 | 0 |
| 9 | *Escherichia coli inactive* | 0 | 0 | 0 | 1 | 0 |
| 10 | *Providencia stuartii* | 0 | 0 | 1 | 0 | 0 |
| 11 | *Proteus mirabilis* | 0 | 0 | 0 | 1 | 0 |
| 12 | *Enterobacter agglomerans* | 0 | 0 | 0 | 1 | 0 |
| 13 | *Klebsiella pneumonia* | 0 | 0 | 0 | 1 | 0 |
| 14 | *Providencia alcalifaciens* | 0 | 1 | 0 | 0 | 0 |

**Keys:** G/nut- Roasted Groundnuts, AG/nut- Unpeeled Roasted Groundnuts.

**Table 4.5 Distribution of Enterobacteriaceae isolates by markets**

| S/N | ORGANISM | NUMBER OF ISOLATES | | | | |
|---|---|---|---|---|---|---|
| | | SAMARU | SABON-GARI | TUDUN-WADA | DANMAGAJI/ WUSASA | ZARIA CITY |
| 1 | *Citrobacter fruendii* | 1 | 1 | 0 | 3 | 1 |
| 2 | *Klebsiella oxytoca* | 0 | 0 | 2 | 1 | 0 |
| 3 | *Salmonella arizonae* | 0 | 0 | 0 | 1 | 1 |
| 4 | *Salmonella spp* | 0 | 0 | 0 | 2 | 0 |
| 5 | *Acinetobacter lwoffi* | 1 | 0 | 0 | 0 | 1 |
| 6 | *Proteus vulgaris* | 0 | 1 | 0 | 0 | 1 |
| 7 | *Serratia marcescens* | 0 | 0 | 1 | 1 | 0 |
| 8 | *Acinetobacter hemolyticus* | 0 | 2 | 0 | 0 | 0 |
| 9 | *Escherichia coli inactive* | 0 | 0 | 0 | 1 | 0 |
| 10 | *Providencia stuartii* | 0 | 1 | 0 | 0 | 0 |
| 11 | *Proteus mirabilis* | 0 | 0 | 0 | 0 | 1 |
| 12 | *Enterobacter agglomerans* | 0 | 1 | 0 | 0 | 0 |
| 13 | *Klebsiella pneumoniae* | 0 | 1 | 0 | 0 | 0 |
| 14 | *Providencia alcalifaciens* | 1 | 0 | 0 | 0 | 0 |

### 4.5 Distribution of Gram Positive Cocci

The initial number of Gram positive isolates was 125.

Table 4.6 shows the categorization of the Gram positive isolates using the catalase and coagulase tests.

### 4.6 Distribution of Staphylococcus Isolates

Microgen$^{TM}$ Staph ID kit was used to identify the 69 isolates that were Coagulase positive.

The distribution of the isolates is as shown in Table 4.7. Table 4.8 indicates the distribution of the Staphylococci isolates by source while Table 4.9 shows their distribution by markets.

**Table 4.6 Biochemical tests of Gram positive cocci**

| Test | No. of Positive Isolates (%) | No. of Negative Isolates (%) |
|---|---|---|
| Catalase | 117(93.6%) | 8(6.4%) |
| Coagulase | 69(55.2%) | 56(44.8%) |

Keys: No- Number

**Table 4.7 Distribution of Staphylococcus Isolates**

| S/N | ISOLATES | NUMBER | PERCENTAGE (%) |
|---|---|---|---|
| 1 | *Staphylococcus aureus* | 28 | 40.6 |
| 2 | *Staphylococcus chromogenes* | 5 | 7.2 |
| 3 | *Staphylococcus xylosus* | 16 | 23.2 |
| 4 | *Staphylococcus schleiferi* | 4 | 5.8 |
| 5 | Staphylococcus haemolyticus | 2 | 2.9 |
| 6 | *Staphylococcus intermedius* | 6 | 8.7 |
| 7 | *Staphylococcus epidermidis* | 2 | 2.9 |
| 8 | *Staphylococcus hyicus* | 6 | 8.7 |
| | | 69 | 100 |

### 4.6.1 Distribution of the Staphylococci isolates by source

**Table 4.8: Distribution of the Staphylococci isolates by source**

| S/N | ORGANISM | NUMBER OF ISOLATES | | | | |
|---|---|---|---|---|---|---|
| | | KULI-KULI | G/NUT | AG/NUT | HAND SWAB | ENVIRON |
| 1 | *Staphylococcus aureus* | 2 | 2 | 0 | 16 | 8 |
| 2 | *Staphylococcus xylosus* | 2 | 0 | 0 | 13 | 1 |
| 3 | *Staphylococcus intermedius* | 1 | 0 | 1 | 3 | 1 |
| 4 | *Staphylococcus hyicus* | 2 | 1 | 0 | 2 | 1 |
| 5 | *Staphylococcus chromogenes* | 0 | 1 | 0 | 1 | 3 |
| 6 | *Staphylococcus schleiferi* | 1 | 0 | 0 | 2 | 1 |
| 7 | *Staphylococcus haemolyticus* | 1 | 0 | 0 | 1 | 0 |
| 8 | *Staphylococcus epidermidis* | 1 | 0 | 0 | 1 | 0 |

**Keys:** G/nut- Groundnuts, AG/nut- Unpeeled Roasted Groundnuts.

### 4.6.2 Distribution of the Staphylococci isolates by markets

**Table 4.9: Distribution of the Staphylococci isolates by markets**

| S/N | ORGANISM | NUMBER OF ISOLATES | | | | |
|---|---|---|---|---|---|---|
| | | SAMARU | SABON-GARI | TUDUN-WADA | DANMAGAJI/ WUSASA | ZARIA CITY |
| 1 | *Staphylococcus aureus* | 8 | 3 | 5 | 6 | 6 |
| 2 | *Staphylococcus xylosus* | 8 | 2 | 2 | 1 | 3 |
| 3 | *Staphylococcus intermedius* | 2 | 0 | 0 | 1 | 3 |
| 4 | *Staphylococcus hyicus* | 0 | 1 | 0 | 2 | 3 |
| 5 | *Staphylococcus chromogenes* | 0 | 1 | 1 | 3 | 0 |
| 6 | *Staphylococcus schleiferi* | 2 | 0 | 0 | 2 | 0 |
| 7 | *Staphylococcus haemolyticus* | 2 | 0 | 0 | 0 | 0 |
| 8 | *Staphylococcus epidermidis* | 0 | 1 | 0 | 0 | 1 |

## 4.7 Antibiotics Resistance of Isolates

### 4.7.1 Resistance of Enterobacteriaceae isolates to tested antibiotics

The resistance of the Enterobacteriaceae isolates to the tested antibiotics is shown in Fig 4.2

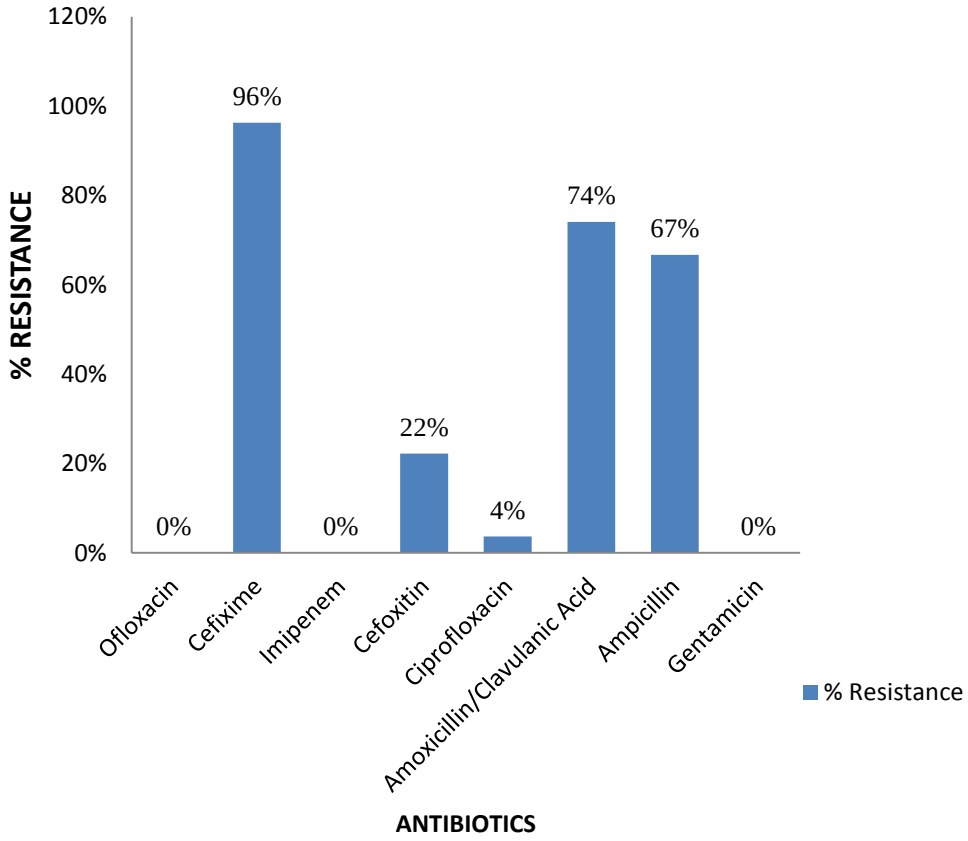



**Fig.4.1: Resistance of Enterobacteriaceae isolates to the tested antibiotics**

### 4.7.2 Resistance of *Staphylococcus Aureus* isolates to tested antibiotics

All the isolates were susceptible to Imipenem (100.0%), Gentamicin (100.0%) and Ciprofloxacin (100.0%) but showed high resistance to Cefixime (82%) as shown in Figure 4.3.

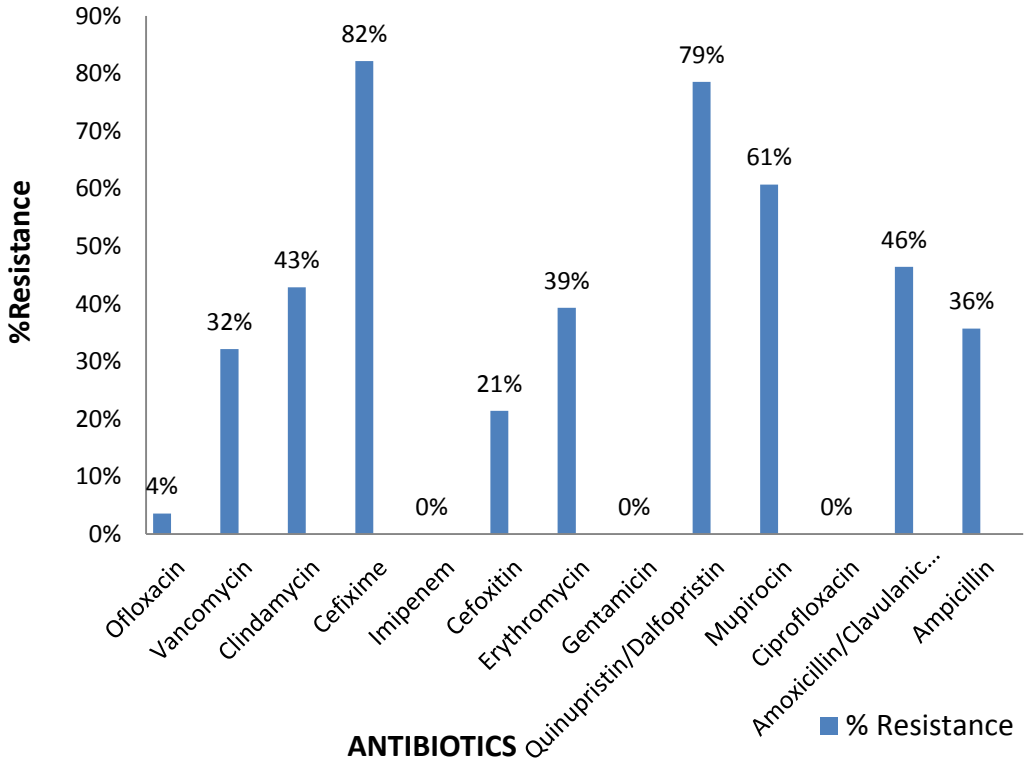



**Fig 4.2: Percentage of Antibiotics Resistance of *Staphylococcus aureus* Isolates**

## 4.8 Resistance Pattern of Isolates

### 4.8.1 Resistance pattern of Enterobacteriaceae isolates

The resistance pattern of the Enterobacteriaceae isolates to the tested antibiotics is shown in Fig 4.4

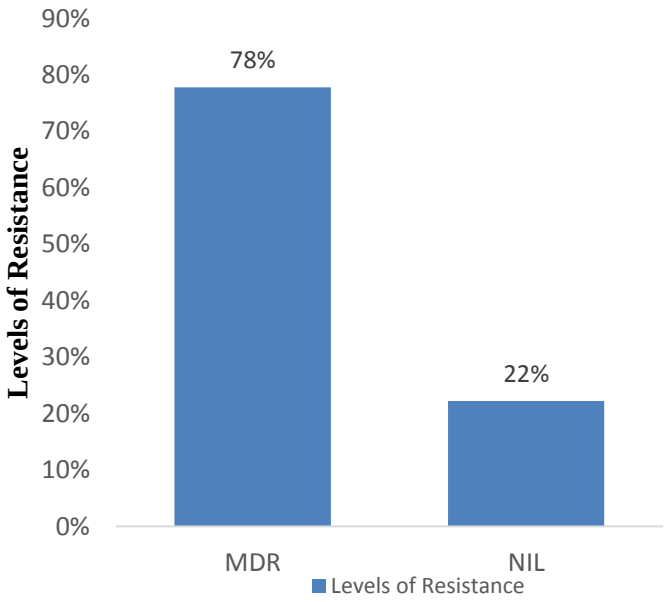



**Fig.4.3 Resistance Pattern of Enterobacteriaceae Isolates**

Table 4.10 shows the antibiotic resistance pattern of the Enterobacteriaceae isolates and the Enterobacteriaceae isolates that were multidrug resistant.

**Table 4.10: Antibiotics resistance pattern of the Enterobacteriaceae isolates**

| S/N | ISOLATE CODE | ANTIBIOTICS RESISTANCE PATTERN | NAR | CLASS OF ANTIBIOTIC RESISTANCE | NCAR | RESISTANCE CATEGORY |
|---|---|---|---|---|---|---|
| 01 | K13(2) | CFM | 1 | CEPH | 1 | NIL |
| 02 | K70(2) | CFM, | 1 | CEPH | 1 | NIL |
| 03 | AG22(1)(2) | MUP, DA, CFM, AMP, QD | 5 | MUP, LIN, CEPH, PEN, STREP | 5 | MDR |
| 04 | AG25(1) | VA, MUP, CFM, AMP, QD, AMC | 6 | GLY, MUP, CEPH, STREP, PEN | 5 | MDR |
| 05 | AG67(m) | CFM | 1 | CEPH | 1 | NIL |
| 06 | GE1 (1) | MUP, CFM, AMP, QD, AMC | 5 | MUP, CEPH, PEN, STREP | 4 | MDR |
| 07 | SmSw10(1) | MUP, AMP, AMC | 3 | MUP, PEN | 2 | NIL |
| 08 | Sw14(2) | MUP, CFM, AMP, AMC | 4 | MUP, CEPH, PEN | 3 | MDR |
| 09 | Sw15(2) | VA, MUP, CFM, AMP, AMC, QD | 6 | GLY, MUP, CEPH, PEN, STREP | 5 | MDR |
| 10 | Sw16(2) | MUP, DA, CFM, QD, FOX | 5 | MUP, LIN, CEPH, STREP | 4 | MDR |
| 11 | Sw20(2) | MUP, CFM, AMP, AMC | 4 | MUP, CEPH, PEN | 3 | MDR |
| 12 | Sw24(2) | MUP, CFM, AMP, AMC | 4 | MUP, CEPH, PEN | 3 | MDR |
| 13 | TwSw29(2) | MUP, CFM, AMP, AMC | 4 | MUP, CEPH, PEN | 3 | MDR |
| 14 | TwSw32(2) | MUP, CFM, AMP, AMC, QD | 5 | MUP, CEPH, PEN, STREP | 4 | MDR |
| 15 | TwSw36(2) | MUP, QD, CFM, AMP, AMC, FOX | 6 | MUP, STREP, CEPH, PEN, | 4 | MDR |
| 16 | DmSw37(2) | MUP, CFM, AMP, AMC | 4 | MUP, CEPH, PEN | 3 | MDR |
| 17 | DmSw38(2) | CFM,AMP,AMC | 3 | CEPH, PEN | 2 | NIL |
| 18 | DmSw39(1) | MUP, CFM, QD, AMC | 4 | MUP, CEPH, STREP, PEN | 4 | MDR |
| 19 | DmSw41(2) | CIP, MUP, CFM, AMC | 4 | QUIN, MUP, CEPH, PEN | 4 | MDR |
| 20 | DmSw43(2) | CFM, AMP, QD, AMC, FOX | 5 | CEPH, PEN, STREP | 3 | MDR |
| 21 | DmSw43(m) | VA, MUP, CFM, AMP, AMC, FOX | 6 | GLY, MUP, CEPH, PEN | 4 | MDR |
| 22 | DmSw44(2) | MUP, CFM, AMP, AMC, QD, FOX | 6 | MUP, CEPH, PEN, STREP | 4 | MDR |
| 23 | DmSw45(2) | MUP, CFM, AMP, AMC, QD, FOX | 6 | MUP, CEPH, PEN, STREP | 4 | MDR |
| 24 | DmSw46(2) | VA, MUP, QD, CFM, AMP, AMC | 6 | GLY, MUP, CEPH, PEN, STREP | 5 | MDR |
| 25 | ZcSw51(1) | VA, MUP, CFM, AMC, QD | 5 | GLY, MUP, CEPH, PEN, STREP | 5 | MDR |
| 26 | ZcSw52(2)(m) | CFM, QD | 2 | CEPH, STREP | 2 | NIL |
| 27 | ZcSw54(2) | VA, MUP, DA, CFM, QD | 5 | GLY, MUP, LIN, CEPH, STREP | 5 | MDR |

**Keys:** QUIN = Quinolone, MUP = Mupirocin, AMP = Ampicillin, CEPH = Cephalosporin, PEN = Penicillin, CIP = Ciprofloxacin, QD = Quinopristin/Dalfopristin, CFM = Cefixime, FOX = Cefoxitin, AMC = Amoxicillin/ Clavulanic acid, DA = Clindamycin, VA = Vancomycin, LIN = Lincosamide, STREP = Streptogramin, GLY = Glycopeptide, MDR = Multidrug-resistant, NIL: not MDR, NAR = Number of antibiotics resistant to, NCAR = Number of Class of antibiotics each isolate is resistant to, MDR: non-susceptible to ≥1 agent in ≥3 antimicrobial categories

### 4.8.2: Resistance pattern of *Staphylococcus Aureus* isolates

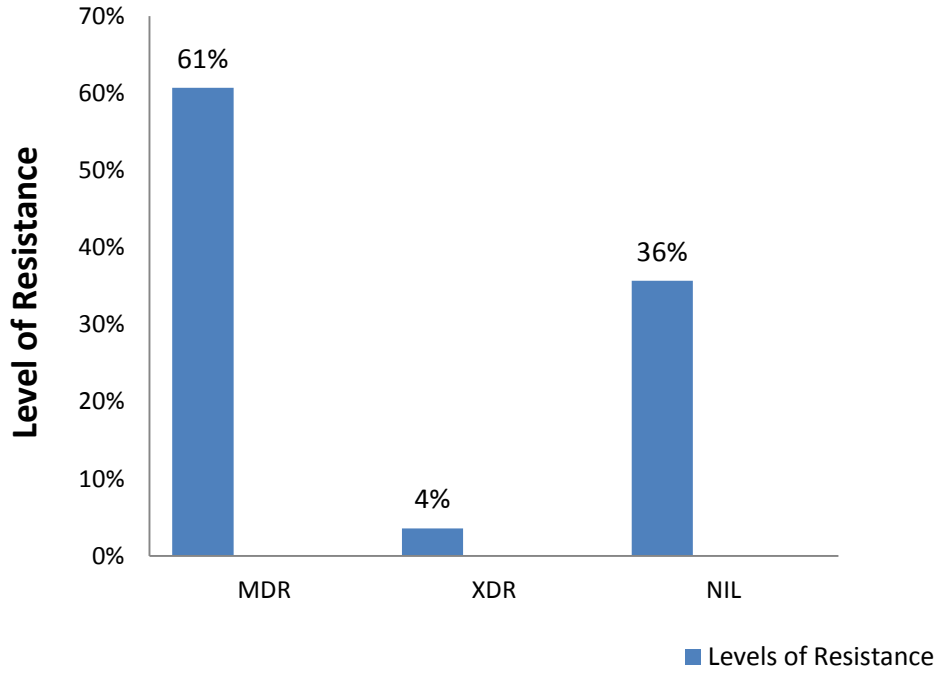



Fig 4.4: Level of Antibiotics Resistance of *Staphylococcus aureus* Isolates

Table 4.11 shows the antibiotic resistance pattern of the Staphylococcal isolates and the Staphylococcal isolates that were multidrug resistant.

**Table 4.11: Antibiotics resistance pattern of Staphylococci isolates**

| S/N | ISOLATE CODE | ANTIBIOTICS RESISTANCE PATTERN | NAR | CLASS OF ANTIBIOTIC RESISTANCE | NCAR | RESISTANCE CATEGORY |
|---|---|---|---|---|---|---|
| 01 | K4(2) | DA, MUP | 2 | LIN, MUP | 2 | NIL |
| 02 | K43(1)(2) | CFM,QD | 2 | CEPH, STREP | 2 | NIL |
| 03 | G1(1) | CFM,QD | 2 | CEPH, STREP | 2 | NIL |
| 04 | G23(m) | CFM, E, QD | 3 | CEPH, MAC, STREP | 3 | MDR |
| 05 | $KE_4$ (1)(2) | CFM,QD,MUP, | 3 | CEPH, STREP, MUP | 3 | MDR |
| 06 | $KE_5$ (2) | VA,CFM | 2 | GLY, CEPH | 2 | NIL |
| 07 | $GE_2$(1) | CFM,QD,MUP | 3 | CEPH, STREP, MUP | 3 | MDR |
| 08 | $GE_5$(1) | VA, DA, E, QD, MUP, AMP, AMC | 7 | GLY, LIN, MAC, STREP, MUP, PEN | 6 | MDR |
| 09 | $AGE_1$(1) | CFM,QD,MUP, AMP, AMC | 5 | CEPH, STREP, MUP, PEN | 4 | MDR |
| 10 | $AGE_1$(2) | CFM, E,QD, MUP, AMP, FOX, AMC | 7 | CEPH, MAC, STREP, MUP, PEN | 5 | MDR |
| 11 | $AGE_3$(1)(2) | CFM | 1 | CEPH | 1 | NIL |
| 12 | $AGE_4$(1)(2) | DA,QD | 2 | LIN, STREP | 2 | NIL |
| 13 | SmSw2(m) | CFM,QD | 2 | CEPH, STREP | 2 | NIL |
| 14 | SmSw10(m) | CFM,QD | 2 | CEPH, STREP | 2 | NIL |
| 15 | SmSw11(m)(1) | QD | 1 | STREP | 1 | NIL |
| 16 | SmSw12(m) | CFM,QD,AMC | 3 | CEPH, STREP, PEN | 3 | MDR |
| 17 | Sw23(m) | CFM, E,QD, MUP, AMP, FOX, AMC | 7 | CEPH, MAC, STREP, MUP, PEN | 5 | MDR |
| 18 | TwSw28(2) | VA, DA, CFM, E, QD, MUP, AMC, FOX, AMP | 9 | GLY, LIN, CEPH, MAC, STREP, MUP, PEN | 7 | MDR |
| 19 | TwSw30(2) | CFM, MUP, AMP, FOX, AMC | 5 | CEPH, MUP, PEN | 3 | MDR |
| 20 | TwSw33(m) | VA, DA,CFM,E, QD, MUP, AMC | 7 | GLY, LIN, CEPH, MAC, STREP, MUP, PEN | 7 | MDR |
| 21 | DmSw38(m) | DA, CFM, MUP, AMP, AMC | 5 | LIN, CEPH, MUP, PEN | 4 | MDR |

**Table 4.11 Continued**

| | | | | | | |
|---|---|---|---|---|---|---|
| 22 | DmSw40(m) | VA, DA, E,QD, MUP, AMP, AMC | 7 | GLY, LIN, MAC, STREP, MUP, PEN | 6 | MDR |
| 23 | DmSw41(m) | OFX,VA,DA,CFM,E,QD, MUP, AMP, FOX, AMC | 10 | QUIN, GLY, LIN, CEPH, MAC, STREP, MUP, PEN | 8 | XDR |
| 24 | DmSw42(m) | VA, DA,CFM,E,QD, MUP, AMP, FOX, AMC | 9 | GLY, GLY, CEPH, MAC, STREP, MUP, PEN | 7 | MDR |
| 25 | ZcSw49(1) | VA, DA, CFM, QD, MUP, AMC | 6 | GLY, LIN, CEPH, STREP, MUP, PEN | 6 | MDR |
| 26 | ZcSw49(m) | CFM | 1 | CEPH | 1 | NIL |
| 27 | ZcSw52(2) | VA, DA, CFM, E, QD | 5 | GLY, LIN, CEPH, MAC, STREP | 5 | MDR |
| 28 | ZcSw53(m) | DA, CFM, E, QD, MUP, | 5 | LIN, CEPH, MAC, STREP, MUP | 5 | MDR |

**Keys:** QUIN = Quinolone, MUP = Mupirocin, AMP = Ampicillin, CEPH = Cephalosporin, PEN = Penicillin, CIP = Ciprofloxacin, QD = Quinopristin/Dalfopristin, CFM = Cefixime, FOX = Cefoxitin, AMC = Amoxicillin/ Clavulanic acid, DA = Clindamycin, VA = Vancomycin, LIN = Lincosamide, STREP = Streptogramin, GLY = Glycopeptide, XDR = Extensively drug-resistant, MDR = Multidrug-resistant, NIL: not MDR, NAR = Number of antibiotics resistant to, NCAR = Number of Class of antibiotics each isolate is resistant to, MDR: non-susceptible to ≥1 agent in ≥3 antimicrobial categories

## 4.9 Determination of Multiple Antibiotics Resistance Index (MARI) for Isolates

### 4.9.1 Determination of multiple antibiotics resistance index (MARI) for Enterobacteriaceae isolates

Determination of the MAR index of the Enterobacteriaceae isolates showed that most of the isolates (88.9%) had MAR index of ≥0.3. The formula for calculating MARI is as given below (Osundiya *et al.,* 2013) and Table 4.12 shows MARI for the enterobacteriaceae isolates.

$$MARI = \frac{\text{number of antibiotics to which organism is resistant}}{\text{total number of antibiotics to which organism is exposed}}$$

Out of the 27 Enterobacteriaceae Isolates, 3 (11.10%) were resistant to one antibiotic agent, 1(3.70%) also was resistant to two antibiotic agents, 2 (7.41%) were resistant to three agents and 7(25.93%) were resistant to four, five and six antibiotic agents.

**Table 4.12: Multiple Antibiotic Resistance (MAR) Index of Enterobacteriaceae Isolates**

| Number of antibiotics to which resistant | MAR Index | No. of isolates (%) | |
|---|---|---|---|
| 1 | 0.1 | 3 (11.10) | 11.10% |
| 2 | 0.3 | 1(3.70) | 88.90% |
| 3 | 0.4 | 2 (7.41) | |
| 4 | 0.5 | 7 (25.93) | |
| 5 | 0.6 | 7(25.93) | |
| 6 | 0.8 | 7(25.93) | |

**Table 4.13: Multiple Antibiotic Resistance (MAR) Index of *Staphylococcus aureus* Isolates**

| Number of antibiotics to which resistant | MAR Index | No. of isolates (%) | |
|---|---|---|---|
| 1 | 0.1 | 3(10.7) | 50.0% |
| 2 | 0.2 | 7(25.0) | |
| 3 | 0.2 | 4(14.3) | |
| 5 | 0.4 | 5(17.9) | 50.0% |
| 6 | 0.5 | 1(3.6) | |
| 7 | 0.5 | 5(17.9) | |
| 9 | 0.7 | 2(7.1) | |
| 10 | 0.8 | 1(3.6) | |

Out of the 28 *Staphylococcus aureus* Isolates, 7(25.0%) were resistant to two antibiotics, 5(17.9%) to seven antibiotic agents, 5 (17.9%) also were resistant to five agents, 4(14.3%) resistant to three agents, 3(10.7%) resistant to one antibiotic agent, 2(7.1%) were resistant to nine agents, 1(3.6%) to six agents and only 1(3.6%) was resistant to ten antibiotic agents.

## 4.10 Determination of Beta-Lactamase Production

Of the 28 *Staphylococcus aureus* isolates that were used in this test, 19 (67.9%) were beta-lactamase positive while 9 (32.1%) of these isolates were beta-lactamase negative.

Fig 4.6 is a pie chart showing beta-lactamase production of the isolates.

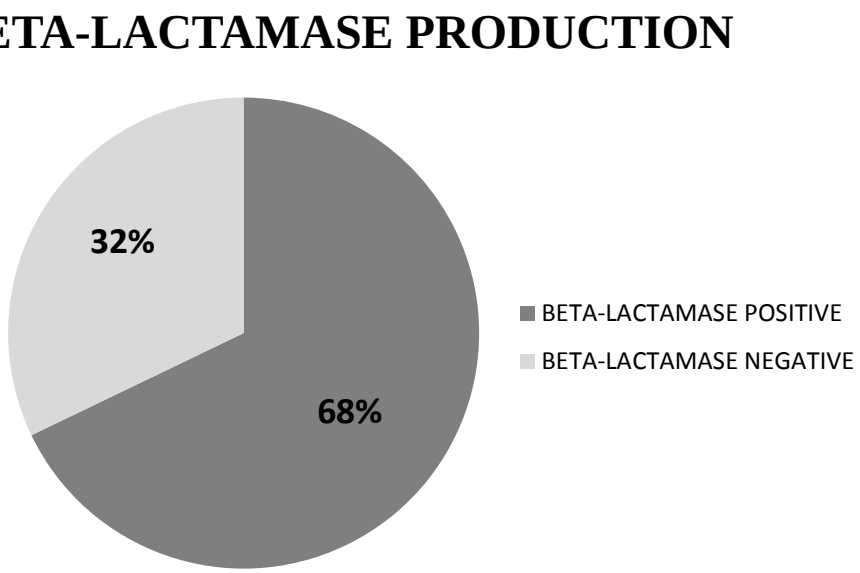



**Fig 4.5: Beta-lactamase Production**

### 4.11 Result of Oxacillin/Cefoxitin Test

Of the 19 *Staphylococcus aureus* isolates that were beta-lactamase positive, 31.6% (6) were positive for the cefoxitin test. This is shown in Table 4.14. The sources of the nineteen *Staphylococcus aureus* isolates are shown in Table 4.15 and those resistant to Cefoxitin are shown in Table 4.16.

**Table 4.14: Oxacillin/Cefoxitin Test**

| PARAMETERS | NUMBER | PERCENTAGE (%) |
|---|---|---|
| No of Resistant Isolates | 6 | 31.6 |
| No of Susceptible Isolates | 13 | 68.4 |

**Keys:** No-Number

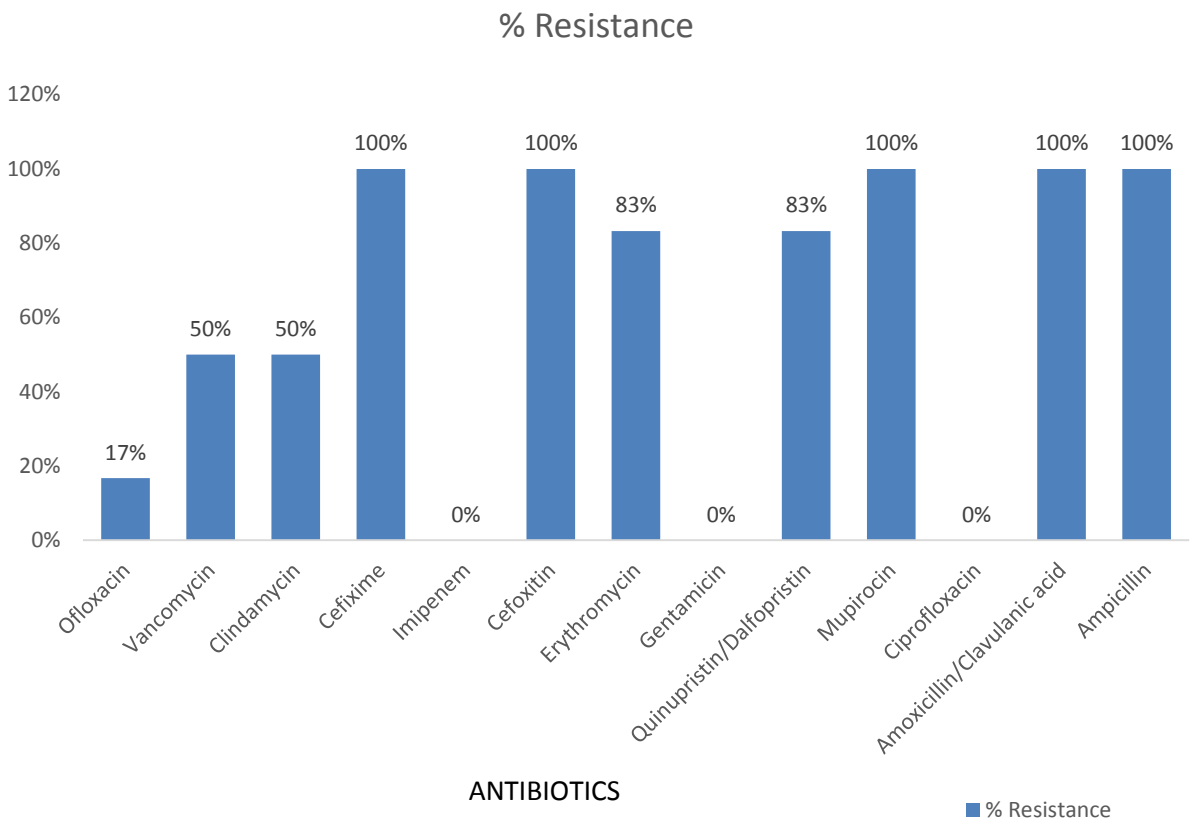
**Table 4.15: Number of *Staphylococcus aureus* isolates by source**

| S/NO | SOURCE | NUMBER OF *Staph aureus* ISOLATES |
|---|---|---|
| 01 | Kuli-kuli | 01 |
| 02 | Groundnut | 02 |
| 03 | Environment | 04 |
| 04 | Hand swabs | 12 |
| 05 | Total | 19 |

**Table 4.16: Sources of the Six (6) Phenotypic MRSA Resistant to Cefoxitin**

| S/NO | SOURCE | NUMBER OF ISOLATES |
|---|---|---|
| 01 | Environment | 01 |
| 02 | Hand swabs | 05 |
| | Total | 06 |

**Fig 4.6: Percentage Antibiotics Resistance of Methicillin Resistant *Staphylococcus aureus* isolates**




The six (6) *Staphylococcus aureus* isolates that were phenotypically taken to be MRSA were found to be resistant to Cefixime(100%), Cefoxitin(100%), Mupirocin(100%), Amoxicillin/Clavulanic acid(100%), Ampicillin(100%) and were susceptible to Imipenem, Gentamicin and Ciprofloxacin with 0% resistance (Fig. 4.7).

### 4.12 Detection of *mecA, vanA* and *blaZ* Genes

Only the six (6) isolates that were β-lactamase positive were used for the *mecA, blaZ* and *vanA* tests. The results are shown in fig.4.8 and Table 4.17.

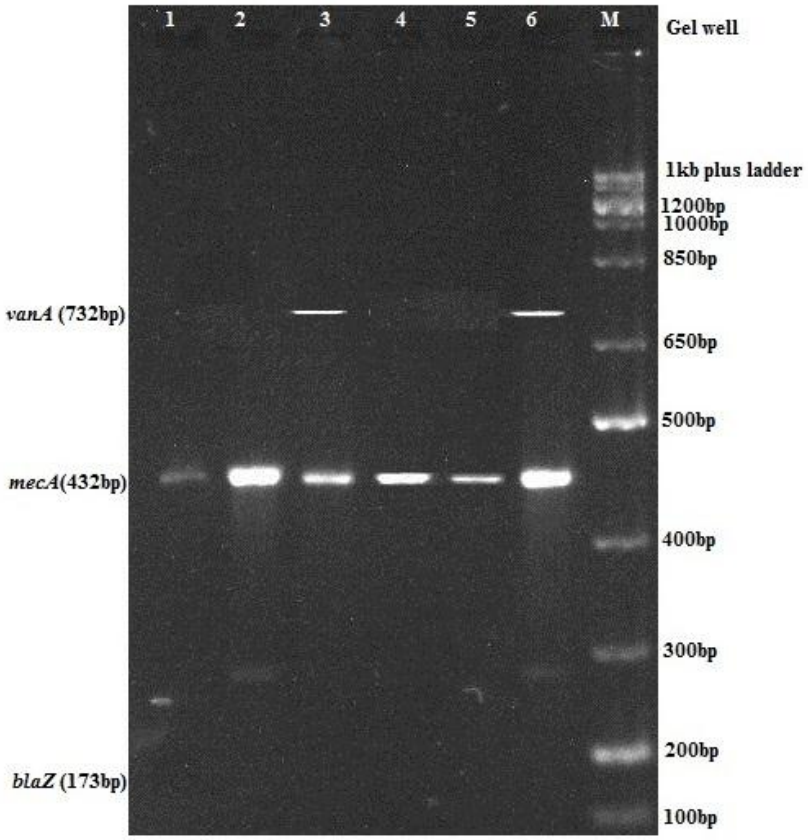



**Fig.4.7: Expression of *vanA, mecA* and *blaZ* genes**

Key:

Lane 1 Isolate from environment

Lane 2 Isolate from hand swab

Lane 3 Isolate from hand swab

Lane 4 Isolate from hand swab

Lane 5 Isolate from hand swab

Lane 6 Isolate from hand swab

Lane M Ladder

**Table 4.17: Expression of *vanA*, *mecA* and *blaZ* genes**

| S/N | LANES | GENE EXPRESSED | BASE PAIR (bp) |
|---|---|---|---|
| 1 | 1 | *mecA* | 432 |
| 2 | 2 | *mecA* | 432 |
| 3 | 3 | *VanA* and *mecA* | 732, 432 |
| 4 | 4 | *mecA* | 432 |
| 5 | 5 | *mecA* | 432 |
| 6 | 6 | *VanA* and *mecA* | 732, 432 |
| 7 | M | Gel Ladder | |

# CHAPTER FIVE

## 5.0: Discussion

Many factors can contribute to microbial contamination throughout production and packaging of groundnut produce. These include poor workers hygiene, and poor equipment sanitation (Beuchat, 1996). Practically all of the food samples purchased harbor a variety of microorganisms and there was high isolation of organisms from both the hand swab samples and samples from the environment where the food products were purchased. This is because microorganisms are ubiquitous and are especially plentiful in soil and the environment making contamination of food samples easy. The occurrence of pathogenic microorganisms has always been attributed to several factors, which include contamination through water, soil, food processing equipments, food contact surfaces and most importantly food handlers (Shamsuddeen and Ameh, 2008; Aboloma, 2008; Kawo and Abdulmumin, 2009). Exposure of the foods to air or dust at the point of sale is likely to increase the counts of the bacteria as virtually most of the bacteria are carried in aerosols by dust and air (Food and Drug Administration, 2009). When food handlers do not practice proper personal hygiene or correct food preparation, they may become vehicles for microorganisms, through their hands, mouth, and skin among others (Silva *et al.,* 2003: Bukar *et al.,* 2009).

Of the enterobacteriaceae isolated, *Citrobacter freundii* was the most prominent. Most of the enterobacteriaceae isolates were present in the hand swabs.

The *Enterobacteriaceae* is a large family of Gram negative bacteria that includes, more familiar pathogens, such as *Escherichia coli*, *Salmonella*, *Shigella*, *Klebsiella* and *Yersinia*. Most of the members of *Enterobacteriaceae* cause gastrointestinal problems in human

beings (Paterson, 2006). The diseases are caused by either toxin from the disease-causing microbes, or by the human body's reactions to the microbe itself (Teplitski *et al*., 2009). The disease is sometimes difficult to treat due to the emergence and spread of resistance in *Enterobacteriaceae* (Paterson, 2006).

*Citrobacter freundii* are commonly found in the environment, mainly in soil, water, and sewages. They are an indicator of potential contamination of water (Wang *et al.,* 2000). *C. freundii* plays an important role in the nitrogen cycle. In the environment, *C. freundii* can convert nitrate or the ammonium ion (which is a nitrogen atom combined with four hydrogen atoms) to nitrite; this reaction occurs in the environment as well as within the digestive tract of humans and other animals (Puchenkova, 1996). After it converts nitrate to nitrite in the environment, the nitrite is converted to nitrogen, and this final step completes the nitrogen cycle in the earth's atmosphere, which is made up of 85% nitrogen (Puchenkova, 1996). The high occurrence of *Citrobacter freundii* could be as a result of contamination of the groundnut which is a legume crop during harvest, processing and probably packaging of the food products by the food handlers.

*Citrobacter* spp and *Klebsiella* spp are members of the normal intestinal flora (Guentzel, 1996). Presence of these organisms in food samples and hands of food handlers could be as a result of poor hygiene which leads to contamination of food products by the organisms (Khan *et al*., 2015).

Gram-negative Enterobacteriaceae may cause severe infections and unfortunately several of the most important members of this family are becoming progressively more resistant to currently available antimicrobials (Fritsche *et al.,* 2005; Paterson, 2006; Denton, 2007). Antibiotic susceptibility pattern of Enterobacteriaceae isolated from this study showed that

high resistance was seen to the Cephalosporin but Imipenem, Gentamicin and the Quinolone antibiotics showed good activity on the isolates. Enterobacteriaceae have been reported to be resistant to most antibiotics especially the Cephalosporins and beta-lactam antibiotics (Thiolas *et al.*, 2005).

Of the 69 coagulase positive Staphylococci, *Staphylococcus aureus* was the most isolated 28/69 (40.6%), most of which were present in the hand swabs. *Staphylococcus aureus* is an important cause of food intoxication throughout the world. This bacterium can contaminate several foods, including minimally processed ready-to-eat vegetables and processed meat products and produce several types of enterotoxins (Balaban and Rasooly, 2000). Most food poisoning cases have been reported to be often caused by eating food contaminated with infectious agents such as *Escherichia coli, Salmonella, Shigella* and *Staphylococcus* (Tan *et al.*, 2013).

In a study in Ilorin by Ajao and Atere (2009), similar microorganisms were isolated from the hand-swab of the food handlers working in canteens. This is an indication that food handlers have poor hand-washing practices. Also Mosupye and von Holy (1999) in Johannesburg isolated similar microorganisms from street-vended foods. *Staphylococcus aureus* and *Escherichia coli* have also been isolated from ready to eat vegetable food samples in Cairo, Egypt (El-Hadedy and El-Nour, 2012). A study carried out by Okareh and Erhahon (2015) isolated *Staphylococcus aureus* (38.3%), *Staphylococcus epidermidis* (21.7%), *Escherichia coli* (16.7%), amongst others from the hand-swab samples of the food handlers, similar microorganisms were isolated from hand-swab samples in this current study. The presence of these microorganisms on the hands of the food vendors is a cause for concern as it could lead to transfer of microorganisms to the food and the utensils being

used. The contaminated food, when consumed by the populace could cause food-borne illness.

MRSA strains are usually resistant to several groups of broad spectrum antibiotics that are used on a large scale in the hospital. The mechanism of increased spreading under antibiotic pressure may have contributed to the worldwide increase in the prevalence of MRSA in hospitals (Voss and Doebbeling, 1985). Out of 28 isolates of *S. aureus* tested, 19 (67.9%) were beta-lactamase producing and a total of 6/19 (31.6%) were found to be methicillin resistant, so phenotypically MRSA. Of the 6 MRSA isolates 1(16.7%) was obtained from the environment where the groundnut products were sold and 5 (83.3%) were from hand-swab samples of the local sellers.

The antibiotic sensitivity profile of MRSA to various antibiotics showed that the isolates showed high resistance to Cefixime, Cefoxitin, Amoxycillin/Clavulanic acid, Mupirocin and Ampicillin. This is in agreement with a work carried out by Gundogan *et al.* (2005) who isolated MRSA from *Staphylococcus aureus* isolates gotten from meat and chicken samples. Using the modified Kirby-Bauer disc diffusion test, they found out that the MRSA were resistant to multiple antibiotics. Three (50%) of the MRSA in this current study were resistant to Vancomycin, this does not agree with the report of Gundogan *et al*. (2005) who reported no resistance to Vancomycin. This might be due to the different geographical locations and/or the type of antibiotics commonly misused in both areas.

Reports regarding prevalence of MRSA in food and in particular in groundnut products are generally scarce. Considering MRSA identification in other food stuffs, many reports have identified its prevalence. In a study carried out by Kwon *et al.* (2006), retailed chicken meats were examined and a total of 766 *Staphylococcus aureus* were isolated from the samples. Out of these *S. aureus* isolates, 4 were found to be *mecA* – carrying MRSA.

*Staphylococcus aureus* has also been isolated from swabs from the brain, eyes and kidneys of cage cultured tilapia. In the study, 198 *S. aureus* were isolated, 98 (50%) of the *S. aureus* were identified to be MRSA (Atyah *et al.,* 2010). In another study carried out by Hanson *et al*. (2011), *S. aureus* were isolated from fresh raw pork, chicken, beef and turkey. Of the 27 *S. aureus* isolates, 2 were found to be MRSA. Although none of the MRSA was isolated from the roasted groundnut or kuli-kuli samples, MRSA can be transferred from the hands of local sellers to the products they sell during processes of packaging and distribution.

The antibiotic susceptibility results of MRSA in this study to the quinolones and the aminoglycoside was quite encouraging and compared favorably well with the study from Ilorin by Taiwo *et al*., (2004) and in Kano by Nwankwo *et al.,*(2010). The quinolones particularly Ciprofloxacin have been suggested for the treatment of MRSA infections and colonization (Blumberg *et al.,* 1991; McDougal *et al.,* 2003). This result will be of local clinical relevance in the treatment of MRSA in this environment especially when vancomycin is not commonly available and with the emergence of Vancomycin resistant *Staphylococcus aureus* (VRSA) in this area.

Multidrug resistant Enterobacteriaceae which was confined to the hospital environments is now emerging in the domestic food related environments as well (Azevedo *et al.,* 2015). From the result of this study, 78% of the Enterobacteriaceae were multidrug resistant (MDR) while 61% of the *Staphylococcus aureus* were multidrug resistant (MDR). The high percentage of MDR might be due to the carriage of resistance genes by the isolates and also, a combination of microbial characteristics such as selective pressure on antimicrobial usage, societal and technological changes that enhance the transmission of drug resistant organisms might be the cause of this high resistance (Orozova *et al.,* 2008).

Most of the isolates had Multiple Antibiotic Resistance (MAR) index of 0.3 and above. This observation suggests that the isolates in this study might have originated from an environment where antibiotics are often used indiscriminately (Paul *et al.*, 1997). Broad-spectrum antibiotics are sometimes reported to be given in place of narrow-spectrum antibiotics as a substitute for culture and sensitivity testing, with the consequent risk of selection of antibiotic-resistant mutants (Nahum *et al*., 2006; Norwitz and Greenberg, 2009).

Food is regarded a significant factor for transferring and spreading of antibiotic resistance genes and food handlers may constitute a reservoir of virulent strains of *Staphylococcus aureus* and may be vehicles of their transmission to food (Mashouf *et al.,* 2015).

In this study, 6/19 (31.6%) of the isolates were molecularly characterized as MRSA. This is higher than the study in US where Ge *et al*. (2017) found a prevalence rate of 1.9 % in retail meats, and what was obtained in Iran by Arfatahery (2016) who reported a prevalence rate of 28.2% in fishery products. In the result of this study, all the MRSA isolates expressed *mecA* gene of 432bp (fig 4.8). In a work carried out by Mashouf *et al.* (2015), on 98 *S. aureus* isolates, *mecA* gene was detected in all MRSA strains. Presence of *mecA*-carrying *S. aureus* in food products especially groundnut and kuli-kuli produced locally and among food handlers can lead to wide spread of resistant strains which can create a public health problem in a case of outbreak of infection by the organism.

Thirty three point three percent (33.3%) of the MRSA expressed the *vanA* gene which codes for Vancomycin resistance (fig 4.8). A study carried out by Whitener *et al.* (2004) who reviewed a patient with foot ulcer carrying both Vancomycin Resistant Enterococcus

(VRE) and MRSA and reported the horizontal transfer of *vanA* gene from VRE to the MRSA. The expression of *vanA* gene among MRSA isolates in this study could be as a result of horizontal transfer of the gene from another isolate harboring the *vanA* gene. Increase in MRSA lead to the use of Vancomycin for treatment of infections caused by MRSA. Carriage of Vancomycin resistance gene among these MRSA isolates can escalate, rendering Vancomycin less effective for treatment of infection when they occur.

In this study the MRSA did not harbor the *blaZ* gene and phenotypic tests showed beta-lactamase activity. In a study by Robles *et al*. (2014), beta-lactamase detection was performed using different tests (PCR, Clover leaf test, Nitrocefin disk, and in vitro resistance to Penicillin). The *S. aureus* isolates showed phenotypic activity for beta-lactamase production but did not harbor *blaZ* gene. Beta-lactamase phenotype can be as a result of the expression of more than one gene, and there is more than one mechanism that grants Staphylococci beta-lactam resistance other than the expression of *blaZ* gene (Malik *et al.,* 2007). Also the detection of a gene does not necessary mean it is expressed (Robles *et al.,* 2014).

The occurrence of resistance to Quinupristin/dalfopristin among the isolates in this study could possibly be due to transfer to humans, of some rare resistance genes like Lincosamide-Streptogramin-A-Pleuromutilin resistance genes *vga* (C) and *vga* (E) that are located on multiresistance plasmids in Livestock-Associated Methicillin-resistant *Staphylococcus aureus* (LA-MRSA) (Kadlec, *et al.*, 2012). Graveland *et al*., (2011) reported that persons in direct contact with LA-MRSA- positive animals have an increased risk of becoming MRSA positive. The risk of carriage is mainly related to the intensity of animal contact and with MRSA prevalence among animals on the farm. This transfer could

occur due to the common practice of humans living in close quarters with livestocks in the study area.

# CHAPTER SIX

## 6.0 Summary, Conclusion and Recommendation

### 6.1 Summary

A total of 294 samples consisting of kuli-kuli (75), roasted groundnut (75), unpeeled roasted groundnut (75), hands of the sellers (54) and the environments (15) were collected from five markets and 152 isolates were obtained from the five markets sampled in the study.

Full identification of Enterobacteriaceae isolates using Microgen$^{TM}$GNA-ID kit showed that the most prevalent organisms were *Citrobacter freundii* (22.2%) and *Klebsiella oxytoca* (11.1%). The initial number of Gram positive isolates was 125, 69 of these isolates were coagulase positive. Microgen$^{TM}$ Staph ID kit was used to confirm that out of the 69 isolates that were Coagulase positive only 28 were *Staphylococcus aureus*.

Antibiotics susceptibility testing showed that all the isolates were susceptible to Imipenem (100.0%), Gentamicin (100.0%) and Ciprofloxacin (100.0%) but showed high resistance to Cefixime (82%). Determination of the MAR index for the Enterobacteriaceae isolates showed that most of the isolates (81.5%) had MAR index of ≥0.3 and that for *Staphylococcus aureus* isolates showed that 50.0% had MAR index of ≥0.3.

Of the 28 *Staphylococcus aureus* isolates that were used for the beta-lactamase test, 19 (67.9%) were positive while 9 (32.1%) of these isolates were negative. Of the 19 *Staphylococcus aureus* isolates that were beta-lactamase positive, 6 (31.6%) were resistant to cefoxitin (MRSA). All the isolates that were phenotypically MRSA carried *mecA* gene,

and 33.3% of these isolates expressed *vanA* gene but none of the isolates expressed *blaZ* gene.

## 6.2 Conclusion

*S. aureus* accounts for frequent contamination of food products and is a well-known clinical and epidemiological pathogen. The increasing prevalence of Community Associated-MRSA and its emerging antibiotic resistance in foods is a serious problem for public health. Contaminated food products during the processing stage are the most frequent transmission routes in food contamination with *S. aureus* or MRSA. Therefore, continuous surveillance and monitoring of pathogens with potential to infect foods and the emergence of MRSA and antibiotic resistance is necessary for public health.

This study implicates existence of high carriage of MRSA in the sampled food related environments and among local sellers of groundnuts and kuli-kuli in Zaria metropolis. Contact with food samples by the local sellers during packaging and distribution can lead to the transfer of MRSA throughout the community. This could cause a public health risk of outbreak of infections caused by MRSA in Zaria and the world at large. Vancomycin resistance was observed among the MRSA indicating an emergence of Vancomycin among MRSA isolates from local sellers of groundnuts and kuli-kuli in Zaria metropolis. This resistance phenomenon requires continual vigilance.

This study confirmed our null hypothesis that there is no occurrence of multidrug-resistant *Staphylococcus aureus* and Enterobacteriaceae in isolates from kuli-kuli and roasted groundnuts obtained from Zaria metropolis. However, there was an occurrence of MRSA, multidrug-resistant *Staphylococcus aureus* and Enterobacteriaceae among isolates from hand-swab samples of some of the local sellers and the food-related environment. These

bacteria could eventually get transferred to the food products during the processes of packaging and distribution.

### 6.3 Recommendation

Although Vancomycin resistant MRSA is not yet common in this part of the world, the rate of spread of this pathogen and its unique ability to acquire and transfer antibiotic resistance calls for:

4 Urgent and well-coordinated surveillance program to combat this situation.

5 Strict antibiotic prescription policies to be enforced by the appropriate authorities to contain the abuse of antibiotics and reduce acquisition of resistance by pathogens.

6 Educational awareness to be encouraged to update local sellers and all food handlers of the consequences of food contamination with Multidrug-resistant *Staphylococcus aureus* and Enterobacteriaceae.

7 Standard hygiene by local sellers.

8 Awareness for local sellers.

### 6.4 Contribution to Knowledge

- This study reported the occurrence of MRSA with *mecA* gene of 432bp from hand swabs samples of local sellers of roasted groundnut and kuli-kuli in Zaria metropolis.
- Isolation of MRSA with *vanA* of 732bp from hand swabs.
- Sixty one percent (61%) of the *Staphylococcus aureus* and seventy-eight percent (78%) of the Enterobacteriaceae isolates were multidrug resistant.

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# APPENDIX I

| S/No | Antimicrobial Agent | Disk Content | Zone Diameter Interpretative Criteria(nearest whole mm) | | |
|---|---|---|---|---|---|
| | | | S | I | R |
| 01 | Amoxicillin/Clavulanic acid | 20/10µg | ≥18 | 14-17 | ≤13 |
| 02 | Ampicillin | 10µg | ≥17 | 14-16 | ≤13 |
| 03 | Cefixime | 5µg | ≥19 | 16-18 | ≤15 |
| 04 | Cefoxitin | 30µg | ≥18 | 15-17 | ≤14 |
| 05 | Ciprofloxacin | 5µg | ≥21 | 16-20 | ≤15 |
| 06 | Clindamycin | 2 µg | ≥21 | 15-20 | ≤14 |
| 07 | Erythromycin | 15 µg | ≥23 | 14-22 | ≤13 |
| 08 | Gentamicin | 30µg | ≥15 | 13-14 | ≤12 |
| 09 | Imipenem | 10µg | ≥23 | 20-22 | ≤19 |
| 10 | Mupirocin | 200 µg | ≥14 | - | ≤13 |
| 11 | Ofloxacin | 5µg | ≥16 | 13-15 | ≤12 |
| 12 | Quinupristin/Dalfopristin | 15 µg | ≥19 | 16-18 | ≤15 |
| 13 | Vancomycin | 30 µg | ≥17 | 15-16 | ≤14 |

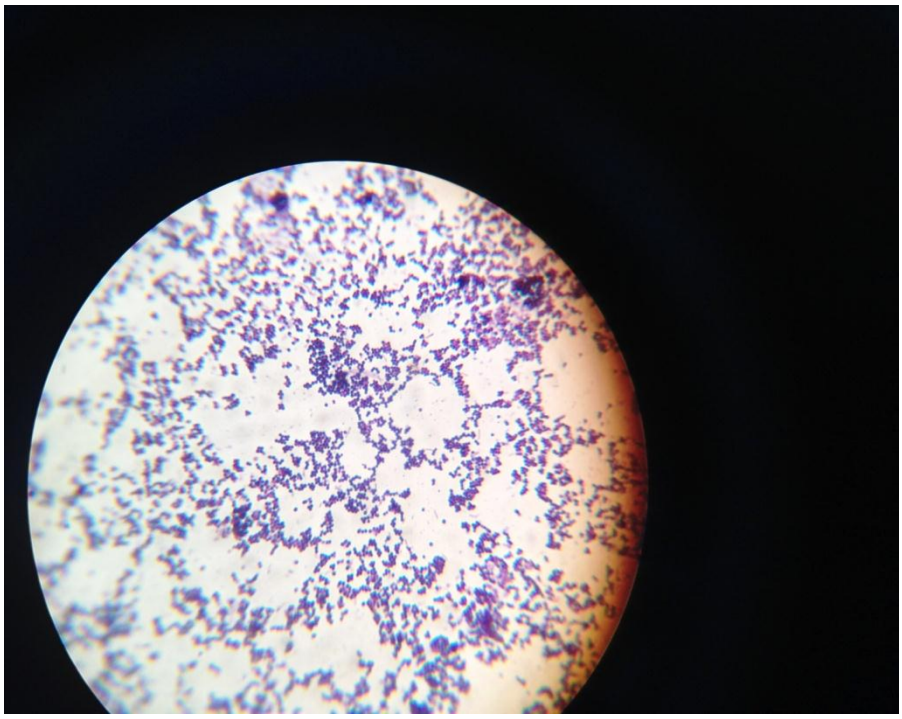
CLSI INTERPRETATIVE CHART

Key:

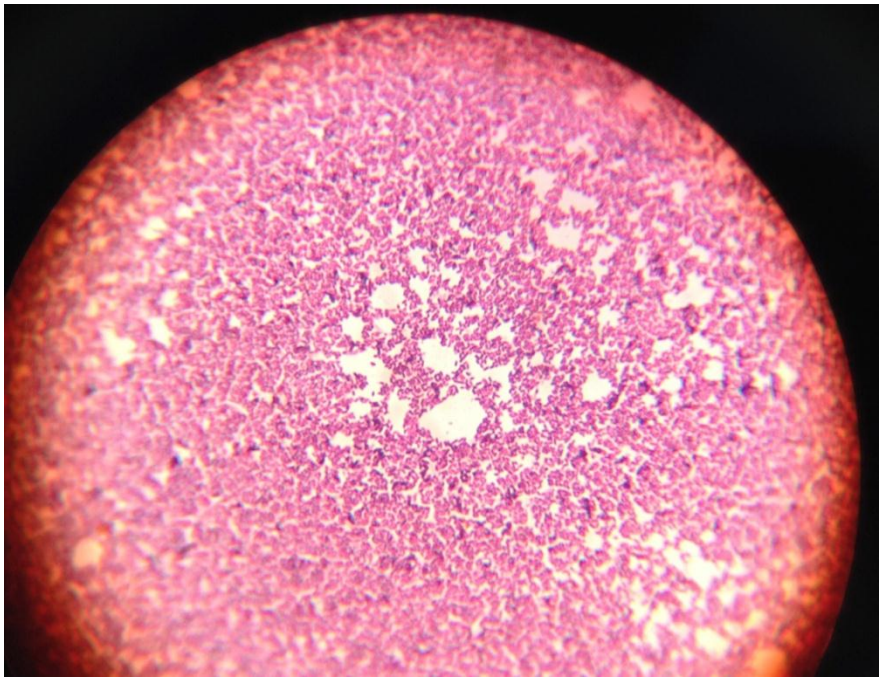
S = Susceptible, I = Intermediate/Moderately susceptible, R = Resistant

Clinical and Laboratory Standards Institute (2006); Clinical and Laboratory Standards Institute (2014).

APPENDIX II



Gram positive cocci

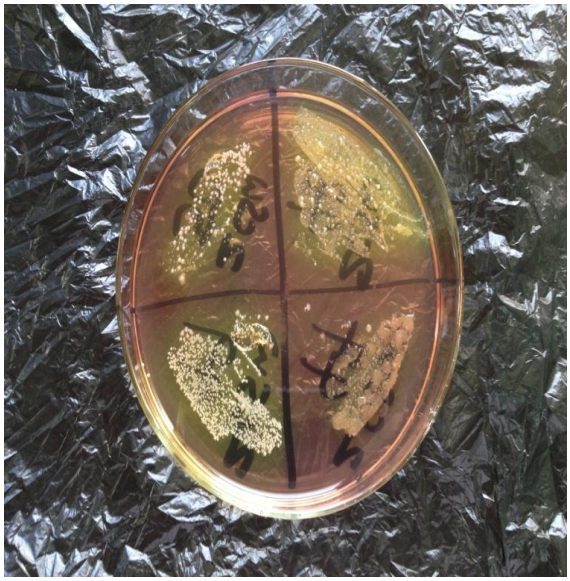


Gram negative rods

APPENDIX III



Isolates on MacConkey agar

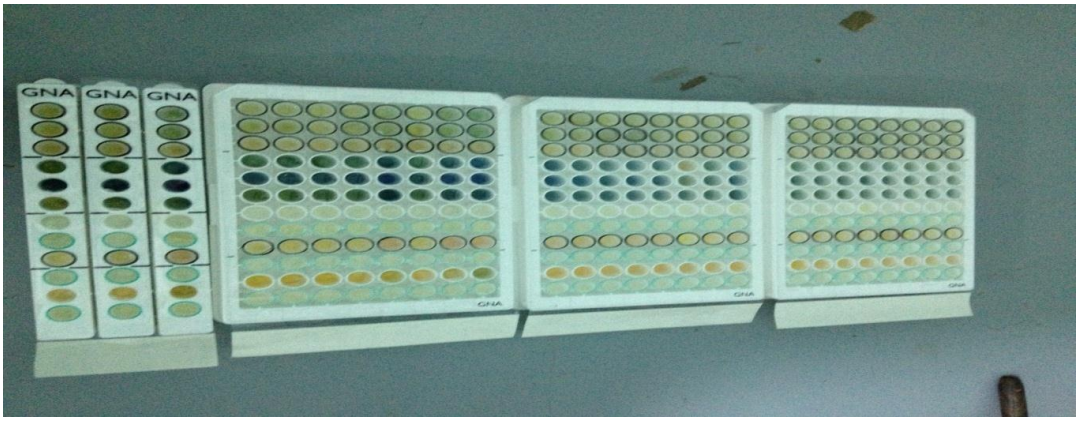


Isolates on Mannitol salt agar



Colonies of organisms from food related environment on Nutrient agar

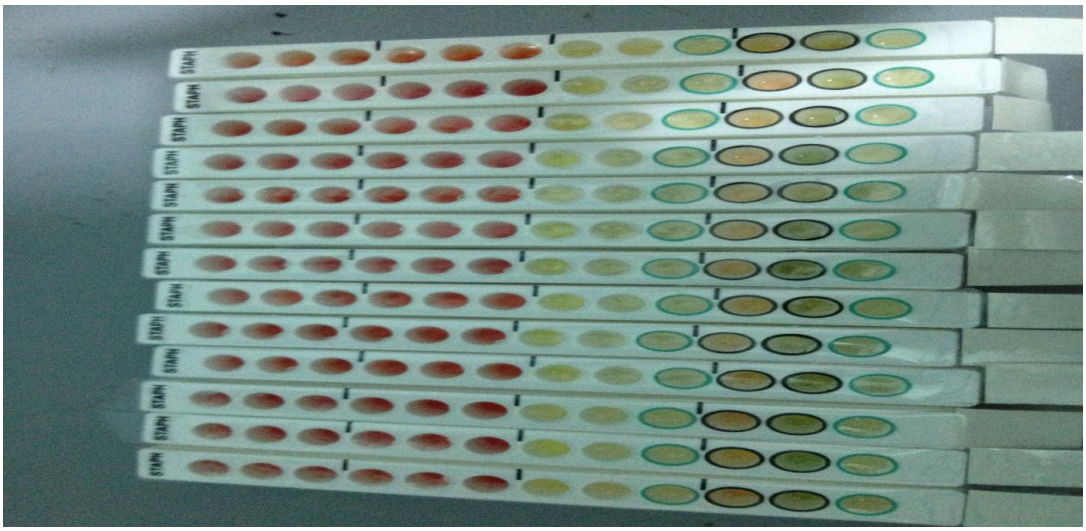
APPENDIX IV




Identification of Enterobacteriaceae isolates using  Microgen kit (before and after incubation)



APPENDIX V



Identification of Staphylococci isolates using Microgen Staph ID kits (before and after incubation)

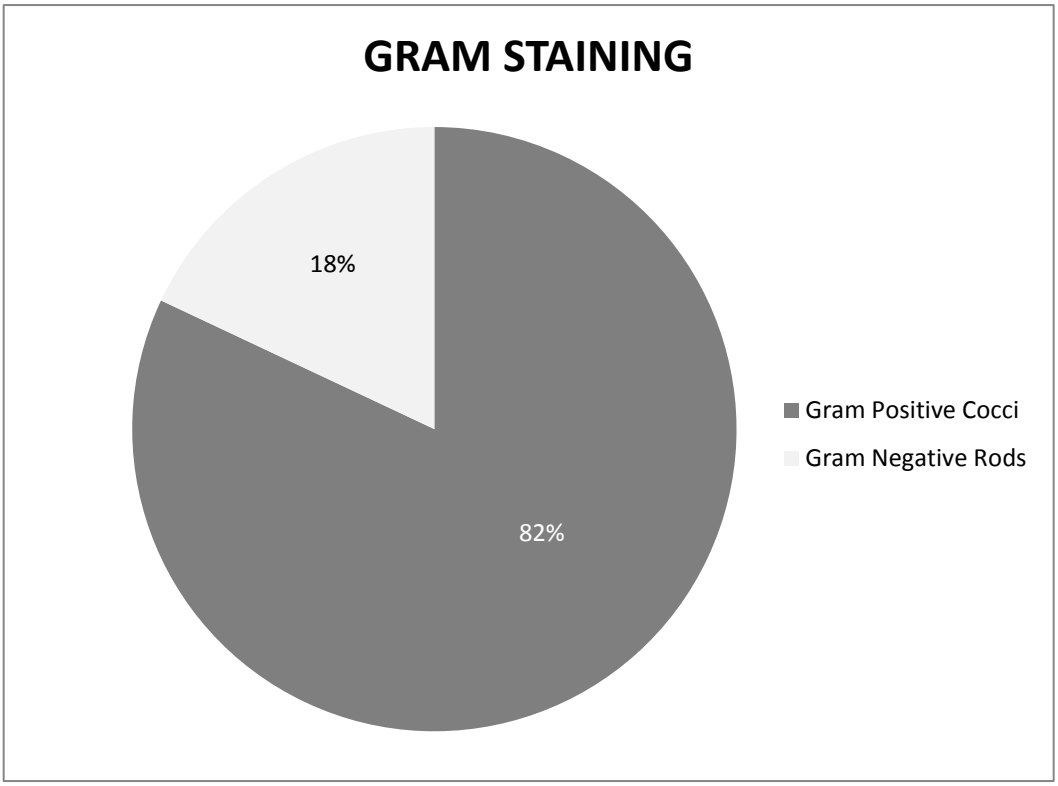


APPENDIX VI



A plate showing zones of inhibition to different antibiotic discs.

APPENDIX VII




Gram Staining of Isolates