ANTIMICROBIAL SUSCEPTIBILITY AND MOLECULAR CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* FROM MASTITIC COWS

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ABSTRACT

This study investigated the antimicrobial susceptibility and molecular characterization of *Staphylococcus aureus* in mastitic cows in southern part of Kano State of Nigeria. A total of two hundred and thirty four (234) milk and swab samples were collected from six (6) local government areas of Kano State in northern part of Nigeria. *Staphylococcus aureus* was isolated from samples and identified using standard microbiological procedures. The antimicrobial susceptibility of the isolates was determined using Kirby- Bauer disc diffusion technique as described by the Clinical and Laboratory standards institute (CLSI). Resistant isolates were tested for Beta-lactamase production. A total of 200 (85.5%) have staphylococcal isolates, of which 141 (70.5%) were *Staphylococcus aureus* (coagulase positive staphylococci, CPS), 59 (29.5%) were coagulase negative staphylococci (CoNS) and 6 (2.9%) were non- Staphylococcal (rods) isolates. The antibiotic susceptibilities of the *Staphylococcus aureus* isolates were in the following order: ofloxacin (99.3%) > ciprofloxacin (98.6%) > gentamicin (91.5%) > cephalexine (56%) > sulphamethoxazole/trimethoprim (46.1%) > tetracycline (43.3%) > cefuroxime (33.3%) > cefoxitin (2.1%) > amoxicillin (1.4%) > ampicillin (1.4%). Resistance to three or more antimicrobials was presented in 98.6% of the *Staphylococcus aureus* isolates. Of the 141 *Staphylococcus aureus* isolates tested for β-lactamase production, 31 (22.5%) were β lactamase producers. However, of the 31 β-lactamase producers, only 13 (41.9%) were multidrug resistant to cefoxitin, Ampicillin and cephalexine. PCR assay was used to detect *mecA* and *blaZ* genes in multi-drug resistant *Staphylococcus aureus* isolates that were β-lactamase producers. The MDR-*Staphylococcus aureus* was also tested by latex agglutination for presence of PBP2a. PCR results indicated none of the isolates showed any amplification to both *mecA* gene and *blaZ* gene. All the Beta-lactamase producing MDR- *Staphylococcus aureus* isolates tested showed negative reaction to latex agglutination test which was also an indication of absence of Pinicillin binding protein

(PBP2a). In conclusion, high isolation of *Staphylococcus aureus* (70.5%) causing bovine mastitis was observed in this study in which both *mecA* and *blaZ* genes were absent.

Key words*:* *Staphylococcus aureus*, CoNS, CPS, Mastitis, β-lactamase, PCR, Disk diffusion test

CHAPTER ONE

1.0: INTRODUCTION

Mastitis is the inflammation of the parenchyma of the mammary gland regardless of the cause. It is characterized by a range of physical and chemical changes in the milk and pathological changes in the glandular tissue. The most important changes in the milk include discoloration, the presence of clots and the presence of large number of leukocytes. There is swelling, heat, pains and indurations in the mammary gland in many clinical cases (Roberson *et al.,* 1994).

Bovine mastitis is a multifactorial disease that results in reduced milk production, changes in milk composition and milk discard. It imposes serious economic losses to the farmers and the dairy industry (Ribeiro *et al.,* 2003; Pitkala *et al.,* 2004). Mastitis can be moderate or severe, and can be caused by many different bacterial species but more commonly by *Staphylococcus* and *Streptococcus* strains (Pyorala, 2002). Coagulase-negative staphylococci (CoNS) are of great interest in veterinary medicine because they are currently being considered emerging pathogens of bovine mastitis. Although CoNS are not as pathogenic as the other principal mastitis pathogens and infection mostly remains subclinical. They can however result in persistent infections, which result in increased milk somatic cell count (SCC) and decreased milk quality (Pyorala, 2002). Prevalent CoNS species vary according to the geographical region under scrutiny (Huxley *et al.,* 2002). Antibiotic resistance is the most puzzling question of public health concern in the earlier decade of this 21st century. Mastitis is the single most common reason for the use of antimicrobials in dairy cattle husbandry. Use of antimicrobial treatment is required for clinical mastitis, persistent infections and in heifers before calving (Taponen *et al.,* 2006). Therefore, antimicrobial resistance of mastitis pathogens has received much interest over

the past few years. Carriage of antimicrobial resistance genes by CoNS species in cattle may also be relevant because it potentially poses a human health hazard. It can happen both through lateral transfers of resistance genes between *Staphylococcus* species and through direct transmission of resistant pathogens (Walther and Perreten, 2007).

Among the antimicrobial agents approved for use in bovine mastitis, β-lactams, such as penicillins and cephalosporins, play a key role. Resistance to β-lactams in *Staphylococcus* infection is mediated by either β-lactamases codified by *bla*Z gene or *mecA*-encoded alternative penicillin binding protein, PBP2a. This shows a reduced binding to β-lactams antibiotics currently available for mastitis therapy. According to recommendations of the CLSI, oxacillin-resistant *Staphylococcus* isolates shall be reported as resistant to other β-lactam antibiotics (Aarestrup and Schwarz, 2006). Humans and dairy cattle may share CoNS strains, implying that bovine staphylococcal multidrug resistant might be a zoonotic pathogen. It is difficult to demonstrate the direction of interspecies transmission, but it has been suggested that CoNS is more likely to spread from humans to dairy cattle than vice versa (Thorberg *et al.,* 2006).

Staphylococcus aureus causes one of the most common types of chronic mastitis. Though some cows may flare up with clinical mastitis (especially after calving) the infection is usually subclinical, causing elevated somatic cell counts (SCC) but no detectable changes in milk or the udder. The bacteria persist in mammary glands, teat canals, and teat lesions of infected cows and shed in milk. The infection is spread at milking time when *Staphylococcus aureus* contaminated milk from an infected gland comes in contact with an uninfected gland, and the bacteria penetrate the teat canal. Once established, *Staph aureus* infections do not respond well to antibiotic therapy and infected cows must be segregated or culled from the herd (Roberson *et al.*,1994).

Successful management, prevention and treatment of bovine mastitis are necessary task for dairy producers. Because of the importance of *Staphylococcus aureus* as a major mastitis pathogen which is very difficult to treat, this work focused on studying staphylococci causing clinical and subclinical mastitis in bovine and their antibiotic resistance.

Staphylococcal mastitis is a major concern in dairy farming and critical source of subclinical and clinical intra-mammary infections in dairy cows leading to severe economic losses to the dairy industry, worldwide (Momtaz *et al.*, 2010; Atasever, 2012; Hussain *et al.*, 2012a). Naturally, *Staphylococcus aureus* isolates are inhabitants of mucous epithelia and skin of human, dairy cattle and other mammalians (Chu *et al.*, 2012), and spread by virtue of milker's hand and milking machines (Seki *et al.*, 1998). β-lactam antibiotics are frequently used for treatment of *Staphylococcus aureus* mastitis as well as intra-mammary infusion for preventive measures in cows. Injudicious use of antimicrobials has resulted in augmenting the bacterial resistance mechanism including the β-lactamase production. The *Staphylococcus aureus* resistance to methicillin (MRSA) was first reported in 1960, and with the MRSA gradually developed multiple resistances and became a source of causing serious nosocomial infections, worldwide (David and Daum, 2010). The pathogenic potential of *Staphylococcus aureus* depends on numerous cell surface virulence factors such as colonization of the epithelium of the teat and the streak canal, invading bovine mammary epithelial cells in culture, phagocytic dysfunction and production of a variety of exotoxins and cell surface-associated proteins that enhance the cellular attachment, organism invasion of host immune system and stimulation of toxic tissue reactions (Kalorey *et al.*, 2007; Hussain *et al.*, 2012b). It has been reported that in divergent geographical areas a limited diversity of *Staph aureus* strains is involved in mastitis infection (Moon *et al.*, 2007). Therefore, genotyping of isolates is necessary to identify the genetic relatedness of strains

and their source of spread; and one of the reliable and broad genotyping methodologies is repetitive element sequence- based PCR or REP-PCR (Del Vecchio *et al.,* 1995). *Staphylococcus aureus* strains are capable of mutation, clonal evolution and horizontal gene transfer that increase up the virulence and drug resistance (Brody *et al.*, 2008). Hence, identification of pathogenic and resistant *Staph aureus* from intra mammary infection at herd level is of vital importance for successful treatment, because of scarcity of information on diversity of bovine mastitis *Staph aureus* isolates in Nigeria, particularly in Northern region, this study was concerned.

1.1: STATEMENT OF RESEARCH PROBLEMS

Bovine mastitis produces a wide variety of problems in the dairy farm. The treatment of this disease is based on the use of antibiotics which are not always effective. These drugs are also responsible for the presence of residues in the milk and the increase of antibiotic-resistant strains. Probiotic products were proposed as a valid alternative to antibiotic therapies and are also useful for the prevention of infectious syndromes (Getahun *et al.,* 2008). *Staphylococcus aureus* is the most predominant contagious pathogen responsible for clinical and subclinical infections in lactating cows (Le Marechal *et al.,* 2011).

Mastitis, the most expensive disease of dairy cows, continues to be a persistent problem in the dairy industry (Bedidi-Madani *et al.,* 1998; Lima *et al.,* 1993). Mastitis, inflammation of the mammary gland with local and or general symptoms that occasionally result in a systemic infection, can be caused by a wide range of microorganisms, including Gram-negative and Gram-positive bacteria (Lima *et al.,* 1993). This disease is considered to be the most frequent and most costly production disease in dairy herds in developed countries (Henri *et al*., 2003).

1.2: JUSTIFICATION OF THE RESEARCH

Historically, *Staphylococcus aureus* was one of the most common causes of bovine mastitis in dairy cattle worldwide. In the last 25 years, the prevalence of infection and occurrence of clinical mastitis due to *Staph. aureus* has decreased in herds using effective mastitis control measures. However surveys indicated that 50-100% of the herds may be infected. (Jones *et al.*,1984).

It has been shown that 3% of all animals are infected with *Staphylococcus aureus* (Schukken *et al.,* 2009). However, *Staph aureus* represents 10 to 12 % of all clinical mastitis infections (Tenhagen *et al.,* 2009). It has also been noted that cows infected with *Staph aureus* do not necessarily have elevated somatic cell count (SCC). During 1978-1980, about 27,000 milk samples from 28 herds showed that 10 percent of cows were infected with *Staph. aureus* (Jones *et al.*,1984).

Heifers are also a reservoir for *Staph. aureus* infections. In several research trials, 12 to 15 % of first-lactation cows were found infected with *Staph aureu*s at calving (Boddie *et al.*,1987; Trinidad *et al.,* 1990a; Trinidad *et al.,* 1990b). Furthermore, infected heifers left untreated produce 10 percent less milk in early lactation when compared with those who received dry cow antibiotic treatment prior to calving (Owens *et al.,* 1991). Many animals remain infected throughout the first lactation and act as reservoirs for infecting other cows in the herd. Although as many as half of the cows with high SCC may be infected with *Staph. aureus*, SCC alone are not sensitive enough to positively diagnose *Staph. aureus* infections.

The majority of intramammary infection due to *Staph. aureus* is sub clinical. At calving 20-50% of heifers may have intramammary infections due to coagulase negative *Staph aureus* (Kirk *et al.,* 1997). Most of these infections are eliminated spontaneously or with antimicrobial therapy during early lactation.

1.3: RESEARCH AIM

This study was conducted to isolate and investigate the phenotypic and genotypic characteristics of *Staph. aureus* involved in dairy cow mastitis in southern region of Kano State, North-West Nigeria: To find out if methicillin resistant *Staph. aureus* are the major cause of mastitis in bovine mastitis considering the potential risk to the animal and public health.

1.4: RESEARCH OBJECTIVES

The specific objective of this study is to:

1. Isolate and identify staphylococcal organisms from cows manifesting symptoms of mastitis.
2. Carry out Susceptibility of the isolates to commonly prescribed antibiotics.
3. Determine those isolates that are resistant to Methicillin/Cefoxitin.
4. Determine β lactamase producers among the resistant isolates
5. Detect the presence or absence of mecA and blaZ genes by PCR analysis and Latex agglutination.

1.5: HYPOTHESIS

Null Hypothesis (H_0)

Staphylococcus aureus do not carry resistance *mec A* gene in mastitic cow.

Alternative hypothesis (H_1)

Staphylococcus aureus carry resistance *mec A* gene in mastitic cow.

CHAPTER TWO

2.0: LITERATURE REVIEW

2.1: STAPHYLOCOCCI

2.1.1: Scientific classification

Domain: Bacteria

Kingdom: Eubacteria

Phylum: Firmicutes

Order: Bacillates

Family: Staphylococcaceae

Genus: *Staphylococcus*

Species: *aureus*

The family *Staphylococcaceae* contains four genera, the most important of which is the genus *Staphylococcus*. Member of this genus are facultatively anaerobic, non motile, Gram positive cocci that usually form irregular clusters or microscopic clusters resembling grapes.

They are catalase positive, oxidase negative, ferment glucose, and have teichoic acid in their cell walls. Bacteriological culture of the nose and skin of normal humans invariably yields staphylococci (Duguid, 1973; Todar, 2004).

2.1.2: General Characteristics of *Staphylococcus aureus*

Staphylococcus aureus is the most important human staphylococcal pathogen and cause boils, styes, pustules, impetigo, infection of wounds (cross-infections), ulcers and burns, osteomyelitis, mastitis, septicemia, meningitis, pneumonia and pleural empyema. Also, causes toxic food-poisoning (rapid onset, no fever), toxic shock syndrome and toxic skin exfoliation. *Staph. aureus*

is carried in the nose and on the skin of many healthy people. It is easily spread in hospitals, particularly on surgical wards. Strains of methicillin resistant *Staph. aureus*, (MRSA, formally methicillin) and vancomycin-resistant *Staph. aureus* are among the most threatening antibiotic-resistant pathogens known (Cheesbrough, 2012).

The organism is a typical mesophile with a growth temperature range of between 7℃ to 48℃ and an optimum at 37℃. Growth occurs optimally at pH values of 6-7. A feature of *Staph. aureus* is its tolerance of salt and reduced water activity. It grows readily in media containing 5-7% Nacl. Nearly all strains of *Staph. aureus* produce enzyme coagulase. *Staphylococcus aureus* can be isolated from faeces and from a wide range of materials such as oil, marine, fresh water, plant surfaces etc. It is normally harmless parasite of the human body surface where it plays an important function, metabolizing skin product and possibly preventing skin colonization by pathogens. *Staphylococcus aureus* is distinguished from other staphylococcal species on the basis of the gold pigmentation of colonies and positive results of coagulase, mannitol-fermentation and deoxyribonuclease tests (Duguid, 1973; Wilkinson, 1997; Lowy, 1998; Todar 2004).

2.2: EPIDEMIOLOGY OF STAPHYLOCOCCUS AUREUS

Staphylococcus aureus has its natural reservoir in humans. It colonizes the nares, axillae, virgina, and pharynx and damaged skin surfaces. Thirty (30) to fifty (50) percent of healthy adults are colonized (Noble *et al.,* 1967; Casewell and Hill, 1986). Approximately 60% of women harbour this organism intermittently at one or more body sites (Von Eiff *et al.,* 2001). Both Methicillin sensitive and Methicillin resistant isolates are persistent colonizers (Sanford *et al.,* 1994). Persons colonized with *Staph. aureus* are at risk for subsequent infections (Wenzel and Perl

1995). Rates of staphylococcal colonization are high among patients with Type-1 diabetes, intravenous drug users, and patients undergoing haemodialysis, surgical patients and patients with acquired immune-deficiency syndrome (Tuazon *et al.*, 1975; Tuazon and Sheagren, 1974; Yu *et al.*, 1986; Kluytmans *et al.*, 1995; Weinke *et al.*, 1992).

Staphylococcus aureus causes a variety of suppurative (pus forming) infections and toxinoses in humans. It causes serious superficial skin lesions such as boils, styles and furunculosis. The more serious infections are pneumonia, mastitis, phlebitis, meningitis and urinary tract infections; and deep-seated infections, such as osteomyelitis and endocarditis. *Staphylococcus aureus* is a major cause of hospital acquired infection of surgical wounds and infections associated with indwelling medical devices (Lowy, 1998; Weems, 2001; Todar, 2004).

Staphylococci produce numerous toxins that are grouped on the basis of their mechanisms of action. Cytotoxins cause pore formation and induce pro-inflammatory changes in mammalian cells. The consequent cellular damage may contribute to the manifestations of the sepsis syndrome (Bhakdi and Tranum-jensen 1991). The pyrogenic toxin-superantigens are structurally related and different domains of the exotoxin molecule are responsible for the two diseases caused food poisoning by releasing enterotoxins into food and toxic syndrome by releasing superantigens into the blood stream.

Toxic shock syndrome is an acute systematic illness associated with infection by toxic shock syndrome toxin (TSST) producing strains of *Staph. aureus*. It came into prominence in 1980/81, when numerous cases were associated with the introduction of super absorbent tampons for use during menstruation. The disease is characterized by a fulminant onset, often in previously healthy persons. The diagnosis is based on clinical findings that include high fever, vomiting,

diarrhea, hypertension, erythematous rash with subsequent desquamation and multi-organism damage. The toxic shock syndrome often develops from a site of colonization rather than infection (Chesney *et al.,* 1981; Lowy, 1998).

An alarming number of necrotizing fasciitis – a life threatening infection requiring urgent surgical and medical therapy has been recently noted to be caused by strains of Methicillin-resistant *Staph. aureus* (MRSA) that produces Panton-Valentine leukocidin. These strains are associated with soft tissue infections and necrotizing Pneumonia (Vandenesch *et al.,* 2003; Nordmann and Nass 2005; Miller *et al.,* 2005). These strains are community-acquired Methicillin-resistant *Staph. aureus* (CA-MRSA) usually affecting healthy people.

The numbers of both community-acquired and hospital-acquired staphylococcal infections have increased (Lowy, 1998). Data from the National Nosocomial infection surveillance system (NNIS) for the period from 1987 to 1997 showed that the number of MRSA infections in intensive care units has continued to increase. Methicillin-resistant strains have also become resistant to other antimicrobial agents (Speller *et al.,* 1997).

2.3: ANTIBIOTICS

2.3.1: Definition and History of Antibiotics

Antibiotics are substances produced by microorganisms or produced wholly or partly by chemical synthesis that kill or inhibit the growth of other organism at low concentration (Russell 2000; Todar 2004; Mayer 2003). Antibiotics are products of the earth, more specifically of the soil; that are byproducts of cellular metabolism (Todar, 2004).

Sir Alexander Fleming who observed inhibition of *Staphylococcus* on an agar plate contaminated by *Penicillium* mold discovered the first antibiotic, penicillin, in 1929. Penicillins became generally available for the treatment of bacterial infections especially those caused by staphylococci and streptococci, about 1946. Initially, the antibiotic was effective against all sorts of infections caused by these two Gram-positive bacteria. Penicillin had ability to kill these bacterial pathogens without harming the host that harbored them. This brings to light the fundamental principle of antimicrobial chemotherapy i.e. selective toxicity. Selective toxicity means that antibiotics used in the treatment of disease must be effective against pathogenic microorganisms not the host (Lamikanra, 1999; Todar, 2004).

Resistance to penicillins in some strains of staphylococci was recognized almost immediately after the introduction of the drug. Resistance to penicillin today occurs in as many as 90% of all strains of *Staph. aureus* (Brett, 1999; Umolu *et al.,* 2000; Ehinmidu, 2003; Olayinka *et al.*, 2004). Surprisingly, *Streptococcus pyogenes* have never fully developed resistance to penicillin; therefore, penicillin remains a reasonable choice antibiotic for many types of streptococcal infections.

However penicillin has never been effective against most Gram negative pathogens (e.g. *Salmonella, Shigella, Pseudomonas*) with the exception of *Neisseria gonorrhoeae*. Gram-negative bacteria are inherently resistant to penicillin because their vulnerable cell wall is protected by another outer membrane that prevents permeation of the penicillin molecule (Lamikanra, 1999; Hugo, 2000; Todar, 2004).

The period of the late 1940s and early 1950s saw the discovery and introduction of streptomycin, chloramphenicol and tetracycline and the age of antibiotic chemotherapy came into full being. These antibiotics were effective against the full array of bacteria pathogens including Gram-positive and Gram-negative bacteria, intracellular parasites and tuberculosis bacillus. However by 1953, a strain of the dysentery bacillus was isolated which was multi-drug resist (Todar, 2004).

Apart from the intrinsic activity of antibiotics, the activity of these drugs in combating infections depends largely on their pharmacodynamic and pharmacokinetics properties. An antibiotic which is concentrated in the urine will be more likely to be useful in the treatment of urinary tract infections than one which is concentrated in the gut because of the basic requirement that concentration of an antibiotic must be present at the locus of infection (in an unchanged form) for antimicrobial effects to occur (Lamikanra, 1999; Finch, 2000).

The past 50 years have shown how successful the pharmaceutical industry has been in developing antimicrobial agents. Until the 1980s a steady stream of new agents (or modified older agents) had became available. The 1980s saw little investment in new antibiotics, but this is changing now (Wise, 1998). There is the need for new agents to take care of the problems with the existing one. The knowledge obtained from bacterial genetics, along with improvements in biotechnology has given the impetus to the search of new compounds, as an understanding of the

genome allows new targets to be identified. Vaccine development also holds promise for the prevention of common diseases (Wise, 1998).

2.3.2 Antibiotics Commonly Used in the Treatment of *Staphylococcus Infections*

There are many antibiotics currently being used in medical practice in the treatment of staphylococcal infections and it is convenient to separate them into different classes based on their different characteristics. Some of these characteristics are: spectrum of antibacterial activity, chemical characteristic, target structure or enzyme within the microbial cells, and whether the antibiotic is bactericidal or bacteriostatic (Lamikanra, 1999).

2.3.2.1 Beta-lactam Antibiotics

These are group of antibiotics or compounds that are characterized by the possession of a thiazoline ring (penicillin) or dihydrothiazine ring (cephalosporin) fused with a beta-lactam ring.

(a). The Penicillins

(1). Benzyl penicillin (crystalline penicillin)

This is the first naturally occurring penicillin to be used effectively in the treatment of bacterial infections. It is not acid stable thus administered intravenously or intramuscularly. It is very rapidly excreted hence the need for frequent administration of injection. It has narrow spectrum of activity.

(2). Ampicillin

It is semi-synthetic penicillin with a broad-spectrum of activity. It is not only active against all the Gram-positive organisms that are sensitive to benzyl penicillin but also against some Gram-negative organisms. It is virtually sensitive to the activity of beta-lactamase enzymes and is therefore hardly effective in the presence of organisms that produce these enzymes.

(3). Methicillin

It is resistant to beta-lactamases and is used only in the treatment of infections due to beta lactamase producing *Staphylococcus.* The usefulness of this antibiotic is severely compromised by the emergence of Methicillin resistant *Staphylococcus* both in hospital and environment (Lamikanra, 1999; Hussain *et al.*, 2000; Chambers, 2001; Olayinka and Olayinka, 2003).

(b). The Cephalosporins

Cephalosporins have considerable activity against many Gram positive organisms but in addition, they are also quite active against a broad spectrum of Gram-negative organisms. As with penicillin, many cephalosporins are sensitive to beta-lactamase, although some of them are relatively resistant. The older cephalosporins like cephalothin are administered as injections and are rapidly eliminated from the body. The newer cephalosporin like cefuxime, ceftazine are longer acting and have the advantages of being beta-lactamases resistant. Cephalexine is an oral cephalosporin used in the treatment of *Staphylococcus* infections (Lamikanra, 1999; Russel, 2000)

Mode of action

Beta-lactam antibiotics are bactericidal, which are only active against growing cells. They inhibit the formation of the cross links between the polypeptide side chains in the last step in the peptidoglycan synthesis called transpeptidation. Peptidoglycan is the main structure of the cell wall that envelope the bacteria cell from being attacked by foreign bodies and high internal osmotic pressure generated by the cell.

Beta-lactam antibiotics also bind and alter the action of other cytoplasmic membrane protein that has a play in the peptidoglycan synthesis. When the peptidoglycan is destroyed, the bacteria burst and die, making beta-lactam antibiotics bactericidal (Lamikanra, 1999; Lambert, 2000).

2.3.2.2: Glycopeptides Antibiotics

Glycopeptides antibiotics are vancomycin and teicoplanin. Vancomycin is active against most Gram-positive bacteria, including methicillin-resistant strain of *Staph epidermidis*, *Enterococcus faecalis, Clostridium difficile* and Gram-negative cocci. Gram-negative bacilli, mycobacteria and fungi are not susceptible. However, vancomycin-resistant *entrococci* are now posing a clinical problem in hospital (Russel, 2000). The emergence of vancomycin resistance in *Staph. aureus* has since been widely reported (Martin and Wilcox, 1997; Smith *et al.*, 1999; Denis *et al.*, 2002; Olayinka *et al.*, 2005). This situation reinforces the recommendation of the centre for disease control and others to test all strains of *Staphylococcus* for resistance to vancomycin and to use vancomycin prudently.

Mode of action

Vancomycin is bactericidal to most susceptible bacteria at minimum inhibition concentration (MIC) and it is an inhibitor of bacterial cell wall peptidoglycan synthesis at a site different from that of beta-lactam antibiotics. It acts at the third and final stage (last two final steps) of peptidoglycan synthesis (Lambert, 2000). Vancomycin is indicated in potentially life-threatening infections that cannot be treated with other effective, less toxic antibiotics.

2.3.2.3: Lincosamides Antibiotics

Examples of Lincosamide antibiotics are lincomycin and clindamycin. They are active against Gram-positive cocci, while Gram-negative cocci tend to be less sensitive and Enterobacteria are resistant.

Mode of action

They are bacteriostatic antibiotics. They bind selectively to P and A sites in the 23s rRNA of the 50s of the bacterial ribosome. They inhibit proteins synthesis of the bacteria by inhibiting the peptide chain (Russel, 2000; Lambert, 2000).

2.3.2.4: Aminoglycosides Antibiotics

Aminoglycosides antibiotics contain amino sugars in their structure and members include neomycin, gentamycin, kanamycin and streptomycin etc. Gentamycin is active against many strains of Gram-positive and Gram-negative bacteria, including some strains of *Ps. aeruginosa*. Gentamycin is the most important amino glycoside antibiotics and is widely used for treating serious infections (Russel, 2000).

Modes of action

Aminoglycosides antibiotics target the bacterial ribosome, which is different from that of the mammals. They bind to the 30s sub unit of the bacteria and distort the shape of the A site on the ribosome thus preventing the 50s from joining the 30s to form active ribosome and hence proper proteins synthesis cannot occur. They are bactericidal because of the formation of toxic non-functional proteins through the misreading of the codons (Lambert, 2000).

2.3.2.5: Quinolone Antibiotics

They are group of synthetics antimicrobial agents and are very active against a broad spectrum of Gram-negative organisms. They are active against *Staph. aureus* especially the methicillin-resistant strains, which are resistant to many other antibacterial substances (Lamikanra, 1999; Olayinka *et al.,* 2005). Norfloxacin, ofloxacin, ciprofloxacin, pefloxacin and sparfloxacin are not stable members of these antibiotics.

Modes of action

They act by inhibiting DNA gyrase. The inhibition of these enzymes prevents super coiling and cell elongation before dividing hence inhibits DNA replication. They are bactericidal and act by binding to the beta-sub unit of the DNA gyrase. They penetrate macrophages better than other antibiotics (Lambert, 2000; Onaolapo, 2004).

2.4: ANTIBIOTIC RESISTANCE

The period before the introduction of antibiotics was marked with infectious diseases being the leading cause of death in the United States and the world (Lieberman and Wootan, 1998). The discovery of antibiotics brought many of these diseases under control in the US. They have played a major role in the decreasing rates of tuberculosis, curing children meningitis, ensuring the recovery of burn victims and reshaping the treatment of syphilis and gonorrhea (Lieberman and Wootan, 1998; Lamikanra, 1999).

The optimism generated by the dawn of antibiotics era in the mid 1940s was on quenched by the emergence of penicillin-resistance *Staph. aureus*. Resistance was usually noted first among isolates from patients in the hospital, an environment characterized by heavy antimicrobial use and proximity of patients that favours cross contamination (Gold and Moellering, 1996; Swartz 1997). Initially, there was a distinction between hospital and community acquired *Staph. aureus* strain, the former being predominantly resistant to penicillin (by virtue of B- lactamase production) and the latter being largely susceptible to penicillin. Some reports showed that penicillin resistant strains are commonly found in the community (Swartz, 1997; Brett, 1999; Umolu *et al.,* 2002; Ehimindu 2003).

However, it was soon evident that bacterial pathogens were unlikely to surrender unconditionally, because some pathogens rapidly became resistant to many of the first effective drugs (Gold and Moellering, 1996). For example the development of resistant to penicillin in *Staph. aureus* by production of β- lactamase quickly decreased the usefulness of penicillin for serious Staphylococcal infections especially among hospitalized patients, in whom resistant strains are frequently found before they spread to the community (Murray and Moellering,

1978). The introduction of numerous antimicrobial agents was followed, after varying intervals by the emergence of resistance among bacteria species.

Currently, the chief drug-resistant nosocomial pathogens include methicillin-resistant *Staph. aureus*, *Enterococcus faecalis, E. faecum* and multi drug resistant species such as *Pseudomanas aeroginosa* and Acinetobacter species. The association of intense antibiotic use with specific drug resistance in a given hospital has been demonstrated repeatedly over the past three decades (Swartz, 1997). Among the community acquired pathogens that currently create problems of antimicrobial resistance are multi drug resistant *Staph. aureus* (Brett, 1999; Ehinmidu, 2003; Olayinka *et al.*, 2004) *Mycobacterium tuberculosis, Neisseria gonorrhoeae* and *Salmonella* (Swartz, 1997).

The development of antibiotic resistance is an inevitable consequence of the clinical use of antimicrobial agents. The variety of mechanisms by which bacteria acquire resistance to antimicrobial drugs is astonishing. More research is urgently needed to define mechanisms of resistance, to look for new targets for antimicrobial drugs, to discover more effective ways of using our existing drugs, to minimize the development of resistance, to ascertain the most useful therapy for infections due to multi-drug resistant organisms and to learn how to prevent these infections (Gold and Moellering, 1996).

2.5: MECHANISM OF ANTIBIOTIC RESISTANCE

Antibiotics resistant bacteria variant are easily isolated in the laboratory and they display a variety of mechanisms underlying their resistance (File, 1999; Todar, 2004; Lowy, 1998; Olayinka and Olayinka 2003). Antibiotic resistance can be classified into two broad types: Inherent and acquired.

2.5.1: Inherent (Natural) Resistance: Bacteria may be inherently resistant to an antibiotic, for example a streptomyces has some gene that is responsible for resistance to its own antibiotic; or a Gram negative bacterium has an outer membrane that establishes a permeability barrier against the antibiotic; or an organism lacks a transport system for the antibiotic; or it lacks the target or reaction that is hit by the antibiotic (Todar, 2002).

2.5.2: Acquired Resistance: Bacteria can develop resistance to antibiotic e.g. bacterial populations previously sensitive to antibiotics become resistant. This type of resistance results from changes in the bacterial genome. Two genetic processes in bacteria lead to acquired resistance: Mutation in the chromosome (sometimes called vertical evolution) and exchange of genes coding for resistance between strains and species (sometimes called horizontal evolution) (Power, 2000; Todar, 2004).

2.5.2.1: Genetic Basis of Acquired Resistance

2.5.2.1.2: Vertical Evolution

This is strictly by principles of natural selection; a spontaneous mutation in the chromosome imparts resistance to a member of the bacterial population. In the selective environment of the antibiotic, the non- mutants are killed and the resistant is allowed to grow and flourish. Since the bacteria grow to reach the population density far in excess, such a mutant could develop from a single generation during 30 minutes to 1hr of growth (Todar, 2002).

2.5.2.1.3: Horizontal Evolution

This is the acquisition of gene for resistance from other organism. Some bacteria develop genetic resistance through the process of mutation and selection and then donate these genes to

some other bacterium through one of several processes for genetic exchange that exist in bacteria (Power, 2000; Todar, 2004). Bacteria are able to exchange genes in nature by three processes; conjugation, transduction, and transformation (Hugo, 2000).

2.5.2.1.3.1: Conjugation

Conjugation involves the active passage of genetic material from one cell to another by means of sex pili. It requires cell-to-cell contact and it involves the transfer of DNA from a donor cell to a recipient cell. Conjugation is not limited to the same or related species; both Gram- negative and Gram positive bacteria have the ability to conjugate. Bacterial conjugation is always mediated by plasmids.

2.5.2.1.3.2: Transduction

Transduction is a process whereby DNA is transferred by bacteriophages between mating bacteria. Bacteriophages or simply termed phages are virus that has bacteria as their host cells. On the basis of the response in their host cells, phages can be classified as virulent or temperate. The attack of virulent phage on a sensitive bacterium involves the injection of viral DNA into the cell which then proceeds to replicate new virus particles, lyses the cell and release new infection progeny phage particles. Temperate phages do not often cause this lytic response when they infect their host, but their nucleic acid becomes incorporated into bacteria chromosome where it is termed prophage. Temperate phages occasionally act as vectors for the transfer of bacterial genes between cells of the same or related species. Transduction plays an important role in the transfer of antibiotic resistance in Gram-positive bacteria such as *Staph. aureus, Streptococcus pyogenes* and the enterococci.

2.5.2.1.3.3: Transformation:

Transformation is the ability of certain microorganism to acquire naked DNA from the environment. This is limited to certain notably *Neisseria gonorrhoeae*, which is naturally competent to acquire DNA in this manner. *Neisseria gonorrhoeae* strains have the ability to recognize DNA from their species, and thus selective in their acquisition of naked DNA from the environments (Hugo 2000, Todar 2004). Three genetic elements are responsible for acquired resistance: Chromosomal genes, plasmids and transposons.

(i) Chromosomal Genes

Resistance to certain antibiotics can arise as consequences of mutations to chromosomal gene because of changes in DNA sequence. Mutation can occur due to single base pair changes. Transitions involve substitution of a purine (A or G) for another or single base or one pyrimidine (C or T) for another. Transversions involve a change from pyrimidine to a purine or vice versa. Frame shift mutations occur when one or two bases are inserted into the DNA sequence resulting in an altered reading frame and altered gene product. Some extensive changes in the DNA sequence such as deletions (loss of part of DNA sequence), insertions (add of extra base pairs to a gene) and duplications (when a segment of the DNA is repeated) also result in frame shifts (Power, 2000; Todar, 2004; Mayer, 2003).

(ii) Plasmids

Plasmids are extra chromosomal genetic elements, which are capable of replicating and transferring independently of the host chromosome. The bacterial chromosome contains all the genes necessary for the growth and replication of the cells but many bacteria also possess these circular elements of DNA called plasmids. Plasmids are made up of resistance transfer factors

(RTFs) and resistance factor markers (Todar, 2004; Onaolapo, 2004). Plasmids have ability to transfer within and between species and can therefore be acquired from other bacteria. The property makes the plasmids acquired resistance much more threatening in terms of spread of antibiotic resistance than resistance acquired due to chromosomal mutation. Plasmids also harbor transposons, which enhance their ability to transfer antibiotic resistance genes in vivo. Plasmids transfer normally by conjugation or transduction (Power, 2000; Todar, 2004; Lamikanra, 1999). Those that carry resistance plasmids (R-Plasmids) are able to survive environments containing the antibiotics to which the plasmids mediate resistance. It has been discovered that drug resistance could be transferred by mixed cultivation of sensitive and resistant strains (Onaolapo, 2004).

(iii) Transposons

Transposons are mobile genetic elements capable of transferring or transposing independently from one DNA molecule to another. The DNA molecules may be chromosomes or plasmids. Transposition is the ability of transposons to transfer and integrate into the recent DNA molecule. The central region of the transposons often codes for antibiotics resistance genes. Transposons do not require homologous regions of DNA in order to integrate into a DNA molecule and are therefore a major cause of the transfer and spread of antibiotic resistance genes among difference bacterial species. It is possible for bacteria to acquire a series of transposons coding for different antibiotic resistance by insertion in existing plasmids or the chromosome (Lamikanra, 1999; Power, 2000; Todar, 2004).

2.6: DRUG RESISTANCE IN *STAPHYLOCOCCUS AUREUS*

The prevalence of penicillinase producing strains of *Staph. aureus* within hospitals soon began to rise shortly after penicillin became rapidly available for treatment of serious staphylococcal infections in the late 1940s after the World War II. Within a few years, resistance to this antibiotic emerged and rapidly spread among strains of *Staph. aureus* and CoNS mostly in the hospitals (Barber *et al.,* 1948). It was observed later that previous treatment with penicillin increased the chances of isolating penicillin-resistant strain. Colonization of hospital staff by penicillin-resistant strains and their role in transmission also were noTable features of these early reports (Chamber, 2001).

By 1970s, it was apparent that high prevalence of penicillin resistance among community isolates was not limited to Denmark. A remarkable constant 70% to 85% prevalence of penicillinase-producing strains was found regardless of location in inner cities, suburbs and rural areas within and outside the United States (Ross *et al.,* 1974; Hughes *et al.,* 1976; Hahn and Baker, 1980). In New Zealand by 1999, the prevalence of penicillin resistance among community isolates was 91% (Brett, 1999) and in Nigeria (Zaria) the prevalence of resistance among community isolates to penicillin (100%) and ampicillin (70% to 85%) was widely reported (Ehinmidu, 2003; Olayinka *et al.,* 2004). Moreso, it is known that epidemic strains of *Staph. aureus* are commonly resistant to many antibiotics (Chamber, 2001; Grisold *et al.,* 2002).

2.6.1 Methicillin-resistant *Staph. aureus* (MRSA)

Methicillin-resistant *Staph. aureus* (MRSA), an organism resistant to many drugs, is seen with increasing frequency in hospitals and long term care facilities. It can cause life-threatening disease, and treatment options are limited (Simor, 2001).

Currently, about 90% of *Staph. aureus* isolates are resistant to penicillins. Penicillinase stable semi-synthetic penicillins (e.g methicillin, cloxacillin sodium) and cephalosporins (e.g cephalothin sodium) were developed in the late 1950s and early 1960s to create β-lactam antibiotics that are not inactivated by beta-lactamase. Methicillin was among the first of these agents to be introduced in clinical practice, but strains of MRSA were identified as early as 1961. Since then, MRSA has become increasingly prevalent in many countries around the world (Simor, 2001; Chambers, 2001).

MRSA can cause life-threatening infections, however this organism is of particular clinical significance because it is also predictably cross-resistant to all penicillins and cephalosporins, and typically resistant to multiple other antibiotics. In addition, it easily spreads from patient to patient, causing outbreaks of infection with important implications for healthcare facilities (Simor, 2001; Chambers, 2001). The common sites of infection caused by MRSA are identical to those of susceptible strains of *Staph. aureus* namely skin, soft tissues and surgical sites. More invasive infection including pneumonia, osteomylitis and endocarditis may also occur. However, most persons with MRSA are not infected. MRSA may colonize mucosal and epithelial surfaces without causing any signs of inflammation or infection (Simor, 2001; Weems, 2001). MRSA is transmitted most often from patient to patient by direct contact with asymptomatic healthcare providers or others who carry the organism. These carriers have been implicated in nosocomial transmission of MRSA (Simor, 2001).

Majority of MRSA strains are not susceptible to macrolides and aminoglycocides, because the genes *ermA* and *aadD* encoding resistance to these drugs are usually conserved within *mec* DNA, and located upstream and downstream respectively of the *mecA* gene (Chambers, 1997).

Resistance in MRSA is related to a chromosomal *mecA* gene that specifies the production of an abnormal penicillin binding protein called PBP2a (Hackbarth and Chambers, 1989). Penicillin-binding proteins are membrane-bound enzymes that have an important role in bacterial cell wall synthesis; they are also the targets for all beta-lactam antibiotics. PBP2a has a decreased affinity to binding beta-lactam antibiotics resulting in resistance not only to methicillin but also to all beta-lactam antibiotics including penicillins, cephalosporins and carbapenems. The *mec* gene complex also contains insertion sites for plasmids and transposons that facilitate acquisition of resistance to other antibiotics. As a result, cross-resistance to non-beta lactam antibiotics, such as erythromycin, clindamycin, gentamicin, cotrimoxazole and ciprofloxacin is common (Simor 2001; Chambers, 2001).

2.6.2: Transmission of *Staphylococcus aureus* mastitis Infections

The major reservoirs of *Staphylocpoccus aureus* are infected udders, teat canals, and teat lesions, but these bacteria also have been found on teat skin, muzzles, and nostrils. The bacteria are spread to uninfected quarters by teat cup liners, milkers' hands, washcloths, and flies. Staphylococci do not persist on healthy teat skin but readily colonize damaged skin and teat lesions. The organisms multiply in infected lesions and result in increased chance of teat canal colonization and subsequent udder infection.

Heifers infected during gestation that carry infections through calving represent an important reservoir from which *Staph. aureus* can spread to uninfected herd mates. There is considerable debate surrounding the route of *Staph. aureus* infection in heifers prior to first calving, but calves fed colostrum from *Staph. aureus* infected dam is a likely source. Early work suggested *Staph. aureus* infected colostrum was not a culprit for first-calf heifers calving with the infection (Barto *et al.*,1982). Though the data is limited, if *Staph. aureus* problem exists on a farm, careful

colostrum selection, e.g., pasteurization, is certainly one area to consider. Clearly, good mastitis control programs will address the presence of this disease in heifers.

2.6.3: Damage Caused by *Staphylococcus aureus* in Mastitis

Staph. aureus produce toxins that destroy cell membranes and can directly damage milk-producing tissue. White blood cells are attracted to the area of inflammation, where they attempt to fight the infection. Initially, the bacteria damage the tissues lining the teats and gland cisterns within the quarter, which eventually leads to formation of scar tissue. The bacteria then move up into the duct system and establish deep-seated pockets of infection in the milk secreting cells (alveoli). This is followed by the formation of abscesses that wall-off the bacteria to prevent spread but allow the bacteria to avoid detection by the immune system. The abscesses prevent antibiotics from reaching the bacteria and are the primary reason why the response to treatment is poor (Jones *et al.*,1984).

However, bacteria can also escape the killing effects of some antibiotics by hiding within neutrophils and other host cells. As the neutrophils attempt to remove bacteria, many organisms survive and become dormant within them, preventing contact with antibiotics. When the white blood cells die (usually in one to two days) the bacteria are released to resume the infection process (Jones *et al.*,1984).

During *Staph. aureus* infection, destruction of alveolar and ductal cells reduces milk yield. These damaged cells may combine with leukocytes and clog the milk ducts that drain the alveolar areas, contributing to further scar tissue formation, occlusion of ducts, and decreased milk production (Kirk *et al.*,1997). The ducts may reopen at a later time, but this usually results in the release of the organisms to other areas of the mammary gland. The spread of *Staph. aureus*

within the gland results in the formation of additional abscesses that can become quite large and detecTable as lumps within the udder.

Though most cases of mastitis caused by *Staph. aureus* are subclinical, chronic cows usually have high Somatic Cell Count, abnormal mammary tissue, and recurrent cases of clinical mastitis. Clinically infected quarters often show moderate swelling and visible clots (chunks) in the milk, especially in forestrippings. Acute *Staph. aureus* infections generally develop late in the lactation. However, the clinical symptoms (udder swelling or hardness, changes in appearance of milk) do not show up until calving or early in the next lactation. It becomes difficult to successfully treat the infection because drugs are not able to penetrate to all infection sites and because the bacteria can avoid contact with antibiotics while residing inside leukocytes (Jones *et al.*,1984). Many strains of *Staph. aureus* have acquired antibiotic resistance for example – the ability to produce an enzyme that inactivates penicillin-based and other antibiotics therefore rendering the treatment ineffective (Wilson *et al.,* 1995).

2.6.4: Antibiotic Treatment of Cow Mastitis Caused by *Staph. aureus*

Antibiotic treatment will not control this disease but it may, in certain cases, shorten the duration of the infection. Treatment effectiveness decreases as the cow becomes older and even as the first lactation progresses. Cure rates were 34% when 89 cows in 10 Dutch herds were treated for subclinical *Staph. aureus* mastitis (Sol *et al.*,1997). The results showed that the probability of cure was lower in older cows with high SCC and in cows infected in hindquarters during early and midlactation. *Staph. aureus* infections were found in 36% of clinical mastitis cases in Finnish herds (Pyorala and Pyorala, 1997). Of these, 39% responded to treatment. Cows with an SCC of less than 1 million were more likely to cure an infection compared with those over the

cut-off point. Successful treatment during lactation is greater if detected and treated early, whereas the response is lower when treating chronic infections. Use of a strip cup or similar device is strongly recommended for detecting abnormal milk. New clinical infections should be treated promptly and appropriately, especially in first-lactation cows. Tissue damage can be minimized if animals are treated during the early stages of infection. The use of DHI program SCC records in addition to visual observation of forestripped milk and milk culture results will indicate effectiveness of treatment.

Many researchers have looked at the efficacy of pirlimycin treatment both in heifers prior to calving and in all animals as an extended therapy treatment during lactation. According to the manufacturer, pirlimycin is one of the most effective compounds against *Staph. aureus* because its chemical nature allows it to penetrate mammary tissues. In heifers, a single tube of pirlimycin treatment in each quarter six to 12 days prior to calving significantly reduced *Staph. aureus* infections at calving (Roy *et al.*,2007). Furthermore, mastitis data presented to the FDA suggest that two tubes, administered 24 hours apart to infected quarters of cows during lactation, resulted in a cure rate of 36.6%, whereas only 1.1% of nontreated controls recovered spontaneously. In field cases, the rate of cows cured during lactation increased to 49.4%. However, trials using the same treatment scheme at Louisiana State University and Iowa State University found cure rates of only 12% or less for chronically infected *Staph. aureus* cows during lactation.

Single-quarter, extended therapy with repeated label doses of pirlimycin has been examined as a means of providing drug levels beyond the expected life of the leukocytes that naturally fight off this infection. This protocol has been widely adopted for new intramammary infections with *Staph. aureus*, as it increases cure rates. Four-quarter extended treatment with repeated label doses will provide adequate therapeutic concentrations for many *Staph. aureus* bacteria. A cure

rate of 50% at four weeks after treatment was found in more than 100 treated cows (Belschner *et al.*,1996). Whether these cure rates justify the additional expenses and effort, not to mention the potential risk of extra-label use and antibiotic residue, is unknown

CHAPTER THREE

3.0: MATERIALS AND METHODS

3.1: MATERIALS

3.1.1: Specimens

Milk or swab from lesion of Cows with clear symptoms of mastitis.

3.1.2: Growth Media

Nutrient broth (NB) (Fluka Chemie GmbH CH-9471 Buchs), Nutrient agar (NA) (Fluka Chemie GmbH CH-9471 Buchs), Mannitol salt agar (MSA) (Merik KGA 64271 Darmstalt, Germany), Luria Bertani (LB) (Oxoid, UK), Mueller Hinton agar (MHA) (Oxoid, UK)

3.1.3: Antibiotics Discs

Ofloxacin (5µg), Sulphamethoxazole/Trimethoprim (25µg), Cefuroxime (30µg), Gentamicin (10µg), Amoxicillin (10µg), Cephalexine (30µg), Ciprofloxacin (5µg), Tetracycline (30µg), Ampicillin (10µg), Cefoxitin (30µg). All from Oxoid Ltd., Basingstone, London, UK.

3.1.4 Chemicals

Hydrogen peroxide (SKG, Nig. Plc), EDTA (TAE) buffer (May & Baker Nig. Ltd), Gram's staining reagent (Laboratory Stocks), Ethidium bromide (Laboratory Stocks),Peptone water (Oxoid L.t.d, Basingstone, London), DNA extraction kits (Norgen Biotek Corp.), Dilute NAOH (BDH Chemicals l.t.d. poole, England.), 0.5% Phenol Red Indicator (BDH Chemicals l.t.d. poole, England.),Dilute H_2SO_4 (BDH Chemicals l.t.d. poole, England.), Barium Chloride Solution (BDH Chemicals Ltd. Poole, England.), Latex Agglutination test Kit (Oxoid).

3.1.5 Equipment

Autoclave (Aldelphi mfg. Co. Ltd, UK), Balance (Top loading) (W&T Avery Ltd, Birmingham), Water bath (Gallenkamp Co. England), Incubators (Baird and Tatlock Ltd. Essex), Light Microscope (Wild Mill, Switzerland), Oven (Hot air) (Baird and Tatlock Ltd. Essex), Micropipette (Huawei Chemical, Zhejiang Chin), Microcentrifuge (Ependorff Ltd.), Syringes and Needles (1ml, 2ml, 5ml, 10ml,20ml) (BD Fraga, Spain), Swab stick (BD Fraga, Spain), Pasteur Pipette or Dropper (Vola, U.K)

3.1.6: Glassware

Sample collection bottles, Glass slides, Petri dishes, Stirring rods, Measuring cylinder, Test tubes, Universal bottles, Volumetric Standard flasks (100ml), Wide-necked bottles (100-300ml). All from pyrex Ltd.

3.1.7: Plasma

Undiluted pooled human plasma was collected from the Blood Bank of Murtala Muhammad Specialised Hospital, Kano and stored at $4^{O}C$ until required.

3.2: METHODS

3.2.1: Study Area

The study was carried out in Rano, the zonal veterinary area of southern part of Kano State. The study involved cows from six (6) local governments. Samples were collected within three (3) days when the animals were brought for immunization program organized by Kano State Ministry for Agriculture and Water resources in May 2012.The sample collection was achieved with the help of the Zonal Veterinary Officer. The animals (cows) with clear symptoms of

mastitis such as discoloration of milk and swelling/discharge/wound on the udder or teat were used for samples.

The samples were collected based on the type of symptoms that appeared and including lactating and non lactating ones. Milks sample were collected from lactating cows with clear symptoms, while swabs were collected from wound and secretion of the udder from lactating or non lactating cows, depending on the sign shown.

3.2.2: Sample Collection and Transportation

The samples were collected using sterile swab sticks or using 20 ml sterile, dry, wide-necked, leak-proof sample bottles for milk from the cows with visible symptoms of mastitis. The area around the wound or the teat of the milk was cleaned, dried and disinfected. The bottles containing the milk samples were then tightly closed, labeled, kept in an ice-park (to keep the organisms fresh and alive) and transported from their respective collection centers to the microbiology laboratory within 4-6 hrs of collections.

3.2.3: Preparation of Growth Media

The growth media were aseptically prepared into various (5 ml-20 ml) portions according to the manufacturers' descriptions and stored at refrigerating temperature.

3.2.4: Treatment of Samples

The laboratory-working bench was aseptically treated and labeled milk and swab samples were brought out from the ice-bag for some minutes to attain room temperature.

3.2.5: Isolation and Characterization of the Isolates

One mililiter of the milk sample was aseptically transferred into a sterile 9 ml nutrient broth using automatic micropipette and the wound swab was placed in 5 ml sterile nutrient broth and vortexes to dislodge the isolates into the broth. The nutrient broth mixture was capped aseptically and incubated at 37℃ of the resulting overnight broth culture was carefully streaked aseptically on dried surface of sterile Mannitol salt agar (MSA), MacConkey agar and blood agar plates to give well district colonies after incubation at 37℃ for 48h. Growth from 48h incubation was observed and characteristic colour of the resulting colonies was noted. The isolates (discrete colonies with distinct colors) on MSA with characteristic deep golden yellow coloration were selected and sub cultured into nutrient broth incubated at 37˚C for 18h. This was then inoculated onto sterile nutrient agar slants aseptically and incubated at 37℃ for 24h and stored at refrigerating temperature (4 ℃) for further investigations.

3.2.6: Preliminary identification Test for *Staphylococcus* species

3.2.6.1: Gram Staining

Gram's stain technique described by Cheesbrough (2006) was used to classify the isolates into Gram positive or negative. A smear of the isolate was made on a clean glass slide and heate-fixed. The smear was stained with crystal violet for 30 seconds, fixed with lugol's iodine for 30 seconds and decolorized with 95% ethanol for 30 seconds after which it was counterstained with dilute carbol fuchsin solution 1 minute. On examination microscopically, the isolates that produced violet cocci (Gram positive) predominantly in clusters were selected for further identification procedures.

3.2.6.2: Biochemical Test

The following biochemical tests were carried out on all the isolates that were Gram positive cocci as described by chessbrough (2006).

a. Catalase

This was used to differentiate those bacteria that produce the enzyme catalase such as *Staphylococci* from non-catalase producing bacteria such as streptococci. Two milliliters (2 mls) of a 3% hydrogen peroxide solution were added on a 24 h culture of the isolates on nutrient agar slant. Rapid effervescent of gas bubbles indicating the breaking of hydrogen peroxide into oxygen and water in the presence of enzyme catalase represents positive result.

b. Coagulase Test

This was used to differentiate *Staph. aureus* from *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* (which do not produce coagulase) using the method described by Cheesbrough (2006). The pooled human plasma was brought out of refrigerator to attain room temperature. Two hundred microlitres (200 µl) of the plasma was added to 0.8 ml of 24 h nutrient broth culture of the isolate and mixed thoroughly. The mixture was incubated at 37 ℃ for 3 h. At interval of 1 hr, the test tubes were observed for clotting. Both positive control (test tube containing 24hrs of *Staph. aureus* ATCC 13709 with the plasma) and negative control (test tube containing only sterile nutrient broth) were set up alongside with the test isolates. Test isolates that were positive to the coagulase test were considered as *Staph. aureus* and selected for further investigation.

3.3.0: ANTIBIOTICS SUSCEPTIBILITY TESTING OF THE ISOLATES

3.3.1: Preparation of Inoculums

A single isolated colony was picked using sterile wire loop and carefully streaked on the surface of sterile nutrient agar plate to give well distinct isolated colonies after incubation at 37 ℃ for 18hr.

3.3.2: Standardization of the inoculums

Overnight culture (24 h) of the isolates on nutrient agar was aseptically transferred into a 5 ml sterile physiological saline, shake vigorously and its turbidity compared to 0.5 McFarland standards (approximately 1.0×10^8 cfu/ml). This is done for each of the test bacterial isolate. The mixture is used for the susceptibility testing (Cheesbrough, 2006).

3.3.3: Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was carried out on each purified *Staph. aureus* isolates using CLSI modified disc diffusion method as described by Cheesbrough (2006). Two milliliters of the standardized inoculums of each isolate were aseptically poured on a fairly dried surface of sterile Mueller Hinton agar plate to evenly cover the surface of the agar, excess was drained off and the surface of the agar was allowed to be absorbed within the agar with the petri dish lid in place for 10 minutes.

Six single antibiotics disc were aseptically distributed evenly on the inoculated plate with each disc lightly pressed down to ensure its contact with the Mueller Hinton agar. Each plate contains maximum of six different antibiotics. Within 30 minutes of applying the disc, the plate was inverted and incubated aerobically at 30 degrees Celsius for 18 h. After the 18 h incubation, the diameters of the zone of inhibition for each of the isolates were measured underside of the plate to the nearest

millimeter (mm) (BSOP 45, 2003). The same procedures were carried out for the other isolates. The antibiotics discs used were as earlier mentioned. (materials, 3.13)

3.3.4: Antimicrobial Susceptibility Test Interpretation Chart

The Table below shows the recommended CLSI zone of inhibitions interpretation chart for antimicrobial susceptibility testing results in mm.

Table 3.1: CLSI Inhibition Zone Interpretation Chart for Selected Antibiotics

Antibiotics	Disc content	Susceptible	Intermediate	Resistance
Ampicillin	10µg	≥29	-	≤28
Ciprofloxacin	5µg	≥21	16-17	≤14
Gentamicin	10µg	≥15	13-14	≤12
Ofloxacin	5µg	≥16	13-15	≤12
Cephalexine	30µg	≥18	15-17	≤14
Tetracycline	30µg	≥19	15-18	≤14
Amoxicillin	10µg	≥28	-	≤28
Cefuroxime	30µg	≥18	15-17	≤14
Sulphamethoxazole/Trimethoprim	25µg	≥16	11-15	≤10
Cefoxitin	30µg	≥22	-	≤21

Note; All values are in millimeters (mm) unit

3.3.5: Determination of Multiple Antibiotics Resistance (MAR) Index

The multiple Antibiotic Resistance (MAR) Index was determined for each isolate by dividing the number of antibiotics to which the organisms is resistant to by the total number of antibiotics tested (Krumperman, 1983; Paul *et al.*, 1997).

$$\text{MAR Index} = \frac{\text{Number of antibiotics to which resistant}}{\text{Total Number of antibiotics tested.}}$$

3.4.0: TEST FOR β- LACTAMASE PRODUCTION

From the antibiogram result, the isolates that showed resistance to three or more antibiotics (multidrug resistant) especially penicillins and cephalosporins were selected for β-lactamase production test using the acidimetric (David and Derek, 2005).

Eighteen point six mililitres (18.6 ml) of phenol-red water solution was added to the vial of 20 million units of crystalline Benzyl penicillin G using sterile syringe to which 1N NaOH was added drop wise to the acidic solution until it developed a violet colour (pH 8.5).

The reagent was then dispensed in aliquots 0.1 ml into sterile tubes and frozen at -20˚C. The desired number of tubes were removed from the freezer and thawed at room temperature and with a sterile loop; colonies of the isolates were added to the test solutions to make an opaque, milky suspension.

A colour change form milky to yellow indicated positive reaction while from milky to violet indicated negative reaction. A positive reaction was observed within 15 minutes.

3.5.0: MOLECULAR CHARACTERISATION OF THE ISOLATES

Isolates that were multidrug resistant to four antibiotics; cefoxitin, ampicillin, amoxicillin and cephalexine and at the same time showed positive reaction to β-lactamase production test were selected for molecular detection of *mec A* and *blaZ* genes.

3.5.1: Gram- Positive Bacteria Genomic DNA extraction

Procedure

About 2×10^9 bacterial cells were harvested in a 2 ml micro centrifuge tube by centrifugation for 10 minutes at 5000×g. The supernatant was discarded. The pellet was resuspended in 180 µl of Gram- positive bacteria lysis buffer. A 200 µl of lysis buffer solution and 20 µl of Proteinase K were added and mixed thoroughly by vortexing to obtain a uniform suspension. The samples were incubated at 56℃ while vortexing occasionally and rocked platform until the cells were completely lysed. Twenty microlitre (20 µl) of RNase A solution was added and vortexed. The mixture was incubated for 10 minutes at room temperature. Four hundred microlitres (400 µl) of 50% ethanol was added and mixed by vortexing. The prepared lysate was transferred to a GeneJET (Genetic DNA Purification Column). The column was centrifuged for 1 min at 6000 × g. The collection tubes containing the flow-through solution were discarded. The GeneJET was placed into a new 2 ml collection tube (included). Five hundred microliter (500 µl) of washing buffer 1 was added, centrifuged for 1 min at 8000×g.The flow-through was discarded and the purification column was placed back into the collection tube. Five hundred microliter (500 µl) of washing buffer 1 was added to the GeneJET, centrifuge for 3 minutes at 12000×g. The collection tubes containing the flow-through solution were discarded. The GeneJET Genotic DNA Purification Column was transferred to a sterile 1.5 ml microcentrifuge tube. Two hundred (200 µl) of elution Buffer was added to the center of the GeneJET Genomic DNA Purification Column membrane to elude the genomic DNA. It was incubated for 2 minutes at room temperature and centrifuged for 1 minute at 8000×g. The purification column was then discarded. The purified DNA was stored at -20℃.

3.5.2: PCR Analysis of *mecA* and *blaZ* genes.

The DNA templates obtained were subjected to multiplex PCR using the following sets of primers.

***mecA* 1**: **Forward primer**, 5' AAA ATC GAT GGT AAA GGT AAA GGT TGG C 3';
Reverse primer, 5'AGT TCT GCA GTA CCG GAT TTG C 3'; (533bp) (Zhang *et al.,* 2005)

***mecA* 2:** **Forward primer:** 5' GTG AAG ATA TAC CAA GTG ATT 3'
Reverse primer: 5' ATG CGC TAT AGA TTG AAA GGA T 3'(143bp) (Zhang *et al.,* 2005)

***mecA* 3:** **Forward primer:** 5' GTG GAA TTG GCC AAT ACA GG 3'
Reverse primer: 5' TGA GTT CTG CAG TAC CGG AT 3'(1319bp) (Zhang *et al.,* 2005)

***blaZ*:** **Forward primer**: CCT AGT AAA GCT CCG GAA 3'
Reverse primer CTA GTC CAT TCG GTC CA 3'(414bp) (Vesterholm-Nielsen *et al.,* 1999)

3.5.3: Multiplex Polymerase Chain Reaction (PCR)

The PCR mixture was prepared using Universal PCR Master Mix (2X) with final PCR mixture volume of 25 µl. Five microlitres (5 µl) of template DNA and 30 p/mole of each primer and 7.5 p/mol of probe was added to each test. A negative control was prepared by the addition of the same contents to the tube with water instead of the isolates. Amplification was performed using MX3000P TM (Stratagene) PCR System programmed to hold at 95 for 10 min for Ampli Taq gold activation and 30 cycles of denaturation at 95 for 15 sec and annealing and extension at 60℃ for 1 minute with end point fluorescence detection. DNA isolated from *Staph. aureus*

ATCC 25922 was used as positive control while water was used as negative control. Both positive and negative controls were included in each PCR run to exclude both amplification failures due to presence of inhibitors and cross contamination. Amplification products were electrophoresed in 1.5% agarose gel at 70 volts for 60 minutes and visualized by ultraviolet light. To assure that the amplification products were of the expected size a 1000 bp DNA marker was run simultaneously as a DNA marker.

3.6.0: DETECTION OF PENICILLIN-BINDING PROTIEN (PBP2a) BY LATEX AGGLUTINATION TEST

This test is a rapid latex agglutination assay, detecting PBP2a in isolates of *Staphylococcus*, as an aid of identifying methicillin-resistant *Staph. aureus* (MRSA) and methicillin- resistant Coagulase- negative staphylococci.

Isolates that were multidrug resistant specifically to four antibiotics namely cefoxitin (MRSA), ampicillin, amoxicillin (β-lactamase producers) and cephalexine and at the same time showed positive reaction to β-lactamase production test were selected for penicillin-binding protein (PBP2a) test.

3.6.1: PBP2 Extraction Procedure

Four drops of extraction Reagent 1 were added into a micro centrifuge tube. Approximately 1.5 × 10^9 cells were tested. This was achieved by using a sterile wire loop to remove sufficient growth to fill the internal diameter of the loop. The tubes were placed on heating block (over 90℃) and heated for three minutes. The tubes were removed and allowed to cool to room temperature. One drop of extraction reagent 2 was added into the tubes and mixed well. The tube was centrifuged at 1500× g for five minutes. The supernatant was used for the test.

For each of the supernatant to be tested, one cycle of the test card was labeled with test latex and another for testing control latex. The latex reagent was mixed well by inversion several times and one drop of the test latex or control latex was added to each labeled circle. 50 µl of supernatant was placed on the test circle and the control circle. The latex and the supernatant were mixed thoroughly in each circle with a mixing stick. The cards were picked up and rocked for up to

three minutes and checked for agglutination under normal lighting conditions. The results of the test and control reactions were recorded.

3.6.2: Reading and Interpretation of results

Agglutination with rapid clotting within three (3) minutes was taken as a positive result indicating the presence of PBP2a.

CHAPTER FOUR

4.0: RESULTS

4.1: Collection and Analysis of Samples

A total of 234 samples were collected from six different local governments of Rano, Bunkure, Dawakin Kudu, Kumbotso, Tudun wada and Kibiya of Kano state in Northern part of Nigeria. Out of the 234 samples collected, 210 were milk samples and 24 were wound swabs. Out of the 210 milk samples collected from the six local governments, 62, 34, 24, 30, 35 and 25 were from Rano, Bunkure, Dawakin kudu, Kumbotso, Tudun wada and Kibiya local governments respectively. For the 24 swab samples, 7, 4, 2, 5, 4 and 2 were collected from Rano, Bunkure, D/kudu, Kumbotso, T/wada and Kibiya respectively (Table 2).

Table 2: Distribution of the Milk and Swab Samples Collected from Different Centers.

Sources	Type of Sample	Number	Percentage (%)
Rano	**Milk**	**62**	**29.5**
	Swab	7	29.2
Bunkure	**Milk**	**34**	**16.2**
	Swab	4	16.7
D/kudu	**Milk**	**24**	**11.4**
	Swab	2	8.3
Kumbotso	**Milk**	**30**	**14.3**
	Swab	5	20.8
T/wada	**Milk**	**35**	**16.7**
	Swab	4	16.7
Kibiya	**Milk**	**25**	**11.9**
	Swab	2	8.3
		ϰ = 210, ψ = 24	

Key:

ϰ = Total Number of Milk is 210

ψ = Total Number of Swab is 24

Grand Total Number of samples is 234

4.2: Isolation and Identification *Staph. aureus* Isolates

4.2.1: Colony Morphology

The colonial morphology [shape, colour (pigment production), elevation, transparency, edge and surface texture] of the isolates were observed. Isolates that produced colonies exhibiting characteristic deep golden colouration on mannitol salt agar were selected for Gram staining as shown in Appendix 4.

4.2.2: Isolation, Identification and Characterization of *Staph. aureus* Isolates

The results of physical characteristics of the milk and swab samples, the characteristics growth on the blood agar, mannitol salt agar, Gram stain and biochemical tests are summarized in appendix 4. The isolates that were Gram-positive, cocci, catalase and coagulase positive were considered as *Staph. aureus* while those that were Gram positive cocci and catalase positive but negative to coagulase test were considered as coagulase negative staphylococci (CoNS).

Among the 234 samples of milk and wound swab collected, 228 (97.4%) isolates grew on blood agar, 206 (88.0%) of the total isolates were cocci and 22 (9.4%) were rods. Out of the 206 cocci, 200 (97.1%) were staphylococcal strains and 6 (2.9%) non-staphylococcal strains. Among the 200 Staphylococcal strains, 141 (70.5%) were coagulase positive staphylococci and these were taken as *Staph. aureus* while 59 (29.5%) were coagulase negative staphylococci (Table 3).

For the 141 (70.5%) *Staph. aureus* isolates recorded from different centres, 27 (19.2%), 31 (22%), 25 (17.7), 17 (12.1%), 21 (14.9%), and 20 (14.2%) were from Rano, Bunkure, D/kudu, Kumbotso, T/wada and Kibiya respectively (Table 3).

Out of the 141 *Staph. aureus* isolates, 120 (85.1%) were from milk samples while 21 (14.9%) were from swab samples as shown in Table 4. Bunkure recorded the highest number of *Staph. aureus* isolates with 31 followed by Rano with 27, Dawakin kudu with 25, Tudun wada with 21, Kibiya 20 and Kumbotso with the lowest 17 (Table 4).

Table 3: Distribution of *Staphylococcus aureus* Isolates According to Different Local Governments

Sources	No of Staphylococci	No of CoNS	No of *Staph. aureus*
Rano	**39**	**12**	**27**
Bunkure	**38**	**7**	**31**
D/kudu	**28**	**3**	**25**
Kumbotso	**23**	**6**	**17**
T/wada	**31**	**10**	**21**
Kibiya	**41**	**21**	**20**
Total	**200(97.1%)**	**59(29.5%)**	**141(70.5%)**

Key:

No of staphylococci = Number of staphylococci

No. of CoNS = Number of Coagulase Negative staphylococci

No. of *Staph. aureus* = Number of *Staph. aureus*

Table 4: Distribution of *Staphylococcus aureus* Isolates at different local Governments in relation to specimen type.

This Table shows the distribution of *Staph. aureus* isolates in the sampled specimens at different local government areas in Kano State.

	Number of *Staph. aureus*						
Sample	**Rano**	**Bunkure**	**D/kudu**	**Kumbotso**	**T/wada**	**Kibiya**	**Total**
Milk	**22**	**25**	**22**	**12**	**21**	**18**	**120**
Swab	**5**	**6**	**3**	**5**	**0**	**2**	**21**
Total	**27**	**31**	**25**	**17**	**21**	**20**	**141**

4.3: Determination of Antimicrobial Susceptibility Test

Ten antimicrobial agents were used to classify the 141 *Staph. aureus* into either susceptible or resistant strain as summarized in Table 5. The susceptibility results were compared with zone size interpretative chart for *Staph aureus* using Mueller Hinton Agar (Table 3.1). The susceptibility of the isolates are as follows: 98.6%, 97.9%, 91.5%, 56% and 46.1% were susceptible to ofloxacin (OFL), ciprofloxacin (CIP), gentamicin (GEN), cephalexine (CL) and sulphamethoxazole/trimethaprim respectively. The other test antibiotics were not as effective with susceptibility of 42.6%, 33.3%, 2.1% 1.4 %, and 1.4% for tetracycline (TET), cefuroxime (CXM), cefoxitin (FOX), amoxicillin (AML) and ampicillin (AMP) respectively (Table 5).

Table 5: Summary of the Percentage Susceptibility of *Staph. aureus* Isolates from Cow Mastitis to Different Antibiotics.

		Number (%) of isolates susceptible
Antibiotics	**Disc strength (µg)**	**Sensitive**
AMP	10	2(1.4)
CL	30	79(56.0)
CIP	5	139(98.6)
GEN	10	129(91.5)
OFL	5	140(99.3)
TET	30	60(42.6)
AML	10	2(1.4)
SXT	25	65(46.1)
FOX	30	3(2.1)
CXM	30	47(33.3)

Key: AMP = Ampicillin, CL= Cephalexine, CIP = Ciprofloxacin, GEN = Gentamicin, OFL = Ofloxacin, TET = Tetracycline, AML = Amoxicillin, SXT = Sulphamethoxazole/trimethaprim, FOX = Cefoxitin, CXM = Cefuroxime

4.4: Antibiotic Resistance Pattern

The multiple drug resistance (MDR) is defined as non-susceptible to at least one agent in the three or more antimicrobial categories (Magiorakos *et al.,* 2012). For the purpose of this work, MDR referred to resistance to at least 3 classes of antibiotics. It was found that 3.6% of the isolates were resistant to three (3) agents, 29.1% were resistant to 4 agents, 26.2% were resistant to 5 agents, 16.3% were resistant to 6 agents, 17.7% were resistant to 7 agents, 4.3% were resistant to 8 agents, 0.7% were resistant to 9 agents and none of the isolates were found to be resistant to all (10) agents as shown in Table 6.

Table 6: Multiple Antibiotic Resistance of the *Staph. aureus* Isolates

Number of antibiotics resistant to	Number (%)	Antibiogram Pattern	Number with each pattern
3	5 (3.6)	Aml, Fox, Cxm	1
		Aml, Sulf, Fox	1
		Amp, Aml, Fox	3
4	41 (29.1)	Amp, Aml, Fox, Cxm,	17
		Amp,Aml,Fox, Sulf	3
		Amp, Aml, Tet, Fox,	18
		Amp, Aml, Cl, Fox	3
5	37 (26.2)	Amp, Aml, Cl, Sulf, Fox,	1
		Amp, Aml, Sulf, Fox, Cxm	14
		Amp, Gen, Cl,Tet, Fox	1
		Amp, Aml, Fox, Cxm	5
		Amp, Aml, Tet, Fox, Cxm	9
		Amp, Aml, Tet, Sulf, Fox	4
6	23 (16.3)	Amp, Aml, Gen, Tet, Fox, Cxm	1
		Amp, Aml, Cl, Sulf, Fox, Cxm	9
		Amp. Aml, Sulf, Fox, Cxm	7
		Amp, Aml, Ofl, Cl, Sulf, Fox	1
		Amp, Aml, Tet, Sulf, Fox, Cl	4
		Amp, Aml, Cl, Tet, Fox, Cxm	1
7	25 (17.7)	Amp, Aml, Gen, Cl, Sulf, Fox, Cxm	2
		Amp, Aml, Cl, Tet, Sulf, Fox, Cxm	10
		Amp,Aml, Gen, Cl, Sulf, Fox, Cxm	8
		Amp, Aml, Gen, Cl, Tet, Sulf, Fox	5
8	6 (4.3)	Amp, Aml, Gen, Cl, Tet, Sulf, Fox, Cxm	6
9	1 (0.7)	Amp, Aml, Cip, Gen, Cl, Tet, Sulf, Fox, Cxm	1

Key: AMP = Ampicillin, CL= Cephalexine, CIP = Ciprofloxacin, GEN = Gentamicin, OFL = Ofloxacin, TET = Tetracycline, AML = Amoxicillin, SXT = Sulphamethoxazole/trimethaprim, FOX = Cefoxitin, CXM = Cefuroxime

4.5: Determination of Multiple Antibiotic Resistance (MAR) Index

The determination of Multiple Antibiotic Resistance (MAR) index of the isolates showed that 95.7% of the isolates had MAR index of 0.4 and above (Table 7).

4.6: Determination of β –lactamase Production.

From the result of multiple antibiotic resistance above, 138 isolates that were resistant to three or more antimicrobials were selected and tested for production of β-lactamase. Out of the 138 isolates tested 30 (21.7%) were β-lactamase producers as shown in Appendix 6. Among those 30 β-lactamase producers, 13(43.3%) were resistant to cefoxitin, ampicillin, amoxicillin and cephalexine (Table 8)

Table 7: Multiple Antibiotic Resistance Index of the Isolates

MAR INDEX	NO. OF ISOLATES (%)
0.3	6 (4.3)
0.4	41 (29.1)
0.5	35 (24.8)
0.6	24 (16.9)
0.7	27 (19.2)
0.8	7 (5.0)
0.9	1 (0.7)
1.0	0(0.0)

Table 8: Antibitic Resistance Pattern of β-lactamase Producing *Staph. aureus* Isolates

Isolates Number	β-lactamase Test	Antibiotics Resistance Pattern
IM001	POSITIVE	Amp, Fox, Aml, Cl
IM003	POSITIVE	Amp, Aml, Cl
IM006	POSITIVE	Amp, Fox, Aml
IM010	POSITIVE	Amp, Fox, Aml, Cl
IS013	POSITIVE	Amp, Fox, Aml, Cl
IM012	POSITIVE	Amp, Fox, Aml
IM014	POSITIVE	Amp, Fox, Aml
IM024	POSITIVE	Amp, Fox, Aml,
IM026	POSITIVE	Amp, Fox, Aml, Cl
IM029	POSITIVE	Amp, Fox, Aml
IM030	POSITIVE	Amp, Fox, Aml
IM031	POSITIVE	Amp,Fox, Aml, Cl
IM042	POSITIVE	Amp, Fox, Aml, Cl
IM044	POSITIVE	Amp, Fox, Aml, Cl
IM047	POSITIVE	Amp, Fox, Aml
IM076	POSITIVE	Amp, Fox, Aml, Cl
IM080	POSITIVE	Amp, Fox, Aml, Cl
IM082	POSITIVE	Amp, Fox, Aml, Cl
IM085	POSITIVE	Amp, Fox, Aml
IM086	POSITIVE	Amp, Fox, Aml
IM094	POSITIVE	Amp, Fox, Aml
IM102	POSITIVE	Amp, Fox, Aml
IM108	POSITIVE	Amp, Fox, Aml
IM131	POSITIVE	Amp, Fox, Aml, Cl
IM135	POSITIVE	Amp, Fox, Aml, Cl
IM137	POSITIVE	Amp, Fox, Aml
IM138	POSITIVE	Amp, Fox, Aml
IM139	POSITIVE	Amp, Fox, Aml
IS008	POSITIVE	Amp, Fox, Aml,Cl
IM054	POSITIVE	Amp, Fox, Aml

Key: AMP = Ampicillin, CL= Cephalexine, CIP = Ciprofloxacin, GEN = Gentamicin, OFL = Ofloxacin, TET = Tetracycline, AML = Amoxicillin, SXT = Sulphamethoxazole/Trimethoprim, FOX = Cefoxitin, CXM = Cefuroxime

4.7: DNA EXTRACTION ANALYSIS

The 13 (43.3%) of 30 Beta-lactamase producing MDR isolates isolates that were positive to β-lactamase production test and at the same time were resistant to cefoxitin, Ampicillin, amoxicillin and cephalexine were subjected to DNA extraction. The result of the PCR amplification is shown in Fig 1.

According to the PCR results, *Staph. aureus* ATCC 25922 (positive control) was Methicillin resistance (MR) carrying the *mecA* gene and also β-lactamase producer (*blaZ* gene positive PCR). Out of the 13 *Staph. aureus* isolates selected for PCR, none carried the *mecA* gene and they did not also carry *blaZ* gene. This suggests that these isolates that showed no amplification for *mecA* and *blaz* genes might have other antibiotics resistance genes which are expressible using other set of primers.

4.8 PCR Analysis of the Resistant *Staph. aureus* Isolates

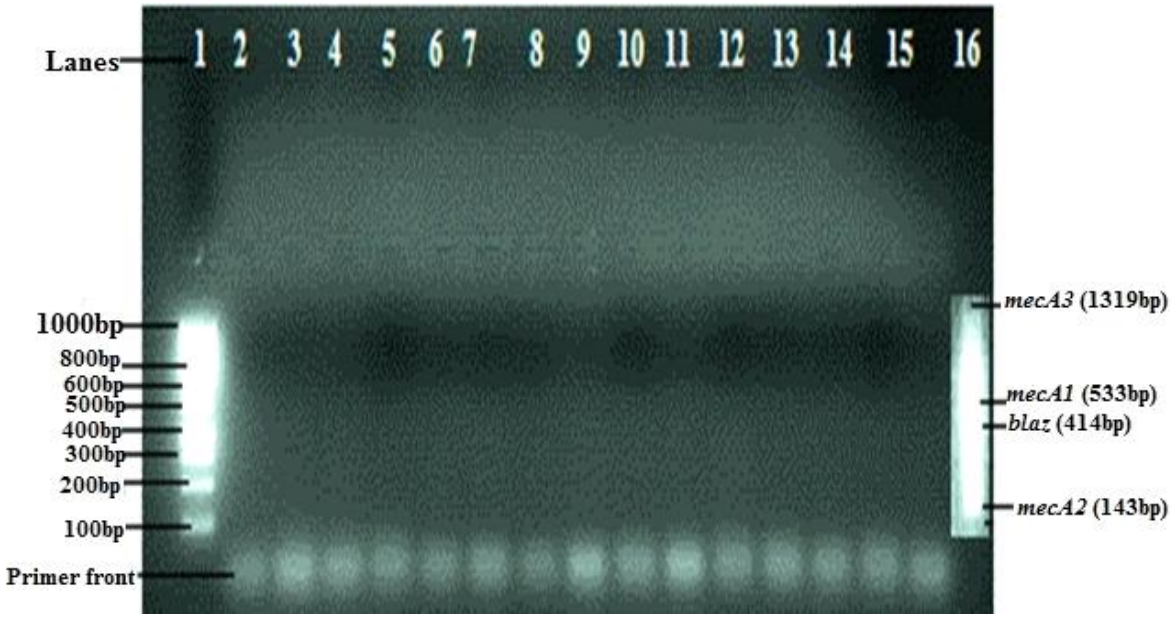

Figure 1.0: Testing for the presence of *mecA* and *blaZ* genes from *Staph. aureus* isolates

Lane1**:** molecular size marker (1000bp DNA ladder), Lane 2: IM001, Lane 3:IM010, Lane 4:IS013, Lane 5: IM026, Lane 6: IM031, Lane 7: IM042, Lane 8:IM044, Lane 9: IM076, Lane 10: IM080, Lane 11: IM082, Lane 12: IS008, Lane 13:IM131, Lane 14: IM135, Lane 15: Negative Control (water), Lane 16: Positive Control *Staph. aureus* ATCC 25922.

CHAPTER FIVE

5.0: DISCUSSION

Staphylococcus. aureus is a major causative organism of mastitis, its emergence as multi drug resistant has become a deep concern for dairy industry worldwide. Because of the importance of Staphylococci specifically *Staph. aureus* as a major mastitis pathogen which is very difficult to be treated, this work focused on studying *Staph. aureus* causing mastitis in bovine and their antibiotic resistance.

The study shows an alarming higher isolation of *Staph. aureus* than Coagulase negative staphylococci from milk, mammary secretion and wound swab of the infected cows from different centers. The isolation rate of *Staph. aureus* is found was 70.5% which support the finding that says that *Staph. aureus* is still considered one of the most common etiological agents associated with clinical and subclinical mastitis in lactating cow (Castro *et al.*, 1992; Espeche *et al.*, 2012; Fourichon *et al.*, 2001).

A rate of 29.5% Coagulase negative staphylococci and 2.9% non-staphylococci were found in this study. Gram negative species found were only 9.4%. These results are similar to those observed by Malinowski *et al.*, 2006.

In this study, it was found that 97.9% of the *Staph. aureus* isolates were multi-drug resistant which is alarming, while comparatively 52% of *Staph. aureus* isolates were reported as multi drug resistant in Ethiopia (Sori *et al.*, 2011). Phenotypic methicillin resistance was found in high percentage among the *Staph. aureus* isolates at 97.9% which is greater than that reported in Korea and India (Moon *et al.*, 2007; Kumar *et al.*, 2010). It is well reported that emergence of drug resistance is the consequence of the improper use of antimicrobials (Kumar *et al.*, 2011; Kenar *et al.* 2012). Reported cases of prevalence of MRSA in Nigeria are high: 43% was

reported by Ikeh (2003) and 34.7% by Taiwo *et al.,* (2004). The percentage (97.9%) of the prevalence of MRSA observed in this study is higher than the earlier report by Onanuga *et al.,* (2005), who reported 69% MRSA in urine of healthy women in Zaria.

In this study there was a high susceptibility of the isolates to ciprofloxacin and ofloxacin. This supports previous reports by Fridkin *et al.* (2005) and Olayinka and Olayinka, (2003). From a study, Kucers and Bennett (1988), reported that quinolones (e.g ciprofloxacin) has an *in vitro* activity against MRSA strains. Ciprofloxacin binds to DNA gyrase (bacterial Topoisomerase II) thereby inhibiting DNA synthesis (Power, 2000).

In this study MRSA isolates show high resistance to ampicillin, amoxicillin and cefuroxime. This supports the findings that MRSA strains are equally resistant to all β lactam antibiotics (Weems, 2001; Gross-Schulman *et al.,* 1998) and that MRSA strains show intrinsic resistance to all other penicillinase resistant penicillins, all cephalosporins such as cephalothin and cephalexin and also to all newer beta lactam antibiotics such as monolactam (Shanson, 1981; Thompson *et al.,* 1982; Hirschl *et al.,* 1984).

As shown in Table 5, out of 141 *Staph. aureus* isolates recovered from cow mastitis cases, 139 (98.6%) were resistant to ampicillin and Amoxicillin, and 138 (97.9%) were resistant to cefoxitin. Resistance was not only the result of destruction of the antibiotic by the enzyme penicillinase produced by some microorganisms, but there are also other mechanisms termed intrinsics (Seligman, 1966). Some authors referred that since the mechanism of methicillin resistance is probably the same for all staphylococci, beta-lactam antibiotics cannot be recommended for any infection caused by these organisms Chambers (1988), however it is evident that there are other mechanisms of resistance. And on the other hand it was observed that

beta-lactams penicillin (penems) and cephalosporins were active *in vitro* against methicillin-resistant strains (Hackbarth and Chambers, 1989).

In this report, the majority of the tetracycline resistant isolates were also resistant to penicillin. This combination of resistance has been previously reported for *Staph. aureus* isolated from intramammary infections (Hareri *et al.,* 2005; Waage *et al.,* 2002; Vintov *et al.,* 2003). It was also concluded from the result that ofloxacin and ciprofloxacin were the most effective antibiotics in-vitro where only 1/141 isolates (0.7%) were resistant to ofloxacin and 3/141 isolates (2.1%) to ciprofloxacin. Similar result was reported by Imran *et al,* (2010) who found that ciprofloxacin was the most effective antibiotic against MRS followed by ofloxacin and chloramphenicol.

Analysis of the results of multiple antibiotic resistance index determined for the isolates showed that 95.7% of the isolates have MAR index greater than 0.3. This suggests that the isolates originated from environment where antibiotics are often used (Krumperman, 1983; Paul *et al.,* 1997). The increasing frequency of drug resistance has been attributed to combination of microbial characteristics, selective pressure of antimicrobial use and societal and technological changes that enhance the transmission of drug resistant organisms (Orozova *et al.,* 2008).

All the isolates did not carry *mecA* gene while showing phenotypic resistance to cefoxitin. As resistance due to β- lactamase in *Staphylococcus* was reported to be mainly mediated by *mecA* gene which is predominate of methicillin resistance in all Staphylococci (Kilic *et al.,* 2006) and/or *blaZ* gene, the determinant of β lactamase production (Vesterholm-Nelsen *et al.,* 1999) so, phenotypic and genotypic methods were directed toward detection of both mechanisms.

Phenotytic prediction of *mecA* gene presence which is historically referred to as methicillin resistance (MR) was conducted by cefoxitin disc diffusion test. On the other hand, prediction of

blaZ gene presence was conducted by Ampicillin and Amoxicillin disk diffusion test (CLSI, 2006; CLSI, 2008). Additionally, phenotypic methods were found to be time consuming and labour intensive (Reischl *et al.,* 2000). Several advantages were reported for genotypic methods in resistance detection compared to convectional susceptibility method, and because of the disadvantages of phenotypic tests and the advantages of genotypic methods, four pairs of primers were included in multiplex PCR. The first three pairs targeted the *mecA* gene, the determinant of Methicillin resistance (Zhang *et al.,* 2005) while the forth pair targeted the blaZ gene, the determinant of β-lactamase production (Vesterholm-nelsen *et al.,* 1999; Hareri *et al.,* 2005). Using novel multiplex PCR assay, detection of *blaZ* and/or *mecA* gene were performed through successful amplification of 414 bp and/or 533 bp, 143bp or 1319 bp for *blaz mecA* 1, *mecA* 2, or *mecA* 3 genes respectively. In the standard *Staph. aureus* ATCC 25922, both *mecA* and *blaZ* genes were detected (Fig. 1). Out of the 13 *Staph. aureus* isolates selected for PCR, none of them carried the *mecA* and *blaZ* genes.

Although the isolates did not carry the *mecA* gene, they were phenotypically resistant to cefoxitin. Non-*mecA* carriage can be attributed to many reasons: The first is the production of modified intrinsic PBPs with altered affinity for methicillin (Tomasz *et al.,* 1989), the second reason can be the inactivation of cefoxitin or methicillin by increased production of β- lactamase which can be declared by detection of *blaZ* gene (Swenson, 2002).

Expression of *mecA* gene yields a penicillin binding protein called PBP2' with reduced affinity for β lactam antibiotic binding. In this study, none of the isolates tested positive for PBP2s which is an indication of the absence of *mecA* gene.

In detailed review by Fluit *et al.* (2001), it was reported that some isolates have been found to be *mecA* negative in the polymerase chain reaction but resistant to methicillin/cefoxitin. These

demonstrated that the isolates had inducible phenotypes which probably may be the situation in this study.

With the absence of *mecA* gene which is the gold standard for MRSA, the phenotypic MRSA prevalence observed in this study will therefore be due to other mechanism like hyper production of β- lactamase (Murakami *et al.*, 1991). Some strains of *Staph. aureus* over express β-lactamase and thus can appear to be resistant to cefoxitin/methicillin despite being *MecA* negative.

CHAPTER SIX

6.0: SUMMARY, CONCLUSION AND RECOMMENDATION

6.1: SUMMARY

This study looked into the antimicrobial susceptibility and molecular characterization of *Staph. aureus* in cow mastitis in southern part of Kano state of Nigeria. One hundred and forty one isolates were confirmed to be Coagulase positive *Staph. aureus* from two hundred and thirty four samples which were from milk and swab from wound. Most of the isolates (97.9%) were found to be resistant to cefoxitin which is an indication of methicillin resistance.

The antibiotic susceptibility pattern showed that the activities of the various antibiotics tested were in the following order: ofloxacin > ciprofloxacin > gentamicin > cephalexine > sulphamethoxazole/trimethaprim > tetracycline > cefuroxime > cefoxitin > amoxicillin > ampicillin.

In parallel to the PCR result which indicated absence of *MecA* and *blaZ* genes in all the studied isolates, the antimicrobial resistance was detected by *in vitro* susceptibility diffusion test with the main antimicrobials used in bovine mastitis therapy as well to some of the commonly used in human staphylococci infection therapy. It was found that 98.6%, 98.6%, 97.9%, 66.7%, 56.7%.53.9%, 44%, 8.5%, 1.7%, 0.8% were resistant to amoxicillin, ampicillin, cefoxitin, cefuroxime, tetracycline, cotrimoxazole, cephaleximе, gentamicin, ciprofloxacin and ofloxacin respectively. Multiple-resistance to three or more antimicrobials was presented in 98.6% isolates. The high MAR index is an indication that the isolates were from area where there is high use of antibiotics. The isolates that were phenotypic shown to be MRSA showed high resistance to cefoxitin (97.9%) but were susceptible to ciprofloxacin and ofloxacin.

Even though altered penicillin binding protein (PBP2a) was not detected in this study (indication of an absence of *mecA* gene) the phenotypic MRSA isolates were multidrug resistant and this might be due to hyper production of β lactamase. The detected resistance, mainly to methicillin/cefoxitin and to ampicillin demonstrated that *Staph. aureus* isolates from bovine mastitis represent a potential hazard to public health.

6.2: CONCLUSION

This study reports increasing prevalence of MRSA isolates without having *mecA* and *blaZ* genes. The prevalence of MRSA from milk and mastitis swab (97.9%) is alarming. The presence of MRSA in the swab of mastitis of cow can cause delay in healing of the wound while isolation of MRSA from the milk confirms that MRSA can colonize a healthy cow and individual. The prevalence of MRSA isolates in milk as discovered from this study is a great risk to both herd mates and milkers because they might become asymptomatic carriers of MRSA through contact with infected cows. This therefore calls for proper hygiene in our herds.

With the high activity of ciprofloxacin and ofloxacin against these isolates compared with the level of resistance observed with cefoxitin and ampicillin in this study, ciprofloxacin and ofloxacin therefore should be recommended for empirical treatment of mastitis associated MRSA strains and there should be reduction on further reliance on ampicillin.

The high MAR index of the isolates which suggested that the isolates are from an environment where antibiotics are often used indicated the possibility of abuse of antibiotics given to the cows even before visiting the hospital.

The detected antibiotics resistance, mainly to cefoxitin and ampicillin demonstrated that *Staph. aureus* isolates from bovine mastitis represent a potential hazard to public health. Therefore, the obtained result is of great concern not only in regard of mastitis therapy but mainly to public health, due to the eventual occurrence of cross infections, as well as, to the possibility of transmission of resistance among the microorganisms by plasmids. We therefore suggest that in Kano State, methicillin resistant *Staph. aureus* is mostly responsible for cow mastitis,

6.3: RECOMMENDATION

1. There is need for further research work to look into the association between the *Staph. aureus* mastitis in the animal (milk and wound swab) and the community, the milkers and the herd workers.

2. This will help to identify the organism and the infection to be classified as zoonotic or not. The resistance pattern between the isolates from the cow and that of the community also need to be compared for proper understanding and treatment of the infection. further intence molecular analysis could be carried out in this area of study to substantiats our findings.

3. Genotypic tests provide resistance profiles rapidly, diminish the biohazard risk associated with the propagation of the microorganisms by culturing and it can be used as a good standard for evaluating new, improved susceptibility methods for testing clinical isolates with difficult to detect resistance profiles where CLSI guidelines now accepted that checking for presence of *mecA* and *blaZ* genes by PCR is the most reliable method for detection of methicillin resistant. Due to the above mentioned disadvantages of phenotypic tests and the advantages of genotypic methods, this proof our alternative hypothesis that MRSA may not necessary responsible for

mastitis in cows. It might be caused by other organisms such as Coagulase negative and some species of Streptococcus.

4. The work supports the above advantages of genotypic tests and also supports the findings of Jorgensen and Ferero (2000).

6.4: CONTRIBUTION TO KNOWLEGE

1. This study reports high phenotypic detection of MRSA isolates without *mecA* and *blaZ* genes from milk and swab of mastitis cows in Kano State.
2. The detected antibiotics resistance, mainly to cefoxitin and ampicillin demonstrated that *Staph. aureus* isolates from bovine mastitis represent a potential hazard to public health.
3. This study also found that ciprofloxacin and ofloxacin may be recommended for the treatment of MRSA associated infection in cows.

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APPENDICES

APPENDIX I

Preparation of Growth Media

I. **Mannitol Salt Agar**

One hundred and eight gram of the powder of mannitol salt agar was dispensed into one litre of distilled water and brought into solubilization by gentle heating and stirring. The medium was then sterilized in 20ml portions, by liquid cycle sterilization (autoclaving) at 121℃ for 15mins. The sterilized 20ml portions were lebeled and stored at 4℃.

II. **Mueller Hinton Agar II**

Thirty-eight grams of the agar (powder) was dispersed into one liter of distilled water and brought into solubilization by gentle heating and stirring. The medium was then sterilized 20ml portions were labeled and stored at 4℃.

III. Nutrient Agar

Twenty eight grams of the powder was dispersed into one litre of distilled water and brought to solubilization by gentle heating and stirring. The medium was then sterilized in 10ml portions by liquid cycle sterilization (autoclaving) at 121℃ for 15 minutes. The sterilized 10ml portions were slanted and stored at 4℃.

IV. Nutrient Broth No 2 (B.P)

Twenty five grams of the powder was dispersed into one litre of distilled water and brought to solubilization by gentle stirring. The medium was sterilized in 5ml portions in bottles by liquid

cycle sterilization (autoclaving) at 121℃ for 15 minutes. The sterilized portions were labeled and store at 4℃.

V. Luria Bertani (LB) Medium

Ten grams of peptone water, five grams of yeast extract and ten grams of sodium chloride powder was dispersed into one litre of distilled water and brought to solubilization by gentle stirring. The medium was sterilized in 5ml portions in bottles by liquid cycle sterilization (autoclaving) at 121℃ for 15 minutes. The sterilized portions were labeled and store at 4℃.

VI. Physiological Saline

Nine grams of sodium chloride was weighed and transferred into a leak-proof bottle premarked to hold 1 litre. Distilled water was then added to the one little mark and mixed until the salt is fully dissolved. The medium was then sterilized in 9ml portions of corked test tubes liquid cycle sterilization (autoclaving) at 121℃ for 15 mins then labeled and stored at 4℃.

VII. Sterile Water

Distilled water was dispensed into various clean wide-necked, leak-proof bottles of volume ranges from 100-300ml., the medium was sterilized by liquid cycle sterilization (autoclaving) at 121℃ for 15 minutes, labeled and stored at room temperature.

VIII. Preparation of Mc-farland Standard

A 1% v/v solution of sulphuric acid was prepared by adding 1ml of concentrated sulphuric acid to 99ml of water and mixed well. A 1% w/v solution of Barium Choloride was also prepared by dissolving 0.5g of dihydrate barium chloride ($BaCl_2.2H_20$) in 49.5ml 0f distilled water. Then

0.6ml of the barium chloride solution was added to 99.4ml of the sulphuric acid solution and mixed (Cheesbrough, 2006).

APPENDIX II

Gram's Stain Technique

Using the Gram's Stain technique described by Cheesbrough (2006), a thin smear of the culture on the nutrient agar slant was applied on a clean glass slide, air dried, stained with crystal violet solution, flooded with Lugol's iodine and decolorized with 95% ethyl alcohol. This was washed immediately in tap water (gentle flow) after which it was counter stained with neutral red stain for two minutes, washed in tap water (gentle flow), blot dried and examined microscopically,. Isolates that produced violet (blue) were taken to be Gram's positive while those with red colour are Gram negative. The isolates that produced Violet cocci predominantly in clusters were selected for further identification procedures.

APPENDIX III

Reaction Setup Table for Primers

PCR Reaction Mixture	20µL	50µL
2× PCR Master Mix	10µL	25µL
Template DNA	1-2µL	1-2µL
Primer F (2.5µM)	1µL	1µL
Primer R (2.5µM)	1µL	1µL
Nuclease-free water	Up to 20µL	Up to 50µl

Suggested PCR Cycle Conditions

PCR Cycle Step	Temperature	Time	No of cycles
Initial Denaturation	94-95ºC	2 min	1
Denaturation	94-95ºC	15-30 sec	30-40
Annealing	50-65ºC	15-30 sec	30-40
Extension	65-72ºC	1 min	30-40
Final Extension	72ºC	5min	1
Hold	4-10ºC	Indefinitely	1

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APPENDIX IV

Table 8: Characteristics of the samples and the isolates

S/N	NATURE OF SAMPLE	N/B	M/A	B/A	MSA	COLOUR OF GROWTH	GRAM'S STAINING	CATALASE	COAGULASE
1	Swab	+	+	+	-	-	+Cocci	+	-
2	Swab	+	+	+	+	Yellow	+cocci	+	+
3	Swab	+	+	+	+	Yellow	+cocci	+	+
4	Swab	+	+	+	+	Yellow	+cocci	+	+
5	Swab	+	+	+	+	Yellow	+cocci	+	+
6	Swab	+	+	+	+	Pink	+cocci	+	-
7	Swab	+	+	+	+	Pink	+cocci	+	-
8	Swab	+	+	+	+	Yellow	+cocci	+	+
9	Swab	+	+	+	+	Yellow	+cocci	+	+
10	Swab	+	+	+	+	Pink	+cocci	+	-
11	Swab	+	+	+	-	-	-Rod	-	-
12	Swab	+	+	+	+	Yellow	+cocci	+	+
13	Milk	+	+	+	+	Yellow	+cocci	+	+
14	Milk	+	+	+	+	Yellow	+cocci	+	+
15	Milk	+	+	+	+	Yellow	+cocci	+	+
16	Milk	+	+	+	+	Yellow	+cocci	+	+
17	Milk	+	+	+	-	Yellow	+cocci	+	+

18	Milk	+	+	+	+	Pink	+cocci	+	-
19	Milk	+	+	+	+	Yellow	+cocci	+	+
20	Milk	+	+	+	+	Yellow	+cocci	+	+
21	Milk	+	+	+	+	Yellow	+cocci	+	+
22	Milk	+	+	+	+	Yellow	+cocci	+	+
23	Swab	+	+	+	+	Yellow	+cocci	+	+
24	Swab	+	+	+	+	Yellow	+cocci	+	+
25	Swab	+	+	+	+	Yellow	+cocci	+	+
26	Milk	+	+	+	-	-	-Rod	-	-
27	Milk	+	+	+	-	-	-Rod	-	-
28	Swab	+	+	+	-	-	+cocci	+	-
29	Milk	+	+	+	-	-	+cocci	+	-
30	Milk	+	+	+	+	Pink	+cocci	+	-
31	Milk	+	+	+	-	-	-Rod	-	-
32	Milk	+	+	+	-	-	+cocci	+	-
33	Milk	+	+	+	-	-	+cocci	+	-
34	Milk	+	+	+	+	Pink	+cocci	+	-
35	Milk	+	+	+	+	Yellow	+cocci	+	+
36	Milk	+	+	+	+	Pink	+cocci	+	-
37	Milk	+	+	+	+	Yellow	+cocci	+	+
38	Milk	+	+	+	+	Yellow	+cocci	+	+

Appendix IV

Continue

39	Milk	+	+	+	+	Yellow	+cocci	+	+
40	Milk	+	+	+	+	Yellow	+cocci	+	+
41	Milk	+	+	+	+	Yellow	+cocci	+	+
42	Swab	+	+	+	+	Yellow	+cocci	+	+
43	Swab	+	+	+	-	-	+cocci	+	-
44	Swab	+	+	+	+	Yellow	+cocci	+	+
45	Swab	+	+	+	-	-	-Rod	-	-
46	Swab	+	+	+	-	-	+cocci	+	-
47	Swab	+	+	+	-	-	-Rod	-	-
48	Milk	+	+	+	+	Pink	+cocci	+	-
49	Milk	+	+	+	+	Yellow	+cocci	+	+
50	Milk	+	+	+	+	Yellow	+cocci	+	+
51	Milk	+	+	+	+	Yellow	+cocci	+	+
52	Milk	+	+	+	+	Yellow	+cocci	+	+
53	Milk	+	+	+	+	Pink	+cocci	+	-
54	Milk	+	+	+	+	Pink	+cocci	+	-
55	Milk	+	+	+	+	Yellow	+cocci	+	+
56	Milk	+	+	+	+	Yellow	+cocci	+	+
57	Milk	+	+	+	+	Yellow	+cocci	+	+

58	Milk	+	+	+	+	Yellow	+cocci	+	+
59	Milk	+	+	+	+	Pink	+cocci	+	-
60	Milk	+	+	+	+	Yellow	+cocci	+	+
61	Milk	+	+	+	+	Yellow	+cocci	+	+
62	Milk	+	+	+	+	Yellow	+cocci	+	+
63	Milk	+	+	+	+	Yellow	+cocci	+	+
64	Milk	+	+	+	+	Yellow	+cocci	+	+
65	Milk	+	+	+	+	Yellow	+cocci	+	+
66	Milk	+	+	+	_	_	-rod	-	-
67	Milk	+	+	+	_	_	-rod	-	-
68	Milk	+	+	+	_	_	-rod	-	-
69	Milk	+	+	+	+	Yellow	+cocci	+	+
70	Milk	+	+	+	+	Yellow	+cocci	+	+
71	Milk	+	+	+	+	Yellow	+cocci	+	+
72	Milk	+	+	+	+	Yellow	+cocci	+	+
73	Milk	+	+	+	+	Yellow	+cocci	+	+
74	Milk	+	+	+	_	_	-rod	-	-
75	Milk	+	+	+	_	_	-rod	-	-
76	Milk	-	+	-	+	Yellow	+cocci	+	+
77	Milk	-	+	-	+	Yellow	+cocci	+	+

78	Milk	-	+	-	+	Yellow	+cocci	+	+
79	Milk	+	+	+	-	-	-rod	+	+
80	Milk	+	+	+	+	Yellow	+cocci	+	+

Appendix IV Continue

81	Milk	+	+	+	+	Yellow	+cocci	+	+
82	Milk	+	+	+	+	Yellow	+cocci	+	+
83	Milk	+	+	+	+	Yellow	+cocci	+	+
84	Milk	+	+	+	+	Yellow	+cocci	+	+
85	Milk	+	+	+	+	Yellow	+cocci	+	+
86	Milk	+	+	+	+	Yellow	+cocci	+	+
87	Milk	+	+	+	+	Yellow	+cocci	+	+
88	Milk	+	+	+	+	Yellow	+cocci	+	+
89	Milk	+	+	+	_	_	-rod	-	-
90	Milk	+	+	+	_	_	-rod	-	-
91	Milk	+	+	+	+	Pink	+cocci	+	-
92	Milk	+	+	+	+	Pink	+cocci	+	-
93	Milk	+	+	+	+	Pink	+cocci	+	-
94	Milk	+	+	+	+	Yellow	+cocci	+	+
95	Milk	+	+	+	+	Yellow	+cocci	+	+
96	Milk	+	+	+	+	Yellow	+cocci	+	+
97	Milk	+	+	+	+	Yellow	+cocci	+	+
98	Milk	+	+	+	+	Yellow	+cocci	+	+

98	Milk	+	+	+	+	Yellow	+cocci	+	+
99	Milk	+	+	+	+	Yellow	+cocci	+	+
100	Milk	+	+	+	+	Pink	+cocci	+	-
101	Milk	+	+	+	+	Yellow	+cocci	+	+
102	Milk	+	+	+	+	Yellow	+cocci	+	+
103	Milk	+	+	+	+	Yellow	+cocci	+	+
104	Milk	+	+	+	+	Pink	+cocci	+	-
105	Milk	+	+	+	+	Yellow	+cocci	+	+
106	Milk	+	+	+	+	Yellow	+cocci	+	+
107	Milk	+	+	+	+	Pink	+cocci	+	-

Appendix IV Continue

108	Milk	+	+	+	+	Pink	+cocci	+	-
109	Milk	+	+	+	+	Yellow	+cocci	+	+
110	Milk	+	+	+	+	Yellow	+cocci	+	+
111	Milk	+	+	+	+	Yellow	+cocci	+	+
112	Milk	+	+	+	+	Yellow	+cocci	+	+
113	Milk	+	+	+	+	Yellow	+cocci	+	+
114	Milk	+	+	+	+	Yellow	+cocci	+	+
115	Milk	+	+	+	+	Pink	+cocci	+	-
116	Milk	+	+	+	+	Yellow	+cocci	+	+
117	Milk	+	+	+	+	Yellow	+cocci	+	+
118	Milk	+	+	+	+	Yellow	+cocci	+	+

119	Milk	+	+	+	+	Yellow	+cocci	+	+
121	Milk	+	+	+	+	Yellow	+cocci	+	+
121	Milk	+	+	+	-	-	-rod	-	-
122	Milk	+	+	+	+	Pink	+cocci	+	-
123	Milk	+	+	+	+	Yellow	+cocci	+	+
124	Milk	+	+	+	+	Yellow	+cocci	+	+
125	Milk	+	+	+	+	Yellow	+cocci	+	+
126	Milk	+	+	+	+	Yellow	+cocci	+	+
127	Milk	+	+	+	+	Yellow	+cocci	+	+
128	Milk	+	+	+	+	Pink	+cocci	+	-
129	Milk	+	+	+	+	Pink	+cocci	+	-
30	Milk	+	+	+	+	Yellow	+cocci	+	+
131	Milk	+	+	+	+	Yellow	+cocci	+	+
132	Milk	+	+	+	+	Yellow	+cocci	+	+
133	Milk	+	+	+	+	Pink	+cocci	+	-
134	Milk	+	+	+	+	Pink	+cocci	+	-
135	Milk	+	+	+	+	Yellow	+cocci	+	+

Appendix IV Continue

136	Milk	+	+	+	+	Pink	+cocci	+	-
137	Milk	+	+	+	+	Yellow	+cocci	+	+
138	Milk	+	+	+	+	Yellow	+cocci	+	+
139	Milk	+	+	+	+	Yellow	+cocci	+	+

140	Milk	+	+	+	+	Yellow	+cocci	+	+
141	Milk	+	+	+	+	Yellow	+cocci	+	+
142	Milk	+	+	+	+	Yellow	+cocci	+	+
143	Milk	+	+	+	+	Pink	+cocci	+	-
144	Milk	+	+	+	+	Yellow	+cocci	+	+
145	Milk	+	+	+	+	Yellow	+cocci	+	+
146	Milk	+	+	+	+	Yellow	+cocci	+	+
147	Milk	+	+	+	+	Yellow	+cocci	+	+
148	Milk	+	+	+	-	-	-rod	-	-
149	Milk	+	+	+	-	-	-rod	-	-
150	Milk	+	+	+	+	Yellow	+cocci	+	+
151	Milk	+	+	+	+	Yellow	+cocci	+	+
152	Milk	+	+	+	+	Yellow	+cocci	+	+
153	Milk	+	+	+	+	Yellow	+cocci	+	+
154	Milk	+	+	+	+	Yellow	+cocci	+	+
155	Milk	+	+	+	+	Pink	+cocci	+	-
156	Milk	+	+	+	+	Pink	+cocci	+	-
157	Milk	+	+	+	+	Yellow	+cocci	+	+
158	Milk	+	+	+	+	Yellow	+cocci	+	+
159	Milk	+	+	+	+	Yellow	+cocci	+	+
160	Milk	+	+	+	+	Yellow	+cocci	+	+
161	Milk	+	+	+	+	Yellow	+cocci	+	+

161	Milk	+	+	+	+	Yellow	+cocci	+	+
162	Milk	+	+	+	+	Yellow	+cocci	+	+

Appendix IV Continue

163	Milk	+	+	+	+	Yellow	+cocci	+	+
164	Milk	+	+	+	+	Pink	+cocci	+	-
165	Milk	+	+	+	+	Yellow	+cocci	+	+
166	Milk	+	+	+	+	Yellow	+cocci	+	+
167	Milk	+	+	+	+	Yellow	+cocci	+	+
168	Milk	+	+	+	+	Yellow	+cocci	+	+
169	Milk	+	+	+	+	Yellow	+cocci	+	+
170	Milk	+	+	+	+	Yellow	+cocci	+	+
171	Milk	+	+	+	+	Yellow	+cocci	+	+
172	Milk	+	+	+	+	Pink	+cocci	+	-
173	Milk	+	+	+	+	Pink	+cocci	+	-
174	Milk	+	+	+	+	Yellow	+cocci	+	+
175	Milk	+	+	+	+	Yellow	+cocci	+	+
176	Milk	+	+	+	+	Yellow	+cocci	+	+
177	Milk	+	+	+	+	Pink	+cocci	+	-
178	Milk	+	+	+	+	Yellow	+cocci	+	+
178	Milk	+	+	+	+	Yellow	+cocci	+	+
179	Milk	+	+	+	+	Pink	+cocci	+	-
180	Milk	+	+	+	+	Pink	+cocci	+	-

181	Milk	+	+	+	+	Yellow	+cocci	+	+
182	Milk	+	+	+	+	Yellow	+cocci	+	+
183	Milk	+	+	+	+	Yellow	+cocci	+	
184	Milk	+	+	+	+	Pink	+cocci	+	-
185	Milk	+	+	+	+	Pink	+cocci	+	-
186	Milk	+	+	+	+	Yellow	+cocci	+	+
187	Milk	+	+	+	+	Yellow	+cocci	+	+
189	Milk	+	+	+	+	Yellow	+cocci	+	+
190	Milk	+	+	+	+	Yellow	+cocci	+	+

Appendix IV Continue

191	Milk	+	+	+	+	Yellow	+cocci	+	+
192	Milk	+	+	+	+	Pink	+cocci	+	-
193	Milk	+	+	+	+	Yellow	+cocci	+	+
194	Milk	+	+	+	+	Yellow	+cocci	+	+
195	Milk	+	+	+	+	Yellow	+cocci	+	+
196	Milk	+	+	+	+	Yellow	+cocci	+	+
197	Milk	+	+	+	+	Pink	+cocci	+	-
198	Milk	+	+	+	+	Yellow	+cocci	+	+
199	Milk	+	+	+	+	Yellow	+cocci	+	+
200	Milk	+	+	+	+	Yellow	+cocci	+	+
201	Milk	+	+	+	+	Pink	+cocci	+	-
202	Milk	+	+	+	+	Yellow	+cocci	+	+

203	Milk	+	+	+	+	Yellow	+cocci	+	+
204	Milk	+	+	+	+	Pink	+cocci	+	-
205	Milk	+	+	+	+	Yellow	+cocci	+	+
206	Milk	+	+	+	+	Yellow	+cocci	+	+
207	Milk	+	+	+	+	Golden yellow	+cocci	+	+
208	Milk	+	+	+	+	Pink	+cocci	+	-
209	Milk	+	+	+	+	Yellow	+cocci	+	+
210	Milk	+	+	+	+	Pink	+cocci	+	-
211	Milk	+	+	+	+	Pink	+cocci	+	-
212	Milk	+	+	+	+	Pink	+cocci	+	-
213	Milk	+	+	+	-	-	-rod	-	-
214	Milk	+	+	+	+	Yellow	+cocci	+	+
215	Milk	+	+	+	+	Yellow	+cocci	+	+
216	Milk	+	+	+	+	Yellow	+cocci	+	+
217	Milk	+	+	+	+	Yellow	+cocci	+	+

Appendix IV Continue

218	Milk	+	+	+	-	-	-rod	-	-
219	Milk	+	+	+	-	-	-rod	-	-
220	Milk	+	+	+	+	Yellow	+cocci	+	+
221	Milk	+	+	+	+	Yellow	+cocci	+	+
222	Milk	+	+	+	+	Yellow	+cocci	+	+

223	Milk	+	+	+	+	Yellow	+cocci	+	+
224	Milk	+	+	+	+	Yellow	+cocci	+	+
225	Milk	+	+	+	-	-	+cocci	+	-
226	Milk	+	+	+	+	Yellow	+cocci	+	+
227	Swab	+	+	+	+	-	-rod	-	-
228	Swab	+	+	+	+	-	-r o d	-	-
229	Milk	+	-	-	-	-	-	-	-
230	Milk	+	-	-	-	-	-	-	-
231	Milk	+	-	-	-	-	-	-	-
232	Milk	+	-	-	-	-	-	-	-
233	Milk	+	-	-	-	-	-	-	-
234	Milk	+	-	-	-	-	-	-	-

Key:

N/A = Nutrient agar

N/B = Nutrient broth

MSA = Mannitol salt agar

MCA = Macconkay agar

B/A = Blood agar

APPENDIX V

Table 9: Antibiotics susceptibilities of *Staph. aureus* isolates from cow mastitis

S/N	ISOLATE	AMP 10µg	CIP 5µg	GEN 10µg	OFL 5µg	CL 30µg	TET 30µg	AML 10µg	SUL/TRI 25µg	FOX 30µg	CXM 30µg
1	S1	R	S	R	S	R	R	S	S	R	S
2	S2	R	S	S	S	R	R	R	R	R	R
3	S3	R	S	R	S	R	R	R	R	R	R
4	S4	R	R	R	S	R	R	R	S	R	R
5	S5	R	S	R	S	S	R	R	S	R	R
6	S6	R	S	R	S	R	S	R	R	R	R
7	S7	R	S	R	S	R	R	R	R	R	R
8	S8	R	S	R	S	R	R	R	R	R	R
9	S9	S	S	S	S	S	S	R	S	R	R
10	S10	R	S	R	S	R	R	R	R	R	R
11	S11	R	S	R	S	R	R	R	R	R	S
12	S12	R	S	S	S	S	S	R	R	R	R
13	M1	R	S	S	S	R	R	R	R	R	R
14	M2	R	S	S	S	R	S	R	R	R	R
15	M3	R	S	S	S	S	S	R	S	R	R
16	M4	R	S	S	S	S	S	R	R	R	S
17	M5	R	S	S	S	R	R	R	R	R	R
18	M6	R	S	S	S	S	S	R	R	R	R
19	M7	R	S	S	S	R	R	R	R	R	R

20	M8	R	R	R	S	R	R	R	R	R	R
21	M9	R	S	S	S	R	R	R	R	R	R
22	M10	R	S	S	S	R	R	R	R	R	R
23	S13	R	S	S	S	R	S	R	R	R	R
24	S14	R	S	S	S	R	R	R	R	R	R
25	S15	R	S	S	S	S	S	R	S	R	R
26	M11	R	S	S	S	S	S	R	S	R	R

Appendix V Continue

27	M12	R	S	S	S	S	R	R	R	R	R
28	S16	R	S	S	S	R	S	R	S	I	R
29	M13	R	S	S	S	S	S	R	R	R	R
30	M14	R	S	S	S	S	S	R	S	R	R
31	M15	R	S	S	S	R	R	R	R	R	R
32	M16	S	S	S	S	S	S	R	R	R	S
33	M17	R	S	S	S	S	R	R	R	R	R
34	M19	R	S	S	S	R	S	R	S	R	R
35	M20	R	S	S	S	R	R	R	R	R	R
36	M21	R	S	S	S	S	S	R	R	R	R
37	M22	R	S	S	S	S	S	R	S	R	R
38	M23	R	S	S	S	S	S	R	S	R	R
39	M24	R	S	S	S	R	S	R	R	R	R
40	M25	R	S	S	S	R	R	R	S	R	S
41	S17	S	S	S	S	S	S	S	S	S	S

42	S18	R	S	S	S	R	R	R	R	R	R
43	S19	R	S	S	S	R	R	R	R	R	R
44	S20	R	S	S	S	S	S	R	S	R	R
45	S21	R	S	S	S	R	R	S	S	R	S
46	M26	R	S	S	S	R	R	R	R	R	R
47	M27	R	S	S	S	S	S	R	R	R	S
48	M28	R	S	S	S	S	R	R	S	R	R
49	M29	R	S	S	S	S	S	R	R	R	R
50	M30	R	S	S	S	S	R	R	S	R	R
51	M31	R	S	S	S	R	S	R	R	R	R
52	M33	R	S	S	S	R	S	R	R	R	R
53	M37	R	S	S	R	R	S	R	R	R	R
54	M38	R	S	S	S	S	S	R	R	R	S
55	M39	R	S	S	S	R	R	R	R	R	R
56	M40	R	S	S	S	S	R	R	R	R	R

Appendix V Continue

57	M41	R	S	S	S	R	R	R	S	R	R
58	M42	R	S	S	S	R	R	R	R	R	R
59	M43	R	S	S	S	R	R	R	R	R	R
60	M44	R	S	R	S	R	R	R	R	R	R
61	M45	R	I	S	I	R	R	R	S	R	R
62	M46	R	S	S	S	R	R	R	R	R	R
63	M47	R	S	S	S	S	S	R	R	R	R

64	M48	R	S	R	S	R	R	R	R	R	R
65	M50	R	S	S	S	R	R	R	R	R	R
66	M51	R	S	S	S	S	S	R	S	R	S
67	M53	R	S	S	S	R	R	R	R	R	R
68	M54	R	S	S	S	S	R	R	R	R	S
69	M55	R	S	S	S	R	S	R	R	R	R
70	M56	R	S	S	S	S	R	R	R	R	S
71	M57	R	S	S	S	R	R	R	R	R	S
72	M59	R	S	S	S	R	R	R	R	R	S
73	M60	R	S	S	S	S	R	R	S	R	S
74	M61	R	S	S	S	R	R	R	R	R	R
75	M63	R	S	S	S	S	R	R	S	R	S
76	M64	R	S	S	S	R	S	R	S	R	S
77	M67	R	S	S	S	S	S	R	R	R	R
78	M68	R	S	S	S	S	R	R	S	R	S
79	M69	R	S	S	S	R	S	R	S	R	R
80	M70	R	S	S	S	R	S	R	S	I	R
81	M71	R	S	S	S	S	S	R	R	R	R
82	M73	R	S	S	S	S	R	R	S	R	S
83	M74	R	S	S	S	S	R	R	S	R	S
84	M75	R	S	S	S	S	S	R	R	R	R
85	M76	R	S	S	S	R	R	R	R	R	S
86	M77	R	S	S	S	R	R	S	S	R	R

Appendix V Continue

87	M78	R	R	S	S	R	R	R	R	R	S
88	M79	R	S	S	S	R	R	R	R	R	R
89	M80	R	S	S	S	R	S	R	R	R	S
90	M81	R	S	S	S	S	S	R	R	R	R
91	M82	R	S	S	S	R	R	R	R	R	S
92	M83	R	S	S	S	S	S	R	R	R	S
93	M85	R	S	S	S	S	S	R	S	R	R
94	M86	R	S	S	S	S	S	R	R	R	R
95	M88	R	S	S	S	S	S	R	R	R	R
96	M89	R	S	S	S	S	S	R	S	R	R
97	M90	R	S	S	S	S	R	R	R	R	R
98	M91	R	S	S	S	S	R	R	R	R	R
99	M92	R	S	S	S	S	R	R	R	R	R
100	M93	R	S	S	S	S	R	R	S	I	S
101	M94	R	S	S	S	S	R	R	S	R	S
102	M95	R	S	S	S	S	S	R	S	R	S
103	M96	R	S	S	S	S	R	R	S	R	S
104	M98	R	S	S	S	R	R	R	R	R	S
105	M102	R	S	S	S	S	S	R	R	R	R
106	M104	R	S	S	S	S	R	R	S	R	S
107	M106	R	S	S	S	S	R	R	S	R	R
108	M107	R	S	S	S	S	R	R	S	R	R

109	M108	R	S	S	S	S	R	R	S	R	S
110	M109	R	S	S	S	R	S	R	S	R	S
111	M110	R	S	S	S	R	S	R	S	R	R
112	M112	R	S	S	S	S	S	R	R	R	R
113	M113	R	S	S	S	S	S	R	S	R	R
114	M114	R	S	S	S	S	R	R	S	R	S
115	M115	R	S	S	S	R	R	R	S	R	S
117	M116	R	S	S	S	S	S	R	S	R	R

Appendix V Continue

118	M118	R	S	S	S	R	S	R	S	R	S
119	M119	R	S	S	S	R	R	R	R	R	R
120	M120	R	S	S	S	S	R	R	S	R	S
121	M121	R	S	S	S	S	R	R	S	R	S
122	M122	R	S	S	S	S	R	R	S	R	R
123	M125	R	S	S	S	S	R	R	S	R	R
124	M126	R	S	S	S	S	R	R	S	R	R
125	M128	R	S	S	S	S	S	R	S	R	S
126	M129	R	S	S	S	S	R	R	S	R	S
127	M131	R	S	S	S	S	R	R	S	R	S
128	M132	R	S	S	S	S	S	R	S	R	R
129	M133	R	S	S	S	S	S	R	R	R	S
130	M134	R	S	S	S	S	S	R	S	R	R
131	M135	R	S	S	S	S	S	R	S	R	R

132	M136	R	S	S	S	S	R	R	R	R	R
133	M137	R	S	S	S	S	R	R	S	R	S
134	M138	R	S	S	S	S	R	R	S	R	R
135	M139	R	S	S	S	S	S	R	S	R	R
136	M157	R	S	S	S	S	R	R	S	R	S
137	M158	R	S	S	S	S	S	R	S	R	S
138	M159	R	S	S	S	S	R	R	S	R	R
139	M160	R	S	S	S	S	R	R	S	R	S
140	M161	R	S	S	S	R	S	R	R	R	R
141	M1162	R	S	S	S	R	S	R	R	R	R

Key:

AMP= Ampicillin, CL= Cephalexine, CIP= Ciprofloxacin, GEN=Gentamicin, OFL= Ofloxacin, TET= Tetracycline, AML= Amoxicillin, SXT= Sulphamethoxazole/trimethaprim, FOX= Cefoxitin, CXM= Cefuroxime

APPENDIX VI

Table 10: β-lactamase production test

Isolate Number	Colour Reaction	Remark
Is001	Violet	_
Is002	Violet	_
Is003	Violet	_
Is004	Violet	_
Is005	Violet	_
Is006	Violet	_
Is007	Violet	_
Is008	Violet	_
Is009	Violet	_
Is010	Violet	_
Is011	Violet	_
Is012	Violet	_
Im001	Yellow	+
Im002	Violet	_
Im003	Yellow	+
Im004	Violet	_
Im005	Violet	_
Im006	Yellow	+
Im007	Violet	_
Im008	Violet	_
Im009	Violet	_

Im010	Yellow	+
Is013	Yellow	+
Is014	Violet	_
Im012	Yellow	+
Is016	Violet	_
Im013	Violet	_

Appendix VI Continue

Im014	Yellow	+
Im015	Volet	_
Im016	Violet	_
Im018	Violet	_
Im019	Violet	_
Im020	Violet	_
Im022	Violet	_
Im023	Violet	_
Im024	Yellow	+
Is018	Violet	_
Is019	Violet	_
Im025	Violet	_
Im026	Yellow	+
Im028	Violet	_
Im029	Yellow	+
Im030	Yellow	+

Im031	Yellow	+
Im033	Violet	_
Im037	Violet	_
Im038	Violet	_
Im039	Violet	_
Im040	Violet	_
Im041	Violet	_
Im042	Yellow	+
Im043	Violet	_
Im044	Yellow	+
Im045	Violet	_
Im046	Violet	_
Im047	Yellow	+
Im048	Violet	_

Appendix VI Continue

Im050	Violet	_
Im053	Violet	_
Im054	Violet	_
Im055	Violet	_
Im056	Violet	_
Im057	Volet	_
Im059	Violet	_
Im061	Violet	_

Im063	Violet	_
Im064	Violet	_
Im067	Violet	_
Im069	Violet	_
Im070	Violet	_
Im071	Violet	_
Im075	Violet	_
Im076	Yellow	+
Im077	Violet	_
Im078	Violet	_
Im079	Violet	_
Im080	Yellow	+
Im081	Violet	_
Im082	Yellow	+
Im085	Yellow	+
Im086	Yellow	+
Im088	Violet	_
Im089	Violet	_
Im090	Violet	_
Imo91	Violet	_
Im092	Violet	_
Im093	Violet	_

Appendix VI Continue

Im094	Yellow	+
Im098	Violet	_
Im102	Yellow	+
Im106	Violet	_
Im107	Violet	_
Im108	Yellow	+
Im109	Violet	_
Im110	Violet	_
Im112	Violet	_
Im113	Violet	_
Im114	Violet	_
Im115	Violet	_
Im116	Volet	_
Im118	Violet	_
Im119	Violet	_
Im120	Violet	_
Im121	Violet	_
Im122	Violet	_
Im125	Violet	_
Im126	Violet	_
Im128	Violet	_
Im129	Violet	_
Im131	Yellow	+

Im132	Violet	_
Im133	Violet	_
Im134	Violet	_
Im135	Yellow	+
Im136	Violet	_
Im137	Yellow	+
Im138	Yellow	+

Appendix VI Continue

Im139	Yellow	+
Im140	Violet	_
Im157	Violet	_
Im158	Violet	_
Im159	Violet	_
Im160	Violet	_
Im162	Violet	_
Im134	Yellow	+
Im157	Violet	_
Im158	Yellow	+
Im159	Violet	_
Is020	Violet	_
Is021	Yellow	+
Is015	Violet	_
Im013	Violet	_

Im020	Violet	_
Im060	Violet	_
Im065	Violet	_
Im074	Violet	_
Im073	Volet	_
Im081	Violet	_
Im083	Violet	_
Im103	Violet	_
Im117	Violet	_
Im125	Violet	_
Im133	Violet	_
Im137	Violet	_

Key:

Yellow (+) = positive reaction for β-lactamase production

Violet (-) = negative reaction for β-lactamase production