Molecular Characterization of Nosocomial Methicillin Resistant Staphylococcus aureus in Parts of Kaduna State, Nigeria

BY

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DECLARATION

I declare that the work reported in this dissertation was carried out by me in the Department of Microbiology, Ahmadu Bello University, Zaria, and under the supervision of Prof. O.S. Olonitola, Dr. S.E. Yakubu and Prof. A.J. Nok. The works of other investigators and Authors have been duly acknowledged.

I declare also that work has not been submitted elsewhere for the award of a degree.

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Opaluwa Saraja Ahmodu Sign Date

DEDICATION

I dedicate this work to my late mother who did all she could to get me educated. May her gentle soul, rest in perfect peace.

This dissertation is also dedicated to my beloved wife, Binta S. Ahmed, my children, Ismail, Habib, Hauwa, Munir and Sa'ad for their patience, support, love and encouragement during the course of the research.

CERTIFICATION

This dissertation entitle MOLECULAR CHARACTERIZATION OF NOSOCOMIAL METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS*(MRSA) IN PARTS OF KADUNA STATE,NIGERIA meets the regulations governing the awards of the degree of Doctor of philosophy of Ahmadu Bello University, Zaria and is approved for contribution to acknowledge and literary presentation.

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ABSTRACT

Molecular Characterization of Nosoconial methicillin resistant *Staphylococcus aureus* in three geographical areas of Kaduna state was undertaken. Standard procedure for identification, confirmation, and detection of mec A gene in staphylococci were followed. A total of 880 samples were examined from the geographical areas, between January 2008 to June 2010. An overall prevalence of 8.3% (n=73) was recorded for Staphylococcus infection. Methicillin resistant *Staphylococcus aureus* (MRSA) accounted for 56% (n=41) of all Nosocomial *Staphylococcus aureus* isolates. 95% (n=70) of isolates were recorded from wound, urinogenitals and blood and 5% (n=3) isolates came from other sites (Sputum, and skin). Genarally age groups of peak infection were 1-10 and 41-50. Most of the MRSA isolates came from male, female and surgical wards, but recovery rate of MRSA was more in accident and emergency and Special Care Baby Unit (SCBU). Coagulase negative Staphylococcus (CoNs) accounted for 40% while coagulase positive Staphylococcus accounted for 60% of MRSA isolates. There was a strong association between resistance to antibiotics and degree of coagulase produced ($P < 0.05$). The number of isolates susceptible decreased when the break point interpretative criteria for establishing resistance was employed. Cefoxitin had sensitivity of 100% and specificity of 58%. 9.8% (n=5) of isolates from Zaria resisted all drugs (superbug) while 53% (n=27) were

sensitive to all drugs tested. In Kafanchan, 8.3% (n=1) resisted all drugs tested. 15.7% (n=8), 25% (n=3) and 0% (n=0) of isolates, are of erythromycin-inducible, clindamycin resistant strains in Zaria, Kafanchan and Birnin Gwari respectively. Overall 16.4% (n=12) resisted Microlide lincosamin streptogramin –B (MLS) and so are likely to possess erythromycin ribosomal methylase 'erm' gene. Antibiotic profile results of isolates showed that 31.8% (n=7) were resistant to one antibiotic only. 13.6% (n=3) resisted two antibiotics and 54.5% (n=12) resisted three or more antibiotics. Quinolone and vancomycin resistant MRSA were detected in hospitals studied. 66.7% (n=34), 75% (n=9) and 90% (n=9) produced β-lactamase enzyme in Zaria, Kafanchan and Birnin-gwari respectively. 25% (n=13), 25% (n=3) expressed PBP2 in Zaria and Kafanchan respectively. Capsular polysaccharide 5&8 were found in 86% (n=44), 83% (n=10) and 70% (n=7) of isolates from Zaria, Kafanchan and Birnin-gwari respectively. Overall, Mec A gene was detected in 28.1% (n=9), comprising 18.8% (n=6) in Zaria and 9.3% (n=3) in Kafanchan. Mec A gene was not detected among isolates from Birnin-gwari and so were its product-PBP2. This result established the existence of Methicillin Resistant *Staphylococcus aureus* (MRSA) in all the hospitals studied and that mec A gene were detected in two of the three Hospitals studied.

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ABBREVIATIONS AND SYMBOLS

WORDS	ABBREVIATION
Antenatal Clinic…….. ……	ANC
Paediatric Medical Ward……..	PMW
Paediatric Surgical Ward……..	PSW
Male Surgical Ward……..	MSW
Male Medical Ward……...	MMW
Female Medical Ward……	FMW
Female Surgical Ward……	FSW
Special Care Baby Unit……	SCBU
Accident & emergency…… …….....	A/E
Urology Unit……..	U I
Emergency Paediatric Unit……. ……	EPU
Surgical Outpatient Department…….	SOPD
General Outpatient Department…….	GOPD
Coagulate positive staphylococcus…… ……	COPS
Coagulase negative staphylococcus……	CONS
Sensitive…… …… ……	SENS
Intermediate……..	INT
Resistant……	RES

Methicillin Resistant Staphylocuccu aureus……MRSA

Methicillin Sensitive Staphylocuccus aureus…….MSSA

Vancomycin intermediate Staphylocuccus aureus…….....VISA

Vancomycin Resistant Staphylocuccus aureus……..VRSA

CHAPTER ONE

INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) is a major problem around the world causing hospital acquired infections and, more recently, infections in the community. The prevalence of MRSA colonization in healthy persons in the community has been shown to be low even when MRSA is highly endemic in hospital settings (Diekam *et al*., 2001). The glycopeptides, particularly vancomycin, have been the mainstays of therapy for MRSA, and the emergence of resistance to this and the new generation antibiotics- the fluoroquinolones is of great concern (Diekam *et al*.,2001;Weber *et al*.,2003).A prevalence of 1.5% was reported for MRSA in Ile- Ife (Adebayo *et al*.,2006). However in other parts of Nigeria, higher prevalence of between 9-50% has been documented (Rotimi *et al*., 1987; Okesola *et al*., 1999). In some U.S. hospitals, MRSA already accounts for 30% to 50% of all nosocomial *S. aureus* isolates. In Portugal and Italy, it is >50% and in Turkey and Greece it is >30%. Methicillin resistance rate was low, 2.0% in Netherlands, 1.8% in Switzerland and < 1% in Scandinavia. These countries with low prevalence are noted for innovative interventions that improve hand hygiene in hospitals and thereby reducing MRSA transmission (Naimi *et al*., 2001). Established risk factors for MRSA infection include recent hospitalization or surgery, residence in long-term care facility, dialysis, and indwelling percutaneous medical devices and catheters. Recently, however, cases of MRSA have been documented in healthy community-dwelling persons without established risk factors for MRSA acquisition. Because they are apparently acquired in the community, these infections are referred to as community- acquired (CA) – MRSA (Naimi *et al*., 2001). Two distinct groups of MRSA have been recognized: - one representing multi-resistant epidemic strains and the other Beta-lactam resistant strains. Genetic background of MRSA

isolates from different continents varies but share type IV SCC mec cassette and the PVL locus (Baba *et al*., 2002), which was found to be associated with skin infections and severe necrotizing pneumonia (Jaroud *et al.,* 2002). The genetic background of MRSA isolates also varies between hospital and community acquired strains all of which show varying degrees of susceptibility to antibiotics (Weber *et al*., 2003). Very little is known of the prevalence of the MRSA in nosocomial cases in our hospitals. Little is also known of the anti-biogram of MRSA in our locality in the light of the development of resistance to new generation anti-biotics and their roles in treatment failure. The study thus, aims to:

i) Determine the strain type (MRSA) in Hospitals in parts of Kaduna State.

ii) Determine prevalence of MRSA in Nosocomial infections in parts of Kaduna State.

iii) Determine anti-biogram of MRSA isolated in Hospitals in parts of Kaduna State.

iv) Establish existence of fluoroquinolone, Vancomycin and Beta-lactam resistant MRSA in Hospitals studied.

1.1 Research Justification:

During the past few years, news on MRSA has usually been discouraging. Clinician and infection control practitioners appear to have lost confidence in their capability to control the nosocomial spread of this pathogen. This is because methicillin resistant *Staphylococcus aureus* (MRSA) are resistant to methicillin and related penicillin's and are particularly difficult to treat because they are also resistant to most other common antibiotics. Infections caused by MRSA may have a worse outcome than those resulting from methicilin sensitive strains. This has lead to increased cost of treatment, longer hospitalization and spread of infective agents. Antibiogram of MRSA is poorly understood in the light of development of resistance to new generation

antibiotics. Prescription policy is virtually non existence in our hospitals and where they exist, patients hardly comply with dosage regimen.

CHAPTER TWO

2.0 LITERATURE REVIEW:

2.1 Epidemiology:

Methicillin- resistant Staphylococcus aureus (MRSA) isolates came into being in 1961 following the introduction of methicillin in 1959. MRSA was first detected in hospitals and is one of the most common causes of nosocomial infections worldwide. A Europian study demonstrated that 50% of the infections in ICU are due in part to MRSA (Diekam *et al*., 2001). Antibiotic surveillance studies have shown that the prevalence of MRSA has increased steadily worldwide reaching 25-50%(Diekam *et al*.,2001).However, in some countries that have taken active preventive measures to avoid MRSA spread-such as comprehensive approaches to patient isolation, the incidence of MRSA in hospitals can remain low(Diekam *et al.,*2001). Much of the increase in prevalence is caused by the spread of epidemic strains of MRSA (EMRSA). The first EMRSA was recognized in the UK in the early 1980s and to date, 17 EMRSA have been identified (Aucken *et al*., 2002). In South Western Nigeria, only 3% of isolates were confirmed as MRSA based on detection of Mec-A gene and of one these MRSA was clonally related to the epidemic MRSA-15(Adebayo *et al*., 2006). During the period from March 1992 to March 1994, the patients admitted to the intensive care unit of the University of Maryland Shock- Trauma Center were monitored for the development of *S. aureus* infections (Nawas *et al*., 1998). Among the 776 patients eligible for the study, 60 (7.7%) patients developed 65 incidents of nosocomial *S. aureus* infections. Of the clinical isolates, 43.1% possessed a polysaccharide type 5 capsules, 44.6% possessed a type 8 capsule, and the remaining 12.3% had capsules that were not typed by the type 5 or type 8 antibodies (Nawas *et al*., 1998). Six antibiogram types were noted among the infection-related isolates, with the majority of the types being resistant only to penicillin and

ampicillin. It was noted that the majority of cases of pneumonia were caused by relatively susceptible strains, while resistant strains were isolated from patients with bacteremia and other infections (Nawas *et al*., 1998). Only 16 (6.3%) of the isolates were found to be methicillin-resistant *S. aureus* (MRSA). DNA fingerprinting by pulsed-field gel electrophoresis showed 36 different patterns, with characteristic patterns being found for MRSA strains and the strains with different capsular types. Clonal relationships were established, and the origins of the infection-related isolates in each patient were determined. Nawas *et al*.,(1998) concluded that (i) nosocomial infection-related isolates from the shock trauma patients did not belong to a single clone, although the predominance of a methicillin-resistant genotype was noted, (ii) most infection-related *S. aureus* isolates were relatively susceptible to antibiotics, but a MRSA strain was endemic, and (iii) for practical purposes, the combination of the results of capsular and antibiogram typing can be used as a useful epidemiological marker (Nawas *et al*., 1998). Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) appeared rapidly and unexpectedly in the United States, resulting in an epidemic caused primarily by isolates classified as USA300 (Adam *et al*., 2008). The evolutionary and molecular understanding of this epidemic is poorly understood. Specifically, it is unclear whether there has been clonal emergence of USA300 isolates or evolutionary convergence toward a hypervirulent phenotype resulting in the independent appearance of similar organisms. To definitively resolve this issue and understand the phylogeny of USA300 isolates, Adam *et al*., (2008) used comparative whole-genome sequencing to analyze 10 USA300 patient isolates from eight states in diverse geographic regions of the United States and multiple types of human infection. Eight of 10 isolates analyzed had very few single nucleotide polymorphisms (SNPs) and thus were closely related, indicating recent diversification rather than convergence. Unexpectedly, 2 of the

clonal isolates had significantly reduced mortality in a mouse sepsis model compared with the reference isolate ($P = 0.0002$), providing strong support to the idea that minimal genetic change in the bacterial genome can have profound effects on virulence (Adam *et al*., 2008). Taken together, the results demonstrate that there has been recent clonal expansion and diversification of a subset of isolates classified as USA300. The findings add an evolutionary dimension to the epidemiology and emergence of USA300 and suggest a similar mechanism for the pandemic occurrence and spread of penicillin-resistant *S. aureus* (known as phage-type 80/81 *S. aureus*) in the 1950s (Adam *et al*., 2008).World- wide, the Literature on the occurrence of methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals and long-term-care facilities in various countries of America, Africa, Asia, Australia/New Zealand, and Europe was reviewed(Voss and Doebbeling,1995). It is concluded that the increasing prevalence of MRSA is a worldwide problem, affecting both affluent and poor countries, and that infection control guidelines are needed in all regions affected. In Africa, Kesah *et al.*, (2003) investigated the prevalence and antibiotic susceptibility pathern of MRSA in eight African Hospitals and Malta). Of the total 1440 clinical isolates of *S. aureus*, methicillin resistance was detected in 213 (15%) of the 1440 isolates tested. The rate of MRSA was relatively high in Nigeria, Kenya, and Cameroon (21–30%), and below 10% in Tunisia, Malta, and Algeria. All MRSA isolates were sensitive to vancomycin, with MICs ≤ 4 mg/L. The isolates were also highly sensitive to ciprofloxacin, except in Kenya, Morocco, and Tunisia, where relative resistance to this drug was noted. Susceptibility to rifampin and fusidic acid seems to be correlated with the clinical use of these compounds. Only 46% of 59 MRSA strains analyzed were susceptible to rifampin, fusidic acid, and ciprofloxacin. The majority (> 60%) of MRSA strains were multi-resistant. There is a need to maintain surveillance and control of MRSA infections in Africa (Kesah *et al.*, 2003).

Heysell *et al*., (2011) investigated prevalence of MRSA nasal carriage among hospitalized patient with tuberculosis in rural Kwazulu-natal. Of 52 patients with an admission nasal swab, 11 (21%) were positive for MRSA. An additional 4 (10%) of patients with negative admission swabs were positive for MRSA on repeat testing. MRSA carriage on admission was more common among patients with previous hospitalisation, and among HIV-infected patients was significantly associated with lower CD4 counts (p=0.03). All MRSA isolates were resistant to cotrimoxazole, and 74% were resistant to ≥5 classes of antibiotics; all retained susceptibility to vancomycin.

Heysell *et al*.,(2011) concluded that there was a high prevalence of multidrug-resistant MRSA nasal carriage among patients. Studies are needed to validate nosocomial acquisition and to evaluate the impact of MRSA on morbidity and mortality among TB patients in similar settings.

2.2Transmission:

Transmission of MRSA occurs primarily from colonizers or infected patients via the hands of healthcare workers (Simor, 2001) Air-borne transmission appears to be important in the acquisition of nasal carriage (Solberg, 2002).

A greater risk of infection with MRSA exists if the patient has history of frequent hospital admissions, or admission to hospital within the last 6 months. At risk, are also patients known to have had previous MRSA infection or exposure to MRSA-colonized patients. Equally at risk are those who had undergone surgery or admission to an ICU within the last 6 months and those who had suffered from skin diseases or had extensive wounds and/or burns (Lina *et al*., 1999). Despite progress in our understanding of infectious disease biology and prevention, the conditions that select for the establishment and maintenance of microbial virulence remain enigmatic. To address this aspect of pathogen biology, (Massey *et al.,* 2006), focused on two

members of the Staphylococcus genus - *Staphylococcus aureus* and *Staphylococcus epidermidis* - and consider why *S. aureus* has evolved to become more virulent than *S. epidermidis*. Several hypotheses to explain this phenomenon are discussed and a mathematical model is used to argue that a complex transmission pathway is the key factor in explaining the evolution and maintenance of virulence in *S. aureus* (Massey *et al.,* 2006). In the case of *S. epidermidis*, where skin contact affords easier transmission between hosts, high levels of virulence do not offer an advantage to this pathogen (Massey *et al*., 2006). Out of 15 clients from whom MRSA was isolated in the previous study, 9 became MRSA positive (60.0%), and this was the case for 6 family members, living with 4 of them. The antibiotic resistance patterns coincided among the family members of 3 families, suggesting MRSA transmission among the client and his/her family member(s). MRSA transmission was shown not to be influenced by the activities of daily living (ADL) of a client nor by the content or time of the care provided by family member(s). As for visiting nurses, MRSA was isolated from 1 out of 148 (detection rate: 0.7%). The practice rate for hand washing was 91.2% after visiting as compared to 22.1% before visiting, and 93.4% after care as compared to 37.5% before care; the differences were significant ($P < 0.001$). The most frequently used hand washing procedures included hand washing with soap, povidone iodine and other disinfectants (Eaton, 2005). The practice rates were 94.9% in visiting nurse stations and 91.2% in clients' homes (Eaton, 2005). There was no significant difference between the procedures in these settings. To determine the carrier rate of methicillin-susceptible mecA-positive *Staphylococcus aureus* (dormant MRSA) among healthcare workers (HCWs), 447 nurses and physicians from 13 general wards and intensive care units were investigated for nasal or oropharyngeal S. aureus carriage during one year whenever an MRSA patient was treated(Kampf *et al*.,2003). Induction of phenotypic resistance in all mecA-positive oxacillin-

susceptible Staphylococcus aureus was attempted by 24 h exposure to oxacillin and cefotaxime. Organisms from the broth tube with the highest antibiotic concentration and visible growth after incubation were re-exposed for a total of seven repetitive exposures. Two mecA-negative oxacillin-susceptible *S. aureus* served as negative control. A population analysis before and after antibiotic exposure was performed by Kampf *et al*.,(2003). A third of the Health CareWorkers were found to be *S. aureus* carriers. Only three nurses were MRSA positive (0.7%). Seven isolates of dormant MRSA were isolated in six nurses and one doctor (1.6%). After four days of repetitive antibiotic exposure six of seven dormant MRSA were highly resistant to oxacillin. Resistance of the two controls *S. aureus* without the Mec A gene was not changed by repetitive antibiotic exposure (kampf *et al*., 2003). Two of the seven dormant MRSA were clonally related as shown by pulsed-field gel electrophoresis (PFGE). The pattern of another dormant MRSA was indistinguishable from an MRSA isolated from a patient who was treated at the same time on the same ward suggesting transmission from the Health Care Worker to the patient. Dormant MRSA may be isolated twice as often as MRSA from Health Care Workers. Transmission to patients is possible, which may lead to clinical infections. It might be useful to screen methicillin-susceptible *S. aureus* isolates from Health Care Workers for the mecA gene when recurrent infections with MRSA occur on a ward and the source cannot be found (Kampf *et al*., 2003). **Heterosexual transmission of community-associated methicillin-resistant** *Staphylococcus aureus* **has not been documented. As part of a survey conducted in northern Manhattan, (Heather *et al*., 2007) encountered 3 households in which heterosexual transmission was responsible for new community-associated methicillin-resistant** *S. aureus* **infection. The vaginal and inguinal isolates obtained from the sexual partners were USA 300. This report documents an important and previously unrecognized means of community-associated**

methicillin-resistant *S. aureus* colonization and transmission for these potentially invasive strains (Heather *et al*., 2007).

2.3 MRSA Clones: *Staphylococcus aureus* is still one of the most feared pathogens because of its ability to cause serious infections. The emergence and spread of methicillin-resistant *Staphylococcus aureus* (MRSA) has been documented in almost every continent and it is associated both with hospital and community-acquired infections. Although *S. aureus* has been found to express 13 capsular types, only two capsular polysaccharides (CPs), type 5 and type 8, accounted for about 80% of infectious isolates. A renewed interest emerged following the observation that both CPs are essential virulence factors and putative protective antigens (Von Eiff *et al*., 2006). The predominance of two capsular polysaccharides, type 5 and 8, on the surface of clinical isolates led to the developement of a conjugate vaccine (StaphVAX(TM)) based on capsular polysaccharides type 5 and 8 conjugated to a carrier protein. Verdier *et al*., (2006), studied capsular phenotype and genotype of 195 isolates representative of all clinical syndromes which encompassed both hospital and community-acquired infections. These isolates were mainly detected in France between January 2001 and December 2004. In this population, most of clinical isolates (87%) expressed either capsular polysaccharide type 5 (42%) or 8 (45%), while 13% were non-typeable by serotyping method with antibodies specific to capsular polysaccharide types 5 or 8. These 26 non-typeable strains were further serotyped and were demonstrated to express a cell wall surface antigen 336, a poly-ribitol phosphate N-acetylglucosamine, which resembles cell wall teichoic acid (Verdier *et al*., 2006). Among MRSA, Verdier *et al.,(*2006)found a predominance of serotype 5 for 64% of strains, whereas MSSA were predominated by capsular serotype 8 (60%). All *S. aureus* clinical isolates included

in this study have been investigated by PCR method, demonstrating that all isolates carried either the cap5 or cap8 locus (Verdier *et al*., 2006).

The evolutionary origins of MRSA are poorly understood, no rational nomenclature exists, and there is no consensus on the number of major MRSA clones or the relatedness of clones described from different countries (kreiswirth *et al*., 1993). Restriction fragment length polymorphisms in methicillin-susceptible and methicillin-resistant (MRSA) strains of *Staphylococcus aureus* isolated in the same hospital over a 4-month period were studied by using SmaI and ApaI digestion of genomic DNA and pulsed-field gel electrophoresis (Carles-Nurit *et al*., 1996). Each of the 20 methicillin-susceptible strains had a unique SmaI pattern, but the 27 MRSA strains showed only seven SmaI patterns. More than half of the SmaI fragments in all of these seven patterns were identical, as were those in the patterns from two unrelated MRSA strains. Digestion with ApaI, which cuts staphylococcus DNA into at least twice as many fragments, confirmed the results obtained with SmaI. Lastly, the plasmid contents of MRSA strains showing identical SmaI and ApaI electrophoretic patterns were not identical. These results are interpreted as supporting the hypothesis that all MRSA strains arose from a single clone and emphasize the need to use several methods in epidemiological investigations of MRSA outbreaks (Carles-Nurit *et al*., 1996).Soon after methicillin was introduced into clinical practice in the early 1960s, resistant strains of *Staphylococcus aureus* (MRSA) appeared, bearing a newly acquired resistance gene, Mec A, that encodes a penicillin binding protein, PBP2a (Kreiswirth *et al*., 1993). MRSA have spread throughout the world, and an investigation of the clonality of 472 isolates by DNA hybridization was performed. All 472 isolates could be divided into six temporally ordered Mec A hybridization patterns, and three of these were subdivided by the chromomosomal transposon Tn554. Each Tn554 pattern occurred in association with one and

only one Mec A pattern, suggesting that Mec A divergence preceded the acquisition of Tn554 in all cases and therefore that mecA may have been acquired just once by *S. aureus* (Kreiswirth *et al*., 1993).

It has been proposed(Kreiswirth *et al.,*1993;Carles-Nurit et al.,1996)that all MRSAs were descended from a single ancestral *S. aureus* strain that acquired *Mec A*, but studies elsewhere (Musser and Kapur, 1992, Fitzgerald *et al*.,2001) showed that some MRSAs are very divergent, implying that *Mec A* has been transferred between *S. aureus* lineages. A DNA microarray representing >90% of the *S. aureus* genome was used to characterize genomic diversity, evolutionary relationships, and virulence gene distribution among 36 strains of divergent clonal lineages, including methicillin-resistant strains and organisms causing toxic shock syndrome(Fitzgerald*etal*.,2001).Genetic variation in *S. aureus* is very extensive, with 22% of the genome comprising of dispensable genetic material. Eighteen large regions of difference were identified, and 10 of these regions have genes that encode putative virulence factors or proteins mediating antibiotic resistance (Fitzgerald *et al*., 2001). They found that lateral gene transfer played a fundamental role in the evolution of *S. aureus*. The *Mec* gene has been horizontally transferred into distinct *S. aureus* chromosomal backgrounds at least five times, demonstrating that methicillin-resistant strains have evolved multiple independent times, rather than from a single ancestral strain. This finding resolves a long-standing controversy in *S. aureus* research. The epidemic of toxic shock syndrome that occurred in the 1970s was caused by a change in the host environment, rather than rapid geographic dissemination of a new hypervirulent strain (Fitzgerald *et al*., 2001). DNA microarray analysis of large samples of clinically characterized strains provides broad insights into evolution, pathogenesis, and disease emergence (Fitzgerald *et al*., 2001).

Enright *et al*., 2002, resolved all of these issues and provide a more thorough and precise analysis of the evolution of MRSA clones than has previously been possible. Using multilocus sequence typing and an algorithm, BURST, Enright *et al*., (2002) analyzed an international collection of 912 MRSA and methicillin-susceptible *S. aureus* (MSSA) isolates. They identified 11 major MRSA clones within five groups of related genotypes. The putative ancestral genotype of each group and the most parsimonious patterns of descent of isolates from each ancestor were inferred by using BURST, which, together with analysis of the methicillin resistance genes, established the likely evolutionary origins of each major MRSA clone, the genotype of the original MRSA clone and its MSSA progenitor, and the extent of acquisition and horizontal movement of the methicillin resistance genes (Enright *et al*., 2002). Major MRSA clones have arisen repeatedly from successful epidemic MSSA strains, and isolates with decreased susceptibility to vancomycin, the antibiotic of last resort, are arising from some of these major MRSA clones, highlighting a depressing progression of increasing drug resistance within a small number of ecologically successful *S. aureus* genotypes (Enright *et al*., 2002).

MRSA is also emerging in community (Chamber, 2001; Hiramatsu *et al*., 2001).Although no systemic population based surveillance studies exist of *S. aureus* in the community, one hospital based study found that more than 40% of MRSA infections were acquired before hospital admission (Layton *et al*., 1995). In that particular study community acquisition of MRSA was associated with recent hospitalization, previous antibiotic therapy, residence in a long term care facility (e.g Nursing home) and intravenous drug abuse (Layton *et al*., 1995). However, colonization and transmission of community acquired MRSA has also been described in individuals(including children) lacking these risk factors(Chambers,2001;Simor,2001).It is now recognized that large numbers of MRSA strain causing infections in the community are

microscopically distinct from those associated with infections in hospitals and long-term care facilities(Naimi *et al*.,2003). Only a limited number of clones are responsible for epidemic spread of MRSA (Jaroud *et al*., 2002)

Vancomycin resistance has emerged recently in S. aureus. Since the first report of vancomycin intermediate-resistant S. aureus(VISA,MIC 18-16mg/l) in Japan in 1996,there have been reports of intermediate resistant strains worldwide. However, in 2002 Vancomycin-resistant S. aureus(VRSA,MIC32mg/l) was reported in USA(Sievert *et al*.,2002) although such strains are very rare.

2.4 Drug Resistance:

Methicillin resistance in staphylococci is caused by an acquired penicillin-binding protein, PBP2a, which is encoded by the chromosomally located Mec-A gene. This alteration in the target site confers resistance to all β-lactam and their derivatives. The Mec A gene is carried on a mobile genetic element, the staphylococcal cassette chromosome Mec(SCCmec) of which four forms have been described that differ in size and genetic composition(Haramatsu *et al.,*2001). Many MRSA isolates are resistant to multiple antimicrobial agents. Ciprofloxacin resistance among MRSA is a common problem worldwide and is a result of at least two resistance mutations, usually in the parc, gyr A genes (Hooper, 2002). Mutations in these genes not only render the organism highly resistant to ciprofloxacin, but also cross-resistant to other quinolones. The first reported case of MRSA showing intermediate resistance to vancomycin and teicoplanin appeared in the medical literature in1997 (Hiramatsu, 1997). Adebayo *et al*., (2006) studied the antibiotic susceptibility of 200 non duplicate *S. aureus* isolates from clinical samples. All the isolates were susceptible to teicoplanin, vancomycin, fusidic acid and rifampicin and less than

3% were resistant to erythromycin, clindamycin, neomycin and monocycline. The high rates of Meticillin-sensitive *S. aureus* (MSSA) resistance to sulphonamides and tetracycline could be attributed to widespread use of these antibacterial agents in Nigeria (Ako-Nai et al.,1991). Gianneechini *et al*., (2002) reported a 47.6% resistance to penicillin for coagulase positive staphylococcus isolates while a 27% resistance was reported for CoNs.

2.4.1Vancomycin Resistant Staphylococcus aureus(VRSA).

The mechanisms of vancomycin resistance in *S. aureus* are not fully understood but it appears to be different for VISA and VRSA (Fridkin *et al*., 2003). VISA strains synthesis increased quantities of peptidoglycan leading to a thickened cell wall. There is also decreased cross-link of peptidoglycan strands trapping vancomycin in the cell wall; VRSA strains acquire Van A from enterococci by a conjugative process. This alters the efficient means of disseminating resistant genes among staphylococci, increasing the likelihood that VRSA will become more prevalent. Cross-resistance is common, particularly with Methicillin and other glycopeptides such as teicoplanin.

VISA and VRSA are not specifically associated with ICUs, but are associated with prolonged exposure to antibiotics, especially vancomycin therapy for MRSA infection (Fridkin *et al*., 2003). Fortunately, VISA and VRSA is usually susceptible to other antibiotics and transmission from patient to patient or patient to Health care worker is rare.

2.4.2 **Quinolone Resistance:**

Quinolones were first introduced for the treatment of gram-negative bacteria infections in the 1980s. Compared with older quinolones, however, the newer generations of agents have a broad spectrum of activity and are used to treat gram-positive infections. As a result, there has been widespread use of quinolones (Fridkin *et al*., 2002).Resistance to quinolones is a growing

problem in hospitals not only among patients in ICUs but also in non-ICU patients (Fridkin *et al*., 2002). In the USA, overall susceptibility to ciprofloxacin of Gram-negative aerobic isolates from ICU patients decreased from 86% in 1994 to 76% in 2000. This rise in quinolone resistance was associated with the increased use of quinolones (Neuhauser *et al*., 2003). Louiselle *et al*.,(2006) investigated the role of antimicrobial drugs in facilitating Healthcare- associated Methicillin-resistant *Staphylococcus aureus*(MRSA) colonization and infection and compared this role with their effects on Methicillin susceptible S. aureus infection. Of the 150 episodes of MRSA colonization and 23 of MRSA infection, fluoroquinolones were the only antimicrobials that increased risk for colonization.

There are several mechanisms of quinolone resistance: decreased membrane permeability, active drug efflux, quinolone resistance developes mainly through spontaneous step wise chromosomal mutations causing alterations in the target enzymes-DNA gyrase and topoisomerase iv. These amino acid alterations occur in critical regions of the enzyme-DNA complex called the quinolone resistance determining region and reduce the drugs affinity for its target (Schmitz *et al*., 2002). The par c subunit of topoisomrase -iv and gyr A subunit of DNA gyrase are the most common sites for resistance mutations to occur. Single amino acid mutation may be sufficient to confer clinical resistance, but additional mutations are usually necessary to develop resistance against the more active quinolones (Jones and Pfaller, 1998). Quinolone resistance can also develop by induction of a multi drug efflux pump and or decrease in the intracellular concentration of the drug. These resistance mechanisms can occur alone or in combination and it is thought that several mechanisms must occur in combination to result in high level resistance. The mechanisms of resistance vary with the quinolone used and the species of organism involved (Jones and Pfaller, 1998).

2.5 Pathogenesis:

Though a number of microbial agents have been implicated in septicaemia, *Staphylococcus aureus* was considered a leading cause of many hospital and community infections including hospital acquired bacteremia, pneumonia and surgical wound infections (Opaluwa *et al*., 2009).The molecular mechanism of *Staphylococcus aureus* phathogenicity is complex and involves several toxins, including the famous staphylococcal enterotoxin (SE) and toxic shock syndrome toxin-1 (TSST-1). Although these toxins were discovered in specific clinical contexts of food poisoning and menstrual toxic shock syndrome, they share common biochemical and biological properties (Damien *et al*., 2007). As superantigens they are able to massively activate mononuclear cells and T cells regardless of the antigenic specificity of the T cells. To date, 19 different enterotoxins and related toxins have been described in *S. aureus* with some differences in structure and biological activity (Damien *et al*., 2007). It has been clearly demonstrated that most human *S. aureus* isolates harbour at least one gene encoding for these toxins. It is suspected that *S. aureus* produces SEs and TSST-1 in humans from colonization to infection, whatever the clinical situation. It is proposed that the production of SEs plays a role not only in classical staphylococcal infections but also in noninfectious diseases. This review focused on recent findings related to staphylococcal superantigens and their impact on human diseases (Damien *et al*; 2007). A single toxin, Panton-Valentine leukocidin (PVL), has been linked by epidemiological studies to community-associated MRSA (CA-MRSA) disease. However, the role that PVL plays in the pathogenesis of CA-MRSA has not been tested directly. To that end, (Jovanka *et al*; 2006) used mouse infection models to compare the virulence of PVL-positive with that of PVL-negative CA-MRSA representing the leading disease-causing strains.

Unexpectedly, strains lacking PVL were as virulent in mouse sepsis and abscess models as those containing the leukotoxin. Isogenic PVL-negative (lukS/F-PV knockout) strains of USA300 and USA400 were as lethal as wild-type strains in a sepsis model, and they caused comparable skin disease. Moreover, lysis of human neutrophils and pathogen survival after phagocytosis were similar between wild-type and mutant strains (Jovanka *et al*., 2006). Although the toxin may be a highly linked epidemiological marker for CA-MRSA strains, Jovanka *et al*., (2006) concluded that PVL is not the major virulence determinant of CA-MRSA.

Mortality attributed to MRSA was significantly higher than for methicillin-sensitve *S. aureus*(MSSA), however, there was no significant diference in terms of the rate of disseminated infection caused by MRSA and MSSA .MRSA strains may, therefore, not have increased virulence compared with MSSA strains (Melzaer *et al*., 2003).Potential reasons MRSA may cause worse outcome are: possible enhanced virulence of the antibiotic- resistant pathogen, decreased effectiveness of vancomycin , which is invariably used to treat MRSA, delay in appropriate antibiotic selection(Cosgrove *et al*.,2003)

Since MRSA is resistant to other antimicrobial agents, including β-lactams and non β-lactam, MRSA infections are often treated with glycopeptides (vancomycin and teicoplanins (Hiramatsu *et al*., 1997; Weber *et al*., 2003). However, glypeptide resistance in *S aureu*s has emerged recently, and should it arise in more multi-resistant strains of MRSA (such as EMRSA 17), this will make successful treatment much more difficult (Hiratmasu *et al*., 2001).

Quinolone resistant is associated with marked co-resistance to other frequently used antibiotics. High rates of resistance among nosocomial pathogens such as MRSA and VRSA mean that even newer more potent quinolones are unlikely to be clinically useful (Jones and Pfaller, 1998; Weber, *et al*., 2003).

2.6 **Diagnosis of MRSA:**

There are many studies comparing the abilities of different methods to detect methicillin resistance in *S. aureus*. Early detection through regular antibiotic susceptibility surveillance is important. Traditional methods for the detection of MRSA from surveillance and screening specimens have included the use of a variety of selective and differential solid media(Davies *et al*.,2000;Safdar *et al*.,2003) They usually employ colonies of previously identified *S. aureus* isolates (Davies *et al*.,2000,) that have been screened for growth on selective agar with methicillin or oxacillin (Gardam *et al*.,2001), or the use of different standardized susceptibility testing methods for the discrimination of MRSA from susceptible or borderline-resistant *S. aureus* (Gardam *et al*.,2001;Diekam *et al*.,2001).Other studies have evaluated the simultaneous detection and identification of MRSA isolates by plating clinical surveillance specimens in selective agar media (Davies *et al*.,2000). The use of chromogenic media for detection of MRSA was also described elsewhere (Kluytmans *et al*., 2002;Flayhart *et al*.,2005). Monoclonal antibodies (MAbs) reactive with serotype 5 and 8 capsular polysaccharides of *Staphylococcus aureus* have been used to test, by enzyme-linked immunosorbent assay (ELISA), blood culture fluids for thepresence of *S. aureus*(Boutonier *et al*.,1989). A total of 748 blood cultures from 665 patients yielding 706 bacterial isolates belongingto more than 26 bacterial species were studied. Ail blood cultures containing bacterial strains belonging to species other than *S. aureus* were negative in ELISA. All 23 blood cultures containing serotype 5 *S. aureus* were positive in ELISA with the corresponding MAb. Out of 20 blood cultures containing serotype 8 *S. aureus*, 19 were positive with the corresponding MAb. AIl 5 blood cultures containing nontypeable *S. aureus* were negative in ELISA with both MAbs. This method provides reliable identification of

serotypes 5 or 8 *S. aureus* by direct testing of blood culture fluids with ELISA (Boutonier *et al*., 1989).

Soft Salt-Mannitol Agar–Cloxacillin Test: a Highly Specific Bedside Screening Test for Detection of Colonization with Methicillin-Resistant *Staphylococcus aureus was described Mir et al., 1998.* In this study, tubes containing soft salt-mannitol agar with cloxacillin (6 μg/ml) (SSMAC) were prepared for inoculation of clinical samples at patients' bedsides by personnel of an intensive-care unit. A total of 1,914 swabs from different sample sites of 81 patients were dipped into SSMAC tubes, and after 24 h of incubation (in an incubator located near the intensive-care unit), an evident color change was considered by the intensive-care-unit personnel to be an MRSA alarm (Mir *et al*., 1998). Sixty-three (3.3%) SSMAC tubes were considered positive for MRSA, 1,827 (95.4%) were considered negative, and 24 (1.2%) were considered intermediate. Compared with values for parallel conventional surveillance cultures for MRSA, excluding tubes with intermediate results, the SSMAC test had a sensitivity of 72.7%, a specificity of 99.2%, a positive predictive value of 76.2%, and a negative predictive value of 99.0%. When intermediate tubes were considered positive, the corresponding values were 75.3, 98.2, 63.2, and 99.0%, respectively. The sensitivity and specificity values of the test to identify MRSA-colonized patients were 89.4 and 100%, respectively (Mir *et al*., 1998). Oropharyngeal and naris specimens were the most reliable samples for MRSA detection. False-negative results were frequent in bronchial aspirates with low ($<10^3$ to 10^6 CFU/ml) MRSA counts. False-positive results were mainly due to methicillin-resistant *Staphylococcus haemolyticus*. The SSMAC tube is a useful, rapid, and inexpensive tool for the early identification of MRSA-colonized patients and, consequently, for the implementation of measures to prevent the spread of MRSA (Mir *et al*., 1998). Manitol salt with cefoxitin in screening for MRSA has also been

described (Smyth and Kahlmeter, 2005; Stoakes *et al*., 2006) and more recently, Salwa *et al*.,(2009) rapidly detected MRSA in swabs using MRSA-select medium and PCR for mec A. The MRSA-select agar detected the majority of cases of MRSA but when compared, the mec A gene PCR assay was more specific, provided significantly faster turnaround times, and resulted in more prompt isolation of MRSA in clinical specimens (Salwa *et al*., 2009). Siripornmongcolchai *et al*.,(2002);Oberdofer *et al*.,(2006) evaluated the diagnostic values of multiple and single locus in the diagnosis of MRSA. The results were compared with those of convectional culture swabs from several body sites. When compared with culture swabs from the nose, throat and wounds, the diagnostic values of the real time PCR test from nasal swabs were as follows;92.3% sensitive,98.6% specific 75% positive predictive value, and 99.6% negative predictive value(Oberdofer *et al*.,2006).A number of recent studies(Fermandes *et al*.,2005,Anand *et al*.,2009) have indicated that the use of cefoxitin-a cephamycin has higher sensitivity and specificity than other compounds traditionally recommended for detecting mec A resistance in staphylococci. Cefoxitin could serve as a surrogate marker for mec A gene detection among staphylococci particularly in resource constrained localities (Fermandes *et al*., 2005), An unusual strain of methicillin-resistant *Staphylococcus aureus* (MRSA) was repeatedly isolated from infants in a newborn special care unit (NBSC) and a newborn intensive care unit. Between January 1989 and March 1990(Wanger *et al*., 1992), approximately 100 isolates from infected or colonized infants were recovered. Surveillance cultures taken during this time revealed a 20% colonization rate, which was defined as recovery of MRSA from the nares, umbilicus, or groin (Wanger *et al*., 1992; Mir *et al*., 1998; Von Eiff *et al*., 2006). Isolates were identified as *S. aureus* by tube coagulase reactivity and heat-stable nuclease production but were unreactive in a latex agglutination assay (Wanger *et al*., 1992). Representative isolates that were collected

during the outbreak and that were found to share the latex agglutination assay-negative phenotype were compared by antibiogram (12 isolates), bacteriophage typing (20 isolates), capsular polysaccharide typing (30 isolates), and plasmid as well as chromosomal DNA analyses (20 isolates). All isolates known to be associated with the outbreak had nearly identical antibiograms (Wanger *et al*., 1992), and were notably susceptible to clindamycin (Siberry *et al.,* 2003). Staphylococcal bacteriophage typing was not useful in determining the relatedness of the isolates, since the majorities were nontypeable (Davies and Martins, 1987). Plasmid pattern analysis revealed one large plasmid (approximately 100 kb) of equivalent size among the isolates. Capsular polysaccharide typing revealed that 14 of 30 isolates tested were type 5(Wanger *et al*., 1992, Von Eiff *et al*., 2006). Isolates identified in children at two other hospitals in the city which were also unreactive by the latex agglutination assay and clindamycin susceptible had plasmid and antibiogram patterns identical to those of isolates from the New Born Special Care (Wanger *et al*., 1992). Pulsed-field gel electrophoresis of restriction enzyme-digested genomic DNAs from the outbreak isolates demonstrated identical patterns which could be clearly differentiated from those of other unrelated MRSA (Tenover *et al.,* 1995). The strain from the New Born Special Care is, therefore, unique and underscores the need for caution in interpreting the latex agglutination reactivities of MRSA isolates (wanger *et al*., 1992). Cuevaso *et al*., (2003) compared the PBP2a test (OXOID. Hampshire. UK), with the POS Combo 1S panels of the MicroScan (MS) system (Dade Behring, Sacramento, CA), in the analysis of 137 MRSA and 151 methicillin-susceptible *Staphylococcus aureus* (MSSA) obtained from 143 Spanish hospitals. PCR for the MecA gene was used as the "gold standard" assay. MSSA (ATCC 25923 and MRSA ATCC 33591) were used as control strains. The PBP2a test had a sensitivity of 100% and a specificity of 98%. Three isolates that lacked MecA were positive with the PBP2a

test (Cuevaso *et al*., 2003). The MS system with readings after 24 h. of incubation had a sensitivity of 96% and a specificity of 98%. Of 27 borderline oxacillin-resistant isolates (BORSA) for which the MIC of oxacillin was 1 to 2 mg/L and which were MecA negative, three tested positive by the PBP2a test (90% specificity). Against these 3 isolates, the oxacillin MIC was 2 mg/L. They concluded that MRSA-Screen Latex Agglutination Test for detection of PBP2a is a rapid, easy to perform, and accurate method for the detection of MRSA. This test provides an alternative to the detection of the MecA gene by PCR in clinical laboratories when this technique is unavailable (Cuevaso *et al*., 2003). Methicillin resistance, particularly hetero-methicillin resistance, in *Staphylococcus aureus* can be difficult to detect by phenotypic methods. Subsequently detection of mecA gene using PCR methods are generally considered the gold standard (Merlino *et al*., 2010).The National Committee for Clinical Laboratory Standards recently, changed the oxacillin breakpoint from ≥4 mg/liter to ≥0.5 mg/liter to detect methicillin-resistant coagulase-negative staphylococci (CoNS) because the previous breakpoint lacked sensitivity (Hussain *et al*.,2000). To determine the correlation between the new oxacillin breakpoint and the presence of the *MecA* gene, 493 CoNS of 11 species were tested. The presence of the *MecA* gene was determined by PCR, and oxacillin susceptibility was determined by the agar dilution method with Mueller-Hinton agar containing 2% NaCl and oxacillin (0.125 to 4.0 mg/liter). The new breakpoint correctly classified all CoNS strains with *MecA* as methicillin resistant and strains of *Staphylococcus epidermidis*, *S. haemolyticus*, and *S. hominis* without *MecA* as methicillin susceptible(Hussain *et al*;2000). The breakpoint of ≥0.5 mg/liter was not specific for *S. cohnii*, *S. lugdunensis*, *S. saprophyticus*, *S. warneri*, and *S. xylosus*, in that it categorized 70 of 74 strains of these species without *mecA* (94.6%) as methicillin resistant. The results of this study indicate that the new oxacillin breakpoint accurately identifies strains of

CoNS with *MecA* but is not specific for strains of certain species of CoNS without *MecA(*Hussain *et al.,2000)*.

2.7 Control of MRSA.

Infection measures include screening and isolation of newly admitted patients suspected of carrying MRSA or *S. aureus* with intermediate resistance vancomycin, implementing infection control programes to prevent resistant strains being transmitted between patients and Hospital staff, and establishing an adequate antibiotic policy to minimize the development of antibiotic resistance(Solberg, 2000). Infections caused by MRSA may have worse outcome than those resulting from methicillin-susceptible strains. In one study, mortality rates were significantly increased in patients who had MRSA bacteriemia compared with methicillin susceptible *Staphylococcus aureus*(Cosgrove *et al*.,2003)

Minimizing the antibiotic pressure that favours selection of resistant strains is essential to slow the emergence of MRSA in the hospital and the community; improved knowledge and education on the best way to ensure favourable infection control practices are needed. Various recommendations for preventing the spread of VISA and VRSA have been made including careful detection of *S. aureus* unrelated to vancomycin resistance. Education of healthcare workers about infection control, strict adherence to infection control measures and surveillance for the emergence of VISA and VRSA (Fridkin *et al*., 2003)

Continued overuse of quinolones will promote the spread and selection of resistance and may limit the effectiveness of these agents. It is important to use them at appropriate doses. Borer *et al*., (2002) investigated the control of a community- acquired outbreak of MRSA skin infections in a closed community of institutionalized adults with developmental disabilities.

20(71%) of the 28 residents had 73 infectious episodes. Of the cultures, 60% and 32% obtained from residents and personnel, respectively, grew *S. aureus;* 96% and 27% were MRSA. Infection control measures, antibiotic restriction, and appropriate therapy resulted in successful termination of this out-break (Borer *et al*., 2002).

It is well known that Methicillin-resistant *Staphylococcus aureus* (MRSA) is hardly controllable organism among pathogens of nosocomial infection.(Jin *et al*.,1999) The MRSA infection control measures have been initiated at a brand new tertiary care teaching hospital which was opened in June, 1994. However, the control measures did bring out little effect. In 1997, reinforcement of all control measures were practiced in intensive care units (Jin *et al*., (1999). The measures brought out a significant improvement in reducing the incidence of MRSA infection, subsequently the same control measures were implemented through-out the entire inpatient area. The following control measures have been reinforced since March 1997: first, application of thorough surveillance of confirmed MRSA infected patients: second, providing cohort care: third, enforcing hand washing practices after patient contact; fourth, establishing infected patients isolation zone: fifth, tagging infected patient's bed and medical record, providing disinfectant spray for washing hands, identifying and treating carriers among patient contact staffs, separate disposal of contaminated wastes, and finally repeating education of nursing staff and family members of the patients(Jin *et al*.,1999). Each month the number of incidence in MRSA nosocomial infection were followed and the leu supervisors were notified the outcome. Showed that the incidence of MRSA infection started to decline soon after the initiation of the control measures, from 132% in March 1997 to 5.8% in July 1997. In 1998, the infection rate maintained close to 2-3%. There had been 467 MRSA infected cases (5.7%) out of 8,253 discharges during the study period; among them 319 cases were infected once; 40 cases

twice; 15 cases three times: four cases four times and 1 case seven times (Jin *et al*., 1999). The orders of preference of organs infected are lungs (56.3%), wounds (11.8%), blood (7.9%), and urinary tract (1.9%). The highest incidence of this infection was found in Medicine (34.8%) and Neurosurgery (22.8%).It was concluded that the implementation and reinforcement of infection control measures are key to successful control of nosocomial infection, in particular, hand washing of patient contact staffs and eradication of carriers could be the most effective measures (Jin *et al*., 1999).

CHAPTER THREE

3.0 MATERIALS AND METHODS:

3.1 Materials:

3.1.1 Media:

i) Mannitol-salt Agar (OxoidCM0085) Basingstoke, Hampshire, England.

ii) Blood agar (Oxoid CM271)Basingstoke,Hampshire, England.

iii) DNASe Agar (oxoidCM0321)Basingstoke,Hampshire, England.

iv)MuellerHintonAgar(OxoidCM0337)Basingstoke,Hampshire,England.

3.1.2 DISC: Standard antibiotic discs were used. This included:

i)Oxacillin(1µg),

ii)Cefoxitin(30µg),

iii)Vancomycin(5µg,

iv)Ciprofloxacin(5µg,

v) Erythromycin (5µg) and

vi) Clindamycin(2µg).

3.1.3Standard Strains of *Staphylococcus aureus*:

i) ATCC------25923

ii) ATCC-----12228

iii) ATCC----29213

3.1.4Equipment and glassware's:

i) Refrigerator,

ii) Incubator,

iii) Hot air oven,

iv)Test tubes,

v) Microscope,

vi)Slide/cover slips,

vii) Autoclave

viii) Sterile Disposable petridishes

ix) Standard pippetes

x) Standard wire loops

3.1.5 Stains and Reagents:

i) Gram stains

ii) Plasma

iii) Hydrogen peroxide

iv)Normal saline

iiv) 1N Hydrochloric acid

3.1.6 Kits:

i) Staphytec plus (BR0066 Oxoid)^{R}

ii) β-Lactamase strip (Oxoid).

iii) RT- PCR (Roche Diagnostics).

iv) Latex test for PBP2A Denka Seiken(DR900A).

3.1.7 Culture Media and preparation of culture media:

All media used were prepared according to manufacturers specifications. Appropriate amounts of the medium powder were weighed, hydrated with the required volume of distilled water and shaken in a conical flask to allow complete dissolution. They were then sterilized in an autoclave at 121^{o}C for 15minutes at the pressure of 15 Ibs. Media were prepared and dispensed under strict aseptic conditions.

3.1.8 **Preparation of Mannitol Salt Agar**:

About 111g of agar powder was aseptically weighed into a sterile conical flask. This was suspended in 1litre of distilled water and brought to boil to dissolve completely. It was then sterilized by autoclaving at121^{o}C for15minutes.It was mixed well, poured, flamed to remove bubbles and allowed to set. Plates were then packed and stored in the refrigerator until ready to use.

3.1.9 Preparation of Blood Agar:

About 37g of blood agar base was aseptically weighed in a sterile conical flask. Using sterile water, the content was made up to the 1 liter mark. The suspension was brought to boil to dissolve completely. When completely dissolved, the mouth of the flask was plugged with cotton wool and later covered with foil to make it waterproof. It was mixed and sterilized by autoclaving at 121^{o}C for 15minutes.It was cooled to 45^{o}C and 7% sterile blood added. The

content was mixed thoroughly, poured, flamed to remove air bubbles and allowed to set. The prepared blood agar plates were stored in refrigerator until ready to use.

3.1.10 Preparation of Mueller Hinton Agar:

About 38g of agar powder was aseptically weighed and suspended in 1 liter of distilled water in a conical flask. It was brought to boil to dissolve completely. The flask mouth was plugged with cotton wool and foil to make it waterproof. The content was then sterilized by autoclaving at 121^{o}C for 15 minutes. The medium was poured aseptically into sterile petridishes to the level of about 4mm.Plates were flamed to remove air bubbles and allowed to set. Prepared Mueller Hinton agar plates were packed and stored in the refrigerator until ready to use.

3.1.11 Preparation of DNAse Agar:

About 39g of powder was aseptically weighed and suspended in 1 litre of distilled water. This was brought to boil to completely dissolve. Mouth of flask was plugged with cotton wool and foil to make it water proof and sterilized by autoclaving at 121^{o}C for 15 minutes. The medium was poured, flamed to remove air bubbles and allowed to set. The prepared plates were the stored until ready to use.

3.1.12Brain Heart Infusion broth:

About 37g of powder was aseptically weighed and suspended in 1litre of distilled water in a conical flask. This was soaked for 10 minutes, swirled to mix and warmed gently to dissolve. The content was then dispensed into universal bottles and sterilized by autoclaving at 121^{o}C for 15 minutes. The prepared broth was stored until ready to use.

3.2Methodology:

3.2.1 Study Area: The study was conducted in Hospitals from three geographical area: Zaria (Kaduna-central),Kafanchan (KadunaEast)andBirnin-Gwari(Kaduna-West).

3.2.3 Sample size: A total of 880 samples were examined from the 3 geographical areas, 560 in Zaria, 160 each from Kafanchan and Birnin-gwari respectively.

3.2.4 Isolation:

All samples (Bronchial aspirates, Urinogenital, oropharyngeal, rectal, Pus from wounds, naris, axilla, and groin samples) entering Medical Microbiology department of the various hospitals in which study was carried out were screened for *Staphylococcus aureus*. Age, sex, and sources of specimen (inpatient or outpatient) were recorded. Suspected colonies were directly inoculated onto Mannitol salt agar and incubated at 37oc for <48hrs. These were sub cultured on nutrient agar slants and stored for characterization. Staphylococci were identified by colony shape, gram stain, catalase and coagulase.

3.2.5 Confirmatory test:

Characteristics: Colonies of *S. aureus* are approximately 1µ in diameter, spherical, smooth with entire edge. They are non-sporing and non-motile Gram positive cocci in "grape-like" clusters. They are facultative anaerobes, Can grow on nutrient agar, on which golden yellow colonies is produced. Complete haemolysis of blood agar is produced by *Staphylococcus aureus* while *Staphylococcus epidermidis* is usually non-haemolytic. They are Catalase, DNase and coagulase positive.

3.2.6 Gram Stain: A portion of suspected colony was emulsified in a drop of normal saline placed on a clean grease free slide, allowed to air dry and fixed. The smear was flooded with crystal violet for 1 minute and washed with water. Lugols iodine was added for 1minute and washed with water. Acetone was applied briefly to decolorize the complex and then washed with water. A counter stain- neutral red was applied for 2 minutes, washed with water and air dried. The slide was examined using x100 oil immersion objective.

3.2.7 Slide Coagulase (clumping factor): A homogenous suspension of suspected *Staphylococcus* colonies were made in a drop of saline, on a clean grease free slide. This was mixed with a drop of undiluted human plasma and examined for clump formation. Clump formation within 15 seconds constitutes a positive test. In the control test only the suspension of organism was prepared. No plasma was added.

3.2.8 **Tube Coagulase test (Free coagulase)**: Ability to clot plasma continues to be the most widely used and generally accepted criterion for identification of pathogenic staphylococci.

The tube coagulase test was performed by mixing 0.1ml of an overnight culture in brain heart infusion broth with 0.5ml of reconstituted plasma (preferably in glass tube), incubating the mixture at 37^{o}C in water bath or heat block for 4hr, and observing the tube for clot formation by slow tilting the tube 90^{o} from the vertical. Any degree of clotting constitutes a positive test.

3.2.9. Catalase test:

Using a wooden spatula, a portion of the suspected colony was emulsified in a drop of hydrogen peroxide placed on a clean grease-free slide. Sudden bubble formation indicates a positive test.

3.2.10 **Haemolysis on Blood Agar:**

Zones produced around colonies of *Staphylococcus aureus* on blood agar was used to determine whether strain was haemolytic or not. Blood agar, freshly prepared and stored in refridgerator were brought out and dried in the incubator. Blood agar plates were divided into about 4 portions using grease pencil. Each *Staphylococcus aureus* isolate was inoculated on a portion; plates were incubated at 37^{o}C overnight and examined for haemolysis.

3.2.11 DNase Test:

Suspect colonies of *Staphylococcus* were inoculated on DNase agar; plates were incubated overnight at 37^{0}C. presense of DNase was tested by flooding plates with a weak 1N HCl.

Polymerized DNA precipitated in the presence of 1N HCl giving an opaque medium. A positive organism shows clear zones around colonies.

3.3 Characterization:

3.3.1 Susceptibility testing:

3.3.2 Preparation of 0.5 Mc Farland Standards:

About 1%v/v solution of sulphuric acid was prepared by adding 1ml of sulphuric acid in 99mls of water. A1% solution of barium chloride was prepared by adding 0.5g $BaCl_2$ in 50ml of water. 0.6ml of $BaCl_2$ solution was then added to 99.4ml of H_2SO_4. The turbid solution was transferred into a tube, caped and stored at room temperature.

Antimicrobial drug susceptibility was done using the Disc Diffusion method as described by Kirby-Bauer. Mueller Hinton Agar and discs of high potency were used. The inoculum was standardized by adjusting the turbidity of an actively- growing broth culture of *Staphylococcus* to match that of barium sulphate standard (0.5 Mc Farland standard). Mueller Hinton agar were removed from the refrigerator and dried in the incubator. The Mueller Hinton Agar plates were then inoculated with cotton-wool swabs moistened in this suspension. Each of the tested susceptibility discs were placed at least 20 mm apart. The plates were incubated immediately at 37^oC overnight. Zone diameters were measured in millimeter and compared with the standard table of interpretative criteria. Positive and Negative control strains ATCC (25923) and ATCC (29213) respectively of *Staphylococcus* were included in the test. Antibiotics tested include oxacillin, cefoxitin, ciprofloxacin, vancomycin, erythromycin and Clindamycin. Break point interpretative criteria were used to determine resistance or sensitivity.

3.3.2.1 Determination of Multiple Antibiotic Resistant (MAR) indexes of isolates.

The MAR index for isolates was calculated using the formula below.

MAR=Number of antibiotics to which resistant divided by number of antibiotics tested. The percentage of isolates with 0.5 MAR index was then determined.

3.3.3 B-lactamase testing: (penicillinase producing *Staphylococcus*): (Chromogenic cephalosporin Method): Nitrocephin sticks (BROO66A) (Oxoid^{R}) was used. A suspension of representative colony growing around Oxacillin or Cefoxitin disk on a Mueller Hinton agar was prepared in a 2ml normal saline in a tube. These colonies were already induced to produce B-lactamase. The impregnated end of stick was then dipped into suspension of test isolate and then observed for colour changes. The test is positive if colour changes from yellow to pink-red after 1hour and negative if no colour changes. Positive and negative control strains were included in the test.

3.3.4 **PBP2A Detection:**

3.3.4.1**PBP2 Extraction:**

About four drops of extraction reagent 1 was placed in a micro centrifuge tube. Approximately $1.5x10^{9}$ cells were picked using standard loop and suspended in the micro centrifuge tube. The tube was placed into boiling water and heat for three minutes. The micro centrifuge tube was removed and allowed to cool to room temperature. A drop of extraction reagent 2 was added and mixed well. It was then centrifuged at 1500xg for 5minutes, the supernatant removed and used for PBP2 test.

3.3.4.2 **PBP2 latex test:**

The circled spots on test card were labeled “test” and “control” respectively. 50µl each of supernatant was placed on the test spot and control spot. A drop of test latex was added to the test spot and similarly, a drop of control latex on the control spot. Using the stick provided, these

were mixed together thoroughly. The card was picked up and rocked for three minutes and observed for agglutination. The test is positive if agglutination occurs in test spot but not in control spot. Negative, if in both test and control spots there was no agglutination. Test is indeterminate, if agglutination occurs in control spot and not in the test spot.

3.3.5 Polysaccharide Typing:

3.3.5.1Staphytec Plus (DR0100-Oxoid^{R}) Test Principle:

Dryspot Staphytect Plus uses blue latex particles coated with both porcine fibrinogen and rabbit IgG including specific polyclonal antibodies raised against capsular polysaccharides of *Staphylococcus aureus(von Eiff et al.,2006).*The reagent is dried onto the reaction card. When the reagent is mixed on the card with colonies of *Staphylococcus aureus* emulsified in saline rapid agglutination occurs through the reaction between (i) fibrinogen and clumping factor, (ii) Fc portion of IgG and Protein A, (iii) specific IgG and capsular polysaccharide. Agglutination may also occur with other species which possess clumping factor or Protein A such as *Staphylococcus hyicus* and *Staphylococcus intermedius*. Absence of clumping factor, Protein A and specific capsular polysaccharides means, agglutination will not occur and the result will be regarded as negative. The most frequent coagulase and Protein A negative isolates of Staphylococci are *Staphylococcus epidermidis.*

3.3.5.2Procedure:

About (50 µl) of saline (0·85%) was dropped into the small rings (at the bottom of each oval) in both the test and control reaction areas. This was done ensuring that the liquid does not mix with the dried latex reagents. Using a sterile loop, equivalent of 5 average-sized suspect Staphylococcal colonies were picked from a culture medium plate and carefully emulsified in the

saline drop, ensuring that the resulting suspension was smooth. The suspension was mixed into the dry Control Latex spots until completely suspended and spread to cover the reaction area using the loop. The loop was then discarded appropriately. Using a separate loop, the procedure was repeated in the same way with the Test Latex. The card was picked up and rocked for up to 20 seconds and observed for agglutination under normal lighting conditions. The reaction cards were completely disposed of in disinfectant.

3.3.5.3Elisa method:

Type 5 and type 8 capsular polysaccharides were detected in isolates grown on agar slants. Each isolate was suspended in phosphate buffered saline and autoclaved at 121^{o}C for 1hr. After centrifugation for 10min; at 10,000×g, the polysaccharides were separated by decanting the supernatant. Polysaccharides were detected using enzyme-linked immunosorbent assay (Boutonnier *et al*; 1989).Strains lacking both types 5 and 8 capsular polysaccharide, were designated non-typable (Von Eiff *et al*., 2006).

3.3.6Detection of Mec A gene (Real-Time PCR):

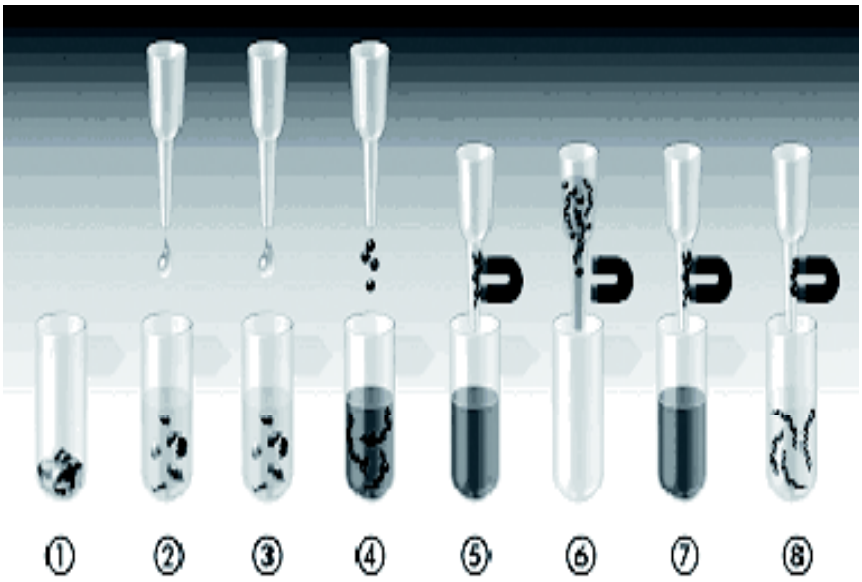
3.3.6.1**Primers used:**

Primers for PCR were synthesized with one of the primers (5´AAAATCGATGGTAAAGGTTGGC3´) corresponding to nucleotides128-1303 and the other (5´AGTTCTGCAGTACCGGATTTGC3´) was complementary to nucleotides 1793-1814. The 533 bp fragment was amplified using Real Time PCR (Murakami et al., 1991; Salwa et al., 2009).

3.3.6.2 DNA Isolation and amplification:

This was done using Mag NA pure LC instrument. The principle of test is based on magnetic-bead technology. The samples were lysed by incubation with a special buffer that contains chemotropic salts and proteinase K. Magnetic glass particles were added and the nuclei bound to their surfaces. Unbound substances were removed by several washing steps, and then the purified nucleic acid eluted.

3.3.6.3MagNA pure LC DNA Isolation Procedure:



1. The sample material was placed into wells of the sample cartridge.
2. Lysis/binding buffer were added to the sample. This was to allow complete cell lysis and release of nucleic acid.
3. Proteinase k was then added to digest protein.

4. Magnetic Glass Particles (MGPs were then added and nucleic acid binds to it.
5. The MGPs with bound nucleic acid were magnetically separated from the residual lysed sample.
6. The MGPs with bound nucleic were washed repeatedly with wash buffer to remove unbound substances.
7. Magnetic separation of nucleic acid bound MGPs was repeated.

The purified nucleic acid was eluted at 70^{o}C from the MGPs in the wells of the elution cartridge.

3.3.6.4Preparation of the PCR Mix:

In a 1.5ml reaction tube on ice, PCR mix for 20µl reaction was prepared by adding 3µl of water, PCR-grade, 2µ lPCRprimer(10xconc.), and10µl master mix (2xconc). This was mixed carefully by pippetting up and down and not votexed. 15µl of the PCR mix was pipetted into each well of the light cycler^{R}480 multiwell plate. About 5µl of the DNA template was added and multiwall plate sealed with foil. The multiwall was placed in a centrifuge, balanced and centrifuged at 1500xg for 2minutes. The multiwall plate was then loaded into the light cycler^{R}480 instrument and real time PCR program started as described by manufacturer (Roche Diagnostic, 1999).

CHAPTER FOUR

4.0 RESULTS:

4.1.0 Confirmatory tests:

On manitol salt agar, Staphylococcus isolates produced pink raised colonies with entire edge. Some isolates produce yellow pink colonies on mannitol salt agar. On blood agar, isolates produced white smooth colonies, some haemolytic while others were not.

All isolates were Gram positive cocci, were arranged in clusters, some were coagulase positive, catalase positive and were found to produce DNase (Appendices 4 and 5).

4.1.1 Prevalence distribution of isolates:

A total of 880 samples were examined from the 3 geographical areas, 560 in Zaria, 160 each from Kafanchan and Birnin gwari. An overall prevalence of 8.3% was recorded. Prevalence of *Staphylococcus* was 51(9.1%) in (Zaria), 12(7.5%) in Kafanchan and 10(6.3%) in B-Gwari. Of the Zaria isolates, 16 (31.3%) were from wound, 26(50.9%) from Urinogentals, 7(13.7%) from blood and 2(3.9%) from other sites (sputum, skin, pus). Of these, 33(64.7%) were MRSA, while 18(35.3%) were MSSA.

Females were more infected than males and age group of peak infection being1-10 and 41-50 years (Table4.1).

Table.4.1. Age / gender distribution of subjects studied.

AGE GROUP (YRS)	MALE	FEMALE
1 – 10	4 (19%)	2 (6.6%)
11-20	2 (9.5%)	2 (6.6%)
21-30	1 (4.8%)	4 (13.3%)
31-40	3 (14.5%)	5 (16.6%)
41-50	5 (23.8%)	8 (26.6%)
51-60	3 (14.0%)	6 (20.0%)
61-70	2 (9.5%)	3 (10.0%)
TOTAL	**21 (41.0%)**	**30 (58.8%)**

21(41.2%) of Male and 30(58.8%) female had Staphylococci infection in Zaria. In Kafanchan, 6(50%), 4(33.3%) and 2(16.6%) of isolates came from wounds, urinogenitals and blood samples respectively. In Birnin Gwari, 7(70%) and 3(30%) of isolates came from wounds and urinogenitals respectively. 9(75%) male,3(25%)female had the infection in Kafanchan and 6(60%)males,4(40%) females had it in Birnin-Gwari.

Age groups of peak infections are 1-10 and 41-50 respectively. The surgical male and female wards had the highest number of cases of MRSA but isolate recovery was highest at the Special Care Baby Unit (SCBU).

Most MRSA came from Surgical/Medical wards and outpatient department but recovery of MRSA isolates was more in special care baby unit (SCBU) and Accident and emergency(Table 4.2).

Table.4.2. Percentage distribution of isolates by ward

Ward	NE	NP (%)	MRSA (%)	MSSA (%)	% Prev.
A	115	6 (11.7%)	3 (9.1%)	3 (16.6%)	5.2%
B	106	10 (19.6%)	8 (24.2%)	2 (11.1%)	9.4%
C	80	2 (3.9%)	2 (6.1%)	0 (0%)	2.5%
D	44	3(5.8%)	2 (6.1%)	1 (5.5%)	6.8%
E	98	5 (9.8%)	3 (9.1%)	2 (11.1%)	5.1%
F	117	25 (49%)	15 (45.5%)	10 (55.5%)	21.4%
TOTAL	560	51 (100%)	33 (100%)	18 (100%)	9.1%

KEY :
A. ANC, PMW and PSW
B. MSW, MMW, FMW and FSW
C. SCBU.
D. Accident and Emergency.
E. Urology, EPU.
F. SOPD GOPD
NE No examined
NP No positive
%prev . % prevalence.

4.1.2 **Susceptibility of isolates to antibiotics:**

In Zaria, 13(39.4%) of the coagulase negative *Staphylococcus* (CoNs) and 20(60.6%) of coagulase positive *Staphylococcus* (CoPs) were MRSA. On the other hand, 1(5.6%) of CoNs and 17(94.4%) of CoPs were MSSA.

Coagulase positive isolates were more resistant to antibiotics than the coagulase negative isolates (Table4.3).

Results of Table 4.4, shows that while some coagulase positive *Staphylococcus* were sensitive to oxacillin, No coagulase negative *Staphylococcus* was considered sensitive to Oxacillin when break point interpretative criteria was employed.

There was a strong association between resistance to antibiotics and degree of coagulase produced ($P<0.05$) Using the break point criteria for oxacillin, 40(78.4%) were resistant, 6(11.6%) were intermediate while 5(9.8%) were sensitive (Table4.4)

Table.4.5. shows that there was no intermediate resistance for Cefoxitin and that number of isolates considered to be sensitive reduced drastically (11.7%) when break point criteria were employed.

In Zaria, 38(74.5%) were sensitive to Cefoxitin while 13(25.5%) were resistant to Cefoxitin. 41(80.4%) were sensitive to Vancomycin while 10(19.6%) were resistant to Vancomycin(Fig.4.1).Results of Fig.4.1 shows the resistance of the isolates to vancomycin(a glycopeptides) was highest in Kafanchan, followed by Zaria and then Birnin-Gwari.

Table.4.3. Coagulase production and susceptibility of Staphylococcus isolates:

Coagulase.	No resistant (%)	No sensitive. (%)	Total. (%)
Negative	13(39.4)	1(5.6)	14(27.5)
Slightly. (+)	4(12.1)	2(11.1)	6(11.8)
Moderately. (++)	10(30.3)	12(66.7)	22(43.1)
Strongly(+++)	6(18.2)	3(16.6)	9(17.6)
Total	33(100)	18(100)	51(100).

Table.4.4. Break point interpretation criteria for Oxacillin (1 µg)

SUSCEPTIBILITY	COPS	CONS	TOTAL
Sensitive.	15 (29.4%) ≥13mm	0 (0%) ≥18mm	15 (29.4%)
Intermediate.	2 (3.9%) 11 – 12mm	-	2 (3.9%)
Resitant.	20 (39.2%) ≤ 10mm	14 (27.5%) ≤ 17mm	34 (66.6%)
TOTAL	**37 (72.5%)**	**14 (27.5%)**	**51 (100%)**

Key:

COPS: Coagulase positive *Staphylococcus.*

CONS: Coagulase negative *Staphylococcus*.

Table4.5. Break point interpretative criteria for Cefoxitin (30µg)

SUSCEPTIBILITY	COPS	CONS	TOTAL
Sensitive	6 (11.7%)	0 (0%)	6 (11.8%)
	≥20mm	≥25mm	
Resistant	31 (60.8%)	14 (27.5%)	45 (88.2%)
	≤ 19mm	≤ 24mm	
TOTAL	**37 (72.5%)**	**14 (27.5%)**	**51 (100%)**

Key: COPS -Coagulase positive *Staphylococcus*
CONS –Coagulase Negative *Staphylococcus*

Table.4.6. Susceptibility of Isolates to antibiotics**.**

Susceptibility.	**Oxacillin (%)**	**Cefoxitin (%)**	**Vancomycin. (%)**	**Ciprofloxacin. (%)**
Resistant	33(64.7)	13(25.5)	10(19.6)	19(37)
Sensitive	18(35.3)	38(74.5)	41(80.4)	32(63)
Total	51(100)	51(100)	51(100)	51(100)

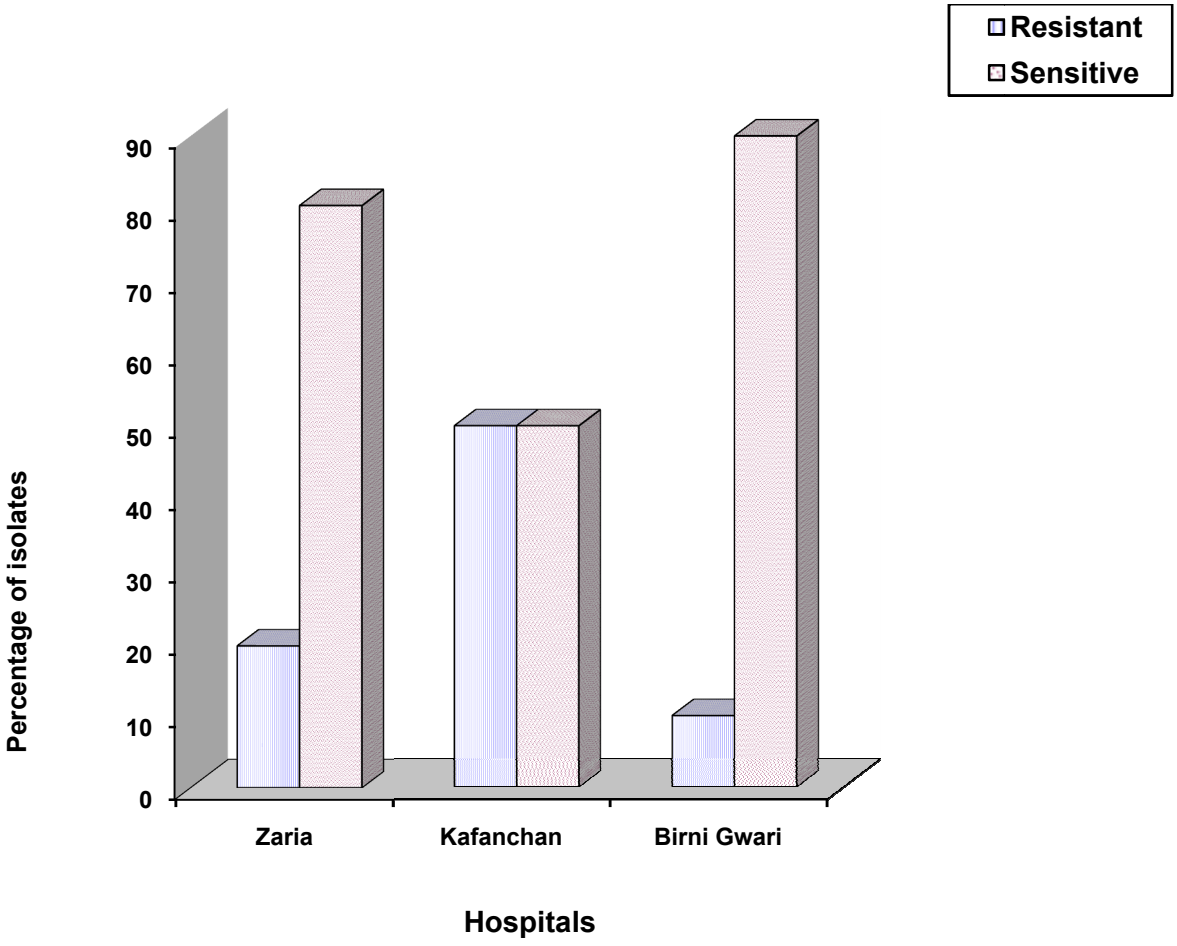


Fig.4.1.Bar chart showing % susceptibility of isolates to Vancomycin

32(63%) were sensitive to ciprofloxacin while 19(37%) were resistant to ciprofloxacin. 6(11.7%) were sensitive to both Cefoxitin and Vancomycin, not ciprofloxacin. 10(19.6%) were sensitive to both cefoxitin and ciprofloxacin, but not Vancomycin. 5(9.8%) were resistant to all the antibiotics tested while 27(53%) were sensitive to all the antibiotics. In Kafanchan 6(50%) were resistant to both Vancomycin and ciprofloxacin. In Birnin-gwari however, 1(10%) were resistant to vancomycin while 2(20%)were resistant to ciprofloxacin. 8(15.7%), 3(25%) and 4(40%)were resistant to Clindamycin in Zaria, kafanchan and Birnin gwari respectively. 6(50%) and 3(30%) of isolates from Kafanchan and Birnin-gwari repectively were resistant to erythromycin (Figure4.2).

Results of Fig.4.2, shows that resistance to ciprofloxacin (quinolone) was highest in Kafanchan than in Zaria, and than in Birnin-Gwari.

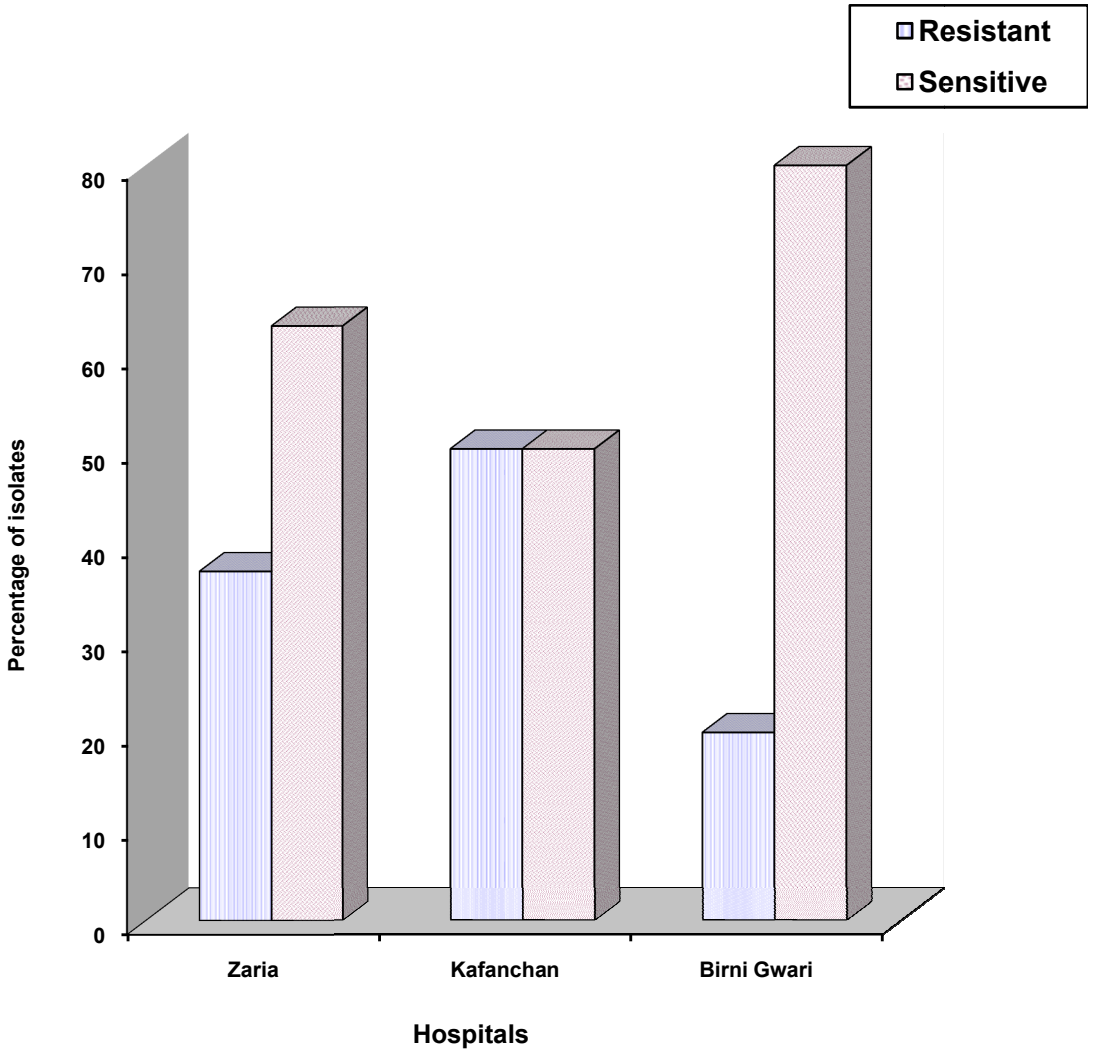


Fig.4.2.Bar chart showing % susceptibility of isolates to Ciprofloxacin

Antibiotic profile results among isolates from kafanchan and Birnin-gwari shows that 31% (n=7) were resistant to only 1 antibiotic, 13% (n=3) were resistant to 2 antiobiotics while 54% (n=12) were resistant to more than 2 antibiotics; an isolate from Kafanchan (K13) was resistant to all six antibiotics tested. Majority of isolates in the study area have multiple antibiotic resistance index of greater than 0.5(Fiq.4.3, 4.4 and 4.5).This implied that majority of isolates are resistant to more than half of antibiotics tested.

From the results of Tables 4.7 and 4.8, oxacillin is the antibiotic to which most isolates are resistant to in both Birnin-Gwari and Kafanchan while Vancomycin and ciprofloxacin are the drugs to which they are most sensitive to in Birnin-Gwari. In Kafanchan isolates are most sensitive to Cefoxitin and Clindamycin and not Vancomycin and Ciprofloxacin.

From the result of Table 4.9, twelve number isolates resisted three or more antibiotics with*Isolate K13 from Kafanchan demonstrating resistance to all antibiotics tested.

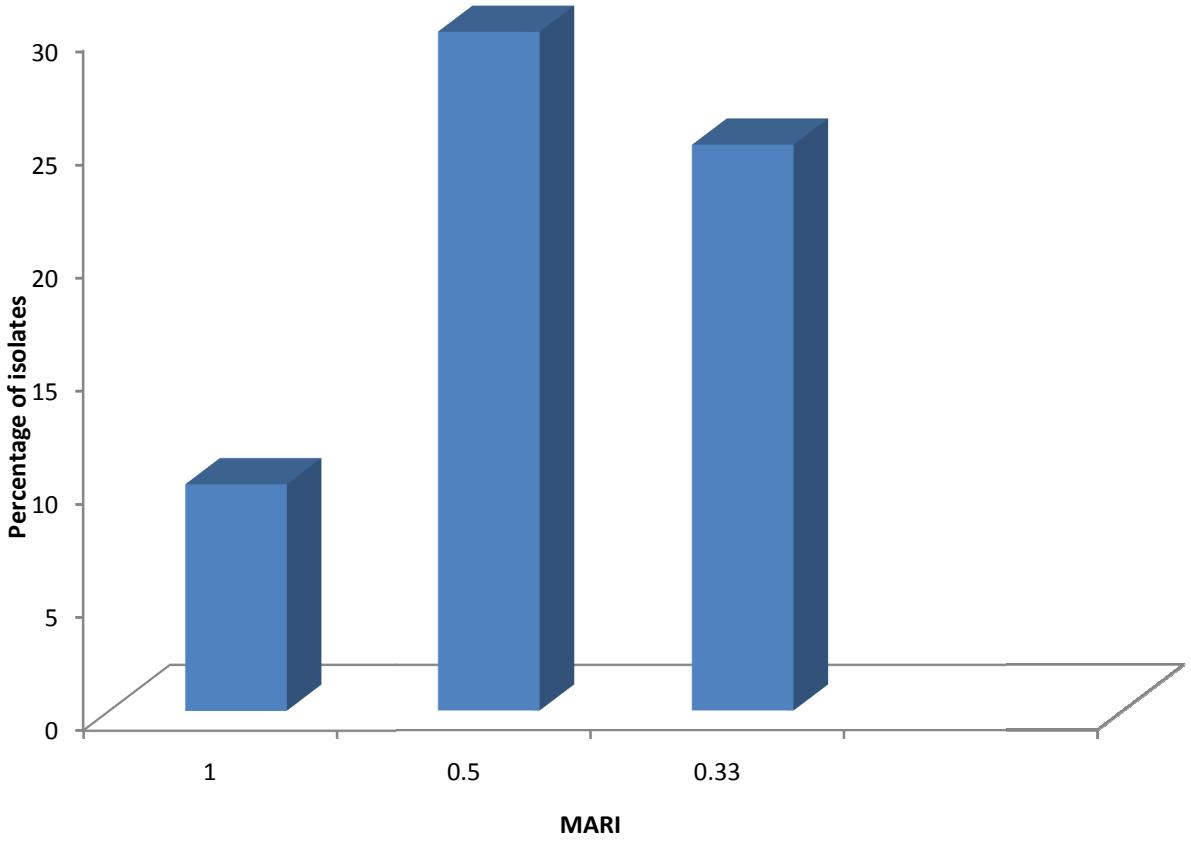


Fig. 4.3 Percentage of isolates with MARI in Birni Gwari

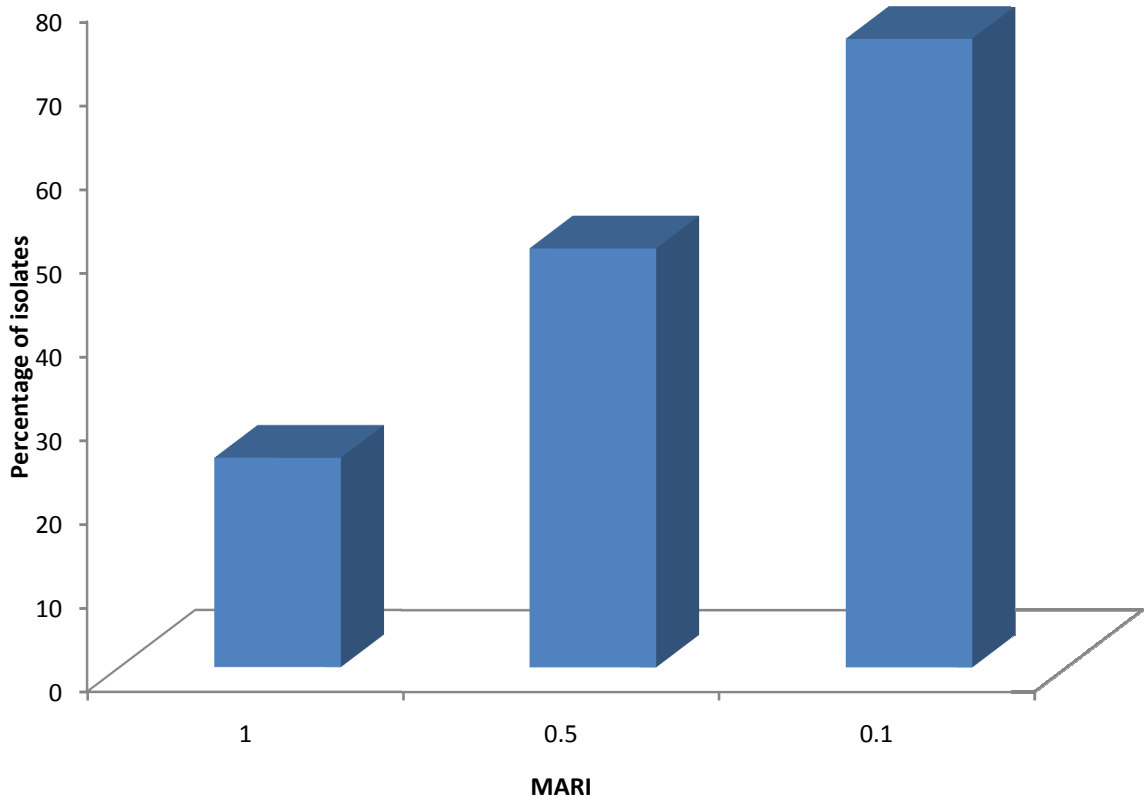


Fig.4.4 Percentage of isolates with MARI in Kafanchan

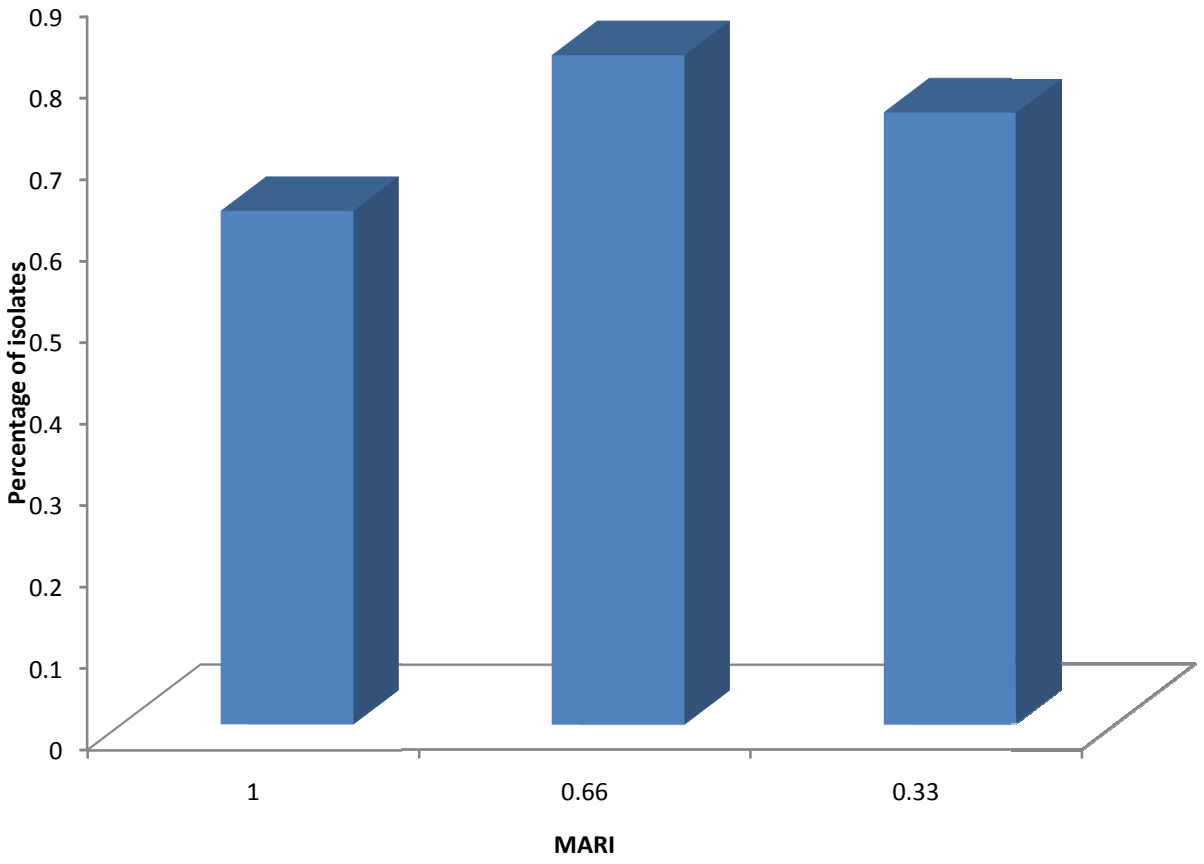


Fig. 4.5 Percentage of isolates with MARI in Zaria

Table.4.7. Antibiotic profile for Kafanchan isolates.

Antibiotics	Kafanchan		Total
	No of isolates Sens.	No of isolates Res.	
Oxacillin	3(25%)	9(75%)	12(100%)
Cefoxitin	9(75%)	3(25%)	12(100%)
Vancomycin	6(50%)	6(50%)	12(100%)
Ciprofloxacillin	6 (50%)	6(50%)	12(100%)
Erythromycin	6(50%)	6(50%)	12(100%)
Clindamycin	9(75%)…….	3(25%)	12(100%)

Key:

Sens. = Sensitivity

Res = Resistant

Table.4.8. Antibiotic profile for Birnin-gwari isolates.

Antibiotics	Birnin gwari		Total
	No of isolate Sens.	No of isolates Res.	
Oxacillin	1(10%)	9(90%)	10(100%)
Cefoxitin	6(60%)	4(40%)	10(100%)
Vancomycin	9(90%)	1(10%)	10(100%)
Ciprofloxacillin	8(80%)	2(20%)	10(100%)
Erythromycin	7(70%)	3(30%)	10(100%)
Clindamycin	6(60%)……	.4(40%)	10(100%)

Key:

Sens. = Sensitivity

Res = Resistant

Table4.9. Antibiotic susceptibility pattern among isolates

No of isolates	Antibiotic resistant to	Isolate source
7	Erythromycin.	K3
7	Oxacillin.	K4
7	Oxacillin.	K16
7	Vancomycin.	K20
7	Oxacillin.	B13
7	Oxacillin.	B14
7	Oxacillin.	B15
2	Oxacillin,Ciprofloxacin.	K2
2	Vancomycin,Erythromycin.	K14
12	Oxac;Cip;Eryt.	K5
12	Oxac;Van;Cip;Clind.	K9
12	Oxac;Cef;Van;Cip;Eryt;Clind.	K13*
12	Oxac;Van;Cip;Eryt.	K15
12	Oxac;Ceef;Cip;Eryt;Clind	K18
12	Oxac;Cef;Van.	K19
12	Oxac;Van;Cip.	B1
12	Oxac;Cef;Clind.	B4
12	Oxac;Cef;Eryt.	B9
12	Oxac;Cef;Eryt;Clind.	B10
12	Oxac;Cef;Cip.	B11
12	Oxac;Eryt;Clind.	B12

4.1.3**Results of the PBP2 test**:

Of the 51 isolates of *Staphylococcus aureus* tested in Zaria, 13(25.5%) were positive for Penicillin Binding Protein 2(PBP2). 3(25%) of the 12 isolates tested in Kafanchan were positive. None of the isolates from Birnin- gwari tested positive for the PBP2 (Fig.4.6).

From the results in Fiq.4.6, 25% of isolates from Zaria and Kafanchan produced penicillin binding protein 2. None of the isolates in Birnin gwari produced PBP2.

4.1.4 **Results of the β-lactamase test:**

34(66.7%) were positive for β-lactamase production in Zaria, 9(75%) in Kafanchan, and 9(90%) in Birnin-gwari. This implied that isolates are likely to be more resistant to penicillins, cephalosporin and carbepenem in Birnin-gwari than in Kafanchan and than in Zaria (Fig.4.7).

From result in Fig.4.7, β-lactamase production was more among isolates in Birnin-gwari followed by isolates in Kafanchan and then Zaria.

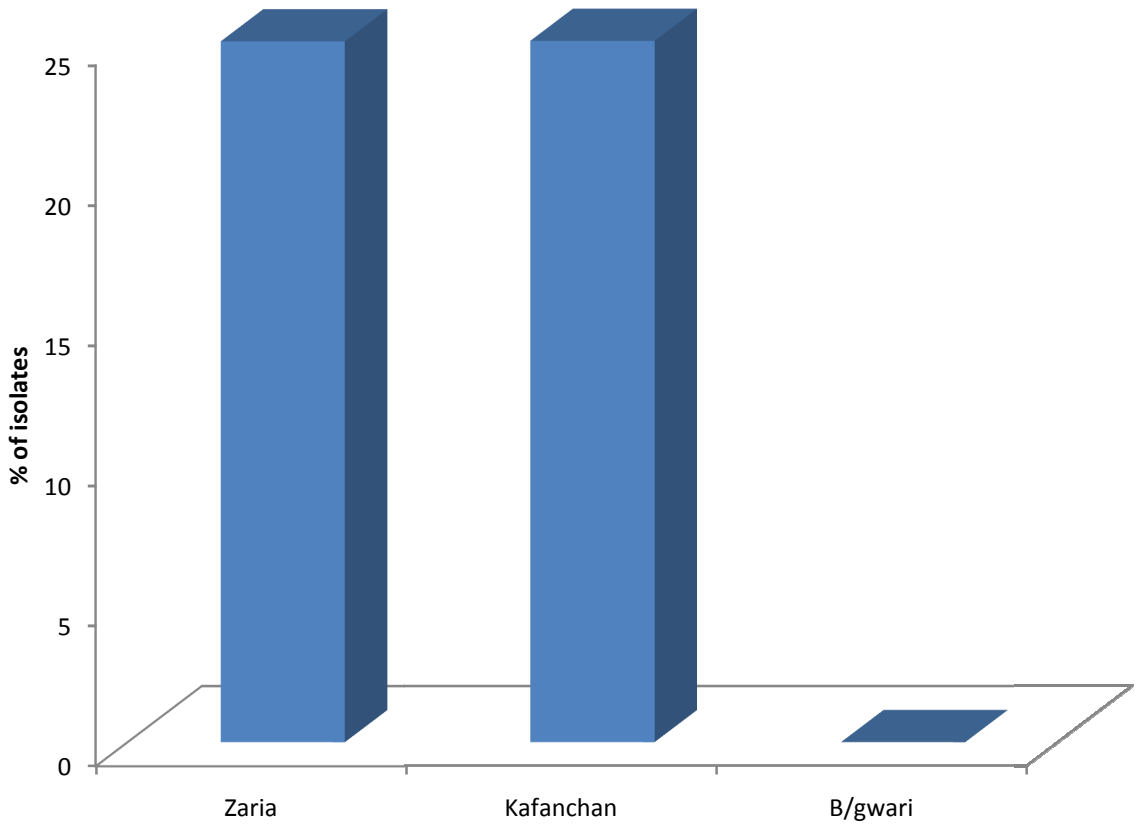


Fig. 4.6.Percentage of isolates producing Penicillin Binding Protein 2.

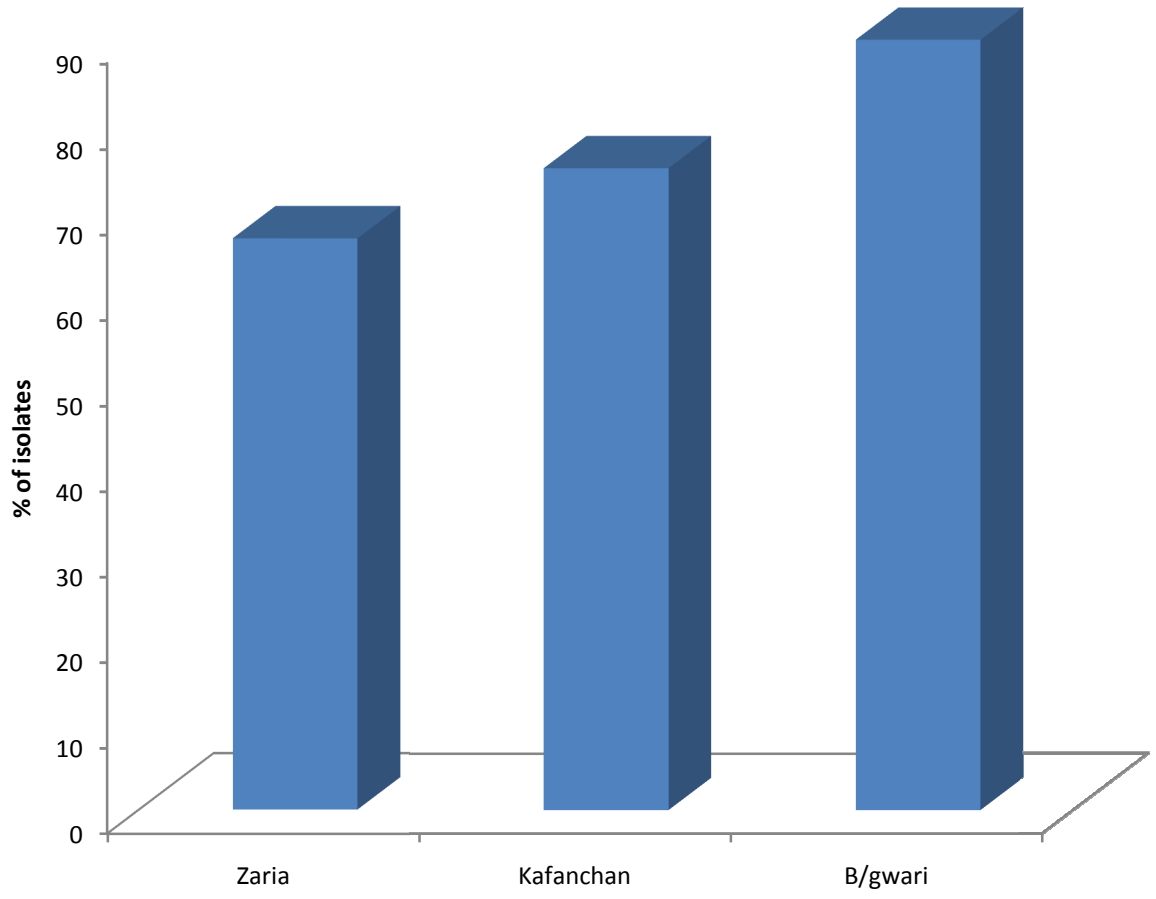


Fig. 4.7: Percentage of isolates producing B-lactamase

4.1.5 **Results of the D-test**:

Of the 51 isolates tested, 8(15.7%) were of clindamycin inducible erythromycin resistant strain in Zaria, 3(25%) of the 12 isolates were of this strain in Kafanchan, and none of the isolates in Birnin-gwari is of this strain. Treatment failure is more likely in kafanchan(25%) than in Zaria (15.7%)when clindamycin is used to treat infections resulting from this strains(Fig.4.8).

From result of Fiq.4.8, Inducible clindamycin resistant strains were prevalent in Zaria and Kafanchan with more of such strains in Kafanchan than Zaria. None of such isolates in Birnin-gwari

4.1.6 **Results of the capsular typing test:**

Of the 51 isolates tested in Zaria, 29(85%) were of capsular type 5 and 8; 10(83.3%) of the12 isolates in Kafanchan, and 7(70%) of 10 isolates from Birnin-Gwari. From result of Fig.4.9, 75%, 85% and 90% of isolates have capsular type 5 &8 respectively from Birnin-Gwari, Kafanchan, and Zaria.

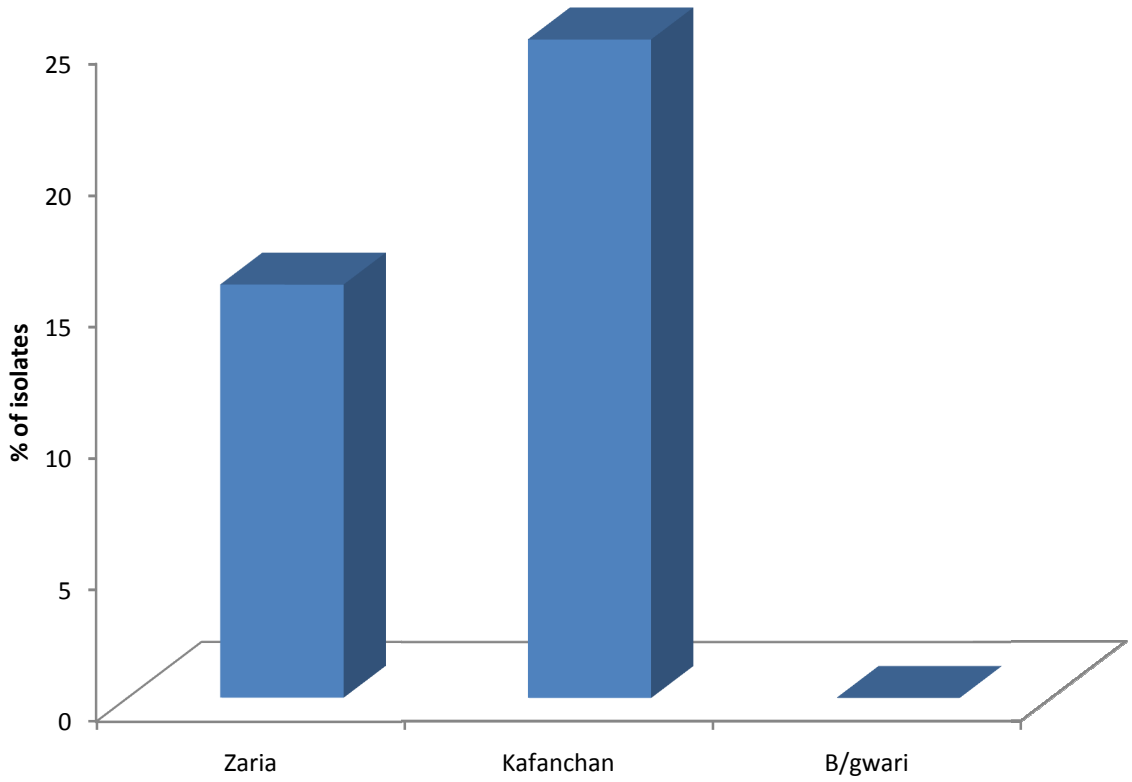


Fig. 4.8 Percentage of isolates that are of inducible clindimycin resistant strain

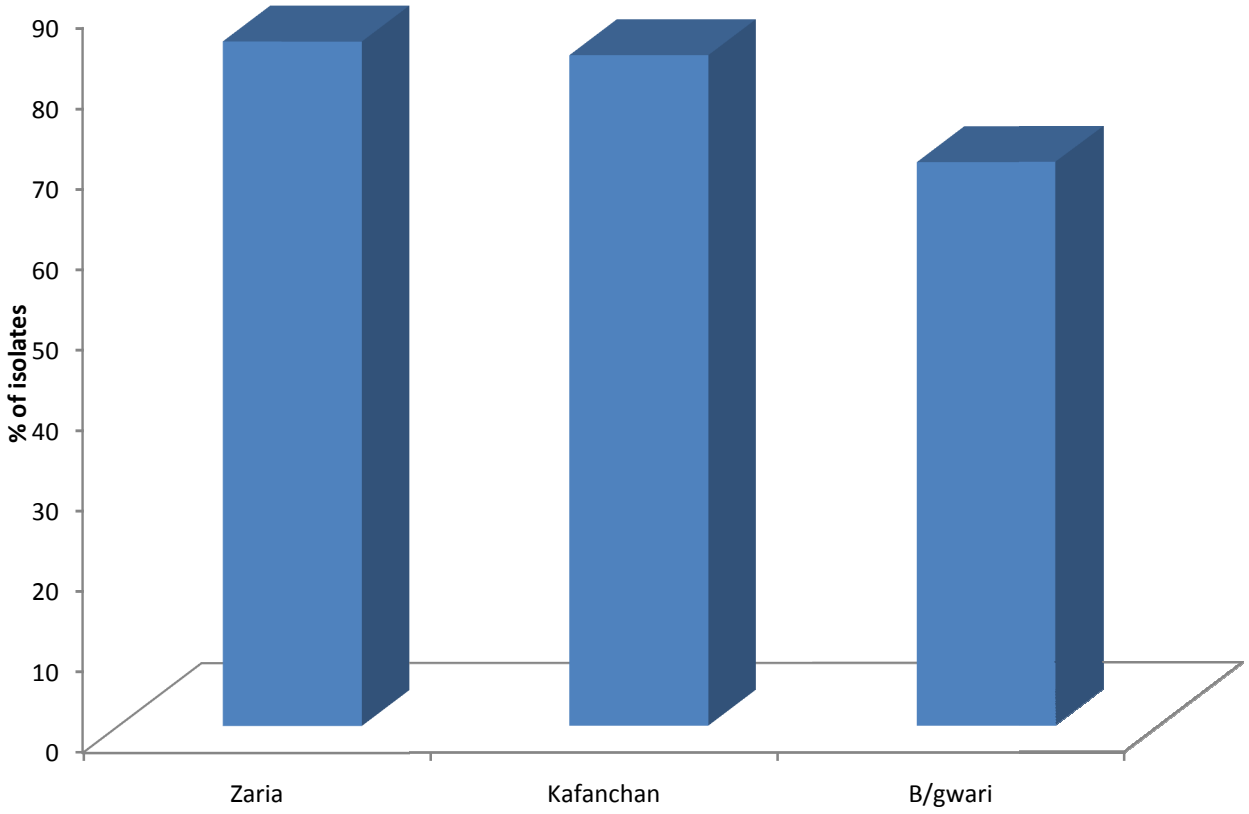


Fig.4.9 Percentage of isolates with 5 and 8 capsular type

4.1.7 **Results of Mec A detection with Real Time PCR:**

A total of 32 isolates were selected for Mec A detection, 21 from Zaria, 6 from Kafanchan and 5 from Birnin Gwari. Overall, 9(28.1%) had mec A in the study area, these Comprised 6(18.8%) in Zaria, 3(9.3%) in Kafanchan. No isolate in Birnin-Gwari had detectable mec A gene using Real time PCR (Fig.4.10).

.From the results in fig.4.10, amplification of the target gene (Mec A) was demonstrated by increase in fluorescence reporter.

Isolate no 1-22 are from Zaria, 23-26 from B/gwari and 27-32 are from kafanchan. Positive isolate numbers are highlighted. Flourescence below.0014 threshold was considered negative for Mec A .Melting point peak for most positive isolates lied between 81^{o}C-83^{o}C. .At this temperature maximum amount of target DNA were amplified.

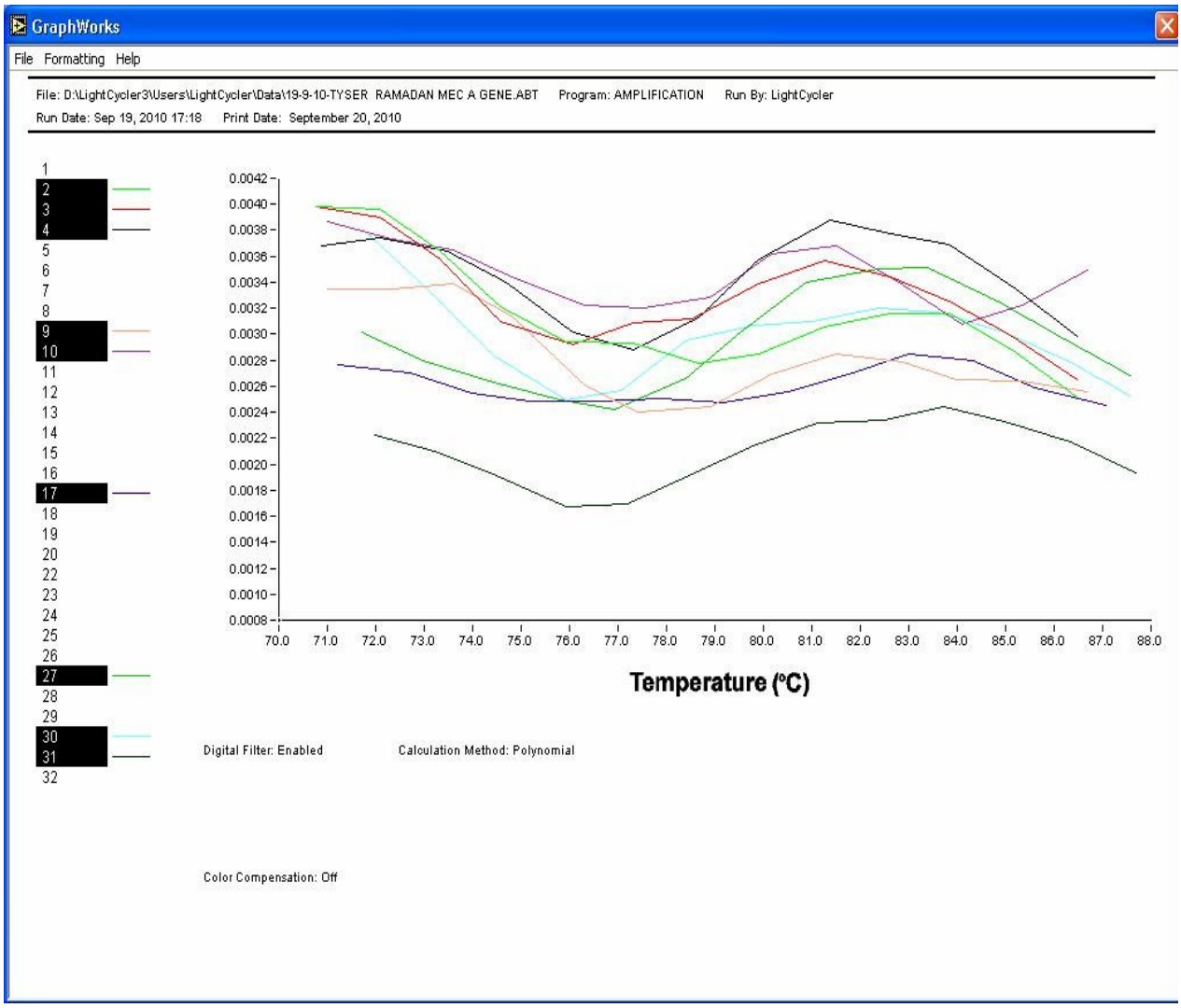


Fig.4.10. Melting point curve for positive isolates.

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSIONS

An overall prevalence of 8.3% was recorded for Nosocomial Staphylococcus infection. This was in agreement with work done by Adebayo *et al*.,(2006);Taiwo *et al.,(*2004);Rotimi *et al*.,(1987)and Okesola *et al*.,(1999) Prevalence of Staphylococcus infection is however not as high as reported in other part of the world where prevalence as high as 50% was reported(Gebhart,2003;Kesah *et al*.,2003;Diekam *et al*.,2001).The low prevalence recorded in this study compared to what was obtained elsewhere may have to do with awareness in hand washing particularly among the hospital staffs in the study area. General hygiene practice in hospital could also explain the low prevalence for nosocomial *Staphylococcus* infection and the fact that antibiotic use policies are already in place in some of the hospitals studied. Methicillin resistant *Staphylococcus aureus*(MRSA) accounted for 41(56%) of all nosocomial *Staphylococcus aureus* isolates. This finding again agreed with work done by Adebayo *et al*., (2006) and Taiwo *et al*.,(2004) in south and western part of Nigeria respectively.

About 96.4% isolates were recorded from wound, urinogenital and blood while 3.6% came from other sites. This observation was also made by Jaroud *et al*.,(2002) in their work on relationship between *Staphylococcus aureus*, genetic background, virulence factors, age groups (alleles) and human diseases. Isolation of Staphylococcus from these sites may have to do with the fact that the organism, naturally establish a benign relationship with host and so are found on the skin and mucous membranes; but when the natural cutaneous barrier is damaged by trauma, inoculation by needles or direct implantation of medical device, organism easily gain entry.

Females were more infected than males and age group of peak infection was 1 – 10 years and 41 – 50 years. This observation was similarly made by Jaroud *et al*.,(2002) in which the young

and the elderly were observed to be more susceptible to infection. Other reasons why female are more infected than male may have to do with the fact that MRSA is associated with frequent hospitalization visit, long hospital stay and with patients on indwelling percutaneous cartheter. These risk factors are more associated with female sex, the young and the elderly who for one reason or the other, visit hospital more often than male. The female sex, the young and the elderly are also believed to be immunologically weak.

The surgical medical wards had the highest number of cases of MRSA but recovery rate was highest from the special care baby unit (SCBU). This was also in agreement with work done by Naimi *et al*., (2001) in which risk factors for acquisition of MRSA was mentioned to include recent hospitalization in long-term care facility, surgery, dialysis and use of indwelling percutaneus medical devices and catheters. In this study, wards from which MRSA were recovered, some patients were on catheters, and others had stayed long on admission while some had undergone surgery.

There was a strong association between resistance to antibiotics and degree of coagulase produced ($P<0.05$).These findings agreed with the work done by Ako Nai *et al*.,(1991); Gianeechini *et al*., (2002). In their work, clinical isolates were characterized by their ability to clot plasma which was used for the identification of pathogenic Staphylococcus (*Staphylococcus aureus*). Coagulase positive *Staphylococcus aureus*(CoPs) were more resistant to antibiotics than coagulase negative Staphylococcus(CoNs).

In this study MRSA isolates with multiple drug resistance were isolated. Majority resisting more than two antibiotics Majority has multiple antibiotic resistant indexes of greater than 0.5. This implies that isolates resisted more than half of the antibiotics tested. The reason for this may have to do with the fact that isolates are of nosocomial source where drugs are frequently being

used. This has serious implication in therapy and may explain cases of treatment failure encountered in hospitals studied .This was also consistent with work of Weber *et al*.,(2003) and Diekam *et al*.,(2001) in which two distinct groups of MRSA were recognized-one representing multi resistant epidemic strains and the other β-lactam resistant strains. Twenty-five percent(25%) each of isolates from Zaria and Kafanchan were positive for penicillin binding protein 2(PBP2).Non of the isolates from Birnin-Gwari was positivie for PBP2.This result is consistent with work of Skov *et al*.,(2003) in which penicillin binding protein –a product of mec A gene was used as the Gold standard in confirming Methicillin resistance. Though some MRSAs were detected in Birnin-Gwari with the phenotypic disc method, none of them produced PBP2. The explanation for this may be that these "super bugs" known to utilize different mechanism of resistance-enzymatic (β-Lactamase production), genetic through possession of genes like Mec A, erythromycin ribosomal methylase (erm) gene and mrs^A gene, does so and consequently resist oxacillin or cefoxitin(MRSA) without necessary carrying the Mec A gene. 66.7% of Zaria, 75% of Kafanchan and 90% of Birnin-gwari -MRSA isolates were positive for β-lactermase. This finding showed that all of MRSA isolates from Birnin-Gwari expressed resistance through β-lactamaes production. This observation was consistent with that made by Olonitola *et al.,(*2007) in which Community Acquired MRSA isolates, lacking Mec A gene were observed to express resistance phenotypically rather than genotypically. The low recovery of mec A among isolates in the study Area , implies that prevalence of mec A- carrying MRSA is low and this may have to do with the fact that majority of isolates express resistance through phenotypic means. The emergence of this super bug in Zaria and Kafanchan hospitals is consistent with the pressure of drug usage in these two Hospitals. These findings were in agreement with that of Weber *et al*.,(2003) in which indiscriminate prescription of the

quinolones was reported to be a major factor for emergence of MRSA. Access to drug by patients, on or before admission, to hospitals is not regulated. When prescriptions are given, regimen dosages are hardly complied with. This observation was similar to that made by Olonitola *et al*.,(2007) on the unregulated access to drugs amongst populations with CA-MRSA. Another explanation as to the emergence of this bug in the two Hospitals reported may have to do with use of disinfectants at sub minimal concentrations. The use of disinfectants at sub minimal concentration can induce resistance. Some of the chemical agents contained are mutagenic. Disinfectant not discarded as at when due also contributes to emergence of multidrug resistant organism. An organism exposed to such an agent over a long period of time develops resistance as they mutate. This observation calls for isolation and monitoring of patients infected with staphylococcus, particularly the MRSA.

Results of the D- test showed that 15.7% and 25%of MRSA isolates from Zaria and Kafanchan respectively were of clindamycin inducible erythromycin resistant strain. These strains were not found in Birnin-Gwari. The result suggests that more treatment failure is likely in Kanfanchan than in Zaria if clindamycin is suggested for treatment of infections due to these MRSA. This findings was consistent with that of Johnson, (2006);Siberry *et al*.,(2003) in which failure of clindamycin in treating MRSA expressing inducible clindamycin resistance was reported and to prevent such failure, a D-test was suggested as second step test when MRSA cultures come back showing erythromycin resistance, clindamycin susceptible.

Results of the capsular typing showed that 85%, 83% and 70% of MRSA isolates from Zaria, Kafanchan and Birnin-gwari respectively possessed capsular type 5&8.Von Eiff *et al*; (2006) made similar observation in which it was reported that between 80-90% of MRSA posseses type 5 or 8 capsular polysaccharide .About 10-20% were regarded as the non typable group and this

group, were said to posses antigen 336. Both capsular polysaccharides are essential virulence factor and putative protective antigens.

Of the 32 isolates screened using the Real Time PCR, 28.1% (n=9) MRSA isolates in the study area had Mec A gene. This comprised of 18.8% (n=6) in Zaria, 9.3% (n=3) in Kafanchan. No Mec A carrying MRSA was detected in Birni-Gwari. More mec A-carrying MRSA came from Zaria than Kafanchan. This may have to do with the fact that Zaria being urban to Kafanchan –a peri urban setting, inhabitants have access to non prescribed antibiotics.more than those in peri-urban and rural settings. In addition drugs linked to inducing resistance are more affordable by urban dwellers to those in rural settings. This may explain the non detection of Mec A carrying MRSA in Birnin-Gwari- a rural setting. Detection of Mec A gene which is considered the "gold standard" in establishing methicillin resistance amongst *Staphylococcus* isolates from the study area proves beyond all doubt the existence of MRSA in our hospitals. Though percentage of MRSA with Mec A (28.1%) reported in this work is high compared to the (1.4 %) reported in Southern part of Nigeria (Adebayo *et al*., 2006), Presence of MRSA with Mec A has been documented in southern part of the country. In work done by Olonitola *et al*.,(2007) on CA-MRSA in Northern Nigeria,, absence of Mec A gene was reported. In this work, nosocomial isolates were examined for Mec A. This may explain the detection of Mec A gene among isolates. This findings are consistent with that of Diekam *et al.,*(2001), Weber,(2003), where MRSA was reported to be highly endemic in hospital settings. The high endemicity of MRSA in hospitals may be due to high concentration of antibiotic usage in hospitals, unchecked transmission among and between workers and patients.

In this study, isolates that were screened as Methicillin resistant (cefoxitin) and those that were D-test positive were all positive for mec A gene. This result was consistent with that of

Fermandes *et al*.,(2005) in which cefoxitin disc was reported to serve as a surrogate marker for Mec A detection in resource constrained environment.

To overcome problem of treatment failure, break point interpretative criteria which considers higher zone diameter as susceptible is considered. The use of break point implies that lesser organism are actually sensitive than initially thought. This observation was equally made by Anand *et al*.,(2009) where they observed that heterogenous expression of resistance of Mec A gene could result in MIC that appear to be borderline and consequently, isolates are regarded as susceptible. This has serious implication in therapy. The use of break point interpretative criteria to establish susceptibility may also explain why isolates considered to be MRSA do not posses Mec A gene.

5.1 Conclusion/Recommendation

Findings:

Mec A carrying methicillin resistant *Staphylococcus aureus* (MRSA) exists in two of the hospitals in the three geographical areas studied. β-lactamase producing strains as well as Vancomycin and quinolone resistant strains were detected in the study area. Majority of the isolates demonstrated multiple resistance to antibiotics tested. Though some express resistance through phenotypic means, others do so through genotypic means as mec A gene were detected in some but not all MRSA isolates.

Erythromycin inducible clindamycin resistant strains were also detected and all these put together may explain cases of treatment failure encountered in our hospitals. The findings have broadened our knowledge of antibiogram pathern of MRSA isolates in the study area. This in a way will inform the prescription policy to be adapted by the hospitals

studied. Existence of vancomycin resistant MRSA has also been reported in these findings. Though its transmission from person to person was been said to be rare and that they do respond to other antibiotics (Fridkin *et al*., 2003), a particular isolate from Kafanchan (K13) resisted all antibiotics including vancomycin. These findings call for concern.

Quinolone and β-lactamase strains were also reported in this study and all of these points to the fact that quinolones have been overused in hospitals studied. Detection of β-lactamase strains implies that isolates will not respond to ampicillins, carbepenems and the cephalosporins. With this knowledge cost effective treatment can be effected thereby preventing longer hospitalization and easy management of infections resulting from such isolates.

Erythromycin-inducible clindamycin resistant strains were also detected in the study. This implied that infections resulting from these strains cannot be treated with clindamycin even when susceptibility test suggest clindamycin sensitive, erythromycin resistant. A D-test is suggested if treatment failure from clindamycin usage is to be avoided.

MRSA were detected in the study area though not all possess mec A gene. Isolates screened with cefoxitin disc to be MRSA and those that are D-test positive are more likely to posses MecA gene. The combination of cefoxitin disc with D-test is therefore suggested for screening for methicillin resistance.

Recommendations:

It is thus appropriate to institute in hospitals where MRSA were discovered, a guided drug-prescription policies that regulates use of broad spectrum antibiotics in order to reduce drug pressure that selects multidrug resistant strains.

Health education on the dangers of the spread of the super bug is suggested particularly in Kafanchan where an MRSA isolate was seen to resist all antibiotics tested

Hand washing by both patients and health practitioners should be encouraged and regular antibiogram check for MRSA is suggested.

Infection control units should be established in these hospitals and their roles should include among other things, search for emerging infections like MRSA.

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Appendix1:

Isolates and Zones of inhibition(mm)

Isol.	Oxac.	Cef.	Van.	Cip.	Eryt.	Clin.	Dtest.	MR-oxa.	MR-cef.
K[2]	7 R	16 S	10 S	10 R	10 S	11 S	---	MRSA	MSSA
K[3]	15 S	20 S	10 S	25 S	7 R	25 S	+ve	MSSA	MSSA
K[4]	7 R	16 S	10 S	20 S	22 S	23 S	---	MRSA	MSSA
K[5]	7 R	16 S	13 S	7 R	7 R	18 S	+ve	MRSA	MSSA
K[9]	7 R	20 S	7 R	7 R	15 S	7 R	---	MRSA	MSSA
K[13] ■	7 R	7 R	7 R	9 R	7 R	7 R	---	MRSA	MRSA
K[14]	11 S	25 S	8 R	24 S	7 R	9 S	+ve	MSSA	MSSA
K[15]	7 R	22 S	7 R	20 R	7 R	20 S	+ve	MRSA	MSSA

K^{16}	7^{R}	14^{S}	10^{S}	15^{S}	30^{S}	24^{S}	---	MRSA	MSSA
K^{18}	7^{R}	10^{R}	10^{S}	9^{R}	7^{R}	7^{R}	---	MRSA	MRSA
K^{19}	7^{R}	28^{R}	8^{R}	20^{S}	22^{S}	24^{S}	---	MRSA	MRSA

Key:

K = Kafanchan isolates

R = Resistant

S = Susceptible

Appendix 2: Isolates and zones of inhibition (mm).

K 20	18 S	20 S	7 R	20 S	15 S	20 S	---	MRSA	MRSA
B 1	7 R	23 S	8 R	22 R	20 S	18 S	---	MRSA	MSSA
B 4	7 R	7 R	11 S	18 S	15 S	7 R	---	MRSA	MRSA
B 9	7 R	7 R	10 S	20 S	7 R	7 S	---	MRSA	MRSA
B 10	7 R	7 R	12 S	20 S	7 R	7 R	---	MRSA	MRSA
B 11	9 R	24 R	10 S	12 R	20 S	18 S	---	MRSA	MRSA
B 12	7 R	16 S	10 S	15 S	7 R	7 R	---	MRSA	MSSA
B 13	7 R	20 S	15 S	20 S	15 S	20 S	---	MRSA	MSSA
B 14	8 R	24 S	11 S	22 S	25 S	30 S	---	MRSA	MSSA
B 15	7 R	10 S	10 S	21 S	24 S	25 S	---	MRSA	MSSA
B 16	15 S	20 S	12 S	18 S	20 S	24 S	---	MSSA	MSSA

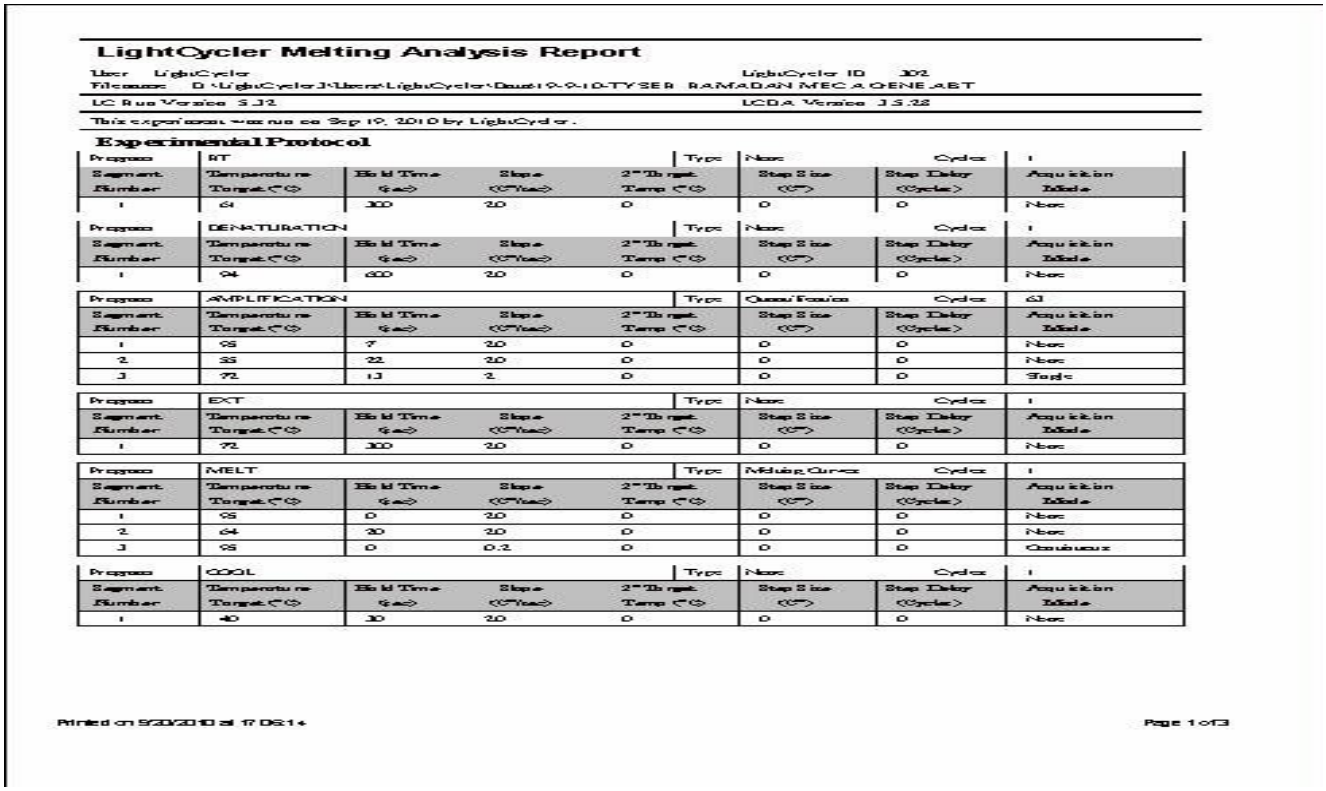
Key:

B = Birnin-Gwari isolates

R = Resistant

S = Susceptible

Appendix 3 Melting point analysis



LightCycler Melting Analysis Report

User: LightCycler — LightCycler ID: 202

Filename: D:\LightCycler3\Users\LightCycler\Data\19-9-10-TYSER RAMADAN MECA GENEART

LC Run Version: 5.32 — LCDA Version: 3.5.28

This experiment was run on Sep 19, 2010 by LightCycler.

Experimental Protocol

Program	RT				Type:	None		Cycles:	1
Segment Number	Temperature Target (°C)	Hold Time (s)	Slope (°C/s)	2nd Target Temp (°C)		Step Size (°C)	Step Delay (Cycles)		Acquisition Mode
1	61	300	20	0		0	0		None

Program	DENATURATION				Type:	None		Cycles:	1
Segment Number	Temperature Target (°C)	Hold Time (s)	Slope (°C/s)	2nd Target Temp (°C)		Step Size (°C)	Step Delay (Cycles)		Acquisition Mode
1	94	600	20	0		0	0		None

Program	AMPLIFICATION				Type:	Quantification		Cycles:	50
Segment Number	Temperature Target (°C)	Hold Time (s)	Slope (°C/s)	2nd Target Temp (°C)		Step Size (°C)	Step Delay (Cycles)		Acquisition Mode
1	95	7	20	0		0	0		None
2	55	22	20	0		0	0		None
3	72	13	2	0		0	0		Single

Program	EXT				Type:	None		Cycles:	1
Segment Number	Temperature Target (°C)	Hold Time (s)	Slope (°C/s)	2nd Target Temp (°C)		Step Size (°C)	Step Delay (Cycles)		Acquisition Mode
1	72	300	20	0		0	0		None

Program	MELT				Type:	Melting Curves		Cycles:	1
Segment Number	Temperature Target (°C)	Hold Time (s)	Slope (°C/s)	2nd Target Temp (°C)		Step Size (°C)	Step Delay (Cycles)		Acquisition Mode
1	95	0	20	0		0	0		None
2	64	20	20	0		0	0		None
3	95	0	0.2	0		0	0		Continuous

Program	COOL				Type:	None		Cycles:	1
Segment Number	Temperature Target (°C)	Hold Time (s)	Slope (°C/s)	2nd Target Temp (°C)		Step Size (°C)	Step Delay (Cycles)		Acquisition Mode
1	40	30	20	0		0	0		None

Printed on 9/20/2010 at 17:06:14 — Page 1 of 3

Appendix 4:

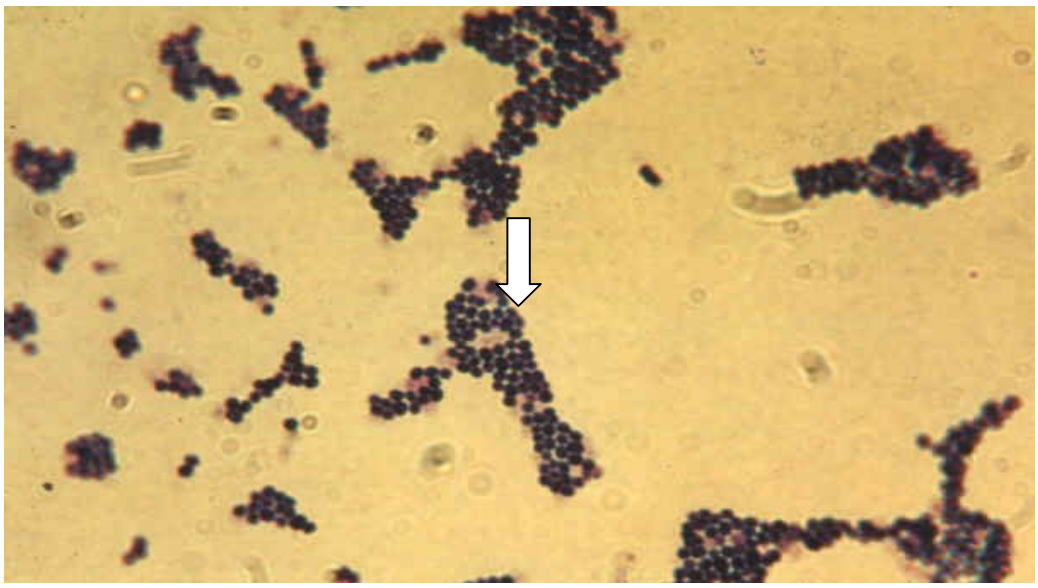


Plate 1: showing typical Gram positive cocci in clusters.

Appendix 5

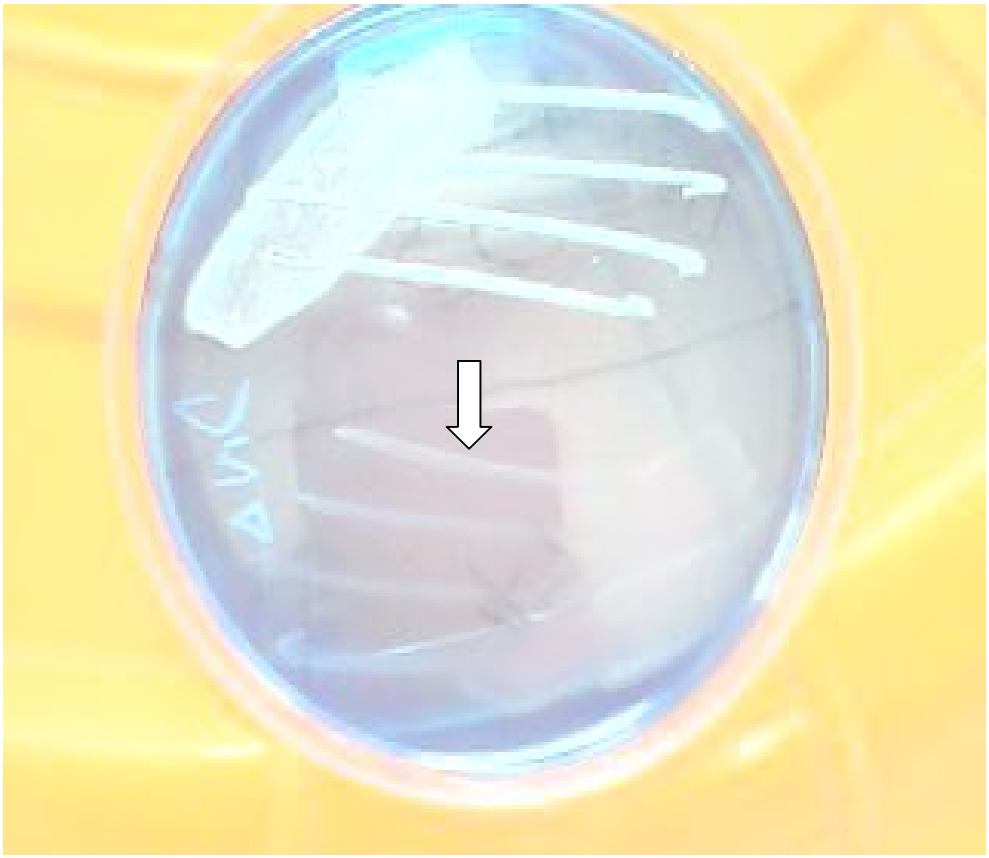


Plate 2: Demonstrating DNase production by an isolate. Note the clear zones around colonies as a result of hydrolysis of DNA by a DNase producing isolate.

Appendix 6

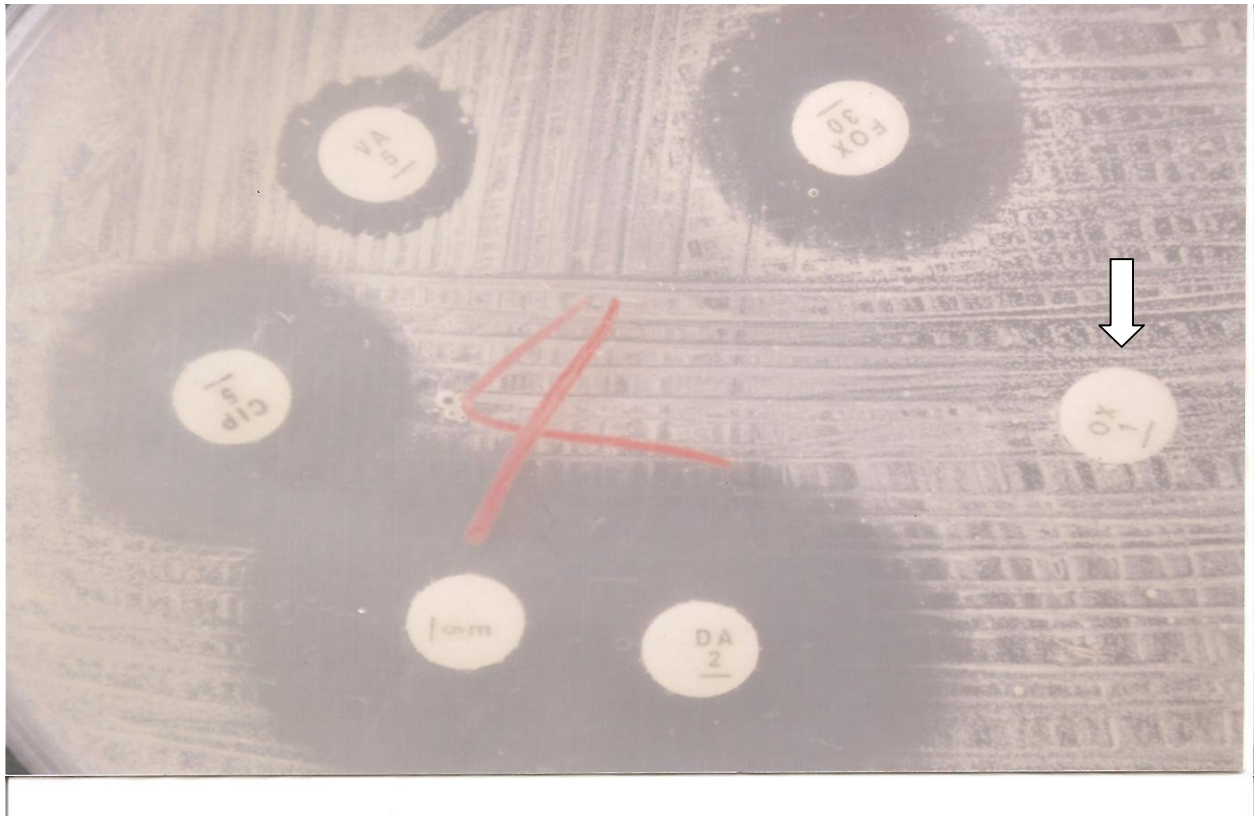


Plate 3: Showing a Zaria isolate demonstrating oxacillin resistance, Cefoxitin, Vancomycin, Ciprofloxacin, Clindamycin and Erythromycin sensitivity (Negative D- test). From the result

above isolate was characterized as MRSA by oxacillin and as MSSA by Cefoxitin.

Appendix 7

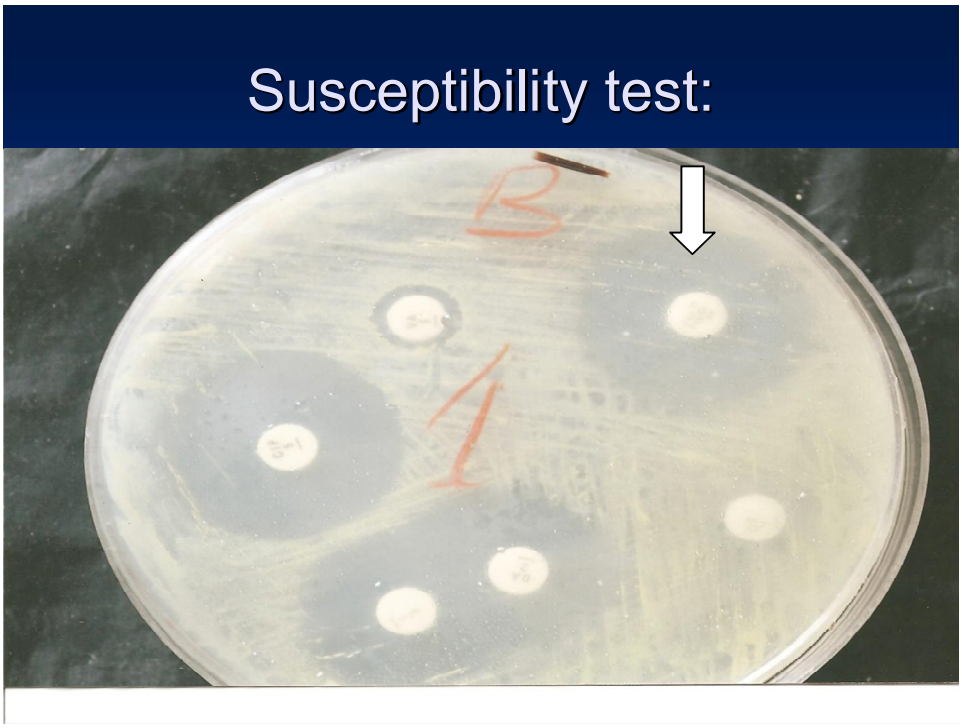


Plate 4: Showing a Birnin-Gwari isolate demonstrating oxacillin resistance, Cefoxitin resistance (MRSA) (Haze of growth around colonies), Vancomycin, Ciprofloxacin, Erythromycin and Clindamycin sensitivity.

Appendix 8

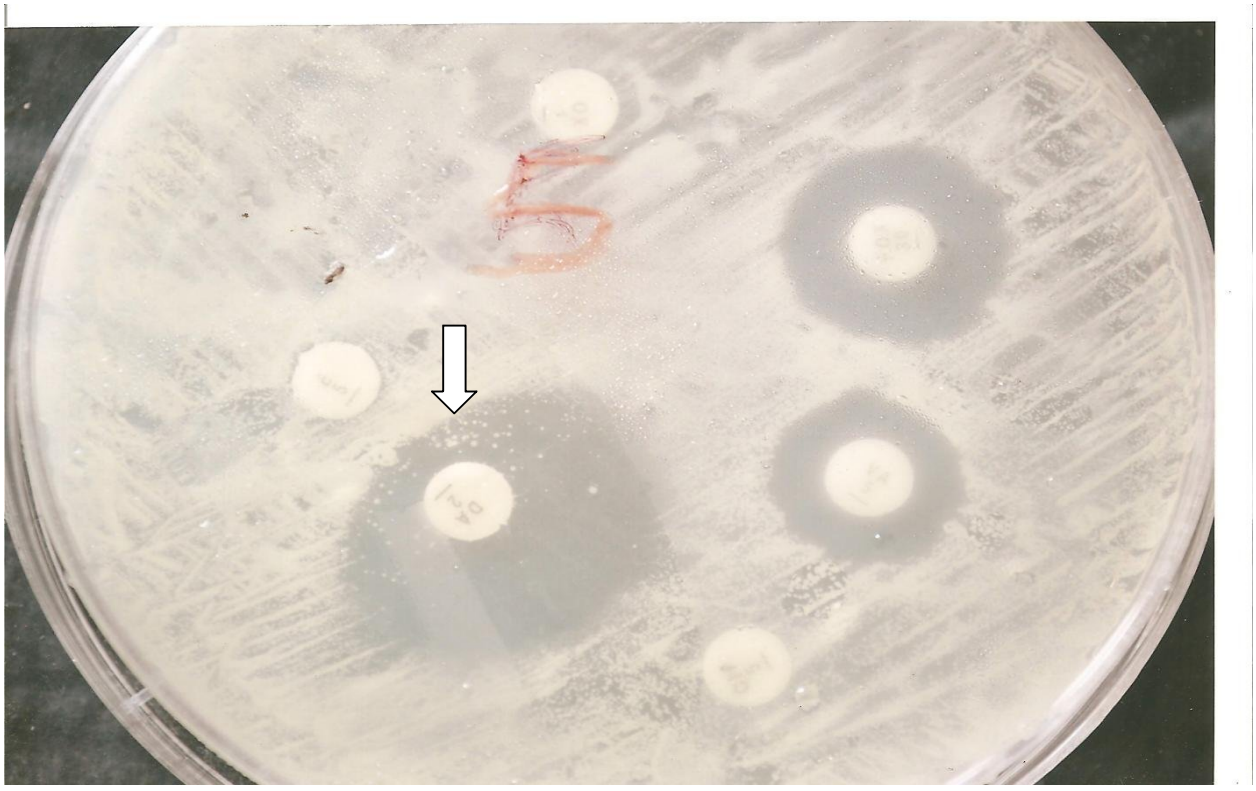


Plate 5: showing inducible clindamycin resistance (a positive D-test).Isolate is erythromycin resistant but clindamycin sensitive.See induced strains growing towards clindamycin disc.

Appendix 9

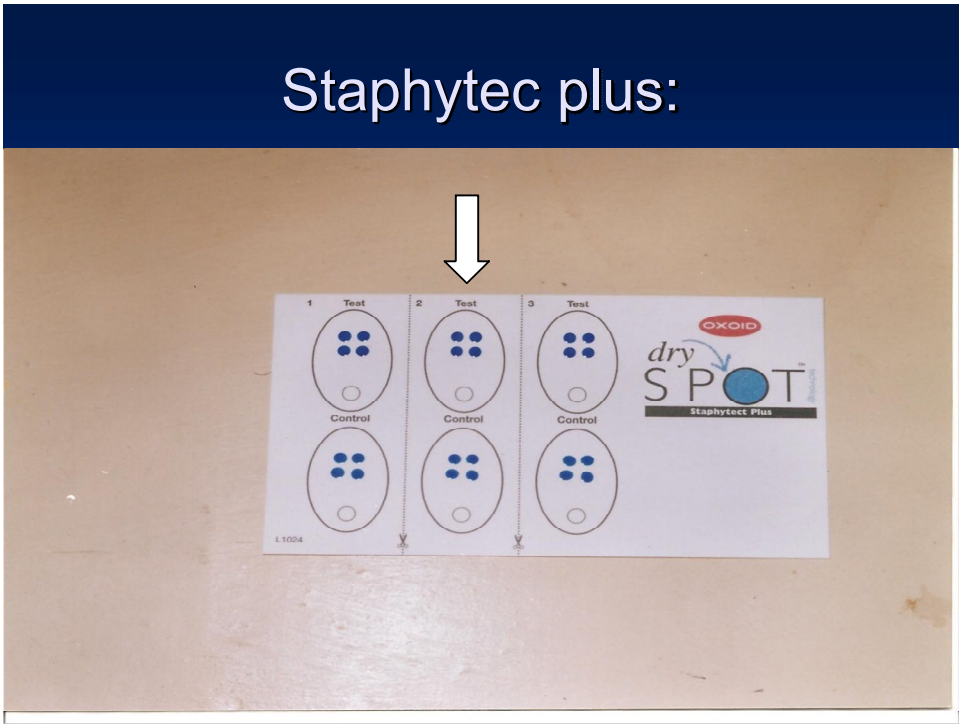


Plate 6: Showing dry spot test used to confirm MRSA that possesses capsular polysaccharide. It has a test spot and a control spot.

Appendix 10

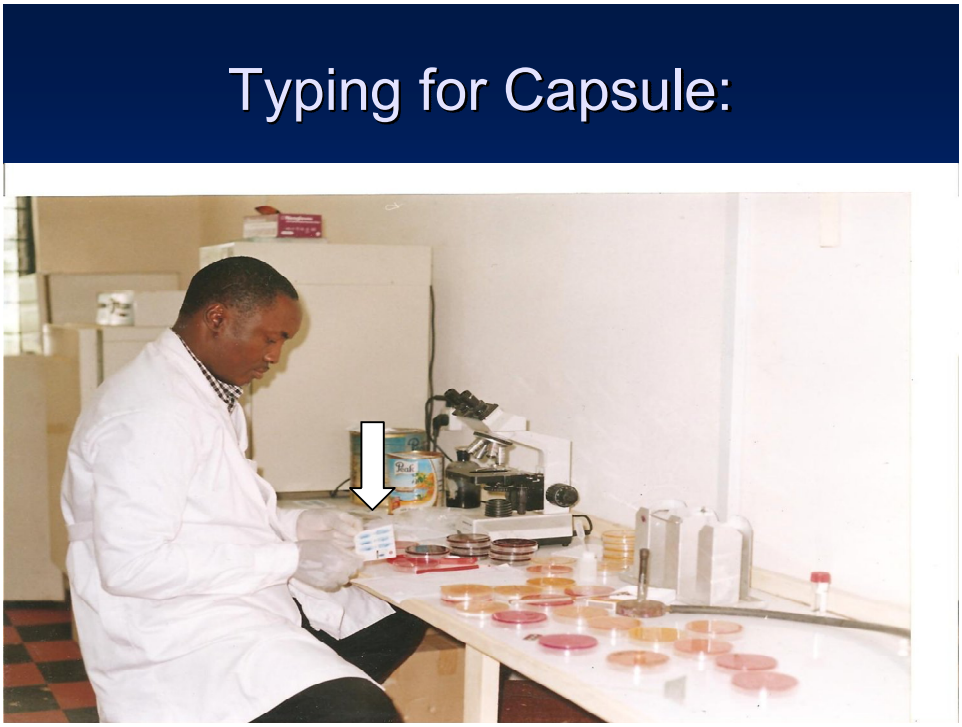


Plate7: Showing the researcher rocking for agglutination on test card.

Appendix 11

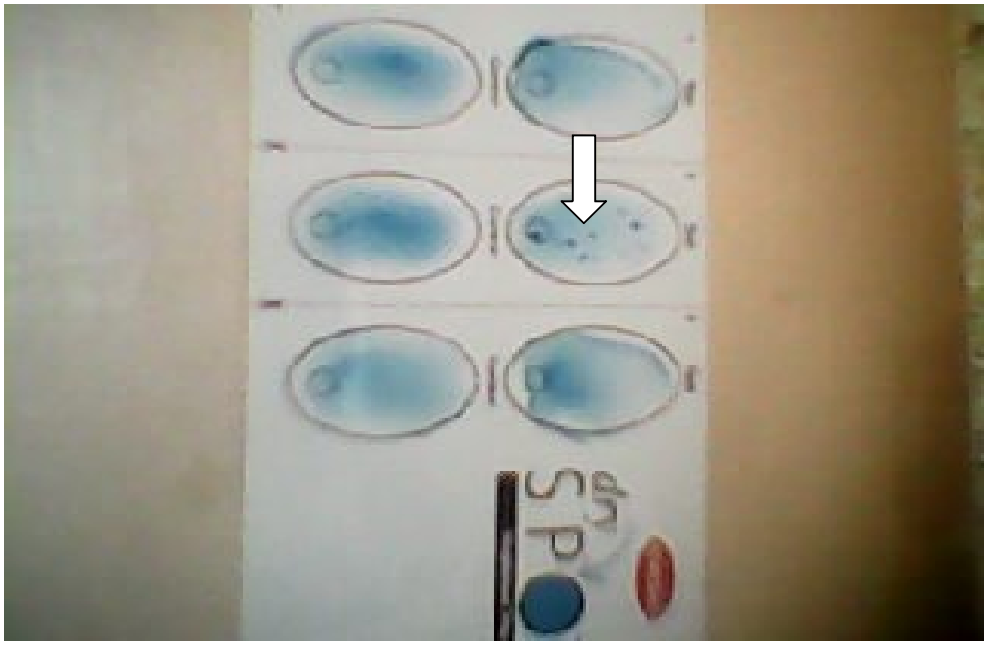


Plate8: Showing an MRSA isolates positive for the capsular types5&8.

Appendix 12



Plate9: showing impregnated end of Nitrocephin Sticks dipped in broth suspension of isolates. A colour change from pink to red indicates an isolate is β-lactermase producer i.e. resistant to ampicillin, amoxicillin, penicillin, carbenicillin, ticarcillin, mezlocillin and pipercillin.

Appendix 13

ISOLATES * OXAC

Crosstab

			OXAC		
			S	R	Total
ISOLATES	K	Count	3	9	12
		Expected Count	2.2	9.8	12.0
	B	Count	1	9	10
		Expected Count	1.8	8.2	10.0
Total		Count	4	18	22
		Expected Count	4.0	18.0	22.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	0.825	1	0.364		
Continuity Correction(a)	0.125	1	0.724		
Likelihood Ratio	0.864	1	0.353		
Fisher's Exact Test				0.594	0.368
Linear-by-Linear Association	0.788	1	0.375		
N of Valid Cases	22				

a. Computed only for a 2x2 table
b. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 1.82.

ISOLATES * CEF

Crosstab

			CEF		
			S	R	Total
ISOLATES	K	Count	9	3	12
		Expected Count	8.2	3.8	12.0
	B	Count	6	4	10
		Expected Count	6.8	3.2	10.0
Total		Count	15	7	22
		Expected Count	15.0	7.0	22.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	0.566	1	0.452		
Continuity Correction(a)	0.086	1	0.770		
Likelihood Ratio	0.565	1	0.452		
Fisher's Exact Test				0.652	0.384
Linear-by-Linear Association	0.540	1	0.462		
N of Valid Cases	22				

a. Computed only for a 2x2 table
b. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.18.

ISOLATES * VAN

Crosstab

			VAN		Total
			S	R	
ISOLATES	K	Count	6	6	12
		Expected Count	8.2	3.8	12.0
	B	Count	9	1	10
		Expected Count	6.8	3.2	10.0
Total		Count	15	7	22
		Expected Count	15.0	7.0	22.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	4.023	1	0.045		
Continuity Correction(a)	2.390	1	0.122		
Likelihood Ratio	4.384	1	0.036		
Fisher's Exact Test				0.074	0.059
Linear-by-Linear Association	3.840	1	0.050		
N of Valid Cases	22				

a. Computed only for a 2x2 table
b. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.18.

ISOLATES * CIP

Crosstab

			CIP		
			S	R	Total
ISOLATES	K	Count	6	6	12
		Expected Count	7.6	4.4	12.0
	B	Count	8	2	10
		Expected Count	6.4	3.6	10.0
Total		Count	14	8	22
		Expected Count	14.0	8.0	22.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	2.121	1	0.145		
Continuity Correction(a)	1.023	1	0.312		
Likelihood Ratio	2.198	1	0.138		
Fisher's Exact Test				0.204	0.156
Linear-by-Linear Association	2.025	1	0.155		
N of Valid Cases	22				

a. Computed only for a 2x2 table
b. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.64.

ISOLATES * ERYTH

Crosstab

			ERYTH		
			S	R	Total
ISOLATES	K	Count	6	6	12
		Expected Count	7.1	4.9	12.0
	B	Count	7	3	10
		Expected Count	5.9	4.1	10.0

Total	Count	13	9	22
	Expected Count	13.0	9.0	22.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	0.903	1	0.342		
Continuity Correction(a)	0.265	1	0.607		
Likelihood Ratio	0.914	1	0.339		
Fisher's Exact Test				0.415	0.305
Linear-by-Linear Association	0.862	1	0.353		
N of Valid Cases	22				

a. Computed only for a 2x2 table
b. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 4.09.

ISOLATES * CLIND

Crosstab

			CLIND		
			S	R	Total
ISOLATES	K	Count	9	3	12
		Expected Count	8.2	3.8	12.0
	B	Count	6	4	10
		Expected Count	6.8	3.2	10.0
Total		Count	15	7	22
		Expected Count	15.0	7.0	22.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	0.566	1	0.452		
Continuity Correction(a)	0.086	1	0.770		
Likelihood Ratio	0.565	1	0.452		
Fisher's Exact Test				0.652	0.384
Linear-by-Linear Association	0.540	1	0.462		
N of Valid Cases	22				

a. Computed only for a 2x2 table
b. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.18.