EFFECT OF FLUOROQUINOLONES ANTIBIOTICS ON VANCOMYCIN AND OXACILLIN RESISTANT STAPHYLOCOCCUS SPECIES IN EASTERN CAPE PROVINCE

By

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DECLARATION

I, Oludotun Soyege declare that this dissertation and the work contained herein being submitted to the University of Fort Hare for the degree of Masters of science in Microbiology in the Faculty of Science and Agriculture, is my original work with the exception of the citations. I also declare that this work has not been submitted to any other University in partial or entirety for the award of any degree.

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GENERAL ABSTRACT

This study shows the susceptibility profile of some Staphylococcus species isolated from commensal Staphylococci in Nkonkobe municipality, South Africa. A total number of 120 Staphylococcal isolates were screened for their susceptibilities to various classes of antibiotics such as Aminoglycoside (Gentamycin), Aminoglycoside (Kanamycin), Macrolide (Erythromycin), Tetracycline (Minocycline), Anti-tuberculous (Rifampicin), Lincosamides (Clindamycin), Cephalosporin and Fluoroquinolones in general. During the study, 32 (26%) the test organisms were susceptible to both methicillin and vancomycin, while 12 (10%) had co-resistance to the antibiotics. Furthermore, Gentamycin (an Aminoglycoside) had a relatively high potency against the isolates with 107 (89.17%) of the bacteria being susceptible to it, while 10 (8.33%) were resistant. On the other hand, Erythromycin (a Macrolide) was active against 72 (60%) of the isolates, while 5 (4.17%) and 74 (61.67%) of the isolates yielded intermediate and resistant responses respectively. In addition , 51 (42.5%) of the isolates were susceptible to rifampicin, while 1 (0.83%) and 17 (14.17%) were intermediate and resistant respectively.

Ten percent of the isolates screened for their antibiotic susceptibility pattern in this study were positive for *mecA* gene among the vancomycin-oxacillin resistant strains while *van* gene was not detected in any of the isolates. This shows how the synergy of both vancomycin and oxacillin contribute to some resistance nature of Staphylococci. In order to overcome this resistance attributes of Staphylococci, to the commonly used antibiotics as discussed under this context, various types of fluoroquinolones were tested. The result shows that less than 10% of the isolates were generally resistant to the fluoroquinolones except against Nalidix acid to which all the isolates were resistant.

Other antibiotics had relatively higher resistance patterns as observed for minocycline (39.51%), clindamycin (12.75%), gentamycin (12.31%) and vancomycin (12.3%). The new generation fluoroquinolones including Gatifloxacin, Levofloxacin, Moxifloxacin and Ciprofloxacin to which less than 5% of the bacteria are resistant gives some clinical advantage over the Methicillin and Vancomycin resistant strains. About 31% of the isolates had multiple antibiotic resistance index of ≥1 and suggests animals in the community as potential reservoirs of antibiotic resistance determinants in the environment. Data obtained in this study is of epidemiological importance and valuable for disease control.

CHAPTER ONE

GENERAL INTRODUCTION

There are many diseases causing organism in humans and animals among which Staphylococcus is involved as a typical virulent microbial agent. Hence, the need to curb the menace caused by this group of organism that usually develop resistance to commonly used antibiotics. *Staphylococcus* is a Gram positive bacteria that is widely distributed in nature especially as a normal flora of the skin. Under the microscope they appear round (cocci) and form grape-like clusters (Ryan and Ray, 2004). *Staphylococcus* species are non-motile, non-spore forming cocci occurring singly, in pairs and in irregular clusters. Colonies may be white, cream, yellow or orange. The optimum growth temperature is 30^0C to 37^0C. They are facultative anaerobes. The genus *Staphylococcus* contains both pathogenic and commensal organisms in humans and other animals. Most staphylococci are found on the surface of the skin around the nose, mouth, genitals, and anus. Breaks in skin and mucous membranes allow entrance of these organisms into the body where they may cause disease. Most staphylococus infections are caused by the species *Staphylococcus aureus. Staphylococcus aureus* is the cause of a wide range of pathogenic infections, though also a commensal of human skin and boils, impetigo, and cellulitis that are limited to a small area of a person's skin (Stryjewski and Chambers, 2008; Boucher *et. al.,* 2010). There are several species in the genus. For example *S. aureus, S. auricularis, S. capitis, S. epidermidis, S. felis, S. intermedius, S. saprophyticus, S. sciuri, S. simulans, S. vitulus, S. xylosus* etc (Holt *et al,* 1994).

Until the isolation and discovery of *Staphylococcus intermedius* all coagulase positive *Staphylococcus* were identified as *S. aureus* (Hajek 1976). *S. intermedius* is commonly found as a transient colonizer in dogs and has been reported in several animals including, cats, foxes, horses, and pigeons (Rachal *et al.,* 2009). *Staphylococcus* aureus is by far the most

common agent of infection with about 20% of the human population and between 60-90% of the population being transiently colonized (Foster 2005). *Staphylococcus Aureus* is an opportunistic pathogen that causes various infections including skin lesions, abscesses, endocarditis, septicaemia (Rachal *et al.*, 2009) and toxic shock syndrome, with some strain producing *staphylococcal* enterotoxins that are involved in food- borne poisoning outbreaks (Jarraud *et al.*, 2002).

In the same premise, *Staphyloccoccus epidemidis* is a commensal bacterium of the human skin, with seemingly low pathogenic potential. *S. epidermidis* rarely causes diseases in immunocompetent patients. *S. epidermidis* isolates are characterized by their pronounced résistance against many of today's commonly used antibiotics including methicillin (Ziebuhr *et al*, 2006). *S. epidermidis* infections preferentially affect immunocomprized, long term hospitalized and critically ill patients. S. epidermis as a commensal bacterium is often difficult for a clinician to decide whether an isolate represents the causative agent of an infection or an unspecified culture contamination (Ziebuhr, 2006; Li, 2009). *Staphylococcus xylosus* is also one of the species of *Staphylococcus* and is a commensal of the skin of humans and animals and a ubiquitous bacterium naturally present in food. It is one of the major starter cultures used for meat fermentation, but a few strains could potentially be hazardous and are related to in animal opportunistic infections (Dordet-Frisoni et al, 2007).

Staphylococci are common natural commensals that inhabit the body of humans and warm-blooded animals. Most of them are found on the skin on mucosal surfaces surrounding openings in the body surface (Archer, 1998). They are Gram positive cocci, catalase positive, aerobic and/or facultative anaerobics, non motile, non spore forming, occurring singly, in pair or in regular clusters having got its name from the Greek words " Staphyle" and " kokkos" which mean "bunch of grapes" and "granules" respectively (Van Der Zwet *et al.*, 2002) about forty species and 17 subspecies of *Staphylococcus* are recognized (De Buyser *et.al.*, 1992;

Bannerman, 2003) and they are broadly differentiated on the basis of coagulase production. Coagulase-positive *Staphylococcus aureus* are the best known and have been frequently implicated in the etiology of infections and toxicity in animals and humans, as against many coagulase-negative staphylococci (CNS), considered to be saprophytic, commensals and/or rarely pathogenic when present in large numbers (Kloos and Scleifer 1980). *S. hominis, S. warneri, S. capitis, S. simulans., S. cohnii, S. xylosus* and *S. saccharolyticus* are examples of the CNS that may be referred to as commensals as they are mostly non invasive, though may also be opportunistic pathogens of both human and animals preferentially affecting the immunocomprised, long-term hospitalized and critically ill patients (Ziebuhr et al, 2001; McCann, 2008).

Recent times have seen a burgeoning literature on some characteristics that used to be the exclusive preserves of clinical *staphylococcal* isolates, but now in the commensal subgroups. Typical examples include the formation of thick, multi-layered biofilms on inert surfaces, such as polymers or metals known to be attributes of nosocomial pathogens (Gotz, 2002) and pronounced resistance against many commonly used antibiotics including methicillin. Clinical isolates obtained as commensal strains were formerly susceptible to antibiotics (Kozitskaya et al, 2005). Methicillin resistance as in *S. aureus* (well known clinical pathogen) mediated by the *mecA* gene encoding a penicillin-binding protein disrupt the activity of β-lactam antibiotics (Bignardi *et. al.,* 1996; Ramos-Trujillo *et. al.,* 2003).

Rationale for the Research

The scourge of Staphylococcal infection continues unabated. The bacteria within the genus have been implicated in various types of infections and progression of the infection becomes worsened by resistance to many antibiotics. *Staphylococcus* aureus is the leading cause of septicaemia, with high levels of morbidity and mortality arising from complications (Lodise *et al.,* 2009; Bosso *et al.,* 2011). Complication rates increase with the duration of bacteraemia and delay in appropriate therapy (Khathi *et al.,* 2003). Members of the genus *Staphylococcus* are major human pathogens (Kampen *et. al.,* 2005). Coagulase-positive *Staphylococcus epidermidis* strains cause a variety of nosocomial infections, including postoperative septicemia. Recent experience has also emphasized the importance of coagulase-negative species. *Staphylococcus epidermidis* has risen to prominence through its frequent association with haemodialysis shunts, indwelling catheters, artificial heart valves, and other prosthetic implants that are used increasingly in modern medicine (Pfaller and Herwaldt, 1988). Similarly, *Staphylococcus saprophyticus* is now recognized as a common cause of female urinary tract infections (Anderson *et. al.,* 1976).

The efficacy of fluoroquinolone in veterinary fields against the *S. intermidius* infection has been approved. For example, fluoroquinolones are recommended as a primary antibiotic regimen for canine pyoderma caused by *S. intermedius* (Ihrke et al., 1999). However, gradual increases of fluoroquinolone resistance have been reported in *S. intermedius* isolates from canine pyoderma, otitis external (Ganièrea *et.al.,* 2004; Lloyd et al., 1999; Intorre et al., 2007; Jones et al., 2007), and urinary tract infections (Shoemaker *et. al.,* 2006; Cohn et al., 2003), which might be due to extensive administration, unnecessary overdose, and/or prolonged misuse of fluoroquinolones (Shakir, 2012; Ruiz, 2003). Several fluoroquinolones including enrofloxacin, difloxacin, orbifloxacin, and marbofloxacin are licensed for veterinary use (Ihrke et al., 1999). Although ciprofloxacin and ofloxacin have been licensed for veterinary use in Korea, it is known that their use has been restricted to

humans in many countries including the United States. Generally, antibacterial fluoroquinolones such as ciprofloxacin have been effective in treating *staphylococcal* infections, especially those caused by methicillin-resistance strains (Aldridge *et. al.,* 1985; Hooper and Wolfson, 1991). This research is designed to assess this trend in commensal S*taphylococcus* species.

Hypothesis

We hypothesize that the commensal *staphylococcus* species in the Nkonkobe Municipality in Eastern Cape Province that exhibit resistance to both vancomycin and methicillin possess methicillin and vancomycin-resistant genes but may exhibit appreciable susceptibilities to new fluoroquinolone antibiotics.

Aims and objectives

The aim of this study is to assess the prevalence of fluoroquinolone resistance among vancomycin and Oxacillin Resistant *Staphylococcus* species in the Nkonkobe Municipality in the Eastern Cape Province, South Africa. The specific objectives include:

1. To assess methicillin and vancomycin susceptibility profile of commensal *Staphylococcus* species isolated from Nkonkobe Municipality environment.
2. Assessment of the susceptibility profile of methicillin and vancomycin resistant commensal *Staphylococcus* species to some fluoroquinolone antibiotics such as ciprofloxacin, ofloxacin, levoflaxic and moxifloxacin.
3. Determination of the prevalence of multiple resistance MET-VAN-FLU order among the commensal *Staphylococcus* species.

CHAPTER TWO

LITERATURE REVIEW

2.1 Factors influencing multidrug resistance in Staphylococcus species

Antibiotic resistance in bacteria that causes infection in man is an issue of major concern. Although the misuse of antibiotics in human medicine is the principal cause of this problem, resistance is a type of drug resistance where a microorganism has the ability to withstand exposure to an antibiotic. Spontaneous or induced mutation in bacteria may encourage resistance to antimicrobials. Genes that are responsible for resistance can be transferred between bacteria in a horizontal fashion by conjugation, transduction, or transformation. Many antibiotic resistant genes reside on plasmids, facilitating their transfer. If a bacterium harbours many resistant genes it is termed multi-drug resistant or a super bacterium. Before the wide spread use of antibiotics there were low levels of pre-existing antibiotic resistant bacteria. Evolutionary pressure from the use of these antimicrobials has played a significant role in the development of multi-drug resistant varieties and the spread of resistance between bacterial species. Antibiotics are often used in rearing animals for food and this too could be responsible for the creation of the resistant strains in bacteria. The question of whether use of antibiotics and resistant isolates of bacteria from animals have an impact on human health has been under scrutiny since the Swann (1969) report was published Lucas (1972). Some of these issues have been reviewed recently (Witte, 1998a and 1998b). Some animal, health, farming, and pharmaceutical groups are reluctant to accept that there are limits between antibiotic use, resistance in animal isolates and resistance in human pathogens (Shryock and Richwine, 2010).

Fluoroquinolone resistant strains of *Staphylococcus aureus* were isolated from human subjects soon after enrofloxacin started to be used in poultry in Europe

(*Jacobs-Reitma et al*. 1994). A study in the United States of America documents found not only a temporary association between the use of fluoroquinolones in treating children and resistance in human isolates, but also that molecular sub-typing indicated that resistant strains from children were very similar to resistant strains from human adult subjects (Smith et al.,1999). There is observational evidence from case studies indicating direct spread of commensal bacteria from animals to man (Hoekstra and Paulton, 2002).

2.2 Drug resistant gene of Staphylococcus species

Early evidence of the resistant gene to vancomycin a glycopeptide the last line of treatment for *Staphylococcal* infection from animals came from observational studies (Anono, 1996) but scientific evidence based on molecular analysis shows the distribution of the vancomycin A or B gene as the resistant determinant (*Van den Braak et al., 1998)*. Molecular studies have helped clarify the presence of the resistance genes, although there seem to be heterogeneity in *vanA* positive gene from human and animal isolates, Indistinguishable patterns are found among pigs, poultry and human isolates. Resistance mediated by *vanA* gene is the commonest form of vancomycin resistance in human isolates in Europe, whereas *vanB* resistance is more common in the United States of America and Australia.

Antibiotic resistance serves as a major challenge in human medicine (Turnidge, 1998; Williams and Heymann, 1998). Antibiotic resistance problems in human commensals and animals include methicillin resistant, vancomycin resistant and fluoroquinolone resistant *Staphylococcus* species. *mecA* gene also contributes to the resistant nature of *Staphylococcus* species. Methicillin resistance to *Staphylococcus aureus* was becoming a challenge and the need to find an effective treatment for this organism led to a rapid increase in vancomycin use

particularly in the United States of America. Kirst *et. al.,* (1998) reported that vancomycin use in the United States of America increased from 2000kg in 1984 to over 11,000kg in 1996. However, in the Netherlands medicinal use of vancomycin increased from 9kg to 60kg over the same period.

Vancomycin resistance started to emerge as a problem in Europe in the 80s particularly in the United States of America. Vancomycin is the drug of last resort in the treatment of *Staphylococcal* infections, strains of Staphylococcus isolates with reduced susceptibility to vancomycin has also emerged (*Woodford et al., 1991*). It is worth considering that Current damage has been done with the currently available antibiotics, that is, Methicillin and Vancomycin and to some extent the fluoroquinolones as resistance is already well established in the use of this moieties in bacterial populations in man, animals, and the environment. Salyers and Cuevas (1997) pointed out that although withdrawal of an antibiotic can lead to a decline in resistance to that antibiotic, resistance levels rapidly rebounds if the antibiotic is re-introduced.

2.3 Epidemiology of methicillin resistant Staphylococcal infections

In recent years there has been several reports of methicillin resistant *Staphylococcus* infection in horses, these reports suggest that the incidence of resistant *Staphylococcal* infection is on the rise. Orsini *et. al.,* (2005) reported on the successful treatment with Vancomycin on Methicillin resistant *Staphylococcus epidermidis* infection in a horse either alone, or in combination with other antimicrobial drugs, usually an aminoglycoside. Most strains of Methicillin resistant *Staphylococcus aureus* are susceptible to vancomycin. Vancomycin intermediate *Staphylococcus aureus* and vancomycin resistant *Staphylococcus aureus* are widely reported resistant isolates in human and farm animals. The combination of

an aminoglycoside and vancomycin causes enhanced nephrotoxicity, this combination is therefore contra-indicated in patients pre-disposed to renal failure. (Hartmann *et. al.*, 1997) suggested that methicillin resistant *Staphylococcus aureus* should be considered only in post operation *Stapyloccocus aureus* infections that do not respond to routine antimicrobial treatment. Because methillin resistant *Staphylococcus aureus* has become such a common pathogen and treatment options are limited, good hygiene is highly recommended both in clinical and community settings.

Staphyloccocus is a common natural commensal which inhabit the body of human beings and warm blooded animals. They inhabit the skin, mucosal surfaces and surrounding openings in the body surface (Archer 1998). It is extremely adaptable to antibiotic pressure, and was one of the earlier bacteria in which penicillin resistance was detected in 1947. Methicillin resistance was first detected in Britain in 1961 and have been responsible for over 37% of fatal cases of sepsis in the United Kingdom in 1999 up from 4% in 1991. They are gram positive cocci, catalase positive, aerobic and or facultative anaerobes, non motile, non-spore forming, occurring singly, in pairs or in irregular clusters having obtained its name from the greek words "staphyle" and "kokkos" which literally means bunch of grapes and granules respectively (Van der Zwet et al., 2002, Bannermann 2003). They are broadly differentiated on the basis of coagulase positive *Staphylococcus. Staphylococcus* is best known and has been frequently implicated in the etiology of infections and toxicity in animals and humans, as against many coagulase negative *Staphylococcus* which is a saprophyte, a commensal and rarely pathogenic when present in their large numbers (Kloos and Schleifer 1980). *Staphylococcus xylosus, Staphylococcus capitis, Staphylococcus hominis, Staphylococcus warneri, Staphylococcus cohnii*, *Staphylococcus pseudointermedius* are examples of the coagulase negative *Staphylococcus* that may be referred to as commensals, Mostly they are non-invasive , they may be opportunistic pathogens of both

human and animals preferentially affecting the immunocompromised long term hospitalised and critically ill patient (Ziebuhr 2001; Bannermann et al., 2003). *Staphylococcus pseudointermedius* was firstly described in the year 2005 it predominantly colonises dogs and cats. It is the leading cause of skin infections in dogs and cats (Weese ; 2010). It has zoonotic potential. In the past five years *Staphylococcus pseudointermedius* has posed a therapeutic challenge because of its limited treatment options. Methicillin resistant *Staphylococcus pseudointermedius* isolates are characterised by the presence of *mecA* gene. In addition to the resistance to β-lactams methicillin resistant *Staphylococcus pseudointermedius* display resistance to other class of antimicrobials such as the aminoglycosides, tetracyclines, lincosamides, trimethoprim, chloramphenicol and fluoroquinolones. Methicillin resistant *Staphylococcus pseudointermedius* colonises healthy animals and people handling animals who then act as reservoirs and enhances spread among the human and animal population. It has also been reported from studies that human infections occur with *Staphylococcus pseudintermedius* have been reported. Several species of Gram positive cocci warrant special consideration. A lot are so virulent that incorrect choice of antibiotic may rapidly be fatal to the patient. The more traditional nosocomial methicillin resistant *Staphylococcus aureus* has also been identified in the community in increasing numbers. This stream of methicillin resistant *Staphylococus aureus* is multi-drug resistant making drug administration a challenge (Howden et al, 2011). This strain agent is resistant to methicillin but susceptible to other antimicrobials.

In recent past there has been documentation to show the prevalence and characteristics of *Staphylococcus* clinical isolates manifesting in the commensal sub group. A typical example is the formation of a thick multi-layered biofilm on inert surfaces such as polymers or metals known to be attributes of nosocomial pathogens (Gotz, 2002) and pronounced resistance against many of today commonly used antibiotics including

methicillin. Methicillin resistance in the commensal strain isolates is mediated by the *mecA* gene decoding a penicillin-binding protein with reduced affinity for β-lactam antibiotics (Bignardi *et. al.,* 1996; Ramos-Trujillo *et. al.,* 2003).

So far only four vancomycin resistant strains of S*taphylococcus aureus* have been reported from the United States (Chang *et al.,* 2003), (Courvalin 2006). In Asia there has been no report of vancomycin mediated resistance, except for vancomycin intermediate resistance in japan (CDC, 1997) and korea (Kim *et al.,* 2000). Recently, Tiwari and Sen (2006) reported a Vancomycin resistant Staphylococcus isolate which is *van* negative.

More than 90% of *Staphylococcus* strains are resistant to penicillins (Chambers 2001), followed by increasing resistance to methicillin, aminoglycoside, macrolides, and lincosamides. The second vancomycin resistant isolate was isolated in pennisylvania and reported by Clarke *et al.,* 2005 and the third vancomycin resistant isolate was isolated in New York. While Intergenomic transfer of high level vancomycin resistance from *enterococcus faecalis* to *Staphylococcus aureus* was reported, and resistant gene transfer from *Staphylococcus aureus* to *Staphylococcus aureus* (Servin 2004*;* Pawa 2000) was reported. This has alarming implication in a clinical and community set up and may soon become a global problem unless antimicrobial agents are used more prudently. Scientists, clinicians and other health care professionals are encouraged to report promptly vancomycin resistant *Staphylococcus aureus* cases for appropriate care and treatment of patients and quick implementation of infection control precautions to prevent the spread of vancomycin resistant *Staphylococcus aureus.*

2.4 Advent of Fluoroquinolones in controlling Methicillin-Vancomycin resistant Staphylococcus infections

Fluoroquinolones was introduced into the clinical setting about ten years ago (Acar and Goldstein, 1997), it offers clinicians oral and parenterally administrable compounds with a broad spectrum of activity and therapeutic results unseen before for a wide range of infections including complicated gastrointestinal infections, sexually transmitted disease, respiratory tract infections and chronic osteomyelitis. Extensive use and misuse of this compound in both human and veterinary medicine has led to the emergence and spread of

effective against both Gram positive and Gram negative bacteria (Morrow *et. al.*, 2010).

The incidence of fluoroquinolone resistance in *Staphylococcus aureus* is not easy to delineate since its origin is not known. Methicillin resistant *Staphylococcus aureus* is an important pathogen as it frequently causes nosocomial infections resistant to various antimicrobial agents. Few years after the introduction of fluoroquinolone for clinical use, most methicillin resistant isolates from clinical specimens were resistant to fluoroquinolones (Acar and Goldstein, 1997). In all cases, the resistance is due to spread of one to two clones among five to eight different clones present in the clinical setting. In cases of community acquired infections such organisms are rarely isolated. In infections of *Staphylococcus aureus* the primary target of fluoroquinolones is the A sub–unit of Deoxyribonucleic acid topoisomerase iv and the secondary target is the A subunit of Deoxyribonucleic acid gyrase. Increased use of fluoroquinolone has led to a greater number of fluoroquinolone resistance *Staphylococcus* species (Hoekstra and. Paulton, 2002). Increased resistance may be attributed to a reduction in the affinity of the drug to the target site through mutation. This has led to the need for the research to determine the susceptibility profile of new generation

fluoroquinolone to community acquired commensal Staphylococcus specie isolates from animals in the Nkonkobe municipality of the Eastern Cape Province of South Africa.

The result of the global problem that antibiotic resistance is causing has made it a major cause of death worldwide, and various international organizations are taking steps to contain antimicrobial resistance (*WHO, 2011)*. Most countries of the world data from consumption and resistance to antibiotics are limited and the relationship of resistance to morbidity and mortality is quantitatively unclear. The situation even gets worse where use of antibiotics is available without prescriptions. What is of paramount significance is data on consumption of antibiotics in designated areas such as hospitals, for example chloramphenicol was infrequently used in a hospital, but was routinely prescribed in a particular ward where more than half of the chloramphenicol resistant hospital staphylococcal isolates originated. In 1994, a threshold hypothesis was proposed that antibiotic resistance could be minimised if total antibiotic use in a particular environment stays below a critical quantitative level. Both the antibiotic in use and how it is used contribute to the development of resistance, while the use of a broad spectrum antibiotic rather than narrow spectrum is known to favour the emergence of resistance. There is the need to put in place control measures such as education of health care workers in the hospital environment, and an effective control programme/policy that will be a way out of the continued resistant nature of some Staphylococcus species (Chowers *et. al.,* 2009).

Due to the characteristic feature of methicillin resistance which is heterogeneous in nature, new molecular techniques such as deoxyribonucleic acid probe hybridisation, polymerase chain reaction and pulse field gel electrophoresis should be used to monitor drug resistant strains. Patients at high risk of methicillin resistant *Staphylococcus aureus/Staphylococcus epidermidis* or those with a history of methicillin resistant *Staphylococcus* aureus/*Staphylococcus epidermidis* colonisation should be evaluated on

admission to hospitals. Patients who are at risk of persistent methicillin resistant *Staphylococcus aureus* carriage are those with skin lesions or sources of infection such as orthopaedic implants, drains or catheters. Additional infection control measures like the decolonisation of staff and patient who are methicillin resistant *Staphylococcus aureus*/*Staphylococcus epidermidis* carriers is of utmost importance (Sotozono *et. al.,* 2002).

A successful antibiotic control programme requires a multi-disciplinary approach. This should include periodical review of prescribing patterns of physicians, recommendation for first line antibiotics, education of prescribing physician and appropriate microbiological testing and reporting. Current study by Dryden, (2010) suggests that for a community acquired staphylococcal infection, initial empiric therapy should comprise a combination of antibiotics until results of *in vitro* susceptibility test are available. With regard to nosocomial infections, the glycopeptides have become one of the last remaining options where methicillin resistant *Staphylococcus aureus*/*Staphylococcus epidermis* are suspected. Since they remain one of the very few classes of antibiotics for the treatment of methicillin resistant *Staphylococcus* aureus/*Staphylococcus epidermidis* it is important they are used carefully to prevent the development of resistance as strains with reduced susceptibility to *Staphylococcus aureus* and *Staphylococcus epidermidis* have been reported as well as vancomycin resistant isolates to methicillin resistant *Staphylococcus aureus* when a glycopeptide is prescribed blood levels is advised to be monitored to ensure that effective concentration is achieved. For vancomycin, the level should be 5-10 mg per litre. In view of the poor extra vascular penetration of the glycopeptide in the central nervous system, bones and joints coupled with the emergence of resistance to vancomycin in methicillin resistant *Staphylococcus aureus*/methicillin resistant *Staphylococcus epidermidis,* there is an urgent need for effective alternative antimicrobials for the effective treatment of infections caused by these pathogens. A number of antibiotics currently under clinical development may be

suitable and this include the following ; the streptogranins, third generation fluoroquinones, oxazolidinones and the carbapenems. The streptogramins act by disrupting the translation of messenger–ribonucleic acid into protein. Several new third generation fluoroquinolones moxifloxacin and trovafloxacin are active against Gram positive cocci. However, ciprofloxacin resistant and methicillin resistant *Staphylococcus aureus* strains often exhibit decreased susceptibility to other fluoroquinolones and it is unclear whether this newer class will be effective against multi-drug resistant strains of *Staphylococcus*. For the oxazolidinones class of antibiotics, they are active against methicillin resistant *Staphylococcus aureus* but the mechanism of action is not yet fully understood. A new semi-synthetic glycopeptide ly333328 a glycopeptide derivative with a key n-alkylation substitution is currently under trial, highly bactericidal in vitro, and tolerability in humans is yet to be established (Biavasco *et. al.*, 1997).

The world health organisation has set the year 2015 as the year of achieving it health related millennium development goals but whether the target can be achieved is a cause for concern because antibiotic resistance is currently a cause for global concern because infections caused by resistant microorganisms often fail to respond to standard treatment resulting in prolonged illness and greater risk of death, reduction in the effectiveness of treatment because patients remain infectious for longer periods and serving as a reservoir and potentially spreading resistant microbes to others, many infectious diseases become uncontrollable and could derail the achievement made towards achieving the 2015 millenium development goals, it increases the cost of health care because resistance to first line medicines will result in the use of more expensive medicines and the longer stay in hospital also attracts extra costs, achievements of modern medicine are put at risk because of antibiotic resistance (WHO, 2001), antibiotic resistance also threatens health, security, and damages trade and economy, as a result of this the World health organisation has concluded

that antibiotics used as growth promoters in animal feeds should be prohibited in the absence of risk assessment. Generally, effective diagnostic a

et al, 1998). In 1998 the European Union health ministers voted to ban four antibiotics widely used to promote animal growth. Regulation banning the use of antibiotics in animal feeds in Europe with the exception of two antibiotics in poultry feeds which became effective in 2006. Evidence of the impact of this low prevalence of antibiotic resistance was attested to in the Scandinavian countries. In 2001 the union of concerned scientists estimated that greater than 70% of the antibiotics in the United States of America are given to food animals e.g chickens, pigs and cattle in the absence of disease. In 2000 the United States Food and Drug Agency announced their intention of revoking the approval of fluoroquinolone use in poultry production because of substantial evidence linking it to the emergence of fluoroquinolone resistant camphylobacter disease condition in humans. The final decision to ban fluoroquinolone use in poultry production was effected five years after, although there was stiff opposition from the food and pharmaceutical industries. Two bills have been passed in 2007 aimed at phasing out non-therapeutic antibiotics in the United States food animal production.

2.5 **The use and mechanism of action of fluoroquinolones**

Fluoroquinolones inhibits bacterial DNA synthesis by interfering with DNA gyrase (bacterial topoisomerase 2) which is an enzyme necessary for DNA replication (Hooper, 2001a). it is rapidly bactericidal has concentration dependent bactericidal activity which means its extent of bacterial killing increases as drug concentration increases, well absorbed after oral administration (50% to > 95%). Absorption of ciprofloxacin and ofloxacin result in blood levels comparable to their intravenous preparations. If the patient can take oral

medications, it is advisable that oral dosage formulation is taken. Fluoroquinolone is widely distributed into the urine, kidney, prostate tissue, lung, bone, stool, neutrophils, and macrophages. They are very useful in the treatment of infection located in the parts of organs mentioned above. Elimination is primarily from the kidneys hence extent of elimination by the kidneys varies between agents. For renal dysfunction specific dosage reduction is highly recommended. Fluoroquinolones are useful for infections caused by Gram negative bacilli. Currently marketed drugs in this class have only moderate activity against Gram positive bacteria and are therefore not the drugs of choice for infections caused by these bacteria.

CHAPTER THREE

ASSESSMENT OF VANCOMYCIN AND OXACILLIN SYNERGY FOR METHICILLIN-RESISTANT COMMENSAL STAPHYLOCOCCI IN NKONKOBE MUNICIPALITY, SOUTH AFRICA

3.0 ABSTRACT

I evaluated vancomycin and oxacillin synergy in methicillin-resistant commensal Staphylococci in Nkonkobe Municipality, South Africa. Of the 120 Staphylococcus isolates screened, 32 (26%) were susceptible to both methicillin and vancomycin, while 12 (10%) of the isolates had co-resistance to the antibiotics, which is still on the high side both clinically and epidemiologically. Gentamycin (an Aminoglycoside) had a relatively high potency against the isolates with 107 (89.17%) of the Bacteria being susceptible to it, while 10 (8.33%) were resistant. On the other hand, Erythromycin (a Macrolide) was active against 72 (60%) of the isolates, while 5 (4.17%) and 74 (61.67%) of the isolates yielded intermediate and resistant responses respectively. Similarly, 51 (42.5%) of the isolates were Susceptible to rifampicin, while 1 (0.83%) and 17 (14.17%) were intermediate and resistant respectively. Ten percent of the isolates were positive for *mecA* gene among the vancomycin-oxacillin resistant strains while *van* gene was not detected in all the isolates.

3.1 INTRODUCTION

Resistance to antibiotics has become a notable trend in clinical control of many diseases that deserves scientific intervention to bring about some control measures. Thus the issue of vancomycin- methicillin co-resistance in commensal staphylococcus species in any locality can be summarily tackled by determining the range of pathogenicity and seeking effective antimicrobial agent against such resistant strains. Most Staphphylococcal infections are caused by *Staphlylococcus aureus.* This bacteria is the cause of a wide range of pathogenic infections, though also a commensal of human skin and nares. *Staph. aureus* most commonly cause skin infections like folliculties, boils, impetigo, and cellulitis that are limited to a small area of a person's skin (Stryjewski and Chambers, 2008; *Boucher et. al.,* 2010). Other known types of Staphylococcus species include *Staph. albus, Staph. epidermidis, Staph. intermedius, Staph. auricularis, Staph. capitis, Staph. felis, Staph. saprophyticus, Staph. sciuri, Staph. vitulus, Staph. xylosus, Staph. simulan, Staph. viridans* and *Staph. pneumoniae* (Holt *et al,* 1994; Prescott *et. al.,* 2008 and Adebayo *et. al.,* 2011).

According to Mahajan (1979), the role of *Staphylococcus aureus* in pathology in any organ of the body, including the eye, is not disputed. Also, the acceptance of *Staphylococcus albus* as a pathogen in different sites in the human body is gradually growing (Meers *et al.,* 1975). It has been reported as the second most common pathogen in bacteriologically proven cases of urinary tract infection, and novobiocin-resistant micrococci have been claimed to have a special predilection for the urinary tract of human beings (Gallagher *et. al.,* 1965; Meers, 1974; Maskell, 1975). Such cocci have also been recovered from lesions of the eye which regressed when appropriate antimicrobial agents were administered (Khosla *et. al.,* 1964; Valenton *et. al.,* 1973). Generally, various health care organizations worldwide have developed various channels available in educating the public and making some research

funding to tackle problems that emanates from multi drug resistant *Staphylococcus aureus* (CDC, 2012; WSDH, 2012). In this study, we report on the vancomycin and oxacillin synergy in methicillin-resistant commensal Staphylococci in Nkonkobe municipality, South Africa as part of our larger study on the surveillance of reservoirs of antibiotic resistance in the South African environment.

3.2 MATERIALS AND METHODS

3.2.1 Isolates Source

The *Staphylococcus* species were obtained from the culture collections of the Applied and Environmental Microbiology Research Group (AEMREG), University of Fort Hare, Alice, South Africa and were isolated from the environments of Nkonkobe Municipality in the Eastern Cape Province.

3.2.2 Standardization of inoculums and inoculation of plates

The stock cultures were re-activated by subculturing into tubes containing nutrient broth and incubated for 24 hour at 37^{o}C with shaking and thereafter subcultured onto nutrient agar plates and again incubated for 24 hour at 37^{o}C. Three to five colonies from each plate were then suspended in tubes containing 5 ml of sterile distilled water and vortexed thoroughly to achieve a uniform suspension. The turbidity of the suspension was compared to that of the 0.5 McFarland standards and adjusted as required. The standardized inoculums were used in the antibiotic susceptibility tests within 15 minutes. Freshly prepared Mueller-

Hinton agar plates were inoculated with the bacterial suspension using a sterile swab with which even lawns of the bacteria were produced. Antibiotic discs were then applied to the surface of the agar using sterile forceps and the plates incubated at 35^{o}C for 18-24 hour. The zones of inhibition were then measured in millimetres using a ruler and interpreted using available interpretive charts.

3.2.3 DNA Extraction

The extraction of the bacterial DNA was done using the boiling method. A fresh colony of the *Staphylococcus* culture was suspended in 500 µl of DEPC-treated (DNase-RNase free) and boiled for 10 min using a heating block. Thereafter the suspension was centrifuged at 10,000 rpm for 5 mins. The supernatant containing bacterial DNA was used as a template for subsequent PCR reactions.

3.2.4 Assessment of putative *mecA* gene and *van* genes

To detect the presence of *mecA* and *vanA* and *vanB* genes in the resistance *Staphylococcus* species the following primer sets in Table 1 were used:

Table 3.1: Primers used for the polymerase chain reaction detection of *Mec*A and *Van* genes in the *Staphylococcus* species.

Target genes	**Primer Sequence**	**Ref**
MecA	Forward: 5'-GGTCCCATTAACTCTGAAG-3'	Geha *et al.* (1994)
	Reverse: 5'-CCA ATT CCACAT TGT TTC GGT CTA A-3'	
VanA	Forward: 5'-GGGAAAACGACAATTGC-3'	Dutka–malen *et al*. (1995)
	Reverse: 5'-GTACAATGCGGCCGTTA-3'	
VanB	Forward:5'-GTGCTGCGAGATACCACAGA-3'	Ramos-trujillo *et al.* (2003)
	Reverse: 5'-CGAACACCATGCAACATTTC-3')	

3.3 Result and Discussion

This study shows the susceptibility patterns of vancomycin-methicillin co-resistance in commensal staphylococcus species in Nkonkobe Municipality, Eastern Cape, South Africa and the public health implications. As is evident in Table 2, about thirty two isolates (26.67%) tested were susceptible to both methicilin and vancomycine, while 12 (10%) of the isolates from these sources had co-resistance to the antibiotics, which is still on the high side both clinically and epidemiologically. Recent times have seen a burgeoning literature on some characteristics that used to be the exclusive preserves of clinical *staphylococcal* isolates, but now in the commensal subgroups. Typical examples include the formation of thick, multilayered biofilms on inert surfaces, such as polymers or metals known to be attributes of nosocomial pathogens (Gotz, 2002) and pronounced resistance against many of today's commonly used antibiotics including methicillin.

Table 3.2: Profile of vancomycin-methicillin co-resistance in commensal Staphylococcus species isolated from Nkonkobe Municipality environment.

METHICILLIN	**VANCOMYCIN**				
	Susceptible	Moderately Susceptible	Intermediate	Resistant	*Resistant strains (%)
Susceptible	32	2	6	7	5.83
Intermediate	12	-	1	6	5
Resistant	10	14	1	12	10

*Resistant strains (%): Percentage (%) of isolates that are positive for the resistant genes out of the 120 isolates.

Other classes of antibiotics were used for further clarification of the resistance pattern. Table 3 shows β-lactam antibiotic profile of the Staphylococcus isolates. One hundred and fifteen of the bacteria isolates were susceptible to Meropenem (MEM), while about 90.83% were susceptible to Sulbactam Ampicillin (SAM). On the other hand, 93 (77.5%) of the isolates were moderately susceptible to Ceftriaxone (CRO) while only 7 (5.83%) of the isolates were fully susceptible to it and 12 (10%) were resistant. Also, 10 (8.33%) of the isolates were susceptible to Ceftazidime (CAZ), while 22 (18.33%) of the isolates were susceptible to it (ATM) and 94 (78.33%) of the isolates were resistant to it.

Table 3.3: Susceptibility profile of Staphylococcus species isolated from Nkonkobe Municipality environment.to some β- lactam antibiotics.

β-lactam antibiotics	**Susceptible**	**Moderately susceptible**	**Intermediate**	**Resistant**	**Moderately resistant**	***Resistant strains (%)**
CRO	7	93	-	12	7	15.83
AUG	75	-	-	4	-	3.33
CAZ	10	19	36	37	-	30.83
CTX	37	52	6	19	-	15.83
SAM	109	-	2	13	-	10.83
PEN-G	53	-	-	53	-	44.17
MEM	115	-	1	4	-	3.33
ATM	22	1	-	94	-	78.33

KEY: AUG- Augmentin; CTX- Cefotaxime; SAM- Sulbactam Ampicillin; PEN G - Penicillin G; MEM- Meropenem; ATM- Aztreonam; CAZ- Ceftazidime; CRO- Ceftriaxone. *Resistant strains (%): Percentage (%) of isolates that are positive for the resistant genes out of the 120 isolates.

In addition, 107 (89.17%) of the isolates were susceptible to the Aminoglycoside (Gentamycin), while 10 (8.33%) were resistant (Table 4). In comparison, 72 (60%) of the isolates were susceptible to the Macrolide (Erythromycin) while 5 (4.17%) and 74 (61.67%) isolates showed intermediate and resistant responses respectively. Similarly, 51 (42.5%) of the isolates were susceptible to the anti-tuberculous agent – Rifampicin, while 1 (0.83%) and 17 (14.17%) isolates showed intermediate and resistant responses respectively. Molecular detection of *MecA* and *Van* genes revealed that 10% of the isolates were positive for *MecA* gene among the isolates that has co-resistance to both vancomycin and oxacillin. *Van* gene was not detected in all the isolates.

Table 3.4: Susceptibly pattern of Staphylococcus species isolated from Nkonkobe Municipality environment against some non β-lactam antibiotics.

Antibiotic class	**Name**	**Susceptible**	**Intermediate**	**Resistance**	***Resistant strains (%)**
AMINOGLYCOSIDE	GENTAMYCIN	107	-	10	8.33
AMINOGLYCOSIDE	KANAMYCIN	107	37	14	11.67
MACROLIDE	ERYTHROMYCIN	72	5	74	61.67
TETRACYCLINE	MINOCYCLINE	87	44	22	18.33
ANTI-TUBERCULOUS	RIFAMPICIN	51	1	17	14.17
LINCOSAMIDES	CLINDAMYCIN	102	1	11	9.17

*Percentage (%) of isolates that were positive for the resistant genes out of the 120 isolates.

According to Bignardi *et. al.* (1996) and Wielders *et. al.* (.2002), most clinical isolates of methicillin-resistant *Staphylococcus aureus* harbours the *mecA* gene which encodes production of PBP2a, a modified penicillin binding protein with low affinity for β-lactam antibiotics (de Lencastre *et al.,* 1994). However, the emergence of resistance *in vitro* as a result of mutations during subculture on media with increasing methicillin concentrations has also been documented (Berger-Bachi, *et. al.,* 1989; Kozitskaya, 2005).

The study of Domaracki *et al.* (2000), shows that vancomycin is the drug of choice for most methicillin-resistant staphylococcus infections, and therefore, the recent emergence of decreased vancomycin susceptibility in methicillin-resistant staphylococci presents a significant clinical problem. Furthermore, reduced susceptibility to vancomycin in *Staphylococcus* species appears to occur on exposure to vancomycin and under selective pressure, rather than by gene transfer as in enterococci (Sieradzki and Tomasz, 1997; Domaracki *et. al.,* 2000). *In vitro* experiments have demonstrated that selective pressure can produce vancomycin resistance but have also revealed that increases in vancomycin resistance can induce concurrent decreases in resistance to β-lactams in both methicillin-resistant coagulase-negative staphylococci (MRCNS) and methicillin-resistant *Staphylococcus aureus* (MRSA). The study of Domaracki *et. al.* (2000) further shows that clinical isolates of vancomycin-susceptible MRCNS and MRSA become increasingly susceptible to oxacillin when grown in the presence of a sub-MIC of vancomycin. However the present study demonstrated specifically the potency of the β-lactam antibiotics-Meropenem (MEM), Sulbactam Ampicillin (SAM) and Gentamycin (an Aminoglycoside) to be very effective for the control of multiple resistant commensal Staphylococci in Nkokobe municipality, South Africa. The findings of this study could be a good guide in infectious diseases control especially with respect to Staphylococcus infections in the community.

CHAPTER FOUR

FLUOROQIUNOLONE SUSCEPTIBILITY CHARACTERISTICS OF SOME STAPHYLOCOCCUS SPECIES ISOLATED FROM NKONKOBE MUNICIPALITY IN THE EASTERN CAPE PROVINCE, SOUTH AFRICA

4.0 ABSTRACT

I assessed the fluoroquinolones susceptibilities of 120 Staphylococcus isolates recovered from the Nkonkobe Municipality in the Eastern Cape Province, South Africa. Less than 10% of the isolates were generally resistant to the fluoroquinolones except against Nalidix acid to which all the isolates were resistant. Other antibiotics had relatively higher resistance patterns as observed for minocycline (39.51%), clindamycin (12.75%), gentamycin (12.31%) and vancomycin (12.3%). Complementary to this, methicillin and vancomycin resistant isolates had minimal resistances to the fluoroquinolones and especially the new generation fluoroquinolones including Gatifloxacin, Levofloxacin, Moxifloxacin and Ciprofloxacin to which less than 5% of the bacteria are resistant. About 31% of the isolates had multiple antibiotic resistance index of ≥1 and that suggests animals in the community as potential reservoirs of antibiotic resistance determinants in the environment.

4.1 INTRODUCTION

There are many disease causing organisms that are increasingly becoming health threats to humans and other living organisms, among which staphylococcus play a prominent role. *Staphylococcus* species are Gram positive bacteria existing as grape-like clusters of cells that appear roundish under the microscope (Holden *et.al.*, 2006; Yugueros *et. al.*, 2001; Prescott, 2008). *Staphylococcus* can be differentiated from other aerobic and facultative anaerobic, Gram-positive cocci by several simple tests. They are facultative anaerobes; can grow in the presence of bile salts and all are catalase-positive with other diversified biochemical testing needed to identify them to the species level (Sun, 1996; Kateete *et. al.,* 2010). One of the most important phenotypical features used in the classification of staphylococci is their ability to produce coagulase, an enzyme that causes blood clot formation. Six species are currently recognised as being coagulase-positive: *Staphylococcus. Aureus, Staphylococcus. delphini, Staphylococcus. Hyicus, Staphylococccus. Intermedius, Staphylococccus. lutrae, Stapylococcus. Pseudintermedius* and *Staphylococcus. Schleiferi Staphylococcus. Coagulans*. These species belong to two separate groups – the *Staphylococcus Aureus* (which stands alone) group and the *Staph. hyicus-intermedius* group (the remaining five). A seventh species has also been described – *Staphylococcus leei* – from patients with gastritis (Jin *et. al.*, 2004).

Hoekstra and Paulton (2002) reported on the contagious nature of Staphylococcus species and routine isolation from domestic animals in veterinary clinical practice (Euzeby, 1997). Besides their role as commensals on mucosal surfaces and the skin, *Staphylococci* are often involved in a wide variety of diseases in animals (Kloos *et. al.,* 1976; Kloos, 1980). Previous investigations (Seibert *et. al.,* 1992; Sieradzki and Tomasz 1997; Sieradzki *et. al.,* 1998) reported that this group of organisms exhibit multiple resistances to antibiotics including the commonly used ones such as vancomycin and oxacillin. Howden *et al.,* (2011) further reiterated that the antimicrobial resistance in some Staphylococcus species is a major

public health threat, compounded by emergence of strains with resistance to vancomycin and daptomycin and this has posed a therapeutic challenge in treating patients in recent times. In this context, mutation, unregulated provision of antibiotics, dispensing of insufficient doses, reduced adherence to complete dose regimens and the poor quality of the drug supply, generally encourages antibiotic resistance in disease causing organisms (Goossens *et al*., 2005; Blomberg *et al*., 2007; Cars *et al*., 2008). Concisely, the advent of Staphylococci resistance to methicillin and vancomycin necessitated the introduction of fluoroquinolones for Staphylococcal infections chemotherapeutic uses today.

Fluoroquinolones are the only class of antimicrobial agents in clinical use that are direct inhibitors of bacterial DNA synthesis. By binding to the enzyme-DNA complex, Fluoroquinolones stabilize DNA strand breaks created by DNA gyrase and topoisomerase IV (Hooper, 2001b). Thus they inhibit two bacterial enzymes (DNA gyrase and topoisomerase IV), which have essential and distinct roles in DNA replication. The quinolones bind to the complex of each of these enzymes with DNA; the resulting complexes, including the drug, block progress of the DNA replication enzyme complex. This action results in damage to bacterial DNA and consequently bacterial cell death. Thus, fluoroquinolones are bactericidal agents (Hooper, 2012) and includes such antibiotics as Ciprofloxacin, Ofloxacin (floxin, oxaldin, tarivid), Rufloxacin (uroflox), Clinafloxacin, Gatifloxacin, Genufloxacin and Moxifloxacin. Others include Nalidixic acid (which are known to be genotoxic and carcinogenic) and Flumequine which is a carcinogen of veterinary use. These hazardous nature disadvantages the uses of some fluoroquinolones antibiotics in disease treatment (Blondeau ., 2004; Surviving ; eMedExpert, 2011 and Hooper, 2012). In this Research, we report on the antibiogram characteristics of some commensal Staphylococcus isolates against some fluoroquinolones as part of our surveillance of reservoirs of antibiotic resistance in the Eastern Cape Province, South Africa.

4.2 MATERIALS AND METHOD

4.2.1 Sources of sample collection

The *Staphylococcus* species were obtained from the culture collections of the Applied and Environmental Microbiology Research Group (AEMREG), University of Fort Hare, Alice, South Africa and were isolated from nasal and inner ear swab samples of domestic animals including pigs, cattle, cows and goats in the environments of Nkonkobe Municipality in the Eastern Cape Province, South Africa.

4.2.2 Phenotypic Antibiotic Susceptibility Test

The phenotypic antibiotic susceptibility test was done using the disc-diffusion technique of National Committee for Clinical Laboratory Standards by Brown (1987). The antibiotics used include methicillin (5 ug), vancomycin (30 µg), ciproflaxin (5 µg), oflaxacin (5 µg), levoflaxin (5 µg), Gatifloxacin (5 µg) and moxifloxacin (5 µg).

4.2.3 Preparation of McFarland Standard

McFarland standard was prepared for use in the antibiogram assay. Briefly, about 0.5 ml of 0.048 M $BaCl_2$ (1.17% w/v $BaCl_2.2H_2O$) was added to 99.5 ml of 0.18 M H_2SO_4 (1% w/v) while constantly stirring. The suspension was thoroughly mixed to ensure homogenization. Water was used as a blank standard, the absorbance of water and the chemical suspension was measured in a spectrophotometer at a wavelength of 625nm. The acceptable absorbance range for the standard is 0.08-0.13. The standard was then stored in tubes of the same size and volume as those that were used in standardizing the inoculums and stored at room temperature in the abscence from light.

4.2.4 Antibiotic sensitivity test

The antibiotic sentivity test was done using the Bauer-Kirby agar diffusion method (Bauer *et al*., 1966). Related studies that were used to update this methodology include the report of Beathy *et al.,* (2004) as described in this context. Appropriate cultures of the bacterial isolates that were standardized to 10^8 cfu/ml were spread on the surface of Mueller-Hinton agar plates using sterile swab . The plates were allowed to dry for 5 min before placing the multidisc antibiotics on the inoculated plates. Contact between the antibiotic discs and the culture was ensured by gently pressing the disc with sterile forceps. Within 30 min of applying the discs, the plates were incubated at 37^oC for 18 hour. The antibiotic discs used were methicillin (5 µg), vancomycin (30 µg), ciproflaxin (5 µg), oflaxacin (5 µg), levoflaxin (5 µg), Gatifloxacin (5 µg) and moxifloxacin (5 µg). At the end of the incubation period, the plates were observed for zones of inhibition and the diameters of the zones were measured and interpretation of the results was done . (CLSI, 2008).

4.2.5 Multiple Antibiotic Resistance Index (MARI)

MAR index was calculated as the ratio of the number of the antibiotics to which resistance occurred by the isolates (a) to the total number of antibiotics to which the isolates were expressed (b), as follows: MARI= a/b (Krumpermann, 1983). In the context of this study, (a) represents the aggregate resistance of antibiotics to all isolates, while (b) represents the total number of antibiotics to get MAR index estimate.

4.3 RESULTS AND DISCUSSION

This study shows the antibiotics susceptibility patterns of some Staphylococcus isolates obtained from a typically rural community in the Eastern Cape Province, South Africa against some fluoroquinolone antibiotics. As shown in Table 4.1, less than 10% of the isolates were resistant to the fluoroquinolones in general except against Nalidix acid to which all were resistant. Also, 39.51% of the isolates were resistant to minocycline, while 12.75%, 12.31% and 12.3% were resistant to clindamycin, gentamycin and vancomycin respectively. The multiple antibiotic resistance generally observed in this study is relatively high and in support of previous reports (Sato *et. al.,* 2004; Sahoo *et. al.,* 2012) on susceptibility patterns of some multiple resistant *Staphylococcus* species.

Table 4.1: Antibiotic susceptibility patterns of some Staphylococcus isolates against selected fluoroquinolones and other classes of antibiotics.

ANTIBIOTIC	**% Response**				
	S%	**R%**	**I%**	**MS%**	**MR%**
Fluoroquinolones					
NALIDIXIC ACID	-	100	-	-	-
OFLOXACIN	85.23	8.504	4.1	2.21	
CIPROFLOXACIN	66.6	9.25	24.1	-	-
GATIFLOXACIN	91.2	-	8.8	-	-
LEVOFLOXACIN	92.7	4.10	3.20	-	-
MOXIFLOXACIN	77.7	9.5	13.6	-	-
Others					
GENTAMYCIN	87.4	12.31	-	-	-
KANAMYCIN	90.8	8.94	-	-	-
ERYTHROMYCIN	66.97	8.43	24.5	-	-
MINOCYCLINE	50.08	39.51	10.20	-	-
RIFAMPICIN	92.5	7.5	-	-	-
CLINDAMYCIN	62.3	12.75	25.00	-	-
VANCOMYCIN	69.2	12.3	18.69	-	-

LEGEND: S%- Percentage sensitive to antibiotics; R%- Percentage resistant to antibiotics; I%- Percentage of intermediate resistance; MS%- Percentage of moderately susceptible strains; MR% - Percentage of moderately resistant.

Table 4.2 articulates a pair-wise susceptibilities the *Staphylococcus species* to the fluoroquinolones. The antibiotics generally considered for this purpose include the following classes of antibiotics namely Aminolglycoside (Gentamycin), minolglycoside (Kanamycin), Macrolide (Erythromycin), Tetracycline (Minocycline), Anti-tuberculous (Rifampicin) and Lincosamide (Clindamycin). The highest susceptibility of 98.5% was observed against the Erythromycin/Ofloxacin,pair followed by 96.8% susceptibility against Erythromycin/Moxifloxacin combination. On the other hand, 97.5% of the isolates were susceptible to Kanamycin/Ciprofloxacin pair, followed by 85.7% for Minocycline/Moxifloxacin (Table 2). The multiple antibiotic resistance observed is consistent with the reports of Waters *et. al.* (2011) and Panda (2012) which highlighted multiple antibiotic resistance in some bacteria isolated from environmental sources and domestic animals.

Table 4.2: Pair-wise comparison of susceptibilities of some *Staphylococcus* species to Fluoroquinolone and other classes of antibiotics.

ANTIBIOTIC CLASS	**NAME**	**Response**	**Fluoroquinolone Antibiotic**									
			Mox		**Lev**		**Cip**		**Ofl**		**Gat**	
			R	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**
AMINOLGLYCOSIDE	GENTAMYCIN	R	12.5	5.3	50	5.4	50	3.44	40	4.80	100	5.4
		S	87.5	94.6	50	94.5	50	96.5	60	95.1	----	94.5
AMINOLGLYCOSIDE	KANAMYCIN	R	16.6	6.3	50	5.4	50	97.5	40	3.9	100	5.4
		S	83.3	93.6	50	94.5	50	2.4	60	96	----	94.5
MACROLIDE	ERYTHROMYCIN	R	20	3.17	---	4.16	50	3.7	50	1.47	----	4.3
		S	80	96.8	100	95.8	50	96.2	50	98.5	100	95.6
TETRACYCLINE	MINOCYCLINE	R	85.7	8.79	50	19.2	50	24	12.5	20.8	---	90.3
		S	14.3	91.2	50	80.7	50	75.9	87.5	79.1	100	9.7
ANTI-TUBERCULOUS	RIFAMPICIN	R	12.5	7.5	66.6	7.4	----	2.4	37.5	6.9	--	8.2
		S	87.5	92.4	33.3	92.5	100	97.5	62.5	93	100	91.7
LINCOSAMIDES	CLINDAMYCIN	R	14.2	16.6	50	24.6	33.33	19.6	33.3	23.7	---	25.3
		S	85.7	83.3	50	75.3	66.66	80.3	66.6	76.2	100	74.6

KEY: R = Resistance; S = Susceptible; Mox = Moxifloxacin; Lev = Levofloxacin; Cip = Ciprofloxacin; Ofl = Ofloxacin; Gat = Gatifloxacin.

It is highlighted in Table 4.3 that the Methicillin and Vancomycin resistant isolates respectively had minimal resistance to fluoroquinolones suggesting that they are generally susceptible to the new generation fluoroquinolones such as Gatifloxacin, Levofloxacin, Moxifloxacin and Ciprofloxacin to which less than 5% of the bacteria are resistant. The resistance to the commonly used antibiotics - Methicillin and Vancomycin (Table 4.3) is in corroboration with previous reports (Sato *et. al.,* 2004; Alam *et al.,* 2011 and Pai *et. al.,* 2006) and further highlights the clinical significance of emerging resistances to antibiotics of first choice. About 31% of the isolates had multiple antibiotic resistance index of 1 and above (MARI > 1) (Fig.4.1). MAR index of relative ratio >1 in this context shows a potential risk source of resistant strains from the environment (Suresh *et. al.,* 2000) that should be safeguarded. The value of MAR index (0.200) differentiates the low and high risk (Riaz *et. al.,* 2011). If the value is between 0.200 and 0.250, it becomes a very risky phase where there are equal chances that MAR may fall in the high risk and low risk phases (Krumperman et al., 1983). MAR is considered as a good tool for risk assessment. According to Riaz *et. al.* (2011), this also gives an idea of the proportion of bacteria showing antibiotic resistance in the risk source, which are the animal reservoir in this study.

Table 4.3: Effect of new generation fluoroquinolones on Methicillin/Vancomycin resistant Staphylococcus species from Nkonkobe Municipality South Africa.

Fluoroquinolone antibiotics	**Response**	**Number of methicillin resistant isolates (%)**	**Number of vancomycin resistant isolates**
Moxi Floxacin	S	34 (28.33)	13 (10.83)
	R	-	1 (0.83)
Levo floxacin	S	43 (35.83)	1 (0.83)
	R	1 (0.83)	1 (0.83)
Ciprofloxacin	S	11 (9.17)	6 (5)
	R	8 (6.67)	2 (1.67)
Ofloxacin	S	37 (30.83)	21 (17.5)
	R	6 (5)	5 (4.17)
Gatifloxacin	S	41 (34.17)	27 (22.5)
	R	1 (0.83)	-

Legend: S –Sensitive; R –Resistant; Percentage of resistant strains in parenthesis

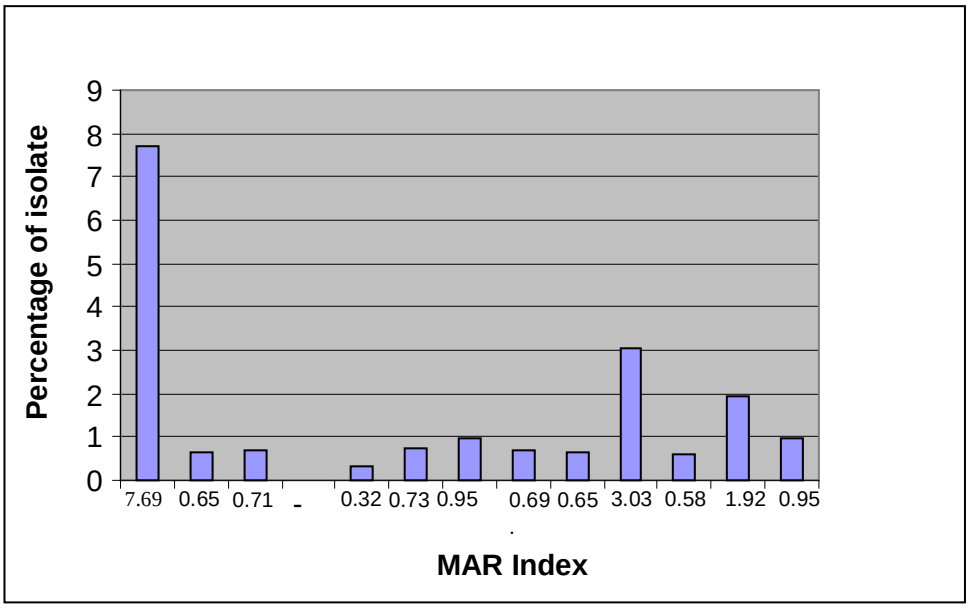

Fig 4.1: Multiple antibiotic resistant index (MARI) of isolates.

In conclusion, the antibiotic susceptibility profile of *Staphylococcus* species against the Fluoroquinolones used in this study shows that first generation Fluoroquinolone, that is, Nalidixic acid is non effective against Staphylococcus species. Resistance to the second generation Fluoroquinolones - Ofloxacin and Ciprofloxacin and the new generation Fluoroquinolone namely Gatifloxacin, levofloxacin, and Moxifloxacin were very low and supports the chemotherapeutic value of Fluoroquinolones in the treatment of *Staphylococcal* infections.

CHAPTER FIVE

GENERAL DISCUSSION AND CONCLUSION

Staphylococcal infections have been one of the major widespread diseases in the tropics and the world at large. Various attempts to control these infections through the administration of some common drugs like methicillin and vancomycin has been complicated with multidrug resistant strains (Thorburn *et. al.*, 2006) as intensified in this study whereby, about thirty two isolates (26.67%) tested were susceptible to both methicillin and vancomycin, while 12 (10%) of the isolates from these sources had co-resistance to the antibiotics.

Various antibiotics classes of antibiotics such as Aminoglycoside (Gentamycin), Aminoglycoside (Kanamycin), Macrolide (Erythromycin), Tetracycline (Minocycline), Anti-tuberculous (Rifampicin), Lincosamides (Clindamycin), Cephalosporin and Fluoroquinolones in general were tested during this study. There were varying range of resistance responses to these antibiotics. Gentamycin (an Aminoglycoside) had a relatively high potency against the isolates with 107 (89.17%) of the bacteria being susceptible to it, while 10 (8.33%) were resistant. On the other hand, Erythromycin (a Macrolide) was active against 72 (60%) of the isolates, while 5 (4.17%) and 74 (61.67%) of the isolates yielded intermediate and resistant responses respectively. In addition to this, 51 (42.5%) of the isolates were susceptible to Rifampicin, while 1 (0.83%) and 17 (14.17%) were intermediate and resistant respectively. This is consistent with the study of Howden *et al.* (2011) who shows how S. aureus evolved intermediate vancomycin resistance by acquiring mutations in the important regulator WalKR and these strains also demonstrated daptomycin non-susceptibility even though this drug had never been used for treatment. In their study, experiments to replace the mutated walK or walR into the parent strain and vice versa confirmed that these mutations were responsible for

the antibiotic resistance, but also led to significant changes in virulence, biofilm formation, and regulation of metabolism within the organism (Howden *et al.,* 2011).

Some clarifications were made in this study to determine the cause of this resistance nature of the isolates whereby each of the 120 Staphylococcus isolates were subjected to *mecA* gene and *van* gene screening. Ten percent of the isolates screened for their antibiotic susceptibility pattern in this study were positive for *mecA* gene among the vancomycin-oxacillin resistant strains while *van* gene was not detected in all the isolates. The subdued synergy of both vancomycin and oxacillin contribute to some resistance nature of Staphylococci. This is consistent with the study of Climo, *et. al.,* (1999) who demonstrated the synergy in combination of vancomycin and oxacillin by all test methods against isolates used and their varying resistant responses. In order to overcome this resistance attributes of Staphylococci, to the commonly used antibiotics as discussed under this context, various types of fluoroquinolones were tested.

The quinolones are a family of synthetic broad spectrum antibacterial drugs, An alkaloid having a quinolone structure was first prepared by Prince *et. al.*, (1949) but had no biological activity. In 1960, Boston *et. al.,* isolated 6-chloro-1h-ethyl-4-oxo-quinoline-3-carboxylic acid during antimalarial research. The first generation began with the introduction of Nalidixic acid in 1962 for the treatment of urinary tract infections in humans. Nalidixic acid was discovered by George Lesher and co- workers in a distillate during an attempt at chloroquine synthesis. They prevent bacterial DNA from unwinding and duplicating. Other quinolones in this generation include pipermidic acid, oxolinic acid, and cinoxin this class of quinolone drugs were introduced in the 1970"s. They proved to be only marginal improvements over nalidixic acid. Since the introduction of nalidixic acid in 1962 more than 10,000 analogs have been synthesized but only a handful are now currently in use in clinical practice. Quinolones in comparison to other antibiotic classes have among the

highest risk of causing colonization with MRSA and *Clostridium difficille*. Majority of quinolones in clinical use belong to the sub-set fluoroquinolones which posesss a fluorine atom attached to the central ring system typically at the sixth and seventh carbon position respectively. The quinolone class of antimicrobial agents has generated considerable interest since its discovery >40 years ago as discussed above (Andriole, 2005).

In this study, the report of resistant Staphylococcus strains obtained from mucosal animal sources in Nkonkobe municipality, Eastern Cape was given. Complementary to this various classes of antibiotics that can enhance the control of this aetiologic agent was tested. This is consistent with the universal surveillance to control Staphylococcal infections demonstrated by Robicsek *et. al.,* (2008) who gave reports of resistant nature of some Staphylococcal strains encountered in selected hospitals of their study area as part of control measures for this diseases.

In conclusion, previous investigations show that researchers divided the quinolone into generations based on their antibacterial spectrum (Naber,1998, Anderson ., 2003, and Blondeau., 2004.). The earlier generation agents are in general more narrower in spectrum than the latter, no standardized basis unfortunately is used to determine which drug belong to which generation, the only minimal standard applied is the grouping of the non-fluorinated drugs found within this class. This fluoroquinolone classification and their activity have some bearing with observations made in this study whereby the new generation fluoroquinolones including Gatifloxacin, Levofloxacin, Moxifloxacin and Ciprofloxacin to which less than 5% of the bacteria are resistant gives some clinical advantage over the Methicillin and Vancomycin resistant strains. Generally, some fluoroquinolones also comes with certain disadvantages as described by Golid ., (2003) and Goldman , (2010) that quinolone associated injury in weight bearing joints juvenile animals resulted not only in apparent contra-indication to their use in human infants and children but also completely

described their formal development by pharmaceutical companies for use in paediatrics, while this situation resulted from a genuine concern for safety seemly supported by relevant experimental findings, it served to remove a potential class of antibiotics from paediatric use. Consequently, this study still uphold the use of new generation fluoroquinolones as discussed , taking into consideration the age group involved and the dosage according to manufacturers specification. This will enhance limiting the reservoir for resistant strains of Staphylococcus in Nkonkobe municipality, South Africa and generate a database for sustainable healthcare delivery systems in controlling the menace of this resistant Staphylococcus disease surge in this part of the world.

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