COMMENSAL BACTERIA BELONGING TO THE *STAPHYLOCOCCUS*, *ACINETOBACTER* AND *STENOTROPHOMONAS* GENERA AS RESERVOIRS OF ANTIBIOTIC RESISTANCE DETERMINANTS IN THE ENVIRONMENT OF NKONKOBE MUNICIPALITY, EASTERN CAPE PROVINCE, SOUTH AFRICA

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DECLARATION

I, the undersigned, declare that this thesis, submitted to the University of Fort Hare in fulfilment of the requirements of the degree of Doctor of Philosophy in Microbiology in the Faculty of Science and Agriculture, School of Biological and Environmental Sciences, is my own work. The work contained herein is original with exception of those citations that have been accredited to their sources. This work has not been submitted at any other University, either partially or entirely for the award of any degree.

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Dedication

This thesis is dedicated to my mother, Princess (Mrs.) D. T. Adegoke and my sons, Anthony Ayomide, Favour Olanrewaju and Victor Oluwapelumi.

LIST OF ABBREVIATIONS

AEMREG	-	Applied and Environmental Microbiology Research Group
CFU	-	Colony Forming Unit
CLSI	-	Clinical and Laboratory Standard Institute
DNA	-	Deoxyribonucleic Acid
ESBLs	-	Extended Spectrum Beta Lactamases
et al.	-	(et alii) and others
EU	-	European Union
EFSA	-	European Food Safety Authority
FAO	-	Food and Agricultural Organization
HIV	-	Human Immunodeficiency Virus
MARI	-	Multiple Antibiotic Resistance Index
MRSA	-	Methicillin Resistant *Staphylococcus aureus*
No	-	Number
PBS	-	Phosphate Buffered Saline
PCR	-	Polymerase Chain Reaction
PFGE	-	Pulse-Field Gel Electrophoresis
UK	-	United Kingdom
VRSA	-	Vancomycin Resistant *Staphylococcus aureus*
USA	-	United States of America
WHO	-	World Health Organization

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GENERAL ABSTRACT

A study to assess the potentials of some commensal bacteria that belong to *Staphylococcus*, *Acinetobacter* and *Stenotrophomonas* genera as reservoirs of antibiotic resistance determinants in the environment of Nkonkobe Municipality of the Eastern Cape Province, South Africa, was carried out using standard microbiological and molecular techniques. A total of 120 *Staphylococcus* isolates which consisted of *Staphylococcus haemolyticus* (30%), *Staphylococcus aureus* (23.3%) from pig; *Staphylococcus capitis* (15%) from goat; *Staphylococcus heamolyticus* (5%) and *Staphylococcus xylosus* (15%) from cattle and other Staphylococci (11%) from dead chicken and pigs were isolated. About 23.3% of these isolates were coagulase positive and 76.7% were coagulase negative. This difference in prevalence along coagulase production divide was statistically significant ($p \leq 0.05$). Eighty-six *Acinetobacter* species (*Acinetobacter baumannii/calcoaceticus* and *Acinetobacter haemolyticus*) were also isolated from Alice and Fort Beaufort towns samples, while 125 *Stenotrophomonas maltophilia* isolates were from grass root rhizosphere (96%) and soil butternut root rhizosphere (4%). Between 75-100% of the *Staphylococccus* species were resistant to Penicillin G, tetracycline, sulphamethaxole and nalidixic acid; about 38 % were methicillin resistant, consisting of 12.6% methicillin resistant *Staphylococcus aureus* (MRSA) from pig and a total of 12% vancomycin resistant were observed. Also, 12% of the isolates were erythromycin resistant while 40.2 % were resistant to the third generation cephalosporin, ceftazidime. The antibiotic resistance genes *vanA, VanB, eryA, eryB, eryC* were not detected in all the phenotypically resistant *Staphylococccus* species, but *mec A* gene and *mph* genes were detected. In the *Acinetobacter* species, a wide range of 30-100% resistance to penicillin G, ceftriazone, nitrofurantoin, erythromycin, and augmentin was observed. Polymerase chain reaction (PCR) revealed the presence of *Tet(B)* and *Tet(39)* genes in these species, while *Tet (A), Tet(M)* and *Tet(H)* were absent. Also, 9.3% of the *Acinetobacter* species showed phenotypic production of extended spectrum beta lactamases

(ESBLs) while 3.5% were positive for the presence of $bla_{CTX-M-1}$ genes. The *Stenotrophomonas maltophilia* isolates showed varying resistance to meropenem (8.9%), cefuroxime (95.6 %), ampicillin-sulbactam (53.9%), ceftazidime (10.7%), cefepime (29.3 %), minocycline (2.2%), kanamycin (56.9%), ofloxacin (2.9%), levofloxacin (1.3%), moxifloxacin (2.8%), ciprofloxacin (24.3%), gatifloxacin (1.3%), polymyxin B (2.9 %), cotrimoxazole (26.1%), trimethoprim (98.6%), aztreonam (58%) and Polymyxin B (2.9 %). The isolates exhibited significant susceptibility to the fluoroquinolones (74.3-94.7 %), polymycin (97.1%) and meropenem (88.1%). Only *sul*3 genes were the only sulphonamide resistance gene detected among the trimethoprim-sulphamethoxazole resistant isolates. The observed multiple antibiotic resistance indeces (MARI) of >2 for *Staphylococcus* species, *Acinetobacter* species and *Stenotrophomonas maltophilia* suggest that they have arisen from high-risk sources where antibiotics are in constant arbitrary use resulting in high selective pressure. The presence of tetracycline resistance genes in *Acinetobacter* species justifies the observed phenotypic resistance to oxytetracycline and intermediate resistance to minocycline. High phenotypic resistance and the presence of some resistance genes in *Staphylococcus* species is a possible threat to public health and suggests animals to be important reservoirs of antibiotic resistance determinants in the environment. Indiscriminate use of antibiotics induces this kind of antibiotic resistance and should be discouraged. Personal hygiene is encouraged as it reduces the load of *Acinetobacter* species contacted from the environment that may be difficult to control. Commensal *Stenotrophomonas maltophilia* are as important as their clinical counterparts due to their roles in opportunistic infection, antibiotic resistance and their associated genes, especially *sul* gene. Personal hygiene is hereby advocated especially when in contact with soil, plants and plants' rhizospheric soil.

CHAPTER ONE

GENERAL INTRODUCTION

1.0 Introduction

Commensal bacteria are becoming increasingly important in the emergence of antibiotic resistance (Marshall *et al*., 2009; Halawani, 2011). Recent epidemiological reports on some bacteria have shown that many seemingly non-pathogenic (commensal) bacteria have been implicated as aetiology of extended spectrum drug resistant infections (Marshall *et al*., 2009). These have been described as acquired traits among such commensals which might have originated from their pathogenic counterparts (Pallechi *et al*., 2008). They, thereby, feed on the antibiotics meant to kill or inhibit them (Dantas *et al*., 2008). It is true that the previously known determinant of antibiotic resistance is believed to be mainly nosocomial while less consideration is accorded to the environmental reservoirs (Nwosu, 2001; Seveno *et al*., 2002). A thorough analysis of the human commensal and/or his environment will reveal their implications as reservoirs of antibiotic resistance gene(s). Some schools of thought believe that commensals take up their antibiotic resistance genes from the environment (D'Costa *et al*., 2006) where they exist in large amounts (Seveno *et al*., 2002). In any location, culturable bacteria are usually considered the source of the antibiotic resistance genes while non culturable bacteria (sometimes non-pathogenic) which are in the majority (Head *et al*., 1998; Torsvik *et al*., 1998; Whitman *et al*., 1998; Beja *et al*., 2002) are less considered (Giovannoni *et al*., 1990; Ward *et al*., 1990; Amann *et al*., 1995; Suzuki *et al*., 1997; Hugenholtz *et al*., 1998). This might position the environment as a possible custodian of antibiotic resistance genes since most of these non culturable reside there. Using culturable microbiota is justifiable as it gives an idea of the resident gene pools within the environment in question. Meanwhile, this does not rule out the residence of these genes in humans as considerable antibiotic resistance genes may be transferred from the human or animal microflora to pathogens (Salyers *et al.,* 2004; Dethlefsen *et al*., 2007). Either in cultured or non cultured bacteria, resistance genes and their phenotypic expression remains a challenge to overcome in

environment, animals and humans. This review focuses on the commensal bacteria as reservoirs of antibiotic resistance genes with specific emphasis on *Staphylococcus* spp., *Acinetobacter* spp. and *Stenotrophomonas maltophilia* which are of peculiar epidemiological importance as flora and pathogens of man.

Resistance to antibiotics by bacteria and its intrinsic factors like resistance genes remain a concern to public health around the globe (Levy, 2000; Deshpande and Joshi, 2011). The distribution and/or dissemination of such highly resistant commensal bacteria are also of paramount concern in human, farm animals and his environments, either cultivated or uncultivated; remote (Sjolund *et al*., 2008) or near and in pathogens on the infected or convalescent (Jury *et al*., 2010). Whichever the case, commensal or pathogen, each has been implicated as possible reservoir of antibiotic resistance genes (de-Araujo *et al*., 2006; Upadhyaya *et al*., 2011).The only difference is perhaps in the recognition previously accorded them. While pathogenic species have been acknowledged adequately in their carriage of antibiotic resistance genes and subsequent phenotypic expression of the genes, which have made treatment difficult (Lipsky, 2007) or limited therapeutic options available; less recognition is accorded the role of commensals (Marshall *et al*., 2009), yet they have been reservoirs of myriads of virulence and drugs resistance genes. Therefore, due recognition of both commensals and pathogens becomes imperative in the fight against antibiotic resistance.

A brief survey showed that commensal bacteria play vital roles as reservoirs of antibiotic resistance genes and their transmission (Blake *et al*., 2003). Byarugaba *et al*. (2011) reported a high level resistance exhibited by certain commensal bacteria of animal origin with the range of 46.8% -96% resistances to tetracycline, erythromycin and ampicillin. Epstein *et al*. (2008) also reported 17% prevalence of methicillin resistant *Staphylococcus intermedius*

which showed about 2% higher than earlier observations (Morris *et al*., 2006; Vengust *et al*., 2006; Abraham and Hans, 2007), showing the rise in resistance in this commensal subgroup just like their pathogenic counterparts. Class 1 integrons (mobile genetic elements) are some of the major contributors to the horizontal dissemination of antibiotic resistance genes in a diversity of enteric bacteria (Frost *et al*., 2005). Hence, the need for the identification of bacterial antibiotic resistance reservoirs in the environment and the determination of the transfer rate of antibiotic resistance genes into other bacteria becomes relevant (IFT, 2006). Sommer *et al*. (2009) observed that most of the antibiotic resistance genes harboured by the human microflora were distantly related (60.7% at the nucleotide level and 54.9% at the amino acid level) to antibiotic resistance genes so far detected in pathogenic isolates. This observation justifies the need for perspective assessment of the antibiotic resistance genes among such important commensal bacteria as *Staphylococcus* spp., *Acinetobacter* spp. and *Stenotrophomonas maltophilia,* due to their proximity as commensal to human and their implication in the life threatening multiple drug resistant infections (Lo *et al*., 2002; Kobashi *et al*., 2007; Rasheed and Awole, 2007).

Quite a number of attributes of pathogenic strains of Staphylococci reside in commensal strains and position them as pertinent entities in infection control. Besides, a recognized commensal organism can become pathogenic under conducive condition *in vivo* (Yan and Polk, 2004). By-passing the host's non-specific immune system to establish an infection by commensals follows about the same trend as their pathogenic counterparts and depend on the original site of the flora and/or the route of entry to the site of infection, the intrinsic pathogenic attributes (virulence) of the bacterium, the inoculums' size which determines the survival quotients and the host (s)' immune status (Li *et al*., 2005). Injury to the skin allows the seemingly harmless skin-resident commensal *Staphylococcus* spp. to

exhibit their difficult-to-resist instincts in peritoneum and joints (Ibrahem, 2010). These attributes generate a notion that commensalism is just a phase in the pathogenicity cycle (Fig 1.1), especially in *Staphylococcus aureus.*

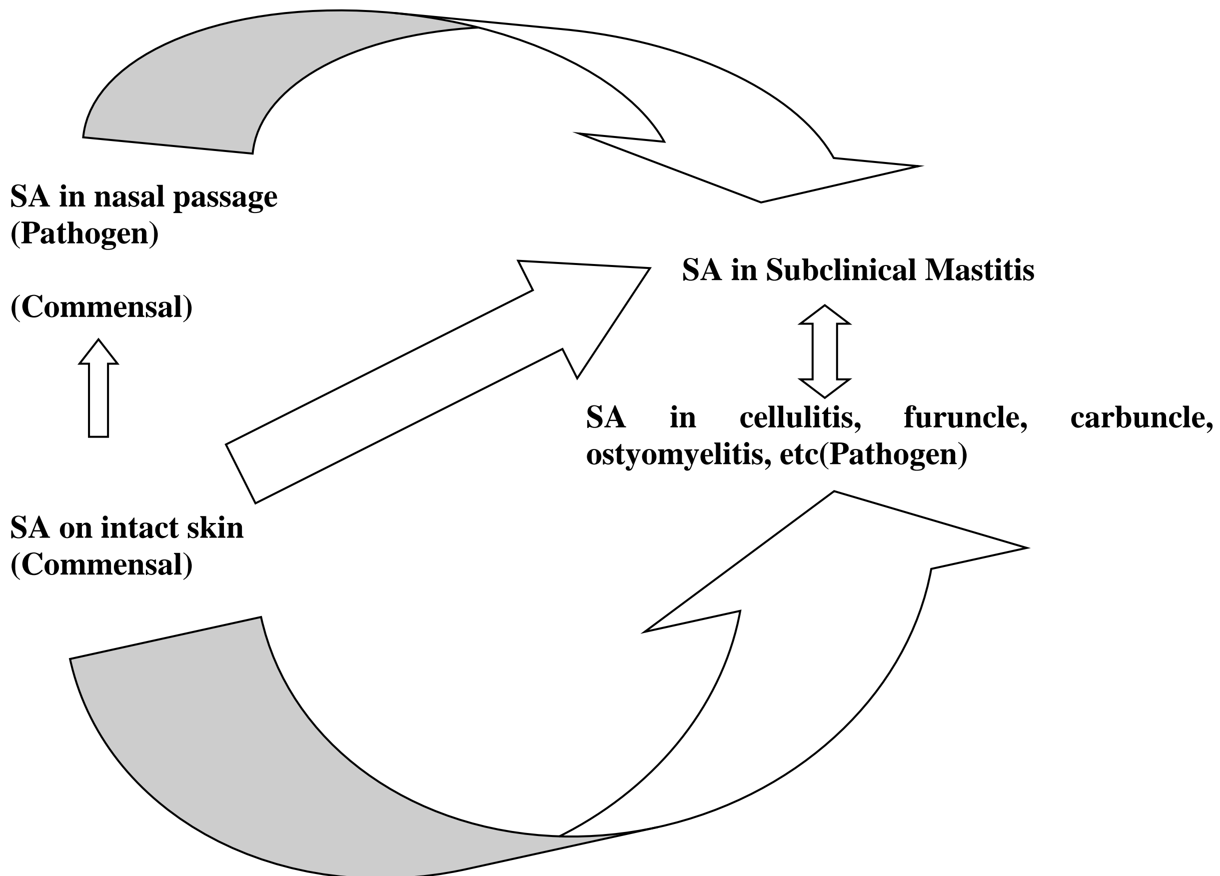

Fig 1.1: Simple illustration of shift in phase by *Staphylococcus aureus* (SA).

The schematic is a complex one in reality. However, Fig 1 is a possibility which justifies that commensal *Staphylococcus* species on healthy skin appear as flora waiting for opportunity to exhibit the intrinsic pathogenic tendencies. This scenario is also true for coagulase negative Staphylococci (CNS), especially *S. epidermidis* that have been described

as an “accidental pathogen” of man (Otto, 2009), and *S. haemolyticus* which is a notorious commensal and pathogen of farm animals (Fischetti *et al*., 2000; Rasheed and Awole, 2007). The former is a known commensal in endodontic region and pathogen of endodontic infection (Vianna *et al*., 2005). More importantly, these organisms have been reported as repositories of resistance genes, even in their commensal phase (Kozitskaya *et al*., 2004; Otto, 2009).

The antibiotic resistance among staphylococci is undoubtedly a major global public health problem in both hospitals and communities. The ubiquity of the human commensal *S. epidermidis* makes it a successful carrier and reservoir of antibiotic resistance genes, which are sometimes transferred to *S. aureus,* the trend noted to influence the rise in the spread of community acquired methicillin resistance *Staphylococcus aureus* (MRSA) (Ma, 2002). Rising skin colonization by ciprofloxacin resistant strains of *S epidermidis* is usually accompanied by the excretion of ciprofloxacin, among other antibiotics in sweat during chemotherapy (Dancer, 2004). This encourages an increased skin colonization by ciprofloxacin-resistant *S. epidermidis* (Raad, 1998) as ciprofloxacin-sensitive *S. epidermidis* would have been wiped out. Sometimes, a repertoire of *mecA* gene presence translates into the expression of resistance to the β-lactams by commensal *S. aureus* (Antignac and Tomaz, 2009). In another instance involving *S. sciuri*, only the inactivation of penicillin binding protein brings about the expression of phenotypic resistance with *mecA* genes’ availability (deLencastre *et al*., 2007; Zapun *et al*., 2009). The proximity of various *Staphylococcus* spp. to humans makes the resistance gene in them a concern (Dethlefsen *et al*., 2007; Cohn and Middleton, 2010). Antibiotic use and environmental factors contribute to the emergence and spread of such resistance, especially in *S. aureus*, which is a common cause of life-

threatening infections in both human and farm animals (Cohn and Middleton, 2010). Therefore, animal-derived products remain a potential source of MRSA (EFSA, 2008).

The presence of the peculiar resistance genes in ready-to-eat food stuff has immense epidemiological importance (EFSA, 2008); as they may contribute to human or animal microflora resistance gene load. Going down memory lane, the effect of the beta lactamase enzyme had resulted in resistance to some beta lactam antibiotics by some bacteria including Staphylococci. Methicillin was discovered and introduced into infection control arsenal in the 1960s. It was observed to have stability against the enzyme with accompanying good therapeutic outcomes until the emergence of MRSA. This scenerio soon extended to vancomycin later introduced for treating MRSA (Hiramatsu *et al*., 1997; Olayinka *et al*., 2005), and was only thought to be limited to clinical strain but was later discovered to have extended to community acquired strains or commensals (Olayinka *et al*., 2004). Concomitant MRSA and vancomycin resistance *Staphylococcus aureus* (VRSA) have resulted in therapeutic failure in about 85.7% orthopedic procedures (Ariza *et al*., 1999). Hence, resistance genes and the phenotypic expression of resistance in Staphylococci has long and to date been a cause for global concern as an epidemiological threat (Finland *et al*., 1950; Finland, 1955; Shittu *et al*., 2011) deserving priority attention. However, records of resistance gene assessment among commensal *Staphylococcus* species are not available in many regions of the world including South Africa.

The presence of antibiotic resistance genes in large proportions in either commensal or pathogenic species of *Acinetobacter* make the organism of immense concern (Deshpande and Joshi, 2011). This is owing to its potentials as a pathogen in immunocompromised individuals (Rise, 2006; Chen *et al*., 2008). Resistance to many conventional antibiotics considered to be in the last line of defence has been observed in large percentage of *A.*

baumannii (Zarakolu *et al.*, 2006) which poses a great challenge for selecting the appropriate therapeutic option (Rise, 2006). This *Acinetobacter* which is usually a commensal but sometimes a pathogen has been reported to harbour sulphonamide resistance genes (*sulII* gene) in its commensal state in the environment (Agerso and Petersen, 2007), and tetracycline resistance genes (Segal *et al.*, 2005) through any of the existing two-way mechanisms of tetracycline resistance (Lau *et al.*, 2008). Despite this potential, the organism is least considered in antimicrobial drug studies involving medicinal plants. Future research in this area is hereby encouraged to consider the use of *Acinetobacter* spp. in the overall public health interest.

Antibiotic resistance genes, either inherent or acquired, are major internal forces behind the antibiotic resistance exhibited by *S. maltophilia* (Zhang *et al.*, 2001; Mckay *et al.*, 2003; Alonso *et al.*, 2004). Various strains of *S. maltophilia* including commensals from the environment, opportunistic pathogens from the immunocompromised, sick or convalescent and those linked with persistent terminal clinical conditions bear resistant genes (Nicodemo and Paez, 2007) that serve as a clog in the chemotherapeutic wheel. The detection of erythromycin resistance genes from *S. maltophilia* from the trapped air in the Canadian hospital rooms was a good example (Di-Bonaventura *et al.*, 2004). Various observations of the resistance genes in *Stenotrophomonas maltophilia* have been made. Song *et al.* (2010) in Korea discovered the antibiotic resistance gene sul1 in class 1 intergron in place of sul gene which determines cotrimoxazole (Trimethoprim-sulfamethazole) resistance in *S. maltophilia* isolates and that resistance to antibiotics might be as a result of multiple antibiotic resistance genes. Sanchez *et al.* (2009) remarked that the presence of genes coding for long existing Qnr determinant in *S. maltophilia* confer antibiotic resistance on the organism against the supposed drug of choice. He also emphasized that the organism has proven proficient in the

acquisition of novel antibiotic resistance genes via horizontal transfer. This is evident in the reports that myriad of genes found in *S. maltophilia* Sm777 possess including a cluster of genes for antibiotic and heavy metal resistance (Pages *et al*., 2008). These genes are purportedly transferred from Gram-positive bacteria (Alonso *et al*., 2000), for the first time, to the best of our knowledge. In the same premise, the *efflux* pump D, E, F, (SmeDEF) multidrug efflux pump contributes to the intrinsic multidrug resistance in *Stenotrophomonas maltophilia* and justifies the need to access the bacteria from time to time for effective planning.

Emphatically, some of these genes are inherent while others are acquired intra-specifically and inter-specifically. This affirmative presence of pools of genes, especially for antibiotic resistance among others in commensal (Schwarz *et al*. 2001) and their transfer to other commensals or pathogens through various means (Ray *et al*., 2009) emphasizes their importance in epidemiology and infection control (Marshall *et al*., 2009). A good instance here as mentioned earlier is the antibiotic resistance gene transfer from Gram positive to Gram negative bacteria and *vice versa* reported by Alonso *et al*. (2004). The indirect hazard arises through transfer of resistance genes which are easily accomplished naturally by the organism, bypassing certain difficult steps and passing the gene to bacteria pathogenic for humans, either directly, or via another commensal bacterium (Popa *et al*., 2011).

In the United States, the inappropriate use of antibiotics is identified as a selective force for this harzard. About 50 % of the antibiotics being used are not only for therapy but also for enhancing growth (IFT, 2006; Pruden *et al*. 2006). Tetracycline, for example, has been used extensively in veterinary medicine, besides its normal application in human medicine (Chopra and Roberts 2001) in such a way that it has hastened the emergence of resistance. Consequently, widespread resistance has been reported in various communities of

human and non-human animals (Institute of Food Technologists 2006; Pruden *et al*. 2006), though most of these were discovered to be supported by efflux mechanism and protein production (Chopra and Roberts 2001). A study conducted by Yang *et al*. (2010) on antibiotic resistance owing to the effect of agriculture in Colorado showed among other things large counts of tetracycline-resistant bacteria and tetracycline resistance genes like *tet (B), tet (C), tet (W)*, and *tet (O)* in wastewater samples and non-farm environments. This study pointed to the fact that wastewater from animal breeding farms may spread antibiotic resistance genes to the environment.

For most animal-based antibiotic resistant bacteria, the number of animals per space and their feeding platform and compositions affect their bacterial strain carriage, for example, Dhlamini (2002) reported that 87% of subsistent poultry systems in KwaZulu-Natal incorporate herbal formula along with trace amounts of commercially prepared antibiotics in the poultry feed for treatment. This suggests that the observed resistance commensal strains and genes found in farm animals from developing and developed countries would differ due to differences in farm approach.

The non availability of proper records on the assessment of antibiotic resistance genes among the commensal bacteria belonging to *Staphylococcus* spp., *Acinetobacter* spp. and *Stenotrophomonas maltophilia* is a recurring decimal in developing countries including South Africa. The ongoing studies in our group in the Nkonkobe Municipality of the Eastern Cape Province of South Africa will shed some light and provide insights into this phenomenom. The use of *Acinetobacter* species and *Stenotrophomonas maltophilia* as test organisms in antimicrobial researches is hereby advocated due to their impact on public health, while intermittent assessment of their antibiotic resistance gene(s), to foster adequate planning in

preventing sudden emergence of multiple drug resistant infections in large proportions, is here advocated as subject of intensive investigation.

1.2 Aim and Objectives

To broad aim of this study was to assess the potentials of some commensal bacteria belonging to *Staphylococcus*, *Acinetobacter* and *Stenotrophomonas* genera as reservoirs of antibiotic resistance determinants in the environment of Nkonkobe Municipality of the Eastern Cape Province, South Africa. Specific objectives include:

- To isolate, identify and characterize some commensal bacteria belonging to *Staphylococcus* spp., *Acinetobacter* spp. and *Stenotrophomonas maltophilia* from the environments of Nkonkobe Municipality of the Eastern Cape Province, South Africa.
- To determine the prevalence of the isolates and their frequencies of occurrence.
- To determine the antibiotic susceptibility profiles and the multiple antibiotic resistant index (MARI) of the isolates.
- To provide information on persistence of the resistant strains across various samples and infer the highest reservoir(s) of resistant determinants.
- To assess the presence of antibiotic resistance genes in the bacterial isolates.

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CHAPTER TWO

Ubiquitous Acinetobacter spp. as Beneficial Commensals but gradually emboldening with Antibiotic Resistance genes

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ABSTRACT

Acinetobacter spp. are ubiquitous obligate aerobic bacteria which occur mostly as commensals on the skin, in the soil, water and plants' rhizosphere. Though the species in this genus have been implicated as aetiologies in some nosocomial infections, their versatility covers biodegradation or dissolution leading to bioremediation, catalysis leading to synthesis of high molecular weight, life sustaining polymers; and enhancement of growth in agriculture. The challenge of antibiotic resistance and mediatory genes is a cause for concern but should not deter the beneficial application of the bacteria especially in the synthesis of novel compounds that would be of relevance to overcoming some global ecological challenges. This review addresses important beneficial attributes of *Acinetobacter* species and gives some insight into emerging trends in their resistance to antibiotics.

2.1 INTRODUCTION

Acinetobacter is formed from coinage of the words, "a-cineto-bacter" which means "no-movement-rod". The genus is made up of 17 clearly named and 14 unnamed species (Dijkshoorn, 2008; Anon., 2011). Species within the genus are obligate aerobes, non-fermentative Gram-negative bacilli that exhibit cocco-bacillary morphology on nutrient agar and rod in fluid media (Kurcik-Trajkovska, 2009). Some species in the genus *Acinetobacter* include *A. baumanni, A. iwofii, A. junii, A. calcoaceticus, A. radioresistens* and *A. haemolyticus*. (Ecker *et al*., 2006) and they are often observed as commensals which are considered non-pathogenic to immuno-competent humans and animals (Dubay *et al*., 2011). However, several species of these ubiquitous bacteria persist in hospital environments and cause severe, life-threatening nosocomial infections in immune compromised patients (Wisplinghoff *et al*., 2000; Towner, 2006). The reported extended spectrum of antibiotic resistances and resilience that bring about high survival capabilities have placed them as a threat to hospitalized patients, especially those in intensive care (Manchanda *et al*., 2010; Kart *et al*., 2011). They also have records of antibiotic resistance in cases of nosocomial infection which makes their dissemination of immense concern to the clinician (Gaynes and Edwards, 2005). In spite of these, their beneficial roles cannot be over emphasized. This chapter reviews those beneficial attributes of *Acinetobacter* species, amidst their role as reservoirs of antibiotic resistance genes and recommends the way forward.

2.2 ENVIRONMENTAL AND BIOTECHNOLOGICAL APPLICATIONS

Some species within the genus *Acinetobacter* biodegrade various pollutants like amino acids derivatives, phenol, biphenyl, benzoic acid, organic nitrile and crude oil (Liu *et al*., 2007; Ahmad *et al*., 2009). They have also been effective agents in the removal of phosphate and its

derivatives or heavy metals (Towner, 2006; Rajkumar *et al*., 2007) and serve as a biological catalyst in remediating the environment and biotechnological advancements.

2.2.1 Environmental Detoxication and Bioremediation

A number of chemical-based industrial effluents contain toxic compounds that eliminate numerous vital aquatic lives and cause a devastating shift in the ecological balance (Pathan *et al*., 2010; Reza and Singh, 2010; Kenny, 2011). Some of these compounds are recalcitrant, attack the liver and continually exterminate several lineages of the susceptible species within the ecosystem (Pathan *et al*., 2010). The application of microbial sources of detoxifying such environments has removed the peculiar problem of cost and time associated with conventional physical and chemical methods (Chiacchierini *et al*., 2004). Biological remediation is also poised with the benefit of low technology, with high public acceptance and can often be executed *in situ*. Harmless toxoids are also produced when appropriate microbes are employed. An array of organic and inorganic toxic compounds can easily be degraded by *Acinetobacter* spp. yielding a non toxic product while utilizing the toxic compound as the sole carbon source (Xu *et al*., 2003; Zhan *et al*., 2008; Zhan *et al*., 2009).

Acinetobacter spp. also produces a multi-component enzyme known as aniline dioxygenase which has potential uses in bioremediation of aromatic amines. It also has activity as an agent of biorefining in the carbazole denitrogenation (Lui, 2007). Detailed characterization of this enzyme is hereby solicited to enhance its application as biocatalyst at an economically viable magnitude.

2.2.2 Degradation of Xenobiotics and Recalcitrant Compounds

Recalcitrants like Quaternary ammonium compounds (QACs), which are lethal substances widely used as disinfectants, are biodegraded by *Acinetobacter* spp (Al-Ahmad *et*

al., 2000). Some species of the genus are also effective in the biodegradation of benzalkonium chloride (Sutterlin *et al.,* 2008). This attribute might be a result of adaptation by the bacteria over a period of exposure to the recalcitrants (Hingst *et al*. 1995). Phenol and its derivatives exhibit environmental toxicity and are perpetual pollutants in rivers, industrial effluents, and landfill runoff waters (Lee *et al*., 2006). Phenol degradation by the bacteria has been observed in various ecosystems around the globe. A good example is the observed four species of *Acinetobacter* in various Egyptian ecosystems (Abd-El-Haleem *et al*., 2002). Two of these species have reportedly been applied for environmental studies by Beshey *et al*. (2002). In this light, Zaki (2006) observed high activity of phenol degradation by *Acinetobacter* strains W-17 and DF-4 via ortho-cleavage pathway using two enzymes namely, phenol hydroxylase and catechol-1,2-dioxygenase. This reiterates the huge inherent potential of *Acinetobacter* spp. as a formidable tool in phenol remediation from environments and industrial wastewater.

Another study by Prasad *et al*. (2010) on the bioremediation potential of *Acinetobacter baumannii* in batch culture using synthetic phenol in water in the concentration range of '125 – 1000 mg/L' as a limiting substrate buttressed the *Acinetobacter* potentials. Five consumption rate and kinetic study models were used (viz: Haldane, Yano and Koga, Aiba *et al*., Teissier and Webb models), of which Monod model turned out as the best. The study revealed the potentials of *A baumannii* to bioremediate sites with various concentrations of phenol pollutants, with just an extended lag phase for the very high concentrations. This *Acinetobacter* potential was applied in the construction of bioflorescent *Acinetobacter* strains DF4/PUTK2 to study the phenol toxicity (Zaki *et al*., 2008).

Due to their wide substrate specificity and ability to oxidize a variety of substrates, some species of *Acinetobacter* have been applied in the degradation of lignin and amino acids

(Buchan *et al*., 2001; Kim *et al*., 2001; Kahng *et al*., 2002). Ghodake *et al*. (2009) purified a dimeric lignin peroxidise with molecular weight of about 55-65 kDa from *Acinetobacter calcoaceticus* NCIM 2890. The enzyme exhibited versatile oxidative activity and was able to oxidize a variety of substrates including Mn^{2+}, tryptophan, mimosine, L-Dopa, hydroquinone, xylidine, n-propanol, veratryl alcohol, and ten textile dyes of various groups. The presence of amino acid tryptophan in this reaction is seen as an added advantage for stability. This makes *Acinetobacter calcoaceticus* NCIM 2890 a novel bacteria of interest to environmentalists as synthetic textile dyes are harmful pollutants and perpetual components of industrial effluents (Jadhav and Govindwar, 2006). The dyes belong to the chromophoric groups which are mostly mutagenic, carcinogenic and recalcitrant (Eichlerova *et al*., 2006)

2.2.3 Degradation of Crude and Mineral Oil

Acinetobacter has been reported as one of the most connected genera with oil contamination (Abu and Atu, 2008; Nkwelang *et al*., 2008). Their ability to utilize diesel, for instance as a sole carbon source, is justified by their reported increase in diesel impacted soil within short growth cycle (Chao and Hsu, 2004). They are therefore established beneficial commensal in oil biodegradation when compared to *Ralstonia picketti* and *Alcaligenes piechaudii* in crude oil degradation and biosulfactant production (Hamme *et al*., 2003). In an experiment to assess the isolates in diesel-contaminated sandy soil for instance, species of *Acinetobacter* were observed as most abundant (Gallego *et al*., 2001). To buttress this was a multi-method research conducted by Satpute *et al*. (2008) to assess the biosulfactant producing marine bacteria where 40% of the bacteria were *Acinetobacter*. In this research, various strains of *Acinetobacter* were reported to have shown High Emulsification Units

(HEU) to xylene, diesel, petrol and crude oil; with highest HEU on petrol. One added value was the degradation of kerosene and hexadecane by *Acinetobacter*.

Acinetobacter genus along with Acidovorax, Sphingomonas and Thiobacillus among others was earlier detected from environments impacted with mineral oil hydrocarbon (Popp *et al*., 2006). This is why a pilot plant used to treat waste water that contains mineral oil bears *Acinetobacter calcoaceticus* strain (Pleshakova *et al*., 2001). The TM-31 *Acinetobacter calcoaceticus* strain bears transferrable plasmids and degrades alkane and its derivatives, arene portion and alkyl residues of the naphthene which are all from mineral oil. In a study involving *Acinetobacter*, *Alcaligenes*, *Flavobacterium*, *Micrococcus*, and *Bacillus* among others, Pleshakova *et al*. (2001) observed that most strains could not utilize the native mineral oil except the strain *Acinetobacter calcoaceticus* TM-31. The outcome of a research by Gomez *et al*. (2011) that used n-hexadecane as a sole carbon source while observing *Acinetobacter* dominantly, suggested the indispensable roles of the culturable bacteria especially *Acinetobacter* in remediating polluted sites. The research was fortified by analysis of the culturable fraction and noted that the nature, prevailing physicochemical conditions and the depth of pollutants, especially the hydrocarbon, determine the attendant bacterial diversity present (Fierer *et al*., 2003; Holden, 2005; Hansel *et al*., 2008). This is because hydrocarbon imparted soil prevalently bears *Acinetobacter* (Proteobacteria) which are succeeded by Actinobacteria in dominance with reduction in concentration of pollutants (Bordenave *et al*., 2007).

2.2.4 Perspective Biodiesel Catalysis

Besides the earlier noted attributes of crude oil degradation, the prospect seems bright for micro-diesel production (Kalscheuer *et al*. 2006) in line with the gradual shift from crude oil to biological sources of energy production. The lipase from *A. baylyi* is presumed a

biocatalyser in this respect (Uttatree *et al*., 2010). Bacteria like *A. baylyi* is further supported by its being a non-fastidious microbe with the ability to secrete esterolytic enzymes (Snellman and Colwell, 2004; Kwang-Woo *et al*., 2006), a heat resistant lipase that is stable to organic solution and the ability to change organic group R of the ester to organic group R of alcohol (which is main reaction in converting oil to biodiesel) (Dayong *et al.,* 2011). Apart from the observed favourable phenotypic factors, *A baylyi* bears the gene atfA which codes for acyltransferase that esterify ethanol with the acyl moieties of CoA which can be harnessed effectively for industrial biofuel production (O'Connell, 2006). In this case, however, more research input is solicited to bring about high production efficiency.

2.2.5 *Acinetobacter* baumannii as a growth Promoter

Acinetobacter spp. can be applied in Agriculture to improve yield and remove delay in plants' maturity. On a general note, Dursun *et al*. (2010) observed that improvements can be brought about on mineral contents of tomato and cucumber fruit by bacterial applications and this specially exerts appreciable effects on elemental (mineral) contents like K, Na, Ca, Zn, Mg, Fe, N and P fruit. Although the soil bacteria, *Acinetobacter calcoaceticus*, *Agrobacterium* sp., *Enterobacter sakazakii*, and *Caulobacter/Asticcacaulis* are phosphate solubilizers (Verma *et al*., 2001; Kuklinsky-Sobral *et al*., 2004), these attributes have not been connected with the accessibility of their host plants with the solubilised phosphates. More research input is advocated to ascertain the effect of bacterial activity on plant access to phosphate in a heavy, metal imparted soil. Beside this, bacterial application significantly promotes growth and numerical increase in flowers, for example with the strain: *Acinetobacter baumannii* CD-1. Dursun *et al*. (2010) concluded that *Acinetobacter baumannii* CD-1 among other bacterial isolates can be considered when matters of boosting yield, improving mineral contents and growth are of concern.

Furthermore, a number of researches prior to Dursun *et al*. (2010) have ascertained that a number of versatile rhizospheric bacteria support growth and are known as 'Plant Growth Promoting Rhizobacterium (PGPR)' including strains in the genera *Alcaligenes, Azospirillium, Azotobacter, Arthrobacter, Bacillus* and *Acinetobacter* (Bashan and de-Bashan, 2005). Later, Erturk *et al*. (2011) noted *Acinetobacter* as one of the Plant Growth Promoting Rhizospheric (PGPR) bacteria that can improve the growth of hazelnut seedling in a soil with low nutrient content. *Acinetobacter* was in this case observed as the next most effective after *Pseudomonas macquariensis* in promoting growth and growth parameter of hazelnut. This is achieved through the ability of the PGPR to provide nutrients to the resident plant within the rhizosphere deplete of nutrients. (Vessey, 2003; Lucy *et al*., 2004; Cakmakci *et al*., 2009)

2.2.6 Polymer synthesis, enzyme screening and optimization

Most commercial extracellular polysaccharides and enzymes have microbes as their source (Ceyhana and Ozdemir, 2008; Asad *et al.,* 2011). So, their short growth and reproduction cycles serve as additional benefits of short production time and cheap technology that brings about bogus economic outcome (Sasikala and Ramana, 1995). A wide range of microbial enzymes have been reported to have shown activity in catalyzing a wide variety of reactions in aqueous and non-aqueous phases (Saxena *et al*., 2003). An example of such an enzyme by *Acinetobacter* is lipase which has been accorded much attention. Li *et al*. (2005) produced lipase by *Acinetobacter radioresistens* with Tween 80 as the carbon source in a repeated fed-batch culture system. The researchers observed that lipase production rate could reach as high as 42,000 U/h in a 2.5 l tank fermentor. Similarly, Japtap *et al*. (2010) utilized human skin resistant *Acinetobacter haemolyticus* TA 106 for the optimized

production of lipase under controlled physicochemical conditions of culture and he discovered that 3% (v/v) inoculums density, 1% (w/v) sucrose and 5mM manganese sulphate will yield a peak output of 55 U/ml. This observation did not only recommend *Acinetobacter* spp. as a veritable source for lipase production but suggested the industrial viability of incorporating the bacteria in lipase producing arsenal.

Similar to lipase is the enzyme cyanobacterial cyanophycin synthetases (Krehenbrink *et al*., 2002; Ziegler *et al*., 2002). Krehenbrink *et al*. (2002) made the first novel characterization of cyanophycin synthetase from *A. calcoaceticus* ADP1. So, cyanophycin is not only synthesized in cyanobacteria but also in *A. calcoaceticus* ADP1. The genomes of many non-cyanobacteria possess genes for proteins with high sequence similarity to cyanophycin synthetases. One overriding advantage of cyanobacterial cyanophycin synthetases synthesized by *Acinetobacter baylyi* is their flexibility and activity for a wide varieties of substrate, hence their wide application (Hai *et al*., 2006).

Several strains of *Acinetobacter* produce large sized extracellular polysaccharides (Pyroh *et al*., 2002; Chamanrohk *et al*., 2008). Sometimes *Acinetobacter* strains are cultured on ethanol to produce ethapolan, a polysaccharide (Johri *et al*., 2002; Pyroh *et al*., 2007). Pirog *et al*. (2007) chose to use glucose and fumarate as the carbon source unlike most earlier researchers and found that ethapolan was produced with greater intensity following the glucose-fumarate joint carbon source. The same observation (of emulsan, lipopolysaccharides) has been made through the bacterial specie even from crude oil (Chamanrohk *et al*., 2008).

2.3 *ACINETOBACTER* AS A RESERVOIR OF ANTIBIOTIC RESISTANT GENES

Despite the myriad of potentials embedded in *Acinetobacter* spp., its attribute of resistance to control via antibiotics, owing to the presence of antibiotic resistance genes is of paramount concern. For instance, Zarakolu *et al*. (2006) observed that 67% of the *A. baumannii* strains exhibited multiple antibiotic resistances to cefepime, tobramycin, ciprofloxacin, carbapenem, and ceftazidime. Meanwhile, the extent of resistance genes borne by an *Acinetobacter* sp. may depend on the environment and the neighbouring bacteria. For instance, sulphonamide resistance gene *sulII* was found to be widely distributed in isolates from both fish ponds and manure. In South Africa, Segal *et al*. (2004) observed *su1II* gene in an *A. baumannii* isolate from a hospital and class I integrons and *tet(A)* gene in *Acinetobacter* spp. of animal origin which may explain the spread of class I integron and *tet(A)* to flocks of chicken and aquatic water ponds respectively. Generally speaking, *A. baumanii* exhibits resistance to tetratracycline resistance mechanisms. The efflux pumps which are transposons' associated with *tet(A)* and *tet(B)*. *Tet(A)* is responsible for efflux of tetracycline alone while *tet(A)* and *tet(B*) are responsible for both tetracycline and minocycline (Guardabassi *et al*., 2000; Huys *et al*., 2005). The second mechanism shields the ribosome from tetracycline by the protein it produces. This protein encoded by *tet(M)* gene screens tetracycline, doxycycline and minocycline from reaching the ribososme. This protein in *A. baumannii* is totally homologous to *tet(M)* protein of *S. aureus* (Ribera *et al*., 2003). This trend of resistance also applies to cephalosporin, sulphonamides, fluoroquinolone and other antibiotic groups which are lined with relevant resistance genes (Bonomo and Szabo, 2006; Agerso and Petersen, 2007; Higgins *et al*., 2010; Kadriye *et al*., 2011). Somewhat worrisome is the lack of highly innovative agents against the Gram negative nosocomial, sometimes of commensal origin (Erasme Hospital, 2002), to *A. baumanni* with pan-resistance to conventional agents (Livermore, 2003; Norrby *et al*., 2005). This serves to affirm the dynamism of bacteria:

commensal or pathogens; and the need to handle them with care when being put to beneficial use.

The dissemination of antibiotic resistance genes, antibiotics and antibiotic resistant bacteria is aided by linking various sections of the environments together (Schluter *et al*., 2007). Factors that clump together large density of bacteria in the same environment, thereby enhancing biofilm formation (Wolf-Rainer, 2011) may equally enhance the potential for exchange of resistance genes (Schluter *et al*., 2007; Hansen *et al*., 2011). Since the prophylactic use of antibiotics has been licenced to farm animals in many countries, wastewater from such farms may contain the residual antibiotics (Kummerer, 2003) which can serve as a selective force for the emergence of antibiotic resistant bacteria *in situ.*

Kobashi *et al*. (2007) isolated 350 tetracycline resistant diverse bacterial species from livestock faeces, farmyard manure and soil. Their assessment of resistance genes showed that the tetracycline resistance genes were evenly distributed across distantly related species. This type of commensal bacteria with resistance genes may be difficult to treat when they become opportunistic pathogens in immunocompromised individuals. In fact, they might be the source of community-based *Acinetobacter* infection just as the report from Erasme Hospital (2002) showed that commensal microbes belonging to *Staphylococcus* spp., *Enterococcus* spp., *Klebsiella* spp., are responsible for hospital acquired infections (Fig 2.1)

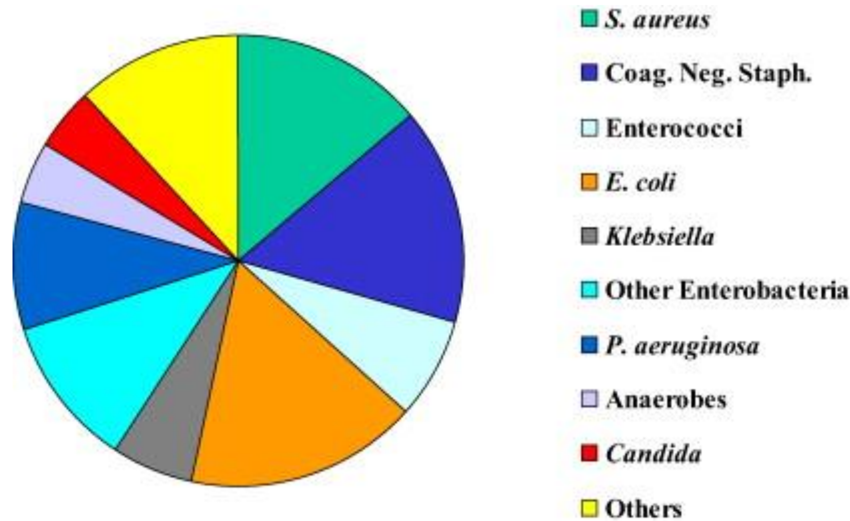

Fig. 2.1. Microorganisms causing nosocomial bacteraemia, (Erasme Hospital, 2002).

The challenge being faced by immunocompromised patients infected by this commensal (*Acinetobacter* with resistance genes) include therapeutic failure due to the phenotypic expression of the resistance, prolonged hospital admission which culminates in economic loss or loss of human labour at work and increased mortality rates and costs of treatment (Paladino *et al*., 2004). However, antibiotic resistant genes are being put to beneficial use as marker genes, being co-transformed together with the gene of interest into genetically transformed fruits (FAO/WHO, 2000), so that the marker genes insertion is guaranteed.

2.4 CONCLUSION

Acinetobacter species serve as veritable tools in Environmental and Industrial Biotechnology to remove recalcitrant and toxic xenobiotics, degrade oil and catalyze crude oil formation, and synthesize various polymers. Further studies are recommended to affirm the

Acinetobacter role as Plant Growth Promoting Rhizosphere bacteria outside the laboratory (ie in natural field condition). Meanwhile, the effect of inducible antibiotic resistance which limits industrial application of these bacteria can be mitigated by abstinence from arbitrary use of such vital antibiotics. So, the use of extended spectrum antibiotics should be reserved for only highly resistant species. Industrial effluents containing chemicals that can induce resistance should be treated before being released to the pool of natural waters. Compartmentalizing the ecosystems limits the spread of antibiotic resistance genes and should be encouraged. Also, bearing in mind the environmental pollution frequently brought about by synthetic fertilizers and expensive cost of inorganic fertilizer, *Acinetobacter* species are good alternative sources of bio fertilizer for sustainable food production.

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CHAPTER THREE

Stenotrophomonas maltophilia, a commensal of importance to biotechnology

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ABSTRACT

Stenotrophomonas maltophilia (Sm) is endowed with immense prowess that can be exploited beneficially in Agriculture, Nutrition, Medicine, Biodegradation, Bioremediation and Phytoremediation. The bacterium possesses multitudinous extracellular proteins and enzymes lined by inherent and acquired mechanisms and/or genes which are primarily responsible for adaptation and survival in its niche. Accessibility to the versatility and synthetic dynasty embedded in the bacterium is however threatened by ease of contamination with toxic product(s) of the same bacterium and the bacterial implication in life threatening multidrug resistant infections promoted by the presence of resistance genes. High level technology and expertise, with collaboration by scientists from all walks of life is advocated to safely harness the biotechnological potentials of the organism at an economically viable magnitude.

3.1 Introduction

Stenotrophomonas maltophilia is a common ubiquitous commensal (Bollet *et al*., 1995) that is readily isolated from water, soil, sewage and regularly on plant or within plant's rhizosphere where they play key roles in biogeochemical cycling of nitrogen, sulphur and other important elements. Though it has been implicated as an opportunistic pathogen (Mendosa *et al*., 2007; Gnanasekaran and Bajaj, 2009) and true pathogen ((Kim *et al*., 2002; Pruvost *et al*., 2002; Thomas *et al*., 2010) due to its role as aetiology of life threatening infections (Gales *et al*., 2001; Pathmanathan and Waterer, 2005), its beneficial roles in its niche vis-a-vis its importance in biotechnological advancement cannot be underestimated (Zhang and Yuen, 2000; Idris *et al*., 2007; Farzaneh *et al*., 2010). This chapter reviews the potentials of *S. maltophilia* in their niche and in biotechnological advancement, the inherent genes predicating the bacterium's attributes including antibiotic resistance genes and other selective properties.

3.2 *STENOTROPHOMONAS MALTOPHILIA* (SM) IN AN ECOLOGICAL NICHE: ADAPTABILITY AND RESILIENCE

Stenotrophomonas maltophilia is a Gram negative commensal bacterium found in myriads of habitats where it occupies a vital niche ranging from terrestrial to aquatic habitats (Borner *et al*., 2003) including the irrigation solutions used in hospitals (Minkwitz and Berg, 2001). While attempting to assess the safety of drinking water in the process of treatment until delivery, Newcombe *et al*. (2004) detected *Stenotrophomonas maltophilia* among other opportunistic pathogens in International Space Station by qPCR. Some other studies have revealed that the bacterium is notable among the rhizosphere bacterial inhabitants (Hartmann

et al., 2008; Ryan *et al*., 2009; Taghavi *et al*., 2009). Its fitness in this domain is dependent on the environment. A peculiarly related example is the resistant mutants of the bacterium that produce the multidrug efflux pump SmeDEF excessively (Alonso and Martinez, 2000; Sanchez *et al*., 2002) but less competitive in a slime mold infection model. Meanwhile, *Stenotrophomonas maltophilia* generally has immense adaptability to its natural environment, even in the face of unsuitable stress or at conditions below optimal. As already known, plants secrete a diverse class of polyphenolic compounds called flavonoids to ascertain interaction between microorganisms and the plant (Shaw *et al*., 2006). This compound poses a challenge for less adaptable bacteria as they have been proven to have antimicrobial activities against extended spectrum beta lactamase producers (Ozcelik *et al*., 2008; Adegoke and Adebayo, 2009; Talib and Mahasneh, 2010; Siddiqi *et al*., 2011), thus creating additional stress for rhizospheric bacteria including *Stenotrophomonas maltophilia* to combat. *Stenotrophomonas maltophilia* however withstands this stress and stands among the most successful rhizosphere bacteria.

It has been equally reported that the development of association with plants built up by *Stenotrophomonas maltophilia* encourages their abilities to survive in soils that are deplete of nutrients (Hartmann *et al*., 2008). This association is facilitated by a number of anatomical structures like flagella (Krzewinski *et al*., 2001) with which the bacteria moves in response to chemo-attraction from the root; pilli, fimbriae and biofilm for adhesion and adaptation against adverse chemicals and ions (Elver *et al*., 2001). The bacterium obtains nutriment and shelter in this mutual association just as its extracellular proteins (enzymes) expel or exhibit lethal effect e.g. lipases, chitinases, nucleases, elastases and proteases (Du *et al*., 2011), exterminating the root borers within the rhizosphere and leaving the plants protected. Extracellular polymers produced by *Stenotrophomonas maltophilia* therefore appear to have

potentials as possible source of antibiotics, even if it demands the incorporation of halogen moieties to reduce toxicity and enhance potency as does fluorine in fluoroquinolone (Robinson *et al*., 1992).

Worth noting also is the resilience of this Gram negative rod, that results in its adaptation in diverse habitats and biomes across the globe (Harris and Rogers, 2001;). Botes *et al*. (2007) reported the survival of *Stenotrophomonas maltophilia* in South African antimony mine which is an environment that had been heavily impacted with high dosage of arsenic occasioned by refining activities. The resilience to withstand the effect of this supposedly adverse chemical in large concentrations (10 mmol l^{-1} arsenite and 20 mmol l^{-1} arsenate) might have informed the authors' reference to its"hyper-resistance to arsenic". This adaptation extends to human and non human animals as a habitat for *Stenotrophomonas maltophilia*. For instance, Bollet *et al*. (1995) noted that in France, the frequency of isolating *Stenotrophomonas maltophilia* from clinical samples has been on the increase since 1987, at time of the emergence of imipenem, the parenteral carbapenem with high pharmacokinetic profile. This adaptive attribute is due to its high resistance profile and unique physio-pathological attributes with which it avoids the non specific anatomical barrier of the immune system (Oliveira-Garcia, 2003). Also, *Stenotrophomonas maltophilia* exhibits mono-cultural growth and multiplies widely utilizing the accessible nutrients in the phyllosphere environment. To achieve this, it alters the cuticle of leaf surface to which it attaches e.g. in *Hedera helix* and *Prunus laurocerasus* (Schreiber *et al*., 2005) to increase the availability of water and dissolved compounds. *Stenotrophomonas maltophilia* also maintains good ecological relationships with other phyllospheric bacteria. Its effective alteration of plants cuticle benefits the epiphytic bacteria in the environment (Krimm *et al*., 2005). Previous study also revealed that *Stenotrophomonas maltophilia* strain BP1 and *Pseudomonas*

syringae TLP2dell jointly exhibit a high level of coexistence with "respect" for each other, despite the seeming preponderance of *Stenotrophomonas maltophilia* (Wilson and Lindow, 1994).

3.3 BIOTECHNOLOGICAL IMPORTANCE OF *STENOTROPHOMONAS MALTOPHILIA*

3.3.1 *Stenotrophomonas maltophilia* as agent in biological control

Another dimension to the importance of *Stenotrophomonas maltophilia* is its role in biological control. An example of such role is found in the biological control of *Bipolaris sorokiniana* on Tall Fescue by *Stenotrophomonas maltophilia* Strain C3 (Zhang and Yuen, 1999). In Zhang-Yuen's research, strain C3 was observed in growth chamber experiment to prevent the germination of conidial on the surfaces of leaves. When compared with non-treated control, noticeable reduction in lesion and infected area by *B. sorokiniana* was observed by Zhang and Yuen (1999) which was proportional to the dosage used. This has been explained to have been predicated by the production of chitinase which prevents the conidial germination (Zhang and Yuen, 2000). The lytic activity affected by the enzymes produced by this bacterium is a notable mechanism the organism explores for biocontrol (Giesler and Yuen, 1998; Idris *et al*., 2007). Enzyme systems that brought about the lytic activities have caught the attention of researchers on biocontrol agents especially those that are active in disrupting fungi cell wall e.g. chitinase (Zhang and Yuen, 2000). Hence, *Stenotrophomonas maltophilia* is an important rhizosphere bacterium which can be explored for agricultural improvement against fungal infection (Messiha *et al*., 2007).

Attributes of *Stenotrophomonas maltophilia* that can be explored in biocontrol arsenal include ease of colonization of rhizosphere, production of antimicrobial compounds and

extracellular proteins (enzymes) (Zhang and Yuen, 2000; Zhang *et al*., 2001; Jorquera *et al*., 2008), some of which have been discussed earlier in this review. Notable examples in this respect are four isolates designated as PD3531, PD3532, PD3533 and PD3534 which suppressed potato brown rot caused by *Ralstonia solanacearum* in Egyptian clay soil (Messiha *et al*., 2007) and the *Stenotrophomonas maltophilia* strain 34S1 that was identified as a biocontrol against the fungus *Magnaporthe poae* which is an agent for patch disease of Kentucky bluegrass (*Poa pratensis*) (Kobayashi *et al*., 1995; Kobayashi *et al*., 2002), though more research input is advocated in these biocontrol activities to further define the roles of the participating agents.

3.3.2 *Stenotrophomonas maltophilia* in biogeochemical cycling

Stenotrophomonas maltophilia has been implicated in the biogeochemical cycling of vital elements like Nitrogen, Sulphur, Phosphorus and a number of others. Dungan *et al*. (2003) reported the transformation of selenate and selenite by the bacterium; the attribute which made the authors suggests the role of this bacterium in the entire biogeochemical cycling. Since their research focussed on Agricultural pond sediment containing selenium, their conclusion affirmed the bacterium's importance, not only for nutrient cycling, but also for bioremediation (Dungan *et al*., 2003). In the meantime, the roles of the bacterium amidst other bacteria in nitrogen fixation have been observed by Park *et al*. (2005) in Korea. The study was conducted in high inorganic fertilizer impacted soil within the rhizospheres of rice, maize and wheat, and the bacterium was observed to exhibit appreciable potentials for nitrogen fixation. Other studies have also confirmed *Stenotrophomonas maltophilia* as a good solubilizer of phosphate and phytate, and as a biological fertilizer (Suckstorff and Berg, 2003; Vessey, 2003; Lim *et al*., 2007). Mineralization of phytate in the biosphere with phytases

produced by the soil microorganisms including *Stenotrophomonas maltophilia* stands as a formidable process of phosphorus recycling (Lim *et al*., 2007). This attribute is of particular interest to crop breeders and soil scientists as the bacterial solubilisation will not only recycle nutrients but also convert them from various forms (e.g. tricalcium phosphate in phosphate min) (Xiao *et al*., 2009) into utilizable forms by plants.

The phytases produced by *Stenotrophomonas maltophilia* which are important in phosphate conversion have other numerous biotechnological applications. They reduce phytate contents in animal feed as well as human food and improve phosphorus' availability (Konietzny and Greiner, 2002; Oh *et al*., 2004). The enzymes are peculiarly incorporated into feeds of farm animals including poultry, swine, and fish diets, amino acids, and energy. *Stenotrophomonas maltophilia* ability to produce this enzyme in large deposits can serve as additional benefit similar to the cases of *Bacillus* sp. (Choi *et al*., 2001), *Raoultella* sp. (Sajidan *et al*., 2004), *Citrobacter braakii* (Kim *et al*., 2003).

3.3.3 *Stenotrophomonas maltophilia* in Biodegradation and Bioremediation

The impacts of *Stenotrophomonas maltophilia* in various forms of degradation processes stand as an indispensable prowess in nature's self cleansing dynamics (Farzaneh *et al*., 2010). Recalcitrants of various forms with tendency to choke up some low forms of life in various habitats are easily degraded by *Stenotrophomonas maltophilia*. Studies in laboratory scale and their subsequent applications in larger scales showed these remarkable decomposition properties. These attributes have been utilized in many quarters of human endeavour and may be applied in the removal of clogging that obstruct water filtrations in water treatment plants (Ryu *et al*., 2008). Biofilm produced by *Stenotrophomonas maltophilia* has been employed to biodegrade branched anionic surfactants from activated

sludge (Farzaneh *et al*., 2010) just as the bacterium has equally been recognized as a potent agent in bioremediation. Aromatic industrial emissions like toluene, xylene, benzene and ethylbenzene have been degraded using the *Stenotrophomonas maltophilia* strain T3-c (Juhasz and Naidu, 2000; Lee *et al*., 2002; Ryu *et al*., 2008). *Stenotrophomonas maltophilia* strain M1 degrades methomyl, an oxime carbamate which though used extensively for the control of insects and nematode, is a toxic xenobiotics that disrupts the balance in the ecosystem killing vital primary consumers (Mohammed, 2009). According to Mohammed (2009), this plasmid based degradation exhibited by this organism was discovered through multiphase-coupled mass spectrometry, and this bioremediation potential promises to be of collossal advantage as one or more pesticides often detected in 95% of surface water systems' samples in USA could be treated with *Stenotrophomonas maltophilia*. Guan *et al*. (2008) observed the degradation of aflatoxin B1 by *Stenotrophomonas maltophilia* 35-3 with highest degradation index of 0.84 at slightly alkaline pH (pH of 8). This enzymatic degradation has great industrial application. Also, many studies have reported the degrading potentials of various strains of *Stenotrophomonas maltophilia* with huge successes (Gilliom *et al*., 1999; Ryan *et al*., 2009; Gren *et al*., 2010; Zhao *et al*., 2011).

In the same vein, this important role in bioremediation encompasses heavy metals removal and phyto-remediation. Vallini *et al*. (2005) reported selenium precipitation by *Bacillus mycoides* and *Stenotrophomonas maltophilia*. Antonioli *et al*. (2007) also observed that *Stenotrophomonas maltophilia* strain SeITE02 can detoxify a selenite contaminated environmental matrix aerobically, reducing selenite to selenium. This attribute is also true for other heavy metals. Non-viable cells of *Stenotrophomonas maltophilia* can be utilized with higher effectiveness to remove Cu (II) from aqueous solutions than a viable one (Ting and Choong, 2009). This makes *Stenotrophomonas maltophilia* a rare bacterium that is beneficial

both as viable cell culture as well as its cell-free extracts or non viable cells. The use of non viable cells can stand as a unique dimension with less demand for cell maintenance in the management of wastewater to eliminate or at least, reduce the heavy metals especially discharged copper wastewater in the environment. Also, *Stenotrophomonas maltophilia* have been observed to play an active role in phyto-remediation of crude oil impacted soil. This attribute was closely connected with their nitrogen fixing potentials as all the bacteria isolated from the plants rhizosphere and used for the study were equally phyllospheric nitrogen-fixing (diazotrophic) bacteria, *Stenotrophomonas maltophilia* inclusive (Al-Awadhi *et al*., 2009) .

3.3 GENETIC BASIS FOR THE ATTRIBUTES OF *STENOTROPHOMONAS MALTOPHILIA*

Observed beneficial attributes of *Stenotrophomonas maltophilia* are orchestrated by inherent and acquired repository of genes (Alonso *et al*., 2000; Kobayashi *et al*., 2002; Zhao *et al*., 2011), of which phenotypic expressions are primarily important for the survival of the bacteria in the natural environment. In terms of relatedness of some strains expressing these "wonders", Rocco *et al*. (2009), observed that the chromosomes of *Stenotrophomonas maltophilia* K279a and R551-3 strains bear same GC content (67%), but different in length, i.e. K279a DNA has the length 4,851,126 bp while R551-3 DNA has 4,573,969 bp. This author further reported higher potential gene products in K279a than in R551-3. Meanwhile, the clusters of type I pili genome are distributed in a unique manner throughout the bacterial gene sequence. This may be interpreted as a similar colonization strategy by *Stenotrophomonas maltophilia* in plants and animals. *Stenotrophomonas maltophilia* carries a number of biosynthetic genes for lipopolysaccharide and/or exopolysaccharide which include rmlA, rmlC and xanB (Huang *et al*., 2006). The phenotypic expression of the genes in producing lipopolysaccharide, of course is imperative in cell function, cell integrity and

bacterial adaptation, bringing about resistance to antibiotics and neutral detergents (Michel, 2000; Poole, 2002). Some clusters of these genes (including the antimicrobial resistant genes) might have been transferred from other bacteria, even those belonging to distant species (Alonso *et al*., 2000). This is possible as many *Stenotrophomonas maltophilia* strains have been found to have identical BOX-PCR patterns with some endophytic isolates and acquire genes for the earlier observed beneficial attributes by horizontal gene transfer (HGT). A good example is trans-conjugation that has been observed in *Stenotrophomonas maltophilia* and *Enterobacter* sp (Taghavi *et al*., 2005).

With regards to antibiotic resistance genes, Alonso *et al*. (2000) showed that a *Stenotrophomonas maltophilia* strain acquired a cluster of genes coding for antibiotic and heavy metal resistance from Gram positive bacteria. This was equally observed by Ojo *et al*. (2006) in *Stenotrophomonas maltophilia* and two other Gram negative rod bacteria. *Stenotrophomonas maltophilia* is equally viewed as a reservoir for disseminating the resistance gene to other Gram negative rods. For example, Gordon and Wareham (2010) following their observation of considerable diversity within plasmid-borne quinolone resistance gene, Smqnr alleles in *Stenotrophomonas maltophilia*, suggested that the bacterium might be a reservoir for the spread of quinolone resistant factors to the Enterobacteriacea family. Some schools of thought believe that commensal bacteria including *Stenotrophomonas maltophilia* are reservoirs of antibiotic resistance genes (Knezevic and Petrovica, 2008; Sanchez *et al*., 2009), out of which some antibiotic producers are “developers” of antibiotic resistance and transfer same to pathogenic species by HGT. The presence of such large antibiotic resistance determinants and/or genes in soil actinomycetes was quoted as evidence to support their assertions (D’Costa *et al*., 2006; Wright, 2007).

For quinolone resistance, Hernandez *et al*. (2011) noted that quite a number of "contributors" include protein protecting target sites, enzymes that modify fluoroquinolone and efflux pumps which also contribute immensely. Zhao and Drlica (2001) and Drlica (2003) showed mutation as the applicable yardstick to determine quinolone resistance measure. Quinolone resistance, being plasmid-borne, can be transferred. Fear correlation exists between the plasmid-borne quinolone resistance gene, Smqnr allele and quinolones resistance phenotype among the *Stenotrophomonas maltophilia* isolates (Sanchez *et al*., 2008). Hence, the reservoir for quinolone-resistance genes and the risk of patients' compliance to antibiotics regimen in inducible antibiotic resistance in non-clinical environments is of immense interest to the clinical epidemiologist.

3.5 CONCLUSION

A careful consideration of the adaptation, 'prowess' and multitudinous applications of this bacterium reveal the inherent benefits and challenges. The attending challenges like concomitant production of toxic products and growing trend of pathogenicity should be tackled through a multidisciplinary approach. This step becomes imperative due to the active role the organism plays in nitrogen fixation, biodegradation, biological control, bioremediation and its high potential for use as a source of novel enzymatic activities in biotechnology. Also, how widespread the role of the bacterium is with regards to acting as a reservoir of antibiotic resistance genes especially in underdeveloped countries should be of interest and is a subject of on-going investigation in our group.

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CHAPTER FOUR

Staphylococcus species and emerging traits in the commensal subgroup: A call to arms

Submitted to Archive of Biological Sciences

ABSTRACT

Staphylococcus [Greek *Staphyle* (bunch of grape) and *kokkos* (granules)] is a Gram positive cocci, catalase positive, aerobic and/or facultative anaerobe, non-motile, non-spore forming, occurring singly, in pair or in irregular clusters. A number of clinical and research approaches are employed to study the bacteria with greater emphasis on the clinical isolates. The most prominently studied and most virulent in their genus is the coagulase positive *Staphylococcus aureus*. Others are mostly commensals and used to be referred to as less virulent. Though they have both beneficial roles or can act as infectious agents, the emergence of dynamic virulent traits among the commensal Staphylococci and their implication in serious life-threatening multidrug resistant infections qualifies them as true “grapes of wrath”. Such emerging traits might have arisen due to interplay of multiple factors like the concomitant effect of *cap* and *ica* operons, mutation and/or horizontal gene transfer (HGT), genetic recombination or other less well defined intrinsic tendencies. There is an urgent need to keep in check the potential menace that emerging traits in commensal *Staphylococcus* incur to public health.

4.1 INTRODUCTION

Staphylococci are common natural commensals that inhabit the body of humans and warm-blooded animals. Most of them are found on the skin mucosal surfaces surrounding openings in the body surface (Archer, 1998; Adegoke and Komolafe, 2008). They are Gram positive cocci, catalase positive, aerobic and/or facultative anaerobes, non motile, non spore forming, occurring singly, in pair or in irregular clusters having got its name from the Greek words " Staphyle" and "kokkos" which mean "bunch of grape" and "granules" respectively (Van Der Zwet *et al*., 2002). About forty species and 17 subspecies of *Staphylococcus* are recognized (Trulzsch *et al*., 2002; Bannerman, 2003) and they are broadly differentiated on the basis of coagulase production. Coagulase-positive Staphylococci (CPS) e.g. *Staphylococcus aureus* are the best known and have been frequently implicated as 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 their large numbers (Kloos and Schleifer 1975). *S. hominis*, *S. warneri*, *S. capitis*, *S. simulans*, *S. cohnii*, *S. xylosus*, and *S. sac-charolyticus* are examples of the CNS that may be referred to as commensals as they are mostly non invasive (http://www.cehs.siu.edu/fix/medmicro/staph.htm), though may also be opportunistic pathogens of both human and animals preferentially affecting the immunocompromized, long-term hospitalized and critically ill patients (Ziebuhr, 2001; Bannerman *et al*., 2003).

Species within the *Staphylococcus* genus are known to ferment mannitol, but a few do not. So, reliance on cultural characteristics alone might not be enough to identify all the variants of *Staphylococcus* species in a natural environment (Bello and Qahtani, 2005). Also, they exhibit variations in cell sizes which depend on the nutrient composition of the cultivating media. In some, this might be due to dynamic genetic polymorphism (Stephens *et al*., 2006).

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. Clinical isolates obtained as commensal strains were formerly mostly susceptible to antibiotics (Kozitskaya, 2004). Methicillin resistance is, just like in *S. aureus* (well known clinical pathogen) mediated by the mecA gene encoding a penicillin-binding protein with reduced affinity to β-lactam antibiotics (Hiramatu *et al*., 2001; Hiramatu *et al*., 2002). In this review, we attempt to overview Staphylococus species as well as some emerging trends in the commensal subgroup.

4.2 *STAPHYLOCOCCUS* SPECIES AS BENEFICIAL MICROORGANISMS

Both CNS and CPS occupy specific niche in their ecosystem (Brumell, 2002) and as such are important in the maintenance of ecological balance. The presence of some commensals in a niche creates microbial antagonism against pathogens (Kostrzynska and Bachand, 2010); inhibits pathogen colonization of the niche; and diminshes infection in the host. Iwase *et al.* (2010) demonstrated that the commensal *Staphylococcus epidermidis* occupies a niche in the nasal cavity and secretes serine protease Esp which inhibits the formation of *S. aureus* biofilms and reduces *S. aureus* nasal colonization. The commensal, in this case confers a non-specific immunity against *Staphylococcus aureus* colonization/infection. This characteristic is also being exploited to reduce contamination by pathogens on produce and meat products (Kostrzynska and Bachand, 2010).

Beside the aforementioned, some commensal species of Staphylococci are considered to be of biotechnological importance in food fermentation. *S. xylosus, S. carnosus,* and *S. equorum* are used as starters for the manufacture of fermented sausages (Mauriello *et al*., 2003; Cocolin *et al*., 2006). These bacteria ensure colour stabilization during sausage ripening as well as contribute to fragrance formation (Sondergaard and Stahnke, 2002). *S. xylosus, S. pulvereri, S. succinus, S. pasteuri* and *S. equorum* are prevalently found in naturally fermented products and in the natural environment of traditional workshops manufacturing dry sausage without using starters (Blaiotta *et al*., 2004).

4.3 *STAPHYLOCOCCUS* SPECIES AS INFECTIOUS AGENTS.

Despite their industrial usage, many species of Staphylococci are commonly implicated as the etiological agent in infections of humans and animals. They have been cross-implicated in many superficial and systemic infections (Adegoke and Komolafe, 2008; Komolafe and Adegoke, 2008; Adegoke and Komolafe, 2009) and this has brought such serious concern that Holden *et al*. (2006) referred to them as “grapes of wrath”. The most virulent is *S. aureus* (Melzer *et al*., 2003), the most common cause of hospital-acquired bacteremia, though CNS are, as a group, the most frequently encountered bacteria in Medical Microbiology laboratories (Cerca *et al* 2005; Arciola *et al* 2006; Brigante *et al*., 2008; El-Shekh *et al*., 2010). CNSs are the most common cause of bacterial colonization of indwelling devices leading to bacteremia (Jeske *et al*., 2003; El-Shekh *et al*., 2010). Specific examples are S. *epidermidis, S. saprophyticus* and *S. haemolyticus* which have repeatedly been associated with human infections (Heikens *et al*., 2005; Holden *et al*., 2006). S. *epidermidis* has been consistent aetiology in nosocomial infections (Rashed and Awole, 2007) while native valve endocarditis in neonates and other patients with internal prosthetic devices,

peritonitis in patients undergoing continuous ambulatory peritoneal dialysis (CAPD) and urinary tract infection (UTI) in general have long been attributed to *S. saprophyticus* (Rupp and Archer, 1994). Also, Agvald-Ohman *et al*. (2004) reported that 14/20 patients were involved in at least one and up to eight probable nosocomial CNS transmission events.

In a more severe trend, the scourge of CNS in immunocompromised individuals is becoming enormous, and evidence from literature suggests the need for more concerted effort at salvaging the situation. Some instances include native valve endocarditis caused by *S. epidermidis*, myelodysplasia with severe neutropenia, recurrent infections and a Mediport (Miele *et al*., 2001; Moore *et al*., 2001), a pathetic case of persistent omphalitis in infants with severe congenital neutropenia (Lee *et al*., 2010), acute leukemia linked with *S. epidermidis* (D'Apollo *et al*., 2003) and *Staphylococcus* related community acquired pneumonia among HIV-infected patients (Watanabe, 2008). Nevertheless, these do not suggest that the CPS has less impact. In fact, *Staphylococcus aureus* is the predominant pathogen in non limb-threatening foot infections of pretreated diabetic patients (Lipsky, 1990), osteomyelitis (Mandal, 2002), the vast majority of skin and soft tissue infections (SSTIs) and localized pus-producing lesions like boils, abscesses, carbuncles and localized wound sepsis (Dryden, 2010).

4.4 INFECTION PATHOGENESIS AND PATHOGENICITY

Members of the genus *Staphylococcus* utilize diverse virulence factors that play a part in the disease process. These factors can be grouped into three: factors that mediate adhesion of bacteria to host cells (Jett and Gilmore, 2002); those that produce tissue damage (Diep *et al*., 2010); and those that protect the staph and/or other pathogen concomitantly present against the host's immune system (Peschel, 2002; Begun *et al*., 2007; Kraus and Peschel, 2008) and antibiotics. Staphylococcal coagulase promotes adhesion and reacts with prothrombin in the

blood to form staphylothrombin which enables serine, cysteine- and metalloprotease (Dubin, 2002) to convert fibrinogen to fibrin and hence, clotting of the blood. Coagulase can coat *S. aureus* surface with fibrin upon contact with blood to resist phagocytosis, the primary host defense mechanism making the bacteria more virulent.

The polysaccharide capsule also facilitates resistance to phagocytosis against *S. aureus* (Lowy, 2002). Surface proteins mediate Staphylococcal attachment to selected host surfaces via tissue matrix molecules. Enterotoxins produce a sepsis syndrome by functioning as superantigens. *S. aureus* in this case produces the superantigen that causes damage by stimulating a T-cell response (Chang *et al*., 2005), that can result in the development of toxic shock syndrome (TSS). The superantigen may also lead to the production of interleukin 4 and 10 which activate T helper 2 (TH2) cells leading to a reduced clearance of microbial pathogens (Burton and Erskine 2003). Paradoxically, the *S. epidermidis* earlier noted to prevent *S. aureus* infection also plays a role as a significant opportunistic pathogen that disrupts skin integrity; weakens hosts defenses and permits bacteremia and internal tissues' invasion (Casadevall and Pirofski, 1999). The aforementioned virulence factors among others bring about the clinical manifestation observed in animals.

Besides Koch's postulates, identification of virulence gene in *Staphylococcus* isolates from a specific clinical situation enable identification of them as the disease aetiology. Akineden *et al*. (2001) noted that severity of mastitis is related to virulence factors produced by *S. aureus.* This virulence varies in various species of organisms and influences their degree of pathogenicity (Thomas and Elkinton, 2004). Turkyilmaz and Kaya (2006) reported their observation in bovine mastitis, dog's external ear infection and chicken infections that CNS are more virulent than CPS and have been known for rapid onset of infection. So, there is need to be cautious about the CNS as much as CPS since they have been implicated as

aetiologies of skin infections, abscesses, septicemia/bacteremia, gastroenteritis, endocarditis, toxic shock syndrome (TSS) and certain food intoxications of both human and farm animals.

4.5 DIAGNOSIS OF *STAPHYLOCOCCUS* SPECIES

Staphylococcus spp. is diagnosed primarily in cultures (Cheesbrough, 2006). Most but not all species within this genus ferment mannitol. So, absolute reliance on cultural characteristics and cell sizes might be insufficient in identifying all the variants of *Staphylococcus* spp. from a natural environment as these depend on the nutrient compositions of the cultivating media. Diagnostic kits include VITEK 2, the BD Phoenix system and the Analytical Profile Index (API) Staph identification kit. The use of API STAPH to identify species level by comparing the biochemistry of the isolates with the existing database is a notable landmark in bacterial identification. However, this may place limitations on innovations, as new isolates different from those within the existing database (Almeida and Jorgensen, 1983) might be regarded as having unacceptable profile. In clinical laboratories unlike in research, due to clinical emergencies, the cultures are not usually employed for thorough confirmation, but to provide a medium to test for antibiotic susceptibility testing on the presumed aetiologies (monomicrobial or polymicrobial) and effect prompt treatment. Research laboratories, however utilizes culture, morphology, biochemistry immunochemistry and genetics of organisms for their characterization and identification.

The genetic perspectives for identification include genus-specific identification and specie-specific identification and are more reliable. Polymerase Chain Reaction (PCR) is used for the identification and can identify isolates or the presence of species of interest from highly contaminated samples (Deepak *et al*., 2007; Abd-Jamil *et al.,* 2010). Generally, one

remarkable achievement of PCR is revelation of many organisms that are non-culturable or difficult to culture or isolate (Crawford *et al*., 2006).

Epidemiological investigation of Staphylococci employs other techiques. In this regard, numerous molecular techniques have been employed over the past decade, though with some shortcomings (Lauri and Mariani, 2009). These methods include multilocus enzyme electrophoresis, phage typing, random amplified polymorphic DNA ribotyping, plasmid DNA restriction patterns and coagulase genotyping. Subtyping is an important investigative tool (Lauri and Mariani, 2009), for example, Zang *et al*. (2008) applied Real-time PCR to detect nuc gene as a specific marker for *S. aureus*, mecA gene encoding methicillin resistance and 5 other genes encoding Staphylococcal enterotoxins. Notable enough, these methods are not without their limitations (Tang *et al*., 1997). There is the limitation of failure of some techniques during isolate typing; hence the need for more robust and simpler typing assays (Zang *et al*., 2005). Phenotypic characterization thus maintains a vital role in the overall management of infectious organisms (Singh *et al*., 2006), and methodological review and improvement are pertinent steps for successful epidemiological tracking of *Staphylococcus* species (Ramsay *et al*., 2003). While less consideration is given to the control of less virulent species, their ability to acquire virulent gene(s) (Hacker *et al*., 2003) should not be overlooked. Hence, improved safety measures should concomitantly be incorporated with the methodological review to accommodate the potential for horizontal transfers of virulent gene(s) between clinical and commensal organisms.

4.6 CONTROL OF STAPHYLOCOCCAL INFECTION

Due to their tendencies to be pathogenic either by acquired or intrinsic potentials, *Staphylococcus* spp. should be controlled, irrespective of their role(s) in a niche. Their control

may be prophylactic or therapeutic. The prophylactic measure includes general rules of hygiene that reduce the bacterial load on their (animal) host (Blancou *et al*., 2005; Bretan, 2009). This sanitary prophylaxis should be given preference in Staphylococcal control arsenal as medical prophylaxis may predicate antibiotic resistance (Tagoe and Attah, 2010). Adequate washing of hands (and the entire body in humans), ensuring grazing of farm animal in controlled hygienic vegetation, proper disinfection of the skin with methylated spirit before administering injections or vaccines etc will prevent the opportunity for commensals to exhibit their pathogenic potentials *in vivo* (Gajadhar *et al*., 2003). Emphatically, arbitrary administration of antibiotics for prophylaxis should be discouraged as this encourages the emergence of resistance (Tagoe and Attah, 2010) by the organism as a means of adaptation.

During the therapy of *Staphylococcus* infection, penicillins, macrolides, fusidic acid, vancomycin, and cephalosporins are antibiotics active against many species of Staphylococci, but most strains of *S. aureus* (particularly the clinical strains) are resistant to penicillin due to the production of plasmid-coded β-lactamase (Cheesebrough, 2006). For these infections, therapy using stable antibiotics to β-lactamase is encouraged. Methicillin is a baseline recommended drug in this regard, although antibiotic susceptibility test should always precede the choice of best and cost effective antibiotic. Vancomycin was the recommended last line of Staphylococcal control (Bhalakia and Morris, 2005) especially against methicillin resistant *S. aureus* (MRSA), but more recently even Vancomycin-resistance has been widely observed (Sievert *et al*., 2008; Lowy, 2011)

4.7 CLINICAL VS COMMENSAL STAPHYLOCOCCI: EMERGING TRAITS

The expansion in the acquired resistance of *S. aureus* extends to methicillin (first by *S. aureus*: 1960, by the commensal *S.epidermidis*: 1962 (Jones, 2008), to third-generation

penicillins, and now to other antibiotics, including vancomycin. *S. epidermidis* and other coagulase-negative Staphylococci have developed interesting strategies in conquering the hospital environment as a novel ecological niche and they have been living at the edge between commensalism and pathogenicity. Thus commensal Staphylococci common in hospitals spread in the natural environment and has had its resistant attributes recycled among commensals (Zolezzi *et al*., 2004). So in the early 1990s, there were reports of cases of Methicillin resistant *Staphylococcus aureus* (MRSA) among healthy persons without health care contact. Beam and Buckley (2006) also reported that 47.5% of a group of healthy community members colonized with MRSA was found to have at least health care–associated risk factors and these infections were labeled community-associated MRSA (CA-MRSA). This means that hospital strains may still get to the community though with at least one risk factor, yet few strains replicate within short periods of times, given conducive condition(s), and/or transfer their attributes of pathogenicity to the commensal which in turn spreads rapidly within the community. Since 2002, the rate of infections by non clinical isolates or commensals (CA-MRSA) has increased in adults and children; they now account for most community-acquired skin and soft-tissue infections diagnosed in casualties (Moran *et al*., 2006). Effort to cushion the effect of resistance to methicillin and vancomycin by the introduction of an oxazolidinone drug linezolid which was approved for clinical use in 2000 (Hutchinson, 2003) came with transient success; not only among the clinical isolates (Toh *et al*., 2007) but also the supposed non-invasive commensal Staphylococci (Araujo *et al*., 2006).

The commensal *Staphylococcus* spp. acquire appropriate virulence genes as a result of Horizontal Gene Transfer (HGT) and genome segments known as pathogenicity islands are the landmarks of pathogenic processes (Groisman and Casadesus, 2005). So, the

expression of serious multidrug resistant infection with a seemingly high level of virulence (an attribute of clinical isolates) expressed by some commensal *Staphylococcus* species might be adduced to the capacity of *S. epidermidis* for example, to form biofilms by adhering to the surfaces of foreign bodies and to matrix proteins of the host (Mack *et al*., 2007; Otto, 2008). It can also be as a result of simultaneous presence of any of *cap* operon encoding the polyglutamate capsule which have been recognized as a major virulence factor in *Bacillus anthracis* (Kocianova *et al*., 2005); sesI gene with the phenotypic SesI protein (virulence factor of *S. epidermidis* or a marker of invasive capacity); and the *ica* operon that produces the biofilm exopolysaccharide (Li *et al*., 2005) in them. Toh *et al*. (2007) reported that linezolid resistance in a methicillin-resistant *Staphylococcus aureus* hospital strain from Colombia is determined by the presence of the cfr gene whose product, Cfr methyltransferase modifies adenosine at position 2503 in 23S rRNA in the large ribosomal subunit. Besides this form of mutation, mobile genetic elements (MGEs), such as plasmids, phages, pathogenicity islands, and genomic islands, could be responsible for transmission. (Zolezzi *et al*., 2004).

Two distinct mechanisms are employed for MGEs distribution (Ranking *et al*., 2010). They may be passed on to daughter cells by vertical transmission (Lindsay and Holden, 2006). Alternatively, they can be horizontally transferred between different bacteria lineages despite high metabolic load implication (Lindsay and Holden, 2006), though the latter places higher metabolic cost on the bacteria. So, the fitness of MGEs is better when it codes for traits that enhance vertical transmission (Ferdy and Godelle, 2005). In the presence of certain restrictions on horizontal transmission however, MGEs are conspicuously absent among some clonal complexes (Kuroda *et al*., 2001). Therefore the distributions of MGEs are employed to explain the emergence of some virulent clones of bacteria that resulted in their epidemiological changes (Henry-Arnaud *et al*., 2007).

Acquisition and transfer of the antibiotic resistance genes through horizontal gene transfer (HGT) is one of the most common ways through which clinical pathogens and commensals develop antibiotic resistance (Franceschi *et al*., 2004), although this may occur through the uptake of exogenous DNA by transduction, transformation and conjugation in food-borne pathogens (Kelly *et al*., 2008). In some cases, the virulence genes are encoded by a bacteriophage genome (Kaneko *et al*., 1998). The frequency of interspecies and even intraspecies HGT are reduced by efficient restriction system (Tock and Dryden, 2005; Hosskinson and Smith, 2007). Factors such as lack of adaptive DNA in the environment, bacterial competence development, specificity of DNA uptake and DNA sequence compatibility for integration into replicating genetic unit are also significant. Natural resistance genes can spread rapidly among *Staphylococcus* strains thus reducing the clinical effectiveness of commonly used drugs (Bozdogan *et al*., 2004; Reyes *et al*., 2007; Robicsek *et al*., 2006).

The spread of resistance among the commensal Staphylococci around the world leaves more to be desired. In United States hospital laboratories with studies on central nervous system specimens from 2000 to 2002, Jones *et al*. (2004) reported about 23.7% coagulase positive *Staphylococcus aureus* (CPS) and 3.1% coagulase negative *Staphylococcus* (CNS); with high resistance of 22.8% to levofloxacin, 27.8% to ceftriaxone and 32.9% to oxacillin by *Staphylococcus aureus* and 5.3% to levofloxacin, 64.9% to ceftriaxone and 67.2% oxacillin by CNS. Obviously, higher resistance was observed among the CNS than CPS and one could logically observe that the development of resistance in this case might not be at the site of the isolation bearing in mind the idea of blood brain barrier. The organisms in their previous niche as the commensals were probably exposed to the antibiotics the host used non-medically and developed resistance (Davies and Davies, 2010).

In case of methicillin resistance exhibited by *Staphylococcus* spp., existing database between 1997 and 1999 showed 70% in Canada, USA, Latin America, Europe and the West Pacific (Diekema *et al*., 2001). The co-resistance of trimethoprim-sulfamethoxazole (SXT) resistance in methicillin sensitive Coagulase Negative *Staphylococcus* (MSCNS) was about 17% compared with about 57% in methicillin resistant Coagulase Negative *Staphylococcus* (MRCNS). The trend was similar in US and the remaining four locations for clindamycin, ciprofloxacin, gentamycin, and erythromycin.

In a study on the trends of resistance in clinical isolates of CNS in Spain over a period of five years from 1986 to 2002 (Cuevas *et al.,* 2004), it was discovered that 28% of strains were community in origin (commensal) and 72% were nososcomial: a trend similar to the observation of Rasheed and Awole (2007). In 2002, a steady rise in oxacillin resistance from 32.5% in 1986 to 61.3% was observed while the peak of gentamicin resistance was 41.4% in 1994, though it dropped in 2002 to 27.85%. A rise of 1.1% to 44.9% in ciprofloxacin resistance observed in 1986 and 2002 respectively was alarming and called for close watch on the commensal Coagulase Negative *Staphylococcus* control by concerned Public Health Experts.

One author's acclaimed first comprehensive data on antibiotic susceptibility patterns for MRSA in South Africa revealed that large resistance was exhibited by the MRSA to erythromycin, tetracycline, trimethoprim/sulfamethoxazole, gentamicin and ciprofloxacin ranged between 55% and 78%, but all isolates were susceptible to teicoplanin, linezolid, vancomycin and quinopristin/dalfopristin (Marais *et al*., 2009). Another study (Lin and Biyela 2005) reported the presence of 58% class 1 integron especially the beta lactamase genes among the commensals from Mhlathuze River in Kwazulu-Natal, South Africa while identifying the river as a major reservoir of resistance genes in that area.

4.8 CONCLUSION

Commensal Staphylococci lead to life threatening infections with high virulence. Virulence genes include *sesI* gene, *cap* operon encoding the polyglutamate capsule in *B. anthracis* virulence, and *ica* operon that leads to production of a biofilm exopolysaccharide necessary for biofilm production. Biofilm production among the organisms accentuates therapeutic intervention. The search for more novel antibiotics becomes imperative in view of the emergence of resistance to the existing ones. Advocacy against the abuse of antibiotics (Anon, 2011) should be adopted and the use of more traditional remedies encouraged. Many plant extracts and constituents being used by traditional healers to treat *Staphylococcus*-related infections over the last century are still effective where no bacterial resistance has been observed (Fenical, 2006). Many of them have been tested scientifically and their efficacy confirmed (Adebayo-tayo and Adegoke, 2008; Adegoke and Adebayo-tayo, 2009) but phytopharmaceuticals are only slowly becoming incorporated into orthodox medicine.

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CHAPTER FIVE

***Stenotrophomonas maltophilia* an opportunistic, yet true pathogen: a need for strict adherence to the rules of therapeutic**

Submitted to Journal of Biomedical Research

ABSTRACT

Stenotrophomonas maltophilia is no doubt an emerging nosocomial pathogen earlier noted in broad spectrum life threatening infections among the vulnerable, and more recently as a pathogen in immune-competent individuals. Its well endowed intrinsic antibiotic resistance factors have made its control a herculean task worldwide. Low outer membrane permeability, natural MDR efflux systems and/or resistance genes, resistance mechanisms like the production of two inducible chromosomally encoded β-lactamases, and lack of patient history are factors that pose great challenges to the *S. maltophilia* control arsenals. New fluoroquinolone, Trimethoprim-sulphamethaxole (TMP-SMX) have been reported as antibiotic regimen with good therapeutic outcomes. A save combination therapy following proper diagnosis is a relative step in combating resistance. Since it is established that *S. maltophilia's* high antibiotic resistance profile, among other factors is predicated by repertoire of genes, proper attention in its control to avoid the spread of such genes intra- and inter-specifically would make epidemiological sense.

5.1 INTRODUCTION

Stenotrophomonas maltophilia, formerly known as *Pseudomonas* or *Xanthomonas maltophilia*, has emerged as an important nosocomial pathogen in clinical environments (Senol, 2004) and the cause of morbidity and mortality in hospitalized patients especially those with underlying debilitating conditions such as immunosuppression, malignancies and implantation of foreign devices (Calza *et al*., 2003; Cernohorska and Votava, 2004; Ruzicka *et al.,* 2004; Walencka *et al.,* 2006). They are aerobic, glucose non-fermentative (but oxidize glucose and maltose), Gram-negative bacillus with slightly smaller size than other members of the genus. They are motile with the aid of polar flagella and produce pigmented colonies on MacConkey agar. *S. maltophilia* are catalase-positive, oxidase-negative (distinguishing feature from most other members of the genus) and have a positive reaction for extracellular DNase and lysine decarboxylase (Gilligan *et al*., 2003). They are frequently isolated from abiotic milieu like water and soil, living entities like animals and plant materials (Lo *et al*., 2002; Borner *et al*., 2003; Smeet *et al*., 2007); and frequently colonize fluids used in the hospital settings (e.g., irrigation solutions, intravenous fluids etc) and patient secretions (e.g., respiratory secretions, urine and wound exudates) (Minkwitz and Berg, 2001). This review article attempts an overview of the implication of the commensal *S. maltophila* in infections; their antibiotic regimen; therapeutic outcomes; and reported genetic basis of observed resistances.

5.2 THE *STENOTROPHOMONAS MALTOPHILIA* AS AN INFECTIOUS AGENT

Stenotrophomonas maltophilia is acknowledged as a commensal organism of supposedly low virulence, yet vibrant as an opportunistic pathogen (Gnanasekaran and Bajaj, 2009). As a frequent coloniser of fluids used in the hospital settings, such as nebulisers, water

baths, dialysis machines and intravenous fluids, it utilizes the irrigation solution it colonizes and/or invasive medical devices to bypass normal host defences to cause human infection (Oliveira-Garcia, 2003). Else, there seem to be no difference between the pathophysiology of this non-fermentative aerobic Gram-negative bacillus and other non-fermentative aerobic organisms. This in a way makes consultation cumbersome (Chang and Huang, 2000). Thus, the bacteria have been implicated as aetiology of a wide spectrum of serious infections especially in those with underlying debilitating conditions such as immunosuppression, malignancies and implantation of foreign devices (Calza *et al*., 2003; Cernohorska and Votava, 2004; Walencka *et al.,* 2006).

Stenotrophomonas maltophilia has been implicated in bacteraemia, endocarditis and respiratory tract infections, especially in patients with cystic fibrosis, urinary tract infections (usually secondary to urinary tract surgery or instrumentation), meningitis, ophthalmologic infections, skin and soft tissue infections and bone and joint infections with rare cases of pyomyositis (Gales *et al*., 2001; Kim *et al*., 2002; Platsouka, 2002; Sakhnini *et al*., 2002; Arora *et al*., 2005; Pathmanathan and Waterer, 2005; Al-Anazi *et al*., 2006; Yemisen *et al*., 2008; Thomas *et al*., 2010). More recognition is being accorded to the skin and soft tissue manifestations of *Stenotrophomonas maltophilia*. Clinical skin presentations include primary cellulitis, cellulitis-like cutaneous metastasis or cellulitis or metastatic nodular skin lesions, gangrenous cellulitis, ecthyma gangrenosum, soft tissue necrosis and infected mucocutaneous ulcers (Denton and Kerr, 1998; Foo *et al*., 2002; Teo *et al*., 2006; Smeet *et al*., 2007). The organism has been isolated with increasing frequency from cystic fibrosis as an emerging potential pathogen (Talmacius *et al*., 2000). Out of all, the most frequent clinical manifestation of *S. maltophilia* infection is pneumonia (Pathmanathan and Waterer, 2005). This organism, which usually occurs freely in the environment, has been implicated in

nosocomial infections and community based infections (Koseoglu *et al*., 2004; Meyer *et al*., 2006; Falagas *et al*., 2009).

5.3 EPIDEMIOLOGY OF *S. MALTOPHILIA* INFECTION

As *S. maltophilia* is well distributed worldwide in the environment as commensal, its scourge in serious infections is equally global. In Germany, a research conducted between 2001 and 2004 to investigate changes in the number of *S. maltophilia* per 1000 persons as nosocomial infection in intensive care unit (ICU) revealed as high as 165 isolates per 1000 in some study locations (Meyer *et al*., 2006). Earlier, Apisarnthanarak *et al*. (2003) in a six weeks surveillance study in Washington, USA, reported the prevalence rate of 9.4 % from stool samples. An outbreak of *Stenotrophomonas maltophilia* bacteremia in controlled allogenic bone marrow transplant patients was observed by Labarca *et al*. (2000) in Los Angeles, USA, just as 44 strains were isolated from 41 hospitalized patients in Turkey in a study from June 2000 to December 2001 (Caylan *et al*., 2004). Following an epidemiological typing, Caylan *et al*. (2004) reported that the 3 outbreaks in the study area were caused by 12 strains. Apisarnthanarak *et al*. (2003) noted that patients colonized with *S. maltophilia* had received a greater number of different types of antibiotics than noncolonized patients.

In Africa, *S. maltophilia* infection cases in 2 patients as early as 1977 (when the organism was still known as *Pseudomonas maltophilia*) was observed by Denis *et al*. (1977). To date, not many reports of the organism in infection have been made from Africa, but Botes *et al*. (2007) reported the hyper-resistance of *Stenotrophomonas maltophilia* (and some other bacteria) to arsenic confirming the 'resilience of the bacteria' and the picture of its potentials in immunocompromised individuals. Meanwhile, *S. africana* of the same genus

with *S. maltophilia* has been observed as an opportunistic human pathogen in Africa (Drancourt *et al*., 1997).

5.4 INFECTION PATHOGENESIS AND PATHOGENICITY

Clinical manifestation from *Stenotrophomonas maltophilia* does not usually arise by infection but rather by colonization (Pathmanathan and Waterer, 2005). Where it does, contaminated irrigation solutions and/or invasive medical devices in hospital settings are the primary "vehicle" with which it by-passes the non-specific immunity and cause human infections. Some other arrays of conditions can also predispose an individual to the infection (Agvald-Ohman, 2007). Such conditions include prolonged hospitalisation especially in intensive care units, foreign body implants and mechanical ventilation, intravenous drug abuse, exposure to broad-spectrum antimicrobial agents (such as the carbapenems), extended-spectrum cephalosporins, and fluoroquinolones, as well as malignancy (Rolston *et al*., 2005). Kim *et al*. (2010) reported the establishment of *S. maltophilia* infection leading to endocarditis in a patient that had a replacement of valve with 27 mm Carbo Medics metallic due to severe rheumatic valvular disease. Also, the duration of hospitalization before the onset of the clinical features and /or diagnosis is an important factor. A case study that considered the duration of hospitalization before the onset of *S. maltophilia* bacteremia, for instance, reported that it ranged from 11.5 to 24 days (Friedman *et al*., 2002; Senol., 2002; Lai *et al*., 2004) and about 3 weeks in other centres (Tsai *et al*., 2006). The burn patients developing *S. maltophilia* bacteremia mostly happened 1 week after hospitalization (Valdezate *et al*., 2001; Krecmery *et al*., 2001).

Though the detail of pathogenesis of *Stenotrophomonas maltophilia* is not fully known, a number of researches have thrown light on certain pertinent dimensions. De

Oliveira-Garcia *et al*. (2002) reported the observation of appreciable sequence identity to the flagellin of *Proteus mirabilis*, *Serratia mercensen*, *Escherichia coli*, etc. in *S. maltophilia* flagella by analysing N-terminal amino acid sequence. Also unlike earlier studies which focused only on *Staphylococcus* and *Pseudomonas* species, *S. maltophilia* produces biofilm with which it colonizes medical devices and other abiotic surfaces (Elvers *et al*., 2001). This biofilm facilitates their attachment to cultured airway epithelial cells (De Vidipo *et al*., 2001; Di Bonaventura *et al*., 2007) and their spread in an abiotic environment is made easier by the production of flagella (De Oliveira-Garcia *et al*., 2002). This biofilm production coded for, by biosynthetic genes rmlA, rmlC, and xanB and flagella, are important in colonization and motility (Huang *et al*., 2006). The biofilm contributes to bacterial virulence as it protects the bacteria against antibiotics (Monroe, 2007; Hunter, 2008). The organism is also endowed with DNase, RNase, arbutinase, acetase, esterases, lipases, mucinase, acid and alkaline phosphatases, hyaluronidase, phosphoamidase, elactase, leucine arylamidase and β-glucosidase which play vital roles in their pathogenesis (Windhorst *et al*., 2002; Nicoletti *et al*., 2011). Windhorst *et al*. (2002) describes the *StmPr1* protease from *Stenotrophomonas maltophilia* that is able to degrade several human proteins from serum and connective tissues. This *Stmpr1* protease has been described as a virulence factor in the bacteria against which the development of theurapeutic agents should focus (Windhorst *et al*., 2002; Nicoletti *et al*., 2010)

The bacteria behave as true pathogens in some cases (Kim *et al*., 2002). This is reflected in their ability to infect immunocompetent individuals. Although this does not happen regularly, it is an occurrence peculiar to true pathogens. Thomas *et al*. (2010) reported a case of *Stenotrophomonas maltophilia* as a cause of pyomyositis in an immunocompetent adult. Earlier in another research, Pruvost *et al*. (2002) also described a

case of community-acquired superficial pyoderma due to this bacterium in an immunocompetent host. It has also been observed in other immunocompetent patients with cases of community-acquired meningitis and plantar pyoderma (Libanore *et al*., 2004). This trend has been equally reported where *S. maltophilia* acted as a key agent amidst polymicrobial infections (Meyer *et al*., 2006). This confirms the dual nature of this Gram-negative rod bacterium and the need to handle it as potential pathogen even when isolated from the environment as commensal.

5.5 DIAGNOSIS OF *STENOTROPHOMONAS MALTOPHILIA* AND ITS CHALLENGES

A correct diagnosis is important in choosing appropriate therapy (Preud'homme *et al*., 1990). The main challenge confronting proper diagnosis (and even control) of *Stenotrophomonas maltophilia* in most clinical manifestations is absence of patient history due to initial rarity (Das *et al*., 2009). A patient history should include clear explanation of new site-specific symptoms, prophylactic antimicrobial usage and subsequent contraindication experience (if any), risk level (by occupation or any relative predisposing factor), prior documented infections or pathogen colonization, information on co-existence of non-infectious fever by patient, (Freifield *et al*., 2011) allergy and family history. The similarity in pathophysiology between the bacteria and other Gram negative aerobic rod also contributes to the hurdles in early diagnosis. Therefore, misdiagnosis of the *Stenotrophomonas maltophilia* cases for other possible aetiology often leads to development of fatal complications and high mortality (Rello *et al*., 1999). In a number of cases, the prescription of prolonged antibiotic therapy interferes with non specific immunity, giving room for the organism to colonize more rapidly (Mamedova and Karaev, 1979; Labro, 2000). Addressing the presence of the organism in sputum as infection and subsequent use of

antibiotic therapy might equally be a wrong approach, since this might just be colonization and antibiotic therapy will disrupt microbial antagonistic effect (non specific immunity) on the *S. maltophilia* and make it adapt better to resist the drug (Drancourt and Raoult, 1997).

Laboratory diagnosis of *Stenotrophomonas maltophilia* is simple. Conventional cultural methods on nutrient agar support the growth, although certain strains require methionine (O'Malley, 2009). Isolation from natural sources (Ting and Choong, 2009) including inanimate colonization or animal sources can easily be done with MacConkey agar supplemented with imipenem antibiotic. The imipenem, being a broad spectrum antibiotic to which *S. maltophilia* is resistant removes most other bacteria (Rudlof *et al*., 2006). Further characterization on the small Gram negative, oxidase negative rod is done using the Analytic Profile Index, API 20E and BD Phoenix (Becton Dickinson, France) systems (Aydemir *et al*., 2008). Since API identification may not be 100% accurate, confirmation of the actual specie can be carried out using molecular techniques such as Genus-specific and specie-specific hybridization (Kempf *et al*., 2000). The beauty of molecular identification is the possibility of culture independent direct detection of the bacteria diversity in the environment (Cottrell *et al*., 2005). *In vivo* studies utilize lipid peroxidation, lactate dehydrogenase activity and histopathological examination of tissue homogenate to measure the effect of *S. maltophilia* on tissue (Naika *et al*., 2004; Ibrahim and Nassar, 2008).

Reference laboratories employ protein electrophoresis, transmission and scanning electron microscopy, immunological assay, western blotting and N-terminal amino acid sequence analysis to confirm the identity of the organism (De Oliveira-Garcia *et al*., 2002; Chhibber *et al*., 2008). The genetic make-up is determined using randomly amplified polymorphic DNA PCR (Krzewinski *et al*., 2001). Epidemiological study of *Stenotrophomonas maltophilia* utilizes other dynamics. A polymerase chain reaction (PCR)

test with total sensitivity and specificity approach has been developed for the detection of *S. maltophilia* (Whitby *et al*., 2000). Pulsed field gel electrophoresis technique (Denton *et al*., 1998) is employed for typing during the molecular epidemiological study of *Stenotrophomonas maltophilia*. The use of NCCLS recommended Standard Broth Microdilution (SBM), a dried-down form of broth microdilution (DMD), E-Test (ET), agar disk diffusion (DD) and agar dilution (AD) methods for studies of antibiotic susceptibility of *Stenotrophomonas maltophilia* with Trimethoprim/Sulfonamethoxazole (Turng *et al*., 1999) provide epidemiology work base data for use in retrospective Sm-control arsenal. The improvement in laboratory identification has brought about the recognition of Sm prevalence in cystic fibrosis, though the organism has been supposed to have limited clinical significance in this case (Goss *et al*., 2004)

5.6 INFECTION PROGNOSIS AND/OR THERAPEUTIC OUTCOME

The chance of co-infection makes the treatment of *S. maltophilia* more difficult and cumbersome. Prognostic factors that include therapy-based immunosuppression, blood-based carcinoma, neutropaenic, transplantation etc. are also important to determine recovery or mortality. Conditions that remove myelosuppression and invasive indwelling catheter, and prompt treatment with pre-confirmed antibiotic have been reported to determine the chance of recovery (Elsner *et al*., 1997). Johnson (2000) noted that nearly all mucocutaneous complications involving *S. maltophilia* of HIV disease either improve or resolve if improved immune function is achieved by highly active antiretroviral drugs.

Although primary cellulitis, disseminated cutaneous nodules, and mucocutaneous ulcers caused by *Stenotrophomonas maltophilia* are often associated with underlying malignancies, some complications of *S. maltophilia* infection accompanied with metastatic skin nodules and/or systemic inflammatory response syndrome (sepsis), mucocutaneous

infections in neutropaenic patients with cancer are poor prognostic. This can be adduced to the fact that many of these patients would have died of their infections and of causes that were probably secondary to their severe immunosuppression. Marchac *et al*. (2004) stated that *A. fumigatus* was much more frequently isolated in the *S. maltophilia* patients. In a study in which 51% cases was compared with 9% controls, the effect of *A. fumigatus* co-infection with *S. maltophilia* was independent of oral steroid use. Also, that allergic bronchopulmonary aspergillosis was diagnosed in 5 of 17 (30%) patients with *A. fumigatus* in the sputum and taking oral steroids.

High mortality often resulting from mucocutaneous *Stenotrophomonas maltophilia* infections in neutropaenic patients with cancer makes the effect of secondary immunosupression a worrisome trend in the infection prognosis (Hanes *et al*., 2002; Tseng *et al*., 2009; Wakino *et al*., 2009). Accompanying widespread injury to vital somatic tissues might be a relative factor to this. Clinical effort to reduce this alarming mortality rate from various forms of this bacterial infection and its attending complications is imperative. For instance, *S. maltophilia* has emerged as a significant cause of morbidity and mortality in cancer patients (Micozzi *et al*., 2000) and the mortality brought about by the organism in the cases of bacteremia in nonburn patients was reported as 10–69% (Micozzi *et al*., 2000; Friedman *et al*., 2002; Lai *et al*., 2004). Tsai *et al*. (2006) also observed a mortality rate of 30.7% in burn patients colonized by *S. maltophilia* while all (100%) the patients in nosocomial meningitis involving *S. maltophilia* were reported dead by Yemisen *et al*. (2008).

5.7 CONTROL OF *STENOTROPHOMONAS MALTOPHILIA*

Since *S. maltophilia* is not only limited to being an opportunistic pathogen of the vulnerable, but also implicated in immunocompetent individuals (Kim *et al*., 2002; Pruvost *et al*., 2002; Libanore *et al*., 2004; Thomas *et al*., 2010), its control is quite essential. Removal

of the invasive indwelling devices without change of medication, hygienic handling of breached skin and self-fix medical devices and proper quality control measure in the preparation of irrigation solution or intravenous fluid are imperative in the control and management of *S. maltophilia* infection. Elsner *et al*. (1997) observed that a patient with fatal pulmonary hemorrhage, acute leukemia and fulminant pneumonia recovered immediately the catheter was removed but the same ciprofloxacin remained as the antibiotic regimen used.

5.8 Antibiotic Regimen

Treatment of patients infected with *S. maltophilia* is generally complicated and difficult because this pathogen shows high levels of intrinsic or acquired resistance to multiple antibiotics, limiting the available therapeutic options (Denton and Kerr, 1998; Koseoglu, 2004). This is worsened by co-infection which makes the treatment of *S. maltophilia* more difficult and cumbersome. *S. maltophilia* is resistant to several antibiotics used empirically for nosocomial infections. It is imperative to remember that some of the antibiotics used in the treatment of ESBL producers like *S. maltophilia* are broad spectrum. Hence, utmost care must be taken in its selection, as consideration to patient's ability to withstand drug contra-indication(s) is imperative even in some polymicrobial cases. Non medical usage of the extended spectrum antibiotics may lead to selection of highly resistant *Stenotrophomonas maltophilia* strains. Co-trimoxazole is the treatment of choice in symptomatic infections but no available information exists on the best management of co-trimoxazole-resistant infections. Ciprofloxacin and other older quinolones have been reported to have 50% efficacy against *S. maltophilia in vitro* (Denton and Kerr, 1998). Observation was also made by Weiss *et al*. (2000) that trovafloxacin, clinafloxacin and morxifloxacin have appreciable *in vitro* activity against the organism and have been employed to treat

terminal-tending infections by it. Trimethoprim – sulphamethoxazole, TMP-SMX have been recommended by a number of researchers as initial therapeutic option for serious *S. maltophilia* infections. Dalamaga *et al*. (2003) reported improvement in the *S. maltophilia* infection in burn patients following the administration of TMP-SMX. Fluoroquinolone stands a better therapeutic choice in case of cystic fibrosis as the drug has been reported to have much higher peak lung concentration than peak plasma concentration (Schubert *et al*., 2005). However, exploiting the benefit of synergy in combination therapy using the fluoroquinolone antibiotics or TMP-SMX might be absolutely advantageous, due to the ease with which the organism acquires resistance to monotherapy (Weiss *et al*., 2000). Zelenitsky *et al*. (2005) reported that TMP-SMX combined with other antimicrobial agents, such as ceftazidime, produced a net bacterial kill and provided significant benefit over monotherapy.

Even then, consideration must be accorded to secondary drug interaction with body metabolism. Some drugs without damaging primary contra-indication might interfere with other existing drugs in plasma (Dikinson *et al*., 2001). Carbapenem antibiotic with oestrogen affect the effectiveness of contraceptive *in vivo*. Some patients' intolerant of TMP-SMX should be noted (Archer and Archer, 2002). Careful consideration must be accorded to the antibiotic regimen prescribed in *Stenotrophomonas* control arsenal. Tesoro *et al*. (2010) recommended the combination of co-trimoxazole with ticarcillin-clavulanate due to their synergism and the reported bactericidal effect against the ticarcillin-clavulanate resistant strains. This might be considered for the patients who are TMP-SMX intolerant.

The high resistance profile *S. maltophilia* to antibiotics has been predicated on a myriad of factors. Inducible beta-lactamase activity (2 inducible chromosomally encoded-lactamases, L1 and L2, and an aminoglycoside acetyltransferase) (Poole *et al*., 2001), poor outer membrane permeability and efflux mechanism (McKay *et al*., 2003), horizontal gene

transfer (HGT) (Alonso *et al*., 2000), biofilm formation and/or production of extracellular slime or glycocalyx are responsible for its resistance to multiple antibiotics (Di Bonaventura *et al*., 2004; Nicodemo and Paez, 2007). Furushita *et al*. (2005) observed intercluster divergence in beta lactamase gene among six strains of *S. maltophilia,* suggesting horizontal gene transfer (HGT) among them. So, antibiotic resistance gene is of specific interest due to the transferability from one species to another (Alonso *et al*., 2000).

Studies have revealed that *S. maltophilia* exhibits high antibiotic resistance profile due to both inherent and acquired antibiotic resistant genes (Alonso *et al*., 2004; Shimizu *et al*., 2008; Gilbert *et al*., 2010; Song *et al*., 2010). All the *S. maltophilia* strains including commensals from the environment, opportunistic pathogens from the vulnerable and those implicated as true pathogens in certain clinical cases have been shown to harbour resistant genes (Alonso *et al*., 2000; Gilbert *et al*., 2010; Song *et al*., 2010). In a Canadian hospital environment for instance, erythromycin resistance genes were detected in 100% air samples collected (containing *S. maltophilia*) from hospital rooms, and tetracycline resistance genes were detected sporadically (Gilbert *et al*., 2010). In Korea, Song *et al*. (2010) observed that antibiotic resistance gene sul1 within class 1 intergron rather than sul2 were responsible for TMP-SMX resistance in *S. maltophilia* isolates and that resistance to antibiotics might be as a result of multiple antibiotic resistance genes also within the Class 1 integron. Antibiotic resistance gene, macrolide phosphotransferase (mphBM) amidst cluster of genes like heavy metal tolerance gene, cadmium efflux determinant (cadA) together with its transcriptional regulator gene (cadC) was reported in *S. maltophilia* D457 by Alonso *et al*. (2000). In the study, the *S. maltophilia* (a Gram negative) acquired a cluster of antibiotic and heavy metal resistance genes from gram-positive bacteria, for the first time. Similarly, the role of *S. maltophilia* efflux pump D,E,F, (SmeDEF) multidrug efflux pump cannot be overlooked as it

contributes to the intrinsic multidrug resistance in *Stenotrophomonas maltophilia.* Zhang *et al*. (2001) noted that *S. maltophilia* efflux pump F, SmeF in a hyperexpressed form along with additional multidrug efflux components can promote multidrug resistance in *S. maltophilia*.

5.9 CONCLUSION

S. maltophilia has a very dynamic characteristic. The organism is not only an opportunistic pathogen in a severe life threatening infection in vulnerables but it is also reported as true a pathogen in immunocompetent individuals. This bacterial species is responsible for myriads of diseases accompanied by morbidity and mortality including respiratory tract infections, especially in clinical conditions like cystic fibrosis, bacteremia and/or urinary tract infections. Appropriate diagnosis with adequate caution is imperative as arbitrary administration of an antibiotic might result in increase in myelosuppression and/or selection of resistant strains of the species. *S. maltophilia* possesses inherent resistance to antimicrobials predicated by low outer membrane permeability, natural MDR efflux systems, and resistance mechanisms like the production of two inducible chromosomally encoded β-lactamases. Imminent danger in *S. maltophilia* control arsenal should be avoided by strict adherence to rules of hygiene, quality control in hospital units and pharmaceutical companies, avoidance of non medical use of antibiotics etc, as these conditions predispose the organism to antibiotic resistance. Resistance genes from the organisms may be transferred to other species and cause serious public health crises if care is not taken.

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CHAPTER SIX

Species Diversity and Antibiotic Susceptibility Profile of Staphylococcus of Animal Farm Origin in Nkonkobe Municipality, South Africa

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ABSTRACT

The occurrence and antibiotic susceptibility profile of *Staphylococcus* species isolated from healthy animals in Nkonkobe Municipality as well as the prevalence of associated antibiotic resistance genes were investigated using both phenotypic and molecular methods. A total of 120 *Staphylococcus* species were recovered and consisted of *Staphylococcus haemolyticus* (30%) and *Staphylococcus aureus* (23.3%) from pig; *Staphylococcus capitis* (15%) from goat; *Staphylococcus heamolyticus* (5%); and *Staphylococcus xylosus* (15%) from cattle and other Staphylococci (11%) from dead chicken and pigs. About 23.3% of these isolates were coagulase positive and 76.7% were coagulase negative. Between 75-100% of the isolates were resistant to Penicillin G, tetracycline, sulphamethaxole and nalidixic acid; about 38 % were methicillin resistant consisting of 12.6% methicillin resistant *Staphylococcus aureus* (MRSA) from pig and 12% vancomycin resistant. Also, 12% of the isolates were erythromycin resistant while 40.2 % were resistant to ceftazidime. The antibiotic resistance genes *vanA, VanB, eryA, eryB, eryC* were absent in all the phenotypically resistant isolates, but *mecA* gene and *mph* genes were detected. The high phenotypic antibiotic resistance and the presence of some of the associated resistance genes is a potential threat to public health and suggests the animals to be important reservoirs of antibiotic resistance determinatnts in the environment.

6.1 INTRODUCTION

The role of commensals in the spread of resistance among bacteria around the globe cannot be overemphasized (Levy, 1986; Summers, 2002). They have been fingered as the possible sources of antibiotic resistance genes which are now rampant among many systemic bacterial pathogens of suppurative and non-suppurative infections (Saha *et al*., 2007; Kitara *et al*., 2011). The prior exposure of the commensals to antibiotics during chemotherapy for other infections in their hosts might be a selective force for antibiotic resistance (Cohen, 1992), especially in human and in animal husbandry where antibiotics are used in large quantities (Moulin, 2001). A number of glycopeptides being used as growth promoters in animal husbandry have been reported as inducers of antibiotic resistance in the animal body flora (Perrier-Gros-Claude *et al*., 1998). The subsequent emergence of resistance has heightened bacterial colonization and/or invasion of the animals, with reduced response to control leading to their persistence as contaminants in animal products such as unpasteurized milk (Kaur and Pathania, 2010; Ogbodo *et al*., 2011). Zoonotic transfer of such difficult-to-treat bacterial species is becoming a worrisome trend as it increases the disease burden in sub-Saharan African countries, leads to higher cost of procuring treatment options and increases infant mortality (WHO, 1999)

Staphylococcus species are among the important commensals of farm animals that harbour resistance genes, especially to methicillin. It is worthy to recall that the emergence of methicillin resistance was reported in *Staph. aureus* following the antibiotic introduction in the 1960s (Grundmann *et al*., 2006). This attribute was first reported in hospitals but later in the community. Today, the methicillin resistant *Staphylococcus aureus* is still a threat to

global health and wellness (Van Loo *et al*., 2007). Other previously known commensal *Staphylococcus* species are now being observed as emerging threats to global health. There have been observation of some coagulase-negative staphylococci (CNS) that colonize animal skin and mucous membrane as flora (Kloos and Bannerman, 1994) now implicated in - skin and soft tissue infection of man (Shittu *et al*., 2004), bacteraemia and Septicaemia (Komolafe and Adegoke, 2008; D'mello *et al*., 2008). Specific examples of these include *Staph. haemolyticus*, *Staph. capitis* and *Staph. xylosus* which are commensals of farm animals but have been implicated in sub-clinical mastitis (Thorberg, 2008).

Weese *et al*. (2005) and Walther *et al*. (2008) reported the notable presence of MRSA in animals. Other studies also examined various animals including herbivores like cattles, goat, sheep and domestic carnivores like cats and dogs (Walther *et al*., 2008; Saleha *et al*., 2010). Pigs are known to harbour typical MRSA, and this spreads rapidly among the entire swine (Cuny *et al*., 2010; VandenBroek *et al*., 2009). These strains have been found among piggery workers, suggesting a zoonotic transfer (Denis *et al*., 2009; Van den Broek *et al*., 2009).

While the MRSA strains in North America (Smith *et al*., 2009) and Singapore (Sergio *et al*., 2007) are well documented, information on their counterparts in sub-saharan Africa especially in the Eastern Cape Province of South Africa is very scarce. In this chapter, we report on the prevalence of *Staphylococcus* species in healthy animals in the Nkonkobe Municipality, Eastern Cape Province, South Africa, as well as the antibiogram and associated resistance gene characteristics of the *Staphylococcus* species.

6.2 MATERIALS AND METHODS

6.2.1 Study Location

The study location is the Nkonkobe Municipality, a highly populated domain in the Eastern Cape Province, South Africa, with a population of about 128 658 on a 3 724 square kilometres of land.

6.2.2 Isolation and prelimanry identification of *Staphylococcus* species

The *Staphylococcus* species were isolated from 150 samples containing nasal, mouth wash and ear swabs of pigs (including the piglets), cattle, goats and chickens from various animal farms within the municipality. Preliminary isolation of *Staphylococcus* species was initiated by inoculating the swabs directly in nutrient broth and incubated at 37°C for 24 hours (Cheesebrough, 2006). Thereafter, a loopful of inoculum was transferred from each turbid tube to mannitol salt agar and incubated also for 24 hour at 37°C (Cheesebrough, 2006). At the end of the incubation period, distinct colonies were picked from these primary plates, purified by repeated subculturing of the isolated colonies and subjected to gram-staining, catalase test, coagulase test and lysostaphin susceptibility. The Staphylococci were preliminarily speciated using the analytical profile index (API) Staph (BioMe´rieux). The results were read following the incubation of inoculated strips between 18-24 hours.

6.2.3 Genus and Specie-specific Identification of *Staphylococcus* species

All isolates identified earlier using the API Staph Kit were confirmed by genus specific and specie-specific polymerase chain reactions (PCR) using the primers listed in Table 6.1. The PCR conditions employed for the genus level identification include 3 mins at 96°C, 40 cycles of 30 s at 95°C, 60 s at 55°C, 30 s at 72°C and a final extension of 3 mins at

72°C(Martineau *et al.,* 2001). *Staph. aureus* ATCC 25923 was used as the positive control while nuclease free water was used as the negative control. The amplicons were electrophoresed on 2.5% agarose gel stained with ethidium bromide and visualized under UV light. Species specific identification was done by multiplex PCR targeting *Staph. saprophyticus*, *Staph. epidermidis, Staph. xylosus* and *Staph. aureus*, and the PCR condition include: 15 min at 4ºC, 3 min at 94ºC, then 40 cycles of 1 s at 95ºC, 30 s at 55ºC, 30 s at 72ºC and a final hold of 3 min at 72ºC (Corbiere *et al*., 2004). The amplicons were electrophoresed in 1 X TBE Buffer on 2% agarose gel stained with ethidium bromide and visualized under UV light. PCR condition include initial enzyme activation step 10 min at 94°C, followed by 35 cycles of 15 s at 94°C, 30 s at the appropriate annealing temperature of 59°C, 50°C and 60°C for *Staph. capitis*, *Staph. haemolyticus* and *Staph. warneri* respectively and 30 s at 72°C were employed (Iwase *et al*., 2007).

Table 6.1: Genus and Species specific Identification Primers used

Organisms		Primers	Primer Sequence (5′-3′)	Size (bp)	References
Staphylococci	Genus	TStaG422	GGC CGT GTT GAA CGT GGT CAAATCA	370	(Martineau *et al*., 2001)
		TStag765	TIA CCA TTT CAG TAC CTT CTG GTA		
	S. capitis	Scap F	GCTAATTTAGATAGCGTACCTTCA	208	Iwase *et al*., 2007
		Scap R	CAGATCCAAAGCGTGCA		
	S. haemolyticus	ShaeF	GTTGAGGGAACAGAT	85	
		ShaeR	CAGCTGTTTGAATATCTT		
	S. warneri	SwarF	TGTAGCTAACTTAGATAGTGTTCCTTCT	63	
		SwarR	CCGCCACCGTTATTTCTT		
	S. xylosus	Xyl F	AACGCGCAACGTGATAAAATTAATG	539	Morot-Bizot *et al*. (2004)
		Xyl R	AACGCGCAACAGCAATTACG		
	S. saprophyticus	Sap1	TCAAAAAGTTTTCTAAAAAATTTAC	221	
		Sap2	ACGGGCGTCCACAAAATCAATAGGA		
	S. aureus	Sa442-1	AATCTTTGTCGGTACACGATATTCTTCACG	1 108	
		Sa442-2	CGTAATGAGATTTCAGTAGATAATACAACA		
	S. epidermidis	Se705-1	ATCAAAAAGTTGGCGAACCTTTTCA	1 124	
		Se705-2	AAAAGAGCGTGGAGAAAAGTATCA		

Table 6.2: Primers used to assess the antibiotic resistance genes

Genes	Primer	Sequence (5´→3´)	Amplicon size	References
ery(A)	erm(A)-1	GCGGTAAACCCCTCTGAG	434 bp	Werckenthin and Schwarz (2000)
	erm(A)-2	GCCTGTCGGAATTGG		
ery(B)	erm(B)-1	CATTTAACGACGAAACTGGC	425 bp	Jensen *et al*. (1999)
	erm(B)-2	GGAACATCTGTGGTATGGCG		
ery (C)	erm(C)-1	ATCTTTGAAATCGGCTCAGG	295 bp	Jensen *et al*. (1999)
	erm(C)-2	CAAACCCGTATTCCACGATT		
msr(A)	*msr*(A)-1	GCAAATGGTGTAGGTAAGACAACT	400 bp	Wondrack *et al.* (1996)
	msr(A)-2	ATCATGTGATGTAAACAAAAT		
mph(C)	*mph (C)-1*	GAGACTACCAAGAAGACCTGACG	722 bp	Luthje and Schwarz (2006)
	mph (C)-2	CATACGCCGATTCTCCTGAT		
mec A	*mecA1*	GTAGAAATGACTGAACGTCCG ATAA	310bp	Geha *et al*. (1994)
	mecA2	CCAATTCCACATTGTTTCGGTCTAA		
van A	*van A1*	GGGAAAACGACAATTGC	732 bp	Dutka-Malen *et al.* 1995
	van A2	GTACAATGCGGCCGTTA		
van B	*van B1*	GTGCTGCGAGATACCACAGA	1145 bp	Ramos-Trujillo *et al.* (2003)
	van B2	CGAACACCATGCAACATTTC		

6.2.4 Phenotypic Antibiotic Susceptibility Profile

The standard disc diffusion technique was employed to determine the antibiotic susceptibility pattern of the isolates and this was performed in accordance with standards described by the National Committee for Clinical Laboratory Standards (NCCLS) (1999) and Cheesebrough (2006). The antibiotics used include penicillinG (11 unit), ampicillin (10 µg), oxytetracycline (10 µg), minocycline (10 µg), streptomycin (10 µg), cotrimoxazole (25 µg), cefotaxime (10 µg), colistin (10 µg) erythromycin (10 µg) gentamycin (10 µg), clindamycin (2 µg), ceftriaxone (30 µg), methicillin (5 µg)/ oxacillin (5 µg), ceftriaxone (30 µg), ceftazidime (30 µg), vancomycin (5µg), cephalothin (25µg), imipenem (10 µg), meropenem (10 µg), ofloxacin (5 µg), levofloxacin (5 µg) and ciprofloxacin (5 µg). *Staph. aureus* ATCC 25923 was used as the positive control. This result was interpreted using the approved standards (NCCLS, 1999; CLSI, 2008).

6.2.4.1 Standardization of Inocum and Plates Inoculation

About four colonies from each of the fresh plates were suspended in tubes containing 5 ml of sterile distilled water and vortexed to homogenize the suspension. The turbidity of the suspension was appropriately adjusted to 0.5 McFarland standards equivalent and used within 15 mins. The bacterial suspension was inoculated onto freshly prepared Muller-Hinton agar using a sterile swab. This suspension was carefully spread all over the plates to ensure uniform growth. Antibiotic discs were then applied to the surface of the agar using sterile forceps and the plates incubated at 35°C for 18-24 hour. At the end of incubation, the zones of inhibition were measured and interpreted using available interpretive charts.

6.2.5 Multiple Antibiotic Resistance Index (MARI)

The MARI 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 exposed (b), i.e:

MARI= a/b (Krumperman, 1983)

6.2.6 PCR detection of Antibiotic Resistance Genes

Table 6.2 summarised the list of primers used for PCR detection of *erm(A), erm(B), erm(C), msr(A)* and *mph(C)* genes following the protocol of Sauer *et al*. (2008). PCR cycles involve an initial denaturation step of 94°C for 5 min followed by 30 amplification cycles including denaturation at 94°C for 60 s, annealing at 51°C for *erm(A), erm(B), erm(C)* or 55°C for *msr(A), mph(C)* for 60 s, and extension at 72°C for 60 s. A final extension at 72°C for 5 min in one cycle then ended the PCR. For *mecA, van A* and *van B* genes, PCR conditions include an initial denaturation step at 94°C for 5 min will be followed by 10 cycles of amplification (denaturation at 94°C for 30 s, annealing at 64°C for 30 s, and extension at 72°C for 45 s), and another 25 cycles of amplification (denaturation at 94°C for 30 s, annealing at 50°C for 45 s, and extension at 72°C for 2 min), ending with a final extension step at 72°C for 10 min.

6.2.7 Statistical Analysis

The observed variables were converted into easily interpretable data by ensuring that no data two decimal places. The significance of these data was determined using chi-square. A *p*-value $p \leq 0.05$ was regarded as being statistically significant while *p*-values ≥ 0.05 was interpreted as being statistically non-significant (Dahiru, 2008)

6.3 RESULTS

Following phenotypic and molecular identification, one hundred and twenty isolates of Staphylococci were recovered. The analytic profile index results showed well over 95 % agreement for genus based identification, when compared with PCR based genus specific identification. Species-specific PCR revealed the following Staphylococcal identities: *Staphylococcus xylosus* (15%), *Staphylococcus aureus* (23.3%), *Staphylococcus haemolyticus* (35%), *Staphylococcus capitis* (15%), and other *Staphylococcus* species (11.7%). (Table 6.3)

For clarity, Table 6.3 specifically showed the recovery of *Staphylococcus* spp. with respect to their animal source(s) while the ability of the species to produce coagulase as a virulent factor was shown in Table 6.4. Sixty-four (55.6%) of the isolates were recovered from pig, 18 (15.7%) from goat and 24 (20.9%) from cow. Twenty eight (23.3%) of the *Staphylococcus* species were coagulase positive while the remaining were non coagulase producers (Table 6.4).

The results of antibiotic susceptibility assay are as shown in Table 6.5. Resistances of 40.2% to ceftazidime, 75% to penicillin G, 83.3% to tetracycline, 100% to nalidixic acid and Sulphamethaxole were observed. Also, 38% of the *Staphylococcus* spp. were resistant to oxacillin, while 12 % were resistant to vancomycin. The presence of *mecA* genes was observed among the methicillin resistant *Staphylococcus* species as shown in Table 6.6. No vancomycin resistance genes (*van A* and *van B*) were detected in these organisms. Also, 12% of the bacteria were resistant to erythromycin, while 40.2 % were resistant to ceftazidime. Over 68.4% of the isolates had multiple antibiotic resistant index (MARI) > 2 (Fig 6.1)

Table 6.3: Prevalence/Frequency of occurrence of the *Staphylococcus* spp. with respect to sample source.

S/N	Animal Source(s)	Species	No (%) of Occurrence	Antibiotics of interest to which Resistance occurred
1	Pig	*S. haemolyticus*	36 (30.0)	Methicillin/oxacillin (38%), Vancomycin (12%)
		S. aureus	28 (23.3)	
2	Goat	*S. capitis*	18 (15.0)	
3	Cattle	*S. haemolyticus*	6 (5.0)	
		S. xylosus	18 (15.0)	
4	Dead Chicken Pig	Other Staphylococcal specie	14 (11.7)	
	TOTAL		**120 (100)**	

Table 6.4: Percentage Isolates' Recovery Based on Coagulase Production (Virulence factor).

Bacterial Isolates	No of isolates from animals	Percentage of the isolates
Coagulase Positive	28	23.3
Coagulase Negative	92	76.7
Total	**115**	**100**

Table 6.5: Antibiotic Susceptibility Profile of the *Staphylococcus* species.

Antibiotic	S (%)	I (%)	R (%)
Penicillin G	25.0	0	75.0
Meropenem	97.7	0	2.3
Vancomycin	83.0	1.0	12.0
Cefotaxime	78.0	9.0	13.0
Ceftazidime	26.5	33.3	40.2
Oxacillin	44.0	18.0	38.0
Minocycline	16.0	11.0	16.0
Tetracycline	16.7	0	83.3
Erythromycin	73.0	15.0	12.0
Clindamycin	53.0	31.0	16.0
Chloramphenicol	91.7	8.3	0
Sulphamethaxole	0	0	100.0
Nalidixic Acid	0	0	100.0
Ciprofloxacin	74.0	23.0	3.0
Ofloxacin	83.0	12.0	5.0
Levofloxacin	98.0	0	2.0

Key: S=Sensitive, R=Resistance, I=Intermediate

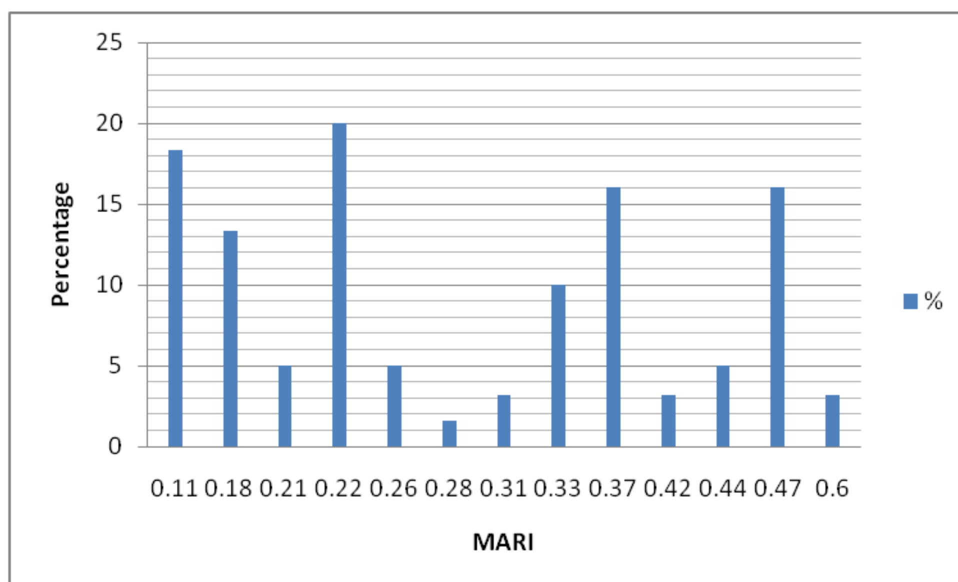

Fig 6.1: Multiple antibiotic resistant index (MARI) and the percentage of isolates involved

Table 6.6: Presence or otherwise of some resistance genes in the *Staphylococcus* species

S/N	RESISTANCE GENES	DETECTION
1.	*mec (A)*	+
2.	*van (A)*	-
3.	*van (B)*	-
4.	*mph (C)*	+
4	*Msr (A)*	-
5.	*ery (A)*	-
6.	*ery (B)*	-
7.	*ery (C)*	-

Key: + means detected, - means not detected

6.4 DISCUSSION

The inherent ability to withstand unfavourable osmotic conditions, pressure and slightly elevated temperature support the survival of Staphylococci on animals (Le-Loir *et al*., 2003). Of particularly interested were those species that can affect humans and out of 30% of the *Staphylococcus* species from pigs was *Staphylococcus haemolyticus*, while 23.3% was *Staphylococcus aureus.* Pig was the only source of *Staph. aureus* isolated in this study and this is in line with the observation reported elsewhere (de Neeling *et al*., 2007). Besides, *Staphylococcus haemolyticus* and some unidentified *Staphylococcus* species were also observed from pig sources. About 15% of the entire Staphylocococcal isolates were *Staph. capitis* and they were isolated from goat. This organism is a known flora of human scalp and

skin, but is also a frequently observed aetiology of endocarditis (Van der Zwet *et al*., 2002; Iwase *et al*., 2007; D'mello *et al*., 2008). About 5% to15% of the total *Staphylococcus* spp. from the animal sources were *Staph. haemolyticus* and *Staph.* xylosus respectively, and they were isolated from cattle. The total occurrences of *Staph. haemolyticus* observed in this study agrees with earlier reports on animal and animal products' as reservoir of bacteria pathogens (Bagcigil *et al*., 2007; Schlegelova *et al*., 2008). Since there are at least 40 recognized *Staphylococcus* species (Trulzsch *et al*., 2002; Bannerman, 2003), assaying for all of them may not be feasible in this study (Deurenberg and Stobberingh, 2008). Hence, 11.7% of the *Staphylococcus* species identified to genus level and recovered from pigs and chicken could not be speciated into any of the target species listed in Table 3. With regards to coagulase production, 24.3 % of the commensal *Staphylococcus* isolates were positive. This difference in prevalence along the divide of coagulase production was statistically significant ($p \leq 0.05$). The recovery of more coagulase-negative species (76.7 %) corroborates previously reports (Gortel *et al*., 1999; Kania *et al*., 2004), This elaboration of Staphylocoagulase has been described as a very important factor in determining the inherent pathogenicity of a bacteria even when found in commensal phase of life (Fairbrother, 2005).

The observed resistance patterns to some of the conventional antibiotics which are usually frequently prescribed within the study area calls for attention considering that the isolates are non-clinical. This further reaffirms the critical role of commensals in public health. The observed high level (75-100%) resistances to sulphamethaxole, nalidixic acid, tetracycline and improved penicillin G; all of which are broad spectrum antibiotics might be due to consumption of antimicrobials (Moulin, 2001) as growth promoters (Perrier-Gros-Claude *et al*., 1998) as extensively practiced in the study area. Also, of special interest are the responses to methicillin. Of the 38% resistance to methicillin/oxacillin observed, 12.6 % was

Staphylococcus aureus from pig. Though oxacillin is more stable than methicillin during storage, laboratory diagnosis of methicillin resistance depends on the testing of oxacillin, and methicillin/oxacillin-resistant *Staphylococcus* isolates are supposed to be reported as being resistant to β-lactam antibiotics (CLSI, 2008). Vancomycin used to be the last antibiotic for treating infections caused by such resistant isolates (Fitzgerald *et al*., 2001; Boucher *et al*., 2010). In fact, it was a drug in the last line of defence (Bhalakia and Morris, 2005). In this study, 12 % of the *Staphylococcus* species were vancomycin-resistant and were recovered from the various animals. Unfortunately, there is usually a close link between the resistance to vancomycin and to other extended spectrum beta-lactam drugs like meropenem and imipenem, with a tendency to worsen the difficulty in the choice of therapeutic options (Chang *et al*., 2003; Boucher *et al*., 2010). Tenover and Goering (2009) also reported the presence of community based MRSA, just as Bhalakia and Morris (2005) also reported the presence of plasmid mediated vancomycin resistance in fomite.

The observed phenotypic methicillin/oxacillin resistance in this study was backed up by the presence of *mecA* genes (Appendix 2). Meanwhile, the presence of *mecA* gene brings about resistance to improved penicillin and all other β -lactam antibiotics (Pinho *et al*., 2001; Weese *et al*., 2005). This is because *mecA* determines the availability of penicillin binding protein PBP2a which substitutes the inactivated PBPs to enhance the stability of the cell wall in the presence of β-lactam antibiotics (Pinho *et al*., 2001). The observed absence of *mecA* gene among few methicillin resistant *Staphylococcus* spp. in this study supports the observation of Montanari *et al.* (1990). The presence of this gene in the commensal organisms might render them difficult to control, given the opportunity to zoonotically infect immunocompromised individuals. This gene is usually housed in a large mobile genetic element known as chromosomal cassette mec (SCCmec) (Weese *et al*., 2005). There have

been eight recognized SCCmec types which are different in occurrence (Weese *et al*., 2005; Otter and French, 2010); some of which are found in humans as hospital-associated and/or community-associated MRSA (Otter and French, 2010). We therefore proposed that the observed MRSA in the pig and methicillin resistant *Staphylococcus haemolyticus* (MRSH) could have been transferred from human sources to the animals as they are also possible colonizers of human hosts. However, the presence of *mecA* gene is required for buttressing the susceptibility of the *Staphylococcus* spp. to methicillin and other lower beta-lactam antibiotics (Duquette and Nuttall, 2004), even if seeming susceptible profile is observed.

Twelve percent of the *Staphylococcus* species were resistant to erythromycin while 40.2% were resistant to the third generation cephalosporin, ceftazidime, confirming the earlier reported better activity of some lower class β-lactam antibiotics relative to some exalted third generation cephalosporin to gram-positive bacteria (Essack, 2001). The detection of *mph(C)* gene justifies the phenotypic resistance to erythromycin and serves as representative of more of such genes among commensals in the study area. Meanwhile, the wide range of multiple antibiotic resistance indexes showed a divergence between the static-use and the adaptive-use which may imply consistent use of various antibiotics in these farms on the animal, to achieve a non chemotherapeutic advantage (Laxminarayan and Klugman, 2011). This implies that the organisms might have developed resistance over a period of exposure without medical prescription. An observation of MAR index > 0.2 means that the isolate source is high-risk source where antibiotics are in constant abuse and the act is bringing about high selective pressure (Suresh *et al*., 2000).

Therefore, this exposure of the animal bacterial flora to antibiotics appears to be encouraging emergence of resistance across a wide range of antibiotics. It is therefore

important to control the misuse or any other non-therapeutic use of antibiotics. Piggery workers should be diligently hygienic as the animal is a consistent source of MRSA. Regular PCR based assessment of MRSA prevalence in various aspects of natural life and hospitals is hereby advocated to bring about appropriate control strategies and to reduce the present scourge of MRSA in multidrug resistant outbreaks.

6.5 CONCLUSION

The study supports the need to assess the roles of commensal in infection control. *Staphylococcus aureus, Staph. xylosus, Staph. capitis, Staph. haemolyticus* and other *Staphylococcus* species which are of public health importance were identified in commensal mode from the animals. Their resistance to methicillin, vancomycin, sulphamethoxazole, tetracycline, nalidixic acid and cephalosporins; especially the presence of *mec A* and *mph(C)* genes positioned them as threats to the farm personels and to immune compromised individuals that contact them. Opportunistic zoonotic infection by these bacterial species may be difficult to treat by most conventional antibiotics, making the choice of expensive antibiotic in the last line of defence compulsory. Improved farm hygienes is hereby solicited to reduce the spread of antibiotic resistance bacterial species that may be difficult to treat.

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CHAPTER SEVEN

Antibiogram characteristics of Acinetobacter baumannii/calcoaceticus isolates recovered from freshwater and soil environment in Nkonkobe Municipality and their extended spectrum beta-lactamase status

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Abstract

This study assessed the antibiogram characteristics of *Acinetobacter* species isolated from Nkonkobe Municipality environment in Eastern Cape Province, South Africa. The study also assessed the occurrence of relevant tetracycline resistance genes in the bacteria genomes as well as their extended spectrum beta-lactamase (ESBL) production status. Eighty-six presumptive *Acinetobacter* species were isolated, out of which 41% were from Alice and all identified as *Acinetobacter baumannii-calcoaceticus*; while 59% consisting of *Acinetobacter baumannii/calcoaceticus* and *Acinetobacter haemolyticus* were isolated from Fort Beaufort environment. Between 30 and 100% of the *Acinetobacter* species were resistant to penicillin G, ceftriazone, nitrofurantoin, erythromycin and augmentin, while about 9% showed intermediate response to minocycline, and 10% were resistant to oxytetracycline. The tetracycline resistance genes (*Tet(B)* and *Tet(39)*) were detected in 66.7% and 44.4% of the resistant and intermediately resistant *Acinetobacter* species respectively, while *Tet (A), Tet(H)* and *Tet(M)* were not detected in them. Also 9.3% of the bacterial isolates showed phenotypic production of ESBLs while 3.5% were positive for $bla_{CTX\text{-}M\text{-}1}$ gene. All the isolates including ESBLs producers were susceptible to the third generation fluoroquinolone antibiotics used in this study and are hereby recommended as antibiotics of choice.

7.1 Introduction

Acinetobacter species are non-motile, nonfermentative, catalase-positive, oxidase negative Gram-negative coccobacilli (Visca *et al.,* 2011). They are found in waterbodies, soil, sewage, humans and non-human animals' bodies, fast foods and hospital fomites (Peleg *et al*., 2008; Easa, 2010). More than 30 species are now known, but *Acinetobacter baumannii* is the most common and is of high clinical and sub-clinical importance (Peleg *et al*., 2008). The bacteria is highly regarded as a successful pathogen due to its role as aetiologies of soft tissues disease in soldiers contracted via contact with soil during war (Camp *et al*., 2011). As a commensal, *A. baumannii* have been reported to reside on animal skins as a non-infectious organism (Mindolli *et al.,* 2004). However when contracted by immunocompromised individuals the organism can cause various types of opportunistic infections (Perez *et al*., 2007; Peleg *et al*., 2008). Other species belonging to the *Acinetobacter* genus include *A. iwofii, A. junii, A. calcoaceticus, A. radioresistens, A. haemolyticus* etc (Ecker *et al*., 2006). These examples are predominantly commensals and are not usually harmful to immuno-competent humans and animals (Dubay *et al*., 2000).

There have been several reports of outbreaks of *A. baumannii* infections; most of which have been found among hospitalized intensive care unit patients with cases of immune suppression and debilitation (Peleg *et al*., 2008; Towner, 2009; Klatt, 2011). These outbreaks are mostly caused by multiple antibiotic resistant isolates, thus narrowing therapeutic options (Peleg *et al*., 2008; Dent *et al*., 2010), prolonging duration of hospitalization (Garcia-Garmendia *et al*., 1999) and increasing mortality rate (Joly-Guillou, 2005; Perez *et al*., 2007; Munoz-Price and Weinstein, 2008; Dent *et al*., 2010). Hence, *Acinetobacter* species are increasingly becoming threats to public health (Towner, 2009). Several reports have implicated commensal *Acinetobacter baumannii* isolated from hospital fomites in various

forms of nosocomial infection ranging from superficial to systemic usually through surgical wounds (Prashanth and Badrinath, 2006).

In chemotherapy involving this bacterium and several others, tetracycline and its derivatives are mostly considered as choice antibiotics especially in veterinary medicine (Boatman/FEDESA, 1998). This is because of its broad spectrum activity against myriads of pathogenic bacteria and/or cost effectiveness (Chopra and Robert, 2001). Numerous other advantages such as low toxicity and their bioavailability in plasma informed their prevalent use in human and animal therapy (Yang *et al.,* 2008).

While several antibiotics have been used in *Acinetobacter* infections therapy, resistance to the antibiotics, however, serves as a major setback (Rahbar *et al.,* 2010). The bacteria exhibit resistance to a wide range of antibiotics, from β-lactams including the penicillin group, cephalosporin and carbapenems, to aminoglycosides and quinolones (Bonomo and Szabo, 2006; Cha et al., 2006). Zarakolu *et al*. (2006) reported incidence of 67% multiple antibiotic resistant *A. baumannii*, which was also reported as the cause of therapeutic failure in another study (Poirel and Nordmann, 2006). The emergence of tetracycline resistance and the presence of tetracycline resistance genes, especially among the environmental strains, suggest the possible presence of such genes among the biotic constituents in the area (Jury *et al*., 2010). This also serves as a backward slide in the progress made in the control of infections using tetracycline, as about 2 294 tonnes of the antibiotic were administered in the European Union in 1997 (Boatman/FEDESA, 1998) while in United States, 3 000 tonnes were administered in 2000 and 3200 tonnes in 2001 (AHI, 2002). Widely used derivatives in Europe and Czechoslovakia are tetracycline, doxycycline, chlortetracycline and oxytetracycline (EMEA, 1999; AISLP, 2003). Farm and other domestic animals like cattle, sheep, pigs, goats, horses, dogs, cats, fowls, rabbits and fishes are usually administered with therapeutic and prophylactic dosage of tetracycline. However, the frequent

uncontrolled use of these antibiotics may promote the distribution of resistant bacteria in both aquatic and terrestrial ecosystems (Kummerer, 2004; Karthikeyan and Meyer, 2006; Baquero *et al*., 2008; Martinez, 2008; Zhang *et al*., 2009).

Acinetobacter species possess intrinsic potentials for the emergence of resistance to antibiotics and acquire novel resistance genes from possibly distantly related species; thus positioning them as important candidates for the evaluation of reservoirs of antibiotic resistance in the environment or even in human subjects (Fetiye *et al*., 2004; Kim *et al*., 2011). The diverse uses of tetracycline have encouraged extensive studies into the resistance mechanisms. Several reports which encompass efflux- and ribosome-based resistance mechanisms relates also to first- and second-generation tetracyclines (Chopra *et al*., 1992; Acar, 1997; Roberts, 1997; Levy *et al*., 1999) and acquisition of new genes has been recognized as a factor responsible for the emergence of the resistances, which have also been observed in isolates from aquatic sources, vegetables, sewage, and the hospital environment (Berlau *et al*., 1999; Dhakephalkar and Chopade, 1994; Guardabassi *et al*., 1998, 1999; Hujer *et al*., 2006; Perez *et al*., 2007).

Five classes of tetracycline resistance genes have been observed in *Acinetobacter* species and includes *tet(A), tet(B)* (Sambrook *et al*., 1989), *tet(H)*, *tet(39)* (Agerson and Peterson, 2007) and *tet (M)* (Chee-Sanford, 2001). Resistance to tetracycline, cephalosporins and some other antibiotics is mediated by some determinants; of which the production of extended-spectrum β-lactamases (ESBLs) is one. Naas *et al*. (2008) reported the presence of PER-1 type ESBLs in *A. baumannii*. CTX-M-2 type ESBLs has also been reported in the bacteria by Nagano *et al*. (2004) in Japan. The presence of CTX-M ESBLs suggests that the bacteria are resistant to cefotaxime and sometimes to ceftazidime. This enzyme enhances the ability of the bacteria to inactivate the antibiotics and as such resist even such high profile extended spectrum antibiotics like carbapenem (Zhanel *et al.,* 2005). The CTX-M β-

lactamases are plasmid-borne. Resistance to β-lactam antibiotics including cephalosporins is imminent in the presence of appropriate extended-spectrum β-lactamases (Poirel *et al*., 2001; Tzouveleki *et al*., 2000). Also, VEB-1 type ESBLs ESBLs has been found in *A. baumannii* where it is chromosomally borne on integron similar to those in *Pseudomonas aeruginosa* (Girlich *et al*., 2002). The integron determines the source and methods of dissemination among *A. baumannii*. (Girlich *et al*., 2002; Poirel *et al*., 2003). VEB-1 type ESBLs has been reported in many isolates from hospital environments in Europe including France, Belgium and Argentina (Peleg *et al*., 2008). Struelen *et al*. (2004) ascertained that commensal *Acinetobacter baumannii* among other commensal bacteria are implicated in hospital infection such as reported by Peleg *et al.* (2008). This study evaluates the antibiogram characteristics of commensal *Acinetobacter* species isolated from the Nkonkobe Municipality environment, as well as the presence of *tet(A), tet(B), tet(H), tet(M)* and *tet(39)* genes in their genomes and their ESBLs status

7.3 Materials and Methods

7.3.1 Study Location and samples collection

Nkonkobe Municipality is a highly populated domain of the Eastern Cape Province, South Africa, with a population of about 128 658 on the 3 724 square kilometres area of land. Only about 20% of the population of Nkonkobe reside in urban settlements, mostly in Alice and Fort Beaufort towns. Twenty five samples each of water and soil were collected from each of the two study towns. Soil samples of about 15 g were collected aseptically into sample bottles while about 1 litre of water from sampling locations (Alice and Fort Beaufort) was collected andtransported to the laboratory under ice. A measure of 10% (w/v) soil

suspension was made and shaken for 15 min on a rotary shaker (Baumann, 1968) in preparation for preliminary isolation.

7.3.2 Preliminary Isolation

Preliminary isolation of the target bacteria was done following the description of Culbreath *et al.* (2011) with modification in volumes. About 5 ml of both water and the prepared soil suspension were inoculated into 10 ml sterile nutrient broth and incubated at 37°C for 24 hours. At the end of incubation, the broth cultures were aseptically streaked onto CHROMagar™ *Acinetobacter* for preliminary isolation of *Acinetobacter* species. *Acinetobacter* species appear as large red colonies on CHROMagar™ *Acinetobacter*, while other Gram negative bacteria, Gram positive bacteria and yeasts are inhibited. Occasionally, *Stenotrophomonas maltophilia* may grow on this medium, but with exceptionally smaller colonies than *Acinetobacter* species (Bollet *et al.,* 1995)

7.3.3 Characterization of the isolates

The presumptive *Acinetobacter* colonies from the CHROMagar™ *Acinetobacter* plates were subcultured on fresh plates of CHROMagar, purified on nutrient agar plates and Gram stained (Cheesebrough, 2006). The Gram negative rods were further characterized for oxidase production, using the oxidase test kit. The oxidase negative isolates was then subjected to speciation using analytic profile index (API 20 NE) (Bio'Merieux).

7.3.4 Antibiotic Susceptibility Test (AST)

The phenotypic antibiotic testing was done in line with Kirby-Bauer disc diffusion method (CLSI 2005; Cheesebrough, 2006). Thirteen standard antibiotic discs (MAST Diagnostics,

Merseyside, United Kingdom) were employed in this assay and include Penicillin G (11u), imipenem (30 µg), meropenem (30µg), Amoxycillin-clavulanic acid (20 µg+16µg), trimethoprim-sulphamethoxazole (1.25µg+23.75µg), nalidixic acid (5 µg), ofloxacin (5µg), ciprofloxacin (5µg), levofloxacin (5µg), ceftriaxone (30µg), cefotaxime (30µg), augmentin (30µg), erythromycin (10 µg), chloramphenicol (30 µg), minocycline (10 µg) and oxytetracycline (10 µg).

7.3.4.1 Standardization of inoculums

About four colonies from each of the fresh plates were suspended in tubes containing 5 ml of sterile distilled water and vortexed to homogenize the suspension. The turbidity of the suspension was appropriately adjusted to 0.5 McFarland standards equivalent and used within 15 mins.

7.3.4.2 Inoculation of plates

The bacterial suspension was inoculated onto freshly prepared Muller-Hinton agar using a sterile swab. This suspension was carefully spread all over the plates to ensure uniform growth. Antibiotic discs were then applied to the surface of the agar using sterile forceps and the plates incubated at 35ºC for 18-24 hour. At the end of incubation, the zones of inhibition were measured and interpreted using available interpretive charts. *Acin* DSM 30007 was used as positive control strain.

7.3.5 Multiple Antibiotic Resistance Index (MARI)

The MARI 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 exposed (b), i.e:

MARI= a/b (Krumperman, 1983)

7.3.6 Phenotypic Extended Spectrum Beta-Lactamase (ESBLs) activity

The double disk synergy test (DDST) for phenotypic assessment of ESBLs production was employed for this study in line with the protocol of Bradford (2001). An amoxicillin-clavulanate disc was placed at the center and the 4 third generation cephalosporins which includes ceftazidime, cefotaxime, ceftriaxone, cefpodoxime (30 mg each) were placed at distance of 15 mm from the centre and incubated for 24 hours at 37ºC. The isolates that showed enhancement between clavulanic acid bearing disc and any of the third generation cephalosporins were interpreted as positive for ESBLs production. Those without such enhancement are interpreted as non-ESBLs producers.

7.3.7 Polymerase chain reaction (PCR) assessment of CTX-M-1- and VEB-1-ESBLs Production

Due to the observed phenotypic expression of ESBLs production, attempts were made to assess the presence of ESBLs genes using the primers listed in Table 7.1. The PCR condition began with initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 60°C and extension at 72°C for 1 min, and a final extension at 72°C for 3 min (CTX-M-1). The second PCR (VEB-1) began with denaturation at 95ºC for 15 min, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 46ºC for 1 min

and extension at 72°C followed by a further extension at 72°C for 10 min (Schlensurger *et al*., 2005).

7.3.8 PCR-based Assessment of Tetracycline Resistance genes

All the following tetracycline resistance genes were assessed using the primers in Table 7.2 at the appropriate PCR conditions:

7.3.8.1 Tet (A) gene

The samples' amplification began with an enzyme activation step within 3 min at 94°C followed by 25 cycles of 1 min at 94°C, 1 min at 57°C and 1 min at 72°C. This was concluded with a final extension within 10 min at 72°C (Sambrook *et al*., 1989)

7.3.8.2 Tet (B) gene

The PCR conditions for *tet (B)* gene amplification include initial denaturation of 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 52°C and 1 min at 72°C followed by final extension of 10 mins at 72°C. The PCR products were analyzed by electrophoresis through 1.5% agarose gels and staining with ethidium bromide (Sambrook *et al*., 1989).

7.3.8.3 Tet (39) gene

PCR condition used for the assessment of *tet (39)* gene begins with an initial denaturation of 3 mins at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 52°C and 1 min at 72°C followed by final extension of 10 mins at 72°C. The PCR products were analyzed by

electrophoresis through 1.5% agarose gels and staining with ethidium bromide (Agerso and Peterson, 2007).

7.3.8.4 Tet (H) gene

The PCR began with an initial denaturation at 94°C for 5 mins, which was followed by 30 cycles consisting of 94°C for 30 s, annealing at 65ºC for 30 s, and extension at 72°C for 30 s. It was concluded with a final extension at 72°C for 7 min. (Agerso and Peterson, 2007).

7.3.3.4 Tet (M) gene

The PCR began with an initial denaturation at 94°C for 5 mins, which was followed by 30 cycles consisting of 94°C for 30 s, annealing at 64ºC for 30 s, and extension at 72°C for 30 s. It was concluded with a final extension at 72°C for 7 min. (Chee-Sanford *et al.*, 2001).

Table 7.1: Primers for detection of CTX-M 1 and VEB Extended spectrum beta-lactamase genes in *Acinetobacter* spp

Primer	Primer Sequence	Size
CTXM-1f	5’ GACGATGTCACTGGCTGAGC -3’	490 bp
CTXM-1r	5’- AGCCGCCGACGCTAATACA -3’	
VEB-f	F:5’-ACGGTAATTTAACCAGATAGG-3’	970 bp
VEB-r	R:5’-ACCCGCCATTGCCTATGAGCC-3’	

Table 7.2: Primers for the Assessment of *Tet B gene* in *Acinetobacter* spp.

Target genes	Primer name	Sequence 5'→3'	Amplicon size (bp)
tet(A)	tet(A)-1	GTAATTCTGAGCACTGTCGC	957
	tet(A)-2	CTGCCTGGACAACATTGCTT	
tet(B)	tet(B)-1	CTCAGTATTCCAAGCCTTTG	
	tet(B)-2	ACTCCCCTGAGCTTGAGGGG	415
tet (39)	tet(39)-1	CTCCTTCTCTATTGTGGCTA	701
	tet(39)-2	CACTAATACCTCTGGACATCA	
tet(H)	tet(H)-1	ATACTGCTGATCACCGTATAGATG	1175
	tet(H)-2	TCCCAATAAGCGACGC	
tet(M)	tet(M)-1	GTTAAATAGTGTTCTTGGAG	700
	tet(M)-2	CTAAGATATGGCTCTAACAA	

7.4 Results

A total of eighty-six *Acinetobacter* isolates were isolated. All the isolates from Alice town samples belonged to the *Acinetobacter baumannii-calcoaceticus* complex, which in turn formed 41% of the total *Acinetobacter* isolates in this study. About 85.2% of the Fort Beaufort town samples isolates were *Acinetobacter baumannii/calcoaceticus* constituting 59% of the total *Acinetobacter* species isolated in this study, while all the *Acinetobacter haemolyticus* isolates were also from the Fort Beaufort samples and constituted 14.8% of the total *Acinetobacter* species identified (Table 7.3).

The results of the antibiotic susceptibility assay revealed that all the isolates were resistant to penicillin G, 90% resistant to nitrofurantoin and 44.4% resistant to third generation cephalosporin, ceftriaxone. Also, 20% of the isolates showed intermediate resistance to erythromycin, while 10% were resistant to each of imipenem, meropenem and chloramphenicol. All the isolates were susceptible to the fluoroquinolone antibiotics viz. ciprofloxacin, ofloxacin and levofloxacin, while 85% of were susceptible to nalidixic acid. Similarly, 80% of the isolates were susceptible to cotrimoxazole, chloramphenicol and meropenem, as 70% were susceptible to imipenem and augmentin (Table 7.4). Similarly, 9 isolates were resistant to oxytetracycline, out of which 8 were intermediate in response to minocycline (Table 7.4). All the bacterial isolates showed high level MAR index (>0.2) ranging from 0.22-0.67 (Fig 7.1).

With respect to the tetracycline resistance genes, six isolates were positive to *Tet B*. With respect to the phenotypic expression of the resistance, 66.7 % of the phenotypically resistant were positive for the gene. Similarly, the presence of recently described novel tetracycline genes, *Tet 39* in 44.4 % of the phenotypically resistant was observed (Table 7.5).

Also, 12 of the isolates showed phenotypic extended spectrum beta-lactamases (ESBLs) activity. However, when the 12 phenotypic ESBLs positive isolates were assessed, three were positive for *bla*$_{CTX-M-1}$ genes, while none was positive for *bla*$_{VEB-1}$ gene (Fig 7.2).

Table 7.3: Results of *Acinetobacter* speciation.

Location/ Species	**Alice No (%)**	**Fort Beaufort No (%)**	**Total**
A. baumannii /calcoaceticus	32 (41.0)	46 (59.0)	78
A. *haemolyticus*	-	8 (100)	8
Total	**32**	**54**	**86**

Table 7.4: Antibiogram Characteristics of the *Acinetobacter* isolates.

Antibiotics	**S (%)**	**I (%)**	**R(%)**
Penicillin G	0	0	100
Ceftriazone	56.6	0	44.4
Meropenem	80	10	10
Imipenem	70	10	20
Nalidixic Acid	85	5	10
Ciprofloxacin	100	0	0
Ofloxacin	100	0	0
Levofloxacin	100	0	0
Erythromycin	50	20	30
Chloramphenicol	80	10	10
Augmentin	70	0	30
Nitrofurantoin	10	0	90
Cotrimoxazole	80	0	20
Minocycline	91	9	0
Oxytetracycline.	90	0	10

KEY: S=Sensitive, I=Intermediate, R=Resistance

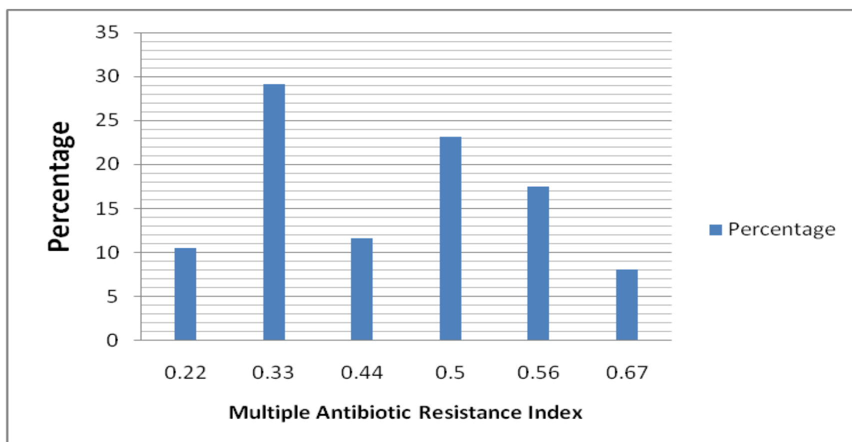

Fig7.1: Multiple Antibiotic Resistant Index and the corresponding percentage of *Acinetobacter* Isolates.

Table 7.5: Occurrence of tetracycline resistance genes in the phenotically resistant isolates.

S/N	Tetracycline Resistance Genes	Detection of genes	Percentage of the resistant isolates with genes
1	*Tet(A)*	_	0
2	*Tet(B)*	+	66.7
3	*Tet(H)*	_	0
4	*Tet(M)*	_	0
5	*Tet(39)*	+	44.4

Key: - (absent); + (present).

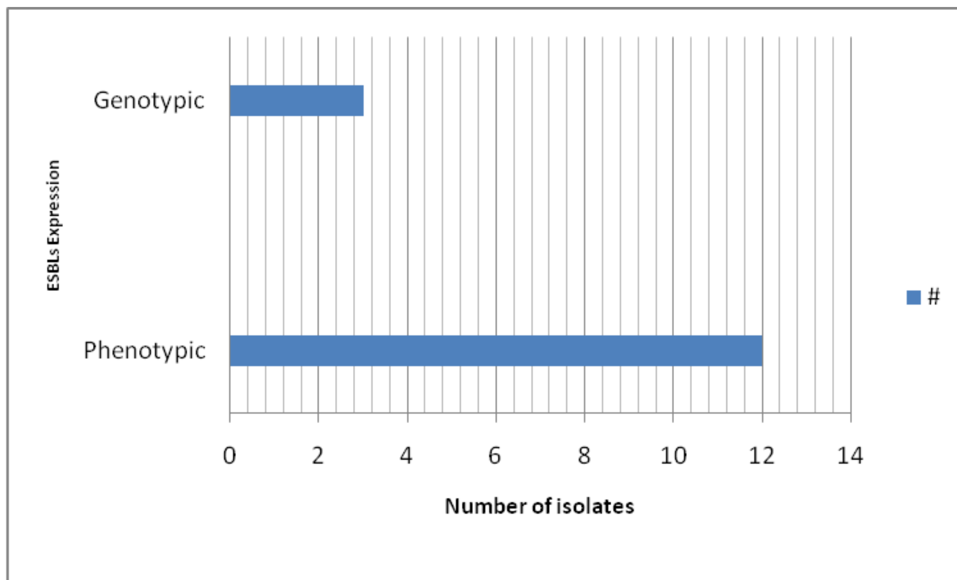

Fig 7.2: Phenotypic and genotypic expression of ESBLs

7.5 Discussion

The possibility of contracting a non-vector based infection depends on the proximity to the infective agent and their prevalence in the environment. For this reason and because of the growing population of the immune-compromised, commensal *Acinetobacter* species becomes very relevant. The strong adaptive ability and resilience of the bacteria contributes to its survival in the environment (Manchanda *et al*., 2010) as observed in this study, but this attribute could be counter-productive to humans and farm animals as the bacteria has potential to be pathogenic as well. *Acinetobacter* species reside on skin surfaces until there is a breach in anatomical barrier of the host.

The commensal *Acinetobacter* species observed in this study have been severally implicated in nosocomial infection, especially *Acinetobacter baumannii* and *Acinetobacter haemolyticus* (Garcia-Garmendia *et al*., 1999; Falagas and Rafailidis, 2006; Jamulitrat *et al*., 2009; Peleg *et*

al., 2008). *Acinetobacter* species mostly exhibit health threatening antibiotic resistance (Grabe *et al*., 2008) and they have been nicknamed "Gram negative MRSA" (Rello *et al.,* 1999). The wide range resistance observed in this study is a cause for concern as it has a tendency to narrow therapeutic options in favour of expensive drugs in the last line of defence, should these commensals become pathogenic (Lahiri *et al.,* 2004). The high incidence of resistance to β-lactam antibiotics observed in this study corroborates that reported elsewhere (Hassan *et al*., 2010). Previous reports (Chopra *et al.,* 2001; Suzuki, 2010) highlighted the importance of resistance to tetracycline by isolates from non animal sources to public health. This study gives a pensive hope of continuous success in the use of minocycline due to intermediate resistance observed against it especially considering that it has been reported as an effective alternative against strains resistant to doxycycline, tetracycline and imipenem (Coelho *et al*., 2006; Halstead *et al*., 2007). This is further corroborated by Bishburg and Bishburg (2009) who reported that *Acinetobacter baumanni* exhibited 86.9% susceptibility to minocycline and 81% susceptibility to imipenem.

The resistance of the *Acinetobacter* species to the β-lactam antibiotics used in this study including the cephalosporins and the carbapenems might be due to the presence of Extended Spectrum Beta-Lactamase (ESBLs) (Bonnin *et al.,* 2011). Some $bla_{CTX\text{-}M}$ alleles are of special concern when their distribution in various geographical regions is considered. CTX-M-2 for instance is found in many places like Argentina in South America and Japan in Asia (Bouvet and Jeanjea, 1989; Simor *et al*., 2002). In most of these places, there were concomitant reports of fluoroquinolone resistance (Poirel *et al*., 2003; Esterly *et al*., 2011), However in this study, high fluoroquinolone susceptibilty by the *Acinetobacter* species including the ESBLs producers were observed. This makes the antibiotic a drug of choice in clinical situations involving these isolates, depending on the age of the patients, following

appropriate susceptibility testing. The observed responses to the Fluoroquinolone agree with the observation of Hoban *et al.* (2001). The MAR index of > 0.2 observed in this study suggests that the isolates emerged from high-risk sources that were exposed to persistent residual antibiotics probably from the wastewater leading to high antibiotic resistance selective pressure (Suresh *et al*., 2000).

The *Tet(B)* and *Tet(39)* genes observed in this study fairly justify the phenotype. This is because resistance or indeterminate profile might also be due to the presence of underlining resistance genes that have been acquired and are being gradually expressed (Martinez and Baquero, 2002). In any case, the presence of only one isolate with resistance genes means a lot in infection control, considering the short replication cycle of bacteria leading to large clones of such isolates (Harrison *et al*., 2006; Inglis *et al*., 2009). The *Tet(B)* gene and tet(39) gene code for resistance to tetracycline and its derivatives have been demonstrated in *Acinetobacter* species (Agerso and Guardabassi, 2005). *Tet(39)* has been earlier reported in *Acinetobacter* isolates from water samples and is usually spread by horizontal transmission of plasmids (Vila *et al*., 2007). *Tet(B)* has also been reported earlier in clinical isolates of *A. baumannii* (Guardiabasi *et al*., 2000). *Tet(B)* genes are specifically important in conferring resistance to tetracycline and minocycline (Chopra *et al.,* 1992).

These identified determinants are not only of concern in the *Acinetobacter*, their gene transfer by any method to other bacteria are of great concern to human health (Normark and Normark, 2002). Another factor that might be responsible for the observed resistances in this study might be the use of antibiotics in agriculture and fish farming (Schmidt *et al.,* 2000), which increase residual antibiotics in agricultural wastewater and induce the emergence of extensive drug resistant bacteria (Austin *et al.,* 1999).

7.6 CONCLUSION

The results of this study showed that the commensal *Acinetobacter* species present in the soil and water environment of Nkonkobe municipality, South Africa, were resistant to many conventional antibiotics. High MAR index and production of extended spectrum beta lactamase suggest their sources to be of potential threat to public health while the presence of tetracycline resistance genes and the $bla_{CTX\text{-}M\text{-}1}$ genes among the bacteria showed them as reservoirs for resistance genes transferable to other bacteria in the environment. This emphasizes the need to adhere to strict rules of personal and general hygiene to reduce the risk of opportunistic infection by such difficult to control bacteria.

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CHAPTER EIGHT

Assessment of antibiotic characteristics and Sulphonamide Resistance determinants in Stenotrophomonas maltophilia isolated from Plant Root Rhizospheres in Nkonkobe Municipality, Eastern Cape Province, South Africa

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ABSTRACT

The antibiotic characteristics and sulphonamide resistance determinants of several *Stenotrophomonas maltophilia* isolates recovered from plant rhizospheres in Nkonkobe Municipality were assessed. A total of 125 isolates were identified, containing 120 (96%) from grass root rhizosphere and 5 (4%) from soil butternut root rhizosphere. *In vitro* antibiotic susceptibility tests showed varying resistances to meropenem (8.9%), cefuroxime (95.6 %), ampicillin-sulbactam (53.9%), ceftazidime (10.7%), cefepime (29.3 %), minocycline (2.2%), kanamycin (56.9%), ofloxacin (2.9%), levofloxacin (1.3%), moxifloxacin (2.8%), ciprofloxacin (24.3%), gatifloxacin (1.3%), polymyxin B (2.9 %), cotrimoxazole (26.1%), trimethoprim (98.6%) and aztreonam (58%). The isolates were susceptible to the fluoroquinolones (74.3-94.7 %), polymycin (97.1%) and meropenem (88.1%). *sul*3 gene was detected among the trimethoprim-sulphamethoxazole (cotrimoxazole) resistant isolates while *sul*2 gene was not detected. This study suggests that commensal *Stenotrophomonas maltophilia* isolates in the Nkonkobe Municipality environment appears to be as important as their clinical counterparts, especially from the perspective of reservoirs of antibiotic resistance determinants.

8.1 INTRODUCTION

Stenotrophomonas maltophilia is a readily available commensal of importance (Alfieri *et al*., 1999) found in water, soil, sewage and frequently on plant or within plant's rhizosphere (Ryan *et al*., 2009). They are commensals known for multitudinous applications in biotechnology (Adegoke *et al*., 2012). The bacteria explore the depression of immune systems to cause infection (Denton *et al*., 1999; Mendosa *et al*., 2007; Gnanasekaran and Bajaj, 2009), though they have also been implicated in infection of immunocompetent subjects (Kim *et al*., 2002; Pruvost *et al*., 2002; Thomas *et al*., 2010). They are therefore important considering their infectivity and the morbidity they initiate (Gales *et al*., 2001; Pathmanathan and Waterer, 2005), which range from nosocomial to community acquired infections. They cause a wide range of human systemic infections (Munter *et al*., 1998; Labarca *et al*., 2000) after entering through the respiratory pathway (Fujita *et al*., 1996; Denton *et al*., 1999). Falagas *et al*. (2009) reported high mortality rate of 37·5% from *Sten. maltophilia* infections.

Multidrug resistance by *Sten. maltophilia* have been well documented (Denton *et al*., 1996; Zhang *et al*., 2001; Brooke, 2012; Vartivarian *et al*., 1994) raising the mortality in some area to as high as 44.4% (Maningo and Watanakunakorn, 1995). Although the drug of choice for *Stenotrophomonas maltophilia* infections is the Sulfonamides (Abdulhak *et al*., 2009), especially the synergistic form (cotrimoxazole or trimethoprim-sulphamethoxazole), resistance to these antibiotics is rampant around the world among human and non-human animals (Grape *et al*., 2003; Guerra *et al*., 2003; 2004) and is mediated by the sulphonamide resistance (*sul*) gene. In this study, *Stenotrophomonas maltophilia* isolates from plants' rhizosphere in the Nkonkobe Municipality, Eastern Cape Province, South Africa, were

assessed for their antibiogram characteristics and the presence of sulphonamide resistance genes in their genomes.

8.2 MATERIALS AND METHODS

8.2.1 Study Location and samples collection

This study was conducted within the Nkonkobe Municipality of the Eastern Cape Province, South Africa. The Municipality is situated in the Amathole District Municipality, bordering the Nxuba Municipality to the west and the Amahlathi Municipality to the east. The municipality has a predominantly rural population and has a total of twenty-one wards. About 80% of the population of Nkonkobe resides in rural settlements. Forty-five root and rhizospheric soil of both soil butternut and grasses in Alice Town environment were carefully uprooted and aseptically cut with a sterile scissors into sterile containers containing 20 ml nutrient broth and transported in ice to the laboratory for bacteria isolation. Large numbers of isolates were isolated from these after 24 hours incubation at 37°C.

8.2.3 Isolation of test bacteria

The isolation of the bacteria from root rhizospheres was done following the methods of Bollet *et al*. (1995) with slight modifications. About 1 g of the plants' root sections were collected and inoculated into 10 ml of nutrient broth (bio-Merieux, Marcy-l'Etoile, France) supplemented with 0.5 mg of DL-methionine (Sigma Chemicals) per ml. After 24 hours of incubation at 37°C, 0.1 ml was inoculated unto a McConkey agar and spread to dry using a glass spreader, and allowed to stand for 15 min. Thereafter, 4 discs of 10 µg imipenem (MAST Diagnostics, Merseyside, United Kingdom) were asceptically placed on the surface

of the inoculated agar. After 18 hours of incubations at 37°C, colonies that grew around the disc were subcultured for purity and were subjected to preliminary identification.

8.2.4 Preliminary Identification of the presumptive *Stenotrophomonas* isolates

The purified isolates were Gram stained and observed under a light microscope. Isolates that were Gram negative were subjected to oxidase test, and the oxidase negative isolates were subjected to preliminary speciation using analytic profile index 20E (API 20 E). Also, the carbon assimilation tests and other biochemical tests were carried out in the identification process. The tests of importance on the kit were nitrate/nitrite reduction, and utilization of L-arginine, L-lysine, L-ornithine, trisodium citrate, sodium thiosulfate, urea and 2-nitrophenyl-βD-galactopyranoside, indole production, gelatine dissolution, and fermentation of 9 sugars. *Stenotrophomonas* genus positive isolates were then selected for specie confirmation.

8.2.5 PCR confirmation of *Stenotrophomonas maltophilia* isolates

Differenction of *Sten. maltophilia* isolates amongst the genus isolates identified above were done using specie-specific polymerase chain reaction using the primer sets SM1 (5′-CAGCCTGCGAAAAGTA-3′) and SM2 (5′-TTAAGCTTGCCACGAACAG-3′) (Whitby *et al.,* 2000). The PCR condition is as follows: an initial denaturation of 95°C for 5 min, a subsequent 30 cycle amplification annealing at 58°C for 10 s, extension at 72°C for 60 s, and denaturation at 95°C for 10 s. For the last cycle, the extension step was 2 mins (Whitby *et al.,* 2000). *Sten. maltophilia* DSM 50170 was used as the control.

8.2.6 Phenotypic Antibiotic Susceptibility test

The disc diffusion technique was employed to determine the antibiotic susceptibility pattern of the isolates. The test antibiotics include meropenem, cefuroxime, ampicillin, ceftazidime, cefepime, minocycline, kanamycin, ofloxacin, levofloxacin, moxifloxacin, ciprofloxacin, gatifloxacin, polymyxin B, cotrimoxazole, trimethoprim and aztreonam. *Stenotrophomonas maltophilia* DSM 50170 was used as the positive control, and the antibiogram was performed in accordance with standards described by the National Committee for Clinical Laboratory Standards (1999) and Cheesebrough (2006).

8.2.7 Multiple Antibiotic Resistance Index (MARI)

The MARI 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 exposed (b), i.e:

MARI= a/b (Krumperman, 1983).

8.2.8 Assessment of Trimethoprim-Sulphamethaxole Resistance Genes

Trimethoprim-sulphamethaxole is the drug of choice in the treatment of infections caused by *Stenotrophomonas maltophilia*. This, along with our initial observation of resistance to this antibiotic informed the need for the assessment of the presence of *sul*2 and *sul*3 genes in the resistant isolates and these were done in accordance with the descriptions of Blahna *et al.* (2006) using the primers listed in Table 8.1. The PCR condition for *sul*2 detection began with an enzyme activation (denaturation) stage at 94°C for 5 min, followed by 30 cycles of

denaturation at 94°C for 40 s, annealing at 55°C for 40 s and extension at 72°C for 1 min. A final extension at 72°C was run for 7 min. For *sul* 3 detection, PCR condition was as follows: heating at 94°C for 5min, 30 cycles at 94°C for 60s, 55°C for 60s and 72°C for 60s, followed with one cycle at 72°C for 7 min (Blahna *et al.,* 2006).

Table 8.1: Primers for the assessment of Trimethoprim/sulphamethazole genes.

Primers	Primer Sequence	Size
Sul 2f	5′-GCGCTCAAGGCAGATGGCATT-3′	285
Sul 2r	5′-GCGTTTGATACCGGCACCCGT-3′	
Sul3f	5′- GAGCAAGATTTTTGGAATCG -3′	799
Sul3r	5′- CATCTGCAGCTAACCTAGGGCTTTGGA -3′	

8.3 RESULTS

One hundred and twenty (96%) *Stenotrophomonas maltophilia* isolates were recovered from grass root rhizosphere, while 5 (4%) were recovered from soil butternut rhizospere (Table 8.2). About 8.9% of the isolates were resistant to meropenem, while resistance to the other antibiotics were as follows: cefuroxime (95.6%), ampicillin-sulbactam (53.9%), ceftazidime (10.7%), cefepime (29.3 %), minocycline (2.2%), kanamycin (56.9%), ofloxacin (2.9%), levofloxacin (3%), moxifloxacin (2.8%), ciprofloxacin (24.3%), gatifloxacin (1.3%), polymyxin B (2.9%) and aztreonam (58%) (Table 8.3).

Variable susceptibilities to the cephalosporins (with carbapenem) were observed. About 88% of the isolatesd were susceptible to meropenem and ceftazidime, while 58.7% were susceptible to cefepime. Also, 97.8% and 97.1% of the isolates were susceptible to minocycline and polymycin B respectively. With regards to the fluoroquinolone, about 94.7% of the isolates were susceptible to both gatifloxacin and levofloxacin, while, 90% and 87.1% were susceptible to moxifloxacin and ofloxacin respectively (Table 8.3). A lower resistance (26.1%) to cotrimoxazole was observed in comparison to 98.6% resistance to trimethoprim (Table 8.3), and the MAR index ranged from 0.32-0.9 (Fig 2). Also, four isolates were positive for *sul3* genes while none were for *sul2* gene (Table 8.4).

Table 8.2: Total number and percentage of *Stenotrophomonas maltophilia* recovered per source.

Source	No Recovered	% Recovered
Grass Root Rhizosphere	120	96.0
Soil Butternut Rhizosphere	5	4.0
Total	**125**	**100**

Table 8.3: Antibiotic Susceptibility profile of the *Stenotrophomonas maltophilia* isolates

Antibiotics	Responses (%)		
	Susceptible	**Intermediate**	**Resistant**
Meropenem	88.1	3.0	8.9
Cefuroxime	1.5	2.9	95.6
Ampicillin-sulbactam	44.6	1.5	53.9
Ceftazidime	88.0	1.3	10.7
Cefepime	58.7	12.0	29.3
Minocycline	97.8	0.0	2.2
Kanamycin	38.5	4.6	56.9
Ofloxacin	87.1	10.0	2.9
Levofloxacin	94.7	4.0	1.3
Moxifloxacin	90.0	7.2	2.8
Ciprofloxacin	74.3	1.4	24.3
Gatifloxacin	94.7	8.0	1.3
Polymyxin B	97.1	0.0	2.9
Aztreonam	14.5	27.5	58.0
Cotrimoxazole	63.8	10.1	26.1
Trimethoprim	0	11.4	98.6

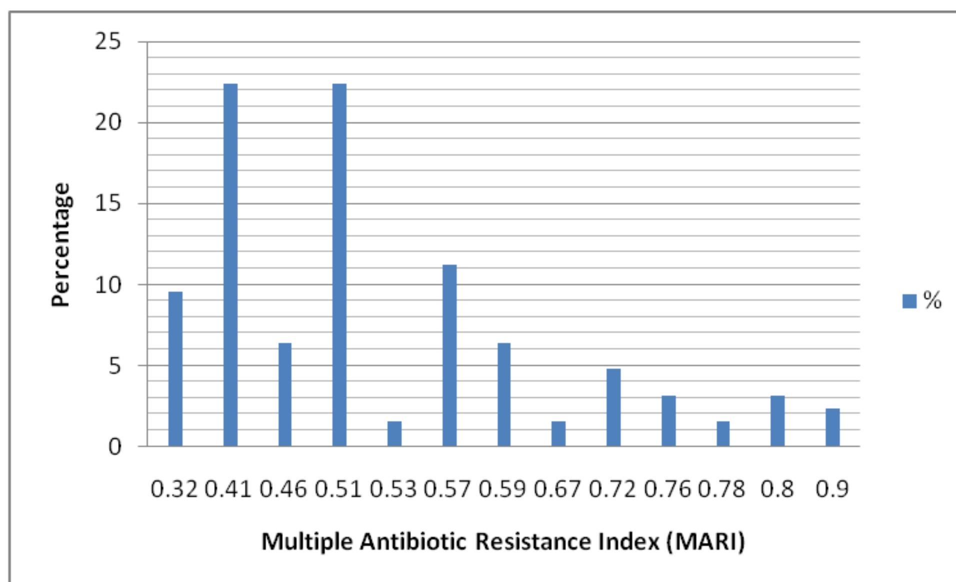

Fig 8.1: Percentage of isolates versus specific multiple antibiotic resistance index

Table 8.4 Sulphonamide resistance genes (*sul*) detected from the *Sten. maltophilia* resistant isolates.

Resistance Genes	Percentage Detected/ phenotypic resistance (%)
Sul3	12.1
Sul2	0

8.5 Discussion

Commensal *Stenotrophomonas maltophilia* may end up as opportunistic pathogen (Nyc and Matejkova, 2010). As revealed in this study, the bacteria are easily culturable, and appear ubiquitous, probably due to their resilience in the face of environmental stress (Borner *et al*., 2003). Our experience in this study suggests that the recovery of the organisms varies from place to place. As some studies have reported isolation of this bacteria from soil butternut and wallnut rhizosphere (Rettenmaier and Lingens 1985; Kan et al., 2007), only 5 isolates (4%) were isolated from the soil butternut rhizosphere compared to 120 (96%) from grass rhizosphere. The intrinsic resistance of this organism to imipenem was exploited for their isolation and identification as it allowed convenient discrimination between the *Stenotrophomas* species and other imipenem resistant bacteria only (Bollet *et al*., 1995). The recovery rate of this bacterium appears to be increasing with time compared to when the bacteria was initially discovered. This scenario is buttressed by our findings as well as those by Gulmez and Hascelik (2005) which showed a higher frequency of occurrence of this specie than previously observed.

Sten. maltophilia has been reported to be resistant to myriads of antibiotics (Alonso *et al*., 2004; Song *et al*., 2010). This high resistance characteristic which was peculiar to clinical isolates has now been observed among environmental strains (Liaw *et al*., 2002; Tan *et al*., 2008). The resistance observed to kanamycin and trimethoprim in this study is in agreement with the report of Musa *et al.* (2008) on commensal *Sten. maltophilia* from *Osphronemus goramy.* Similarly, *Sten. maltophilia r*esistance to cephalosporin is higher in this study compared to that reported previously (Jones *et al.,* 2003). Berg *et al*. (2005) and Crossman *et al.* (2008) also noted that resistance to conventional antibiotics would have helped *Sten. maltophilia* to compete with other rhizospheric bacteria and made them survive in their

habitat. This assertion is pertinent as all the isolates here showed MAR index > 0.2 which implies that they have arisen from high-risk sources where antibiotics is in constant arbitrary use resulting in high selective pressure as reported by Suresh *et al*. (2000).

Fluoroquinolone and polymycin B, both of which showed good activities against the *Sten. maltophilia* isolates are usually antibiotics of choice in the treatment of infections by the bacteria. The activities of these antibiotics against the bacteria have been similarly reported by Gales *et al*. (2001) and Tripodi *et al.* (2001). Valdezate *et al.* (2001) observed that >95% (94.7% in this study) of the bacterial isolates in their study were susceptible to a fluoroquinolone. However, it is known that trimethoprim-sulphamethoxazole is the drug of therapeutic choice against *Stenotrophomonas maltophilia* infections (Denton and Kerr, 1998; Betriu *et al.,* 2001; Gales *et al.*, 2001; Krueger *et al.*, 2004), but several reports have shown that the prevalence of *Stenotrophomonas maltophilia* strains that are resistant to TMP-SXT are increasing (Micozzi *et al.,* 2000; Tsiodras *et al.,* 2000; Al-jasser, 2006). In this study, about 26% of the *Stenotrophomonas maltophilia* isolates were resistant to this antibiotic as against 2% reported elsewhere (Gales *et al*., 2001). The trend continues to threaten public health of individuals, especially in an HIV/AIDS infested populations where the immune system is weakened.

Resistance to trimethoprim-sulphamethoxazole is mediated by sulphonamide resistance *sul* genes among other determinants (Toleman *et al.,* 2007). A study in Portugal by Antunes *et al*. (2005) detected *sul1, sul2, or sul3* genes in some Gram negative isolates. This *sul*3 gene was observed to meditate trimethoprim-sulphamethoxazole resistance (Enne *et al.,* 2002). This gene was earlier detected in some gram negative isolates recovered from animals and food in Switzerland and German (Grape *et al*., 2003; Guerra *et al*., 2003; 2004), suggesting commensal *Stenotrophomonas maltophilia* to be as important as its clinical

counterpart. The presence of *sul3* genes in this study may imply that the endophytic and clinical strains possess a similar level of antibiotic resistance, which may be more extensive among some endophytic strains of *Sten. maltophilia* (Ryan *et al.,* 2009). This probably explains the resistance against cotrimoxazole (trimethoprim-sulphamethoxazole, SXT) observed in this study. The potential threat that such resistant isolates could be to public health informed the call for a surveillance study of *sul* gene and phenotypic SXT by Toleman *et al.* (2007).

8.6 CONCLUSION

Commensal *Sten. maltophilia* appears to be an important commensal with comparable antibiogram characteristics to its clinical strains. It also appears to be abundant in grass and soil butternut rhizosphere in the Eastern Cape Province of South Africa. The multiple antibiotic resistance index of the bacterial isolates suggest their sources have been under antibiotics selective pressure that could be related to abuse of antibiotics. Their antibiogram characteristics also suggest the bacterium is an important reservoir of antibiotic resistant determinants (especially sulphonamide resistance genes) in the environment.

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CHAPTER NINE

General Discussion and Conclusion

9.1 Discussion

This study explained the ubiquitous nature of members of the test bacterial genera as the commensal *Staphylococcus*, *Acinetobacter* and *Stenotrophomonas* species were isolated from such diverse sources as animals (goat, cattle, pig and chicken), soil, water, and plants' rhizospheres. The inherent potentials to withstand unfavourable osmotic condition, pressure and slightly elevated temperature have been reported to support the survival of Staphylococci on animals (Harris *et al.,* 2002; Le-Loir *et al*., 2003), as their isolation in this study and elsewhere (de Neeling *et al*., 2007; Stegmann and Perreten, 2010; Tulinsky *et al*., 2012) suggests. *Staph. capitis* is one of the commensal Staphyloccocus species isolated in the study. The organism is a known flora of human scalp and skin, and it has been frequently reported as aetiology of endocarditis (Van der Zwet *et al*., 2002; Iwase *et al*., 2007; D'mello *et al*., 2008). The isolation of *Staph. haemolyticus* in this study also aligns with previous reports (Bagcigil *et al*., 2007; Schlegelova *et al*., 2008), and the bacteria have been implicated in meningitis, cellulitis, prosthetic joint infections, or bacteremia (Falcone *et al.,* 2007). Hence, *Staphylococcus* species are increasingly being recognized and appreciated for their dual characteristic as pathogen and commensal (Trulzsch *et al*., 2002; Bannerman, 2003, Deurenberg and Stobberingh, 2008).

The observed resistances to some of the conventional antibiotics which are also frequently prescribed within the study area is worrisome considering that the isolates are non-clinical, and this further reaffirms the critical role of some commensals in public health. In particular, resistances to sulphamethaxole, nalidixic acid, tetracycline and penicillin G, all of which are broad spectrum antibiotics could be due to misuse of the antimicrobials (Moulin, 2001) especially as growth promoters (Perrier-Gros-Claude *et al*., 1998; FEDESA, 1998; Philip et al., 2004). Smith *et al.* (2009) observed that 44% of all the *Staphylococcus* species were

resistant to most antibiotics used in their study and they highlighted drug abuse as a contributing factor. The methicillin resistance observed, no doubt, is of potential threat to the animals and farm personnel (Aubry-Damon *et al.,* 2004) in the study area and around the globe. Adequate information is unavailable about MRSA colonization of healthy cattle thus underscoring the importance of this study. A Dutch study observed that 28% of calves were colonized by MRSA (Graveland et al. 2008), while 1.3% calves and 0.4% adult cows were also colonised in Switzerland (Huber et al. 2009).

Vancomycin used to be the antibiotic in the last line of defence in the treatment of infections caused by such resistant isolates (Fitzgerald *et al*., 2001; Boucher *et al*., 2010). Some species of Staphylococci were vancomycin-resistant and were recovered from the various animals in this study. Considering that there is a close link between resistance to vancomycin and other extended spectrum beta-lactam antibiotics like meropenem and imipenem (Paterson and DePestel, 2009), the tendency to worsen difficulty in the choice of therapeutic options (Chang *et al*., 2003; Boucher *et al*., 2010) becomes apparent. Tenover and Goering (2009) reported the presence of community based MRSA, just as Bhalakia and Morris (2005) also reported the presence of plasmid mediated vancomycin resistance in fomite. These resistant organsims could infect farm personnel in a zoonotic infection and increase the risk to public health, more so with the confirmed presence of the resistance markers - *mecA* and *mph(C)* in some of the Staphylococcal isolates suggesting them to be reservoirs of antibiotic resistance determinants as reported elsewhere (Lee, 2006; Schlegelova *et al.,* 2008).

Resident commensals *Acinetobacter* species are important as biotechnologically useful commensals and dreadful opportunistic pathogens (Villers *et al*., 1998; Chen *et al*., 2005).

They are sometimes threats to immunocompromised wandering farm animals and farm workers. In this case, the animal skin acts as temporary residence for the bacteria until there is a breach in the skin or when an immunocompromised farmer gets infected zoonotically (Bester and Essack, 2010). In this study, about 91% of all the species of *Acinetobacter* isolated was the frequently implicated aetiology of nosocomial infection - *Acinetobacter baumannii* (Garcia-Garmendia *et al*., 1999; Falagas and Rafailidis, 2006; Jamulitrat *et al*., 2009). *Acinetobacter haemolyticus* which usually affects debilitating individuals was only isolated from the Fort Beaufort samples in this study. Peleg *et al*. (2008) reported *Acinetobacter baumannii* and *Acinetobacter haemolyticus* prevalence rate of 25% in healthy individuals. This, along with our findings suggest the need for commitment to strict rules of hygiene that could reduce the bacterial load on animal skin in contact with soil and contaminated water sources (Ecker *et al.,* 2006).

Acinetobacter species mostly exhibit health threatening antibiotic resistance (Grabe *et al*., 2008). The observed resistance to the third generation cephalosporin (ceftazidime, cefotaxime, ceftriaxone, cefpodoxime) in this study tends to narrow therapeutic choice of antibiotics in the last line of defence. In other studies, Zarakolu *et al*. (2006) and Hassan *et al*. (2010) similarly reported high rates of antibiotic resistant *Acinetobacter* species. A more alarming resistance rate of about 90% to ceftriaxone was reported by Rhabar *et al*. (2010) in a hospital environment in Tehran, Iran. One of the possible determinants of the extended spectrum resistance exhibited by the *Acinetobacter* species in this study is the observed CTX-ESBLs production. Bacteria producing such *CTX-M-1*-type ESBLs have been extensively reported among many clinical isolates from humans (Komatsu *et al.,* 2001; Bonnet, 2004) and cattle (Shiraki *et al.,* 2004). Immuno-compromised individuals tend to suffer higher

mortality, complicated therapy and morbidity rates by ESBLs producing *Acinetobacter* species (Chastre *et al.,* 1996; Ramphal and Ambrose, 2006)

Tetracycline resistance remains one of the main determinants in assessing resistance genes in natural environments (Sandalli *et al.,* 2010). The low cost, good diffusion, less toxicity, availability, therapeutic advantage of tetracycline as drug of preference against infection has led to its indiscriminate use (Chopra and Roberts, 2001); hence the emergence of tetracycline resistance. As a result, tetracycline resistance is being used as a model for studying the ecology of antibiotic resistance and the presence of the genes responsible for resistance to the antibiotic is suggestive of the resistance characteristics in an organism (Aminov *et al*., 2001) or environment (Rahube and Yost 2010). Previous reports (Chopra *et al.,* 2001; Suzuki, 2010) highlighted the importance to public health of resistance to tetracycline by isolates from non animal sources. In this study, oxytetracycline resistance was observed but the high susceptibilities to minocycline re-affirm its therapeutic preference to oxytetracycline, doxycycline, tetracycline and imipenem (Coelho *et al*., 2006; Halstead *et al*., 2007; Bishburg and Bishburg 2009).

The phenotypic expression of resistance genes occurs after an appropriate internal mechanism has been fully accomplished (Duval et al., 2010). In this study, while *Tet(A), Tet(B), Tet(39), Tet(H)* and *Tet(M)* genes were assessed, the *Tet (B)* gene and *Tet*(*39*) gene were detected. *Tet(B)* confers resistance to tetracycline and minocycline (Chopra and Roberts, 2001) while the *Tet(39)* have been previously linked to oxytetracycline resistance in fish farming in Thailand (Agerso and Peterson, 2007). Schmitt *et al.* (2006) linked the presence of *tet* genes with exposure to tetracycline, which may imply that the *Acinetobacter* species in this study area harbouring *Tet (B)* and *Tet (39)* genes might have been exposed to

residual tetracycline from wastewater. However, as earlier observed by Enne *et al.* (2006), the *Tet(B)* genes in this study were unexpressed against the minocycline, as no resistance was observed despite the presence of the gene. Schmitz *et al.* (2001) had also observed that many bacterial isolates were susceptible to tetracycline despite the presence of the tetracycline resistance genes.

The expression of intrinsic pathogenic potentials among commensals often justifies the ascertion that commensalism is a phase in pathogenic cycle (Towner, 2009). Hence, commensal *Stenotrophomonas maltophilia* is important as it may end up as an opportunistic pathogen (Nyc and Matejkova, 2010) due to its resilience in the face of environmental stress (Borner *et al*., 2003).

Sten. maltophilia is resistant to myriads of antibiotics (Alonso *et al*., 2004; Song *et al*., 2010). This high resistant characteristic which is peculiar to clinical isolates has now been observed among environmental strains (Liaw *et al*., 2002; Tan *et al*., 2008). The resistance to kanamycin and trimethoprim observed in this study is in agreement with the report of Musa *et al.* (2008) on commensal *Sten. maltophilia* from *Osphronemus goramy.* Lower resistances to trimethoprim-sulphamethoxazole (cotrimoxazole) compared to trimethoprim observed in this study reiterates the advantage of fixed dose combination therapy (in synergy) over single dosage in antibiotic administration, especially in bacteremia or neutropenia (Gautam and Saha, 2008). However, resistance to the trimethoprim-sulphamethoxazole (TMP-SXT) known to be effective therapeutic alternative for *Stenotrophomonas maltophilia* (Denton and Kerr, 1998; Betriu *et al.,* 2001; Gales *et al.*, 2001; Krueger *et al.*, 2001) is mediated by *sul* genes, among other determinants. Several reports (Micozzi *et al.,* 2000; Tsiodras *et al.,* 2000; Enne *et al.,* 2002) have shown that the prevalence of strains that are resistant to TMP-SXT is

increasing. Resistance to this antibiotic in this study was about 26% compared to 2% that was reported earlier in Canada and Latin America (Gales *et al*., 2001). In this study, *sul3* was detected among the TMP-SXT resistant isolates. This genes *sul3* was earlier detected in animals and food in Switzerland and Germany among some gram negative isolates (Grape *et al*., 2003; Guerra *et al*., 2003; 2004) that showed resistance to TMP-SXT, thus suggesting commensal *Stenotrophomonas maltophilia* to be as important as its clinical counterpart.

The multiple antibiotic resistance index (MARI) > 2 observed in this study explains the sources of the *Staphylococcus* species, *Acinetobacter* species and *Stenotrophomonas maltophilia* isolates as potential threats to public health (Suresh *et al.,* 2000), the commensal nature of the organisms notwithstanding. The MAR index also suggests that all the three groups of bacteria are important reservoirs of antibiotic resistance determinants in the Nkonkobe Municipality environment. While antibiotic resistance remains a global challenge, the main factor responsible for it is the arbitrary use of antibiotics (Smith *et al.,* 2009) which invariably impact on the commensal bacteria of both human and veterinary origins during therapy, more so as about half of the antibiotics in some parts of the world are administered on animals (FEDESA, 1998; Philip et al., 2004) to treat, prevent infection and promote growth. It is established that in Europe, approximately one-third of all veterinary use of antibiotics are channelled as growth promoters (FEDESA, 1998). The growth factors used in Europe exhibit cross resistance but are mostly active against Gram-positive bacteria, while few are against Gram-negatives (Philips *et al*., 2004; Khardori, 2006). This process however encourages development of resistance to the therapeutic antibiotics in bacteria and should be banned.

Other approved methods have been used on similar studies like this with results similar to our observations. The use of agar diffusion also recommended by CLSI (2008) is also as effective in determination of antibiotic susceptibilty profile of bacteria. The use of real time PCR and pyrosequence has been very effective in bacterial identification and assessment of genes (Halse et al., 2010), and can be used for this kind of study.

9.2 Conclusion

In this study, commensal *Staphylococcus* species, *Acinetobacter* species and *Stenotrophomonas maltophilia* were successfully recovered from Nkonkobe Municipality in the Eastern Cape Province, South Africa. The resistance by these commensals to methicillin, vancomycin, erythromycin, clindamycin, oxytetracycline, carbapenem, trimethoprim, sulphamethoxazole, cotrimoxazole, nalidixic acid, among others suggests that they are potential threats to public health, especially during opportunistic infections. The detection of resistance genes in the commensals re-affirms their roles as reservoirs of antibiotic resistance determinants in the environment. Future research prospect could involve a comparative analyses of clinical and environmental isolates as reserviors of specific antibiotic resistance determinants in the province and nationally.

9.5 References

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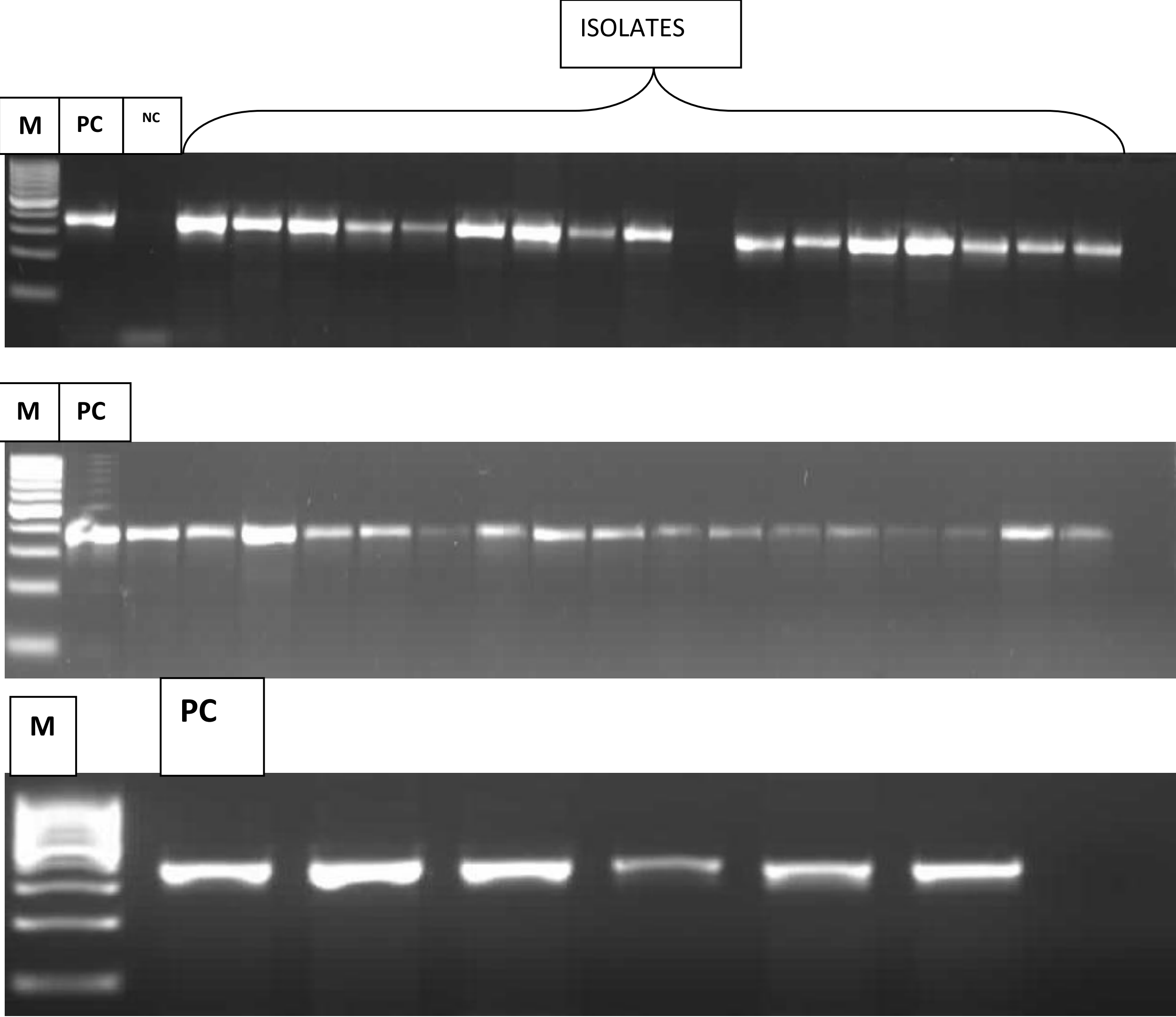
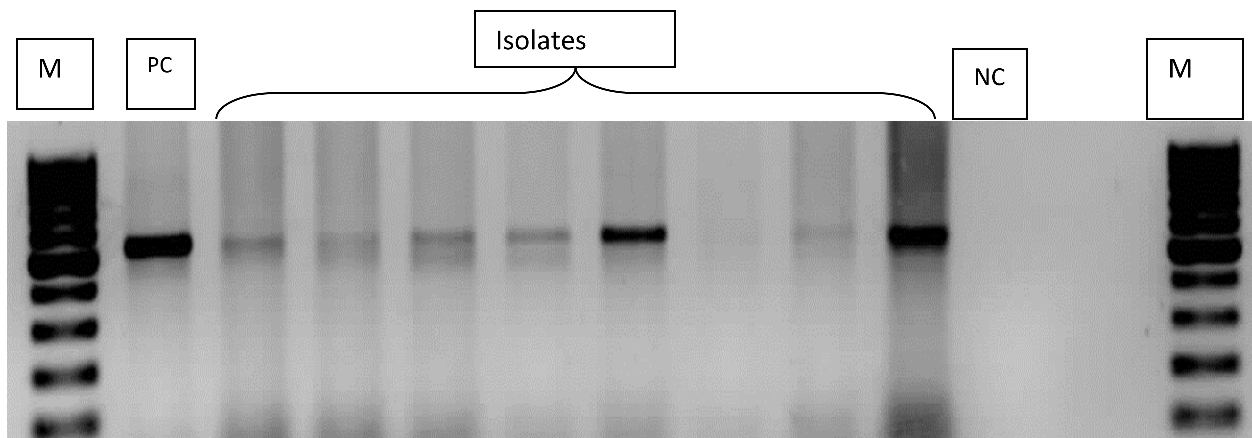
APPENDICES

Appendix 1: Identification gel Pictures (Sample)



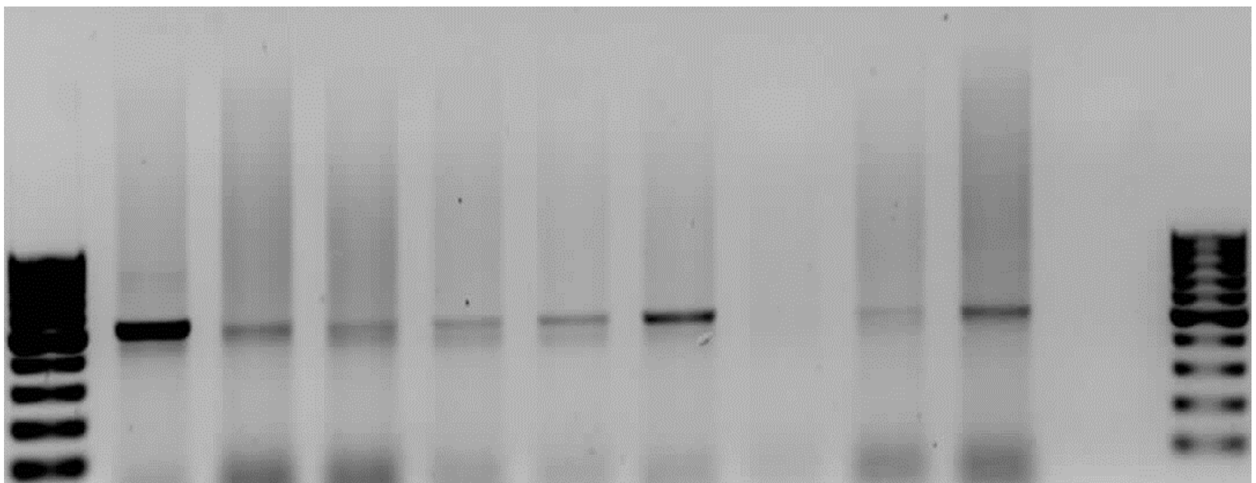
a. **Gel Electrophoresis showing with *Staphylococcus* amplicon at 370 bp**

KEY: M=Marker (100bp ladder), PC=Postive control (*Staph. aureus* ATCC 25923) **,**
NC=Negative control (DNAse free water)



Gel Electrophoresis with the *Stenotrophomonas maltophilia* amplicon at 550bp

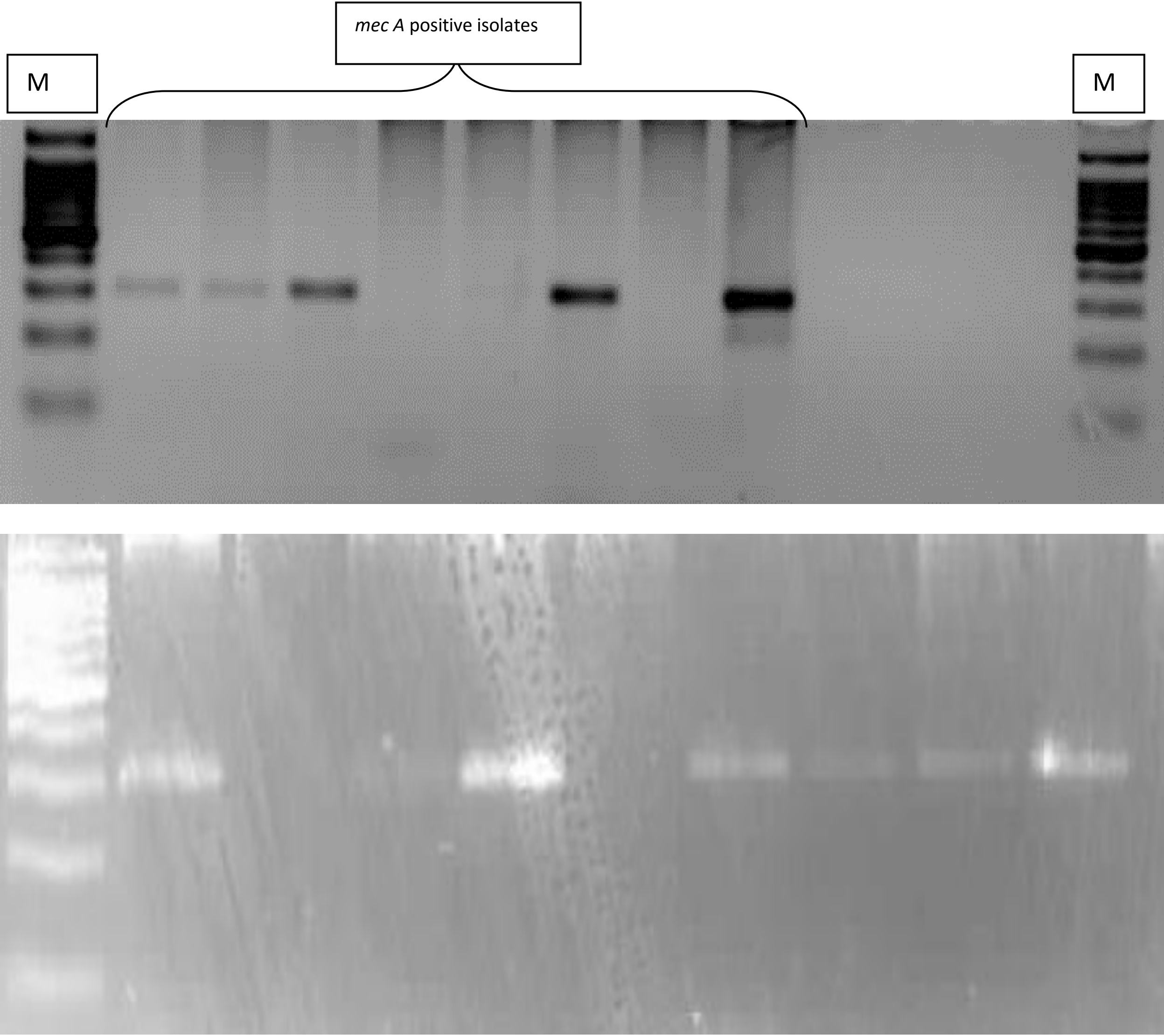
KEY: M=Marker (100bp ladder), PC=Positive control (DSM 50170), **NC= Negative control (DNAse free water)**



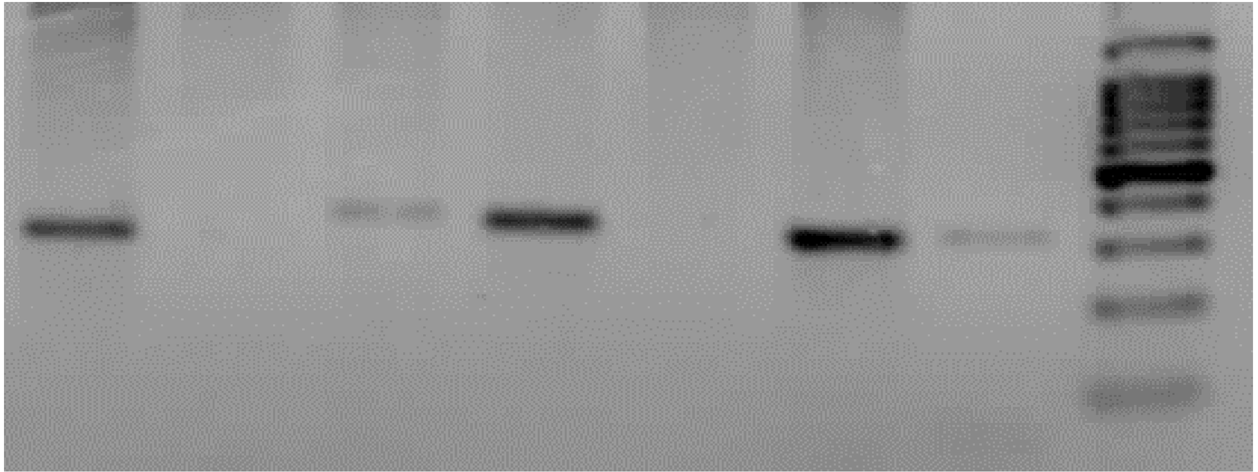
b. Gel Electrophoresis with the *Stenotrophomonas maltophilia* amplicon at 550bp

KEY: M=Marker (100bp ladder), PC=Positive control (DSM 50170), **NC= Negative control (DNAse free water)**

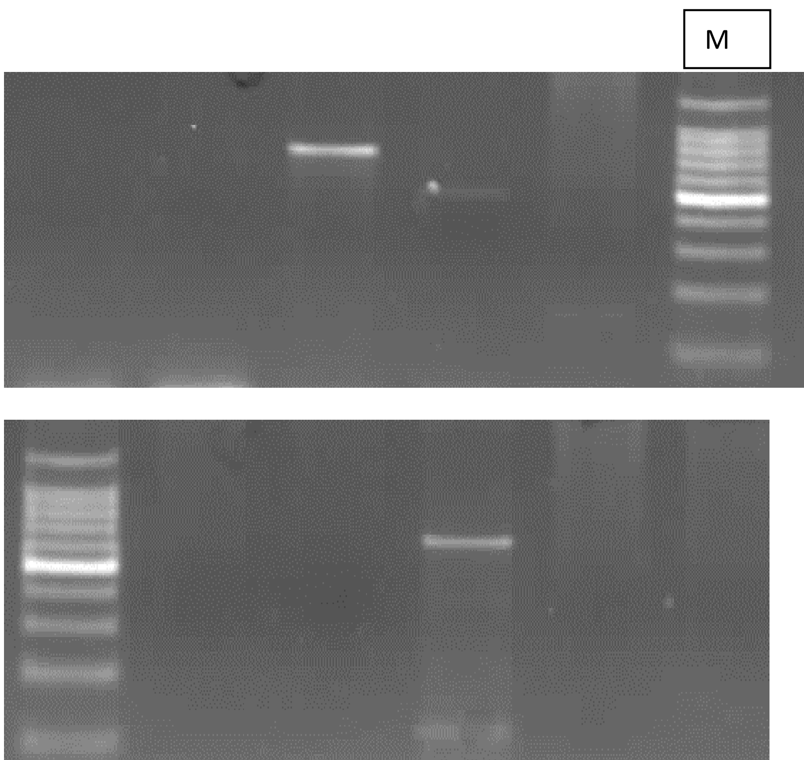
Appendix 2: Some Resistance Genes Gel Pictures



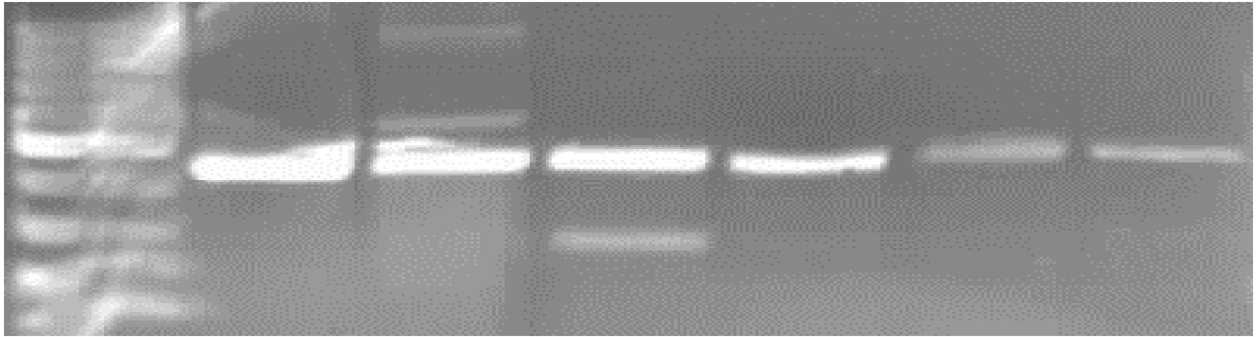
a. Gels showing *mec A* gene at amplicon size of 310 bp (*Staphylococcus* species)



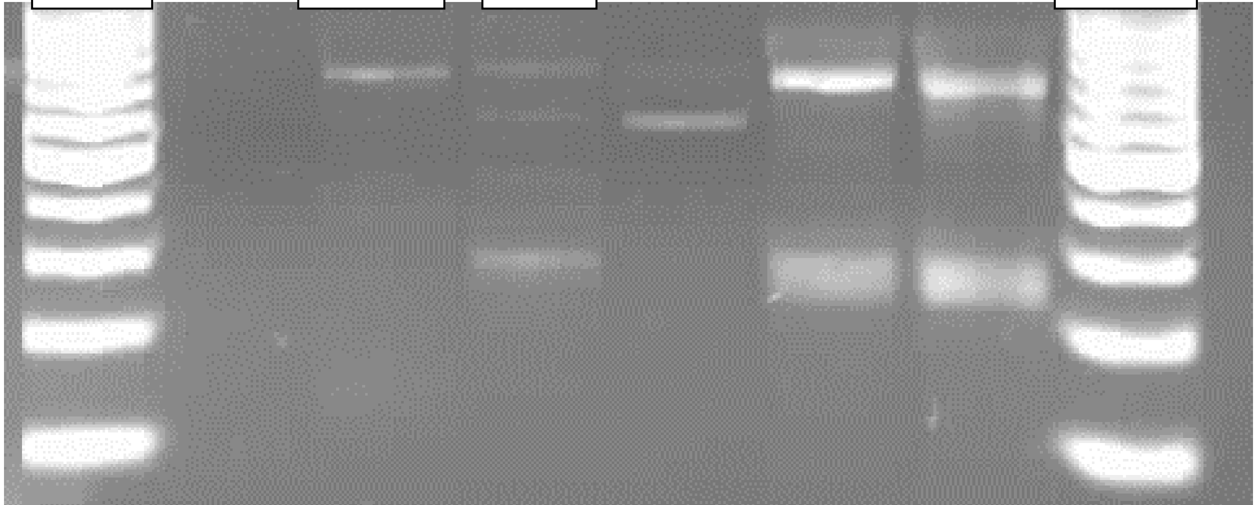
b. Gels showing *mec A* gene at amplicon size of 310 bp (*Staphylococcus* species)



c. Genes coding for inactivation mechanism (mph gene)(*Staphylococcus* species)

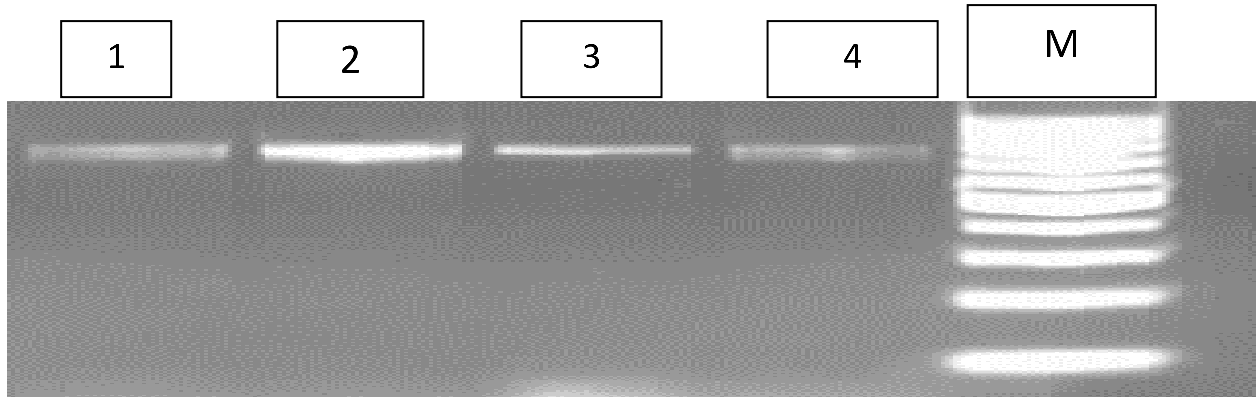


d. Gel electrophoresis showing bands of *Tet B* at 415bp (*Acinetobacter*)



e. Gel Electrophoresis showing <u>four</u> *Tet 39* gene at 701 bp (*Acinetobacter* species)

f. Gel Electrophoresis showing three CTX-M-1-genes at 490 bp (*Acinetobacter* species)



g. **Sulphonamide Resistance genes (*Sul 3*) among *Stenotrophomonas maltophilia* at 799 bp**

Appendix 3: Isolates and Preservation Details

Isolate code	Isolate identity	Preservation condition	Where store
A01	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80ºC	AEMREG culture collection
A02	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80ºC	AEMREG culture collection
A03	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80ºC	AEMREG culture collection
A04	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80ºC	AEMREG culture collection
A05	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80ºC	AEMREG culture collection
A06	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80ºC	AEMREG culture collection
A07	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80ºC	AEMREG culture collection
A08	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80ºC	AEMREG culture collection
A09	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80ºC	AEMREG culture collection
A10	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80ºC	AEMREG culture collection
A11	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80ºC	AEMREG culture collection
A12	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A13	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A14	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A15	*Acinetobacter*	20% glycerol stock @ -80oC	AEMREG culture collection

	baumanni/calcoaceticus		
A16	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A18	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A19	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A20	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A21	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A22	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A23	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A24	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A25	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A26	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A27	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A28	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A29	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A30	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A31	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A32	*Acinetobacter*	20% glycerol stock	AEMREG culture

	baumanni/calcoaceticus	@ -80oC	collection
A33	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A34	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A35	*Acinetobacter haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A36	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A37	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A38	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A39	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A40	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A41	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A42	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A43	*Acinetobacter haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A44	*Acinetobacter haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A45	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A46	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A47	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A48	*Acinetobacter*	20% glycerol stock	AEMREG culture

	haemolyticus	@ -80oC	collection
A49	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A50	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A51	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A52	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A53	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A54	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A55	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A56	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A57	*Acinetobacter haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A58	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A59	*Acinetobacter haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A60	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A61	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A62	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A63	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A64	*Acinetobacter*	20% glycerol stock	AEMREG culture

	baumanni/calcoaceticus	@ -80oC	collection
A65	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A66	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A67	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A68	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A69	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A70	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A71	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A72	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A73	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A74	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A75	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A76	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A77	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A78	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A79	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A80	*Acinetobacter*	20% glycerol stock	AEMREG culture

	baumanni/calcoaceticus	@ -80oC	collection
A81	*Acinetobacter haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A82	*Acinetobacter haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A83	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A84	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A85	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection
A86	*Acinetobacter baumanni/calcoaceticus*	20% glycerol stock @ -80oC	AEMREG culture collection

Staphylococcus species

Isolates code	**Stocke d as**	**Isolate identity**	**Preservation condition**	**Where store**
1	S1	*Staphylococcus aureus*	20% glycerol stock @ -80°C	AEMREG culture collection
2	S2	*Staphylococcus haemolyticus*	20% glycerol stock @ -80°C	AEMREG culture collection
3	S3	*Staphylococcus haemolyticus*	20% glycerol stock @ -80°C	AEMREG culture collection
4	S5	*Staphylococcus haemolyticus*	20% glycerol stock @ -80°C	AEMREG culture collection
5	S6	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
6	S7	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
7	S8	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
8	S9	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
9	S10	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
10	S11	*Staphylococcus capitis*	20% glycerol stock @ -80oC	AEMREG culture collection
12	S12	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection

13	S13	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
14	S14	*Staphylococcus xylosus*	20% glycerol stock @ -80oC	AEMREG culture collection
15	S15	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
16	16	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
17	S17	*Staphylococcus xylosus*	20% glycerol stock @ -80oC	AEMREG culture collection
18	S18	*Staphylococcus capitis*	20% glycerol stock @ -80oC	AEMREG culture collection
19	S19	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
20	S20	*Staphylococcus captis*	20% glycerol stock @ -80oC	AEMREG culture collection
21	S21	*Staphylococcus capitis*	20% glycerol stock @ -80oC	AEMREG culture collection
22	S22	*Staphylococcus capitis*	20% glycerol stock @ -80oC	AEMREG culture collection
23	S23	*Staphylococcus capitis*	20% glycerol stock @ -80oC	AEMREG culture collection
24	S25	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
25	S26	*Staphylococcus capitis*	20% glycerol stock @ -80oC	AEMREG culture collection
26	S27	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
27	S28	*Staphylococcus capitis*	20% glycerol stock @ -80oC	AEMREG culture collection
28	S29	*Staphylococcus xylosus*	20% glycerol stock @ -80oC	AEMREG culture collection
29	S30	*Staphylococcus* capitis	20% glycerol stock @ -80oC	AEMREG culture collection
30	S31	*Staphylococcus xylosus*	20% glycerol stock @ -80oC	AEMREG culture collection
31	S32	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
32	S33	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
33	S33	*Staphylococcus capitis*	20% glycerol stock @ -80oC	AEMREG culture collection
34	S34	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
35	S34	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
36	S45	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection

37	S46B	*Staphylococcus capitis*	20% glycerol stock @ -80oC	AEMREG culture collection
38	S47	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
39	S48	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
40	S48B	*Staphylococcus capitis*	20% glycerol stock @ -80oC	AEMREG culture collection
41	S49	*Staphylococcus capitis*	20% glycerol stock @ -80oC	AEMREG culture collection
42	S50	*Staphylococcus* spp.	20% glycerol stock @ -80oC	AEMREG culture collection
43	S51B	*Staphylococcus capitis*	20% glycerol stock @ -80oC	AEMREG culture collection
44	S52	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
45	S52B	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
46	S53	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
47	S55	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
48	S56	*Staphylococcus* spp.	20% glycerol stock @ -80oC	AEMREG culture collection
49	S57	*Staphylococcus* spp.	20% glycerol stock @ -80oC	AEMREG culture collection
50	S59	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
51	S59C	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
52	S60	*Staphylococcus* spp.	20% glycerol stock @ -80oC	AEMREG culture collection
53	S61	*Staphylococcus* spp.	20% glycerol stock @ -80oC	AEMREG culture collection
54	S61B	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
55	S62	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
56	S62B	*Staphylococcus xylosus*	20% glycerol stock @ -80oC	AEMREG culture collection
57	S63	*Staphylococcus* spp.	20% glycerol stock @ -80oC	AEMREG culture collection
58	S63B	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
59	S64	*Staphylococcus* spp.	20% glycerol stock @ -80oC	AEMREG culture collection
60	S64B	*Staphylococcus* spp.	20% glycerol stock @ -80oC	AEMREG culture collection

61	S65B	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
62	S66	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
63	S66B	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
64	S67	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
65	S68	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
66	S69	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
67	S70	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
68	S72	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
69	S73B	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
70	S74	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
71	S74B	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
72	S74	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
73	S76	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
74	S77B	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
75	S78	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
76	S79	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
77	79B	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
78	S79d	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
79	S80	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
80	S80B	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
81	S81	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
82	S83	*Staphylococcus capitis*	20% glycerol stock @ -80oC	AEMREG culture collection
83	S85	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
84	S85B	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection

85	S87	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
86	S88	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
87	S89	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
88	S90	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
89	S92	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
90	S93	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
91	S94	*Staphylococcus* aureus	20% glycerol stock @ -80oC	AEMREG culture collection
92	S99	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
93	S100	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
94	S108	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
95	S110	*Staphylococcus* spp.	20% glycerol stock @ -80oC	AEMREG culture collection
96	S129	*Staphylococcus xylosus*	20% glycerol stock @ -80oC	AEMREG culture collection
97	S130	*Staphylococcus xylosus*	20% glycerol stock @ -80oC	AEMREG culture collection
98	S130C	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
99	S131	*Staphylococcus xylosus*	20% glycerol stock @ -80oC	AEMREG culture collection
100	S131B	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
101	S132	*Staphylococcus xylosus*	20% glycerol stock @ -80oC	AEMREG culture collection
102	S132B	*Staphylococcus* spp.	20% glycerol stock @ -80oC	AEMREG culture collection
103	S133	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
104	S133C	*Staphylococcus haemolyticus*	20% glycerol stock @ -80oC	AEMREG culture collection
105	S134	*Staphylococcus aureus*	20% glycerol stock @ -80oC	AEMREG culture collection
106	S135	*Staphylococcus xylosus*	20% glycerol stock @ -80oC	AEMREG culture collection
107	S136	*Staphylococcus xylosus*	20% glycerol stock @ -80oC	AEMREG culture collection
108	S137	*Staphylococcus xylosus*	20% glycerol stock @ -80oC	AEMREG culture collection

109	S138	*Staphylococcus xylosus*	20% glycerol stock @ -80oC	AEMREG culture collection
110	S139	*Staphylococcus xylosus*	20% glycerol stock @ -80oC	AEMREG culture collection
111	S140	*Staphylococcus xylosus*	20% glycerol stock @ -80oC	AEMREG culture collection
112	S141	*Staphylococcus capitis*	20% glycerol stock @ -80oC	AEMREG culture collection
113	S142	*Staphylococcus xylosus*	20% glycerol stock @ -80oC	AEMREG culture collection
114	S143	*Staphylococcus xylosus*	20% glycerol stock @ -80oC	AEMREG culture collection
115	S144	*Staphylococcus xylosus*	20% glycerol stock @ -80oC	AEMREG culture collection
116	S145	*Staphylococcus xylosus*	20% glycerol stock @ -80oC	AEMREG culture collection
117	S146	*Staphylococcus capitis*	20% glycerol stock @ -80oC	AEMREG culture collection
118	S147	*Staphylococcus capitis*	20% glycerol stock @ -80oC	AEMREG culture collection
119	S148	*Staphylococcus xylosus*	20% glycerol stock @ -80oC	AEMREG culture collection
120	S149	*Staphylococcus xylosus*	20% glycerol stock @ -80oC	AEMREG culture collection

Stenotrophomonas maltophilia

Isolates code	**Isolate identity**	**Preservation condition**	**Where store**
Sn 01	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80°C	AEMREG culture collection
Sn 02	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80°C	AEMREG culture collection
Sn03	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn04	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn05	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn06	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection

Sn07	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn08	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn09	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn10	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn11	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn12	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn13	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn14	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn15	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn16	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn17	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn18	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn19	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn20	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn21	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn22	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection

Sn23	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn24	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn25	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn26	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn27	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn28	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn29	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn30	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn31	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn32	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn33	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn34	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn35	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn36	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn37	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn38	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection

Sn39	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn40	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn41	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn42	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn43	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn44	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn45	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn46	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn47	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn48	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn49	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn50	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn51	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn52	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn53	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn54	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection

Sn55	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn56	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn57	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn58	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn59	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn60	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn61	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn62	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn63	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn64	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn65	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn66	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn67	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn69	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn69	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn70	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection

Sn71	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn72	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn73	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn74	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn75	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn76	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn77	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn78	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn79	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn80	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn81	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn82	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn83	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn84	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn85	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn86	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection

Sn87	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn88	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn89	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn90	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn91	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn92	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn93	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn94	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
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Sn97	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn98	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn99	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
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Sn101	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
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Sn103	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn104	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
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Sn111	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn112	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn113	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn114	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
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Sn121	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn122	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn123	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn124	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection
Sn125	*Stenotrophomonas maltophilia*	20% glycerol stock @ -80oC	AEMREG culture collection