COMPARATIVE IN-VITRO ACTIVITIES OF TRIMETHOPRIM-SULFAMETHOXAZOLE AND THE NEW FLUOROQUINOLONES AGAINST CONFIRMED EXTENDED SPECTRUM BETA-LACTAMASE PRODUCING *Stenotrophomonas maltophilia* IN NKONKOBE MUNICIPALITY, EASTERN CAPE ENVIRONMENT

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DECLARATION

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

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Now to God eternal, my source, my pillar, and refuge be all the thanks and glory for His grace and favour every step of the way.

DEDICATION

This work is dedicated to my parents, Mr and Mrs P.O. Adesemoye, who laid the foundation for all my successes with their love and sacrifice.

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GENERAL ABSTRACT

Stenotrophomonas maltophilia is increasingly emerging as an opportunistic pathogen of global concern. Due to its inherent resistance to several classes of antibiotics including carbapenems and its ability to acquire mobile resistance elements, treatment of infections caused by *S. maltophilia* is a constant challenge for clinicians. Trimethoprim-sulphamethoxazole (TMP-SMX) is the generally accepted antibiotic of choice for the treatment of infections caused by this organism, but resistance to the drug is increasingly being reported; hence, the need for alternative therapeutic options. In this study, the antimicrobial susceptibility profile of 110 commensal *S. maltophilia* isolates obtained from Nkonkobe municipality, Eastern Cape Province, Republic of South Africa was investigated. Twenty-one antibiotics including TMP-SMX and the newer fluoroquinolones; levofloxacin, gatifloxacin and moxifloxacin were included in the antibiotic panel. About 63.4% of the isolates were susceptible to TMP-SMX with a resistance rate of 28.2%. The fluoroquinolones were more effective with susceptibilities ranging from 76% to 94.7%. Resistance to the fluoroquinolones ranged from 1.3% to 2.7%. Levofloxacin was the most effective fluoroquinolone tested. Phenotypic dectection of extended spectrum β-lactamases (ESBLs) showed double disc synergy test (DDST) positivity in 59.5% of the isolates. Cefepime was the most sensitive indicator cephalosporin in the DDST with 77.3% of suspected ESBL-producing isolates showing cefepime-clavulanic acid synergy. Isolates exhibited nine different ESBL phenotypes, however, PCR amplification of the *bla* genes revealed four isolates that possessed genes belonging to the CTX-M group (CTX-M-1 and CTX-M-8 groups). ESBL genes are usually carried on mobile elements such as plasmids and transposons which may also bear genes that mediate resistance to aminoglycosides, tetracyclines, TMP-SMX and fluoroquinolones. ESBL positive isolates appeared more susceptible to the fluoroquinolones compared to TMP-SMX but there was no significant

relationship between ESBL production and susceptibility to these drugs ($p > 0.05$). The newer fluoroquinolones are a possible alternative treatment option for *S. maltophilia* infections in this environment but further studies and clinical investigations are needed to determine the *in vivo* efficacy of these drugs.

CHAPTER ONE

GENERAL INTRODUCTION

The struggle between antibiotics and microorganisms dates as far back as the discovery of antibiotics themselves (Levy and Marshall, 2004; Tenover, 2006; Davies and Davies; 2010; D'Costa *et al*., 2011). Following Fleming's discovery of the penicillin-producing mould, tremendous progress has been made in the last seven decades in the development of new antimicrobial agents. The β-lactam group which includes broad spectrum penicillins, narrow and extended spectrum cephalosporins, monobactams and carbapenems are among the most widely prescribed all over the world because of their efficacy and low toxicity (Elander, 2003). However, even before penicillin was introduced for clinical use in the late 1930s, Abraham and Chain (1940) reported their discovery of an enzyme called penicillinase which could inactivate penicillin and as succinctly described by Dr Lederberg in his article, the future may very well remain a battle between "our wits and their genes" (Lederberg, 2000). In recent times, many authors, scientists and clinicians have expressed fears that infectious pathogens presently kept in check by the use of antibiotics will again become major sources of mortality (Nwosu, 2001; Livemore, 2007). Modern medical procedures such as transplants and immunosuppressive treatments which depend heavily on our ability to treat infection may collapse (Livermore, 2009).

The indiscriminate, heavy use of the β-lactam antibiotics both for medical and non-medical purposes has played a monumental role in the emergence of resistant bacteria (Nwosu, 2001; Levy and Marshall, 2004). This is particularly so with the employment of antibiotics in agriculture, animal and livestock rearing and the sale of antibiotics over the counter especially in some developing countries. Incorrect prescription and use of antibiotics by both medical personnel and the general public have also been a major contributory factor

(Nwosu, 2001; Perchere, 2001; Todar, 2008). The β-lactams usually act by inhibiting the penicillin-binding proteins which catalyse the final cross-linking of peptidoglycan in the bacterial cell wall leading to weakening of the cell wall and eventually lysis and cell death (Ghuysen, 1991; Bayles, 2000; Bush, 2001). The production of β-lactamases by the bacteria, however, leads to hydrolysis of the antibiotics and ineffective compounds (Bush, 2001).

Gram-negative commensal and/or opportunistic pathogens are of importance and interest to clinicians. Though these organisms are thought to have low inherent pathogenicity, they are gradually becoming a problem in intensive care units worldwide (Livermore, 2009). These bacteria have evolved from being multi-drug resistant (resistance to three or more antimicrobial groups) to extreme drug resistance (susceptibility to two or fewer classes of antibiotics) and now to pan drug resistance (diminished susceptibility to all classes) (Senekal, 2010). This might be due to the evolution of resistance genes as new members of the β-lactam group of antibiotics have been developed with species shifts in favour of β-lactamase producers (Pfaller and Segreti, 2006). This has also occurred among the Gram-negative opportunists with organisms such as *Enterobacter* spp. and *Pseudomonas aeruginosa* having become more common hospital pathogens in the last few decades. Many of these Gram-negative organisms possess naturally occurring chromosomally encoded β-lactamases which are thought to have developed as a result of selective pressure exerted by β-lactam producing soil microorganisms (Ghuysen, 1991). Antibiotic resistance genes apart from being chromosomal can also be carried on mobile genetic elements, such as plasmids and integrons, which facilitate the transfer of resistance between different species of bacteria by transformation, conjugation and transduction (Nwosu, 2001; Madigan *et al*., 2003; Livermore, 2007). It is therefore imperative to evaluate and monitor resistance genes in commensal, pathogenic and environmental bacteria in order to assess the environmental

resistance pool and better understand the ecology of antibiotic resistance (Goni-Urriza *et al*., 2000; Ammore *et al*., 2001).

The mechanisms of resistance in Gram-negative bacteria can be as a result of reduced accumulation of the drugs within cells subsequent to loss of porins or up-regulation of the bacterial efflux systems or both; but the most common cause of resistance against β-lactam antibiotics is the production of β-lactamases (Bonnet, 2004; Jacoby and Munoz-Price, 2005). According to Jones (2001), "the most important resistance problems that impact on nosocomial infections are extended spectrum β-lactamases (ESBLs) in *Klebsiella pneumoniae*, *Escherichia coli* and *Proteus mirabilis*, high level third generation cephalosporin (AmpC) β-lactamase resistance among *Enterobacter* and *Citrobacter freundii* and multi-drug resistant *P. aeruginosa*, *Acinetobacter* spp. and *S. maltophilia*".

Infections caused by multi-drug resistant Gram-negative bacteria, especially extended spectrum β-lactamase producers, are associated with higher mortality and morbidity (Rahal, 2000; Salma, 2008). This often leads to higher healthcare costs compared with their drug susceptible counterparts due to longer intensive care unit stay and total length of stay in hospital, especially when ineffective empirical antimicrobial therapy is prescribed (Daxboeck *et al*., 2006; Salma, 2008; Tan *et al*., 2008; Wright and Eiland III, 2008). The impact of antibiotic resistance in non-fermentative Gram-negative bacilli may be even more significant because of their tendency to cause infections in intensive care and immunocompromised patients and the lack of effective antibiotics against the most resistant organisms currently and even in the future (Livermore, 2007; Tan *et al*., 2008).

S. maltophilia is one of these nosocomial pathogens that have garnered interest because of evidence of its increasing role in nosocomial and opportunistic infections

worldwide particularly in debilitated and immunocompromised persons (Denton and Kerr, 1998; Betriu *et al.*, 2002; Koseoglu *et al.*, 2004; McGowan, 2006). It has been suggested that this increase is due to expansion of the patient population at risk due to advances in medical therapeutic interventions such as invasive therapeutic devices and increased utilization of broad spectrum antimicrobials (Senol, 2004; Al-Jasser, 2006b). *S. maltophilia* is inherently resistant to most β- lactam antibiotics including the carbapenems and aminoglycosides (Denton & Kerr, 1998; Friedmann *et al*., 2002). This multi-drug resistance has been attributed to several mechanisms including outer membrane protein alterations associated with efflux or reduced permeability and the production of multiple inducible β-lactamases (Looney, 2005; Toleman *et al*., 2007).

Trimethoprim-sulfamethoxazole (TMP-SMX) is therefore, currently the recommended treatment of choice for serious *S. maltophilia* infection but its effect is only bacteriostatic and there have been reports of resistance to the drug (Hohl *et al*., 1991; Fang and Madinger, 1996; Denton and Kerr 1998; Toleman *et al*., 2007). The newer fluoroquinolones have however, been recommended as possible alternative treatment for infections caused by this organism. The improved availability and activity of the fluoroquinolones make them a suitable therapeutic option (Weiss *et al*., 2000). This study intends to compare the antimicrobial activity of the third and fourth generation fluoroquinolones and TMP-SMX against confirmed ESBL-producing strains of *S. maltophilia* in the Eastern Cape environment with the purpose of providing retrospective information on the *in vitro* susceptibility of the organism to fluoroquinolones as an alternative treatment option should this environmental commensal become implicated in nosocomial or community acquired infections.

Traditionally, studies on antibiotic resistance have focused mainly on clinically important pathogenic bacteria. Conversely, according to Wright (2007), scientists need to broaden their view to include the entire pan-microbial genome. This refers to resistance genes in pathogenic bacteria, non-pathogenic bacteria and even the study of genes that have the potential to become resistance genes (Wright, 2007; Jayaraman, 2009). D'Costa *et al*. (2006) in their study of antimicrobial resistance among *Actinomycetes* isolated from the soil resistome found that the isolates were resistant to an average of 7-8 antibiotics out of the 21 tested. This is particularly significant because soil microbes have been an under-appreciated and under-reported reservoir of antibiotic resistance genes and because the majority of organisms in soil are unculturable, the resistance potential could be even greater. It is also impossible to rule out the horizontal transfer of genes from environmental organisms to pathogenic bacteria making it even more important to study the antibiotic resistome of these environments (Jayamaran, 2009).

1.1 AIMS AND OBJECTIVES

The main aim of this study was to compare the *in vitro* activity of the third and fourth generation fluoroquinolones (levofloxacin, gatifloxacin and moxifloxacin) and trimethoprim-sulfamethoxazole against confirmed extended spectrum β-lactamase producing strains of *Stenotrophomonas maltophilia* found in the Eastern Cape environment.

Specific objectives include:

- To determine the antibiogram characteristics of *S. maltophilia* isolates obtained from plants rhizosphere samples in the Nkonkobe district municipality of the Eastern Cape

Province, South Africa as part of the culture collection of the Applied and Environmental Microbiology Research Group (AEMREG).

- To select for putative extended spectrum β-lactamase producing *S. maltophilia* isolates.
- To assess the resistance genes responsible for ESBL production in confirmed isolates.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Stenotrophomonas maltophilia*

Stenotrophomonas maltophilia, formerly known as *Pseudomonas maltophilia* and then *Xanthomonas maltophilia* was eventually classified as the sole species of the genus *Stenotrophomonas* following a long period of uncertainty about its taxonomic classification (Palleroni and Bradbury, 1993). It was first isolated in 1943 and was named *Bacterium bookeri* at the time. The genus name derived from the Greek words **stenus** meaning *narrow*, **trophus** meaning *one who feeds* and **monas** meaning *a unit*; was intended to demonstrate the limited nutritional status of the organism (Palleroni and Bradbury, 1993; Berg *et al.*, 1999) but several studies have subsequently shown that the genus is "capable of great metabolic diversity and intraspecific heterogeneity" (Ryan *et al*., 2009)

Although only one species was described initially, the genus currently comprises of eleven species: *S. maltophilia* (Hugh, 1981) (Palleroni and Bradbury, 1993), *S. nitritireducens* (Finkmann *et al.*, 2000), *S. rhizophilia* (Wolf *et al.*, 2002), *S. acidaminiphilia* (Assih *et al.*, 2002), *S. koreensis* (Yang *et al.*, 2006), *S. terrae and S. humi* (Heylen *et al.*, 2007), *S. gisengisoli* (Kim *et al.*, 2010), *S. daejeonensis* (Lee *et al*., 2010), *S. panacihumi* (Yi *et al*., 2010) and *S. pavanii* (Ramos *et al*., 2011). *S. maltophilia* is the dominant species of the genus and is the only one that has been implicated in human disease (Paton *et al.*, 1994; Coenye *et al.*, 2004).

2.1.1 Morphology, Culture and Biochemical characterisitics

Stenotrophomonas are straight or slightly curved non-sporulating Gram-negative bacilli measuring 0.5 to 1.5µm long. This organism is motile with several polar flagella and may produce fimbriae. The colonies appear as smooth, glistening with entire margins and are white, greyish or pale yellow (Denton and Kerr, 1998). *S. maltophilia* occur singly or in pairs and do not accumulate poly-β-hydroxybutyrate as intracellular granules (Denton and Kerr, 1998).

S. maltophilia are obligate aerobes and do not grow at temperatures lower than 5^{o}C or higher than 40^{o}C. Growth is optimal at 35^{o}C. Most strains require methionine or cysteine for growth but this is not a universal requirement. Some strains exhibit a brownish discolouration on clear media which has been attributed to a secondary chemical reaction of extracellular products but this is more intense when these strains are incubated at 42^{o}C (Blazevic, 1976) or grown on media with high tyrosine content (Hugh and Gilardi, 1980). *S. maltophilia* is not considered to be beta-haemolytic but some studies have shown that when cultured on blood agar both aerobically and anaerobically, it produces a greenish hue (alpha haemolysis) around confluent growth (Bonny *et al.*, 2010).

It carries somatic O antigens and flagellar H antigens which have been identified and used in typing *S. maltophilia* in epidemiological studies and the structure of the most common serotype; O3 has been determined along with six others (Denton and Kerr, 1998). Several studies have reported cross-reactions of the somatic antigens with the O antigens of other organisms including *Brucella sp*. (Corbel *et al.*, 1984), *Renibacterium salmoninarum* (Brown *et al.*, 1995), *Legionella pneumophilia* and *Shigella dysenteriae* type 8 (Bonny *et al.*, 2010).

S. maltophilia also possesses a unique fatty acid profile which has been of use in identification of the bacterium (Norman *et al.*, 1997). Studies by Moss *et al*. (1973) revealed the presence of large amounts of 13-methyltetradecanoic acid (a branched chain 15-carbon fatty acid) and three additional branched chain hydroxyl-fatty acids not present in the other bacteria investigated.

Table 2.1: Biochemical characteristics of *Stenotrophomonas maltophilia* (Denton and Kerr, 1998).

TEST	REACTION
Indophenol oxidase	-
Catalase	+
Growth	
• 5°C	-
• 18°C	+
• 37°C	+
Motility	
• 18°C	+
• 37°C	V
Indole	-
Lysine hydroxylase	+
Orthinine decarboxylase	-
Methyl red	-
Voges-Proskauer	-
Hydrogen sulphide	-
Reduction of nitrate to nitrite	V
Citrate	V
Phenylalanine deaminase	-
β-Galactosidase (ONPG)	V
Hydrolysis	
• Esculin	+
• Gelatin	+
• Tween 80	+
• DNA	+
• Starch	-
• Urea	-
Carbon sources for growth	
• Adonitol	-
• Arabinose	-
• β-hydroxybutyrate	-
• Cellobiose	V
• Dulcitol	-
• Glucose	+
• Fructose	V
• Galactose	-
• Mannitol	V
• Rhamnose	-
• Salicin	-
• Sorbitol	-
• Trehalose	-

+ = >85% strains positive v = 16 to 84% strains positive - = ≤ 15% strains positive

2.1.2 Habitat

S. maltophilia is ubiquitous and found in a wide variety of environments and geographical areas. It has been isolated from different water sources including rivers, wells, lakes, bottled water and sewage (Kerr and Wilkinson, 1998; Hauben *et al.,* 1999; Whitby *et al.,* 2000). It is also present in a variety of soil and plant rhizosphere environments like grasses, sugarcane and palms, wheat, cabbage, rape, mustard, corn, beet, bananas, cotton, beans, tobacco, rice paddies, citrus plants, orchids, irises, legume inoculants that use non-sterile peat as a carrier and stored timber (Denton and Kerr, 1998). There have been reports of isolation from oil brines and other related materials from oil fields in Japan (Iizuka and Komgata, 1963) and it has also been reported to grow on loofah sponges (Bottone *et al.,* 1994). It can be found in food sources like frozen fish, milk, poultry eggs and lamb carcasses (Hugh and Ryschenkow, 1961; Juffs, 1973).

Over the years, *S. maltophilia* has evolved into an important nosocomial pathogen (Denton and Kerr, 1998; Whitby *et al*., 2000; Koseoglu *et al.,* 2004). It has been isolated from a variety of nosocomial sources which include: blood sampling tubes, central venous/arterial pressure monitors, contact lens care systems, deionized water dispensers, dialysis machines, disinfectant solutions, hands of health care personnel, hydrotherapy pools, ice-making machines, nebulizers and inhalation therapy equipment, necropsy specimens, oxygen analysers, oxygen humidifier reservoirs, shaving brushes, shower heads, sink traps, water faucets, sphygmomanometers and ventilator circuits (Denton and Kerr, 1998; Senol, 2004).

2.1.3 Pathogenicity

S. maltophilia had long been thought to be an organism with limited pathogenicity because of the failure to differentiate colonisation from infection and the inability to directly associate infections with mortality in affected patients (Looney, 2005). The organism was often linked with clinically overt infection only when acting in synergy with other more virulent pathogens and attempts to induce infection in an experimental animal model by injecting whole bacteria into mice failed to cause serious sepsis. In addition, the organism has been distinguished more for its multiple antibiotic resistance than for invasiveness and tissue destruction (Looney, 2005)

S. maltophilia produces several extracellular enzymes including DNase, RNase, arbutinase, acetase, esterases, lipases, mucinase, acid and alkaline phosphatases, phosphoamidase, leucine arylamidase, β-glucosidase, elastase and hyaluronidase; some of which have been associated with the pathogenesis of *S. maltophilia* infections (O'Brien and Davies, 1982; Travassos *et al.*, 2004; Looney *et al.*, 2009). The production of proteases and elastase plays a significant role in bacterial pathogenesis, participating in invasion, tissue damage and evasion of the host's natural defences. Production of lipases seems to contribute to the virulence of some species associated with pulmonary infections, either by hydrolysing lipid-rich pulmonary tissue components or by triggering an intense inflammatory response (Travassos *et al*., 2004).

Fimbriae or pili are important in the adherence of bacteria to epithelial cells for the initiation of colonisation or invasion of the host cells (Denton and Kerr, 1998). This process is mediated by fimbrial adhesins which aid the direct binding of bacteria to the target cells or indirect binding by the forming of cross linkages within the bacteria that aid colonisation.

The fimbriae produced by *S. maltophilia* have been found to aid its adhesion to epithelial cells and also inert surfaces resulting in biofilm formation (De Oliveira-Garcia *et al.,* 2003). The formation of biofilms protects the bacteria from the hosts' natural immune system and from inactivation by antimicrobial compounds. The ability of *S. maltophilia* to adhere to plastic and other abiotic surfaces such as medical implants and catheters and also its ability to survive in intravenous fluids and production of an extracellular elastase may contribute to the pathogenesis of intravenous line related infections (Denton and Kerr, 1998; De Oliveira-Garcia *et al.*, 2003).

Production of an outer membrane lipopolysaccharide (LPS) is also an important virulence factor and this is supported by the possession of the *spg*M gene which codes for the LPS production and also confers resistance to complement-mediated killing on isolates that possess the gene (McKay *et al.,* 2003). Finally, *S. maltophilia* also has the potential for indirect pathogenicity, as it can aid the virulence of other pathogens. It has been shown to modify the biofilm formation and polymyxin B sensitivity of *P. aeruginosa* (Livermore, 2009). Kataoka *et al.* (2003) have also shown that β-lactamases produced by *S. maltophilia* can increase the survival of *P. aeruginosa* that are normally susceptible to imipenem when the two organisms grow in culture together.

2.1.4 Resistance mechanisms of *Stenotrophomonas maltophilia*

S. maltophilia possess several mechanisms which contribute to its multi-drug resistant status. It is intrinsically resistant to various groups of antibiotics including β-lactams, carbapenems, quinolones, aminoglycosides, tetracyclines, disinfectants and heavy metals through the possession of multi-drug efflux pumps, low permeability of the outer membrane, chromosomal β-lactamases and aminoglycoside-modifying enzymes. It can also acquire resistance genes by the acquisition of integrons, transposons and plasmids (Toleman *et al*., 2007; Looney *et al.*, 2009).

β-lactam resistance is mediated by the expression of two inducible β-lactamases, L1 and L2. L1 is an Ambler class B zinc-dependent metalloenzyme that hydrolyses all classes of β-lactams except the monobactams while the L2 enzyme is an Ambler class A serine β-lactamase which is inhibited by clavulanic acid (Kataoka *et al*., 2003). Other studies have, however, reported the production of other β-lactamases in *S. maltophilia* (Cullman and Dick, 1990; Paton *et al*., 1994; Payne *et al.,* 1994; Al-Naiemi *et al*., 2006; Lavigne *et al*., 2008). Resistance to TMP-SMX has been attributed to the presence of *sul*1 genes located on class 1 integrons and *sul*2 genes carried on insertion sequence common region (ISCR) elements. Overexpression of efflux pumps and low outer membrane permeability are the features responsible for resistance to the quinolones (Looney *et al.*, 2009). In addition to being an opportunistic pathogen in humans, *S. maltophilia* strains have also been used in biotechnology and in the control of plant pathogens but it has been demonstrated that all strains share the intrinsic resistance phenotype irrespective of whether they are clinical or environmental isolates (Berg *et al.,* 1999; Sanchez *et al.,* 2009).

2.1.5 *Stenotrophomonas maltophilia* as an opportunistic human pathogen

Little is known about the origin of pathogenic strains of *S. maltophilia* responsible for nosocomial infections. Molecular studies done on nosocomial isolates suggest the environment may be a major source of acquisition of the bacteria by patients as identical strains have been found in both hospital and environmental sources (Muder, 2007) and genetic analysis suggests that *S. maltophilia* has adapted to human colonisation by losing certain plant pathogenic traits and gaining potential human virulence factors (Crossman *et al*., 2008). It has been implicated in several different infections including urinary tract infections, ocular infections, respiratory tract infections, meningitis, bacteremia, skin and soft tissue infections often affecting immunocompromised patients and patients with cystic fibrosis. It can however, also cause community acquired infections (Heath and Currie, 1995; Denton and Kerr, 1998; Falgas *et al.,* 2009).

Significant risk factors that have been associated with *S. maltophilia* infections include: prolonged hospitalization, admission to intensive care unit, central venous catheterization, neutropenia, mechanical ventilation and prior antibiotic therapy (Vartivarian *et al.,* 1994; Senol *et al.*, 2002; Senol, 2004; Looney *et al.*, 2009). It is also being increasingly reported in opportunistic infections affecting patients with HIV (Calza *et al.,* 2003).

Infections caused by *S. maltophilia* are difficult to treat because the organism is resistant to majority of the available broad spectrum antibiotics including most antipseudomonal β-lactam antibiotics, aminoglycosides and the older fluoroquinolones. It is particularly inherently resistant to the carbapenems (imipenem and meropenem) (Looney, 2005) which gives it an increased chance of survival over other potential pathogens in the hospital environment (Avison *et al.,* 2000).

The most active single drugs against the organism are ticarcillin/clavulanate and trimethoprim-sulfamethoxazole but TMP-SMX is still the recommended therapy of choice for the treatment of *S. maltophilia* infections (Hanberger *et al.*, 2001). However the action of TMP-SMX is bacteriostatic and several studies have reported an increasing prevalence of *S. maltophilia* strains that show resistance to this drug of choice (Tsiodras *et al.*, 2000; Gales *et al.*, 2001; San Gabriel *et al.*, 2004). The emergence of TMP-SMX resistance has prompted the use of the newer fluoroquinolones either alone or in combination with other antibiotics (Krueger *et al.*, 2001; Friedman *et al.*, 2002). A study by Weiss *et al.* (2000) reported better *in vitro* activity of the newer quinolones; trovafloxacin, clinafloxacin and moxifloxacin compared to ciprofloxacin and levofloxacin.

2.2 EXTENDED SPECTRUM BETA LACTAMASES

Extended spectrum β-lactamases (ESBLs) are a recent and increasingly important problem in the area of infectious diseases (Wright and Eiland III, 2008). Their production is the predominantly recognized mechanism of resistance to β-lactam antibiotics in clinically important Gram-negative bacteria (Bonnet, 2004; Bush and Jacoby, 2010).

The TEM-1 enzyme was the first plasmid-mediated β-lactamase to be described originally in a single strain of *E. coli* isolated from the blood culture of a patient in Greece and the assignation TEM was derived from the patient's name; Temoniera. Another plasmid-borne β-lactamase was also found at about the same time in *Klebsiella pneumoniae* and *E. coli* and was designated SHV-1 (sulfhydryl variable) (Turner, 2005). The spread of the enzyme was facilitated by the fact that it is plasmid- and transposon-mediated. It is now found worldwide in several organisms including but not limited to members of the family

Enterobacteriaceae, *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Neisseria gonorrhoeae* (Bradford, 2001; Turner, 2005; Wright and Eiland III, 2008). The enzymes are thought to have evolved from penicillin binding proteins with which they show some sequence homology (Bradford, 2001). Many Gram-negative bacteria possess a naturally occurring chromosomally mediated β-lactamase which probably assists the bacteria in finding a niche when faced with competition from other bacteria that naturally produce beta-lactams (Bradford, 2001; Turner, 2005).

The discovery and spread of these enzymes drove the development and introduction of novel antibiotics of the β-lactam class by the pharmaceutical industry in an attempt to tackle the resistance problems posed by the newly discovered beta lactamases. However, new beta-lactamases emerged with the introduction of each new class of drugs. The development of the oxymino-cephalosporins was a major achievement and these drugs became the treatment of choice for serious Gram-negative infections because of resistance to hydrolysis by the TEM-1 and SHV-1 enzymes (Schwaber *et al*., 2005). The result has, however, been the development of a more diverse and potentially more devastating group of beta-lactamases which have progressed from ESBLs to AmpC enzymes (Wright and Eiland III, 2008).

Resistance to the new expanded spectrum cephalosporins emerged rapidly and was first described in a *K. pneumoniae* isolate in Germany and was named SHV-2 because of its amino acid similarity to the SHV-1 enzyme. This and several other enzymes subsequently discovered were then named extended spectrum beta-lactamases (ESBLs) because of their ability to hydrolyse these expanded spectrum β-lactam antibiotics (Turner, 2005).

2.2.1 Classification of beta lactamases

Beta-lactamases as a whole are classified according to two schemes: the Ambler molecular classification system and the Bush-Jacoby-Medieros functional classification system (Ambler *et al.,* 1991; Bush *et al.,* 1995). The Ambler classification divides the beta lactamases into four classes: A through D based on amino acid similarities. Classes A, C and D are referred to as serine beta lactamases because these enzymes hydrolyse their substrates by forming an acyl enzyme through the serine active site while the class B enzymes employ at least one active site zinc ion to hydrolyse their substrates and are therefore referred to as metallo-enzymes (Bush and Jacoby, 2010). Though this structural classification appears to be the simplest way to classify this diverse group of enzymes, the functional classification based on their different substrate profiles assists in relating the properties of the enzymes to their clinical implications and observed antibiogram characteristics.

In an attempt to more fully and efficiently describe the growing group of beta lactamase enzymes, Bush and Jacoby (2010) published an updated classification system (shown in Table 2.2) which is essentially an updated functional classification scheme that considers the substrate and inhibitor profiles while correlating the major groupings with the simpler molecular classification. This assists in grouping the enzymes in ways that relate with observed clinical phenotypes. The ESBLs are, therefore, defined as beta-lactamases capable of hydrolysing oxymino-cephalosporins that are inhibited by clavulanic acid and are placed in functional group 2be. The majority of ESBLs have an active serine site and belong to the Ambler class A which have a molecular mass of approximately 29kDa and preferentially hydrolyse penicillins (Bradford, 2001).

Class A ESBLs consists of a heterogeneous molecular cluster which comprises beta-lactamases that share 20 to 99% of their identities (Bradford, 2001). The enzymes are derived from the originally plasmid-mediated TEM-1/2 and SHV-1 penicillinases and differ from them by one to four point mutations. These mutations are responsible for the expanded hydrolytic spectra of the ESBLs. TEM and SHV ESBLs now consist of more than 500 different types; most of which are ceftazidimases and are found worldwide (Bradford, 2001; Bonnet, 2004; Bush and Fisher, 2011). The class D enzymes also belong to group 2 and include the OXA-type ESBLs which are so called because they hydrolyse oxacillin but are poorly inhibited by clavulanic acid. OXA-type ESBLs are predominantly found in *Pseudomonas aeruginosa* but have also been reported in other Gram-negative bacteria (Deepthi and Deepthi, 2010).

Many other non-TEM and non-SHV plasmid-mediated ESBLs have been reported. The most widespread of these are the CTX-M types which have been reported in organisms in every geographical area of the world (Bonnet, 2004; Paterson and Bonomo, 2005). CTX-M ESBLs are potent hydrolysers of cefotaxime from which this group of enzymes derive their name while minimum inhibitory concentration of ceftazidime may be in the susceptible range. It should, however, be noted that the same organism may have both SHV and CTX-M types and AmpC types which may alter the antibiotic resistance pattern (Paterson and Bonomo, 2005; Bush and Fisher, 2011).

***Table 2.2: Classification schemes for bacterial β-lactamases (*Bush and Jacoby, 2010).**

Bush-Jacoby group (2009)	Bush-Jacoby-Medeiros group (1995)	Molecular class (subclass)	Distinctive substrate(s)	Inhibited by		Defining characteristic(s)	Representative enzyme(s)
				CA or TZB[a]	EDTA		
1	1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins	*E. coli* AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1
1e	NI[b]	C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often other oxyimino-β-lactams	GC1, CMY-37
2a	2a	A	Penicillins	Yes	No	Greater hydrolysis of benzylpenicillin than cephalosporins	PC1
2b	2b	A	Penicillins, early cephalosporins	Yes	No	Similar hydrolysis of benzylpenicillin and cephalosporins	TEM-1, TEM-2, SHV-1
2be	2be	A	Extended-spectrum cephalosporins, monobactams	Yes	No	Increased hydrolysis of oxyimino-β-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	2br	A	Penicillins	No	No	Resistance to clavulanic acid, sulbactam, and tazobactam	TEM-30, SHV-10
2ber	NI	A	Extended-spectrum cephalosporins, monobactams	No	No	Increased hydrolysis of oxyimino-β-lactams combined with resistance to clavulanic acid, sulbactam, and tazobactam	TEM-50
2c	2c	A	Carbenicillin	Yes	No	Increased hydrolysis of carbenicillin	PSE-1, CARB-3
2ce	NI	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime, and cefpirome	RTG-4
2d	2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	NI	D	Extended-spectrum cephalosporins	Variable	No	Hydrolyzes cloxacillin or oxacillin and oxyimino-β-lactams	OXA-11, OXA-15
2df	NI	D	Carbapenems	Variable	No	Hydrolyzes cloxacillin or oxacillin	OXA-23, OXA-48

Bush-Jacoby group (2009)	Bush-Jacoby-Medeiros group (1995)	Molecular class (subclass)	Distinctive substrate(s)	Inhibited by		Defining characteristic(s)	Representative enzyme(s)
				CA or TZB[a]	EDTA		
						and carbapenems	
2e	2e	A	Extended-spectrum cephalosporins	Yes	No	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam	CepA
2f	2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyimino-β-lactams, cephamycins	KPC-2, IMI-1, SME-1
3a	3	B (B1)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, CcrA, IND-1
		B (B3)					L1, CAU-1, GOB-1, FEZ-1
3b	3	B (B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, Sfh-1
NI	4	Unknown					

a CA, clavulanic acid; TZB, tazobactam.

b NI, not included.

The other types of ESBLs that have been described include: PER, VEB, GES/IBC, TLO-1, and SFO-1 and BES-1 types. The PER type ESBLs share 25 to 27% similarity with the TEM- and SHV-types. These enzymes hydrolyse penicillins and cephalosporins effectively and are inhibited by clavulanic acid. These other ESBL types are not simple point mutations of any known beta-lactamases and have varying degrees of similarity with them. They have also been found in a wide variety of environments (Paterson and Bonomo, 2005). The GES/IBC ESBLs have been reported from hospital pathogens in South Africa and France (Weldhagen *et al.,* 2003) but there is limited data on the epidemiology of ESBL-producing organisms in South Africa and Africa as a whole. Recent studies which characterized ESBLs from South Africa have however revealed the presence of a diverse number of enzymes in various organisms (Pitout *et al*., 1998; Hanson *et al*., 2001; Goviden *et al.,* 2008; Ehlers *et al.,* 2009, Peirano *et al.,* 2011).

2.2.2 TEM β-lactamases

This group of β-lactamases was the first to be described in the 1960s and TEM-1 is the most common beta-lactamase responsible for resistance to β-lactam antibiotics in Gram-negative organisms worldwide (Ghuysen, 1991). It hydrolyses penicillins and the first generation cepahalosporins such as cephalothin and cephalodrine but has no activity against the extended spectrum cephalosporins. Its location on the Tn3 transposon and several transposition and rearrangement events has aided the migration into other species such as *H. influenzae* and *N. gonorrhoeae* (Livermore, 1995).

TEM-2, the first derivative from TEM-1 had a single amino acid substitution which caused a shift in the isoelectric point from pI of 5.4 to 5.6 but had no effect on the substrate

profile. TEM-3, originally described in 1989 was the first variant with the ESBL phenotype and since then >90 additional derivatives have been described. The ESBL phenotype occurs as a result of several amino acid substitutions which include glutamate to lysine at position 104, arginine to serine or histidine at position 164, glycine to serine at position 238 and glutamate to lysine at position 240. Different combinations of these substitutions result in subtle changes in the substrate profiles of the enzymes or a change in the isoelectric points which may range from pI 5.2 to 6.5 (Bradford, 2001). Figure 2.1 shows the relevant amino acid substitutions and the various derivative phenotypes

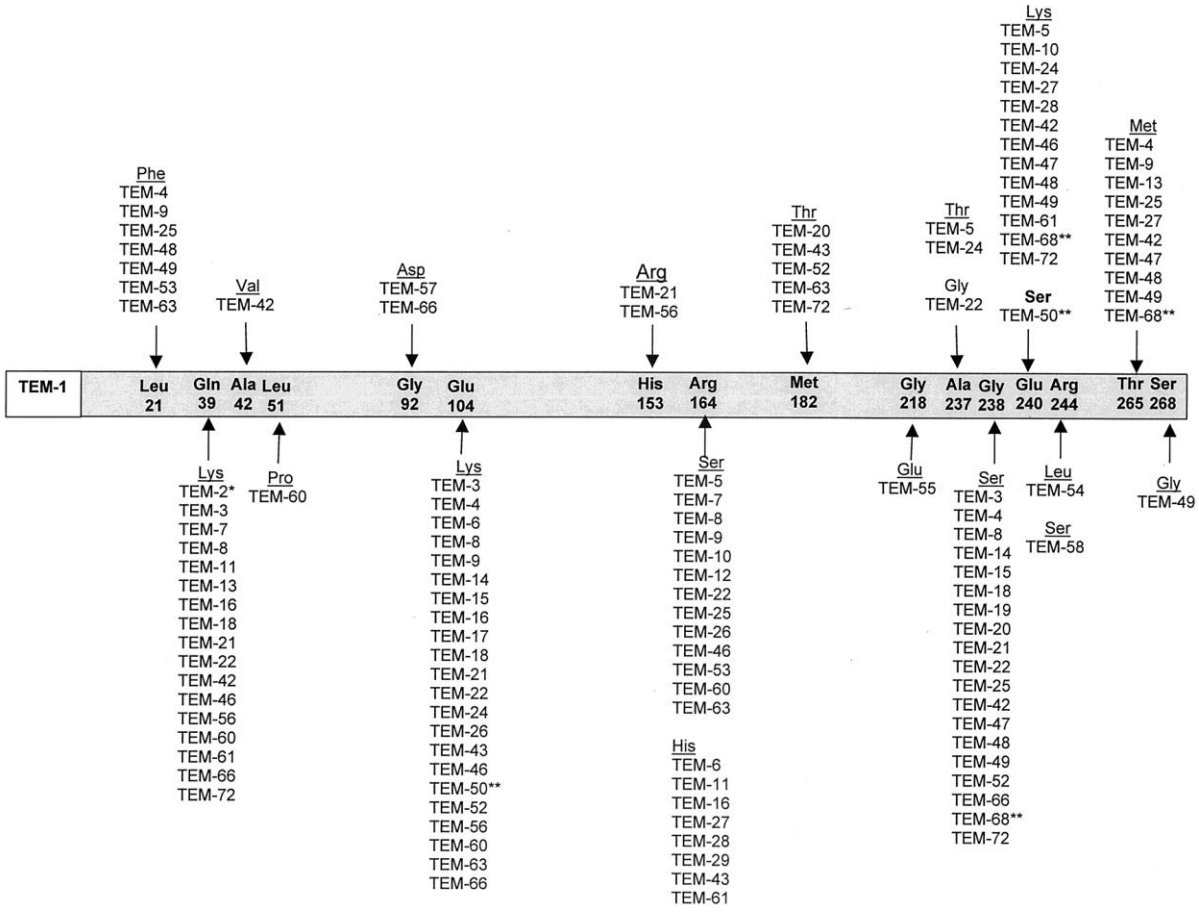

Figure 2.1: Showing the common amino acid substitutions with the resulting TEM phenotypes. Adapted from Bradford P.A. (2001).

Laboratory mutations of TEM-1 that contain substitutions at positions other than those that occur naturally have been described (Bradford, 2001) but naturally occurring TEM-type ESBLs are thought to have been the result of varying selective pressure from several β-lactams rather than selection with a single agent (Blazquez *et al.*, 2000). The ESBL phenotype changes the configuration of the active site of the enzyme enabling it to hydrolyse oxymino-cephalosporins while also enhancing its susceptibility to β-lactamase inhibitors such as clavulanic acid (Jacoby and Munoz-Price, 2005). Some TEM variants have been found to have ESBL type mutations identified together with those that confer resistance to β-lactamases inhibitors but this combination is rare as inhibitor-resistant β-lactamases are inefficient in hydrolysing the extended spectrum cephalosporins (Jacoby and Munoz-Price, 2005). Some derivatives have, however, been found to retain both activities at significant levels (Fiett *et al*., 2000).

Although TEM-type β-lactamases are most often found in *E. coli* and *K. pneumoniae*, they are also found in other species of Gram-negative bacteria such as *Enterobacter aerogenes, Morganella morganii, Proteus mirabilis, Proteus rettgeri*, and *Salmonella* spp. (Palzkill *et al.*, 1995, Morosini *et al.*, 1995, Mugnier *et al*., 1996, Bonnet *et al.*, 1999, Marchandin *et al.*, 1999). They have also been reported in non-*Enterobacteriaceae* Gram-negative bacteria like *Pseudomonas aeruginosa* (Bradford, 2001).

2.2.3 SHV β-lactamases

The SHV type ESBLs are probably the most commonly found in clinical isolates worldwide. About 50 derivatives of SHV-1 have been described with majority of them possessing an ESBL phenotype which is characterised by substitution of serine for glycine at position 238

and lysine for glutamate at position 240 (Al-Jasser, 2006*a*). These substitutions mirror those seen in TEM-type ESBLs at these positions (Bradford, 2001) and the serine residue is essential for the efficient hydrolysis of ceftazidime while the lysine residue is essential for the hydrolysis of cefotaxime (Huletsky *et al.*, 1993). One variant, SHV-10 has been found to have an inhibitor-resistant phenotype with an additional substitution of glycine for serine at position 130 (Bradford, 2001). These enzymes have been described mostly in *Enterobacteriaceae* but also in *P. aeruginosa* and *Acinetobacter* spp.

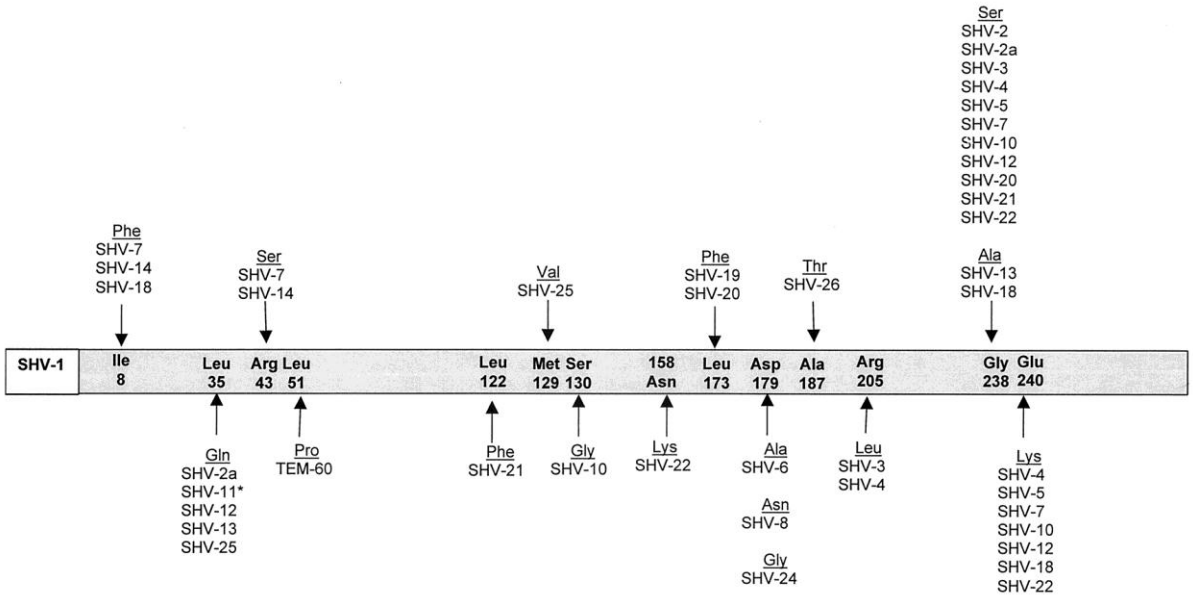

Figure 2.2: Showing the common amino acid substitutions in the SHV-1 enzyme with the resulting SHV phenotypes. Adapted from Bradford P.A. (2001).

2.2.4 CTX-M β-lactamases

The CTX-M enzymes are a group of plasmid-mediated enzymes that preferentially hydrolyse cefotaxime with variable activity against ceftazidime. These β-lactamases are thought to have originated from the soil organism; *Kluyvera ascorbata* (Humeniuk *et al.*, 2002) and show

only approximately 40% homology with the TEM or SHV β-lactamases (Bradford, 2001). Over 50 types of these enzymes have now been described and have been classified into five families based on phylogenetic studies: CTX-M-1 group consists of six plasmid-mediated enzymes: CTX-M-1, CTX-M-3, CTX-M-10, CTX-M-12, CTX-M-15 and FEC-1 including some unpublished enzymes. CTX-M-2 group consists of eight plasmid-mediated enzymes viz; CTX-M-2, CTX-M-4, CTX-M-4L, CTX-M-5, CTX-M-6, CTX-M-7, CTX-M-20 and Toho-1. CTX-M-8 is the sole member of its group. CTX-M-9 group consists of CTX-M-9, CTX-M-13, CTX-M-14, CTX-M-16, CTX-M-17, CTX-M-19, CTX-M-21, CTX-M-27 and Toho-2. CTX-M-25 group has two members: CTX-M-25 and CTX-M-26 (Bonnet, 2004).

The serine residue at position 237 is thought to play a role in the extended spectrum activity of these β-lactamases. They are also better inhibited by tazobactam than sulbactam and clavulanate. The CTX-M β-lactamases have been found in different species of Gram-negative bacteria worldwide and are now the most common enzymes in community isolates (Bush and Fisher, 2011; Peirano *et al.*, 2011).

2.2.5 OXA β-lactamases

This group of class D β-lactamases are characterised by the ability to hydrolyse cloxacillin and oxacillin and are poorly inhibited by clavulanate (Bush *et al.*, 1995). There is only about 20-30% amino acid homology between the members of the group so the enzymes are quite a diverse group in terms of structure (Bradford, 2001). OXA β-lactamases are found mainly in *P. aeruginosa* but have also been described in other Gram-negative bacteria. The ESBL variants have one of two amino acid substitutions: asparagine for serine at position 73 or

aspartate for glycine at position 157 which may be particularly necessary for high level resistance to ceftazidime (Danel *et al.,* 1999).

2.2.6 Laboratory detection of Extended Spectrum beta lactamases

The laboratory detection of ESBLs in microorganisms can be broadly classified into: (i) Phenotypic methods (screening and confirmatory tests) which are based on the ability of the ESBLs to hydrolyse third generation cephalosporins and be inhibited by β-lactamase inhibitors such as clavulanic acid. (ii) Genotypic methods which employ molecular techniques to determine the genes responsible for ESBL production in various organisms. The phenotypic tests are easy to do, cost-effective and have been incorporated into several automated systems for ESBL detection (Wiegand *et al*., 2007) but these tests are unable to differentiate between the types of ESBL enzymes hence, the need for genotypic tests. The molecular methods are also able to detect low levels of enzyme production which may otherwise be missed by the phenotypic tests (Woodford and Sundsfjord, 2005). Genotypic tests can also be carried out directly on clinical specimens eliminating the need for culturing and as a result reducing detection time significantly (Tenover, 2007). These genotypic methods are however cost intensive and are not readily available to smaller diagnostic laboratories. The screening test involves screening with an indicator cephalosporin which is based on resistance or diminished susceptibility of likely ESBL producers to these β-lactams. The confirmatory tests are based on synergy demonstrated between the oxymino cephalosporins and the β-lactamase inhibitor, clavulanic acid, confirming the production of extended spectrum beta-lactamases by those isolates.

2.2.6.1 Screening Tests

The disk diffusion method can be used to screen for the presence of ESBLs by including one or more indicator cephalosporins (cefpodoxime, ceftazidime, cefotaxime, aztreonam or ceftriaxone) in the antibiotic panel for susceptibility tests. However, because the substrate profiles for the ESBLs differ, the Centre for Laboratory Standards Institute (CLSI) (formerly NCCLS) recommends the use of more than one cephalosporin to improve the sensitivity of detection (NCCLS, 2005). It is sufficient to use ceftazidime which is a consistently good substrate for the TEM and SHV variants and cefotaxime which is a substrate for the CTX-M enzymes. Cefpodoxime has been reported as the best single indicator drug (if only a single drug can be used) (Jarlier *et al.*, 1988) but it can yield a high number of false positive results due to other mechanisms of β-lactam resistance (Livermore and Paterson, 2006). Isolates that show resistance or diminished susceptibility to any of these agents should be further subjected to the confirmatory test.

Dilution methods can also be used to screen for ESBL production. Ceftazidime, aztreonam, cefotaxime and ceftriaxone are used at a screening concentration of 1µg/ml or cefpodoxime at a concentration of 1µg/ml for *Proteus mirabilis* and 4µg/ml for the others. The presence of growth at or above these concentrations is indicative of possible ESBL production (NCCLS, 2005).

2.2.6.2 Phenotypic confirmatory tests

2.2.6.2.1 Combination disk tests

This involves the use of antimicrobial disks containing a cephalosporin with or without clavulanate. The test is performed following the same procedures as for normal disc diffusion tests on Mueller-Hinton agar plates inoculated with the test organism and incubated for 16-18h. The zone diameters are then measured and a difference of >5mm between the zone diameters of the cephalosporin alone and the combined cephalosporin/clavulanate disk phenotypically confirms the production of ESBLs (NCCLS, 2005).

2.2.6.2.2 Broth microdilution

Broth microdilution tests are carried out according to the standard methods as recommended by CLSI using ceftazidime (0.25-128µg/ml), ceftazidime plus clavulanic acid (0.25/4-128/4µg/ml), cefotaxime (0.25-64µg/ml) or cefotaxime plus clavulanic acid (0.25/4-64/4µg/ml) (Queenan et al., 2004). A decrease of $\geq$ 3 twofold serial dilution in minimum inhibitory concentration (MIC) of any of the cephalosporins in combination with clavulanic acid compared to the MIC of the cephalosporin alone confirms ESBL production (NCCLS, 2005).

2.2.6.2.3 Double disk synergy test (DDST)

This test is also based on demonstrable synergy between cephalosporins and clavulanic acid which is a β-lactamase inhibitor. It was described by Jarlier *et al.* (1988) and is technically easy to perform but requires experienced interpretation. Two to four disks of third generation cephalosporins are placed on either side of a centrally placed augmentin (amoxicillin+clavulanic acid) disk at a distance of 30mm centre to centre on a Mueller-Hinton agar plate. An extension of the zone of inhibition of any of the cephalosporins towards the augmentin disk confirms ESBL production (Jarlier *et al.,* 1988).

Several studies have compared the various methods of ESBL detection and have reported the sensitivity of DDST as ranging between 79%-97% to 94% - 100% (Vercauteren *et al.,* 1997; Randegger *et al.,* 2001). The sensitivity is also low when extended spectrum β-lactamase activity is very low causing wide zones of inhibition around the cephalosporin and aztreonam disks (Revathi and Singh, 1997). False negative results have been reported in organisms harbouring SHV-2, SHV-3 or TEM-12 enzymes (Thomson and Sanders, 1992; Vercauteren *et al.,* 1997; Ho *et al.,* 1998). False positive results also occur in *S. maltophilia* because aztreonam is not a substrate for the metalloenzymes and clavulanic acid inhibits other β-lactamases produced by this organism and not just the ESBLs (Munoz-Bellido and Garcia-Rodriguez, 1998). In order to improve sensitivity of the test, some authors have reduced the distance between the disks to 20mm in isolates strongly suspected to harbour ESBLs but producing negative results (Thomson and Sanders, 1992; Ho *et al.,* 1998; Tzelepi *et al.,* 2000) or the distance can also be increased to 40mm in those that produce wide zones of inhibition.

2.2.6.2.4 Three dimensional test

The three dimensional test was first described by Thomson and Sanders (1992) and it provides evidence of inactivation of extended spectrum cephalosporins or aztreonam by ESBLs without the use of β-lactam inhibitors. The surface of a Mueller-Hinton agar plate is inoculated as per standard procedure for diffusion susceptibility tests and in addition a circular slit is cut in the agar along the axis of the margin. A heavy inoculum of the test organism (10^9 to 10^{10} CFU/ml) is then pipetted into the slit and disks impregnated with the β-lactams are placed on the surface of the agar 3mm from the slit. Inactivation of the drug due to beta lactamase activity is visualised as a distortion of the circular inhibition zone or the presence of discrete colonies near the slit (Thompson and Sanders, 1992). This test is however, technically challenging to execute though highly sensitive (Bradford, 2001).

2.2.6.2.5 Inhibitor-potentiated disk diffusion tests

Disks containing ceftazidime, cefotaxime, ceftriaxone and aztreonam are placed simultaneously on Mueller-Hinton agar plates supplemented with 4µg/ml clavulanic acid and those without clavulanic acid and incubated. A difference of >10mm in the zone diameters in the two media denotes a positive result for ESBL production (Ho *et al.,* 1998). The clavulanate containing plates, however, need to be freshly prepared as the potency of clavulanic acid begins to decrease after 72 hours.

2.2.6.2.6 Cephalosporin/Clavulanate combination disks on Iso-sensitest agar

This is the method recommended by the British Society for Antimicrobial Chemotherapy (BSAC) which interprets semi-confluent growth on Iso-sensitest agar using ceftazidime-clavulanate and cefotaxime-clavulanate combination disks as positive for ESBL production. A ratio of cephalosporin/clavulanate size to cephalosporin zone size of 1.5 or more signifies ESBL activity. The sensitivity is reported to be 93% for both ceftazidime and cefotaxime (M'zali *et al*., 2000; Deepthi and Deepthi, 2010).

2.2.6.2.7 Disk approximation tests

A cefoxitin disk is placed at a distance of 2.5cm from the cephalosporin disk and production of inducible β-lactamases is indicated by flattening of the zone of inhibition of the cephalosporin disk towards the inducer disk by >1mm. There are also commercial methods available for ESBL detection (Revathi and Singh, 1997).

2.2.6.2.8 E-Test

The E-test (AB Biodisk, Solna, Sweden) is based on the MIC method and consists of a test strip which carries two gradients on opposite ends. One side contains the third generation cephalosporin while the other end carries the cephalosporin/clavulanic acid combination. The MIC is taken as the point where the ellipse of inhibition intersects with the E-test strip edge and a ratio of cephalosporin MIC to cephalosporin/clavulanic acid MIC ≥8 indicates ESBL

production. The sensitivity of the E-test ranges from 87-100% and specificity from 95-100% (Cormican *et al.*, 1996).

2.2.6.2.9 Vitek ESBL test

This test also makes use of cefotaxime or ceftazidime (0.5µg/ml) alone and in combination with clavulanic acid (4µg/ml) for ESBL detection. The Vitek cards (bioMerieux Vitek, Hazelton, Missouri) are inoculated as per manufacturer's instructions and analysis of the wells is performed automatically after 4-15h of incubation at a set threshold. A predetermined reduction in the growth of wells containing cephalosporins with clavulanic acid compared with the wells containing cephalosporin alone indicates the presence of ESBLs (Sanders *et al*., 1996).

2.2.6.2.10 Phoenix Automated Microbiology System (Benton Dickinson Biosciences, Sparks, Md).

This method has been recommended as a rapid method for ESBL detection. It uses growth response to cefpodoxime, ceftazidime, ceftriaxone and cefotaxime with or without clavulanic acid to detect ESBL production. Results are usually available within 6 hours (Park *et al.*, 2007).

2.2.6.3 Genotypic detection of ESBLs

The molecular method most commonly used for ESBL detection is Polymerase chain reaction (PCR) amplification of *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$ genes using specific oligonucleotide primers and subsequent sequencing of the PCR amplicons. Nucleotide sequencing is the gold standard for identification of ESBL genes especially *bla*$_{TEM}$ and *bla*$_{SHV}$. It is necessary to differentiate between the non-ESBL parent enzymes (TEM-1, TEM-2, SHV-1) and other variants of the ESBL enzymes as point mutations around the active site of the parent enzymes have led to amino acid substitutions that have expanded their spectrum of activity (Bradford, 2001; Al-Jasser, 2006a).

Other methods that do not require sequencing have been developed and these include: PCR with restriction fragment length polymorphism (PCR-RFLP) (Arlet *et al.,* 1995), PCR with single strand conformational polymorphism (PCR-SSCP) (M'zali *et al.,* 1998), ligase chain reaction (Kim & Lee, 2000), restriction site insertion PCR and real time PCR (RT-PCR). The applications of these are, however, limited because of the ever-increasing number of ESBL subtypes and continuous evolution of the enzymes. Sequencing is also not always straightforward because some isolates may carry multiple copies of ESBL genes.

However, PCR amplification of *bla*$_{CTX-M}$ gene is often sufficient to conclude that a CTX-M enzyme is responsible for the ESBL phenotype. Methods that have been developed for rapid screening of isolates for bla$_{CTX-M}$ include PCR assay with four sets of primers to amplify group-specific CTX-M genes, amplification of a universal DNA fragment specific for the different groups of CTX-M β-lactamases, duplex PCR, multiplex PCR, RT-PCR, pyrosequencing and reverse-line hybridisation (Pitout *et al.,* 2004; Pitout and Laupland, 2008).

Molecular techniques play a huge role in the screening, tracking and monitoring of the spread of ESBL-producing organisms especially CTX-M enzyme producers in the community and hospital settings and it is hoped that the recent developments of more easily accessible and cost-effective molecular methods such as microarrays and rapid-cycle sequencing will make the detection and identification of these genes possible in real time (Pitout and Laupland, 2008).

2.2.6.4 Challenges of ESBL detection

The increased prevalence of ESBL-producing bacteria in the hospital and community makes it imperative to have laboratory testing methods that provide adequate and accurate identification of these enzymes especially in clinical isolates. This, however, remains a huge challenge as the detection of ESBL-producing organisms is complex due to multiple factors which include production of multiple different β-lactamase types by a single bacterial isolate, their varying substrate affinities, the production of ESBLs by organisms that constitutively produce the AmpC β-lactamase, the confounding factors that modify the expression of the enzymes and the inoculum effect (Gniadkowski, 2001; Al-Jasser, 2006*a*).

The MIC of extended spectrum cephalosporins rises dramatically as the inoculum size increases beyond that used in routine susceptibility testing resulting in false negative results when lower inocula are used (Deepthi and Deepthi, 2010). Studies done have showed that isolates which appear susceptible at the standard inoculum of 10^5/ml have highly elevated MICs at higher inocula of 10^7/ml or 10^8/ml (Jacoby, 1997). The coexistence of both AmpC β-lactamase and ESBLs in the same organism often results in elevated cephalosporin MICs leading to false negative test results for the detection of ESBLs and this may be due to the

fact that AmpC type β-lactamases resist inhibition by clavulanate thereby obscuring the synergistic effect of clavulanate and cepahalosporins (Deepthi and Deepthi, 2010).

2.2.7 Extended spectrum beta lactamases produced by *Stenotrophomonas maltophilia.*

S. maltophilia produces at least two clinically important beta lactamases designated L1 and L2 (Krueger *et al*., 2001). The L1 enzyme is produced by virtually all strains of the organism and belongs to the metallo-enzyme family. It is a zinc-dependent carbapenemase (Bush Group 3) which is not inhibited by clavulanic acid. It hydrolyses most beta lactam antibiotics including the carbapenems (imipenem and meropenem) which confers *S. maltophilia* with its characteristic resistance to this class of drugs. It is, however, unable to hydrolyse aztreonam and therefore, aztreonam may serve as a competitive inhibitor of the L1 enzyme (Munoz Bellido *et al*., 1997). The L2 enzyme belongs to Bush group 2e and is a cephalosporinase which is inhibited by clavulanic acid. It possesses a serine active site, exists as a dimer in its natural state and is most closely related to the TEM beta lactamases. It has the ability to hydrolyse third generation cephalosporins and aztreonam (Munoz Bellido *et al.,* 1997; Denton and Kerr, 1998).

Several studies have shown the existence of other beta lactamases produced by *S. maltophilia*. Cullman and Dick (1990) described the presence of six distinct enzymes in 20 clinical strains and other reports have confirmed the heterogeneity of beta lactamase production in *S. maltophilia* (Paton *et al*., 1994; Payne *et al.*, 1994). Al Naiemi *et al*. (2006) also described the presence of a CTX-M type beta lactamase in a strain of *S. maltophilia* isolated from the sputum of a neonate. As previously mentioned, the presence of these ESBLs

provides the organism with an even more powerful resistance mechanism with serious clinical implications and further limited options for therapy. The ESBL genes are often found carried on plasmids, chromosomes and transposons accompanied by genes conferring resistance to other classes of antibiotics (Jacoby and Munoz-Price, 2005). The ability of these genes to be transferred easily poses a serious threat to the community (Wright and Eiland III, 2008) and this can be compared to the situation with methicillin resistant *Staphylococcus aureus* where the resistance mechanisms were first noted in important nosocomial pathogens followed by appearance of different clones in the community (Pitout *et al*., 2005).

A study by Kataoka *et al*. (2003) reports that the beta lactamases produced by *S. maltophilia* have the ability to aid the survival of strains of *P. aeruginosa* which are normally susceptible to imipenem when the two organisms grow together in culture. This further highlights the importance of studying the beta-lactamase enzymes produced by this organism in the environment.

2.3 TRIMETHOPRIM-SULFAMETHOXAZOLE

Trimethoprim-sulfamethoxazole (TMP-SMX), a fixed combination of two antimicrobials was first introduced in 1968 following the recognition of several advantages of the two drugs in combination over the use of each one individually (Kielhofner, 1990; Eliopoulous and Huovinen, 2001; Masters *et al.*, 2003). Trimethoprim is a diaminopyrimidine, a structural analog of the pteridine portion of dihydrofolic acid while sulfamethoxazole is a sulfonamide, a structural analog of para-aminobenzoic acid (PABA) (Rubin and Swartz 1980, Masters *et al.*, 2003). The antibiotic is available for use in a fixed drug ratio of one part TMP to five parts SMX (Rubin and Swartz, 1980).

2.3.1 Mechanism of action

Bacteria are obligate folic acid synthesizers and both TMP and SMX inhibit the synthesis of tetrahydrofolic acid thereby preventing the production of thymidines, purines and bacterial DNA effectively leading to a disruption of bacterial replication (Masters *et al.*, 2003). Sulfamethoxazole blocks the synthesis dihydrofolic acid from para-aminobenzoic acid by inhibiting dihydropteorate synthetase while in the subsequent step trimethoprim prevents the formation of tetrahydrofolic acid from dihydrofolic acid by inhibiting the action of dihydrofolic acid reductase. This sequential blockade of two enzymes in the same crucial pathway is efficiently bactericidal (Eliopoulos and Huovinen, 2001; Masters *et al.*, 2003). It was also hoped that the use of these two drugs in one combination would prevent the development of bacterial resistance to any of the individual components alone (Masters *et al.*, 2003).

2.3.2 Resistance to TMP-SMX

Resistance of bacteria to TMP-SMX is thought to be mediated by five main mechanisms: the development of permeability barriers and/or efflux pumps, naturally unresponsive target enzymes, regulational changes in the target enzymes, mutations or recombinational changes in the target enzymes and acquired resistance by drug-resistant target enzymes (Eliopoulos and Huovinen, 2001). Resistance can also be plasmid or transposon-mediated, particularly the plasmid-mediated production of TMP-resistant forms of dihydrofolate reductase (Kielhofner, 1990; Masters *et al*., 2003).

2.4 THE FLUOROQUINOLONES

The fluoroquinolones are a group of synthetic antimicrobial agents whose origins lie in the use of chloroquine for the treatment of malaria. During the commercial production of chloroquine, a by-product found to have antibacterial activity was isolated and modified to produce the first quinolone, nalidixic acid (Appelbaum and Hunter, 2000; Anderson and McGowan, 2003; Enami *et al.*, 2005). The use of nalidixic acid was, however, limited due to the need for frequent administration, a high tendency to select for resistant Gram-negative bacilli, poor activity against Gram-positive bacteria, photosensitivity reactions in patients and the potential to cause convulsions in patients with seizure disorders (Zhanel *et al.*, 1999).

Since then, several modifications to the basic structure have led to the development of new members of the class which have evolved from compounds with limited spectrum to the newer agents with remarkable broad spectrum activity (Anderson and McGowan, 2003; Bolon, 2009; Sharma *et al.*, 2009). The fluoroquinolones have become the only synthetic drugs to rival the β-lactams in terms of impact in clinical usage. This increased usage has been attributed to their rapid bactericidal effect against most organisms that are susceptible which is of growing importance clinically because of the increasing number of patients who are critically ill or immunosuppressed. The fluoroquinolones also penetrate tissues and mammalian cells effectively; important when the offending organisms are intracellular (Appelbaum and Hunter, 2000).

2.4.1 Structure of Quinolones

All members of the quinolone group possess one of two types of ring structures: a naphythyridone nucleus which has nitrogen at position 1 and 8 or a quinolone nucleus with only nitrogen at position 1. They also have an exo-cyclic oxygen and carboxylic acid side chain at position 3 (Appelbaum and Hunter, 2000). Most of the new fluoroquinolones are analogs of the basic quinolone nucleus with important structural modifications occurring at positions 1, 5, 7 and 8 leading to differences between the various agents (Zhanel *et al.*, 1999; Enami *et al.*, 2005; Sharma *et al.*, 2009).

2.4.2 Classification of fluoroquinolones

Fluoroquinolones can be classified into four generations based on their activity against microorganisms. The first generation fluoroquinolones include nalidixic acid and cinoxin which have moderate Gram-negative activity and are no longer commonly used in the treatment of infections because frequent dosing is required and bacteria easily acquire resistance to these agents (King *et al*., 2000). Lomefloxacin, norfloxacin, enoxacin, ofloxacin and ciprofloxacin are the second generation fluoroquinolones which have improved Gram-negative coverage as well as some Gram-positive and atypical pathogen coverage. These drugs have wider clinical application than their first generation counterparts (King *et al.*, 2000; Oliphant and Green, 2002).

The newer fluoroquinolones are, therefore, those of the third and fourth generation which include levofloxacin, sparfloxacin, moxifloxacin, gatifloxacin and trovafloxacin. They retain the improved Gram-negative coverage of the second generation drugs but also have

improved Gram-positive activity particularly against penicillin-susceptible and penicillin-resistant *Streptococcus pneumoniae* and atypical pathogens such as *Mycoplasma pneumoniae* (King *et al.*, 2000). The fourth generation drug trovafloxacin differs only in the fact that it also covers anaerobic organisms and has an even more improved Gram-positive coverage (Oliphant and Green, 2002)

2.4.3 Mechanism of action

The fluoroquinolones act by inhibiting the bacterial DNA gyrase and topoisomerase IV enzymes which are involved in bacterial DNA synthesis thereby enabling the drugs to be both specific and bactericidal (Blondeau, 2004; Bolon, 2009; Sharma *et al.*, 2009). DNA gyrase inserts negative superhelical twists in the double helix of bacterial DNA ahead of the replication fork thereby aiding the separation of daughter chromosomes. This is crucial for the initiation of DNA replication and the binding of initiation proteins. It is a type II topoisomerase composed of two GyrA and two GyrB monomeric subunits which are encoded by the *gyr*A and *gyr*B genes (Hooper, 1995; Blondeau, 2004). It has been proposed that the fluoroquinolones bind to the DNA binding groove between the A and B subunits leading to conformational changes to DNA gyrase molecule (Hooper, 1995).

The second target for the fluoroquinolones is the topoisomerase IV enzyme. Topoisomerase IV is responsible for decatenation which refers to the removing of the interlinking daughter chromosomes thereby allowing separation into two daughter cells at the end of a round of replication (Kato *et al.,* 1990). It consists of four homologous monomeric subunits, two ParC and two ParE subunits which are encoded by the parC and pare genes respectively. The fluoroquinolones interact with the enzyme bound DNA complex to create

conformational changes that result in the inhibition of normal enzyme activity. Consequently, the new drug-enzyme-DNA complex blocks progression of the replication fork, thereby inhibiting normal bacterial DNA synthesis and resulting ultimately in rapid bacterial cell death (Blondeau, 2004).

2.4.4 Advantages of the newer fluoroquinolones

Unlike the earlier quinolones that required frequent dosing and had several unwanted side effects, the newer agents display a high bioavailability, low protein binding and longer elimination half-lives (Applebaum and Hunter, 2000). Once daily administration also significantly increases patient compliance. Resistance to fluoroquinolones can arise from mutations or alterations in the target sites; DNA gyrase and topoisomerase IV and mutations affecting the accumulation of the drugs in the bacteria such as variations in the uptake reflux system (Applebaum and Hunter, 2000; Emmerson and Jones, 2003; Sharma *et al*., 2009). Data from studies on resistant mutant strains suggests that the newer generation fluoroquinolones may have a dual binding mechanism of action inhibiting both DNA gyrase and topoisomerase IV and owing to the rarity of double mutations, the preferential use of the newer drugs could potentially limit the emergence of fluoroquinolones resistance (Blondeau, 2004).

Several studies have reported the *in vitro* activity of the newer fluoroquinolones compared with older fluoroquinolones against *S. maltophilia* (Sanchez-Hernandez *et al.,* 1999; Munoz-Bellido *et al.,* 2000; Weiss *et al.,* 2000; Ribera *et al*, 2002). The study of Valdezate *et al*. (1999) reported that the minimum inhibitory concentrations of the newer fluoroquinolones were eight fold lower than those for ofloxacin and ciprofloxacin as well as

16-128 fold lower than those of perfloxacin, norfloxacin and nalidixic acid. The fluoroquinolones may also provide the option of choice for patients who are intolerant to the sulphur content of sulphamethoxazole.

CHAPTER THREE

ANTIBIOGRAM CHARACTERISTICS OF COMMENSAL *Stenotrophomonas maltophilia* ISOLATED FROM NKONKOBE MUNICIPALITY ENVIRONMENT IN THE EASTERN CAPE

3.0 ABSTRACT

Stenotrophomonas maltophilia has become an increasingly important nosocomial and opportunistic pathogen worldwide. Trimethoprim-sulphamethoxazole (TMP-SMX) is the recommended treatment for *S. maltophilia* infections but its effect is only bacteriostatic and resistance to the drug is being reported, hence the need for effective alternative treatment options. The fluoroquinolones have been proposed as a possible alternative and have shown good activity *in vitro*. This study reports the antimicrobial susceptibility profile of *S. maltophilia* obtained from plants rhizosphere in Nkonkobe municipality environment of Eastern Cape Pronvince of South Africa. 110 isolates were examined of which 63.38% were susceptible to TMP-SMX. The new fluoroquinolones, levofloxacin, gatifloxacin and moxifloxacin all showed good activity with susceptibilities of 94.7%, 92.3% and 88% respectively. Ciprofloxacin was the least effective fluoroquinolone with susceptibility of 75.9%. Resistance to the fluoroquinolones ranged from 1.3 - 2.7%. All TMP-SMX resistant isolates were susceptible to four or five of the fluoroquinolones. The new fluoroquinolones provide a possible alternative for the treatment of TMP-SMX resistant *S. maltophilia* infection in the Eastern Cape environment, but further studies are necessary in order to determine their effects *in vivo.*

Keywords: *Stenotrophomonas maltophilia*, trimethoprim-sulphamethoxazole resistance, fluoroquinolones, commensal.

3.1 INTRODUCTION

Stenotrophomonas maltophilia, a non-fermentative Gram-negative bacillus, which was initially thought to be a predominantly non-pathogenic environmental organism, is becoming progressively recognised as an important nosocomial organism responsible for significant morbidity and mortality in immunocompromised and debilitated patients (Denton and Kerr, 1998; Valdezate *et al.*, 2001; Hu *et al*., 2011). The increase in *S. maltophilia* infections has been attributed to advances in cancer treatment, the use of invasive therapeutic devices and the widespread use of broad spectrum antibiotics (Denton and Kerr, 1998; Koseoglu *et al*., 2004; Al-Jasser, 2006*a*; Falgas *et al.*, 2008) and potential risk factors for infection include chronic respiratory disorders (particulary cystic fibrosis), presence of an indwelling catether, prolonged endotracheal intubation, malignancies, previous exposure to antibiotics and prolonged hospitalisation (Hu *et al*., 2011). It has been implicated in several forms of infections including bacteremia, wound infections, skin infections, pulmonary infections, urinary tract infections, endocarditis and meningitis but treatment poses an immense challenge for clinicians as the bacteria is highly resistant to several classes of antibiotics further limiting treatment options (Alonso *et al.*, 2000).

S. maltophilia displays a vast array of resistance mechanisms which contribute independently or collectively to its multi-drug resistant status (Toleman *et al*., 2007). These include the expression of multiple β-lactamases, inducible efflux pumps, outer membrane impermeability (Valdezate *et al.*, 2001) and also the acquisition of integrons, plasmids and transposons which carry various resistance genes (Hu *et al*., 2011). It does not, however, show mutations in the quinolone-resistance determining regions (Toleman *et al*., 2007)

Trimethoprim-sulfamethoxazole (TMP-SMX) has been recommended as the treatment of choice for *S. maltophilia* infections based on results of *in vitro* susceptibility testing confirming effectiveness and favourable clinical outcomes observed in patients treated with this drug (Denton and Kerr, 1998; Berg *et al.*, 1999; Betriu *et al.*, 2002; Gales *et al.*, 2001; Krueger *et al.*, 2001). However, the effect of the drug is only bacteriostatic and it is not convenient to use in severely ill patients especially those who have polymicrobial infections that need complicated antibiotic regimens (Weiss *et al*., 2000). Furthermore, strains resistant to this drug have been reported although the mechanism of resistance is not well understood (Vartivarian *et al.*, 1994; Chang *et al.*, 2007; Nicodemo and Paez, 2007; Hu *et al.*, 2011). There are also issues relating to allergic reactions from the use of TMP-SMX due to the accumulation of the nitroso metabolite of sulphamethoxazole leading to the formation of sulphamethoxazole-specific antibodies as well as gluthathione and ascorbic acid deficiency (Cheng *et al.*, 2008; Falgas *et al*., 2008).

Several studies have proposed that the new quinolones could be an alternative therapeutic option for the treatment of infections caused by this organism (Weiss *et al.*, 2000; Valdezate *et al*., 2001; Giamarellos-Bourboulis *et al.*, 2002; Nicodemo *et al.*, 2004). This study was thus carried out in order to determine the antibiotic susceptibility profile of *Stenotrophomonas maltophilia* isolates in the Eastern Cape environment and compare the effects of the newer fluoroquinolones and TMP-SMX on these isolates.

3.2 MATERIALS AND METHODS

3.2.1 Source of Isolates

One hundred and ten (110) *S. maltophilia* isolates were obtained from the culture collection of the Applied and Environmental Microbiology Research Group (AEMREG), University of Fort Hare for use in this study. The bacteria had been previously isolated from the rhizosphere of plants in the Eastern Cape environment and identified using standard biochemical methods and confirmed using the API 20NE identification kit. The isolates were then confirmed up to species level using species specific PCR and stored at -20^{o}C.

3.2.2 Antibiotic susceptibility tests

The antibiogram characteristics of the isolates was determined according to the Kirby-Bauer method for disc diffusion susceptibility testing following the CLSI (formerly NCCLS) (NCCLS, 2005) standardized guidelines for susceptibility testing using commercial antibiotic discs. A total of 21 antibiotic discs (MAST Diagnostics, Merseyside, United Kingdom) were used which include: meropenem (30µg), ampicillin (10µg), ampicillin-sulbactam (30µg), cefuroxime (30µg), aztreonam (30µg), trimethoprim-sulphamethoxazole (1.25µg+23.75µg), trimethoprim (5µg), minocycline (30µg), kanamycin (30µg), ofloxacin (5µg), ciprofloxacin (5µg), levofloxacin (5µg), gatifloxacin (5µg), moxifloxacin (5µg), ceftazidime (30µg), cefpodoxime (10µg), cefepime (30µg), cefotaxime (30µg), augmentin (30µg), colistin sulphate (10µg) and polymyxin B (300U).

3.2.3 Preparation of McFarland Standard

Exactly 0.5 ml of 0.048 M $BaCl_2$ (1.17% w/v $BaCl_2.2H_2O$) was added to 99.5 ml of 0.18 M H_2SO_4 (1% w/v) while constantly stirring. The suspension was thoroughly mixed to ensure that it is even and then using a 1cm light path, a matched cuvette and water as a blank standard, the absorbance was measured in a spectrophotometer at a wavelength of 625nm. The acceptable absorbance range for the standard is 0.08 – 0.13. The standard was then stored in tubes of the same size and volume as those that were used in standardizing the inoculums and stored at room temperature away from light.

3.2.4 Standardization of inocula

The stock cultures were re-activated by subculturing into tubes containing nutrient broth and incubated for 24h at 37^{o}C with shaking. The organisms were further subcultured onto nutrient agar plates prepared according to manufacturer's instructions and again incubated for 24h at 37^{o}C. Three to five colonies from each plate were then suspended in tubes containing 5ml of sterile distilled water and vortexed thoroughly to achieve a uniform suspension. The turbidity of the suspension was compared to that of the 0.5 McFarland standard and adjusted as required. The standardized inocula were used in the antibiotic susceptibility tests within 15 minutes (NCCLS, 2005).

3.2.5 Inoculation of plates

Freshly prepared Mueller-Hinton agar plates were inoculated with the bacterial suspension using a sterile swab. The plates were carefully streaked all over in three directions to ensure uniform growth. Antibiotic discs were then applied to the surface of the agar using sterile forceps and the plates incubated at 35^{o}C for 18-24h. The zones of inhibition were then measured using a ruler and interpreted using available interpretive charts. *S. maltophilia* DSM 50170 was used as a control strain.

3.3 RESULTS

The antibiogram profile of the *Stenotrophomonas maltophilia* isolates tested is as shown in the Table 3.1. The antibiogram results showed that only 63.4% of the isolates were susceptible to trimethoprim-sulfamethoxazole. Susceptibility to the fluoroquinolones, however, ranged from 80% to 94.7% with levofloxacin showing the highest susceptibility and ciprofloxacin being the least effective of the fluoroquinolones. Resistance ranged between 1.3% and 2.7%. Isolates also showed high susceptibility to the polymyxins, colistin sulphate and polymyxin B, with both having 97.2% susceptibility rates.

Susceptibility to the cephalosporins was variable with ceftazidime being the most effective with 81.8% of the isolates being susceptible to the drug. The isolates were, however, highly resistant to cefotaxime and particularly cefpodoxime with 81.6% and 98.7% resistance respectively. Despite *S. maltophilia* isolates being inherently resistant to the carbapenems, 87.3% of the isolates in this study showed zone diameters within the susceptible range for meropenem according to the interpretive charts. Resistance to trimethoprim and cefuroxime

was 94.4% and 95.8% respectively. All the isolates that were interpreted as resistant or intermediate to TMP-SMX were susceptible to the fluoroquinolones tested. However, four of the isolates only showed intermediate susceptibility to ciprofloxacin and all were susceptible to the remaining fluoroquinolones.

The multiple antibiotic resistance (MAR) index, when applied to a single isolate, is defined as [a/b], where [a] represents the number of antibiotics to which the isolates was resistant and [b] represent the number of antibiotics against which the isolate was tested. MAR index higher than 0.2 identifies organisms that originate from high-risk sources of contamination, where antibiotics are often used (Krumperman, 1983). The *S. maltophilia* isolates in this study showed multiple drug resistance to between one and up to thirteen antibiotics in the panel tested with the MAR indices ranging from 0.06 to 0.62 (Fig 3.1). Over 80% of the isolates had an MAR index greater than 0.2 and about 1% of the isolates were resistant to thirteen of the twenty-one antibiotics tested resulting in an MAR index of 0.7.

Table 3.1: Antibiogram of the commensal *Stenotrophomonas maltophilia*.

ANTIBIOTICS	SUSCEPTIBLE (%)	INTERMEDIATE (%)	RESISTANT (%)
Meropenem	87.3	3.8	8.9
Ampicillin	42.6	1.5	55.9
Minocycline	93.7	0	6.3
Kanamycin	40.6	7.2	52.2
Ofloxacin	89.2	8.1	2.7
Ciprofloxacin	76.0	21.5	2.5
Levofloxacin	94.7	4.0	1.3
Moxifloxacin	88.0	9.3	2.7
Gatifloxacin	92.3	6.4	1.3
Ceftazidime	81.8	5.2	13.0
Cefpodoxime	0	1.3	98.7
Cefepime	58.4	10.4	31.2
Cefotaxime	5.2	13.2	81.6
Augmentin	59.7	7.8	32.5
Colistin sulphate	97.2	0	2.8
Polymyxin B	97.2	0	2.8
Trimethoprim-sulfamethoxazole	63.4	8.4	28.2
Cefuroxime	2.8	2.8	94.4
Trimethoprim	2.8	1.4	95.8
Aztreonam	14.3	27.1	58.6
Ampicillin-sulbactam	73.0	4.0	23.0

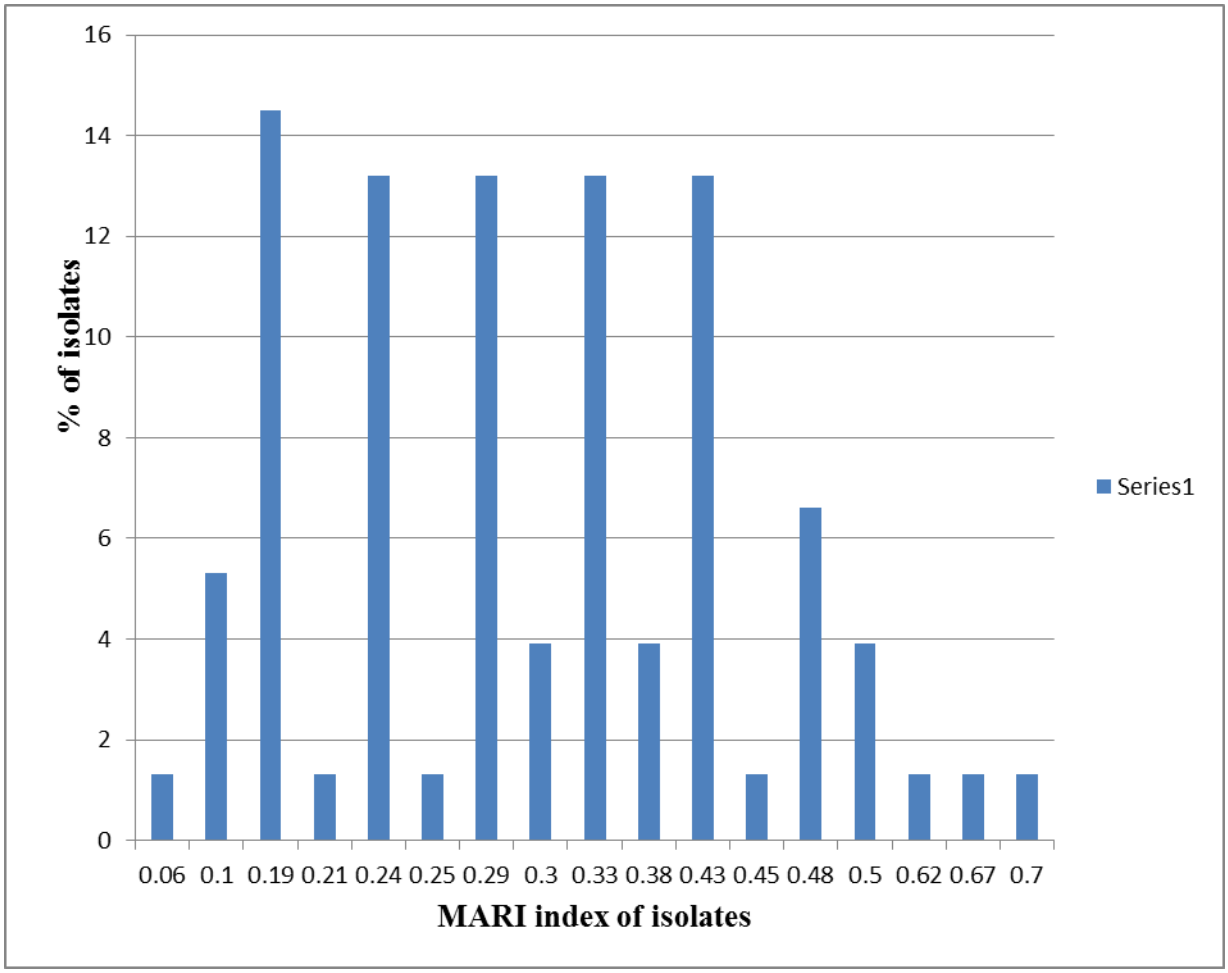

Fig 3.1: Multiple Antibiotic Resistant Index (MARI) of *Stenotrophomonas maltophilia*.

3.4 DISCUSSION

S. maltophilia is characterised by resistance to different classes of antibiotics. Generally, in this study which was in agreement with most other studies that have investigated the antimicrobial susceptibility profile of this organism, isolates tested were inherently resistant to several antibiotic groups including β-lactams, macrolides and aminoglycosides (Valdezate *et al.*, 2001). Interestingly though, the results show that 87.3% of the isolates tested were susceptible to meropenem but these isolates cannot be considered

truly susceptible because resistant stable mutants are easily and quickly selected when *S. maltophilia* isolates are exposed to carbapenems rendering the drugs ineffective against them (Howe *et al.*, 1997).

Trimethoprim-sulfamethoxazole (TMP-SMX) is generally accepted worldwide as the antibiotic of choice for the treatment of infections caused by this organism. Numerous studies have found the antibiotic to be the most effective amongst those tested (Tripodi *et al.*, 2001; Betriu *et al.*, 2002; Al-Jasser, 2006*b*) and in this study, 63.4% of the isolates tested were susceptible to the drug while resistance was observed in 28.2%. This is similar to the results obtained by Hu *et al.* (2011) in their study in which 66.7% of the 102 isolates tested were susceptible to TMP-SMX while 30.4% of them were resistant. This was attributed to the selection pressure caused by extensive use of the drug in these isolates which were mainly from patients with serious debilitating diseases and poor immunity. Chang *et al.* (2007) also reported a resistance rate of 25% to TMP/SMX and Valdezate *et al.* (2001) in their study of unique *Stenotrophomonas maltophilia* strains reported a resistance of 26.2%. These values are significantly higher than the reported worldwide resistance rates of less than 10%. Results from the SENTRY study (Gales *et al.*, 2001) encompassing data from Europe, Latin America and North America indicates that the level of resistance to TMP-SMX ranges from 2% to 10% depending on location and therefore Valdezate *et al.* (2001) suggest that these differences may be due to the use of different methodologies and varying breakpoints.

The presence of TMP-SMX-resistant *S. maltophilia* strains has significant implications. A study by Tsiodras *et al.* (2000) investigated the clinical implications of TMP/SMX resistant strains and found that these infections occurred in severely ill patients with extensive exposure to the health care system and often required invasive procedures such as surgery or catheter removal for effective treatment. Infections were directly

associated with severe morbidity but exposure to appropriate therapy tended to be protective against death.

It has been suggested that newer fluoroquinolones may be a suitable alternative treatment option for *S. maltophilia* infections. In agreement with other studies (Biedenbach *et al.*, 1999; Vartivarian *et al.*, 1994; Valdezate *et al.*, 1999; Weiss *et al.*, 2000; Valdezate *et al.*, 2001; Giamarellos-Bourboulis *et al.*, 2002), the new fluoroquinolones, levofloxacin, gatifloxacin and moxifloxacin all showed good activity against the isolates in the present study with susceptibility ranging from 88% to 94.67% whereas the susceptibility to ciprofloxacin was 76%. Valdezate *et al.* (1999) reported that more than 95% of the isolates tested in their study were susceptible to the newer fluoroquinolones and Weiss *et al.* (2000) in a comparison of seven quinolones found that clinafloxacin, moxifloxacin and trovafloxacin had significantly better *in vitro* activity compared to ciprofloxacin and levofloxacin. Quinolones were also effective against some of the isolates that were resistant to TMP-SMX. This is comparable to the results in this study in which all the TMP-SMX-resistant isolates were susceptible to the fluoroquinolones tested except for four TMP-SMX-resistant isolates that showed only intermediate susceptibility to ciprofloxacin. Giamarellos-Bourboulis *et al.* (2002) also reported that moxifloxacin was bactericidal against genetically distinct *S. maltophilia* isolates that were resistant to TMP-SMX. Furthermore, the newer fluoroquinolones can reach a concentration that is at least five times their serum concentration in the lungs (Zhanel *et al.*, 1999) and since they exert concentration-dependent killing, the fluoroquinolones provide an option for the treatment of respiratory tract infections caused by *S. maltophilia* particularly in patients with cystic fibrosis (Weiss *et al.*, 2000).

More than 80% of the *S. maltophilia* isolates in this study showed a MAR index >0.2. This infers that these isolates originated from an environment that has been contaminated

through the use of antibiotics. MAR indexing may allow the possibility of distinguishing the source of contamination leading to the development of multiple antibiotic resistances in these organisms (Krumperman, 1998). The Eastern Cape Province is a rural environment with large agriculture and livestock-rearing concerns. These multi-drug resistant isolates may have emerged as a result of high selective pressure in the environment due to sub-optimal use of antibiotics but this remains to be determined.

Recent studies done have indicated that resistance genes responsible for TMP-SMX resistance are linked to insertion sequence common region (ISCR) elements which are DNA sequences found beyond and close to the 3' conserved sequences of class 1 integrons (Chang *et al.,* 2007). These ISCR elements have been identified in numerous Gram-negative bacteria and are thought to be responsible for the mobility and dissemination of many antibiotic resistance genes including extended spectrum β-lactamases, carbapenemase genes and aminoglycoside, chloramphenicol and quinolone genes (Avison *et al*., 2002; Barbolla *et al.,* 2004; Li and Nikaido, 2004; Toleman *et al.*, 2007). In this study, all the isolates resistant to TMP-SMX showed a high level of resistance to the penicillins, cephalosporins, and the aminoglycoside tested, further confirming the multi-drug resistant phenotype of these isolates.

Majority of the studies done to evaluate the antimicrobial susceptibility profile of *S. maltophilia* have been carried out on clinical isolates and no further information is available on the antimicrobial susceptibilities of environmental isolates in South Africa. With mounting evidence on the interactions between human pathogenic/commensal bacteria and environmental bacteria (Seveno *et al.,* 2002; Martinez, 2008; Canton, 2009), the need for surveillance of these resistance reservoirs cannot be underestimated.

3.5 CONCLUSION

This study clearly shows the multidrug resistant nature of even commensal *S. maltophilia* and provides insight into the susceptibility profile of this organism in the Eastern Cape environment. With all the evidence that suggests that there is interaction between commensal organisms and pathogenic bacteria, this information may provide relevant information for empirical therapy should these organisms become implicated in clinical infections. TMP-SMX remains an effective drug for the treatment of *S. maltophilia* infections but with the increase in resistance among environmental strains of this organism, it is important to consider alternative, effective treatment options. The new fluoroquinolones provide such an option and further studies are required to determine their therapeutic effectiveness *in vivo*. With continued advances in the field of medicine and improved access to healthcare, the problem with nosocomial and opportunistic pathogens such as *S. maltophilia* is not going to go away and it is important that clinicians and microbiologists are aware of the resistance phenotypes of these organisms and keep a tight rein of surveillance on antibiotic use so that our treatment options do not continue to dwindle; while stepping up research on the development of new antimicrobial agents.

CHAPTER FOUR

CTX-M ESBLS IN *Stenotrophomonas maltophilia* ISOLATES FROM THE EASTERN CAPE PROVINCE ENVIRONMENT

4.0 ABSTRACT

Resistance of Gram-negative organisms to β-lactam antibiotics is predominantly mediated by the production of chromosomal, induced or acquired β-lactamases. Based on the double disc synergy tests (DDST), 59.5% of the *Stenotrophomonas maltophilia* in this study were phenotypically confirmed to be producers of extended spectrum β-lactamases (ESBLs). Cefepime was the most sensitive indicator cephalosporin with 77% sensitivity. Nine different ESBL phenotypes were observed. PCR amplification of the *bla* genes revealed the presence of CTX-M ESBLs belonging to CTX-M-1 group and CTX-M-8 group. The presence of ESBLs in these commensal organisms is significant because of the possibility of the spread of these genes in the environment and community and the implications of transfer to other pathogenic organisms.

Keywords: *Stenotrophomonas maltophilia*, extended spectrum beta lactamase (ESBL), CTX-M group

4.1 INTRODUCTION

β-lactamases remain the leading cause of resistance to the β-lactam group of antibiotics among Gram-negative bacteria and the prevalence and incidence of these enzymes have been increasing worldwide (Bradford, 2001; Bonnet, 2004; Al-Jasser, 2006a; Deepthi and Deepthi, 2010). β-lactamases are found in a wide variety of organisms and most Gram-negative organisms are thought to possess a chromosomal β-lactamase or acquire these from plasmids and transposons in other organisms (Deepthi and Deepthi, 2010). These β-lactamases have been extensively studied and are divided into four classes: A through D based on differences in their amino acid sequences according to Ambler *et al.* (1991). The spread of these enzymes and increasing resistance to β-lactam antibiotics led to the development of the oxymino-cephalosporins which were less susceptible to inactivation by the β-lactamases (Livermore and Hawkey, 2005). Increased use of these new agents, however, has led to the rapid emergence of resistant strains which produced extended spectrum β-lactamases (ESBLs) (Bradford, 2001; Paterson and Bonomo, 2005).

The ESBLs are a rapidly evolving group which belong predominantly to class A and D in the Ambler structural classification but the functional classification by Bush *et al.* (1995) clusters them in group 2be. The enzymes hydrolyse the extended-spectrum cephalosporins and the monobactams and are generally susceptible to β-lactamase inhibitors such as clavulanate, sulbactam and tazobactam. The CTX-M ESBLs are a subgroup of this class of ESBLs.

S. maltophilia produces two inducible β-lactamases designated L1 and L2 which mediate its natural resistance to the β-lactam group of antibiotics. L1 is a metallo-enzyme, a Zn+-dependent carbapenemase belonging to Bush group 3 and hydrolyses most penicillins,

cephalosporins, carbapenems but not monobactams. It is not inhibited by clavulanic acid. The L2 β-lactamase is a serine β-lactamase belonging to Bush group 2be. It hydrolyses the cephalosporins and the monobactams and is inhibited by clavulanic acid and other β-lactamase inhibitors. However, several studies have reported the heterogeneity of β-lactamase production in *S. maltophilia*. Payne *et al*. (1994) reported the presence of seven metallo-enzymes and eight serine β-lactamases in clinical isolates investigated while Paton *et al*. (1994) reported one metallo-enzyme and three serine β-lactamases. More recently, Al-Naeimi *et al*. (2006) and Lavigne *et al.* (2008) using phenotypic and genotypic methods were able to identify ESBLs belonging to the CTX-M group in clinical isolates of *S. maltophilia*. These are the first reports confirming ESBL production in this nosocomial organism. The purpose of this study was to identify, using phenotypic and molecular methods, the prevalence of ESBL-producing environmental isolates of *S. maltophilia* in the Eastern Cape Province.

4.2 MATERIALS AND METHODS

4.2.1 Phenotypic detection of extended spectrum beta lactamases

Isolates that showed resistance or intermediate susceptibility to the indicator cephalosporins (ceftazidime 30µg, cefotaxime 30µg, cefepime 30µg and cefpodoxime 10µg) in the susceptibility tests previously reported (Section 3.3) were subjected to the phenotypic confirmatory tests using the double disc synergy test (DDST) as described by Jarlier *et al.* (1988) with modifications. The inoculum was standardized as previously described (NCCLS, 2005) and streaked on freshly prepared Mueller-Hinton agar plates. Antibiotic discs (MAST Diagnostics, Merseyside, United Kingdom) containing ceftazidime (30µg), cefotaxime (30µg), cefpodoxime (10µg) and cefepime (30µg) were placed around a central amoxicillin-

clavulanic acid (30µg) disc at a distance of 25mm centre to centre. Isolates that showed synergy towards the central clavulanate disc were phenotypically confirmed to be ESBL producers.

4.2.2 Extraction of genomic DNA

Genomic DNA was extracted using the method of Alzahrani *et al.* (2010) with modifications. The *S. maltophilia* isolates were subcultured on Luria-Bertani agar plates and incubated at 37^{o}C overnight. Three to five colonies of the bacteria was then suspended in 250 µl of sterile nuclease-free water in sterile eppendorf tubes and vortexed to achieve a uniform suspension. The cells were then lysed by heating to 100^{o}C for 15 min on a heating block (Accublock Digital Dry Bath, Labnet). The suspension was then centrifuged (Thermo scientific, Haraeus Fresco 17) at 15,000 rpm for 15 min at 4^{o}C to remove cell debris and the lysate was stored at -20^{o}C until used for the PCR reactions.

4.2.3 PCR amplification

Conventional Polymerase chain reaction (PCR) was used to amplify possible extended spectrum β-lactamase genes from the isolates that showed DDST positivity. PCR amplification was carried out with specific primers based on already published sequences of the ESBL enzyme groups (Table 4.1) as described in the method by Schlesinger *et al*. (2005) with modifications to screen for *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{IBC}$, *bla*$_{PER}$, *bla*$_{OXA}$, *bla*$_{VEB}$ and *bla*$_{SFO}$ genes. All reactions were performed at a final volume of 25 µl containing 12.5 µl PCR master mix 2x (Fermentas), 0.5 µl of each primer (Inqaba Biotec), 3 µl template DNA and

8.5 µl nuclease free water (Fermentas) under the following conditions in a Biorad Mycycler Thermal Cycler: 15 min at 95^{o}C, 35 cycles of 1 min at 94^{o}C, 1 min at the annealing temperature published for each primer set, 1 min at 72^{o}C followed by a further extension for 10 min at 72^{o}C. Ten microlitres of each PCR amplicon was then loaded on 1.5% agarose (Pronadisa, low grade EEE agarose) gel containing 0.5x TBE buffer along with 100 bp DNA ladder (Fermentas, GeneRuler, 0.5 µg/µl). Electrophoresis was carried out at a voltage of 100 V for 75 min. The gel was stained with 0.5 µg/ml ethidium bromide and the DNA was then visualised with an ultraviolet transilluminator and captured with the documentation software Alliance 4.7 (Uvitec).

Table 4.1: Primer sequences, annealing temperature, expected product size and references.

Primer type and gene family	Sequence	Annealing temp (^{o}C)	Reference or source	PCR product size (kb)
TEM	F:5'-TCAACATTTCCGTGTCG-3' R:5'-CTGACAGTTACCAATGCTTA-3'	42	Schlesinger *et al.*, 2005	0.86
SHV	F:5'-ATGCGTTATATTCGCCTGTG-3' R:5'-AGATAAATCACCACAATGCGC-3'	47	Schlesinger *et al.*, 2005	0.78
CTX-M 1	F:5'-GACGATGTCACTGGCTGAGC-3' R:5'-AGCCGCCGACGCTAATACA-3'	53	Pitout *et al.* (2004)	0.49
CTX-M 2	F:5'-ATGATGACTCAGAGCATTCG-3' R:5'-TGGGTTACGATTTTCGCCGC-3'	55	Saladin *et al.* (2002)	0.87
CTX-M 8	F:5'-CTGGAGAAAAGCAGCGGGGG-3' R:5'-ACCCACGATGTGGGTAGCCC-3'	51	Minarini *et al.* (2007)	0.58
CTX-M 9	F:5'-ATGGTGACAAAGAGAGTGCA-3' R:5'-CCCTTCGGCGATGATTCTC-3'	55	Pitout *et al.* (2004)	0.87
OXA	F:5'-ACACAATACATATCAACTTCGC-3' R:5'-AGTGTGTTTAGAATGGTGATC-3'	42	Schwaber *et al.* (2005)	0.81
IBC	F:5'-GGGCGTACAAAGATAATTTCC-3' R:5'-GAAGCAACGTCGGCTTGAACG-3'	47	Schlesinger *et al.* (2005)	0.94
VEB	F:5'-ACGGTAATTTAACCAGATAGG-3' R:5'-ACCCGCCATTGCCTATGAGCC-3'	46	Schlesinger *et al.* (2005)	0.97
SFO	F:5'-GTTAATCCATTTTATGTGAGG-3' R:5'-CAGATACGCGGTGCATATCCC-3'	44	Schlesinger *et al.* (2005)	0.94
PER	F:5'-ATGAATGTCATTATAAAAGC-3' R:5'-AATTTGGGCTTAGGGCAGAA-3'	42	Weldhagen *et al.* (2003)	0.93

4.3 RESULTS

For the ESBL confirmatory phenotypic tests, 59.5% of the isolates tested positive with the double disc synergy test. One or more of the cephalosporins showed synergy towards the central clavulanic acid disc for each of these isolates. Cefepime appeared to be the most sensitive indicator cephalosporin with 77.3% of these isolates showing cefepime-clavulanate synergy. This was closely followed by 75% ceftazidime-clavulanate synergy and 54.5% and 0.1% for cefotaxime and cefpodoxime respectively. The *Stenotrophomonas* isolates exhibited nine distinct ESBL phenotypes (Fig 4.1) of which 31.8% showed synergy between clavulanic acid and cefepime, ceftazidime and cefotaxime (CPM-CAZ-CTX-AUG).

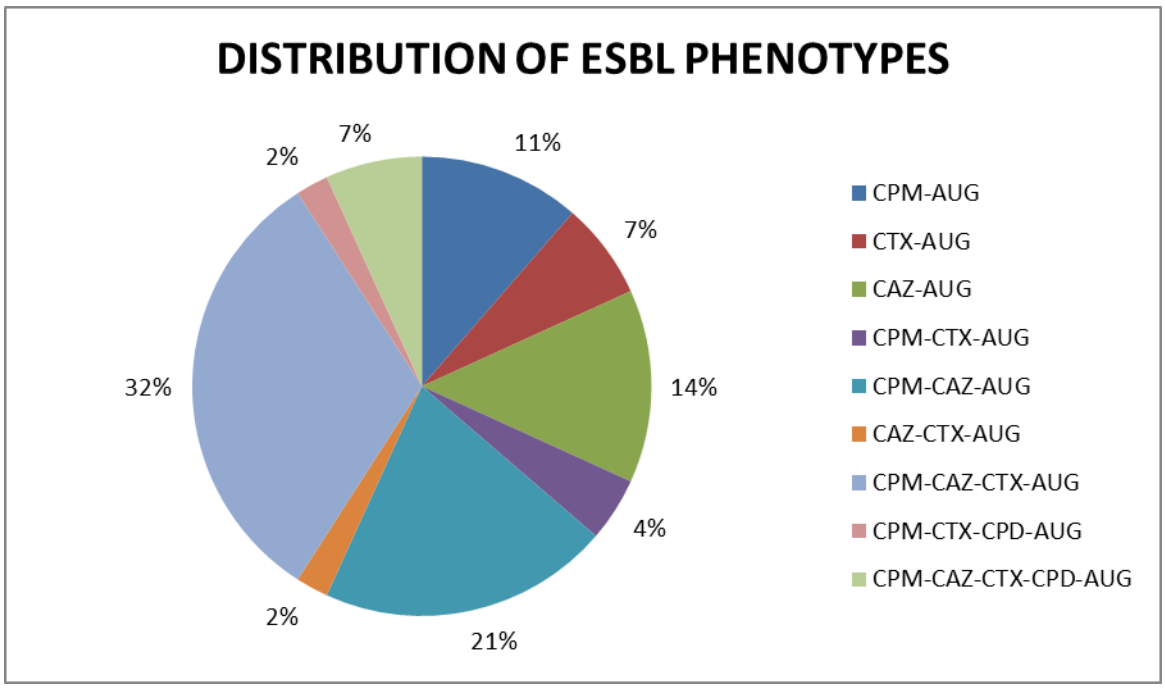

Fig 4.1: Pie chart showing distribution of different ESBL phenotypes exhibited by the *Stenotrophomonas maltophilia* isolates using the double disc synergy tests.

As previously reported (Section 3.3), 58.6% and 27.1% of the isolates were resistant and intermediately susceptible to aztreonam respectively, of which 55% of these also displayed a positive DDST further confirming the possible production of ESBLs by these isolates. However, PCR amplification only allowed identification of the CTX-M group of ESBLs in four of these isolates (Fig 4.2 and Fig 4.3) all of which had the same ESBL phenotype (CPM-CAZ-AUG). The CTX-M ESBLs amplified belonged to the CTX-M-1 and CTX-M-8 groups. Each of the four isolated showed amplification of genes from both groups.

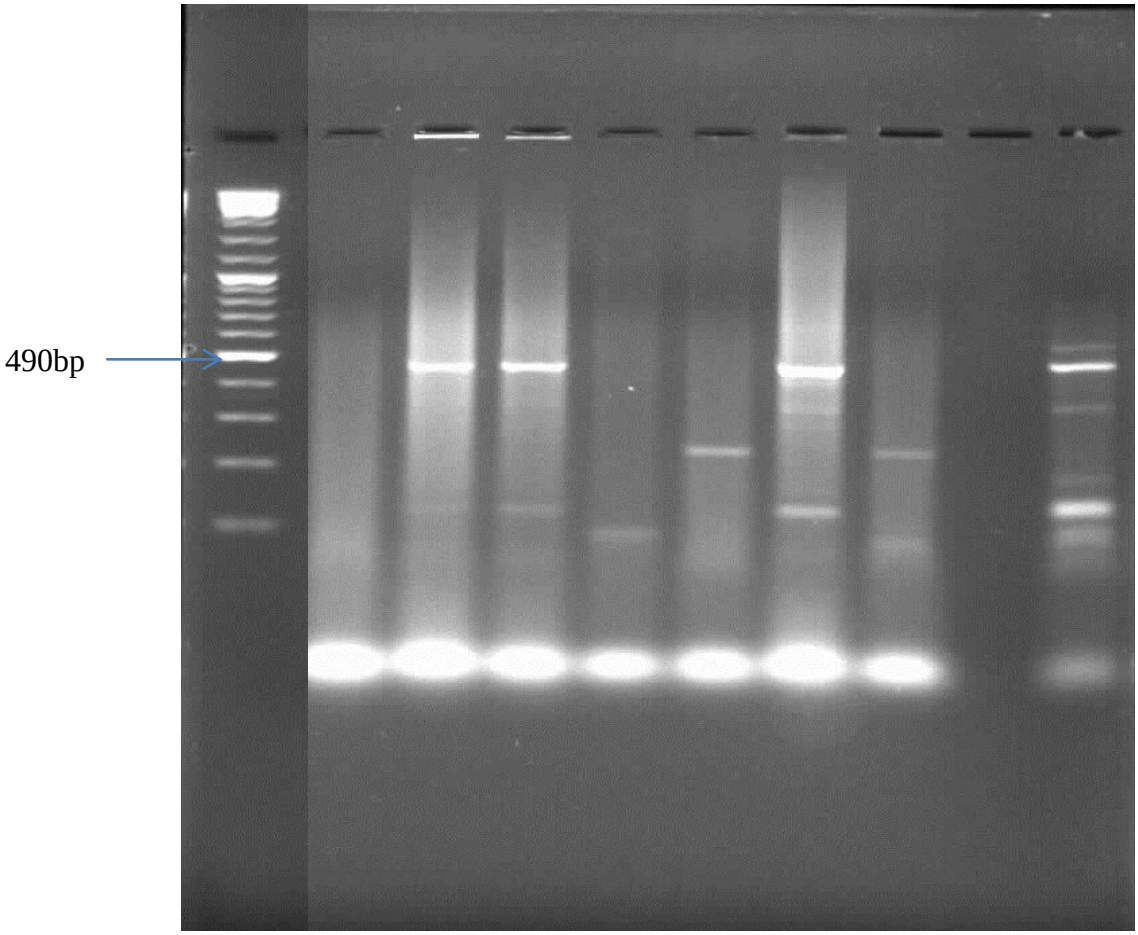

Fig 4.2: Gel picture of amplification of genes belonging to CTX-M 1 group. Amplicons are of the expected size of 490bp. Lane 1: molecular marker; Lane 2: nuclease-free water; Lanes 3-8, 10: template DNA; Lane 9: empty

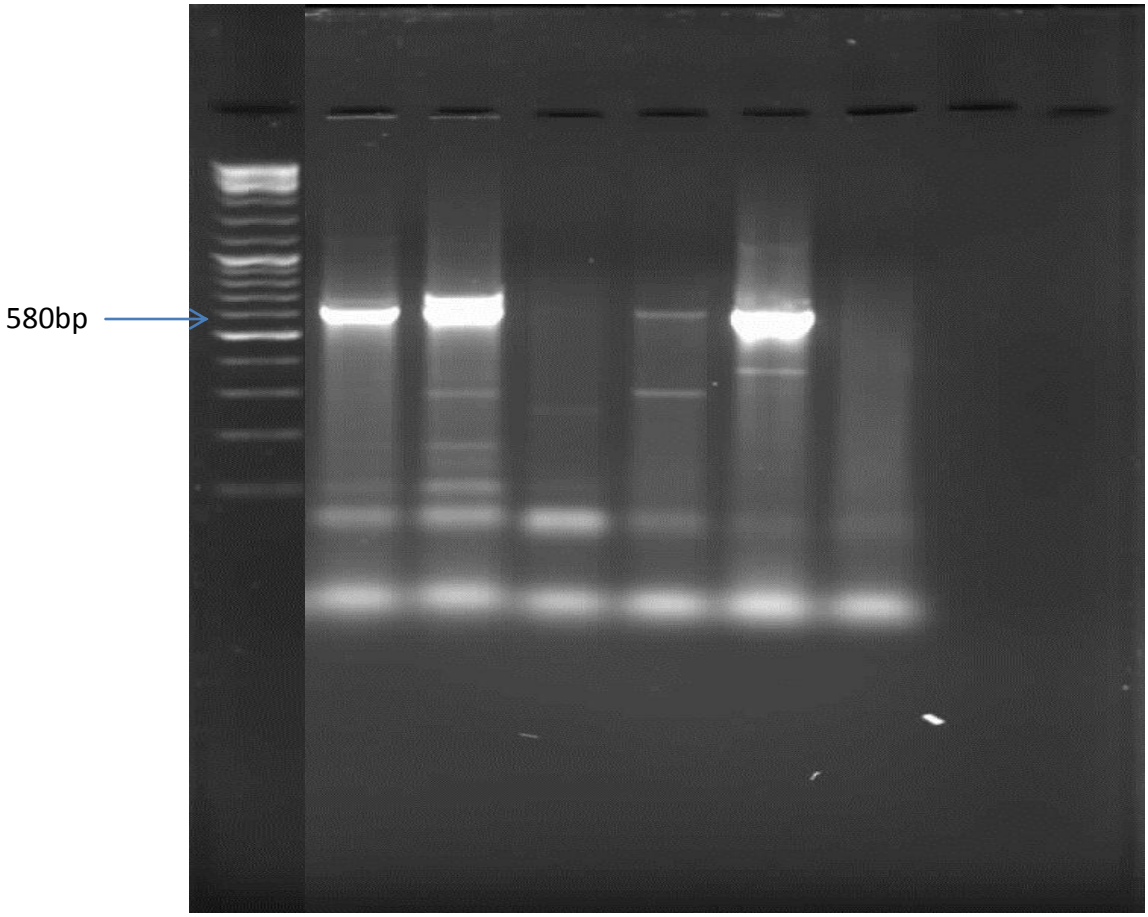

Fig 4.3: Gel picture showing amplification of ESBLs belonging to CTX-M 8 group. Amplicons show expected amplicon size of 580bp. Lane 1: molecular marker; Lanes 2-6: template DNA; Lane 7: nuclease-free water.

4.4 DISCUSSION

In this study, we report the presence of CTX-M ESBLs in *S. maltophilia* isolated from the Eastern Cape environment. The issue of ESBL production in *S. maltophilia* is a controversial one. Several studies have reported double disk synergy test positivity in clinical isolates of this organism (Blahova *et al.,* 1998; Canton *et al.,* 1999; Hejnar *et al.,* 2004) but Munoz Bellido and Garcia-Rodriguez (1998) suggest that these reactions could be explained by other properties of the L2 β-lactamase of *S. maltophilia*. More recent studies, making use of phenotypic and molecular methods have, however, further confirmed the presence of ESBLs in this species (Al-Naiemi *et al*., 2006; Hu *et al.,* 2009). In this study, 59.5% of the isolates that showed resistance to the indicator cephalosporins were positive for ESBL production using the DDST (Jarlier *et al.*, 1988).

The Clinical and Laboratory Standards Institute (CLSI) recommends the use of more than one cephalosporin when using the disc diffusion method for the detection of ESBLs in order to increase the sensitivity of the tests because of the variable substrate affinities of different ESBL enzymes (CLSI, 2009). Cefotaxime is thought to be an adequate substrate for CTX-M enzymes which are more potent hydrolysers of cefotaxime than ceftazidime, while ceftazidime is a suitable substrate for the TEM and SHV variants. However some authors have suggested that if only one drug can be used, then cefpodoxime may be a more effective single indicator (Jarlier *et al.,* 1988; Steward *et al.,* 2001). In this study, about 99% of the isolates were resistant to cefpodoxime in the initial screening tests and only 0.1% of those isolates displayed cefpodoxime-clavulanic acid synergy. This may indicate that resistance is due to the L1 β-lactamase or other non-ESBLs or possibly other resistance mechanisms in *S. maltophilia* (Blahova *et al.,* 1998).

The inclusion of cefepime in double disc synergy testing is thought to improve the sensitivity of the test especially in organisms that also produce Amp-C β-lactamases. Comparably with other studies (Tzelepi *et al.*, 2000; Gupta *et al.*, 2007; Mohanty *et al.*, 2010), cefepime was found to be the most effective indicator of ESBL production in the DDST in this study. No studies have confirmed the production of AmpC β-lactamases in *S. maltophilia* but given its multi-resistant nature, production of several β-lactamases, and ability to acquire resistance genes from other organisms, it may be worthwhile to include cefepime in the screening and confirmation of ESBLs in this organism to improve the sensitivity of the test. In addition, CTX-M ESBLs hydrolyse cefepime more efficiently than other ESBL types often with MICs in the resistant range (Sridhar, 2011).

A little over a decade ago, investigations into extended spectrum β-lactamase enzymes almost exclusively revealed TEM and SHV types of ESBLs. However, the dynamics have changed with the CTX-M ESBLs increasingly becoming the predominant enzyme type isolated from Gram-negative organisms and it is being found more frequently in community isolates (Patterson and Bonomo, 2005; Livermore and Hawkey, 2005; Livermore *et al*., 2007). The PCR amplification of *bla* genes in this study revealed the presence of CTX-M ESBLs in four of the *S. maltophilia* isolates. The amplicons detected were the expected size for enzymes belonging to the CTX-M 1 and CTX-M 8 groups. This is similar to results obtained by Al-Naeimi *et al*. (2006) and Lavigne *et al*. (2008) who reported the presence of CTX-M enzymes in clinical isolates of *S. maltophilia*. Al-Naeimi *et al*. (2006) identified the CTX-M gene as CTX-M-1 after sequencing while the CTX-M enzyme in the French study by Lavigne *et al*. (2008) was identified as CTX-M-15 following plasmid analysis.

The CTX-M group of ESBLs is divided into five major groups based on amino acid sequences. Members of each group share greater than 94% amino acid similarity while there

is less than 90% similarity between distinct groups. CTX-M-1 group consists of six plasmid mediated enzymes: CTX-M-1, CTX-M-3, CTX-M-10, CTX-M-12, CTX-M-15 and FEC-1 including some unpublished enzymes. CTX-M-2 group consists of eight plasmid mediated enzymes namely: CTX-M-2, CTX-M-4, CTX-M-4L, CTX-M-5, CTX-M-6, CTX-M-7, CTX-M-20 and Toho-1. CTX-M-8 is the sole member of its group. CTX-M-9 group consists of CTX-M-9, CTX-M-13, CTX-M-14, CTX-M-16, CTX-M-17, CTX-M-19, CTX-M-21, CTX-M-27 and Toho-2. CTX-M-25 group has two members: CTX-M-25 and CTX-M-26 (Bonnet, 2004).

Both enzymes in the Al-Naeimi *et al*. (2006) and Lavigne *et al*. (2008) studies belong to the CTX-M-1 group which correlates with our finding of ESBLs of the CTX-M-1 group. CTX-M-15 is by far the most frequently reported CTX-M ESBL around the world in both hospital and community isolates of *Enterobacteriaceae,* especially *Escherichia coli* urinary isolates (Woodford *et al*., 2004; Pitout *et al*., 2005; Pitout *et al*., 2007; Lewis II *et al*., 2007; Padimini *et al*., 2008). In South Africa, several studies have also reported the presence of CTX-M ESBLs in several Gram-negative organisms including *P. aeruginosa* and *E. coli* (Paterson *et al*., 2003; Ehlers *et al*., 2009; Peirano *et al.,* 2011) but to the best of our knowledge this is the first description of CTX-M ESBLs in environmental isolates of *S. maltophilia*. The detection of these ESBLs in only four of the isolates tested may be due to several factors. The template DNA used in the PCR reactions were genomic DNA and as earlier discussed extended spectrum β-lactamase genes can be carried on plasmids, integrons or transposons. Further studies need to be done to determine if the isolates possess such mobile elements and the resistance genes they carry.

4.5 CONCLUSION

Phenotypic detection of ESBLs in non-fermentative Gram negative bacilli is complex but with the use of molecular methods such as PCR, we have been able to confirm the presence of ESBLs in *S. maltophilia* isolated from this environment. The presence of these genes has significant implications for infection control specialists and microbiologists because laboratories do not routinely screen for the presence of ESBLs in this group of organisms. The occurrence of ESBLs in these organisms may therefore, be seriously underreported making nosocomial and opportunistic organisms such as *S. maltophilia* an un-monitored reservoir of these genes with the potential to spread them to both community and hospital isolates. The possession of extended spectrum β-lactamases also further limits the already severely limited therapeutic options for the treatment of infections caused by this organism.

CHAPTER FIVE

COMPARATIVE *IN-VITRO* ACTIVITY OF TRIMETHOPRIM-SULFAMETHOXAZOLE AND THE NEW FLUOROQUINOLONES AGAINST EXTENDED-SPECTRUM BETA- LACTAMASE PRODUCING *Stenotrophomonas maltophilia*

The production of extended-spectrum β-lactamases by any organism suggests that such organism is resistant to β-lactam group of antibiotics including penicillins, broad-spectrum and extended-spectrum cephalosporins and the monobactam, aztreonam while they remain susceptible to the β-lactamase inhibitors (Bradford, 2001, Falgas and Karageogopoulos, 2009; Deepthi and Deepthi, 2010). Non-fermentative Gram-negative bacilli such as *S. maltophilia* have become increasingly important as causes of nosocomial and opportunistic infections in the last two decades and the production of ESBLs by these organisms poses an immense challenge for effective therapy (Looney *et al.,* 2009). The ability of these bacteria to acquire various β-lactamases with different susbrate profiles and the selection of complex mutant enzymes that possess inhibitor-resistant phenotypes renders virtually all β-lactams ineffective (Al-Jasser, 2006a; Bush, 2010).

Extended-spectrum β-lactamase genes are often carried on plasmids which also bear genes that encode for resistance to other classes of antibiotics such as aminoglycosides, fluoroquinolones, tetracyclines (excluding glycycyclines) and trimethoprim-sulfamethoxazole (Al-Jasser, 2006a; Bush, 2010; Falgas and Karageogopoulos, 2009; Pitout and Laupland, 2008). Resistance to TMP-SMX is being described increasingly in *S. maltophilia* infections with reported resistance rates varying from different areas of the world (Toleman *et al.,* 2007; Falgas *et al.,* 2008). This has been linked to the possession of class 1 integrons bearing *sul*1

genes and insertion sequence (ISCR) common region elements bearing *sul*2 genes which are responsible for TMP-SMX resistance (Hu *et al.*, 2011). This class 1 integrons have also been associated with production of ESBLs (Al-Jasser, 2006a, Perez *et al.*, 2007; Bush, 2010; Phongpaichit *et al.*, 2011)

In this study, we compared the *in-vitro* susceptibilities of TMP-SMX and the fluoroquinolones, ofloxacin, ciprofloxacin, levofloxacin, moxifloxacin and gatifloxacin against confirmed extended spectrum beta-lactamase producing *S. maltophilia*. The antibiotic susceptibilities were determined by disc diffusion method as previously reported (Section 3.3) and phenotypic detection of ESBLs was done by the double disc synergy method (Section 4.3). As shown in Table 5.1 below, about 59.5% of the isolates were phenotypically confirmed as ESBL producers. Of the ESBL positive isolates, 70.5% were susceptible to TMP-SMX while 22.7% of them were resistant. Susceptibility to the fluoroquinolones ranged from 72.7% to 95.5% while resistance rate was 2.3% to each of the drugs. Levofloxacin was the most effective fluoroquinolone against the ESBL positive isolates while ciprofloxacin was the least effective with susceptibility of 72.7%.

Table 5.1: Percentage susceptibilities of ESBL +ve and ESBL –ve isolates to TMP-SMX and the fluoroquinolones.

		TMP-SMX	**OFX**	**CIP**	**LEV**	**MFX**	**GAT**
ESBL +VE	S (%)	70.5	86.4	72.7	95.5	88.6	93.2
	I (%)	6.8	11.4	25.0	2.3	9.1	4.5
	R (%)	22.7	2.3	2.3	2.3	2.3	2.3
ESBL – VE	S (%)	54.5	93.9	78.9	93.9	90.9	90.9
	I (%)	15.2	3.0	21.1	6.0	6.0	9.0
	R (%)	30.3	3.0	0	0	3.0	0

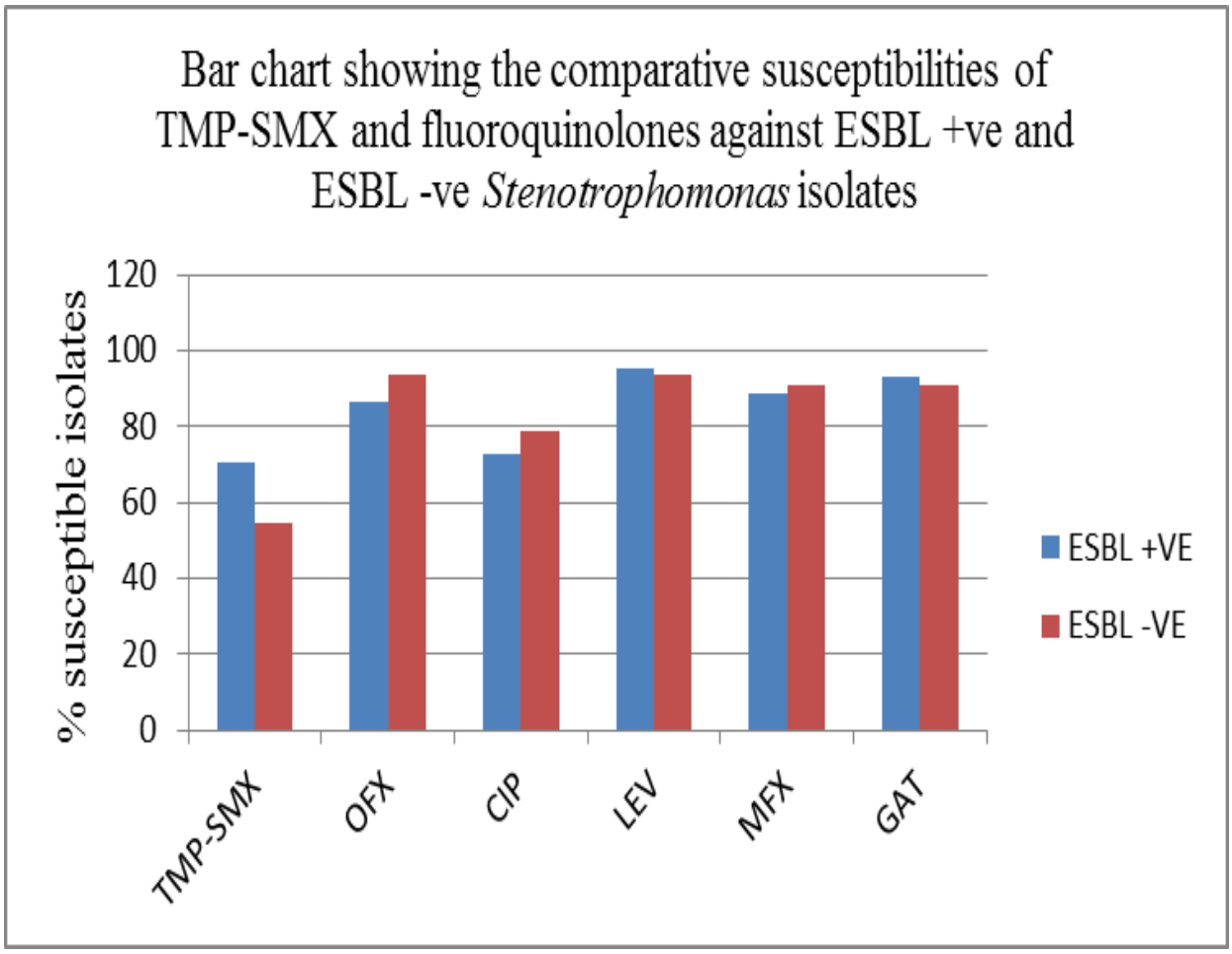

Fig 5.1: Bar chart showing the comparative susceptibilities of TMP-SMX and fluoroquinolones against ESBL-positive and ESBL-negative *S. maltophilia* isolates.

This data was analysed using Fischer's exact test and p-value < 0.05 was considered as significant. The presence or absence of ESBL production did not have a significant effect on the susceptibilities of these *S. maltophilia* isolates to TMP-SMX or the fluoroquinolones (p-value >0.05 in all categories). Figure 5.1 is a graphical representation of the susceptibilities of the ESBL-positive and ESBL-negative isolates and it clearly shows the fluoroquinolones are almost equally effective against ESBL-positive and ESBL-negative isolates.

Although this study did not find any significant relationship between ESBL production and resistance to TMP-SMX and the fluoroquinolones, other studies and surveys done around the world have shown the tendency of associated resistance in community isolates of ESBL producing organisms (Woodford *et al.*, 2004; Pitout *et al.*, 2007; Pitout and Laupland, 2008). Studies examining the susceptibility profile of CTX-M-producing *E. coli* reported resistance to TMP-SMX up to 64% and resistance to cephalosporins up to 68%. Empirical therapy with cephalosporins and fluoroquinolones were also found to be associated with increased mortality (Ben-Ami *et al.*, 2006; Rodriguez-Bano *et al.*, 2006; Pitout and Laupland, 2008). In the present study, only one of the isolates found to possess CTX-M ESBLs after PCR was resistant to TMP-SMX.

Some studies have determined that fluoroquinolone resistance in *S. maltophilia* is mediated by the chromosomal *qnr* gene and the genes that encode for the SmeDEF efflux pumps that lead to extrusion of fluoroquinolones (Shimzu *et al*., 2008, Sanchez *et al.*, 2009; Sanchez and Martinez, 2010; Hernandez *et al.*, 2011). However, in the extensive review of quinolone resistance by Hernandez *et al.* (2011) the authors describe the emergence of plasmid encoded *qnr* genes in various environmental bacterial pathogens. The genetic environment surrounding these genes reveals that they are integrated in complex *sul*1 type integrons and are associated with *ISCR*1 (Nordmann and Poirel, 2005).

The presence of ESBLs, therefore, poses a unique challenge for the selection of appropriate empirical therapy. Prompt and effective use of the appropriate empirical antibiotics affects eventual treatment outcome but clinicians are now faced with the attendant co-existence of ESBLs and resistance to major antibiotic groups making the “magic bullet” even more difficult to find (Tumbarello *et al.*, 2006; Perez *et al*., 2007)

CHAPTER SIX

GENERAL DISCUSSION AND CONCLUSION

Bacterial resistance to antimicrobial treatment is an emerging major public health threat facilitated by the widespread indiscriminate use of these antimicrobials in health care and community settings (Colodner, 2005). Subsequent to this, multidrug resistant organisms evolve due to increased antimicrobial selection pressure, from inherently or relatively susceptible organisms to more resistant strains thereby increasing the proportion of resistant strains of a particular organism. This selection of less susceptible strains tends to favour the emergence of free-living opportunistic pathogens that are resistant to antibiotics and even sensitive organisms begin to acquire new resistance mechanisms usually by the transfer of plasmids and transposons from less virulent organisms in the environment (French, 2010). The identification of putative resistance genes in environmental organisms is an issue that is just beginning to receive attention (Martinez, 2008).

Opportunistic pathogens are pathogens that cause disease only in individuals who are susceptible to illness due to a compromise in their immune systems such as severely debilitated patients, cystic fibrosis patients and those infected with HIV (Berg *et al*., 2005). The non-fermentative Gram negative bacteria are playing an increasingly important role in these infections (Enoch *et al.,* 2007). *P. aeruginosa* has long been the most important organism in this group but *S. maltophilia* has been named the third most important non-fermentative Gram negative bacillus in opportunistic infections (Barchitta *et al.*, 2009; Jones *et al.*, 2003; Nyc and Matejkova, 2010). It is often difficult to differentiate between true infection and colonisation by this organism however; mortality rates attributable to *S. maltophilia* bacteremia are as high as 60% (Senol *et al.*, 2002; Friedman *et al.*, 2002; Enoch *et al.,* 2007). The population at risk of infections due to this organism have also increased

considerably due to advances in medical therapeutic interventions such as the use of invasive therapeutic devices (central venous catethers, mechanical ventilation, haemodialysis) and increased utilization of broad spectrum antimicrobials (Senol, 2004; Al-Jasser, 2006b). It is also being reported in patients infected with HIV/AIDS (Calza *et al*., 2003)

Treatment of infections due to *S. maltophilia* is challenging due to its multi-resistant status with the production of multiple β-lactamases which confer resistance to the β-lactams, aminoglycoside-modifying enzymes, multidrug efflux pumps and low outer membrane permeability which are responsible for resistance to aminoglycosides, quinolones and polymyxins. Recently the presence of *sul* genes responsible for resistance to TMP-SMX has also been described (Toleman *et al.,* 2007; Chang *et al.,* 2007). According to Sanchez *et al.* (2009) this intrinsic resistance phenotype is exhibited by both clinical isolates and environmental strains of the organism.

The success of TMP-SMX as the agent of choice for the treatment of these infections has been attributed not only to its antibacterial effect but also to its immunomodulatory action which is associated with the induction of suppression of TNF-α (Nyc and Matejkova, 2010). Its efficacy can however be compromised by selection of resistant strains during long term or repeated administration or by colonization by an already resistant strain. Several studies have reported variable rates of resistance to TMP-SMX around the world (Betriu *et al.,* 2002) and in this study, 28.2% of the isolates were resistant to the drug. This has significant implications in the Eastern Cape environment. TMP-SMX is used extensively as prophylactic treatment against *Pneumocystis carinii* pneumonia in HIV/AIDS patients and unfortunately, this group of patients are also susceptible to *S. maltophilia* infections due to their immunocompromised status. This may lead to the selection of resistant strains of *S. maltophilia* in these individuals due to prolonged exposure to TMP-SMX subsequently

making them more vulnerable to other opportunistic infections by TMP-SMX resistant *S. maltophilia*. On the other hand, as previously discussed the genes responsible for TMP-SMX resistance have been found on moblile elements associated with resistance genes to a variety of other antibiotics including fluoroquinolones.

In conclusion, the growing burden of *S. maltophilia* as an important opportunistic pathogen cannot be overlooked. The high level of TMP-SMX resistance seen in these environmental isolates further buttresses the need for special attention on these organisms as possible reservoir of antibiotic genes. Furthermore, with the ongoing pandemic of HIV/AIDS and the continuous selective pressure exerted on these commensal organisms, community acquired multidrug resistant infections may increase. TMP-SMX still remains an effective drug for the management *S. maltophilia* infections if a tight rein is kept on its appropriate use. The newer fluoroquinolones however provide a suitable alternative in cases where TMP-SMX is either unsuitable or ineffective.

It remains to be determined how these environmental isolates acquired the CTX-M ESBLs. Their presence may indicate the increasing number of CTX-M positive isolates of other Gram negative bacteria in the community or the transfer of these resistance genes from hospital pathogens to environmental bacteria. The problem of multi-drug resistant Gram negative bacteria and extended-spectrum β-lactamases is not going to go away and infectious disease specialists can only stay a step ahead by continuous surveillance and the prudent use of antibiotics while research is ongoing on the development of new antibiotic compounds.

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