The Demographic and Microbiological Profile of Cystic Fibrosis in

Public and Private Sectors in KwaZulu-Natal

by

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Declaration

I hereby declare that this dissertation is my own work, except where specifically acknowledged in the text. Neither the present dissertation nor any part there of has been submitted to any other university for a degree.

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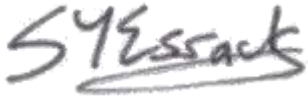

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DEDICATION

The journey of my Masters degree would not have begun and surely not have ended without the continued support and encouragement of my mother and my late brother who recently passed away.

Romans 8:28

ABSTRACT

Background: Cystic fibrosis necessitates long-term treatment with multiple antibiotics creating selection pressure for the development of antibiotic resistance in infecting and/or colonizing organisms, impacting on disease management, morbidity and mortality.β-lactamase mediated resistance, in particular, was investigated in isolates from cystic fibrosis patients from the public and private health sectors in Durban, South Africa. **Methods:** Sputum samples were obtained from patients attending public and private cystic fibrosis clinics. The patient demographics and clinical data were recorded. Bacterial isolates were subjected to minimal inhibitory concentration (MIC) determinations, phenotypic screening for extended spectrum-beta-lactamases (ESBLs), AmpC beta-lactamases and metallo-beta-lactamases (MBLs), and, PCR and sequencing for bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, bla_{CMY}, bla_{PER}, bla_{VEB}, bla_{OXA}, bla_{KPC}, bla_{GES}, bla_{IMP}, bla_{VIM},and bla_{NDM} genes. **Results:**The most common genotype was F508del and the most common pathogen was *Pseudomonas aeruginosa* with susceptibility to antibiotics ranging from 14-100% with marginal differences between mucoid and non-mucoid phenotypes. All *P aeruginosa* isolates were putative ESBL producers and 75% were putative MBL producers. All but one isolate carried multiple beta-lactamases from 2 or more different Ambler classes. Novel TEM-205 (GenBank Accessionno. KC900516) was found in a single isolate in combination with NDM-1, reported for the first time in *P. aeruginosa* in South Africa. TEM-205 showed 5 amino acid changes compared with TEM-1; viz.,V84I,E104K,R164S,M182T and A184V while novel TEM-213 (GenBank Accession no.KC663615), identified in three isolates, showed a single amino acid change Y105F. Resistance phenotypes did not routinely correlate with genotypes. This is the first report of NDM-1 from *Burkholderiace pacia complex* (Bcc) in South Africa. **Conclusion:**The co-expression and/or co-carriage of Ambler classes A, B and C β-lactamases in various permutations in single isolates severely restricts the clinical management of CF not only with beta-lactam antibiotics but also aminoglycosides and fluoroquinolones, the resistance genes of which commonly occur on the same genetic determinants of resistance. The presence of NDM-1 in combination with the CMY AmpC β-lactamases, TEM, SHV and CTX-MESBLs is of grave concern leaving colistin as the sole remaining treatment option in this pathogen. The incidence, prevalence and susceptibility patterns of different microorganisms in the sputa of CF patients should be closely monitored to optimize management and treatment options in a disease requiring chronic antibiotic therapy which increases the propensity for the development of antibiotic resistance.

CONTENTS

ABBREVIATIONS

ASL Air way surface liquid

BMI Body mass index

cAMP Cyclic adenosine monophosphate

CA-MRSA Community-associated Methicill in-resistant *Staphylococcus aureus*

CF Cystic fibrosis

CFTR Cystic fibrosis transmembrane conductance regulator

CLSI Clinical and Laboratory Standards Institute

CTX-M Cefotaximase-Munich

EDTA Ethylenediamine-tetra-acetic acid

ENaC Epical membrane epithelial sodium channel

ESBL Extended-spectrum-beta-lactamase

GES Guiana extended-spectrum-beta lactamase

HA-MRSA Hospital-associated Methicill in-resistant *Staphylococcus aureus*

IBC Integron-associated beta-lactamase

KPC *Klebsiella pneumonia e*carbapenemase

MBL Metallo-beta-lactamase

MDR Multi drug resistance

MIC Minimum inhibitory concentration

MRSA Methicillin-resistan t*Staphylococcus aureus*

NAD Nicotinamide adenine dinucleotide

NDM New Delhi metallo-beta-lactamase

OXA Oxacillinase

PBP Penicillin-binding protein

PCL Peri-ciliary liquid

PCR Polymerase chain reaction

PVL Panton Valentine leukocidin

RND Resistance nodulation division

SCV Small colony variant

SHV Sulfhydryl variable-beta-lactamase

TEM Temoneira-beta-lactamase

VIM Verona integron-encoded metallo-beta-lactamase

Chapter 1

Introduction

Cystic fibrosis (CF) is the most common and best known genetic disease involving a defect intrans-epithelial chloride (Cl^-) transport by mutations in the CF gene on chromosome 7, which codes for the cystic fibrosis transmembrane conductance regulator protein (CFTR). CF is characterized by chronic lung malfunction, pancreatic insufficiency and high level of chloride in sweat [1]. Since the discovery of the CF gene in 1989, more than a thousand mutations have been described. CF is caused by the inheritance of two mutated CF genes, one from each parent and is inherited in an autosomal recessive manner where each parent of a child with CF is a carrier of one abnormal CF gene, but is individually healthy [2]. This disease affects persons without distinction of age or sex but can b a symptomatic in a number of cases [1]. In South Africa approximately in 20 individuals in the white population, 1 in 55 in the population of mixed ancestry and up to 1 in 90 black Africans carry a CFTR mutation according to the South African Cystic Fibrosis Consensus Document 2012. CFTR mutations vary considerably between populations and regions of the world with ΔF508 constituting approximately 66% of all CF mutations globall y[3]. The F508DEL mutation further accounts for up to 81% of all CF alleles in the South African white population[4], 53% in South Africans of mixed-race but is rarely detected in black African populations[5].

The CFTR protein is a cyclica denosine monophosphate (cAMP) mediated chloride channel that regulates the ion and water balance across epithelia. Absence of functional CFTR protein results in an increased viscosity of the exocrine secretions leading to ciliary dysfunction, mucus impaction and chronic endo-bronchial infection in the lungs. The normal mucus clearance is mediated by a two-layer liquid system called the air way surface liquid (ASL). The upper phase is a mucus layer generated by mucins ecretion. The lower phase is a poly-anionic watery layer known as a peri-ciliary liquid (PCL). This two layer system permits efficient ciliary beating and mucus clearance. The height of the PCL layer is regulated by homeostatic mechanisms which include co-ordinated activities of different ion channels. The apical membrane epithelial sodium channel (ENaC) together with the CFTR channel is responsible form a intaining the PCL height. In the absence of CFTR function, unrestrained sodium ions (Na^+) absorption occurs together with failure of active chloride ion (Cl^-) secretion

which leads to failure of mucus clearance and decrease of airway surface liquid volume if no compensational mechanism exists [2].

Mutations in CFTR gene are classified into five groups according to their consequences in the CFTR protein synthesis and its chloride channel function. The presence of large deletions and stop codon categorized under Class I result intruncated and mostly non-functional CFTR. Class II mutations, including the common F508del, lead to aberrantly folded CFTR protein that is recognized by the cell quality control mechanism and subsequently degraded, resulting in the absence of mature CFTR protein at the apical cell membrane. Class III mutations lead to the full-length CFTR protein being incorporated into the cell membrane, but with defective regulation so that no CFTR function is present. These three classes usually lead to a classic CF phenotype with pancreatic insufficiency, although the severity of lung disease is highly variable. CFTR mutations leading to defective chloride conductance are grouped Into Class IV. Class V mutations involve transcription dys-regulation, resulting in a decreased amount of otherwise normal CFTR. The latter two classes are often associated with a milder phenotype and pancreatic in sufficiency [2].

The primary cause of long term complication and frequently death among CF patients is chronic bacterial infection of the respiratory tract. The respiratory pathogens most commonly associated with CF patients are *Staphylococcus aureus*, *Haemophilus influenzae* and *Pseudomonas aeruginosa*. *S. aureus* and *H. I nfluenza* are isolated early in life of patients with CF while nearly all CF patients become colonized with *P. aeruginosa* later in life. Other bacterial pathogens isolated from CF respiratory tract re generally not persistent colonizers. These intermitted species include *Streptococcus pneumonia*, *Escherichia coli, Klebsiella* spp., *Proteus* spp.,*Serratia* spp., *Enterobacter* spp.,and *Citrobacter* spp. Many other opportunistic bacterial pathogens belonging to Gram- negative non-fermenters have also been isolated in the respiratory tract of CF patients, including other *Pseudomonas* spp., *Acinetobacter anitratus*, *Achromobacter* spp., *Stenotrophomonas maltophilia* and *Burkholderia cepacia complex (Bcc)*. Less often and later on in life, CF patients can be become infected with organisms like *Aspergillus* spp., *Candida albicans* and atypical mycobacteria [6]. *P. aeruginosa* and *Bcc* are the most problematic bacterial infections and are characterized by low responsiveness to antibiotic therapy and significant reduction in

patient's lung function. Both bacteria pose the risk of epidemic spread within the CF community, with the *Bcc* being distributed among CF patients to much smaller extent (3-30% ; compared to *P. aeruginosa* 70-80%) [7].

S. aureus is a Gram-positive coccus that has been describeds a major pathogen in CF and is usually the first pathogen to infect and colonize airways of CF patients. *S. aureus* is the predominant pathogen in children, reaching a prevalence rate of nearly 50% by the age of 10 years [8]. Nasal carriage rates of *S. aureus* are significantly higher in patients with CF (66%) than in patients without CF (32%) [9]. Selective media such as mannitol salt agar may be used for isolation of *S. aureus*. Positive result of coagulase and deoxyribonuclease tests can also be used to distinguish is organism from other *Staphylococcus* spp[10]. This pathogen may cause epithelial damage which leads to adherence of other pathogens. Other studies suggest that *S. aureus* is a co-infective pathogen associated with *P. aeruginosa*. Both pathogens lead to more intense inflammatory response. Before the use of antibiotics in treatment of *S. aureus* infections, *S. aureus* was causative f several deaths in children with CF. Today, this risk is not so serious, but CF patients not given the correct antibiotic therapy show high prevalence of *S. aureus* in nasal epithelium. Young patients with mild lung disease and colonized with *S. aureus* or *H. influenzae* are often treated with oral antibiotics, including amoxicillin-clavulanate,t rimethoprim-sulfamethoxazole and cephalexin. If colonized with *S. aureus* alone, patients often receive oxacillin, nafcillin or Cephalothin monotherapy [11**].** Kahl and colleagues (1998) reported that *S. aureus* sub-population, small colony variant (SCV) phenotypes could be recovered from up to 50% of CF patients harboring *S. aureus*. In contrast to *S. aureus*, SCVs yield small, non-hemolytic, non-igmented and slowly growing colonies on enriched media such as sheep blood or chocolate agars, thus making these isolates difficult to recognize as *S. aureus* [12].

Methicillin-resistant *S.aureus* (MRSA) has shown a progressive increase in prevalence in CF populations. MRSA have been found in both healthcare and community associated *S. aureus* infections. Hospital-associated MRSA (HA-MRSA) show high prevalence in older CF patients, while community-associatedMRSA(CA-MRSA) strains have been associated with younger CF patients[12]. CA-MRSA have the *mecA* gene on a small mobile staphylococcal cassette chromosome and a unique virulence factor in the form of Panton-Valentine

leukocidin (PVL). PVL is a two component pore-forming toxin encoded by two co-transcribed genes that cause tissue necrosis and leukocyte destruction[9].There are several resistance mechanisms used by *S. aureus* which include enzymatic inactivation of the antibiotic (penicillinase and aminoglycoside-modification enzymes), alteration of the target with decreased affinity for the antibiotic, trapping of the antibiotic (for vancomycin and possibly daptomycin) and efflux pumps (fluoroquinolones and tetracycline) [13]. Three distinctly different mechanisms of methicillin resistance have been described in *S. aureus*. The best documented and probably most important mechanism is production of a unique, low affinity penicillin-binding protein, PBP 2a. Strains possessing PBP 2a are resistant to methicillin, oxacillin, and probably all other currently available beta-lactam antibiotics. The second mechanism of reduced susceptibility to methicillin is the hyper-production of *Staphylococcus* penicillinase. Anti-staphylococcal penicillins were developed to resist the hydrolytic action of staphylococcal penicillinase, but it appears that some contemporary strains produce such large amounts of the enzyme that methicillin and oxacillin are slowly but appreciably degraded. A third mechanism describes an intermediate level of resistance to methicillin due to production of modified, normal PBPs with reduced affinity for beta-lactams.These strains produce PBPs1 and 2 of normal molecular size, but with low affinity for beta-lactam antibiotics [14].

H. influenzae is a Gram-negative cocco-bacillus that requires special growth factors, hemin (factor X) and nicotinamide-adenine-dinucleotide (NAD also known as V factor). *H. influenzae* strains are divided into two groups depending on presence or absence of a polysaccharide capsule [15]. Non-encapsulated *H. influenzae* is the one that is mostly associated with chronic lung infections and acute exacerbations in CF patients [16].Chocolate agar plate with an antimicrobial disk of bacitracin can be used to isolate *H. influenzae* in respiratory secretion on CF patients. *H. influenzae* can be differentiated from most other species of *Haemophilus* by its specific requirement for both hemin and NAD for growth. *H. haemolyticus* is the other species that also requires both hemin and NAD factors for growth. To differentiate between two species hemolysis must be checked on blood agar. *H. haemolyticus* usually causes hemolysis on these media, while *H. influenzae* does not; although it has been reported that a substantial proportion of *H. haemolyticus* are non-hemolytic.. Many non-hemolytic *H. haemolyticus* strains are mis-identified as *H. influenzae.*

Thus, analysis of 16rRNA or conserved P6 genes or IgA protease genes can be used for differentiate these two organisms [15].

H. influenzae usually infects younger CF patients. The in ability to detect *H.influenzae* in adult patients with CF could be explained by this organism being obscured by mucoid *P. aeruginosa* [17]. Young patients with mild lung disease and colonized with *H. influenzae* or *S.aureus* are often treated with oral antibiotics including amoxicillin-clavulanate, trimethoprim-sulfamethoxazole and cephalexin. Patients colonized with both *H. influenzae* and *S. aureus* may receive combination therapy with both a narrow-spectrum penicillin and an aminoglycoside such as gentamicin [11]. *H. influenzae* under goes hyper-mutation which is associated with resistance to many antibiotics. The outer membrane f *H.influenzae* provides very little resistance to the penetration of beta-lactams in to the cell compared to enterobacteriaceae. This explains the lower beta-lactam minimum inhibitory concentration (MIC) of both susceptible and resistance strains. Resistance of ampicillin and other β-lactam antibiotics is mediated by production of beta-lactamases or alteration of the penicillin binding protein in strains lacking beta-lactamases.Some strains possess both mechanisms. Susceptibility to all beta-lactamsin *H.influenzae*is generally predicted bys usceptibilityt to ampicillin as defined by the Clinical and Laboratory Standards Institute (CLSI) MIC breakpoints [18].

P. aeruginosa is an oxidase-positive Gram-negative motile rod [1].This bacterium is an opportunistic pathogen which only causes diseases in patients with impaired host defenses [19]. It is more prevalent in adult CF patients, as infection has been shown in 20% CF patients 0–2 years old while in 81% of adult groups (>18 years old) [1]. *P. aeruginosa* isolated from patients with CF can be differentiated in terms of their morphotypes and susceptibility profiles. The initial strains that infect CF patients are described as rough or planktonic strains. These strains are sensitive to a variety of antibiotics, are motile and prototrophic, and have smooth lipopolysaccharide. The mucoid *P. aeruginosa* is associated with development of chronic infection in CF patients. These strains are non-motile, have rough lipopolysaccharide and are frequently auxotrophic. An examination of sputa from patients reveals that mucoid strains are Gram-negative rods in small clusters surrounded by amorphous material that stains Gram-negative. This material isapolysaccharide polymer referred to as alginate which forms the biofilm matrix and renders the embedded

Pseudomonas species difficult to clear by the immune system. *P. aeruginosa* cells in biofilms are resistant to antibiotics. The mechanisms of resistance in biofilms are unclear but high concentration of β-lactamases, penetration barriers and slow growth are so me of the factors involved in resistance mechanisms. There is growing consensus that lung pathology occurring during the chronic *P. aeruginosa* infection is due to a large extent to the immune response directed against pseudomonal biofilms [12].

Isolation of *P. aeruginosa* from respiratory secretions of CF patients is easily accomplished, with both rough and mucoid isolates being recovered on agar selective for Gram-negative organisms, such as MacConkey and eosin methylene blue agar. Identification of *P. aeruginosa* can be accomplished by positive oxidase test, pigment production and growth at 42^{o}C. Some strains lose their phenotypic appearance as chronic infection progresses. The loss of these phenotypes is most likely an evolutionary change in which corresponding genes are either down-regulated or los t in a nutrient-rich environment [12]. The intrinsic and acquired antibiotic resistance makes *P. aeruginosa* one of the most difficult to treat [20].

Several mechanisms are involved inantibiotic resistance of *P.aeruginosa*. Efflux pump mechanisms have become broadly recognized as major components of resistance to many classes of antibiotics. Some efflux pumps selectively extrude specific antibiotics, while others, referred to as multidrug resistance (MDR) pumps, expel avariety of structurally diverse compounds [20]. Efflux systems of the resistance nodulation division (RND) family, MexAB-OprM, MexEF-OprN, MexCD-OprJ, and MexXY-OprM are well characterized in *P. aeruginosa* and contribute significantly to antibiotic resistance [21].

Beta-lactam antibiotics are the most common treatment for pseudomonal bacterial infections. Production of beta-lactamases is the main mechanism of bacterial resistance to this class of antibiotic. Many Gram-negative bacteria possess naturally occurring, chromosomally mediated beta-lactamases [22]. AmpC beta-lactamase is characteristically chromosomally encoded in *P. aeruginosa*. Some a ntibiotics, such as the carbapenems, are strong inducers of this beta-lactamase but are, stable to its hydrolytic effects. Clavulanate can induce expression of the AmpC beta-lactamase, result in antagonism of the bactericidal activity of ticarcillin. This has led some authors to suggest that ticarcillin-clavulanate be avoided when selecting an anti-pseudomonal beta-lactam antibiotic. Stably derepressed mutants that hyper-produce the AmpC beta-lactamase may lead to resistance to ticarcillin, piperacillin, and third-generation

cephalosporins [21]. Hyper-production of the inducible AmpC beta-lactamase is mostly due to the inactivation of the amidase AmpD and two additional AmpD homologues leading to an increase of inducer molecules. Hyper-mutationis characterized by an increased spontaneous-mutation rate and seems to be an advantage for fast adaptation to a heterogeneous and fluctuating environment, like the lung of a chronically infected CF patient. Among *P. aeruginosa* strains from CF patients, high proportions are hyper-mutable. Recently, studies found hyper-mutation to be the key factor in development of mutation-mediated multi-resistance in patients with chronic *P. aeruginosa* lung infections [23].

Some beta-lactamases are acquired such as TEM and SHV plasmid mediated beta-lactamases. Extended-spectrum-beta-lactamases (ESBLs) are beta-lactamases that hydrolyze extended spectrum cephalosporins with an oxyimino side chain. These cephalosporins include cefotaxime, ceftriaxone, and ceftazidime, as well as the oxyimino-monobactam, aztreonam. TEM and SHV ESBLs have been detected in *P. aeruginosa* and have minor substitutions that greatly extend their hydrolytic spectra with resistance to oxyimino-aminothiazolyl-cephalosporins, monobactams, and penicillins but not to carbapenems [24]. CTX-M is a recently described family of the extended-spectrum-beta-lactamases. The name CTX reflects the potent hydrolytic activity of these beta-lactamases against cefotaxime [22]. CTX-M-1 was described for the first time in 2006, in a *P .aeruginosa* strain which was isolated from the sputum of a 21-year-old cystic fibrosis patient in Amsterdam [24]. PER-1 beta-lactamase efficiently hydrolyzes penicillins and cephalosporins and is susceptible to clavulanic acid inhibition. The PER-1 beta-lactamase was first detected in strains of *P. aeruginosa* isolated from Turkey. Later, it was found among the isolates of *Salmonella enterica, Proteus mirabilis* and *Alcaligenes faecalis*. The OXA-type beta-lactamases confer resistance to ampicillin and cephalothinand are characterized by their high hydrolytic activity against oxacillin and cloxacillin and the fact hat they are poorly inhibited by clavulanic acid. Amino acid substitutions in OXA enzymes can also give the ESBL phenotype. OXA-type ESBLs have been found mainly in *P. aeruginosa*. Other uncommon ESBLs such as VEB, GES and integron-associated beta-lactamase (IBC)beta-lactamases are found mainly in *P. aeruginosa* [22].

Antibiotic-resistant bacteria linfections are a major public health problem worldwide. Few disease states have as high a prevalence of antibiotic–resistant infections as does CF. Estimates suggest that 25-45% of adult CF patients are chronically infected with multi-

resistant bacteria within their airways [25]. Patients with CF are at risk of multi-resistant infections because they have endo-bronchial bacterial infections that in most cases cannot be eradicated [26] and frequent high dose antibiotic therapy is an essential part of CF management [27]. Patients are exposed to multiple courses of antibiotics both chronically and intermittently, and this introduces selective pressure for the development of antibiotic resistance in infecting and/or colonizing organisms impacting on disease management, morbidity and mortality [26]. Regular surveillance of sputum cultures is thus essential. This prospective study describes the microbiological profile of cystic fibrosis in the public and private health sectors in Durban, South Africa, specifically, the bacterial/fungal identity of isolates from sputum cultures and the antibiotic susceptibility of bacterial isolates. The phenotypic and genotypic antibiotic resistance mechanisms were also delineated.

1.4Aims and Objectives

Aim

To describe the profile of cystic fibrosis in the public and private health sectors in Durban, in terms of patients demographics, and the phenotypic and genotypic resistance to β-lactam antibiotics.

Objectives

1. To describe the demographic profile of cystic fibrosis patients in the public and private healthcare sectors of Durban, KwaZulu-Natal in terms of patient age, date of diagnosis and CF mutation from patient clinical records.
2. To describe the microbiological profile of sputum obtained from CF patients in terms of the dentity of the isolates as determined by the Vitek 2 System.
3. To phenotypically ascertain antibiotic susceptibility profiles against antibiotic panels recommended by the CLSI by MIC determinations.
4. To phenotypically screen for ESBL production using the double disc synergy test, AmpC beta-lactamase production using the cefoxitin disc sensitivity test, inducible AmpC beta-lactamase production using the disk antagonism test and MBL production using the imipenem-EDTA combined disk test as appropriate, on the basis of MIC results.

5. To delineate the genotypic mechanisms of beta-lactamase-mediated antibiotic resistance by PCR and sequencing of bla_{TEM}, bla_{SHV} $bla_{CTX\text{-}M}$, bla_{CMY}, bla_{PER}, bla_{VEB}, bla_{OXA},bla_{KPC}, bla_{GES},bla_{IMP},bla_{VIM},andbla_{NDM}genes

CHAPTER 2

Two papers and a conference presentation emanated from this study as follows:

Mhlongo, N., Govinden, U., Egner, J., Essack, S.Y. (2014). *Demographic and microbiological profile of cystic fibrosis in Durban, South Africa.* African Journal of Microbiology Research 8 (33):3118-3122.

Mhlongo, N., Govinden, U., Essack, S.Y.(2015). *NDM-1, Novel TEM-205, Novel TEM-213 and Other ESBLS Co-expressed inIsolates from Cystic Fibrosis Patients*. Southern African Journal of Infectious Diseases (in press).

Mhlongo, N., Govinden, U., Essack, S.Y. Novel TEM-205 isolated in *Pseudomonas aeruginosa* from a cystic fibrosis patient in South Africa. Presented at the 53rd Interscience Conference on Antimicrobial Agents and Chemotherapy, Denver, Colorado, September 2013

Contributions:

- Ms N Mhlongo, as the principle investigator, wrote the protocol for the study, undertook the data acquisition, laboratory work and data analysis, and, drafted the journal articles and conference poster.
- Professor S Y Essack, as principle supervisor, conceptualized the study,contributed to data analysis and undertook critical revision of the journal articles and conference poster.
- Dr U Govinden, as co-supervisor, designed the study, facilitated data acquisition, laboratory work and data analysis, and contributed to the writing and critical revision of the journal articles and conference poster.
- Dr J Egner provided the specialist clinical expertise and undertook critical revision of the journal article.

NB: The culturing, identification and susceptibility testing was undertaken by collaborators at Lancet Laboratories and corroborated by the National Health Laboratory Services at Inkosi Albert Luthuli Central Hospital and acknowledged accordingly. Ms Mhlongo undertook the phenotypic and genotypic characterization of the beta-lactamases.

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Demographic and microbiological profile of cystic fibrosis in Durban, South Africa

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Cystic fibrosis (CF) necessitates long-term treatment with multiple antibiotics creating selection pressure for the development of antibiotic resistance in infecting and/or colonizing organisms, impacting on disease management, morbidity and mortality. Sputum samples were obtained from patients attending the only two CF clinics in Durban over a year. The patient demographics and clinical data were recorded. Bacterial isolates were subjected to identification, susceptibility testing and phenotypic screening for extended spectrum β-lactamases (ESBLs), AmpC β-lactamases and metallo-β-lactamases (MBLs). Twenty-five patients constituted the study sample. The most common genotype was F508del and the most common pathogen was *Pseudomonas aeruginosa* with susceptibility to antibiotics ranging from 14-100% with marginal differences between mucoid and non-mucoid phenotypes. All *P. aeruginosa* isolates were putative ESBL producers and 75% were putative MBL producers. The incidence, prevalence and susceptibility patterns of bacterial pathogens and colonizers isolated from cystic fibrosis patients should be closely monitored to optimize management and treatment options in a disease requiring chronic antibiotic therapy which increases the propensity for the development of antibiotic resistance.

Key words: *Pseudomonas aeruginosa*, cystic fibrosis, extended spectrum β-lactamases (ESBLs), metallo-β-lactamases (MBLs).

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease caused by a mutation in the gene of the CF transmembrane regulator (CFTR) resulting in high morbidity and early mortality (Coutinho et al., 2008). In South Africa, approximately 1 in 20 individuals in the white population, 1 in 55 in the population of mixed-race and 1 in 90 black Africans carry a CFTR mutation (The South African Cystic Fibrosis Consensus Document, 2012). CFTR mutations vary considerably between populations and regions of the world with F508del constituting approximately 66% of all CF mutations globally (Saleheen and Frossard, 2008). The F508del mutation further accounts for up to 81% of all CF alleles in the South African white/caucasian population (Goldman et al., 2001), 53% in South Africans of mixed-race but is rarely detected in black African populations (Maseka et

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al., 2013).

The primary cause of long term complication and frequently death among CF patients is chronic bacterial infection of the respiratory tract with *Pseudomonas aeruginosa, Staphylococcus aureus* and *Haemophilis influenzae* as common causative bacteria (Hauser et al., 2011), although many other opportunistic bacteria have also been isolated, notably *Burkholderia cepacia* complex (BCC), *Stenotrophomonas maltophilia* and *Acinetobacter* spp., while, *Aspergillus* spp., non-tuberculosis mycobacteria and respiratory viruses have also been implicated (Saiman and Siegel, 2004). The most problematic bacterial infections are caused by *P. aeruginosa* and BCC and are characterized by significant reduction in lung function and low responsiveness to antibiotic therapy because of antibiotic resistance (Drevinek et al., 2008). Few disease states have high prevalence of antibiotic–resistant infections as does CF with 25-45% of adult CF patients estimated to be chronically infected with multi-resistant bacteria within their respiratory tracts (Cystic Fibrosis Foundation, 1994). Both bacteria also pose the risk of epidemic spread within the CF community, with the BCC being distributed among CF patients to much smaller extent (3 – 30%) as compared to *P. aeruginosa* (70 – 80%) (Drevinek et al., 2008). Neonates with CF have structurally normal lungs and no *P. aeruginosa*, but non-mucoid *P. aeruginosa* is acquired after variable time periods (LiPuma, 2010). The prevalence of *P. aeruginosa* increases with age and non mucoid *P. aeruginosa* is converted to mucoid *P. aeruginosa* which is usually associated with increasing lung deterioration with time (Govan and Deretic, 1996; Li et al., 2005).

This cross sectional observational study describes the demographic and microbiological profiles of CF in patients attending the only two dedicated CF clinics in the public and private health sectors in Durban, South Africa.

MATERIALS AND METHODS

Ethical considerations

This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu Natal, South Africa (BE148/11).

Study sample

Patients attending the only two CF clinics (one public and the other private) in Durban, South Africa on scheduled clinic days over a 12 months period from June 2012-June 2013 formed the study sample and only 1 sputum sample (after expectoration) was sourced from each patient. The demographic and clinical data recorded included race, gender, age, age of first diagnosis and CFTR genotype (if known).

Microbiology

Twenty – five sputum samples were obtained (15 from the public sector and 10 from the private sector clinic). Bacterial and fungal isolates were identified and minimum inhibitory concentrations (MICs) for the bacterial isolates were determined using the VITEK MS and the VITEK 2 systems (bioMérieux, USA) with MICs analyzed according to CLSI guidelines (CLSI, 2012) for benzyl penicillin, oxacillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefuroxime, cefotaxime, cefoxitin, ceftazidime, cefepime, ertapenem, imipenem, meropenem, amikacin, gentamicin, tobramycin, ciprofloxacin, moxifloxicin, erythromycin, clindamycin, teicoplanin, vancomycin, tetracycline, tigecycline, fusidic acid, mupirocin, nitrofurantoin, colistin and trimethoprim/ sulfamethoxazole appropriately. *Streptococcus pneumoniae* ATCC 49619 and *Enterococcus faecalis* ATCC 29212 served as controls for Gram-positive identification and susceptibility respectively, *Shigella sonnei* ATCC 25931 and *Escherichia coli* ATCC 25922 served as controls for Gram-negative identification and susceptibility respectively and *Candida albicans* ATCC 14053 was the control for fungal identification.

All isolates were screened for extended-spectrum β-lactamase (ESBL) production using the double disc synergy method (Begum et al., 2013), AmpC β-lactamase production using the cefoxitin disc sensitivity test, inducible AmpC β-lactamase production using the disk antagonism test (Upadhay et al., 2010) and metallo-β-lactamase (MBL) production using the imipenem-EDTA combined disk test (Yong et al., 2002).

RESULTS

Twenty-five out of a total of 42 patients currently registered with the CF clinics constituted the study sample, representing 60% of the known patient cohort. There were 14 adults and 11 children, 14 males and 11 females, ranging in age from 2-33 years. Twenty-three (92%) of patients were white, 1 was of mixed-race and 1 was Indian. Of the 23 white patients, 18 were homo-zygous and 2 heterozygous for the F508del mutation, one showed the 3659 del C, 1 showed 1 copy each of the Δ1507 and 1 the E60X mutations and 1 was unknown. The CFTR genotypes of the other patients were unknown. Most of the patients were diagnosed in infancy or *in utero*. Only 1 patient was diagnosed in adulthood (Table 1).

Twenty-two bacterial and 6 fungal isolates were identified (Table 1). Patients harboured 3 different *Candida* spp. and 6 bacterial species other than the mucoid and non-mucoid *P. aeruginosa* in 12 different permutations. *P. aeruginosa* constituted the vast majority of bacterial isolates at 15 (68%).

The antibiotic susceptibility of *P. aeruginosa* isolates is shown in Table 2 with a marginal difference in susceptibility between the mucoid and non-mucoid phenotypes. *E. cloacae* was resistant to ampicillin, amoxicillin/clavulanate and cefoxitin; *K. pneumonia* was resistant to amoxicillin /clavulanate, cefoxitin and tobramycin; *B. cepacia* was resistant to meropenem and ciprofloxacin, *S. aureus* was resistant to benzyl penicillin and rifampicin and *Streptococcus mitis* showed intermediate resistance to erythromycin. All isolates were susceptible to colistin, used against multi-drug resistant isolates as a last resort because of its nephrotoxicity. All 20 (100%) Gram-negative isolates yielded a positive test for ESBLs and 15

Table 1. Patient demographics, age of CF diagnosis, CFTR genotype and microorganisms isolated from sputum.

Patient	Age	Gender	Race	Age of diagnosis	Genotype	Microorganisms Isolated from Sputum
1	2	M	W	Birth	Homozygous F508del	Normal respiratory tract bacterial flora
2	3	M	C	1 Year	Unknown	Normal respiratory tract bacterial flora; *Candida dubliniensis*
3	3	F	W	Birth	Homozygous F508del	Normal respiratory tract bacterial flora
4	4	F	W	1 Year	homozygous F508del	Normal respiratory tract bacterial flora; *C. dubliniensis*
5	5	F	W	Birth	homozygous F508del	Normal respiratory tract bacterial flora
6	7	M	W	3 months	homozygous F508del	*S. mitis S. maltophilia; Candida albicans*
7	8	F	W	13 Months	homozygous F508del	Normal respiratory tract bacterial flora
8	8	M	W	Birth	homozygous F508del	Normal respiratory tract bacterial flora
9	10	M	W	17 months	F508del /G551D	Mucoid *P. aeruginosa*
10	10	M	W	1 week	homozygous F508del	Non-mucoid *P. aeruginosa*
11	12	F	W	3 months	homozygous F508del	Non Mucoid *P. aeruginosa*
12	15	M	W	*In vitro*	Unknown	Mucoid and non-mucoid *P. aeruginosa; E. cloacae; C. albicans*
13	16	M	W	13 months	3659 del C	Mucoid and non-mucoid *P. aeruginosa*
14	18	F	W	22 months	homozygous F508del	*E. cloacae*
15	19	F	W	*In vitro*	homozygous F508del	Mucoid *P. aeruginosa; K. pneumonia; C. albicans*
16	20	M	W	Birth	Heterozygous F508del	Mucoid *P. aeruginosa*
17	21	F	W	9 months	homozygous F508del	Mucoid and non-mucoid *P. aeruginosa*
18	21	M	W	1 week	homozygous F508del	Normal respiratory tract bacterial flora
19	24	M	W	6 weeks	homozygous F508del	Normal respiratory tract bacterial flora; *C. albicans*
20	25	M	I	4 years	Unknown	*S. aureus*
21	25	F	W	1 month	Δ1507; E60X (1 copy each)	*B. cepacia*
22	26	F	W	*In vitro*	homozygous F508del	Non-mucoid *P. aeruginosa*
23	28	M	W	17 months	homozygous F508del	Mucoid *P. aeruginosa*
24	32	F	W	20 years	homozygous F508del	Normal respiratory tract bacterial flora
25	33	M	W	1 year	homozygous F508del	Mucoid and non-mucoid *P. aeruginosa; Candida glabrata*

F- female; M- male; I- Indian; C- mixed race; W- white.

Table 2. Susceptibility of *P. aeruginosa* isolates to selected antibiotics.

Antibacterial agent	Susceptibility (%)	
	Mucoid *P. aeruginosa* (n= 8)	Non-mucoid *P. aeruginosa* (n= 7)
Piperacillin/-tazobactam	6 (75)	7 (100)
Ceftazidime	7 (88)	7 (100)
Cefepime	6 (75)	6 (86)
Imipenem	8 (100)	6 (86)
Meropenem	7 (88)	7 (100)
Amikacin	4 (50)	3 (43)
Gentamicin	2 (25)	1 (14)
Tobramycin	7 (88)	5 (71)
Ciprofloxacin	6 (75)	4 (57)
Colistin	8 (100)	7 (100)

(75%) were positive for MBLs. Although 18 (90%) of isolates screened were positive for AmpC β-lactamase production on the basis of resistance to cefoxitin on the disc sensitivity test, none were inducible according to the

Table 3. Results of phenotypic screening for β-lactamases.

β-lactamase	*P. aeruginosa* (n=15)	*E. cloacae* (n=2)	*K. pneumonia* (n=1)	*B. cepacia* (n=1)	*S. maltophilia* (n=1)
ESBL	15	2	1	1	1
AmpC	13	2	1	1	1
Inducible AmpC	0	0	0	0	0
MBL	12	1	0	1	1

disk antagonism test (Table 3).

DISCUSSION

Although CF occurs in all South African population groups, it is better described in the white and mixed race populations while its prevalence in the black population is less well known (Westwood et al., 2006) indicating potential under-diagnosis as the black population group comprises greater than 80% of the total KwaZulu Natal population. Notwithstanding the fact that CF patients may be managed outside of the two CF clinics, possible under-diagnosis may be attributed to CF being omitted from differential diagnoses in this population group, poor access to medical care and misdiagnosed as malnutrition, indicative of CF, is very common in the black population for poverty-related reasons (Maseka et al., 2013). Further, just two of the 13 CF clinics in South Africa are located on the coast in Durban and may not be easily accessible to people from the inner and rural areas. The predominant CFTR genotype was F508del as recorded previously for the South African population (Maseka et al., 2013).

Many organisms that are isolated from sputa of CF patients are pathogens (e.g. *S .aureus*) that often progress to colonize the upper respiratory tract or are common environmental organisms that behave as opportunistic pathogens (e.g. *P. aeruginosa*) (Valenza et al., 2008; Cardoso et al., 2008). *S. aureus* is usually the first pathogen to infect and colonize the airways of CF patients (Hauser et al., 2011), while *P. aeruginosa* occurs in early childhood with prevalence increasing with age such that as many as 80% of patients with CF are infected with *P. aeruginosa* by the time they reach the age of 20 (Li et al., 2005). The median age recorded in this study was 16 explaining the predominant isolation of *P. aeruginosa*.

Patients with CF are at risk of multi-resistant infections as a result of endo-bronchial bacterial infections that in most cases cannot be eradicated (Aaron, 2007) and frequent high dose antibiotic therapy is an essential part of CF management. Patients are exposed to multiple courses of antibiotics both chronically and intermittently, and this introduces selective pressure for the development of antibiotic resistance in infecting and/or colonizing organisms (The South African Cystic Fibrosis Consensus Document, 2007).

In comparison, non-mucoid *P. aeruginosa* showed lesser susceptibility to imipenem, ciprofloxacin and the aminoglycosides while mucoid *P. aeruginosa* were less susceptible to meropenem, the cephalosporins and the piperacillin-tazobactam inhibitor combination. Although differences in antimicrobial susceptibility between mucoid and non-mucoid *P. aeruginosa* have been documented in many studies, the significance is yet to be ascertained. Notwithstanding the marginal differences in susceptibility observed in this study, it is postulated that the exo-polysaccharide/alginate compromises access to antibiotics such that the mucoid isolates are exposed to sub-inhibitory concentrations of antibiotics facilitating the evolution of resistance (Hauser et al., 2011).

All *P. aeruginosa* isolates in this study were putative ESBL producers and were resistant to most of cephalosporin generations. Infections with ESBL-producing pathogens occur in patients who have recently received broad spectrum antibiotics, particularly third-generation cephalosporins and quinolones as is the case with chronic therapy in CF. Multi-drug resistance to the aminoglycoside, fluoroquinolone and β-lactam antibiotic classes was also evident and attributed to the co-carriage of resistance genes on the same genetic determinants of resistance, whether plasmids, transposons or integrons, severely limiting treatment options (Kanj and Kanafan, 2011).

The incidence, prevalence and susceptibility patterns of different microorganisms in the sputa of CF patients should be closely monitored to optimize management and treatment options in a disease requiring chronic antibiotic therapy to reduce morbidity and mortality. The complexity and diversity of β-lactamase expression in *P. aeruginosa* from CF patients, necessitates early detection to inform efficacious antibiotic therapy as antibiotic options are limited not only in the treatment of CF but in the treatment of all infections globally.

Conflict of Interests

The authors have not declared any conflict of interests.

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NDM-1, Novel TEM-205, Novel TEM-213 and Other ESBLS Co-expressed in Isolates from Cystic Fibrosis Patients from South Africa

Running title: NDM-1, TEM-205, TEM-213 in cystic fibrosis from South Africa

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Abstract

Background: β-lactamase mediated resistance was investigated in isolates from cystic fibrosis patients attending clinics in the public and private health sectors in Durban, South Africa.

Methods: Fifteen *Pseudomonas aeruginosa*, 2 *Enterobacter cloacae* and 1 each of *Klebsiella pneumoniae*, *Burkholderia cepacia* complex (Bcc) and *Stenotrophomonas maltophilia* were subjected to MIC determinations, PCR and sequencing for bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, bla_{CMY}, bla_{PER}, bla_{VEB}, bla_{OXA}, bla_{KPC}, bla_{GES}, bla_{IMP}, bla_{VIM}, and bla_{NDM} genes.

Results: All but one isolate carried multiple β-lactamases from 2 or more different Ambler classes. Novel TEM-205 (GenBank Accession no. KC900516) was found in a single isolate in combination with NDM-1, reported for the first time in *P. aeruginosa* in South Africa. TEM-205 showed 5 amino acid changes compared with TEM-1; viz., V84I, E104K, R164S, M182T and A184V while novel TEM-213 (GenBank Accession no. KC663615), identified in 3 isolates, showed a single amino acid change Y105F. Resistance phenotypes did not routinely correlate with genotypes. This is the first report of NDM-1 from *Bcc* in South Africa.

Conclusions: The co-expression and/or co-carriage of Ambler classes A, B and C β-lactamases in various permutations in single isolates severely restricts the clinical management of CF not only with beta-lactam antibiotics but also aminoglycosides and fluoroquinolones, the resistance genes of which commonly occur on the same genetic determinants of resistance. The presence of NDM-1 in combination with the CMY AmpC β-lactamases, TEM, SHV and CTX-M ESBLs is of grave concern leaving colistin as the sole remaining treatment option in this pathogen.

Introduction

Cystic fibrosis (CF) is an inherited, recessive, autosomal disease affecting multiple organ systems. Its impact on the respiratory system is the leading cause of morbidity and mortality and the primary cause of death is by respiratory failure from chronic pulmonary infection with *Pseudomonas aeruginosa* as the commonest causative organism.[1-2] While early infections in CF are largely attributable to *Staphylococcus aureus* and *Haemophilis influenzae*, *P. aeruginosa* infection increases with age such that the vast majority of adult CF patients are chronically infected[2] necessitating long-term combination therapy with aminoglycosides, β-lactams and fluoroquinolones in inhaled, oral and intravenous dosage forms to circumvent resistance.[1] The irony of standard, chronic combination therapy in CF is the fact that these very antibiotic classes co-select for resistance with β-lactam antibiotics, aminoglycosides and fluoroquinolones resistance genes commonly occurring on the same genetic determinants of resistance whether plasmids, transposon, integrons or gene cassettes.

Further, *P. aeruginosa* is one of the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, P. aeruginosa, Enterobacter spp.*) pathogens responsible for significant morbidity and mortality because of innate and acquired resistance.[3] The evolution of resistance is facilitated by the host where purulent airway secretions and impaired mucociliary clearance compromise the penetration of antibiotics[2] together with the pathogen's ability to switch to the mucoid phenotype; both resulting in exposure to sub-inhibitory antibiotic concentrations.[4] Resistance to the β-lactam antibiotics, in particular, may be mediated by reduced permeability because of altered porin profiles, active efflux and the production of plasmid-mediated AmpC β-lactamases, extended-spectrum β-lactamases and carbapenemases, particularly metallo β-lactamases.[5]
This study presents CF as a microcosm of selection pressure with a focus on β-lactamase-mediated resistance. We report on two novel TEM β-lactamases and the appearance of NDM-1 in *P. aeruginosa* and *Burkholderia cepacia* complex (Bcc) from cystic fibrosis patients in South Africa.

Material and Methods

Ethical considerations

This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu Natal (BE148/11).

Study sample

Fourteen of a total of 25 patients attending the only two CF clinics in Durban on scheduled clinic days provided a total of 22 bacterial isolates from sputum samples over a 12 month period from June 2012-June 2013. Of these bacterial isolates, all but 2 of the Gram-positive isolates, viz., *S. aureus* and *Streptococcus mitis* constituted the final microbiological sample of 20.

Identification and susceptibility testing

Bacterial isolates were identified and minimum inhibitory concentrations (MICs) were determined using the Vitek 2 identification system (bioMérieux, USA) with MICs analyzed according to the CLSI (2012).[6] *Shigella sonnei* ATCC 25931 and *Escherichia coli* ATCC 25922 served as controls for Gram-negative identification and susceptibility respectively *Streptococcus pneumoniae* ATCC 49619 and *Enterococcus faecalis* ATCC 29212 served as controls for Gram-positive identification and susceptibility respectively and *Candida albicans* ATCC 14053 was the control for fungal identification.

Phenotypic detection of β-lactamases

Isolates were screened for extended-spectrum beta-lactamase (ESBL) production using the double disc synergy method[7], AmpC beta–lactamase production using the cefoxitin disc sensitivity test, inducible AmpC beta–lactamase production by the disk antagonism test[5] and metallo beta-lactamase (MBL) production by the imipenem-EDTA combined disk test.[8]

Genotypic characterization of β-lactamases

Isolates were screened for the presence of bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{CMY}, bla_{PER}, bla_{VEB}, bla_{OXA}, bla_{KPC}, bla_{GES}, bla_{IMP}, bla_{VIM} and bla_{NDM} genes using specific primers. Bacterial strains were grown on Muller-Hinton agar (Biolab, Johannesburg, South Africa) overnight and DNA extraction was performed using the ZR fungal/bacterial DNA MiniPrep kit (The Epigenetic, CA, USA). PCR amplification mixture was prepared in a final volume of 50 µl, containing sterilized distilled water, 2 µl template DNA, 10 µM of each primer (Inqaba Biotechnology, Pretoria, South Africa) and 25 µl of master mix (Applied Biosystems, Foster City, CA). The

PCR amplification for *bla*$_{TEM}$ and *bla*$_{SHV}$, *bla*$_{CTX-M}$ and *bla*$_{CMY}$ was then performed in a Gene Amp 2700 PCR system (Applied Biosystems, Forster City, CA) as described by Essack *et al.*[9], Edelstein *et al.*[10] and Zhao *et al.*[11]. The PCR amplification for *bla*$_{PER}$, *bla*$_{VEB}$, *bla*$_{OXA}$, *bla*$_{KPC}$, *bla*$_{GES}$, *bla*$_{IMP}$, *bla*$_{VIM}$, and *bla*$_{NDM}$ was performed as described by De Champs *et al.*[12], Bert *et al.*[13], Weldhagen *et al.*[14] and Nordmann *et al.*[15] with some modifications. *Enterobacter cloacae* producing GES-5, *K. pneumoniae* producing IMP -1, *E. coli* producing KPC-2, *K. pneumonia* producing NDM-1, *E. coli* producing OXA – 48 and *K. pneumonia* producing VIM -1 obtained from P. Nordmann were used as control strains[16] while in-house controls were used for *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$ and *bla*$_{CMY}$. PCR products were separated in 1.5% agarose gel for 40 min at 120 V, stained with ethidium bromide (0.5 μg ml^{-1}) and detected by UV trans-illumination. Sequencing of the PCR positive products was carried out by using the BigDye version 3.1 dye terminator cycle sequencer from Applied Biosystems and the sequences were analyzed using BLAST 2.0 (Basic Local Alignment Search Tool) software available on the website of National Center for Biotechnology information (http://www.ncbi.nhlm.nih.gov/blast /BLAST.cgi).

Results

Sputum samples from 14 patients yielded 8 mucoid *P. aeruginosa*, 7 non-mucoid *P. aeruginosa*, 2 *Enterobacter cloacae* and 1 each of *Klebsiella pneumoniae*, *Burkholderia cepacia* complex (Bcc), *Stenotrophomonas maltophilia*, constituting a final sample of 20 isolates for this study. (Six fungal isolates consisting of 3 different *Candida spp.* and the 2 Gram-positive isolates, viz. *S. aureus* and *S. mitis* were not subjected to further study). Four patients carried both mucoid and non-mucoid *P. aeruginosa*, 1 patient carried mucoid and non-mucoid *P. aeruginosa* together with *E. cloacae* and 1 patient carried a mucoid *P. aeruginosa* with *K. pneumoniae*.

All but two isolates carried multiple β-lactamases from 2 or more different Ambler classes. Resistance phenotypes did not routinely correlate with genotypes, in that the MICs did not evidence the typical resistance profiles of the β-lactamases identified (Tables 1 and 2). For example, just isolates 7b and 24b showed raised carbapenem MICs despite a 70% occurrence of MBLs in the form of NDM.

Novel TEM-205 (GenBank Accession no. KC900516) was found in a single isolate and showed 5 amino acid changes compared with TEM-1; viz., V84I, E104K, R164S, M182T

and A184V while novel TEM-213 (GenBank Accession no. KF663615) showed a single amino acid change Y105F (Table 3). To our knowledge, this is the first report of NDM-1 in *P. aeruginosa* and Bcc in South Africa.

Discussion

Cystic fibrosis typifies a microcosm of selection pressure for the evolution of resistance in individual patients who are exposed to multiple courses of antibiotics both chronically and acutely. Resistance is thus the inevitable consequence of the necessary repeated courses of antibiotics during pulmonary exacerbations caused by chronically infecting pathogens, particularly *P. aeruginosa*. Few disease states have as high a prevalence of antibiotic–resistant infections as does CF with a 10-19% prevalence of multiple drug resistance (MDR) defined as resistance to all agents in two or more antibiotic classes.[2]

The multiplicity, complexity and diversity of β-lactamases resulting in poor correlation between resistance phenotypes (MICs) and genotypes (identification of β-lactamase genes by PCR and DNA sequencing) in this study was attributed to one or more of the following: (1) high intra-species diversity, in that, sub-populations of phenotypically distinct and diverse mucoid and non-mucoid *P. aeruginosa* are common in cystic fibrosis with phenotypes displaying significant variation even within isolates of the same colony morphotype from the same sample and despite being from single clonal lineages[3], (2) *P. aeruginosa* in cystic fibrosis maintain only a small fraction of the population in the hyper-mutative state[18], (3) silent or minimally functional genes[19], (4) the presence of hetero-resistance (mixed populations of drug-resistant and drug-susceptible cells of a single strain), the detection of which may be influenced by the screening method, test conditions, local epidemiology, antibiotic selection pressure and the unstable nature of the resistance phenotype.[20] Further, low-level resistance and even susceptibility has been reported for several carbapenemases.[15] Microbial communities in the respiratory tracts of CF patients are thus complex ecosystems with extensive microbial diversity[1] and changing phenotypes during chronic infection.[21] A delineation of the genetic environment is recommended to explore and understand the lack of enzyme production despite the presence of ESBL and carbapenemase genes. Sufficed to say, this lack of correlation confounds susceptibility-informed antibiotic therapy, which, in our opinion necessitates routine genotypic investigations to confirm phenotypic observations

should resistant sub-populations and/or silent/minimally expressed genes become fully functional during therapy.

The co-expression and/or co-carriage of Ambler classes A, B and C β-lactamases, specifically TEM, CTX-M, NDM and CMY, in various permutations in single isolates severely restricts the clinical management of CF not only with beta-lactam antibiotics but also aminoglycosides and fluoroquinolones, the resistance genes of which commonly occur on the same genetic determinants of resistance whether plasmids, transposon, integrons or gene cassettes, leaving colistin as the sole remaining treatment option.

bla$_{NDM}$ occurs in non-clonally associated isolates and is present on a variety of plasmids carrying several other resistance genes such as those of other carbapenemases (OXA-48 and VIM-types), plasmid-mediated cephalosporinases, ESBLs, as well as aminoglycoside, macrolide, rifampicin and sulphamethoxazole resistance genes. Consequently, prior use of any of these antibiotic classes may select for carbapenemase-producing isolates. The acquisition of *bla*$_{NDM}$ has, further, largely been associated with travel to the Indian sub-continent.[15] This study however demonstrates the presence of NDM-1 in one or more cystic fibrosis patients with possible dissemination from patient to patient in the CF clinics resulting in the 70% NDM-1 found in isolates from these CF patients confined to South Africa. NDM may have further evolved as a result of the selection pressure imposed by chronic treatment with β-lactam, aminoglycoside, fluoroquinolone antibiotics as the mainstay of CF treatment. NDM-1, described in another case report on MBLs from a different province in South Africa further corroborated MICs below the resistance breakpoint despite genotypic detection.[22] Low-level resistance and even susceptibility has been reported for most carbapenemases.[15] CF patients present a risk for the dissemination of NDM in the community as the vast majority are managed on an out-patient basis, with hospitalization for severe, acute exacerbations only. Routine screening for carbapenemases in *P. aeruginosa* isolates from CF patients is thus advised.

TEM-205 is a combination of TEM-63 and TEM-116, the former first reported in South Africa. The mutation in TEM-213 is not on a recognised "hotspot". Both enzymes should be subjected to biochemical studies to elucidate their kinetic characteristics.

Conclusions

Our conclusions are two-fold, the first relating to the management of cystic fibrosis and the second to the general proliferation of β-lactamases. The increasing incidence, prevalence and resistance patterns of bacterial pathogens and colonizers isolated from cystic fibrosis patients should be closely monitored to optimize management and treatment options and contain dissemination in the community especially as CF is a disease requiring chronic antibiotic therapy which increases the propensity for the development of antibiotic resistance - a vicious circle especially when the commonest chronic pathogen is of the ESKAPE group. β -lactamases continue to proliferate in response to antibiotic selection pressure of the beta-lactam, aminoglycoside and fluoroquinolone antibiotic classes making it imperative to conserve the efficacy of existing antibiotics while redoubling efforts to discover new ones.

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276 Table 1: MICs and β lactamases produced in *P. aeruginosa*

Isolate	Isolate no.	TZP	CAZ	FEP	IPM	MEM	AKM	GEN	TOB	CIP	COL	β-lactamases
P.aeruginosa												
mucoid	1a	≤4	4	16	2	≤0.25	16	≥16	2	1	≤0.5	TEM-63; CMY-2
	5a	≤4	2	2	≤0.25	≤0.25	16	8	2	1	≤0.5	CTX-M-37; NDM-1
	7a	32	16	8	2	1	≤2	≤1	≤1	0.5	≤0.5	TEM-63; CTX-M-37; CMY-2; NDM-1
	12a	16	4	4	≤0.25	≤0.25	32	8	16	1	≤0.5	TEM-205; NDM-1
	15	32	4	2	1	4	≤2	≤1	≤1	≤0.25	≤0.5	TEM-63; CTX-M-37; CMY-2
	23	≤4	4	4	1	≤0.25	≥64	≥16	4	≥4	≤0.5	CTX-M-37; NDM-1
	24a	≤4	4	16	2	≤0.25	32	≥16	2	≥4	≤0.5	CTX-M-37; NDM-1
	32	≤4	4	8	2	0.5	32	8	4	0.5	2	TEM-213
non-mucoid	1b	≤4	4	8	2	0.5	16	≥16	2	2	≤0.5	TEM-63; CMY-2; NDM-1
	5b	≤4	4	2	1	≤0.25	32	≥16	2	1	≤0.5	CMY-2; NDM-1; CTX-M-37
	7b	16	8	8	≥16	1	≥64	≥16	≤1	2	≤0.5	TEM-63; NDM-1
	21	≤4	2	≤1	≤0.25	≤0.25	16	8	≤1	≤0.25	≤0.5	CTX-M-37; NDM-1
	24b	≤4	4	16	2	≤0.25	32	≥16	16	≥4	≤0.5	CTX-M-37; CMY-2; NDM-1
	26	≤4	≤1	4	0.5	0.5	32	≥16	16	≤0.25	≤0.25	CTX-M-37; NDM-1
	28	≤4	4	4	≤0.25	≤0.25	4	4	≤1	0.5	≤0.5	CTX-M-37; NDM-1

277 TZP, piperacillin/tazobactam; CAZ, ceftazidime; FEP, cefepime; IPM, imipenem; MEM, meropenem; AMK, amikacin; GEN, gentamicin; TOB, tobramycin; CIP,

278 ciprofloxacin; COL, colistin

279 Table 2: MICs and β lactamases produced in Enterobacteriaceae, *B. cepacia and S.maltophilia*

Isolate		AMP	AMC	TZP	CXM	FOX	CTX	CAZ	FEP	IPM	MEM	AKM	GEN	TOB	CIP	TGC	SXT	β-lactamases
E. cloacae	1c	≥32	≥32	≤4	4	≥64	≤1	≤1	≤1	≤0.25	≤0.25	≤2	≤1	≤1	≤0.25	1	≤20	CTX-M-37; CMY-2
	29	≥32	≥32	≤4	4	≥64	≤1	≤1	≤1	≤0.25	≤0.25	≤2	≤1	≤1	≤0.25	1	≤20	TEM 213; NDM-1
K.pneumonia	12b	≥32	≥32	≤4	4	≥64	≤1	≤1	≤1	≤0.25	≤0.25	≤2	≤1	≥16	≤0.25	≤0.5	≤20	
e																		TEM-63; CMY-2; SHV-12
B. cepacia	27	-	-	-	-	-	-	≤1	-	-	≥16	-	-	-	16	-	≤20	TEM-63; CTX-M-37; NDM-1
S.maltophilia	30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	≤20	TEM 213

280 AMP, ampicillin; AMC, amoxicillin/clavulanic acid; TZP, piperacillin/tazobactam; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime;, IPM,

281 imipenem; MEM, meropenem; AMK, amikacin; GEN, gentamicin; TOB, tobramycin; CIP, ciprofloxacin; TGC, tigecycline; SXT, trimethoprim/

282 sulfamethoxazole

283 Table 3: Amino Acids Changes of TEM-205 and TEM-213

Enzyme	Amino acid positions[17]						
	21	84	104	105	164	182	184
TEM-1	L	V	E	Y	R	M	A
TEM-63	F		K		S	T	
TEM-116		I					V
TEM-205		I	K		S	T	V
TEM-213				F			

284 A, alanine; E, glutamic acid; F, phenylalanine; I, isoleucine; K, lysine; L, leucine; M,
285 methionine; R, arginine; S, serine; T, threonine; V, valine; Y, tyrosine
286

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INYUVESI YAKWAZULU-NATALI

NOVEL TEM-205 ISOLATED IN *PSEUDOMONAS AERUGINOSA* FROM A CYSTIC FIBROSIS PATIENT IN SOUTH AFRICA

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Abstract

Introduction: β-lactamase mediated resistance was investigated in *Pseudomonas aeruginosa* isolates from patients attending cystic fibrosis clinics in the public and private health sectors in Durban, South Africa. **Methods:** Fifteen *P aeruginosa* isolated from 25 sputum samples were subjected to MIC determinations, PCR and sequencing for bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{CMY}, bla_{VEB}, bla_{PER}, bla_{GES}, bla_{KPC}, bla_{OXA}, bla_{VIM}, bla_{IMP} and bla_{NDM} genes. **Results:** All but one isolate carried multiple β-lactamases from 2 or more different Ambler classes. Novel TEM-205 (GenBank Accession no. KC900516) was found in combination with NDM-1 in a single isolate. TEM-205 showed 5 amino acid changes compared with TEM-1; viz., V84I, E104K, R164S, M182T and A184V. **Conclusion:** β-lactamases of the TEM family continue to proliferate in response to antibiotic selection pressure of the beta-lactam, aminoglycoside and fluoroquinolone antibiotic families, common chronic therapy in cystic fibrosis patients. The apparent de novo evolution of NDM-1 in CF patients is of grave concern.

Introduction

- Cystic fibrosis (CF) is an autosomal recessive disease caused by a mutation in the gene of the CF trans-membranous conductance regulator (CFTR) resulting in significant morbidity and early mortality [1].
- The primary cause of long term complication and frequently death among CF patients is acute and chronic bacterial infection of the respiratory tract with *Pseudomonas aeruginosa, Staphylococcus aureus* and *Haemophilus influenzae* as common causative pathogens [2] although many other opportunistic bacterial pathogens have also been isolated, notably *Burkholderia cepacia* complex (Bcc), *Stenotrophomonas maltophilia* and *Acinetobacter* spp. [3].
- The most problematic bacterial infections are caused by *P. aeruginosa* and Bcc, and are characterized by significant reductions in a patient's lung function and low responsiveness to antibiotic therapy as a result of antibiotic resistance [4].
- Few disease states have as high a prevalence of antibiotic-resistant infections as does CF with 25-45% of adult CF patients estimated to be chronically infected with multi-resistant bacteria within their respiratory tracts [5].

Material and Methods

Ethical approval number BE148/11 (Biomedical Research Ethics Committee, UKZN)

Fifteen *P. aeruginosa* isolates from 25 sputum samples were subjected to:

- MIC determinations using the VITEK 2 identification system (bioMérieux, USA) with MICs analyzed according to CLSI guidelines [6].
- Phenotypic screening for extended-spectrum beta-lactamase (ESBL) production using the double disc synergy method [7], inducible AmpC beta-lactamase production using the disk antagonism test [8] and metallo-beta-lactamase (MBL) production using the imipenem-EDTA com bined disk test [9].
- PCR detection of bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{CMY}, bla_{VEB}, bla_{PER}, bla_{GES}, bla_{KPC}, bla_{OXA}, bla_{VIM}, bla_{IMP} and bla_{NDM} genes[10-16].
- Sequencing of PCR positive products using the BigDye 3.1 dye terminator cycle sequencer (Applied Biosystems) with analysis using BLAST 2.0 (http://www.ncbi.nlm.nih.gov/blast /BLAST.cgi).

Results

Table 1 : MICs and β lactamases produced in *P. aeruginosa* with resistance in red and intermediate susceptibility in blue

Isolate	Isolate no.	TZP	CAZ	FEP	IPM	MEM	AMK	GEN	TOB	CIP	CST	β-lactamases
P. aeruginosa												
Mucoid	1a	≤4	4	16	2	≤0.25	16	≥16	2	1	≤0.5	TEM-63; CMY-2
	3a	≤4	2	2	≤0.25	≤0.25	16	8	2	1	≤0.5	CTX-M-37; NDM-1
	7a	32	16	8	2	1	≤2	≤1	≤1	0.5	≤0.5	TEM-63; CTX-M-37; CMY-2; NDM-1
	13a	16	4	4	≤0.25	≤0.25	32	8	16	1	≤0.5	TEM-205; NDM-1
	15	32	4	2	1	1	≤2	≤1	≤1	≤0.25	≤0.5	TEM-63; CTX-M-37; CMY-2
	23	≤4	4	4	1	≤0.25	≥64	≥16	4	≥4	≤0.5	CTX-M-37; NDM-1
	24a	≤4	4	16	2	≤0.25	32	≥16	2	≥4	≤0.5	CTX-M-37; NDM-1
	37	≤4	4	8	2	0.5	32	8	4	0.5	2	TEM-213
Non-mucoid	1b	≤4	4	8	2	0.5	16	≥16	2	2	≤0.5	TEM-63; CMY-2; NDM-1
	5b	≤4	4	2	1	≤0.25	32	≥16	2	1	≤0.5	CMY-2; NDM-1; CTX-M-37
	7b	16	8	4	≥16	1	≥64	≥16	≤1	2	≤0.5	TEM-63; NDM-1
	21	≤4	2	≤1	≤0.25	≤0.25	16	8	≤1	≤0.25	≤0.5	CTX-M-37; NDM-1
	24b	≤4	4	16	2	≤0.25	32	≥16	16	≥4	≤0.5	CTX-M-37; CMY-2; NDM-1
	26	≤4	≤1	4	0.5	0.5	32	≥16	16	≤0.25	≤0.25	CTX-M-37; NDM-1
	28	≤4	4	4	≤0.25	≤0.25	4	4	≤1	0.5	≤0.5	CTX-M-37; NDM-1

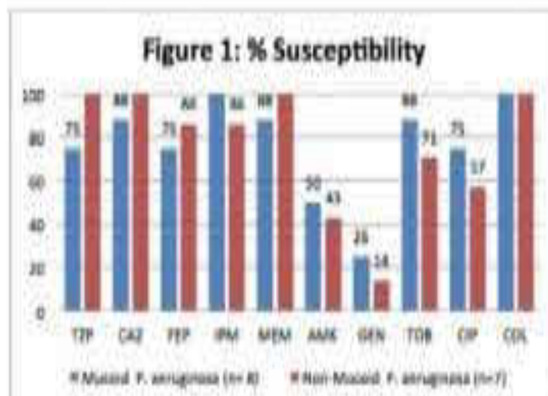

Table 2: Amino Acids Changes of TEM-205 and TEM 213

Enzyme	Amino acid positions [17]						
	21	84	104	105	164	182	184
TEM-1	L	V	E	Y	R	M	A
TEM-63	F		K		S	T	
TEM-116		I					V
TEM-205		I	K		S	T	V
TEM-213				F			

A, alanine; E, glutamic acid; F, phenylalanine; K, lysine; L, leucine; M, methionine; R, arginine; S, serine; V, valine; Y, Tyrosine

Discussion

- Cystic fibrosis typifies a microcosm of selection pressure for the evolution of resistance in individual patients who are exposed to multiple courses of antibiotics both chronically and acutely [18].
- The co-expression and/or co-carriage of Ambler classes A, B and C β-lactamases in various permutations in single isolates severely restricts the clinical management of CF not only with beta-lactam antibiotics but also aminoglycosides and fluoroquinolones, the resistance genes of which commonly occur on the same genetic determinants of resistance whether plasmids, transposon, integrons or gene cassettes.
- TEM-205 is a combination of TEM-63 and TEM-116, the former first reported in South Africa.
- The postulated de novo evolution of NDM-1 in 80% of isolates in apparent response to repeated courses of carbapenem therapy and its existence in combination with the TEM and CTX-M ESBLs as well as the CMY AmpC β-lactamases is of grave concern leaving colistin as the sole remaining treatment option.
- The multiplicity, complexity and diversity of β-lactamases confounds the correlation of resistance phenotypes with genotypes. Inadequate correlation between the MICs and the β-lactamase genes identified may be attributed to one/more of the following reasons:
  - Sub-populations of phenotypically distinct and diverse mucoid and non-mucoid *P. aeruginosa* are commonplace in cystic fibrosis with phenotypes displaying significant variation even within isolates of the same colony morphotype from the same sample and despite being from single clonal lineages [19].
  - *P. aeruginosa* in cystic fibrosis maintain only a small fraction of the population in the hyper-mutative state [20].
  - Silent or minimally functional genes [21].
  - Possible resistance suppression of cephalosporins with aminoglycosides as reported of cefepime and tobramycin [22] in the case of the very low ceftazidime MICs.
- Kinetic studies of variants is academic as β-lactamases belonging to different Ambler classes are increasingly co-expressed and/or co-carried in pathogenic bacteria in clinical settings and occur concomitantly with other resistance mechanisms such as efflux.

Conclusions

- β-lactamases of the TEM family continue to proliferate in response to antibiotic selection pressure of the beta-lactam, aminoglycoside and fluoroquinolone antibiotic families, common chronic therapy in cystic fibrosis patients.
- The incidence, prevalence and susceptibility patterns of bacterial pathogens and colonizers isolated from cystic fibrosis patients should be closely monitored to optimize management and treatment options in a disease requiring chronic antibiotic therapy which increases the propensity for the development of antibiotic resistance.

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References

1–22. [illegible]

GenBank Accession number in progress

Paper 1 fulfills objectives 1 – 4 while paper 2 further elaborates on these objectives and fulfils objective 5 as does the conference poster.

Chapter 3

This prospective, descriptive study describes the demographic and microbiological profiles of cystic fibrosis in patients attending the only two dedicated CF clinics in the public and private health sectors in Durban, South Africa. It further presents CF as a microcosm of selection pressure with a focus on beta-lactamase-mediated resistance and reports on two novel TEM beta-lactamases, and the appearance of NDM-1 in *P. aeruginosa* and *Bcc* from cystic fibrosis patients in South Africa.

Conclusions

- The most common genotype in the study sample which represented 60% of the known patient cohort was F508del.
- The most common pathogen was *P. aeruginosa* with susceptibility to antibiotics ranging from 14-100% with marginal differences between mucoid and non-mucoid phenotypes.
- All *P aeruginosa* isolates were putative ESBL producers and 80% were putative MBL producers.
- All isolates were susceptible to colistin.
- All but one isolate carried multiple β-lactamases from two or more different Ambler classes.
- Novel TEM-205 (GenBank Accession no. KC900516) was found in a single isolate in combination with NDM-1, reported for the first time in *P. aeruginosa* in South Africa. TEM-205 showed five amino acid changes compared with TEM-1; viz., V84I, E104K, R164S, M182T and A184V.
- Novel TEM-213 (Gen Bank Accession no. KC663615), identified in 3 isolates, showed a single amino acid change Y105F.
- Resistance phenotypes did not routinely correlate with genotypes.

The co-expression and /or co-carriage of Ambler classes A, B and C β-lactamases in various permutations in single isolates severely restricts the clinical management of CF not only with beta-lactamantibiotics but also aminoglycosides and fluoroquinolones, the resistance genes

Of which commonly occur on the same genetic determinants of resistance. The presence of NDM-1 in combination with the CMY AmpC β-lactamases, TEM, SHV and CTX-M ESBLs is of grave concern leaving colistin as the sole remaining treatment option in this pathogen. The incidence, prevalence and susceptibility patterns of different microorganisms in the sputa of CF patients should be closely monitored to optimize management and treatment options in a disease requiring chronic antibiotic therapy which increases the propensity for the development of antibiotic resistance.

Limitations

- The study sample was limited to patients that attended the CF clinics during the study period. The results cannot be extrapolated to KZN nor South Africa as a whole.
- Patient records, particularly records in the public sector were often incomplete precluding in-depth correlations of pulmonary function parameters (e.g.FEV_1) and body nutritional status (BMI) with variables such as CFTR genotype, age at time of CF diagnosis and chronic *P. aeruginosa* infection.

Recommendations

- A data base of CF patients needs to be created to obtain an accurate demographic and microbiological profile that is representative of the CF patient cohort in KwaZulu-Natal and South Africa.
- Future molecular biology studies should focus on distinct sub-populations of *P. aeruginosa* isolates as there is evidence of significant variation even within isolates of the same colony morphotype from the same sample and despite being from single clonal lineages [28].
- Molecular typing may help to detect the presence of common resistant strains circulating among patients to prevent spread of these strains, particularly in CF patients who frequently share the same environments.

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