Characterisation of fosfomycin resistance in urinary pathogens from the Western Cape, South Africa.

by

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Thesis presented in fulfilment of the requirements for the degree of Master of Science in the Faculty of Medicine and Health Sciences at Stellenbosch University

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March 2021

Declaration

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Abstract

Introduction: Urinary tract infections (UTI) are the most commonly acquired bacterial infections worldwide. The South African Department of Health advised that fosfomycin, nitrofurantoin and gentamicin be used for the treatment of uncomplicated UTI due to other antibiotics showing adverse side effects. Fosfomycin has effectively been utilised in the management of UTI, however resistance has been detected in urinary pathogens at the Tygerberg Hospital National Health Laboratory Service (NHLS) Medical Microbiology diagnostic laboratory. This study aimed to determine the prevalence of fosfomycin resistance among community-acquired urinary pathogens in the Western Cape and to characterise fosfomycin resistance mechanisms in fosfomycin resistant *Escherichia coli* and *Klebsiella pneumoniae* isolates.

Methods and Materials: Two-hundred urinary isolates (Enterobacterales and *Enterococcus spp*.) from antenatal clinics in the Western Cape were collected from the Tygerberg Hospital NHLS Medical Microbiology laboratory during 2019 and 2020 and used to determine the prevalence of fosfomycin resistance. Fosfomycin susceptibility was determined using disc diffusion and Etest®. Fosfomycin resistant *E. coli* and *K. pneumoniae* isolates from the prevalence study and another set of fosfomycin resistant isolates (5 *E. coli* and 19 *K. pneumoniae*) collected from urine samples submitted to the NHLS at Tygerberg Hospital in 2017 (Ethics #: U17/05/026) were used to characterise fosfomycin mechanisms. FosA mediated resistance was determined using a phenotypic assay and *fosA* genes were detected by PCR. Mutations in the fosfomycin target gene *murA* and transporter genes, *glpT* and *uhpT*, were characterised by polymerase chain reaction (PCR) and Sanger sequencing.

Results: Fosfomycin resistance was detected in 3.5% of community-acquired urinary pathogens. Fosfomycin resistance rates were 2.2% in *E. coli* (3/139) and 12.9% in other Enterobacterales. All *Enterococcus spp*. isolates were susceptible to fosfomycin. In the combined sample set of 31 fosfomycin resistant isolates, the phenotypic assay detected FosA in only 7 isolates, while *fosA* genes were detected by PCR in 25. Chromosomal mutations were identified in 6 isolates, of which three isolates (1 *K. pneumoniae* and 2 *E. coli*) had deletions in the *uhpT* gene, which has previously been reported to confer fosfomycin resistance. The role of other mutations found in the *glpT* gene of *E. coli* and the *murA* and *glpT* of *K. pneumoniae* isolates has not been determined.

Conclusion: The fosfomycin resistance rate in community-acquired UTI was low, which supports the careful ongoing use of fosfomycin for the treatment of uncomplicated community-acquired UTI. FosA mediated resistance was the most common mechanism of fosfomycin resistance identified in this population.

Opsomming

Inleiding: Infeksies van die urinêre traktus is die mees algemene bakteriële infeksies wat wêreldwyd opgedoen word. Die Suid-Afrikaanse Departement van Gesondheid beveel aan dat fosfomisien, nitrofurantoïen en gentamisien die gewenste middels is vir die behandeling van ongekompliseerde infeksies van die urinêre traktus (UTI) weens ander middels se newe-effek profiele. Alhoewel fosfomisien tans effektief vir behandeling aangewend word, is daar weerstandige patogene gevind by Tygerberg Hospitaal Nasionale Gesondheidslaboratoriumdiens (NGLD) se Mediese Mikrobiologie diagnostiese laboratorium. Hierdie studie poog om die prevalensie van fosfomisien-weerstandige gemeenskapsverwante urinêre patogene in die Wes-Kaap te bepaal, asook om die weerstandigheidsmeganismes in fosfomisien-weerstandige *Escherichia coli* en *Klebsiella pneumoniae* te karakteriseer.

Metodes en materiale: Tweehonderd urinêre isolate (Enterobacterales en *Enterococcus spesies*) vanaf voorgeboorte klinieke in die Wes-Kaap is vanuit Tygerberg Hospitaal NGLD se Mediese Mikrobiologie laboratorium versamel tydens die periode 2019 tot 2020 met die doel om die fosfomisien-weerstandigheidsprevalensie te bepaal. Fosfomisien-vatbaarheid is bepaal met skyfdiffusie en die Etest® toets. Alle fosfomisien-weerstandige *E. coli* en *K. pneumoniae* isolate van hierdie prevalensie studie asook isolate vanaf ‘n vooraf versamelde stel (5 *E. coli* and 19 *K. pneumoniae*) is bestudeer om die weerstandigheidsmeganismes te karakteriseer Die vorige stel isolate is verkry in 2017 (Etiese #: U17/05/026) vanuit urinêre monsters wat by Tygerberg Hospitaal NGLD laboratorium ingedien is. FosA-gemedieërde weerstandigheid is bepaal met behulp van ‘n fenotipiese toets en *fosA* gene is met ‘n polimerase kettingreaksie (PKR) opgespoor. Mutasies in die fosfomisien teikengeen *murA* en die vervoerdergene *glpT* en *uhpT*, is met PKR en Sanger volgorde bepalingstoetsing bespeur.

Resultate: Fosfomisien-weerstandigheid is gewaar in 3.5% van die gemeenskapsverwante urinêre patogene. Die fosfomisien-weerstandigheidskoers was 2.2% in *E. coli* (3/139) en 12.9% in ander Enterobacterales spesies. Alle Enterococcus spesies was vatbaar vir fosfomisien. In die gekombineerde monsterstel van 31 fosfomisien-weerstandige isolate het die fenotipiese toets FosA in slegs sewe isolate opgespoor terwyl PKR die *fosA* geen in 25 isolate opgespoor het. Chromosomale mutasies is in ses isolate geïdentifiseer waarvan drie isolate (1 *K. pneumoniae* en 2 *E. coli*) genetiese skrappings in die *uhpT* geen getoon het.

Hierdie skrappings is bekend om weerstandigheid te kan verleen. Die rolle van die ander waargeneemde mutasies is steeds onbekend.

Afsluiting: Die fosfomisien-weerstandigheidskoers in gemeenskapsverwante UTI is steeds laag en bevorder dus die versigtige voortdurende gebruik van fosfomisien vir die behandeling van ongekompliseerde gemeenskapsverwante UTI. FosA-gemedieërde weerstandigheid is die mees algemene fosfomisien-weerstandigheidsmeganisme in hierdie populasie en word gewoonlik met plasmiede bemiddel wat maklike oordrag tussen bakteriële selle deur middel van konjugasie in staat stel.

Acknowledgements

I would firstly like to thank God for the love, sanity, and peace He has granted me throughout the course of this study. I also want to pass my greatest gratitude to my supervisors; Dr Pieter Nel and Dr Mae Newton-Foot for their profound guidance, patience and going the extra mile. The Medical Microbiology NHLS staff for their assistance in isolate collection and sorting. The Medical Microbiology division HOD, Prof Andrew Whitelaw who guided this project to success. I want to thank my mother for being my strength and support system in all ways. The NRF has granted me a personal bursary and I appreciated that, along with my project funding organisations; Harry Crossley Foundation and NHLS Research Trust Fund.

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AMC	Ampicillin-clavulanic acid
AMP/AMX	Ampicillin/amoxicillin
AMR	Antimicrobial resistance
AST	Antimicrobial susceptibility testing
ATCC	American Type Culture Collection
CA	Community-acquired
cAMP	Cyclic adenosine monophosphate
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CO_2	Carbon dioxide
CPE	Carbapenemase-producing Enterobacteriaceae
CREs	Carbapenem resistant Enterobacteriaceae
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
EAU	European Association of Urology
EC	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ESBLs	Extended spectrum β-lactamases
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	The Food and Drug Administration
G-6-P	Glucose-6-phosphate

IV	Intravenous
KP	*Klebsiella pneumoniae*
MDR	Multidrug resistant
MH	Mueller-Hinton agar
MIC	Minimum inhibitory concentration
NC	Non-template control
NHLS	National Health Laboratory Service
NIT	Nitrofurantoin
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
STI	Sexually transmitted infections
TAE	Tris, acetic acid, EDTA
TMP-SMX	Trimethoprim-sulfamethoxazole
USA	United States of America
UTI	Urinary tract infections
VRE	Vancomycin-resistant *Enterococcus*
WHO	World Health Organization

Chapter 1

Literature review

1.1 *Urinary tract infections*

Urinary tract infections (UTI) are considered to be the most commonly acquired bacterial infections worldwide, with about 150 million people affected per year (Mueller *et al.*, 2019). UTI are a major public health problem with high morbidity and increasing rates of antimicrobial resistance that are expected to impact greatly on health care expenditures. These infections can affect any part of the urinary tract including the kidneys, ureters, bladder and urethra (Tan, 2016), and they usually occur as a result of retrograde ascending infection following periurethral colonisation with gastro-intestinal tract flora (Lewis *et al.*, 2013; Flores-mireles *et al.*, 2016; Nimri, Sulaiman and Hani, 2017). This can lead to cystitis (infection of the bladder or lower UTI) or pyelonephritis (infection of the kidneys or upper UTI) (Coetzer, 2004; Amin, Mehdinejad and Pourdangchi, 2009; Raz, 2011; Flores-mireles *et al.*, 2016).

UTI are known to be common in children and infants, both in the community and hospital settings (Tan, 2016). Approximately 50 – 60% of women develop a UTI at some point in their lives, with adult women being more likely to develop infections compared to males (Ahmed and Ghadeer, 2013; Tan, 2016). Additional risk factors that may lead to the acquisition of UTI include extremes of age, pregnancy, spinal cord injuries and urinary catheters (Foxman, 2003; Tan, 2016).

UTI are regarded as being either complicated or uncomplicated. Uncomplicated UTI are infections that occur in individuals with a structurally and neurologically normal urinary tract and who do not have identified risk factors (Tan, 2016). Uncomplicated UTI are the most common infections that require prescription of antimicrobials. If a UTI occurs in the presence of a condition involving structural or functional abnormalities of the urinary tract, resulting in a persistent or relapsing infection, it is considered to be complicated (Nicolle and AMMI Canada Guidelines Committee, 2005; Tan, 2016). This includes cases where a patient has

an immunocompromising condition, the presence of foreign objects in the urinary tract and hospital-acquired infections. UTI in men and in pregnancy are always considered to be complicated (Foxman, 2003; Tan, 2016). Infections of the renal parenchyma by blood borne organisms can also occur, especially in cases of *Staphylococcus aureus* bacteraemia where the kidney can be a site for abscess formation (Tong *et al.*, 2015). Possible complications of pyelonephritis include sepsis, premature delivery with increased foetal mortality and renal damage with resultant renal failure, especially amongst paediatric patients.

Patients with nosocomial (hospital-acquired) UTI are more likely to experience recurrent infections or even further health complications (Nimri, Sulaiman and Hani, 2017). Recurrent UTI per definition is experiencing three urinary tract infections in a period of a year or two infections in a period of six months (Albert *et al.*, 2004; Ahmed and Ghadeer, 2013). Nosocomial UTI are usually catheter associated and can account for more than a million cases per year, with MDR organisms contributing to more severe infections such as bloodstream infections (Jacobsen *et al.*, 2008; CDC, 2013). Approximately 15-25% of hospitalised patients with urinary catheters in situ develop catheter associated UTI which result in an extended catheterisation period (Majumder *et al*., 2016).

1.2 *Causative agents of UTI*

The most common causes of UTI are members of the Enterobacterales order which are also a major cause of opportunistic infections (Nimri, Sulaiman and Hani, 2017). *Escherichia coli,* which forms part of the human gut microbiota and has a commensal relationship with the intestinal tract, causes up to 80% of community-acquired and 30% of hospital-acquired UTI (Tsukamoto *et al.*, 2014). Other members that commonly cause UTI include *Klebsiella pneumoniae, Enterobacter* and *Proteus species.* Apart from organisms in the order of Enterobacterales, *Pseudomonas* species, *Staphylococcus saprophyticus* and *Enterococcus* species are also common causes of UTI (Raz, 2011; Tan, 2016).

Enterococci are Gram-positive organisms which form part of the normal gut microbiota, oral cavity and female genital tract (Goel *et al.*, 2016). They are also known to be opportunistic pathogens and are more common in hospital-acquired UTI. However, community-acquired

UTI caused by *Enterococci* are increasing due to the overuse of broad-spectrum antibiotics. *Enterococcus faecalis* and *Enterococcus faecium* are reported to be the most common enterococcal species isolated from clinical samples with a high prevalence in hospital settings (Goel *et al.*, 2016; Ou and Nadeau, 2017). Vancomycin-resistant *Enterococcus* (VRE) is mainly associated with hospital-acquired UTI, especially during prolonged hospitalization; whereas community-acquired UTI caused by VRE are suspected to result from association with sewage material, farm animal products and stools of healthy farm animals.

1.3 *UTI antimicrobial treatment*

The management of UTI depends on whether they are complicated or uncomplicated. Simple uncomplicated cystitis is mostly managed very well with oral antibiotics. However, treatment for complicated UTI requires early diagnosis and proper treatment to prevent sepsis (Tan, 2016). As recommended by the European Association of Urology's (EAU) 2017 guidelines, possible first line options for the treatment of uncomplicated UTI include fosfomycin trometamol, nitrofurantoin and pivmecillinam. The Asian Association of UTI and Sexually Transmitted Infections (STI) also recommends fosfomycin trometamol, nitrofurantoin and pivmecillinam in addition to trimethoprim sulfamethoxazole (TMP-SMX) and fluoroquinolones as treatment for uncomplicated cystitis (Development of Asian Guidelines for UTI/STI, 2015). UTI in men are always considered complicated, therefore a treatment duration of at least seven days with TMP-SXM or a fluoroquinolone is advised to allow penetration of the antimicrobial into the prostate tissue (Burkhard *et al.*, 2018), whereas treatment with oral beta-lactams would require 10 to 14 days of treatment (Kang *et al.*, 2018).

Ultimately the choice of empiric treatment of UTI depends on the epidemiology of resistant strains in the given population. According to the Standard Treatment Guidelines and Essential Medicines List for South Africa (2018), nitrofurantoin is prescribed for pregnant women and children, while ciprofloxacin is prescribed for adults. In cases of complicated cystitis, amoxicillin/clavulanic acid is recommended for children weighing 35 kg or less. According to literature, ciprofloxacin and amoxicillin/clavulanic acid are predominately prescribed in public facilities in South Africa, whereas in private facilities treatment is more varied (Lewis *et al.*, 2013). In addition to ciprofloxacin and amoxicillin/clavulanic acid, private

facilities use cefuroxime, cefpodoxime, levofloxacin, amoxicillin and fosfomycin. Fosfomycin is less expensive than other broad spectrum antibiotics, has greater susceptibility rates and requires shorter treatment durations (Ou and Nadeau, 2017).

It is important to treat bacteriuria in pregnant women even when they are not showing symptoms, the choice of treatment should be safe for both the mother and the foetus. The majority of UTI in healthy pregnancies are community acquired, with *E. coli* being the main causative agent (Nilsson et al., 2003). The empiric treatment of *E. coli* bacterium in UTI in pregnancy is ampicillin however, resistance has been increasing over the years thus more safe options are needed. UTI is always considered complicated in pregnancies but nitrofurantoin has a high urinary concentration and does not affect the foetus, thus it is safe to be orally given to pregnant women with asymptomatic bacteriuria and acute cystitis.

The Food and Drug Administration (FDA) has recently reported fluoroquinolones (including ciprofloxacin) to have permanent side effects such as ruptured tendons, swollen muscles and joints, nerve damage, as well as central nervous system side effects like anxiety and confusion (United States Food and Drug Administration, 2018). It was advised by the South African Department of Health in April 2019 that ciprofloxacin should not be used for non-severe bacterial infections where other antibiotics are effective; neither should it be used for non-bacterial infections or prophylaxis of complicated UTI, due to its severe side effect profile. The South African Department of Health recommends the use of fosfomycin and nitrofurantoin for oral treatment and gentamicin as intravenous treatment for uncomplicated cystitis (Circular H53/2019, Western Cape Department of Health).

In recent years, strains of *E. coli* and *K. pneumoniae* that produce extended spectrum β-lactamases (ESBLs) and/or are resistant to fluoroquinolones have been isolated, with cross-resistance to aminoglycosides being very common (Takahata *et al.*, 2010; Tsukamoto *et al.*, 2014; Li *et al.*, 2015, WHO, 2011). Infections with these multidrug-resistant organisms have limited therapeutically active antimicrobial options (Falagas *et al.*, 2010). Due to concerns such as cost, toxicity, resistance and side effects of antimicrobials such as fluoroquinolones, the use of fosfomycin is being favoured (Ou and Nadeau, 2017). Despite

reported resistance in various countries, fosfomycin remains an option for the treatment of ESBL-producing Gram-negative bacteria (Oteo *et al.*, 2009; Falagas *et al.*, 2010; Takahata *et al.*, 2010; Mueller *et al.*, 2019; Ramos and Llet, 2019).

1.4 *Fosfomycin*

Fosfomycin (formerly known as phosphonomycin) is a bactericidal antibiotic discovered in Spain, in 1969, during a search for organisms that produce broad-spectrum antibiotics (Hendlin *et al.*, 1969). It was found to be produced by strains of *Streptomyces fradiae* (ATCC 21096), *Streptomyces viridochromogenes* (ATCC 21240) and *Streptomyces wedmorensis* (ATCC 21239) (Hendlin *et al.*, 1969). Fosfomycin is now synthetically produced using phosphonic acid as a starting material (Zhanel, Zhanel and Karlowsky, 2018). Fosfomycin is used as a first line oral agent for uncomplicated UTI in women (Knottnerus *et al.*, 2008; Maraki *et al.*, 2009). It has a wide antibacterial spectrum including most enteric Gram-negative bacteria, methicillin susceptible and resistant *S. aureus* as well as glycopeptide susceptible and resistant *Enterococci* (Raz, 2011).

Fosfomycin has a unique structure (Figure 1.1) and inhibits bacterial cell wall synthesis with a mechanism of action that is different from other antimicrobial agents (Cattoir and Guérin, 2018). Fosfomycin has a low molecular weight of 138 Da and can easily penetrate bacterial cells and biofilms (Reffert and Smith, 2014; Aghamali *et al.*, 2019). It is well tolerated and little cross-resistance has been shown to other agents (Coetzer, 2004; Knottnerus *et al.*, 2008; Maraki *et al.*, 2009; Raz, 2011; Li *et al.*, 2015; Nimri, Sulaiman and Hani, 2017; Mueller *et al.*, 2019).

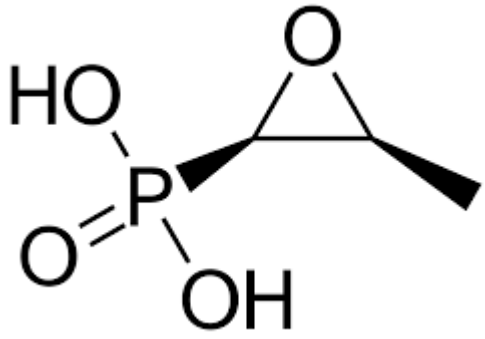

Figure 1.1: Fosfomycin molecular structure (Molecular Formula: C3H7O4P). (www.chemspider.com/Chemical-Structure.394204.html)

1.4.1 Use of fosfomycin

Fosfomycin can be administered both orally as well as intravenously. Fosfomycin is a calcium salt polar molecule, therefore water soluble. It is absorbed by the gastrointestinal tract and excreted by the kidneys in an unchanged form (Monurol, fosfomycin tromethamine; Hendlin *et al.*, 1969; Raz, 2011). Fosfomycin is available in two oral formulations: fosfomycin trometamol and fosfomycin calcium. Fosfomycin trometamol has an improved bioavailability as it is more readily absorbed into the bloodstream as compared to fosfomycin calcium and is therefore the preferred oral formulation (Falagas *et al.*, 2008). Fosfomycin disodium is a form of fosfomycin used intravenously, it is unstable in acidic conditions which explains the poor oral absorption of the disodium salt.

Fosfomycin has the added benefit that it can be administered as a single dose of Monurol (fosfomycin tromethamine) and can be safely prescribed for children older than 5 years of age (Tsukamoto *et al.*, 2014). The FDA-USA has classified fosfomycin as a pregnancy category B drug, as safety on pregnant women has not been established. Fosfomycin has effectively been utilised in the management of UTI caused by ESBL-producing Enterobacterales and can also be used in combination with colistin in UTI caused by carbapenem resistant Enterobacterales (CREs) (Giamarellou and Poulakou, 2009; Falagas *et al.*, 2010; Takahata *et al.*, 2010; Raz, 2011; Benzerara *et al.*, 2017). Furthermore, parenteral formulations of fosfomycin have successfully been used in the management of life threatening infections due to CREs (Li *et al.*, 2015).

Intravenous (IV) fosfomycin is used for hospitalised patients with multi-drug resistant Gram-positive and Gram-negative infections not responding to other antimicrobial agents or if there is resistance or severe side effects (Putensen *et al.*, 2019). It is used clinically for the treatment of a range of infections including those of the urinary, respiratory and gastrointestinal tracts, the central nervous system, infective endocarditis, septic arthritis and bloodstream infections (Falagas *et al.*, 2008; Karageorgopoulos *et al.*, 2012).

In 2017, the World Health Organization (WHO) added intravenous fosfomycin to its list of essential 'last-resort' medicines; this is a group of antibacterial medicines reserved as a treatment option for highly specific patients and settings where there is no alternative treatment option. Other antimicrobials in the 'reserve' group include aztreonam, cefepime, cefazoline, polymyxin B, colistin, linezolid, tigecycline, and daptomycin (WHO, 2017). Biofilm related infections, abscess formation, severe allergy and resistance to standard antimicrobial therapy are some of the examples where intravenous fosfomycin could be implemented (Zhanel, Zhanel and Karlowsky, 2018), however intravenous administration could lead to further hospital-acquired infections and sepsis.

1.4.2 Fosfomycin mechanism of action

Fosfomycin molecules enter the cell through two main transport systems; the glycerol-3-phosphate transporter (GlpT) and the hexose phosphate (G6P) uptake system (UhpT) (Figure 1.2) (Nicolle and AMMI Canada Guidelines Committee, 2005; Maraki *et al.*, 2009; Raz, 2011; Li *et al.*, 2015). Fosfomycin binds to Cys-115 on the active site of MurA (UDP-N-acetylglucosamine enolpyruvyl transferase), mimicking phosphoenolpyruvate (PEP), thereby inactivating the enzyme (Takahata *et al.*, 2010; Li *et al.*, 2015). MurA is responsible for the formation of UDP-N-acetylglucosamine enolpyruvate in the biosynthesis of peptidoglycan; therefore inhibition of MurA blocks cell wall synthesis leading to cell lysis and death (Nicolle and AMMI Canada Guidelines Committee, 2005; Knottnerus *et al.*, 2008; Maraki *et al.*, 2009).

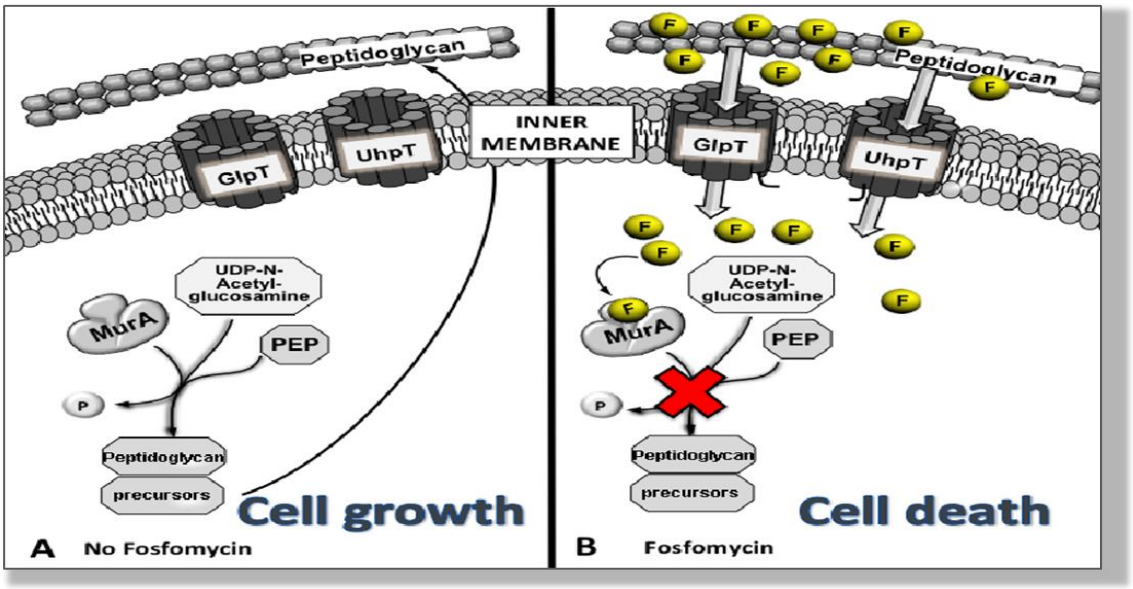

Figure 1.2: Fosfomycin mechanism of action. (A) Bacterial cell with normal production of peptidoglycan precursors and a peptidoglycan layer promoting cell growth. (B) Bacterial cell being treated with fosfomycin, inhibiting the production of peptidoglycan precursors and therefore blocking the production of the cell wall and resulting in cell death. (www.mdpi.com/journal/antibiotics)

1.4.3 Fosfomycin resistance mechanisms

Fosfomycin resistance primarily occurs by modification of the antibiotic target due to mutations in the *murA* gene (Giamarellou and Poulakou, 2009; Anton *et al.,* 2010; Falagas *et al.*, 2010). Mutations in the *murA* gene reduce the affinity between the MurA protein and the fosfomycin molecule (Li *et al.*, 2015). Fosfomycin resistance may also be due to the inactivation of the hexose phosphate (UhpT) and glycerol-3-phosphate (GlpT) transport systems, thereby decreasing uptake of the antibiotic. Expression of these genes (*glpT* and *uhpT*) is regulated by cAMP levels, which can be affected by mutations in the *ptsI* or *cyaA* genes, thereby decreasing antibiotic uptake. The expression of the *uhpT* gene is also regulated by the *uhpA*, *uhpB* and *uhpC* regulatory genes for high-level expression (Nilsson *et al.*, 2003; Cattoir and Guérin, 2018).

Another major mechanism of fosfomycin resistance involves the fosfomycin resistance proteins encoded by the *fos* genes; these encode enzymes such as FosA, FosB, FosC and FosX (Figure 1.3) (Martinez and Silley, 2010; Elliott *et al.*, 2019). FosA proteins are the most

frequently reported Fos related fosfomycin resistance conferring proteins and are common in Enterobacterales (Elliott *et al.*, 2019). FosB has been detected in Gram-positive pathogens such as *S. aureus* (Fu *et al.*, 2016). FosC was first reported in *Pseudomonas syringae* in 1995, and *fosC2* has been reported in fosfomycin resistant *E. coli* in Japan (Garcia, Arca and Suarez, 1995; Wachino *et al.*, 2010). FosX has been identified in the chromosome of several microorganisms such as *Listeria monocytogenes* and *Clostridium botulinum* (Castañeda-García, Blázquez and Rodríguez-Rojas, 2013).

FosA confers resistance by hydrolysing fosfomycin and inactivating it. FosA proteins include FosA1, FosA2, FosA3, FosA4, FosA5, FosA6 and FosA7, which have conserved key amino acid residues in the active site (H7, T9, W46, C48, S50, H64, K90, N92, S94, E95, G96, S98, Y100, E110, and R119) (Ito *et al.*, 2017). *fosA3* gene is the most predominant variant in *E. coli* isolates, co-occurring with genes conferring resistance to other antibiotics such as β-lactams, aminoglycosides, fluoroquinolones, sulphonamides and tetracyclines (Yang, Lu and Tseng, 2017; Cattoir and Guérin, 2018). *fosA* genes have been reported in the chromosomes of *K. pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Klebsiella aerogenes*, *Serratia marcescens*, *Morganella morganii*, *Providencia stuartii*, and *Pseudomonas aeruginosa*, which suggests intrinsic resistance or reduced susceptibility to fosfomycin. *E. coli* however does not carry the *fosA* gene on its chromosome and FosA conferred resistance is likely to be plasmid mediated (Ito *et al.*, 2017).

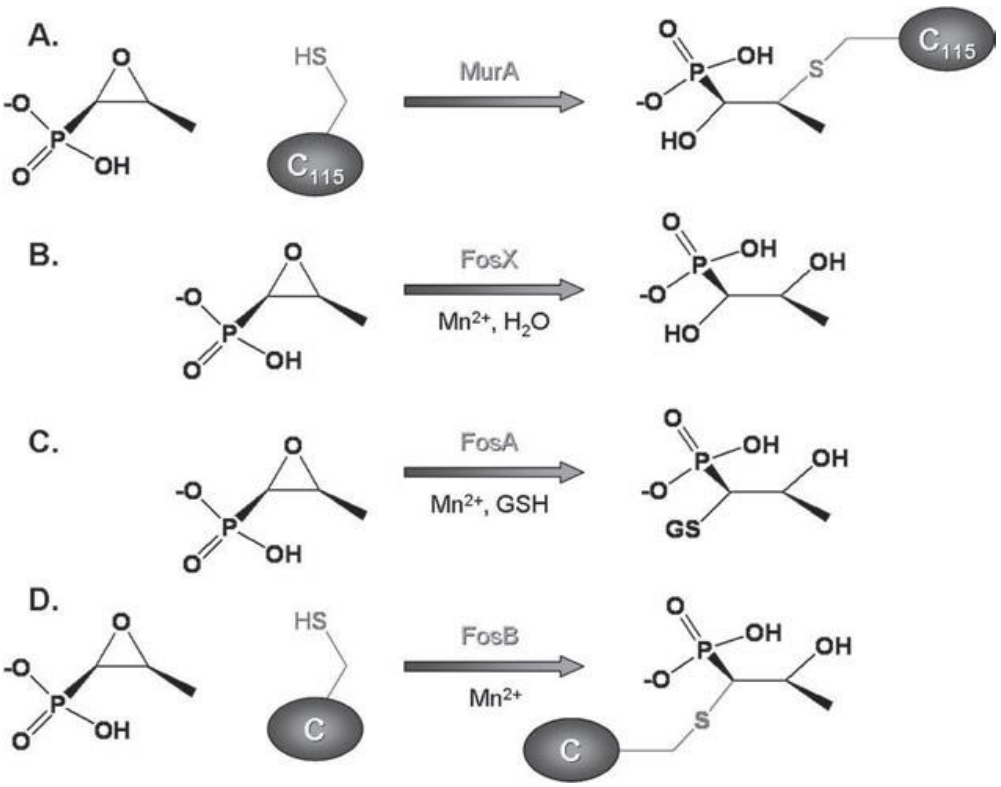

Figure 1.3: Mechanism of fosfomycin action and inactivation. (**A**) Fosfomycin targets the active-site Cys residue of MurA. (**B**) FosX-mediated inactivation of fosfomycin results in the formation of a diol. (**C**) FosA-mediated fosfomycin inactivation results in the acquisition of a glutathione. (**D**) FosB-mediated resistance to fosfomycin results in a ring-opened inactivated product with free Cys. (Source: Martinez and Silley, 2010)

In *E.coli*, a biological cost, usually seen as decreased growth rate and virulence of a bacterium, has been reported to be associated with fosfomycin-modifying enzymes encoded by *fosA* genes and chromosomal mutations that change the form and functionality of either one or two transport systems (GlpT and/or UhpT) (Silver, 2017; Cattoir and Guérin, 2018). Resistance is one of the main determinants of how quickly and to what extent a resistant mutant will establish itself in a host under selective pressure, but this fitness cost might be the reason fosfomycin resistance in clinical *E. coli* isolates remains low (Cattoir and Guérin, 2018).

1.4.4 Epidemiology of fosfomycin resistance

Although fosfomycin resistance rates in *E. coli* in Europe, Asia and USA are still low, with an approximate resistance rate of 3.2%, other countries such as Spain and Israel have reported increasing fosfomycin resistance (Mueller *et al.*, 2019). Resistance increased from 2.2% to 21.7% between 2004 and 2008 in Spain, as clinical use of fosfomycin increased by 50% during this period (Oteo *et al.*, 2009; Falagas *et al.*, 2010).

A study done in Japan found 1.6% of *E. coli* isolates to be resistant to fosfomycin, with the resistance mechanisms being amino acid substitutions in MurA and ineffectiveness of the transporters (GlpT or UhpT) (Takahata *et al.*, 2010). Mueller *et al*. (2019) reported a 1.4% prevalence of fosfomycin resistance in *E. coli* isolates from community-acquired UTI in Switzerland. They reported that 29% of the fosfomycin resistant *E. coli* isolates possessed a *fosA* gene, of which 80% were *fosA3*.

A fosfomycin resistance rate of 16% was reported in vancomycin-resistant *E. faecium* in a study done in Canada. A study done in Pittsburgh, USA, reported a 1.3% resistance rate, also in vancomycin-resistant *E. faecium* (Guo *et al.*, 2017; Ou and Nadeau, 2017). A South African study found fosfomycin resistance in 2% of *E. faecalis,* and 100% fosfomycin susceptibility in *E. faecium* isolates at a Johannesburg hospital (Mothibi, Bosman and Nana, 2020). There are few studies on the epidemiology and mechanisms of fosfomycin resistance among *Enterococcus spp*.

South African studies have reported 95.5 – 95.7% fosfomycin susceptibility in UTI isolates from Gauteng (Lewis *et al.*, 2013; Mothibi, Bosman and Nana, 2020). Similar rates were described in a review study published in 2019, where a resistance rate of <5% was reported in South Africa (Figure 1.4) (Aghamali *et al.*, 2019); however this was based on only two studies. There is a lack of data on fosfomycin susceptibility in urinary pathogens on the African continent. This highlights the importance of improved surveillance systems for fosfomycin susceptibility in urinary pathogens.

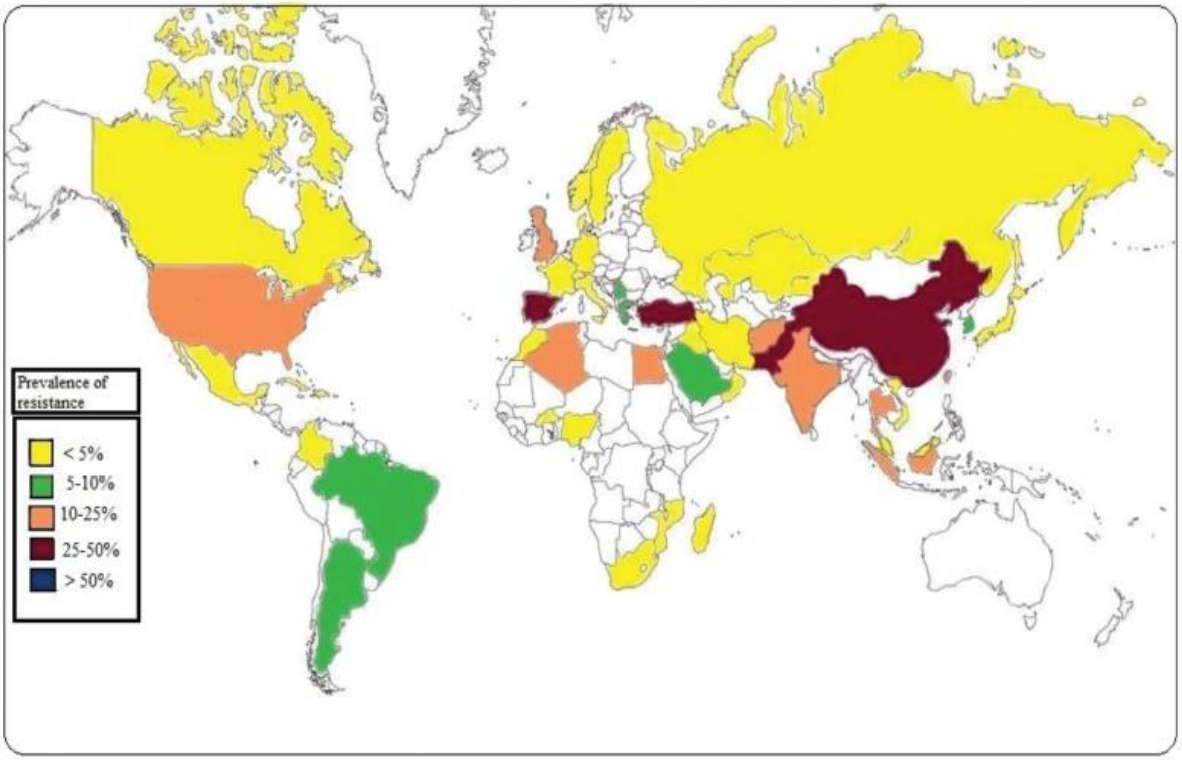

Figure 1.4: Prevalence of fosfomycin resistance globally. (Aghamali *et al*., 2019)

1.5 *Problem statement*

Few studies on fosfomycin resistance and mechanisms of resistance in urinary pathogens have been done in South Africa. Fosfomycin susceptibility testing was introduced at the National Health Laboratory Service (NHLS) Medical Microbiology diagnostic laboratory at Tygerberg Hospital in 2016. It is performed on urinary isolates where commonly available oral therapeutic drugs are not feasible options due to resistance, allergies or if there are concerns regarding the side effect profiles. Due to increasing concern regarding the side effect profiles of some drugs and increasing antibiotic resistance in urinary tract infections, fosfomycin has become a common alternative treatment.

Fosfomycin resistance has been detected in urinary pathogens isolated at the NHLS Medical Microbiology diagnostic laboratory at Tygerberg Hospital, but the prevalence of fosfomycin resistance amongst urinary pathogens in this population remains unknown and the underlying mechanisms of fosfomycin resistance have not been described.

1.6 *Aims and objectives*

1. To determine the prevalence of fosfomycin resistance among community-acquired urinary pathogens in the Western Cape of South Africa.
    - To collect Enterobacterales and *Enterococcus* isolates from urine samples from women at antenatal clinics.
    - To determine the fosfomycin susceptibility of these isolates.

2. To characterise the fosfomycin resistance mechanisms in fosfomycin resistant isolates from the Western Cape of South Africa.
    - To detect FosA-mediated fosfomycin resistance.
    - To identify chromosomal fosfomycin resistance mechanisms.

Chapter 2

Prevalence of fosfomycin resistance in community-acquired urinary pathogens in the Western Cape, South Africa

2.1 *Introduction*

Antimicrobial resistance (AMR) is an important global problem which accounts for more than 700,000 deaths annually (WHO, 2014; O'Neill J:, 2016). AMR is a natural process that occurs when infectious microorganisms survive exposure to antimicrobials that would normally kill them or inhibit their growth. It is estimated that AMR will cost approximately 10 million lives and about US$100 trillion per year by 2050 (O'Neill J:, 2016). According to the World Health Organisation (WHO), Africa and South East Asia are the regions most lacking in AMR surveillance systems. The lack of quality data is problematic as it often leads to the development of treatment guidelines that are not appropriate for the local situations which in turn can lead to the possible emergence of multidrug-resistant bacteria.

Urinary tract infections (UTI) are the most commonly acquired bacterial infections worldwide, affecting approximately 50 – 60% of women annually. The first line oral agents for the treatment of urinary tract infections (UTI) in our setting include trimethoprim-sulfamethoxazole, ciprofloxacin, amoxicillin, amoxicillin-clavulanic acid, cefuroxime and nitrofurantoin, depending on the regional susceptibility patterns of causative pathogens. The South African Department of Health recommends fosfomycin, nitrofurantoin and gentamicin as treatment options for uncomplicated UTI due to other antibiotics showing adverse side effects. Fosfomycin is commonly effective in treating Gram-negative bacteria, methicillin susceptible and resistant *Staphylococcus aureus* as well as glycopeptide-susceptible and resistant *Enterococci* (Raz, 2011).

A South African study published in 2013 has reported 98.3% and 72.7% fosfomycin susceptibility in Gram-negative and Gram-positive UTI isolates, respectively (Lewis *et al.*, 2013), however there is not enough surveillance data in South Africa regarding the prevalence of fosfomycin resistance. This chapter describes the rate of fosfomycin resistance in community-acquired urinary isolates in the Western Cape population.

2.2 *Materials and methods*

2.2.1 Study samples

Urine samples from patients attending different antenatal clinics in the Western Cape are referred to the National Health Laboratory Service (NHLS) Medical Microbiology Laboratory daily. These samples are processed on the request of a treating physician and microscopy, culture and susceptibility testing are performed as part of routine diagnostic laboratory work. Two hundred Enterobacterales and *Enterococcus spp*. isolates cultured from urine specimens from antenatal clinics were collected from the NHLS Medical Microbiology Laboratory over a period of 4 months (October 2019 – January 2020). Urinary pathogens isolated from antenatal specimens are representative of community-acquired infections, therefore these isolates were used to investigate the prevalence of fosfomycin resistance in community-acquired UTI in the Western Cape population.

Ethical approval was obtained from the Stellenbosch University Health Research Ethics Committee in October 2019 (Ethics #: S19/08/168; Addendum A).

2.2.2 Routine microbiology investigation

Isolation, identification and antimicrobial susceptibility testing of urinary pathogens was done as part of routine diagnostic procedures in the NHLS Medical Microbiology Laboratory. A calibrated loop method was used to inoculate 1 µl of the urine sample onto UriSelect™ agar medium (BIO-RAD UriSelect™, Diagnostic Media Production, Green Point, South Africa), followed by incubation at 35-37 °C for 18-24 hours in the presence of 5% carbon dioxide (CO_2). UriSelect™ is a non-selective chromogenic agar medium used for the isolation and differentiation of urinary tract pathogens.

Organisms suggestive of *Escherichia coli* were represented by pink colonies on the UriSelect™ agar and their identification was confirmed by performing a spot indole test (Diagnostic Media Production, Sandringham, South Africa). *Klebsiella pneumoniae*,

Enterobacter cloacae, *Serratia marcescens* and *Citrobacter spp*. were represented by blue-purple colonies, *Enterococcus spp*. was represented by turquoise-blue colonies and *Proteus mirabilis* was represented by orange/brown colonies on the UriSelect™ agar; the identification of these organisms was confirmed biochemically by the automated VITEK® 2 (Biomerieux, United States of America) platform as part of routine diagnostic processing.

Antimicrobial susceptibility testing was performed on all urine isolates using the automated VITEK® 2 platform as part of routine diagnostic processing. The VITEK® 2 platform assists the clinical microbiology laboratory to provide increased levels of automation. It improves laboratory turn-around time in organism identification and simultaneously performs antimicrobial susceptibility testing (AST) for multiple antibiotics and reports the results as susceptible, intermediate or resistant. It provides AST results and detects resistance for Gram-positive cocci, Gram-negative bacilli and yeasts, and interprets the results using Clinical and Laboratory Standards Institute (CLSI) criteria (Pincus, 2010). The VITEK® 2 antimicrobial susceptibility testing system includes the following antimicrobials for Gram-negative isolates: nitrofurantoin, trimethoprim-sulfamethoxazole, ciprofloxacin, ampicillin/amoxicillin, amoxicillin-clavulanic acid, piperacillin/tazobactam, cefuroxime (oral), cefuroxime (IV), cefotaxime, ceftazidime, cefepime, ertapenem, imipenem, meropenem, gentamicin and amikacin. The Gram-positive AST antimicrobial spectrum includes penicillin/ampicillin, erythromycin, ciprofloxacin, tetracycline, high-level streptomycin, high-level gentamicin, vancomycin, teicoplanin, linezolid and tigecycline.

2.2.3 Fosfomycin susceptibility testing

Enterobacterales and *Enterococcus* spp. isolates were collected from the microbiology diagnostic laboratory and subjected to fosfomycin susceptibility testing using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (MH) (Diagnostic Media Production, Green Point Cape Town, South Africa). Disc diffusion was performed by preparing 0.5 McFarland standard solutions (bacterial suspension of 1.5 X 10^8 CFU/ml) from pure colonies and saline; this was inoculated onto a MH agar plate to form a lawn. A fosfomycin disc (Mast Group Ltd, United Kingdom) containing 200 µg fosfomycin and 50 µg glucose-6-phosphate (G-6-P) was placed onto the MH-agar and incubated at 37°C for 18 hours in the presence of CO_2. Fosfomycin penetrates the cell via 2 main channels: GlpT and UhpT. The UhpT

requires activation by G-6-P (Falagas *et al.*, 2016; Díez-aguilar and Cantón, 2019). Therefore, the presence of G-6-P in the disc allows fosfomycin to enter the bacterial cell more effectively thus mimicking the actual physiological conditions at the site of infection and giving a more accurate susceptibility result (Díez-aguilar and Cantón, 2019).

The results were interpreted according to the CLSI 2019 criteria and reported as either susceptible, intermediate or resistant (Table 2.1). Fosfomycin minimum inhibitory concentrations (MICs) of the fosfomycin resistant isolates were determined using fosfomycin Etest® strips (Liofilchem, Italy). A 0.5 MacFarland solution was prepared as described above and a lawn was cultured on a MH plate with a sterile Etest® strip placed on the agar. These were incubated at 37°C for 16 hours in the presence of CO_2 and the results were interpreted according to the CLSI criteria (Table 2.1). The CLSI criteria only has breakpoints for *E. coli* amongst Enterobacterales and *Enterococcus faecalis* amongst *Enterococcus spp*. For the purpose of this study, *E. coli* breakpoints were used for all Enterobacterales, and *Enterococcus faecalis* breakpoints for all *Enterococcus spp.*

Table 2.1: Fosfomycin antibiotic susceptibility testing breakpoints. (Clinical and Laboratory Standards Institute, 2019).

	Disc diffusion zone diameter breakpoint (mm)		
Organism	Susceptible	Intermediate	Resistant
E. coli	≥ 16	13 – 15	≤ 12
E. faecalis	≥ 16	13 – 15	≤ 12
	MIC breakpoints (µg/ml)		
Organism	Susceptible	Intermediate	Resistant
E. coli	≤ 64	128	≥256
E. faecalis	≤ 64	128	≥256

2.3 *Results*

2.3.1 Study samples and species distribution

A total of 200 isolates were collected and were predominantly *E. coli* (n=138; 69%) followed by *E. faecalis* (n=24; 11.5%), *K. pneumoniae* (n=16; 8%), *P. mirabilis* (n=9; 5%) and *E. cloacae* (n=5; 3%) (Figure 2.1). The remaining organisms were *Klebsiella oxytoca* and *Klebsiella aerogenes* at 1% (n=2) each, and *Citrobacter freundii*, *Citrobacter koseri*, and *Morganella morganii* with a 0.5% (n=1) occurrence of each.

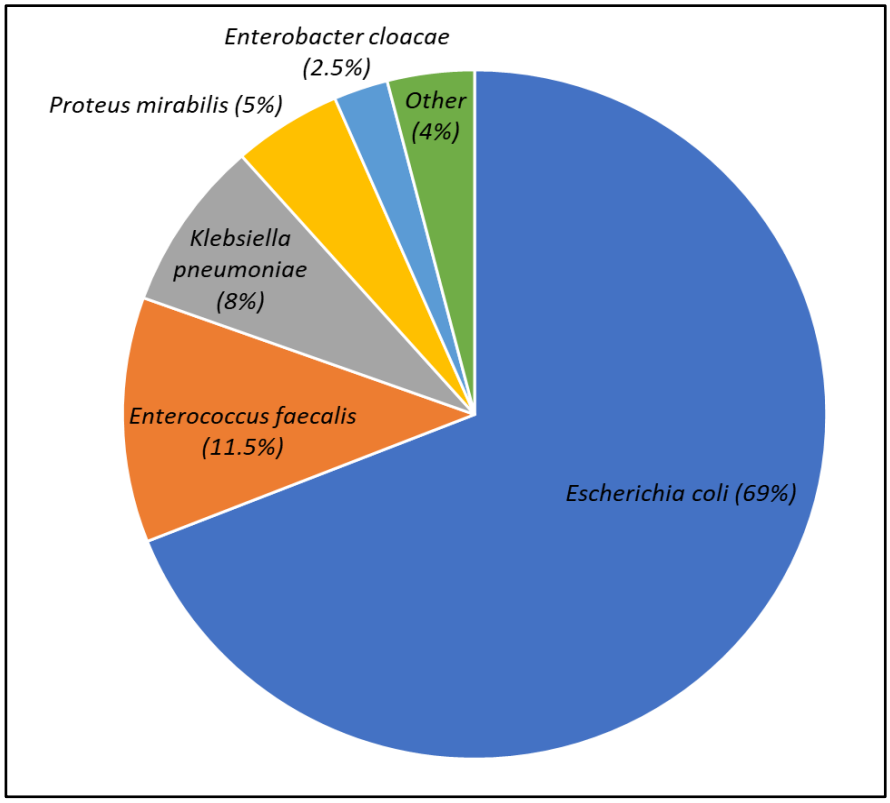

Figure 2.1: Species distribution of 200 urinary isolates collected from patients at antenatal clinics in the Western Cape.

2.3.2 Antimicrobial susceptibility profiles of urinary isolates

Figures 2.2 and 2.3 show the percentage of resistant Enterobacterales and *Enterococcus* isolates, respectively, to different treatment options, as reported by VITEK®. A high resistance rate was seen for ampicillin/amoxicillin (65%) and for trimethoprim-sulfamethoxazole (44%) amongst Enterobacterales, and tetracycline (88%) and erythromycin (100%) amongst *Enterococcus spp*. All *Enterococcus spp*. were susceptible to tigecycline, linezolid and vancomycin with a 4% resistance rate seen for penicillin/ampicillin among *Enterococcus spp*. (Figure 2.3).

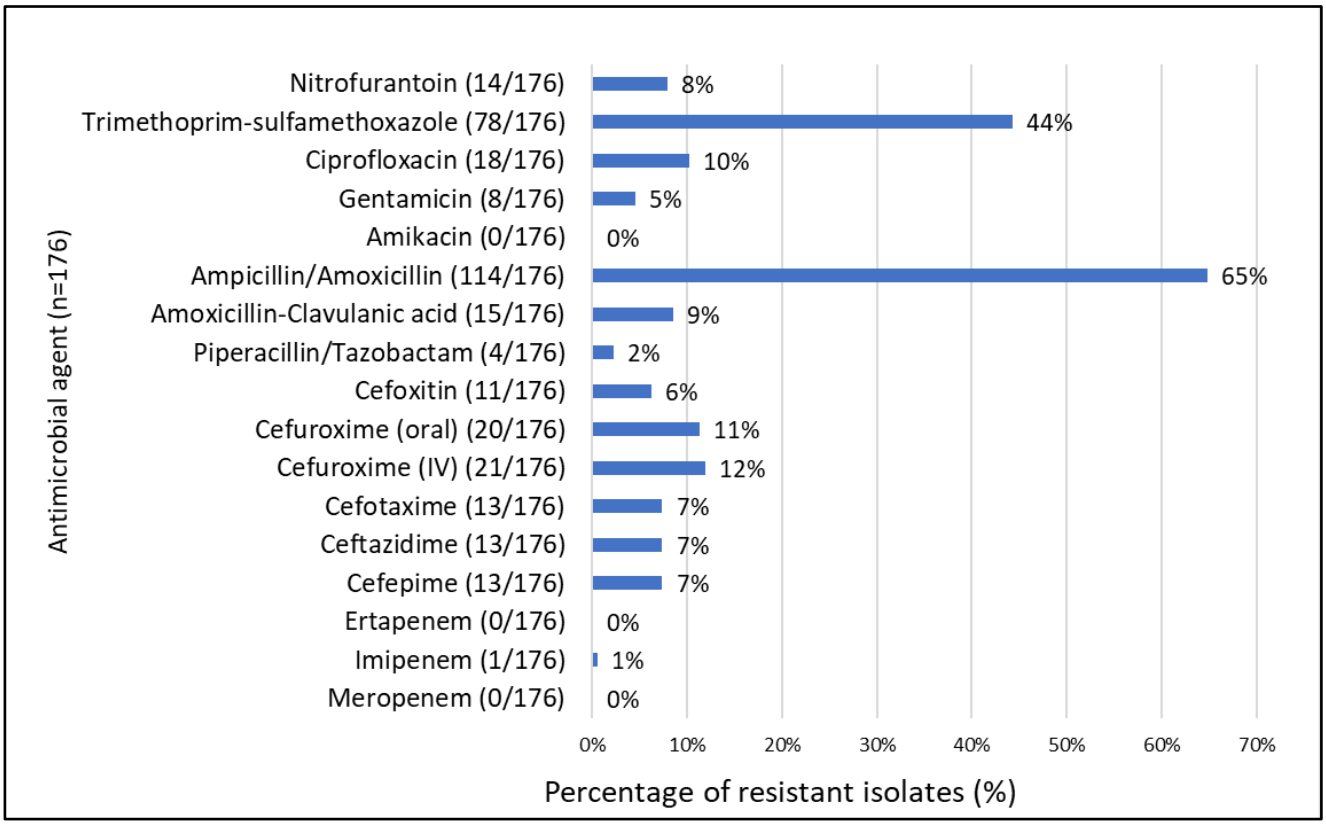

Figure 2.2: Antimicrobial resistance rates of community-acquired Enterobacterales pathogens (n = 176) isolated from urine samples.

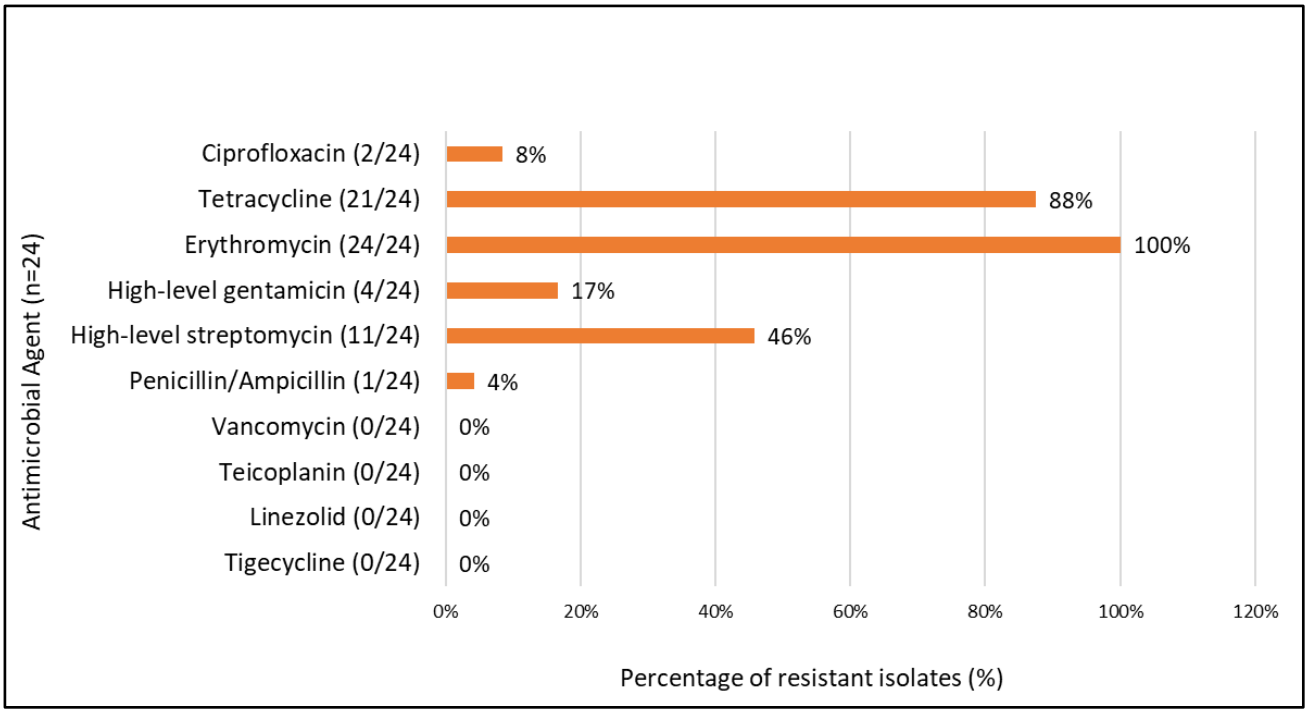

Figure 2.3: Antimicrobial resistance rates of community-acquired *Enterococcus* pathogens (n=24) isolated from urine samples.

2.3.3 Fosfomycin susceptibility

Of the 200 isolates, seven were resistant to fosfomycin based on disc diffusion: three *E. coli,* two *E. cloacae,* one *K. pneumoniae* and one *P. mirabilis* isolate. This translates to a fosfomycin resistance rate of 3.5%. MICs of the fosfomycin resistant isolates were determined by gradient diffusion. One isolate (*E. cloacae)* had an MIC of 512 µg/ml and the rest of the isolates had MICs of >1024 µg/ml.

2.3.4 Antimicrobial susceptibility profiles of fosfomycin resistant organisms

Table 2.2 presents the antibiograms of fosfomycin resistant isolates only and includes appropriate antimicrobials available in oral formulation, as they are first treatment options for community-acquired UTI. All isolates were susceptible to ciprofloxacin and trimethoprim-sulfamethoxazole. Most isolates were intermediately resistant or susceptible to nitrofurantoin, which is frequently prescribed for UTI and is one of the first line empiric agents as suggested by the South African Department of Health for the treatment of UTI.

Table 2.2: Vitek® 2 antibiograms of fosfomycin resistant isolates

Organism	NIT	CIP	CXM	AMC	TMP-SMX	AMP/AMX
Enterobacter cloacae	I	S	R	R	S	R
Enterobacter cloacae	I	S	S	S	S	R
Escherichia coli	S	S	I	S	S	S
Escherichia coli	I	S	R	S	S	R
Escherichia coli	S	S	R	S	S	R
Klebsiella pneumoniae	S	S	S	S	S	R
Proteus mirabilis	R	S	S	S	S	S

Nitrofurantoin (NIT), ciprofloxacin (CIP), cefuroxime (CXM), ampicillin-clavulanic acid (AMC), trimethoprim-sulfamethoxazole (TMP-SMX), ampicillin-amoxicillin (AMP/AMX). S – susceptible; I – intermediate; R – resistant.

2.4 *Discussion*

UTI are one of the major bacterial infections that require antibiotic prescription. Fosfomycin is one of the oral drugs recommended by the South African Department of Health for the treatment of uncomplicated UTI. Resistance has however been detected in urinary pathogens isolated at Tygerberg Hospital NHLS Medical Microbiology laboratory. In this study we used bacterial isolates from urine specimens from pregnant women attending antenatal clinics in the Western Cape province of South Africa as a representation of community-acquired urinary isolates. Women are at a high risk of UTI due to the anatomical structure of the female urinary system, however, pregnant women are at an even higher risk of ascending infection due to the weight of the foetus and dilation of the ureters and renal pelvis (Lee *et al.*, 2019). For this reason, it is important to screen pregnant women regularly for urinary pathogens, especially during the early stages of pregnancy (Gilbert *et al.*, 2013; Tan and Chlebicki, 2016; Lee *et al.*, 2019). In most healthy pregnancies, UTI are community-acquired which makes this target group an appropriate representation of community-acquired urinary pathogens.

E. coli comprised 69% of the urinary pathogens, followed by *E. faecalis* (11.5%), *K. pneumoniae* (8%), *P. mirabilis* (5%) and *E. cloacae* (3%). The species distribution among Enterobacterales in our study is similar to a study done in Turkey by Tulin Demir and Tuncay Buyukguclu. They have described 66.9% *E. coli*, 11.1% *K. pneumoniae*, 3% *P. mirabilis*, 1.4% *E. cloacae* amongst isolates from hospital and community-acquired UTI in Turkey (Demir and Buyukguclu, 2013). The majority of the urinary pathogens were *E. coli*, which is in keeping with other studies investigating the most common pathogens in community-acquired UTI (Kabugo *et al.*, 2016: WHO, 2011).

We found 3.5% (7/200) fosfomycin resistance amongst community urinary pathogens: 2.2% among *E. coli* (3/138) and 12.9% (4/31) among other Enterobacterales (*K. pneumoniae, P. mirabilis* and *E. cloacae)*. The *E. coli* resistance rate of 2.2% is similar to the 3.2% fosfomycin resistance rate recently reported in community-acquired uropathogens from Europe, Asia and the United States (Mueller *et al.*, 2019). A study done in Charlotte Maxeke hospital in Johannesburg reported fosfomycin resistance in 2% of *E. coli* (Mothibi, Bosman and Nana, 2020); although it was in hospital-acquired urinary pathogens, it is in keeping with our results. *E. coli* is not routinely tested for fosfomycin resistance in the Tygerberg Hospital NHLS Medical Microbiology diagnostic laboratory due to the presumed low prevalence of resistance. The reported resistance rate of 2.2% supports this ongoing practice, but ongoing surveillance is advised.

Enterococcus spp. are commonly isolated from urinary specimens from hospitalised patients, with *E. faecalis* and *E. faecium* being the most frequent (Ou and Nadeau, 2017). There is little information on fosfomycin resistance in *E. faecalis,* however resistance has been reported in vancomycin-resistant *E. faecium* acquired from hospital settings. Yan Guo *et al*. reported a fosfomycin resistance rate of 1.3% in vancomycin-resistant *E. faecium* from rectal screening cultures from 2012–2016 in Pennsylvania, USA (Guo *et al.*, 2017). Three hospitals in Canada have reported an even higher fosfomycin resistance rate of 19% in vancomycin-resistant *E. faecium* from urine samples (Ou and Nadeau, 2017). Our study reports 100% fosfomycin susceptibility in *Enterococcus spp*. from community-acquired urinary isolates, which were all vancomycin susceptible.

All fosfomycin resistant isolates were susceptible to ciprofloxacin and trimethoprim-sulfamethoxazole and these drugs thus remain treatment options for fosfomycin resistant isolates in this study. Fosfomycin resistant *K. pneumoniae* and *P. mirabilis* were found to be susceptible to most oral options except ampicillin/amoxicillin and nitrofurantoin, respectively. Regardless of the high susceptibility, ciprofloxacin and trimethoprim-sulfamethoxazole have both been proven to have unfavourable side effect profiles.

The use of ciprofloxacin can lead to swollen or ruptured tendons, and muscle, joint and nerve damage (United States Food and Drug Administration, 2018). Due to this severe side effect profile, it is advised by the South African Department of Health that ciprofloxacin not be used for non-severe bacterial infections. Trimethoprim-sulfamethoxazole is known to cause loss of appetite, nausea, and a Stevens Johnson reaction of the skin and mucous membranes. In early stages of pregnancy, trimethoprim-sulfamethoxazole can also cause neural tube and cardiovascular defects (Lee *et al.*, 2008).

Fosfomycin has no known adverse side effects and displays little cross resistance to other antimicrobial agents. Fosfomycin is also cheaper compared to other broad-spectrum antibiotics and therefore remains a valuable drug for multidrug resistant isolates. The fosfomycin resistance rate (3.5%) found in this study is low and encourages careful ongoing use of fosfomycin for the treatment of community-acquired UTI.

Chapter 3

Phenotypic and molecular assays to detect FosA mediated fosfomycin resistance

3.1 *Introduction*

Fosfomycin is an old oral agent that has been re-visited as a therapeutic option in the treatment of multidrug resistant (MDR) Gram-negative pathogens, including infections caused by carbapenemase-producing Enterobacterales (CPE). Fosfomycin resistance most commonly occurs via mutations in the *murA*, *glpT* and *uhpT* genes, but has also been reported to be conferred by the expression of Fos proteins: FosA (FosA1, FosA2, FosA3, FosA4, FosA5, FosA6, and FosA7), FosB, FosC and FosX, with FosA being most commonly reported among Enterobacterales (Martinez and Silley, 2010; Elliott *et al*., 2019). Fos proteins induce fosfomycin resistance by hydrolysing the fosfomycin drug rendering it ineffective.

It has been reported that different *fosA* variants may be chromosomally located in species such as *Klebsiella*, *Enterobacter* and *Serratia marcescens,* as opposed to being plasmid mediated as in *Escherichia coli* (Ito *et al.*, 2017). The first *fosA* gene described is likely to have originated from *Enterobacter cloacae* and was carried by conjugative plasmids to *Serratia marcescens* (Ito *et al.*, 2017; Yang, Lu and Tseng, 2017). FosA3 is commonly reported to be plasmid mediated in *E. coli* isolates and shares 93% amino acid identity with FosA4, of which the origin remains unknown. FosA5 and FosA6 most likely originated from the chromosome of *Klebsiella pneumoniae,* although it has also been found on plasmids in *E. coli* (Ma *et al.*, 2015; Guo *et al.*, 2016). FosA7 was reported to be chromosomally located in *Salmonella enterica* serovar Heidelberg isolated from broiler chickens (Rehman *et al.*, 2017). There are still more *fosA* genes which are yet to be identified.

This study aimed to detect FosA mediated resistance in fosfomycin resistant Enterobacterales isolated from community-acquired urinary tract infections (UTI), as described in Chapter 2, and other fosfomycin non-susceptible urinary pathogens previously

isolated at the Tygerberg Hospital National Health Laboratory Service (NHLS) Medical Microbiology diagnostic laboratory.

3.2 *Materials and Methods*

3.2.1 Study samples

Two sets of isolates were used for this study; fosfomycin resistant isolates collected in the prevalence study described in Chapter 2, and a set of previously collected fosfomycin non-susceptible *E. coli* and *K. pneumoniae* isolates from 2017 (Ethics #: U17/05/026; Addendum B). Fosfomycin resistant isolates from the prevalence study include *E. cloacae* (n=2), *K. pneumoniae* (n=1), *E. coli* (n=3), and *Proteus mirabilis* (n=1). The 2017 fosfomycin non-susceptible isolates include five *E. coli* and 19 *K. pneumoniae* isolates collected from urine samples submitted to NHLS Medical Microbiology diagnostic laboratory at Tygerberg Hospital for microscopy, culture and susceptibility testing.

All isolates collected in 2019-2020 were fosfomycin resistant but the 2017 sample-set included one *K. pneumoniae* isolate that was intermediately susceptible to fosfomycin on the gradient diffusion test (E-test). For this reason, the 2017 sample-set was referred to as ‘non-susceptible’. Isolation, identification and antimicrobial susceptibility testing of urinary pathogens was done as part of routine diagnostic procedures as described for other isolates in Chapter 2.

3.2.2 Bacterial culture

An aseptic method was used to culture fosfomycin resistant isolates on a tryptose blood agar plate (Diagnostic Media Production, Green Point Cape Town, South Africa) to acquire pure single colonies. The culture plate was incubated at 35 - 37 °C for 18 - 24 hours in the presence of 5% carbon dioxide (CO_2).

3.2.3 Phenotypic detection of FosA expression

The phenotypic assay was used for the detection of FosA expression, by assessing the impact of a FosA-inhibitor on the disc diffusion zone of inhibition. Sodium phosphonoformate is a FosA-inhibitor which allows phenotypic differentiation between FosA-mediated fosfomycin resistance and fosfomycin resistance through other mechanisms. In isolates expressing FosA, sodium phosphonoformate inoculated onto a fosfomycin disc was expected to increase the zone size and reduce growth of colonies within the zone of inhibition.

As described in Chapter 2, disc diffusion was performed by preparing 0.5 McFarland standard solutions from pure colonies in saline. This was inoculated onto Mueller-Hinton agar (MH) (Diagnostic Media Production, Green Point Cape Town, South Africa) to form a lawn of growth. A fosfomycin disc (Mast Group Ltd, United Kingdom), containing 200 µg fosfomycin and 50 µg glucose-6-phosphate, was inoculated with 20 µl (1 mg) of a 50 mg/ml sodium phosphonoformate (Sigma-Aldrich, Johannesburg, South Africa) solution, and placed onto the MH-agar. A second fosfomycin disc without the sodium phosphonoformate solution was placed on the same culture plate as a control, after which the media plates were incubated at 35 - 37°C for 18 hours in the presence of 5% CO_2. The results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI 2019) criteria. An increase in the size of the zone of inhibition (≥ 5 mm) in the presence of sodium phosphonoformate was indicative of FosA inhibition thus the isolate was presumed to have FosA expression (Nakamura *et al.*, 2014; Mueller *et al.*, 2019).

3.2.4 Molecular detection of fosA genes

A crude method of DNA extraction was performed: a loop full of pure colonies was inoculated into 400 µl nuclease free water and vortexed to make a homogeneous solution. This was heated at 96 °C for 30 minutes to allow heat damage to the cell membrane thus releasing the nucleic material. This was then frozen at -80 °C for a further 30 minutes and the suspension was centrifuged at 16 000 x g for 10 minutes to precipitate the cellular debris. Extracted DNA was stored in microcentrifuge tubes at -20 °C until further use.

The glutathione S-transferase (fos) genes, *fos*A1, *fosA2*, *fosA3*, *fosA4*, *fosA5*, *fosA6* and *fosA7* were detected by polymerase chain reaction (PCR) (ProFlex™ PCR System) using primers described in Table 3.1. A master mix was prepared using 12.5 µl KAPA Taq ready-mix (KAPA Biosystems, South Africa) buffer, 0.5 µl of 10 pmol/µl of each primer (Inqaba Biotec™, South Africa) and 9.5 µl ddH_2O, to make up 23 µl per sample; 2 µl of DNA was added to each reaction and the PCR was run under conditions described in Table 3.2. The first positive PCR products for each target underwent Sanger sequencing (Inqaba Biotec™) and were used as positive controls for subsequent PCR reactions. A non-template control was included in each PCR run. PCR products were detected by agarose gel electrophoresis: 5 µl each PCR product was mixed with 1 µl of Novel juice (GeneDirex, USA) and loaded into a 2% agarose gel; this was run in 1X TAE (40 mM Tris, 20 mM acetic acid, 1 mM Ethylenediaminetetraacetic acid) at 100 volts for 45 minutes. Five microlitres of a 100bp DNA marker (500 µg/ml) was used as a size marker (New England Biolabs Inc.).

Table 3.1*: fosA1* – 7 amplification primers

Primer name	Target gene	Amplicon size (bp)	Sequence 5'→ 3'	Reference
*fosA*1-2_Fw	*fosA1*/*fosA2*	176	5'-STAYGACGAGGCGCGCSRKTACGTG-3'	(Nordmann, Poirel and Mueller, 2019)
*fosA*3-4_Fw	*fosA3*/*fosA4*	175	5'-CTGGATGMGCAGCGGCGTAAAACG-3'	(Nordmann, Poirel and Mueller, 2019)
*fosA*5-6_Fw	*fosA5*/*fosA6*	168	5'-CCGCAGCGGCGCGTTACTCCGCCG-3'	(Nordmann, Poirel and Mueller, 2019)
*fosA*_Univ_Rv*	*fosA1-6*	Dependant on forward primer	5'-CRTCVGGRTCGAGRAAATAG-3'	(Nordmann, Poirel and Mueller, 2019)
*fosA*7_Fw	*fosA7*	218	5′-TGTGGCGACCTTTGGGTCTG-3′	(Mueller *et al.*, 2019)
*fosA*7_Rv			5′-AGTTCCAGCTTGTGGCCATC-3′	

* *fosA*_Univ_Rv primer was used as a reverse primer with each specific forward primer *fosA1 - 6* in singleplex PCR reactions. Final concentration of each primer was 0.2 µM.

Table 3.2: PCR conditions for *fosA* gene amplification.

PCR steps	PCR cycle program (˚C)	Time	Number of cycles
Activation	98	1 min	X1
Denaturation	98	1 sec	X35
Annealing	(55 – 58) *	30 sec	
Extension	72	15 sec	
Final extension	72	3 min	X1
End/Hold	4	∞	

* *fosA1-2* Tm – 58˚C; *fosA3-4* Tm - 55 ˚C; *fosA5-6* Tm - 55 ˚C; *fosA7* Tm - 58 ˚C.

3.3 *Results*

3.3.1 Phenotypic detection of FosA expression

Disc diffusion with sodium phosphonoformate was performed on five fosfomycin non-susceptible *E. coli* (EC1 – EC5) and 19 *K. pneumoniae* (KP1 – KP19) isolates collected in 2017 and seven fosfomycin resistant isolates (CA1 – CA7; three *E. coli,* two *E. cloacae,* one *K. pneumoniae* and one *P. mirabilis*) described in Chapter 2. EC2 – EC5 displayed an increase in the size of the zone of inhibition of over 5 mm in the presence of sodium phosphonoformate (Table 3.3) which is consistent with FosA expression. EC1 did not show an increase in zone size when treated with sodium phosphonoformate. No significant increase in the zone of inhibition was found for *K. pneumoniae* isolates from the 2017 sample set (KP1 – KP19) in the presence of sodium phosphonoformate. From the 2020 isolates, one *E. coli* (CA5), one *E. cloacae* (CA1), and one *K. pneumoniae* (CA6) had an increase in zone size of more than 5 mm in the presence of sodium phosphonoformate; indicative of FosA expression.

3.3.2 Molecular detection of fosA genes

fosA genes were detected by PCR in all fosfomycin non-susceptible clinical isolates. All 5 *E. coli* isolates from the 2017 set were PCR positive for *fosA3/4* (Figure 3.1). All 19 *K. pneumoniae* isolates from 2017 contained *fosA5/6* (Figure 3.2). From the community-acquired fosfomycin resistant Enterobacterales isolates collected in 2019/2020, only one *E. coli* isolate was FosA positive, and presented with the *fosA7* gene.

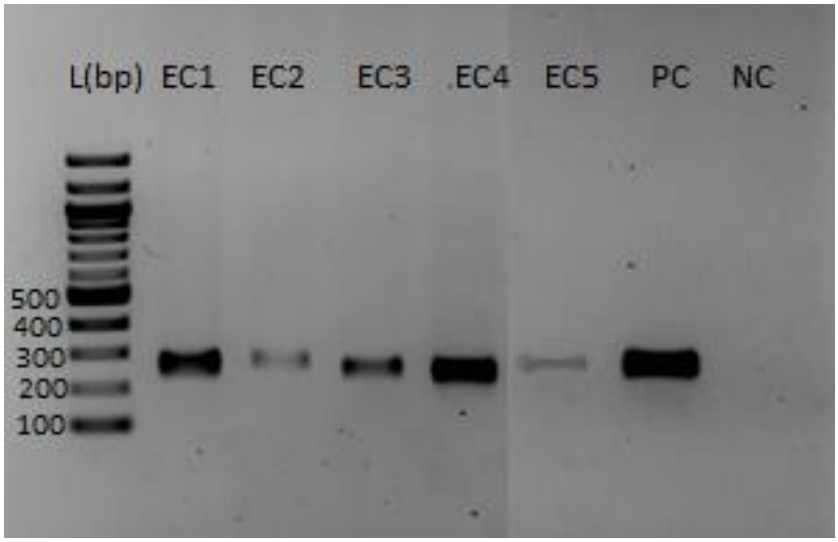

Figure 3.1: Representative gels for *fosA3/4* detection in fosfomycin non-susceptible *E. coli* isolates (EC1 - EC5 are *E. coli* isolates from the 2017 sample set) run in 2% agarose gel. PC – Positive control; NC – Non-template Control; L – Ladder (New England Biolabs Inc. 100bp DNA marker).

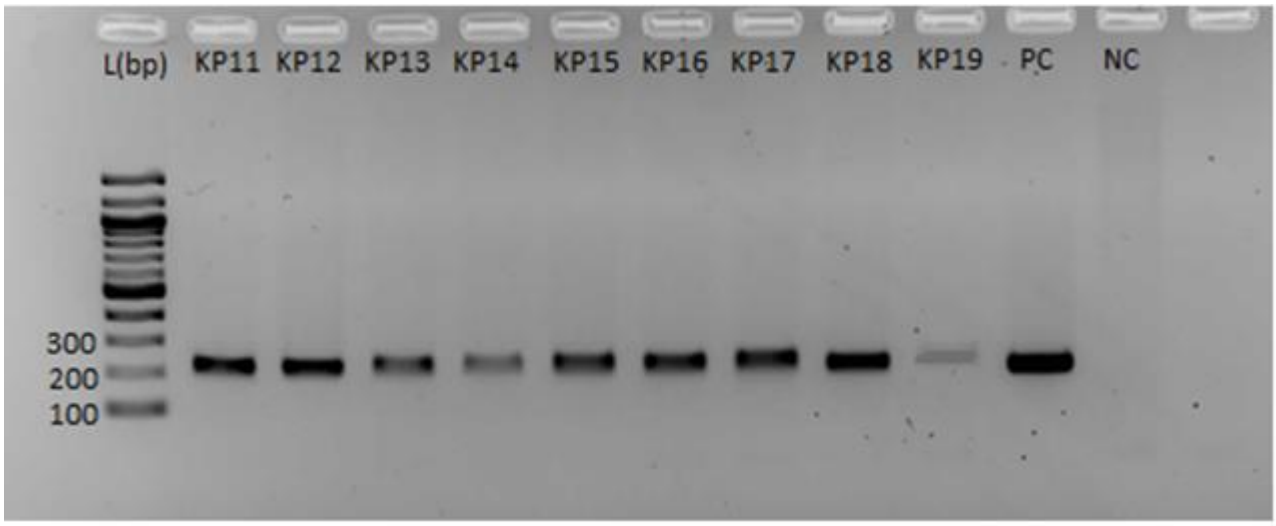

Figure 3.2: Representative gels for *fosA5/6* detection in fosfomycin non-susceptible *K. pneumoniae* isolates. All isolates loaded in the 2% gel are *K. pneumoniae* isolates from the 2017 sample set. PC – Positive control; NC – Non-template Control; L – Ladder (New England Biolabs Inc. 100bp DNA marker).

Table 3.3: Detection of FosA production in fosfomycin resistant Enterobacterales isolates.

2017 isolates				
Sample ID	**Organism**	**Fosfomycin (mm)**	**Fosfomycin + sodium phosphonoformate (mm)**	**Molecular detection**
EC1	*Escherichia coli*	No Zone*	No Zone	*fosA3/4*
EC2	*Escherichia coli*	7	20	*fosA3/4*
EC3	*Escherichia coli*	No Zone	18	*fosA3/4*
EC4	*Escherichia coli*	No Zone	13	*fosA3/4*
EC5	*Escherichia coli*	No Zone	17	*fosA3/4*
KP1	*Klebsiella pneumoniae*	No Zone	No Zone	*fosA5/6*
KP2	*Klebsiella pneumoniae*	No Zone	No Zone	*fosA5/6*
KP3	*Klebsiella pneumoniae*	No Zone	No Zone	*fosA5/6*
KP4	*Klebsiella pneumoniae*	No Zone	No Zone	*fosA5/6*
KP5	*Klebsiella pneumoniae*	No Zone	No Zone	*fosA5/6*
KP6	*Klebsiella pneumoniae*	No Zone	No Zone	*fosA5/6*
KP7	*Klebsiella pneumoniae*	No Zone	No Zone	*fosA5/6*
KP8	*Klebsiella pneumoniae*	14	15	*fosA5/6*
KP9	*Klebsiella pneumoniae*	No Zone	No Zone	*fosA5/6*
KP10	*Klebsiella pneumoniae*	No Zone	No Zone	*fosA5/6*
KP11	*Klebsiella pneumoniae*	No Zone	No Zone	*fosA5/6*

KP12	*Klebsiella pneumoniae*	No Zone	No Zone	*fosA5/6*
KP13	*Klebsiella pneumoniae*	No Zone	No Zone	*fosA5/6*
KP14	*Klebsiella pneumoniae*	7	No Zone	*fosA5/6*
KP15	*Klebsiella pneumoniae*	7	7	*fosA5/6*
KP16	*Klebsiella pneumoniae*	No Zone	No Zone	*fosA5/6*
KP17	*Klebsiella pneumoniae*	No Zone	10	*fosA5/6*
KP18	*Klebsiella pneumoniae*	No Zone	No Zone	*fosA5/6*
KP19	*Klebsiella pneumoniae*	No Zone	No Zone	*fosA5/6*
2019 – 2020 isolates				
Sample ID	**Organism**	**Fosfomycin (mm)**	**Fosfomycin + sodium phosphonoformate (mm)**	***fosA* detection**
CA1	*Enterobacter cloacae*	No Zone	15	ND
CA2	*Enterobacter cloacae*	No Zone	No Zone	ND
CA3	*Escherichia coli*	No Zone	No Zone	ND
CA4	*Escherichia coli*	No Zone	9	*fosA7*
CA5	*Escherichia coli*	10	20	ND
CA6	*Klebsiella pneumoniae*	No Zone	12	ND
CA7	*Proteus mirabilis*	No Zone	8	ND

*"No Zone" is defined as 6 mm, the size of fosfomycin disc used; ND – none detected

3.3.3 Correlation of phenotypic and molecular results

A total of 31 fosfomycin non-susceptible isolates were investigated (n=8 *E. coli*, n=20 *K. pneumoniae*, n=2 *E. cloacae* and n=1 *P. mirabilis).* Seven of these isolates were FosA positive by phenotypic assay and twenty-five had *fosA* detected by PCR. Only 7/31 isolates had correlating results between the two methods. Four *E. coli* isolates were positive on phenotypic assay, and *fosA3/4* positive by PCR*;* and three (1 *E. coli*, 1 *E. cloacae* and 1 *P. mirabilis*) isolates were negative on both assays.

3.4 *Discussion*

This chapter investigated the contribution of FosA production to fosfomycin resistance in urinary Enterobacterales isolates, using molecular and phenotypic assays. FosA is one of the major mechanisms of fosfomycin resistance in Enterobacterales (Silver, 2017; Elliott *et al.*, 2019). *fosA* genes have been previously identified in the chromosomes of *K. pneumoniae (fosA5/6)*, *Klebsiella oxytoca (fosA)* and *E. cloacae (fosA1/2)* and naturally reduce susceptibility to fosfomycin when expressed. Plasmid mediated *fosA3* is the most common *fosA* gene found among *E. coli* isolates (Ito *et al.*, 2017; Yang, Lu and Tseng, 2017; Cattoir and Guérin, 2018).

Of the 31 fosfomycin resistant isolates studied in this chapter, 25 harboured *fosA* genes. Nineteen of those were *K. pneumoniae* isolates harbouring *fosA5/6* genes, five were *E. coli* isolates harbouring *fosA3/4* genes and one *E. coli* isolate harboured *fosA7 gene.* The detection of *fosA5/6* genes in 19 of our *K. pneumoniae* isolates may support *fosA* being chromosomally located. However, one of the *K. pneumoniae* (CA6) isolates did not harbour the *fosA* gene in this study. Nontombi Mbelle and colleagues reported in a study done in Pretoria, that a *fosA* gene was present in 95% of their *K. pneumoniae* isolates although they were all fosfomycin susceptible and Elliott *et al*. identified *fosA6* in 23/24 of their *K. pneumoniae* isolates. This may support the phenomenon of the genes being chromosomally mediated in other strains but not always expressed (Mbelle *et al.*, 2020).

There was a clear difference in *fosA* prevalence between the two sets of isolates investigated in this chapter. The first set (2017) of fosfomycin resistant isolates all (n=24) harboured *fosA* genes compared to only 1/7 fosfomycin resistant isolates from the 2019-2020 set. The main difference between the two sample sets is the different collection time points; between which changes in the distribution of resistance mechanisms may have occurred. A more likely explanation: the 2019-2020 isolates were all isolates from community-acquired urinary tract infections from pregnant women with no known complications, whereas 2017 isolates were a mixture of community and hospital-acquired infections.

FosA-inhibitor sodium phosphonoformate was used to detect FosA expression. Seven isolates were FosA positive on the phenotypic assay and 25 isolates were positive on the molecular assay. Of the seven phenotypic FosA positive isolates, only four *E. coli* isolates contained the *fosA3/4* (*E. coli*) gene based on PCR. The other three isolates (*E. coli*, *E. cloacae* and *K. pneumoniae*) were positive for FosA on phenotypic assay but PCR negative. Two *E. coli* isolates harboured *fosA7* and *fosA3/4* genes but were negative on the phenotypic assay. We observed that FosA inhibition by sodium phosphonoformate disc diffusion method works well for *E. coli* isolates harbouring *fosA3/4,* since one of the two *fosA* positive *E. coli* isolate which was negative on the phenotypic assay harboured the *fosA7* gene.

Between the two assays, molecular results did not correlate well with the phenotypic results. PCR detected *fosA* genes that were not detected on the phenotypic assay, which could mean that FosA was not expressed in these isolates and does not confer fosfomycin resistance, or that there are chromosomal mutations involved in conferring resistance. FosA-inhibitor sodium phosphonoformate may also work better for *E. coli,* as compared to *K. pneumoniae* and other organisms. Another reason for the poor correlation, that has been suggested previously, is that this method may be more specific for FosA3 or FosA4 inhibition rather than all FosA variants, as the phenotypic results for *E. coli* isolates harbouring *fosA3/4* (4/5) seemed to correlate better with the molecular results, than other organisms harbouring other *fosA* genes (Nakamura *et al.*, 2014; Elliott *et al.*, 2019).

This chapter indicated that the inclusion of FosA-inhibitor sodium phosphonoformate, in disc diffusion assays, could be a good indicator of FosA3/4 expression in fosfomycin resistant *E. coli*. The molecular assay is a sensitive technique and is reliable for the detection of *fosA* genes in bacterial organisms although it does not indicate if the genes are expressed or if the protein is functional. This can be investigated by real-time PCR which is a method that measures the amount of FosA PCR products generated in real time. Recombinant expression could determine if the genes are active. Specific *fosA* genes could be ligated into an expression vector and expressed in *E. coli*. Transformants can be selected on agar media and susceptibility to fosfomycin determined. Studies to determine the expression of *fosA* genes in fosfomycin resistant urinary pathogens can be of importance to determine the impact of *fosA* on clinical outcomes in the Western Cape community and hospital settings.

In conclusion, *fosA* genes were detected in 95% of the fosfomycin resistant *K. pneumoniae* isolates and 75% of the *E. coli* isolates, suggesting that this may be the predominant fosfomycin resistance mechanism in this population.

Chapter 4

Characterisation of chromosomal fosfomycin resistance mechanisms in *E. coli* and *K. pneumoniae.*

4.1 *Introduction*

Fosfomycin is a bactericidal drug that inhibits the biosynthesis of peptidoglycan in both Gram-negative and -positive bacteria leading to cell lysis and death. Fosfomycin enters the cell through two main transport systems: the glycerol-3-phosphate transport (GlpT) and hexose phosphate uptake transport (UhpT) systems (Nicolle and AMMI Canada Guidelines Committee, 2005; Maraki *et al.*, 2009; Raz, 2011; Li *et al.*, 2015). Fosfomycin then acts as a phosphoenolpyruvate (PEP) analogue and binds to the Cys115 on the active site of the MurA (UDP-N-acetylglucosamine enolpyruvyl transferase) target protein. MurA plays a role in the formation of UDP-N-acetylglucosamine enolpyruvate in the biosynthesis of peptidoglycan; blocking its activity inhibits cell wall synthesis resulting in cell death (Knottnerus *et al.*, 2008; Takahata *et al.*, 2010; Martin-Gutiérrez *et al.*, 2018; Aghamali *et al.*, 2019).

Reduced drug uptake due to mutations in chromosomal genes is reported to be the main mechanism of fosfomycin resistance in clinical *Escherichia coli* and *Klebsiella pneumoniae* isolates (Nilsson *et al.*, 2003; Takahata *et al.*, 2010; Lu *et al.*, 2016; Cattoir and Guérin, 2018). Mutations occurring in the transporter genes (*glpT* and *uhpT*) as well as their regulatory genes (*ptsI*, *cyaA* and *uhpA*) result in the loss of function or decreased expression of the GlpT and UhpT transport proteins and may decrease antibiotic uptake (Ma *et al.*, 2015; Lu *et al.*, 2016). Mutations in *murA* result in modification of the antibiotic target protein MurA, which reduces its affinity to the drug, resulting in fosfomycin resistance.

Mutations occurring in chromosomal genes are infrequently detected in *E. coli* due to biological cost, hence fosfomycin resistance is uncommon amongst *E. coli* isolates (Karageorgopoulos *et al.*, 2012; Martin-Gutiérrez *et al.*, 2018). Mutations conferring fosfomycin resistance may decrease the virulence of *E. coli* in urinary tract infections, as the growth rate and adherence to the endothelial cells in the urinary system will be reduced

(Karageorgopoulos *et al.*, 2012). Only a few mutations have been reported in the *murA* gene of *E. coli* as most of them drastically reduce cell viability (Herring and Blattner, 2004). Mutations causing fosfomycin resistance have been reported to occur at a higher frequency in strains of *K. pneumoniae* (Demir and Buyukguclu, 2017). This chapter describes mutations occurring in the chromosomal *murA*, *glpT* and *uhpT* genes of fosfomycin non-susceptible *E. coli* and *K. pneumoniae* isolates from urine samples collected in the Western Cape.

4.2 *Methods and Materials*

4.2.1 Study samples

Two sets of isolates were screened for fosfomycin resistance mechanisms; fosfomycin resistant *E. coli* (n=3) and *K. pneumoniae* (n=1) isolates collected in the prevalence study described in Chapter 2, and a set of previously collected fosfomycin non-susceptible *E. coli* (n=5) and *K. pneumoniae* isolates (n=19) from 2017 as described in Chapter 3. Being the most common urinary pathogens in the Western Cape population that showed resistance to fosfomycin, *E. coli* and *K. pneumoniae* were screened for possible chromosomal mutations that may confer fosfomycin resistance.

4.2.2 Amplification of chromosomal genes

A crude method was used for DNA extraction, as described in Chapter 3. Amplification of the chromosomal genes *murA, glpT* and *uhpT* in *E. coli* and *K. pneumoniae* was done by singleplex polymerase chain reactions (PCR) on the ProFlex™ PCR System (ThermoFisher Scientific, South Africa). A master mix was prepared using 12.5 µl KAPA Taq ready-mix (KAPA Biosystems, South Africa) buffer, 1 µl of 10 pmol/µl of each primer (Whitehead Scientific (Pty) Ltd, South Africa or Inqaba Biotec™, South Africa) and 8.5 µl nuclease free water to make up 23 µl per sample. Two microlitres of DNA was added to each reaction and the PCR was run under conditions described in Table 4.1.

Table 4.1: PCR conditions for the *murA*, *glpT* and *uhpT* singleplex PCR reactions

PCR steps	PCR cycle program (˚C)	Time	Number of cycles
Activation	95	3 min	X1
Denaturation	95	30 sec	X30
Annealing	(52 – 68) *	30 sec	
Extension	72	1 min	
Final extension	72	5 min	X1
End/Hold	4	∞	

**E. coli: murA* Tm - 52 ˚C; *glpT* Tm - 68 ˚C; *uhpT* Tm - 60 ˚C. *K. pneumoniae: murA* Tm - 52 ˚C; *glpT* Tm - 55 ˚C; *uhpT* Tm - 55 ˚C.

Primers used for PCR amplification of chromosomal genes in *E. coli* and *K. pneumoniae* are described in Table 4.2. The second *K. pneumoniae glpT* primer set (Liu *et al.*, 2020) was used when the first *glpT* primer set (Lu *et al*., 2016) produced insufficient product for Sanger sequencing.

Table 4.2: Primers used for PCR amplification and sequencing of *murA*, *glpT* and *uhpT* in *E. coli* and *K. pneumoniae.*

Target gene/ Species	**Primer name**	**Sequence (5'–3')**	**Amplicon size (bp)**	**Reference**
murA *K. pneumoniae*	KP-murA-F	GCGAGACCGCAAACTCAATG	1439	(Lu *et al.*, 2016)
	KP-murA-R	CGACAGAACGCAGATGATGC		
glpT *K. pneumoniae*	KP-glpT-F	ATTTTATCAGCCGCTGTCGC	1642	(Lu *et al.*, 2016)
	KP-glpT-R	ATTCGCGCGTTATTTGAGCG		
glpT (2) *K. pneumoniae*	KP-glpT-F	CACCAGGTCTTGCTCAAGGT	1643	(Liu *et al.*, 2020)
	KP-glpT-R	TATTGCCTACCATCGCGACC		
uhpT *K. pneumoniae*	KP-uhpT-F	GCTGTCGACTGTCGGACCT	1583	(Lu *et al.*, 2016)
	KP-uhpT-R	GCGCTGGTTTTTACAATGCC		
murA *E. coli*	murA-F	AAACAGCAGACGGTCTATGG	1541	(Takahata *et al.*, 2010)
	murA-R	CCATGAGTTTATCGACAGAACG		
glpT *E. coli*	glpT-F	GCGAGTCGCGAGTTTTCATTG	1825	(Takahata *et al.*, 2010)
	glpT-R	GGCAAATATCCACTGGCACC		
uhpT *E. coli*	uhpT-F	TTTTTGAACGCCCAGACACC	1667	(Takahata *et al.*, 2010)
	uhpT-R	AGTCAGGGGCTATTTGATGG		

PCR products were detected by agarose gel electrophoresis: 5 µl of each PCR product was mixed with 1 µl of Novel juice (GeneDirex, USA) and loaded into a 2% agarose gel (Lonza, South Africa) which was run in 1X TAE (40 mM Tris, 20 mM acetic acid, 1 mM Ethylenediaminetetraacetic acid) buffer at 120 volts for 50 minutes. DirectLoad™ Wide Range DNA Marker (Sigma-Aldrich Co. LLC, South Africa) was used as a DNA ladder. Fosfomycin susceptible *E. coli* and *K. pneumoniae* were used as positive controls; these isolates were fosfomycin susceptible by disc diffusion. Non-template controls were included in each PCR run. All positive PCR products, including susceptible controls, were subjected to Sanger sequencing at Inqaba Biotec™ and DNA sequences were analysed using the BioEdit Sequence Alignment Editor 20 to identify potential mutations (Hall, 1999). *E coli* strain K-12 substr. MG1655 (ref: NC_000913.3) and *K. pneumoniae* subsp. pneumoniae HS11286 (ref: NC_016845.1) were used as reference sequences.

4.3 *Results*

The *murA, glpT* and *uhpT* genes were successfully amplified in all eight fosfomycin resistant *E. coli* isolates. Figure 4.1 shows representative agarose gel images for the three PCR reactions in *E. coli*. Amplification of *murA, glpT* and *uhpT* genes was successful for all 20 *K. pneumoniae* isolates, although in some isolates *glpT* was only successfully amplified using the second primer set (Liu *et al.*, 2020) (Figure 4.2). Some variation in PCR product size was observed on the gels, however the products were confirmed to be the correct gene and size, based on Sanger sequencing.

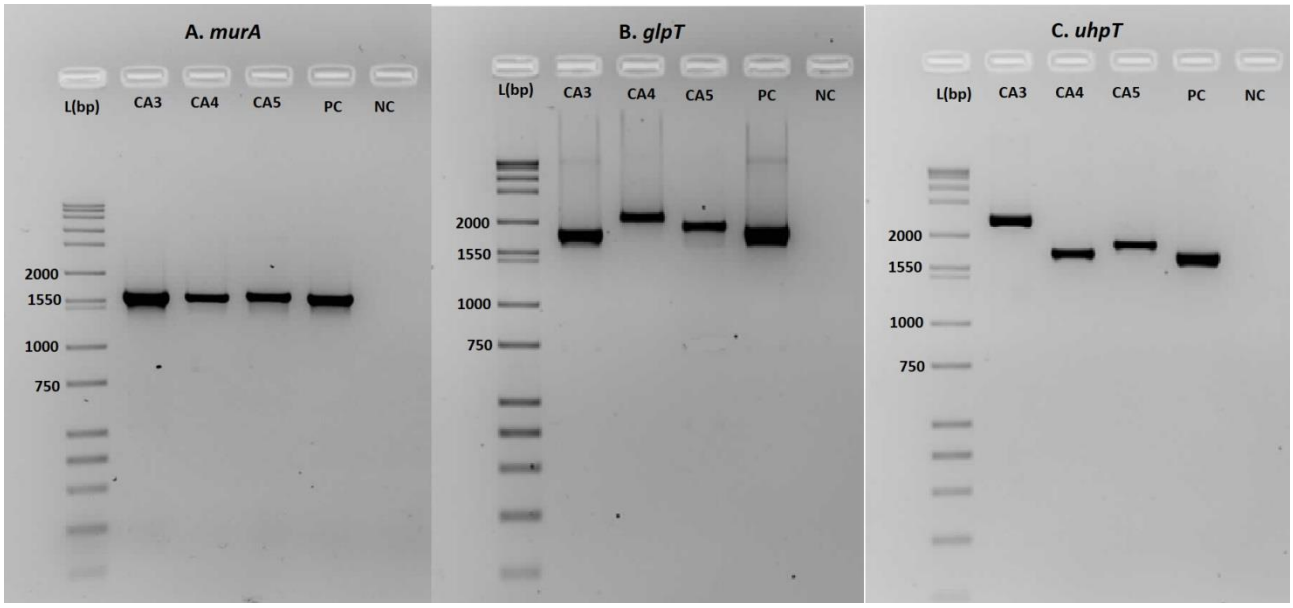

Figure 4.1: Representative gels for PCR amplification of *murA* (A), *glpT* (B) and *uhpT* (C) genes in fosfomycin resistant *E. coli* isolates. CA3-5 are fosfomycin resistant *E. coli* isolates from 2019-2020 sample set run in 2% agarose gel. PC – Positive control; NC – Non-template Control; L – Ladder (DirectLoad™ Wide Range DNA Marker).

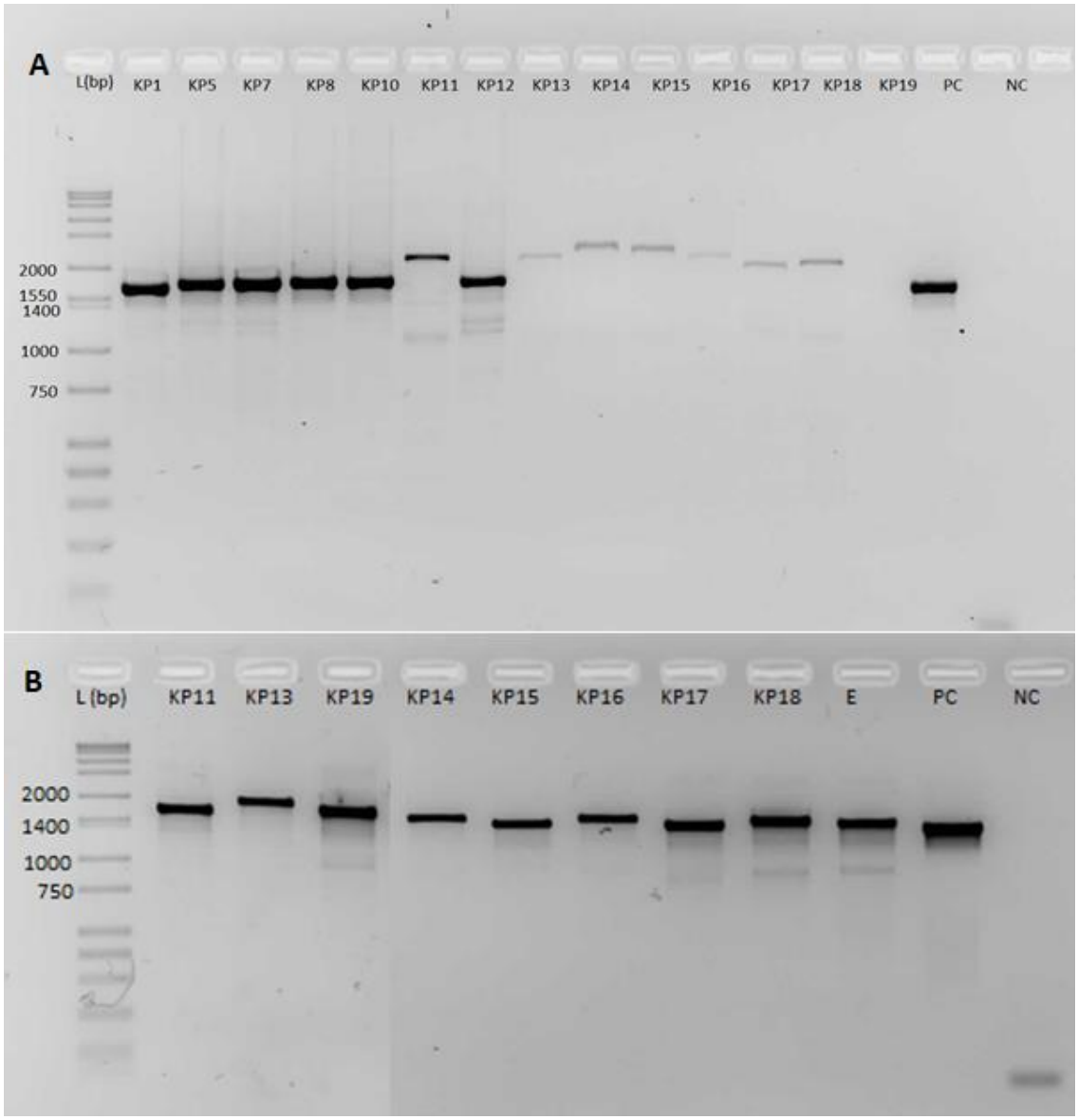

Figure 4.2: Representative gel of PCR amplification of *glpT* in fosfomycin resistant *K. pneumoniae,* from the 2017 sample set, using A) the first "*glpT*" primer set (Lu *et al.*, 2016) and B) the second "*glpT* (2)" primer set (Liu *et al.*, 2020) run in 2% agarose gel. PC – Positive control; NC – Non-template Control; L– Ladder (DirectLoad™ Wide Range DNA Marker).

Chromosomal mutations were detected in only four of the eight fosfomycin resistant *E. coli* isolates (Table 4.3). Two isolates contained deletions of multiple base pairs in the *uhpT* gene, and all four contained previously undescribed mutations or previously described mutations of unknown functional role in the *glpT* gene (Table 4.3). Other variants, Leu297Phe, Glu443Gln and Gln444Glu, were detected in the *glpT* gene of three of the *E. coli* isolates, however the mutations have been reported to have no impact on fosfomycin

susceptibility (Takahata *et al.*, 2010; Martin-Gutiérrez *et al.*, 2018). No mutations were identified in the *murA* genes of any of the *E. coli* isolates.

Mutations were detected in the *murA* and *uhpT* genes of only two of the 20 *K. pneumoniae* isolates. One contained a deletion in the *uhpT* gene as well as previously undescribed mutations in the *murA* and *uhpT* genes (Table 4.3). The other isolate contained only a previously undescribed mutation in the *uhpT* gene. The remaining 18 *K. pneumoniae* isolates did not have any mutations detected in the chromosomal *murA, uhpT* and *glpT* genes.

Table 4.3: Mutations detected in fosfomycin resistant *K. pneumoniae* and *E. coli*.

Organism	Sample set	Sequence variants		
		murA	***glpT***	***uhpT***
E. coli (CA3)	2020	ND	Thr348Asn	Deletion (68bp)[a]
E. coli (CA4)	2020	ND	Glu374Ala	ND
E. coli (CA5)	2020	ND	Gly415Asp; Asn450Thr	Deletion (96bp)[b]; Thr425Ala
E. coli (EC1)	2017	ND	Pro114Leu	ND
K. pneumoniae (CA6)	2020	Ser148Asn; Thr206Lys; Ser210Thr	ND	Deletion (38bp)[c]; Val434Ile
K. pneumoniae (KP13)	2017	ND	ND	Lys10Glu

[a]Position 985 – 1053; [b]Position 1293 – 1389; [c]Position 1117- 1155. Red – mutations that have not been described previously; Purple – mutation that have been previously described and are known to confer resistance; Blue – mutations that have been previously described but role in fosfomycin resistance in unknown. ND – none detected

4.4 *Discussion*

Chromosomally mediated fosfomycin resistance is often due to the alteration of the target protein MurA or loss of function of the transporters GlpT and UhpT. The chromosomal genes, *murA, glpT* and *uhpT*, were successfully amplified in fosfomycin resistant *E. coli* and *K. pneumoniae* isolates and sequenced by Sanger sequencing to identify potentially resistance-causing mutations.

The *murA*, *glpT* and *uhpT* PCR products showed differences in size between isolates and in comparison to the controls, however sequencing confirmed that the PCRs were specific and the products were the appropriate sizes, suggesting that this was not due to large insertions or deletions. The size variation may be attributed to various technical factors, such as the use of unpurified DNA for amplification, loading of unpurified PCR products on the gel, incorrect loading of the gel, or uneven distribution of voltage power by the gel electrophoresis instrument, which could affect the migration of PCR products. The size discrepancy has, however, previously been shown in our laboratory to be due to the use of Novel juice for DNA visualisation, where the same products visualised with ethidium bromide migrated similarly.

None of the *E. coli* isolates in our sample set had mutations in the *murA* gene, however four had mutations identified in the *glpT* and two had mutations identified in the *uhpT* transporter genes (Table 4.3). Mutations in the *murA* gene of *E. coli* are not very common because they are associated with a biological cost (Karageorgopoulos *et al.*, 2012; Martin-Gutiérrez *et al.*, 2018). *glpT* mutations that have not been described before in *E. coli*, were identified in three *E. coli* isolates (Table 4.3) and it is unknown if they confer fosfomycin resistance. Two *E. coli* isolates presented partial deletions in the *uhpT* gene, one of which had an additional substitution (Thr425Ala) in UhpT. Deletions are reported to be the most common mutations involved in gene inactivation in both clinical and in vitro generated fosfomycin resistant isolates (Takahata *et al.*, 2010; Castañeda-García, Blázquez and Rodríguez-Rojas, 2013).

Other mutations such as Leu297Phe, Glu443Gln and Gln444Glu, that have been previously described, were detected in the *glpT* gene of three *E. coli* isolates, but they have previously been proven to not confer resistance (Takahata *et al.*, 2010; Sorlozano-Puerto *et al.*, 2020). Takahata *et al*. (2010) grew fosfomycin resistant *E. coli* isolates harbouring these mutations in *glpT* in minimal agar supplemented with G-3-P (glucose-3-phosphate) and found that these mutations in the *glpT* gene do not have an impact on the proper function of GlpT and therefore do not confer fosfomycin resistance in *E. coli*. This finding was also supported by Sorlozano-Puerto *et al*. (2020), who detected the three *glpT* mutations (Leu297Phe, Glu443Gln and Gln444Glu) in *E. coli* but found them to have no impact on the biological function of the GlpT protein when grown in minimal agar supplemented with G-3-P (Takahata *et al.*, 2010; Sorlozano-Puerto *et al.*, 2020). The Thr348Asn mutation detected

on the *glpT* gene of one of the *E. coli* isolates in this study was also reported by Martín-Gutiérrez and colleagues, however its role in fosfomycin resistance has not been established (Martin-Gutiérrez *et al.*, 2018).

Among the 20 fosfomycin resistant *K. pneumoniae* isolates, two presented chromosomal mutations. One *K. pneumoniae* (CA6) presented three mutations (Ser148Asn, Thr206Lys and Ser210Thr) in the *murA* gene, of which none have been described previously. A partial deletion in the *uhpT* gene was also detected in the same isolate. The other *K. pneumoniae* (KP13) only had one substitution mutation (Lys10Glu) in UhpT, which has not been described previously. None of the *K. pneumoniae* isolates had mutations in the *glpT* genes and no mutations were detected in the chromosomal genes of the rest of the 18 *K. pneumoniae* isolates. The novel variants identified in resistant *E. coli* and *K. pneumoniae* isolates may not be responsible for fosfomycin resistance as they may also be present in susceptible isolates, which were not included in this study.

Some of the detected mutations have not been described before and their role in fosfomycin resistance could be further investigated. Growth of organisms in minimal agar supplemented with G-6-P (glucose-6-phosphate) will only allow isolates with a functional UhpT to grow, and if supplemented with G-3-P only isolates with a functional GlpT transporter will grow; this can thus give an indication of which mutation alters the function of its respective transporter protein. Growing these organisms in supplemented agar will however only indicate the role of the mutations in the transporters and cannot be used to determine the role of *murA* mutations. Allelic exchange could be used to determine the role of these mutations. Allelic exchange introduces a specific mutation in a host by using homologous-recombination to replace a genomic region with a foreign sequence of interest; the strains with the confirmed mutations can be subjected to fosfomycin susceptibility testing to determine if the mutation confers fosfomycin resistance.

Fosfomycin resistance could also result from mutations occurring in regulatory genes like *ptsI*, *cyaA* or *uhpA* which were not screened for in this study. Screening of these genes could form part of future studies, which can be done by individually amplifying these genes by PCR and sequencing to screen for mutations. Another method, that is more reliable in

screening and identifying mutations, is whole genome sequencing by Illumina, which allows screening of mutations in chromosomal genes as well as detection of other potential resistance conferring genes. Illumina has a low error rate and produces high copy numbers of short contigs which are more reliable for the detection of mutations than long read technologies.

In conclusion, of the 28 fosfomycin resistant *E. coli* and *K. pneumoniae* isolates screened in this chapter only six isolates had mutations detected in the chromosomal genes and many of those mutations have not been confirmed to confer resistance. The deletions observed in the *uhpT* of two *E. coli* and one *K. pneumoniae* isolate are likely to confer resistance. However, it appears that mutations in these chromosomal genes are not a major contributor to fosfomycin resistance in this population.

Chapter 5

Conclusion

Fosfomycin is a bactericidal drug available as an oral formulation (fosfomycin trometamol). It is used as a first line option for the management of community-acquired uncomplicated urinary tract infections (UTI) and for UTI caused by multidrug resistant bacteria. There is currently not enough data on the prevalence of fosfomycin resistance in South Africa, and the underlying resistance mechanisms have not been described in our setting. This study aimed to determine the prevalence of fosfomycin resistance in community-acquired urinary pathogens in the Western Cape and to characterise the mechanisms of fosfomycin resistance in this population.

Two hundred Enterobacterales and *Enterococcus spp*. isolates from urine samples from patients attending antenatal clinics were used for the purpose of this study. Pregnant women frequently attend antenatal clinics for routine check-ups which include giving urine samples to screen for UTI. Therefore, they are a convenient group that represents community-acquired UTI.

A fosfomycin resistance rate of 3.5% was found among community-acquired urinary pathogens in the Western Cape population. The results are in keeping with what was reported in 2013 by Lewis *et al*., who found 4.5% fosfomycin resistance in Gauteng community-acquired UTI (Lewis *et al.*, 2013). Similar to Lewis' report, a 4.3% fosfomycin resistance was reported in Charlotte Maxeke Johannesburg Academic Hospital on Enterobacteriaceae isolates collected from 2015-2017 (Mothibi, Bosman and Nana, 2020). No fosfomycin susceptibility data was available for other provinces in South Africa including the Western Cape, indicating a worrisome lack of fosfomycin resistance surveillance systems in the country.

This study reported a fosfomycin resistance rate of 2.2% among *Escherichia coli* isolates, which is consistent with what was reported in Johannesburg where they found 2% resistance among *E. coli* isolates (Mothibi, Bosman and Nana, 2020). A study done in Turkey also found a 2.2% fosfomycin resistance among *E. coli* isolates (Demir and Buyukguclu, 2013).

A resistance rate lower than 5% was also seen in other regions like Europe, Asia, the United States and Switzerland, with Switzerland having 1.4% resistance from community associated pathogens isolated in 2012 - 2013 (Mueller *et al.*, 2019). *Enterococcus faecalis* made up 11.5% of our isolates and all were susceptible to fosfomycin. Fosfomycin resistance among our community urinary pathogens is low and thus the results of this study support the ongoing prescription of fosfomycin for community-acquired uncomplicated UTI. The low resistance rate among *E. coli* (2.2%) also supports the practise of not routinely testing *E. coli* for fosfomycin susceptibility; however, we encourage ongoing surveillance.

Mechanisms of fosfomycin resistance were investigated by the detection of *fosA1-7* in fosfomycin resistant isolates and screening of mutations in the *murA*, *glpT* and *uhpT* genes in fosfomycin resistant *E. coli* and *Klebsiella pneumoniae*. FosA proteins inactivate fosfomycin by hydrolysis, resulting in drug inactivation and thus conferring resistance. Five of the 8 *E. coli* isolates harboured *fosA3/4* and one *E. coli* isolate harboured *fosA7*. Nineteen of the 20 *K. pneumoniae* isolates harboured *fosA5/6* genes. No other fosfomycin resistant isolates harboured *fosA* genes. It has been reported that *fosA* is intrinsic in *Klebsiella* isolates (Ito *et al.*, 2017) and in our 2017 sample set *fosA5/6* was detected in 19 *K. pneumoniae* isolates; we did not distinguish between the two *fos* variants. It is however unknown if they are located in the chromosome or plasmid mediated.

It has been reported that deletions in transporter genes influence fosfomycin drug uptake, which in turn confers fosfomycin resistance (Takahata *et al*., 2010; Sorlozano-Puerto *et al*., 2020). Deletions were detected in the *uhpT* genes of three of our isolates (n=2 *E. coli* and n=1 *K. pneumoniae*). These isolates did not harbour any *fosA* genes, thus fosfomycin resistance in these isolates may be conferred by these deletions (Table 5.1). One *E. coli* (EC1) isolate harbouring a *fosA3/4* additionally presented a mutation (Pro114Leu) in GlpT; this mutation has not been previously reported but may have caused the discrepancy between the phenotypic and molecular assay. Another *E. coli* (CA4) isolate presenting *fosA7* gene also presented with a mutation in *glpT*.

No other chromosomal mutations known to confer fosfomycin resistance were identified in resistant *E. coli* and *K. pneumoniae*, however it is of importance to determine the role of the

novel mutations that were identified and described in Chapter 4. Amplification of chromosomal genes from fosfomycin resistant *Proteus mirabilis* and *Enterobacter cloacae* was not done, and their resistance mechanisms remain unknown.

Table 5.1: Mutations identified and *fosA* genes detected in fosfomycin resistant isolates by molecular assay.

2017 isolates					
Sample ID	**Organism**	***fosA* detection**	**Mutations**		
			murA	***glpT***	***uhpT***
EC1	*E. coli*	*fosA3/4*	ND	Pro114Leu	ND
EC2	*E. coli*	*fosA3/4*	ND	ND	ND
EC3	*E. coli*	*fosA3/4*	ND	ND	ND
EC4	*E. coli*	*fosA3/4*	ND	ND	ND
EC5	*E. coli*	*fosA3/4*	ND	ND	ND
KP1	*K. pneumoniae*	*fosA5/6*	ND	ND	ND
KP2	*K. pneumoniae*	*fosA5/6*	ND	ND	ND
KP3	*K. pneumoniae*	*fosA5/6*	ND	ND	ND
KP4	*K. pneumoniae*	*fosA5/6*	ND	ND	ND
KP5	*K. pneumoniae*	*fosA5/6*	ND	ND	ND
KP6	*K. pneumoniae*	*fosA5/6*	ND	ND	ND
KP7	*K. pneumoniae*	*fosA5/6*	ND	ND	ND
KP8	*K. pneumoniae*	*fosA5/6*	ND	ND	ND
KP9	*K. pneumoniae*	*fosA5/6*	ND	ND	ND
KP10	*K. pneumoniae*	*fosA5/6*	ND	ND	ND
KP11	*K. pneumoniae*	*fosA5/6*	ND	ND	ND
KP12	*K. pneumoniae*	*fosA5/6*	ND	ND	ND
KP13	*K. pneumoniae*	*fosA5/6*	ND	ND	Lys10Glu
KP14	*K. pneumoniae*	*fosA5/6*	ND	ND	ND
KP15	*K. pneumoniae*	*fosA5/6*	ND	ND	ND
KP16	*K. pneumoniae*	*fosA5/6*	ND	ND	ND

KP17	*K. pneumoniae*	*fosA5/6*	ND	ND	ND
KP18	*K. pneumoniae*	*fosA5/6*	ND	ND	ND
KP19	*K. pneumoniae*	*fosA5/6*	ND	ND	ND
2019-2020 isolates					
Sample ID	**Organism**	***fosA* detection**	**Mutations**		
			murA	***glpT***	***uhpT***
CA1	*E. cloacae*	ND	NS	NS	NS
CA2	*E. cloacae*	ND	NS	NS	NS
CA3	*E. coli*	ND	ND	Thr348Asn	Deletion (68bp)[a]
CA4	*E. coli*	*fosA7*	ND	Glu374Ala	ND
CA5	*E. coli*	ND	ND	Gly415Asp; Asn450Thr	Deletion (96bp)[b]; Thr425Ala
CA6	*K. pneumoniae*	ND	Ser148Asn; Thr206Lys; Ser210Thr	ND	Deletion (38bp)[c]; Val434Ile
CA7	*P. mirabilis*	ND	NS	NS	NS

[a]Position 985 – 1053; [b]Position 1293 – 1389; [c]Position 1117- 1155; ND – none detected; NS – not screened.

Although fosfomycin resistance was rare with only 7/200 isolates being resistant, fosfomycin resistant mechanisms in *E. coli* and *K. pneumoniae* from community urinary pathogens (2019-2020 sample set) seem to be primarily caused by loss of function of the UhpT transporter due to partial deletions in the gene. In the 2017 sample set, which consists of both community and hospital-acquired UTI isolates, fosfomycin resistance was mainly *fosA* mediated (Table 5.1). Community and hospital-acquired UTI would be expected to have different resistance mechanisms as hospital strains are more likely to be multidrug resistant and more prone to acquisition of plasmid genes.

Limitations and future studies

Limitations to the study include not screening for mutations in all genes (including regulatory genes) that have been previously reported to contribute to fosfomycin resistance. Screening of mutations in the transporter regulatory genes such as *ptsI*, *uhpA* and *cyaA* and detection of other *fos* genes is important to identify all mutations and genes that may contribute to fosfomycin resistance. Whole genome sequencing could be incorporated in future studies, this will enable the identification of mutations in all chromosomal genes and detection of all *fos* related genes. It would additionally determine if the *fos* genes are plasmid mediated or chromosomally located, which could help determine if some organisms in our population have intrinsic resistance to fosfomycin.

Whole genome sequencing on the Illumina platform and Oxford Nanopore platforms could be utilised and a comparison between the two methods could also be considered. Illumina sequencing produces high copy numbers of short reads with a lower error rate (<1%) and is thus a highly reliable method for mutation identification. Nanopore produces long reads and has an error rate of 10%, but it generates sequences that are easier to assemble and to distinguish plasmid and chromosomal sequences. In addition to screening for all the genes involved in fosfomycin resistance, whole genome sequencing also enables identification of possible novel resistance mechanisms.

Another limitation was that we did not determine if the mutations in the *murA*, *glpT* and *uhpT* genes that have not been previously described confer resistance. Investigations to determine their role in fosfomycin resistance using an allelic exchange method to introduce a mutation in a host can be done. To determine if the mutant confers fosfomycin resistance, the strains with the confirmed specific mutations can be subjected to fosfomycin susceptibility testing.

An observation that was made during this study, but that was not thoroughly investigated, was the growth of single colonies in the inhibition zone of the disc diffusion susceptibility plates. The Clinical and Laboratory Standards Institute (CLSI) has not given any recommendation on how to handle growth of single colonies within the inhibition zone, however the European Committee on Antimicrobial Susceptibility Testing (EUCAST)

suggested not taking those colonies into consideration. These colonies make interpretation difficult and operator dependant. Elliot *et al*. (2019) suggested that the growth of single colonies within the inhibition zone may be caused by the presence of chromosomal *fosA* genes rather than chromosomal mutations. The role of *fos* genes in the single colony growth within the inhibition zone could be investigated further, also by whole genome sequencing, where a wide range of *fos* genes such as *fosA*, *fosB*, *fosC* and *fosX* could be screened in order to determine if a specific gene has an influence in the growth of single colonies within the inhibition zone. Sequencing of scattered colonies' genomes may indicate the common genes that are harboured by these colonies and could improve the interpretation of diffusion methods in the laboratory.

Another limitation is that the two sample sets, collected in different time periods (2017 and 2019-2020), were investigated in a single study making it difficult to collectively describe and link the findings.

In conclusion, the prevalence of fosfomycin resistance in community-acquired UTI is still low and the main mechanism of fosfomycin resistance identified in this population was FosA mediated. This study supports the careful ongoing use of fosfomycin to treat community-acquired UTI and encourages further surveillance of fosfomycin susceptibility in both community and hospital-acquired UTI.

References

Aghamali, M. *et al.* (2019) 'Fosfomycin: Mechanisms and the increasing prevalence of resistance', *Journal of Medical Microbiology*, 68(1), pp. 11–25. doi: 10.1099/jmm.0.000874.

Ahmed, A. B. and Ghadeer, A. S. (2013) 'Recurrent Urinary Tract Infections Management in Women: A review.', Sultan Qaboos University medical journal, 13(3), pp. 359–67. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23984019%0Ahttp://www.ncbi.nlm.nih.gov/pubmed/23984019.

Albert, X. *et al.* (2004) 'Antibiotics for preventing recurrent urinary tract infection in non-pregnant women', *Cochrane Database of Systematic Reviews*. doi: 10.1002/14651858.cd001209.pub2.

Amin, M., Mehdinejad, M. and Pourdangchi, Z. (2009) 'Study of bacteria isolated from urinary tract infections and determination of their susceptibility to antibiotics', *Jundishapur Journal of Microbiology*.

Anton Y. Peleg, M.B., B.S., M.P.H. and David C. Hooper, M. . (2010) 'Hospital-Acquired Infections Due to Gram-Negative Bacteria', *The New England Journal of Medicine*, 362(19), pp. 1804–1813. doi: 10.1056/NEJMra0904124.Hospital-Acquired.

Asian Association of UTI and Sexually Transmitted Infections. (2015) 'Development of Asian Guidelines for UTI / STI'.

Benzerara, Y. *et al.* (2017) 'Emergence of plasmid-mediated fosfomycin-resistance genes among *Escherichia coli* isolates, France', *Emerging Infectious Diseases*. doi: 10.3201/eid2309.170560.

Burkhard, F. et al. (2018) 'Urinary Incontinence: EAU Guidelines 2018', European Association of Urology, Presented at the EAU Annual Congress Copenhagen p.20. Available at: http://uroweb.org/guideline/urinary-incontinence/.

Castañeda-García, A., Blázquez, J. and Rodríguez-Rojas, A. (2013) 'Molecular Mechanisms and Clinical Impact of Acquired and Intrinsic Fosfomycin Resistance', *Antibiotics*, 2(2), pp. 217–236. doi: 10.3390/antibiotics2020217.

Cattoir, V. and Guérin, F. (2018) 'How is fosfomycin resistance developed in *Escherichia*

coli?', *Future Microbiology*, 13(16), pp. 1693–1696. doi: 10.2217/fmb-2018-0294.

CDC (2013) 'Antibiotic resistance threats in the United States, 2013', Current, p. 114. doi: CS239559-B.

Clinical and Laboratory Standards Institute (2019) *M100 Performance Standards for Antimicrobial Susceptibility Testing*. doi: 10.1108/08876049410065598.

Coetzer, E. (2004) 'Urinary Tract Infection in Adults', *Cme*, 22(4), pp. 182–188. doi: 10.4172/2327-5073.1000e120.

Demir, T. and Buyukguclu, T. (2013) 'Evaluation of the in vitro activity of fosfomycin tromethamine against Gram-negative bacterial strains recovered from community- and hospital-acquired urinary tract infections in Turkey', *International Journal of Infectious Diseases*. International Society for Infectious Diseases, 17(11), pp. e966–e970. doi: 10.1016/j.ijid.2013.04.005.

Demir, T. and Buyukguclu, T. (2017) 'Fosfomycin: In vitro efficacy against multidrug-resistant isolates beyond urinary isolates', *Journal of Global Antimicrobial Resistance*. Taibah University, 8(55), pp. 164–168. doi: 10.1016/j.jgar.2016.11.011.

Díez-aguilar, M. and Cantón, R. (2019) 'New microbiological aspects of fosfomycin', *Journal of the Spanish Society of Chemotherapy*, 32, pp. 8–18.

Elliott, Z. S. *et al.* (2019) 'The Role of *fosA* in Challenges with Fosfomycin Susceptibility Testing of Multispecies Klebsiella pneumoniae Carbapenemase- Producing Clinical Isolates', 57(10), pp. 1–8.

Falagas, M. E. *et al.* (2008) 'Fosfomycin: Use beyond urinary tract and gastrointestinal infections', *Chinese Journal of Infection and Chemotherapy*, 10(2), p. 103. doi: 10.1086/527442.

Falagas, M. E. *et al.* (2010) 'Fosfomycin for the treatment of multidrug-resistant, including extended-spectrum β-lactamase producing, Enterobacteriaceae infections: a systematic review', *The Lancet Infectious Diseases*, 10(1), pp. 43–50. doi: 10.1016/S1473-3099(09)70325-1.

Falagas, M. E. *et al.* (2016) 'Fosfomycin', *Clinical Microbiology Reviews*, 29(2), pp. 321 LP – 347. doi: 10.1128/CMR.00068-15.

Flores-mireles, A. L. *et al.* (2016) 'and treatment options', 13(5), pp. 269–284. doi: 10.1038/nrmicro3432.Urinary.

Foxman, B. (2003) 'Epidemiology of urinary tract infections: Incidence, morbidity, and economic costs', *Disease-a-Month*, 49(2), pp. 53–70. doi: 10.1016/S0011-5029(03)90000-9.

Fu, Z. *et al.* (2016) 'Characterization of Fosfomycin Resistance Gene, *fosB*, in Methicillin-Resistant *Staphylococcus aureus* Isolates', *PLoS ONE*, 11(5), pp. 4–11. doi: 10.1371/journal.pone.0154829.

Garcia, P., Arca, P. and Suarez, J. E. (1995) 'Product of fosC, a gene from *Pseudomonas syringae*, mediates fosfomycin resistance by using ATP as cosubstrate', *Antimicrobial Agents and Chemotherapy*, 39(7), pp. 1569–1573. doi: 10.1128/AAC.39.7.1569.

Giamarellou, H. and Poulakou, G. (2009) 'Multidrug-resistant Gram-negative infections: What are the treatment options?', *Drugs*, pp. 1879–1901. doi: 10.2165/11315690-000000000-00000.

Gilbert, N. M. *et al.* (2013) 'Urinary Tract Infection as a Preventable Cause of Pregnancy Complications: Opportunities, Challenges, and a Global Call to Action', *Global Advances in Health and Medicine*, 2(5), pp. 59–69. doi: 10.7453/gahmj.2013.061.

Goel, V. *et al.* (2016) 'Community Acquired Enterococcal Urinary Tract Infections and Antibiotic Resistance Profile in North India', *Journal of Laboratory Physicians*, 8(01), pp. 050–054. doi: 10.4103/0974-2727.176237.

Guo, Q. *et al.* (2016) 'Glutathione-S-transferase FosA6 of *Klebsiella pneumoniae* origin conferring fosfomycin resistance in ESBL-producing *Escherichia coli*', *The Journal of antimicrobial chemotherapy*, 71(9), pp. 2460–2465. doi: 10.1093/jac/dkw177.

Guo, Y. *et al.* (2017) 'High-level fosfomycin resistance in vancomycin-resistant *Enterococcus faecium*', *Emerging Infectious Diseases*, 23(11), pp. 1902–1904. doi: 10.3201/eid2311.171130.

Hall, T. (2004) 'Bio Edit 取説.Pdf'.

Hendlin, D. et al. (1969) 'Phosphonomycin , a New Antibiotic Produced by Strains of Streptomyces', American Association for the Advancement of Science Stable, 166(3901), pp. 122–123. https://www.jstor.org/stable/1727759 .

Herring, C. D. and Blattner, F. R. (2004) 'Conditional Lethal Amber Mutations in Essential *Escherichia coli* Genes', *Journal of Bacteriology*, 186(9), pp. 2673–2681. doi: 10.1128/JB.186.9.2673-2681.2004.

Ito, R. *et al.* (2017) 'Widespread fosfomycin resistance in Gram-negative bacteria attributable to the chromosomal *fosA* gene', *mBio*, 8(4), pp. 1–9. doi: 10.1128/mBio.00749-17.

Jacobsen, S. M. et al. (2008) 'Complicated Catheter-Associated Urinary Tract Infections Due to Escherichia coli and Proteus mirabilis', Clinical Microbiology Reviews, 21(1), pp. 26–59. doi: 10.1128/CMR.00019-07.

Kabugo, D. *et al.* (2016) 'Factors associated with community-acquired urinary tract infections among adults attending assessment centre, Mulago Hospital Uganda.', *African Health Sciences*, 16(4), pp. 1131–1142. doi: 10.4314/ahs.v16i4.31.

Kang, C. *et al.* (2018) 'Clinical Practice Guidelines for the Antibiotic Treatment of Community-Acquired Urinary Tract Infections', 50(1), pp. 67–100. doi: 10.3947/ic.2018.50.1.67.

Karageorgopoulos, D. E. *et al.* (2012) 'Fosfomycin: Evaluation of the published evidence on the emergence of antimicrobial resistance in Gram-negative pathogens', *Journal of Antimicrobial Chemotherapy*, 67(2), pp. 255–268. doi: 10.1093/jac/dkr466.

Knottnerus, B. J. *et al.* (2008) 'Fosfomycin tromethamine as second agent for the treatment of acute, uncomplicated urinary tract infections in adult female patients in The Netherlands?', *Journal of Antimicrobial Chemotherapy*, 62(2), pp. 356–359. doi: 10.1093/jac/dkn177.

Lee, A. C. C. *et al.* (2019) 'Urinary tract infections in pregnancy in a rural population of Bangladesh: Population-based prevalence, risk factors, etiology, and antibiotic resistance', *BMC Pregnancy and Childbirth*. BMC Pregnancy and Childbirth, 20(1), pp. 1–11. doi: 10.1186/s12884-019-2665-0.

Lee, M. *et al.* (2008) 'Motherisk Update Urinary tract infections in pregnancy', *Canadian Family Physician*, 54, pp. 914–915. Available at: http://www.cfp.ca/content/cfp/54/6/853.full.pdf.

Lewis, D. A. *et al.* (2013) 'Antimicrobial susceptibility of organisms causing community-acquired urinary tract infections in Gauteng Province, South Africa', *South African Medical Journal*, 103(6), pp. 377–381. doi: 10.7196/samj.6722.

Li, Ya *et al.* (2015) 'Antimicrobial susceptibility and molecular mechanisms of fosfomycin resistance in clinical *Escherichia coli* isolates in mainland China', *PLoS ONE*, 10(8), pp. 1–7. doi: 10.1371/journal.pone.0135269.

Liu, P. *et al.* (2020) 'Journal of Global Antimicrobial Resistance Mechanisms of fosfomycin resistance in clinical isolates of carbapenem-resistant *Klebsiella pneumoniae*', *Integrative Medicine Research*. Taibah University, 22, pp. 238–243. doi: 10.1016/j.jgar.2019.12.019.

Lu, P. L. *et al.* (2016) 'Characterisation of fosfomycin resistance mechanisms and molecular epidemiology in extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* isolates', *International Journal of Antimicrobial Agents*. Elsevier B.V., 48(5), pp. 564–568. doi: 10.1016/j.ijantimicag.2016.08.013.

Ma, Y. *et al.* (2015) 'Characterization of *fosA5*, A new plasmid-mediated fosfomycin resistance gene in *Escherichia coli*', *Letters in Applied Microbiology*, 60(3), pp. 259–264. doi: 10.1111/lam.12366.

Majumder, M. I. et al. (2016) 'Microbiology of Catheter Associated Urinary Tract Infection', Intech, i(tourism), p. 13. doi: http://dx.doi.org/10.5772/57353.

Maraki, S. *et al.* (2009) 'Susceptibility of urinary tract bacteria to fosfomycin', *Antimicrobial Agents and Chemotherapy*, 53(10), pp. 4508–4510. doi: 10.1128/AAC.00721-09.

Martin-Gutiérrez, G. *et al.* (2018) 'Urinary tract conditions affect fosfomycin activity against *Escherichia coli* strains harboring chromosomal mutations involved in fosfomycin uptake', *Antimicrobial Agents and Chemotherapy*, 62(1), pp. 1–9. doi: 10.1128/AAC.01899-17.

Martinez, M. and Silley, P. (2010) 'Antimicrobial drug resistance', *Handbook of Experimental Pharmacology*, 199(January 2009), pp. 227–264. doi: 10.1007/978-3-642-10324-7_10.

Mbelle, N. M. *et al.* (2020) 'Pathogenomics and Evolutionary Epidemiology of Multi-Drug Resistant Clinical *Klebsiella pneumoniae* Isolated from Pretoria, South Africa', *Scientific Reports*. Springer US, 10(1), pp. 1–17. doi: 10.1038/s41598-020-58012-8.

'Monurol fosfomycin tromethamine'.

Mothibi, L. M., Bosman, N. N. and Nana, T. (2020) 'Fosfomycin susceptibility of uropathogens at Charlotte Maxeke Johannesburg Academic Hospital', *Southern African Journal of Infectious Diseases; Vol 35, No 1 (2020)DO - 10.4102/sajid.v35i1.173* . Available at: https://sajid.co.za/index.php/sajid/article/view/173/390.

Mueller, L., Cimen, C., Poirel, L., Descombes, M. C., et al. (2019) 'Prevalence of fosfomycin resistance among ESBL-producing Escherichia coli isolates in the community, Switzerland', European Journal of Clinical Microbiology and Infectious Diseases. European Journal of Clinical Microbiology & Infectious Diseases, pp. 6–10. doi: 10.1007/s10096-019-03531-0.

Nakamura, G. *et al.* (2014) 'Practical agar-based disk potentiation test for detection of fosfomycin-nonsusceptible *Escherichia coli* clinical isolates producing glutathione S-transferases', *Journal of Clinical Microbiology*, 52(9), pp. 3175–3179. doi: 10.1128/JCM.01094-14.

Nicolle, L. E. and AMMI Canada Guidelines Committee, A. C. G. (2005) 'Complicated urinary tract infection in adults.', *The Canadian journal of infectious diseases & medical microbiology = Journal canadien des maladies infectieuses et de la microbiologie medicale*, 16(6), pp. 349–60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18159518%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2094997.

Nilsson, A. I. *et al.* (2003) 'Biological costs and mechanisms of fosfomycin resistance in *Escherichia coli*', *Antimicrobial Agents and Chemotherapy*, 47(9), pp. 2850–2858. doi: 10.1128/AAC.47.9.2850-2858.2003.

Nimri, L., Sulaiman, M. and Hani, O. B. (2017) 'Community-acquired urinary tract infections caused by *Burkholderia cepacia* complex in patients with no underlying risk factor', *JMM Case Reports*, 4(1), pp. 10–13. doi: 10.1099/jmmcr.0.005081.

Nordmann, P., Poirel, L. and Mueller, L. (2019) 'Rapid Detection of Fosfomycin Resistance in *Escherichia coli*', *Journal of Clinical Microbiology*, 57(1), pp. 16–20. doi: 10.1128/JCM.01531-18.

O'Neill J: (2016) 'Tackling Drug-Resistant Infections Globally: Final Report and Recommendations', (May), pp. 1–80.

Oteo, J. *et al.* (2009) 'CTX-M-15-producing urinary *Escherichia coli* O25b-ST131-phylogroup B2 has acquired resistance to fosfomycin', *Journal of Antimicrobial Chemotherapy*, 64(4), pp. 712–717. doi: 10.1093/jac/dkp288.

Ou, L. B. and Nadeau, L. (2017) 'Fosfomycin susceptibility in multidrug-resistant enterobacteriaceae species and vancomycin-resistant *Enterococci* urinary isolates', *Canadian Journal of Hospital Pharmacy*, 70(5), pp. 368–374. doi: 10.4212/cjhp.v70i5.1698.

Pincus, D. H. (2010) 'Microbial identification using the bioMérieux VITEK® 2 system', *Encyclopedia of Rapid Microbiological Methods*, pp. 1–32.

Putensen, C. *et al.* (2019) 'Current clinical use of intravenous fosfomycin in ICU patients in two European countries', *Infection*. Springer Berlin Heidelberg, 47(5), pp. 827–836. doi:

10.1007/s15010-019-01323-4.

Ramos, R. and Llet, M. S. (2019) 'Fosfomycin in infections caused by multidrug- resistant Gram-negative pathogens', 32, pp. 45–54.

Raz, R. (2011) 'Fosfomycin : an old — new antibiotic', pp. 4–7.

Reffert, J. L. and Smith, W. J. (2014) 'Fosfomycin for the treatment of resistant Gram-negative bacterial infections: Insights from the society of infectious diseases pharmacists', *Pharmacotherapy*, 34(8), pp. 845–857. doi: 10.1002/phar.1434.

Rehman, M. A. *et al.* (2017) 'First detection of a fosfomycin resistance gene, *fosA7*, in *Salmonella enterica* serovar Heidelberg isolated from broiler chickens', *Antimicrobial Agents and Chemotherapy*, 61(8), pp. 1–6. doi: 10.1128/AAC.00410-17.

Silver, L. L. (2017) 'Fosfomycin: Mechanism and resistance', *Cold Spring Harbor Perspectives in Medicine*, 7(2). doi: 10.1101/cshperspect.a025262.

Sorlozano-Puerto, A. *et al.* (2020) 'Characterization of fosfomycin and nitrofurantoin resistance mechanisms in *Escherichia coli* isolated in clinical urine samples', *Antibiotics*, 9(9), pp. 1–19. doi: 10.3390/antibiotics9090534.

Takahata, S. *et al.* (2010) 'Molecular mechanisms of fosfomycin resistance in clinical isolates of *Escherichia coli*', *International Journal of Antimicrobial Agents*. Elsevier B.V., 35(4), pp. 333–337. doi: 10.1016/j.ijantimicag.2009.11.011.

Tan, C. W. and Chlebicki, M. P. (2016) 'Urinary tract infections in adults', *Singapore Medical Journal*, 57(9), pp. 485–490. doi: 10.11622/smedj.2016153.

Tong, S. Y. C. *et al.* (2015) '*Staphylococcus aureus* infections: Epidemiology, pathophysiology, clinical manifestations, and management', *Clinical Microbiology Reviews*, 28(3), pp. 603–661. doi: 10.1128/CMR.00134-14.

Tsukamoto, N. *et al.* (2014) 'High prevalence of cross-resistance to aminoglycosides in fluoroquinolone-resistant *Escherichia coli* clinical isolates', *Chemotherapy*, 59(5), pp. 379–384. doi: 10.1159/000361011.

United States Food and Drug Administration (2018) 'FDA drug safety communication: Fluoroquinolones'. Available at: https://www.fda.gov/media/114192/download.

Wachino, J. I. *et al.* (2010) 'Prevalence of fosfomycin resistance among CTX-M-producing *Escherichia coli* clinical isolates in Japan and identification of novel plasmid-mediated

fosfomycin-modifying enzymes', *Antimicrobial Agents and Chemotherapy*, 54(7), pp. 3061–3064. doi: 10.1128/AAC.01834-09.

WHO (2017) 'Report of the 21st WHO Expert Committee on the Selection and Use of Essential Medicines WHO headquarters , Geneva 27-31 March 2017 Table of contents Annexes', (March). Available at: http://www.who.int/medicines/publications/essentialmedicines/EML_2017_ExecutiveSummary.pdf?ua=1.

Yang, T. Y., Lu, P. L. and Tseng, S. P. (2017) 'Update on fosfomycin-modified genes in Enterobacteriaceae', *Journal of Microbiology, Immunology and Infection*. Elsevier Taiwan LLC, 52(1), pp. 9–21. doi: 10.1016/j.jmii.2017.10.006.

Zhanel, G. G., Zhanel, M. A. and Karlowsky, J. A. (2018) 'Intravenous fosfomycin: An assessment of its potential for use in the treatment of systemic infections in Canada', *Canadian Journal of Infectious Diseases and Medical Microbiology*. Hindawi, 2018. doi: 10.1155/2018/8912039.

Addendum A

UNIVERSITEIT
STELLENBOSCH
UNIVERSITY

Approval Notice

New Application

01/10/2019

Project ID :10934

HREC Reference No: S19/08/168

Project Title: Characterisation of fosfomycin resistance in urinary pathogens from the Western Cape, South Africa.

Dear Ms Lesedi Mosime

The **New Application** received on 26/08/2019 10:59 was reviewed by members of **Health Research Ethics Committee** via **expedited** review procedures on 01/10/2019 andwas **approved**.

Please note the following information about your approved research protocol:

Approval date: 1 October 2019

Expiry date: 30 September 2020

Please remember to use your Project ID 10934 and Ethics Reference Number S19/08/168 on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review

Translation of the informed consent document(s) to the language(s) applicable to your study participants should now be submitted to the HREC.

Please note you can submit your progress report through the online ethics application process, available at: Links Application Form Direct Link and the application should be submitted to the HREC before the year has expired. Please see *Forms and Instructions* on our HREC website (www.sun.ac.za/healthresearchethics) for guidance on how to submit a progress report.

The HREC will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility, permission must still be obtained from the relevant authorities (Western Cape Departement of Health and/or City Health) to conduct the research as stated in the protocol. Please consult the Western Cape Government website for access to the online Health Research Approval Process, see: https://www.westerncape.gov.za/general-publication/health-research-approval-process. Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.

For standard HREC forms and instructions, please visit: Forms and Instructions on our HREC website https://applyethics.sun.ac.za/ProjectView/Index/10934

If you have any questions or need further assistance, please contact the HREC office at 021 938 9677.

Yours sincerely,

Mrs. Melody Shana

Coordinator

HREC1

National Health Research Ethics Council (NHREC) Registration Number:

REC-130408-012 (HREC1) •REC-230208-010 (HREC2)

Federal Wide Assurance Number: 00001372
Office of Human Research Protections (OHRP) Institutional Review Board (IRB) Number: IRB0005240 (HREC1) •IRB0005239 (HREC2)

The Health Research Ethics Committee (HREC) complies with the SA National Health Act No. 61 of 2003 as it pertains to health research. The HREC abides by the ethical norms and principles for research, established by the World Medical Association (2013). Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects;the South African Department of Health (2006). Guidelines for Good Practice in the Conduct of Clinical Trials with Human Participants in South Africa (2nd edition); as well as the Department of Health (2015). Ethics in Health Research: Principles, Processes andStructures (2nd edition).

The Health Research Ethics Committee reviews research involving human subjects conducted or supported by the Department of Health and Human Services, or other federal departments or agencies that apply the Federal Policy for the Protection of Human Subjects to such research (United States Code of Federal Regulations Title 45 Part 46); and/or clinical investigations regulated by the Food and Drug Administration (FDA) of the Department of Health and Human Services.

Addendum B

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Approved with Stipulations
New Application

29 June 2017

Mosime, Lesedi

Ethics Reference #: U17/05/026
Title: Detection, molecular characterization and epidemiology of fosfomycin resistance mechanisms in Enterobacteriaceae from urinary isolates at Tygerberg hospital

Dear Ms Lesedi Mosime

The **Response to Modifications** received on **19 June 2017**, was reviewed by members of **Health Research Ethics Committee 2** via Minimal Risk Review procedures on **22 June 2017**.

Please note the following information about your approved research protocol:

Protocol Approval Period: **22-June-2017** -**21-June-2018**

The Stipulations of your ethics approval are as follows:

Request for waiver of consent

The criteria for waiving of consent have been met and therefore the request has been approved.

Budget: Clarification of funding

The Protocol (Budget, page 10) states that a grant application has been submitted to the NHLS Research Trust for funding; however, in the Payment Instruction form (Section 3, page 1), the funder is indicated as the National Health Laboratory Service Research Fund, which implies that the application has been approved. Please clarify if it is the NHLS Research Trust or Fund, and whether the application for funding has already been approved or not. The information should correspond between these two documents. Please clarify.

Administrative issues

- In the Application Form (Section 4, page 2), Investigator's Declaration and Checklist (Section 2, page 2), Prof. Andrew Whitelaw is indicated as a Sub/Co-investigator; however, in the Protocol (page 1), he is indicated as a Co-Supervisor. Please clarify his role.
- In the Application Form (Section 4, page 2) and Protocol (page 1), Dr. Mae Newton-Foot is indicated as a Co-Supervisor; however, in the Investigator's Declaration and Checklist (Section 2, page 2), she is indicated as a Sub/Co-investigator. Please clarify her role.
- In the Protocol (Ethics, page 9, end of 2nd paragraph), the word "Committee" was omitted after "Health Research Ethics". Please amend.
- In the substantiation for waiver of consent request letter (Heading of #1) a., page 1), the word "practically" should be "practicability". Please amend.

Please remember to use your **protocol number** (**U17/05/026**) on any documents or correspondence with the HREC concerning your research protocol.

Please note that this decision will be ratified at the next HREC full committee meeting. HREC reserves the right to suspend UREC approval and to request changes or clarifications from student applicants. The UREC coordinator will notify the applicant (and if applicable, the supervisor) of the changes or suspension within 1 day of receiving the notice of suspension from HREC. HREC has the prerogative and authority to ask further

questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review:
Please note a template of the progress report is obtainable on www.sun.ac.za/rds and should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

Translation of the consent document to the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2015 (Department of Health).

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Helene Visser at City Health (Helene.Visser@capetown.gov.za Tel:+27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.
For standard HREC forms and documents please visit: www.sun.ac.za/rds
If you have any questions or need further assistance, please contact the HREC office at 0219389207.

Included Documents:

New application:
Application form new protocol
Cover letter
CV 2017 Dr A Lourens
CV Dr M Newton-Foot
General Checklist (Eng)
Investigator declaration AL signed
Investigator declaration MNF
L Mosime Investigator declaration
L Mosime CV
Synopsis
Modifications required:
20150224 Investigator declaration
AW CV
Informed consent checklist

Payment instruction amended
Protocol – amended ethics
Substantiation of waiver

Sincerely,

Debbie Marais
UREC Coordinator
Undergraduate Research Ethics Committee