THE LEVEL AND PERSISTENCE OF ANTIBIOTIC RESISTANT STRAINS OF BACTERIA IN WASTEWATER BEFORE, DURING AND AFTER TREATMENT AT A MUNICIPAL WASTEWATER TREATMENT PLANT IN STELLENBOSCH

By

SINETHEMBA HOPEWELL YAKOBI

Thesis submitted in fulfillment of the requirements for the degree

Master of Technology: Biomedical Sciences

In the Faculty of Health and Wellness Sciences

At the Cape Peninsula University of Technology

Supervisor(s): Prof Sehaam Khan & Dr Wesaal Khan

Bellville

December 2015

DECLARATION

I, Sinethemba Hopewell Yakobi, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination toward any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

February 2016

Signed **Date**

ABSTRACT

Wastewater treatment plants (WWTPs) are designed to remove/decrease conventional pollution parameters from the wastewater influent, so that the final effluent (run off) does not compromise the receiving surface water source. However, as hospital and clinical effluent may form part of the initial influent at a WWTP, bacteria may be exposed to various antibiotics or pharmaceuticals throughout the various stages of primary, secondary and tertiary processes utilised to remove or reduce the level of pollutants. Numerous studies have then indicated that WWTPs have become potential reservoirs for antibiotic resistant bacteria (ARB) and due to ineffective treatment practices, antibiotics are being released into the environment. Consequently, research has shown that relatively low concentrations of these compounds still promotes the development of bacterial resistance, which potentiates the rapid spread of ARB in the environment. The primary aim of this study was thus to identify and trace the antibiotic resistant strains of *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) throughout the Stellenbosch WWTP. This was done in order to determine the persistance of the ARB organisms at the various stages of treatment and to ascertain which identification and antibiotic resistance detection methods are ideal for the routine application and detection of these organisms.

The first phase of the project thus focussed on the identification of the target organisms (*S. aureus*, *E. coli* and *K. pneumoniae*) using the Matrix-assisted laser desorption/ionization Time of Flight Mass Spectrometry (MALDI-TOF MS), VITEK 2 identificaiton system and 16S rRNA polymerase chain reaction analysis. Two litres (L) of wastewater were collected for three cycles from the WWTP in Stellenbosch at the influent, aeration tank, secondary settling tank and effluent sites. MALDI-TOF MS positively identified 63% (44/70) of the target organisms to the species level from all cycles. In contrast the 16S rRNA amplification followed by amplicon sequencing positively identified 99% (69/70) of the target bacteria from all cycles to species level. However, the VITEK 2 identification system positively identified to species level an average of 83% (58/70) of the target bacteria from all cycles. Cochran's Q test probability analysis for the identification of *E. coli* was then equal to 0.00091, *K. pneumoniae* was equal to 0.000081 and *S. aureus* probability analysis was equal to 0.049787. This then resulted in the rejection of the null hypothesis and acceptance of the alternative hypothesis, which states that, there is a significant difference between the three identification techniques in identifying a particular species of the target bacteria to species level. Based on these results obtained it is therefore recommended that 16S rRNA gene sequencing technique can be utilised for the

routine analysis of wastewater quality. However, for laboratories that are not set up for PCR analysis the VITEK 2 system is recommend.

Since antibiotic resistant bacteria and genes encoding antibiotic resistance are commonly detected in wastewater, often at higher rates and concentrations compared to surface water, the 44 *E. coli* isolates were screened for possible resistance to quinolones, the 20 *K. pneumoniae* isolates were screened for possible resistance to carbapenems and the six *S. aureus* isolates were screened for possible resistance to methicillin. The PCR technique was used to amplify the *gyrA* and *parC* (DNA gyrase and DNA topoisomerase IV, respectively) genes in *E. coli*, the presence of bla_{KPC} genes (β-lactam *Klebsiella pneumoniae* carbapenemases) in *K. pneumoniae* and the presence of *mecA* genes in *S. aureus*. The VITEK 2 system was used to detect antibiotic profiles of *E. coli* isolates resistant to quinolones, *K. pneumoniae* isolates showing resistance to carbapenems and *S. aureus* isolates showing resistance to methicillin. Using the PCR technique, the *gyrA* gene and *parC* gene was detected in all *E.coli* isolates (n = 44); the bla_{KPC} gene was detected in five *K. pneumoniae* isolates (n = 20) and the *mecA* gene was detected in one *S. aureus* isolate (n = 6). Since, resistance is caused by missense mutations that occur within *gyrA* gene and *parC* gene of *E. coli* respectively, single nucleotide polymorphisms (SNPs) within this region was compared to a wild-type strain to detect possible missense mutations. From the genomic DNA extraction, 40 *E. coli* isolates had the wild-type *gyrA* gene and four isolates presented with mutations when compared to the wild-type. From the plasmid DNA extraction, 42 *E. coli* isolates had amino acid sequences identical to the wild-type *gyrA* gene and two isolates presented with missense mutations. From the genomic DNA extraction, 43 *E. coli* isolates had amino acid sequence similar to the wild-type *parC* gene with one isolate presenting with a mutation. From the plasmid DNA extraction, 40 *E. coli* isolates had amino acid sequence similar to the wild-type *parC* gene and four isolates presented with mutations. Analyses of the antibiotic resistant profiles using the VITEK 2 system yielded six *K. pneumoniae* isolates and one *E. coli* isolate with resistance profiles against their respective antibiotics. No antibiotic resistant profile was detected in any of the *S. aureus* isolates.

Though *E. coli* was detected at all the sampling sites throughout the study period, the quinolone resistant isolate, which had a mutation on the hot spot of the *gyrA* gene, was detected in the secondary settling tank. No *E. coli* isolates with a mutation on the target genes or that had a quinolone resistant profile was detected at the effluent point. *Klebsiella pneumoniae* isolates were also detected at all sampling sites. Two *K. pneumoniae* isolates with the bla_{KPC} genes and carbapenem resistance profiles were detected at the aeration tank, one *K. pneumoniae* isolate that did not have the bla_{KPC} genes but showed carbapenem resistance was also detected at the

aeration tank. One *K. pneumoniae* isolate with the *bla*$_{KPC}$ gene and a carbapenem resistance profile was detected at the secondary settling tank and two *K. pneumoniae* isolates with the *bla*$_{KPC}$ gene and a carbapenem resistant profile was detected in the effluent. Since, the effluent from the Stellenbosch Waterworks flows into the Veldwachters River which streams into the Eerste River and the water of this river is used by neighbouring agricultural farms for irrigation purposes, it was important that the effluent be screened for the presence of antibiotic strains and antibiotic resistant bacteria since this study showed that a *K. pneumoniae* isolate with the *bla*$_{KPC}$ gene and a carbapenem resistant profile was detected in the effluent.

ACKNOWLEDGEMENTS

Now unto **Him** that is able to keep us from falling, and to present us faultless before the presence of his glory with exceeding joy, **to the only wise God our Saviour**, be glory and majesty, dominion and power, both now and ever. Amen.

I wish to give my deepest thanks to the following people and institution:

Prof Sehaam Khan, Department of Biomedical Science at Cape Peninsula University of Technology. Profy, my supervisor and mother. You were never hesitant in sharing your wisdom. At the time of the weakest point of my life, you gave me a hefty 'talk', through which I got motivated to get focussed and move on. For accepting me as your student, for caring and for your guidance, devotion and support throughout the research project, Thank you.

Dr Wesaal Khan, Department of Microbiology at Stellenbosch University. Thank you for your your guidance. I appretiate the hard work that you have put in toward the success of this research project. Because of such contributions I have managed to complete my M.Tech programme.

Thando Ndlovu and Micheal Tobin for your assistance and support during the period of this research project.

Cape Peninsula University of Technology and the National Research Foundation, for their financial support. The financial assistance of the National Research Foundation towards this research is acknowledged. Opinions expressed in this thesis and the conclusions arrived at, are those of the author, and are not necessarily to be attributed to the National Research Foundation.

Water Services Department Stellenbosch Municipality, Vivian Kloppers (Superintendent: Wastewater), for allowing me to do my research project using samples from their site.

Students in Lab B119 (Nkosikho Sogwagwa and Tinashe Magagani), for your assistance and encouragements.

My Mother, Simnikiwe and Sethu, for your support, inspirations and love. You are all swimming in my heart.

My Father, Dr B.L Yakobi, you're a constant encouragement, I wouldn't be where I am today if it were not for you, the love and support which you have shown me has profoundly impacted in my life. I LOVE YOU. Shosho Skhokho

DEDICATION

THIS THESIS IS DEDICATED TO MY SISTER THE LATE BULELWA CHRISTINA YAKOBI AND THE ENTIRE YAKOBI FAMILY

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

APPENDICES

GLOSSARY

ARB	Antibiotic Resistant Bacteria
ARG	Antibiotic Resistant Gene
bla_{KPC}	β-lactam *Klebsiella pneumoniae* carbapenemases
BLAST	Basic Local Alignment Search Tool
HGT	Horizontal Gene Transfer
MALDI-TOF MS	Matrix-assisted laser desorption/ionization Time of Flight Mass Spectrometry
MRSA	Methicillin-Resistant *Staphylococcus aureus*
PCR	Polymerase Chain Reaction
SNP	Single Nucleotide Polymorphism
QRDR	Quinolone-resistance-determining region
WHO	World Health Organisation
WWTPs	Wastewater Treatment Plants

KEYWORDS

16S rRNA

Antibiotic resistance genes

Antibiotic resistant bacteria

bla$_{KPC}$

Effluent

Genomic DNA

gyrA

Health hazard

Matrix-assisted laser desorption/ionization Time of Flight Mass Spectrometry

mecA

parC

Plasmid DNA

Polymerase Chain Reaction

VITEK 2 system

Wastewater Treatment Plants

CHAPTER ONE

LITERATURE REVIEW

CHAPTER ONE

1. LITERATURE REVIEW

1.1 INTRODUCTION

The growth in the human population, urbanisation, rising fertiliser prices and insufficient good quality water resources, are the driving forces behind the advancing trend in the use of wastewater for agriculture and aquaculture. Wastewater is defined as water that has been adversely affected in quality by environmental pollution and pollutants composed of domestic, agricultural and industrial wastewater, groundwater leakage and storm water entering the municipal sewage network (McKay and Moeller, 2000). There are two subclasses of wastewater, namely grey water and black water. Grey water contains no human waste and therefore it is considered to be of lesser health risk to the environment and human population. This type of wastewater is water dispelled from bathroom and kitchen sinks, bath tubs, showers and laundry, etc. Black water is wastewater dispelled from household toilets, institutions and commercial buildings. This type of wastewater generally contains human excreta, which poses a public health risk if not treated appropriately (Food and Agriculture Organization, 1997). There is a great variation in wastewater composition but generally it is composed of 95% water (that is frequently used to flush waste down the drain), while the other 5% is pathogenic bacteria, non-pathogenic bacteria, organic particles, soluble organic and inorganic particles, toxins, macro-solids, pharmaceuticals and hormones (Rizzo et al., 2013).

In the 1850's, life-threatening disease outbreaks caused by the exposure to untreated wastewater were reported. Since that time the practice of wastewater collection and treatment was developed (Adbio, 2015). The purpose of wastewater treatment plants (WWTPs) are to dispose of human and animal waste, extract wet wastes from manufacturing industries, pesticide residues and all organic and inorganic contaminants present in the wastewater (Clara et al., 2005). In essence, WWTPs are purposed to treat and remove harmful substances from the water, so that the water quality is at a level where it can be reused and no harm will be caused to the environment or any human life that may come in contact with the water. While WWTPs have to fulfill the desired effluent standards, there may be some problems that may affect the quality of treatment, such as sewage system damage, inefficiency of the design of plants technical equipment and impaired hydraulics. Inadequately treated effluents from urban WWTPs are thus alleged to be among the main anthropogenic sources for the environmental spread of antibiotics, antibiotic resistant genes (ARGs) and antibiotic resistant bacteria (ARB) (Rizzo et al, 2013).

It is hypothesised that, the increase in antibiotic use by humans, agricultural and veterinary practices, has reduced the therapeutic potential of these drugs against disease causing pathogens (Li et al., 2014). Although WWTPs decrease the population number of bacteria, research has shown that the treatment process increases the proportion of bacteria resistant to certain antibiotics (Zhang et al., 2009). In 2014, the World Health Organisation (WHO) report highlighted that if there is no urgent coordinated action against this rapid development of microbial resistance to antibiotics, the world is rapidly moving toward a post-antibiotic era; a period were minor injuries and common infection, which were once treatable, will then become incurable and may lead to the death of many (WHO, 2014).

The large concentration of antibiotics found in municipal wastewater is due to the disposal of unused drugs or human excretions containing drugs that has not been completely metabolised (Munir et al., 2011). Bacteria may come in contact with antibiotics/pharmaceuticals before or during water treatment and while this may result in the elimination of some bacteria, it may also result in other bacteria developing and spreading resistance (Bouki et al., 2013). Furthermore, the presence of these antibiotics and antibiotic resistant strains of bacteria in the treated wastewater, released with the effluent, may pose a significat health risk to humans (Leclercq et al., 2013). The effluent from WWTPs is also used for irrigation purposes and therefore if ARB are present in the effluent, farm workers may be at great risk of being infected by these ARB. The primary aim of the current study was thus to firstly compare automated and molecular methods for the identification of the Gram-positive pathogen *Staphylococcus aureus* (*S. aureus*) and the Gram-negative pathogen *Klebsiella pneumoniae* (*K. pneumoniae*) as well as the bacterium commonly used as an indicator organism, *E. coli,* in wastewater sampled at various points of the Stellenbosch WWTP. The antibiotic resistance profiles of these isolates were then determined using gene specific and VITEK 2 analysis.

1.2 WASTEWATER TREATMENT PLANT

The primary purpose of wastewater treatment is to protect the health as well as the well-being of the community. The principle of wastewater treatment is thus to dispose of any hazardous or harmful agents originating from a point-source or non-point source of pollution that may have contaminated already used water, which may endanger human health or the natural environment. The designs of the WWTPs (Figure 1.1) are based on the necessity to reduce organic, inorganic substances and other suspended solids, so as to limit pollution and the level of toxic waste being released in the effluent and ultimately the environment (Fao.org, 1997). Additional treatment steps and control measures can thus be incorporated into the WWTP as required, in order to reduce high levels of organic load.

The initial preliminary stage (physical treatment) of the wastewater treatment process removes all big inert solid materials using screens or mesh wiring. The sludge remains are collected, concentrated and pumped out for further treatment. Following physical treatment, the wastewater goes through the primary settling tanks where the solid organic elements are separated from the water suspension by gravity settling (SA water, 2014).

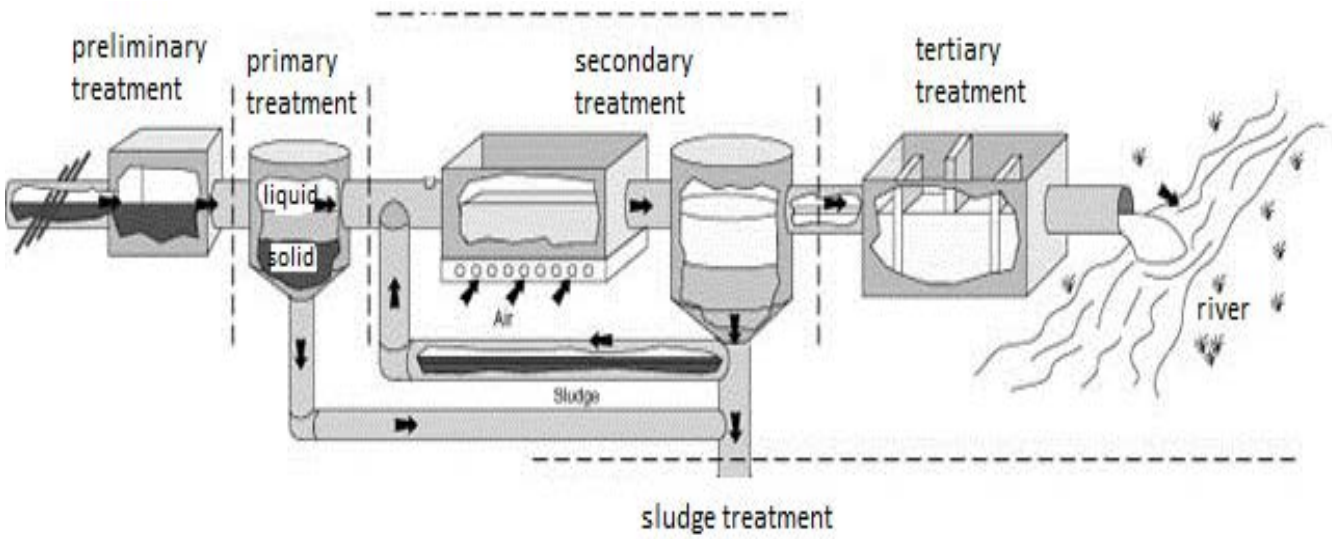


Figure 1.1: The multi-stage treatment process at WWTPs (Rensselaer Polytechnic Institute, 2015).

The wastewater then goes through a biological process where the dissolved and suspended solid organic elements are broken down using micro-organisms. From here the wastewater flows to the aeration tanks where air is forcefully blown into the water to provide oxygen for the bacteria to proliferate. Both the wastewater and the bacterial population in it flow into secondary settling tanks (clarifiers), where the biomass settle at the bottom of the tanks by gravity settling and are concentrated as sludge.

The clarified wastewater may flow to a second secondary settling tank, depending on the design of the WWTP, before flowing to the tertiary treatment. At the tertiary treatment stage most WWTP use disinfection as a means of treatment. Dependent on the effluent load, disinfection may also be achieved by the addition of chlorine to the effluent treated wastewater stream.This should eliminate or significantly reduce the number of pathogens that will be released in the treated effluent. If the treated wastewater is reused for food crop irrigation purposes or any use that may expose humans to the wastewater, additional treatment may be required. The efficiency of the water treatment process is also greatly reduced when the WWTP receives greater volumes of influent than it has been designed to manage (Pescod, 1992).

The wastewater treatment process comprises of both biological and physico-chemical treatment procedures. Biological treatment is used to breakdown organic materials as well as nutrients. Since many pharmaceuticals have been shown to have very low biodegradability propensities, biological procedures cannot effectively remove these drugs (Sim et al., 2010). The focus of the physico-chemical treatment procedure in wastewater treatment is primarily on the division of colloidal particles, which is accomplished by the addition of chemicals such as coagulants as well as flocculants. During the three stages of water treatment processes, considerable variations occur in the spread of the bacterial population and it is generally assumed that during treatment there should be a significant reduction of bacterial numbers, together with the total number of resistant bacteria (Guardabassi et al., 2002). However, ARB and antibiotic resistant genes (ARGs) have been identified widely in wastewater sampled from all three treatment stages at WWTPs (Kim and Aga, 2007). Recent studies have also shown that the difference and degrees of WWTP designs and their process may have an effect on the fate of ARB in wastewater (Bouki et al., 2013). In addition, increased concentrations of ARB have been found in raw and treated wastewater and according to research conducted the conditions in WWTPs are favourable for the ARB spread and WWTPs are identified as potential sources for resistance spread (Goni-Urriza et al., 2000).

1.3 ANTIBIOTICS

Antibiotics are chemical compounds designed to improve the health of human beings, animals and plants by preventing and treating infections caused by pathogenic microorganisms. They either kill bacteria directly (bacteriocidal) or slow down their ability to multiply and grow (bacteriostatic) (Kohanski et al., 2010). Antibiotics are categorised into classes such as b-lactams, aminoglycosides, carbapenems, cephalosporins, quinolones, macrolides, sulfonamides, and tetracyclines, based on their structure and function (bioMérieux Corporate Website, 2008). These compounds are discharged into the environment deliberately and accidentally in the course of their manufacturing processes and through ingestion or disposal (Gulkowska et al., 2008). When bacteria have invaded a host and the host's immune system is overwhelmed, an antibiotic attacks the invading bacteria to a level where the immune system can recover (Munir et al., 2011). However, when bacteria become resistant to these antibiotics, the bacteria can no longer be eliminated by the antibiotic that was initially designed for its control.

Resistance in bacteria can occur via two pathways, namely the acquired pathway or intrinsic pathway. Acquired resistance occurs when a bacterium that was once susceptible to a specific antibiotic becomes resistant to it due to acquiring new DNA or its own DNA mutates. Intrinsic

resistance occurs naturally when a bacterium trait arises from the organism's biology (Hawkey, 1998). Mutation may occur spontaneously regardless of whether antibiotics are present or not, depending on which genes these mutations occur in (Brown, 2002). These mutations may result in antibiotic resistance and eventually the resistance is transferred to sub-populations. Transferable resistance via plasmids was first acknowledged in 1959, where a resistant gene originally found in *Shigella* sp. was identified in an *Escherichia coli* isolate (Hawkey, 1998). The frequent use of antibiotics then prompts antibiotic resistance development in some bacteria. Moreover, resistance development resulting in multidrug resistant bacteria is due to the improper use of antibiotics and inappropriate doses used.

The extensive administration of antibiotics in medical centres exerts a huge selective pressure for the development and spread of ARB. Consequently, bacteria associated with nosocomial infections have been found to be antibiotic-resistant and some of these bacteria are resistant to nearly all broad and narrow spectrum antibiotics currently approved for their elimination and control (Rea et al., 2010). The release of antibiotic residues in the environment or WWTPs is another major concern, because of the possible adverse effects and the potential to transfer the resistance to other microorganisms (Gulkowska et al., 2008). Much attention has thus been given to the occurrence of antibiotics in the environment. In a study conducted by Costanzo et al. (2005), it was found that bacteria isolated from WWTP bioreactors were resistant to antibiotics including trimethoprim (TMP), erythromycin (ERY), tetracycline (TET), ciprofloxacin and ampicillin. In addition, streams receiving urban and industrial wastewater may have up to 15 different types of antibiotics present (Ternes et al., 2002). It has also been reported by McArdell et al. (2003) that some antibiotics are poorly absorbed by humans and thus when excreted via urine of faeces, they are either unchanged or untransformed. This could lead to an underestimation of the average antibiotics released in urban and industrial wastewater today, considering that techniques for sample preparation and extraction are still being developed, and that antibiotic variety and usage has significantly increased (Kolpin et al., 2002). In 2013, the CDC published a report outlining the major antibiotic resistant bacteria which included the multidrug-resistant *Acinetobacter,* vancomycin resistant *Enterococcus,* methicillin resistant *Staphylococcus aureus,* carbapenem-resistant *Klebsiella pneumoniae* and quinolone-resistant *E. coli* (Cdc.gov, 2015).

1.4 ANTIBIOTIC RESISTANCE

When an antibiotic no longer has the ability to successfully eliminate bacteria directly (bacteriocidal) or slow down their ability to multiply as well as grow (bacteriostatic) and targeted bacteria in the presence of therapeutic dosages of an antibiotic can still continue to proliferate, this suggests that the bacteria has developed resistance toward the antibiotic. The antibiotic and ARB are discharged in different ways into wastewater as a result of the increased as well as often careless use of antibiotics in medical, veterinary, and agricultural practices. One of the major global health issue is that parts of the environment that are most directly influenced by human or agricultural actions presented greater concentrations of ARB as well as ARGs (Pruden et al., 2006).

Bacteria become resistant to antibiotics through a variety of mechanisms. One of these mechanisms is chromosomal mutations (Alekshun and Levy, 2007). These mutations occur as a result of errors of replication or an improper repair of impaired DNA and these are referred to as spontaneous mutations. These mutations commonly determine resistance to structurally related compounds (Woodford and Ellington, 2007). While a greater percentage of mutations occur during mitosis, conversely they can occur in cells that are not dividing and even cells with a slow mitosis process. This genetic change can alter the protein targeted by the antibiotic, preventing the antibiotic from binding or inactivating the protein of which it is engineered to target. The mutation can code for an antibiotic-inactivating enzyme or the bacterium may change its cell membrane/wall's permeability to prevent the entry of the antibiotic into the cell binding or inactivating the target protein. Mutations that only occur during the period of non-lethal selection of microbes are referred to as adaptive mutations. The adaptive process is the major cause of antibiotic-resistant mutation development under standard conditions (Martinez and Baquero, 2000). Antibiotic resistant genes (ARGs) can be found in both genomic and plasmid DNA and non-resistant bacteria can also acquire these genes by another mechanism referred to as horizontal gene transfer (HGT) (Todar, 2004). This is the transfer of resistant genetic material from one bacterium to another. There are various forms of transportable hereditary elements by which bacteria accomplish gene transfer, namely plasmids, transposons, bacteriophages and integrons (Figure 1.2) (Rizzo et al., 2013). The transfer of resistance genes is more effective and common amongst bacterial populations developing resistance than chromosomal mutations (Alekshun and Levy, 2007). Therefore there may be interactions between the bacteria at WWTPs before or during wastewater treatment bacteria and this may result in the transfer of resistant genetic material between individual bacteria of the same species or even across species (Todar, 2004).

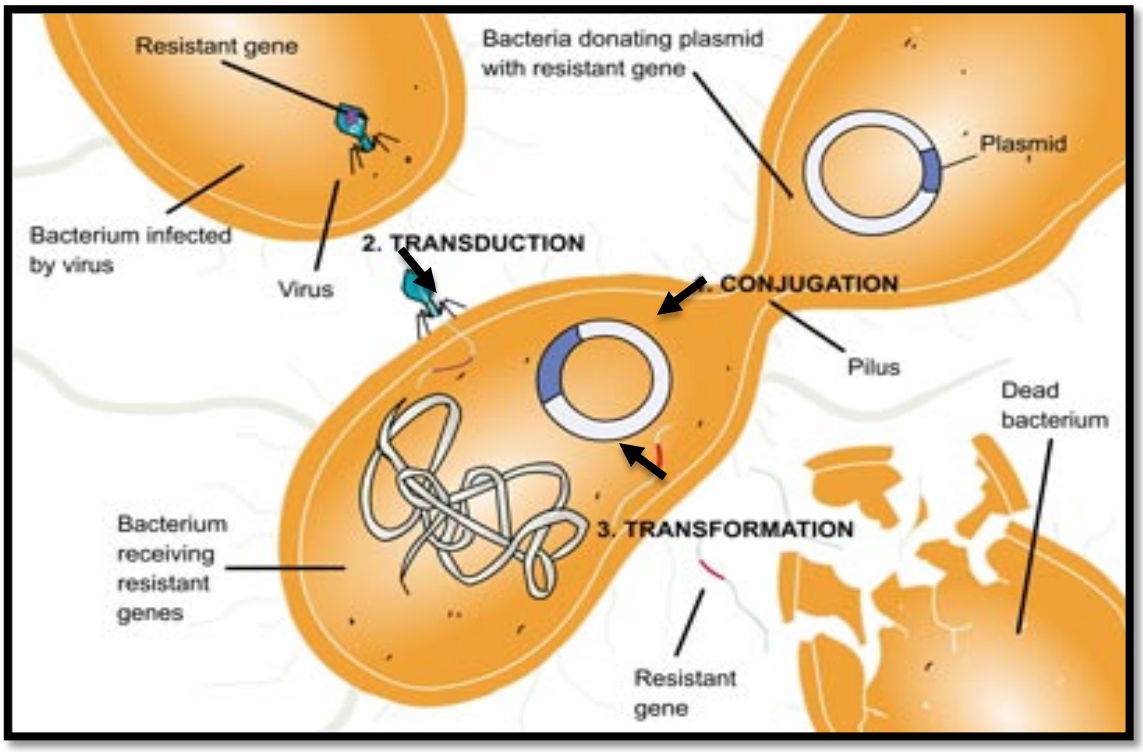


Figure 1.2: Horizontal gene transfer (HGT) processes, conjugation, transduction and transformation (Fenner et al., 2013).

The ARGs can be transferred in three main ways, namely conjugation (via conjugative transposons and plasmids), transduction (via bacteriophages and integrons) and transformation (via integration of chromosomal DNA or plasmids into a chromosome). The genetic material that is most commonly transferred during the conjugation process is in the form of a plasmid and the hereditary material transported often offers the recipient bacterium a genetic advantage (Nature Publishing Group, 2014). Mobile antibiotic resistant genetic elements have been detected constantly in WWTP's (Szczepanowski et al., 2008; Schlüter et al., 2008). It has therefore been assumed that transformation is the direct application of ARG transfer in WWTPs. However, the technical or methodological difficulties in the study of these transfer mechanisms in WWTPs are the main reason for the lack of direct evidence regarding ARG transfer at these sites (Rizzo et al., 2013). In addition, studies have indicated that the biological procedures at WWTPs during wastewater treatment have a positive effect on ARB strain emergence, selection and ARG transfer (Kim et al., 2007).

Regardless of the suggestions that link the occurrence of ARG in populations of bacteria to antibiotic use and concentration (Fridman et al., 2014), the resistance could be from either or both, the advanced enhancement of ARB (mutation), and the transmission of resistant genetic material amongst bacteria of a given population. This spreading of resistant genetic factors in multifaceted settings remains challenging to validate (Rizzo et al., 2013). The WWTPs are considered to be one of the environments that are likely hotspots for ARG transfer because of the vast number of different bacterial populations in the wastewater. According to research conducted by, Rizzo et al. (2013), ARGs that are resistant to all classes of antibiotics could be found in the effluent of the WWTP. Transferable resistant genes in coliform bacteria were studied at the various stages of wastewater treatment (primary, secondary and tertiary). On average it was found that the number of genes slightly decreased in the progression stages of treatment, with the greatest reduction achieved by progressive treatment of biofiltration and sand filtration (Rizzo et al., 2013). A study conducted by Marcinek et al. (1998) determined that the rate of gene transfer amongst bacteria of the same population at a WWTP ranged from 105 to 108 events every four hours. Other sources found plasmids resistant to different antibiotics in a vast range of bacteria at specific sites and thus suggested that gene transfer takes place at these sites (Pawlowski et al., 2013). Findings by Ghosh et al. (2009) showed that 12% of the plasmids isolated from wastewater carried resistant introns. However resistant introns were decreased by an effluent treatment process called thermophilic anaerobic digestion, which reduces resistant introns by 80% - 95%. It was later shown that during aerobic digestion processes there is an increased occurrence of resistant introns and this further suggested to the researchers that HGT takes place throughout the wastewater treatment process (Rizzo et al., 2013). Regardless of the treatment course, technology or functional factors, studies still show a lack in complete eradication or inactivation of ARB and ARGs in wastewater.

Ansari et al. (2008) conducted a study in India and found bacteria resistant to antibiotics downstream from a WWTP, while Chitnis et al. (2004) found multi-resistance genes in drinking water and Parvathi et al. (2011) detected multi-resistant *Salmonella* in water sprayed on vegetables. In Europe, Rubino et al. (2011) reported on the multi-resistant enterohaemorrhagic *E. coli* (EHEC) epidemic that originated from water sprayed vegetables. Today, the increasing incidence of the development and release of ARB and their genes in WWTPs is a major concern facing modern medicine and public health.

1.5 TARGET BACTERIA

1.5.1 *Escherichia coli*

Escherichia coli (E. coli) are Gram-negative, rod-shaped bacteria, first isolated from the faeces of newborns in 1885 (Todar, 2004). The bacterium is regarded as part of the normal flora of the large intestine. In 1935 there was an outbreak of diarrhea and it was found that the causative organism was *E. coli* (Bettelheim and Goldwater, 2014). However many strains are not harmful and the harmless strains profit the hosts by producing vitamin K2, and inhibit the proliferation of other pathogenic bacteria in the intestine (Prevention, 2015). The conditions in the gastrointestinal (GI) tract of humans are suitable for *E. coli* colonisation and this takes place within 40 hours of child birth as *E. coli* can at this stage attach to the mucus membrane covering the large intestine, through ingestion of food, water or from individuals handling the infant (Todar, 2004). The organism has a remarkable response to environmental signals and change (e.g. pH, chemicals, temperature, osmolarity), bearing in mind that it is a unicellular organism. Reacting to the change in conditions (temperature and osmolarity), the species regulate the diameter of the pores in their outer cell membrane, to either allow or prohibit molecules into the cell (Todar, 2004). *Escherichia coli* is also widely used as indicators of faecal contamination in various water sources (Prevention, 2015).

The quantification of *E. coli* in surface waters serves to assess the performance of the WWTPs in its efficiency in removing microorganisms from the wastewater before it released into the environment. In Southern Africa, *E. coli* (virulent strains) was found to be one of the leading disease causing pathogens in humans (Ateba and Bezuidenhout, 2008). In this region countries are mostly populated by immunocompromised individuals, especially in rural areas where no proper medical services are available. Bacteria associated with waterborne outbreaks may be a huge health risk and in severe infections these waterborne diseases cause chronic illness and even death (Theron and Cloete, 2002). A study was conducted in 2001 to investigate the occurrence of pathogenic *E. coli* in designated river water samples in South Africa. The water at these sites was directly and indirectly used for human drinking and for domestic use. It was found that in the water there were *E. coli* strains that exhibited virulence properties, which could cause chronic health problems if ingested (Muller et al., 2001).

Escherichia coli emanates from human and animal wastes and can be carried away into brooks, rivers, streams or dams and eventually end up in WWTPs (Mara and Horan, 2003). The plants are designed to eliminate these pathogens. However even with the widespread practice of chlorination aimed at removing pathogens before treated water is released into the surrounding environment, *E. coli* is released from the WWTPs in levels that could cause risk to the public

health (Anastasi et al., 2010). Inadequate or even poorly operated disinfection processes may result in most bacteria surviving treatment processes and subsequently being released in the effluent, which occurs more frequently in developing countries (Anastasi et al., 2012).

1.5.1.1 Quinolone-resistant *E. coli*

In an *E. coli* infection, quinolones (synthetic antibiotics) have been administered to treat such contagions. These antibiotics are used not only for *E. coli* infections but for most of the Gram-negative bacteria, and as a result they have become common in the fields of human and veterinary medicine (Ito et al., 2008). Quinolones have an excellent in-vitro activity and on discovering this antibiotic it was assumed that resistance against it would be rare (Webber and Piddock, 2001). Strains of Gram-negative bacteria, including *E. coli*, have however become resistant since the introduction of quinolones in human and veterinary medicine (Garau et al., 1999). Research conducted in West Africa before 2004 on quinolone-resistance in commensal and pathogenic *E. coli* showed that the resistance was very low; however, more recent studies have shown that there is a huge emergence of quinolone-resistant bacteria in West Africa (Namboodiri et al., 2011). However while quinolone-resistance has been reported in most parts of central and northern Africa, little data is available on the prevalence of this type of resistance in the sub-Saharan Africa (Lamikanra et al., 2011).

Many mechanisms of acquiring resistance have been suggested and quinolone-resistance in *E. coli* can also be acquired if the bacteria are under sufficient selective pressure (Webber and Piddock, 2001). Quinolones inhibit the proliferation of *E. coli* by inhibiting the action of DNA gyrase (*gyrA*) and DNA topoisomerase IV (*parC*) proteins vital for bacterial cell replication (Lamikanra et al., 2011). On entering the cell, the quinolones bind to the *gyrA/parC* compound and DNA respectively, resulting in the topoisomerase-quinolone-DNA ternary complex leading to breakages within the double-strands of DNA and the blockage of progressions of the DNA replication enzyme complex. The outcome of this reaction is bacterial DNA damage and bacterial death (Dalhof, 2012). In instances of quinolone-resistance development, if a DNA sequence variation occurs in one of the two target genes (*gyrA* and *parC*) this can result in conformational alterations of these enzymes. This will inhibit quinolones from adhering to the DNA- substrate complex, conversely the enzymes still maintain their enzymatic function (Lamikanra et al., 2011). Research shows that resistance-conferring transmutations are primarily determined by *gyrA* and then *parC* (Liu et al., 2012). While genetic information for target sites which confer resistance is usually encoded in the chromosome, reports have shown the development of plasmid-mediated quinolone-resistance. The development of this type of resistance is a huge setback, as resistance may now not only be limited to *E. coli* and may be

transferred to other organisms (Dalhof, 2012). Thus the transfer of plasmid-mediated quinolone-resistance genes in quinolone susceptible cells (Gram-negative and Gram-positive) may foster the development of quinolone-resistance, while in cells that already have resistance genes on the chromosome, the bacteria's resistance potency may be increased (Namboodiri et al., 2011). These genes can also be harboured by the bacteria, by integrating them into genetic units characterised by their ability to capture and incorporate gene cassettes by site-specific recombination (Dalhof, 2012).

Previous studies have shown that topoisomerase IV (*parC*) is not as sensitive to quinolones as DNA gyrase is (Hoshino et al., 1994; Alt et al., 2011), therefore, topoisomerase IV is a secondary target for quinolones-resistance in Gram-negatives (Khodursky et al., 1995). According to Hopkins et al. 2005 mutations in the *parC* gene of *E. coli* have always been consistent with mutations detected on the *gyrA* gene. Suggesting the mutations that occur in the *parC* gene, occur only when there is a reduced sensitivity of the DNA gyrase to quinolones (Saenz, 2003). Nucleotide sequences of the quinolone-resistant *parC* mutant genes were determined, these mutations are located at amino acids Ser63 to Glu84 (Kumagai et al., 1996). Most missense mutations appear at codon Gly-78, Ser-80, or Glu-84 (Kumagai et al., 1996). A study conducted by Simone (1999) showed that a single mutation in the *parC* gene alone does not affect the quinolone susceptibility of the bacteria. However, mutation in the *parC* gene with a mutation in the *gyrA* gene within the QRDR results in a high level resistance to quinolone (Bachoual et al., 1998).

1.5.2 *Klebsiella pneumoniae*

Klebsiella pneumoniae (K. pneumoniae) are small Gram-negative coccobacilli that are commonly found in the mouth, skin and intestines of humans. The organisms are named after a 19th century German microbiologist by the name of Edwin Klebs (Emedicine.medscape.com, 2014). The bacteria is non-motile, non-spore forming and has a capsule that coats the complete surface structure of the cell and enables the bacteria to confer resistance to agents that act to eliminate the organism. The bacteria may be found singularly, in pairs or cluster arrangements and it is easily stained with aniline dyes (*Klebsiella-pneumoniae*, 2014). *Klebsiella pneumoniae, Klebsiella oxytoca,* and *Klebsiella granulomatis* are the three species in the genus of *Klebsiella* that cause disease in humans. Most recorded hospital cases around the world have been linked to *K. pneumoniae,* causing infections of the urinary tract and the respiratory tract (Dizbay et al., 2014). *Klebsiella pneumoniae* emanates from human and animal wastes as well as the different types of precipitation, but they may also be releases in the effluent of hospitals into water systems where they will eventually end up in WWTPs.

In hospital settings, the most significant source of *K. pneumoniae* patient infection occurs through contact with faeces and contaminated instruments. *Klebsiella pneumoniae* is an opportunistic pathogen and infections caused by these bacteria most often affect individuals with compromised immune systems (Leavitt et al., 2009). The host's innate immunity is overcome by the bacteria in several ways. Their pathogenicity is mainly determined by the polysaccharide capsule and this capsule also prevents death that would be caused by bactericidal serum factors (Schembri et al., 2005). The bacteria also produce adhesins with specific receptor traits that assist the bacteria to bind to the cells of the host during the infection process. Even with antimicrobial therapy, the mortality rate resulting from *K. pneumoniae* infections is estimated to be 50% and nearly all the deaths were associated with alcoholism and bacteremia (Emedicine.medscape.com, 2014). The increasing rates of antibiotic resistant *K. pneumoniae* are of major public health concern worldwide.

Klebsiella pneumoniae is one of the major causes of disease as well as deaths in South African children and this burden has been worsened by the HIV epidemic (Brink et al., 2011). It is hard to treat these infections because of the bacterial capsule and it was thus suggested that *K. pneumoniae* infections are best treated with third- and fourth-generation carbapenems (Prince et al., 1997). Consequently, the rapid increase in carbapenem use was accompanied by a rapid extended-spectrum of β-lactamases resistance (Drawz and Bonomo, 2010). These antibiotics are a class of beta-lactum, broad spectrum which acts by inhibiting the cell wall synthesis and they are used to target most infections caused by Gram-negative bacteria. Resistant *K. pneumoniae* produce an enzyme called carbapenemases (Limbago et al., 2011). The enzymes enable the bacteria to be resistant to a wide-range spectrum of beta-lactam substrates and they are the most versatile of the β-lactamases. Some researchers have chosen to call these enzymes carbapenem-hydrolysing enzymes, suggesting that these enzymes have a broader substrate spectrum than just one segment of carbapenems (Queenan and Bush, 2007). These enzymes are not only found in *K. pneumoniae* but they have spread rapidly among the family of *Enterobacteriaceae* and thus poses a global threat (Limbago et al., 2011).

1.5.2.1 Carbapenem-resistant *K. pneumoniae*

Carbapenem-resistance in *K. pneumoniae* is an acquired trait. The earliest record of carbapenem-hydrolysing enzymes or *Klebsiella pneumoniae* carbapenemases (KPCs) detection was in North Carolina in 2001 (Leavitt et al., 2009). In 2004 researchers collected samples from a clinical laboratory, with all the isolated *K. pneumoniae* exhibiting carbapenem-resistance (Leavitt et al., 2009). All the positive isolates were analysed for the presence of the carbapenem-hydrolysing beta-lactam (bla_{KPC}) gene, which is responsible for the synthesis of KPCs. The

results obtained suggested that *K. pneumoniae* had developed resistance toward carbapenems and that the resistance was rapidly spreading, affecting many clones and leading to the emergence of carbapenem-resistance (Leavitt et al., 2009). Few treatment options are available to treat infection caused by carbapenem-resistant strains and more that 50% patients with carbapenem-resistant *K. pneumoniae* infections ended up dead (Leavitt et al., 2009). As from the 1st March 2010 the Molecular Biology Laboratory at the Ampath National Reference Laboratory screened the genome of *Enterobacteriaceae* checking for the novel genes and New Delhi metallo-β-lactamases (NDM). For the first time on the African continent KPCs were identified in medical samples of hospitalised patients in Johannesburg and Pretoria, South Africa, respectively, from *K. pneumoniae* and *Enterobacter cloacae* isolates (Brink et al., 2011). Carbapenem-hydrolysing enzyme producing *K. pneumoniae* is commonly misidentified in standard susceptible testing. To prevent this misidentification the Clinical and Laboratory Standards Institute recommended phenotypic confirmation tests and carbapenemase inhibitor tests should be conducted, despite the time-consuming nature of such tests (Mosca et al., 2013).

The bla_{KPC} genes have been frequently identified in plasmids and these plasmids also have aminoglycosides, which are resistance determinants (Sacha et al., 2010). Resistance spectrum offered by these genes is increasing worldwide. A novel Tn4401 was identified by Naas et al. (2008) in KPC-producing *K. pneumoniae*. In addition, a study conducted in China (Shen et al., 2009) found an insertion of the same transposon at a different location as compared to the previous findings. The identification of this transposon at different locations and on different plasmids indicated a frequent and dynamic transposition process. It has also been suggested that these transposons are the origin of bla_{KPC} gene acquisition and spread (Naas et al., 2008). The KPC producing *K. pneumoniae* are rapidly spreading worldwide and this is a major clinical and public health concern, especially if these bacteria are not effectively eradicated at WWTPs.

1.5.3 *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) is a facultative, non-motile, non-spore forming, Gram-positive cocci, usually found to have a grape-like clustered arrangement and is commonly found on the skin and hair as well as in the nasal and throat passages of people and animals (Stoppler and Sheil, 2015). *Staphylococcus aureus* was first identified in 1880 in pus from clinical abscesses in Aberdeen, Scotland by the specialist Sir Alexander Ogston (Mandal, 2010). *Staphylococcus aureus* is classified under the genus Staphylococcaceae*,* which are common in the environment. Infection of *S. aureus* may be due to a primary invasion that causes injury or

disease to the host. The bacteria can produce proteins, called fibrinogen/fibrin binding protein, that aid the bacteria attaching to the blood clots causing tissue trauma and resulting in wound infection. This is mostly witnessed in post-surgery infections. The bacteria can affect most of the known mammalian species and due to this ability *S. aureus* can easily be transmitted from one species to the next (between animal and humans) (Mandal, 2010). Transmission occurs when the host coughs or sneezes and the bacteria will be carried via aerosols to the next individual or transmission can occur through direct contact with the bacteria. *Staphylococcus aureus* has a remarkable range of virulence factors that enables it to withstand the extreme conditions presented by the human body (Liu, 2009).

1.5.3.1 Methicillin-resistant *S. aureus*

To treat infections caused by the genus *Staphylococcus* an antibiotic called methicillin has been widely administered. The antibiotics adds an acyl group to transpeptidase domain active site of penicillin-binding proteins (PBPs) and blocks PBPs from binding to the substrate and inhibits binding of peptidoglycan strands during cell wall synthesis (Macheboeuf et al., 2006). The mechanism of action is similar to that of the penicillin (Emedexpert, 2012) but methicillin contains a modification from the original penicillin. This modification enables the antibiotic to repress the gene of the bacteria that encodes for penicillinase (beta-lactamase), an enzyme that causes bacteria to be resistant to penicillin (Autiero et al., 2009). In the early 1960s shortly after the production of the penicillin modification (methicillin), the first methicillin-resistant *S. aureus* isolate was reported by the late professor Patricia Jevons of the United Kingdom (UK) (Sakoulas and Moellering, 2008). Methicillin-resistant *Staphylococcus aureus* (MRSA) over the years has become a global pathogen, it causes nosocomial infections and it is resistant to a wide spectrum of antibiotic drugs (Davies and Davies, 2010). The subsequent emergence of MRSA has been associated with healthcare settings. Hospitalised human immuno-deficiency virus (HIV) patients with tuberculosis (TB) have an increased risk of MRSA colonisation and infection (Sydnor and Perl, 2011). In a research study conducted in Cape Town, South Africa, 291 patients infected with HIV and TB were tested for MRSA infection. Results showed that 18% of these patients were co-infected with MRSA (Sydnor and Perl, 2011). In Johannesburg, South Africa 23% of patients with *S. aureus* bacteraemia had been infected with MRSA. In addition, research showed that from 2001 - 2002 South Africa had a MRSA incidence of 33.3% (Marais et al., 2009). Evidence on the antibiotic sensitivity behaviours of MRSA isolates is vital for following the developments in antibiotic resistance, for therapeutic methodologies, and recognising environmental reservoirs of MRSA in the community. Plano et al. (2011) identified WWTPs as possible sources of MRSA exposure, this suggestion was prompted by findings at a Swedish

municipal wastewater treatment plant by Börjesson et al. (2009), where they identified MRSA resistance genes at all treatment steps (Goldstein et al., 2012).

In a study conducted by Goldstein et al. (2012), a high percentage of MRSA was detected in *S.aureus* isolates obtained from the influent samples and the percentages of MRSA detected in the samples decreased as the wastewater treatment process progressed. Although the MRSA isolates had persisted during the early phases of wastewater treatment, no MRSA were detected in the water sampled at the tertiary phase (chlorination) (Goldstein et al., 2012). The studies suggest that individuals working at WWTPs and individuals that live near by farming and recreational locations, irrigated with the treated wastewater, might be exposed to MRSA (Goldstein et al., 2012). This raises health risk concerns, as the world, more specifically Africa has a water shortage crisis and treated wastewater is increasingly used for landscaping and crop irrigation.

The resistance to methicillin in *S.aureus* is produced by the *mecA* gene. The genetic segment is a mobile genetic element which is part of a 21kb – 60kb staphylococcal chromosome cassette *mec* (Wielders et al., 2002). The *mecA* gene provides MRSA with a broad range of resistance to all *β*-lactam antibiotics. The resistance is mainly caused by mutations and other antibiotic resistance genes may also be present in the cassette enabling MRSA to be resistant to multiple antibiotics (Choffnes et al., 2010). A research study conducted by Wu et al. (2001) suggested that the antibiotic pressure aids in the selection of a unique structural change of the *mecA* homologue in the regulator sequence (Wu et al., 2001). Mutations that lead to resistance are usually found in the chromosome (Lupo et al., 2012), but the spread and emergence of resistant genes occurs due to horizontal gene transfer (HGT) (Wielders et al., 2002). However, harbouring the *mecA* gene is not sufficient for methicillin-resistance and some *S. aureus* (<2%) strains with the *mecA* gene are susceptible to methicillin (Hiramatsu, 1995). The expression of methicillin-resistance is determined by the regulatory components that control the expression of the β-lactamase genes (*blaI, blaRI, blaZ*) which, because of sequence similarities, also can down regulate *mecA* gene transcription (Haddadin, 2002). Genetic exchange of plasmids between bacteria is believed to be a crucial part in antibiotic resistance spread in *S. aureus* (Ibe, 2014). However, there is little evidance on the epidemiology of MRSA as the majority of MRSA strains do no contain a plasmid (Alli et al., 2010).

1.6 BACTERIAL IDENTIFICATION

Wastewater reuse is utilised predominantly in developing countries such as Morocco, Tunisia, Egypt, Sudan, Namibia, amongst others, where the effluent from the wastewater plant may be utilised to irrigate a variety of crops (Kivaisi, 2001). In South Africa, the concept of wastewater

reuse is indirectly applied, as the effluent from the wastewater treatment plants flows into river systems utilised by neighbouring farms to irrigate crops. A problem however, arises in that wastewater laboratories traditionally screen the effluent samples for the general faecal indicator groups such as, *Escherichia coli* (*E. coli*), enterococci, etc. to monitor the efficiency of the treatment process. Various pathogenic bacteria such as *Salmonella* sp., *Staphylococcus aureus*, *Klebsiella pneumoniae*, etc. may then persist throughout the wastewater treatment process and are released in the effluent from the wastewater treatment plant into the river systems. The problem is exacerbated as the current routine diagnostic methods used to screen for the faecal indicator organisms, as well as pathogenic microorganisms, are often time-consuming and require approximately 24 hours or more, for definite results to be obtained.

Recently, Bourbeau and Ledeboer (2013) stated that automation in microbiology laboratories was progressing, as this technology had replaced many of the techniques used for the routine identification of species. In addition, the ability to distinguish between different strains of the same species is important in research laboratories (Bourbeau and Ledeboer, 2013). For genus and species identification the choice of a system is influenced by the accuracy and speed of identification, the susceptibility of that particular system, the spectrum of organisms that can be identified and various quality control costs (Clark et al., 2013). Traditionally the methods used for bacterial identification rely solely on the organisms phenotypic characteristics (fermentation, morphology, morbidity, staining etc.). However, multiple tests need to be conducted in order to confirm the correct identification of a specific bacteria and most phenotypic factors are not sensitive for strain differentiation (Tang et al., 1997). These limitations can nonetheless be circumvented by molecular biology methods (Poretsky et al., 2014), or automated processes such as the VITEK 2 system and the use of the MALDI-TOF MS, which can be utilised for species identification, the identification of non-culturable microorganisms and to identify organisms with slow proliferation rates (Moter and Göbel, 2000).

Bacterial identification can be credibly achieved through conventional polymerase chain reaction (PCR)-based techniques (Adzitey and Corry, 2011). These molecular techniques are rapid, less laborious, more sensitive, specific and efficient in comparison with conventional phenotypic methods (Adzitey, et al., 2012). In bacteria the 16S rRNA gene is the DNA segment most commonly targeted and is used for taxonomic purposes (Clarridge, 2004). The 16S rRNA sequence identification technique is ideal as the ribosomal small subunit is universally present in bacteria (Mignard and Flandrois, 2006). In addition, the 16S rRNA gene includes segments with species specific variability, which allows for bacterial identification to genus level. The species

level identification can then be confirmed by PCR using species specific primers (Clarridge, 2004).

The VITEK 2 system can be used to analyse general pathogens such as *E. coli* and *Salmonella typhi* and has been found to be advantageous with regard to speed and accuracy (Funke and Funke-Kissling, 2004), in comparison to routine diagnostic techniques. The VITEK 2 system allows for kinetic analysis. The optical system combines photometer and multichannel fluorimeter readings and records results based on the sample's qualities of turbidity, fluorescence and colorimetric indicators (Ligozzi et al., 2002). This system is able to simultaneously perform tests on more than one sample (Joyanes et al., 2001), yields rapid and reliable results (Ling et al., 2001) and routine clinical laboratories profit from the systems decreased turnaround time. In a study conducted by Ling et al., (2013) the VITEK 2 system was used to achieve rapid bacterial identification of Gram-negative enteric bacilli in blood cultures. A total of 118 strains were investigated and of these, 97 (82.2%) of the strains were correctly identified to the species level and 21 (17.8%) strains were not identified.

In 1975, Anhalt and Fenselau used pyrolysis mass spectrometry (MS) to classify microbes. They noticed that unique mass spectra were produced from bacterial extracts of different genera and of different species (Biswas and Rolain, 2013). Thus, among the more recent developments in bacterial identification analyses is the use of protein profiles obtained by MALDI-TOF MS directly from colonies (Wolk and Dunne, 2011). The MALDI-TOF MS for bacterial identification offers rapid results and minimal sample preparation. In addition, when MALDI-TOF MS is utilised for bacterial identification, reproducible mass spectra can be acquired using intact cells and developing algorithms for interpretation and comparison of these spectra. This technique is also considered more sensitive than conventional assays as it allows for the detection of human pathogens, which are often poorly identified by phenotypic identification techniques (Nagy et al., 2012; Sauer and Kliem, 2010). Samples are preferably grown on a nutrient rich medium and the system analyses the profile of bacterial macromolecules, with the identification of the organism achieved in a matter of minutes (Murray, 2012). A study conducted by Biswas and Rolain (2013) then suggested that the identification of anaerobes, fastidious bacteria and slow growing bacteria, has been improved by the arrival of MALDI-TOF-MS in clinical laboratories.

1.7 HYPOTHESIS AND OBJECTIVE(S) OF STUDY

Incomplete elimination of bacteria and pharmaceutical drugs present (antibiotics) during wastewater treatment at the Stellenbosch Waterworks may result in the entry of antibiotic-resistant bacteria at the receiving stream of the wastewater effleunet. The effluent from the Stellenbosch Waterworks flows into the Veldwachters River which streams into the Eerste River and the water of this river is used by neighbouring agricultural farms for irrigation purposes. The presence of antibiotic resistant strains in treated wastewater is a huge factor that may be relevant to public health issues. The primary aim of the project was to identify and trace the antibiotic resistant strains of *Staphylococcus aureus* (methicillin-resistant), *Escherichia coli* (quinolone-resistant) and *Klebsiella pneumoniae* (carbapenem-resistant) throughout the Stellenbosch WWTP. This aim was achieved as follows:

Aim one:

- Polymerase Chain Reaction (PCR) using 16S rRNA gene specific primers, the MALDI-TOF MS and the VITEK 2 analyser were compared for the identification of the Gram-positive pathogen *Staphylococcus aureus* (*S. aureus*) and the Gram-negative pathogen *Klebsiella pneumoniae* (*K. pneumoniae*) as well as the bacterium commonly used as an indicator organism, *E. coli,* in wastewater sampled at the Stellenbosch wastewater treatment plant (WWTP).
- Species specific primers (*S. aureus, K. pneumoniae* and *E. coli)* were then utilised to clarify any discrepancies in the results obtained, i.e. dissimilar results were obtained for two or more analysis techniques.
- The results obtained from the three bacterial identification techniques were compared and assessed using Cochran's Q test.

Aim two:

- PCR was used to detect single nucleotide polymorphisms (SNP) in *gyrA* and *parC* (DNA gyrase and DNA topoisomerase IV, respectively) genes in *E. coli*, the presence of bla_{KPC} genes (β-lactam *Klebsiella pneumoniae* carbapenemases) in *K. pneumoniae* and the presence of *mecA* genes in *S. aureus*. In both genomic and plasmid DNA.
- The VITEK 2 system was used detect antibiotic resistant profiles of *E. coli* isolates resistance to quinolones, *K. pneumoniae* isolates showing resistance to carbapenems and *S. aureus* isolates showing resistance to methicillin.
- The presence of an antibiotic gene and the antibiotic profile of the bacteria were compared and assessed using the McNemar test

CHAPTER TWO

MATERIALS AND METHODS

CHAPTER TWO

2. MATERIAL AND METHODS

2.1 SAMPLE SITE AND COLLECTION

Wastewater samples were collected from the wastewater treatment plant (WWTP) in Stellenbosch (GPS co-ordinates: 33.943505, 18.824584) at four sampling sites. Three sampling sessions were conducted from February 2014 to July 2014, with a total of 12 wastewater samples collected at the influent point (site one; n = 3), the aeration tank (site two; n = 3), the secondary settling tank (site three; n = 3) and the effluent point (site four; n = 3) (Figure 2.1). Samples were collected by immersing a 2 L sterile schott bottle into the wastewater. The samples were transported to the laboratory on ice to maintain a cool temperature.

Figure 2.1: Stellenbosch wastewater treatment plant (WWTP) sampling sites, Stellenbosch (GPS: -33.9447525, 18.8231056) (Google Maps, 2015).

2.2 CULTURING OF TARGET BACTERIA ON SELECTIVE MEDIA

In order to obtain pure cultures, and because of high contamination levels expected, 1 ml of wastewater from each of the four respective sampling sites (for each sampling cycle) was serial diluted up to a dilution factor of 10^{-4}. This was to reduce the probability of having an over growth on the culture plates, as the wastewater is presumed to be highly contaminated with target bacteria. From the dilution factors 100 µl of the 10^{-3} and 10^{-4} dilutions from each respective sampling site was spread plated onto selective media as indicated in sections 2.2.1 to 2.2.3. After the incubation period, morphologically distinct colonies from the respective selective media plates (sections 2.2.1 to 2.2.3) were then streaked at least twice onto nutrient agar (NA) (Biolab, South Africa) plates to obtain pure cultures. The NA plates were incubated at 37°C for 18 to 24 h. The isolates were then assigned a code with a number to denote the isolate obtained, for example the first isolate obtained from the first cycle on an Mannitol Salt Agar (MSA) plate was encoded $MSA_1 1$, while the twenty fourth isolate obtained from the third cycle on the Chromocult Coliform Agar (CCA) plate was coded $CCA_3 24$. The isolate number for a particular cycle thus ranged from one till however many the isolates were obtained from a respective media for that particular cycle.

2.2.1 *Escherichia coli* culturing

Escherichia coli isolation was accomplished by spread plating 100 µl of the dilution factors 10^{-3} and 10^{-4} from each respective sampling site in duplicate onto CCA (Fluka, India) plates, which were incubated at 37°C for 18 to 24 h. The CCA is a differential chromogenic culture medium used in microbiology for the detection of *E. coli* (Alonso et al., 1998). A careful selection of inhibitors used in the selective media is required to ensure the growth and recovery of *E. coli*. *Escherichia coli* cleaves the Salmon-GAL and X-glucuronide in the media and develops a dark-blue to violet colour. The positive colonies are thus distinguished from the other coliform colonies by the formation of the distinct colour. The control *E. coli* ATCC 25922 was used as a reference to confirm morphology of the isolates (Yue et al., 2008).

2.2.2 *Klebsiella pneumoniae* culturing

Klebsiella pneumoniae isolation was accomplished by spread plating 100 µl of the dilution factors 10^{-3} and 10^{-4} from each respective sampling site onto the HiCrome *Klebsiella* Selective Agar (KSA) (Fluka, India) plates, which were incubated at 37°C for 18 to 24 h. The KSA has a chromogenic substrate incorporated, which is specific for *Klebsiella* species (Sigma-Aldrich, 2013). Most of the frequently encountered Gram-negative faecal contaminants are inhibited on this media using a selective supplement. The bile salts mixture and sodium lauryl sulphate (SLS) inhibits most of the accompanying flora and the NaCl maintains the osmotic equilibrium of the

medium. Peptone and yeast extract provide the essential nutrients required for the growth of *K. pneumoniae*. The *K. pneumoniae* then produces a purple-magenta coloured colony (Sigma-Aldrich, 2013) on the agar plate. The control *K. pneumoniae* ATCC BAA-1705 was used as a reference to confirm the morphology of the isolates (Wang et al., 2012).

2.2.3 *Staphylococcus aureus* culturing

Staphylococcus aureus isolation was accomplished by spread plating 100 µl of the dilution factors 10^{-3} and 10^{-4} from each respective sampling site in duplicate onto MSA (Biolab, South Africa) plates, which were incubated at 37°C for 18 to 24 h. Currently, MSA is commercially available and recommended for the recovery of *S. aureus* as the mannitol fermentation offer advantages in differentiation (Kateete et al., 2010). In high concentrations, sodium chloride (NaCl) inhibits bacteria growth other than staphylococci (Shields and Tsang, 2013). *Staphylococcus aureus* growth on the agar was confirmed by the mannitol fermentation, where the Phenol Red pH indicator changes from red to yellow forming yellow colonies with yellow zones (Shields and Tsang, 2013). The control *S. aureus* ATCC 33591 was used as a reference to confirm the morphology of the isolates (Pereira et al., 2009).

2.3 MALDI-TOF Mass Spectrometry versus Biomerieux VITEK 2 and Molecular Techniques for the Identification of *Escherichia coli, Klebsiella pneumoniae* and *Staphylococcus aureus* in Wastewater

2.3.1 Genomic DNA Extraction and 16S rRNA Polymerase Chain Reaction Analysis

The pure cultures of the bacterial isolates obtained from each sampling site per sampling session (n = 70), were grown on nutrient agar (NA) at 37°C for 18 to 24 h, and a single colony was inoculated into 4 ml Luria Bertani (LB) (Biolab, South Africa) broth and incubated for 18 to 24 h at 37°C. Total genomic DNA was then extracted from 200 µl of the respective LB broth cultures using the High Pure Template Preparation Kit (Roche, Germany) as per the manufacturer's instructions.

The Genomic DNA extracted from the wastewater isolates (n = 70) was then subjected to conventional PCR to amplify the 16S rRNA conserved regions. The primers used for 16S rRNA are able to amplify the V3 hypervariable region within most bacteria (Table 2.1). The amplification reaction mixtures were performed in a total volume of 50 µl, containing 1X Go Taq buffer, 2 mM dNTPs, 1.5 mM $MgCl_2$, 1 µM V3F and V3R (primers), respectively, 0.25 U Go Taq polymerase (Promega, USA) and 5 µl of template DNA. A negative control was included which contained all reagents but no template DNA and the *S. aureus* ATCC 33591 strain was used as the positive control.

Table 2.1: 16S rRNA primer sequences (universal primers)

Name	Primer Sequence (5' – 3')	Product size	Gene and (reference)
V3F	CCAGACTCCTACGGGAGGCAG	200bp	V3 (334–537) (Chakravorty et al., 2007)
V3R	CGTATTACCGCGGCTGCTG		

The Polymerase Chain Reaction was completed using the Bio-Rad C1000^{TM} Thermal Cycler (Biorad, USA), with the following programme conditions; an initial denaturation of 95°C for 3 min followed by 35 cycles of 95°C for 60 s, 55°C for 60 s, 72°C for 70 s and a final extension of 72°C for 5 min. The amplified product was visualised on 1.5% agarose gel stained with ethidium bromide (10 mg/ml). The DNA bands corresponding to the correct 200 bp size were excised from the agarose gel and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and the purified products were sent for sequencing at the University of Stellenbosch, Central Analytical Facility (DNA Sequencing Facility). The obtained sequences were analysed using the online Basic Local Alignment Search Tool (BLAST), available at http://blast.ncbi.nlm.nih.gov/Blast.cgi, to find the closest match of local similarity of the isolates to the online international database in GenBank, EMBL, DDBJ and PDB sequence data (Barker, 1998).

2.3.1.1 SPECIES SPECIFIC PCR

Once the results for the three primary identification techniques has been analysed, species specific primers (*S. aureus, K. pneumoniae* and *E. coli)* were utilised to clarify any discrepancies in the results obtained, i.e. dissimilar results were obtained for two or more analysis techniques.

Pure cultures of the specific bacterial isolates obtained from each sampling site per sampling session (seven *E. coli* isolates, 17 *K. pneumoniae* and four *S. aureus*), were grown on nutrient agar (NA) at 37°C for 18 to 24 h. and a single colony was inoculated into 4 ml Luria Bertani (LB) broth and incubated for 18 to 24 h at 37°C. Total genomic DNA was then extracted from 200 µl of the respective LB broth cultures using the High Pure Template Preparation Kit (Roche) as per the manufacturer's instructions. The Genomic DNA extracted from the wastewater isolates (n = 28) was then subjected to conventional PCR using primers specific for *E. coli*, *S. aureus* and *K. pneumoniae* (Table 2.2).

For *E. coli* the *PhoA* gene (903 bp) was amplified (Wei et al., 2013). The amplification reaction mixtures were performed within a total volume of 50 µl, containing 1X Go Taq buffer, 2 mM dNTPs, 1.5 mM $MgCl_2$, 1 µM *pho*F and *pho*R (primers) respectively, 0.25 units Taq polymerase (Promega) and 2 µl of template DNA. A negative control was included which contained all reagents but no DNA template and the *E. coli* ATCC 25922 strain was used as the

positive control. The Polymerase Chain Reaction amplification was completed using the Bio-Rad C1000^{TM} Thermal Cycler, with the following programme conditions; an initial denaturation of 94°C for 10 min followed by 40 cycles of 94°C for 30s, 50°C for 20s, 72°C for 20s and a final extension of 72°C for 10 min.

The 16S – 23S internal transcribed spacer gene for *K. pneumoniae* (130 bp) (Liu et al., 2008) was amplified. The amplification reaction mixtures were performed within a total volume of 50 µl, containing 1X Go Taq buffer, 2 mM dNTPs, 1.5 mM $MgCl_2$, 3 µM *ITS*F and *ITS*R (primers) respectively, 0.25 units Taq polymerase (Promega) and 10 µl of template DNA. A negative control was included which contained all reagents but no DNA template and the *K. pneumoniae* ATCC BAA-1705 strain was used as the positive control. The Polymerase Chain Reaction amplification was completed using the Bio-Rad C1000^{TM} Thermal Cycler, with the following programme conditions; an initial denaturation of 94°C for 2 min followed by 35 cycles of 94°C for 60s, 50°C for 60s, 72°C for 60s and a final extension of 72°C for 10 min.

The *vicK* gene for *S. aureus* (300 bp) (Liu et al., 2007) was amplified. The amplification reaction mixtures were performed within a total volume of 50 µl, containing 1X Go Taq buffer, 2 mM dNTPs, 1.5 mM $MgCl_2$, 2.5 µM *vicK*F and *vicK*R (primers) respectively, 0.25 units Taq polymerase (Promega) and 2 µl of template DNA. A negative control was included which contained all reagents but no DNA template and the *S. aureus* ATCC 33591 strain was used as the positive control. The Polymerase Chain Reaction amplification was completed using the Bio-Rad C1000^{TM} Thermal Cycler, with the following programme conditions; an initial denaturation of 94°C for 5 min followed by 35 cycles of 94°C for 40s, 50°C for 40s, 72°C for 60s and a final extension of 72°C for 10 min.

The amplified products were then visualised on 1.5% agarose gel (*K. pneumoniae and S. aureus* samples) and 0.8% agarose gel (*E. coli* samples) stained with ethidium bromide. PCR bands corresponding to the correct respective bands were excised from the gel and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) and the purified products were sent for sequencing at the University of Stellenbosch, Central Analytical Facility (DNA Sequencing Facility). The obtained sequences were analysed using the online Basic Local Alignment Search Tool (BLAST), available at http://blast.ncbi.nlm.nih.gov/Blast.cgi, to find the closest match of local similarity of the isolates to the online international database in GenBank, EMBL, DDBJ and PDB sequence data (Barker, 1998).

Table 2.2: Species specific primers for target bacterial identification

Target Bacteria	Primer (5' – 3')	Product size	Gene (Reference)
S. aureus	F - CTAATACTGAAAGTGAGAAACGTA R - TCCTGCACAATCGTACTAAA	300 bp	*vicK* gene (Liu et al., 2007)
E. coli	F - GTGACAAAAGCCCGGACACCAGAAATGCCT R - TACACTGTCATTACGTTGCGGATTTGGCGT	903 bp	*phoA* gene (Wei et al., 2013)
K. pneumoniae	F -- ATTTGAAGAGGTTGCAAACGAT R - TTCACTCTGAAGTTTTCTTGTGTTC	130 bp	16S–23S internal transcribed spacer (Liu et al., 2008)

2.3.2 MALDI-TOF MS

A single colony from the pure cultures of the bacterial isolates obtained from each sampling site per sampling session (n = 70), was streaked onto NA plates and incubated at 37°C for 18 to 24 h prior to analysis by MADLI-TOF MS (Bruker Daltonics, Bremen, Germany), as outlined in Loff et al. (2014), at the Proteomics Unit of the University of the Western Cape, South Africa. The Biotype software has a database which is used as a reference to compare each sample mass. The subjective unit score value is calculated between 0 and 3, on the bases of similarities in properties of the sample and the reference spectrum. The ten best matching references are then displayed on the database records (Schulthess et al., 2013). The unit score value determines the extent of identification of the sample, a score between ≥1.7 and <2 will identify the genus of the organism, and a score of ≥2 will identify the species of the organism (Loff et al., 2014).

2.3.3 Biomerieux VITEK 2 system identification

A single colony from the pure cultures of the bacterial isolates obtained from each sampling site per sampling session (n = 70), was streaked onto blood agar (BA) (Alibaba, China) and incubated for 18 to 24 h overnight at 37°C. These samples were then analysed using the VITEK 2 system (Biomerieux, USA) at the PathCare N1 City Microbiology Laboratory, where a sterile swab was used to prepare a homogenous suspension (0.5 McFarland standard) of each isolate by transferring a pure colony from the BA plates into 4 ml tube of sterile saline. Identification cards were placed into each tube on the cassette and the complex was placed into the Biomerieux VITEK® 2 Compact 60 system for analysis. For isolates suspected to be Gram-negative (isolated from the KSA and CCA media plates) VITEK 2 GN cards were used and for Gram-positive bacteria (isolated from the MSA media plates) VITEK 2 GP cards were used. To correctly identify the bacteria, the identification cards use fluorescence, turbidity, and colorimetric

signals that are analysed by the system using a combination of multichannel fluorimeter and photometer readings (Wallet et al., 2005). The VITEK 2 system analyses the result and identifies the organism on the bases of biochemical reactions (colorimetric tests).

2.3.4 COCHRAN Q test

The results obtained from the three bacterial identification techniques were assessed using the Cochran's Q test. The test is designed to assess the differences between three or more matched sets of treatments or quantities (Griffith, 2001). A typical design for this scenario involves the total number of the respective target bacteria (N), a binary measurement of a success (1) or a failure (0) for each identification result on each of the identification techniques (*k*) where *k* is more than two (Berg, 2014). The null hypothesis (H_0) states that there is no difference between the identification techniques in identifying a particular species of the target bacteria ($p_{MALDI\text{-}TOF\ MS} = p_{PCR} = p_{VITEK}$), while the alternative hypothesis (H_1) states that there is a difference between the identification techniques in identifying a particular species of the target bacteria. The null hypothesis is therefore rejected when $p < 0.05$, and thus to identify the differences between the three techniques pairwise analysis was completed. The pairwise test p-values were Bonferroni corrected (an adjustment made to P values when several dependent or independent statistical tests are being performed simultaneously on a single data set) to reduce the chances of obtaining false-positive results (type I errors) when multiple pairwise tests are performed on a single set of data. In all tests a p-value smaller than 0.05 was considered as statistically significant (Dunn and Clark, 1974). The computed value Q is interpreted as a chi-square value (Sheskin, 2003).

2.4 THE CORRELATION BETWEEN GENES THAT CONFER ANTIBIOTIC RESISTANCE AND THE ANTIBIOTIC RESISTANCE PROFILES OF BACTERIA AT A MUNICIPAL WASTEWATER TREATMENT PLANT IN STELLENBOSCH, SOUTH AFRICA.

2.4.1 Antibiotic resistance Gene Specific Polymerase Chain Reaction

After pure cultures were obtained, a colony from each respective plate was inoculated into 4 ml Luria-Bertani (LB) broth, and incubated at 37°C for 18 to 24 hours. Total genomic DNA was then extracted from 200 µl of the respective LB broth cultures using the High Pure Template Preparation Kit (Roche) as per the manufacturer's instructions. A further 600 µl from the resuspended broth was used for plasmid DNA isolation using the PureYield^{TM} Plasmid Miniprep System Kit (Roche) as per the manufacturer's instructions. Specific primers were used to identify antibiotic resistant genes in the target microorganisms (Table 2.3), both in the genomic DNA and plasmid DNA, respectively.

Table 2.3: Primer sequences for the antibiotic resistant genes of the target bacteria

Target Bacteria	Antibiotic	Primer (5' – 3')	Product size	Gene (Reference)
S. aureus	Methicillin	F - TAGAAATGACTGAACGTCCG R - TTGCGATCAATGTTACCGTAG	154 bp	*mecA* (Pereira et al., 2010)
E. coli	Quinolones	F - GGATAGCGGTTAGATGAGC R - CGTTCACCAGCAGGTTAGG	521 bp	*gyrA* *(*Yue, et al., 2008)
		F - AATGAGCGATATGGCAGAGC R - TTGGCAGACGGGCAGGTAG	450 bp	*parC* *(*Yue, et al., 2008)
K. pneumoniae	Carbapenems	F - TTGTTGATTGGCTAAAGGG R - CCATACACTCCGCAGGTT	106 bp	bla_{KPC} (Wang et al., 2012)

2.4.1.1 Quinolone resistant *E. coli*

A total of 44 *E. coli* isolates were analysed for mutations within the *gyrA* and *parC* genes in both genomic and plasmid DNA. The major mechanism of resistance in *E. coli* occurs due to mutations in the quinolone-resistance-determining regions, *gyrA* and *parC,* respectively. The wild type organism used for the basis of comparison was the *E. coli* strain ATCC 25922 (Yue et al., 2008). For genomic DNA the amplification reaction mixtures for both the *gyrA* and *parC* genes were performed in a total volume of 50 µl, containing 1X GoTaq buffer, 2 mM dNTPs, 3 mM $MgCl_2$, 1 mM of each respective primer (Table 2.3), 0.25 units Taq polymerase (Promega) and 5 µl of genomic DNA. For plasmid DNA the amplification reaction mixtures were performed in a total volume of 50 µl, containing 1X GoTaq buffer, 2 mM dNTPs, 1.5 mM $MgCl_2$, 0.4 mM of each respective primer (Table 2.3), 0.25 units Taq polymerase (Promega) and 2 µl of plasmid DNA (adapted from Yue et al., 2008). A negative control was included which contained all reagents but no DNA template, and *E. coli* ATCC 25922 was used as the positive control.

The Polymerase Chain Reaction amplification was completed using the Bio-Rad C1000^{TM} Thermal Cycler, with the following programme conditions; an initial denaturation of 95°C for 5 min followed by 30 cycles of 95°C for 45 sec, 54°C for 45 sec, 72°C for 45 sec and finally 72°C for 5 min (adapted from Yue et al., 2008).

2.4.1.1.1 SNP detection within *gyrA* and *parC* genes

All sequences obtained for the *gyrA* and *parC* genes were evaluated for nucleotide substitutions that would result in missense mutations when comparing the amino acid seqences to the wild type sequence corresponding to amino acids Ala67 to Gln106 (Yoshida et al., 1990) for the *gyrA* gene, and Ser63 to Glu84 for the *parC* gene (Kumagai et al., 1996 and Deguchi et al., 1997).

2.4.1.2 Carbapenem-resistant *K. pneumoniae*

A total of 20 *K. pneumoniae* isolates were analysed for the presence of the bla_{KPC} gene in both the genomic and plasmid DNA. The major mechanism of resistance in *K. pneumoniae* occurs due to the presence of the bla_{KPC} gene that encodes for the enzyme carbapenemase. For genomic DNA the amplification reaction mixtures were performed in a total volume of 50 µl, containing 1X GoTaq buffer, 2 mM dNTPs, 2.5 mM $MgCl_2$, 0.4 mM of each respective primer (Table 2.3), 0.25 units Taq polymerase (Promega) and 10 µl of genomic DNA. For plasmid DNA the amplification reaction mixtures were performed in a total volume of 50 µl, containing 1X GoTaq buffer, 2 mM dNTPs, 2.5 mM $MgCl_2$, 0.4 mM of each respective primer (Table 2.3), 0.25 units Taq polymerase (Promega) and 2 µl of plasmid DNA (adapted from Wang et al., 2012). A negative control was included which contained all reagents but no DNA template and *K. pneumoniae* ATCC BAA-1705 was used as the positive control.

The Polymerase Chain Reaction amplification was completed using the Bio-Rad C1000^{TM} Thermal Cycler, with the following programme conditions; an initial denaturation of 94°C for 3 min followed by 40 cycles of 94°C for 30 sec, 50°C for 45 sec, 72°C for 45 sec and finally 72°C for 2 min (adapted from Wang et al., 2012).

2.4.1.3 Methicillin-resistant *S. aureus*

A total of six *S. aureus* isolates were analysed for the presence of the *mecA* gene in both the genomic and plasmid DNA. The major mechanism of resistance in *S. aureus* occurs due to the presence of the *mecA* gene that triggers methicillin-resistance. For genomic DNA the amplification reaction mixtures were performed in a total volume of 50 µl, containing 1X GoTaq buffer, 2 mM dNTPs, 2.5 mM $MgCl_2$, 0.4 mM of each respective primer (Table 2.3), 0.25 units Taq polymerase (Promega) and 10 µl of genomic DNA. For plasmid DNA the amplification reaction mixtures were performed with the total volume of 50 µl, containing 1X GoTaq buffer, 2 mM dNTPs, 1.5 mM $MgCl_2$, 0.4 mM of each respective primer (Table 2.3), 0.25 units Taq polymerase (Promega) and 2 µl of plasmid DNA (adapted from Pereira et al., 2009). A negative control was included which contained all reagents but no DNA template and *S. aureus* ATCC 33591 was used as the positive control.

The Polymerase Chain Reaction amplification was completed using the Bio-Rad C1000^{TM} Thermal Cycler, with the following programme conditions; an initial denaturation of 92°C for 3 min followed by 30 cycles of 92°C for 60 sec, 53°C for 60 sec, 72°C for 60 sec and finally 72°C for 3 min (adapted from Pereira et al., 2009).

The amplified products were then visualised on 1.5% agarose gel (all samples) stained with ethidium bromide. PCR bands corresponding to the correct respective bands were excised from the gel and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) and the purified products were sent for sequencing at the University of Stellenbosch, Central Analytical Facility (DNA Sequencing Facility). The obtained sequences were analysed using the online Basic Local Alignment Search Tool (BLAST), available at http://blast.ncbi.nlm.nih.gov/Blast.cgi, to find the closest match of local similarity of the isolates to the online international database in GenBank, EMBL, DDBJ and PDB sequence data (Barker, 1998).

2.4.2 VITEK 2 system

A single colony from the pure cultures of the bacterial isolates obtained from each sampling site per sampling session (n = 70), was streaked onto blood agar (BA) (Alibaba, China) and incubated for 18 to 24 h overnight at 37°C. These samples were then analysed using the VITEK 2 system (Biomerieux, USA) at the PathCare N1 City Microbiology Laboratory, where a sterile swab was used to prepare a homogenous suspension (0.5 McFarland standard) of each isolate by transferring a pure colony from the BA plates into 4 ml tube of sterile saline. Susceptibility cards were placed into each tube on the cassette and the complex was placed into the Biomerieux VITEK® 2 Compact 60 system for analysis. For isolates suspected to be Gram-negative (isolated from the KSA and CCA media plates) VITEK 2 AST-N cards were used and for Gram-positive bacteria (isolated from the MSA media plates) VITEK 2 AST-P cards were used. The VITEK 2 system analysis the results and identifies antibiotics that the organism is resistant to based on the biochemical reactions (colorimetric tests).

2.4.3 McNemar's test

The McNemar test was performed to assess the difference between the the resistant profiles when the resistant gene was carried in the plasmid or in the genome of the bacterial cell. The McNemar test examines the difference between the proportions (presence of gene in the plasmid vs resistance profile and presence of gene in the genome vs resistance profile) and if the two proportions significantly differ and when the p-value is less than the conventional 0.05, the conclusion is that there is a significant difference between the two proportions.

CHAPTER THREE

RESULTS

CHAPTER THREE

3. RESULTS

3.1 MALDI-TOF MASS SPECTROMETRY VERSUS BIOMERIEUX VITEK 2 AND MOLECULAR TECHNIQUES FOR THE IDENTIFICATION OF *ESCHERICHIA COLI, KLEBSIELLA PNEUMONIAE* AND *STAPHYLOCOCCUS AUREUS* IN WASTEWATER

3.1.1 MALDI-TOF MS identification

Three sampling cycles were conducted at various points of the Stellenbosch WWTP. Based on morphological analysis (selective media), for the first cycle, 24 isolates (Table 3.1) were presumed to be the target bacteria (i.e. either *E.coli*, *K. pneumoniae* or *S. aureus*), while in cycle two, 20 isolates (Table 3.2) were presumed to be the target bacteria and in the last cycle, 26 isolates (Table 3.3) were presumed to be the target bacteria. In total 70 target isolates were obtained for the entire sampling period from the respective selective media, with the colonies presumed to be either *E. coli* (n=44), *K. pneumoniae* (n=20) or *S. aureus* (n=6), analysed using MALDI-TOF MS, the VITEK 2 system and PCR targeting the 16S rRNA region. The results for the MALDI-TOF MS analysis are outlined in Tables 3.1 to 3.3, with the samples coded *CCA* presumed to be *E. coli* isolates, the samples coded *KSA* presumed to be *K. pneumoniae*, and samples coded *MSA* were presumed to be *S. aureus.*

The MALDI-TOF MS showed peak patterns, with each peak representing a peptide. The detailed sequence of these peptides could be found by selecting a specific peptide and ionizing it to obtain a complete amino acid sequence, and when the peaks are high, the unit score value increases and the species of the bacteria may be identified (Calderaro et al., 2014). The detailed sequences of these peptides are the illustration of the mass-to-charge (m/z) values which show the protein densities and molecular masses. The best matched samples thus have a score of ≥2 (species identification), while samples with a score between ≥1.7 and <2 are only identified to the genus level. Samples with a unit score of <1.7 have low matching references on the database records (Schulthess et al., 2013). Tables 3.1, 3.2 and 3.3 illustrate tabulated results obtained from the MALTI-TOF, with the respective unit score, for sampling cycles one, two and three, respectively.

Table 3.1: Cycle one, MALDI-TOF MS, PCR and VITEK identification results

Site	Code Name	MALDI-TOF MS	Score	16S rRNA (PCR)	ID %	VITEK	Species Specific (PCR)
Influent	*CCA$_1$30*	*Escherichia* sp.	1.9	*E. coli*	100%	*E. coli*	*E. coli*
	CCA$_1$31	*E. coli*	2	*E. coli*	99%	*E. coli*	
	CCA$_1$40	*Escherichia* sp.	1.9	*E. coli*	98%	*E. coli*	*E. coli*
	CCA$_1$62	*E. coli*	2	*E. coli*	98%	*E. coli*	
	CCA$_1$70	*E. coli*	2.1	*E. coli*	99%	*E. coli*	
	KSA$_1$7	*K. oxytoca*	2.2	*K. pneumoniae*	100%	*K. pneumoniae*	*K. pneumoniae*
	KSA $_1$16	*Klebsiella* sp.	1.8	*K. pneumoniae*	100%	*K. pneumoniae*	*K. pneumoniae*
Aeration Tank	*CCA$_1$14*	*E. coli*	2.1	*E. coli*	96%	*E. coli*	
	CCA$_1$15	*E. coli*	2.2	*E. coli*	99%	*E. coli*	
	CCA$_1$17	*E. coli*	2.1	*E. coli*	99%	*E. coli*	
	CCA$_1$18	*E. coli*	2.2	*E. coli*	99%	*E. coli*	
	CCA$_1$19	*Escherichia* sp.	1.9	*E. coli*	100%	*E. coli*	*E. coli*
	KSA $_1$38	*Klebsiella* sp.	1.8	*K. pneumoniae*	100%	*K. oxytoca*	*K. pneumoniae*
	KSA $_1$67	*Klebsiella* sp.	1.8	*K. pneumoniae*	100%	*K. oxytoca*	*K. pneumoniae*
Secondary Settling tank	*MSA$_1$14*	*S. aureus*	2	*S. aureus*	91%	*S. aureus*	
Effluent	*CCA$_1$1*	*E. coli*	2	*E. coli*	97%	*E. coli*	
	CCA$_1$2	*E. coli*	2	*E. coli*	99%	*E. coli*	
	CCA$_1$8	*E. coli*	2.2	*E. coli*	100%	*E. coli*	
	CCA$_1$9	*E. coli*	2.1	*E. coli*	100%	*E. coli*	
	CCA$_1$13	*Escherichia* sp.	1.7	*E. coli*	98%	*E. coli*	*E. coli*
	KSA $_1$79	*Klebsiella* sp.	1.8	*K. pneumoniae*	100%	*K. oxytoca*	*K. pneumoniae*
	KSA $_1$80	*Klebsiella* sp.	1.9	*K. pneumoniae*	100%	*K. oxytoca*	*K. pneumoniae*
	KSA $_1$82	*Klebsiella* sp.	1.9	*K. pneumoniae*	100%	*K. oxytoca*	*K. pneumoniae*
	KSA $_1$84	*K. oxytoca*	2	*Klebsiella* sp.	96%	*K. oxytoca*	*K. pneumoniae*

Table 3.2: Cycle two, MALDI-TOF MS, PCR and VITEK identification results

Site	Code Name	MALDI-TOF MS	Score	16S rRNA (PCR)	ID %	VITEK	Species Specific (PCR)
Influent	*$CCA_2$1*	*Escherichia* sp.	1.9	*E. coli*	97%	*E. coli*	*E. coli*
	$CCA_2$2	*Escherichia* sp.	1.9	*E. coli*	100%	*E. coli*	*E. coli*
	$CCA_2$3	*E. coli*	2.1	*E. coli*	100%	*E. coli*	
	$CCA_2$8	*Escherichia* sp.	1.9	*E. coli*	100%	*E. coli*	*E. coli*
	$CCA_2$9	*E. coli*	2	*E. coli*	100%	*E. coli*	
	$CCA_2$13	*E .coli*	2.1	*E. coli*	100%	*E. coli*	
	$KSA_2$5	*Klebsiella* sp.	1.8	*K. pneumoniae*	91%	*K. pneumoniae*	*K. pneumoniae*
	$MSA_2$2	*Staphylococcus* sp.	1.7	*S. aureus*	99%	*S. aureus*	*S. aureus*
	$MSA_2$3	*S. aureus*	2.1	*S. aureus*	99%	*S. aureus*	
Aeration	*$CCA_2$17*	*E. coli*	2	*E. coli*	100%	*E. coli*	
	$CCA_2$18	*E. coli*	2.3	*E. coli*	100%	*E. coli*	
	$CCA_2$19	*E. coli*	2.1	*E. coli*	100%	*E. coli*	
	$CCA_2$20	*E. coli*	2	*E. coli*	100%	*E. coli*	
	$CCA_2$21	*E. coli*	2.1	*E. coli*	100%	*E. coli*	
	$KSA_2$11	*K. oxytoca*	2	*K. pneumoniae*	100%	*K. pneumoniae*	*K. pneumoniae*
Secondary Settling Tank	*$KSA_2$16*	*Raoultella planticola*	2.2	*K. pneumoniae*	98%	*K. pneumoniae*	*K. pneumoniae*
Effluent	*$CCA_2$26*	*E. coli*	2	*E. coli*	100%	*E. coli*	
	$CCA_2$27	*E. coli*	2.1	*E. coli*	99%	*E. coli*	
	$KSA_2$23	*K. pneumoniae*	2.4	*K. pneumoniae*	96%	*K. pneumoniae*	*K. pneumoniae*
	$KSA_2$24	*K. oxytoca*	2.1	*K. pneumoniae*	97%	*K. oxytoca*	No product

Table 3.3: Cycle three, MALDI-TOF MS, PCR and VITEK identification results

Site	Code Name	MALDI-TOF MS	Score	16S rRNA (PCR)	ID %	VITEK	Species Specific (PCR)
Influent	*$CCA_3$1*	*E. coli*	2	*E. coli*	100%	*E. coli*	
	$CCA_3$2	*E. coli*	2.3	*E. coli*	99%	*E. coli*	
	$CCA_3$3	*E. coli*	2.1	*E. coli*	99%	*E. coli*	
	$CCA_3$6	*E. coli*	2.1	*E. coli*	99%	*E. coli*	
	$CCA_3$7	*E. coli*	2.1	*E. coli*	99%	*E. coli*	
	$CCA_3$8	*E. coli*	2	*E. coli*	99%	*E. coli*	
	$CCA_3$9	*E. coli*	2	*E. coli*	100%	*E. coli*	
	$KSA_3$4	*K. pneumoniae*	2.1	*K. pneumoniae*	95%	*Raoultella planticola*	*K. pneumoniae*
	$KSA_3$7	*Klebsiella* sp.	1.9	*K. pneumoniae*	99%	*K. pneumoniae*	*K. pneumoniae*
	$MSA_3$1	*NRI*	1.6	*S. aureus*	97%	*E. coli*	*No product*
	$MSA_3$2	*NRI*	1.6	*S. aureus*	95%	*E. coli*	*S. aureus*
Aeration Tank	*$CCA_3$11*	*E. coli*	2.2	*E. coli*	99%	*E. coli*	
	$CCA_3$12	*E. coli*	2.1	*E. coli*	99%	*E. coli*	
	$CCA_3$14	*E. coli*	2.1	*E. coli*	99%	*E. coli*	
	$CCA_3$15	*E. coli*	2.1	*E. coli*	98%	*E. coli*	
	$KSA_3$9	*Raoultella ornithinolytica*	2.2	*K. pneumoniae*	93%	*Raoultella ornithinolytica*	*No product*
	$KSA_3$13	*K. pneumoniae*	2.1	*K. pneumoniae*	98%	*K. pneumoniae*	*K. pneumoniae*
	$KSA_3$14	*K. pneumoniae*	2.1	*K. pneumoniae*	97%	*K. pneumoniae*	*K. pneumoniae*
Secondary Settling Tank	*$CCA_3$17*	*E. coli*	2.1	*E. coli*	99%	*E. coli*	
	$CCA_3$18	*E. coli*	2.3	*E. coli*	100%	*E. coli*	
Effluent	*$CCA_3$20*	*E. coli*	2.1	*E. coli*	100%	*E. coli*	
	$CCA_3$23	*E. coli*	2.1	*E. coli*	100%	*E. coli*	
	$CCA_3$24	*E. coli*	2.2	*E. coli*	99%	*E. coli*	
	$KSA_3$21	*K. pneumoniae*	2	*K. pneumoniae*	99%	*Raoultella ornithinolytica*	*K. pneumoniae*
	$KSA_3$22	*Klebsiella* sp.	1.9	*K. pneumoniae*	98%	*K. pneumoniae*	*K. pneumoniae*
	$MSA_3$6	*NRI*	1.3	*S. aureus*	99%	*S. aureus*	*S. aureus*

**NRI – no reliable identification*

In cycle one, 24 isolates were sent for identification using MALDI-TOF MS analysis at the facility in the Department of Biotechnology, University of the Western Cape (UWC), (Table 3.1). Of these isolates, 15 were presumed to be *E. coli,* eight isolates were presumed to be *K. pneumoniae* and one isolate was presumed to be *S. aureus.* Fourteen (58%) isolates then had a unit score of ≥2 (species identification), 10 (42%) isolates had a unit score of between ≥1.7 and <2 (genus identification), and no isolates had a unit score of <1.7 (no reliable identification) (Table 3.1). In addition, 12 (50%) isolates were positively identified as the target bacteria. These 12 samples had a unit score of ≥2 and were identified to the species level. From the results obtained 11 (73%) of the 15 presumptive *E. coli* isolates were positively identified using MALDI-TOF MS (Table 3.1). The four (27%) remaining isolates were only identified to the genus level as *Escherichia* sp.

From the eight samples presumed to be *K. pneumoniae*, two samples had a unit score of ≥2 (species identification) and both were identified as *Klebsiella oxytoca* (*K. oxytoca*). Six isolates were identified to genus level with a unit score of between ≥1.7 and <2 and these were identified as *Klebsiella* sp. In addition, the identification of the *S. aureus* isolates were confirmed with a unit score of ≥2 obtained (species identification) (Table 3.1).

In cycle two, 20 isolates were sent for identification using MALDI-TOF MS analysis (Table 3.2). Thirteen of these isolates were presumed to be *E. coli,* five isolates were presumed to be *K. pneumoniae* and two isolates were presumed to be *S. aureus.* After analysis, 15 (75%) isolates had a unit score of ≥2 (species identification), five (25%) isolates had a unit score of between ≥1.7 and <2 (genus identification), and no isolates had a unit score of <1.7 (no reliable identification) (Table 3.2). In addition, from this cycle, 12 (60%) samples were positively identified as the target organisms. These isolates had a unit score of ≥2 (species identification). Ten (77%) of the presumptive positive *E. coli* isolates were then positively identified using MALDI-TOF MS. The other three isolates (23%) were identified to the genus level as *Escherichia* sp. (Table 3.2). For the five isolates presumed to be *K. pneumoniae* in cycle two, four isolates had a unit score of ≥2 (species identification) and one isolate had a unit score of between ≥1.7 and <2 (genus identification). From the four isolates with a unit score of ≥2, two were identified as *K. oxytoca*, one was identified as *K. pneumoniae* and one isolate was identified as *Raoultella planticola* according to MALDI-TOF MS identification. The remaining isolate was identified as *Klebsiella* sp. with a unit score of between ≥1.7 and <2 (identified to genus level). For the two isolates presumed to be *S. aureus*, one isolate had a unit score of ≥2 (species identification) and was positively identified as *S. aureus* and one isolate had a unit score of ≥1.7 and <2 (genus identification) and was identified as *Staphylococcus* sp. (Table 3.2).

In cycle three, 26 samples were sent for identification using MALDI-TOF MS analysis (Table 3.3). Sixteen of these isolates were presumed to be *E. coli,* seven isolates were presumed to be *K. pneumoniae* and three isolates were presumed to be *S. aureus.* After analysis, 21 (81%) isolates had a unit score of ≥2 (species identification), two (8%) isolates had a unit score of between ≥1.7 and <2 (genus identification), and three (11%) isolates had a unit score of <1.7 (no reliable identification) (Table 3.3).

From this cycle, 20 (77%) isolates were then positively identified as the target organisms, with a unit score of ≥2 (species identification). The MALDI-TOF MS was able to positively identify 100% of the presumed *E. coli* isolates, with all 16 isolates (100%) having a unit score of ≥2 and were identified to species level. Seven samples were presumed to be *K. pneumoniae*, and of these five isolates had a unit score of ≥2 (species identification) and two had a unit score of between ≥1.7 and <2 (genus identification). From the five isolates with a unit score of ≥2, four were identified as *K. pneumoniae* and one was identified as *Raoultella ornithinolytica* according to MALDI-TOF MS identification. Of the two samples which had a unit score of between ≥1.7 and <2, both were identified as *Klebsiella* sp. All three isolates presumed to be *S. aureus* had a unit score of <1.7 as these isolates had low matching references on the database records and the results were regarded as non-reliable (Table 3.3).

3.1.2 VITEK 2 IDENTIFICATION

The results for the VITEK 2 identification of the isolates (n = 70) obtained throughout the sampling period are outlined in Tables 3.1 to 3.3. In cycle one, 24 isolates were sent for identification using the VITEK 2 system (Table 3.1) and *Escherichia coli* (n = 15) and *S. aureus* (n = 1) isolates were all (100%) confirmed as the target bacteria. However, from the presumed *K. pneumoniae* isolates (8), six isolates (75%) were identified as *K. oxytoca* and two isolates were identified as *K. pneumoniae* (Table 3.1).

In cycle two, 20 isolates were sent for identification using the VITEK 2 system (Table 3.2). The *E. coli* (n = 13) and *S. aureus* (n = 2) isolates were again 100% positively identified as the target bacteria. However, from the presumed five *K. pneumoniae* isolates, one isolate (20%) was identified as *K. oxytoca* and the other four isolates were identified as *K. pneumoniae* (Table 3.2).

From cycle three, 26 isolates were sent for identification using the VITEK 2 system (Table 3.3). All (100%) of the presumed *E. coli* isolates (n = 16) were positively identified by the VITEK 2 system to the species level. However, of the seven isolates presumed to be *K. pneumonia*e, four (57%) were positively identified as *K. pneumoniae*, while two of the isolates

were identified as *Raoultella ornithinolytica* (29%) and one isolate was identified as *Raoultella planticola* (14%). Only one out of the three (33%) presumed *S. aureus* isolates was positively identified as the target bacteria, while the other two isolates (67%) were identified as *E. coli* by the VITEK 2 system (Table 3.3).

3.1.3 Polymerase Chain Reaction (16S rRNA) identification

The results for the 16S rRNA analysis of the isolates (n = 70) obtained throughout the sampling period are outlined in Tables 3.1 to 3.3. The hypervariable region of 200 kb was amplified using PCR. From the 24 isolates obtained in cycle one, 23 isolates (96%) were positively identified as being the target bacteria (Table 3.1). A total of 15 presumptive *E. coli* isolates were then analysed for cycle one, with all (100%) isolates positively identified after blast analysis. Of the eight samples presumed to be *K. pneumoniae,* seven isolates (88%) were positively identified to the species level and one isolate (12%) was identified as *Klebsiella* sp. The isolate presumed to be *S. aureus*, was also positively identified after blast analysis. In addition, for cycle two, 100% of the presumed *E. coli* isolates (13) were again positively identified using 16S rRNA analysis and all (5) isolates presumed to be *K. pneumoniae,* were positively identified. In addition, the two samples presumed to be *S. aureus,* were both positively identified to the species level (Table 3.2). For cycle three, PCR was performed on 26 isolates using the V3 primers. The molecular technique was able to positively identify 100% of the 16 presumed *E. coli* isolates. In addition, all seven isolates were identified as *K. pneumoniae* and the identification of the three samples presumed to be *S. aureus* were confirmed to species level (Table 3.3).

3.1.4 SPECIES SPECIFIC PCR

As indicated, once the results for the three primary identification techniques has been analysed, species specific primers were then utilised to clarify any discrepancies in the results obtained, i.e. dissimilar results were obtained for two or more analysis techniques. *Escherichia coli* isolates were identified utilising the *phoA* gene (903 bp), *S. aureus* isolates were identified using the *vicK* gene (300 bp) and the 16S internal transcribed spacer region (130 bp) was utilised to identify *K. pneumoniae* isolates.

All the isolates presumed to be *E. coli* (n = 7) were positively identified as *E. coli* strains (Figure 3.1). Polymerase chain reaction artefacts of 400 bp were also observed in Figure 3.1. From the 20 isolates presumed to be *K. pneumoniae* (Figure 3.2), (representative isolates shown), 18 of the isolates were positively identified as *K. pneumoniae* and only two isolates (*$KSA_2$24* and *$KSA_3$9)* were not amplified. From the four isolates presumed to be *S. aureus* (Figure 3.3), three of the isolates were positively identified as *S. aureus* and only one isolate (*$MSA_3$1*) was not amplified.

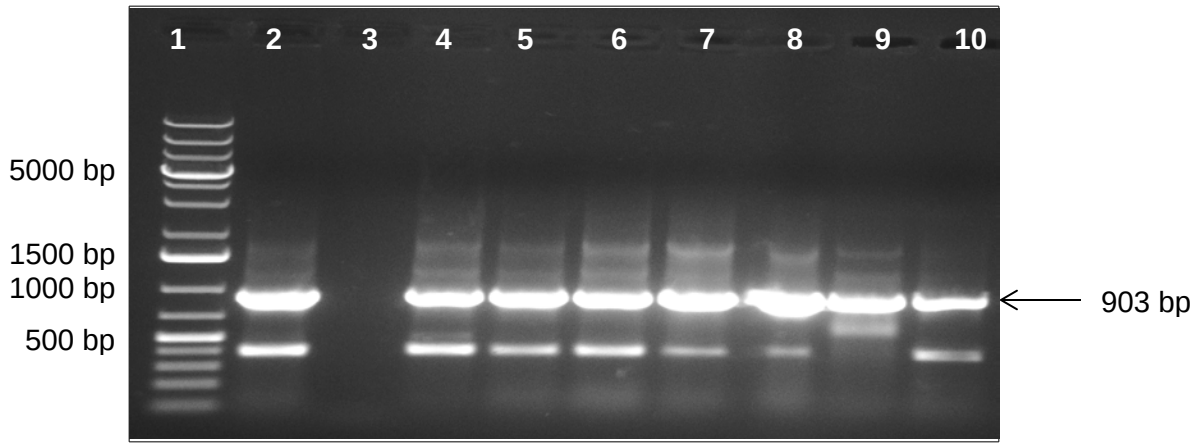


Figure 3.1: PCR amplification of genomic DNA for the identification of the *phoA* gene of 903 bp from isolates presumed as *E. coli*. Lane 1. Marker (GeneRuler 1kb Plus DNA Ladder #SM 1333); Lane 2. Positive control (*E. coli* ATCC 25922); Lane 3. Negative control; Lane 4. $CCA_2 1$; Lane 5. $CCA_2 2$; Lane 6. $CCA_2 8$; Lane 7. $CCA_1 30$; Lane 8. $CCA_1 40$; Lane 9. $CCA_1 19$; Lane 10. $CCA_1 13$.

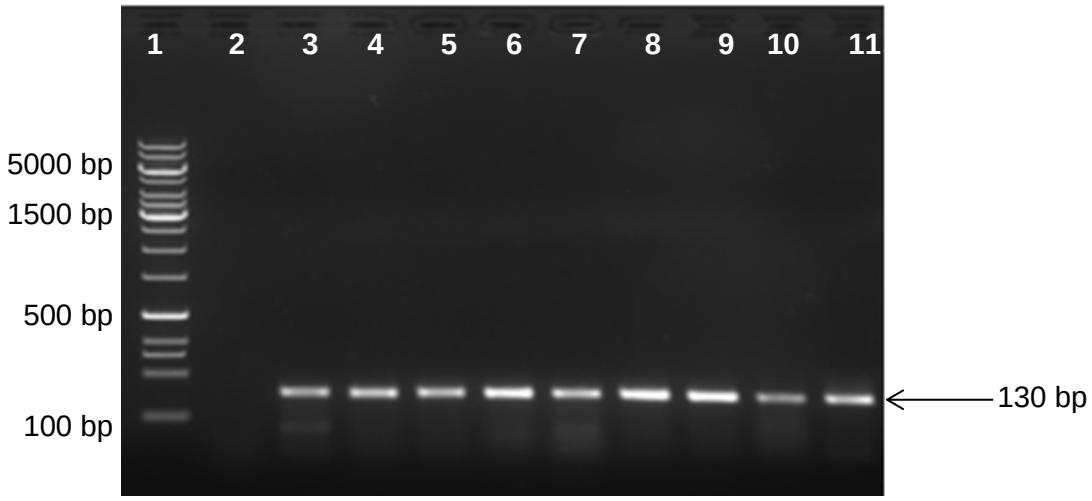


Figure 3.2: PCR amplification of genomic DNA for the identification of the *16S-23S internal transcribed spacer* gene of 130 bp from isolates presumed as *K. pneumoniae*. Lane 1. Marker (GeneRuler 1kb Plus DNA Ladder #SM 1333); Lane 2. Negative control; Lane 3. Positive control (*K. pneumoniae* ATCC *BAA-1705*); Lane 4. $KSA_1 7$; Lane 5. $KSA_1 16$; Lane 6. $KSA_1 38$; Lane 7. $KSA_1 67$; Lane 8. $KSA_1 79$; Lane 9. $KSA_1 80$; Lane 10. $KSA_1 82$; Lane 11. $KSA_1 84$.

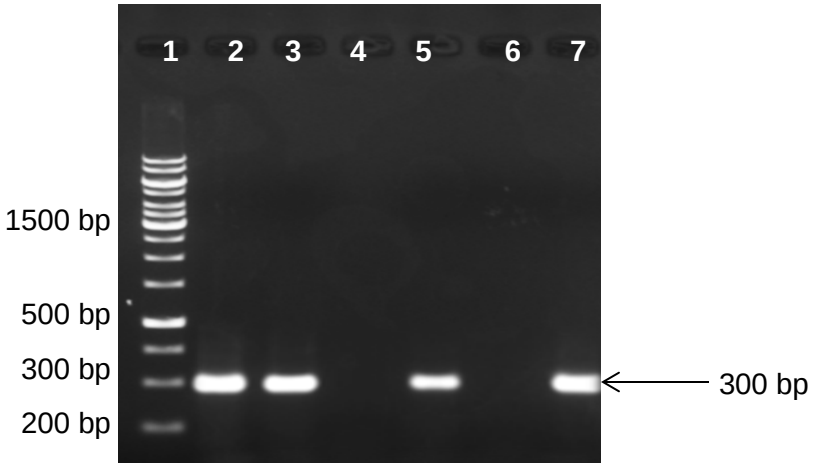


Figure 3.3: PCR amplification of genomic DNA for the identification of the *vicK* gene of 300 bp from isolates presumed as *S. aureus*. Lane 1. Marker (GeneRuler 1kb Plus DNA Ladder #SM 1333); Lane 2. *MSA$_3$6*; Lane 3. M*SA$_3$2*; Lane 4. *MSA$_3$1*; Lane 5. *MSA$_2$2*; Lane 6. Negative control; Lane 7. Positive control.

3.1.5 COCHRAN Q results

An overall comparison of the percentage efficiency of the three techniques employed in the current study in identifying the target organisms to species level is indicated in Table 3.4. The Cochran's Q test results for *E. coli* identification by the three identification techniques is also indicated in Table 3.5. Forty four *E. coli* isolates (obtained from all three sampling cycles) were analysed, with the MALDI-TOF MS technique positively identifying 37 *E. coli* isolates, resulting in 15.9% failure and 84.1% success (Table 3.5). In comparison, both the 16S rRNA PCR technique and VITEK 2 system positively identified all 44 isolates as *E. coli*. The calculated probability of MALDI-TOF MS, PCR and VITEK was found to be 0.000912, with a Q value of 14 obtained, thus indicating that there was a significant difference between the three identification techniques in identifying *E. coli*, and the null hypothesis was rejected. However, the pairwise comparison of PCR versus VITEK indicated that there was no difference (p = 1) between these two techniques in the identification of *E. coli*. In contrast, the pairwise comparison of MALDI-TOF MS versus PCR had a calculated probability of 0.008151 and MALDI-TOF MS versus VITEK also had a calculated probability of 0.008151, this revealed that there was a significant difference between MALDI-TOF MS versus PCR and MALDI-TOF MS versus VITEK in identifying *E. coli* strains. This result was expected as MALDI-TOF MS was only able to identify 84.1% of the presumptive *E. coli* isolates, while 16S rRNA PCR and VITEK 2 analysis successfully identified all the isolates as *E. coli*. When the pairwise test p-values were Bonferroni corrected, this result was maintained indicating that there was a significant difference between the three identification

techniques in identifying *E. coli* isolates and that there was a significant difference between MALDI-TOF MS versus PCR and MALDI-TOF MS versus VITEK in identifying *E. coli* strains.

The Cochran's Q test results for *K. pneumoniae* (n = 20) identification by the three techniques are indicated in Table 3.6. From the 20 isolates obtained throughout the sampling period, the MALDI-TOF MS positively identified five *K. pneumoniae* isolates, resulting in a 75% failure and a 25% success rate, while the VITEK 2 system positively identified 11 *K. pneumoniae* isolates resulting in a 45% failure and a 55% success rate and the 16S rRNA PCR analysis positively identified 19 *K. pneumoniae* isolates, resulting in a 5% failure and a 95% success rate (Table 3.4). The calculated probability of MALDI-TOF MS, PCR and VITEK was found to be 0.000096, with a Q value of 18.5, thus indicating that there is a significant difference between the techniques in identifying *K. pneumoniae* isolates, and the null hypothesis was rejected. The pairwise comparison of PCR versus VITEK then resulted in a p = 0.004678, indicating that there was a significant difference between the 16S rRNA PCR technique and the VITEK 2 system in identifying *K. pneumoniae* isolates. In addition, the pairwise comparison of MALDI-TOF MS versus 16S rRNA PCR resulted in p = 0.000183, indicating that there was also a significant difference between the MALDI-TOF MS and PCR in identifying *K. pneumoniae* isolates. In contrast, the pairwise comparison of MALDI-TOF MS vs. VITEK (p = 0.057780) indicated that there is no significant difference between these two techniques in the identification of the *K. pneumoniae* isolates. When the pairwise test p-values were Bonferroni corrected, the results still reflected that there was a significant difference between the three identification techniques in identifying *K. pneumoniae* isolates and that there was a significant difference between the MALDI-TOF MS and 16S rRNA PCR technique in identifying the target bacterium. When Bonferroni corrected, the pairwise test between MALDI-TOF MS versus VITEK again reflected that there is no significant difference between the two identification techniques in identifying *K. pneumoniae.*

It should be noted that the Cochran's Q test results for the identification of *S. aureus* isolates could not calculated because of the small number of isolates (n = 6) obtained throughout the sampling period. Six *S. aureus* isolates were then analysed, with the MALDI-TOF MS technique positively identifying two *S. aureus* isolates, resulting in a 66% failure and a 33% success rate, while the VITEK technique positively identifying four *S. aureus* isolates resulting in a 33% failure and a 66% success rate. In comparison the 16S rRNA PCR technique positively identified all six *S. aureus* isolates, resulting in a 100% success rate (Table 3.4).

Table 3.4: Comparison of techniques in identifying the target organism to species level

ORGANISM	MALDI-TOF MS	PCR	VITEK
E. coli	37	44	44
Escherichia sp.	7	0	0
Other	0	0	0
Target bacteria % id	84%	100%	100%
K. pneumoniae	5	19	10
Klebsiella sp.	9	1	0
Other	6	0	10
Target bacteria % id	25%	95%	50%
S. aureus	2	6	4
Staphylococcus sp.	1	0	0
Other	3	0	2
Target bacteria % id	33%	100%	66%

Table 3.5: The Cochran Q test results of MALDI-TOF MS vs PCR vs VITEK for the identification of *E. coli*

VARIABLE	TARGET BACTERIA POSITIVELY IDENTIFIED	FAILURE % (0'S)	SUCCESS % (1'S)
MALDI-TOF MS	37	15.9	84.1
PCR	44	100	100
VITEK	44	100	100
$N = 44$	$Q = 14.00$	df = 2	$p < 0.000912$

Table 3.6: The Cochran Q test results of MALDI-TOF MS vs PCR vs VITEK for the identification of *K. pneumoniae*

VARIABLE	TARGET BACTERIA POSITIVELY IDENTIFIED	FAILURE % (0'S)	SUCCESS % (1'S)
MALDI-TOF MS	5	75	25
PCR	19	5	95
VITEK	10	50	50
$N = 20$	$Q = 18.5$	df = 2	$p < 0.000096$

3.2 THE CORRELATION BETWEEN GENES THAT CONFER ANTIBIOTIC RESISTANCE AND THE ANTIBIOTIC RESISTANCE PROFILES OF BACTERIA AT A MUNICIPAL WASTEWATER TREATMENT PLANT IN STELLENBOSCH, SOUTH AFRICA.

3.2.1 PCR detection of resistant genes

Polymerase Chain Reactions was performed on both genomic and plasmid DNA extracted from all 44 *E. coli* isolates obtained from all sampling cycles (15 isolates from cycle one, 13 isolates from cycle two and 16 isolates from cycle three) (Figure 3.4 & 3.5). The *gyrA* gene and *parC* gene was detected in the plasmid and genomic DNA of all 44 *E. coli* isolates, respectively. Since, resistance is caused by missense mutations that occur in the quinolone resistance-determining region (QRDR) found within the N-terminus of the *gyrA* gene of *E. coli*, single nucleotide polymorphisms within this region were compared to a wild-type strain to detect possible missense mutations (Appendix A and B). These mutations are located at amino acids Ala67 to Gln106 (Yoshida et al., 1990) for the *gyrA* gene. Furthermore, quinolone resistance-determining mutations of the DNA gyrase gene often appear at codon Ser83 and Asp87 (Hopkins et al., 2005 and Yoshida et al., 1990) which are positioned near the active site of the DNA gyrase gene. Mutations in the *parC* gene of *E. coli* have always been consistent with mutations detected on the *gyrA* gene. Nucleotide sequences of the quinolone-resistant *parC* genes were determined (Ser63 to Glu84) (Appendix C and D).

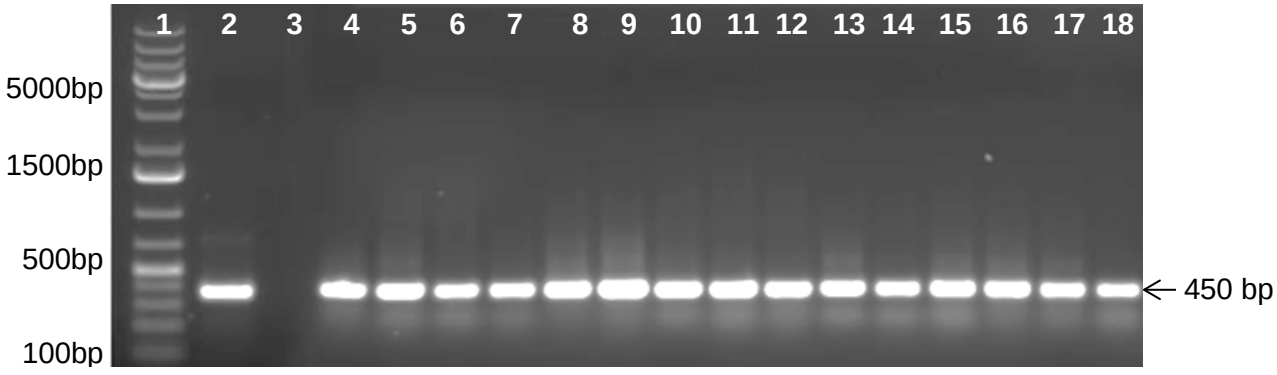


Figure 3.4: Cycle One, PCR amplification of genomic DNA for the identification of the *parC* gene of 450 bp from isolates positively identified as *E. coli*. Lane 1. Marker (GeneRuler 1kb Plus DNA Ladder #SM 1333); Lane 2. Positive control (*E. coli* ATCC 25922); Lane 3. Negative control; Lane 4. $CCA_1 1$; Lane 5. $CCA_1 2$; Lane 6. $CCA_1 8$; Lane 7. $CCA_1 9$; Lane 8. $CCA_1 13$; Lane 9. $CCA_1 14$; Lane 10. $CCA_1 15$; Lane 11. $CCA_1 17$; Lane 12. $CCA_1 18$; Lane 13. $CCA_1 19$; Lane14. $CCA_1 30$; Lane 15. $CCA_1 31$; Lane 16. $CCA_1 40$; Lane 17. $CCA_1 62$; Lane 18. $CCA_1 70$.

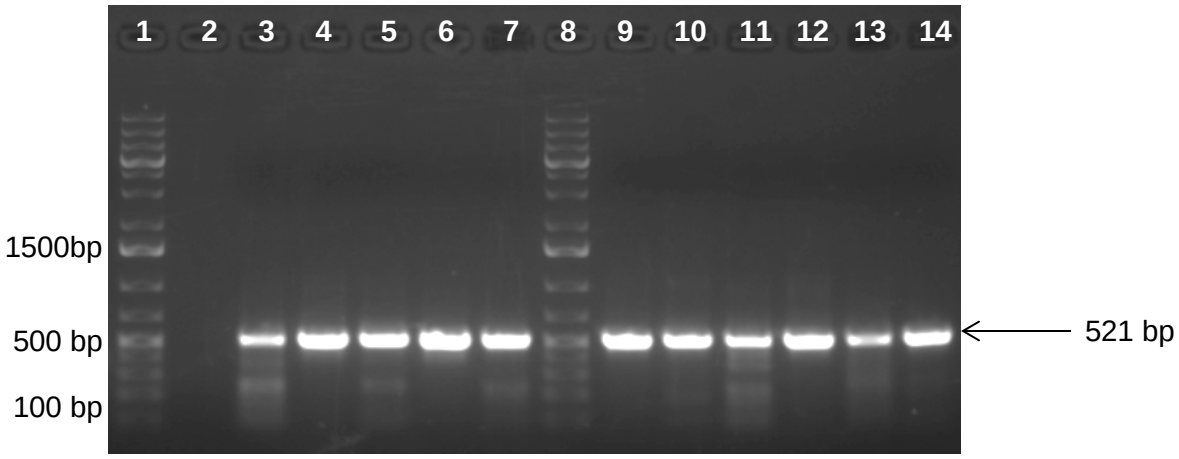


Figure 3.5: Cycle One, PCR amplification of plasmid DNA for the identification of the *gyrA* gene of 521 bp from isolates positively identified as *E. coli*. Lane 1. Marker (GeneRuler 1kb Plus DNA Ladder #SM 1333); Lane 2. Negative control; Lane 3. Positive control (*E. coli* ATCC 25922); Lane 4. $CCA_1 1$; Lane 5. $CCA_1 2$; Lane 6. $CCA_1 8$; Lane 7. $CCA_1 9$; Lane 8. Marker (GeneRuler 1kb Plus DNA Ladder #SM 1333); Lane 9. $CCA_1 13$; Lane 10. $CCA_1 14$; Lane 11. $CCA_1 15$; Lane 12. $CCA_1 17$; Lane 13. $CCA_1 18$; Lane 14. $CCA_1 19$.

Escherichia coli ATCC 25922 was used as a quality control strain in susceptibility testing (Yue et al., 2008 and Hao et al., 2012). For the genomic DNA PCR analysis, 40 *E. coli* isolates had the wild-type *gyrA* gene and four isolates presented with mutations when compared to the wild-type (Table 3.7). Both isolate $CCA_2 1$ and $CCA_2 2$ had a mutation at codon Gly75, which was changed to Cys. Isolate $CCA_3 17$ had codon changes at Glu94 to Gln, Gly75 to Arg, Iso74 to Met and Val73 to Thr while isolate $CCA_3 18$ presented with changes at codon Leu98 to Val, Ala93 to Thr and Asp87 to Hst (Table 3.7; Appendix A and B). From the plasmid DNA extraction, 42 *E. coli* isolates had amino acid sequences identical to the wild-type *gyrA* gene and two isolates presented with missense mutations. Isolate $CCA_2 2$ had a mutation at codon Gly75 to Cys and isolate $CCA_3 18$ had changes at codon Leu98 to Val and Ala67 to Asp (Table 3.7). For the genomic DNA PCR analysis, 43 *E. coli* isolates had amino acid sequence similar to the wild-type *parC* gene with one isolate, ($CCA_3 8$) presenting with a mutation at codon Asp69 to Tyr. For the plasmid DNA PCR analysis, 40 *E. coli* isolates had amino acid sequence similar to the wild-type *parC* gene and four isolates presented with mutations. Isolate $CCA_1 9$ had a mutation at codon Gly78 to Asp, isolate $CCA_1 18$ had a change at codon Leu71 to Glu, isolate $CCA_2 18$ had a change at codon Ala64 to Ser while $CCA_3 14$ had a change at codon Gly68 to Arg (Table 3.7; Appendix C and D).

Polymerase Chain Reactions were performed on both genomic and plasmid DNA extracted from 20 *K. pneumoniae* isolates obtained from all sampling cycles (eight isolates from cycle one, five isolates from cycle two and seven isolates from cycle three) to detect the presence of the *bla*$_{KPC}$ gene. Carbapenem resistance in *K. pneumoniae* is an acquired trait (Leavitt et al., 2009). It was suggested that the presence of the *bla*$_{KPC}$ genes (β-lactam *Klebsiella pneumoniae* carbapenemases) frequently found on plasmids may be associated with resistance, since these plasmids code for aminoglycosides, which are resistance determinants (Sacha et al., 2009). For the genomic DNA PCR analysis, the *bla*$_{KPC}$ gene was detected in five *K. pneumoniae* isolates (*KSA*$_2$*11, KSA*$_2$*16, KSA*$_2$*23, KSA*$_2$*24* and *KSA*$_3$*13*) (Figure 3.6; Table 3.8). While for the plasmid DNA, the *bla*$_{KPC}$ gene was detected in three of the same *K. pneumoniae* isolates (*KSA*$_2$*24*, *KSA*$_2$*23* and *KSA*$_3$*13*) (Figure 3.6 and Table 3.8). Sequence and BLAST analysis results confirmed the presence of the *bla*$_{KPC}$ gene the respective isolates (Altschul et al., 1997).

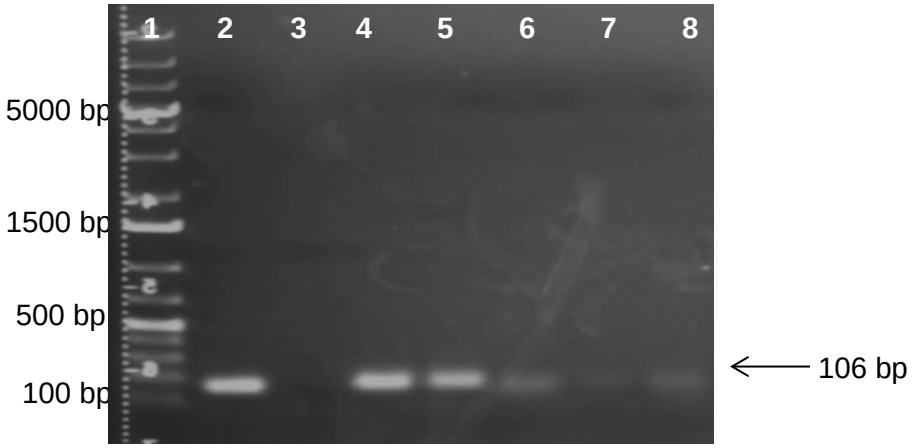


Figure 3.6: Cycle Two, PCR amplification of plasmid DNA for the identification of the *Carbapenemase gene* of 106bp from isolates positively identified as *Klebsiella* sp. Lane 1. Marker (GeneRuler 1kb Plus DNA Ladder #SM 1333); Lane 2. Positive control (*K. pneumoniae* ATCC *BAA-1705*); Lane 3. Negative control; Lane 4. *KSA*$_2$*24*; Lane 5. *KSA*$_2$*23*; Lane 6. *KSA*$_2$*1*6; Lane 7. *KSA*$_2$*11*; Lane 8. *KSA*$_2$*5.*

Polymerase Chain Reactions was performed on both genomic and plasmid DNA extracted from six *S. aureus* isolates obtain from all sampling cycles (one isolate from cycle one, two isolates from cycle two and three isolates from cycle three) (Table 3.9) to detect the presence of the *mecA* gene. The *mecA* gene provides MRSA with a broad range of resistance to all *β*-lactam antibiotics such as methicillin, oxacillin, nafcillin, cloxacillin, and dicloxacillin. The resistance is mainly caused by mutations and other antibiotic resistance genes may also be present in the cassette enabling MRSA to be resistant to multiple antibiotics (Sajith et al., 2012).

The expression of methicillin resistance is determined by the regulatory components that control the expression of the β-lactamase genes (*blaI, blaRI, blaZ*) which, because of sequence similarities, also can down regulate *mecA* gene transcription (Haddadin, 2002). For the genomic DNA PCR analysis, the *mecA* gene was not detected in any of the six *S. aureus* isolates. However, for the plasmid DNA PCR analysis, the *mecA* gene was detected in one *S. aureus* isolate (*MSA$_3$2*) (Figure 3.7; Table 3.9). Sequence and BLAST analysis results confirmed the presence of the *mecA* gene in the one isolate (Altschul *et al.,* 1997).

No *E. coli* isolates with a mutation on the target genes or that had a quinolone resistant profiles were detected in the effluent (Table 3.7). However, two *K. pneumoniae* isolates with the *bla*$_{KPC}$ genes and carbapenem resistant profiles were detected in the effluent (Table 3.8).

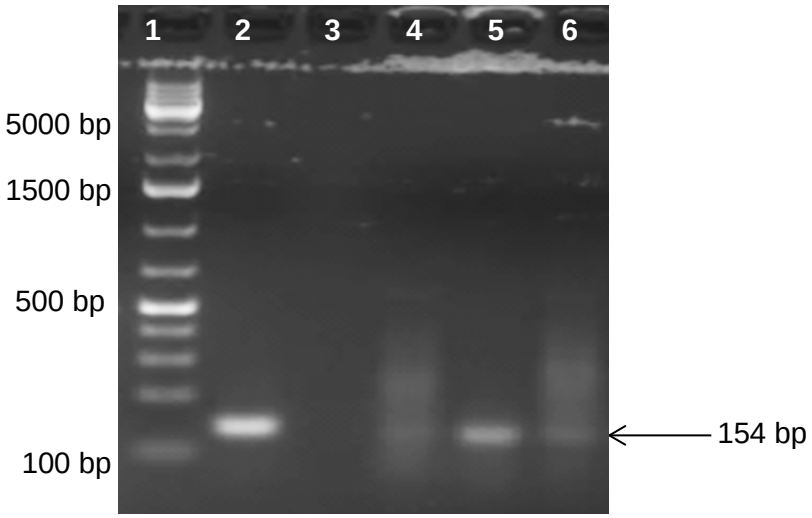


Figure 3.7: Cycle Three, PCR amplification of plasmid DNA for the identification of the *mecA gene* of 154bp from isolates positively identified as *S. aureus*. Lane 1. Marker (GeneRuler 1kb Plus DNA Ladder #SM 1333); Lane 2. Positive control (*S. aureus* ATCC *33591*); Lane 3. Negative control; Lane 4. *MSA$_3$1*; Lane 5. *MSA$_3$2;* Lane 6. *MSA$_3$6.*

Table 3.7: Cycle 1, 2 and 3, Genomic and plasmid *gyrA* and *parC* gene and antibiotic resistance profile identification from *E. coli* isolates

Sampling site	Code name	Polymerase Chain Reaction				VITEK
		(Genomic) gene gyrA	*(Plasmid) gene gyrA*	*(Genomic) gene parC*	*(plasmid) gene parC*	
INFLUENT	*$CCA_1$1*	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_1$2	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_1$8	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_1$9	*WT*	*WT*	*WT*	*Gly78 - Asp*	Susceptible
	$CCA_1$13	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_2$1	*Gly75 – Cys*	*WT*	*WT*	*WT*	Susceptible
	$CCA_2$2	*Gly75 – Cys*	*Gly75 - Cys*	*WT*	*WT*	Susceptible
	$CCA_2$3	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_2$8	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_2$9	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_2$13	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_3$1	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_3$2	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_3$3	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_3$6	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_3$7	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_3$8	*WT*	*WT*	*Asp69 - Tyr*	*WT*	Susceptible
	$CCA_3$9	*WT*	*WT*	*WT*	*WT*	Susceptible
AERATION TANK	*$CCA_1$14*	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_1$15	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_1$17	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_1$18	*WT*	*WT*	*WT*	*Leu71 - Glu*	Susceptible
	$CCA_1$19	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_2$17	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_2$18	*WT*	*WT*	*WT*	*Ala64 - Ser*	Susceptible
	$CCA_2$19	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_2$20	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_2$21	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_3$11	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_3$12	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_3$14	*WT*	*WT*	*WT*	*Gly68 - Arg*	Susceptible
	$CCA_3$15	*WT*	*WT*	*WT*	*WT*	Susceptible
SETTLING TANK	*$CCA_3$17*	*Glu94 – Glm* *Gly75 – Arg* *Iso74 – Met* *Val73 – Thr*	*WT*	*WT*	*WT*	Susceptible
	$CCA_3$18	*Leu98 –Val* *Ala93 – Thr* *Asp87 - Hst*	*Leu98 – Val* *Ala67 - Asp*	*WT*	*WT*	Resistant

EFFLUENT	*$CCA_1$30*	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_1$31	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_1$40	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_1$62	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_1$70	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_2$26	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_2$27	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_3$20	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_3$23	*WT*	*WT*	*WT*	*WT*	Susceptible
	$CCA_3$24	*WT*	*WT*	*WT*	*WT*	Susceptible

[a]*WT* – wild type, [b]*ARG* – antibiotic resistant gene

Table 3.8: Cycle 1, 2 and 3, Genomic and plasmid antibiotic resistance identification of *K. pneumoniae* isolates

Sampling site	**Code name**	***(Genomic) Carbapenemase gene***	***(Plasmid) Carbapenemase gene***	**VITEK**
INFLUENT	*$KSA_1$7*	*NP*	*NP*	Susceptible
	$KSA_1$16	*NP*	*NP*	Susceptible
	$KSA_2$5	*NP*	*NP*	Susceptible
	$KSA_3$4	*NP*	*NP*	Susceptible
	$KSA_3$7	*NP*	*NP*	Susceptible
AERATION TANK	*$KSA_1$38*	*NP*	*NP*	Susceptible
	$KSA_1$67	*NP*	*NP*	Susceptible
	$KSA_2$11	*ARG*	*NP*	Resistant
	$KSA_3$13	*NP*	*NP*	Susceptible
	$KSA_3$13	*ARG*	*ARG*	Resistant
	$KSA_3$14	*NP*	*NP*	Resistant
SETTLING TANK	*$KSA_2$16*	*ARG*	*NP*	Resistant
EFFLUENT	*$KSA_1$79*	*NP*	*NP*	Susceptible
	$KSA_1$80	*NP*	*NP*	Susceptible
	$KSA_1$82	*NP*	*NP*	Susceptible
	$KSA_1$84	*NP*	*NP*	Susceptible
	$KSA_2$23	*ARG*	*ARG*	Resistant
	$KSA_2$24	*ARG*	*ARG*	Resistant
	$KSA_3$21	*NP*	*NP*	Susceptible
	$KSA_3$22	*NP*	*NP*	Susceptible

[a]*ARG* – antibiotic resistant gene detected, [b]*NP* – no product

Table 3.9: Cycle 1, 2 and 3, Genomic and plasmid antibiotic resistance identification of *S. aureus* isolates.

Sampling site	Code name	*(Genomic) mecA gene*	*(Plasmid) mecA gene*	VITEK
INFLUENT	*$MSA_2$2*	*NP*	*NP*	Susceptible
	$MSA_2$3	*NP*	*NP*	Susceptible
	$MSA_3$1	*NP*	*NP*	Susceptible
	$MSA_3$2	*NP*	*ARG*	Susceptible
SETTLING TANK	*$MSA_1$14*	*NP*	*NP*	Susceptible
EFFLUENT	*$MSA_3$6*	*NP*	*NP*	Susceptible

[a]*ARG* – antibiotic resistant gene detected, [b]*NP – no product*

3.2.2 VITEK 2 susceptibility test

The VITEK 2 system susceptibility test was used to analyse 44 *E. coli* isolates obtained from all sampling cycles (15 isolates from cycle one, 13 isolates from cycle two and 16 isolates from cycle three). One *E. coli* isolate (*$CCA_3$18*) was shown to be quinolone resistant, while the other 43 isolates were found to be susceptible to the antibiotic quinolone (Table 3.7). The VITEK susceptibility test also showed that 16 of the *E. coli* isolates were resistant to ampicillin and 20 *E. coli* isolates were resistant to trimethoprim (results not shown). The isolate *$CCA_3$18* that showed resistance to quinolone, also presented with the Asp87 to Hst mutation on the genomic DNA which has been shown to be important in the conference of resistance to quinolone. All isolates were sensitive to cephalosporins, carbapenems and aminoglycosides.

When analysing the results obtained from the VITEK 2 analysis for the 20 *K. pneumoniae* isolates obtained from all sampling cycles (eight isolates from cycle one, five isolates from cycle two and seven isolates from cycle three), carbapenem resistant profiles were detected in six *K. pneumoniae* isolates (*$KSA_2$11, $KSA_2$16, $KSA_2$23, $KSA_2$24, $KSA_3$13* and *$KSA_3$14*) (Table 3.8). When comparing the PCR results to the VITEK result, five of the isolates contained the *bla_{KPC}* gene (*$KSA_2$11, $KSA_2$16, $KSA_2$23, $KSA_2$24, $KSA_3$13)* while the *bla_{KPC}* gene was not detected in isolate *$KSA_3$14.* The VITEK susceptibility test also showed that all the *K. pneumoniae* isolates (n=20) were resistant to ampicillin (results not shown). In addition, the five *K. pneumoniae* isolates which were resistant to carbapenems were also resistant to quinolones with one *K. pneumoniae* isolate was resistant to trimethoprim (*$KSA_3$13*). All isolates were sensitive to colistin sulphate.

When the six *S. aureus* isolates from all sampling cycles (one isolates from cycle one, two isolates from cycle two and three isolates from cycle three) were analysed using the VITEK 2 system, no methicillin resistance profiles were detected (Table 3.9) even in isolate *$MSA_3$2* wherein the *mecA* gene was amplified. The VITEK susceptibility test also showed that all the *S. aureus* isolates were resistant to ampicillin and sensitive to trimethoprim, cephalosporins, carbapenems and aminoglycosides.

No *E. coli* isolates with a mutation on the target genes or that had a quinolone resistant profile was detected in the effluent (Table 3.7). Two *K. pneumoniae* isolates with the *bla_{KPC}* genes and carbapenem resistance profiles were detected in the aeration tank, while one *K. pneumoniae* isolate that did not have the *bla_{KPC}* genes but showed carbapenem resistance was also detected at the aeration tank. One *K. pneumoniae* isolate with the *bla_{KPC}* gene and a carbapenem resistance profile was also detected at the secondary settling tank and two *K. pneumoniae* isolates with the *bla_{KPC}* gene and carbapenem resistant profiles were detected in the effluent (Table 3.8)

3.2.3 Statistical Analysis

Out of the 70 isolates investigated for the presence and expression of antibiotic resistance genes, 15 isolates (21.4%) were positive for either missense mutations within the QRDR (*E. coli*) or the presence of genes that conferred antibiotic resistance (*K. pneumonia* and *S. aureus*). However, only seven isolates (10%) exhibited antibiotic resistance profiles when analysing the VITEK 2 system results.

In all three cycles, using the VITEK 2 system analyser, only one *E. coli* isolate (2%) (*$CCA_3$18*) out of the 44 *E. coli* isolates obtained was found to quinolone resistant and, no statistical analysis could be performed. Similarly, as none of the *S. aureus* (17%) isolates were found to be methicillin resistant, no statistical analysis could be performed. Six *K. pneumoniae* (30%) isolates (*$KSA_2$11; $KSA_2$16; $KSA_2$23; $KSA_2$24; $KSA_3$13* and *$KSA_3$14*) where found to be carbapenem resistant. Five of the isolates contained the *bla_{KPC}* gene (*$KSA_2$11, $KSA_2$16, $KSA_2$23, $KSA_2$24, $KSA_3$13)*, with the *bla_{KPC}* gene not detected in *$KSA_3$14.*

The McNemar test was performed to assess the difference between the presence of the *bla_{KPC}* gene on the genome (PCR) vs. the expression of the gene (VITEK 2 system) and to assess the difference between the presence of the *bla_{KPC}* gene on the plasmid (PCR) vs. the expression of the gene (VITEK 2 system). Since the p-value in both instances was greater than 0.05, no significant difference between the two proportions respectively, was found. .

CHAPTER FOUR

DISCUSSION

CHAPTER FOUR

4. DISCUSSION

4.1 MALDI-TOF Mass Spectrometry versus Biomerieux VITEK 2 and Molecular Techniques for the Identification of *Escherichia coli, Klebsiella pneumoniae* and *Staphylococcus aureus* in Wastewater

In total 70 presumptive *E. coli* (n = 44), *K. pneumoniae* (n = 20) and *S. aureus* (n = 6) isolates were obtained from various points of the Stellenbosch WWTP during the three sampling cycles. The effectiveness of MALDI-TOF MS, VITEK 2 and molecular methods for the routine identification of these isolates was then analysed. The MALDI-TOF MS technique successfully identified an average of 84% of the *E. coli* isolates, an average of 25% *K. pneumoniae* isolates and an average of 33% of the *S. aureus* isolates. The technique was unable to differentiate between closely related organisms and if the organism tested was not in the database, unreliable results were produced. However, results obtained in the current study confirmed that the MALDI-TOF MS technique was reliable for the identification of *E. coli* isolates obtained from wastewater samples. This data corresponds to a study conducted by Loff et al. (2014), where the MALDI-TOF MS was able to identify *E. coli* isolated from wastewater using selective media. The MALDI-TOF MS is considered one of the most widely used and recommended microbiological identification procedures (Juiz et al., 2011), as it reduces the identification process by approximately 24 hours. Researchers have also used the MADLI-TOF MS to determine unknown protein masses and peptides and it is suggested that it assists in the diagnosis of allergies, Alzheimer's disease, rheumatoid arthritis and tumours by identifying specific biochemical markers (Marvin et al., 2003). Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is thus considered by some to have revolutionized routine identification in microbiology laboratories.

However, some research groups have indicated that the use of MALDI-TOF MS system should be practiced with caution as many bacteria are poorly identified by this technique (Hrabak, et al., 2013). This was corroborated in the current study where low percentage identifications were obtained for presumptive *K. pneumoniae* (25%) and *S. aureus* (33%) isolates with the MALDI-TOF MS technique. In cycle one and two, four isolates (*$KSA_1$7*, *$KSA_1$84, $KSA_2$11* and *$KSA_2$24*) were identified as *K. oxytoca* by the MALDI-TOF MS technique. Isolates *$KSA_1$7* and *$KSA_2$11* were then positively identified as *K. pneumoniae* by 16S rRNA and VITEK 2 analysis, which was corroborated by species specific analysis, while no identifiable result could be obtained for isolates *$KSA_1$84* and *$KSA_2$24*. This result suggested that the MALDI-

TOF MS possibly had a narrow database spectrum and was unable to differentiate between *Klebsiella pneumoniae* and *Klebsiella oxytoca.* However, Monnet and Freney (1994) reported that the API system and biological procedures at times also fails to distinguish between the species of *Klebsiella pneumoniae* and *Klebsiella oxytoca* (Ohkusu, 2000). *Klebsiella oxytoca* is described as a respiratory pathogen and in 1963 the organism was accepted as part of the *Klebsiella* genus but was misclassified as *K. pneumoniae* (Power and Calder, 1983). However, biochemical tests based on the phenotypic distinction between *K. pneumoniae* and *K. oxytoca* are said to be unreliable and are considered time-consuming (Monstein et. al, 2009). A distinct difference however, is that *Klebsiella oxytoca* is indole-positive and it is able to grow in melezitose but not in 3-hydroxybutyrate (Maslow and Arbeit, 1993).

In addition, in cycle two *Raoultella planticola* (*R. planticola*) was positively identified by MALDI-TOF MS, however the same isolate was identified as *K. pneumoniae* by the other two identification techniques as well as by utilising the species specific primers. *Raoultella planticola* are Gram-negative bacteria of the Enterobacteriaceae genus. They are found in water and soil, and these species are associated with histamine-producing bacteria which cause fish poisoning (Kim et al., 2012). The DNA sequences of *Klebsiella* and *Raoutella* are very similar and therefore until the 1990s *Raoultella planticola* was part of the *Klebsiella genus*. It was first named *Klebsiella planticola* (Nada and Areej, 2014), but the new genus of *Raoultella* was formed after the 16S rRNA gene and *rpoB* gene sequences were analysed, and *Klebsiella planticola* was subsequently re-classified as *R. planticola* (Nada and Areej, 2014).

Similarly, in cycle two, one isolate (*$MSA_2 2$*) was identified as *Staphylococcus* sp. using MALDI-TOF MS analysis, while 16S rRNA and VITEK 2 analysis identified the isolate as *S. aureus* (confirmed by species specific analysis). In addition, in cycle three, no reliable identification was obtained for three isolates (*$MSA_3 1$, $MSA_3 2$* and *$MSA_3 6$*) using the MALDI-TOF MS technique. Isolates *$MSA_3 6$* and *$MSA_3 2$* were however identified as *S. aureus* by 16S rRNA and VITEK 2 analysis (confirmed by species specific PCR), while conflicting results were obtained for isolate *$MSA_3 1$* using all analysis techniques. However, based on the overall results obtained in the current study, MALDI-TOF MS is able to analyse and identify multiple unknown isolates simultaneously and reduces the time span and laboratory resources required.

Identification of unknown bacteria can be obtained within 3 to 8 hours when using the VITEK 2 fluorescence reading of Gram-negative (ID-GNB) or Gram-positive (ID-GPC) identification cards (Wallet et al., 2005). The technology uses a database system to compare bacterial traits represented on the respective identification card to the reference databank. The identification cards have been improved over the years to also identify non-fermenting bacteria (Wallet et al.,

2005). The VITEK 2 system was able to positively identify 100% of all the *E. coli* isolates (n = 44), four (67%) out of the six *S. aureus* isolates and 50% of the *K. pneumoniae* isolates (n = 10). Seven of the ten isolates that were not identified as *K. pneumoniae,* were identified as *K. oxytoca,* while three isolates were identified as *Raoultella* spp. As indicated *Klebsiella pneumoniae* and *Klebsiella oxytoca* as well as *Raoultella* spp. are very closely related. Eight of these isolates were then positively identified as *K. pneumoniae* by species specific PCR and Blast analysis (identity of 96% or higher obtained), while conflicting results were obtained for two isolates using all the analyses techniques. These results suggest that the VITEK 2 system could also not efficiently distinguish between closely related organisms such as *K. pneumoniae* and *K. oxytoca.* However, in the current study, the VITEK cards were found to be an easy tool to use in the laboratory and this technique was able to identify all the *E. coli* isolates and 66% of the *S. aureus* isolates.

The use of the 16S rRNA region for bacterial identification is commonly used in microbiology laboratories as this region is present in all bacteria and is frequently presented as operons. The region is long enough for use in informatics and over time the function of the region has not changed (Janda and Abbott, 2007). 16S rRNA Polymerase Chain Reaction (V3 hypervariable region) was then performed on the 70 bacterial isolates presumed to be either *E. coli* (n=44), *K. pneumoniae* (n=20) or *S. aureus* (n=6). Through sequencing and Blast analysis the 16S rRNA technique identified the highest percentage (98%) of the target bacteria from all cycles. In cycle one, an isolate presumed to be *K. pneumoniae* was however, identified as a *Klebsiella sp.* using 16S rRNA analysis and while MALDI-TOF MS and VITEK 2 analysis identified the organism as *K. oxytoca,* the isolate was positively identified as *K. pneumoniae* using species specific PCR and Blast analysis.

When comparing the identification technique results for each isolated target organism respectively, it was noted that for seven isolates conflicting results were obtained for all identification techniques [PCR (16S rRNA and species specific), MALDI-TOF and VITEK]. Further analysis will thus be required to confirm the identity of these isolates. However, for the presumptive 70 target isolates it was also noted that the results obtained for 43 (61%) of the isolates were comparable for all three identification techniques and for the remaining 20 isolates (29%), two techniques had comparable results. The Cochran's Q test results however showed that a significant difference between the three identification techniques in identifying *E. coli* and *K. pneumoniae* was obtained and the null hypothesis was rejected. However, the pairwise comparison of PCR versus VITEK indicated that there was no difference (p = 1) between these two techniques in the identification of *E. coli*. In contrast, the pairwise comparison of PCR

versus VITEK for the identification of *K. pneumoniae* resulted in a p = 0.004678, indicating that there was a significant difference between these two techniques in identifying this organism. In addition, while the pairwise comparison of MALDI-TOF MS versus 16S rRNA PCR also showed that there was a significant difference between these two techniques in identifying *K. pneumoniae* isolates, the pairwise comparison of MALDI-TOF MS vs. VITEK (p = 0.057780) indicated that there is no significant difference between these two techniques in the identification of the *K. pneumoniae* isolates.

4.2 The correlation between genes that confer antibiotic resistance and the antibiotic resistance profiles of bacteria at a municipal wastewater treatment plant in Stellenbosch, South Africa.

For the 44 *E. coli* isolates obtained throughout the study period, SNPs were found in nine isolates (*$CCA_1$9, $CCA_2$1, $CCA_2$2, $CCA_3$8, $CCA_1$18, $CCA_2$18, $CCA_3$14, $CCA_3$17* and *$CCA_3$18*), within either the DNA gyrase gene or topoisomerase gene, respectively. Two isolates had mutations within the hot spots of the QRDR, (Hopkins et al., 2005; Yoshida et al., 1990 and Kumagai et al., 1996). Isolate *$CCA_1$9* had a mutation on the *parC* gene at codon Gly78, however results from the VITEK susceptibility test showed that the organism was still susceptible to quinolone. This is due to the fact that topoisomerase IV is a secondary target for quinolones resistance in Gram-negatives (Khodursky et al., 1995) and mutations in the *parC* gene of *E. coli* have always been consistent with mutations detected on the *gyrA* gene. Mutations that occur on the *parC* enhance the resistance that is primarily conferred by mutations on the hot spots of the *gyrA* gene (Hopkins et al., 2005). Since this isolate did not have any missense mutations within the *gyrA* gene, quinolone susceptibility was not unexpected. Isolate *$CCA_3$18* was found to have a mutation on the *gyrA* gene at codon Asp87 and no mutation was found on the *parC* gene. The results from the VITEK susceptibility test showed that the organism was resistant to quinolone. The resistance could be caused since this missense mutations occurs in the QRDR found within the N-terminus of the *gyrA* gene and specifically at Asp87 (Hopkins et al., 2005 and Yoshida et al., 1990), which is positioned near the active site of the DNA gyrase gene.

For the 20 *K. pneumoniae* isolates obtained throughout the study period, the carbapenem resistant gene (*bla_{KPC}* gene) was detected in five *K. pneumoniae* isolates using the PCR technique. The *bla_{KPC}* gene was detected on the genomic DNA for all five *K. pneumoniae* isolates (*$KSA_2$11, $KSA_2$16, $KSA_2$23, $KSA_2$24* and *$KSA_3$13).* Furthermore, the gene was also detected on the plasmid DNA of three of the same *K. pneumoniae* isolates (*$KSA_2$23, $KSA_2$24* and *$KSA_3$13*). Comparison of the VITEK 2 system resistance profiles showed that the same five *K. pneumoniae* isolates (*$KSA_2$11, $KSA_2$16, $KSA_2$23, $KSA_2$24* and *$KSA_3$13*) exhibited resistance

to carbapenem. A sixth isolate (*KSA$_3$14)* which was not positive for the presence of the *bla*$_{KPC}$ gene, also showed carbapenem resistance. Research shows that there are other non-enzymatic mechanisms in which *K. pneumoniae* can be resistant to carbapenems (Netikul and Kiratisin, 2015). A decrease in expression of outer membrane proteins (OMPs), especially *OmpK35* and *OmpK36* porins, has been shown to be associated with increased minimum inhibitory concentrations (MICs) of both cephalosporins and carbapenems in *K. pneumoniae*. This could explain why even though isolate *KSA$_3$14* did not have the *bla*$_{KPC}$ gene, it was still resistant to carbapenem.

For the six *S. aureus* isolates obtained throughout the study period, the methicillin resistant gene was detected on plasmid DNA in one *S. aureus* isolate (*MSA$_3$2)* using the PCR technique. The VITEK 2 system did not however, detect any resistant profiles in any of the six *S. aureus* isolates against methicillin. Harbouring the *mecA* gene is not sufficient for methicillin resistance as some *S. aureus* (<2%) strains with the *mecA* gene are susceptible to methicillin (Hiramatsu, 1995). The expression of methicillin resistance is determined by the regulatory components that control the expression of the β-lactamase genes (*blaI, blaRI, blaZ*) which, because of sequence similarities, also can down regulate *mecA* gene transcription (Haddadin, 2002).

Resistant profiles were detected most frequently in the *K. pneumoniae* isolates. From the 20 *K. pneumoniae* isolates, the carbapenem-resistant gene was detected in five *K. pneumoniae* isolates using the PCR technqiue. All five carbapenem-resistant genes were detected on the genomic DNA of *KSA$_2$11, KSA$_2$16, KSA$_2$23, KSA$_2$24* and *KSA$_3$13,* however on the plasmid DNA the carbapenem-resistant gene was detected in only three *K. pneumoniae* isolates (*KSA$_2$23, KSA$_2$24* and *KSA$_3$13*). On the other hand the VITEK detected resistant profiles on six *K. pneumoniae* isolates (*KSA$_2$11, KSA$_2$16, KSA$_2$23, KSA$_2$24, KSA$_3$13* and *KSA$_3$14*). From the six *S. aureus* isolates, the methicillin-resistant gene was detected in one *S. aureus* isolate using the PCR technique. The methicillin-resistant gene was not detected on the genomic DNA however, the methicillin-resistant gene was detected on the plasmid DNA of *MSA$_3$2*. The VITEK system did not detect any resistance profiles in any of the six *S. aureus* isolates. From this result it can be suggested that the presence of an antibiotic gene, is not necessarily an indication of antibiotic resistance. For the 44 *E. coli* isolates, mutated genes (DNA gyrase and topoisomerase) were found in nine isolates (*CCA$_1$9, CCA$_2$1, CCA$_2$2, CCA$_3$8, CCA$_1$18, CCA$_2$18, CCA$_3$14, CCA$_3$17* and *CCA$_3$18*). Two isolates had mutations on the hot spots of the QRDR. Isolate *CCA$_1$9* had a mutation on the *parC* gene on codon Gly78, however results from the VITEK susceptibility test showed that the organism is susceptible to quinolone. This is due to the fact that topoisomerase

IV is a secondary target for quinolones-resistance in Gram-negatives (Khodursky et al., 1995) and mutations in the *parC* gene of *E. coli* has always been consistent with mutations detected on the *gyrA* gene. Mutations that occur on the *parC* enhance the resistance that is primarily conferred by mutations on the hot spots of the *gyrA* gene (Hopkins et al., 2005). Isolate *$CCA_3$18* was found to have a mutation on the *gyrA* gene on codon Asp87, and results from the VITEK susceptibility test showed that the organism is resistant to quinolone.

Results for the McNemar test showed that there was no significant difference between detecting an antibiotic resistant gene on the genomic DNA and the expression of the gene and between detecting an antibiotic resistant gene on the plasmid DNA and the expression of that gene. Furthermore there is also no significant difference between detecting an antibiotic resistant gene on the genomic DNA and detecting the same antibiotic resistant gene on the plasmid DNA. Thirty present (30%) of the isolated *K. pneumoniae* isolates (6/20) were found to be resistant to carbapenems, while only 2% of the *E. coli* isolates (1/44) were found to be resistant to quinolones and 17% *S. aureus* isolates (1/6) were found to be resistant to methicillin.

Though *E. coli* was detected at all the sampling sites throughout the study period, the quinolone resistant isolate, which had a mutation on the hot spot of the *gyrA* gene, was detected in the secondary settling tank. No *E. coli* isolates with a mutation on the target genes or that had a quinolone resistant profile was detected at the effluent point. *Klebsiella pneumoniae* isolates were also detected at all sampling sites. Two *K. pneumoniae* isolates with the bla_{KPC} genes and carbapenem resistance profiles were detected at the aeration tank, one *K. pneumoniae* isolate that did not have the bla_{KPC} genes but showed carbapenem resistance was also detected at the aeration tank. One *K. pneumoniae* isolate with the bla_{KPC} gene and a carbapenem resistance profile was detected at the secondary settling tank and two *K. pneumoniae* isolates with the bla_{KPC} gene and a carbapenem resistant profile was detected in the effluent. While the current study did not readily detect ARBs and ARGs within the Stellenbosch WWTP (within the four sites), recent studies have shown that they are detected in wastewater samples and studies have shown that the difference and degrees of WWTP designs and treatment process does have an effect on the fate of ARB in wastewater (Bouki et al., 2013). A study by Gatica et al., 2015 confirmed that WWTPs significantly decrease the levels of bacterial population in the wastewater, however, relatively high levels of antibiotic resistant bacteria and antibiotic resistant genes are being released from the WWTPs. These were detected in effluent samples and soil samples that are downstream from the WWTP. These resistant elements can persist in the environment for long periods.

The WWTP design does not only have an effect on the quality of wastewater treatment but also has an effect on the fate of antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs). In recent studies ARB and ARGs have been detected in wastewater samples and studies have shown that the difference and degrees of WWTP designs and treatment process does have an effect on the fate of ARB in wastewater (Bouki et al., 2013). Antibiotic resistance is considered to be amongst the most significant public health threats of the twenty-first century. Previously antibiotic resistance was considered to be limited to hospitals and other clinical facilities. But now there is a growing insight that it is also associated with environmental reservoirs. Wastewater treatment plants are considered to be significant reservoirs of antibiotic resistance and that there are high possibilities that bacteria in the wastewater may acquire resistance. Wastewater combines extremely high levels of faecal and environmental bacteria together with high concentrations of antibiotic compounds that are believed to induce selection.

CHAPTER FIVE

CONCLUSION

CHAPTER FIVE

5. CONCLUSION

Wastewater treatment plants have been suspected to be potential reservoirs for antibiotic resistance development. The presence of antibiotic resistant strains in treated wastewater is a huge factor that may be relevant to public health if significantly high antibiotic resistance bacteria are continually released into the environment. The effluents from urban WWTP are alleged to be among the main anthropogenic sources for the environmental spread of antibiotics, antibiotic resistant genes (ARGs) and antibiotic resistant bacteria (ARB). A recent study conducted by Kristiansson et al. (2011), showed that the effluent streaming from a WWTP in India promoted the development and emergence of multi-resistant genes in the river into which it streamed into and elements associated with genetic mobility were detected in significantly higher frequencies down-stream from the treatment plant. This further proves that WWTPs are potential reservoirs for antibiotic resistance development and spread. Antibiotic resistance development during the treatment processes in an alarm but a bigger concern is the antibiotic resistant bacteria that are released from the WWTPs into the environment.

5.1 MALDI-TOF Mass Spectrometry versus Biomerieux VITEK 2 and Molecular Techniques for the Identification of *Escherichia coli, Klebsiella pneumoniae* and *Staphylococcus aureus* in Wastewater

The MALDI-TOF MS technique positively identified an average of 63% of the target bacteria to species level for all isolates obtained for the duration of the sampling period. In addition, the MALDI-TOF MS identification technique was the least cost-effective and test repetition was required for certain organisms in order to confirm results. Furthermore, results obtained using this method suggests that the technique could not differentiate between organisms that are closely related, namely *K. pneumoniae* and *K. oxytoca* (Patel, 2012). Moreover, if the protein profile of the organism tested was not in the database, unreliable results were produced (Biswas and Rolain, 2013). On performance and speed MALDI-TOF MS systems for bacterial identification is fast, has less procedural steps and the turnaround time from isolation to obtaining final results is approximately 24 hours. Thus based on the results obtained, the MALDI-TOF MS would be preferable when identifying *E. coli* and slow growing bacteria.

The VITEK 2 system identified an average of 83% of the target bacteria to species level and this identification technique was found preferable based on the speed of analysis. The VITEK 2 identification test was also found to have better identification accuracy levels/percentages than the MALDI-TOF MS technique. Advantages include a closed system that avoids cross

contamination and environmental contamination, the ability to simultaneously perform tests on more than one sample and easy preparation and loading of the bacterial samples (Funke and Funke-Kissling, 2004). It is recommended, that the VITEK 2 identification system be used in microbiology laboratories that need fast, accurate and species level identification of bacteria. The VITEK 2 system also provides traceability, safety and minimal biohazardous waste.

However, for other activities such as research projects, that are more focused on accuracy rather than turnaround time, the 16s rRNA amplification gene sequencing technique is recommended as in the current study the 16S rRNA gene sequencing technique identified the highest percentage (98%) of the target bacteria to species level for all cycles.. The results obtained from the species specific PCR also correlated well with the 16S rRNA PCR results. Thus it is recommended that 16S RNA PCR be employed for bacterial identification. This technique is cost-effective, can identify slow-growing or fastidious bacteria and a great number of bacteria can be identified simultaneously.

5.2 The correlation between geneS that confer antibiotic resistance and the antibiotic resistance profiles of bacteria at a municipal wastewater treatment plant in Stellenbosch, South Africa

Out of the 70 isolates investigated for the presence and expression of antibiotic resistance 15 isolates (21.4%) were positive for either missense mutations within the QRDR (*E. coli*) or the presence of genes that conferred antibiotic resistance (*K. pneumonia* and *S. aureus*). However, only seven isolates (10%) actually exhibited antibiotic resistance when analysing the VITEK 2 system results. Within this study, the PCR technique was used to detect the presence of antibiotic resistance gens or mutations within regions. The technique can thus not give an indication of resistance, but merely allows for amplification of target area to analyse nucleotide sequences and the presence of genes or mutations within regions that could confer resistance. This however, must be confirmed with techniques that analyse resistance profiles, like the VITEK 2 system. When using the VITEK 2 system to detect antibiotic resistance, it was found that the testing system gave rapid, reliable, and highly reproducible results.

Certain limitations were however, experienced when utilising the PCR technique for the detection of the resistance genes. Some PCR products produced a very faint band which could not be adequately sequenced and thus yielded an unreliable BLAST analysis result. However when using the VITEK 2 system such limitations were not encountered. In contrast, even though the VITEK 2 susceptibility test yielded rapid, reliable, and highly reproducible results, it couldn't identify whether the resistance was mediated from the genomic DNA or plasmid DNA. Results obtained in the current study thus indicated that both the PCR analysis as well as the VITEK 2

system susceptibility technique should be utilised in combination to obtain a complete antibiotic resistance profile for various bacterial strains

Two of the eight isolated target bacteria that were found to be resistant against the respective antibiotics were identified at the effluent point. Wastewater Treatment Plants (WWTPs) are designed to eliminated or greatly reduce the bacterial percentage in the wastewater. It is important to select wastewater treatment processes that reduce pathogen numbers. Effective pathogen removal requires a very careful design of treatment processes that has to be established since several pathogen groups have to be removed to varying degrees and this must be achieved at the lowest possible cost, in developing countries. During the three stages of wastewater treatment, considerable variation occurs in the spread of the bacterial population and it was observed that during treatment though the bacteria were not completely eliminated, there was a reduction of bacterial population observed in the number of isolated pathogens from site one (influent) to site four (effluent) (Sim et al., 2010). Most municipal wastewater treatment facilities use primary and secondary levels of treatment only, but some also use tertiary treatments. The type and order of treatment may vary from one treatment plant to another.

Factors that may have an effect on the level or quality of wastewater treatment at the Stellenbosch Waterworks include the fact that this WWTP is designed to receive 20 400 000 litres per day (20.4 Ml/d). However, during summer (dry weather) it is reported to receive an average flow of 18 Ml/d, while during winter (wet weather) it is reported to receive an average flow of 26 Ml/d (Kloppers, 2015). Thus, the average water reception during wet weather exceeds the volume that the WWTP is designed to effectively treat by an average of 6 Ml/d (6 000 000 litres per day). This may a contributor to the wastewater possibly being inadequately treated. Furthermore, the WHO (2006) stated that it is critical that WWTPs reduce pathogen levels before wastewater is used for crop irrigation. Since, the effluent from the Stellenbosch Waterworks flows into the Veldwachters River which streams into the Eerste River and the water of this river is used by neighbouring agricultural farms for irrigation purposes, it was important that the effluent be screened for the presence of antibiotic strains and antibiotic resistant bacteria.

REFERENCES

Advance Biotech, 2015. Wastewater History - Old to Modern Treatments.. [online] Available at: http://www.adbio.com/wastewater/ww_history.htm [Accessed 9 Oct. 2015].

Adzitey, F. and Corry, J. (2011). A Comparison between Hippurate Hydrolysis and Multiplex PCR for Differentiating *Campylobacter coli* and *Campylobacter jejuni*. Tropical Life Sciences Research, [online] 22(1), p.91. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3819090/ [Accessed 17 Mar. 2015].

Adzitey, F., Huda, N. and Ali, G. (2012). Molecular techniques for detecting and typing of bacteria, advantages and application to foodborne pathogens isolated from ducks. 3 Biotech, 3(2), pp.97-107.

Alekshun, M. and Levy, S. (2007). Molecular Mechanisms of Antibacterial Multidrug Resistance. Cell, 128(6), pp.1037-1050.

Alli, O., Akinloye, O., Rowley, D. and Butcher, P. (2010). A comparative assessment of ribosomal DNA polymorphisms in methicillin resistant *Staphylococcus aureus* (MRSA) epidemiology. African Journal of Biomedical Research, 10(2). pp. 117–125.

Alonso, J., Soriano, A., Amoros, I. and Ferrus, M. (1998). Quantitative determination of *E. coli* , and fecal coliforms in water using a chromogenic medium. Journal of Environmental Science and Health, Part A, 33(6), pp.1229-1248.

Alt, S., Mitchenall, L., Maxwell, A. and Heide, L. (2011). Inhibition of DNA gyrase and DNA topoisomerase IV of *Staphylococcus aureus* and *Escherichia coli* by aminocoumarin antibiotics. Journal of Antimicrobial Chemotherapy, 66(9), pp.2061-2069.

Altschul, S. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, 25(17), pp.3389-3402.

Anastasi, E., Matthews, B., Gundogdu, A., Vollmerhausen, T., Ramos, N., Stratton, H., Ahmed, W. and Katouli, M. (2010). Prevalence and Persistence of *Escherichia coli* Strains with Uropathogenic Virulence Characteristics in Sewage Treatment Plants. Applied and Environmental Microbiology, 76(17), pp.5882-5886.

Anastasi, E., Matthews, B., Stratton, H. and Katouli, M. (2012). Pathogenic *Escherichia coli* Found in Sewage Treatment Plants and Environmental Waters. Applied and Environmental Microbiology, 78(16), pp.5536-5541.

Ansari, M., Grohmann, E. and Malik, A. (2008). Conjugative plasmids in multi-resistant bacterial isolates from Indian soil. Journal of Applied Microbiology, 104(6), pp.1774-1781.

Ateba, C. and Bezuidenhout, C. (2008). Characterisation of *Escherichia coli O157* strains from humans, cattle and pigs in the North-West Province, South Africa. International Journal of Food Microbiology, 128(2), pp.181-188.

Autiero, I., Costantini, S. and Colonna, G. (2009). Modeling of the Bacterial Mechanism of Methicillin-Resistance by a Systems Biology Approach. PLoS ONE, 4(7), p.e6226.

Bachoual, R., Tankovic, J. and Soussy, C. (1998). Analysis of the Mutations Involved in Fluoroquinolone Resistance of In Vivo and In Vitro Mutants of *Escherichia coli*. Microbial Drug Resistance, 4(4), pp.271-276.

Barker, W. (1998). The PIR-International Protein Sequence Database. Nucleic Acids Research, 26(1), pp.27-32.

Berg, R. (2014). SPSS Tutorials | SPSS Cochran Q Test. [online] Spss-tutorials.com. Available at: http://www.spss-tutorials.com/spss-cochran-q-test/ [Accessed 17 Jun. 2015].

Bettelheim, K. and Goldwater, P. (2014). Serotypes of Non-O157 Shigatoxigenic <i>*Escherichia coli*</i> (STEC). Advances in Microbiology, 04(07), pp.377-389.

BioMérieux, 2008. Resistance to antibiotics. [online] Available at: http://www.biomerieux.com/en/resistance-antibiotics [Accessed 11 Mar. 2015].

Biswas, S. and Rolain, J. (2013). Use of MALDI-TOF mass spectrometry for identification of bacteria that are difficult to culture. Journal of Microbiological Methods, 92(1), pp.14-24.

Börjesson, S., Melin, S., Matussek, A. and Lindgren, P. (2009). A seasonal study of the *mecA* gene and *Staphylococcus aureus* including methicillin-resistant *S. aureus* in a municipal wastewater treatment plant. Water Research, 43(4), pp.925-932.

Bouki, C., Venieri, D. and Diamadopoulos, E. (2013). Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: A review. Ecotoxicology and Environmental Safety, 91, pp.1-9.

Bourbeau, P. and Ledeboer, N. (2013). Automation in Clinical Microbiology. Journal of Clinical Microbiology, 51(6), pp.1658-1665.

Bratu, S. (2005). Carbapenemase-producing *Klebsiella pneumoniae* in Brooklyn, NY: molecular epidemiology and in vitro activity of polymyxin B and other agents. Journal of Antimicrobial Chemotherapy, 56(1), pp.128-132.

Brink, A., Coetzee, J., Clay, C., Sithole, S., Richards, G., Poirel, L. and Nordmann, P. (2011). Emergence of New Delhi Metallo-Beta-Lactamase (NDM-1) and *Klebsiella pneumoniae* Carbapenemase (KPC-2) in South Africa. Journal of Clinical Microbiology, 50(2), pp.525-527.

Brown, T. 2002. *Genomes*, New York: Bios Scientific Publishers.

Calderaro, A., Arcangeletti, M., Rodighiero, I., Buttrini, M., Gorrini, C., Motta, F., Germini, D., Medici, M., Chezzi, C. and De Conto, F. (2014). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry applied to virus identification. Scientific Reports, 4, p.6803.

Centers for Disease Control and Prevention, 2015. Biggest Threats| Antibiotic/Antimicrobial Resistance | CDC. [online] Available at: http://www.cdc.gov/drugresistance/biggest_threats.html [Accessed 3 Dec. 2015].

Chakravorty, S., Helb, D., Burday, M., Connell, N. and Alland, D. (2007). A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. Journal of Microbiological Methods, 69(2), pp.330-339.

Chitnis, V., Chitnis, S., Vaidya, K., Ravikant, S., Patil, S. and Chitnis, D. (2004). Bacterial population changes in hospital effluent treatment plant in central India. Water Research, 38(2), pp.441-447.

Choffnes, E., Relman, D. and Mack, A. (2010). Antibiotic resistance. Washington, D.C.: National Academies Press.

Clara, M., Strenn, B., Gans, O., Martinez, E., Kreuzinger, N. and Kroiss, H. (2005). Removal of selected pharmaceuticals, fragrances and endocrine disrupting compounds in a membrane bioreactor and conventional wastewater treatment plants. Water Research, 39(19), pp.4797-4807.

Clark, A., Kaleta, E., Arora, A. and Wolk, D. (2013). Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry: a Fundamental Shift in the Routine Practice of Clinical Microbiology. Clinical Microbiology Reviews, 26(3), pp.547-603

Clarridge, J. (2004). Impact of 16S rRNA Gene Sequence Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases. Clinical Microbiology Reviews, 17(4), pp.840-862.

Costanzo, S., Murby, J. and Bates, J. (2005). Ecosystem response to antibiotics entering the aquatic environment. Marine Pollution Bulletin, 51(1-4), pp.218-223.

Dalhoff, A. (2012). Global Fluoroquinolone Resistance Epidemiology and Implictions for Clinical Use. Interdisciplinary Perspectives on Infectious Diseases, 2012, pp.1-37.

Davies, J. and Davies, D. (2010). Origins and Evolution of Antibiotic Resistance. Microbiology and Molecular Biology Reviews, 74(3), pp.417-433.

Deguchi, T., Yasuda, M., Nakano, M., Ozeki, S., Kanematsu, E., Nishino, Y., Ishihara, S. and Kawada, Y. (1997). Detection of mutations in the *gyrA* and *parC* genes in quinolone- resistant clinical isolates of *Enterobacter cloacae*. Journal of Antimicrobial Chemotherapy, 40(4), pp.543-549.

Dizbay, M., Guzel Tunccan, O., Karasahin, O. and Aktas, F. (2014). Emergence of carbapenem-resistant *Klebsiella* spp. infections in a Turkish university hospital: epidemiology and risk factors. J Infect Dev Ctries, 8(01).

Drawz, S. and Bonomo, R. (2010). Three Decades of -Lactamase Inhibitors. Clinical Microbiology Reviews, 23(1), pp.160-201.

Dunn, O.J. and Clark, V.A., 1974. Analysis of Variance and Regression. Wiley, New York, pp.

99-114.

Emedexpert, 2012. Penicillins. [online] Available at: http://www.emedexpert.com/compare/penicillins.shtml [Accessed 11 Mar. 2015].

Emedicine, 2014. *Klebsiella* Infections. [online] Available at: http://emedicine.medscape.com/article/219907-overview [Accessed 11 Mar. 2015].

Food and Agriculture Organization, 1997. Chapter 2 - Health risks associated with wastewater use. [online] Available at: http://www.fao.org/docrep/w5367e/w5367e04.htm [Accessed 11 Mar. 2015].

Fenner, J., Lemieux, A., Cobb, D., Savastio, R., Smith, M., Ukinski, T. and Smith, M. (2013). Gene Shuffling Microbes Dominate Lake of Antarctica. [online] Guardian Liberty Voice. Available at: http://guardianlv.com/2013/10/gene-shuffling-microbes-dominate-lake-of-antarctica/ [Accessed 11 Mar. 2015].

Fridman, O., Goldberg, A., Ronin, I., Shoresh, N. and Balaban, N. (2014). Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. Nature, 513(7518), pp.418-421.

Funke, G. and Funke-Kissling, P. (2004). Evaluation of the New VITEK 2 Card for Identification of Clinically Relevant Gram-Negative Rods. Journal of Clinical Microbiology, 42(9), pp.4067-4071.

Garau J, Xercavins M, Rodriguez-Carballeira M, Gomez-Vera JR, Coll I, Vidal D, Llovet T, Ruiz-Bremon A. (1999)Emergence and dissemination of quinolone-resistant *Escherichia coli* in the community. Antimicrobial Agents and Chemotherapy, pp.2736–2741.

Gatica, J., Kaplan, E. and Cytryn, E. (2015). Antibiotic Resistance Elements in Wastewater Treatment Plants: Scope and Potential Impacts. The Handbook of Environmental Chemistry.

Ghosh, S., Ramsden, S. and LaPara, T. (2009). The role of anaerobic digestion in controlling the release of tetracycline resistance genes and class 1 integrons from municipal wastewater treatment plants. Applied Microbiology and Biotechnology, 84(4), pp.791-796.

Goldstein, R., Micallef, S., Gibbs, S., Davis, J., He, X., George, A., Kleinfelter, L., Schreiber, N., Mukherjee, S., Sapkota, A., Joseph, S. and Sapkota, A. (2012). Methicillin-Resistant *Staphylococcus aureus* (MRSA) Detected at Four U.S. Wastewater Treatment Plants. Environmental Health Perspectives, 120(11), pp.1551-1558.

Goni-Urriza, M., Capdepuy, M., Arpin, C., Raymond, N., Caumette, P. and Quentin, C. (2000). Impact of an Urban Effluent on Antibiotic Resistance of Riverine *Enterobacteriaceae* and *Aeromonas* spp. Applied and Environmental Microbiology, 66(1), pp.125-132.

Google Maps, (2015). GPS: -33.9447525, 18.8231056. [online] Available at: https://www.google.co.za/maps/@-33.9447525,18.8231056,665m/data=!3m1!1e3 [Accessed 18 Mar. 2015].

Griffith, J. (2001). Moving WebWord > Statistically Analysing Success Rates in WebUsability Testing: The Cochran's Q Test. [online] Webword.com. Available at: http://webword.com/moving/cochransq.html [Accessed 17 Jun. 2015].

Guardabassi, L., Lo Fo Wong, D. and Dalsgaard, A. (2002). The effects of tertiary wastewater treatment on the prevalence of antimicrobial resistant bacteria. Water Research, 36(8), pp.1955-1964.

Gulkowska, A., Leung, H., So, M., Taniyasu, S., Yamashita, N., Yeung, L., Richardson, B., Lei, A., Giesy, J. and Lam, P. (2008). Removal of antibiotics from wastewater by sewage treatment facilities in Hong Kong and Shenzhen, China. Water Research, 42(1-2), pp.395-403.

Haddadin, A. (2002). Methicillin resistant Staphylococcus aureus (MRSA) in the intensive care unit. Postgraduate Medical Journal, 78(921), pp.385-392.

Hao, R., Qiu, S., Wang, Y., Yang, G., Su, W., Song, L., Zhang, J., Chen, J., Jia, L., Wang, L. and Song, H. (2012). Quinolone-Resistant *Escherichia coli O127a*:K63 Serotype with an Extended-Spectrum-Beta-Lactamase Phenotype from a Food Poisoning Outbreak in China. Journal of Clinical Microbiology, 50(7), pp.2450-2451.

Hawkey, P. (1998). The origins and molecular basis of antibiotic resistance. BMJ, 317(7159), pp.657-660.

Hiramatsu, K. (1995). Molecular Evolution of MRSA. Microbiology and Immunology, 39(8), pp.531-543.

Hopkins, K., Davies, R. and Threlfall, E. (2005). Mechanisms of quinolone resistance in *Escherichia coli* and *Salmonella*: Recent developments. International Journal of Antimicrobial Agents, 25(5), pp.358-373.

Hoshino, K., Kitamura, A., Morrissey, I., Sato, K., Kato, J. and Ikeda, H. (1994). Comparison of inhibition of *Escherichia coli* topoisomerase IV by quinolones with DNA gyrase inhibition. Antimicrobial Agents and Chemotherapy, 38(11), pp.2623-2627.

Ibe, C. (2014). Antibiotic Resistance Patterns and Plasmid Profiles of Methicillin Resistant *Staphylococcus aureus* Isolates from Human Samples. British Microbiology Research Journal, 4(2), pp.185-194.

Ito, C., Gales, A., Tognim, M., Munerato, P. and Dalla Costa, L. (2008). Quinolone-resistant *Escherichia coli*. Brazilian Journal of Infectious Diseases, 12(1), pp. 5-9

Janda, J. and Abbott, S. (2007). 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. Journal of Clinical Microbiology, 45(9), pp.2761-2764.

Joyanes, P., del Carmen Conejo, M., Martinez-Martinez, L. and Perea, E. (2001). Evaluation of the VITEK 2 System for the Identification and Susceptibility Testing of Three Species of Nonfermenting Gram-Negative Rods Frequently Isolated from Clinical Samples. Journal of Clinical Microbiology, 39(9), pp.3247-3253.

Juiz, P., Almela, M., Melción, C., Campo, I., Esteban, C., Pitart, C., Marco, F. and Vila, J. (2011). A comparative study of two different methods of sample preparation for positive blood cultures for the rapid identification of bacteria using MALDI-TOF MS. European Journal of Clinical Microbiology & Infectious Diseases, 31(7), pp.1353-1358.

Kateete, D., Kimani, C., Katabazi, F., Okeng, A., Okee, M., Nanteza, A., Joloba, M. and Najjuka, F. (2010). Identification of *Staphylococcus aureus*: DNase and Mannitol salt agar improve the efficiency of the tube coagulase test. Annals of Clinical Microbiology and Antimicrobials, 9(1), pp.23-30.

Khodursky, A., Zechiedrich, E. and Cozzarelli, N. (1995). Topoisomerase IV is a target of quinolones in *Escherichia coli*. Proceedings of the National Academy of Sciences, 92(25), pp.11801-11805.

Kim, S. and Aga, D. (2007). Potential Ecological and Human Health Impacts of Antibiotics and Antibiotic-Resistant Bacteria from Wastewater Treatment Plants. Journal of Toxicology and Environmental Health, Part B, 10(8), pp.559-573.

Kim, S., Jensen, J., Aga, D. and Weber, A. (2007). Tetracycline as a selector for resistant bacteria in activated sludge. Chemosphere, 66(9), pp.1643-1651.

Kim, S., Roh, K., Yoon, Y., Kang, D., Lee, D., Kim, M. and Sohn, J. (2012). Necrotizing fasciitis involving the chest and abdominal wall caused by *Raoultella planticola*. *BMC Infect Dis*, 12(1), p.59.

Klebsiella-pneumoniae, 2014. *Klebsiella pneumoniae*. [online] Available at: http://*klebsiella-pneumoniae*.org/ [Accessed 11 Mar. 2015].

Kivaisi, A. (2001). The potential for constructed wetlands for wastewater treatment and reuse in developing countries: a review. Ecological Engineering, 16(4), pp.545-560.

Kloppers, V. 2015. *Wastewater Treatment Plant, Stellenbosch*, e-mail to S. Yakobi [Online], 8 June 2015. Availible e-mail: Vivian.Kloppers@stellenbosch.gov.za

Kohanski, M., Dwyer, D. and Collins, J. (2010). How antibiotics kill bacteria: from targets to networks. Nature Reviews Microbiology, 8(6), pp.423-435.

Kolpin, D., Furlong, E., Meyer, M., Thurman, E., Zaugg, S., Barber, L. and Buxton, H. (2002). Response to Comment on “Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999−2000: A National Reconnaissance”. Environmental Science & Technology, 36(18), pp.4007-4008.

Kumagai, Y., Kato, J., Hoshino, K., Akasaka, T., Sato, K. and Ikeda, H. (1996). Quinolone-Resistant Mutants of *Escherichia coli* DNA Topoisomerase IV *parC* Gene. Antimicrobial Agents and Chemotherapy, 40(3), pp710-714.

Lamikanra, A., Crowe, J., Lijek, R., Odetoyin, B., Wain, J., Aboderin, A. and Okeke, I. (2011). Rapid evolution of fluoroquinolone-resistant *Escherichia coli* in Nigeria is temporally associated with fluoroquinolone use. BMC Infectious Diseases, 11(1), pp.312-321.

Leavitt, A., Chmelnitsky, I., Colodner, R., Ofek, I., Carmeli, Y. and Navon-Venezia, S. (2009). Ertapenem Resistance among Extended-Spectrum- -Lactamase-Producing *Klebsiella pneumoniae* Isolates. Journal of Clinical Microbiology, 47(4), pp.969-974.

Leclercq, R., Oberle, K., Galopin, S., Cattoir, V., Budzinski, H. and Petit, F. (2013). Changes in Enterococcal Populations and Related Antibiotic Resistance along a Medical Center-Wastewater Treatment Plant-River Continuum. Applied and Environmental Microbiology, 79(7), pp.2428-2434.

Lederberg, J. (1993). Emerging infections: microbial threats to health. Trends in Microbiology, 1(2), pp.43-44.

Li, S., Liu, J., Xiang, Y., Wang, Y., Lee, W. and Zhang, Y. (2014). Therapeutic Potential of the Antimicrobial Peptide OH-CATH30 for Antibiotic-Resistant Pseudomonas aeruginosa Keratitis. *Antimicrobial Agents and Chemotherapy*, 58(6), pp.3144-3150.

Ligozzi, M., Bernini, C., Bonora, M., de Fatima, M., Zuliani, J. and Fontana, R. (2002). Evaluation of the VITEK 2 System for Identification and Antimicrobial Susceptibility Testing of Medically Relevant Gram-Positive Cocci. Journal of Clinical Microbiology, 40(5), pp.1681-1686.

Limbago, B., Rasheed, J., Anderson, K., Zhu, W., Kitchel, B., Watz, N., Munro, S., Gans, H., Banaei, N. and Kallen, A. (2011). IMP-Producing Carbapenem-Resistant *Klebsiella pneumoniae* in the United States. Journal of Clinical Microbiology, 49(12), pp.4239-4245.

Ling, T., Liu, Z. and Cheng, A. (2003). Evaluation of the VITEK 2 System for Rapid Direct Identification and Susceptibility Testing of Gram-Negative Bacilli from Positive Blood Cultures. Journal of Clinical Microbiology, 41(10), pp.4705-4707.

Ling, T., Tam, P., Liu, Z. and Cheng, A. (2001). Evaluation of VITEK 2 Rapid Identification and Susceptibility Testing System against Gram-Negative Clinical Isolates. Journal of Clinical Microbiology, 39(8), pp.2964-2966.

Liu, B., Liao, X., Yang, S., Wang, X., Li, L., Sun, J., Yang, Y., Fang, L., Li, L., Zhao, D. and Liu, Y. (2012). Detection of mutations in the *gyrA* and *parC* genes in *Escherichia coli* isolates carrying plasmid-mediated quinolone resistance genes from diseased food-producing animals. Journal of Medical Microbiology, 61(11), pp.1591-1599.

Liu, G. (2009). Molecular Pathogenesis of *Staphylococcus aureus* Infection. Pediatr Res, 65(5 Part 2), pp.71R-77R.

Liu, Y., Liu, C., Zheng, W., Zhang, X., Yu, J., Gao, Q., Hou, Y. and Huang, X. (2008). PCR detection of *Klebsiella pneumoniae* in infant formula based on *16S–23S internal transcribed spacer*. International Journal of Food Microbiology, 125(3), pp.230-235.

Liu, Z., Shi, X. and Pan, F. (2007). Species-specific diagnostic marker for rapid identification of *Staphylococcus aureus*. Diagnostic Microbiology and Infectious Disease, 59(4), pp.379-382.

Loff, M., Mare, L., de Kwaadsteniet, M. and Khan, W. (2014). 3M™ Molecular Detection system versus MALDI-TOF mass spectrometry and molecular techniques for the identification of *Escherichia coli 0157:H7, Salmonella* spp. & *Listeria* spp. Journal of Microbiological Methods, 101, pp.33-43.

Lupo, A., Coyne, S. and Berendonk, T. (2012). Origin and Evolution of Antibiotic Resistance: The Common Mechanisms of Emergence and Spread in Water Bodies. Frontiers in Microbiology, 3(18), pp. 10-23.

Macheboeuf, P., Contreras-Martel, C., Job, V., Dideberg, O. and Dessen, A. (2006). Penicillin Binding Proteins: key players in bacterial cell cycle and drug resistance processes. FEMS Microbiology Reviews, 30(5), pp.673-691.

Mandal, A. (2010). What is *Staphylococcus aureus*. [online] News-Medical.net. Available at: http://www.news-medical.net/health/What-is-Staphylococcus-Aureus.aspx [Accessed 11 Mar. 2015].

Mara, D. and Horan, N. 2003. *Handbook of water and wastewater microbiology.* London: Academic Press.

Marais E, Aithma N, Perovic O, Oosthuysen W F, Musenge E, Dusé A G. (2009). Antimicrobial susceptibility of methicillin-resistant *Staphylococcus aureus* isolates from South Africa. Original articles - South African Medical Journal, 99 (3), pp. 56-64

Marcinek H, Wirth R, Muscholl-Silberhorn A, Gauer M. (1998). *Enterococcus faecalis* gene transfer under natural conditions in municipal sewage water treatment plants. Applied and Environmental Microbiology, pp.626-632

Martinez, J. and Baquero, F. (2000). Mutation Frequencies and Antibiotic Resistance. Antimicrobial Agents and Chemotherapy, 44(7), pp.1771-1777.

Marvin, L., Roberts, M. and Fay, L. (2003). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in clinical chemistry. Clinical Chemical Acta, 337(1-2), pp.11-21.

Maslow, J. and Arbeit, R. (1993). Relationship between indole production and differentiation of *Klebsiella* species: indole-positive and -negative isolates of *Klebsiella* determined to be clonal. Journal of Clinical Microbiology, [online] 31(8), p.2000. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC265686/ [Accessed 12 Mar. 2015].

McArdell, C., Molnar, E., Suter, M. and Giger, W. (2003). Occurrence and Fate of Macrolide Antibiotics in Wastewater Treatment Plants and in the Glatt Valley Watershed, Switzerland. Environmental Science & Technology, 37(24), pp.5479-5486.

McGinnis, S. and Madden, T. (2004). BLAST: at the core of a powerful and diverse set of sequence analysis tools. Nucleic Acids Research, 32(Web Server), pp.W20-W25.

McKay, J. and Moeller, A. (2000). Statutory Regulation of Water Quality in Modern Australia. Water International, 25(4), pp.595-609.

Mignard, S. and Flandrois, J. (2006). 16S rRNA sequencing in routine bacterial identification: A 30-month experiment. Journal of Microbiological Methods, 67(3), pp.574-581.

Monnet, D. and Freney, J. (1994). Method for differentiating *Klebsiella planticola* and *Klebsiella terrigena* from other *Klebsiella* species. Journal of Clinical Microbiology, [online] 32(4), p.1121. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC267203/ [Accessed 12 Mar. 2015].

Monstein, H., Tärnberg, M. and Nilsson, L. (2009). Molecular identification of CTX-M and *blaOXY*/K1 β-lactamase genes in *Enterobacteriaceae* by sequencing of universal M13-sequence tagged PCR-amplicons. BMC Infectious Diseases, 9(1), p.7-16.

Mosca, A., Miragliotta, L., Del Prete, R., Tzakis, G., Dalfino, L., Bruno, F., Pagani, L., Migliavacca, R., Piazza, A. and Miragliotta, G. (2013). Rapid and sensitive detection of *blaKPC* gene in clinical isolates of *Klebsiella pneumoniae* by a molecular real-time assay. SpringerPlus, 2(1), p.31-36.

Moter, A. and Göbel, U. (2000). Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. Journal of Microbiological Methods, 41(2), pp.85-112.

Müller, E. (2001). The occurrence of *E. coli O157:H7* in South African water sources intended for direct and indirect human consumption. Water Research, 35(13), pp.3085-3088.

Munir, M., Wong, K. and Xagoraraki, I. (2011). Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. Water Research, 45(2), pp.681-693.

Murray, P. (2012). What Is New in Clinical Microbiology—Microbial Identification by MALDI-TOF Mass Spectrometry. The Journal of Molecular Diagnostics, 14(5), pp.419-423.

Naas, T., Cuzon, G., Villegas, M., Lartigue, M., Quinn, J. and Nordmann, P. (2008). Genetic Structures at the Origin of Acquisition of the -Lactamase *blaKPC* Gene. Antimicrobial Agents and Chemotherapy, 52(4), pp.1257-1263.

Nada, B. and Areej, M. (2014). *Raoultella planticola*, a central venous line exit site infection. Journal of Taibah University Medical Sciences, 9(2), pp.158-160.

Nagy, E., Becker, S., Kostrzewa, M., Barta, N. and Urban, E. (2012). The value of MALDI-TOF MS for the identification of clinically relevant anaerobic bacteria in routine laboratories. Journal of Medical Microbiology, 61(10), pp.1393-1400.

Namboodiri, S., Opintan, J., Lijek, R., Newman, M. and Okeke, I. (2011). Quinolone resistance in *Escherichia coli* from Accra, Ghana. BMC Microbiol, 11(1), p.44.

Nature Publishing Group, 2014. *Some Organisms Transmit Genetic Material to Offspring without Cell Division*. [online] Available at: http://www.nature.com/scitable/topicpage/some-organisms-transmit-genetic-material-to-offspring-6524963 [Accessed 11 Mar. 2015].

Netikul, T. and Kiratisin, P. (2015). Genetic Characterization of Carbapenem-Resistant *Enterobacteriaceae* and the Spread of *Carbapenem-Resistant Klebsiella pneumonia* ST340 at a University Hospital in Thailand. *PLOS ONE*, 10(9), p.e0139116.

Nys S, Okeke IN, Kariuki S, Dinant GJ, Driessen C, Stobberingh EE: (2004). Antibiotic resistance of faecal *Escherichia coli* from healthy volunteers from eight developing countries. J Antimicrob Chemother 54(5):952-955.

Ohkusu, K. 2000. *Cost-effective and rapid presumptive identification of gram-negative*. [online] Available at: http://www.ncbi.nlm.nih.gov/pubmed/11101600. [Accessed 12 Mar. 2015].

Parvathi, A., Vijayan, J., Murali, G. and Chandran, P. (2011). Comparative Virulence Genotyping and Antimicrobial Susceptibility Profiling of Environmental and Clinical *Salmonella enterica* from Cochin, India. Current Microbiology, 62(1), pp.21-26.

Patel, R. 2012. *Bacterial Identification by MALDI TOF*. [online] Available at: http://www.mayomedicallaboratories.com/articles/hottopics/transcripts/2012/02-bactid/index.html [Accessed 12 Mar. 2015].

Pawlowski, A., Dudzinska, M. and Pawlowski, L. (2013). Environmental Engineering IV. Hoboken: CRC Press, p.159.

Pereira, V., Lopes, C., Castro, A., Silva, J., Gibbs, P. and Teixeira, P. (2009). Characterization for enterotoxin production, virulence factors, and antibiotic susceptibility of *Staphylococcus aureus* isolates from various foods in Portugal. Food Microbiology, 26(3), pp.278-282.

Pescod, M. (1992). Wastewater treatment and use in agriculture. Rome: Food and Agriculture Organization of the United Nations, pp.47-124.

Petti, C., Polage, C. and Schreckenberger, P. (2005). The Role of 16S rRNA Gene Sequencing in Identification of Microorganisms Misidentified by Conventional Methods. Journal of Clinical Microbiology, 43(12), pp.6123-6125.

Plano, L., Garza, A., Shibata, T., Elmir, S., Kish, J., Sinigalliano, C., Gidley, M., Miller, G., Withum, K., Fleming, L. and Solo-Gabriele, H. (2011). Shedding of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* from adult and pediatric bathers in marine waters. BMC Microbiol, 11(1), p.5-11.

Poretsky, R., Rodriguez-R, L., Luo, C., Tsementzi, D. and Konstantinidis, K. (2014). Strengths and Limitations of 16S rRNA Gene Amplicon Sequencing in Revealing Temporal Microbial Community Dynamics.PLoS ONE, 9(4), pp.93827-93839.

Power, J. and Calder, M. (1983). Pathogenic significance of *Klebsiella oxytoca* in acute respiratory tract infection. Thorax, 38(3), pp.205-208.

Prevention, C. (2015). *E. coli* (*Escherichia coli*)| *E. coli* | CDC. [online] Cdc.gov. Available at: http://www.cdc.gov/ecoli/ [Accessed 11 Mar. 2015].

Prince, S., Dominger, K., Cunha, B. and Klein, N. (1997). *Klebsiella pneumoniae* pneumonia. Heart & Lung: The Journal of Acute and Critical Care, 26(5), pp.413-417.

Pruden, A., Pei, R., Storteboom, H. and Carlson, K. (2006). Antibiotic Resistance Genes as Emerging Contaminants: Studies in Northern Colorado. Environmental Science & Technology, 40(23), pp.7445-7450.

Queenan, A. and Bush, K. (2007). Carbapenemases: the Versatile -Lactamases. Clinical Microbiology Reviews, 20(3), pp.440-458.

Rea, M., Dobson, A., O'Sullivan, O., Crispie, F., Fouhy, F., Cotter, P., Shanahan, F., Kiely, B., Hill, C. and Ross, R. (2010). Effect of broad- and narrow-spectrum antimicrobials on *Clostridium difficile* and microbial diversity in a model of the distal colon. Proceedings of the National Academy of Sciences, 108(Supplement_1), pp.4639-4644.

Rensselaer Polytechnic Institute, 2015. *Wastewater Treatment Principles and Regulations*. [online] Available at: http://www.rpi.edu/dept/chem-eng/Biotech-Environ/Environmental/WWTP_Principles.html [Accessed 2 July. 2015].

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M., Michael, I. and Fatta-Kassinos, D. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Science of The Total Environment, 447, pp.345-360.

Rubino, S., Cappuccinelli, P. and Kelvin, D. (2011). *Escherichia coli* (STEC) serotype O104 outbreak causing haemolytic syndrome (HUS) in Germany and France. The Journal of Infection in Developing Countries, 5(06), pp. 437-440

SA water, 2014. *Wastewater Treatment Process- SA Water*. [online] Available at: http://www.sawater.com.au/sawater/education/ourwastewatersystems/wastewater+treatment+process.htm [Accessed 11 Mar. 2015].

Sacha, P., Ostas, A., Jaworowska, J., Wieczorek, P., Ojdana, D., Ratajczak, J. and Tryniszewska, E. (2010). The *KPC* type beta-lactamases: new enzymes that confer resistance to carbapenems in Gram-negative bacilli. Folia Histochemica et Cytobiologica, 47(4), pp. 543-547

Saenz, Y. (2003). Mutations in *gyrA* and *parC* genes in nalidixic acid-resistant *Escherichia coli* strains from food products, humans and animals. Journal of Antimicrobial Chemotherapy, 51(4), pp.1001-1005.

Sajith, A. K., Preetha, J., Lakshmi, S., Anandi, C. and Ramesh, R. (2012). Detection of mecA genes of *Methicillin-Resistant Staphylococcus aureus* by Polymerase Chain Reaction. International Journal of Health and Rehabilitation Sciences, 1(2), pp. 64-68.

Sakoulas, G. and Moellering, Jr., R. (2008). Increasing Antibiotic Resistance among Methicillin-Resistant *Staphylococcus aureus* Strains. Clinical Infectious Diseases, 46(S5), pp.S360-S367.

Sauer, S. and Kliem, M. (2010). Mass spectrometry tools for the classification and identification of bacteria. Nature Reviews Microbiology, 8(1), pp.74-82.

Schembri, M., Blom, J., Krogfelt, K. and Klemm, P. (2005). Capsule and Fimbria Interaction in *Klebsiella pneumoniae*. Infection and Immunity, 73(8), pp.4626-4633.

Schlüter, A., Krause, L., Szczepanowski, R., Goesmann, A. and Pühler, A. (2008). Genetic diversity and composition of a plasmid metagenome from a wastewater treatment plant. Journal of Biotechnology, 136(1-2), pp.65-76.

Schulthess, B., Brodner, K., Bloemberg, G., Zbinden, R., Bottger, E. and Hombach, M. (2013). Identification of Gram-Positive Cocci by Use of Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry: Comparison of Different Preparation Methods and Implementation of a Practical Algorithm for Routine Diagnostics. Journal of Clinical Microbiology, 51(6), pp.1834-1840.

Shen, P., Wei, Z., Jiang, Y., Du, X., Ji, S., Yu, Y. and Li, L. (2009). Novel Genetic Environment of the Carbapenem-Hydrolyzing -Lactamase KPC-2 among *Enterobacteriaceae* in China. Antimicrobial Agents and Chemotherapy, 53(10), pp.4333-4338.

Sheskin, D. (2003). Handbook of Parametric and Nonparametric Statistical. London: Chapman & Hall/CRC.pp.870.

Shields, P. and Tsang, A. (2013). Mannitol Salt Agar Plates Protocols. [online] Microbelibrary.org. Available at: http://www.microbelibrary.org/component/resource/laboratory-test/3034-mannitol-salt-agar-plates-protocols [Accessed 12 Mar. 2015].

Sigma-Aldrich, 2013. HiCrome™ *Klebsiella* Selective Agar Base for microbiology. [online] Available at: http://www.sigmaaldrich.com/catalog/product/fluka/90925?lang=en&region=ZA [Accessed 12 Mar. 2015].

Sim, W., Lee, J. and Oh, J. (2010). Occurrence and fate of pharmaceuticals in wastewater treatment plants and rivers in Korea. Environmental Pollution, 158(5), pp.1938-1947.

Simone, B. P. (1999). Impact of *gyrA* and *parC* Mutations on Quinolone Resistance, Doubling Time, and Supercoiling Degree of *Escherichia coli*. Antimicrobial Agents and Chemotherapy, [online] 43(4),pp. 868–875

Stoppler, M. and Sheil, W. (2015). Staph Infection: Symptoms, Causes, Pictures & Treatment. [online] MedicineNet. Available at: http://www.medicinenet.com/staph_infection/article.htm [Accessed 11 Mar. 2015].

Sydnor, E. and Perl, T. (2011). Hospital Epidemiology and Infection Control in Acute-Care Settings. Clinical Microbiology Reviews, 24(1), pp.141-173.

Szczepanowski, R., Bekel, T., Goesmann, A., Krause, L., Krömeke, H., Kaiser, O., Eichler, W., Pühler, A. and Schlüter, A. (2008). Insight into the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to antimicrobial drugs analysed by the 454-pyrosequencing technology. Journal of Biotechnology, 136(1-2), pp.54-64.

Tang, Y., Procop, G. and Persing, D. (1997). Molecular diagnostics of infectious diseases. Clinical Chemistry, 43(11), pp.2021-2038.

Ternes, T., Meisenheimer, M., McDowell, D., Sacher, F., Brauch, H., Haist-Gulde, B., Preuss, G., Wilme, U. and Zulei-Seibert, N. (2002). Removal of Pharmaceuticals during Drinking Water Treatment. Environmental Science & Technology, 36(17), pp.3855-3863.

Theron, J. and Cloete, T. (2002). Emerging Waterborne Infections: Contributing Factors, Agents, and Detection Tools. Critical Reviews in Microbiology, 28(1), pp.1-26.

Todar, K. (2004). Todar's online textbook of bacteriology. [S.l.]: Kenneth Todar.

Wallet, F., Loiez, C., Renaux, E., Lemaitre, N. and Courcol, R. (2005). Performances of VITEK 2 Colorimetric Cards for Identification of Gram-Positive and Gram-Negative Bacteria. Journal of Clinical Microbiology, 43(9), pp.4402-4406.

Wang, L., Gu, H. and Lu, X. (2012). Rapid low-cost detection of *Klebsiella pneumoniae* carbapenemase genes by internally controlled real-time PCR. Journal of Microbiological Methods, 91(3), pp.361-363.

Webber, M. and Piddock, L. (2001). Erratum: Quinolone resistance in *Escherichia coli*. Veterinary Research, 32(6), pp. 275-284.

Wei, B., Cha, S., Kang, M., Park, I., Moon, O., Park, C. and Jang, H. (2013). Development and application of a multiplex PCR assay for rapid detection of 4 major bacterial pathogens in ducks. Poultry Science, 92(5), pp.1164-1170.

Who.int, (2014). WHO | Antimicrobial resistance. [online] Available at: http://www.who.int/mediacentre/factsheets/fs194/en/ [Accessed 11 Mar. 2015].

Who.int, (2006). WHO | WHO's first global report on antibiotic resistance reveals serious, worldwide threat to public health. [online] Available at: http://www.who.int/mediacentre/news/releases/2014/amr-report/en/ [Accessed 21 Aug. 2015].

Wielders, C., Fluit, A., Brisse, S., Verhoef, J. and Schmitz, F. (2002). *mecA* Gene Is Widely Disseminated in *Staphylococcus aureus* Population. Journal of Clinical Microbiology, 40(11), pp.3970-3975.

Wolk, D. and Dunne, W. (2011). New Technologies in Clinical Microbiology. Journal of Clinical Microbiology, 49(9 Supplement), pp.S62-S67.

Woodford, N. and Ellington, M. (2007). The emergence of antibiotic resistance by mutation. Clinical Microbiology and Infection, 13(1), pp.5-18.

Wu, S., de Lencastre, H. and Tomasz, A. (2001). Recruitment of the *mecA* Gene Homologue of *Staphylococcus sciuri* into a Resistance Determinant and Expression of the Resistant Phenotype in *Staphylococcus aureus*. Journal of Bacteriology, 183(8), pp.2417-2424.

Yoshida, H., Bogaki, M., Nakamura, M. and Nakamura, S. (1990). Quinolone resistance-determining region in the DNA gyrase *gyrA* gene of *Escherichia coli*. Antimicrobial Agents and Chemotherapy, 34(6), pp.1271-1272.

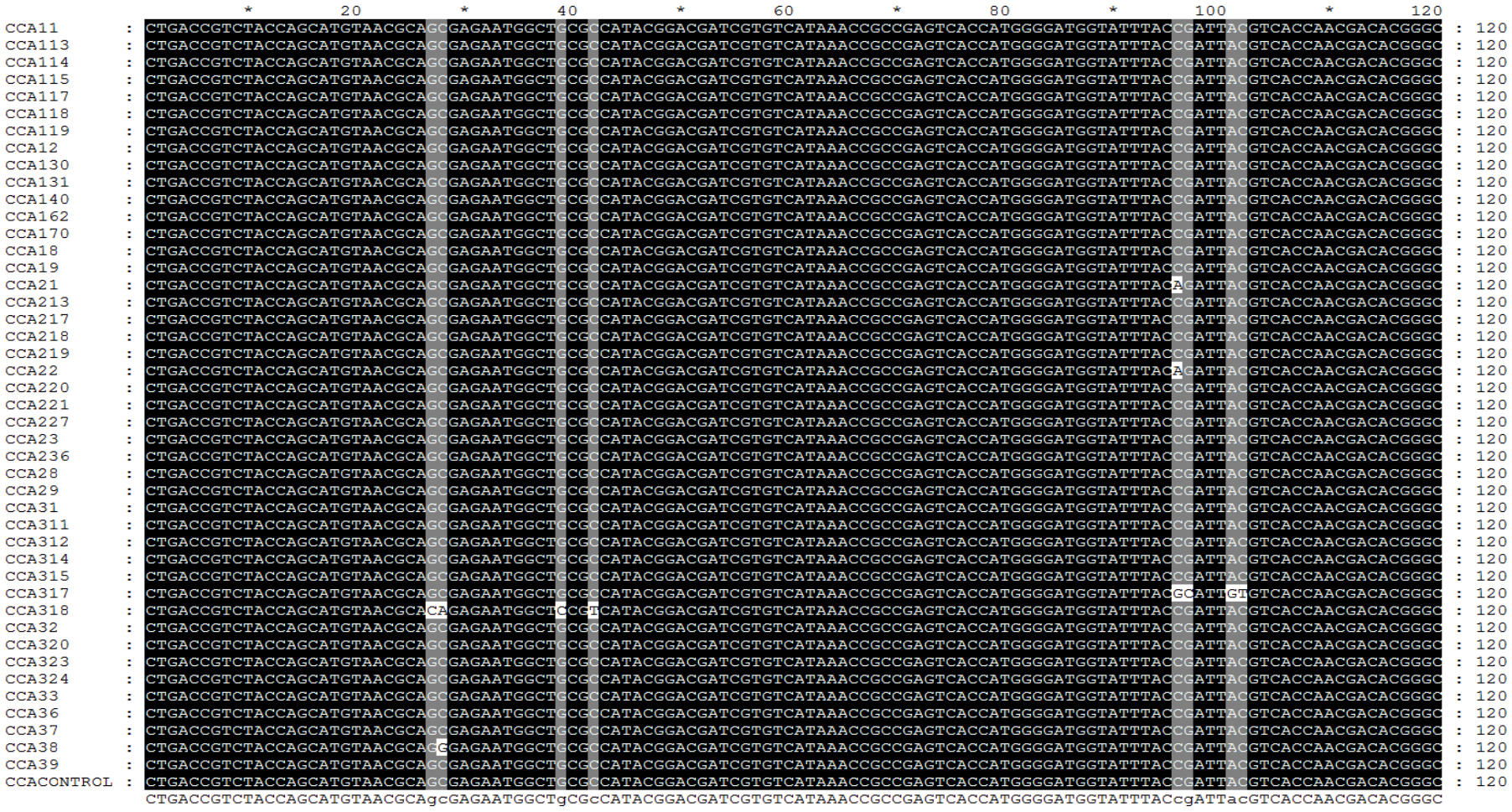
Yue, L., Jiang, H., Liao, X., Liu, J., Li, S., Chen, X., Chen, C., Lü, D. and Liu, Y. (2008). Prevalence of plasmid-mediated quinolone resistance qnr genes in poultry and swine clinical isolates of *Escherichia coli*. Veterinary Microbiology, 132(3-4), pp.414-420.

Zhang, Y., Marrs, C., Simon, C. and Xi, C. (2009). Wastewater treatment contributes to selective increase of antibiotic resistance among *Acinetobacter* spp. Science of The Total Environment, 407(12), pp.3702-3706

APPENDICES

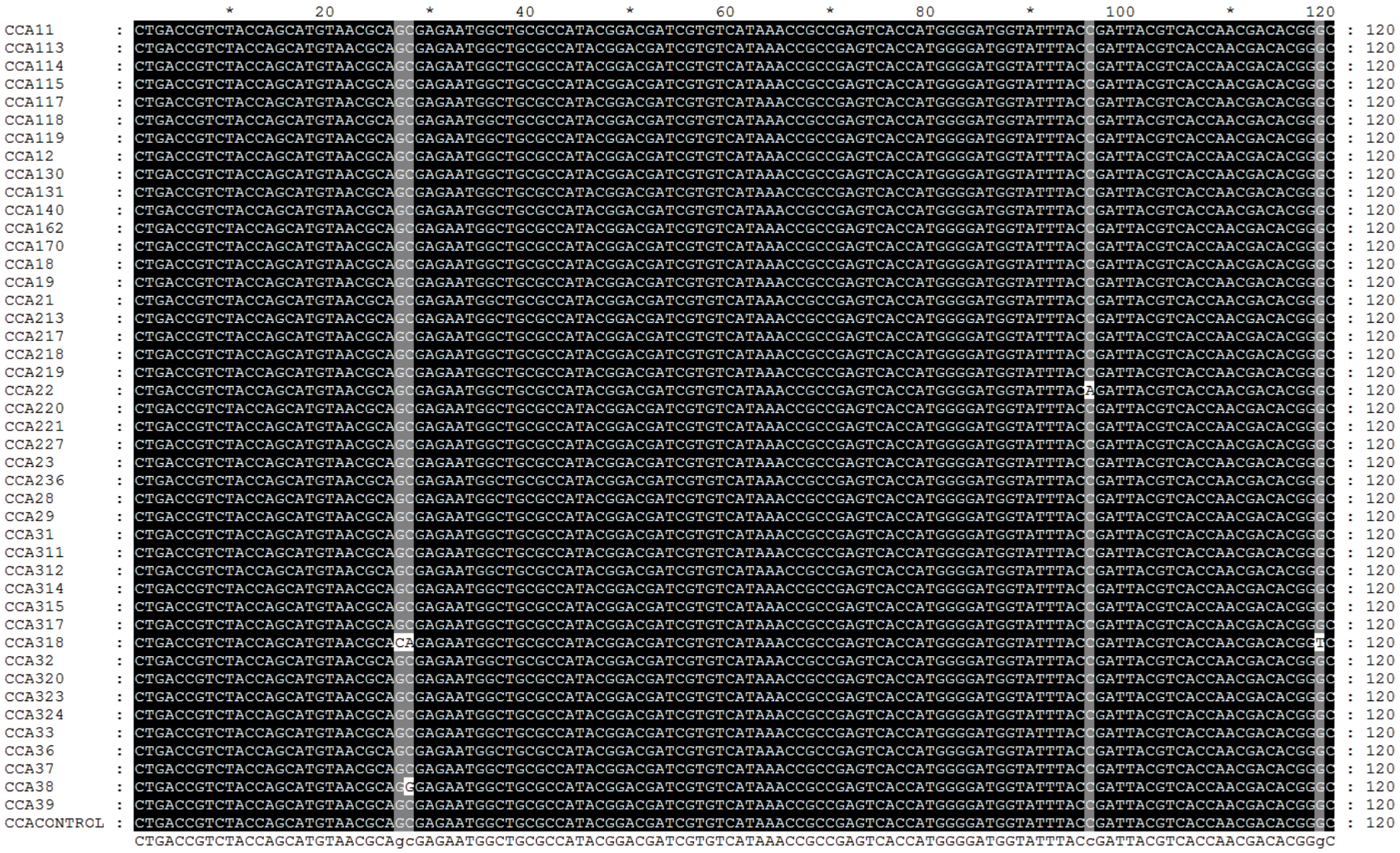
Appendix A

QRDR of *E. coli* isolates, genomic DNA sequence *gyrA* gene, from codon Ala67 to Gln106



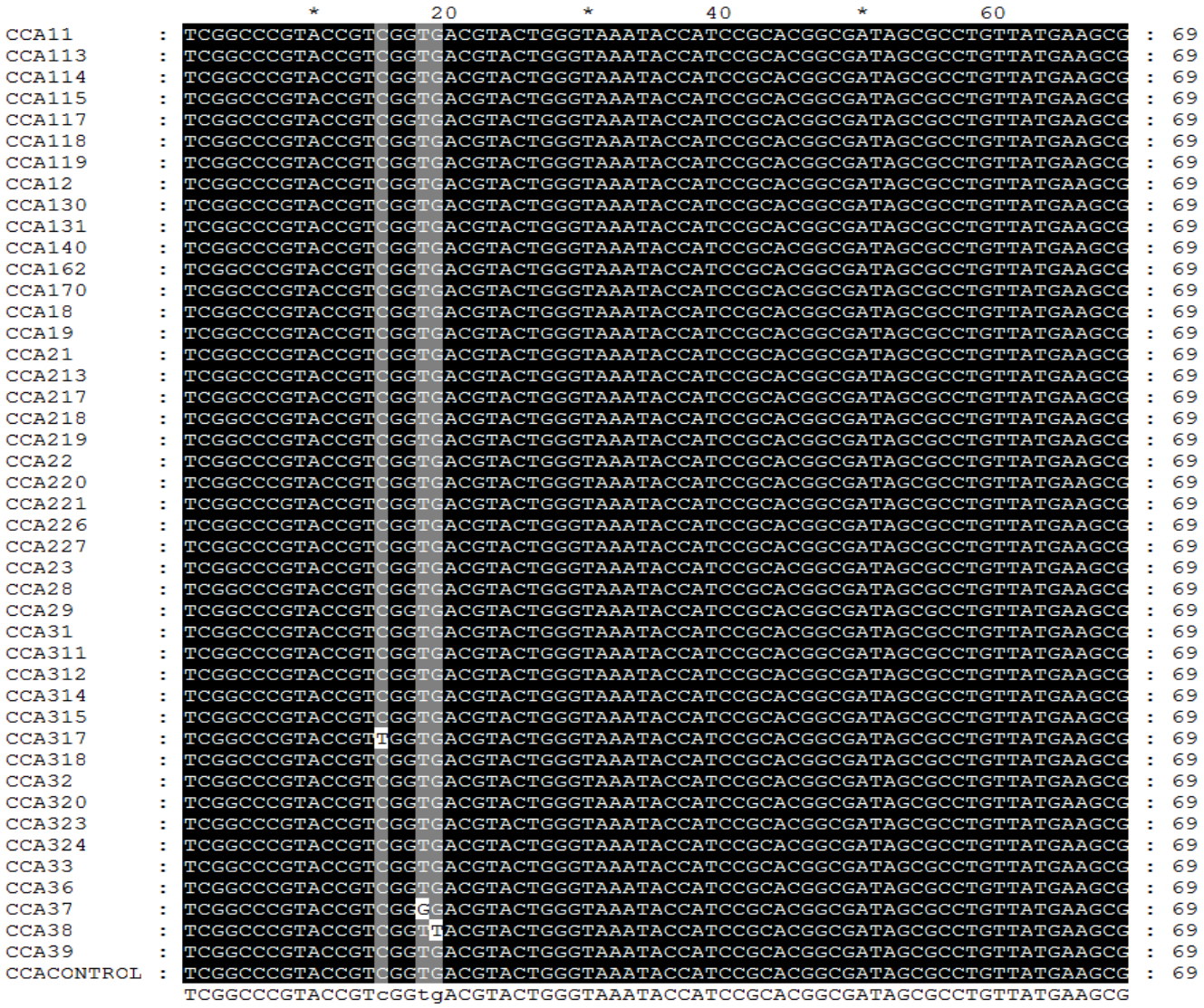
Appendix B

QRDR of *E. coli* isolates, plasmid DNA sequence *gyrA* gene, from codon Ala67 to Gln106



Appendix C

QRDR of *E. coli* isolates, genomic DNA sequence *parC* gene, from codon Ser63 to Glu84



Appendix D

QRDR of *E. coli* isolates, genomic DNA sequence *parC* gene, from codon Ser63 to Glu84