

Characterization of ESKAPE pathogens at WWTPs in the North West province of South Africa

B van Niekerk

 **orcid.org 0000-0003-3534-5394**

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Supervisor: Dr LG Molale-Tom

Co-Supervisor: Prof CC Bezuidenhout

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This Masters project presented me with various challenges, but I have one motto in life, and that is “Refuse To Lose”.

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ABSTRACT

The ESKAPE pathogens is an acronym and consists of: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterococcus* spp. The ESKAPE group of bacteria have presented an alarming increase in MDR (Multi drug resistant) and virulence and have as a consequence been put on the watch list by the World Health Organization (WHO). This study aimed to determine the prevalence and characteristics of ESKAPE pathogens that were isolated from wastewater treatment plants before and after treatment. Samples were collected from the influent and effluent of the WWTPs (Wastewater treatment plants). In order to isolate the ESKAPE pathogens, selective chromogenic media was used. Pure isolates of the presumed ESKAPE pathogens were subjected to antibiotic susceptibility and virulence tests. The present project tested for the production of DNase, lipase, protease, and hemolysin. None of the presumptive ESKAPE organisms illustrated any DNase production, whereas some did exhibit lipase and protease production. Some also exhibited β -hemolysis activity. They presented a high resistance against various antibiotics that are readily used in clinical settings. Presumptive ESKAPE pathogens were also screened for the presence of antibiotic resistance genes including *bla_{KPC}*, *bla_{SHV}*, *bla_{OXA-48}*, *bla_{CTX}*, *bla_{TEM}*, *bla_{VIM}*, FOX and MOX. All genes screened were found within the presumptive ESKAPE organisms, except FOX and *bla_{KPC}*. Since all genera associated with the ESKAPE group were identified by the present study, the results are a cause for concern, given further that most of the nosocomial infections that occur are caused by members of the ESKAPE pathogen group, notably *S. aureus* and *P. aeruginosa*. Some of the prominent health effects include bacterial pneumonia, urinary tract infections (UTIs), bacteremia and infections in soft tissue. Not only are these pathogens a cause for concern in clinical settings, but they are becoming more prevalent in the environment. This may have health implications in communities that utilize these effluent-receiving waters, giving rise to a further cause for concern, as the fact that they are populating the environment may lead to an acceleration of infections, while proper treatment or health care facilities may not be available in the rural communities directly influenced by them due the use of effluent receiving waters.

Keywords: antibiotic resistance, multidrug resistance, ARGs, ESKAPE pathogens, wastewater treatment plants.

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CHAPTER 1: INTRODUCTION

1.1 Background

The discovery of antibiotics has led to an increase in human life expectancy, but organisms that develop resistance against them are natural occurrences, which raises concern about the future (Ma *et al.*, 2020; Renner *et al.*, 2017). There are various reasons for the rapid increase in antibiotic resistance patterns in both the clinical and environmental settings, including over-prescription, overuse in agricultural settings and misuse by patients (Ma *et al.*, 2020). The ESKAPE pathogens group contains the following organisms: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterococcus* spp. (Mulani *et al.*, 2019). These are opportunistic pathogens that cause a wide range of infections within the gastrointestinal tract, urinary tract, respiratory system and soft tissue, while they are also extensively associated with high levels of antibiotic resistance (Odjadjare *et al.*, 2012; Renner *et al.*, 2017).

The development of new antibiotics has substantially slowed down over the years. For instance, only 11 new antibiotics were developed between 2017 and 2019 (De Oliveira *et al.*, 2020). The low rate of antibiotic development is worrisome because the ESKAPE organisms are rapidly growing more resistant towards clinically utilized antibiotics. These ESKAPE organisms utilize a wide range of resistance mechanisms to increase their survival rate, including binding site modification, drug deactivation, mutations or changes in cell permeability (Denissen *et al.*, 2022; Pandey *et al.*, 2021). They have been identified by the World Health Organization (WHO) as a special focus point for the development of new antibiotics, because of their pathogenicity traits and rapid increase in antibiotic resistance (Mulani *et al.*, 2019; De Oliveira *et al.*, 2020).

Wastewater treatment plants (WWTPs) in South Africa remove most of the organic matter from the water, on which the treated water flows back into rivers and are reintroduced into the environment (Ntombela *et al.*, 2016). South African WWTPs are experiencing various challenges, including a lack of technical skills among operators and mismanagement of funds. These aspects cause WWTPs not to meet the standards set by the Department of Water and Sanitation (DWS) (DWS, 2022a; Ntombela *et al.*, 2016). The requirement for a Green Drop report was set in place to audit WWTPs in South Africa so that struggling ones could be identified and strategies implemented to increase their effectiveness (DWS, 2022a; Ntombela *et al.*, 2016). A new Green Drop evaluation was conducted in 2021 and published

in 2022 that illustrated that these plants were in a poor state (DWS; 2022a). Compared to a valuation done in 2013, it was clear that, by 2021, their state were declining: in 2013, five plants were in an average state, 12 were in a poor state and 104 were in a critical state. In contrast, in 2021 two were in an average state, 11 in a poor one and 102 in a critical one (DWS; 2022a). The latter pose a risk to public health caused by the release of improperly treated wastewater back into the environment (DWS, 2022a).

Although *Escherichia coli* numbers are reduced from the influent to the effluent of WWTPs there is still some being released in the effluent of the WWTPs, thus others organisms could evaded treatment and be present in the effluent (Anastasi *et al.*, 2012). This reduction in *E. coli* illustrates that WWTPs do possess the ability to reduce the bacterial load of hazardous wastewater, but they do not completely remove all of the organisms (Anastasi *et al.*, 2012). Because there is sometimes *E. coli* still present in the effluent, it might indicate that some ESKAPE pathogens could moves through the WWTPs and end up in the effluent.

On top of the fact that the WWTPs are in poor condition, these organisms are known to escape most forms of treatment thus it is possible that they could leak into the environment (Slekovec *et al.*, 2012). These ESKAPE pathogens are known to adapt and survive in various environments, including water, sewage, soil and even within or on plants, thus increasing the problematic effect that they may have on communities (Odjadjare *et al.*, 2012; De Oliveira *et al.*, 2020).

They are not just a reason for concern in the clinical health sector, but have been found in environmental reservoirs such as water sources, soil, WWTPs and even, again, in or on plants and other food products (Denissen *et al.*, 2022). These organisms have various strategies to survive unfavourable conditions and accumulate in the environment (Denissen *et al.*, 2022; Odjadjare *et al.*, 2012). They have been shown to be opportunistic pathogens and include some of the most common organisms causing nosocomial infections in patients (Denissen *et al.*, 2022; Ma *et al.*, 2020). These ESKAPE pathogens illustrates high levels of antibiotic resistance, thus treatment is not always available and this increases the mortality rate related to infections that they cause (Denissen *et al.*, 2022; Ma *et al.*, 2020). These organisms have the potential to escape the treatments of patients at hospitals and are subsequently released in the wastewater to reach the WWTPs (De Oliveira *et al.*, 2020; Odjadjare *et al.*, 2012; Slekovec *et al.*, 2012). There they may escape treatment and can be introduced to the environment (Denissen *et al.*, 2022; Ntombela *et al.*, 2016).

Consider that South Africa is reliant on surface water for domestic and industrial use (OECD, 2011). It is a water-scarce country due to a low rainfall average of 500 mm per year (DWS,

2022b). The water quality of its surface waters has been declining because of the large amounts of wastewater effluent that are being reintroduced back into rivers (Verlicchi & Vittoria, 2022). This and rising levels of greywater can lead to harmful ESKAPE organisms that enter and accumulate in the environment, increasing the risk of infections (Denissen *et al.*, 2022).

Developing countries such South Africa are known to make use of untreated open river systems as water sources for household activities (Bakari, 2017; Nkosi *et al.*, 2021). The presence and accumulation of ESKAPE organisms in the river waters of South Africa can cause widespread infections in communities that use these effluent-receiving water for drinking, agriculture or recreation (Denissen *et al.*, 2022; Rosenberg Goldstein *et al.*, 2012).

1.2 Aim and objectives

This study aimed to determine the prevalence and characteristics of ESKAPE pathogens isolated from two wastewater treatment plants in the North-West Province of South Africa before and after treatment.

The objectives of this study were:

- To determine the presence of ESKAPE pathogens at WWTPs.
- To determine the extracellular enzyme activity of the isolated organisms that might pertain to potential pathogenicity
- To identify and determine the antibiotic resistance profiles of the isolated ESKAPE pathogens as well as the presence of selected ARGs.
- To determine if these potentially pathogenic environmentally isolated ESKAPE organisms may have environmental health implications.

CHAPTER 2: LITERATURE REVIEW

2.1 The current state of WWTP in SA

South African WWTPs aim to remove inorganic compounds, biodegradable organic compounds and pathogenic microorganisms from the water so that it can safely flow into rivers again (Ntombela et al., 2016; USAID, 2015). The basic process that the WWTPs use is a four-step treatment that consists of preliminary-, primary-, secondary- and tertiary treatment (USAID, 2015). Preliminary treatment aims to remove large suspended materials such as garbage bags, logs, glass, and other large solid particles (Crini & Lichtfouse, 2018; USAID, 2015). During the primary treatment process, fats and other organic suspended and heavy solids, sink to the bottom of the clarifier tanks while floating solids are removed by skimmers (USAID, 2015). Secondary treatment comprises, among others, activation of biofilters or sludge, while parameters like ammonia and other remaining organic matter that was left in suspension after primary treatment is removed (Crini & Lichtfouse, 2018; USAID, 2015). These substances are mixed with microorganisms that break down some of the remaining organic compounds (Crini & Lichtfouse, 2018; USAID, 2015).

Tertiary treatment is the last phase of the process before the water is discharged back into the environment. During this phase, the remaining organisms and chemicals that can cause environmental eutrophication are removed (Crini & Lichtfouse, 2018; USAID, 2015). This process can use chemicals to remove other chemical compounds such as nitrogen and phosphorus, while also removing potential pathogens either by using UV radiation, ozonation or chlorination (USAID, 2015; WRC, 2015). The wastewater that underwent treatment is released into the environment once the water has been disinfected and tested, or when it is within the necessary limits of permitted faecal coliforms. (Crini & Lichtfouse, 2018; USAID, 2015).

Seventy percent (70%) of South Africa's wastewater treatment plants (WWTP) are small or medium-sized and get overloaded because of the increase in sewage that flows into them (Ntombela et al., 2016). Overloading of the plants is not the only problem centred on WWTPs in South Africa, a lack of funding and technical expertise of operators aggravates the situation (Ntombela et al., 2016). Due to a lack of urban planning and funding means that they are not upgraded to effectively treat the increased sewage from settlements (Ntombela et al., 2016).

The Green Drop programme aims to monitor the performance of WWTPs, in doing this the main problems can be identified and focused on by the management of the various WWTPs in South Africa (DWS, 2022a; Ntombela *et al.*, 2016). As indicated, a large gap was found between the Green Drop reports of 2021 and 2013 (DWS, 2022a). In the report for 2013, a total of 121 systems were audited, and it was found that five WWTPs performed on average standards, whereas 12 showed poor performance and 104 were in critical condition (DWS, 2022a; DWS, 2013). The new report that was conducted in 2021 and released in 2022 audited a total of 115 systems: it was found that two WWTPs were in average condition, 11 in a poor state and 102 in critical condition (DWS, 2022a). From 2013 to 2021, not one of the WWTPs that were audited achieved an excellent or good score. The Green Drop report of 2021 found that, because these treatment plants had not complied with the effluent quality standards, it has a high risk of impacting public health and the effluent-receiving environment (DWS, 2022a).

2.2 ESKAPE pathogens

ESKAPE pathogens comprises of opportunistic pathogens that commonly cause nosocomial infections: they develop high levels of resistance against various antibiotics that are currently used to treat infections (Ma *et al.*, 2020; Mulani *et al.*, 2019; Tolera *et al.*, 2018). The ESKAPE group consists of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. (Santajit & Indrawattana, 2016). Due to overuse of common antibiotics in the clinical and agricultural settings organisms are becoming increasingly resistant (Larsson & Flach, 2022; Mulani *et al.*, 2019). Most of the nosocomial infections are caused by these and, because of the MDR, they "escape" treatment with antimicrobial and antibiotic agents (Mulani *et al.*, 2019).

Consequently, in 2017 the World Health Organization (WHO) listed the ESKAPE organisms among the 12 bacterial pathogens that have a rapid increase in antibiotic resistance (Mulani *et al.*, 2019). It was determined in 2010 that the ESKAPE pathogens have developed complete resistance against commonly used antibiotics, and new data states that they are increasing their resistance (Mulani *et al.*, 2019; Orosz *et al.*, 2022).

Microorganisms have been developing antibiotic resistance at a much higher rate in recent years, since antibiotics are misused in the clinical setting, as indicated, and are also overused in agriculture thus environments are being polluted with antibiotic waste (Larsson & Flach, 2022). This increase of the antibiotics in the environment increases the rate at

which mutations occur causing new bacterial phenotypes to emerge: thus the microorganisms become increasingly resistant to antibiotics (Larsson & Flach, 2022; Polianciuc *et al.*, 2020; Woodford & Ellington, 2007). The genes developed by these organisms that cause their antibiotic resistance also move between species, hence increasing antibiotic resistance within the microbial community (Larsson & Flach, 2022; Polianciuc *et al.*, 2020).

WWTPs are in fact known reservoirs of antibiotic-resistance genes shared among various organisms, thus increasing their resistance, even after having passed through the treatment process (Piotrowska *et al.*, 2019). This means that ESKAPE organisms are becoming an ever-increasing threat to health of humans, animals and the environment (Denissen *et al.*, 2022; Ramsamy *et al.*, 2018a).

2.3 ESKAPE pathogens characteristics and prevalence in the environment

ESKAPE pathogens as indicated, occur in various environments, and can escape some forms of treatment such as water treatment (physical and chemical treatment) and antibiotic treatment of infected humans and animals (Dekic *et al.*, 2019; Henriot *et al.*, 2019; Karimi *et al.*, 2016; Mulani *et al.*, 2019; Slekovec *et al.*, 2012; Thapaliya *et al.*, 2017). They are known to be opportunistic pathogens and may cause infections in soft tissue, the respiratory system, the gastrointestinal tract and the urinary tract. There is an increase in the use of reclaimed wastewater for irrigation in certain parts of the world and the high prevalence of ESKAPE pathogens in the wastewater shows that there is a possibility that they will enter or have already entered the environment, where they may cause infections in certain communities (Rosenberg Goldstein *et al.*, 2012).

2.3.1 *Enterococcus faecium*

Enterococcus faecium forms part of the natural flora within the gut of animals and humans (Lebreton *et al.*, 2014). It is also an opportunistic pathogen that resists a wide range of antibiotics (Gilmore *et al.*, 2014; McBride *et al.*, 2010). *E. faecium* is more resistant to antibiotics than *E. faecalis*, which is another *Enterococcus* species that causes infections. The antibiotic profiles that have been seen in *E. faecium* are reason for considerable concern because they show that the bacterium has grown resistant to antibiotics such as penicillins, glycopeptides and aminoglycosides (Higueta & Huycke, 2014; McBride *et al.*, 2010). Resistance against vancomycin and other new groups of antibiotics such as daptomycin, linezolid and quinupristin/ dalfopristin has been noted in recent years: clearly this will cause problems in future (Higueta & Huycke, 2014; McBride *et al.*, 2010). *E. faecium*

causes a wide range of diseases, including pelvic infections, urinary tract infections (UTIs), intra-abdominal infections, infections in soft tissue and, most commonly, bacteremia and endocarditis (Higuita & Huycke, 2014; Bharti *et al.*, 2016). The mortality rate for the bacteremia that it causes is much higher than other *Enterococci* because of its broad range resistance to antibiotics. Most of the deaths related to this bacteremia occur in patients that have underlying illnesses.

Moreover, endocarditis is an infection caused by *Enterococci* that needs to be attended to immediately because of its seriousness: even when the least resistant *Enterococci* are involved in the infection treatment can be difficult (Higuita & Huycke, 2014). Endocarditis is a heart valve infection, and it occurs most frequently among elderly people or people with underlying heart diseases.

For the treatment of *Enterococcus faecium* infections, a variety of antibiotics can be used but, because of the high resistance that *E. faecium* shows, they need to be chosen carefully (Higuita & Huycke, 2014; Garbutt *et al.*, 2000). McBride *et al.* (2010) demonstrated that amoxicillin was a good treatment for bacteremia, but the risk and the costs involved should be analysed before blindly administering this, as stated by Garbutt *et al.* (2000).

Treatment for enterococcal infections is fairly easy when the right antibiotics are administered but, in the cases where *Enterococci* causes endocarditis, treatment is much harder (Higuita & Huycke, 2014). For this treatment, two drugs that synergize with each other are administered. Without the use of multidrug therapy, treatments are usually ineffective. In cases where the organisms are highly resistant to aminoglycosides, the antibiotic treatment often fails: the patients need to undergo surgery so that the infected valve can be removed (Higuita & Huycke, 2014).

E. faecium has been found in various environments, including beach sand, plant crops and wastewater before and after treatment (Byappanahalli *et al.*, 2012; Karimi *et al.*, 2016). Karimi *et al.* (2016) showed that it is present within hospital wastewater before and after treatment, and the potential for the organisms to escape into the environment does exist. This means that they should be watched closely, because they have the potential to survive for long periods in water and soil, increasing the health risk to the public posed by environmental contamination with *E. faecium* (Karimi *et al.*, 2016).

2.3.2 *Staphylococcus aureus*

Staphylococcus aureus forms part of the natural flora of animals and humans but it is also known to be an opportunistic pathogen that has caused an increase in mortality rates in hospitals (Balaban *et al.*, 2000; Schaumburg *et al.*, 2015). This is a cause for concern, aggravated by the fact that it is one of the leading causes of many health problems, including bloodstream infections (bacteremia), skin and soft tissue infections, osteoarticular infections (Osteomyelitis, Septic Arthritis), UTIs, nosocomial pneumonia, endocarditis and meningitis (Balaban *et al.*, 2000; Tong *et al.*, 2015).

Skin and soft tissue infections are also of a large concern when it comes to dealing with *S. aureus*. This pathogen is isolated most frequently from purulent cellulitis, cutaneous abscesses and surgical infected sites (Tong *et al.*, 2015). The three main groups of osteoarticular infections are septic arthritis, osteomyelitis and prosthetic joint infections. *S. aureus* is the leading cause of infections in these three main groups.

Osteomyelitis occurs when the bones of the patient are infected, which causes inflammatory destruction of the bone and leads to bone necrosis. A total of 30-60% of osteomyelitis cases is caused by *S. aureus*. Septic arthritis is another considerable problem caused by *S. aureus*, septic arthritis is usually a secondary infection, thus it is contracted because of bacteremia seeping from the vascular synovial lining in the joint (Tong *et al.*, 2015). Infection of prosthetics joints is also a major concern here, because it occurs in about 2% of patients that receive prosthetics and, among these, 29-39% are caused by *S. aureus*.

The pathogen is moreover a frequent culprit in endocarditis because of its high resistance to antibiotics and its high rate of infection. The number of endocarditis infections has increased in the past years from 11.4 infections per 100 000 people to 16.6; 16%-34% of cases were caused by *S. aureus* (Tong *et al.*, 2015). *S. aureus* moreover causes an abundance of health problems in patients such as nosocomial pneumonia, meningitis and epidural abscesses. All of these can be treated but, because of the overuse of antibiotics, it has become more difficult (Balaban *et al.*, 2000; Micek, 2007).

Research shows that a considerable number of *S. aureus* isolates are resistant to methicillin: MRSA (methicillin-resistant *Staphylococcus aureus*) strand of infection poses a threat to patients and different treatment strategies should be adopted by using various antibiotics to treat these infections (Liu *et al.*, 2011; Micek, 2007; Tong *et al.*, 2015). In such cases,

vancomycin has been used frequently with great success, but it was found that some infections were not treated effectively in this way, due to increased resistance of *S. aureus* against it (Micek, 2007).

Alternative antibiotics recommended for treatment include linezolid, tigecycline, tetracycline, telavancin, daptomycin, rifampin, glycopeptides, iclaprim, clindamycin, ceftobiprole and ceftaroline (Liu *et al.*, 2011; Micek, 2007). Most of these are approved by the US food and drug administration (FDA), and they hold little to no risk for patients who receive them for severe cases of infections. With a view to MRSA skin and soft-tissue infections (SSTIs), various antibiotics can be used, including linezolid, tigecycline, tetracycline and clindamycin (Liu *et al.*, 2011; Micek, 2007).

In cases where patients suffer from severe infections, antibiotics can be used in parallel with one other form of treatment, and the use of two antibiotics when treating endocarditis caused by *S. aureus* is in fact common practice (Tong *et al.*, 2015). Among patients who suffer from severe cases of endocarditis, surgery is effective, and this greatly increases survival rates. In cases where patients have prosthetic joint infections (PJIs), two strategies have been applied with success: the first is to treat patients with vancomycin or antistaphylococcus penicillin for a short course of six weeks, which enjoys a success rate of up to 70% (Tong *et al.*, 2015). The second is to prescribe a short course of vancomycin or antistaphylococcus penicillin and follow this up that treatment with three to six months of treatment with rifampin and a combination with another oral agent such as fusidic acid or ciprofloxacin. Extant research has established that the exact method of treatment for *S. aureus* infections is very important because wrongful treatments can in these cases lead to a worsening of the health implications involved.

This pathogen is known to be able to escape into the environment, causing infections in humans. Thapaliya *et al.* (2017) showed that these organisms can be found in water and sand. It is also found in higher concentrations in areas that receive treated water from WWTPs. This is alarming, because it means that not all *S. aureus* is removed during the treatment process (Thapaliya *et al.*, 2017).

Thapaliya *et al.* (2017) are not the only researchers to have found that *S. aureus* has the potential to escape WWTP treatment and enter the environment. Thompson *et al.* (2013) found that it can be found in the influent and the effluent of WWTPs.

2.3.3 *Klebsiella pneumoniae*

Klebsiella pneumoniae has been isolated from a multitude of organisms, including plants, animals and humans (Martin & Bachman, 2018). It causes a wide range of infections such as UTIs, pneumonia, bacteraemia and liver abscesses (Keynan & Rubinstein, 2007; Paczosa & Meccas, 2016; Vading *et al.*, 2018). These infections are usually most prevalent in immunocompromised patients but, in recent years, hyper virulent strains which causes health implications in various people have increased (Keynan & Rubinstein, 2007; Paczosa & Meccas, 2016; Vading *et al.*, 2018).

Klebsiella spp. is known for their high resistance to antibiotics, giving rise to an increase in the mentioned hyper virulent strains, which cause major concerns in the medical industry (Rastegar Lari *et al.*, 2013). *Klebsiella pneumoniae* has several mechanisms that enable it to be increasingly resistant to antibiotics. One of the most prevalent of these is the production of β -lactamases, which enables it to hydrolyse various antibiotics such as cephalosporins, penicillins and monobactams (Keynan & Rubinstein, 2007; Vading *et al.*, 2018). Another of these mechanisms is the expression of carbapenemase, which enables it to be carbapenem-resistant (Keynan & Rubinstein, 2007; Paczosa & Meccas, 2016). These mechanisms cause widespread problems in patients and, due to these, the mortality rate in patients have increased when they were infected by *K. pneumoniae* that produces β -lactamase, carbapenemase or both (Paczosa & Meccas, 2016; Rastegar Lari *et al.*, 2013).

The preferred antibiotics treatments for *K. pneumoniae* infections are aminoglycoside and Beta-lactams, but resistance mechanisms make treatment difficult. Various treatment methods are therefore used when the resistant *K. pneumoniae* strains are found to be present in patients (Rastegar Lari *et al.*, 2013). *K. pneumoniae* strains that produce β -lactamase are preferably treated by means of carbapenems (Keynan & Rubinstein, 2007). In the case of the production of carbapenemase, an optimal treatment has not been identified, thus making treatments much harder (Hirsch & Tam, 2010). These infections have nonetheless been treated successfully with aminoglycosides, polymyxins, tetracyclines and tigecycline. Other methods of treatment are the use of cephalosporins and β -lactamase inhibitors, the use of which will allow patients to then be treatable with β -lactam antibiotics (Hirsch & Tam, 2010).

The presence of *K. pneumoniae* in the environment is nothing new, various studies has noted it. Popa *et al.*, (2021) illustrated that multidrug-resistant *K. pneumoniae* has the potential to escape WWTP treatment and enter the environment after chlorination. Another

study found that *K. pneumonia* was present in wetlands that received water from rivers in which upstream WWTPs were present (Henriot *et al.*, 2019). Because these organisms have the potential to escape WWTP treatment and end up in the environment, more research is necessary for the successful removal of these pathogens.

2.3.4 *Acinetobacter baumannii*

The natural habitat of *Acinetobacter baumannii* still has to be determined. It has been found in various environments, including clinical and environmental environments like surface water and soil (Hrenovic *et al.*, 2014; Peleg *et al.*, 2008). It is a common cause of nosocomial infections, which is worrisome in view of the pathogen's multiple resistances against various antibiotics (Cisneros & Rodriguez-Baño, 2002). They can survive in various environments (Maragakis & Perl, 2008).

It causes a wide range of infections in patients, including bacterial pneumonia, UTIs, meningitis and blood-stream infections (Dijkshoorn *et al.*, 2007; Peleg *et al.*, 2008). As indicated, resistance against various antibiotics has been recorded. The most common among these is against all β -lactams, including carbapenems or class three and higher antimicrobials (Maragakis & Perl, 2008; Peleg *et al.*, 2008).

Some *Acinetobacter* isolates are susceptible to polymyxins, but these are not used frequently, because they are potentially toxic. Some strains have shown resistance to all antimicrobial agents, including newly developed drugs that make treatment very difficult (Abbo *et al.*, 2005; Maragakis & Perl, 2008).

This pathogen uses various mechanisms to increase its resistance against antimicrobial agents, including enzyme inactivation, reduction in access to bacterial targets and changes/ mutations in cellular functions (Maragakis & Perl, 2008; Peleg *et al.*, 2008)

As mentioned, the infections caused by these organisms are very hard to treat. Specialised treatment is needed. In cases where the infection is ventilator-associated such as pneumonia, aerosol polymyxins can be used, since resistance to these is still low (Dijkshoorn *et al.*, 2007; Maragakis & Perl, 2008). Sulbactam is a β -lactamase inhibitor. It offers an effective treatment method for *A. baumannii* infections, although it is not recommended for monotherapy (Cisneros & Rodriguez-Baño, 2002; Maragakis & Perl, 2008).

Combination therapy is used effectively for treating infections that have shown high resistance to other therapies. The most effective among these is ampicillin-sulbactam and meropenem combination therapy (Hiraki *et al.*, 2013). Another combination therapy that has been successfully applied is sulbactam combined with β -lactamase inhibitors (Dijkshoorn *et al.*, 2007). Aminoglycosides are further agents used in combination with other antimicrobial agents to treat infections, but an increase in resistance against these has been reported in *A. baumannii* (Maragakis & Perl, 2008).

It has been widely shown that *A. baumannii* has become more and more prevalent in the environment outside of hospitals (Dekic *et al.*, 2019). It has been isolated from a large variety of environments such as hospital wastewater, WWTPs, urban wastewater and rivers (Dekic *et al.*, 2019). The isolation of *A. baumannii* is a relatively new phenomenon, however, since it was mostly isolated from patients in the past (Higgins *et al.*, 2018). The increase of *A. baumannii* in the environment is a cause of concern and should be monitored closely (Dekic *et al.*, 2019; Higgins *et al.*, 2018).

2.3.5 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa causes nosocomial infections such as UTIs, pneumonia, bloodstream infections and skin infections in patients that have suffered severe burns (Driscoll *et al.*, 2007). It frequently causes infections in hospitalised patients; higher mortality rates occur in these cases (Driscoll *et al.*, 2007). It is the second most common cause of pneumonia in ICUs, including paediatric ones (Driscoll *et al.*, 2007). Given the danger that it maintains and its increasing antibacterial resistance, this pathogen will in all likelihood pose an increasing risk for patients that contract this infection.

The pathogen moves around by using a single flagellum and attaches to cell membranes and other surfaces by using type IV pili. When *P. aeruginosa* attaches to cell surfaces, pathogenic mechanisms are activated. *P. aeruginosa* is regularly found in epithelial lung tissue that has been injured before hospitalization (Driscoll *et al.*, 2007).

A variety of treatments are used to treat *P. aeruginosa* infections. However, it is commonly accepted that therapy with a single anti-pseudomonal agent can prove ineffective if the specific strain of *P. aeruginosa* is unknown. It is more effective to treat patients with presumptive *P. aeruginosa* infections by means of two different antipseudomonal agents; once the bacterial isolate has been identified, the therapy can be downscaled to a single anti-pseudomonal agent (Driscoll *et al.*, 2007).

For pneumonia caused by *P. aeruginosa*, guidelines that the American Thoracic Society (ATS) and the Infectious Disease Society of America (IDSA) formulated are available. These state that treatments should consist of two anti-pseudomonal agents from different classes, while therapy with the appropriate anti-pseudomonal agents should last for seven to eight days if the patient's health improves (Driscoll *et al.*, 2007).

Another form of treatment is to inhale anti-pseudomonal agents, which is only given to people who do not have cystic fibrosis and suffer from an acute respiratory infection only. The effectiveness of the routine use of this form of therapy is unknown, but it can be used together with other forms of therapy. Colistin and tobramycin are the agents commonly used for inhalation therapy in the case of this kind of infection (Driscoll *et al.*, 2007).

Pseudomonas is becoming more prevalent because of its ability to live on biofilms that can protect organisms from physical and chemical stress (Odjadjare *et al.*, 2012). There has been evidences that *Pseudomonas* spp. can escape the treatment of the WWTP: an increase in *P. aeruginosa* was found downstream from some WWTPs (Slekovec *et al.*, 2012). Sludge from WWTPs that contains these pathogens are also sometimes used as fertilizer, which causes them to reside in the environment (Slekovec *et al.*, 2012). After heavy rainfall, the pathogens can move into bodies of water from the runoff. Their prevalence after WWTP treatment is again alarming, because it can cause infections in rural communities that use the water received from the effluent of the WWTPs (Slekovec *et al.*, 2012).

2.3.6 *Enterobacter* spp.

Enterobacter cloacae can cause nosocomial infections in immunocompromised patients (Sanders & Sanders, 1997). The most common *Enterobacter* spp. that causes infections in humans are *E. aerogenes* and *E. cloacae*, which are mostly seen to infect patients that have to be in the hospitals for extended periods, especially in the ICU. It has been noticed that *Enterobacter* infections occur in the respiratory tract, surgical wounds, blood and the urinary tract (Sanders & Sanders, 1997).

Enterobacter spp. can be found in water and faeces of animals and humans. Infection can be acquired from an exogenous or endogenous source. Nosocomial infections seem to originate with patients previously infected with an *Enterobacter* spp. and, because of antibiotic treatment received, the normal flora within the body is suppressed; because the species are highly resistant to antibiotics, they can multiply without any other form of suppression (Sanders & Sanders, 1997).

In a paediatric ICU where ampicillin and gentamicin were initially used as the antibiotic treatment, the organisms grew resistant to the previous antibiotics, and it was found that third-generation cephalosporins proved effective for treatment (Sanders & Sanders, 1997). However, after long exposure, the organisms grew increasingly resistant to these. As a result, resistance against other β -lactam antibiotics has also developed. The said study shows the importance of identification of the bacteria before the administration of antibiotics to prevent these organisms to become increasingly resistant to antibiotics (Sanders & Sanders, 1997).

Tamma *et al.* (2013) established that cefepime and carbapenems could be used to treat some of the *Enterobacter* spp. that produced AmpC β -lactamase. Another study found that multidrug-resistant *Enterococcus* spp. were present in river water, the study was done upstream and downstream of a WWTP (Bessa *et al.*, 2014). The study showed that there was no significant release of pathogens from the WWTP and that the treatment process was successful, but high amounts of the pathogens were still encountered in the river after high amounts of rainfall (Bessa *et al.*, 2014). This shows that the pathogens are present in the environment and that the runoff into the river contributed to the increase of pathogens in the river (Bessa *et al.*, 2014).

2.4 Pathogenicity: virulence factors

Virulence factors are the attributes that pathogenic organisms utilize to successfully infect a host and colonize host organisms on a cellular level (Lantz, 1997; Sharma *et al.*, 2017). These include hemolysin, DNase, lipase and protease.

2.4.1 Hemolysin

Hemolysin is considered a virulence factor because it is produced by a range of pathogenic bacteria (Goebel *et al.*, 1988). It disrupts the cell membrane or forms holes in erythrocytes that destroy the erythrocyte, other neighbouring cells and tissue, while releasing nutrients like iron that these pathogenic bacteria utilise (Mogrovejo *et al.*, 2020). Two primary types of hemolysis take place: α -hemolysis and β -hemolysis, of which the former is known to cause complete lysis of erythrocytes causing a clearing to form around the colony producing the hemolysin (Mogrovejo *et al.*, 2020; Vesper & Vesper, 2004). α -Hemolysis forms darker zones around the growing colonies because it causes only partial lysis of the erythrocytes, while it further causes the haemoglobin to be reduced to methemoglobin (Mogrovejo *et al.*, 2020; Vesper & Vesper, 2004). The hemolysin test is done on blood agar, where bacterial

colonies on plates form different coloured zones around the colonies are used, depending on the type of hemolysis activity (Buxton, 2005; Mogrovejo *et al.*, 2020).

2.4.2 DNase

DNase is an extracellular enzyme produced by pathogenic organisms that causes the degradation of DNA (Gerceker *et al.*, 2009). The test for DNase production is done on DNase test agar. This is supplemented with Toluidine Blue O (TBO), which forms a blue colour when it is present (DB, 2006). When the DNA in the agar is hydrolysed by the DNase, the colonies will form pink zones on the agar (DB, 2006).

2.4.3 Lipase

Lipase as a virulence factor is not yet completely understood. The production of lipase by organisms can affect the immune cells of humans (Stehr *et al.*, 2003). Granulocytes are affected by lipase, and it has been noted that, when lipase is added to granulocytes, the rate of bacterial elimination by the granulocytes is lowered. Another observation that shows that lipase is a virulence factor is that anti-lipase IgG antibodies were found in patients who are infected with lipase-producing pathogens (Stehr *et al.*, 2003). The production of lipase by organisms can be determined by tributyrin agar (Millipore, 2018a). Lipase production can be seen on the agar when clear zones form around the growing colonies (Lanka & Latha, 2015).

2.4.4 Protease

Protease is produced by pathogenic organisms, and can directly interact with the patient's tissue, causing lysis of tissue and, while it can also inactivate proteins that are crucial to the immune response of the infected patient (Frees *et al.*, 2013; Lantz, 1997). To detect protease-producing bacteria, skim milk agar is used and casein in the agar is hydrolysed, which causes clear zones to form around the colonies that produce it (Venter, 2010).

2.5 Resistance mechanisms

The pathogens use a wide range of antimicrobial resistance mechanisms. Some of these are drug-altering or inactivation by producing β -lactamases, chloramphenicol acetyltransferases or aminoglycoside-modifying enzymes that inactivate antibiotics (Santajit & Indrawattana, 2016).

2.5.1 β -lactamases

β -lactamases are enzymes produced by various organisms, and their hydrolysis causes the antibiotic to be ineffective, thus enabling the bacterial cell to survive (Bush & Bradford, 2020; Ferreira *et al.*, 2011). Two systems are used to classify β -lactamases: The Ambler scheme and the Bush-Jacoby-Medeiros system. The class A Ambler enzyme can inactivate a wide range of antibiotics such as penicillins, aztreonam, cefoperazone, cefamandole, third-generation oxyiminocephalosporins and methoxy cephalosporins. The genes that code for the enzymes that cause the inactivation of the antibiotics can have high mutation rates that code genes of the SHV, while the TEM genes have very high mutation rates that cause production of a high number of enzymes, which increases the antibiotic resistance in these pathogens (Ferreira *et al.*, 2011; Santajit & Indrawattana, 2016). Class B Ambler enzymes are classified by using the Bush-Jacoby system. The Metallo- β -lactamases (MBLs) require Zn^{2+} to function, and pathogens that produce these enzymes have high resistance to all β -lactams such as β -lactamase inhibitors (except aztreonam), cephalosporins, penicillins and carbapenems. The MBLs coding genes are easily transported to other organisms, because they are found within plasmids (Santajit & Indrawattana, 2016).

Class C Ambler enzymes are composed of enzymes such as penicillinase and cephalosporins and, similar to AmpC β -lactamases, these enzymes cause resistance to cephalosporin drugs, whereas the resistance is low and only applies to a few of the cephalosporin drugs (Bush & Bradford, 2020). These enzymes do not usually cause problems during clinical treatments, because the production is usually very low in organisms such as *Pseudomonas aeruginosa* and *Enterobacter* spp.; nonetheless, these genes can still be transferred to other microorganisms via plasmids (Santajit & Indrawattana, 2016). When other organisms obtain the plasmid, overproduction of AmpC β -lactamases may occur that will cause resistance to all penicillin, aztreonam and most cephalosporins. The class D Ambler enzymes are oxacillin hydrolysing ones (OXA), and the most common enzymes of this group are OXA-11, OXA-14, and OXA-16. These enzymes show high resistance against β -lactamases, except for OXA-18 (Bush & Bradford, 2020; Ferreira *et al.*, 2011; Santajit & Indrawattana, 2016).

2.5.2 Drug binding site modification

Modification of drug binding sites is a further mechanism used to heighten resistance, where binding sites that the antibiotic targets are changed, causing various problems (Lambert, 2005; Santajit & Indrawattana, 2016). The gene that codes for penicillin-binding protease

(PBPs) mutates, and this causes the expression of new penicillin-binding protease that are found in the cytoplasmic membrane. Organisms that use this method of resistance, such as *S. aureus*, shows high resistance to cell wall biosynthesis inhibitors such as β -lactam drugs (Santajit & Indrawattana, 2016).

2.5.3 Porin reduction

Organisms in the ESKAPE pathogen group can decrease the permeability of antibiotics such as β -lactams, fluoroquinolones and chloramphenicol by reducing the amounts of porins that the organism possesses (Hooper, 2005; Kumar & Schweizer, 2005; Santajit & Indrawattana, 2016). Clinical studies have shown that certain antibiotic-resistant organisms have increased their resistance by changing and reducing their porin expression (Santajit & Indrawattana, 2016). It has been noted that organisms that possess porins from the OmpF groups that have relatively large channels, were replaced with porins from the OmpC group that has smaller channels, thus limiting the permeability of the outer membrane and increasing the antibiotic resistance of the organisms (Pagès *et al.*, 2008). Because of the reduction and changes of the porins found on the outer membrane of the organisms, the uptake of antibiotics has been influenced, thus increasing the resistance against antibiotics leaving the bacteria unaffected by certain antibiotic groups (Kumar & Schweizer, 2005)

2.5.4 Efflux pumps

Efflux pumps are used to transport toxic substances to the extracellular environment, and some organisms use these to remove antibiotics, leaving the organism unaffected (Webber & Piddock, 2003). These can be used to remove multiple antibiotics from within the cell, because the antibiotics are removed constantly, which means that the concentration within the organism to have an antibacterial effect is never sufficient (Santajit & Indrawattana, 2016). Both Gram-positive and Gram-negative bacteria utilise efflux pumps to increase their resistance, especially in the case of tetracycline resistance (Hooper, 2005). Most of the pumps that increase multidrug resistance can be located in the cytoplasmic membrane of the bacteria, which allows them to take up the toxins affecting the organisms from the cytoplasm and pump them outwards. Efflux pumps can cause considerable problems in the clinical setting, because certain genes that encode for efflux pumps can be found within plasmids. This means that any organism that successfully incorporates the plasmid will obtain resistance (Hooper, 2005). Most of the organisms of the ESKAPE group utilize at least one of the efflux pump mechanisms to increase resistance to a variety of antibiotics.

2.6 Culturing and isolation

The present study used chromogenic agars to isolate ESKAPE pathogens since they are usually utilised in clinical settings for the isolation of pathogens (Perry, 2017). They utilise the chromogenic enzyme substrates to select certain pathogens based on their extracellular enzyme activity, which facilitates growth with a certain colour in the target organism on the agar plates (Perry, 2017).

2.6.1 *Enterococcus faecium*

For the Isolation of *E. faecium*, chromogenic agar can be used. The present study used *Enterococcus faecium* ChromoSelect Agar. Green colonies will show on the agar (Millipore, 2018b). *Enterococcus* spp. produce β -glucosidase, which reacts with the chromogenic compounds of the agar, which allows the colonies to colour blue. *E. faecium* has the potential to ferment arabinose, which reacts with the chromogenic media to form green colonies: thus, the combination of the blue and the yellow colouring of the *E. faecium* will lead to the formation of green colonies on the agar (Millipore, 2018b).

2.6.2 *Staphylococcus aureus*

For the isolation of this bacterium, *S. aureus* CHROMagar was used. *S. aureus* CHROMagar is a chromogenic agar that allows for the isolation of *S. aureus*: the agar contains chemicals that the *S. aureus* reacts with, causing it to form light pink colonies (Chromagar, 2019a).

2.6.3 *Klebsiella pneumoniae*

For the isolation of *K. pneumoniae*, *Klebsiella* chromoselect agar was used. The use of the agar with the *Klebsiella* selective supplement inhibits the growth of other Gram-negative faecal contaminants (Millipore, 2018c). The agar allows the agent that causes pneumonia to be cleaved, thus forming purple-magenta colour colonies on the agar (Millipore, 2018c).

2.6.4 *Acinetobacter baumannii*

The chromogenic agar that was used for the detection of multidrug-resistant *Acinetobacter* was CHROMagar *Acinetobacter* (CHROMagar, 2019b). It colours *Acinetobacter* red on the agar, and other Gram-negative bacterial species forms blue colonies (CHROMagar, 2019b).

2.6.5 *Pseudomonas aeruginosa*

Cetrimide agar inhibits the growth of other *Pseudomonas* species, thus it is seen as a good medium for the purpose of isolating *Pseudomonas aeruginosa*. The *P. aeruginosa* will form blue-green colonies on the agar because of the production of pyocyanin (Millipore, 2018d).

2.6.6 *Enterobacter* spp.

m-FC and m-ENDO agar are selective and differentiating in nature, and these were used to isolate *Enterobacter* spp. from samples (Millipore, 2018e).

2.7 Molecular identification and antibiotic resistance genes

16S ribosomal RNA (rRNA) can be used to identify unknown bacterial species by comparing their 16S rRNA sequences to a database of known sequences (Chakravorty *et al.*, 2007). The 16S rRNA consists of nine regions (V1-V9) and there is no specific region that can be used to identify and distinguish all bacterial species (Chakravorty *et al.*, 2007). There are limitations in using only the 16S rRNA for identification, since the short read lengths from the sequences can sometimes impair identification onto species level, while there might be differences in identification if different regions of the 16S gene are chosen and, moreover, sequencing errors can lead to misidentification (Poretsky *et al.*, 2014). The 16S rRNA regions contain conserved sequences: therefore, universal polymerase chain reaction (PCR) primers can be used for each of the 16S rRNA regions so as to identify different species of bacteria (Chakravorty *et al.*, 2007). PCR makes use of primers so the specific regions of the DNA can be amplified and further analysed. The PCR is performed cyclically in three phases, that is, denaturation, extension and annealing, at different temperatures (Ramesh *et al.*, 1992).

CHAPTER 3: MATERIALS AND METHODS

3.1 Graphic representation of methods

Figure 1 illustrates the workflow employed by the present project. For the present purpose, this comprised five main components: sampling and isolation, antibiotic resistance testing, extracellular enzyme production (virulence), ARG screening and 16S sequencing for the identification of organisms.

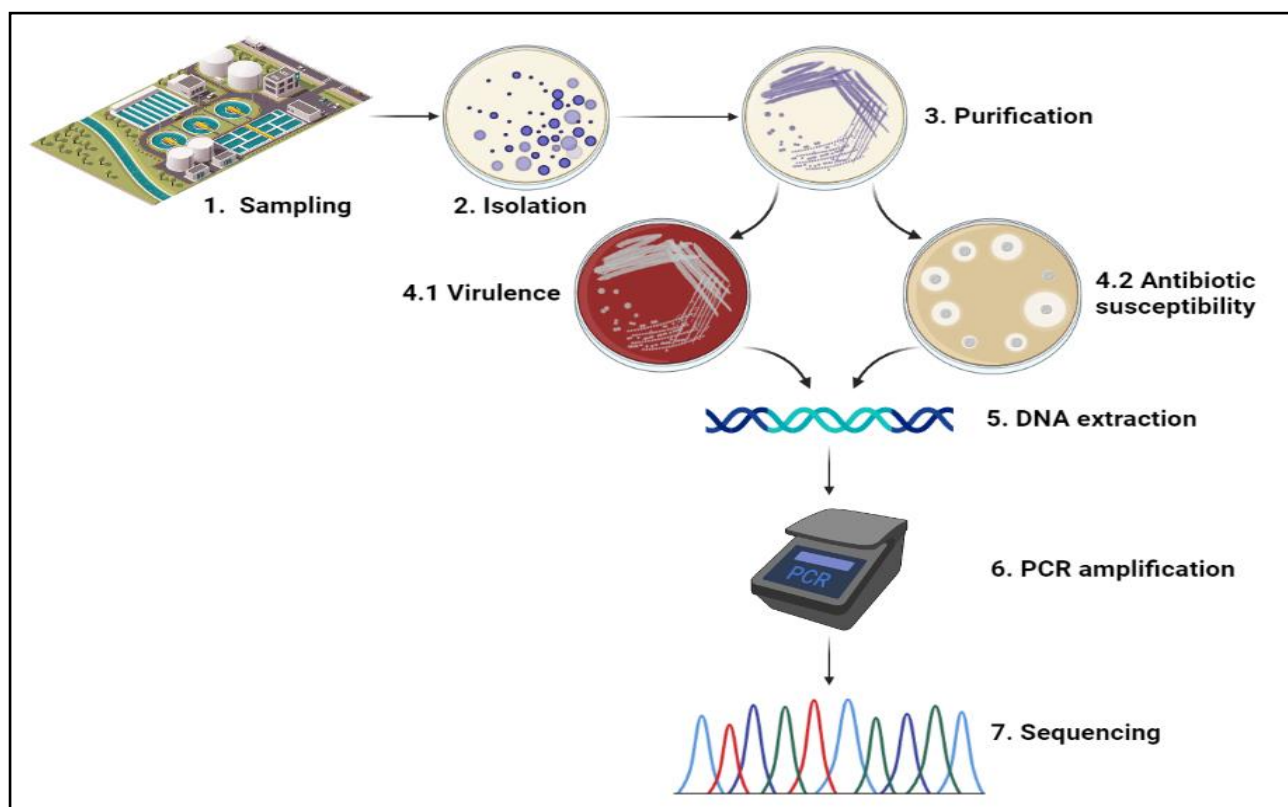


Figure 1: Graphic summary of the methods and the selection criteria for the present study.

3.2 Sample collection from WWTP

Samples were collected from the influent and effluent of two wastewater treatment plants (WWTPs) in the North West province of South Africa. Samples were collected by making use of the direct sampling method (SERAS, 2002). Bottles were sterilized and marked beforehand. Samples were collected aseptically while wearing the appropriate PPE and gloves whenever contact with the bottles occurred. The samples were kept in cooler boxes with ice to prevent any unwanted growth of organisms present in the water and were analysed within four to ten hours after sampling. The samples were collected in 1L sterile bottles and 4L of samples were taken from the influent and the effluent of each WWTP to ensure that if there were any accidents that there was still enough of the samples to analyse. The samples were taken once a month within the first week of that month. Six Samples

runs were conducted and the samples were collected from July 2021 – November 2021 and the final samples were collected in January 2022. There were no samples collected in December 2021 due to logistical issues. The sampling period includes both some of the dry (July 2021 – August 2021) and wet (Sept. 2021 – Jan 2022) seasons (Makuwa *et al.*, 2022).

3.3 Isolation and purification

The influent samples that were collected was used in a dilution series, and the effluent filtered by using the membrane filtration method (ASM, 2015). For the Isolation of the ESKAPE pathogens, chromogenic and differentiating agars were used. Dilutions were done in sterile McCartney bottles containing distilled water. The following dilution series was prepared with the samples, 10^{-1} , 10^{-3} and 10^{-5} . After the dilutions were prepped 100 μ L of the dilutions were added to the respective plates thus increasing the dilution factor on the plates to 10^{-2} , 10^{-4} and 10^{-6} , the plates were then incubated as indicated in the paragraph below. For the filtration of the effluent samples, 100ml of the effluent was filtered through 0.45mm filters using a multi-branch vacuum filtration system and placed on the respective agar plates and incubated as indicated in the paragraph below (ASM, 2015).

E. faecium was isolated after being incubated at 37°C on *Enterococcus faecium* Chromoselect agar and green colonies that changed the agar from red to yellow were selected (Millipore, 2018b).

S. aureus form pinkish colonies on the *S. aureus* CHROMagar, the plates were incubated for 24 hours at 37°C (Ali, 2019; Chromagar, 2019a; Sharp & Searcy, 2006).

For the isolation of *Klebsiella pneumoniae*, *Klebsiella* Chromoselect agar was used, and the plates were incubated at 37°C for 24 hours, while the colonies that formed on the agar were violet and had a mucous appearance (Millipore, 2018c).

For the isolation of *Acinetobacter baumannii*, *Acinetobacter* CHROMagar was used (Karakece, 2015). These plates were incubated at 37°C for 24 hours after the incubation, and the *A. baumannii* formed red colonies on the CHROMagar (Chromagar, 2019b). Cetrimide plates were incubated at 37°C for 24 hours, and *Pseudomonas* was visible because of the formation of yellow-green to blue colonies on the agar (Millipore, 2018d). The m-FC plates were incubated at 37°C for 24 hours instead of 44.5°C, which caused the *Enterobacter* spp. to be visible on the agar because of the formation of blue-to-dark blue and grey-to-grey-blue colonies (Himedia, 2015; Millipore, 2018e). The m-ENDO plates were incubated at 37°C for 24 hours and dark pink colonies formed.

Single colonies were selected from these chromogenic media and streaked out at least three times to obtain pure colonies (Jalal *et al.*, 2006). These purified colonies were then Gram

stained to confirm that they were pure isolates before further tests were done (Jalal *et al.*, 2006).

3.4 Antibiotic susceptibility

Antibiotic resistance profiles of the presumptive isolates were tested by using the disk diffusion method. For this method, Mueller-Hinton (MH) agar plates were used, and the McFarland standard was used to ensure consistency across the tests. The McFarland standard ensures that the bacterium that is being tested in suspension is equal to between 1×10^8 and 2×10^8 CFU/ml of *E. coli* (Hudzicki, 2009). The organisms were inoculated in Nutrient Broth and checked regularly to ensure that they meet the McFarland standard, afterwards 100 μ L of the samples were placed on the MH agar by the use of the spread plate method (Hudzicki, 2009). After the inoculation of the MH plates, five antibiotic discs that were manufactured by Oxoid™ were placed on the inoculated plate and incubated at 35°C for 24 hours (Hudzicki, 2009). Zones around the antibiotic discs were measured and compared to the CLSI standard values to determine if the organism was susceptible, intermediately resistant or resistant to the antibiotics (CLSI, 2020; Hudzicki, 2009). To determine the antibiotic resistance profiles of each of the organisms, Clinical Laboratory Standards Institute 30th edition of *Performance Standards for Antimicrobial Susceptibility* testing was used (CLSI, 2020). Table 1 reflects the antibiotics and concentrations used to determine the antibiotic resistance profiles.

The percentage antibiotic resistant for each of the various ESKAPE organisms were calculated as followed: $\text{Number of organisms resistant to the specific antibiotic} / \text{Total number of organisms from the specific ESKAPE group} \times 100$

Table 5: The antibiotics that were used for each presumptive organism of the ESKAPE group.

<i>Enterococcus faecium</i>	<i>Staphylococcus aureus</i>	<i>Klebsiella pneumoniae</i>	<i>Acinetobacter baumannii</i>	<i>Pseudomonas aeruginosa</i>	<i>Enterobacter spp.</i>
Ampicillin 10 µg (AMP)	Ampicillin 10 µg (AMP)	Ampicillin 10 µg (AMP)	Ceftriaxone 30 µg (CRO)	Ampicillin 10 µg (AMP)	Ampicillin 10 µg (AMP)
Gentamycin 10 µg (CN)	Gentamycin 10 µg (CN)	Ofloxacin 5 µg (OFX)	Gentamycin 10 µg (CN)	Gentamycin 10 µg (CN)	Gentamycin 10 µg (CN)
Chloramphenicol 30 µg (C)	Chloramphenicol 30 µg (C)	Cefazoline 30 µg (KZ)	Amikacin 30 µg (AK)	Ciprofloxacin 5 µg (CIP)	Chloramphenicol 30 µg (C)
Kanamycin 30 µg (K)	Ciprofloxacin 5 µg (CIP)	Norfloxacin 10 µg (NOR)	Cefotaxime 30 µg (CTX)		Netilmicin 30 µg (NET)
Vancomycin 30 µg (VA)		Gentamycin 10 µg (CN)	Meropenem 10 µg (MEM)		Cefazoline 30 µg (KZ)
Erythromycin 15 µg (E)		Imipenem 10 µg (IMP)	Ceftazidime 30 µg (CAZ)		Levofloxacin 5 µg (LEV)
Penicillin G 10 µg (P)		Cefotaxime 30 µg (CTX)			
		Amikacin 30 µg (AK)			
		Ceftriaxone 30 µg (CRO)			
		Nitrofurantoin 300 µg (F)			

3.5 Virulence factors

Virulence factors that were evaluated included hemolysin, lipase, protease and DNase. These were tested by culturing the presumptive ESKAPE pathogens on various media that indicated if the extracellular enzymes were produced by these pathogens. For all of the virulence tests the plates were separated into 10 zones and the presumptive ESKAPE pathogens were spot inoculated on the plate, one organisms in a zone.

For protease, a mixture of skim milk, brain heart broth and agar were used (Venter, 2010). A positive test for protease is indicated by a clear zone that forms in the whiteish agar around the inoculated colonies (Venter, 2010).

DNase agar supplemented with toluidine blue was used to indicate the production of DNase (Millipore, 2018f). A positive test for DNase production is indicated by a bright pink reaction on the agar plate because of the metachromic properties of the toluidine blue (Millipore, 2018f).

Lipase production was detected by using tributyrin agar (Millipore, 2018g). A positive lipase test is indicated by clear zones forming on the turbid agar around the inoculated organisms (Millipore, 2018g).

To test for hemolysin production sheep blood agar is used, where bacterial colonies on plates form different coloured zones around the colonies, depending on the type of hemolysis activity (Buxton, 2005; Mogrovejo *et al.*, 2020). After incubation, clear zones would form on the plates if β -hemolysis was present and darker zones would form on the plates when α -Hemolysis activity was present (Mogrovejo *et al.*, 2020; Vesper & Vesper, 2004). γ - hemolysis activity is identified when the organisms grow on the blood agar plates but no zones form around the colonies (Buxton, 2005; Mogrovejo *et al.*, 2020).

3.6 DNA extraction, PCR, antibiotic resistance genes, purification and sequencing preparation

For DNA extractions the presumptive ESKAPE organisms were inoculated in nutrient broth and incubated for 24 hours. The organisms were then centrifuged at 11000 rpm for 1 minute in Eppendorf tubes until a desirable sized pellet was obtained. After the pellet was obtained the Macherey-Nagel NucleoSpin Microbial DNA extraction kit was utilized and the protocol was followed as instructed by the manufacturer to isolate DNA from the presumptive ESKAPE organisms (Macherey-Nagel, 2022.). The quality of the DNA that was extracted was analysed using a nanodrop spectrophotometer. The desired DNA A260/A280 values ranged between 1.8 and 2.0, this is seen as a high-quality DNA that is not contaminated by proteins or RNA (Matlock, 2015). Thus, if the values were between those values the DNA was used for downstream analysis. For the identification of the organisms, the V1-V9 region of the 16S rRNA gene was amplified by using two universal primers that are commonly used for the identification of bacteria, namely the 27F and the 1492R primers (Frank *et al.*, 2008; Johnson *et al.*, 2019). The annealing temperature for the 16S primers is listed in Table 6 and the thermocycle for the PCR was repeated 30 times.

After the 16S PCR had been completed, a clean-up was done to purify the PCR products. The first clean-up was done by using AMPure magnetic beads and 70% ethanol to remove excess salts, enzymes, primers and nucleotides after the first PCR reaction to purify the PCR product (Beckman Coulter, 2016). The protocol provided by Beckman Coulter (2016) was utilised to perform the first cleanup of the PCR products.

After the first cleanup Sequencing PCR was performed by using the 27F primer and the BigDye Terminator v3.1 cycle sequencing kit protocol as instructed by the manufacturer (Applied Biosystems, 2010). The sequencing PCR cycle was repeated 25 times with the annealing temperature listed in Table 6. After the sequencing PCR had been completed, the final PCR product was purified by using the CleanSEQ Dye-terminator removal protocol as per the manufacturer's instructions (Beckman Coulter, 2017). This method of dye terminator removal is done by using CleanSEQ magnetic beads and 85% ethanol. After the dye terminators had been removed and the product had been purified by following the protocol of Beckman Coulter (2017), sequencing was conducted by using a SeqStudio sanger sequencer. The sequences that were obtained from the SeqStudio were analysed using FinchTV and afterwards compared to the BLAST databases so as to confirm the identity of the organism.

Table 2 depicts the antibiotic-resistance genes (ARGs) screened for during this study and the primers used for the 16S region. It also contains the primer sequences, gene fragment length of the genes and the annealing temperatures that were utilised.

Table 6: 16S and ARG Primer sequences and annealing temperatures.

Gene	Sequence	Fragment length	Annealing temp
16S	F- AGAGTTTGATCCTGGCTCAG R- TACGGYTACCTTGTTACGACTT	1400 bp	72°C
<i>bla</i> _{KPC}	F- CGTCTAGTTCTGCTGTCTTG R- CTTGTCATCCTTGTTAGGCG	819 bp	52°C
<i>bla</i> _{SHV}	F- ATGCGTTATATTCGCCTGTG R- TGCTTTGTTATTCGGGCCAA	392 bp	49°C
<i>bla</i> _{OXA-48}	F- TTCGGCCACGGAGCAAATCAG R- GATGTGGGCATATCCATATTCATCGCA	400 bp	52°C
<i>bla</i> _{CTX}	F- ATGTGCAGYACCAGTAARGTKATGGC R- TGGGTRAARTARDTSACCAGAAYCAGCGG	860 bp	63°C
<i>bla</i> _{TEM}	F- TCGCCGCATACACTATTCTCAGAATGA R- ACGCTCACCGGCTCCAGATTTAT	445 bp	58°C
<i>bla</i> _{VIM}	F- GATGGTGTGTTGGTCGCATA R- CGAATGCGCAGCACCAG	130 bp	60°C
FOX	F- AACATGGGGTATCAGGGAGATG R- CAAAGCGCGTAACCGGATTGG	190 bp	64°C
MOX	F- GCTGCTCAAGGAGCACAGGAT R- CACATTGACATAGGTGTGGTGC	520 bp	59 °C

3.7 Data analysis

DNA sequences were edited by using FinchTV and were run through the BLAST database to determine the identities of the organisms.

The identified ESKAPE pathogens were used for evolutionary analyses that were conducted in MEGA X. Alignment was done by using the MUSCLE alignment algorithm. The evolutionary history was inferred by using the maximum likelihood method and general time reversible model. The trees with the highest log likelihood is illustrated in Figures 10 and 11. The percentage of trees in which the associated taxa clustered together is shown next

to the branches of Figures 10 and 11. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated by using the maximum composite likelihood (MCL) approach and then selecting the topology with superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (five categories (+G, parameter = 0.7329)). The rate variation model facilitated the occurrence that some sites were evolutionarily invariable ([+I], 0.00% sites). Codon positions included were 1st+2nd+3rd+Noncoding.

Excel 2016 Version 16.0.6828.1019 was used to draw up all the tables and some of the graphs.

Heat maps that represent the resistance patterns of each of the different presumptive ESKAPE organisms were drawn up by using Statistica version 14.0.1 published by TIBCO Software Inc.

Canoco version 5.12 published by Petr & Majka Smilauer was used for the multivariate statistical analysis of the final results. A redundancy analysis (RDA) was done by drawing RDA plots, which involves a form of multivariate statistical analysis that can be used to analyse information so as to represent genotypical and phenotypical data, and it can also be used as a tool to predict future trends (Capblancq & Forester, 2021). These RDA plots have been used in extant research as a powerful tool for describing organisms and the correlation they have to other variables such as spatial distribution or other phenotypical data (Capblancq & Forester, 2021). RDA plots generated for this study were used to illustrate the similarities between the different sampling locations and the similarities in the grouping of the organisms at the sampling sites.

CHAPTER 4: RESULTS

This chapter presents the results obtained: the number of presumptive isolates that were obtained, the antibiotic resistance and virulence traits of those presumptive isolates, 16S identification of the organisms and the ARGs that they contained. After having identified the organisms, phylogenetic trees were constructed and multivariate statistics established.

4.1 Cultivation of presumptive ESKAPE pathogens

Table 3 illustrates the number of presumptive ESKAPE organisms that were isolated from each sampling site and the chromogenic media that was utilised to this end. The following total numbers of presumptive ESKAPE isolates were obtained from the influent and effluent of WWTP A and WWTP B: *E. faecium* (40), *S. aureus* (30), *K. pneumoniae* (39), *A. baumannii* (30), *P. aeruginosa* (25) and *Enterobacter* spp. (103). The highest presumptive numbers obtained from the WWTP B influent were *E. faecium* (14), *S. aureus* (14) and *K. pneumoniae* (13). In the case of WWTP A, this number was that of presumptive *A. baumannii* (11) isolates. The influent/ effluent of WWTP A and the influent of WWTP B yielded seven presumptive *P. aeruginosa* isolates. The highest number of presumptive *Enterobacter* spp. isolates, namely 15, were obtained from the influent of both WWTP A and WWTP B.

Table 7: The number of organisms that were selected and purified by means of the chromogenic media from each of the sources.

Organism	Agar used	Colour on agar	Number of selected colonies				
			AI	AE	BI	BE	Total
<i>Enterococcus faecium</i>	Enterococcus chromoselect agar	Green	11	8	14	7	40
<i>Staphylococcus aureus</i>	<i>S. aureus</i> CHROMagar	Pink	10	4	14	2	30
<i>Klebsiella pneumoniae</i>	<i>Klebsiella</i> chromoselect agar	Purple	7	12	13	7	39
<i>Acinetobacter baumannii</i>	<i>Acinetobacter</i> CHROMagar	Red	8	11	6	5	30
<i>Pseudomonas aeruginosa</i>	Cetrimide	Green	7	7	7	4	25
<i>Enterobacter</i> spp.	m-FC	Dark blue/ white blue	11	14	12	9	46
<i>Enterobacter</i> spp.	m-ENDO	Dark/light pink	15	13	15	14	57
Total isolated organisms							268

AI: WWTP A influent, AE: WWTP A effluent, BI: WWTP B influent, BE: WWTP B effluent.

4.2 Antibiotic resistance

Figures 2 to 7 are heat maps that illustrate the antibiotic resistance patterns of the presumptive ESKAPE isolates. The heat maps represent the resistance profiles based on the results of the disc diffusion method, while the resistance profiles were determined by using CLSI standards (see CLSI, 2020; Hudzicki, 2009). The resistance patterns of the presumptive isolates are indicated by colour: green = susceptible (S), yellow = intermediate resistant (I) and red = resistant (R). The heat maps depict the high resistance against several antibiotics among the presumptive ESKAPE isolates.

The organism's IDs can be interoperated as followed:

- The first number is the sampled month (1 = month one, 2 = month two etc.)
- The second number indicates the location where the sample was taken:
1= WWTP A influent; 2= WWTP A effluent; 3= WWTP B influent; 4= WWTP B effluent
- The third number indicates what presumptive ESKAPE organism it is:
1= *Enterococcus faecium*; 2= *Staphylococcus aureus*; 3= *Klebsiella pneumoniae*;
4= *Acinetobacter baumannii*; 5= *Pseudomonas aeruginosa*; 6&7= *Enterobacter* spp.
- The final letter A-D indicates the replicates of the selected organisms.

4.2.1 *Enterococcus faecium* antibiotic resistance

Figure 2 below is a heat map that illustrates the antibiotic resistance patterns of the presumptive *Enterococcus faecium* that was isolated WWTP A (19 isolates) and WWTP B (21 isolates). The resistance patterns of these selected isolates indicate that there were high levels of resistance against ampicillin (10 µg) (R: 42.5%), kanamycin (30 µg) (R: 67.5%) and penicillin G (10 µg) (R: 37.5%). High levels of intermediate resistance were also observed against erythromycin (15 µg) (I: 57.5%; R: 25%). Some resistance and intermediate resistance were exhibited against chloramphenicol (30 µg) (I: 20%; R: 12.5%), gentamycin (10 µg) (I: 22.5%; R: 22.5%) and vancomycin (30 µg) (I: 5%; R: 22.5%).

Figure 2 illustrates that none of the presumptive *E. faecium* isolates was susceptible to all of the antibiotics. Differences in resistance profiles against some antibiotics between the influent and effluent of the WWTPs were noted. The number of influent isolates that exhibited resistance against certain antibiotics greater than that of the effluent for the following antibiotics: kanamycin (30 µg) (INF: 20; EFF: 6) and gentamycin (10 µg) (INF: 9; EFF: 0). The number of effluent isolates that exhibited resistance against a certain antibiotic was greater than that of the influent for: vancomycin (30 µg) (INF: 1; EFF: 8).

Enterococcus faecium

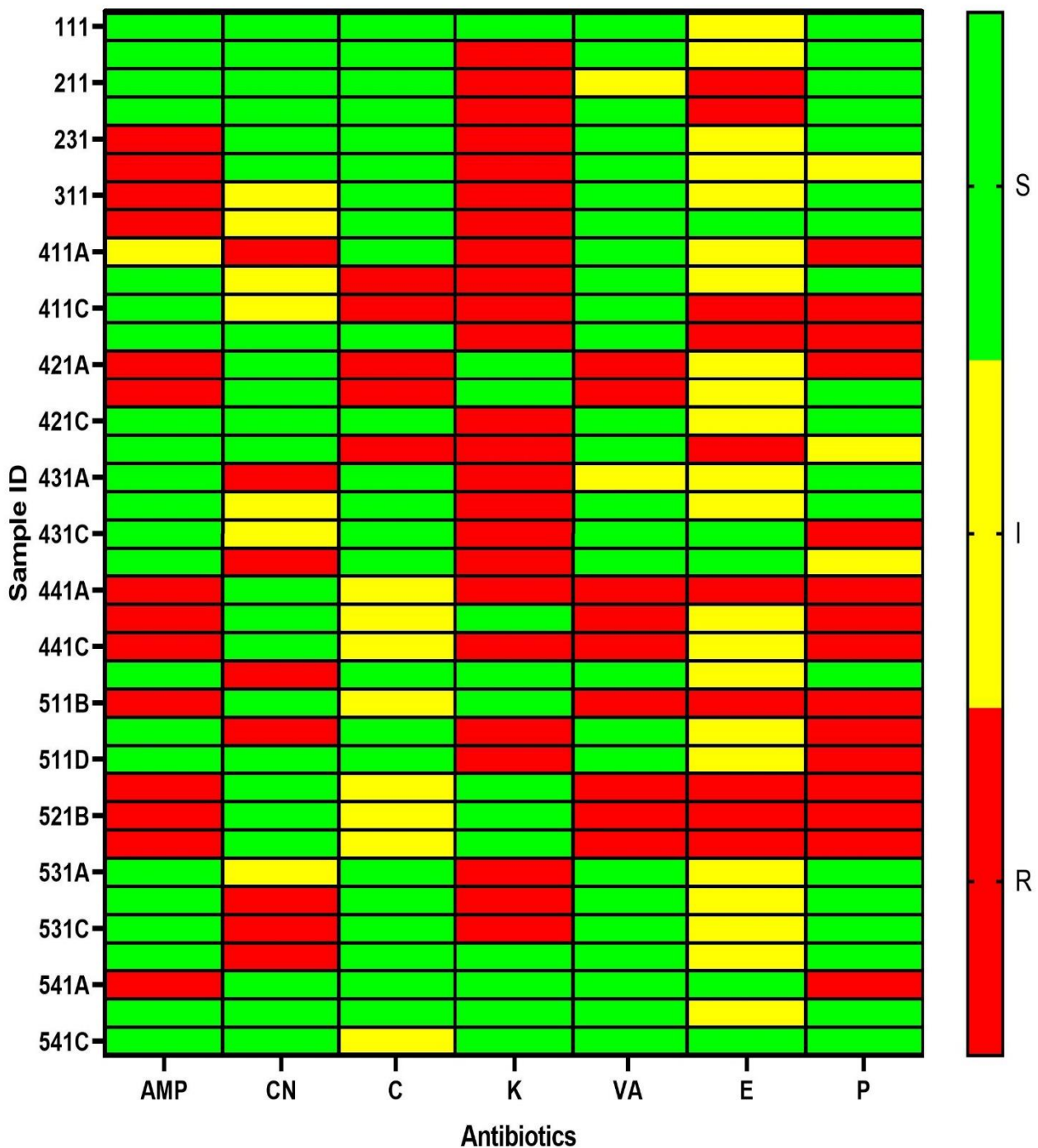


Figure 2: Heat map illustrating the resistance patterns of the presumptive *Enterococcus faecium* isolates. Green = susceptible (S), yellow= intermediate resistant (I) and red = resistant (R); AMP = ampicillin (10 µg), K= kanamycin (30 µg), E= erythromycin (15 µg), P= penicillin G (10 µg), C= chloramphenicol (30 µg), CN= gentamycin (10 µg) and VA= vancomycin (30 µg).

4.2.2 *Staphylococcus aureus* antibiotic resistance

Figure 3 below is a heat map that illustrates the antibiotic resistance patterns of the presumptive *Staphylococcus aureus* isolates. The 30 presumptive isolates that were isolated from WWTP A (14) and WWTP B (16) are represented in this figure. The resistance patterns of these selected isolates indicate that the highest resistance was against ampicillin (10 µg) (R: 60%). Two isolates were resistant to ciprofloxacin (5 µg) (R: 7%) and one isolate to gentamycin (10 µg) (R: 3%). None of the presumptive *S. aureus* isolates illustrated resistance against chloramphenicol (30 µg).

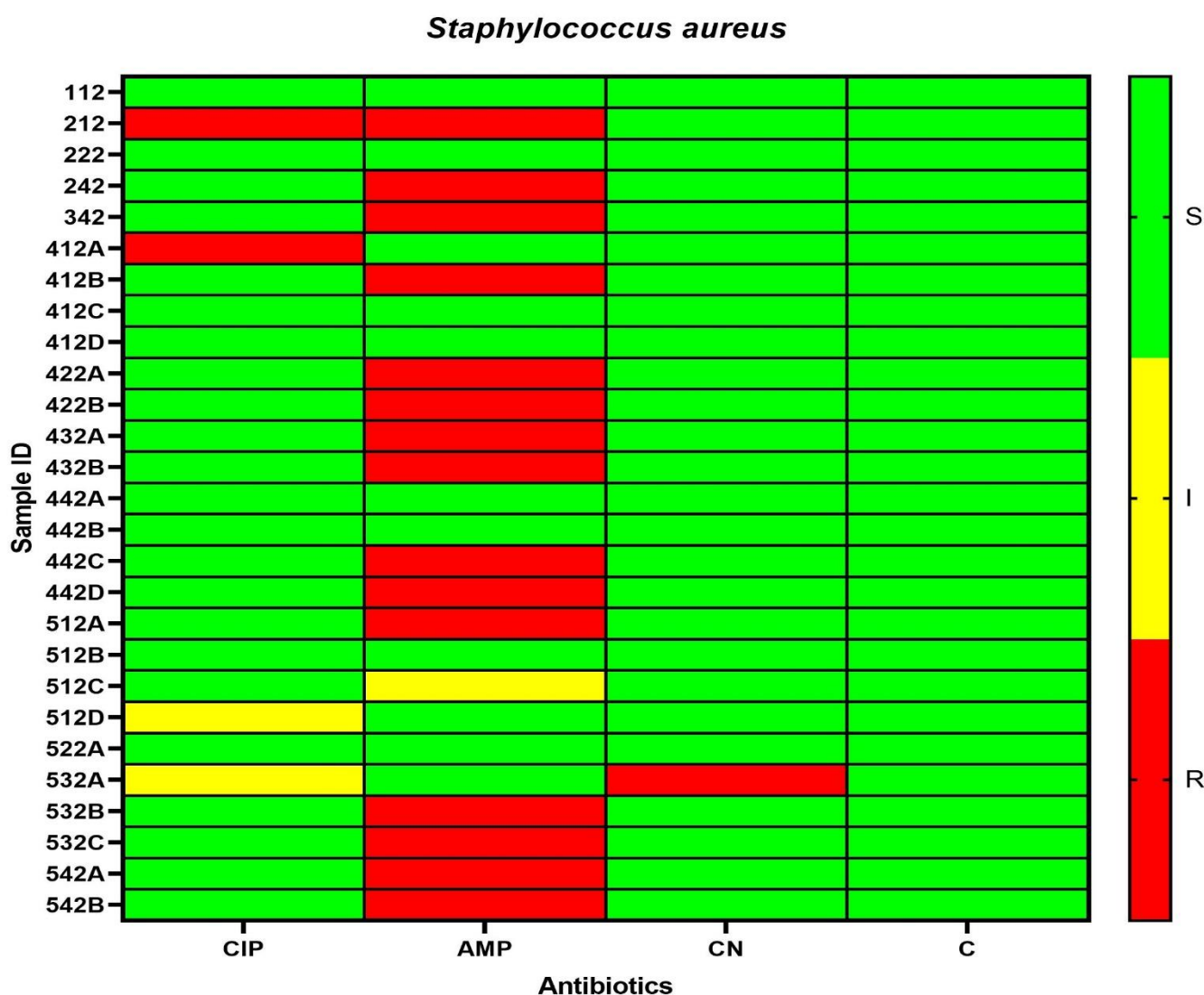


Figure 3: Heat map illustrating the resistance patterns of the presumptive *Staphylococcus aureus* isolates. Green = susceptible (S), yellow = intermediate resistant (I) and red = resistant (R); CIP = ciprofloxacin (5 µg), AMP = ampicillin (10 µg), CN = gentamycin (10 µg) and C = chloramphenicol (30 µg).

4.2.3 *Klebsiella pneumoniae* antibiotic resistance

Figure 4 below is a heat map that illustrates the antibiotic resistance patterns of the presumptive isolates from WWTP A (19) and WWTP B (21). It illustrates that the presumptive *Klebsiella pneumoniae* isolates had illustrated resistance towards cefazoline (30 µg) (R: 55%) and ampicillin (10 µg) (R: 92.5%). The resistance against the eight remaining antibiotics was relatively low but there were signs of emerging resistance: norfloxacin (10 µg) (I: 5%; R: 0%), nitrofurantoin (300 µg) (I: 5%; R: 2.5%), imipenem (10 µg) (I: 12.5%; R: 10%), cefotaxime (30 µg) (I: 32.5%; R: 5%), ofloxacin (5 µg) (I: 0%; R: 2.5%), ceftriaxone (30 µg) (I: 22.5%; R: 7.5%), amikacin (30 µg) (I: 5%; R: 5%) and gentamycin (10 µg) (I: 2.5%; R: 5%). The number of influent isolates that exhibited resistance against a certain antibiotic was greater than that of the effluent for ceftriaxone (30 µg) (INF: 3; EFF: 0). The number of effluent isolates that exhibited resistance against a certain antibiotic was greater than that of the influent for imipenem (10 µg) (INF: 0; EFF: 4).

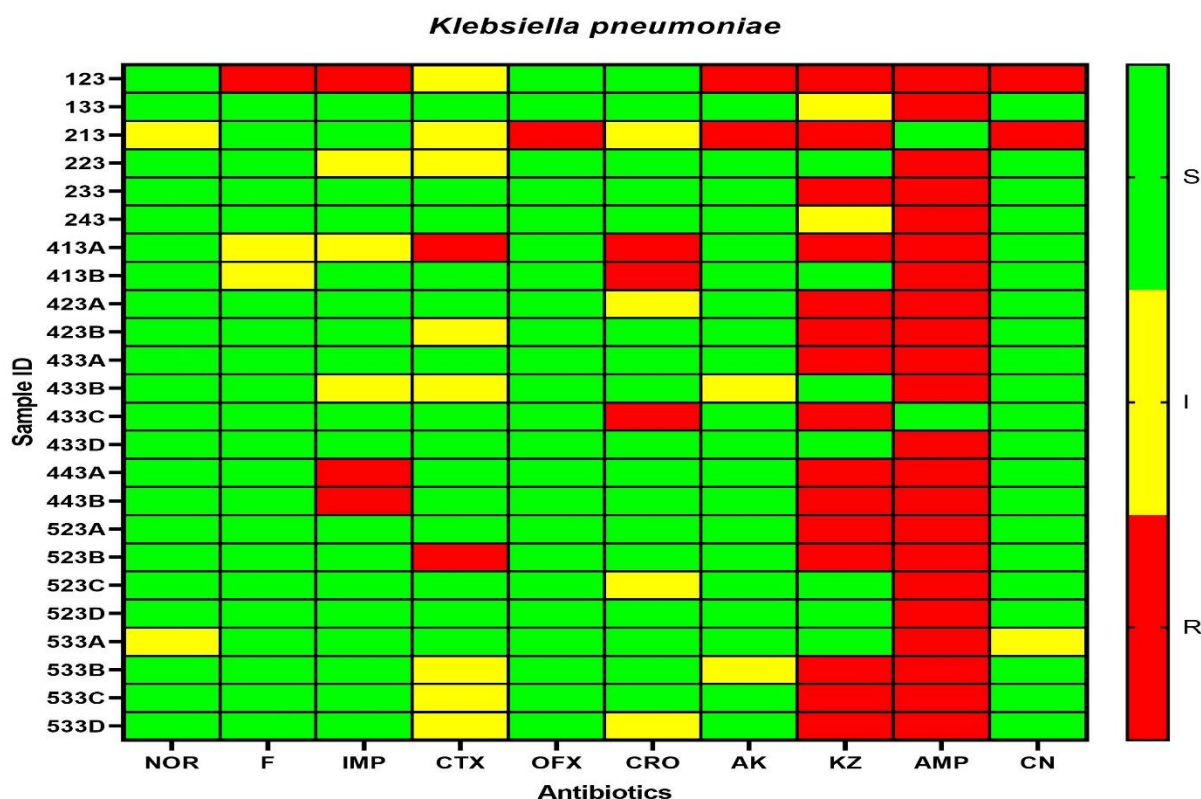


Figure 4: Heat map illustrating the resistance patterns of the presumptive *Klebsiella pneumoniae* isolates. Green = susceptible (S), yellow = intermediate resistant (I) and red = resistant (R); NOR = norfloxacin (10 µg), F = nitrofurantoin (300 µg), IMP = imipenem (10 µg), CTX = cefotaxime (30 µg), OFX = ofloxacin (5 µg), CRO = ceftriaxone (30 µg), AK = amikacin (30 µg), KZ = cefazoline (30 µg), AMP = ampicillin (10 µg) and CN = gentamycin (10 µg).

4.2.4 *Acinetobacter baumannii* antibiotic resistance

Figure 5 below is a heat map that illustrates the antibiotic resistance patterns of the 30 presumptive *Acinetobacter baumannii* isolates from both WWTP A (19) and WWTP B (11). These organisms demonstrated resistance against meropenem (10 µg) (R: 73%) and ceftriaxone (30 µg) (R: 27%), but there was also a large number of isolates that exhibited signs of emerging resistance against ceftriaxone (30 µg) (I: 50%). The number of isolates that were intermediately resistant against ceftriaxone (30 µg) (I: 83%) and ceftazidime (30 µg) (I: 53%) was much higher than the isolates that exhibited complete resistance against these antibiotics. There was only one isolate that exhibited resistance (R: 3%) against gentamycin (10 µg) and amikacin (30 µg). The number of effluent isolates that exhibited resistance against a certain antibiotic was greater than that of the influent for cefotaxime (30 µg) (INF: 0; EFF: 3).

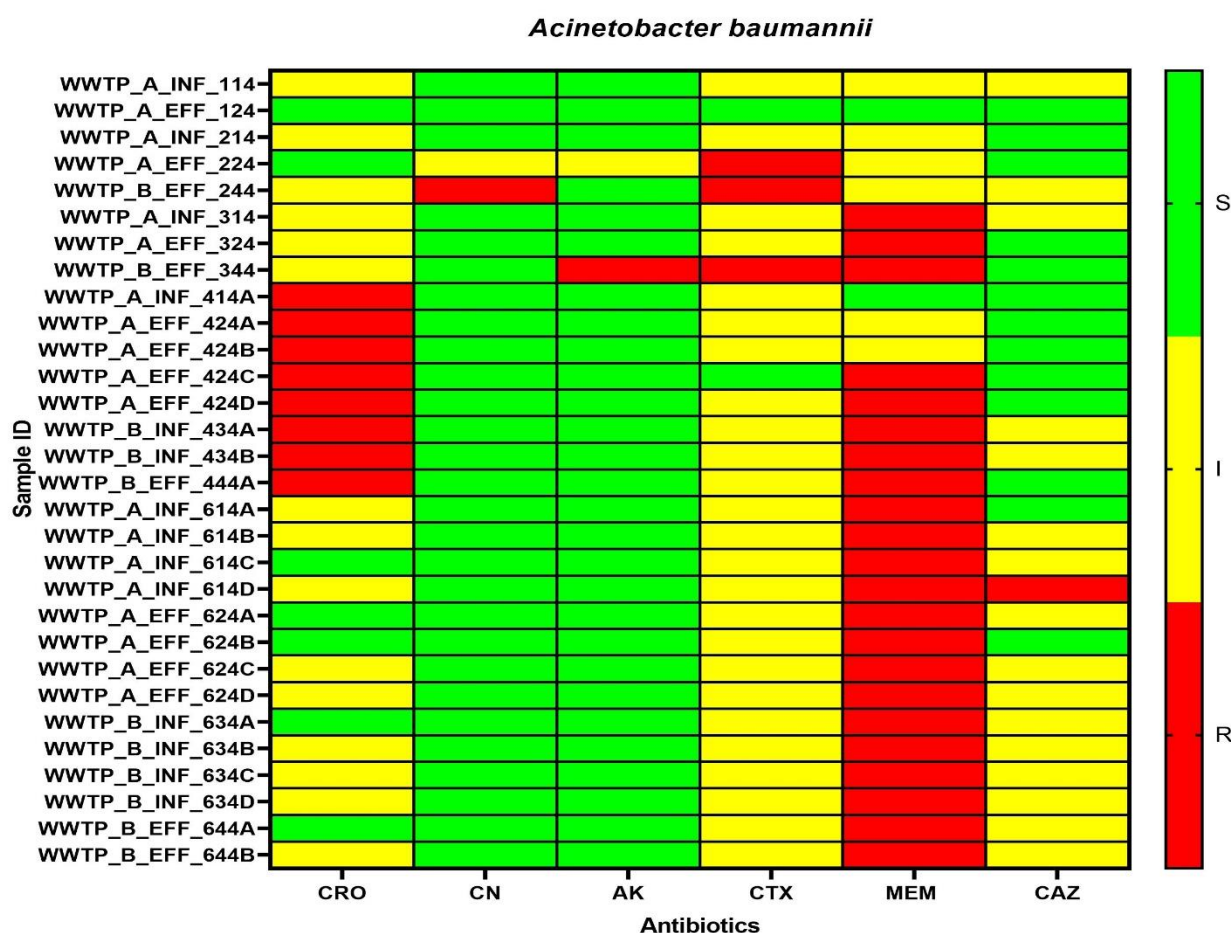


Figure 5: Heat map illustrating the resistance patterns of the presumptive *Acinetobacter baumannii* isolates. Green = susceptible (S), yellow = intermediate resistant (I) and red = resistant (R); CRO = ceftriaxone (30 µg), CN = gentamycin (10 µg), AK = amikacin (30 µg), CTX = cefotaxime (30 µg), MEM = meropenem (10 µg) and CAZ = ceftazidime (30 µg).

4.2.5 *Pseudomonas aeruginosa* antibiotic resistance

Figure 6 below is a heat map that illustrates the antibiotic resistance patterns of the 25 presumptive *Pseudomonas aeruginosa* isolates from WWTP A (14) and WWTP B (11). These isolates exhibited complete resistance against ampicillin (10 µg) (R: 100%) but were highly susceptible to gentamycin (10 µg) (R: 8%) and ciprofloxacin (5 µg) (I: 12%). The number of effluent isolates that exhibited resistance against a certain antibiotic was greater than that of the influent for ampicillin (10 µg) (INF: 11; EFF: 14).

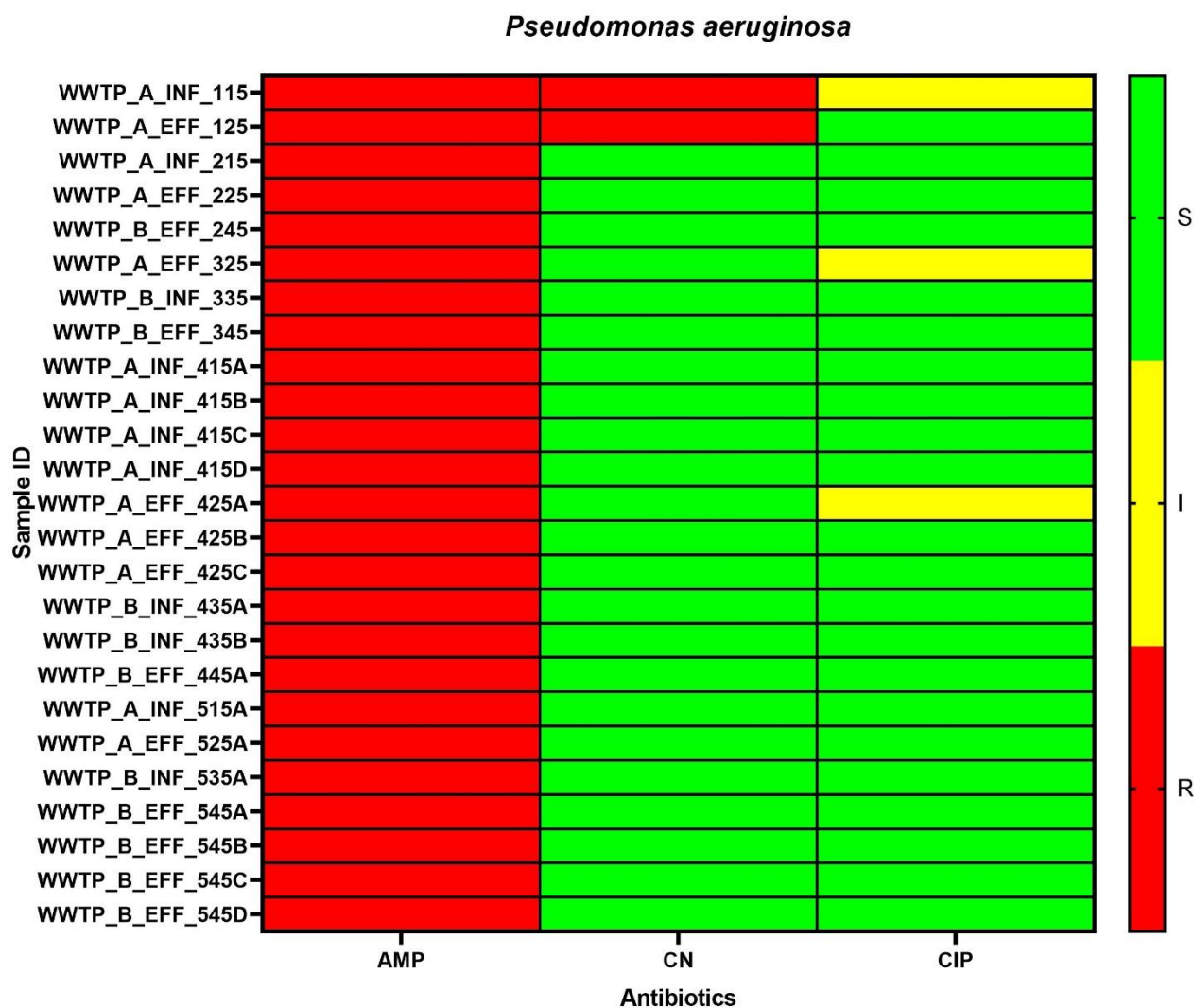


Figure 6: Heat map illustrating the resistance patterns of the presumptive *Pseudomonas aeruginosa* isolates. Green = susceptible (S), yellow = intermediate resistant (I) and red = resistant (R); AMP = ampicillin (10 µg), CN = gentamycin (10 µg) and CIP = ciprofloxacin (5 µg).

4.2.6 *Enterobacter* spp. antibiotic resistance

Figure 7 below is a heat map that illustrates the antibiotic resistance patterns of the 34 presumptive *Enterobacter* spp. isolates from WWTP A (53) and WWTP B (50). These presumptive *Enterobacter* spp. isolates illustrated resistance against ampicillin (10 µg) (R: 86%) and cefazoline (30 µg) (R: 65%). The resistance against chloramphenicol (30 µg) (I: 2%; R: 4%), gentamycin (10 µg) (I: 1%; R: 2%), and levofloxacin (5 µg) (I: 1%; R: 4%) varied, but the majority of the presumptive isolates were susceptible to these antibiotics, seldomly exhibiting resistance or intermediate resistance against the remaining antibiotics. All of the isolates were susceptible to netilmicin (30 µg).

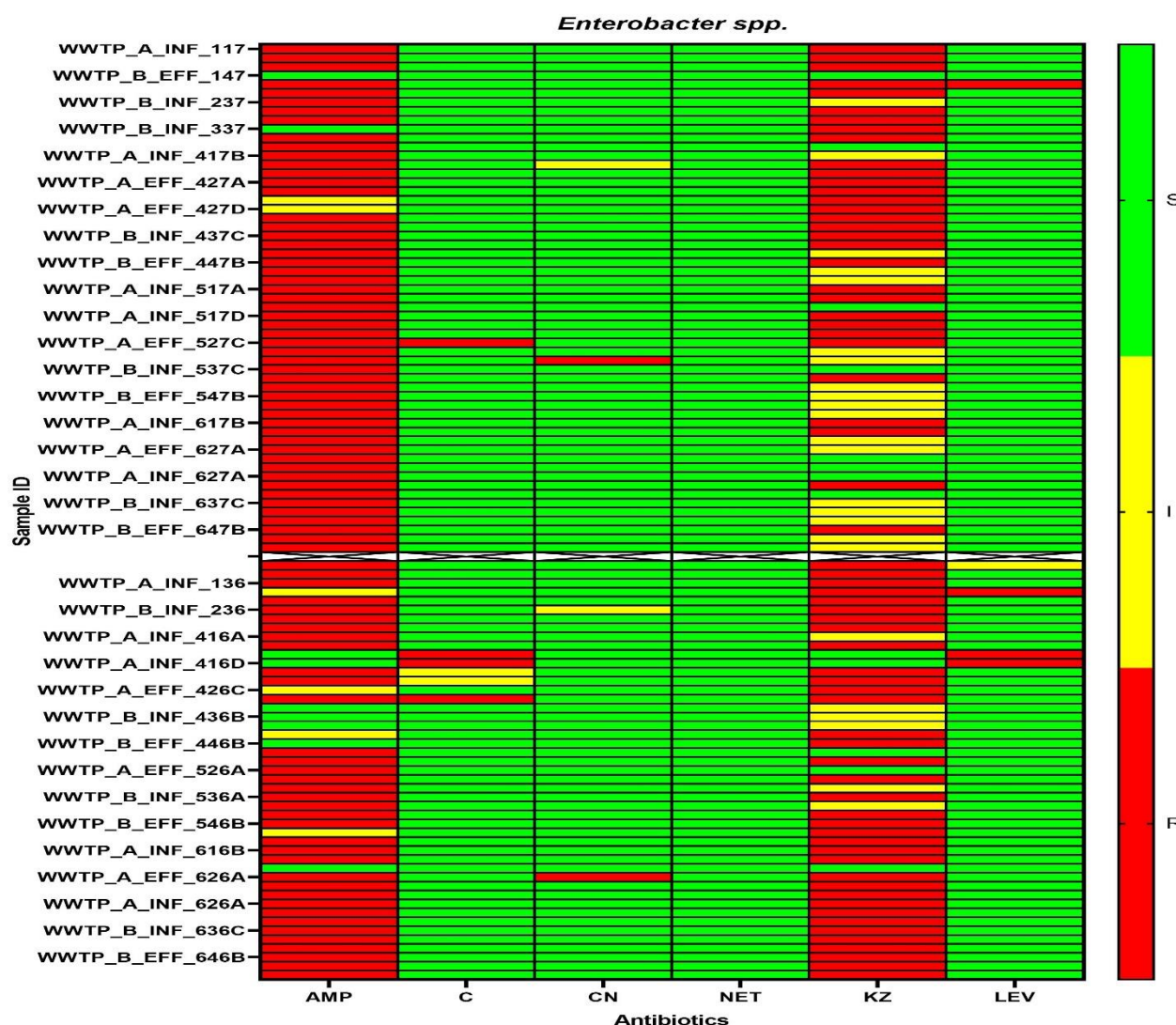


Figure 7: Heat map illustrating the resistance patterns of the presumptive *Enterobacter* spp. Green = susceptible (S), yellow = intermediate resistant (I) and red = resistant (R); AMP = ampicillin (10 µg), C = chloramphenicol (30 µg), CN = gentamycin (10 µg), NET = netilmicin (30 µg), KZ = cefazoline (30 µg) and LEV = levofloxacin (5 µg).

The same antibiotics were not tested on all of the presumptive isolates, but the presumptive ESKAPE pathogens as a whole illustrated the following trends of resistance against the antibiotics that were used in this study: AMP (78%) > MEM (70%) > K (65%) > KZ (63%) > P (32.5%) > E (25%) > VA (22.5%) > CRO (15%) > IMP (10%) > CN (7.4%) > CTX (6%) > C (5.2%) > AK (4%) > LEV (4%) > CIP (3.6%) > CAZ (3%) > F (2.5%) > OFX (2.5%) > NOR (0%) > NET (0%).

4.3 Final selection for identification

The selection criteria for final selection were based on whether the organisms exhibit β hemolytic activity and/ or whether the organism was resistant to two or more classes of antibiotics. If the organisms exhibited either one of these traits, they were selected for 16S rRNA identification. Tables 4 and 5 below list the isolates that were selected. They also illustrate the location of the isolates obtained, the identity of the organism, the hemolysis activity of the isolate, the extracellular enzyme production of the isolate, the antibiotic resistance profile of the isolate and the antibiotic resistance genes (ARGs) identified in the organism. Table 4 illustrates that no ARGs that were targeted were identified within the organisms found in the WWTP A influent, whereas some targeted ARGs were identified from the effluent organisms from WWTP A. Table 5 illustrates no distinct difference in the ARGs found in the influent and effluent of WWTP B.

Table 8: Identity and characteristics of the organisms isolated from WWTP A.

WWTP A					
Influent					
Sample ID	Organism ID	Hemolysis	Extracellular	Antibiotic resistance	ARGs
115	<i>Enterobacter_cloacae</i>	β	Lipase, Protease	AMP, CN	-
117	<i>Enterobacter</i> spp.	γ	-	AMP, KZ	-
211	<i>Bacillus_cereus</i>	γ	-	E, K	-
212	<i>Staphylococcus_gallinarum</i>	β	Protease	AMP, CIP	-
213	<i>Escherichia_coli</i>	γ	Protease	AMP, CIP	-
216	<i>Citrobacter_freundii</i>	γ	Protease	AK, CN, KZ, OFX	-
217	<i>Aeromonas_caviae</i>	γ	-	KZ, LEV	-
411A	<i>Enterococcus_faecium</i>	γ	Protease	AMP, KZ	-
411B	<i>Enterococcus_faecium</i>	γ	-	CN, K, P	-
411C	<i>Enterococcus_faecium</i>	γ	-	AMP, P, VA	-
411D	<i>Enterococcus_faecium</i>	γ	-	C, E, K, P	-
412D	<i>Aerococcus_sp.</i>	β	-	-	-
413A	<i>Klebsiella_pneumoniae</i>	γ	-	E, K, P	-
413B	<i>Escherichia_coli</i>	γ	-	AMP, CRO, CTX, KZ,	-
416B	<i>Enterobacter_sp.</i>	γ	-	AMP, CRO	-
417C	<i>Escherichia_coli</i>	γ	Protease	AMP, KZ	-
511B	<i>Pseudomonas_sp.</i>	γ	-	AMP, KZ	-
516B	<i>Aeromonas_sp.</i>	γ	-	K, P	-
517A	<i>Enterobacter_sp.</i>	γ	-	AMP, KZ	-
517B	<i>Escherichia_coli</i>	γ	Lipase	AMP, KZ	-
517D	<i>Enterobacter_hormaechei</i>	γ	Lipase	AMP, KZ	-
616A	<i>Escherichia_coli</i>	γ	-	AMP, KZ	-
616B	<i>Klebsiella_pneumoniae</i>	γ	Lipase	AMP, KZ	-
616C	<i>Klebsiella_pneumoniae</i>	γ	Lipase	AMP, KZ	-

617C	<i>Enterobacter_cloacae</i>	γ	Lipase	AMP, KZ	-
Effluent					
123	<i>Acidovorax_sp.</i>	γ	Protease	AK, AMP, CN, F, IMP, KZ,	VIM, OXA
125	<i>Proteus_mirabilis</i>	γ	-	AMP, CN	VIM
221	<i>Bacillus_cereus</i>	β	Lipase, Protease	E, K	-
226	<i>Pseudomonas_sp.</i>	γ	Protease	AMP, KZ	VIM
227	<i>Enterobacteriaceae</i>	γ	-	AMP, KZ	VIM
324	<i>Pseudomonas_sp.</i>	β	Protease	MEM	-
325	<i>Pseudomonas_sp.</i>	β	-	AMP	-
326	<i>Bacillus_licheniformis</i>	β	Protease	AMP, KZ	-
327	<i>Escherichia_coli</i>	β	Protease	KZ	-
421A	<i>Pseudomonas_sp.</i>	γ	-	AMP, C, P, VA	VIM
421B	<i>Pseudomonas_sp.</i>	γ	-	AMP, C, VA	VIM
421C	<i>Enterococcus_faecium</i>	γ	-	K	-
422B	<i>Bacillus_sp.</i>	β	-	AMP	-
423A	<i>Klebsiella_oxytoca</i>	γ	-	AMP, KZ	VIM, CTX
423B	<i>Aeromonas_sp.</i>	γ	-	AMP, KZ	-
426A	<i>Pseudomonas_sp.</i>	γ	-	AMP, KZ	VIM
426B	<i>Enterobacter_sp.</i>	γ	-	AMP, KZ	-
427A	<i>Klebsiella_sp.</i>	γ	-	AMP, KZ	-
521A	<i>Pseudomonas_protegens</i>	γ	-	Amp, E, P, VA	VIM, OXA
521B	<i>Pseudomonas_sp.</i>	γ	-	Amp, E, P, VA	VIM, OXA
521C	<i>Pseudomonas_sp</i>	γ	-	AMP, E, P, VA	VIM, OXA, TEM
522A	<i>Bacillus_sp.</i>	β	-	-	-
523A	<i>Aeromonas_salmonicida</i>	γ	-	AMP, CTX, KZ	TEM
525A	<i>Pseudomonas_sp.</i>	β	-	AMP	-
526B	<i>Aeromonas_sp.</i>	γ	Lipase	AMP, KZ	TEM
526A	<i>Aeromonas_sp.</i>	β	-	AMP, KZ	-
527A	<i>Hafnia_paralvei</i>	γ	Lipase	AMP, KZ	VIM
527B	<i>Hafnia_sp.</i>	β	Lipase	AMP, KZ	-
527C	<i>Pseudomonas_protegens</i>	γ	Lipase	AMP, KZ	-
623B	<i>Escherichia_coli</i>	γ	Lipase	AMP, KZ	VIM

626A	<i>Escherichia_coli</i>	γ	Lipase	AMP, CN, KZ	VIM, OXA
626B	<i>Enterobacter_sp.</i>	γ	-	AMP, KZ	VIM
626C	<i>Enterobacter_sp.</i>	γ	-	AMP, KZ	SHV
627B	<i>Enterococcus_faecium</i>	β	-	AMP, KZ	-

Table 5: Identity and characteristics of the organisms isolated from WWTP B.

WWTP B					
Influent					
Sample ID	Organism ID	Hemolysis	Extracellular	Antibiotic resistance	ARGs
233	<i>Escherichia coli</i>	γ	Protease	AMP, KZ	TEM
236	<i>Aeromonas sp.</i>	γ	Protease	AMP, KZ	MOX
237	<i>Aeromonas salmonicida</i>	γ	Protease	AMP, KZ	VIM
431A	<i>Enterococcus faecium</i>	β	-	CN, K	-
431B	<i>Enterococcus faecium</i>	β	-	K	-
431C	<i>Enterococcus faecium</i>	γ	-	K, P	-
431D	<i>Enterococcus faecium</i>	γ	-	CN, K	-
432B	<i>Bacillus sp.</i>	β	-	AMP	-
433A	<i>Klebsiella pneumoniae</i>	γ	-	AMP, KZ	TEM
433C	<i>Citrobacter braakii</i>	γ	Protease	CRO, KZ	-
437A	<i>Klebsiella sp.</i>	γ	-	AMP, KZ	-
437C	<i>Klebsiella pneumoniae</i>	γ	-	AMP, KZ	-
437D	<i>Enterobacter sp.</i>	γ	-	AMP, KZ	-
531B	<i>Enterococcus faecium</i>	γ	-	CN, K	-
531C	<i>Bacillus sp.</i>	γ	-	CN, K	VIM
532B	<i>Staphylococcus aureus</i>	β	-	AMP	-
533A	<i>Escherichia coli</i>	β	-	AMP	-
533C	<i>Aeromonas veronii</i>	γ	Lipase	AMP, KZ	VIM, OXA
533D	<i>Escherichia coli</i>	γ	Lipase	AMP, KZ	VIM, OXA
536A	<i>Aeromonas sp.</i>	γ	-	AMP, KZ	VIM, OXA
537A	<i>Klebsiella pneumoniae</i>	γ	-	AMP, KZ	VIM
631A	<i>Enterococcus faecium</i>	γ	-	AMP, K	-
631C	<i>Enterococcus faecium</i>	γ	-	AMP, CN, K	-
632A	<i>Kurthia populi</i>	β	-	AMP	-

632B	<i>Kurthia populi</i>	β	-	AMP	-
633A	<i>Citrobacter freundii</i>	γ	-	AMP, KZ	-
633C	<i>Citrobacter sp.</i>	γ	-	AMP, KZ	-
636A	<i>Escherichia coli</i>	γ	Lipase	AMP, KZ	-
636B	<i>Klebsiella pneumoniae</i>	γ	Lipase	AMP, KZ	VIM
636C	<i>Pseudomonas aeruginosa</i>	γ	-	AMP, KZ	-
636D	<i>Klebsiella pneumoniae</i>	γ	Lipase, Protease	AMP, KZ	VIM
637	<i>Enterobacter sp.</i>	γ	Protease	AMP, KZ	VIM, OXA
Effluent					
441C	<i>Aeromonas hydrophila</i>	γ	-	AMP, K, P, VA	-
441D	<i>Acinetobacter sp.</i>	γ	-	AMP, K, P, VA	-
442A	<i>Kurthia sp.</i>	β	-	-	-
442C	<i>Bacillus sp.</i>	β	-	-	-
447B	<i>Klebsiella sp.</i>	γ	-	AMP, KZ	-
541A	<i>Bacillus sp.</i>	γ	-	AMP, P	-
542A	<i>Bacillus sp.</i>	β	Protease	AMP	-
542B	<i>Bacillus velezensis</i>	β	-	AMP	-
546A	<i>Acinetobacter sp.</i>	γ	-	AMP, KZ	-
643A	<i>Aeromonas sp.</i>	γ	Lipase	AMP, IMP, KZ	-
643C	<i>Aeromonas sp.</i>	β	-	AMP, KZ	-
644A	<i>Acinetobacter baumannii</i>	β	-	MEM	-
646A	<i>Klebsiella pneumoniae</i>	γ	Lipase	AMP, KZ	VIM, OXA
646B	<i>Klebsiella variicola</i>	γ	-	AMP, KZ	VIM, OXA
646C	<i>Klebsiella pneumoniae</i>	γ	Lipase	AMP, KZ	VIM, OXA, SHV
646D	<i>Klebsiella pneumoniae</i>	γ	-	AMP, KZ	SHV
647B	<i>Escherichia coli</i>	γ	Lipase	AMP, KZ	VIM, OXA

4.4 Media Isolation vs 16S Identification

Figures 8 and 9 below illustrate the success rate of using chromogenic agar. They compare the number of sequenced isolates positively identified as part of the ESKAPE group to the presumptive ESKAPE pathogens that were isolated.

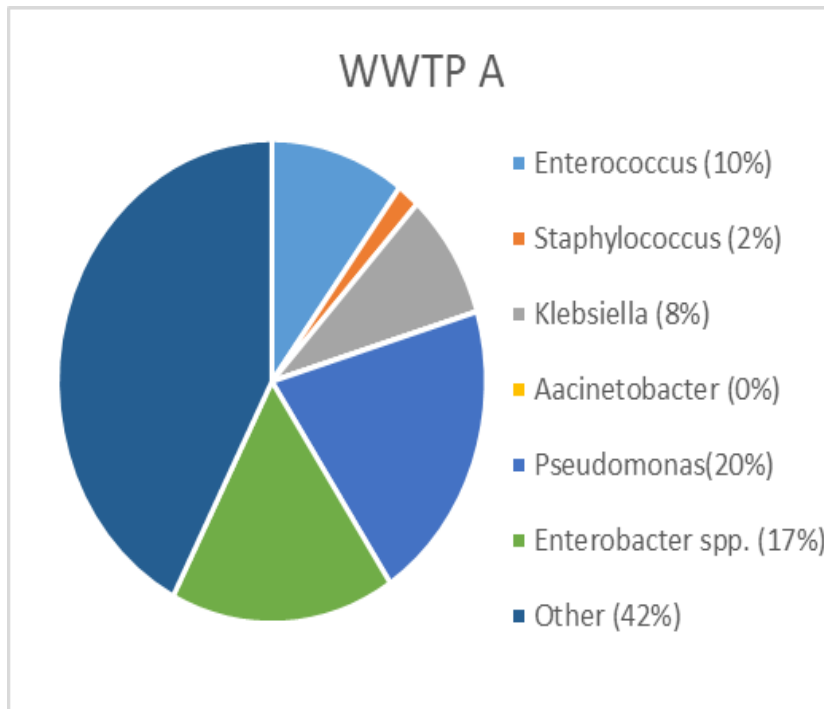


Figure 8: Pie charts illustrating the percentages of organisms that were positively identified and those that did not form part of the ESKAPE pathogen group from WWTP A.

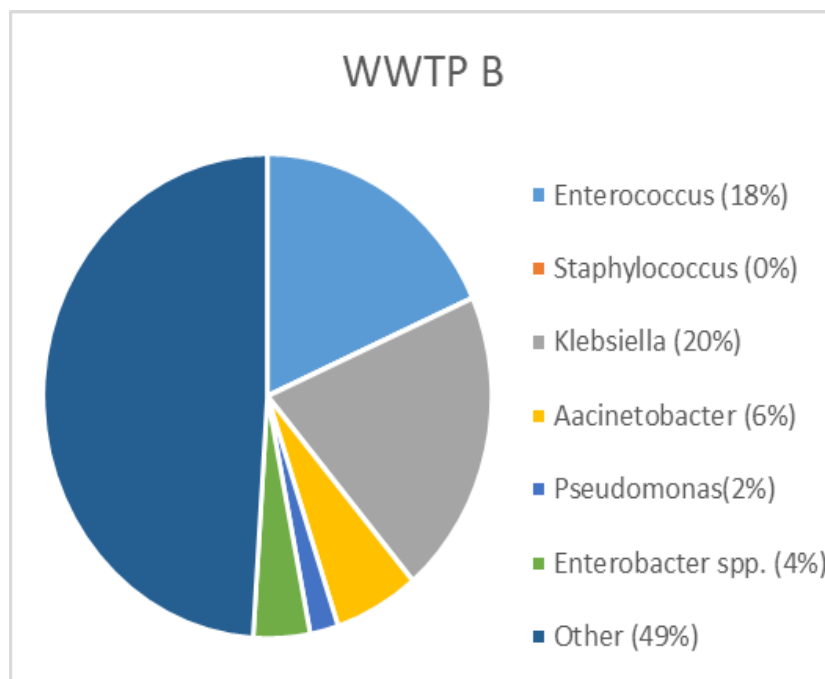


Figure 9: Pie charts illustrating the percentages of organisms that were positively identified and those that did not form part of the ESKAPE pathogen group from WWTP B.

These charts depict the success of the isolation process. They illustrate that 42% of the isolates from WWTP A and 49% from WWTP B were false positives. The numbers of correctly isolated organisms from each of the different chromogenic media differed as depicted in the two figures. The highest number of positively identified isolates from WWTP A was *Pseudomonas* (20%) and *Klebsiella* (20%) in WWTP B. The unreliability of the culturing method is also illustrated in these graphs, which show that there were no *Acinetobacter* isolated from WWTP A and no *Staphylococcus* isolated from WWTP B. There is no correlation between the number of correctly isolated organisms belonging to the ESKAPE pathogen group between WWTPs A and B. Thus Figures 8 and 9 illustrate that the culturing methods were somewhat unreliable.

4.5 Antibiotic resistance genes

Tables 4 and 5 illustrate the 108 organisms that were screened for ARGs and the ARGs that were isolated from the organisms. It was determined that 28 isolates contained the *bla*_{VIM} gene, the the *bla*_{SHV} gene, 13 the *bla*_{OXA} gene, four the *bla*_{TEM} gene, one the *bla*_{CTX} gene and a further one the MOX gene. None of the isolates that were identified contained *bla*_{KPC} or FOX antibiotic resistance genes. Table 4 illustrates that no genes were identified from the isolates obtained from the influent, while genes were identified in the case of the organisms isolated from the effluent of WWTP A. Table 5 shows that no specific pattern could indicate an increase or decrease in the number of genes that were present in the organisms.

4.6 Phylogenetics

Phylogenetic trees were drawn to illustrate the species distribution observed from each of the two sampling sites. The organisms that were identified as non-ESKAPE ones after the identifications had been obtained, were excluded from the phylogenetic tree. Reference strains were obtained from NCBI to serve as positive controls for the phylogenetic trees. The diversity of all the positively identified ESKAPE organisms is illustrated in Figures 10 and 11. From WWTP A and WWTP B, 24 isolates were positively identified as part of the ESKAPE pathogen group and the sequences were used to draw maximum likelihood trees. The correct genus clustering of these isolates on the phylogenetic trees further confirms the identification of the isolates at least on the genus level. It is only accurate to the genus level, because 16S rRNA sequencing cannot always be used to accurately identify organisms at the species level (Janda & Abbott, 2007). The accuracy of identifying organisms at the genus level with 16S rRNA sequences was more than 90%, whereas identification at the species level with 16S rRNA sequences effectivity was about 65% to 85% (see Janda &

Abbott, 2007). Thus, clustering of the organisms into the same clades in Figures 10 and 11 depicts the fact that the organisms were indeed found to belong within the same genus.

Figure 10 illustrates the clustering of the main six genera from the ESKAPE pathogen group isolated from WWTP A, namely *Enterobacter*, *Staphylococcus*, *Klebsiella*, *Acinetobacter*, *Pseudomonas* and *Enterococcus*. The reference strain that was used shows the accession number in front of the species name. *Thermosphaera aggregans* was used as an outgroup sequence for Figure 10. The figure is separated into six distinct clusters of the various organisms, where *Enterobacter* and *Klebsiella* share a cluster; *Pseudomonas* and *Acinetobacter* another; and *Enterococcus* and *Staphylococcus* a further one. The shared clusters and the moderately high bootstrap values illustrate a high similarity among the species and the strains of the organisms, further confirming confidence in their identities.

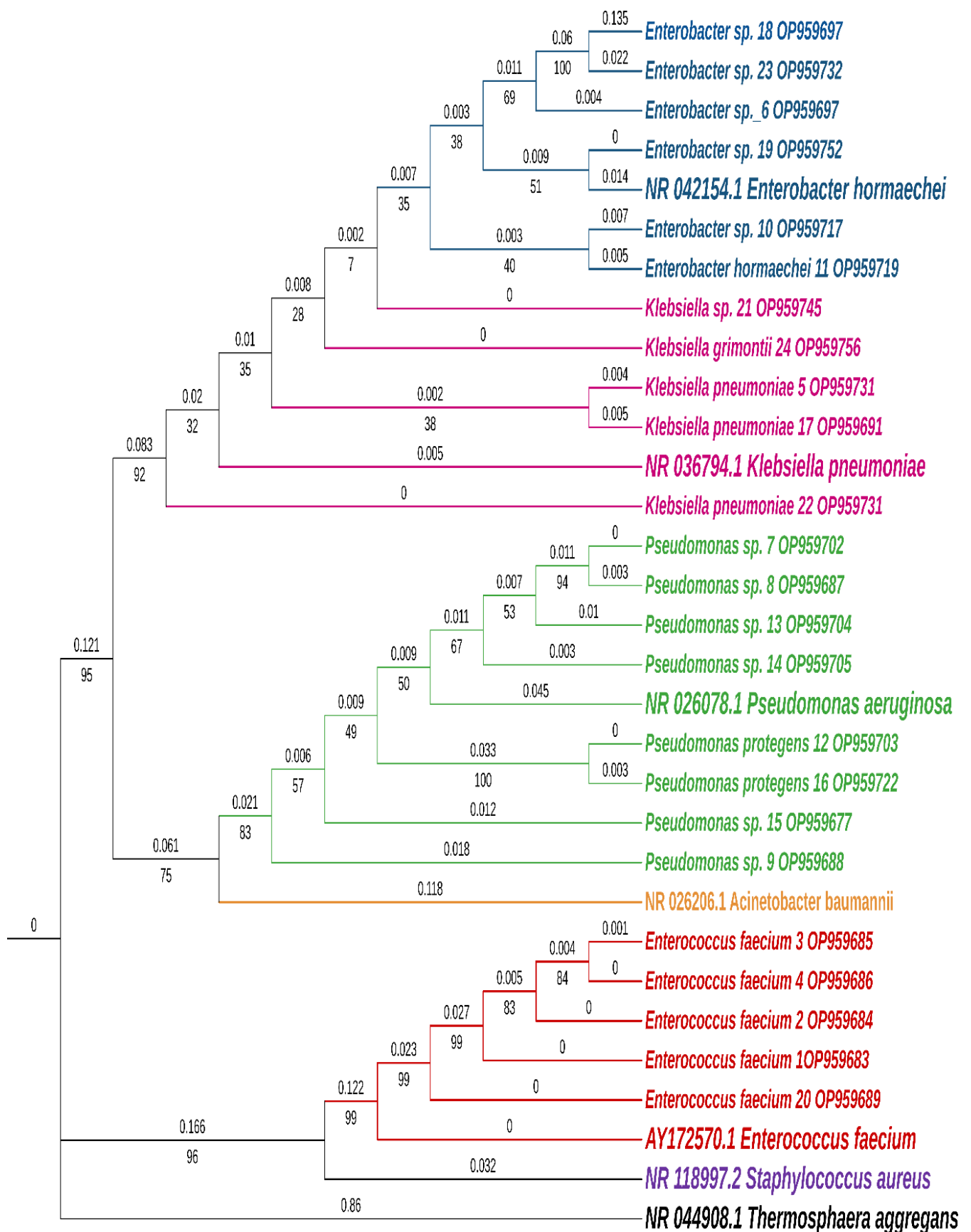


Figure 10: Maximum likelihood phylogenetic tree illustrating the phylogenetic relationship of ESKAPE species isolated from WWTP A. The tree contains 24 isolates from the ESKAPE group. The bootstrap values of the branches are represented at each of the branches. The tree was constructed by using 1000 bootstraps.

Figure 11 illustrates the clustering of the six main genera from the ESKAPE pathogen group isolated from WWTP B, namely *Enterobacter*, *Staphylococcus*, *Klebsiella*, *Acinetobacter*, *Pseudomonas* and *Enterococcus*. The reference strain that was used shows the accession number in front of the species name. *Thermosphaera aggregans* was used as an outgroup sequence. The tree is separated into six distinct clusters of the various organisms, where *Enterobacter* and *Klebsiella* share a cluster; *Pseudomonas* and *Acinetobacter* another; and *Enterococcus* and *Staphylococcus* a further one. The organisms that share clusters and the moderately high bootstrap values illustrate a high similarity among the species and the strains of the organisms, further confirming the confidence in their identities.

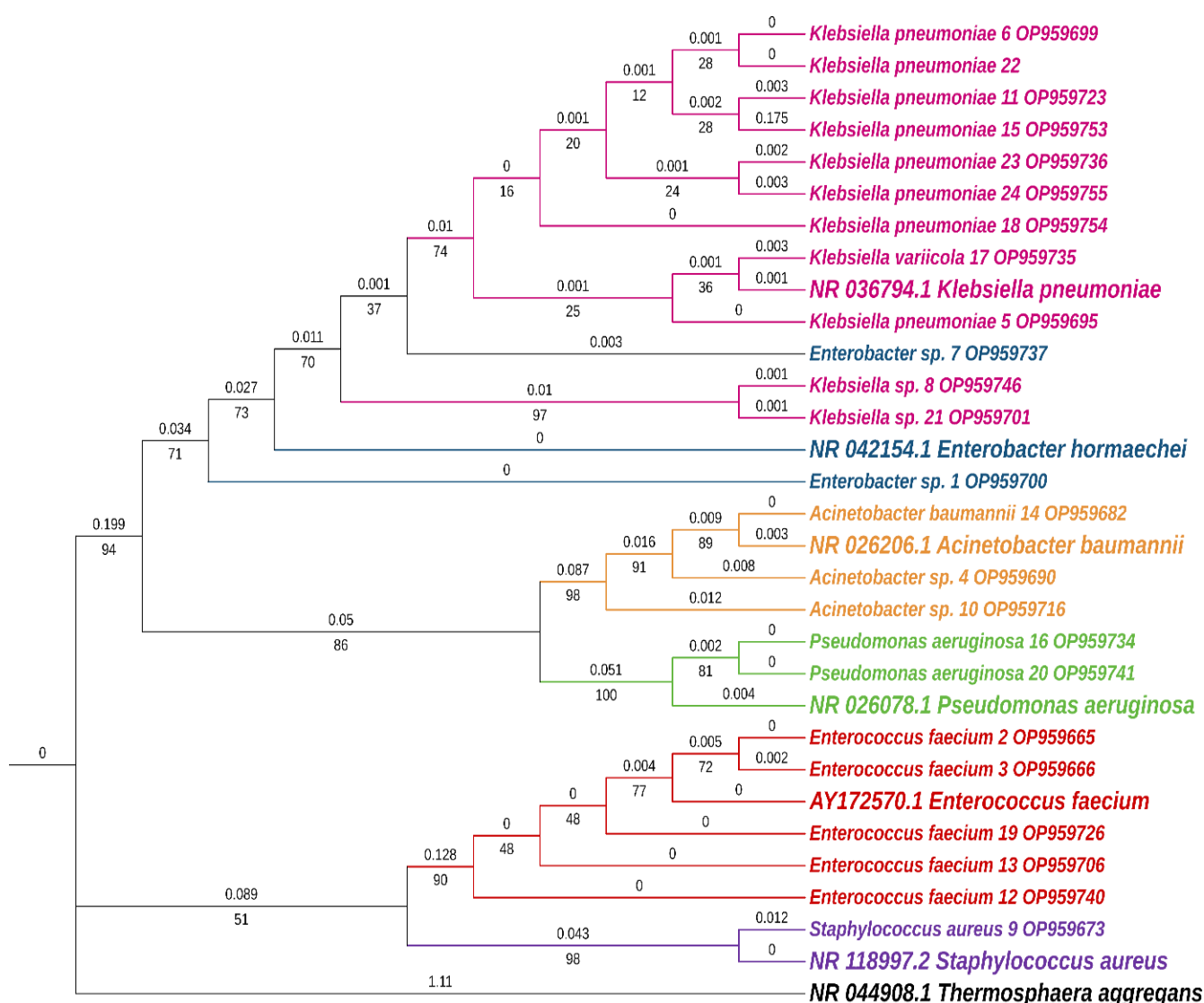


Figure 11: Maximum likelihood phylogenetic tree illustrating the phylogenetic relationship of ESKAPE species isolated from WWTP B. The tree contains 24 isolates from the ESKAPE group. The bootstrap values of the branches are represented at each of the branches. The tree was constructed by using 1000 bootstraps.

4.7 Multivariate statistics

The redundancy analysis (RDA) plots that depict the relationship between the sampling sites, phenotypical characteristics and genotypical characteristics are reflected in Figures 12 and 13. Figure 13 is an RDA plot that illustrates the relationship between all of the organisms that were isolated at both WWTP A and WWTP B, as based on their identification. The results illustrated in Tables 4 and 5, facilitate the drawing of RDA plots to illustrate the relationships among the different wastewater treatment plants, the organisms, the antibiotic resistance patterns and their extracellular enzyme activity.

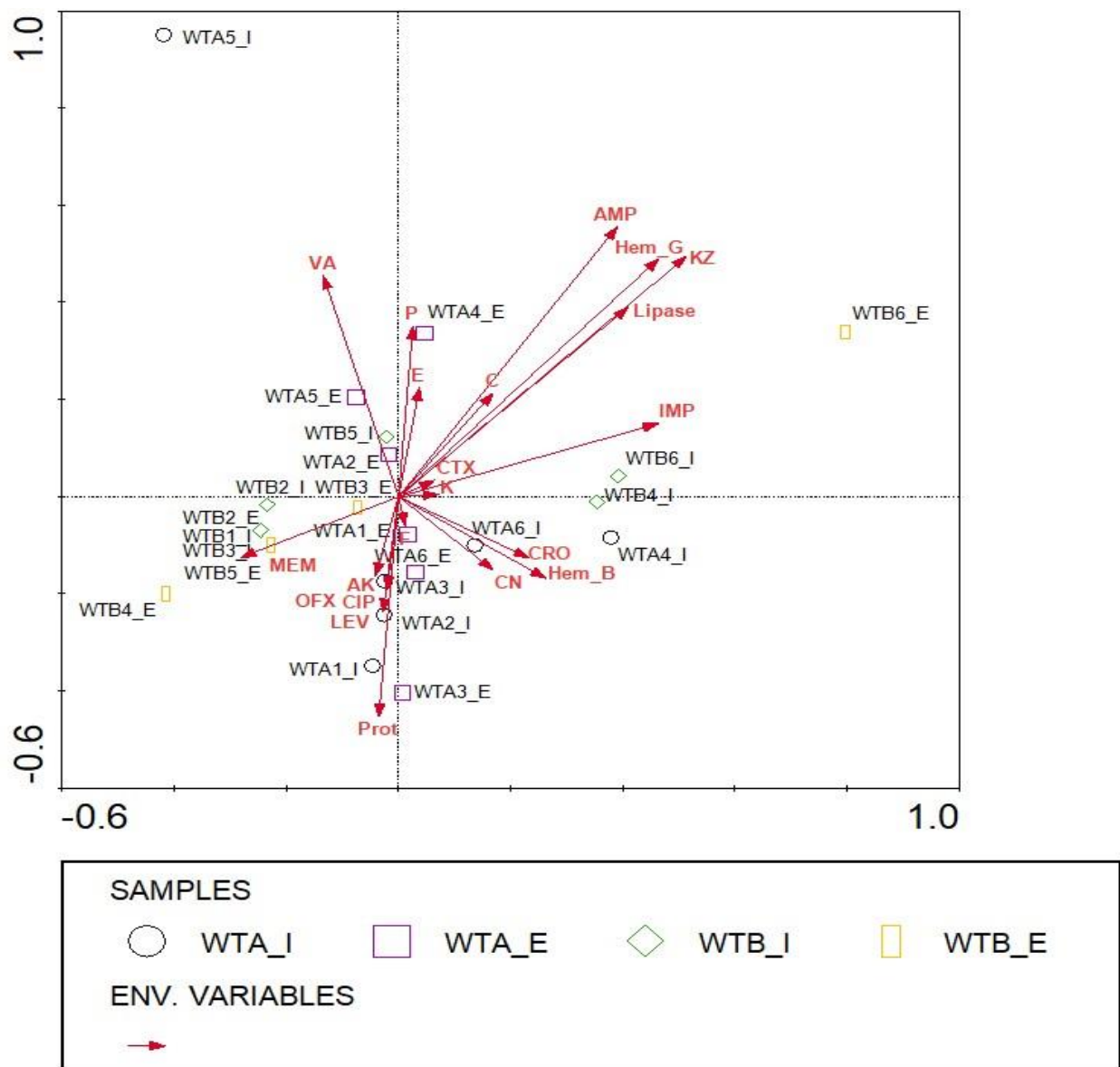


Figure 12: RDA plot illustrates the relationships among different WWTPs influent and effluent based on their species distribution. WTA = WWTPA; WTB = WWTP B; I = influent; E = effluent; 1 = *E. faecium*; 2 = *S. aureus*; 3 = *K. Pneumoniae*; 4 = *A. baumannii*; 5 = *P. Aeruginosa*; 6 = *Enterobacter* spp. In this RDA, the relationship between antibiotic resistance and the virulence factors can also be seen as compared to the respected sampling sites.

Figure 12 illustrates a mostly positive correlation between WWTP A and WWTP B and their influent and effluent. There is a negative relationship between the influent of WWTP A organism 5 and the effluent of WWTP B organism 6 when compared to the cluster of organisms. These clusters illustrate similarities between the sites and the organisms that were isolated from those plants. From Figure 12 a positive relationship between the extracellular enzyme activity and various antibiotic resistance profiles can be inferred.

A positive relationship was found between the species that exhibited gamma hemolysis activity and lipase production and the species that exhibited resistance against AMP, KZ, C, CTX and IMP. A positive relationship can also be observed between the species that exhibited beta hemolysis activity and the species that exhibited resistance against CRO and CN. Another positive relationship can be observed between the species that produced protease and those that exhibited resistance against MEM, AK, OFX, LEV and CIP.

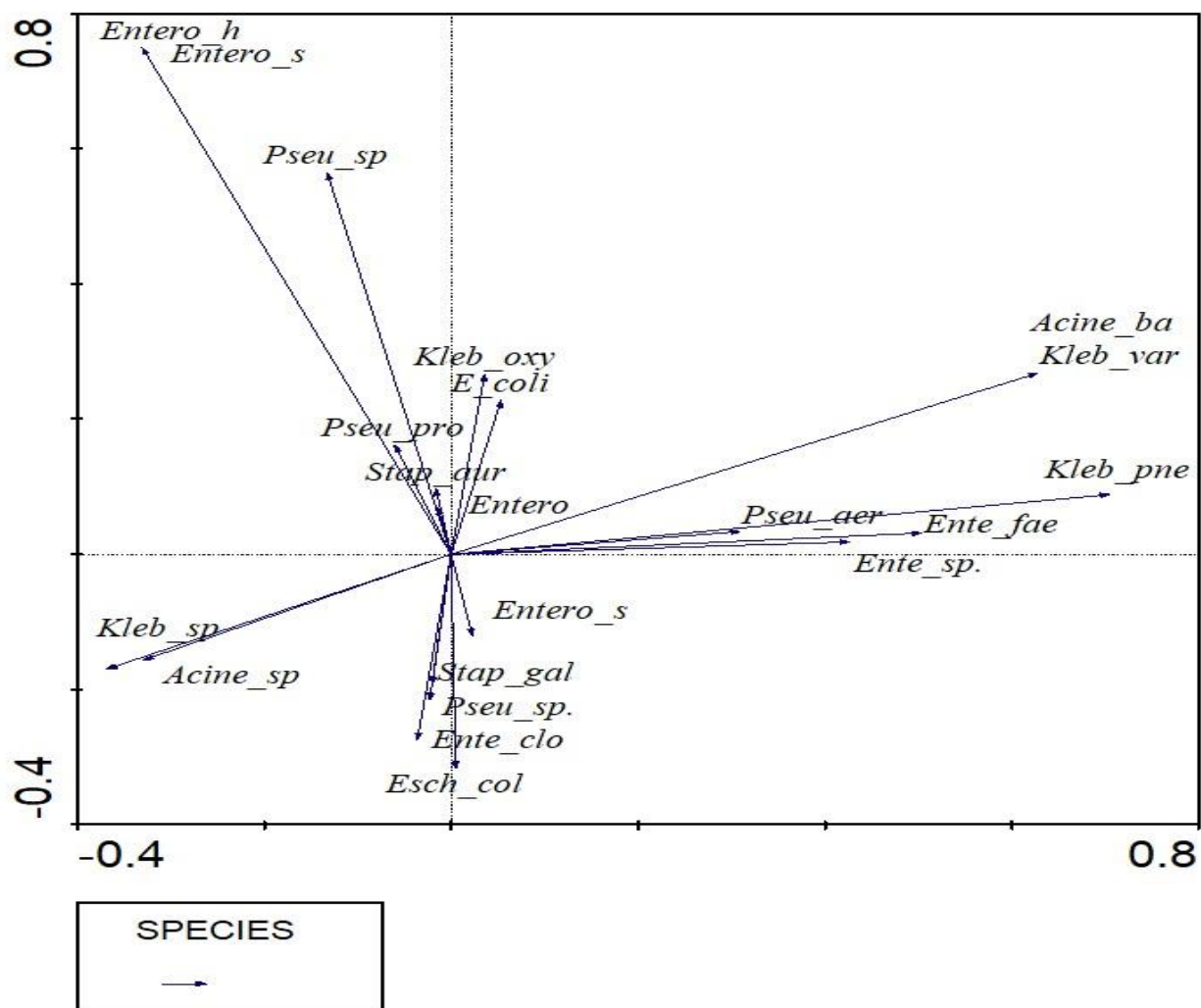


Figure 13: RDA plot depicting the species distribution and the relationships among as found in all of the sampling points and as based on their confirmed identifications as part of the ESKAPE pathogens group.

Figure 13 illustrates the relationship trends among the various species that were identified in the ESKAPE group. A positive relationship occurred among the organisms that were grouped, and most of the identified organisms clustered together with no outliers, which confirms a positive relationship between the various clusters observed, as reflected in the figure.

CHAPTER 5: DISCUSSION

This chapter discusses the results obtained in the present study. It includes the discussion of the rational of selecting the specific WWTPs, the isolation of the organisms and the success rates of this method, the potential virulence of the organisms and their antibiotic resistance traits as well as the effects that these might have on the environment and communities that use the effluent-receiving waters.

5.1 WWTP selection

The present study was conducted at two WWTPs, these two treatment plants were selected based on their differences in:

- The Green Drop status that they obtained.
- The purification processes.
- The treatment capacity of the treatment plants.
- The population it serves, the land activities in the surrounding areas.
- What bodies of water receive the effluent of the plants.

The Green Drop status of WWTP A was listed as a Functional WWTP whereas WWTP B was listed as a Critical risk WWTP.

Different treatment processes are used at these WWTPs, WWTP A utilises three treatment pathways (biological treatment, biofilter treatment and activated sludge treatment) whereas WWTP B only utilises biofilter treatment for the treatment process.

The two treatment plants differ in size, WWTP A has the capacity to treat 45 Mℓ of wastewater per day whereas WWTP B is a smaller plant only having the capacity to treat 3Mℓ of wastewater per day.

The population that the WWTPs serves also differs, WWTP A servers roughly 162 700 members of the community from the surrounding area whereas WWTP B serves roughly 56 700 members of the community of the surrounding area.

Different land-based activities are found around the WWTPs, WWTP A is surrounded by both mining and agricultural activities whereas WWTP B is just surrounded by agricultural activities.

Lastly the effluent receiving waters of these treatment plants also differ, WWTP A's effluent is discharged into two rivers whereas WWTP B's effluent is discharged into only one river.

5.2 Isolation ESKAPE pathogens

Initially, chromogenic agar was used for the isolation of ESKAPE pathogens. However, the identification process, as shown in Figure 1, yielded some difficulty, because a majority of the isolates were identified as not part of the ESKAPE pathogens group. After the 16S rRNA sequencing of these isolates, many were found not to be ESKAPE pathogens. As depicted in Figures 8 and 9, almost half of the isolated organisms were false positives of the ESKAPE group: WWTP A had 42% of these and WWTP B 49%.

Similar findings were made by Perry (2017), where the chromogenic agar used was selected for *Clostridium difficile*, whereas other anaerobic species produced the same dark black colonies. Filius *et al.* (2003), Verhaegen *et al.* (2015) and Messeir *et al.* (2012) also identified limitations that chromogenic agars might have as single sources of identification when it came to various organisms.

The present study encountered similar limitations when it came to using these chromogenic medias as sole preliminary isolations and identification methods. Other phenotypical and genotypical tests are recommended for the identification of these ESKAPE pathogens. By using other methods to correctly identify the organisms, more accurate research on their prevalence can be done.

The percentage of false positive identifications encountered in the present and other projects indicate the unreliability of the chromogenic agars as the sole methods for the isolation and identification of ESKAPE pathogens. As a consequence, the present study focused on presumptive and positively identified ESKAPE pathogens for further analysis.

5.3 Virulence factors

The production of the following four virulence factors was investigated: DNase, protease, lipase and hemolysin. This elucidated the possible pathogenic nature of the organisms, but the final selection was made based on their hemolysis activity (that is, either alpha or beta in nature). Hemolysis activity as a final selection was chosen because it is seen as one of the most important virulence factors that organisms utilise when causing infections and diseases in patients (Sagar *et al.*, 2013). A total of 26 organisms were selected for final identification based on their hemolysis activity, whereas a further six were identified as part of the ESKAPE group. It was found that no isolates produced Dnase, while 22 produced

lipase of which only seven formed part of the ESKAPE group. A total of 19 produced protease of which only five formed part of the ESKAPE group.

Proteas and lipase are extracellular enzymes produced by various ESKAPE pathogens (Jadimurthy *et al.*, 2022; Patil *et al.*, 2021). The lack of extracellular enzyme production from some of the isolates is not an indication of the incapacity of these organisms to produce these extracellular enzymes (Allison *et al.*, 2014; Cruz *et al.*, 2021). It has been commonly noted that extracellular enzyme production could be influenced by the environment and the need for the enzyme to obtain nutrients so as to enable the organism to thrive (Allison *et al.*, 2014; Li *et al.*, 2019). Researchers have described this occurrence as an evolutionary trait in which organisms will suppress enzyme production to conserve energy when they find themselves within an environment where enzyme production is not necessary (Allison *et al.*, 2014; Cruz *et al.*, 2021). Another reason for the lack of enzyme production could be that the organism has not yet obtained the genes responsible for the production of the extracellular enzymes (Deng *et al.*, 2019; Thomas & Wigneshweraraj, 2014).

5.3.1 Protease production of the ESKAPE organism

Protease is produced by most of the organisms that form part of the ESKAPE pathogens group. All confirmed ESKAPE organisms exhibited protease production except for *Acinetobacter baumannii* and *Klebsiella pneumoniae*. Although *A. baumannii* and *K. pneumoniae* did not exhibit any protease production, other studies report that *A. baumannii* and *K. pneumoniae* do have the ability to produce it (Tilley *et al.*, 2014; Al Meani *et al.*, 2020; Mohamed *et al.*, 2017; Bengoechea & Sa Pessoa, 2019). The lack of enzyme production could have been an indication that these organisms did not require the production of the enzyme in question, or they might not have obtained the genes to facilitate the production of this enzyme (Allison *et al.*, 2014; Deng *et al.*, 2019; Li *et al.*, 2019; Thomas & Wigneshweraraj, 2014).

Two *P. aeruginosa* isolates produced protease in the present case, and has been reported also in other studies (see O'callaghan *et al.*, 2019; Oldak & Trafny, 2005; Prasad *et al.*, 2020; Tang *et al.*, 2018). Oldak and Trafny (2005) stated that protease might be the most important virulence factor exhibited by *P. aeruginosa*.

One isolate from *E. faecium*, *S. aureus* and *Enterobacter* spp. illustrated the production of protease. Again, it has been reported in extant literature that these organisms do possess the ability to produce this (Bhagwat & Annapure, 2019; Boukhtache *et al.*, 2017; Gündoğan & Devren, 2010; Hao *et al.*, 2020; Luang-In *et al.*, 2019; Pietrocola *et al.*, 2017; Rom *et al.*, 2021; Saad *et al.*, 2021; Singh & Phukan, 2019).

The results obtained in the present study therefore reinforce those found in other studies (Boukhtache *et al.*, 2017; Hao *et al.*, 2020; Prasad *et al.*, 2020; Saad *et al.*, 2021). The production of protease as a virulence factor has been identified as an essential extracellular enzyme for the successful infection of a host (Ali *et al.*, 2021; Culp & Wright, 2017; Koziel & Potempa, 2013). The successful infection of the host and host cells is not the only pathogenetic property of protease: it also damages soft tissue and cell walls of host cells and inhibit proteins that are important for the host immune response (Frees *et al.*, 2013; Koziel & Potempa, 2013; Lantz, 1997). Protease affects the host directly and indirectly: directly, by destroying soft tissue and breaking apart proteins that are important for soft tissue; indirectly, by causing an excessive inflammatory response in the infected area (Koziel & Potempa, 2013). Protease as a virulence factor can cause lung damage, epidermal damage and can act as a hemolytic agent (Ali *et al.*, 2021; Koziel & Potempa, 2013; Lee *et al.*, 2002).

Because mice and humans share a number of physiological and anatomical similarities, the former are good subjects for demonstrating the potential health implications that pathogens might have on human hosts (Bryda, 2013). Research has described the effect that protease as a virulence factor has on mice: it has the ability to cause blood cell lysis and damage to the host's skin and can lead to lung damage and severe haemorrhages of vital organs (Ali *et al.*, 2021; Koziel & Potempa, 2013; Lee *et al.*, 2002).

The fact that the organisms studied in the present project produce protease increases the potential of infections and could cause problems for communities that use effluent-receiving water (Haldar *et al.*, 2022; Masindi *et al.*, 2022). These pathogens, which are known to survive in various environments such as water, soil and plants, as indicated, could cause problems for the environment and the communities utilising these waters (Denissen *et al.*, 2022; Odjadjare *et al.*, 2012). The impacts that the organisms might have on humans and the environment are discussed further in section 5.2.5.

5.3.2 Lipase production of the ESKAPE organism

Lipase is a common virulence factor utilised by many pathogenic organisms (Stehr *et al.*, 2003). Four of the six groups found within the ESKAPE pathogen group produced lipase. No lipase production was noted for *S. aureus* and *A. baumannii*, although this has been reported in other studies. Lipase production has been noted for *S. aureus* and *A. baumannii* by various researchers, indicating that some strains of these organisms do have the

capability to produce lipase (Awour *et al.*, 2023?; Chen & Alonzo, 2019; Gündoğan & Devren, 2010; Mohammed, 2013; Johnson *et al.*, 2016; Tanaka *et al.*, 2018).

Enterobacter spp. illustrated the highest number of isolates producing lipase. Similar results were reported by various researchers in terms of the lipolytic activity of these organisms (Pham *et al.*, 2021; Joyruth & Growther, 2020). The second highest number of lipase-producing isolates occurred in the case of *K. pneumoniae*, where two isolates exhibited lipase production. Various extant studies reported that the production of lipase by *K. pneumoniae* is a common occurrence (Emmanuel *et al.*, 2020; Kanimozhi *et al.*, 2022; Tanasupawat *et al.*, 2016).

E. faecium and *P. aeruginosa* only had one isolate that tested positive for lipase production. Other studies reported the former case in terms of clinical and foodborne isolates (Bhagwat & Annapure, 2019; Ramakrishnan *et al.*, 2012; Ramakrishnan *et al.*, 2013; Merih-Kivanc, 2021). The production of lipase by *P. aeruginosa* in this study corresponds with studies that have shown that *P. aeruginosa* isolates from soil, food and clinical environments possess the ability to produce lipase (Ali *et al.*, 2022; Hu *et al.*, 2018; Tielen *et al.*, 2013).

The secretion of lipolytic enzymes in order to invade host tissue is widely described for various pathogenetic bacteria (Chen & Alonzo, 2019; Park *et al.*, 2013). Lipase has been recorded to affect the immune cells of humans inhibiting some of the immune responses of the host (Park *et al.*, 2013; Stehr *et al.*, 2003). The secretion of lipase in lipid-rich environments can have a negative effect on the host's health: for instance, the secretion of lipase in the lungs breaks down pulmonary surfactant which, in turn, causes an inflammatory response that accelerates the degradation of lung tissue within the host (Chen & Alonzo, 2019). Pathogenic organisms also utilise lipase to hydrolyse lipids within host cells and use this as an energy source (Park *et al.*, 2013). By utilizing the lipase to infect the host and interfere with its immune responses, the pathogens ensure their own survival, which causes the host's health increasingly to deteriorate as the pathogenetic organisms reproduce (Chen & Alonzo, 2019; Park *et al.*, 2013; Stehr *et al.*, 2003).

ESKAPE pathogens have been reported to produce lipase, which holds health implications to humans and animals when they are introduced into effluent-receiving surface water (Haldar *et al.*, 2022; Odjadjare *et al.*, 2012). The prevalence of the organisms within the environment that produces lipase could lead to an increase in mortality of both animals and humans (Ahmed, 2007; Rodríguez-Tapia & Morales-Novelo, 2017). The implications that these organisms might have for humans and the environment are discussed further in section 5.2.5.

5.3.3 DNase production of the ESKAPE organism

The study found no isolates that exhibited traits of virulence that are characteristic of DNase production. Although the pathogens did not produce any DNase, it does not mean that these organisms are incapable of producing it. In this study, none of the *E. faecium* isolates produced any DNase but a study has reported DNase production by foodborne isolates of *E. faecium* (Nasiri & Hanifian, 2022). Other studies reported no DNase production by foodborne and clinical strains of *E. faecium* (Chakchouk-Mtibaa *et al.*, 2018; Schittler *et al.*, 2019). The fact that there was no DNase production in the present study in the case of *S. aureus* does not indicate that it is incapable of producing it. Studies have illustrated that foodborne isolates of *S. aureus* regularly produce DNase (Şanlıbaba, 2022; Sankomkai *et al.*, 2020). None of the *P. aeruginosa* that was isolated exhibited any DNase production but, again, studies reported that environmental and clinical isolates of the organism are capable of DNase production (Mulcahy *et al.*, 2010; Wilton *et al.*, 2018). Mulcahy *et al.* (2010) reported that DNase is not only seen as a virulence factor, but also that *P. aeruginosa* utilises this enzyme to be able to use DNA as a carbon source for survival. Although none of the isolates in this study produced DNase, it has been reported that *E. faecium*, *S. aureus* and *P. aeruginosa* are some of the organisms within the ESKAPE pathogen group that do have the ability to produce it.

The fact that the organisms did not exhibit production of DNase could be ascribed to the fact that they do not require extracellular enzymes to survive, or that they do not express the genes responsible for the production of this extracellular enzyme (Allison *et al.*, 2014; Cruz *et al.*, 2021; Li *et al.*, 2019; Thomas & Wigneshweraraj, 2014). The production of DNase by these pathogens could have an adverse effect on the infected host, because it is known to cause the degradation of nucleic acids (Gerceker *et al.*, 2009; Haas *et al.*, 2014). It is also believed that DNase improves the organism's ability to escape the immune response of the host increasing the odds of survival for the pathogenetic organism (Gerceker *et al.*, 2009; Haas *et al.*, 2014). This indicates that DNase is an important virulence factor when produced by the pathogenic organisms.

When they are introduced into the environment, they may possess the ability to increase infection rates if they were to produce DNase. This could be problematic, because effluent-receiving water is used for both household and agricultural purposes (Depetris, 2021; Lin *et al.*, 2022; Masindi *et al.*, 2022; Rodríguez-Tapia & Morales-Novelo, 2017). The implications that these organisms might have for humans and the environment are discussed further in section 5.2.5.

5.3.4 Hemolysis activity of the ESKAPE organism

The only organisms that did not exhibit any β -hemolysis activity were *K. pneumoniae* and *A. baumannii*, whereas the other four members of the ESKAPE pathogen group did produce hemolysin and exhibited β -hemolytic activity. One of the main virulence factors in potential pathogenic organisms is their ability to produce hemolysin, which means that they may be pathogens (Mogrovejo *et al.*, 2020; Vesper & Vesper, 2004). Of the four groups of organisms, *P. aeruginosa* exhibited the most hemolytic activity with three isolates exhibiting β -hemolytic activity. This finding dovetails with studies that have recorded that *P. aeruginosa* isolates from clinical and environmental samples exhibited hemolytic activity (Mogrovejo *et al.*, 2020; Wolfmeier *et al.*, 2022).

Only one isolate from the identified *E. faecium*, *S. aureus* and *Enterobacter* spp. exhibited β -hemolysis activity. Several studies recorded that *E. faecium*, *S. aureus* and *Enterobacter* spp. from clinical, foodborne and environmental isolates exhibited hemolytic activity (El Attar, 2007; Camargo *et al.*, 2014; Sanlibaba *et al.*, 2018; Semedo *et al.*, 2003; Wang *et al.*, 2020; Zhang *et al.*, 2016). No hemolysis activity was encountered in the case of the *K. pneumoniae* isolates, which is in accordance with studies that have been conducted on clinical and environmental isolates, which noted the lack of hemolytic activity in those isolates (Barati *et al.*, 2016; Pereira & Vanetti, 2014). *A. baumannii* isolates from the present indicated no hemolytic activity, whereas extant studies have recorded β -hemolysis activity within *A. baumannii* isolated from clinical environments (Dahdouh *et al.*, 2016; Schramm *et al.*, 2019).

β -hemolysis is the complete lysis of cells, which causes them to expel their intracellular compounds, enabling the pathogenic organism to utilise the cell content as nutrients (Mogrovejo *et al.*, 2020; Vesper & Vesper, 2004). The iron-mediating toxin effect is increased when organisms produce hemolysin, which affects nucleated cells and leucocytes that, in turn, reduces the host's immune response against the pathogenic organism (Divyakolu *et al.*, 2019; Egbe & Enabulele, 2014). The production of hemolysin by opportunistic pathogens is vital for their survival and the success of the infection, because it causes cell lysis, which supplies the organisms with nutrients and helps them evade the immune response of the host (Divyakolu *et al.*, 2019; Egbe & Enabulele, 2014). Because of the effect that hemolysin has on the host and the cells, it is seen as a virulence factor that can substantially influence the host when it is infected by a hemolysin-producing organism.

Organisms that produce hemolysin could be problematic to the health of both animals and humans that utilise water downstream of the WWTPs (Chahal *et al.*, 2020; Masindi *et al.*,

2022; Rodríguez-Tapia & Morales-Novelo, 2017). Because these organisms have been reported to exhibit various hemolytic activities, they could easily infect hosts that utilise these (Depetris, 2021; Divyakolu *et al.*, 2019; Masindi *et al.*, 2022). The implications that these organisms might have for humans and the environment are discussed further in section 5.2.5.

5.3.5 Impact of potential pathogens downstream

These ESKAPE organisms are known as opportunistic pathogens: the production of extracellular enzymes pertaining to various virulence factors indicates that they could be potential pathogens, while they were found in the influent and effluent of WWTPs (Driscoll *et al.*, 2007; Gilmore *et al.*, 2014; Peleg *et al.*, 2008; Sanders & Sanders, 1997; Schaumburg *et al.*, 2015; Vading *et al.*, 2018). The virulence factors tested in the present study shows that they help the organism infect a host successfully on a cellular level, evade or suppress the host's immune response and cause damage to the host cells (Chen & Alonzo, 2019; Divyakolu *et al.*, 2019; Lantz, 1997; Sharma *et al.*, 2017). Extant research notes the production of lipase, protease, hemolysin and DNase in members of the ESKAPE pathogens group, illustrating that they do possess the ability to infect a host and cause various health problems (Awour *et al.*, 2023?; Mogrovejo *et al.*, 2020; Prasad *et al.*, 2020; Şanlıbaba, 2022).

The fact that these organisms “escape” the treatment at the WWTPs and are expelled into the surface water means that they could cause various problems not only for humans, but also for ecosystems downstream from WWTPs (Chahal *et al.*, 2020; Masindi *et al.*, 2022; Rodríguez-Tapia & Morales-Novelo, 2017). Pollution of surface water could lead to the death of various animals and might force communities close to the water to relocate or face other adverse effects if they are dependent on the use of these effluent-receiving waters (Ahmed, 2007; Rodríguez-Tapia & Morales-Novelo, 2017). The latter are regularly used for agricultural irrigation, sanitation and drinking in rural communities (Depetris, 2021; Lin *et al.*, 2022; Masindi *et al.*, 2022; Rodríguez-Tapia & Morales-Novelo, 2017). The contaminated surface water is used for irrigation, which compromises the produce and the soil of farms and, because of this, humans and animals that consume the produce irrigated by the contaminated water might be infected (Haldar *et al.*, 2022; Masindi *et al.*, 2022). The movement of the organisms from wastewater to the environment is problematic, because they are known to survive in environments like soil, water, sewage and plants, as indicated (Denissen *et al.*, 2022; Odjadjare *et al.*, 2012). Because they are released into the

environment and the water they reside in is used for various activities, the chances of infection increase (Haldar *et al.*, 2022; Odjadjare *et al.*, 2012).

The increased number of organisms within the environment should not be the only cause for concern, since they also produce various extracellular enzymes that increase their virulence and the success rate of infections (Chen & Alonzo, 2019; Divyakolu *et al.*, 2019; Lantz, 1997; Sharma *et al.*, 2017). Consuming water, plants or other food products contaminated by water containing these potential pathogens could cause organisms to infect a host and further cause adverse health effects caused by the extracellular virulence factors they produce (Haldar *et al.*, 2022; Lin *et al.*, 2022). After these organisms have successfully infected a host, they reproduce and are then excreted back into wastewater, where they again reach the environment after improper wastewater treatment, thus forming a cycle of continuous infections and environmental contamination (Gerba, 2015; Gupta *et al.*, 2017; Pandey *et al.*, 2014)

5.4 Antibiotic resistance and antibiotic resistance genes

The heat maps (Figures 2 to 7) illustrated certain resistance trends for each of the presumptive isolates. A study conducted by Ramsamy *et al.* (2018a) illustrated antibiotic resistance patterns of ESKAPE pathogens, and their results showed similarities to the resistance patterns noted in the present study. The study conducted by Ramsamy *et al.* (2018a) spanned five years and that of Orosz *et al.* (2022) ten (from 2010-2020), and these illustrated the way in which the organisms evolve and develop higher resistance against clinical relevant antibiotics. These two studies illustrated that the resistance of ESKAPE organisms is increasing against various antibiotics that are commonly used in clinical settings, causing the organisms to be more successful in escaping treatments and possibly reaching the environment (Orosz *et al.*, 2022; Ramsamy *et al.*, 2018a).

This increase in resistance against the antibiotics could be attributed to the various mechanisms that the organisms from the ESKAPE pathogen group utilise. One of the most common among these is the use of β -Lactamase production (De Oliveira *et al.*, 2020). It is made possible by the antibiotic resistance genes that the organisms acquire. Organisms can acquire the genes from the WWTPs, because it has been reported that WWTPs are a reservoir for ARGs (Piotrowska *et al.*, 2019). Common types of β -Lactamase genes expressed by ESKAPE pathogens are *bla*_{TEM}, *bla*_{SHV}, *bla*_{KPC} and *bla*_{CTX}, while other β -Lactamase producing genes that have also been frequently identified are *bla*_{FOX} and *bla*_{MOX} (De Oliveira *et al.*, 2020; Piotrowska *et al.*, 2019). These β -Lactamase ARGs are a

considerable problem, especially when they keep spreading, because they have a very high rate of mutation and present the organisms with resistance mechanisms against a broad spectrum of antibiotics (Piotrowska *et al.*, 2019). ARGs were found in both the pathogenic and non-pathogenic isolates from both of WWTPs according to Piotrowska *et al.* (2019). The exchange of genes between non-pathogenic and pathogenic organisms can be a threat to the environment and the public health system. The present study illustrated that many of these organisms contained ARGs.

5.4.1 *Enterococcus faecium*

The presumptive *E. faecium* that was isolated and subjected to antibiotic resistance tests produced results similar to those obtained in other studies (Markwart *et al.*, 2019; Miller *et al.*, 2014; Sattari-Maraji *et al.*, 2019). It displayed resistance against ampicillin, gentamycin, penicillin G and kanamycin. Resistance to various antibiotics, including the ones that have been used in this study, has been identified globally, where studies found that *E. faecium* is growing resistant to more antibiotics over time and could cause complications regarding the effective treatment of patients (Markwart *et al.*, 2019; Miller *et al.*, 2014; Sattari-Maraji *et al.*, 2019). Denissen *et al.* (2022) and Zaheer *et al.* (2020) discussed how *Enterococci* can be found in various environments, while they contain various ARGs that enable them to grow increasingly resistant against various antibiotics. In the present study, none of the *Enterococcus* isolates tested positive for the ARGs that were screened for, whereas extant studies found that the organisms contained various ARGs which could impact the treatments of infected patients (Denissen *et al.*, 2022; Miller *et al.*, 2014; Piotrowska *et al.*, 2019; Sattari-Maraji *et al.*, 2019; Zaheer *et al.*, 2020).

A wide range of life-threatening diseases caused by *E. faecium*, including pelvic infections, UTIs, intra-abdominal infections, infections in soft tissue and, most commonly, bacteremia and endocarditis (Bharti *et al.*, 2016; Gilmore *et al.*, 2014). When these organisms become more resistant because of the misuse of antibiotics or horizontal gene transfer, treatments of these infections are more difficult and could in fact lead to the death of the host.

5.4.2 *Staphylococcus aureus*

The presumptive *S. aureus* that was isolated and subjected to antibiotic resistance tests showed similar results to those obtained in other studies (Denissen *et al.*, 2022; Sineke *et al.*, 2021). They displayed high resistance against ampicillin. Similar trends of resistance are discussed by Denissen *et al.* (2022) and Sineke *et al.* (2021). Denissen *et al.* (2022) reported similar results from their samples in terms of antibiotic susceptibility. Other research recorded that the *S. aureus* isolates were mostly susceptible to chloramphenicol

(Denissen *et al.*, 2022; Sineke *et al.*, 2021; Asante *et al.*, 2021). The isolates from the present study also illustrated that most of the isolates were susceptible to gentamycin which, again, corresponds with other research (Asante *et al.*, 2019; Asante *et al.*, 2021; Sineke *et al.*, 2021).

Only one confirmed *Staphylococcus* isolate was positively identified by means of the 16S rRNA sequencing and it did not contain any ARGs that were screened for, whereas other studies reported that the organism could contain various ARGs that increase their resistance to a broad spectrum of antibiotics such as tetracycline and penicillins (Amoako *et al.*, 2019; Denissen *et al.*, 2022; Piotrowska *et al.*, 2019).

S. aureus forms part of the natural flora of animals and humans but it is also known to be an opportunistic pathogen that has been recorded to be fatal in 35% of cases (Balaban *et al.*, 2000; Schaumburg *et al.*, 2015). It is one of the more prevalent pathogens in hospitals that causes various infections, including bacteremia, skin and soft tissue infections, osteoarticular infections (osteomyelitis, septic arthritis), UTIs, nosocomial pneumonia, endocarditis and meningitis (Balaban *et al.*, 2000; Tong *et al.*, 2015). Since these organisms show an increase in resistance, it is evident that they will become hard to treat in the future if the resistance is not addressed (Denissen *et al.*, 2022; Sineke *et al.*, 2021).

5.4.3 *Klebsiella pneumoniae*

The presumptive *K. pneumoniae* that was isolated and subjected to antibiotic resistance tests showed similar results to those obtained in other studies (Denissen *et al.*, 2022; Ramsamy *et al.*, 2018b). It displayed high resistance against ampicillin and cefazoline. Denissen *et al.* (2022) obtained similar results, and some of their isolates were completely resistant against ampicillin and Cefazolin. The presumptive *K. pneumoniae* isolates exhibited very low resistance against ceftriaxone, which differs from other studies that reported that it exhibits high resistance against ceftriaxone (Agyepong *et al.*, 2019a; Aminul *et al.*, 2021; Ramsamy *et al.*, 2018b). High resistance against the remaining antibiotics was not found, and this corresponds with results obtained in other studies (Denissen *et al.*, 2022; Ramsamy *et al.*, 2018b). In the present study, the presumptive *K. pneumoniae* was highly susceptible to imipenem, which resonates with further work in the field (Effah *et al.*, 2020; Agyepong *et al.*, 2019a; Founou *et al.*, 2018). The theory that these organisms are growing evermore resistant to antibiotics has been noted by Effah *et al.* (2020), who state that resistance against antibiotics like imipenem has been increasing since 2005.

Fifteen *Klebsiella* isolates were confirmed with 16S rRNA sequencing. Among these, four different ARGs were found, namely *bla*_{SHV}, *bla*_{OXA-48}, *bla*_{VIM} and *bla*_{TEM}. Studies conducted

by Piotrowska *et al.* (2019) and Denissen *et al.* (2022) also report the isolation of *bla*_{SHV} and *bla*_{TEM} from *Klebsiella* isolates. Extant research has also found various other ARGs within *Klebsiella* isolates, namely *bla*_{CTX} and *bla*_{KPC}, whereas these were not detected in the present study (see Agyepong *et al.*, 2019a; De Oliveira *et al.*, 2020; Founou *et al.*, 2018; Piotrowska *et al.*, 2019; Ramsamy *et al.*, 2018b).

Infections such as UTIs, pneumonia, bacteraemia and liver abscesses are caused by *K. pneumoniae*, and these are usually most prevalent among immunocompromised patients while, in recent years, an increase in hypervirulent *K. pneumoniae* has developed (Keynan & Rubinstein, 2007; Paczosa & Meccas, 2016; Vading *et al.*, 2018). This present study and others illustrate the various resistance patterns of these organisms (see Aminul *et al.*, 2021; De Oliveira *et al.*, 2020; Ramsamy *et al.*, 2018b). The increase in antibiotic resistance is not the only cause for concern: an increase in hypervirulent strains within communities is also problematic, because treatment will be limited due to the increased resistance (Rastegar Lari *et al.*, 2013).

5.4.4 *Acinetobacter baumannii*

The presumptive *A. baumannii* that was isolated and subjected to antibiotic resistance tests showed similar results to those obtained in other studies (Denissen *et al.*, 2022; Ramsamy *et al.*, 2018a; Moosavian *et al.*, 2020). It displayed high resistance against meropenem and high amounts of intermediate resistance against ceftriaxone, cefotaxime and ceftazidime. Extant studies obtained similar results, where some of their isolates were highly resistant against various antibiotics such as meropenem and ceftazidime (Denissen *et al.*, 2022; Ramsamy *et al.*, 2018a; Moosavian *et al.*, 2020). Denissen *et al.* (2022) state that *A. baumannii* isolates were resistant against ceftazidime. The fact that those found in our study were mostly intermediately resistant could indicate that those organisms might grow to become completely resistant under certain circumstances. The increase in resistance to antibiotics such as meropenems could be due to a reduction in the expression of porins on the outer membrane (Nguyen & Joshi, 2021).

Only three *Acinetobacter* isolates were positively identified with the 16S rRNA sequencing, while no ARGs were identified in these cases. Extant studies report that the organisms are known to contain ARGs such as *bla*_{TEM} and *bla*_{OXA} (Agyepong *et al.*, 2019b; Denissen *et al.*, 2022; De Oliveira *et al.*, 2020; Nguyen & Joshi, 2021; Piotrowska *et al.*, 2019).

A. baumannii causes a wide range of infections that could be life-threatening if left untreated or improperly treated, including bacterial pneumonia, UTIs, meningitis and blood-stream infections (Dijkshoorn *et al.*, 2007; Peleg *et al.*, 2008). These life-threatening infections pose

an even higher risk of mortality because of the antibiotic resistance that has been recorded in this study and various others (Denissen *et al.*, 2022; Ramsamy *et al.*, 2018a; Moosavian *et al.*, 2020). Because these organisms have been recorded to be multidrug-resistant, they might not be able to be treated correctly and this would lead to a higher mortality rate.

5.4.5 *Pseudomonas aeruginosa*

The presumptive *P. aeruginosa* that was isolated and subjected to antibiotic resistance tests showed similar results to those obtained in other studies. It displayed high resistance against ampicillin. Denissen *et al.* (2022) report that *P. Aeruginosa* exhibits high resistance to β -lactams, and other studies describe that it has the ability to be highly resistant to ampicillin (Behzadi *et al.*, 2021). The present study found that the isolates were mostly susceptible to ciprofloxacin and gentamycin. Contrary to these results, various other studies have described the organism to be resistant to other antibiotics, including ciprofloxacin (Founou *et al.*, 2020; Kunz Coyne *et al.*, 2022; Roulová *et al.*, 2022).

Thirteen confirmed *Pseudomonas* isolates were positively identified by means of the 16S rRNA sequencing. In these isolates, three different ARGs were positively screened for: *bla*_{VIM}, *bla*_{OXA} and *bla*_{TEM}. Piotrowska *et al.* (2019) and Behzadi *et al.* (2021) also isolated *bla*_{VIM} from *Pseudomonas* spp. The ARG *bla*_{OXA} was identified in *Pseudomonas* spp. by Founou *et al.* (2020) and Janice *et al.* (2021). The fact that the isolated *Pseudomonas* spp. of the present study contained various genes does not come as a surprise, as these organisms are known to contribute to the transfer of ARGs in the environment (De Oliveira *et al.*, 2020).

Various nosocomial infections such as urinary tract infections, pneumonia, bloodstream infections and skin infections in patients that have suffered severe burns are caused by *P. aeruginosa* (Driscoll *et al.*, 2007). The mortality rate when patients get infected is the highest among bacterial pathogens and has also been recorded as the second highest cause of pneumonia in paediatric ICUs (Driscoll *et al.*, 2007). Because the mortality rate is so alarming, the effective treatment of these infections is crucial, but the increase in antibiotic resistance complicates treatment, causing some cases to be untreatable if the correct antibiotics are not available (Behzadi *et al.*, 2021; Driscoll *et al.*, 2007; Kunz Coyne *et al.*, 2022; Piotrowska *et al.*, 2019).

5.4.6 *Enterobacter* spp.

The presumptive *Enterobacter* spp. isolated and subjected to antibiotic resistance tests showed similar results to those obtained in other studies. It displayed high resistance

against ampicillin and cefazoline. Denissen *et al.* (2022) report that *Enterobacter* spp. exhibited high resistance to β -lactams, but they also found high levels of resistance to levofloxacin, whereas the present study found high susceptibility towards various antibiotics, including levofloxacin. Other studies describe *Enterobacter* spp. as having a high resistance to various clinical antibiotics (Davin-Regli *et al.*, 2019; Ghanavati *et al.*, 2018; Mbanga *et al.*, 2021).

Twelve confirmed *Enterobacter* isolates were positively identified by means of the 16S rRNA sequencing. In these isolates, three different ARGs were positively screened for, namely *bla*_{VIM}, *bla*_{OXA-48} and *bla*_{SHV}. These three genes have also been found in studies conducted by Denissen *et al.* (2022) and De Oliveira *et al.* (2020). Other genes that were screened for, which were however not found in the present study, were in fact found in other studies, namely *bla*_{TEM}, *bla*_{KPC} and *bla*_{CTX} (Denissen *et al.*, 2022; De Oliveira *et al.*, 2020; Piotrowska *et al.*, 2019). Other research identified further genes within these organisms that increase their resistance to a broad spectrum of antibiotics (Ghanavati *et al.*, 2018; Khademi *et al.*, 2022; Mbanga *et al.*, 2021).

Enterobacter spp. infections have been recorded in patients that have been hospitalized for extended periods, especially within the ICUs of hospitals: the species that regularly cause these infections are *Enterobacter aerogenes* and *Enterobacter cloacae* (Sanders & Sanders, 1997). The infections caused by *Enterobacter* spp. are mostly those centred on the respiratory tract, surgical wounds, blood and the urinary tract (Sanders & Sanders, 1997). Since these organisms are known for various infections, their increased antibiotic resistance against broad-spectrum antibiotics is a cause of concern; effective treatments could soon be unavailable if the rapid rate of antibiotic resistance is not addressed (Denissen *et al.*, 2022; Ghanavati *et al.*, 2018; Khademi *et al.*, 2022; Mbanga *et al.*, 2021).

5.4.7 Impact of ARGs and antibiotic resistance

As has been indicated, antibiotic resistance is acquired by gene transfer from other organisms or the poor use of antibiotics by humans for clinical or agricultural purposes (Cangelosi *et al.*, 2005). The improper use of antibiotics in the private and agricultural sectors causes large amounts of antibiotics to be expelled into the environment, and this facilitates development of resistance against antibiotics, which makes treatment of infections difficult (Mann *et al.*, 2021; Manyi-Loh *et al.*, 2018).

Not only does the improper use of antibiotics cause an increase in antibiotic resistance, but the high rate of horizontal gene transfer in wastewater treatment plants and the environment also increases resistance (De Oliveira *et al.*, 2020; Piotrowska *et al.*, 2019). This means not

only do that these organisms exhibit multiple antibiotic resistance traits, but that they also contain ARGs that they might have obtained from various organisms either in the environment or within WWTPs (De Oliveira *et al.*, 2020; Piotrowska *et al.*, 2019).

The organisms, once more, cause various life-threatening infections in hosts, including UTIs, respiratory infections, wound infections and blood infections (Driscoll *et al.*, 2007; Peleg *et al.*, 2008; Tong *et al.*, 2015). Their increased resistance in various environments entails that the effective treatment of infections is limited in various areas that do not have access to adequate healthcare (Morris-Paxton *et al.*, 2020). The infections spread easily to communities downstream from WWTPs, because the effluent-receiving waters are used for agricultural and household purposes (Haldar *et al.*, 2022; Masindi *et al.*, 2022).

Having said this, consider that regular use of these water could impact communities, because prolonged use of the water increases the risk of infection with opportunistic pathogens. This increased risk of infection could affect the whole community, because the rural areas do not have access to adequate healthcare, and diseases are regularly recorded to run rampant (Morris-Paxton *et al.*, 2020). Thus the increase in the antibiotic resistance of these ESKAPE pathogens is a problem for the communities that utilise the water downstream from the WWTPs for household and agricultural uses (Chahal *et al.*, 2020; Masindi *et al.*, 2022; Rodríguez-Tapia & Morales-Novelo, 2017).

5.5 ESKAPE pathogenic phenotypes and ARGs in influent and effluent

The present argument has demonstrated that there is no major difference between the amount of ESKAPE pathogens isolated from the influent and the effluent of the WWTPs. This is reinforced by the data reflected in Tables 4 and 5 and the clustering between the WWTPs' influent and effluent as depicted in Figure 12. The fact of the correlation between these, in addition to the recognition that there are no large changes within the community composition of the influent and the effluent, reinforces the statement that ESKAPE pathogens escape the treatment of the WWTPs and trickle into the environment (see Denissen *et al.*, 2022; Rosenberg Goldstein *et al.*, 2012; Slekovec *et al.*, 2012).

This could contribute to an increase in antibiotic-resistance within the environment, in addition to horizontal gene transfer (Cangelosi *et al.*, 2005; Cavicchioli *et al.*, 2019; Piotrowska *et al.*, 2019). The increase in antibiotic resistance in turn causes the organisms to become harder to treat when they infect a host, causing the organisms to be excreted again into the wastewater (Cangelosi *et al.*, 2005; Gerba, 2015). These organisms will then return to the environment because of ineffective treatment of patients and wastewater

(Cangelosi *et al.*, 2005; Gerba, 2015; Gupta *et al.*, 2017). They will then accumulate in the environment and this will increase the odds that they will be able to reinfect a new host and start a new cycle where they will reproduce, cause an infection and then be reintroduced back into the environment (Cangelosi *et al.*, 2005; Gerba, 2015; Gupta *et al.*, 2017).

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

This chapter presents the conclusion to be reached as based on the objectives that were set out for this study. It also contains recommendations for future research on this subject.

6.1.1 Prevalence

The first objective of this study was to determine the prevalence of ESKAPE pathogens at WWTPs. This was achieved by using various chromogenic media to isolate the pathogens from both the influent and effluent of WWTPs. The organisms that were isolated from the WWTPs were then selected for sequencing, where they were determined to be part of the ESKAPE pathogen group, thus illustrating that they are present in both the influent and the effluent of the WWTPs, as indicated.

6.1.2 Virulence

The second objective was to determine the potential pathogenicity of the isolated ESKAPE pathogens by determining extracellular enzyme activity. The extracellular enzymes that were tested for and produced by these organisms were those that enable the organisms to infect a host effectively ensuring that they can reproduce within the host in the absence of an adverse immune response against the pathogen. After reproducing in the host, they end up in wastewater again, as has been determined. The organisms that were isolated from the WWTPs tested positive for lipase, protease and hemolysin production. Tables 4 and 5 illustrate that they produce extracellular enzymes pertaining to virulence and could be opportunistic and cause health implications in the environment and rural areas.

6.1.3 Antibiotic resistance

The third objective was to determine the antibiotic resistance profiles of the isolated ESKAPE pathogens as well as the presence of selected ARGs. The antibiotic resistance patterns and the ARGs that were observed show similar results to extant studies (Denissen *et al.* 2022; De Oliveira *et al.*, 2020; Mulani *et al.*, 2019; Piotrowska *et al.*, 2019). These studies dovetail with the fact that the isolates found in the present study have similar trends to those found in other countries. The long-term research that was done by Ramsamy *et al.* (2018a) (5 years) and Orosz *et al.* (2022) (10 years) reinforce the statement that these ESKAPE pathogens are continuously adapting to survive multiple forms of antibiotic treatments and that they are growing more resistant to these various antibiotics over time.

A large contributor to increasing antibiotic resistance is the fact that WWTPs have known reservoirs of ARGs that can be incorporated and utilised by the organisms, causing them to

become highly resistant to certain antibiotics before they are released into the environment (Piotrowska *et al.*, 2019; Waśko *et al.*, 2022). Certain ESKAPE pathogens have been known to be good carriers of ARGs, which increases the effectivity and rate at which ARGs spread among various organisms (Piotrowska *et al.*, 2019; Waśko *et al.*, 2022).

In the present study, no large difference was noted between the ARG content of the isolates from the influent and effluent of WWTP B, whereas there was a big difference in the case of WWTP A. There were no ARGs present in the isolates from the influent of WWTP A, but there was a large number of ARGs in the isolates from the effluent of WWTP A.

This can be confirmation of the statement that was made by Piotrowska *et al.* (2019) that WWTPs can be reservoirs for ARGs and that organisms can pick up these genes and become increasingly resistant to antibiotics before being released back into the environment. The increase in antibiotic resistance due to horizontal gene transfer could mean that the organisms are getting more resistant to various antibiotics, which renders infections that could be acquired from the environment much harder to treat in rural areas.

6.1.4 Environmental impact

The final objective of this study was to determine if these potentially pathogenic environmentally isolated ESKAPE organisms may have environmental health implications. The present study importantly established that the health implications that these ESKAPE pathogens could have on people are severe and can even lead to an increase in mortality rates (Mulani *et al.*, 2019). The organisms are very good at adapting to their environment and surviving in unfavourable conditions, thus enabling them to escape the treatments at the WWTPs and trickle into the environment (Denissen *et al.*, 2022; Rosenberg Goldstein *et al.*, 2012; Slekovec *et al.*, 2012).

The fact that these organisms could escape treatments of WWTPs and end up in the environment was confirmed by the present study in view of the high rate of ESKAPE pathogens observed in the effluent of the WWTPs. There was no real large increase or decrease of isolated ESKAPE pathogens that were isolated from the effluent compared to that of the influent. This statement is reflected in Figure 12, which illustrates that there is mostly a positive correlation between the influent and effluent of each of the plants and their respective isolation of ESKAPE pathogens. The study has illustrated that these environmental ESKAPE species are potential pathogens and antibiotic-resistant. Similar results were obtained in other studies (Denissen *et al.*, 2022; Rosenberg Goldstein *et al.*, 2012; Slekovec *et al.*, 2012). This means that these organisms could move into the surface

waters of the environment which, in turn, could cause health complications for the environment and communities that utilise the effluent-receiving waters.

6.2 Recommendations

- During the isolation and purification process, a considerable number of false-positive organisms were identified by the 16S rRNA sequencing. It is recommended that other chromogenic medias are tested to see if they reduce the rate of false positive isolations, so that the isolation can be done with higher accuracy.
- For genetic identification, it is advised that not only the 16S region is sequenced but that, instead, housekeeping genes should be identified and used to identify each of the ESKAPE organisms. Culturing methods are very time-consuming: future research should utilise assays that are culture-independent such as qPCR or nanopore assays to save time when investigating ESKAPE pathogens.
- To analyse the composition of the wastewater and the species distribution it is recommended to overlay results from WWTPs and clinical samples to determine if the pathogens that are present in the hospitals are the same species that are present in the wastewater. Taking samples from hospital wastewater before it is mixed into the general wastewater that is in the influent of the WWTPs could also help to determine if hospitals are large sources of ESKAPE organisms within the wastewater or whether the main sources are urban areas. If those results indicate that the number of ESKAPE organisms in the wastewater is high, a preliminary treatment process of hospital wastewater could be tested to see if this lowers the number of ESKAPE organisms within the wastewater of the WWTPs.
- These organisms are resilient and tend to escape the treatment process at WWTPs. A surveillance program should be put in place to establish the seasonal variation of the organisms within the WWTPs and to see how their antibiotic resistance profiles change over time, because this study found that the ESKAPE pathogens get introduced back into the environment after wastewater treatment. Advanced treatment processes such as UV and ozonation treatment should be considered to analyse the effectiveness of each treatment method.

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