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Prevalence, antibiogram and molecular characterization of methicillin-resistant *Staphylococcus aureus* recovered from treated wastewater effluent and receiving surface water

KERISHA RAMESSAR

Submitted in fulfilment of the academic requirements for the Masters of Science degree in the Discipline of Microbiology, School of Life Sciences, College of Agriculture, Engineering and Science at the University of KwaZulu-Natal (Westville Campus).

As the candidate's supervisor, I have approved the dissertation for submission.

Supervisor: Signed: ______________ Name: ________________ Date: _________________

PREFACE

The experimental work described in this dissertation was carried out in the Discipline of Microbiology, School of Life Sciences, College of Agriculture, Engineering and Science at the University of KwaZulu-Natal (Westville Campus), Durban, South Africa from January 2016 – December 2018, under the supervision of Prof. A.O. Olaniran.

These studies represent original work of the author and have not been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text

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DECLARATION 1– PLAGIARISM

I, Kerisha Ramessar, declare that:

1. The research reported in this dissertation, except where otherwise stated, is my original research.

2. The dissertation has not been submitted for any degree or examination at any other university.

3. This dissertation does not contain other scientists' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other scientists.

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DECLARATION 2– PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this dissertation (include publications in preparation, submitted, in press and published and give details of the contributions of each author to the experimental work and writing of each publication).

Publication 1: Prevalence and fate of Methicillin-resistant *Staphylococcus aureus* in treated effluent and receiving aquatic milieu of two wastewater treatment plants in Durban, South Africa – *Environmental Monitoring and Assessment* (Submitted)

Publication 2: Antibiogram and molecular characterization of methicillin-resistant *Staphylococcus aureus* recovered from treated wastewater effluent and receiving surface water in Durban, South Africa) – *World Journal of Microbiology and Biotechnology* (Submitted)

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Abstract

Inadequately treated wastewater effluent serves as a reservoir of potentially pathogenic bacteria and contributes to the spread of these organisms in the environment, including *Staphylococcus aureus* (*S. aureus*), a faecal bacterium known to cause pneumonia, septicaemia and skin infections in humans. The presence of *S. aureus* in water has become problematic as it has been shown to exhibit resistance towards *β*-lactam antibiotics commonly used to treat infections, including methicillin, leading to the emergence of methicillin-resistant *S. aureus* (MRSA). The current study aimed to determine the prevalence of MRSA and *mecA* (known to induce methicillin resistance) in the influent, treated effluent and receiving surface rivers of two wastewater treatment plants (WWTPs) in Durban. The study also evaluated the antibiogram and virulence gene profiles of MRSA isolates recovered from the treated effluent and receiving surface water using the Kirby-Bauer disc diffusion and PCR assays. Genetic fingerprinting was carried out to determine the phylogenetic relationship between isolates with selected antibiogram profiles. The prevalence of MRSA in WWTP1 ranged from 11.45-85.63% (influent), 16.28-39.36% (before chlorination), 2.16-5.07% (after chlorination), 1.06-7.24% (downstream) and 4.95-14.09% (upstream). In WWTP2, the prevalence of MRSA for the influent ranged from 48.25-86.18%, before chlorination; 23.73-93.75%, after chlorination; 4.28-48.82%, downstream; 1.74-19.31% and upstream; 5.90-28.78%. Correlation studies of selected physico-chemical parameters to the prevalence of MRSA was carried out. The real-time PCR assay showed a reduction in the concentration of *mecA* from the influent to the treated effluent in both WWTPs. The highest resistance was observed towards lincomycin (100%), followed by oxacillin (98.75%), cefoxitin and penicillin (97.50%) and ampicillin (96.25%). Additionally, 72.50%, 66.25%, 52.50%, 40% and 33.75% of the isolates showed resistance against cefozolin, azithromycin, amoxicillin/clavulanic acid, erythromycin and vancomycin, respectively. The following antibiotic resistance genes were detected in resistant isolates: *aac(6')/aph(2")* in 56.25%, *ermC* in 62.50%, *msrA* in 22.50% and *blaZ* and *tetK* in 70%. The virulence genes *hla* and *sea* were detected in 57.50% of the isolates, *hld* in 1.25% and the *lukS P/V* gene was not detected. Thirteen pulsotypes

(designated A-M) was generated for selected isolates using pulse field gel electrophoresis, correlating them to their respective antibiograms. The study revealed a lower prevalence of MRSA and concentration of *mecA* in the treated effluent as compared to the influent of both WWTPs. It also revealed that these multi-drug resistant strains, isolated from the treated effluent and receiving surface waters, are potentially pathogenic and could contribute to the spread of disease in the environment. Hence, the need for more stringent monitoring and evaluation of treatment performance of the WWTPs.

CHAPTER ONE

1.0 Introduction

Water is considered to be an important and precious resource as it supports all life (Reda, 2016). It is used for various domestic and industrial purposes, such as in food production, irrigation and electricity generation (Shukla *et al*., 2013). Access to safe drinking water is vital for the sustainable development and improvement of the quality of life in both developing and developed countries (Reda, 2016). It is estimated that 2.1 billion of the global population do not have access to reliable and safe water and 2.3 billion do not have access to proper sanitation, resulting in the spread of waterborne diseases (WHO and UNICEF, 2017). An increasing population size, high number of immunocompromised patients, poor water supply, inadequate sanitation and interruptions of water supply places the South African population at risk of developing infectious intestinal diseases (Luyt *et al*., 2012; Kumar *et al*., 2015). Additionally, inefficient wastewater management and practices have led to the disposal of improperly treated wastewater effluents into natural water bodies (Kumar *et al*., 2015).

This improperly treated effluent may harbour potentially pathogenic microorganisms, including strains of *Vibrio cholerae, Salmonella* spp., *Shigella* spp., *Escherichia coli* and *Staphylococcus aureus* (*S. aureus*) (Almagro-Moreno and Taylor, 2013). In addition, wastewater treatment plants (WWTPs) could serve as a reservoir for antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Thapaliya *et al*., 2017). Contamination of receiving surface waters with improperly treated effluent may lead to the spread of water-related diseases as well as contribute to the dissemination of ARB and ARGs in the environment (Pandey *et al*., 2014).

S. aureus has been associated with a variety of infections which include; pneumonia, septicaemia, skin and soft tissue infections. Challenges have emerged in treating these infections due to resistance to antibiotics such as methicillin. The first isolated methicillin resistant *S. aureus* (MRSA) was related to clinical settings (Börjesson *et al*., 2009). However,

infections emerged amongst people who were not exposed to this risk factor and cases of unrelated, clinical-associated MRSA infections have since increased (Börjesson *et al*., 2009).

According to Goldstein *et al*. (2012), improperly treated effluent from WWTPs are possible reservoirs for MRSA and that its discharge into surface waters exposes many communities to MRSA. Goldstein *et al*. (2012) and Thompson *et al*. (2013) have detected MRSA in municipal and hospital wastewaters, respectively. Other studies have reported an increased survival span of MRSA in river and sea waters (Thapaliya *et al*., 2017). Few studies have been reported on the detection and characterization of MRSA from treated wastewater effluent and receiving rivers. The discharge of improperly treated wastewater effluent containing some MRSA strains into receiving surface waters may expose individuals and animals who come into contact and/or use this water to MRSA, resulting in the potential rapid spread of antibiotic resistant infections (Goldstein *et al*., 2012; Thapaliya *et al*., 2017). Therefore, effluent of WWTPs and receiving surface waters must be regularly assessed to ensure good quality treated wastewater effluent is discharged into water bodies to protect the health of the general public (Kumar *et al*., 2015).

1.1 Scope of the study:

According to Börjesson *et al.* (2010), treated wastewater effluent and its respective receiving surface waters have become a reservoir for MRSA. The *mecA* gene, which encodes for a penicillin binding protein that confers resistance to methicillin in *S. aureus* (David and Daum, 2010; Al-Abbas, 2012; Rahimi and Shokoohizadeh, 2016), has also been detected in the influent and treated effluent of WWTPs (Börjesson *et al*., 2009). The presence of MRSA in treated effluent and its respective receiving surface waters could pose a health threat to those who rely on these water sources for domestic and agricultural purposes (Goldstein *et al*., 2012). Previous studies carried out by Goldstein *et al*. (2012) not only revealed the presence of MRSA in treated wastewater effluent of four WWTPs, but also showed that isolated strains were multi-

drug resistant and harboured virulence genes. The discharge of MRSA into the environment poses a health threat to those with weakened immune systems as well as promotes the dissemination of antibiotic resistant and virulent bacterial strains in receiving water bodies. Thus far, studies in South Africa have mainly focused on characterization of MRSA in clinical strains (Moodley *et al*., 2010). To the best of our knowledge, no studies have reported on the characterization of antibiotic resistance and virulence gene attributes of MRSA isolates obtained from the treated effluent of WWTPs and receiving rivers in Durban, South Africa.

This project aimed to determine the prevalence of MRSA in treated wastewater effluent and receiving rivers of two independent WWTPs in Durban, Kwa-Zulu Natal. The study also aimed to quantify the *mecA* gene and correlate it to the microbial counts from each respective sampling point to determine if WWTPs contribute to the spread of resistant bacteria and resistance genes in the environment. Further, the study aimed to determine the antibiogram and virulence gene profiles of the MRSA isolates. Screening for the presence of virulence genes will provide information on the potential pathogenicity of MRSA and the role of these WWTPs in the spread of MRSA in communities and the environment through the receiving rivers. The antibiogram analysis will reveal the resistance/susceptibility profiles of the isolates and will allow for better understanding of the treatment regime required to control MRSA-associated infections in affected communities. It also aimed to determine possible correlation between antibiotic resistant phenotype and genotype of the resistant MRSA strains.

1.2 Hypotheses:

It is hypothesized that there is a high prevalence of MRSA in treated wastewater effluent of the investigated WWTPs and their respective receiving surface waters. It is also hypothesized that MRSA isolates from the treated effluent are multi-drug resistant, and harbour antibiotic resistance and virulence determinants. It is further hypothesized that isolates with similar antibiograms may be genetically related.

1.3 Aims:

1.3.1. To determine the prevalence of MRSA in the treated wastewater effluent and receiving surface waters by enumerating presumptive MRSA and *S. aureus* in the water sample using the membrane filtration technique and appropriate selective media over a six-month period and by quantifying the *mecA* gene in the water sample using real-time PCR.

1.3.2. To confirm the identity of MRSA isolates from the water sample using the catalase, oxidase and coagulase tests and PCR amplification of the *mecA* gene.

1.3.3. To determine the antibiotic resistance/susceptibility profile using the Kirby-Bauer disc diffusion assay.

1.3.4. To detect the presence of specific antibiotic resistance genes (*aac(6')/aph(2'')*, *blaZ*, *ermC*, *msrA* and *tetK*) using PCR.

1.3.5. To detect the presence of specific virulence genes (*hla, hld, lukS/F PV* and *sea*) using PCR.

1.3.6. To determine the genetic fingerprint using pulsed field gel electrophoresis.

1.4 Objectives:

1.4.1. To determine the prevalence of MRSA and *S. aureus* in the treated effluent and receiving surface waters of two independent wastewater treatment plants (WWTPs) in the Durban area via plate count method and q-PCR quantification of *mec*A gene.

1.4.2. To isolate, purify and identify MRSA recovered from treated effluent of these WWTPs and receiving rivers using appropriate biochemical and molecular tests.

1.4.3. To determine the antibiotic resistance profiles of the MRSA isolates.

1.4.4. To profile the virulence gene signatures of the MRSA isolates.

1.4.5. To determine the genetic diversity of selected MRSA isolates.

1.5 Layout of the dissertation:

The dissertation is divided into five chapters. Chapter one deals with a general introduction. It also highlights the scope of the study, hypotheses, objectives and aims which were carried out. Literature reviews related to this study is presented in chapter 2, whilst chapter three focuses on the prevalence of MRSA and quantification of the *mecA* gene present in wastewater influent, effluent and receiving surface waters. Chapter four deals with the characterization of MRSA recovered from treated effluent and receiving rivers, focusing on their antibiogram, virulence gene signatures and genetic fingerprints. Chapter five conclude and summarizes the findings of this study and highlights future aspects of the study.

References

Al-Abbas, M. J. A. 2012. Antimicrobial susceptibility of Enterococcus faecalis and a novel Planomicrobium isolate of bacterimia. *International Journal of Medicine and Medical Sciences*. *4*(2): 19-27.

Almagro-Moreno, S., and Taylor, R.K. 2013. Cholera: environmental reservoirs and impact on disease transmission. *Microbiology Spectrum*. **1**: e1004787.

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

Börjesson, S., Matussek, A., Melin, S., Löfgren, S., and Lindgren, P.E. 2010. Methicillin-resistant *Staphylococcus aureus* (MRSA) in municipal wastewater: an uncharted threat? Journal of Applied Microbiology. **108**:1244–1251.

David, M. Z., and Daum, R. S. 2010. Community-associated methicillin-resistant *Staphylococcus aureus*: epidemiology and clinical consequences of an emerging epidemic. *Clinical Microbiology Reviews*. **23**: 616–687.

Goldstein, R.E.R., Micallef, S.A., Gibbs, S.G., Davis, J.A., He, X., George, A., Kleinfelter, L.M., Schreiber, N.A., Mukherjee, S., Sapkota, A., Joseph, S.W., and Sapkota, A.R. 2012. Methicillin-Resistant *Staphylococcus aureus* (MRSA) detected at four U.S. wastewater treatment plants. *Environmental Health Perspectives*. **120**: 1551-1558.

Kumar, D., Asolekar, S.R., and Sharma, S.K. 2015. Post-treatment and reuse of secondary effluents using natural treatment systems: The Indian practices. *Environmental Monitoring and Assessment*. **187**: 612.

Luyt, C. D., Tandlich, R., Muller, W. J., & Wilhelmi, B. S. 2012. Microbial monitoring of surface water in South Africa: an overview. *International journal of environmental research and public health,* **9**(8), 2669-2693.

Moodley, A., Oosthuysen, W.F., Duse, A.G., Marais, E., and the South African MRSA Surveillance Group 2010. Molecular characterization of clinical methicillin-resistant *Staphylococcus aureus* isolates in South Africa. Journal of Clinical Microbiology. **48**(12): 4608-4611.

Pandey, P. K., Kass, P. H., Soupir, M. L., Biswas, S., and Singh, V. P. 2014. Contamination of water resources by pathogenic bacteria. *AMB Express,* **4**(1): 51.

Rahimi, F., and Shokoohizadeh, L. 2016. Characterization of methicillin *resistant Staphylococcus aureus* strains among inpatients and outpatients in a referral hospital in Tehran, Iran. *Microbial pathogenesis*. **97**: 89-93.

Reda, A. H. 2016. Physico-Chemical analysis of drinking water quality of Arbaminch Town. *Ann Chem Open Access,* **2**(006).

Shukla, D., Bhadresha, K., Jain, N.K., and Modi, H.A. 2013. Physicochemical Analysis of Water from Various Sources and Their Comparative Studies. IOSR Journal of Environmental Science. *Toxicology and Food Technology*. **5**: 2319-2402.

Thapaliya, D., Hellwig, E. J., Kadariya, J., Grenier, D., Jefferson, A.J., Dalman, M., Kennedy, K., DiPerna, M., Orihill, A., and Taha, M. 2017. Prevalence and Characterization of *Staphylococcus aureus* and Methicillin-Resistant *Staphylococcus aureus* on Public Recreational Beaches in Northeast Ohio. *GeoHealth,* **1**(10), 320-332.

Thompson, J.M., Gündoğdu, A., Stratton, H.M., and Katouli, H.M. 2013. Antibiotic resistant *Staphylococcus aureus* in hospital wastewaters and sewage treatment plants with

special reference to methicillin-resistant *Staphylococcus aureus* (MRSA). *Journal of Applied Microbiology*. **114**: 44-5.

World Health Organization and UNICEF. 2017. Progress on drinking water, sanitation and hygiene: 2017 update and SDG baselines.

CHAPTER TWO

Literature Review

2.1 Introduction

Water is vital for the existence of life; hence, must be easily accessed to all (Cabral, 2010). Water is used for sanitation, in agriculture and most importantly for drinking (Cabral, 2010). However, water shortages and an insufficient supply of good quality water have led to major concerns in environmental, industrial and municipal sectors (Kumar *et al.,* 2015). In many countries of African continent, little or no access to proper sanitation or functional wastewater treatment systems have led to contamination of water bodies which include rivers and lakes, bearing major consequences for public health and the environment (Bateganyaa *et al*., 2015; Jhansi and Mishra 2013). Faecal contamination of water resources has led to the spread of waterborne diseases (Kumar *et al*., 2015). According to the World Health Organization (WHO) and UNICEF, it is estimated that 159 million people drink water from untreated water sources and 2.3 billion people lack proper sanitation. The death toll for children under 5 years reached 361 000 due to diarrhoeal diseases transmitted through water (WHO and UNICEF, 2017). A sustainable development goal of WHO is to provide people with free to access water, from a safe and reliable water source, without faecal or chemical contamination (Hunter *et al*., 2010). Therefore, it is important that strict regulations be implemented globally to allow people their basic right, to access safe water.

2.2 Overview of water in South Africa

South Africa is a semi-arid country making water a precious natural resource (Pitman, 2011). Hedden and Cilliers (2014) reported that South Africa receives an annual average rainfall of 495 mm, far below the global average of 1033 mm, making it the 30th driest country in the world. This is alarming considering that the water used per capita (253 litres per day) is above the global average of 173 litres per day (Hedden and Cilliers, 2014).

The increase demand on water resources and service delivery of domestic water is highly influenced by the relocation of people into industrialized provinces (such as Gauteng) (Luyt *et al*., 2012). Reasons for poor water quality in South Africa include but are not limited to poor service delivery, the lack of proper infrastructure, acid mine drainage, interruptions of water supply due to burst pipes, low maintenance of communal taps and poor waste and wastewater management practices. With an increasing population size and number of immunocompromised patients, poor water quality places the population at risk for developing waterborne intestinal diseases Little or no access to safe drinking water and improper sanitation also influences the spread for waterborne diseases (Luyt *et al*., 2012). However, in order to assess the microbial quality of water in South Africa, The National Microbial Monitoring Programme for Surface Water (NMMP) was developed and implemented by the Department of Water Affairs (DWA). They aim to regularly collect and analyse water samples from specific sampling sites. These sample sites are based on the following criteria: 1) if the land-use, either directly or indirectly, contributes to faecal contamination (including land used to house informal settlements and for agricultural activities), 2) the size of the population that may use the water resource and 3) the purpose of the water-use in that area (Luyt *et al*., 2012).

2.3 Wastewater

The decline of the quality of water in the environment is influenced by the discharge of improperly treated wastewater into waterbodies including rivers (Popa *et al*., 2012). Wastewater can be defined as industrial, domestic, commercial and storm water runoff, whilst urban wastewater is considered to be a mixture of domestic and industrial wastewater, sewage and rainwater, whilst agricultural wastewater is water originating from farms, agricultural activities and contaminated groundwater (Naidoo and Olaniran, 2013).

Wastewater serves as a source of faecal microorganisms which has led to numerous outbreaks of waterborne diseases affecting numerous people in both developed and developing countries (Cabral, 2010). Potentially pathogenic microorganisms include strains of *Vibrio cholera, Salmonella* spp*., Shigella, Escherichia coli* and *Staphylococcus aureus*. *Vibrio cholera* is responsible for cholera and symptoms of infection include diarrhoea and gastrointestinal tract infections (Almagro-Moreno and Taylor, 2013). *Salmonella* belongs to the family *Enterobacteriaceae* and has been associated with Salmonellosis which results in typhoid or gastroenteritis, while infections mediated by *Shigella* strains causes abdominal cramps and bloody stools (Feasey *et al.,* 2012; Mardaneh *et al.,* 2013). Pathogenic strains of *Escherichia coli* including Enterotoxigenic *E. coli*, Enterohemorrhagic *E. coli* and Enteroinvasive *E. coli* strains have been associated with gastroenteritis, diarrhoea, vomiting, abdominal cramps, chills and bloody stools (Cabral, 2010).

Therefore, it is imperative that regular water microbiological tests are carried out to assess the presence of pathogenic bacteria in wastewater effluent and receiving rivers (Cabral, 2010). Detection of indicator organisms, which are members of the microflora of the human and animal intestinal tracts, are used to assess the microbial load of water (Luyt *et al*., 2012). According to Cabral (2010) an ideal faecal bacterial indicator should meet the following requirements:

1) Should exist in a high concentration in the human intestine and faeces;
2) Should be detected in the receiving waters easily, rapidly and economically;
3) Should not be pathogenic;
4) Should be present in a higher concentration than potential pathogenic bacteria;
5) Multiplication must only occur in the enteric environment;

6) It must not be present in the gut of farm and domestic animals and
7) It should have similar growth patterns to pathogenic bacteria.

Physico--chemical analysis of the water should also be explored as Lokhande *et al*. (2011) demonstrated that physico-chemical characteristics influence the survival of microorganisms. This includes analysis of: pH, temperature, salinity, total dissolved solids (TDS), turbidity, electrical conductivity, resistivity, biological oxygen demand (BOD) and Chemical oxygen demand (COD).

2.4 Wastewater treatment plants

Wastewater is transported to a centralized wastewater treatment plant (WWTP) via underground pipes to receive proper wastewater treatment (Popa *et al*., 2012). According to Hendricks and Pool (2012), the wastewater treatment process consists of three essential steps namely; primary treatment: this involves the pre-treatment of raw wastewater and facilitates the removal of coarse and fine grit by use of sedimentation tanks to allow heavier organic particles to settle to the bottom. Secondary treatment: uses aerated biological digestion by bacteria which allows for the removal of the remaining suspended and dissolved materials. Nitrification and de-nitrification can also be used. Thereafter, the wastewater is separated into the solid and liquid phase in the sedimentation tanks. Wastewater then enters the maturation pond for additional pathogen removal, and; tertiary treatment: which makes use of ultraviolet light or chlorine for disinfection before the release of the treated effluent into receiving surface waters.

Of concern, is the discharge of improperly treated effluent from WWTPs into receiving surface waters which may bear negative consequences for end-users (Bateganyaa *et al*., 2015). Contributing factors include poor operation of the wastewater treatment plants due to unskilled staff or mechanical malfunctions with the WWTP itself (Popa *et al*., 2012). Additionally, the

quantity of wastewater received exceeds the treatment capacity of the WWTP which may result in either inefficient treatment or periodic leaks (Popa *et al*., 2012). The effects of the discharge of improperly treated wastewater on the surrounding environment has been documented by Naidoo and Olaniran (2013). Receiving surface waters are used by humans and livestock for drinking as well as domestic and recreational purposes. Therefore, use of this water may result in illness due to the high bacterial loads found in wastewater. Additionally, the discharge of improperly treated effluent may result in the deposition of organic matter and nutrients which negatively impacts the environment, micro- and macro-fauna (Naidoo and Olaniran, 2013). It may also result in eutrophication and temporary oxygen deficiencies which may disturb the communities of that environment (Naidoo and Olaniran, 2013). Failure to treat waste properly may also lead to turbid effluent discharge which causes a deposition of sand and grit into the water systems, disturbing the sediment characteristics and water flow (Naidoo and Olaniran, 2013).

Considering these factors, it is imperative that the proper measures to be put in place to ensure that wastewater is treated to acceptable standards prior to discharge into the environment (Kumar *et al.,* 2015). The Department of Water Affairs and Sanitation make use of The South African Water Quality Guidelines to determine the quality of water, regardless of its source, and establish if it is acceptable to be used for domestic purposes. They have also developed the Green Drop status to regulate quality of water in wastewater management.

There are 121 WWTPs that are owned and operated by the Department of Public Works Services (DPW) across the nine provinces of South Africa. These WWTPs were assessed and the report showed that none of the WWTPs achieved green drop status. The DPW Green Drop status was reported to be 14.5% indicating the WWTPs performance to be unsatisfactory.

In a report assessing the Green Drop status of WWTPs in South Africa, carried out by The Department of Water Affair in 2013, it was reported that the provincial Green Drop status for all provinces (except for Western Cape) achieved green drop scores below 30%. This means that most WWTPs in each province did not meet the regulatory requirements and the wastewater treatment management were deemed of poor practice. The eThekwini Metro municipality was ranked fourth in the country achieving a green drop score of 90.45%. The green drop report from 2011 showed that out of the 27 WWTPs within the eThekwini Metro municipality, nine had a green drop score >90%, fifteen had a green drop score of >80% and three had a score of >70%. Compared to previous reports carried out by the Department of Water Affairs in 2009, 19/27 WWTPs showed to improve their respective green drop scores whilst 7/27 showed a decline.

2.5 *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) are Gram-positive, cocci shaped cells which are arranged in clusters (Gould, 2011). They are identified by the presence of yellow colonies on Mannitol Salt Agar due to their ability to ferment mannitol (Ayeni *et al.,* 2015). They are commonly found in the nose, mouth, upper respiratory tracts of humans and mucous membranes (Alfatemi *et al.,* 2014) and are causative agents for pneumonia, septicemia, skin infections and urinary tract infections (Goldstein *et al.,* 2012; Dormanesh *et al.,* 2015). *S. aureus* has a wide variety of host niches which suggests that this microorganism is able to adapt and survive in changing environments and establish infections (Burnside *et al.,* 2010). Bacteraemia, a bloodstream infection is prominently associated with *S. aureus*. Risk factors include: surgeries, intravascular devices and patients with immunodeficiencies (de-Smidt *et al*., 2015). Food poisoning is also associated with enterotoxigenic *S. aureus* (Mossong *et al*., 2015). Staphylococcal food poisoning (SFP), associated with an enterotoxigenic strain of *S. aureus*, was ranked as the first cause of foodborne outbreaks in France, is (Mossong *et al*., 2015).

2.5.1 Methicillin-resistant *Staphylococcus aureus*

Previously, penicillin was used to treat bacterial infections caused by staphylococci, however, by the mid-1940s *S. aureus* strains soon exhibited resistance to penicillin (Green *et al*., 2012). Methicillin, a semi-synthetic antibiotic, was introduced in 1959 as an alternative antibiotic for the treatment of *S. aureus* mediated infections (Rahimi and Shokoohizadeh, 2016). However, by 1962, *S. aureus* developed resistance to methicillin, spread globally and became known as Methicillin Resistant *Staphylococcus aureus* (MRSA) (Ventola, 2015). MRSA isolates have also exhibited resistance to other antibiotics such as macrolides, lincosamides, fluoroquinolones, aminoglycosides and trimethoprim-sulfamethoxazole (Green *et al*., 2012).

MRSA was first associated with hospital environments and was therefore regarded as hospital-acquired MRSA (HA-MRSA) (Goldstein *et al.,* 2012). However, in the 1990s, healthy people without risk factors were infected with community-acquired MRSA (CA-MRSA) (Goldstein *et al.,* 2012). CA-MRSA differs from HA-MRSA as it causes infections in people that are not exposed to a health care setting due to a change in epidemiology (David *et al*., 2014; Venniyil *et al*., 2016).

The risk factors for CA-MRSA include individuals with diabetes mellitus, malignancies, cardiovascular renal failure and use of intravenous drugs (Pathare *et al*., 2015). CA-MRSA affects a distinct population compared to HA-MRSA and has distinct clinical syndromes (David and Daum, 2010). CA-MRSA infections occur in younger patients when compared to HA-MRSA which occurs in older patients (David and Daum, 2010). In infancy, this may be passed from the mother (Schaumburg *et al.,* 2014). They are associated with skin and soft tissue infections and may also cause necrotizing pneumonia and severe sepsis (David and Daum, 2010). HA-MRSA causes pneumonia, bacteraemia, and invasive infections and occur in patients that may be infected with another condition (David and Daum, 2010). Studies have

shown that *S. aureus* colonization risk factors in Africa include people with HIV infections, those living in rural areas and those that are hospitalized (David and Daum, 2010).

Methicillin resistant strains are becoming more common in hospitals and communities compared to methicillin susceptible strains (Pathare *et al.*, 2015). The transmission of MRSA from individual to individual, leading to the spread of the microorganism has become a public health concern (Pathare *et al.*, 2015). Sources of community acquired MRSA (CA-MRSA) include schools, prisons, slaughterhouses and retail meat and improperly treated wastewater effluent (Goldstein *et al.*, 2012; Abidatul *et al.*, 2018; Sato *et al.*, 2017).

2.5.2 MRSA in WWTPs and receiving rivers

Goldstein *et al.* (2012) suggests that WWTPs may serve as a reservoir for MRSA. MRSA strains may be disseminated into the environment through hospital wastewater, sewage systems, as well as into surface waters through improper waste management (Thompson *et al.,* 2013). Another contributing factor is rainfall which may contribute to the spread of MRSA as the combination of storm water and sanitary sewers could potentially result in an overflow of untreated wastewater that is discharged into rivers and lakes (Thapaliya *et al.*, 2017). The release of these strains may contribute to the gene pool of multidrug resistant bacteria in the environment (Thompson *et al.,* 2013).

Goldstein *et al.* (2012) revealed the presence of MRSA in 22/44 (50%) of WWTPs in the United States of America. This is alarming considering water shortages in certain areas of South Africa may allow for a greater amount of treated wastewater effluent to be used for agriculture, industry and groundwater recharge (Goldstein *et al.*, 2012). Börjesson *et al.* (2010) reported strains of MRSA with great genetic diversity in wastewater. This study showed that a specific strain did not survive the treatment indicating that new strains of MRSA might be evolving in wastewater. It may also be implied that even though wastewater treatment may

reduce the total number of strains present in wastewater, it may also select for strains with more extensive antibiotic resistance characteristics (Börjesson *et al*., 2010).

The presence of MRSA in wastewater and treated wastewater effluent poses risk for the health of the WWTP workers and individuals who are exposed to this water (Goldstein *et al*., 2012). WWTP workers may be exposed to MRSA through epidermal exposure or inhalation. It is important that the WWTP workers practice proper hygiene methods by frequent washing of hands and wearing gloves when working in the plant (Goldstein *et al*., 2012). Treated wastewater may also be reused as spray irrigation in agricultural areas, public parks and golf courses (Goldstein *et al*., 2012). Public exposure to these areas requires strict regulations for treated wastewater reuse to be implicated (Goldstein *et al*., 2012).

2.6 Antibiotic Resistance

Antibiotics are used to combat microbial infections but also used for agricultural and livestock farming (Zaman *et al*., 2017). They have been used successfully in the treatment or prevention of infections in patients with chronic diseases, those receiving chemotherapy and those who have undergone surgery (Ventola, 2015). In developing countries, antibiotics have helped decrease the morbidity and mortality rates caused by food and water-borne diseases (Ventola, 2015). However, the overuse and misuse of antibiotics and lack of new drug development have led to antibiotic resistance amongst bacteria which has become a global threat (Zaman *et al*., 2017). The World Health Organization (WHO) describes antibiotic resistance as the development of a microorganism that is no longer affected by a drug it was originally sensitive to (WHO, 2014). Epidemiological studies have revealed that development and dissemination of antibiotic resistance bacteria is directly related to antibiotic consumption (Ventola, 2015). Antibiotic resistant bacteria can be transmitted from animals to humans through the food chain as animals may receive antibiotics in their feed to be used as growth promoters (Zaman *et al*.,

2017). Multi-drug resistant bacteria are responsible for many deaths (Zaman *et al.*, 2017). Falagas *et al.* (2006) defines multi-drug resistance as a microorganism's ability to exhibit resistance towards 3 or more classes of antibiotics. The development of multidrug resistant strains limits the treatment options for bacterial infections (Rahimi and Shokoohizadeh, 2016). Reports have shown that 63 000 patients die from hospital-acquired bacterial infections in the USA every year and 25 000 patients die due to multi-drug resistance bacterial infection Europe every year (Zaman *et al.*, 2017). Resistance towards antimicrobial agents make bacterial infections harder to control, increases the risk of the spread of infection from one person to another, prolongs illness and hospital stays, increases economic costs and the risk of death (WHO, 2014). Therefore, it is important the new policies are implemented and research efforts are made to control the emergence of antibiotic resistance in this era.

2.7 Mechanisms of antibiotic resistance in MRSA

The dissemination of antibiotic resistance genes is caused through horizontal gene transfer and can be associated with mobile genetic elements such as gene cassettes, integrons and plasmids (Wan and Chou, 2015).

Integrons are defined by the occurrence of an integrase gene (*intI*) and a proximal primary recombination site (*attI*). They are divided into classes based on their amino sequence of the integrases. Integrons have two conserved segments that can be separated by a variable region which consists of mobile cassettes with antibiotic resistance genes (Wan and Chou, 2015). The 5'-conserved segment harbours the integrase gene and attachment site which allows for the class 1 integrons to apprehend and express resistant genes. The 3'-conserved segment encodes for the resistance to quaternary ammonium compounds and sulphonamides used in disinfectants (Wan and Chou, 2015). Class 1 integrons were detected in MRSA and have been abundantly associated to aqueous environments, including wastewater (Wan and Chou, 2015).

Wan and Chou (2015) expressed the abundance of integrase genes present in wastewater of municipal WWTPs and swine slaughterhouse. These integrons survived the treatment process and were released in the treated effluent. The study suggested that wastewater serves as a hotspot for class 1 integrons and may be a potential source for antibiotic resistant MRSA infections in humans and animals (Wan and Chou, 2015).

Plasmids are disseminated between bacteria through horizontal gene transfer by bacteria. Plasmids are circular DNA structures which replicate independently of chromosomes. They are responsible for distributing antibiotic resistance genes amongst various strains of bacteria (San Millan, 2018). The plasmids present in Staphylococci range in size from 1.2 kb to 100 kb. Based on their size and mechanism of replication they are classified into three classes (Kuntová *et al*., 2012). In Staphylococci, it has been demonstrated that plasmids detected harbour genes that allow for an increased tolerance towards antiseptics such as quaternary ammonium compounds, which are commonly used in environmental disinfectants (Wan and Chou, 2015). Kuntová *et al*. (2012) showed that 89% of the MRSA strains studied contained extrachromosomal plasmids suggesting that the plasmids contribute to the fitness of the MRSA strains. Studies also showed even though the MRSA population evolve, most plasmids circulate in the population for years (Kuntová *et al*., 2012). This was shown by the sequence analysis of the plasmids pDLK1 and pDLK2 and the restriction profile of the enterotoxin D plasmid, which showed structural stability and evolutionary importance (Kuntová *et al*., 2012). This was supported by Shahkarami *et al* (2015) which showed that plasmid profiles show genetic relatedness of bacterial isolates.

Efflux pumps are another antibiotic resistance mechanism which play a role in drug extrusion (Sun *et al*., 2014). Bacterial efflux systems can be specific by removing a single antibiotic or class of antibiotics, or may be multidrug resistance efflux pumps and remove several classes of antimicrobial compounds (Costa *et al*., 2013). The presence of efflux pumps has been

detected in most bacterial species. The genes that encode for these proteins are located on plasmids or chromosomes. Based on their energy sources, substrates and transmembrane spanning regions efflux pumps are grouped into five classes: the resistance-nodulation-division family, the major facilitator superfamily, the ATP (adenosine triphosphate)-binding cassette superfamily, the small multidrug resistance family and the multidrug and toxic compound extrusion family (Sun *et al*., 2014). The regulation of efflux pumps is based on the chemical and physical stress response and the pathogenicity of *S.aureus*. More than ten efflux pumps have been reported for *S. aureus* (Costa *et al*., 2013). In MRSA, it has been reported that efflux pump removes antibiotics such as tetracyclines and macrolides, antiseptics and play a role in transfer of antibiotic resistance genes (Zmantar *et al*., 2013). Efflux pumps contribute to low level antibiotic resistance and emergence of multidrug resistant pathogens (Costa *et al*., 2013).

WWTPs play an important role in the development and spread of antibiotic resistance genes and antibiotic resistant bacteria. The accumulation of antibiotics, with continued activity, in the environment may result in *in vitro* or horizontal gene transfer of antibiotic resistance genes (using these mechanisms) amongst bacteria including, *S. aureus*, in wastewater effluent and receiving surface waters (Börjesson *et al*, 2009; Börjesson *et al*., 2010). Amongst these resistance genes, include the presence of *mecA* which has been detected in hospital and municipal wastewater through cultivation and molecular methods carried out by Börjesson *et al*., 2010.

2.8 Antibiotic resistance genes in MRSA

Infections caused by MRSA are difficult to treat as studies have reported antimicrobial resistance to many other antibiotic classes such as aminoglycosides, fluoroquinolones, lincosamides and macrolides (Kaur and Chate, 2015). Detection of antibiotic resistance genes

is carried out by PCR which allows for rapid and accurate diagnosis, therefore preventing and controlling the spread of infections (Duran *et al*., 2012). Studies by Amoako *et al.* (2016) showed the antibiotic resistance genes present in MRSA strains from the private health sector in South Africa were *mecA* (100%), *blaZ* (100%), *ermC* (48.2%) and *aac(6')/aph(2'')* (92.6%). Other studies reported the presence of *tetK* (89.18%) and *msr* (56.75%) in clinical samples of MRSA (Dormanesh *et al*., 2015).

2.8.1 *mecA*

Amplification of the *mecA* gene is routinely used to identify MRSA strains because the *mecA* gene confers resistance to methicillin in the *S. aureus* (Al-Abbas, 2012; Rahimi and Shokoohizadeh, 2016). The *mecA* gene encodes for a penicillin binding protein (PBP2 and PBP2a) which is a cell wall transpeptidase (David and Daum, 2010), allowing for cell wall synthesis to continue while in the presence of *β*-lactam antibiotics (Paterson *et al,* 2014). *mecA* translation increases with exposure to *β*-lactam antibiotics (David and Daum, 2010). The expression of the *mecA* gene is controlled by the MecI regulator which is a DNA binding repressing protein and the MecR1 regulator which is a sensor/signal transducer as shown in Figure 2.1 (Chovanová e*t al*., 2016).

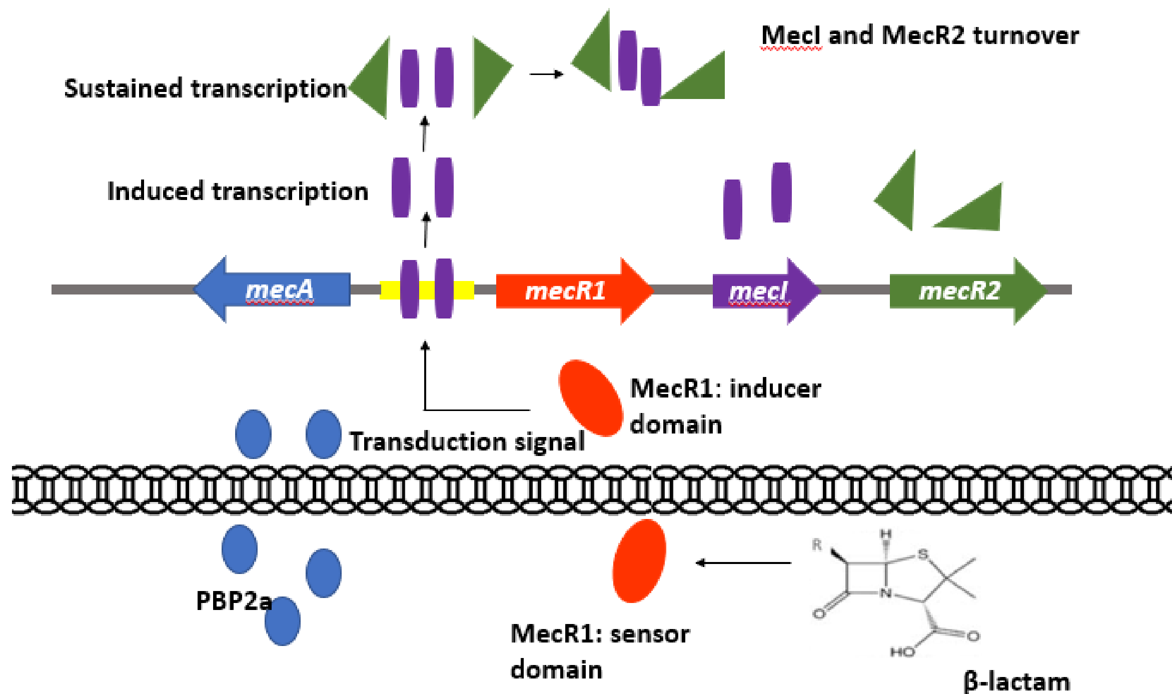

Figure 2.1: Mechanism of action for *mecA* induction (as adapted from Arêde *et al*., 2012) MecR1 is activated in the presence of β-lactam antibiotics. This induces the expression of *mecA* and *mecR1-mecI-mecR2*. The MecR2, an anti-repressor, is needed to allow for the induction of *mecA* as it promotes the inactivation of MecI by proteolytic cleavage. If the β-lactams are absent then the MecR1 won't be activated (Arêde *et al*., 2012).

2.8.2 Staphylococcal Chromosomal Cassette *mec* genetic elements (SCC*mec*)

The expression of the *mecA* gene is regulated by the *mecR1* and *mecI* genes which are carried by SCC*mec* elements (David and Daum, 2010). Most MRSA strains contain the SCC*mec* element which is integrated into a chromosomal site known as *orfx* (David and Daum, 2010). Research has shown that SCC*mec* developed from coagulase negative *Staphylococcus species* and carries the *mecA* gene (David and Daum, 2010). The SCC*mec* are the only vectors that are responsible for the transfer of the *mecA* gene amongst species (Rahimi and Shokoohizadeh, 2016). The SCC*mec* genetic elements are characterized by *mec* and *ccr* gene complexes which serves as two genetic markers (Dormanesh *et al.,* 2015). These elements are classified based

on the nature of the *mec* and *ccr* genes into types I-VII (Valsesia *et al.,* 2010). The distribution of the SCC*mec* elements is important for molecular typing of MRSA strains (Dormanesh *et al.,* 2015).

SCC*mec* types I-III are larger and are present in HA-MRSA strains. These may have been transferred from a commensal staphylococcal species (David and Daum, 2010). The SCC*mec* types IV and V are smaller and have been associated with MRSA infections in patients with the absence of HA-MRSA risk factors (David and Daum, 2010). Type II and III SCC*mec* elements have sites that allow for the insertion of genes that encode non-*β*-lactam resistance phenotypes to the *S. aureus* strains therefore are associated with multidrug phenotypes (David and Daum, 2010). Type IV, V and VII are susceptible to most non-*β*-lactam antibiotics (Valsesia *et al.,* 2010). SCC*mec* types IV and V are also mobile therefore the distribution of MRSA in a community may be accomplished through transfers from carriers to other individuals (David and Daum, 2010). The smaller SCC*mec* elements may also be passed from MRSA strains to MSSA strains (David and Daum, 2010).

Even though the *mecA* gene is used as the gold standard to detect MRSA, studies worldwide have reported the presence of MRSA strains lacking the *mecA* gene (Olayinka *et al*., 2009). A study from Nigeria reported *S. aureus* strains that exhibited phenotypic resistance to methicillin however lacked the *mecA* gene, the five major SCC*mec* types and the gene product of PBP2A (Olayinka *et al*., 2009). However, the study reported hyperproduction of *β*-lactamase which may have resulted in methicillin resistance (Olayinka *et al*., 2009). Ba *et al.* (2014) also showed alterations in the different amino acids present in the PBP cascades resulting in the methicillin resistance. These findings suggest other mechanisms may be responsible for *β*-lactam resistance of MRSA and that amplification of the *mecA* gene is not enough to confirm MRSA strains.

2.8.3 *blaZ*

Resistance to penicillin is mediated by the *blaZ* gene which encodes *β*-lactamase (Zmantar *et al.,* 2013). *β*-lactamase is an extracellular enzyme that is synthesized after the exposure of Staphylococci to *β*-lactam antibiotics (Zmantar *et al.,* 2013). *blaZ* hydrolyzes the *β*-lactam ring which causes the *β*-lactam to become inactive (Zmantar *et al.,* 2013). Regulation of the *blaZ* is achieved by two regulatory genes which are *blaR1,* the anti-repressor and *blaI*, the repressor (Zmantar *et al.,* 2013). The signalling pathway that controls the synthesis of the *β*-lactamase requires the sequential cleavage of the regulatory proteins BlaR1 and BlaI so after the exposure of *β*-lactams, the transmembrane sensor transducer cleaves itself (Zmantar *et al.,* 2013). The expression of *mecA* is also controlled by *β*-lactamase regulators, BlaI and BlaR1, which are structurally and functionally similar. MecI and BlaI co-repress the transcription of *mecA* and *blaZ* (Chovanová e*t al*., 2016). These regulators are alike and can therefore replace each other (Chovanová e*t al*., 2016). MecI and BlaI can bind to the promoter region of the *mecA* and *blaZ*, as homodimers (Chovanová e*t al*., 2016). Upon exposure to antibiotics, a signal transduction brings about sensing through the two transmembrane inducers, MecR1 and BlaR1, which causes proteolytic autocleavage of the cytoplasmic domains of these proteins (Chovanová e*t al*., 2016). This is carried out by the transducers undergoing acylation by the antibiotic which results in conformational changes in the molecule (Chovanová e*t al*., 2016). This is followed by cleavage of the cognate repressors, MecI and Bla then inducing *mecA* and *blaZ* transcription (Chovanová e*t al*., 2016).

2.8.4 Macrolide resistance genes: *ermC* and *msrA*

Macrolide-lincosamide-streptogramin (MLS) antibiotics are used in the treatment of *S. aureus* infections which include bovine mastitis in cattle (Li *et al*., 2015). Antibiotics known as MLS include: clindamycin, erythromycin and spiramycin. Different mechanisms contribute MLS

resistance and includes: 1) the modification of target sites caused by methylation or mutation, 2) efflux pumps and 3) drug inactivation (Li *et al*., 2015).

Erythromycin is classified as a macrolide which is effectively used against Gram-positive and some Gram-negative bacteria however, the widespread utilization of this macrolide antibiotic has resulted in an increase in the resistance of *S. aureus* (Ding *et al*., 2012; Goudarzi *et al*., 2015). Ribosome methylases are enzymes synthesized by *S. aureus* which are encoded by one or more erythromycin resistant methylase genes (*erm*) (Ding *et al*., 2012). Methylation of 23s-rRNA occurs and this is followed by changes on the binding site for macrolide-lincosamidestreptogramin B (MLSB) antibiotics (Ding *et al*., 2012). *ermA, erm B* and *ermC* are the main methylase genes that have been found in *S. aureus* (Ding *et al*., 2012). Adenine at position 2058 of 23s-rRNA undergoes N6-dimethylation due to the production of methylase (Ding *et al*., 2012). It overcomes the inhibitory effect of macrolide on protein synthesis due to conformation changes in the P-site of 23s-rRNA which prevents the binding of macrolides (Ding *et al*., 2012).

msrA is also involved in resistance towards macrolides-streptogramins as it encodes macrolide efflux pumps which belongs to the ABC transporter family (Ding *et al*., 2012). This induces resistance and exports 14-15 membered macrolides and streptogramins A antibiotics from the cells. (Ding *et al*., 2012; Li *et al*., 2015). This allows for the antibiotic concentration within the cell to remain at a sub-toxic level which does not disturb the bacterial cell growth (Piątkowska *et al*., 2012)

2.8.5 *aac(6')/aph(2'')*

Aminoglycosides are broad-spectrum antibiotics used against aerobic Gram-negative bacilli, staphylococci and some mycobacteria (Yuan *et al*., 2013). Aminoglycosides irreversibly bind to the 30S ribosomal subunit of bacteria resulting in the inhibition of protein synthesis. However, MRSA has become resistant to aminoglycosides by altering the structure of

aminoglycosides by aminoglycoside-modifying enzymes (AMEs) (Yuan *et al*., 2013). There are three classes of enzymes which are responsible for the structural modification of aminoglycosides (Yuan *et al*., 2013). These include: aminoglycoside acetyltransferases (AACs), aminoglycoside phosphotransferases APHs) and aminoglycoside adenylyltransferases (ANTs) (Yuan *et al*., 2013). The two most common AMEs in MRSA are the ANT, encoded by *ant(4')-Ia* and AAC(6′)/APH(2″), which is encoded by *aac(6')/aph(2'')*, a bifunctional enzyme (Yuan *et al*., 2013). The modifying enzymes encoded by the *aac(6')/aph(2'')* gene confers resistance to gentamicin. Most of these enzymes are carried on plasmids or transposons and may be combined into SCCmec genetic elements (Shokravi *et al.,* 2015).

2.8.6 *tetK*

Tetracycline is used in human and animal medicine and is considered a broad-spectrum antibiotic (Hedayatianfard *et al*., 2014). It was discovered in the 1940s. Tetracyclines are bacteriostatics used as preventive treatment against infections, including skin infections caused by *S. aureus* in both, humans and animals (Ong *et al*., 2017). However, tetracycline resistance as a result of drug overuse was first reported against *Shigella dysenteriae* (Ong *et al*., 2017) Sequentially, this spread to commensal, opportunistic and pathogenic bacteria due to transfer of tetracycline resistance genes (*tet*) (Ong *et al*., 2017). Tetracyclines prevent the association between the bacterial ribosome and aminoacyl-tRNA, inhibiting protein synthesis (Ullah *et al*., 2012). Mechanisms which confer tetracycline resistance to bacteria are ribosomal protection proteins, enzymatic inactivation or active efflux pumps (Adwan *et al*., 2014).

According to Hedayatianfard *et al*. (2014) there are 40 different tetracycline resistance genes that have been identified. Amongst these genes, *tetK*, *tetM*, *tetO* and *tetS* are commonly reported in Gram-positive bacteria whereas *tetA*, *tetB*, *tetD*, *tetE* and *tetG* are reported in Gram-negative bacteria (Hedayatianfard *et al*., 2014). *tetK* and *tetO* encode for energy-dependent

membrane-associated efflux proteins. They are responsible for preventing the accumulation of tetracycline within the cell (Ullah *et al*., 2012). *tetM* and *tetO* encode for ribosomal protection proteins, which reduces the bonds between tetracycline and the ribosome (Ullah *et al*., 2012).

2.9 Virulence determinants of MRSA

Virulence factors contributes to the ability of MRSA strains to cause diseases and colonize the host (Alfatemi *et al.,* 2014). These factors include haemolysins, exotoxins, leukocidins and superantigens (Burnside *et al*., 2010). The transcription of virulence genes in *S. aureus* is regulated by one component transcriptional regulators and two-component systems (Burnside *et al.,* 2010). Examples include the complex accessory gene regulatory system (agrABCD) which allows for the superantigens, cytotoxins and secreted enzymes to be upregulated and the transcription of cell wall proteins (protein A) to be repressed (Burnside *et al.,* 2010). The pathogenicity of *S. aureus* depends on the production of surface proteins which allows the bacterium to adhere to host tissues, secrete extracellular toxins and enzymes which will destroy the host cell, escape the host immune defence and allow for the growth of bacteria in host cells (Kong *et al*., 2016). The toxins produced are secreted during the post-exponential and early stationary phases, into the extracellular matrix where they will penetrate and invade the host (Kong *et al*., 2016). These toxins are cytolytic and acquire important nutrients from lysed cells promoting bacterial growth (Kong *et al*., 2016). The bacterium may also secrete enzymes which include coagulase and protease which allow for it to evade, invade and penetrate the host cells. *S. aureus* surface proteins may also help with bacterial adhesion (Kong *et al*., 2016).

2.9.1 Staphylococcal enterotoxins

There are nine major serological types of staphylococcal enterotoxins. This includes: SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI and SEJ and their associated genes *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei* and *sej* respectively (Kadariya *et al.,* 2014). These staphylococcal enterotoxins have similar structure and biological activity but differ in antigen characteristics (Alfatemi *et*

al., 2014). Staphylococcal enterotoxins are stable and resistant to various environmental conditions such as heat, freezing and drying (Kadariya *et al.,* 2014). They are resistant to proteolytic enzymes which include pepsin and trypsin and low pH which enables the enterotoxins to function after ingestion (Kadariya *et al.,* 2014). They also belong to a family of pyrogenic toxin superantigens which are responsible for superantigenic activity such as immunosuppression and nonspecific T-cell proliferation (Kadariya *et al*., 2014). It is hypothesized that this activity helps facilitate the entry of the toxins to enter the bloodstream allowing it to interact with T-cells and antigen-presenting cells leading to superantigen activity (Kadariya *et al*., 2014).

They are commonly found in dairy products and raw vegetables and cause nausea, vomiting, diarrhoea and muscular and abdominal pain in humans (Alfatemi *et al.,* 2014). The enterotoxin genes are carried and distributed by different mobile genetic elements such as plasmids, prophages, pathogenicity islands and staphylococcal cassette chromosome (Schelin *et al.,* 2011).

2.9.2 Staphylococcal enterotoxin A

The staphylococcal enterotoxin A, encoded by *sea,* is mainly responsible for staphylococcal food-borne disease. SEA is highly resistant to proteolytic enzymes (Kadariya *et al*., 2014). The *sea* gene is carried by temperate bacteriophages that have a polymorphic nature (Schelin *et al*., 2011). The bacteriophage is inserted as a prophage into the bacterial chromosome and acts as part of the bacterial genome (Schelin *et al*., 2011). When an environmental stress such as food preservation conditions is induced, replication of the phage genome is induced and new bacteriophages are released (Schelin *et al*., 2011). The transcription of *sea* depends on the lifecycle of the SEA-encoding prophages compared to non-phage encoded enterotoxin genes such as *sed.* SEA trains can be grouped into SEA_1 and SEA_2 based on sequence analysis. It has an endogenous promoter region, P_1 found in both groups and a second phage related latent

promoter, P_2, which allows for *sea* to be expressed after second phage related promoter (Schelin *et al*., 2011). Schelin *et al.* (2011) also showed that the SEA_1 group produced high amounts of SEA and the SEA_2 group produced low amounts of SEA

2.9.3 Haemolysins

Another virulence factor harboured by MRSA are haemolysins (Monecke *et al.,* 2014). Haemolysins are proteins that lyse red blood cells allowing the bacterium to scavenge iron compounds. There are three major haemolysins which include alpha, beta and delta (Monecke *et al.,* 2014).

2.9.3.1 Haemolysin alpha

The alpha haemolysin is encoded by the *hla* gene and is situated around base positions 1,110,000 to 1,230,000 in the staphylococcal chromosome genome (Monecke *et al.,* 2014). The alpha toxin is a pore forming toxin which is secreted as a water-soluble 33-kDa monomeric protein which binds to surface receptors on target cells (Kwak *et al.,* 2012). The pore is a polymeric ring which has a diameter of 1-2 nm and is made of seven 33kDa protein molecules which penetrate the host cell membrane (Monecke *et al.,* 2014). This causes osmotic swelling, rupture, lysis followed by cell death. The haemolysin alpha toxin is also neurotoxic and causes necrosis of the skin (Monecke *et al.,* 2014).

The alpha toxin is regulated by the agr and saeR/S systems as the deletion of saeRS and agr results in a weakened expression (Monecke *et al.,* 2014). This was also shown in sarA/Z which indicated that the alpha toxins are upregulated during the dissemination phase however are downregulated during the stationary phase when the factors promote biofilm formation and adhesion (Monecke *et al.,* 2014). Invasive and non-invasive isolates do not differ in the presence or absence of this gene because the *hla* gene is present in most isolates and lineages of *S. aureus* (Monecke *et al.,* 2014).

The intestinal cells form a selectively permeable epithelial barrier which prevents the entry of potentially harmful microorganisms and antigens from the intestinal lumen into internal tissues and blood (Kwak *et al.,* 2012). The tight junctions, which consist of transmembrane proteins promote physical barrier function, regulating the solutes and ions that diffuse between adjacent cells and help maintain cell polarity (Kwak *et al.,* 2012). Adherence junction proteins play an important signalling role which contributes to the mechanical strength of the junctional complexes (Kwak *et al.,* 2012). The interaction between these tight junctions and adherence junctions is important to maintain the epithelial structure and barrier function (Kwak *et al.,* 2012). Enteropathogenic bacteria modify ion transport and disrupt the tight and adherence junctions and cytoskeleton via their toxins and proteases (Kwak *et al.,* 2012). Calcium ions (Ca^{2+}) promotes different cellular functions such as regulation of gene expression, cytoskeletal rearrangements and apoptosis (Kwak *et al.,* 2012). The alpha toxin from *S. aureus* induces an increase of cytosolic free Ca^{2+} in the human airway epithelial cells and endothelial cells (Kwak *et al.,* 2012). However, more research needs to be done to determine the effect of the alpha toxin on human intestinal Caco-2 cells (Kwak *et al.,* 2012). Studies by Kwak *et al.* (2012), showed that free staphylococcal alpha-toxins in the bloodstream causes an intestinal epithelial barrier dysfunction and could worsen the septic condition by discharging intestinal bacteria into underlying tissue and the blood.

2.9.3.2 Haemolysin delta

The delta haemolysin toxin is encoded by the *hld* gene and is a 26 amino acid peptide. Ninety-seven percent of *S. aureus* isolates produce this toxin (Burnside *et al.,* 2010). This toxin lyses erythrocytes, mammalian cells and sub cellular structures which include membrane bound organelles (Burnside *et al.,* 2010). The structural gene is encoded within RNAIII which activates virulence factors such as enterotoxins, toxins and toxic shock syndrome toxins and it

repesses cell surface proteins (Burnside *et al.,* 2010). More investigation needs to be carried out on the contribution to virulence by *hld* (Burnside *et al.,* 2010).

2.9.4 Leukotoxins

MRSA strains may also produce Panton-Valentine Leukocidin (PVL) which is leukotoxin (Motamedi *et al.,* 2015). PVL is a bicomponent leukotoxin that is encoded by bacteriophages (Boakes *et al*., 2011). PVL is encoded by *lukS-PV* and *lukF-PV* genes which are co-localized and co-expressed (Monecke *et al*., 2013). The *lukS-PV* and *lukF-PV* can be found in the genome of the icosahedral or elongated head shaped temperate bacteriophages (Boakes *et al*., 2011).

This increases the virulence and may result in severe necrotic pneumonia and skin and soft tissue infections (Motamedi *et al.,* 2015). PVL is a pore forming toxin which targets the host's leukocytes (Motamedi *et al.,* 2015), disrupts leuckocyte membrane and causes tissue necrosis (Shrestha *et al.,* 2014). The presence of PVL in *S. aureus* has resulted in highly virulent and transmissible strains (Shrestha *et al.,* 2014). PVL related infections may spread to the lungs and may cause necrotizing pneumonia which rapidly destroys lungs tissue (Shrestha *et al.,* 2014). It has proven to be lethal and is emerging as a global health threat (Shrestha *et al.,* 2014). Studies by Breurec *et al.* (2011) showed that western and central African countries have a 57% prevalence rate of PVL positive MRSA serving as a challenge for the control of diseases and infections. The presence of the *pvl* gene serves as a genetic marker for HA-MRSA and CA-MRSA (Motamedi *et al.,* 2015).

The PVL encoding gene are relatively conserved however twelve single nucleotide polymorphisms have been identified such as a nonsynonymous change which allows the conversion of histidine to arginine at amino acid 176 (Boakes *et al*., 2011). The consequence of this change has not been characterized however it is assumed that it does not weaken leukotoxicity (Boakes *et al*., 2011).

The transfer of different PVL phages between strains of *S. aureus* is limited by phage/bacteria specificity factors such as restriction modification systems (Boakes *et al*., 2011). Infection occurs followed by lysogeny of the bacterial chromosome though the integrative pathway however the loci at which lysogeny of the PVL phage occurs and the attachment mechanism is unknown (Boakes *et al*., 2011).

2.10 Concluding Remark

Water, an important and precious resource, is needed for domestic use, irrigation and food production. In order to support sustainable development and improve the quality of life, in both developed and developing countries, safe drinking water should be accessible to all people. The disposal of improperly treated wastewater effluents into natural water bodies due to poor wastewater management has posed a threat to public and environmental health. Studies by Thapaliya *et al*. (2017) have shown that WWTPs serve as a hotspot for antibiotic resistant bacteria and antibiotic resistance genes. Improperly treated effluent may serve as reservoirs for MRSA. This may be discharged into receiving surface waters which may have detrimental consequences to individuals and animals who come into contact with this water. Exposure to MRSA in this water may result in skin and soft tissue infections, necrotizing pneumonia, bacteraemia and severe sepsis. Emerging resistance to commonly used antibiotics may result in ineffective treatment of MRSA infections and poses a cause for concern. Therefore, it is important that proper wastewater management practice and regulations be implemented to improve the quality of wastewater released into receiving surface waters and to prevent the dissemination of MRSA and outbreaks of MRSA infections.

References

Abidatul, A.A., Farhanah, N.M.J., Noramirah, R., Ling, S., New, C.Y., and Son, R. 2018. Prevalence and classification of high antimicrobial resistant *Staphylococcus aureus* in wastewater eluted from poultry slaughterhouse. *Food Research*. **2**(2): 201-207

Adwan, G., Adwan, K., Jarrar, N., and Amleh, A. 2014. Molecular detection of nine antibiotic resistance genes in methicillin resistant *Staphylococcus aureus* isolates. *Romanian Archives of Microbiology and Immunology,* **73**(1-2): 9-18.

Al-Abbas, M. J. A. 2012. Antimicrobial susceptibility of Enterococcus faecalis and a novel Planomicrobium isolate of bacterimia. *International Journal of Medicine and Medical Sciences*. *4*(2): 19-27.

Alfatemi, S.M.H., Motamedifar, M., Hadi, N., and Saraie, H.S.E. 2014. Analysis of virulence genes among methicillin resistant *Staphylococcus aureus* (MRSA) strains, *Jundishapur Journal of Microbiology*. **7**: e10741.

Almagro-Moreno, S., and Taylor, R.K. 2013. Cholera: environmental reservoirs and impact on disease transmission. *Microbiology Spectrum*. **1**: e1004787.

Amoako, D.G., Bester, L.A., Somboro, A.M., Baijnath, S., Chetna, N., Govind, C.N., and Essack, S.Y. 2016. Plasmid-mediated resistance and virulence mechanisms in the private health sector in KwaZulu-Natal, South Africa: An investigation of methicillin resistant *Staphylococcus aureus* (MRSA) clinical isolates collected during a three-month period. *International Journal of Infectious Diseases*. **46**: 38-41.

Arêde, P., Milheiriço, C., de Lencastre, H., and Oliveira, D.C. 2012. The anti-repressor mecr2 promotes the proteolysis of the *mecA* repressor and enables optimal expression of β-lactam resistance in MRSA. *PLoS Pathogens*. **8**: e1002816.

Ayeni, F.A., Gbarabon, T., Andersen, C., and Nørskov-Lauritsen, N. 2015. Comparison of identification and antimicrobial resistance pattern of *Staphylococcus aureus* isolated from Amassoma, Bayelsa state, Nigeria. *African Health Sciences*. **15**: 1282-1288.

Ba, X., Harrison, E.M., Edwards, G.G., Holden, M.T.G., Larsen, A.R., Petersen, A., Skov, R.L., Peacock, S.J., Parkhill, J., Patersen, G.K., and Holmes, M.A. 2014. Novel mutations in penicillin-binding protein genes in clinical *Staphylococcus aureus* isolates that are methicillin resistant on susceptibility testing, but lack the *mec* gene. *Journal of Antimicrobial Chemotherapy*. **69**: 594-597.

Bateganyaa, N.L., Nakalanzic, D., Babud, M., and Heinab, T. 2015. Buffering municipal wastewater pollution using urban wetlands in sub-Saharan Africa: a case of Masaka municipality, Uganda. *Environmental Technology*. **36**: 2149-2160.

Boakes, E., Kearns, A. M., Ganner, M., Perry, C., Hill, R. L., and Ellington, M. J. 2011. Distinct bacteriophages encoding Panton-Valentine Leukocidin (PVL) among international methicillin-resistant *Staphylococcus aureus* clones harboring PVL. *Journal of Clinical Microbiology*. **49**: 684–692.

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

Börjesson, S., Matussek, A., Melin, S., Löfgren, S., and Lindgren, P.E. 2010. Methicillin-resistant *Staphylococcus aureus* (MRSA) in municipal wastewater: an uncharted threat? Journal of Applied Microbiology. **108**:1244–1251.

Breurec, S., Fall, C., Pouillot, R., Boisier, P., Brisse, S., Diene-Sarr, F., Djibo, S., Etienne, J., Fonkoua, M.C., Perrier-Gros-Claude, J.D., Ramarokoto, C.E., Randrianirina, F., Thiberge, J.M., Zriouil, S.B., Garin, B., and Laurent, F. 2011. Epidemiology of

methicillin-susceptible *Staphylococcus aureus* lineages in five major African towns: high prevalence of *Panton–Valentine leukocidin* genes. *Clinical Microbiology and Infection.* **17**: 633-639.

Burnside, K., Lembo, A., de los Reyes, M., Iliuk, A., BinhTran, N.T., Connelly, J. E., Lin, W.J., Schmidt, B.Z., Richardson, A.R., Fang, F.C., Tao, W.A., and Rajagopal, L. 2010. Regulation of hemolysin expression and virulence of *Staphylococcus aureus* by a serine/threonine kinase and phosphatase. *PLoS ONE*. **5**: e11071.

Cabral, J. P. S. 2010. Water microbiology. Bacterial pathogens and water. *International Journal of Environmental Research and Public Health*. **7**: 3657–3703.

Chovanová, R., Mikulášová, M., and Vaverková, Š. 2016. Modulation of *mec*A gene expression by essential oil from *Salvia sclarea* and synergism with oxacillin in methicillin resistant *Staphylococcus epidermidis* carrying different types of staphylococcal chromosomal cassette *mec*. *International Journal of Microbiology*. **2016**: 1-10.

Costa, S. S., Viveiros, M., Amaral, L., and Couto, I. 2013. Multidrug efflux pumps in *Staphylococcus aureus*: an update. *The open microbiology journal,* **7**:59.

David, M.Z., Cadilla, A., Boyle-Vavra, S., and Daum, R.S. 2014. Replacement of HA-MRSA by CA-MRSA infections at an academic medical centre in the Midwestern United States, 2004-5 to 2008. *PLoS ONE*. **9**: e92760.

David, M. Z., and Daum, R. S. 2010. Community-associated methicillin-resistant *Staphylococcus aureus*: epidemiology and clinical consequences of an emerging epidemic. *Clinical Microbiology Reviews*. **23**: 616–687.

Department of Water Affairs. 2011. Greendrop report: Department of public works wastewater systems.

Department of Water Affairs. 2013. Greendrop report: Department of public works wastewater systems. Volume 2

de-Smidt, M.C.F., Moodley, A., Badat, R., Quan, V., Kularatne, R., Nana, T., Lekalakala, R., Govender, N., and Perovic, O. 2015. *Staphylococcus aureus* bacteraemia in Gauteng academic hospitals, South Africa. *International Journal of Infectious Diseases.* **30**: 41-48.

Ding, Z.F., Zhang, H., Tang, W., Tong, C.Y., Li, R.T., Chen, L.X., Pu, L.J., Zhu, Z.B., and Cui, Y.D. 2012. Methylase genes-mediated erythromycin resistance in *Staphylococcus aureus* from Bovine Mastitis in China. *Israel Journal of Veterinary Medicine*. **67**: 170-179.

Duran, N., Ozer, B., Duran, G.G., Onlen, Y., and Demir, C. 2012. Antibiotic resistance genes & susceptibility patterns in staphylococci. *Indian Journal of Medical Research*. **135**: 389-396.

Dormanesh, B., Siroosbakhat, S., Khodaverdi, Darian, E., and Afsharkhas, L. 2015. Methicillin-Resistant *Staphylococcus aureus* isolated from various types of hospital infections in pediatrics: Panton-Valentine Leukocidin, staphylococcal chromosomal cassette *mec SCCmec* phenotypes and antibiotic resistance properties. *Jundishapur Journal of Microbiology*. **8**: e11341.

Falagas, M.E., Koletsi, P.K., and Bliziotis, I.A. 2006. The diversity of definitions of multidrug-resistant (MDR) and pandrug-resistant (PDR) *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. *Journal of Medical Microbiology*. **55**: 1619–1629.

Feasey, N. A., Dougan, G., Kingsley, R. A., Heyderman, R. S., and Gordon, M. A. 2012. Invasive non-typhoidal salmonella disease: an emerging and neglected tropical disease in Africa. *The Lancet,* **379**(9835): 2489-2499.

Goldstein, R.E.R., Micallef, S.A., Gibbs, S.G., Davis, J.A., He, X., George, A., Kleinfelter, L.M., Schreiber, N.A., Mukherjee, S., Sapkota, A., Joseph, S.W., and Sapkota, A.R. 2012. Methicillin-Resistant *Staphylococcus aureus* (MRSA) detected at four U.S. wastewater treatment plants. *Environmental Health Perspectives*. **120**: 1551-1558.

Goudarzi, G., Tahmasbi, F., Anbari, K., and Ghafarzadeh, M. 2015. Distribution of genes encoding resistance to macrolides among *Staphylococci* isolated from the nasal cavity of hospital employees in Khorramabad, Iran. *Iranian Red Crescent Medical Journal*. **18**: e25701.

Gould, I. M. 2011. Clinical activity of anti-Gram-positive agents against methicillin-resistant *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*. **66**: iv.17-iv.21.

Green, B. N., Johnson, C. D., Egan, J. T., Rosenthal, M., Griffith, E. A., and Evans, M. W. 2012. Methicillin-resistant *Staphylococcus aureus*: an overview for manual therapists. *Journal of Chiropractic Medicine*. **11**: 64–76.

Hedayatianfard, K., Akhlaghi, M., and Sharifiyazdi, H. 2014. Detection of tetracycline resistance genes in bacteria isolated from fish farms using polymerase chain reaction. *Veterinary Research Forum*. **5**(4): 269

Hedden, S., and Cilliers, J. 2014. Parched prospects: The emerging water crisis in South Africa. *African Futures*. **11**: 1-16.

Hendricks, R., and Pool, E. J. 2012. The effectiveness of sewage treatment processes to remove faecal pathogens and antibiotic residues. *Journal of Environmental Science and Health. Part A, Toxic/Hazardous Substances & Environmental Engineering*. **47**: 289–297.

Hunter, P. R., MacDonald, A. M., and Carter, R. C. 2010. Water supply and health. *PLoS medicine,* **7**(11): e1000361.

Jhansi, S.C., and Mishra, S.K. 2013. Wastewater treatment and reuse: sustainability options. *Consilience: The Journal of Sustainable Development*. **10**: 1-15.

Kadariya, J., Smith, T.C., and Thapaliya, D. 2014. *Staphylococcus aureus* and staphylococcal food-borne disease: an ongoing challenge in public health. *BioMed Research International*. **2014**: 1-9.

Kaur, D. C., and Chate, S. S. 2015. Study of antibiotic resistance pattern in methicillin resistant *Staphylococcus aureus* with special reference to newer antibiotic. *Journal of global infectious diseases,* **7**(2): 78.

Kong, C., Neoh, H., and Nathan, S. 2016. Targeting *Staphylococcus aureus* toxins: A potential form of anti-virulence therapy. *Toxins*. **8**: 72.

Kumar, D., Asolekar, S.R., and Sharma, S.K. 2015. Post-treatment and reuse of secondary effluents using natural treatment systems: The Indian practices. *Environmental Monitoring and Assessment*. **187**: 612.

Kuntová, L., Pantůček, R., Rájová, J., Růžičková, V., Petráš, P., Mašlaňová, I., and Doškař, J. 2012. Characteristics and distribution of plasmids in a clonally diverse set of methicillin-resistant *Staphylococcus aureus* strains. *Archives of microbiology*. **194**: 607-614.

Kwak, Y.K., Vikström, E., Magnusson, K.E., Vécsey-Semjén, B., Colque-Navarro, P., and Möllby, R. 2012. The *Staphylococcus aureus* Alpha-Toxin Perturbs the Barrier Function in Caco-2 Epithelial Cell Monolayers by Altering Junctional Integrity. *Infection and Immunity*. **80**: 1670–1680.

Li, L., Feng, W., Zhang, Z., Xue, H., & Zhao, X. 2015. Macrolide-lincosamide-streptogramin resistance phenotypes and genotypes of coagulase-positive *Staphylococcus*

aureus and coagulase-negative staphylococcal isolates from bovine mastitis. *BMC veterinary research,* **11**(1): 168.

Lokhande, R. S., Singare, P. U., and Pimple, D. S. 2011. Study on physico-chemical parameters of waste water effluents from Taloja industrial area of Mumbai, India. *International Journal of Ecosystem,* **1**(1), 1-9.

Luyt, C. D., Tandlich, R., Muller, W. J., and Wilhelmi, B. S. 2012. Microbial monitoring of surface water in South Africa: an overview. *International journal of environmental research and public health,* **9**(8), 2669-2693.

Mardaneh, J., Abbas Poor, S., and Afrugh, P. 2013. Prevalence of *Shigella* species and antimicrobial resistance patterns of isolated strains from infected pediatrics in Tehran. *International Journal of Enteric Pathogens*. **1**: 28-31.

Monecke, S., ller, E.M., Buchler, J., Stieber, B., and Ehricht, R. 2014. *Staphylococcus aureus* in vitro secretion of alpha toxin (hla) correlates with the affiliation to clonal complexes. *PLoS ONE*. **9**: e100427.

Mossong, F., Decruyenaere, G., Moris, C., Ragimbeau, C.M., Olinger, S., Johler, M., Perrin, P., Hau, P., and Weicherding, P. 2015. Investigation of staphylococcal food poisoning outbreak combining case control, traditional typing and whole genome sequencing methods, Luxembourg. *Eurosurveillance.* **20**: 1-7.

Motamedi, H., Abadi, S. S. R., Moosavian, S. M., and Torabi, M. 2015. The association of *Panton-valentine leukocidin* and *mecA* genes in methicillin-resistant *Staphylococcus aureus* isolates from patients referred to educational hospitals in Ahvaz, Iran. *Jundishapur journal of microbiology*. **8**:1-5.

Naidoo, S., and Olaniran, A.O. 2013. Treated wastewater effluent as a source of microbial pollution of surface water resources. *International Journal of Environmental Research and Public Health*. **11**: 249-270.

Olayinka, B.O., Olayinka, A.T., Obajuluwa, A.F., Onaolapo, J., and Olurinola, P.F. 2009. Absence of *mecA* gene in methicillin-resistant *Staphylococcus aureus* isolates. *African Journal of Infectious Diseases*. **3**: 49-56.

Ong, M. H.L., Ho, W.Y., Ng, W.W., and Chew, C.H. 2017. High prevalence of *tetM* as compared to *tetK* amongst methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from hospitals in Perak, Malaysia. *Jundishapur journal of microbiology,* **10**(6).

Pathare, N.A., Tejani, S., Asogan, H., Mahruqi, G.A., Fakhri, S.A., Zafarulla, R., and Pathare, A.V. 2015. Comparison of methicillin resistant *Staphylococcus aureus* in healthy community hospital visitors [CA-MRSA] and hospital staff [HA-MRSA]. *Mediterranean Journal of Hematology and Infectious Diseases*. **7**: e2015053.

Paterson, G. K., Harrison, E. M., and Holmes, M. A. 2014. The emergence of mecC methicillin-resistant *Staphylococcus aureus*. *Trends in microbiology*. **22**(1): 42-47.

Piątkowska, E., Piątkowski, J., and Przondo-Mordarska, A. 2012. The strongest resistance of *Staphylococcus aureus* to erythromycin is caused by decreasing uptake of the antibiotic into the cells. *Cellular & molecular biology letters,* **17**(4): 633.

Pitman, W. 2011. Overview of water resource assessment in South Africa: current state and future challenges. *Water SA,* **37**(5): 659-664.

Popa, P., Timofti, M., Voiculescu, M., Dragan, S., Trif, C., and Georgescu, L. P. 2012. Study of physico-chemical characteristics of wastewater in an urban agglomeration in Romania. *The scientific world journal,* **2012**.

Rahimi, F., and Shokoohizadeh, L. 2016. Characterization of methicillin *resistant Staphylococcus aureus* strains among inpatients and outpatients in a referral hospital in Tehran, Iran. *Microbial pathogenesis*. **97**: 89-93.

San Millan, A. 2018. Evolution of plasmid-mediated antibiotic resistance in the clinical context. *Trends in microbiology*

Sato, T., Usui, M., Konishi, N., Kai, A., Matsui, H., Hanaki, H., and Tamura, Y. 2017. Closely related methicillin-resistant *Staphylococcus aureus* isolates from retail meat, cow with mastitis, and humans in Japan. *PloS One*. **12**(10): e0187319

Schaumburg, F., Alabi, A. S., Peters, G., and Becker, K. 2014. New epidemiology *of Staphylococcus aureus* infection in Africa. *Clinical Microbiology and Infection*. **20**(7): 589-596.

Schelin, J., Wallin-Carlquist, N., Thorup Cohn, M., Lindqvist, R., Barker, G. C., and Rådström, P. 2011. The formation of *Staphylococcus aureus* enterotoxin in food environments and advances in risk assessment. *Virulence*. **2**: 580–592.

Shahkarami, F., Rashki, A., and Ghalehnoo, Z. R. 2014. Microbial susceptibility and plasmid profiles of methicillin-resistant *Staphylococcus aureus* and methicillin-susceptible *S. aureus. Jundishapur journal of microbiology*. **7**(7).

Shokravi, Z., Mehrad, L., and Ramazani, A. 2015. Detecting the frequency of aminoglycoside modifying enzyme encoding genes among clinical isolates of methicillin-resistant *Staphylococcus aureus*. *BioImpacts.* **5**: 87.

Shrestha, B., Singh, W., Raj, V.S., Pokhrel, B.M., and Mohapatra, T.M. 2014. High prevalence of *Panton-Valentine Leukocidin (PVL)* genes in nosocomial-acquired *Staphylococcus aureus* isolated from tertiary care hospitals in Nepal. *BioMed Research International*. **2014**: 1-7.

Sun, J., Deng Z., and Yan, A. 2014. Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. *Biochemical and biophysical research communications,* **453**(2): 254-267.

Thapaliya, D., Hellwig, E. J., Kadariya, J., Grenier, D., Jefferson, A. J., Dalman, M., Kennedy, K., DiPerna, M., Orihill, A., and Taha, M. 2017. Prevalence and Characterization of *Staphylococcus aureus* and Methicillin-Resistant *Staphylococcus aureus* on Public Recreational Beaches in Northeast Ohio. *GeoHealth,* **1**(10), 320-332.

Thompson, J.M., Gündoğdu, A., Stratton, H.M., and Katouli, H.M. 2013. Antibiotic resistant *Staphylococcus aureus* in hospital wastewaters and sewage treatment plants with special reference to methicillin-resistant *Staphylococcus aureus* (MRSA). *Journal of Applied Microbiology*. **114**: 44-5.

Ullah, F., Malik, S. A., Ahmed, J., Shah, S., Ayaz, M., Hussain, S., and Khatoon, L. 2012. Investigation of the genetic basis of tetracycline resistance in *Staphylococcus aureus* from Pakistan. *Tropical Journal of Pharmaceutical Research,* **11**(6): 925-931**.**

Valsesia, G., Rossi, M., Bertschy, S., and Pfyffer, G. E. 2010. Emergence of SCC*mec* type IV and SCC*mec* type V methicillin-resistant *Staphylococcus aureus* containing the Panton-Valentine Leukocidin genes in a large academic teaching hospital in Central Switzerland: External invaders or persisting circulators? *Journal of Clinical Microbiology*. **48**: 720–727.

Venniyil, P. V., Ganguly, S., Kuruvila, S., and Devi, S. 2016. A study of community-associated methicillin-resistant *Staphylococcus aureus* in patients with pyoderma. *Indian Dermatology Online Journal*. **7**: 159–163.

Ventola, C. L. 2015. The antibiotic resistance crisis: part 1: causes and threats. *Pharmacy and Therapeutics,* **40**(4): 277.

Wan, M. T., and Chou, C. C. 2015. Class 1 integrons and the antiseptic resistance gene (qacEΔ1) in municipal and swine slaughterhouse wastewater treatment plants and wastewater—associated methicillin-resistant *Staphylococcus aureus*. *International Journal of Environmental Research and Public Health*. **12**: 6249-6260.

World Health Organization. 2014. Antimicrobial resistance global report on surveillance: 2014 summary.

World Health Organization and UNICEF. 2017. Progress on drinking water, sanitation and hygiene: 2017 update and SDG baselines.

Yuan, W., Hu, Q., Cheng, H., Shang, W., Liu, N., Hua, Z., Zhu, J., Hu, Z., Yuan, J., Zhang, X., Li, S., Chen, Z., Hu, X., Fu, J., and Rao, X. 2013. Cell wall thickening is associated with adaptive resistance to amikacin in methicillin-resistant *Staphylococcus aureus* clinical isolates. *Journal of Antimicrobial Chemotherapy*. **68**: 1089–1096.

Zaman, S. B., Hussain, M. A., Nye, R., Mehta, V., Mamun, K. T., and Hossain, N. 2017. A review on antibiotic resistance: alarm bells are ringing. *Cureus,* **9**(6).

Zmantar, T., Bekir, K., Elgarsadi, S.I., Hadad, O., and Bakhrouf, A. 2013. Molecular investigation of antibiotic resistance genes in methicillin resistant *Staphylococcus aureus* isolated from nasal cavity in pediatric service. *African Journal of Microbiology Research*. **7**: 4414-4421.

CHAPTER THREE

This chapter has been formatted and submitted to the Journal of Environmental Monitoring and Assessment

Prevalence and fate of Methicillin-resistant *Staphylococcus aureus* in treated effluent and receiving aquatic *milieu* of two wastewater treatment plants in Durban, South Africa.

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Abstract

Release of improperly treated effluent from municipal wastewater treatment plants may result in detrimental consequence on public and environmental health. The presence of Methicillin-resistant *Staphylococcus aureus* (MRSA), a well-known causative agent of many hospital-acquired infections, is particularly worrisome as MRSA may easily colonize and infect individuals who encounter the contaminated water. This study aimed to determine the impact of treated effluent of two wastewater treatment plants (WWTPs) in Durban, KwaZulu-Natal on the physico-chemical parameters and prevalence of MRSA in the respective receiving surface waters. Correlation studies of selected physico-chemical parameters to the prevalence of MRSA was also carried out. The prevalence of MRSA ranged variously as follows: 11.45-85.63% (Influent), 16.28-39.36% (Before Chlorination), 2.16- 5.07% (After chlorination), 1.06-7.24% (Downstream) and 4.95-14.09% (Upstream) in WWTP1. Similarly, at WWTP2, the prevalence of MRSA ranged from 48.25-86.18%, 23.73-93.75 %, 4.28-48.82 %, 1.74-19.31% and 5.90-28.78% in the influent, before chlorination, after chlorination, downstream and upstream samples, respectively. The study revealed a reduction in the prevalence of MRSA and *mecA* in the treated effluent as compared to the influent samples of both WWTPs. However, MRSA and *mecA* was still detected in the effluent samples after the treatment process, indicating the resistance of selective strains of MRSA to the wastewater treatment process. This finding therefore suggests that the WWTPs investigated in this study may serve as a reservoir for MRSA dissemination into the environment.

Keywords: methicillin-resistant *Staphylococcus aureus***,** prevalence, quantification of *mecA*, real-time PCR, wastewater treatment, wastewater treatment plants

3.1 Introduction

Globally, water is considered a precious resource (Pitman, 2011). It is required for drinking, preparation of food, sanitation and hygiene as well as agricultural and industrial purposes therefore, it is important that a sufficient and safe supply of water is easily accessed by people (Hunter *et al.*, 2010). In 2017, the World Health Organization reported that 842 000 deaths were due to lack of clean drinking water and proper sanitation and the child mortality rate reached 361 000 due to waterborne diseases (WHO and UNICEF, 2017). Access to an inadequate quality of water have led to many infections and disease caused by bacteria, protozoa and viruses, including: *Vibrio cholerae*, *Salmonella spp.*, *Shigella spp.*, *Escherichia coli*, *Cryptosporidium parvum*, Adenovirus 3 and Leptospira that are responsible for causing vomiting, nausea, diarrhea, abdominal pain and fever (Pandey *et al.*, 2014). The lack of potable water is compounded by rapid urbanization, a growing population, poor service delivery, lack of proper infrastructure and lack of proper wastewater management (Naidoo and Olaniran, 2013; Bateganyaa *et al.*, 2015). This is a cause for major concern in industries, agriculture, municipalities and the environment (Kumar *et al.*, 2015). Water scarcity can be combatted by wastewater reuse in agriculture and industry (Jhansi and Mishra, 2013)

Olaniran and Naidoo (2013) define wastewater as industrial or domestic sewage or storm runoff. However, the release of improperly treated wastewater from wastewater treatment plants (WWTPs) may result in the release of organic compounds and nutrients into receiving surface waters, with dire negative impact on the micro and macro-fauna present in the environment (Naidoo and Olaniran, 2013). Chemical agents detected in water, such as lead, copper and nitrate, have also been reported to induce vomiting, skin rashes, cancer, abnormal pregnancies and neurocognitive diseases (Hunter *et al.*, 2010; Rahmaninan *et al.*, 2015). The presence of other pollutants such as pesticides, trihalomethanes and pharmaceuticals have been reported to have adverse health effects on the end-user (Hunter *et al.*, 2010). Wastewater is

also a source for potentially pathogenic microorganisms, including: *Vibrio cholerae, Salmonella spp., Shigella spp*. and *Escherichia coli* (Almagro-Moreno and Taylor, 2013).

Staphylococcus aureus (*S. aureus*) is also commonly detected in wastewater and is responsible for a variety of infections which include septicaemia, pneumonia and skin infections (Goldstein *et al*., 2012; Almagro-Moreno and Taylor, 2013). Treatment of infections caused by this microorganism has become difficult due to strains becoming resistant to commonly prescribed antibiotics such as methicillin (Goldstein *et al*., 2012). Methicillin-resistant *Staphylococcus aureus* (MRSA) is known to induce infections in hospital and community settings (San Sit *et al*., 2017). The presence of the *mecA* in MRSA, carried on the Staphylococcal cassette (SCC*mec*), encodes for a penicillin-binding protein (PBP2a), that induces resistance to β-lactam antibiotics (San Sit *et al*., 2017). MRSA is commonly found within healthcare-settings, which allows for its potential spread into the environment through hospital wastewater (Börjesson *et al*., 2010; Thompson *et al*., 2013). Wastewater has been identified as a potential source for MRSA as *S. aureus* has been found to transfer antibiotic resistance genes *in vitro*, via horizontal gene transfer (Börjesson *et al.* 2009; Goldstein *et al*., 2012).

South Africa is regarded as a semi-arid country, receiving an average rainfall of 465 mm (Pitman, 2011). Thus, water shortages in South Africa remain a crisis, resulting in the use of treated wastewater in agriculture and industries. It is therefore important to characterize the chemical, biological and physical parameters of treated wastewater in order to determine the impact of the discharge of improperly treated effluent on the environment (Naidoo and Olaniran, 2013). By making use of the established parameters and guidelines, the quality of water can be assessed to determine if it is acceptable to be used domestically and to improve the wastewater management system. Numerous studies have implicated treated wastewater as a reservoir of potentially pathogenic microorganisms, including MRSA (Börjesson *et al*., 2010; Goldstein *et al*., 2012). However, to the best of our knowledge, no studies have been carried

out to investigate the prevalence of MRSA in treated effluent of wastewater treatment plants in Durban, South Africa and the consequential impact on the receiving surface waters. This study was therefore carried out to determine the efficiency of two WWTPs in the removal of MRSA in the received influent and to establish the impact of the treated effluent discharge from these WWTPs on the prevalence of MRSA in the receiving rivers.

3.2 Materials and Methods

3.2.1 Collection of water samples

Wastewater samples were collected from two wastewater treatment plants (designated as WWTP1 and WWTP2) in Durban, KwaZulu-Natal. Sample collection was carried out three times for each plant at different points of the WWTP. These points included: (1) influent, (2) before chlorination, (3) after chlorination, (4) upstream of the receiving river and (5) downstream of the receiving river. The water samples were collected in 5 L sterile bottles which were washed with de-ionized water and 70% (v/v) ethanol. To maintain sterility, the ethanol was left in the container and was rinsed using the water sample at the point of collection. The mouth of the container was placed against the water current, if it existed, to permit water to flow into the container. If a current did not exist then water samples were collected using a sampling stick and then poured into each container. Containers were sealed and stored away from sunlight. The samples were stored on ice and transported to the University of KwaZulu-Natal (Westville campus) where it was stored at 4 ˚C. Sample analysis was carried out within 48 h of collection.

3.2.2 Determination of the physico-chemical parameters

The temp. (temperatures) of the water samples were measured on site using a mercury-in-glass thermometer (Lloyds register quality company, U.K.). Other parameters that were tested included: turbidity, using a 2100 P turbidimeter (HACH), pH, using a Hanna HI11310 Edge®

pH meter, BOD/5 (biological oxygen demand), resistivity, EC (electrical conductivity), salinity and TDS (total dissolved solids) using a HQ 40d multi meter and parameter specific probes (HACH) and COD (chemical oxygen demand) using a Spectroquant NOVA 60 (Merck).

3.2.3 Enumeration and presumptive identification of *S. aureus* and MRSA

Wastewater samples were serially diluted with sterile distilled water. Thereafter, 100 ml of each dilution was filtered using a 0.45 µm cellulose nitrate filter in triplicate. Each filter was then placed on Mannitol Salt Agar (MSA) (Remel, ThermoFisher, USA) containing 6 µg/ml of cefoxitin to select for MRSA, and without cefoxitin to select for *S. aureus*. Control plates were incubated at 37°C for 24 h while plates containing cefoxitin were incubated at 37°C for 48 h. The presence of yellow colonies on MSA (with and without antibiotic) were considered presumptive MRSA and *S. aureus*, respectively. These were enumerated and expressed as colony forming units per millilitre (CFU/ml).

3.2.4 Data analysis

The prevalence of MRSA was calculated using the equation described by Tao *et al*. (2010), where A represents the number of presumptive MRSA and B represents the total number of presumptive *S. aureus*.

$$Prevalence\ (\%) = \frac{A}{B} \times 100$$

The removal efficiency of MRSA for each WWTP was calculated according to the equation below (Tao *et al.,* 2010), where B represents the number of presumptive MRSA in the influent and A represents the number of presumptive MRSA in the treated effluent (after chlorination).

$$Removal\ efficiency\ (\%) = \frac{B - A}{B} \times 100$$

3.2.5 Quantification of *mecA* using Real Time-PCR

3.2.5.1 Preparation of chemical competent *E. coli* DH5α cells

The chemical competent *E. coli* DH5α cells were prepared as previously described (Sambrook and Russel, 2001; Li *et al*., 2010). Briefly, a single colony of *E. coli* DH5α cells was inoculated into 5 ml of Luria Bertani (LB) broth and was incubated at 37 °C while shaking for 24 h. This was followed by inoculating 1 ml of the broth into 100 ml of fresh LB broth and was incubation with shaking at 37 °C until the cells reached an OD reading of 0.8 at 610 nm. The culture was transferred into sterile 50 ml tubes and centrifuged at 5000 × *g* for 10 min at 4°C and the supernatant was discarded. While on ice, 25 ml of cold $CaCl_2$ was added to resuspend the cells and this was left to stand for 10 min on ice. This was centrifuged at 5000 × *g* for 10 min at 4°C, the supernatant was discarded and 25 ml of $CaCl_2$ was added to resuspend the cells again. This was then left to stand on ice for 45 min before centrifuging at 5000 × *g* for 10 min at 4°C and 1 ml of $CaCl_2$ with 20% glycerol was mixed with these cells. 50 µl of these competent cells were transferred into 1.5 ml Eppendorf tubes and stored at -80°C. The competent cells were plated on LB agar plates and LB plates with 100 mg/ml Ampicillin to check the presence of contaminations.

3.2.5.2 Cloning of *mecA* into pJET1.2/blunt vector

The *mecA* gene (insert) was prepared by amplifying the gene using 25 µl of High Fidelity Mastermix (0.04 U/µL Phusion High Fidelity DNA Polymerase, 2× Phusion HF Buffer and 400 µM of each dNTP) (Thermo Scientific), 400 µM of *mec A* forward primer, 400 µM of *mecA* Reverse primer, 18 µl of nuclease free water and 10 µl of DNA. The primer sets used to detect the *mecA* gene included Forward: CCTAGTAAAGCTCCGGAA and Reverse: CTAGTCCATTCGGTCCA (Thompson *et al*., 2013). The PCR cycling conditions were: Initial denaturation at 95°C, 35 cycles of denaturation at 95°C for 2 min, annealing at 58°C for

1 min and extension at 72°C for 1 min. This was followed by a final extension at 72°C for 10 min. The amplified product ran on a 1.2% agarose gel at 60V for 90 min. It was excised and then extracted using the gel extraction kit (Thermo Scientific).

This insert was ligated into the vector (pJET1.2/blunt, ThermoFisher, USA). The ligation reaction was done on ice and consisted of 2 µl of 10x reaction buffer, 1µl of T4 DNA Ligase, 3µl of nuclease free water, 1µl of pJET 1.2 BR plasmid and 13 µl of DNA. This was briefly vortexed, centrifuged for 3-5 s and then incubated at 22°C in the T100 Thermal Cycler (Bio-Rad, USA) for 30 min.

The 5µl of ligation reaction mixture was added into the 50 µl of DH5α *E. coli* cells for the transformation of ligated insert and vector. This was left to stand on ice for 30 min followed by heat shock where the mixture was incubated at 42°C for 1 min in a water bath and then was left to stand on ice for 10 min. To this mixture, 750 µl of Luria Broth was added and this incubated at 37°C for 1 h in the shaker. This was centrifuged at 12 000 × *g* and 100 µl of the supernatant was left in the Eppendorf tube while the rest was discarded. The cells were mixed with the supernatant and 100 µl of the cells were spread plated on LB plates with ampicillin (50 µg/ml) and were incubated at 37°C for 16 h.

This procedure was followed by colony PCR where the PCR reaction consisted of: 12.55 µl distilled water, 1× PCR buffer, 250 µM of dNTPs, 3 mM of $MgCl_2$, 0.1 µM of *mecA* forward and *mecA* reverse Primers, 2 U *Taq* polymerase and the cloned colony. PCR amplification occurred under the following conditions: Initial denaturation at 95°C for 5 min, 25 cycles of denaturation at 95°C for 2 min, annealing at 58°C for 1 min and extension at 72°C for 1 min. This was followed by a final extension at 72°C for 10 min. The amplified product was confirmed on a 1.2% agarose at 60V for 90 min.

Following the success of colony PCR amplification, the positive colony was sub-cultured in 5 ml of LB broth with ampicillin and was left to incubate at 37 °C with shaking for 16 h. The broth was centrifuged at 12 000 × *g* for 1 min and the supernatant was discarded. This was followed by plasmid isolation using the plasmid isolation kit (ThermoFisher, USA). Gel electrophoresis on a 1.2% gel was carried out to confirm the cloned gene on the isolated plasmid where three bands were observed. Further confirmation included enzyme restriction using the enzymes *XbaI* and *XhoI*. The plasmid was stored at -20°C.

3.2.5.3 Real-time PCR

The iCycler iQ System (Bio-Rad) was used to run the real-time PCR assay. The total volume of the PCR mastermix was 10 µl which consisted of 5 µl of SYBR Green Premix (Thermo Scientific), 0.5 mM of *mecA* forward and reverse primer, 3 µl of nuclease free water and 1 µl of DNA template. The PCR cycling conditions were: initial denaturation at 95°C for 2 min, 35 cycles of denaturation at 95°C for 5 s, annealing at 58°C for 1 min and elongation at 95°C for 15 s. A standard curve was constructed using plasmid DNA, of a known concentration, from the cloned *mecA* genes obtained through10-fold serial dilutions of the plasmid DNA. This diluted DNA was used to run the real-time PCR assay for the standard curve to obtain the equation: $y = mx + c$. The standard curve that generated a regression value of 0.99 was used in calculating *mec*A concentration in the samples.

The real-time PCR assay was carried out in triplicates using the DNA isolated from the wastewater samples using the PowerWater Kit (DNeasy PowerWater Kit, Qiagen). The threshold cycle (Ct) value for each sample was determined and the gene copy numbers were then calculated using the formula: $copy\ number = 10(\frac{10(Ct-c)}{m})$ (Herrara *et al.*, 2009).

3.3 Results

3.3.1 Physico-chemical Profiles of the Wastewater Samples

The averages and standard deviations of selected physicochemical parameters of water samples obtained from both wastewater treatment plants (WWTPs) are depicted in Tables 3.1 and 3.2. In WWTP1, the temperature for the water samples ranged as follows: 20.33- 27.33 °C in influent (INF); 19.33- 25.67°C before chlorination (BC); 18.00- 28.33°C after chlorination (AC); 18.00- 27.33 °C downstream (DS); and 16.00-26.00 °C upstream (US). The pH ranged from 7.18-7.81 across all points, over all the three sampling periods. A steady decrease in turbidity was observed from the INF (487.67 ± 12.74; 486.33 ± 3.51 and 212.67 ± 4.04) to the AC points (12.40 ± 1.06; 13.34 ± 22.3 and 35.87 ± 5.26) during all three sampling periods, respectively. However, there was a slight increase in turbidity in the DS and US points of the receiving river, except, for the DS point during sampling period 3. Electrical conductivity (in μs/cm) ranged as follows: INF 724.00 - 1002.00, BC 514.00-750.00, AC 563.00-860.67, DS 432.67- 860.00 and upstream 257.67- 837.67, while the TDS (in mg/L) ranged from 414.67-483.67 (INF), 352.33- 366.67 (BC), 396.00-424.33 (AC), 400.67-421.33 (DS) and 257.67-410.00 (US). The resistivity ranged from 1006.30-1869.70 Ω/m and the salinity ranged from 0.35- 0.67% for all points in all three sampling periods. The COD values (in mg/L) ranged from 504.00-1002.00 (INF), 77.33-302.00 (BC), 73.00-306.00 (AC), 59.00-307.33 (DS) and 193.33-294.00 (US). The BOD_5 values (in mg/L) ranged from 143.00-230.67 for INF, 52.50-128.83 for BC, 91.67- 184.50 for AC, 7.54-23.72 for DC and 17.45-129.83 for US.

In WWTP2, the temperature for the water samples ranged as follows: 18.67- 25.33 °C in influent (INF); 18.33- 25.00°C before chlorination (BC); 19.00- 24.67°C after chlorination (AC); 18.67- 25.00 °C downstream (DS); and 17.33-23.33 °C upstream (US). The pH ranged from 7.01-7.88 across all points, over all the three sampling periods. A steady decrease in

turbidity was observed from the INF (454.33 ± 12.50; 314.33 ± 32.51 and 339.00 ± 1.00) to the AC points (95.00 ± 2.65; 100.33 ± 32.33 and 117.33 ± 5.26) during all three sampling periods, respectively. However, there was a slight increase in turbidity in the AC during sampling period 3. A slight increase in turbidity in the DS and US points of the receiving river was observed, except, for the DS point during sampling period 3. Electrical conductivity (in µs/cm) ranged as follows: INF 750.00 - 879.00, BC 532.33-799.00, AC 528.67-753.33, DS 379.00- 608.00 and upstream 317.67- 345.00, while the TDS (in mg/L) ranged from 366.67-436.33 (INF), 25.33- 390.67 (BC), 256.00-364.00 (AC), 182.37-300.00 (DS) and 152.20-345.00 (US). The resistivity ranged from 2.92-3150.00 Ω/m and the salinity ranged from 0.15-0.44% for all points in all three sampling periods. The COD values (in mg/L) ranged from 644.50-825.00 (INF), 256.00-289.33 (BC), 260.00-296.00 (AC), 222.67-276.33 (DS) and 212.00-269.00 (US). The BOD_5 values (in mg/L) ranged from 139.67-268.33 for INF, 58.50-178.25 for BC, 10.00- 199.33 for AC, 23.14-24.93 for DC and 21.10-24.90 for US.

The correlation matrices of the selected physico-chemical parameters with *S. aureus* population for WWTP1 and WWTP2 are shown in Tables 3.3 and 3.4, respectively. For WWTP1, resistivity and salinity had no significant correlations to other parameters in the WWTP. Temperature show significant negative correlation with the electrical conductivity (-0.699 at $p<0.01$), while pH negatively correlated with turbidity (-0.671 at $p<0.01$), COD (-0.585 at $p<0.05$) and BOD (-0.563 at $p<0.05$). Significant positive correlations were shown between MRSA and turbidity (0.563 at $p<0.01$) and between electrical conductivity and total dissolved solids (0.773 at $p<0.01$). There was also a positive correlation between BOD and COD (0.998), BOD and *S. aureus* (0.932), and *S. aureus* and COD (0.924) at $p<0.01$ (Table 3.4).

For WWTP2, *S. aureus*, total dissolved solids and pH had no significant correlations to other parameters in the WWTP (Table 3.4). A significant negative correlation was shown between temperature and resistivity (-0.591 at $p<0.05$). Turbidity showed a significant positive

correlation with electrical conductivity (0.805), salinity (0.811), COD (0.899) and BOD (0.706) at $p<0.01$ and MRSA (0.530 at $p<0.05$). Electrical conductivity showed a significant positive correlation with salinity (0.999 at $p<0.01$), COD (0.622 at $p<0.05$) and BOD (0.805 at $p<0.01$). Salinity showed a significant positive correlation with COD (0.633 at $p<0.05$) and BOD (0.801 at $p<0.01$). Significant positive correlations were shown between BOD and COD (0.504 at $p<0.05$) and between BOD and MRSA (0.561 at $p<0.05$).

Table 3.1 Physico-chemical parameters of water samples from WWTP1 and receiving surface waters

	Sample Point	Temperature (°C)	pH	Turbidity (NTU)	EC (µs/cm)	TDS (mg/L)	Resistivity (Ω/m)	Salinity (%)	COD (mg/L)	BOD (mg/L)
SAMPLING PERIOD	**INF**	27.33 ± 0.58	7.19	487.67 ± 12.74	724.00 ± 5.29	414.67 ± 1.53	1387.33 ± 8.96	0.42 ± 0.01	504.00 ± 165.46	143.00 ± 76.92
	BC	25.67 ± 0.58	7.51	20.63 ± 1.74	514.00 ± 7.00	364.00 ± 2.65	1869.70 ± 156.51	0.37 ± 0.01	77.33 ± 15.95	52.50 ± 27.58
	AC	28.33 ± 0.58	7.62	12.40 ± 1.06	563.00 ± 36.51	424.33 ± 3.79	1154.00 ± 10.44	0.43 ±0.01	222.00 ± 3.00	184.50 ± 0.71
	DS	27.33 ± 0.58	7.68	17.60 ± 0.35	618.00 ± 39.13	432.67 ± 1.15	1818.00 ± 22.87	0.67 ± 0.01	306.33 ± 2.08	23.72 ± 0.17
	US	26.00 ± 0.00	7.70	7.73 ± 1.56	340.33 ± 1.15	257.67 ± 18.77	1134.33 ± 2.89	0.43 ± 0.01	193.33 ± 25.58	25.19 ± 0.87
SAMPLING PERIOD	**INF**	20.33 ± 0.58	7.41	486.33 ± 3.51	1002.00 ± 18.56	483.67 ± 0.58	1006.30 ± 19.35	0.48 ± 0.00	731.50 ± 31.22	230.67 ± 28.38
	BC	19.33 ± 0.58	7.69	17.57 ± 0.15	750.00 ± 1.73	366.67 ± 0.58	1333.00 ± 2.65	0.37 ± 0.01	151.00 ± 18.73	128.83 ± 6.52
	AC	18.00 ± 0.00	7.42	13.40 ± 0.46	860.67 ± 2.31	421.67 ± 0.58	1112.67 ± 87.20	0.42 ± 0.00	73.00 ± 7.81	91.67 ± 6.58
	DS	18.00 ± 0.00	7.81	22.3 ± 0.30	860.00 ± 5.29	421.33 ± 3.06	1163.70 ± 7.37	0.42 ± 0.00	59.00 ± 31.00	7.54 ± 1.53
	US	16.00 ± 0.00	7.52	29.97 ± 0.67	837.67 ± 0.58	410.00 ± 0.00	1194.33 ± 0.58	0.41 ± 0.00	223.67 ± 9.29	129.83 ± 5.03
SAMPLING PERIOD 3	**INF**	21.33 ± 0.58	7.18	212.67 ± 4.04	871.67 ± 2.52	427.67 ± 1.53	1142.70 ± 4.16	0.43 ± 0.00	1002.50 ± 19.92	170.42 ± 5.38
	BC	21.33 ± 0.58	7.57	367.67 ± 20.11	721.67 ± 2.08	352.33 ± 0.58	1385.70 ± 3.21	0.35 ± 0.00	302.00 ± 1.00	87.92 ± 25.03
	AC	21.00 ± 1.00	7.62	35.87 ± 5.26	808.67 ± 10.41	396.00 ± 5.29	1261.00 ± 33.40	0.39 ± 0.01	306.00 ± 2.65	10.00 ± 0.00
	DS	19.00 ± 0.00	7.60	30.00 ± 5.09	818.33 ± 3.06	400.67 ± 1.53	1222.70 ± 5.03	0.40 ± 0.01	307.33 ± 0.58	23.01 ± 0.63
	US	17.33 ± 0.58	7.54	40.87 ± 4.32	812.67 ± 0.58	398.00 ± 0.00	1230.67 ± 0.58	0.41 ± 0.02	294.00 ± 2.65	17.45 ± 0.45

Table 3.2 Physico-chemical parameters of water samples from WWTP2 and receiving surface waters

	Sample Point	Temperature (°C)	pH	Turbidity (NTU)	EC (µs/cm)	TDS (mg/L)	Resistivity (Ω/m)	Salinity (%)	COD (mg/L)	BOD (mg/L)
SAMPLING PERIOD	**INF**	25.33 ± 0.58	7.11	454.33 ± 12.50	879.00 ± 6.24	436.33 ± 2.31	1125.00 ± 6.08	0.44 ± 0.00	825.00 ± 31.18	173.33 ± 55.99
	BC	25.00 ± 0.00	7.30	102.00 ± 3.61	753.33 ± 0.58	368.00 ± 0.00	1328.00 ± 0.00	0.37 ± 0.00	289.33 ± 14.05	178.25 ± 2.00
	AC	24.67 ± 0.58	7.72	95.00 ± 2.65	745.33 ± 33.65	364.00 ± 16.64	1344.00 ± 62.22	0.36 ± 0.02	260.00 ± 21.17	162.83 ± 5.77
	DS	25.00 ± 0.00	7.81	41.00 ± 1.00	608.00 ± 8.66	300.00 ± 6.08	1620.70 ± 32.35	0.30 ± 0.01	222.67 ± 41.88	23.14 ± 0.23
	US	23.33 ± 0.58	7.59	14.00 ± 2.00	345.00 ± 7.00	345.00 ± 7.00	2900.00 ± 60.83	0.17 ± 0.01	212.00 ± 21.79	23.55 ± 0.16
SAMPLING PERIOD	**INF**	18.67 ± 0.58	7.38	314.33 ± 32.15	814.67 ± 5.51	399.00 ± 3.00	1227.67 ± 8.50	0.40 ± 0.00	644.50 ± 53.36	268.33 ± 7.37
	BC	18.33 ± 0.58	7.35	280.67 ± 27.39	799.00 ± 3.00	390.67 ± 1.53	1252.00 ± 5.00	0.39 ± 0.00	274.33 ± 4.04	170.58 ± 1.26
	AC	19.00 ± 0.00	7.01	100.33 ± 32.33	650.33 ± 2.52	316.33 ± 1.53	1538.67 ± 5.51	0.32 ± 0.00	287.33 ± 2.08	126.00 ± 5.34
	DS	18.67 ± 0.58	7.52	38.33 ± 5.51	509.00 ± 3.61	246.67 ± 1.53	1964.00 ± 12.77	0.25 ± 0.01	276.33 ± 6.81	23.42 ± 1.32
	US	18.33 ± 0.58	7.43	18.67 ± 2.08	343.00 ± 1.00	164.70 ± 0.36	2.92 ± 0.02	0.16 ± 0.00	239.00 ± 1.00	21.1 ± 0.60
SAMPLING PERIOD 3	**INF**	22.00 ± 0.00	7.40	339.00 ± 1.00	750.00 ± 12.29	366.67 ± 5.86	1333.33 ± 21.73	0.37 ± 0.01	764.00 ± 27.01	139.67 ± 11.85
	BC	22.00 ± 0.00	7.25	76.67 ± 2.08	532.33 ± 6.66	258.33 ± 3.06	1878.30 ± 22.81	0.26 ± 0.01	256.00 ± 14.00	58.50 ± 1.32
	AC	22.00 ± 0.00	7.32	117.33 ± 1.53	528.67 ± 7.09	256.00 ± 3.61	1892.00 ± 25.24	0.26 ± 0.01	296.00 ± 3.00	199.33 ± 2.47
	DS	21.00 ± 0.00	7.88	44.33 ± 0.58	379.00 ± 6.56	182.37 ± 3.37	2640.00 ± 45.83	0.18 ± 0.00	263.33 ± 29.14	24.93 ± 0.13
	US	21.00 ± 0.00	7.66	26.67 ± 6.35	317.67 ± 1.53	152.20 ± 0.82	3150.00 ± 17.32	0.15 ± 0.00	269.00 ± 27.97	24.90 ± 0.12

Table 3.3 Correlation coefficients for the selected physico-chemical parameters and *S. aureus* population at WWTP1

	Temp.	**pH**	**Turbidity**	**EC**	**TDS**	**Resistivity**	**Salinity**	**COD**	**BOD**	**MRSA**	***S. aureus***
Temp.	1	-0.078	0.097	-0.699**	-0.193	0.473	0.363	-0.020	-0.027	0.406	-0.096
Ph		1	-0.671**	-0.330	-0.358	0.113	0.080	-0.585*	-0.563*	-0.569*	-0.504
Turbidity			1	0.373	0.337	-0.183	-0.038	0.184	0.142	0.563*	-0.005
EC				1	0.773**	-0.396	-0.067	0.230	0.211	-0.015	0.106
TDS					1	-0.140	0.369	0.192	0.169	0.117	0.015
Resistivity						1	0.298	-0.185	-0.176	0.135	-0.079
Salinity							1	0.030	0.012	0.043	-0.083
COD								1	0.998**	0.033	0.924**
BOD									1	0.011	0.932**
MRSA										1	-0.059
S. aureus											1

**. correlation is significant at the 0.01 level (2-tailed).

*. correlation is significant at the 0.05 level (2-tailed).

Table 3.4 Correlation coefficients for the selected physico-chemical parameters and *S. aureus* population at WWTP2

	Temp.	**pH**	**Turbidity**	**EC**	**TDS**	**Resistivity**	**Salinity**	**COD**	**BOD**	**MRSA**	***S. aureus***
Temp.	1	-0.020	-0.236	-0.367	-0.172	-0.591*	-0.379	-0.162	-0.289	-0.091	-0.039
pH		1	-0.495	-0.465	0.046	0.426	-0.476	-0.394	-0.508	-0.134	-0.100
Turbidity			1	0.805**	-0.048	-0.391	0.811**	0.899**	0.706**	0.530*	0.477
EC				1	-0.125	-0.506	0.999**	0.622*	0.805**	0.410	0.319
TDS					1	0.325	-0.110	-0.046	-0.088	-0.008	-0.030
Resistivity						1	-0.495	-0.301	-0.395	-0.222	-0.217
Salinity							1	0.633*	0.801**	0.408	0.315
COD								1	0.540*	0.622*	0.438
BOD									1	0.561*	0.185
MRSA										1	0.234
S. aureus											1

*. correlation is significant at the 0.05 level (2-tailed).

**. correlation is significant at the 0.01 level (2-tailed).

3.3.2 Prevalence of MRSA in the wastewater samples based on presumptive MRSA count

The prevalence of MRSA in WWTP1 ranged from 11.45- 85.63% in INF, 16.28-39.36% in BC, 2.16-5.07% in AC, 1.06-7.24% in DS and 4.95-14.09% in US (Fig. 3.1). WWTP1 showed the highest percentage of presumptive MRSA at the INF (85.63%) for sampling period 1. However, the highest prevalence was observed at BC for sampling periods 2 (22.06%) and 3 (39.36%). The percentage prevalence decreased during all 3 sampling periods at AC (2.82%, 5.07 and 2.16, respectively). An increase in percentage prevalence was noted in DS (7.23%) and US (4.95%) during sampling period 1. A lower percentage prevalence was reported at DS (2.61% and 1.06%) as compared to US (14.09% and 5.38%) for sampling periods 2 and 3, respectively.

The prevalence of MRSA in WWTP2 ranged from 48.25- 86.18% in INF, 23.73- 93.75% in BC, 4.28- 48.82 % in AC, 1.74- 19.31 % in DS and 5.90- 28.78% in US (Fig. 3.1). WWTP2 showed a highest percentage prevalence of presumptive MRSA at the INF (68.38%) for sampling period 1. The percentage prevalence showed to be higher at BC (93.75% and 60.11%) for sampling periods 2 and 3 respectively. The percentage prevalence decreased at AC (4.28% and 4.80) at sampling periods 1 and 2, respectively. However, during sampling period 3, percentage prevalence decreased to 48.82% at AC, which was lower than the percentage prevalence at BC but slightly higher than INF. A decrease in percentage prevalence was noted in DS (1.74 and 19.31%) during sampling periods 1 and 3 but an increase (18.19%) during sampling period 2. The percentage prevalence of MRSA at US was recorded to be 5.90%, 28.78% and 14.98.

The removal efficiency rate of MRSA ranged from 98.91-99.17% and 90.71-99.96% in WWTP1 and WWTP2, respectively during the sampling periods.

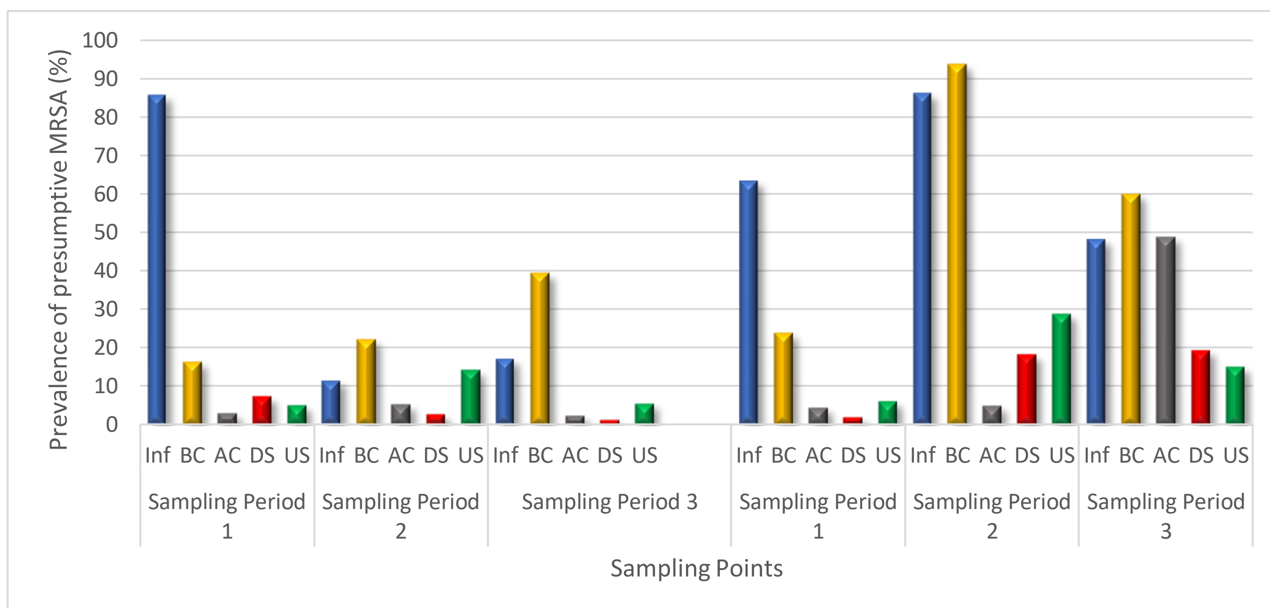


Fig. 3.1 Prevalence of presumptive MRSA in wastewater and receiving rivers at WWTP1 and WWTP2

3.3.3 Profiles of 16S rRNA and *mecA* genes in the wastewater samples

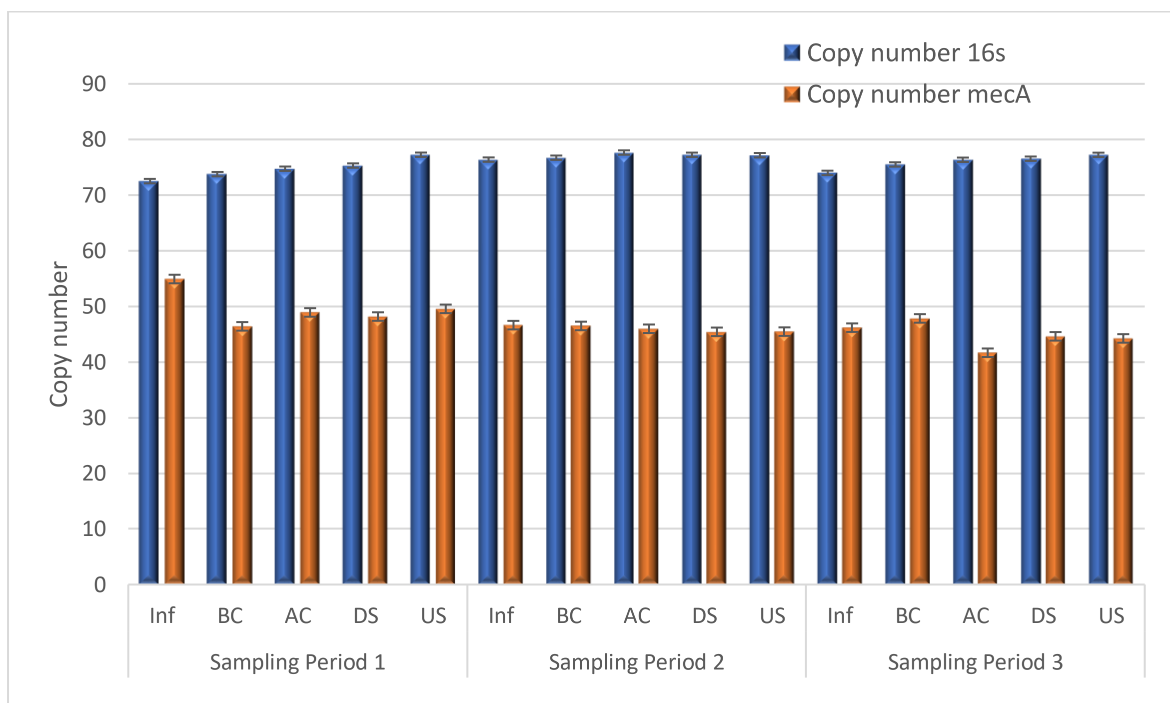
The standard curve for the 16S rRNA generated a formula of $y = -10.555x + 109.54$ with an r^2 value of 0.97, while that for *mecA* generated a formula of $y = -4.06x + 45.54$ with an r^2 value of 0.99. The copy number for 16S rRNA at WWTP1 ranged from 72.52-76.38 (INF), 73.77-76.72 (BC), 74.74-77.65 (AC), 75.31-77.24 (DS) and 76.42-77.25 (US) for all three sampling periods (Fig. 3.2a). The copy number for 16s rRNA at WWTP2 ranged from73.46-75.43 (INF), 74.65-76.91 (BC), 75.69-77.56 (AC), 76.29-77.42 (DS) and 77.10-77.25 (US).

Fig. 3.2a also showed the copy number for *mecA* at WWTP1 that ranged from, for INF 46.17-54.91, BC 46.41- 47.84, AC 41.67- 48.91, DS 44.61- 48.17 and US 44.24- 49.56, for all three sampling periods. Higher concentration of *mecA* was noted at the INF (copy number 54.91 and 46.63) at sampling periods 1 (copy number 54.91) and 2 (copy number 46.63), while BC recorded the higher copy number (47.84) during sampling period 3. At the AC, a slight decrease

in the copy number of the *mecA* was also recorded (48.91, 45.97 and 41.67) during all 3 sampling periods, respectively. However, there was a lower copy number at the DS point (48.17) but a higher copy number at the US point (49.56). Lower copy numbers for the *mecA* gene was recorded at DS (45.42) and US (45.46) during sampling period 2 but higher copy numbers were recorded at DS (44.61) and US (44.24) during sampling period 3.

Fig. 3.2b showed the copy number for *mecA* at WWTP2 ranged from, for INF 44.32- 45.83, BC 39.54- 44.91, AC 41.27- 47.96, DS 43.62- 46.07 and US 42.79- 46.49, for all three sampling periods. Higher concentration of *mecA* was noted at the INF (copy number 44.78 and 45.83) at sampling periods 1 and 2 and AC recorded the higher copy number (47.96) during sampling period 3. At AC, a slight increase in the copy number of the *mecA* was recorded (41.27, 43.96 and 47.97) during all 3 sampling periods, respectively, when compared to BC. However, there was a higher copy number at DS (44.45) during sampling period 1 but there was a lower copy number at the DS point (43.62 and 46.07) at sampling periods 2 and 3 respectively. The copy numbers at US were 46.49, 42.79 and 44.23 for sampling periods 1, 2 and 3 respectively.

a)



b)

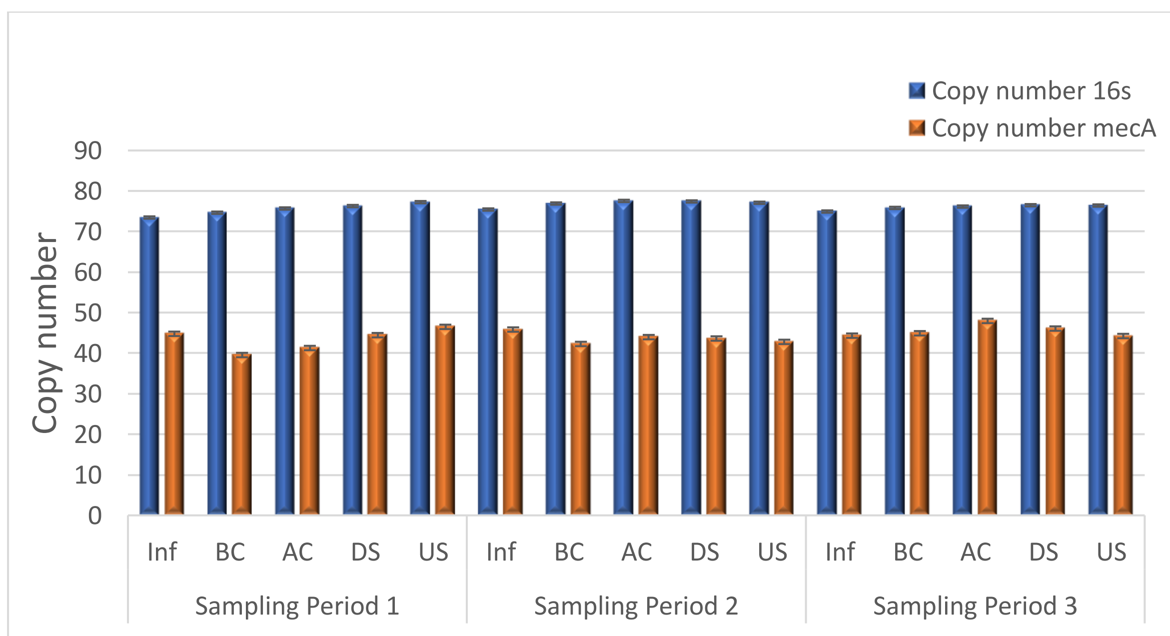


Fig. 3.2 Average copy number of 16S rRNA and *mecA* genes in a) WWTP1 and b) WWTP2

3.4 Discussion

The quality of water is measured by assessing its physical, chemical and bacteriological parameters (Reda, 2016). Therefore, it is important that treated effluent complies with the relevant discharge standards before it is released into the environment, to protect the health of the environment and the general public (Bateganyaa *et al*., 2015).

The physico-chemical parameters of the water samples were determined and represented in Tables 3.1 and 3.2 for WWTP1 and WWTP 2, respectively. The temperature of water is important as it influences the biochemical reactions of organisms present in the water by increasing their rate of activity (Lokandhe *et al*., 2011). However, water temperature may vary depending on the location of the water source, the time of day that samples are collected and the season of the year (Shukla *et al*., 2013). For WWTP1, the highest temperatures recorded were during the first sampling period and ranged from 25.67- 28.33°C. Warmer temperatures observed at the upstream and downstream points at WWTP1 could be attributed to the fact these points are situated near an industrial area. Lokhande *et al*. (2011) explained that water sources may be used for industrial cooling systems, which may result in warm water temperatures. The highest temperature recorded at WWTP2 was 25.33°C at the influent, also during the first sampling period. For the rest of the sampling periods, at both plants, the water temperatures were within the acceptable limit of 25°C (DWAF, 1984).

pH measures acidity or alkalinity of water samples (Rahmanian *et al*., 2015) and is set to range between 5.5-7.5 (DWAF, 1984). It is important that water is not extremely acidic or alkaline as this may have a negative impact on aquatic life and may also affect the operation of WWTPs (Kavitha *et al*., 2012). The pH of the water samples ranged from 7.18- 7.81 and 7.01- 7.88 for WWTP1 and WWTP2, respectively. A slight increase in pH was noted at the upstream and downstream points of the receiving rivers. These results were similar to that of a study which

recorded an average pH of 7.82 for domestic wastewater and the receiving river water (Popa *et al*., 2012). The pH results show that the treated effluent from these WWTPs and water from the receiving river is safe for reuse in irrigation (Kavitha *et al*., 2012).

Turbidity is defined as cloudiness of a liquid due to the various particles suspended in that sample (Rahmanian *et al*., 2015). According to DWAF (1996), the turbidity of domestic water should range from 0-1 NTU. The turbidity of the influent and after chlorination samples ranged from 212.67- 487.68 NTU and 12.40-35.87 NTU, respectively at WWTP1, while it ranged from 314.33-454.33 NTU (influent) and 38.33-117.33 NTU (after chlorination) at WWTP2. Even though the turbidity of the water decreased from influent to treated effluent in both WWTPs, the turbidity of water did not meet the acceptable range of <1 NTU. At the receiving rivers, the turbidity at the downstream points of WWTP1 (17.60- 30.00 NTU) and WWTP2 (38.33-44.33 NTU), and upstream points (7.73- 40.87 NTU) of WWTP1 and (14.00-26.67 NTU) WWTP2 were higher than the recommended limit. DWAF (1996) reported that this may have devasting effects on the taste, odour and appearance of the water, and that consumption of this water may result in infections due to disease causing agents and chemicals present in the water. As shown in Tables 3.3 and 3.4, there is a positive correlation between MRSA and turbidity at both WWTPs. This may be due to the suspension of organic or inorganic matter in the water which provides food and shelter for microorganisms, including MRSA (Altaher and Alghamdi, 2011).

Electrical conductivity is defined as the ability of water to conduct an electric current due to the presence of ionic solids such as magnesium, chloride and calcium (Reda, 2016). The electrical conductivity should not exceed 2500 µs/cm (250 ms/m) in wastewater (DWAF, 1984). The electrical conductivity (EC) for both plants fell below the limit of 2500 µs/cm and ranged between 340.33-1002.00 and 317.67-879.00 for WWTP1 and WWTP2, respectively. Although EC might not have a direct negative impact on human health, it is used to evaluate

the purity of water (Rahmanian *et al*., 2015; Reda, 2016). The industrial plant situated near the downstream point of the receiving river of WWTP1 may have contributed to the increase in the EC of surface waters near this point as compared to the downstream point of the receiving rivers of WWTP2. The release of industrial waste effluent downstream of the river may cause a change in mineral content, impacting the EC (Rahmanian *et al*., 2015). This may have a negative effect on pipes and metal items near or in the water as high EC is the cause of corrosion (Rahmanian *et al*., 2015).

The total dissolved solids (TDS) measures salinity by measuring the organic and low concentrations of inorganic matter present in the water (Rahmanian *et al*., 2015; Surti, 2016). TDS measures salts such as bicarbonate, carbonate, sodium, potassium and manganese. High amounts of these substances may change the density of water, lower the solubility of oxygen and change the osmoregulation of organisms present in the water (Surti, 2016). With the exception of the influent for the second sampling period (483.67 NTU) at WWTP1, the TDS values of all sampling points from both WWTPs as well as downstream and upstream points of the respective receiving rivers were below the acceptable limits of 450 mg/L (DWAF, 1996). Higher values of TDS (1308 mg/l) have been reported in treated effluent (Kavitha *et al*., 2012).

Chemical oxygen demand (COD) can be defined as the amount of oxygen needed to oxidize organic and inorganic compounds in the water (Popa *et al*., 2012; Surti, 2016). According to the Department of Water and Forestry, the COD should not exceed 30 mg/L (DWAF, 1984). The COD ranged from 504.00-1002.50 mg/L and 644.50- 825.00 (in the INF) to 73.00- 306.00 and 260.00- 296.00 (in the AC) for waters at WWTP1 and WWTP2, respectively. As observed in Tables 3.1 and 3.2, the COD for both WWTPs exceeded the acceptable limit of 30 mg/L across all sampling points. The COD of the treated effluent from both WWTPs (except for sampling period 2 at WWTP2) was much higher than findings by Kavitha *et al*. (2012) which reported COD values of 99 mg/L. This current study also reported high COD values for

downstream (59.00-307.33 mg/L for WWTP1 and 222.67-276.33 mg/L for WWTP2) and upstream points (193.33- 294.00 mg/L for WWTP1 and 212.00-269.00 mg/L for WWTP2) of the river. High COD values in the receiving rivers was attributed to industrial waste and external sources of domestic waste (Kavitha *et al*., 2012). The high COD value shows that the treated effluent and receiving river shows the presence toxic substances and biologically resistant substances (Kavitha *et al*., 2012).

The BOD values for INF to AC, ranged from 143.00-230.67 mg/L and 10.00-184.50 mg/L for WWTP1 and 139.67- 268.33 mg/l and 126.00-199.33 mg/L for WWTP2, respectively. BOD values ranged from 7.54-23.72 mg/L and 23.14-24.93 (DS) and from 17.45-129.83 mg/L and 21.10-24.90 mg/L (US) at WWTP1 and WWTP2, respectively. These values were much higher than the maximum value of 5 mg/L, as stipulated by the South African guidelines (DWAF, 1999). Even though the BOD values do not fall within the acceptable limit, this result suggests that both WWTPs are not the only contributors to the BOD of the receiving rivers. This may be due to the industrial area near the receiving river of WWTP1. This can be supported by a study which showed high BOD values of 535.8, 604.8 and 776.2 mg/L for the effluent samples from paint, engineering and dye industries, respectively (Lokhande *et al*., 2011). Similarly, WWTP2 and its receiving river are situated near a nature reserve, which may contribute to the high BOD values. This is supported by Lokhande *et al*. (2011) who stated that other sources of high BOD values may be caused by the leaves, woody debris, animal waste and dead animal or plant matter. The observed positive correlation between BOD and MRSA may be evidence that these sources may serve as reservoirs of MRSA contributing to their prevalence in the waters.

The high BOD values indicated the depletion of oxygen in the water, lowering the availability of oxygen to organisms present in the water which may result in the death of the aquatic life (Lokhande *et al*., 2011)

The spread of MRSA in clinical and environmental settings has posed a global threat to public health (Kim *et al*., 2012). This study was undertaken to determine the prevalence of MRSA in the treated effluent from two WWTPs and to ascertain if treated effluents from these plants contribute to the dissemination and spread of MRSA into their respective receiving rivers. The decrease in MRSA population revealed a removal efficiency of 98.91- 99.17% and 90.71-99.79% during the sampling periods in WWTP1 and WWTP2, respectively. As shown in Fig. 3.1, the highest prevalence of MRSA for each sampling period was recorded to be 85.63% at INF (for sampling period 1) and 22.06% and 39.36% at BC (for sampling period 2 and 3, respectively) at WWTP1 and 68.38 at INF (for sampling period 1) and 93.75% and 60.11% at BC (for sampling period 2 and 3, respectively) at WWTP2. The influent and before chlorination are the primary and secondary stages of the treatment process. There is a decrease in the prevalence of MRSA in the after-chlorination point at both WWTPs, however both plants were not able to completely remove MRSA in the treated effluent. Results varied to a study carried out by Börjesson *et al*. (2009) which showed that high number of MRSA could be isolated from the influent whereas no MRSA could be isolated from the effluent. Goldstein *et al*. (2012) also demonstrated a steady decrease of ten MRSA isolates found in the influent to one MRSA isolate found in the treated effluent. Börjesson *et al*. (2009) suggested that a decrease in MRSA in the effluent is due to treatment efficiency of the plant or, that in wastewater, the strains may enter the viable but non-culturable state. Another reason for the reduction of MRSA in the receiving rivers of WWTP1 may be due to the discharge of chemicals and heavy metals, from the nearby factories, which may hinder the growth of the MRSA present at this point of the river.

Quantitative PCR is a rapid but highly sensitive method to detect the prevalence of MRSA in water samples (Kim *et al*., 2012). This is done by rapid detection of the *mecA* gene which encodes for methicillin resistance (San Sit *et* al., 2017). Figs. 3.2a and b showed the average

copy number of the 16S rRNA and *mecA* at each sampling point of both WWTPs and their receiving rivers. 16S rRNA served as the standard for the total bacteria present at all sampling points of WWTPs and receiving surface waters (Mao *et al*., 2015). The number of cells carrying the *mecA* gene in the influent ranged from 46.17-54.91 and 44.32-45.83 during all three sampling periods for WWTP1 and WWTP2, respectively. The number of cells that carried the *mecA* gene in the treated effluent ranged from 41.67-48.91 and 41.27-47.96 for WWTP1 and WWTP2, respectively. A decrease in these cells from the influent to the treated effluent was observed for all three sampling periods in WWTP1 and for two of the sampling periods at WWTP2. This was similar to the findings of Börjesson *et al.* (2009) showing a reduction in the concentration of the *mecA* gene from the influent to the treated effluent. The findings of the current study indicate that both treatment plants were able to reduce the concentration of the *mecA* gene in the water. The third sampling period showed an increase in cells with the *mecA* gene in the treated effluent. This finding shows that both WWTPs may play a role in the spread of *mecA* to receiving surface waters as previously reported (Börjesson *et al*., 2009).

Results from this study show that the treatments in both WWTPs are effective in reducing the prevalence of MRSA from the influent to the treated effluent. However, no significant decrease in the concentration of *mecA* was observed for each sampling period from the INF to treated effluent. The low reduction of *mecA* may be due to disinfection and tertiary treatment, which could result in cell destruction and subsequent release of DNA into the water (Quach-Cu *et al*., 2018). Studies have reported an increase in antibiotic resistance genes (ARGs) in treated effluent even if the microbial population was reduced during the wastewater treatment (Quach-Cu *et al*., 2018). This suggests that treated wastewater effluent may potentially cause the spread of ARGs into receiving surface waters of the WWTPs. This finding is supported by Czekalski *et al*. (2012) who reported an accumulation of ARGs in receiving surface waters due to wastewater discharge.

Higher prevalence of MRSA was detected in US (14.09% and 5.38%) for sampling periods 2 and 3 at WWTP1 and in US (5.90% and 28.78%) for sampling periods 1 and 2 at WWTP2. Similar results were reported for the *mecA* gene at this point. This shows that both WWTPs are not the only source of antibiotic resistant bacteria or ARGs in the receiving rivers. Animal waste carrying gastrointestinal bacteria, may be discharged into the water through drainage ditches (Xiong *et al*., 2015). Genetic information may be exchanged between these bacteria and the indigenous bacteria present in the waters, including ARGs, such as *mecA* (Xiong *et al*., 2015).

The receiving river of WWTP1 is situated in a recreational area while that of WWTP2 is situated near a nature reserve that are habitats of many animals and birds. Thus. the animals could serve as a reservoir for MRSA and contribute to the spread of microorganisms in the environment (Thapaliya *et al*., 2017). Even though both WWTPs may have shown to decrease the amount of MRSA from the influent to the effluent, the remaining strains may be released into the receiving surface waters, suggesting that WWTPs may potentially select for MRSA strains that will eventually be released into receiving rivers (Börjesson *et al*., 2010).

3.5 Conclusion

The enumeration of MRSA and the quantification of the *mecA* gene showed that both WWTPs were able to reduce the MRSA load in the treated effluent before releasing it into the receiving rivers. Since both *mecA* and *S. aureus* exist in the same environment, Börjesson *et al*. (2009) suggests that horizontal gene transfer may occur in wastewater and give rise to MRSA. The results from this study show that the treated effluent from the WWTPs under investigation act as a reservoir for MRSA and may contribute to its spread in the environment. This is quite alarming as studies by Börjesson *et* al. (2009) showed that people who use such treated effluent for irrigation had a high prevalence of *S. aureus* infections. This may also be a concern to the people who work at the WWTPs through exposure to reclaimed water. They may be exposed to MRSA through direct skin contact with the water (Goldstein *et al*., 2012) as they may be exposed to MRSA. Wearing gloves and hand washing by workers of the WWTPs and those that live near contaminated receiving rivers, is advised to reduce MRSA infections. Furthermore, this study showed that although these WWTPs may reduce the number of bacteria present in wastewater, it may not efficiently remove ARGs from the water. This could lead to the spread of ARGs amongst various strains of bacteria, contributing to the spread and survival of multi-drug resistant bacteria, including MRSA.

References

Almagro-Moreno, S. & Taylor, R.K. (2013). Cholera: environmental reservoirs and impact on disease transmission. *Microbiology Spectrum*. **1**: e1004787.

Altaher, H. & Alghamdi, A. (2011). Enhancement of quality of secondary industrial wastewater effluent by coagulation process: A case study. *Journal of Environmental Protection*. **2**(09): 1250.

Bateganyaa, N.L., Nakalanzic, D., Babud, M., & Heinab T. (2015). Buffering municipal wastewater pollution using urban wetlands in sub-Saharan Africa: a case of Masaka municipality, Uganda. *Environmental Technology*. **36**: 2149-2160.

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

Börjesson, S, Matussek, A., Melin, S., Löfgren, S., & Lindgren, P.E. (2010). Methicillin-resistant *Staphylococcus aureus* (MRSA) in municipal wastewater: an uncharted threat? Journal of Applied Microbiology. **108**:1244–1251.

Czekalski, N., Berthold, T., Caucci, S., Egli, A., & Burgmann, H. (2012). Increased levels of multiresistant bacteria and resistance genes after wastewater treatment and their dissemination into Lake Geneva, Switzerland. *Frontiers in Microbiology*, **2012**(3): 106.

Department of Water and Environmental Affairs (DWAF). (1984). Republic of South Africa, Government Gazette, Requirements for the Purification of Wastewater or Effluent: general and special standards, regulation no. 91. Department of Water and Environmental Affairs, republic of South Africa, Durban, 1984.

Department of Water Affairs and Forestry (DWAF). (1996). South African water quality

guidelines for domestic use. 2nd edition. Department of Water Affairs and Forestry, South Africa, Durban. pp 1-185.

Department of Water and Environmental Affairs (DWAF). (1999). Quality of Domestic Water Supplies. Sampling Guide 2, Department of Water Affairs and Forestry, Department of Health and Water Research Commission.

Goldstein, R.E.R., Micallef, S.A., Gibbs, S.G., Davis, J.A., He, X., George, A., Kleinfelter, L.M., Schreiber, N.A., Mukherjee, S., Sapkota, A., Joseph, S.W., & Sapkota, A.R. (2012). Methicillin-Resistant *Staphylococcus aureus* (MRSA) detected at four U.S. wastewater treatment plants. *Environmental Health Perspectives*. **120**: 1551-1558.

Herrera, M. L., Vallor, A. C., Gelfond, J. A., Patterson, T. F., & Wickes, B. L. (2009). Strain-dependent variation in 18S ribosomal DNA copy numbers in Aspergillus fumigatus. *Journal of clinical microbiology*. **47**(5): 1325-1332.

Hunter, P. R., MacDonald, A. M., & Carter, R. C. (2010). Water supply and health. *PLoS medicine*. **7**(11): e1000361.

Jhansi, S.C., & Mishra, S.K. (2013). Wastewater treatment and reuse: sustainability options. *Consilience: The Journal of Sustainable Development*. **10**: 1-15.

Kavitha, R. V., Murthy, V. K., Makam, R., & Asith, K. A. (2012). Physico-chemical analysis of effluents from pharmaceutical industry and its efficiency study. *International Journal of Engineering Research and Applications*. **2**(2): 103-110.

Kim, J. U., Cha, C. H., An, H.K., Lee, H. J., & Kim, M. N. (2012). A multiplex real-time PCR assay for the detection of methicillin-resistant *Staphylococcus aureus* (MRSA) suitable in high endemic regions. *Journal of clinical microbiology*, JCM-02495.

Kumar, D., Asolekar, S.R., & Sharma, S.K. (2015). Post-treatment and reuse of secondary effluents using natural treatment systems: The Indian practices. *Environmental Monitoring and Assessment*. **187**: 612.

Li, X., Sui, X., Zhang, Y., Sun, Y., Zhao, Y., Zhai, Y., & Wang, Q. (2010). An improved calcium chloride method preparation and transformation of competent cells. *African Journal of Biotechnology*. **9**(50): 8549-8554.

Lokhande, R. S., Singare, P.U., & Pimple, D. S. (2011). Study on physico-chemical parameters of waste water effluents from Taloja industrial area of Mumbai, India. *International Journal of Ecosystem*. **1**(1): 1-9.

Mao, D., Yu, S., Rysz, M., Luo, Y., Yang, F., Li, F., Hou, J., Mu, Q., & Alvarez, P.J.J. (2015). Prevalence and proliferation of antibiotic resistance genes in two municipal wastewater treatment plants. *Water research*. **85**: 458-466.

Naidoo, S., and Olaniran, A.O. (2013). Treated wastewater effluent as a source of microbial pollution of surface water resources. *International Journal of Environmental Research and Public Health*. **11**: 249-270.

Pandey, P. K., Kass, P. H., Soupir, M. L., Biswas, S., & Singh, V. P. (2014). Contamination of water resources by pathogenic bacteria. *AMB Express*. **4**(1): 51.

Pitman W. (2011). Overview of water resource assessment in South Africa: current state and future challenges. *Water SA*. **37**(5): 659-664.

Popa, P., Timofti, M., Voiculescu, M., Dragan, S., Trif, C., & Georgescu, L. P. (2012). Study of physico-chemical characteristics of wastewater in an urban agglomeration in Romania. *The scientific world journal*. **2012**.

Quach-Cu, J., Herrera-Lynch, B., Marciniak, C., Adams, S., Simmerman, A., & Reinke, R. A. (2018). The Effect of Primary, Secondary, and Tertiary Wastewater Treatment Processes on Antibiotic Resistance Gene (ARG) Concentrations in Solid and Dissolved Wastewater Fractions. *Water*. **10**(1): 37.

Rahmanian, N., Ali, S. H. B., Homayoonfard, M., Ali, N., Rehan, M., Sadef, Y., & Nizami, A. (2015). Analysis of physiochemical parameters to evaluate the drinking water quality in the State of Perak, Malaysia. *Journal of Chemistry*. **2015.**

Reda, A. H. (2016). Physico-Chemical analysis of drinking water quality of Arbaminch Town. *Ann Chem Open Access*. **2**(006).

Sambrook, J., and Russell, D.W. (2001). Molecular cloning: A laboratory manual. 3rd edition. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press.pp.132-150.

San Sit, P., Teh, C.S.J., Idris, N., Sam, I.C., Omar, S.F.S., Sulaiman, H., Thong, K.L., Kamarulzaman, A., & Ponnampalavanar, S. (2017). Prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) infection and the molecular characteristics of MRSA bacteraemia over a two-year period in a tertiary teaching hospital in Malaysia. *BMC infectious diseases*. **17**(1): 274.

Shukla, D., Bhadresha, K., Jain, N.K., & Modi, H.A. (2013). Physicochemical Analysis of Water from Various Sources and Their Comparative Studies. IOSR Journal of Environmental Science. *Toxicology and Food Technology*. **5**: 2319-2402.

Surti H. (2016). Physico-Chemical and Microbial Analysis of Waste Water from different Industry and Cod Reduction Treatment of Industrial Waste Water by using Selective Microorganisms. *International Journal of Current Microbiology and Applied Sciences*. **5**(6): 707-717.

Tao, R., Ying, G.G., Su, H.C., Zhou, H.W., & Sidhu, J.P.S. (2010). Detection of antibiotic resistance and tetracycline resistance genes in Enterobacteriaceae isolated from the Pearl Rivers in South China. *Environmental Pollution*. 158: 2101-2109

Thapaliya, D., Hellwig, E. J., Kadariya, J., Grenier, D., Jefferson, A. J., Dalman, M., Kennedy, K., DiPerna, M., Orihill, A., & Taha, M. (2017). Prevalence and Characterization of *Staphylococcus aureus* and Methicillin-Resistant *Staphylococcus aureus* on Public Recreational Beaches in Northeast Ohio. *GeoHealth,* **1**(10): 320-332.

Thompson, J.M., Gündoğdu, A., Stratton, H.M., & Katouli, H.M. (2013). Antibiotic resistant *Staphylococcus aureus* in hospital wastewaters and sewage treatment plants with special reference to methicillin-resistant *Staphylococcus aureus* (MRSA). *Journal of Applied Microbiology*. **114**: 44-5

World Health Organization and UNICEF. (2017). Progress on drinking water, sanitation and hygiene: 2017 update and SDG baselines.

Xiong, W., Sun, Y., Ding, X., Wang, M., & Zeng, Z. (2015). Selective pressure of antibiotics on ARGs and bacterial communities in manure-polluted freshwater-sediment microcosms. *Frontiers in microbiology*. **6**: 194.

CHAPTER FOUR

This chapter has been formatted and submitted to the World Journal of Microbiology and Biotechnology

Antibiogram and molecular characterization of methicillin-resistant *Staphylococcus aureus* recovered from treated wastewater effluent and receiving surface water in Durban, South Africa

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Abstract

Municipal wastewater treatment plants (WWTPs) may serve as a reservoir for potentially pathogenic and antibiotic resistant bacteria. The discharge of improperly treated wastewater effluent may lead to the spread of these bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) which is responsible for causing pneumonia, septicaemia and skin and soft tissue infections, into the receiving surface waters. This study aimed to determine the antibiogram and virulence gene profiles of MRSA isolates recovered from treated wastewater effluent and receiving surface waters. Genetic fingerprinting of the isolates was also carried out to determine the phylogenetic relationship between the isolates and selected antibiogram profiles. Eighty MRSA isolates were obtained from treated effluent and receiving rivers of two WWTPs in Durban, KwaZulu-Natal. Antibiotic resistance was observed towards lincomycin (100%), oxacillin (98.75%), cefoxitin and penicillin (97.50%), and ampicillin (96.25%). In addition, 72.50%, 66.25%, 52.50%, 40% and 33.75% of isolates showed resistance against cefozolin, azithromycin, amoxicillin/clavulanic acid, erythromycin and vancomycin, respectively. Antibiotic resistance genes detected in the isolates tested in this study: *aac(6')/aph(2'')* (56.25%), *ermC* (62.50%), *msrA* (22.50%), and *blaZ* and *tetK* (70%). The frequency of virulence genes: *hla* and *sea* was 57.50%, *hld* was 1.25%, while *lukS P/V* was 0%. Pulse Field Gel Electrophoresis analysis generated 13 pulsotypes (designated A-M) showing a correlation to their respective antibiograms. Findings from this study showed the presence of potentially pathogenic, multi-drug resistant MRSA in the treated effluent and receiving surface waters. This may have detrimental effects on the health of individuals who come into contact with these water resources.

Keywords: Methicillin-resistant *Staphylococcus aureus*, wastewater treatment plants, antibiograms, antibiotic resistance genes, virulence genes, pulse field gel electrophoresis

4.1 Introduction

Staphylococcus aureus is a Gram-positive bacterium that may cause infections which can lead to fatal diseases such as skin and soft tissue infections, pneumonia, food poisoning and toxic shock (Zmanter *et al*., 2013; Alfatemi *et al*., 2014; Velasco *et al*., 2015). Antibiotics have played a major role in the treatment of staphylococcal infections; however, antibiotic resistance has emerged due to the misuse and overuse of antibiotics as well as the evolvement of bacterial strains harbouring antibiotic resistance genes (ARGs) (Ong *et al*., 2017). Methicillin-resistant *Staphylococcus aureus* (MRSA) was first isolated in the 1960s in the hospital environment therefore referred to as hospital-acquired MRSA (HA-MRSA) (Goldstein *et al*., 2012). However, in the 1990s, infections appeared in individuals with no risk factors and were regarded as community-acquired MRSA (CA-MRSA) (Goldstein *et al*., 2012).

Methicillin-resistance in MRSA is caused by the *mecA* gene which is carried on the staphylococcal cassette chromosome *mec* (SCC*mec*) in all MRSA strains (Zmantar *et al*., 2013). The *mecA* gene encodes for a penicillin binding protein (PBP) which induces resistance to *β*-lactam antibiotics in MRSA (Thompson *et al*., 2013; Ong *et al*., 2017). MRSA have been reported to show resistance to multiple antibiotics, including: aminoglycosides, lincosamides, tetracyclines and macrolides. Increasing resistance has been shown against vancomycin which is used as an ultimate option for treatment of MRSA infections (Ong *et al*., 2017). Many studies have shown that ARGs are responsible for causing antibiotic resistance in MRSA. Commonly reported ARGs found in MRSA are those that encode resistance towards macrolides, macrolide– lincosamide–streptogramin B, tetracyclines, *β*-lactams and aminoglycosides (Zmantar *et al*., 2013; Dormanesh *et al*., 2015). These ARGs can be transferred between species through horizontal gene transfer and integrated into the host's DNA influencing the evolution of the strains (Mkize *et al*., 2017). This may be due to the various mobile genetic elements

found in MRSA which include transposons, plasmids, gene cassettes and bacteriophages (Mkize *et al.*, 2017).

A variety of virulence factors in MRSA have been reported to be responsible for its colonization and pathogenicity in a host. *S. aureus* produce low molecular weight toxins, which include staphylococcal enterotoxins (A-Q) associated with their respective genes. The group of staphylococcal enterotoxins A-E are related to food poisoning (Alfatemi *et al.*, 2014). Other toxins include haemolysins (alpha, beta and delta) which are known to lyse red blood cells (Burnside *et al.*, 2010). Leukocidal toxins are caused by the Panton-Valentine Leukocidin (PVL) which serves as a virulence marker in many community-acquired MRSA infections (Thapilya *et al.*,2017). This toxin has also been associated with skin and soft tissue infections, and systemic infections (Dormanesh *et al.*, 2015). Therefore, it is important to identify potential environmental sources of MRSA to prevent and control the spread of infections. Börjesson *et al.* (2009) detected the presence of the *mecA* gene in hospital and municipal wastewater. This suggests that wastewater treatment plants may play a role as a reservoir and dissemination of MRSA into receiving surface waters and its surrounding environment. The use of improperly treated wastewater in agricultural and industrial applications, due to water shortages (Goldstein *et al.*, 2012), may bear harmful consequences to the end-users as they may be exposed to potentially pathogenic and multi-drug resistant MRSA.

This study aimed to characterize MRSA strains recovered from treated wastewater effluent and receiving surface waters of two WWTPs in Durban and to determine their antibiogram and virulence gene profiles. Genetic fingerprinting was carried out on selected isolates, based on their antibiograms, to ascertain their genetic relatedness using pulsed field gel electrophoresis.

4.2 Materials and Methods

4.2.1 Source of Methicillin Resistant *Staphylococcus aureus* isolates and culture conditions

Presumptive Methicillin Resistant *Staphylococcus aureus* (MRSA) strains were isolated from the treated effluent, upstream and downstream points of receiving rivers of two wastewater treatment plants (WWTPs) in the Durban area. The isolates were grown on Mannitol Salt Agar (MSA) (Biolab, Merck) supplemented with cefoxitin. Isolates which grew as yellow colonies were considered presumptive MRSA and were subjected to biochemical testing. Isolates that were catalase positive, oxidase negative and coagulase positive were screened for the presence of the *mecA* gene to confirm them as MRSA. Eighty confirmed MRSA isolates were stored as glycerol stock at -80°C for further characterization.

4.2.2 Molecular confirmation of the MRSA isolates via PCR detection of *mec*A gene

DNA was isolated using the boiling method where 5 colonies were suspended in 70µl of distilled water. The suspension was boiled for 10 minutes then centrifuged at 13 000 rpm. 50 µl of the supernatant was removed and stored at -20ºC (Bai *et al.,* 2010). PCR amplification of the *mecA* gene was performed using the method described by Thompson *et al.* (2013). The 25 µl PCR assay mixture contained: 10.55 µl of distilled water, 1× PCR buffer, 3 mM of $MgCl_2$, 250 µM of dNTPs, 0.1 µM of each of forward and reverse primers, 2 U *Taq* polymerase (SuperTherm) and 2µl of DNA template. The primer sets used to detect the *mecA* gene included Forward: CCTAGTAAAGCTCCGGAA and Reverse: CTAGTCCATTCGGTCCA (Thompson *et al*., 2013).The PCR cycle was performed in a T100 Thermal Cycler (Bio-Rad, USA) under the following conditions: Initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 2 min, amplification at 58°C for 1 min, elongation at 72°C for 1 min and final elongation at 72°C for 10 min. The amplified product was resolved on a 1.5% agarose

gel (Seakem) at 60 V for 90 min. The gel was stained with 1% Ethidium bromide for 15 min and viewed under UV light (420 nm) using the Chemigenius Bioimaging System (Syngiene).

4.2.3 Antibiotic resistance profiling of MRSA isolates

4.2.3.1 Antibiotic susceptibility testing

Antibiotic susceptibility tests were performed using the Kirby-Bauer disc diffusion assay according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2014). All the isolates were inoculated in nutrient broth (Biolab, Merck) and incubated at 37°C for 24 h. Each isolate was standardized to 0.5 McFarland standard and swabbed onto Mueller-Hinton (Biolab, Merck) agar plates. Thereafter, the plates were allowed to dry for about 30 min before placing 5 antibiotic discs equidistant from each other on each plate. Each isolate was tested in duplicate. The plates were incubated at 37 °C for 24 h and thereafter screened for the presence/absence of clear zones. The clear zones diameter, where inhibition of growth around the disc occurs, was observed and measured (in mm). The isolates were then classified as being either susceptible, intermediate or resistant based on the CLSI (2014) guidelines. Isolates that are resistant to three or more classes of antibiotic were considered as being multi-drug resistant (Falagas *et* al., 2006). *E. coli* 25922 was used as a control strain.

4.2.3.2 Detection of antibiotic resistance genes

All isolates were screened for the presence of the following antibiotic resistance genes: *aac(6')/aph(2''), blaZ*, *ermC*, *msrA* and *tetK*. The 25 µl PCR assay mixture contained: 5.9 µl of distilled water, 1 µM of each forward and reverse primer, 200 µM of dNTPs, 1.5 mM of $MgCl_2$, 1× PCR buffer, 1 U *Taq* polymerase and 5 µl of bacterial DNA as template. The primers used for the PCR amplification of these antibiotic resistance genes are shown in Table 4.1.

Table 4.1 Primers used for PCR amplification of antibiotic resistance genes

Gene Target	Sequence (5' ⟶ 3')	Product Size (bp)	Reference
aac(6')/aph(2'')- F	TAATCCAAGAGCAATAAGGGC	227	Kuntová *et al.* (2012)
aac(6')/aph(2'')- R	GCCACACTATCATAACCACTA		
blaZ- F	ACTTCAACACCTGCTGCTTT	173	Kuntová *et al.* (2012)
blaZ- R	TGACCACTTTTATCAGCAAC		
ermC- F	CTTGTTGATCACGATAATTTCC	190	Kuntová *et al.* (2012)
ermC- R	ATCTTTTAGCAAACCCGTATTC		
msrA- F	GGCACAATAAGAGTGTTTAAAGG	940	Dormanesh *et al.* (2015)
msrB- R	AGTTATATCATGAATAGATTGTCCTGTT		
tetK- F	GTAGCGACAATAGGTAATAGT	360	Dormanesh *et al.* (2015)
tetK- R	GTAGTGACAATAAACCTCCTA		

The PCR cycle was performed in a T100 Thermal Cycler (Bio-Rad, USA). The PCR amplification for the *aac(6')/aph(2'')*, *blaZ* and *ermC* was performed under the following conditions: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 2 min, annealing at 55°C for 30 s, elongation at 72°C for 1 min and a final elongation at 72°C for 7

min (Kuntová *et al.,* 2012). The PCR amplification for *msrA* was performed under the following conditions: initial denaturation at 94°C for 6 min, 34 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min 10 s, elongation at 72°C for 1 min 10 s and a final elongation at 72°C for 8 min (Dormanesh *et al*., 2015). The PCR amplification of *tetK* was performed under the following conditions: initial denaturation at 94°C for 5 min, 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min 10 s, elongation at 72°C for 1 min and a final elongation was done at 72°C for 10 min (Dormanesh *et al*., 2015). The amplified product was resolved on a 2% agarose gel (SeaKem) at 60 V for 90 min.

4.2.4 PCR Amplification of virulence genes

PCR amplification was carried out to screen for the presence of the *hla*, *hld*, *lukS/F PV* and *sea* genes. The PCR assay was carried out in a 25 µl reaction mixture, containing 0.9 µl of distilled water, 1 µM of each forward and reverse primer for each gene, 200 µM of dNTPs, 1.5 mM of $MgCl_2$, 1× PCR buffer, 1 U *Taq* polymerase and 5 µl of bacterial DNA as template. The primers used are shown in Table 4.2.

Table 4.2 Primers used for PCR amplification of virulence genes

Gene Target	Sequence (5'⟶ 3')	Product Size (bp)	Annealing Temperature (°C)	Reference
hla- F	CTGATTACTATCCAAGAAATTCGATTG	209	58	Alfatemi *et al*. (2014)
hla- R	CTTTCCAGCCTACTTTTTTATCAGT			
hld- F	AAGAATTTTTATCTTAATTAAGGAAGGAGTG	111	58	Alfatemi *et al*. (2014)
hld- R	TTAGTGAATTTGTTCACTGTGTCGA			
lukS/F PV- F	ATCATTAGGTAAAATGTCTGGACATGATCCA	403	60	Alfatemi *et al*. (2014)
lukS/F PV- R	GCATCAAGTGTATTGGATAGCAAAAGC			
sea- F	GGTTATCAATGTGCGGGTGG	102	60	Alfatemi *et al*. (2014)
sea- R	CGGCACTTTTTTCTCTTCGG			

The PCR cycle was performed in a T100 Thermal Cycler (Bio-Rad, USA) under the following conditions: Initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, elongation at 72°C for 1 min and a final elongation step at 72°C for 7 min (Alfatemi *et al.,* 2014). The amplified products were resolved in a 2% agarose gel (Seakem) at 60 V for 90 min.

4.2.5 Pulse field gel electrophoresis

Twenty-one isolates were subjected to pulsed field gel electrophoresis (PFGE) based on their antibiograms. PFGE was performed according to the protocol described by the Center for Disease Control and Prevention (CDC, 2011) with minor modifications. Colonies from

overnight cultures, grown on nutrient agar at 37°C, were suspended into TE buffer and adjusted to an OD reading of 0.9-1.1 at a wavelength of 610 nm. This was followed by the addition of 2µl of lysostaphin (Sigma, St Louis, MO, USA) and 200 µl of adjusted cell suspension into 200 µl of 1.8% low melting Seakem agarose (Thermo Scientific). The mixture was dispensed into a plug mould and left to solidify for 10-15 min at room temperature. After the plugs were removed from the moulds, they were placed into 500 ml of EC lysis buffer and 20 µl of Proteinase K (Thermo Scientific) and left to incubate overnight at 55 °C. The lysis buffer was discarded and the plugs were washed 4-5 times with TE buffer and then stored at 4 °C. The plugs were cut and restricted with *SmaI* (Thermo Scientific) for 3 h. After digestion, the plugs were loaded into the wells of a 1% agarose gel (SeaKem Gold). PFGE was carried out in the CHEF-DR III system (Bio-Rad Laboratories, Inc, CA, USA) according to the following conditions: 6 V, temperature 14 °C, initial switch time 5 s, final switch time 40 s, included angle 120, with a run time of 20 h. The gel was stained in Ethidium bromide for 30 min and destained in distilled water for 30-45 min. *Salmonella branderup* strain was used as a control and was digested using *XbaI* (Thermo Scientific). Bionumerics software v.53 (Applied Maths and Scientific Software Development, Saint-Martens-Latem, Belgium) was used to perform cluster analysis using the Dice coefficient.

4.3 Results

4.3.1 Confirmation of Methicillin Resistant *Staphylococcus aureus*

A number of isolates that grew as yellow colonies on Mannitol Salt Agar supplemented with Cefoxitin were recovered from the discharge point of the treated effluent as well as upstream and downstream points of receiving surface waters of two wastewater treatment plants in Durban, KwaZulu-Natal. Biochemical tests and PCR amplification of the *mecA* gene (Fig. 4.1) resulted in 80 confirmed Methicillin Resistant *Staphylococcus aureus* (MRSA) isolates.

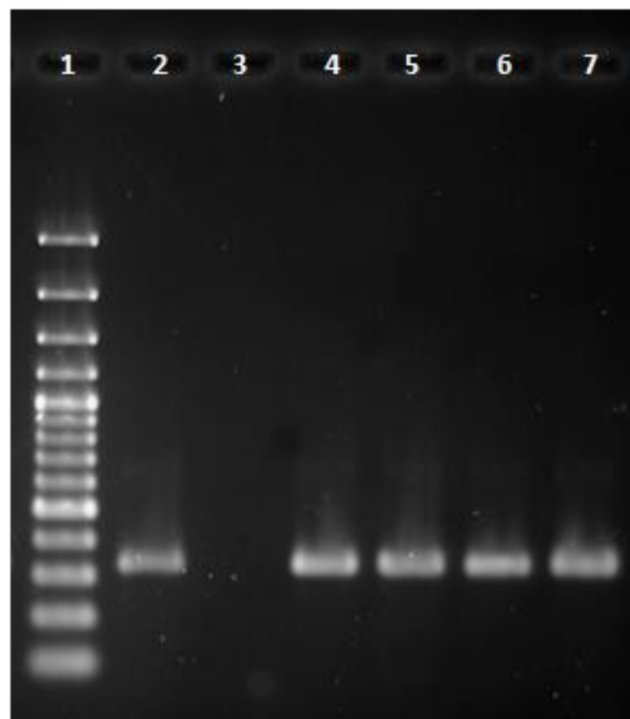

Fig. 4.1 Representative gel of amplified *mecA* gene in the MRSA isolates

Lane 1 contains the 100 bp marker, lane 2 contains the positive control (314 bp), lane 3 contains the negative control and lanes 4-8 contains environmental samples with amplification of *mecA* (314 bp).

4.3.2 Antibiogram analysis

4.3.2.1 Antibiotic resistance profiles of the MRSA isolates

The antibiotic resistance profiles of the MRSA isolates are shown in Table 4.3. The highest resistance was observed against lincomycin (100%), oxacillin (98.75%), followed by cefoxitin and penicillin (97.50%) and ampicillin (96.25%). Additionally, 72.50%, 66.25%, 52.50%, 40% and 33.75% showed resistance to cefozolin, azithromycin, amoxicillin/clavulanic acid, erythromycin and vancomycin, respectively. Lower resistance was observed against clindamycin and rifampicin (22.50%), sulfamethoxazole/trimethoprim (17.50%), tetracycline (11.25%), gentamicin, imipenem and chloramphenicol (5.00%) and amikacin (2.50%) respectively. None of the isolates was resistant to ciprofloxacin. Intermediate resistance was observed for clindamycin (53.75%), sulfamethoxazole/trimethoprim (21.25%), erythromycin and norfloxacin (15.00%), cefozolin (13.75%), chloramphenicol (12.5%) and tetracycline (11.25%). The isolates were susceptible to amikacin (95%), gentamicin (93.75%), ciprofloxacin (91.25%), imipenem (90.00%), chloramphenicol (82.50%), tetracycline (77.50%), norfloxacin and rifampicin (75%), vancomycin (66.25%) and sulfamethoxazole/trimethoprim (61.25%). All isolates showed resistance to three or more antibiotic classes and are therefore regarded as multi-drug resistant.

Table 4.3 Antibiotic resistance profile of MRSA isolates (n=80)

Antibiotic Class	Antibiotic (concentration)	Number of isolates (%)		
		Susceptible	Intermediate	Resistant
Aminoglycosides	Amikacin (30 µg)	76 (95.00)	2 (2.50)	2 (2.50)
	Gentamicin (10 µg)	75 (93.75)	1 (1.25)	4 (5.00)
β-lactam/β-lactamase inhibitor combination	Amoxicillin/clavulanic acid (30 µg)	38 (47.50)	0 (0.00)	42 (52.50)
β-lactam	Ampicillin (10 µg)	3 (3.75)	0 (0.00)	77 (96.25)
	Oxacillin (1 µg)	1 (1.25)	0 (0)	79 (98.75)
	Penicillin (10 U)	2 (2.50)	0 (0)	78 (97.50)
Carbapenem	Imipenem (30 µg)	72 (90.00)	4 (5.00)	4 (5.00)
Cephalosporin	Cefoxitin (30 µg)	2 (2.50)	0 (0)	78 (97.50)
	Cefozolin (30 µg)	11 (13.75)	11 (13.75)	58 (72.50)
Fluoroquinolones	Ciprofloxacin (5 µg)	73 (91.25)	7 (8.75)	0 (0)
	Norfloxacin (10 µg)	60 (75.00)	12 (15.00)	8 (10.00)
Glycopeptides	Vancomycin (30 µg)	53 (66.25)	0 (0)	27 (33.75)
Lincosamide	Clindamycin (2 µg)	19 (23.75)	43 (53.75)	18 (22.50)
	Lincomycin (2 µg)	0 (0)	0 (0)	80 (100)

Table 4.3 continued…

Antibiotic Class	Antibiotic (concentration)	Number of isolates (%)		
		Susceptible	**Intermediate**	**Resistant**
Macrolide	Azithromycin (15 µg)	21 (26.25)	6 (7.50)	53 (66.25)
	Erythromycin (15µg)	36 (45.00)	12 (15.00)	32 (40.00)
Phenicols	Chloramphenicol (30 µg)	66 (82.50)	10 (12.50)	4 (5.00)
Rifamycin B derivative	Rifampicin (5 µg)	60 (75.00)	2 (2.5)	18 (22.5)
Sulfonamide/methoprim	Sulfamethoxazole/trimethoprim (1.25+23.75µg)	49 (61.25)	17 (21.25)	14 (17.50)
Tetracycline	Tetracycline (30 µg)	62 (77.50)	9 (11.25)	9 (11.25)

4.3.2.2 Antibiotic resistance gene signatures of the MRSA isolates

Detection of antibiotic resistance genes: *aac(6’)/aph(2’’)*, *blaZ*, *ermC*, *msrA* and *tetK* via PCR revealed the presence of these genes in some of the isolates (Fig. 4.2). Of the 80 MRSA isolates tested, *aac(6')/aph(2")* was detected in 45 (56.25%) of the isolates, *blaZ* in 56 (70.00%), *ermC* in 50 (62.50%), *msrA* in 18 (22.50%) and *tetK* gene in 56 (70.00%) of the isolates.

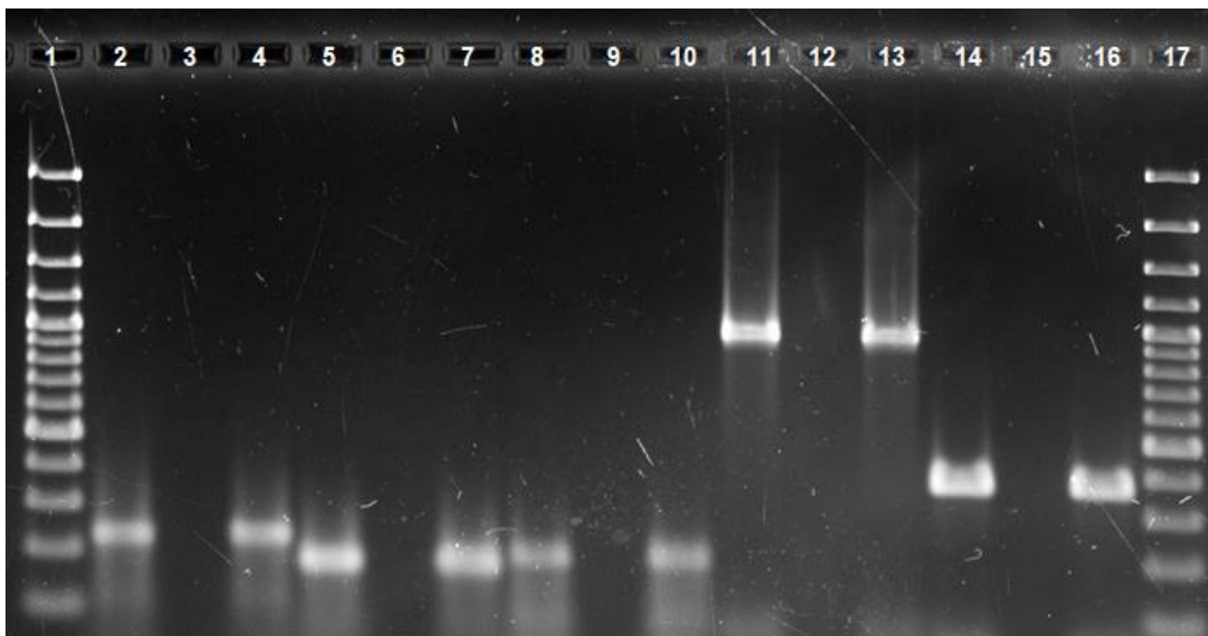

Fig. 4.2 Representative gel of amplified antibiotic resistance genes in the MRSA isolates

Lane 1 and 17 show the 100 bp marker. Lane 2 shows the positive control for *aac(6')/aph(2")* (227 bp), lane 3 shows the negative control and lane 4 shows *aac(6')/aph(2")* detected in the representative environmental isolate (227 bp). Lane 5 shows the positive control for *blaZ* (173 bp), lane 6 shows the negative control and lane 7 shows *blaZ* detected in the representative environmental isolate (173 bp). Lane 8 shows the positive control for *ermC* (190 bp), lane 9 shows the negative control and lane 10 shows *ermC* detected in the representative environmental isolate (190 bp). Lane 11 shows the positive control for *msrA* (940 bp), lane 12 shows the negative control and lane 13 shows *msrA* present in the representative environmental

isolate (940 bp). Lane 14 shows the positive control for *tetK* (360 bp), lane 15 shows the negative control and lane 16 shows *tetK* present in the representative environmental isolate (360 bp).

4.3.2.3 Genotypic and phenotypic characterization of antibiotic resistance amongst the MRSA isolates

The phenotypic and genotypic resistance patterns observed amongst the MRSA isolates are shown in Table 4.4. Fifty-one antibiotic resistance patterns were observed, of which 36 were represented by single isolates. Six of the MRSA isolates demonstrated the same phenotypic antibiotic resistance pattern against: amoxicillin/clavulanic acid, oxacillin, ampicillin, cefoxitin, penicillin and lincomycin with an antibiotic resistance index (ARI) of 0.30. Of these isolates, 3 harboured *aac(6')/aph('')*, 4 harboured *blaZ* and 5 harboured *ermC* and *tetK*. Five isolates have the same phenotypic antibiotic resistance pattern against: vancomycin, amoxicillin/clavulanic acid, oxacillin, ampicillin, cefoxitin, clindamycin, erythromycin, penicillin, rifampicin, cefozolin, lincomycin and azithromycin with an ARI of 0.60. Of the 4 isolates, *aac(6')/aph(2'')* and *tetK* were detected in 1, and *blaZ* and *msrA* in 2 of the isolates. Four isolates showed antibiotic resistance pattern against oxacillin, ampicillin, cefoxitin, penicillin and lincomycin with ARI of 0.25. Of these 4 isolates, *aac(6')/aph(2'')* and *blaZ* were detected in 3, and *ermC* and *tetK* in 4 isolates. Four isolates showed the same antibiotic resistance pattern against: oxacillin ampicillin, cefoxitin, penicillin, cefozolin, norfloxacin, lincomycin and azithromycin (ARI: 0.40). Three of the isolates harboured the *aac(6')/aph(2'')* and *ermC* genes, while *blaZ* and *tetK* were detected in all 4.

Table 4.4 Distribution of antibiotic resistance phenotype and genotype and multiple antibiotic resistance index amongst isolates (n=80)

Phenotype	No. of isolates	Resistance profiles	Antibiotic resistance index	Antibiotic resistance genes				
				aac(6')/ aph(2'')	*blaZ*	*ermC*	*msrA*	*tetK*
A1	1	VA AMC OX AMP FOX P KZ MY	0.40	1	1	0	1	0
A2	1	VA AMC OX AMP FOX P KZ MY AZM	0.45	0	1	1	1	1
A3	2	VA AMC OX AMP FOX E P KZ MY AZM	0.50	1	1	1	1	0
A4	2	VA AMC OX AMP FOX E P SXT KZ MY AZM	0.55	1	1	2	0	0
A5	1	VA AMC OX AMP FOX DA E P SXT RD KZ MY	0.60	0	1	0	1	0
A6	1	VA AMC OX AMP FOX DA E P RD IPM KZ MY AZM	0.65	0	0	0	0	1
A7	1	VA AMC OX AMP FOX DA E P RD KZ MY	0.55	0	1	1	0	0
A8	5	VA AMC OX AMP FOX DA E P RD KZ MY AZM	0.60	1	2	0	2	1
A9	1	VA AMC OX TE AMP FOX E P KZ MY AZM	0.55	1	0	0	1	0
A10	3	VA AMC OX TE AMP FOX DA E P RD KZ MY AZM	0.65	0	2	0	1	1
A11	1	VA AMC C OX AMP FOX DA E P SXT RD IMP KZ MY AZM	0.75	0	0	0	0	1
B1	1	VA OX AMP FOX P RD KZ MY AZM	0.45	1	1	0	1	1
B2	2	VA OX AMP FOX P KZ MY	0.35	1	2	2	0	2
B3	1	VA OX AMP FOX DA E P RD IPM KZ MY AZM	0.60	0	1	0	1	1
B4	1	VA OX AMP FOX DA P RD MY AZM	0.45	0	0	0	1	1
B5	1	VA OX AMP FOX E P KZ MY AZM	0.45	1	1	0	1	0
B6	1	VA OX TE AMP FOX E P MY AZM	0.45	1	0	0	1	1

Key: AK- Amikacin; AMC- Amoxicillin/clavulanic acid; AMP- Ampicillin; AZM- Azithromycin; C- Chloramphenicol; CN- Gentamicin; DA- Clindamycin; E- Erythromycin; FOX- Cefoxitin; IPM- Imipenem; KZ-Cefozolin; MY- Lincomycin; NOR- Norfloxacin; OX- Oxacillin; P- Penicillin; RD- Rifampicin; SXT- Sulfamethoxazole/trimethoprim; TE- Tetracycline; VA- Vancomycin

Table 4.4 continued…

Phenotype	No. of isolates	Resistance profiles	Antibiotic resistance index	Antibiotic resistance genes				
				aac(6')/ aph(2'')	*blaZ*	*ermC*	*msrA*	*tetK*
B7	1	VA OX CN FOX P RD KZ NOR MY	0.45	0	0	0	0	0
C1	1	C OX AMP FOX P KZ MY AZM	0.40	1	1	1	0	1
C2	1	C OX AMP FOX P MY AZM	0.35	1	1	1	0	1
D1	1	KZ MY AZM	0.15	1	1	0	0	1
E1	1	AMC OX AMP FOX P SXT KZ NOR MY AZM	0.50	1	1	0	0	1
E2	1	AMC OX AMP FOX P SXT KZ MY	0.40	1	1	1	0	1
E3	1	AMC OX AMP FOX P SXT MY AZM	0.40	1	1	1	0	1
E4	2	AMC OX AMP FOX P KZ MY	0.35	2	0	2	0	2
E5	3	AMC OX AMP FOX P KZ MY AZM	0.40	3	2	2	0	3
E6	3	AMC OX AMP FOX P MY AZM	0.35	2	1	1	0	2
E7	6	AMC OX AMP FOX P MY	0.30	3	4	5	0	5
E8	1	AMC OX AMP FOX E P KZ MY AZM	0.45	0	1	1	1	0
E9	1	AMC OX AMP FOX DA P SXT MY	0.40	1	1	1	0	1
E10	1	AMC OX TE AMP AK CN FOX DA P RD KZ NOR MY	0.65	0	0	0	0	0
E11	1	AMC OX TE AMP FOX DA E P KZ MY AZM	0.55	1	1	1	0	1
E12	1	AMC C OX AMP FOX P KZ MY AZM	0.45	1	1	1	0	1
F1	1	OX AMP FOX P MY AZM	0.30	0	0	1	0	1

Key: AK- Amikacin; AMC- Amoxicillin/clavulanic acid; AMP- Ampicillin; AZM- Azithromycin; C- Chloramphenicol; CN- Gentamicin; DA- Clindamycin; E- Erythromycin; FOX- Cefoxitin; IPM- Imipenem; KZ- Cefozolin; MY- Lincomycin; NOR- Norfloxacin; OX- Oxacillin; P- Penicillin; RD- Rifampicin; SXT- Sulfamethoxazole/trimethoprim; TE- Tetracycline; VA- Vancomycin

Table 4.4 continued…

Phenotype	No. of isolates	Resistance profiles	Antibiotic resistance index	Antibiotic resistance genes *aac(6')/ aph(2'')*	*blaZ*	*ermC*	*msrA*	*tetK*
F2	4	OX AMP FOX P MY	0.25	3	3	4	0	4
F3	2	OX AMP FOX P KZ MY	0.30	2	2	2	0	2
F4	2	OX AMP FOX P KZ MY AZM	0.35	2	1	2	0	2
F5	4	OX AMP FOX P KZ NOR MY AZM	0.40	3	4	3	0	4
F6	1	OX AMP FOX P NOR MY AZM	0.35	1	1	1	0	0
F7	1	OX AMP FOX P SXT KZ MY	0.35	1	1	1	0	1
F8	1	OX AMP FOX E P KZ MY	0.35	1	1	1	0	1
F9	2	OX AMP FOX E P KZ MY AZM	0.40	0	2	2	2	2
F10	1	OX AMP FOX E P MY AZM	0.35	0	1	1	0	0
F11	1	OX AMP FOX E P SXT KZ MY	0.40	0	1	1	0	0
F12	1	OX AMP FOX E P SXT KZ MY AZM	0.45	0	1	1	1	0
F13	1	OX AMP FOX DA P SXT KZ MY AZM	0.45	0	1	1	0	1
F14	1	OX AMP AK CN FOX E P KZ MY AZM	0.50	1	1	1	0	1
F15	1	OX AMP CN FOX E P MY AZM	0.40	1	1	1	1	1
F16	2	OX AMP E P SXT KZ MY	0.35	1	2	1	0	1
G1	1	OX FOX KZ MY AZM	0.25	1	1	1	0	1
H1	1	OX TE AMP FOX P KZ MY AZM	0.40	1	0	1	0	1

Key: AK- Amikacin; AMC- Amoxicillin/clavulanic acid; AMP- Ampicillin; AZM- Azithromycin; C- Chloramphenicol; CN- Gentamicin; DA- Clindamycin; E- Erythromycin; FOX- Cefoxitin; IPM- Imipenem; KZ-Cefozolin; MY- Lincomycin; NOR- Norfloxacin; OX- Oxacillin; P- Penicillin; RD- Rifampicin; SXT- Sulfamethoxazole/trimethoprim; TE- Tetracycline; VA- Vancomycin

4.3.3 Virulence gene signatures of the MRSA isolates

PCR amplification of virulence genes: *hla*, *hld*, *lukS/F PV* and *sea* revealed their presence in some of the isolates as shown in Fig. 4.3 Of the four virulence genes, *hla*, *hld*, *lukS/F PV* and *sea*, *hld* and *sea* were found to be most prevalent, as they were detected in 46 (57.50%) isolates. The *hla* gene was only detected in 1 (1.25%) isolate while *lukS/F PV* was not detected in any of the isolates.

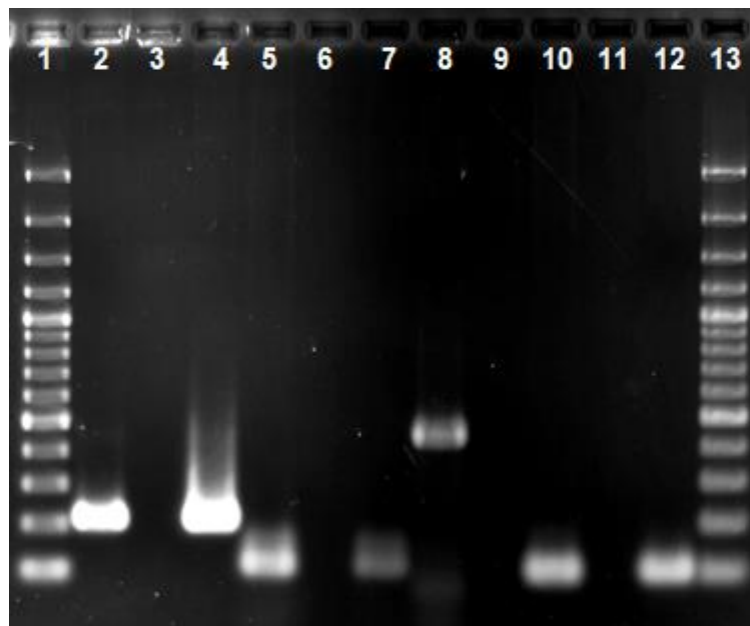

Fig. 4.3 Representative gel of amplified virulence genes

Lanes 1 and 13 show the 100 bp marker. Lane 2 shows the positive control for the *hla* gene (209 bp), lane 3 shows the negative control and lane 4 shows *hla* present in the representative environmental isolate (209 bp). Lane 5 shows the positive control for the *hld* (111 bp), lane 6 shows the negative control and lane 7 shows *hld* gene present in the representative environmental isolate (111 bp). Lane 8 shows the positive control for *lukS/F PV* (443 bp) and lane 9 shows the negative control. Lane 10 shows the positive control for *sea* (102 bp), lane 11 shows the negative control and lane 12 shows *sea* present in the representative environmental isolates (102 bp).

The co-existence of three virulence genes: *hla*, *hld* and *sea* genes was only noted in 1 isolate recovered from DS of WWTP1. This isolate was also found to harbour all the five antibiotic resistance genes tested: *aac(6')/aph(2'')*, *blaZ*, *ermC*, *msrA* and *tetK*

4.3.4 Genetic Fingerprinting of selected multi-drug resistant MRSA isolates

The pulse field gel electrophoresis (PFGE) profiles and dendrogram of 21 selected MRSA isolates are shown in Fig. 4.4. Of the 21 isolates, PFGE grouped the MRSA isolates into 13 pulsotypes designated as A-M with 80% similarity, correlating the isolates to their respective antibiograms. The largest cluster consisted of three isolates with the same pattern. Only pulsotypes J and L had the same patterns amongst the isolates, which also exhibited the same antibiogram profiles. The isolates were clustered into major pulsotypes: A (2/21; %), C (2/21; %), E (2/21; %), G (2/21; %), H (2/21; %), J (2/21; %) and L (3/21). Pulsotypes B, D, F, I, K and M were represented by single isolates.

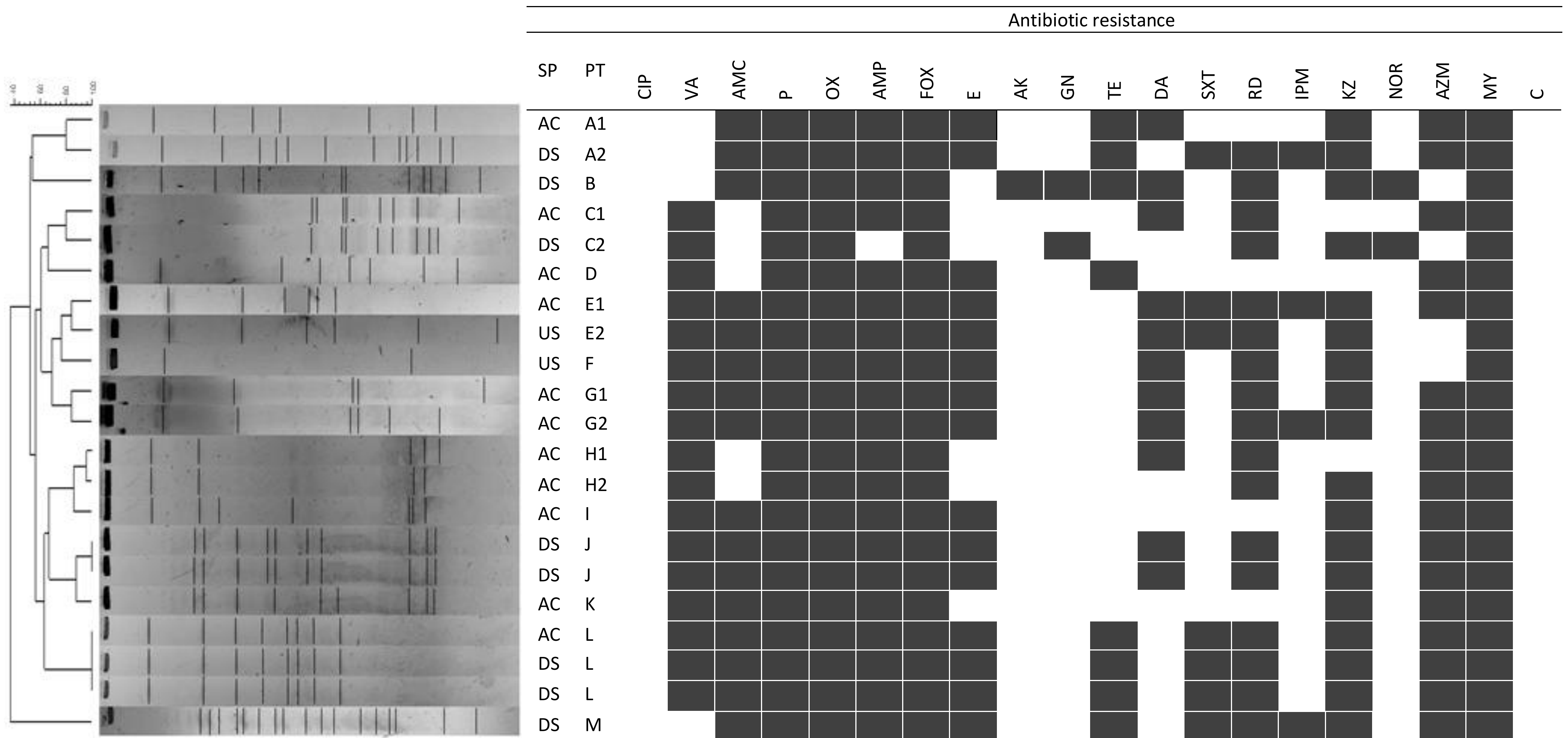

Fig. 4.4 PFGE-based dendrogram, antibiotic resistance profiles and source of selected MRSA isolates recovered from treated wastewater effluent and receiving surface waters

Key: SP- Sampling point; PT- Pulsotype; AC- After chlorination; DS- Downstream; US- Upstream; AK- Amikacin; AMC- Amoxicillin/clavulanic acid; AMP- Ampicillin; AZM- Azithromycin; C- Chloramphenicol; CN- Gentamicin; DA- Clindamycin; E- Erythromycin; FOX- Cefoxitin; IPM- Imipenem; KZ- Cefozolin; MY- Lincomycin; NOR- Norfloxacin; OX- Oxacillin; P- Penicillin; RD- Rifampicin; SXT- Sulfamethoxazole/trimethoprim; TE- Tetracycline; VA- Vancomycin; Black indicates resistance and white indicates intermediate or susceptible.

4.4 Discussion

Improperly treated wastewater may serve as a reservoir of bacteria, including Methicillin Resistant *Staphylococcus aureus* (MRSA), which may have negative consequences on public and environmental health (Bateganyaa *et al*., 2015). These MRSA may be potentially pathogenic causing infections in humans which are difficult to treat due to the emergence of antibiotic resistance (Goldstein *et al*., 2012). In MRSA, the *mecA* gene encodes for a penicillin binding protein (PBP2A/PBP2') resulting in resistance to *β*-lactam antibiotics (Marais *et al*., 2009; Naquin *et al.,* 2014). Through detection of the *mecA* gene by PCR, a total of 80 MRSA strains were confirmed from the treated effluent and receiving surface waters of both WWTPs in Durban. Previous study also confirmed the presence of MRSA in treated wastewater effluent (Goldstein *et al*., 2012).

Antibiotic resistance profiles of the MRSA isolated in this study were determined against 20 antibiotics that are commonly used to treat MRSA associated infections. As shown in Table 4.3, all isolates were resistant to lincomycin. This differed from studies by Dormanesh *et al*. (2015) which reported that only 6.75% (5/74) of tested clinical MRSA strains were resistant to lincomycin. The increased number of lincomycin resistant strains in this study supports the replacement of lincomycin by clindamycin to treat Gram-positive bacterial infections due to its improved pharmacological and therapeutic effects (Li *et al*., 2015). Resistance to oxacillin, ampicillin and penicillin was observed in 98.75%, 97.50% and 96.25% of the MRSA isolates, respectively. This is similar to the observations in previous studies in which all MRSA isolates isolated from poultry (n=30) tested were reported to exhibit resistance towards Oxacillin, Ampicillin and Penicillin (New *et al*., 2016). This is not surprising since these antibiotics are *β*-lactam antibiotics which the *mecA* gene confers resistance to. It was also observed that although all 80 presumptive MRSA isolates grew on Mannitol Salt Agar supplemented with cefoxitin, 78/80 (97.50%) isolates exhibited resistance to cefoxitin. This corroborates a study

by Thompson *et al.* (2013) which showed that cefoxitin resistance is used to help detect the presence of MRSA as it suggests methicillin resistance. Thompson *et al.* (2013) reported on antibiotics resistance of MRSA isolated from wastewater of two hospitals and its receiving sewage treatment plant. In hospital 1, 55%, 65% and 90% of isolates (n=35) were resistant to amikacin, chloramphenicol and gentamicin, respectively. In hospital 2, 60%, 65% and 75% of isolates (n=46) exhibited resistance to amikacin, chloramphenicol and gentamicin, respectively. The reduced number of isolates showed resistance to amikacin, chloramphenicol and gentamicin. This corroborates the finding in this study as a low number of isolates exhibited resistance against amikacin (2.50%), chloramphenicol (5%) and gentamicin (5.00%) by the MRSA isolates. No resistance was observed against ciprofloxacin and low resistance was observed against imipenem (5.00%). Findings by Dormanesh *et al.* (2015) also showed low resistance against ciprofloxacin (24.32%) and imipenem (2.70%) amongst the 70 MRSA isolates tested. High susceptibility to imipenem was expected as it is commonly used to treat human clinical MRSA infections (Dormanesh *et al.*, 2015). Antibiotic resistance was observed against clindamycin (22.50%), erythromycin (40%) and tetracycline (11.25%). Dehkordi *et al.* (2017) reported higher levels of resistance against clindamycin (48.64%), erythromycin (86.48%) and tetracycline (100%) in MRSA (n=37) isolated from hospital food samples. Notable resistance was observed in this study against vancomycin (33.75%) differing from Goldstein *et al.* (2012) which reported 0% resistance against vancomycin from MRSA (n=84) isolated from WWTPs. This is worrisome as vancomycin, a glycopeptide antibiotic, is used as a last resort treatment in severe MRSA infections (Ong *et al.*, 2017). This is of concern as penicillin, ampicillin, clindamycin, tetracycline and erythromycin are used to globally treat MRSA infections due to affordability and accessibility (Rong *et al.*, 2017). The observed low resistance to rifampicin (22.50%) in this study is expected as this antibiotic is used in combination with other antibiotics (such as fusidic acid) to treat MRSA infections (Tang *et al.*,

2013). Amoxicillin/clavulanic acid is a combination of β-lactam and β-lactamase inhibitor that prevents the staphylococcal strain from hydrolysing the β-lactam (Mhmoud *et al.*, 2014). This antibiotic is used to treat MRSA infections as it displays good activity against this strain (Mhmoud *et al.*, 2014). However, 42 (52.50%) MRSA strains in this study exhibited resistance to this antibiotic. This is of concern, as resistance to this antibiotic may lead to challenges in the treatment of MRSA associated infections.

The prevalence of antibiotic resistance genes (ARGs) amongst the MRSA isolates was determined in this study. The *blaZ* gene, detected in 56 (70.00%) of the isolates, confers resistance to *β*-lactam antibiotics (Zmanter *et al.*, 2013). It encodes for the *β*-lactamase, which is responsible for hydrolysing the *β*-lactam ring upon exposure to *β*-lactam antibiotics, leaving the antibiotic inactive (Zmanter *et al.*, 2013). The *tetK* gene, commonly found in Gram-positive bacteria, confers resistance to tetracycline (Zmantar *et al.,* 2013; Ong *et al.*, 2017) and encodes for efflux proteins which prevents the build-up of tetracycline inside the cell (Ullah *et al.*, 2012; Ong *et al.*, 2017). The high prevalence of *blaZ* and *tetK* observed in 70% of isolates in this study is similar to the findings of Dehkordi *et al.* (2017) which showed the presence of *tetK* in 72.97% (n=27) of MRSA isolated from hospital food in Iran. However, Mkhize *et al.* (2017) detected the presence of *blaZ* and *tetK* in only 10.3% and 24% of MRSA (n= 104) respectively, isolated from faecal samples from commercial broiler chickens. Clinical isolates of MRSA strains showed the presence of *tetK* in 63.6% (n=55) (Adwan *et al.*, 2014) and *blaZ* in 100% (n= 39) of MRSA isolated from the nasal cavity of paediatric service (Zmanter *et al.*, 2013). The *aac(6')/aph(2'')* gene, which encodes for resistance against aminoglycosides, was found in 56.25% of isolates. Aminoglycosides are also used to treat MRSA infections as they exhibit anti-staphylococcal activity and inhibit protein synthesis by binding to the 30S ribosomal unit (Gade and Qazi, 2014). The combination of Gentamicin and Tobramycin, belonging to the aminoglycoside antibiotic class, are most effective in treating Staphylococcal infections (Gade

and Qazi, 2014). The *aac(6')/aph(2'')* gene encodes for aminoglycoside modifying enzymes which induces drug inactivation (Gade and Qazi, 2014). A study by Duran *et al.* (2012) revealed the presence of *aac(6')/aph(2'')* in 20.9% (n= 139) of coagulase positive *S. aureus* from a clinical environment. The result in this study differed from that of Gomes *et al.* (2015) which revealed a slightly lower prevalence of the *aac(6')/aph(2'')* gene (44.0%) in 56 MRSA strains isolated from bloodstream infections. Erythromycin belongs to the antibiotic class macrolides, which are used to treat MRSA infections exhibiting effective pharmacokinetic properties by inhibiting protein synthesis (Prabhu *et al.*, 2011). However, due to the increased exposure to macrolides, MRSA strains are acquiring resistance (Prabhu *et al.*, 2011). The *ermC* confers resistance to macrolides by modifying the antibiotic target site in the MRSA strain (Prabhu *et al.*, 2011). This study showed the presence of the *ermC* gene in 62.5% of isolates, whereas it was only detected in 31% of the 41 MRSA isolates tested by Zarfel *et al.* (2013). The *msrA* gene also confers resistance to macrolides and streptogramin B antibiotics by encoding for efflux mechanisms that pumps these antibiotics from the cells (Zmanter *et al.*, 2013; Li *et al.*, 2015). *msrA* was detected in 22.50% of MRSA isolates in this study, whereas Zarfel *et al.* (2013) did not detect this gene in any of the 41 MRSA tested. Adwan *et al.* (2014) showed the presence of *ermC* in only 54.5% (n=55) of clinically isolated MRSA strains. However, in MRSA strains isolated from the nasal cavities of paediatric service, lower levels of *ermC* (22.0%) but higher levels of *msrA* (36.0%) was detected in 39 MRSA isolates (Zmanter *et al.*, 2013).

As indicated in Table 4.4 a total of 51 antibiotic resistant phenotypes were generated, with 6/80 isolates found to be resistant to a combination of the following antibiotics: Amoxicillin/clavulanic acid, Oxacillin, Ampicillin, Cefoxitin, Penicillin and Lincomycin. All isolates in this study exhibited resistance to three or more different antibiotic classes and were classified as multidrug resistant strains as defined by Falagas e*t al.* (2006). Similarly, Goldstein

et al. (2012) reported that 93% (78/84) MRSA isolates from WWTPs were multi-drug resistant. This could serve as a potential threat as end-users of the treated wastewater effluent and water from receiving rivers may be at risk of contracting MRSA infections that are multidrug resistant therefore posing challenges to treatment using conventional antibiotics. Multi-drug resistant MRSA infections may be challenging to treat in children, elders and immunocompromised patients (Akanbi *et al*., 2017). The multi-drug resistant MRSA in poorly treated wastewater and effluents may also leach into seawater through the receiving surface waters (Akanbi *et al*., 2017). This may be fatal to those who engage in recreational activities at the beach especially those with skin lesions (Akanbi *et al*., 2017).

The resistance phenotype observed did not correlate with the presence of ARGs as shown in Table 4.4. It was observed that the presence of *aac(6')/aph(2")* was present in 56.25% (45/80) but four showed phenotypic resistance to an aminoglycoside antibiotic, *ermC* was present in 62.5% (50/80) but 32 showed phenotypic resistant to erythromycin and *tetK* was present in 70% (56/80) but only nine showed resistance to tetracycline. These results indicate that the presence of resistance genes (*aac(6')/aph(2")*, *ermC* and *tetK* did not translate into phenotypic resistance. A possible explanation could be that these ARGs are silent and therefore do not confer resistance to the host strain (Vaz-Moreira *et al*., 2014). This was similar to a study by Amoako *et al.* (2016) which revealed the presence of *aac(6')/aph(2")* and *ermC* genes in MRSA isolates but did not confer phenotypic resistance to aminoglycosides and erythromycin, respectively. However, silent ARGs may be potentially dangerous as they can be transferred from one strain to another via horizontal gene transfer or may be potentially expressed when exposed to an increased concentration of the antibiotics (Zhu *et al*., 2013).

One the other hand, *blaZ* was detected in 70% (56/80) but 98.75% (79/80) exhibited phenotypic resistance towards a *β*-lactam antibiotic and *msrA* was present in 22.50% (18/80) but 53 showed resistance to a tested macrolide antibiotic. Higher number of isolates were phenotypically

resistant towards *β*-lactam and macrolide antibiotics even though the prevalence of the *blaZ* and *msrA* gene was low. This may be due to the presence of the *mecA* gene which generally confers resistance to *β*-lactam antibiotics. Other possible reasons for resistance towards the *β*-lactam and macrolide classes may be the presence of specific ARGs that were not screened for in this study (Shaikh *et al*., 2015).

Investigation of the virulence gene signatures of the MRSA isolates in this study indicated that none of the isolates harboured the *lukS/F PV* gene. This gene encodes for Panton-Valentine leukocidin (PVL), a leukotoxin that result in necrotizing pneumonia which destroys lung tissue (Monecke *et al*., 2014). This is contradictory to the study of Goldstein *et al*. (2012) where *lukS/F PV* gene was detected in 68% (n=236) of MRSA strains isolated from a WWTP in the USA. This PVL toxin is used as a molecular marker to detect the presence of CA-MRSA (Velasco *et al*., 2015). Kane *et al*. (2018) reported that majority of HA-MRSA lack this gene as compared to CA-MRSA. This was confirmed in a study that compared the presence of virulence genes in both CA-MRSA (present in 92% of isolates) and HA-MRSA (absent in all isolates) in Colombia (Portillo *et al*., 2013). This suggests that MRSA present in the wastewater of these two plants could be HA-MRSA. MRSA may be present in blood, body fluids and secretions from skin and soft tissue infections which may be disposed without proper treatment in the hospital waste which eventually ends up in the WWTPs and can be spread into the environment (Thompson *et al*., 2013). The *hla* and *hld* genes encode for alpha-haemolysin and delta-haemolysin respectively, which result in the lysis of red blood cells (Burnside *et al*., 2010). These haemolysins can be responsible for tissue injury, blood infections, renal parenchyma infections which is caused by blood carrying the bacteria (Doughari et al., 2011) and may cause the bacteria to scavenge iron compounds present in blood (Monecke *et al*., 2014). It was observed that 1/80 (1.25%) contained the *hla* gene while 46/80 (57.50%) contained the *hld* gene. These results differed from that of Alfatemi *et* al. (2014) which showed

the presence of *hla* and *hld* in 100% and 84.24% (n=146) of clinical MRSA strains. Contrarily, Xie *et* al. (2016) also detected the presence of *hla* and *hld* in 100% and 0% (n=62) of HA-MRSA and CA-MRSA. The *hla* gene is produced at high levels in CA-MRSA and has been observed to increase the strains pathogenicity (Day *et al*., 2012). Tavares *et al*. (2014) showed that the expression of *hla* in CA-MRSA is higher than in HA-MRSA. This supports the suggestion that the MRSA isolates detected in these WWTPs originated from hospitals due to improper treated hospital wastewater. A possible suggestion for the high prevalence of the *hld* gene is because it encodes for haemolysin that are associated with blood (Burnside *et al*., 2010). This suggests that these MRSA strains are from effluents from hospitals or slaughter houses that enters these WWTPs. (Thompson *et al*., 2013; Abidatul *et al*., 2018). The *sea* gene encodes for staphylococcal enterotoxin A which is responsible for food-borne disease, which may result in nausea, vomiting and diarrhoea (Kadariya *et al*., 2014). The *sea* gene showed to be present in 46/80 (57.50%) isolates which is higher than that of Alfatemi e*t al*. (2014) where *sea* gene was detected in 27.39% of clinical MRSA isolates. However, results from this study are similar to findings of Xie *et al*. (2016) which detected *sea* in 59.7% (n=62) of HA-MRSA and CA-MRSA. In the United States, the staphylococcal enterotoxin A was detected in 77.8% of *S. aureus* strains that were responsible for food-borne diseases (Kadariya *et al*., 2014). MRSA screened positive for staphylococcal enterotoxin A, isolated from stool samples of people affected with gastrointestinal illness (Kadariya *et al*., 2014). In this study, the high prevalence of *sea* gene could be due to the consumption of food contaminated with MRSA, resulting in these MRSA strains finding their way into the WWTPs through faecal wastes. This may include the water used to wash the raw material for food processing or the waste from animal slaughter. One of the isolates had the *hla*, *hld* and *sea* genes and also showed the presence of the antibiotic resistance genes: *mecA, aac(6')/aph(2")*, *blaZ*, *ermC*, *msrA* and *tetK*. This isolate showed phenotypic resistance to the following antibiotics: Oxacillin, Penicillin, Ampicillin,

Gentamicin, Erythromycin, Cefoxitin, Lincomycin and Azithromycin. This can be regarded as the most dangerous strain collected in this study.

The PFGE profiles and dendrograms of 21 selected isolates as shown in Fig. 4.4 revealed the presence of thirteen pulsotypes. This was similar to the 12 pulsotypes obtained for 22 MRSA isolates recovered from wastewater (Goldstein *et al.*, 2012). Two isolates have pulsotype J and three isolates showed to have pulsotype M. These isolates also exhibited identical antibiotic resistant phenotypes which portrays their genetic relatedness. Also, isolates that belonged to the same pulsotype exhibited similar antibiotic resistance patterns. Similar findings were reported by Moghadam *et al*. (2017) based on investigation of the genetic relatedness of MRSA strains isolated from burn patients using PFGE. Pulsotypes G, H and J showed isolates were from the same sampling point suggesting that these points are the origin of these MRSA strains. However, isolates belonging to the same pulsotypes were not necessarily isolated from the same sampling point of the WWTP (as observed in pulsotypes A, C and L) as these strains were found in the treated effluent and downstream points of the river. This suggests that the treated effluent discharge may be responsible for MRSA strains in the receiving rivers and this may pose challenges to environmental and human health (Naidoo and Olaniran, 2013). Pulsotype E was found in the treated effluent and upstream points of the river, suggesting that the treated effluent is not the only source for the MRSA isolates as previously indicated (Sabri *et al*., 2018).

4.5 Conclusion

This study showed that treated effluent from WWTPs located in Durban, KwaZulu-Natal, can potentially serve as a source of genetically diverse, multi-drug resistant and virulent strains of MRSA in the receiving surface waters. This is in agreement with previous study which showed the occurrence of MRSA in treated wastewater effluent and receiving rivers (Goldstein *et al*., 2012). These findings suggest that better wastewater treatment options should be explored to prevent the spread of multi-drug resistant bacteria in the environment and MRSA infections to end-users.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

References

Abidatul AA, Nur Farhanah NMJ, Noramirah R, Ling S, New CY, Son R (2018) Prevalence and classification of high antimicrobial resistant *Staphylococcus aureus* in wastewater eluted from poultry slaughterhouse. *Food Research*. **2**(2): 201-207

Adwan G, Adwan K, Jarrar N, Amleh A (2014) Molecular detection of nine antibiotic resistance genes in methicillin resistant *Staphylococcus aureus* isolates. *Romanian Archives of Microbiology and Immunology*. **73**(1-2): 9-18.

Akanbi OE, Njom HA, Fri J, Otigbu AC, Clarke AM (2017) Antimicrobial Susceptibility of *Staphylococcus aureus* isolated from recreational waters and beach sand in Eastern Cape Province of South Africa. *International journal of environmental research and public health*. **14**(9): 1001.

Alfatemi SMH, Motamedifar M, Hadi N, Saraie HSE (2014) Analysis of virulence genes among methicillin resistant *Staphylococcus aureus* (MRSA) strains, *Jundishapur Journal of Microbiology*. **7**: e10741.

Amoako DG, Bester LA, Somboro AM, Baijnath S, Govind CN, Essack SY (2016) Plasmid-mediated resistance and virulence mechanisms in the private health sector in KwaZulu-Natal, South Africa: An investigation of methicillin resistant *Staphylococcus aureus* (MRSA) clinical isolates collected during a three-month period. *International Journal of Infectious Diseases*. **46**: 38-41.

Bai J, Shi X, Nagaraja TG (2010) A multiplex PCR procedure for the detection of six major virulence genes in *Escherichia coli* O157:H7. *Journal of Microbiological Methods*. **82**: 85-89.

Bateganyaa NL, Nakalanzic D, Babud M, Heinab T (2015) Buffering municipal wastewater pollution using urban wetlands in sub-Saharan Africa: a case of Masaka municipality, Uganda. *Environmental Technology*. **36**: 2149-2160.

Börjesson S, Melin S, Matussek A, Lindgren PE (2009) A seasonal study of the mecA gene and *Staphylococcus aureus* including methicillin-resistant *S. aureus* in a municipal wastewater treatment plant. *Water research*.

43(4): 925-932.

Burnside K, Lembo A, de los Reyes M, Iliuk A, BinhTran NT, Connelly JE, Lin WJ, Schmidt BZ, Richardson AR, Fang FC, Tao WA, Rajagopal L (2010) Regulation of hemolysin expression and virulence of *Staphylococcus aureus* by a serine/threonine kinase and phosphatase. *PLoS ONE*. **5**: e11071.

CDC (Centers for Disease Control and Prevention) (2011) Oxacillin resistant *Staphylococcus aureus* on PulseNet (OPN): Laboratory Protocol for Molecular Typing of *S. aureus* by Pulsedfield Gel Electrophoresis (PFGE).

Clinical and Laboratory Standards Institute (2014) Performance standards for antimicrobial susceptibility testing. 24th Informational Supplement. (M100-S24). Wayne, Pa, USA: Clinical and Laboratory Standards Institute.

Day SR, Moore CM, Kundzins JR, Sifri CD (2012) Community-associated and healthcare-associated methicillin-resistant *Staphylococcus aureus* virulence toward Caenorhabditis elegans compared. *Virulence*. **3**(7): 576-582.

Dehkordi FS, Gandomi H, Basti AA, Misaghi A, Rahimi E (2017) Phenotypic and genotypic characterization of antibiotic resistance of methicillin-resistant *Staphylococcus aureus* isolated from hospital food. *Antimicrobial Resistance & Infection Control*. **6**(1): 104.

Dormanesh B, Siroosbakhat S, Khodaverdi Darian E, Afsharkhas L (2015) Methicillin-Resistant *Staphylococcus aureus* isolated from various types of hospital infections in pediatrics: Panton-Valentine Leukocidin, staphylococcal chromosomal cassette *mec SCCmec* phenotypes and antibiotic resistance properties. *Jundishapur Journal of Microbiology*. **8**: e11341.

Doughari HJ, Ndakidemi PA, Human IS, Benade S (2011) Virulence, Resistance Genes, and Transformation Amongst Environmental Isolates of *Escherichia coli* and *Acinetobacter spp*. *Journal of Microbiology and Technology*. **22**: 25–33.

Duran N, Ozer B, Duran GG, Onlen Y, Demir C (2012) Antibiotic resistance genes & susceptibility patterns in staphylococci. *Indian Journal of Medical Research*. **135**: 389-396.

Falagas ME, Koletsi PK, Bliziotis IA (2006) The diversity of definitions of multidrug-resistant (MDR) and pandrug-resistant (PDR) *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. *Journal of Medical Microbiology*. **55**: 1619–1629.

Gade ND, Qazi MS (2014) Recent trend of aminoglycoside resistance among *Staphylococcus aureus* isolates in tertiary care hospital. *Journal of Microbiology and Antimicrobials*. **6**: 94-96.

Goldstein RER, Micallef SA, Gibbs SG, Davis JA, He X, George A, Kleinfelter LM, Schreiber NA, Mukherjee S, Sapkota A, Joseph SW, Sapkota AR (2012) Methicillin-Resistant *Staphylococcus aureus* (MRSA) detected at four U.S. wastewater treatment plants. *Environmental Health Perspectives*. **120**: 1551-1558.

Gomes RMF, Bomfim MRQ, Trindade MJV, Farias LM, Carvalho MAR, Serufo JC, Santos SG (2015) Potential spread of methicillin-resistant *Staphylococcus aureus* recovered from patients with bloodstream infection. *Chemotherapy Open Access*. **4**: 1-8.

Kadariya J, Smith TC, Thapaliya D (2014) *Staphylococcus aureus* and staphylococcal food-borne disease: an ongoing challenge in public health. *BioMed Research International*. **2014**: 1-9.

Kane TL, Carothers KE, Lee SW (2018) Virulence Factor Targeting of the Bacterial Pathogen *Staphylococcus aureus* for Vaccine and Therapeutics. *Current drug targets*. **19**(2): 111-127.

Kuntová L, Pantůček R, Rájová J, Růžičková V, Petráš P, Mašlaňová, I, Doškař J (2012) Characteristics and distribution of plasmids in a clonally diverse set of methicillin-resistant *Staphylococcus aureus* strains. *Archives of microbiology*. **194**: 607-614.

Li L, Feng W, Zhang Z, Xue H, Zhao X (2015) Macrolide-lincosamide-streptogramin resistance phenotypes and genotypes of coagulase-positive *Staphylococcus aureus* and coagulase-negative staphylococcal isolates from bovine mastitis. *BMC veterinary research*. **11**(1): 168.

Marais E, Aithma N, Perovic O, Oosthuysen WF, Musenge E, Dusé AG (2009) Antimicrobial susceptibility of methicillin-resistant *Staphylococcus aureus* isolates from South Africa. *South African Medical Journal*. **99**: 170-173.

Mkize N, Zishiri OT, Mukaratirwa S (2017) Genetic characterisation of antimicrobial resistance and virulence genes in *Staphylococcus aureus* isolated from commercial broiler chickens in the Durban metropolitan area, South Africa. *Journal of the South African Veterinary Association*. **88**(1): 1-7.

Mhmoud NA, Fahal AH, Mahgoub ES, van de Sande WWJ (2014) The combination of amoxicillin-clavulanic acid and ketoconazole in the treatment of *Madurella mycetomatis* eumycetoma and *Staphylococcus aureus* co-infection. *PLoS Neglected Tropical Diseases*. **8**: e2959.

Moghadam SO, Pourmand MR, Douraghi M, Sabzi S, Ghaffari P (2017) Utilization of PFGE as a Powerful Discriminative Tool for the Investigation of Genetic Diversity among MRSA Strains. *Iranian journal of public health*. **46**(3): 351.

Monecke S, ller EM, Buchler J, Stieber B, Ehricht R (2014) *Staphylococcus aureus* in vitro secretion of alpha toxin (hla) correlates with the affiliation to clonal complexes. *PLoS ONE*. **9**: e100427.

Naidoo S, Olaniran AO (2013) Treated wastewater effluent as a source of microbial pollution of surface water resources. *International Journal of Environmental Research and Public Health*. **11**: 249-270.

Naquin A, Clement J, Sauce M, Grabert R, Sherpa M, Boopathy R (2014) Presence of antibiotic resistant *Staphylococcus aureus* in sewage treatment plant. *Journal of Water Sustainability.* **4**:227-236.

New CY, Amalia AR, Ramzi OSB, Son R (2016) Antibiotic resistance evolution of Methicillin Resistant *Staphylococcus aureus* (MRSA) and colloidal silver as the nanoweapon. *International Food Research Journal*. **23**: 1248-1254.

Ong MHL, Ho WY, Ng WW, Chew CH (2017) High Prevalence of tetM as Compared to tetK Amongst Methicillin-Resistant *Staphylococcus aureus* (MRSA) Isolates from Hospitals in Perak, Malaysia. *Jundishapur journal of microbiology*. **10**(6).

Portillo BC, Moreno JE, Yomayusa N, Álvarez CA, Cardozo BEC, Pérez JAE, Díaz PL, Ibañez M, Mendez-Alvarez S, Leal AL, Gómez NV (2013) Molecular epidemiology and characterization of virulence genes of community-acquired and hospital-acquired methicillin-resistant *Staphylococcus aureus* isolates in Colombia. *International journal of infectious diseases*. **17**(9): e744-e749.

Prabhu K, Rao S, Rao V (2011) Inducible clindamycin resistance in *staphylococcus aureus* isolated from clinical samples. *Journal of Laboratory Physicians*. **3**: 25–27.

Rong D, Wu Q, Xu M, Zhang J, Yu S (2017) Prevalence, virulence genes, antimicrobial susceptibility, and genetic diversity of *Staphylococcus aureus* from retail aquatic products in China. *Frontiers in microbiology*. **8**: 714.

Sabri NA, Schmitt H, Van der Zaan B, Gerritsen HW, Zuidema T, Rijnaarts HHM, Langenhoff AAM (2018) Prevalence of antibiotics and antibiotic resistance genes in a wastewater effluent-receiving river in the Netherlands. *Journal of Environmental Chemical Engineering*.

Shaikh S, Fatima J, Shakil S, Rizvi SMD, Kamal MA (2015) Antibiotic resistance and extended spectrum beta-lactamases: types, epidemiology and treatment. *Saudi Journal of Biological Sciences*. **22**: 90-101.

Tang HJ, Chen CC, Cheng KC, Wu KY, Lin YC, Zhang CC, Weng TC, Yu WL, Chiu YH, Toh HS, Chiang SR, Su BA, Ko WC, Chuang YC (2013) In-vitro efficacies and resistance profiles of rifampin-based combination regimens for biofilm-embedded methicillin-resistant *Staphylococcus aureus*. *Antimicrobial agents and chemotherapy*. **(2013)**: AAC-01236.

Tavares A, Nielsen JB, Boye K, Rohde S, Paulo AC, Westh H, Schønning K, de Lencastre H, Miragaia M (2014) Insights into alpha-hemolysin (Hla) evolution and expression among *Staphylococcus aureus* clones with hospital and community origin. *PloS one*. **9**(7): e98634.

Thapaliya D, Hellwig EJ, Kadariya J, Grenier D, Jefferson AJ, Dalman M, Kennedy K, DiPerna M, Orihill A, Taha M (2017) Prevalence and characterization of *Staphylococcus aureus* and Methicillin-Resistant *Staphylococcus aureus* on public recreational beaches in Northeast Ohio. *GeoHealth,* **1**(10): 320-332.

Thompson JM, Gündoğdu A, Stratton HM, Katouli HM (2013) Antibiotic resistant *Staphylococcus aureus* in hospital wastewaters and sewage treatment plants with special reference to methicillin-resistant *Staphylococcus aureus* (MRSA). *Journal of Applied Microbiology*. **114**: 44-5.

Ullah F, Malik SA, Ahmed J, Shah S, Ayaz M, Hussain S, Khatoon L (2012) Investigation of the genetic basis of tetracycline resistance in *Staphylococcus aureus* from Pakistan. *Tropical Journal of Pharmaceutical Research.* **11**(6): 925-931**.**

Vaz-Moreira I, Nunes OC, Manaia CM (2014) Bacterial diversity and antibiotic resistance in water habitats: searching the links with the human microbiome. *Federation of European Microbiological Sciences*. **38**: 761–18.

Velasco V, Buyukcangaz E, Sherwood JS, Stepan RM, Koslofsky RJ, Logue CM (2015) Characterization of *Staphylococcus aureus* from Humans and a Comparison with İsolates of Animal Origin, in North Dakota, United States. *PLoS ONE* **10**(10): e0140497.

Xie X, Bao Y, Ouyang N, Dai X, Pan K, Chen B, Deng Y, Wu X, Xu F, Li H, Huang S (2016) Molecular epidemiology and characteristic of virulence gene of community-acquired and hospital-acquired methicillin-resistant *Staphylococcus aureus* isolates in Sun Yat-sen Memorial hospital, Guangzhou, Southern China. *BMC Infectious Diseases*. **16**:339.

Zarfel G, Krziwanek K, Johler S, Hoenigl M, Leitner E, Kittinger C, Masoud L, Feierl G, Grisold AJ (2013) Virulence and antimicrobial resistance genes in human MRSA ST398 isolates in Austria. *Epidemiology & Infection*. **141**(4): 888-892.

Zhu YG, Johnson TA, Su JQ, Qiao M, Guo GX, Stedtfeld RD, Hashsham SA, Tiedje JM (2013) Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proceedings of the National Academy of Sciences*. **110**(9): 3435-3440.

Zmantar T, Bekir K, Elgarsadi SI, Hadad O, Bakhrouf A (2013) Molecular investigation of antibiotic resistance genes in methicillin resistant *Staphylococcus aureus* isolated from nasal cavity in pediatric service. *African Journal of Microbiology Research*. **7**: 4414-4421.

CHAPTER FIVE

5.1 General Discussion and Conclusion

Water is essential for the existence of life as it is required for drinking, preparation of food, sanitation, agricultural and industrial purposes. Unfortunately, access to low quality water for drinking and sanitation have led to the spread of many water-borne infections and diseases (Pandey *et al*., 2014; WHO and UNICEF, 2017). Influencing factors include: urbanization, over-population, improper infrastructure and improper wastewater treatment (Naidoo and Olaniran, 2013; Bateganyaa *et al*., 2015). Improperly treated wastewater that is released into receiving rivers may potentially carry contaminants such as chemical agents and microorganisms (Hunter *et al*., 2010). These microorganisms may potentially cause diseases in end-users of this water. Also, they may be resistant to antibiotics that are commonly used in the treatment of infections, posing serious treatment challenges.

The presence of methicillin-resistant *Staphylococcus aureus* (MRSA) detected in hospital and municipal wastewater suggest that wastewater treatment plants (WWTPs) may serve as a reservoir in the dissemination of MRSA into receiving surface waters and its surrounding environment (Börjesson *et al*., 2009). The presence of MRSA in treated effluent and its receiving surface waters may present a health risk to those who rely on this water for domestic and agricultural purposes (Goldstein *et al*., 2012). This is problematic as many strains of multi-drug resistant MRSA have been reported to induce diseases and infections which include: septicaemia, pneumonia and skin infections (Almagro-Moreno and Taylor, 2013; Goldstein *et al*., 2012). This poses negative consequences on those with weakened immune systems and promotes the dissemination of antibiotic resistant and virulent MRSA strains in receiving water bodies. To the best of our knowledge, no studies have reported on the characterization of antibiotic resistance and virulence gene attributes of MRSA isolates obtained from the treated effluent of WWTPs and receiving rivers in Durban, South Africa. This project aimed to determine the prevalence of MRSA, quantify the *mecA* gene and correlate it to the microbial

counts of MRSA in the influent, treated wastewater effluent and receiving rivers of two independent (WWTPs) in Durban, Kwa-Zulu Natal. The study also aimed isolate MRSA from the treated effluent and receiving rivers of both WWTPs and to characterize the antibiograms, virulence gene profiles and genetic fingerprints of MRSA isolates. This provided insight of the potential pathogenicity of these MRSA isolates, if they were multi-drug resistant and if they were genetically related.

The physico-chemical parameters of the wastewater samples and receiving surface waters were determined and results presented in chapter three. For most of the sampling periods, the temperature and pH for both WWTPs were within the acceptable limits (DWAF, 1984). Slightly warmer temperature observed at the downstream point of the river (for WWTP1) may be attributed to the industrial activities near-by which may have resulted in the discard of industrial waste into the rivers or use it for industrial cooling (Lokhande *et al.*, 2011). The results showed a decrease in total dissolved solids, electrical conductivity and turbidity from the influent to treated effluent of both WWTPs, with values obtained in the treated effluent falling within the acceptable limits (DWAF, 1996). Majority of the results for the chemical oxygen demand (COD) and biological oxygen demand (BOD) decreased from influent to treated effluent in both WWTPs. However, both COD and BOD of the treated effluent exceeded the acceptable limits across all sampling points. The observed high BOD and COD of the rivers suggest that both WWTPs are not the only contributors. High COD values in the receiving rivers may be attributed to toxic substances from the industrial area located near WWTP1. High BOD values may be attributed to leaves, woody debris, animal waste and dead animal or plant matter as rivers of WWTP1 and WWTP2 are situated in a park and nature reserve, respectively (Lokhande *et al.*, 2011). These findings suggest that water from the treated effluent and receiving rivers of both WWTPs should not be used for domestic purposes (DWAF, 1996).

The prevalence of MRSA was determined to assess if the treated effluent from the two WWTPs contribute to the dissemination and spread of MRSA into their respective receiving rivers. Removal efficiencies of 98.91- 99.17% and 90.71-99.96% was recorded for WWTP1 and WWTP2, respectively over the sampling periods. Results as presented in chapter three also revealed highest prevalence of MRSA in the primary or secondary stages of each WWTP for all 3 sampling periods. A decrease in the prevalence of MRSA in treated effluent was observed for both WWTPs for all sampling periods and this corroborates the findings of Goldstein *et al.* (2012). This decrease suggests the high efficiency of treatment at both WWTPs or that some strains may enter the viable but non-culturable state (Börjesson *et al.*, 2009). Lower levels of MRSA at the downstream points of the river of WWTP1 (as compared to influent and before chlorination) may be due to near-by located factories that discharge chemicals and heavy metals which may hinder growth of the MRSA. Higher prevalence of MRSA was detected in upstream points (as compared to downstream) for sampling periods 1 and 2 at both WWTPs. This suggests that other sources may also be responsible for the spread of MRSA into the receiving waters. This could include gastrointestinal bacteria present in animal waste being discharged into the river (Xiong *et al.*, 2015).

In addition to enumeration of presumptive MRSA through plate count, quantitative PCR was also used to quantify *mecA* (which encodes for methicillin resistance in *S. aureus*) in treated effluent and receiving surface waters of both WWTPs (Kim *et al.*, 2012; San Sit *et al.*, 2017). A slight decrease in the concentration of *mecA* was observed from the influent to the treated effluent for all three sampling periods in WWTP1 and for the first two sampling periods at WWTP2. The third sampling period (at WWTP2) showed an increase of *mecA*. The low reduction of *mecA* from the influent to the treated effluent show that both WWTPs may play a role in spreading the *mecA* to receiving surface waters (Börjesson *et al.*, 2009). The chlorination process at both WWTPs may cause cell disruption releasing DNA into the water. This is in

agreement with the findings of Quach-Cu *et al*. (2018) indicating a decrease in microbial population, despite the influx of antibiotic resistance genes present in treated effluent. Eighty MRSA strains were isolated from the treated effluent and receiving waters of both WWTPs in Durban and identified through PCR amplification of the *mecA* gene, which induces resistance to *β*-lactam antibiotics by encoding for a penicillin binding protein (Marais *et al*., 2009; Naquin *et al.,* 2014).

The antibiograms of the MRSA isolates showing high resistance towards oxacillin, ampicillin and penicillin is reported in chapter four. This finding was supported by another study where all MRSA isolates (n=30) tested exhibited resistance to these antibiotics (New *et al*., 2016). This may be justified as the presence of *mecA* confers resistance to this class of antibiotics. This study showed that 78/80 (97.50%) isolates exhibited resistance to cefoxitin whilst all 80 presumptive MRSA isolates grew on Mannitol Salt Agar that was supplemented with cefoxitin. This is expected because cefoxitin is used to help detect methicillin resistance in *S. aureus* (Thompson *et al*., 2013). No resistance was observed against ciprofloxacin and low resistance was observed against imipenem which was expected as these are used to treat MRSA infections in individuals. As expected, low resistance was observed against rifampicin as it is used in combination with other antibiotics to treat MRSA infections (Tang *et al*., 2013). However, low resistance was observed against amoxicillin/clavulanic acid (52.50%) and vancomycin (33.75%). This is not surprising as Amoxicillin/clavulanic acid has been reported to display good activity against MRSA and vancomycin is used as a resort treatment in severe MRSA infections (Mhmoud *et al*., 2014; Ong *et al*., 2017). These findings suggest that the continual development of resistance towards a broader category of antibiotic classes may pose a threat to the future treatment of MRSA associated infections. This study showed that all isolates were multi-drug resistant. The presence of multi-drug resistant MRSA in poorly treated wastewater is worrisome as the effluents may also leach into seawater through the receiving rivers and may

be fatal to those who engage in beach activities who may inhale the water or have skin lesions (Akanbi *et al.*, 2017). Treatment of these infections may be challenging in children, elders and immunocompromised patients (Akanbi *et al.*, 2017).

Chapter four also revealed the distribution of selected antibiotic resistance genes amongst isolates. This includes: *aac(6')/aph(2")* (56.25%), *blaZ* (70.0%), *ermC* (62.5%), *msrA* (22.50%) and *tetK* (70.00%) which encode for resistance against aminoglycosides, *β*-lactams, erythromycin, macrolides and streptogramin B and tetracyclines, respectively. By observing the antibiotic phenotypes, many of these antibiotic resistance genes seem to be silent as they did not induce resistance in the host strain as previously reported (Amoako *et al.*, 2016). These silent antibiotic resistance genes may be potentially dangerous as they could be transferred from one host to the next via horizontal gene transfer or upon exposure to a high concentration of antibiotics and may be potentially expressed (Zhu *et al.*, 2013).

The virulence gene profiles were also discussed in chapter four. It was observed that none of the isolates harboured the *lukS/F PV* gene which encodes for a leukotoxin that results in necrotizing pneumonia which destroys lung tissue (Monecke *et al.*, 2014). Higher levels of this gene were detected by Goldstein *et al.* (2012). The PVL toxin (encoded by *lukS/F PV*) is used as a molecular marker to detect the presence of community acquired MRSA (CA-MRSA) as studies have shown the presence of *lukS/F PV* in CA-MRSA but absent in hospital acquired - MRSA (HA-MRSA) strains (Portillo *et al.*, 2013; Velasco *et al.*, 2015). This suggests that MRSA present in both WWTPs may have originated from blood, body fluids and secretions from skin and soft tissue infections discarded in the hospital waste which eventually ends up in the WWTPs (Thompson *et al.*, 2013). Lower detection of *hla* in 1/80 (1.25%) and *hld* in 46/80 (57.50%) was observed in this study contrary to the findings of Alfatemi *et al.* (2014). The presence of the virulence genes in these MRSA is dangerous as these genes encode for haemolysins which are responsible for blood infections and renal parenchyma infections

(Doughari et al., 2011). Since haemolysins are associated with blood, it can be suggested that MRSA isolates detected in these WWTPs originated from hospital wastewater or slaughter houses. The *sea* gene was detected in 46/80 (57.50%) isolates which were similar to findings by Xie *et al*. (2016) which detected *sea* in 59.7% (n=62) of HA-MRSA and CA-MRSA. This gene encodes for the staphylococcal enterotoxin A which is responsible for food-borne diseases (Kadariya *et al*., 2014). MRSA isolated from stool samples of patients with gastrointestinal illness have screened positive for the staphylococcal enterotoxin (Kadariya *et al*., 2014). A possible explanation for the detection of the *sea* gene may be due to the consumption of food contaminated with MRSA amongst individuals resulting in the excretion of MRSA in faecal wastes. Other reasons include wastewater generated from food and agricultural industries that is contaminated with MRSA could be collected at these WWTPs.

Chapter four also revealed the PFGE profiles and dendrograms of 21 selected isolates in which 13 pulsotypes were generated. This was similar to a study that reported 12 pulsotypes of 22 MRSA isolates recovered from wastewater environment (Goldstein *et al*., 2012). It was observed that isolates that belonged to the same pulsotype (suggesting genetic relatedness), had similar antibiotic resistance patterns, which was supported by the findings of Moghadam *et al*. (2017). The results revealed that genetically related isolates were found in the treated effluent and downstream points of the river. This suggests that the treated effluent may be responsible for spread of MRSA strains downstream of the receiving rivers. This result shows that improperly treated wastewater may release MRSA into the receiving rivers bearing negative consequences on its surrounding environment (Naidoo and Olaniran, 2013). However, the same pulsotype was also found in the treated effluent and upstream points of the river, suggesting other sources of MRSA discharge into the rivers, including domestic wastewater and animal waste (Sabri *et al*., 2018).

This study revealed the inefficiency of the WWTPs for complete removal of MRSA and *mecA* in the influent received. This suggests that these WWTPs may serve as hotspot for the dissemination of antibiotic resistant bacteria and antibiotic resistance genes (ARGs) into the environment. The study also revealed the presence of multi-drug resistant and genetically diverse MRSA in treated effluent and receiving rivers. These bacteria have the ability to transfer ARGs via horizontal gene transfer and cause severe diseases in infected individuals. Constant monitoring of the treated wastewater quality is recommended to ensure that these WWTPs comply with the set standards. Better treatment options should also be explored to protect the aquatic ecosystem health and safety of the end-users of this water.

5.2 Potential for future development

Microbial source tracking should be considered to investigate the source of these MRSA pathogens in the receiving rivers of both WWTPs. It is important to assess the chemical and microbial materials, including the presence of human body fluids, that could potentially be present in influent, treat effluent and receiving rivers of both WWTPs. This will help improve the quality of water in both these rivers which will benefit human and environmental health (Rahmanian *et al*., 2015).

It is also important to evaluate the risk of multi-drug resistant MRSA infections from individuals exposed to reclaimed water and the receiving rivers. This could be assessed by increasing the number of virulence and antibiotic resistance genes (ARGs) characterized in the isolated MRSA strains (Goldstein *et al*., 2012). Further studies can help understand the mechanisms of virulence and antibiotic resistance expression in these isolates and the ability to acquire and transfer virulence and ARGs. This will help understand the pathogenicity of each strain and the need to explore rapid and more effective treatment options.

Other molecular subtyping methods used for classification and epidemiological studies, including multi-locus sequence typing and *spa* typing (Holtfreter *et al.*, 2016) can be explored in characterizing the MRSA strains isolated in this study. Whole genome sequencing analysis of multi-drug resistant and virulent MRSA strains detected in this study will provide an in-depth understanding of the metabolic and functional capabilities of these isolates. This will also assist in further differentiation of MRSA strains obtained from treated effluent and receiving rivers at a nucleotide level. It will also help to determine the hierarchical relationship of each strains, their transfer of genetic information and track the strains that could be responsible for outbreaks of infections (SenGupta *et al.*, 2014).

The most pathogenic and antibiotic resistant strain of the study showed to have the presence of: *mecA, aac(6')/aph(2'')*, *blaZ*, *ermC*, *msrA* and *tetK*. It exhibited phenotypic resistance to the following antibiotics: Oxacillin, Penicillin, Ampicillin, Gentamicin, Erythromycin, Cefoxitin, Lincomycin and Azithromycin. A study by Yoshioka *et al* (2014) monitored the various stages of an MRSA infection using a mouse model. It is suggested to further investigate the pathogenicity of this strain using animal models. This technique can be applied to monitor the level of pathogenicity and understand the mechanisms of disease in a mouse model caused by this MRSA strain. This will evaluate current antibiotic therapy and encourage development of treatment options with better efficacy in infected individuals.

References

Akanbi, O. E., Njom H. A., Fri, J., Otigbu, A. C., and Clarke, A. M. 2017. Antimicrobial Susceptibility of *Staphylococcus aureus* Isolated from Recreational Waters and Beach Sand in Eastern Cape Province of South Africa. *International journal of environmental research and public health*. **14**(9): 1001.

Alfatemi, S.M.H., Motamedifar, M., Hadi, N., and Saraie, H.S.E. 2014. Analysis of virulence genes among methicillin resistant *Staphylococcus aureus* (MRSA) strains, *Jundishapur Journal of Microbiology*. **7**: e10741.

Almagro-Moreno, S., and Taylor, R.K. 2013. Cholera: environmental reservoirs and impact on disease transmission. *Microbiology Spectrum*. **1**: e1004787.

Amoako, D.G., Bester, L.A., Somboro, A.M., Baijnath, S., Chetna, N., Govind, C.N., and Essack, S.Y. 2016. Plasmid-mediated resistance and virulence mechanisms in the private health sector in KwaZulu-Natal, South Africa: An investigation of methicillin resistant *Staphylococcus aureus* (MRSA) clinical isolates collected during a three-month period. *International Journal of Infectious Diseases*. **46**: 38-41.

Bateganyaa, N.L., Nakalanzic, D., Babud, M., and Heinab, T. 2015. Buffering municipal wastewater pollution using urban wetlands in sub-Saharan Africa: a case of Masaka municipality, Uganda. *Environmental Technology*. **36**: 2149-2160.

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

Department of Water and Environmental Affairs (DWAF). 1984. Republic of South Africa, Government Gazette, Requirements for the Purification of Wastewater or Effluent:

general and special standards, regulation no. 91. Department of Water and Environmental Affairs, republic of South Africa, Durban, 1984.

Department of Water Affairs and Forestry (DWAF). 1996. South African water quality guidelines for domestic use. 2nd edition. Department of Water Affairs and Forestry, South Africa, Durban. Pp. 1-185.

Doughari, H.J., Ndakidemi, P.A., Human, I.S., and Benade, S. 2011. Virulence, Resistance Genes, and Transformation Amongst Environmental Isolates of *Escherichia coli* and *Acinetobacter spp*. *Journal of Microbiology and Technology*. **22**: 25–33.

Goldstein, R.E.R., Micallef, S.A., Gibbs, S.G., Davis, J.A., He, X., George, A., Kleinfelter, L.M., Schreiber, N.A., Mukherjee, S., Sapkota, A., Joseph, S.W., and Sapkota, A.R. 2012. Methicillin-Resistant *Staphylococcus aureus* (MRSA) detected at four U.S. wastewater treatment plants. *Environmental Health Perspectives*. **120**: 1551-1558.

Holtfreter, S., Grumann, D., Balau, V., Barwich, A., Kolata, J., Goehler, A., Weiss, S., Holtfreter, B., Bauerfeind, S.S., Döring, P., and Friebe, E. 2016. Molecular epidemiology of *Staphylococcus aureus* in the general population in Northeast Germany–results of the Study of Health in Pomerania (SHIP-TREND-0). *Journal of clinical microbiology*. **54**(11): 2774-2785.

Hunter, P. R., MacDonald, A. M., & Carter, R. C. 2010. Water supply and health. *PLoS medicine*. **7**(11): e1000361.

Kadariya, J., Smith, T.C., and Thapaliya, D. 2014. *Staphylococcus aureus* and staphylococcal food-borne disease: an ongoing challenge in public health. *BioMed Research International*. **2014**: 1-9.

Kim, J. U., Cha, C. H., An, H. K., Lee, H. J., and Kim, M. N. 2012. A multiplex real-time PCR assay for the detection of methicillin-resistant *Staphylococcus aureus* (MRSA) suitable in high endemic regions. *Journal of clinical microbiology*, JCM-02495.

Lokhande, R. S., Singare, P. U., & Pimple, D. S. 2011. Study on physico-chemical parameters of waste water effluents from Taloja industrial area of Mumbai, India. *International Journal of Ecosystem*. **1**(1): 1-9.

Marais, E., Aithma, N., Perovic, O., Oosthuysen, W.F., Musenge, E., and Dusé, A.G. 2009. Antimicrobial susceptibility of methicillin-resistant *Staphylococcus aureus* isolates from South Africa. *South African Medical Journal*. **99**: 170-173.

Mhmoud, N. A., Fahal, A. H., Mahgoub, E. S., and van de Sande, W. W. J. 2014. The combination of amoxicillin-clavulanic acid and ketoconazole in the treatment of *Madurella mycetomatis* eumycetoma and *Staphylococcus aureus* co-infection. *PLoS Neglected Tropical Diseases*. **8**: e2959.

Moghadam, S. O., Pourmand, M. R., Douraghi, M., Sabzi, S., and Ghaffari, P. 2017. Utilization of PFGE as a Powerful Discriminative Tool for the Investigation of Genetic Diversity among MRSA Strains. *Iranian journal of public health*. **46**(3): 351.

Monecke, S., ller, E.M., Buchler, J., Stieber, B., and Ehricht, R. 2014. *Staphylococcus aureus* in vitro secretion of alpha toxin (hla) correlates with the affiliation to clonal complexes. *PLoS ONE*. **9**: e100427.

Naidoo, S., and Olaniran, A.O. 2013. Treated wastewater effluent as a source of microbial pollution of surface water resources. *International Journal of Environmental Research and Public Health*. **11**: 249-270.

Naquin, A., Clement, J., Sauce, M., Grabert, R., Sherpa, M., and Boopathy, R. 2014. Presence of antibiotic resistant *Staphylococcus aureus* in sewage treatment plant. *Journal of Water Sustainability.* **4**:227-236.

New, C.Y., Amalia, A.R., Ramzi, O.S.B., and Son, R. 2016. Antibiotic resistance evolution of Methicillin Resistant *Staphylococcus aureus* (MRSA) and colloidal silver as the nanoweapon. *International Food Research Journal*. **23**: 1248-1254.

Ong, M. H.L., Ho, W.Y., Ng, W.W., and Chew, C.H. 2017. High Prevalence of tetM as Compared to tetK Amongst Methicillin-Resistant *Staphylococcus aureus* (MRSA) Isolates from Hospitals in Perak, Malaysia. *Jundishapur journal of microbiology*. **10**(6).

Pandey, P. K., Kass, P. H., Soupir, M. L., Biswas, S., and Singh, V. P. 2014. Contamination of water resources by pathogenic bacteria. *AMB Express*. **4**(1): 51.

Portillo, B.C., Moreno, J.E., Yomayusa, N., Álvarez, C.A., Cardozo, B.E.C., Pérez, J.A.E., Díaz, P.L., Ibañez, M., Mendez-Alvarez, S., Leal, A.L., and Gómez, N.V. 2013. Molecular epidemiology and characterization of virulence genes of community-acquired and hospital-acquired methicillin-resistant *Staphylococcus aureus* isolates in Colombia. *International journal of infectious diseases*. **17**(9): e744-e749.

Quach-Cu, J., Herrera-Lynch, B., Marciniak C., Adams, S., Simmerman, A., and Reinke, R. A. 2018. The Effect of Primary, Secondary, and Tertiary Wastewater Treatment Processes on Antibiotic Resistance Gene (ARG) Concentrations in Solid and Dissolved Wastewater Fractions. *Water*. **10**(1): 37.

Rahmanian, N., Ali, S. H. B., Homayoonfard, M., Ali, N., Rehan, M., Sadef, Y., & Nizami, A. 2015. Analysis of physiochemical parameters to evaluate the drinking water quality in the State of Perak, Malaysia. *Journal of Chemistry.* **2015.**

Sabri, N. A., Schmitt, H., Van der Zaan, B., Gerritsen, H. W., Zuidema, T., Rijnaarts, H. H. M., & Langenhoff, A. A. M. 2018. Prevalence of antibiotics and antibiotic resistance genes in a wastewater effluent-receiving river in the Netherlands. *Journal of Environmental Chemical Engineering*.

San Sit, P., The, C.S.J., Idris, N., Sam, I.C., Omar, S.F.S., Sulaiman, H., Thong K.L., Kamarulzaman, A., and Ponnampalavanar, S. 2017. Prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) infection and the molecular characteristics of MRSA bacteraemia over a two-year period in a tertiary teaching hospital in Malaysia. *BMC infectious diseases*. **17**(1): 274.

SenGupta, D. J., Cummings, L. A., Hoogestraat, D. R., Butler-Wu, S. M., Shendure, J., Cookson, B. T., and Salipante, S. J. 2014. Whole genome sequencing for high-resolution investigation of methicillin resistant *Staphylococcus aureus* epidemiology and genome plasticity. *Journal of clinical microbiology*. JCM-00759.

Tang, H.J., Chen, C.C., Cheng, K.C., Wu, K.Y., Lin, Y.C., Zhang, C.C., Weng, T.C., Yu, W.L., Chiu, Y.H., Toh, H.S., Chiang, S.R., Su, B.A., Ko, W.C., and Chuang, Y.C. 2013. In-vitro efficacies and resistance profiles of rifampin-based combination regimens for biofilm-embedded methicillin-resistant *Staphylococcus aureus*. *Antimicrobial agents and chemotherapy*. **2013**: AAC-01236.

Thompson, J.M., Gündoğdu, A., Stratton, H.M., and Katouli, H.M. 2013. Antibiotic resistant *Staphylococcus aureus* in hospital wastewaters and sewage treatment plants with special reference to methicillin-resistant *Staphylococcus aureus* (MRSA). *Journal of Applied Microbiology*. **114**: 44-5.

Velasco, V., Buyukcangaz, E., Sherwood, J.S., Stepan, R.M., Koslofsky, R.J., and Logue, C.M. 2015. Characterization of *Staphylococcus aureus* from Humans and a Comparison with İsolates of Animal Origin, in North Dakota, United States. *PLoS ONE*. **10**(10): e0140497.

World Health Organization and UNICEF. 2017. Progress on drinking water, sanitation and hygiene: 2017 update and SDG baselines.

Xie, X., Bao, Y., Ouyang, N., Dai, X., Pan, K., Chen, B., Deng, Y., Wu, X., Xu, F., Li, H., and Huang, S. 2016. Molecular epidemiology and characteristic of virulence gene of community-acquired and hospital-acquired methicillin-resistant *Staphylococcus aureus* isolates in Sun Yat-sen Memorial hospital, Guangzhou, Southern China. *BMC Infectious Diseases*. **16**:339.

Xiong, W., Sun, Y., Ding, X., Wang, M., and Zeng, Z. 2015. Selective pressure of antibiotics on ARGs and bacterial communities in manure-polluted freshwater-sediment microcosms. *Frontiers in microbiology*. **6**: 194.

Yoshioka, K., Ishii, K., Kuramoto, T., Nagai, S., Funao, H., Ishihama, H., Shiono, Y., Sasaki, A., Aizawa, M., Okada, Y., and Koyasu, S. 2014. A novel mouse model of soft-tissue infection using bioluminescence imaging allows noninvasive, real-time monitoring of bacterial growth. *PloS one*. **9**(9): e106367.

Zhu, Y.G., Johnson, T.A., Su, J.Q., Qiao, M., Guo, G.X., Stedtfeld. R.D., Hashsham, S.A., and Tiedje, J.M. 2013. Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proceedings of the National Academy of Sciences*. **110**(9): 3435-3440.

APPENDICES

Table 1: Presumptive *S. aureus* from WWTP1 (Sampling Period 1)

Dilution	INF		BC		AC		DS		US	
	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml
1	TMTC		TMTC		TMTC		TMTC		TMTC	
1	TMTC		TMTC		TMTC		TMTC		TMTC	
1	TMTC		TMTC		TMTC		TMTC		TMTC	
10^{-1}	TMTC		TMTC		TMTC		TMTC		TMTC	
10^{-1}	TMTC		TMTC		TMTC		TMTC		TMTC	
10^{-1}	TMTC		TMTC		TMTC		TMTC		TMTC	
10^{-2}	TMTC		328	328	TMTC		TMTC		259	259
10^{-2}	TMTC		337	337	TMTC		TMTC		230	230
10^{-2}	TMTC		304	304	TMTC		TMTC		216	216
10^{-3}	364	3640	47	470	145	1450	378	3780	29	290
10^{-3}	383	3830	42	420	117	1170	334	3340	28	280
10^{-3}	408	4080	50	500	122	1220	360	3600	16	160

Key: INF- influent; BC- before chlorination; AC- after chlorination; DS- downstream; US- upstream; TMTC- too many to count; CFU/ml- colony forming units/ millilitre

Table 2: Presumptive MRSA from WWTP1 (Sampling Period 1)

Dilution	INF		BC		AC		DS		US	
	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml
1	TMTC		TMTC		TMTC		TMTC		TMTC	
1	TMTC		TMTC		TMTC		TMTC		TMTC	
1	TMTC		TMTC		TMTC		TMTC		TMTC	
10^{-1}	TMTC		443	44.3	305	30.5	TMTC		111	11.1
10^{-1}	TMTC		514	51.4	235	23.5	TMTC		83	8.3
10^{-1}	TMTC		520	52	298	29.8	TMTC		81	8.1
10^{-2}	TMTC		58	58	42	42	198	198	17	17
10^{-2}	TMTC		53	53	37	37	214	214	13	13
10^{-2}	TMTC		43	43	22	22	260	260	9	9
10^{-3}	325	3250	9	90	2	20	27	270	1	10
10^{-3}	360	3600	10	100	8	80	33	330	1	10
10^{-3}	304	3040	4	40	4	40	28	280	2	20

Table 3: Presumptive *S. aureus* from WWTP1 (Sampling Period 2)

Dilution	INF		BC		AC		DS		US	
	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml
1			TMTC		TMTC		TMTC		TMTC	
1			TMTC		TMTC		TMTC		TMTC	
1			TMTC		TMTC		TMTC		TMTC	
10^{-1}	TMTC		TMTC		TMTC		TMTC		TMTC	
10^{-1}	TMTC		TMTC		TMTC		TMTC		TMTC	
10^{-1}	TMTC		TMTC		TMTC		TMTC		TMTC	
10^{-2}	TMTC		289	289	152	152	65	65	288	288
10^{-2}	TMTC		331	331	148	148	85	85	364	364
10^{-2}	TMTC		326	326	132	132	78	78	320	320
10^{-3}	TMTC		40	400	17	170	7	70	39	390
10^{-3}	TMTC		32	320	11	110	9	90	48	480
10^{-3}	TMTC		46	460	14	140	8	80	50	500
10^{-4}	78	7800								
10^{-4}	61	6100								
10^{-4}	68	6800								

Table 4: Presumptive MRSA from WWTP1 (Sampling Period 2)

Dilution	INF		BC		AC		DS		US	
	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml
1	TMTC		TMTC		TMTC		88	0,88	TMTC	
1	TMTC		TMTC		TMTC		68	0,68	TMTC	
1	TMTC		TMTC		TMTC		75	0,75	TMTC	
10^{-1}	TMTC		TMTC		111	11,1	17	1,7	TMTC	
10^{-1}	TMTC		TMTC		97	9,7	13	1,3	TMTC	
10^{-1}	TMTC		TMTC		90	9	15	1,5	TMTC	
10^{-2}	TMTC		72	72	7	7	2	2	72	72
10^{-2}	TMTC		78	78	8	8	2	2	55	55
10^{-2}	TMTC		79	79	10	10	3	3	43	43
10^{-3}	91	910	9	90	0	0	0	0	6	60
10^{-3}	89	890	8	80	1	10	0	0	6	60
10^{-3}	74	740	7	70	0	0	1	10	4	40
10^{-4}	6	600								
10^{-4}	9	900								
10^{-4}	7	700								

Table 5: Presumptive *S. aureus* from WWTP1 (Sampling Period 3)

Dilution	INF		BC		AC		DS		US	
	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml
1			TMTC		TMTC		TMTC		TMTC	
1			TMTC		TMTC		TMTC		TMTC	
1			TMTC		TMTC		TMTC		TMTC	
10^{-1}	TMTC		TMTC		TMTC		TMTC		TMTC	
10^{-1}	TMTC		TMTC		TMTC		TMTC		TMTC	
10^{-1}	TMTC		TMTC		TMTC		TMTC		TMTC	
10^{-2}	TMTC		263	263	112	112	43	43	287	287
10^{-2}	TMTC		250	250	105	105	37	37	252	252
10^{-2}	TMTC		229	229	126	126	45	45	267	267
10^{-3}	140	1400	29	290	14	140	6	60	35	350
10^{-3}	152	1520	36	360	8	80	3	30	32	320
10^{-3}	120	1200	30	300	10	100	5	50	38	380
10^{-4}	27	2700								
10^{-4}	17	1700								
10^{-4}	16	1600								

Table 6: Presumptive MRSA from WWTP1 (Sampling Period 3)

Dilution	INF		BC		AC		DS		US	
	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml
1			TMTC		TMTC		59	0,59	TMTC	
1			TMTC		TMTC		72	0,72	TMTC	
1			TMTC		TMTC		62	0,62	TMTC	
10^{-1}	TMTC		TMTC		45	4,5	10	1	137	13,7
10^{-1}	TMTC		TMTC		52	5,2	11	1,1	129	12,9
10^{-1}	TMTC		TMTC		48	4,8	6	0,6	142	14,2
10^{-2}	TMTC		119	119	3	3	1	1	14	14
10^{-2}	TMTC		117	117	4	4	0	0	13	13
10^{-2}	TMTC		90	90	0	0	0	0	12	12
10^{-3}	34	340	8	80	0	0	0	0	3	30
10^{-3}	32	320	14	140	0	0	0	0	2	20
10^{-3}	27	270	12	120	0	0	0	0	2	20
10^{-4}	3	300								
10^{-4}	2	200								
10^{-4}	3	300								

Table 7: Presumptive *S. aureus* from WWTP2 (Sampling Period 1)

Dilution	INF		BC		AC		DS		US	
	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml
1			TMTC		TMTC		TMTC		TMTC	
1			TMTC		TMTC		TMTC		TMTC	
1			TMTC		TMTC		TMTC		TMTC	
10^{-1}	TMTC		TMTC		118	11,8	201	20,1	TMTC	
10^{-1}	TMTC		TMTC		121	12,1	221	22,1	TMTC	
10^{-1}	TMTC		TMTC		95	9,5	266	26,6	TMTC	
10^{-2}	TMTC		250	250	6	6	22	22	26	26
10^{-2}	TMTC		296	296	10	10	33	33	39	39
10^{-2}	TMTC		224	224	17	17	23	23	31	31
10^{-3}	36	360	24	240	1	10	7	70	4	40
10^{-3}	47	470	18	180	2	20	3	30	7	70
10^{-3}	51	510	23	230	2	20	3	30	2	20
10^{-4}	0	0								
10^{-4}	4	400								
10^{-4}	6	600								

Table 8: Presumptive MRSA from WWTP2 (Sampling Period 1)

Dilution	INF		BC		AC		DS		US	
	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml
1			TMTC		28	0,28	49	0,49	TMTC	
1			TMTC		27	0,27	52	0,52	TMTC	
1			TMTC		19	0,19	50	0,5	TMTC	
10^{-1}	TMTC		TMTC		9	0,9	13	1,3	51	5,1
10^{-1}	TMTC		TMTC		4	0,4	7	0,7	41	4,1
10^{-1}	TMTC		TMTC		6	0,6	9	0,9	48	4,8
10^{-2}	337	337	50	50	1	1	1	1	3	3
10^{-2}	289	289	30	30	1	1	1	1	2	2
10^{-2}	305	305	57	57	2	2	0	0	1	1
10^{-3}	27	270	7	70	0	0	0	0	0	0
10^{-3}	31	310	6	60	0	0	0	0	0	0
10^{-3}	32	320	7	70	0	0	0	0	0	0
10^{-4}	0	0								
10^{-4}	2	200								
10^{-4}	5	500								

Table 9: Presumptive *S. aureus* from WWTP2 (Sampling Period 2)

Dilution	INF		BC		AC		DS		US	
	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml
1			TMTC		TMTC		TMTC		TMTC	
1			TMTC		TMTC		TMTC		TMTC	
1			TMTC		TMTC		TMTC		TMTC	
10^{-1}	TMTC		TMTC		198	19,8	215	21,5	TMTC	
10^{-1}	TMTC		TMTC		220	22	198	19,8	TMTC	
10^{-1}	TMTC		TMTC		241	24,1	221	22,1	TMTC	
10^{-2}	TMTC		TMTC		33	33	26	26	188	188
10^{-2}	TMTC		TMTC		38	38	34	34	214	214
10^{-2}	TMTC		TMTC		20	20	27	27	210	210
10^{-3}	TMTC		136	1360	1	10	3	30	23	230
10^{-3}	TMTC		154	1540	3	30	2	20	25	250
10^{-3}	TMTC		158	1580	6	60	3	30	35	350
10^{-4}	41	4100								
10^{-4}	34	3400								
10^{-4}	48	4800								

Table 10: Presumptive MRSA from WWTP2 (Sampling Period 2)

Dilution	INF		BC		AC		DS		US	
	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml
1			TMTC		74	0,74	TMTC		TMTC	
1			TMTC		63	0,63	TMTC		TMTC	
1			TMTC		68	0,68	TMTC		TMTC	
10^{-1}	TMTC		TMTC		17	1,7	46	4,6	TMTC	
10^{-1}	TMTC		TMTC		12	1,2	44	4,4	TMTC	
10^{-1}	TMTC		TMTC		15	1,5	39	3,9	TMTC	
10^{-2}	TMTC		TMTC		4	4	4	4	62	62
10^{-2}	TMTC		TMTC		3	3	9	9	65	65
10^{-2}	TMTC		TMTC		3	3	6	6	68	68
10^{-3}	TMTC		131	1310	0	0	0	0	9	90
10^{-3}	TMTC		146	1460	0	0	0	0	7	70
10^{-3}	TMTC		143	1430	0	0	1	10	6	60
10^{-4}	34	3400								
10^{-4}	29	2900								
10^{-4}	43	4300								

Table 11: Presumptive *S. aureus* from WWTP2 (Sampling Period 3)

Dilution	INF		BC		AC		DS		US	
	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml
1			TMTC		TMTC		TMTC		TMTC	
1			TMTC		TMTC		TMTC		TMTC	
1			TMTC		TMTC		TMTC		TMTC	
10^{-1}	TMTC		TMTC		TMTC		TMTC		TMTC	
10^{-1}	TMTC		TMTC		TMTC		TMTC		TMTC	
10^{-1}	TMTC		TMTC		TMTC		TMTC		TMTC	
10^{-2}	TMTC		264	264	272	272	85	85	85	85
10^{-2}	TMTC		285	285	280	280	73	73	81	81
10^{-2}	TMTC		253	253	301	301	90	90	92	92
10^{-3}	288	2880	33	330	32	320	6	60	4	40
10^{-3}	295	2950	26	260	21	210	4	40	9	90
10^{-3}	308	3080	28	280	27	270	7	70	7	70
10^{-4}	27	2700								
10^{-4}	29	2900								
10^{-4}	35	3500								

Table 12: Presumptive MRSA from WWTP2 (Sampling Period 3)

Dilution	INF		BC		AC		DS		US	
	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml	Colony count	CFU/ml
1			TMTC		TMTC		TMTC		TMTC	
1			TMTC		TMTC		TMTC		TMTC	
1			TMTC		TMTC		TMTC		TMTC	
10^{-1}	TMTC		TMTC		TMTC		130	13	111	11,1
10^{-1}	TMTC		TMTC		TMTC		115	11,5	103	10,3
10^{-1}	TMTC		TMTC		TMTC		126	12,6	115	11,5
10^{-2}	TMTC		140	140	106	106	14	14	19	19
10^{-2}	TMTC		151	151	110	110	17	17	17	17
10^{-2}	TMTC		125	125	101	101	13	13	14	14
10^{-3}	116	1160	21	210	18	180	3	30	0	0
10^{-3}	129	1290	19	190	15	150	0	0	0	0
10^{-3}	104	1040	23	230	16	160	1	10	2	20
10^{-4}	15	1500								
10^{-4}	21	2100								

Table 13: Physico-chemical parameters for WWTP1 (sampling period 1)

	SP.	INF	Ave.	Std Dev	BC	Ave	Std Dev	AC	Ave	Std Dev.	DS	Ave	Std Dev	US	Ave.	Std Dev
Temp (°C)	1	27	27.33	0.58	25	25.67	0.58	29	28.33	0.58	27	27.33	0.58	26	26	0
	2	27			26			28			28			26		
	3	28			26			28			27			26		
PH	1	7.22	7.19	0.03	7.48	7.51	0.03	7.58	7.62	0.03	7.68	7.68	0.01	7.73	7.70	0.04
	2	7.17			7.50			7.63			7.68			7.70		
	3	7.19			7.54			7.64			7.69			7.66		
Turb.	1	494	487.67	12.74	19.3	20.63	1.74	13.6	12.4	1.06	17.5	17.6	0.35	6.32	7.73	1.56
	2	473			22.6			11.6			17.2			7.46		
	3	496			20.0			12			18.1			9.41		
EC µs/cm	1	718	724.00	5.29	511	514.00	7.00	592	563.00	36.51	573	618	39.13	339	340.33	1.15
	2	726			509			575			637			341		
	3	728			522			522			644			341		
TDS (mg/L)	1	413	414.67	1.53	361	364	2.65	420	424.33	3.79	432	432.67	1.15	236	257.67	18.77
	2	415			365			426			432			268		
	3	416			366			427			434			269		

	SP.	INF	Ave.	Std Dev	BC	Ave	Std Dev	AC	Ave	Std Dev.	DS	Ave	Std Dev	US	Ave.	Std Dev
COD (mg/L)	1	442.5	504	165.46	95	77.33	15.95	225	222	3	308	306.33	2.0816 65999	205	193.33	25.58
	2	621			73			219			307			164		
	3	387			64			222			304			211		
Resist.	1	1392	1387.3 3	8.96	1956	1869.6 7	156.51	1166	1154	10.44	1844	1818	22.869 19325	1136	1134.3 3	2.89
	2	1377			1964			1149			1809			1136		
	3	1393			1689			1147			1801			1131		
Sal(%)	1	0.41	0.42	0.01	0.36	0.37	0.01	0.42	0.43	0.01	0.26	0.67	0.0057 73503	0.43	0.43	0.01
	2	0.42			0.37			0.43			0.27			0.43		
	3	0.42			0.37			0.43			0.27			0.44		
BOD_o (mg/L)	1	126	143	76.92	72	52.50	27.58	184	184.5	0.71	23.55	23.72	0.17	24.66	25.19	0.87
	2	227			38.5			185			23.73			24.72		
	3	76			33			158.5			23.88			26.19		

Key: INF- influent; BC- before chlorination; AC- after chlorination; DS- downstream; US- upstream; Ave- average; Std Dev- standard deviation

Table 14: Physico-chemical parameters for WWTP1 (sampling period 2)

	SP.	INF	Ave.	Std Dev	BC	Ave	Std Dev	AC	Ave	Std Dev.	DS	Ave	Std Dev	US	Ave.	Std Dev
Temp (°C)	1	21	20.33	0.58	19	19.33	0.58	18	18	0	18	18	0	16	16	0
	2	20			19			18			18			16		
	3	20			20			18			18			16		
PH	1	7.39	7.41	0.04	7.7	7.69	0.01	7.46	7.42	0.03	7.84	7.81	0.03	7.6	7.52	0.07
	2	7.39			7.69			7.41			7.78			7.47		
	3	7.46			7.68			7.4			7.8			7.49		
Turb.	1	483	486.33	3.51	17.7	17.57	0.15	13.9	13.4	0.46	22	22.3	0.3	30.4	29.97	0.67
	2	486			17.6			13			22.3			30.3		
	3	490			17.4			13.3			22.6			29.2		
EC µs/cm	1	1004	1002.33	18.56	748	750	1.73	858	860.67	2.31	854	860	5.29	838	837.67	0.58
	2	983			751			862			862			838		
	3	1020			751			862			864			837		
TDS (mg/L)	1	484	483.67	0.58	366	366.67	0.58	421	421.67	0.58	418	421.33	3.06	410	410	0
	2	484			367			422			422			410		
	3	483			367			422			424			410		

	SP.	INF	Ave.	Std Dev	BC	Ave	Std Dev	AC	Ave	Std Dev.	DS	Ave	Std Dev	US	Ave.	Std Dev
COD (mg/L)	1	706.5	731.5	31.22	166	151	18.73	77	73	7.81	35	59	31	216	223.67	9.29
	2	766.5			130			78			48			234		
	3	721.5			157			64			94			221		
Resist.	1	984	1006.33	19.35	1336	1333	2.65	1165	1112.67	87.20	1172	1163.67	7.37	1194	1194.33	0.58
	2	1017			1331			1161			1161			1194		
	3	1018			1332			1012			1158			1195		
Sal (%)	1	0.48	0.48	0	0.36	0.37	0.01	0.42	0.42	0	0.42	0.42	0	0.41	0.41	0
	2	0.48			0.37			0.42			0.42			0.41		
	3	0.48			0.37			0.42			0.42			0.41		
BOD$_0$ (mg/L)	1	256	230.67	28.38	126.25	128.83	6.52	99.25	91.67	6.58	9.12	7.54	1.53	131.25	129.83	5.03
	2	236			124			88.25			7.44			134		
	3	200			136.25			87.5			6.06			124.25		

Table 15: Physico-chemical parameters for WWTP1 (sampling period 3)

	SP.	INF	Ave.	Std Dev	BC	Ave	Std Dev	AC	Ave	Std Dev.	DS	Ave	Std Dev	US	Ave.	Std Dev
Temp (°C)	1	22	21.33	0.58	22	21.33	0.58	22	21	1	19	19	0	18	17.33	0.58
	2	21			21			21			19			17		
	3	21			21			20			19			17		
PH	1	7.19	7.18	0.01	7.57	7.57	0.01	7.63	7.62	0.01	7.6	7.60	0.01	7.51	7.54	0.03
	2	7.17			7.58			7.61			7.59			7.55		
	3	7.17			7.56			7.62			7.6			7.57		
Turb.	1	217	212.67	4.04	390	367.67	20.11	29.8	35.87	5.26	24.3	30	5.09	37.4	40.87	4.32
	2	209			362			38.7			31.6			39.5		
	3	212			351			39.1			34.1			45.7		
EC µs/cm	1	869	871.67	2.52	724	721.67	2.08	797	808.67	10.41	819	818.33	3.06	813	812.67	0.58
	2	872			721			812			821			812		
	3	874			720			817			815			813		
TDS (mg/L)	1	426	427.67	1.53	353	352.33	0.58	390	396	5.29	401	400.67	1.53	398	398	0
	2	429			352			398			402			398		
	3	428			352			400			399			398		

	SP.	INF	Ave.	Std Dev	BC	Ave	Std Dev	AC	Ave	Std Dev.	DS	Ave	Std Dev	US	Ave.	Std Dev
COD (mg/L)	1	979.5	1002.5	19.91858429	303	302	1	307	306	2.645751311	308	307.3333333	0.577350269	291	294	2.645751311
	2	1014			301			303			307			295		
	3	1014			302			308			307			296		
Resist.	1	1138	1142.67	4.16	1382	1385.67	3.21	1297	1261	33.41	1222	1222.67	5.03	1230	1230.67	0.58
	2	1144			1387			1255			1218			1231		
	3	1146			1388			1231			1228			1231		
Sal (%)	1	0.43	0.43	0	0.35	0.35	0.00	0.38	0.39	0.01	0.41	0.40	0.01	0.44	0.41	0.02
	2	0.43			0.355			0.39			0.4			0.4		
	3	0.43			0.35			0.4			0.4			0.4		
BOD_0 (mg/L)	1	10	10	0	62.25	87.92	25.03	175.75	170.42	5.38	23.43	23.01	0.63	17.97	17.45	0.45
	2	10			89.25			165			22.29			17.19		
	3	10			112.25			170.5			23.31			17.19		

Table 16: Physico-chemical parameters for WWTP2 (sampling period 1)

	SP.	INF	Ave.	Std Dev	BC	Ave	Std Dev	AC	Ave	Std Dev.	DS	Ave	Std Dev	US	Ave.	Std Dev
Temp (°C)	1	26	25.33	0.58	25	25	0	24	24.67	0.58	25	25	0	24	23.33	0.58
	2	25			25			25			25			23		
	3	25			25			25			25			23		
PH	1	7.11	7.11	0.01	7.28	7.303	0.02	7.71	7.72	0.01	7.73	7.81	0.07	7.58	7.59	0.02
	2	7.11			7.31			7.72			7.86			7.58		
	3	7.12			7.32			7.73			7.85			7.61		
Turb.	1	440	454.33	12.50	105	102	3.61	93	95	2.65	42	41	1	12	14	2
	2	460			103			98			41			16		
	3	463			98			94			40			14		
EC µs/cm	1	886	879	6.24	754	753.33	0.58	707	745.33	33.65	603	608	8.66	337	345	7
	2	877			753			759			618			348		
	3	874			753			770			603			350		
TDS (mg/L)	1	435	436.33	2.31	368	368	0	345	364	16.64	293	300	6.08	161.6	165.7	3.58
	2	435			368			371			304			167.3		
	3	439			368			376			303			168.2		

	SP.	INF	Ave.	Std Dev	BC	Ave	Std Dev	AC	Ave	Std Dev.	DS	Ave	Std Dev	US	Ave.	Std Dev
COD (mg/L)	1	843	825	31.18	304	289.33	14.05	244	260	21.17	176	222.67	41.88	237	212	21.79
	2	789			288			252			257			197		
	3	843			276			284			235			202		
Resist.	1	1129	1125	6.08	1328	1328	0	1415	1344	62.22	1658	1620.67	32.35	2970	2900	60.83
	2	1128			1328			1318			1601			2870		
	3	1118			1328			1299			1603			2860		
Sal (%)	1	0.44	0.44	0	0.37	0.37	0	0.35	0.37	0.02	0.29	0.30	0.01	0.16	0.177	0.01
	2	0.44			0.37			0.37			0.3			0.17		
	3	0.44			0.37			0.38			0.3			0.17		
BOD_o (mg/L)	1	200	173.33	55.99	176.25	178.25	2	156.25	162.83	5.77	22.98	23.14	0.23	23.61	23.55	0.16
	2	109			180.25			165.25			23.4			23.67		
	3	211			178.25			167			23.04			23.37		

Table 17: Physico-chemical parameters for WWTP2 (sampling period 2)

	SP.	INF	Ave.	Std Dev	BC	Ave	Std Dev	AC	Ave	Std Dev.	DS	Ave	Std Dev	US	Ave.	Std Dev
Temp (°C)	1	18	18.67	0.58	18	18.33	0.58	19	19	0	19	18.67	0.58	19	18.33	0.58
	2	19			18			19			19			18		
	3	19			19			19			18			18		
PH	1	7.44	7.38	0.07	7.27	7.35	0.07	7.02	7.01	0.02	7.43	7.52	0.08	7.37	7.43	0.06
	2	7.4			7.37			7.01			7.53			7.43		
	3	7.3			7.4			6.99			7.59			7.48		
Turb.	1	351	314.33	32.15	286	280.67	27.39	71	100.33	32.33	38	38.33	5.51	18	18.67	2.08
	2	291			251			135			44			17		
	3	301			305			95			33			21		
EC µs/cm	1	809	814.67	5.51	796	799	3	648	650.33	2.52	505	509	3.61	342	343	1
	2	820			799			650			510			343		
	3	815			802			653			512			344		
TDS (mg/L)	1	396	399	3	389	390.67	1.53	315	316.33	1.53	245	246.67	1.53	164.3	164.7	0.36
	2	402			391			316			247			164.8		
	3	399			392			318			248			165		

	SP.	INF	Ave.	Std Dev	BC	Ave	Std Dev	AC	Ave	Std Dev.	DS	Ave	Std Dev	US	Ave.	Std Dev
COD (mg/L)	1	682.5	644.5	53.36	272	274.33	4.04	288	287.33	2.08	271	276.33	6.81	239	239	1
	2	667.5			279			285			284			238		
	3	583.5			272			289			274			240		
Resist.	1	1236	1227.67	8.50	1257	1252	5	1544	1538.67	5.51	1978	1964	12.77	2.94	2.92	0.02
	2	1219			1252			1539			1961			2.91		
	3	1228			1247			1533			1953			2.91		
Sal (%)	1	0.4	0.4	0	0.39	0.39	0	0.32	0.32	0	0.24	0.25	0.01	0.16	0.16	0
	2	0.4			0.39			0.32			0.25			0.16		
	3	0.4			0.39			0.32			0.25			0.16		
BOD_o (mg/L)	1	274	268.33	7.37	169.25	170.58	1.26	132	126	5.34	24.03	23.42	1.32	21.78	21.1	0.60
	2	271			171.75			121.75			21.9			20.64		
	3	260			170.75			124.25			24.33			20.88		

Table 18: Physico-chemical parameters for WWTP2 (sampling period 3)

	SP.	INF	Ave.	Std Dev	BC	Ave	Std Dev	AC	Ave	Std Dev.	DS	Ave	Std Dev	US	Ave.	Std Dev
Temp (°C)	1	22	22	0	22	22	0	22	19	0	21	21	0	21	21	0
	2	22			22			22			21			21		
	3	22			22			22			21			21		
PH	1	7.38	7.40	0.02	7.27	7.25	0.02	7.33	7.32	0.01	7.88	7.88	0.03	7.65	7.66	0.01
	2	7.4			7.23			7.31			7.85			7.66		
	3	7.41			7.25			7.32			7.9			7.67		
Turb.	1	338	339	1	79	76.67	2.08	116	117.33	1.53	45	44.33	0.58	23	26.67	6.35
	2	339			75			117			44			34		
	3	340			76			119			44			23		
EC µs/cm	1	759	750	12.29	525	532.33	6.66	521	528.67	7.09	372	379	6.56	316	317.67	1.53
	2	736			538			530			380			318		
	3	755			534			535			385			319		
TDS (mg/L)	1	371	366.67	5.86	255	258.33	3.06	252	256	3.61	178.8	182.37	3.37	151.3	152.2	0.82
	2	360			261			257			182.8			152.4		
	3	369			259			259			185.5			152.9		

	SP.	INF	Ave.	Std Dev	BC	Ave	Std Dev	AC	Ave	Std Dev.	DS	Ave	Std Dev	US	Ave.	Std Dev
COD (mg/L)	1	745.5	764	27.01	240	256	14	296	296	3	296	263.33	29.14	238	269.67	27.97
	2	751.5			262			299			240			291		
	3	795			266			293			254			280		
Resist.	1	1317	1333.33	21.73	1903	1878.33	22.81	1920	1892	25.24	2690	2640	45.83	3170	3150	17.32
	2	1358			1858			1885			2630			3140		
	3	1325			1874			1871			2600			3140		
Sal (%)	1	0.37	0.37	0.01	0.25	0.26	0.01	0.25	0.26	0.01	0.18	0.18	0	0.15	0.15	0
	2	0.36			0.26			0.26			0.18			0.15		
	3	0.37			0.26			0.26			0.18			0.15		
BOD_o (mg/L)	1	147	139.67	11.85	60	58.5	1.32	201	199.33	2.47	24.87	24.93	0.13	24.9	24.9	0.12
	2	126			58			196.5			24.84			24.78		
	3	146			57.5			200.5			25.08			25.02		

Table 19: Antibiotic resistant profiles

	Antibiotic resistant phenotypes																			
Isolate	**CIP**	**VA**	**AMC**	**C**	**OX**	**TE**	**AMP**	**AK**	**CN**	**FOX**	**DA**	**E**	**P**	**SXT**	**RD**	**IPM**	**KZ**	**NOR**	**MY**	**AZM**
1	S	S	S	S	R	S	R	S	S	R	I	S	R	S	S	S	R	R	R	R
2	S	S	S	S	R	S	R	S	S	R	I	S	R	S	S	S	I	R	R	R
3	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	I	R	R
4	S	S	R	S	R	S	R	S	S	R	I	S	R	I	S	S	I	S	R	S
5	S	S	S	S	R	S	R	S	S	R	I	S	R	S	S	S	S	S	R	S
6	S	S	S	S	R	S	R	S	S	R	I	S	R	S	S	S	I	S	R	S
7	S	S	S	I	R	S	R	S	S	R	I	S	R	S	S	S	R	S	R	S
8	S	S	R	S	R	S	R	S	S	R	I	S	R	S	S	S	I	S	R	S
9	S	R	S	S	R	S	R	S	S	R	S	S	R	S	S	S	R	S	R	S
10	S	S	R	S	R	S	R	S	S	R	I	I	R	R	S	S	R	R	R	R
11	S	S	S	S	R	S	R	S	S	S	I	R	R	R	S	S	R	S	R	S
12	S	S	S	S	R	S	R	S	S	R	I	S	R	S	S	S	R	R	R	R
13	S	S	S	S	R	S	R	S	S	R	I	S	R	S	S	S	R	R	R	R
14	S	S	S	S	R	S	R	S	S	R	I	S	R	S	S	S	R	S	R	I
15	S	S	S	S	R	S	R	S	S	R	I	R	R	I	S	S	R	S	R	S
16	S	S	S	S	R	S	R	S	S	R	I	S	R	S	S	S	R	R	R	R
17	S	S	R	S	R	S	R	S	S	R	R	I	R	R	S	S	I	S	R	S
18	S	S	S	S	R	S	R	S	S	R	I	S	R	R	S	S	R	S	R	S
19	S	S	S	S	R	S	R	S	S	R	S	I	R	S	S	I	I	S	R	S
20	S	S	R	S	R	S	R	S	S	R	I	S	R	R	S	S	R	S	R	S
21	S	S	R	S	R	S	R	S	S	R	I	I	R	S	S	S	R	S	R	S
22	S	S	R	S	R	S	R	S	S	R	I	S	R	S	S	S	R	S	R	S

Table 19: Continued…

	Antibiotic resistant profile																			
Isolate	**CIP**	**VA**	**AMC**	**C**	**OX**	**TE**	**AMP**	**AK**	**CN**	**FOX**	**DA**	**E**	**P**	**SXT**	**RD**	**IPM**	**KZ**	**NOR**	**MY**	**AZM**
23	S	S	R	S	R	S	R	S	S	R	I	S	R	I	S	S	I	S	R	S
24	S	S	S	R	R	S	R	S	I	R	I	S	R	S	S	I	R	S	R	R
25	S	S	S	I	R	R	R	S	S	R	I	S	R	S	S	I	R	S	R	R
26	S	S	S	S	R	S	S	S	S	R	I	I	S	I	S	S	R	S	R	R
27	S	S	S	S	R	S	R	S	S	R	I	S	R	I	S	S	R	S	R	R
28	S	S	R	S	R	S	R	S	S	R	I	S	R	S	S	S	I	S	R	S
29	S	S	R	S	R	S	R	S	S	R	I	S	R	S	S	S	S	S	R	R
30	S	S	S	S	R	S	R	S	S	R	I	S	R	I	S	S	S	S	R	I
31	S	S	S	S	R	S	R	S	S	R	I	I	R	S	S	S	R	S	R	R
32	S	S	S	R	R	S	R	S	S	R	S	S	R	I	S	S	S	I	R	R
33	S	S	R	S	R	S	R	S	S	R	I	S	R	R	S	S	S	S	R	R
34	S	S	R	S	R	S	R	S	S	R	I	I	R	I	S	S	R	S	R	R
35	S	S	R	S	R	S	R	S	S	R	I	S	R	S	S	S	I	I	R	R
36	S	S	R	S	R	S	R	S	S	R	I	S	R	S	S	S	R	S	R	R
37	S	S	R	S	R	R	R	S	S	R	R	R	R	S	S	S	R	S	R	R
38	S	S	R	R	R	S	R	S	S	R	I	S	R	S	S	S	R	S	R	R
39	S	S	S	S	R	S	R	S	S	R	R	S	R	R	S	S	R	S	R	R
40	S	S	R	S	R	S	R	S	S	R	I	S	R	S	S	S	S	S	R	S
41	S	S	S	S	R	S	R	S	S	R	I	S	R	I	S	S	S	I	R	R

Table 19: Continued…

Isolate	CIP	VA	AMC	C	OX	TE	AMP	AK	CN	FOX	DA	E	P	SXT	RD	IPM	KZ	NOR	MY	AZM
	Antibiotic resistant profile																			
42	S	S	R	S	R	S	R	S	S	R	I	S	R	S	S	S	R	I	R	R
43	S	S	R	S	R	S	R	S	S	R	I	S	R	I	S	S	S	I	R	R
44	S	S	R	S	R	S	R	S	S	R	I	S	R	S	S	S	S	S	R	S
45	S	S	S	S	R	S	R	S	R	R	S	R	R	S	S	S	S	I	R	R
46	S	S	R	S	R	S	R	R	R	R	S	R	R	S	S	S	R	S	R	R
47	S	R	R	S	R	S	R	S	S	R	S	S	R	S	S	S	R	S	R	R
48	S	R	S	S	R	S	R	S	S	R	I	I	R	S	S	S	R	S	R	I
49	S	S	S	S	R	S	R	S	S	R	S	R	R	S	S	S	R	S	R	R
50	I	S	R	S	R	S	R	S	S	R	S	R	R	S	S	S	R	I	R	R
51	I	S	S	S	R	S	R	S	S	R	S	R	R	S	S	S	R	I	R	R
52	I	R	R	S	R	S	R	S	S	R	S	R	R	S	S	S	R	I	R	R
53	S	R	R	I	R	I	R	I	S	R	R	R	R	I	R	S	R	S	R	R
54	I	R	R	S	R	S	R	S	S	R	S	R	R	R	S	S	R	I	R	R
55	S	R	S	S	R	I	R	S	S	R	R	R	R	I	R	R	R	S	R	R
56	S	R	R	S	R	S	R	S	S	R	I	R	R	S	S	S	R	S	R	R
57	I	R	S	I	R	S	R	S	S	R	R	I	R	S	R	S	S	S	R	R
58	S	R	R	S	R	I	R	S	S	R	R	R	R	S	R	R	R	S	R	R
59	I	R	S	I	R	S	R	I	S	R	I	I	R	S	R	S	R	I	R	R
60	S	R	S	S	R	S	R	S	S	R	S	R	R	I	S	S	R	S	R	R
61	S	R	R	R	R	I	R	S	S	R	R	R	R	R	R	R	R	S	R	R
62	S	R	S	I	R	R	R	S	S	R	I	R	R	S	I	S	I	S	R	R
63	S	R	R	S	R	R	R	S	S	R	S	R	R	S	S	S	R	S	R	R

Table 19: Continued…

	Antibiotic resistant profile																			
Isolate	**CIP**	**VA**	**AMC**	**C**	**OX**	**TE**	**AMP**	**AK**	**CN**	**FOX**	**DA**	**E**	**P**	**SXT**	**RD**	**IPM**	**KZ**	**NOR**	**MY**	**AZM**
64	S	R	R	I	R	I	R	S	S	R	R	R	R	S	R	S	R	S	R	R
65	S	S	S	I	R	S	R	S	S	R	I	R	R	S	S	S	I	S	R	R
66	S	S	R	I	R	R	R	S	S	R	S	R	R	R	R	R	R	S	R	R
67	S	R	R	I	R	I	R	S	S	R	R	R	R	I	R	S	R	S	R	R
68	S	R	R	S	R	R	R	S	S	R	R	R	R	I	R	S	R	S	R	R
69	S	R	R	S	R	R	R	S	S	R	R	R	R	S	R	S	R	S	R	R
70	S	R	R	S	R	I	R	S	S	R	R	R	R	I	R	S	R	S	R	R
71	S	R	R	S	R	I	R	S	S	R	R	R	R	S	R	S	R	S	R	R
72	S	S	R	I	R	R	R	R	R	R	R	I	R	S	R	S	R	R	R	I
73	S	R	S	S	R	S	S	S	R	R	I	I	R	S	R	S	R	R	R	I
74	S	R	R	S	R	R	R	S	S	R	R	R	R	S	R	I	R	S	R	R
75	S	S	S	S	R	S	R	S	S	R	S	R	R	R	S	S	R	S	R	R
76	S	R	R	S	R	I	R	S	S	R	R	R	R	I	R	S	R	S	R	I
77	S	R	R	S	R	S	R	S	S	R	S	R	R	R	I	S	R	S	R	R
78	S	R	R	S	R	S	R	S	S	R	S	S	R	S	S	S	R	S	R	S
79	I	R	R	S	R	S	R	S	S	R	R	R	R	R	R	S	R	S	R	S
80	S	S	S	S	R	S	R	S	S	R	S	R	R	R	S	S	R	S	R	S

Key: AK- Amikacin; AMC- Amoxicillin/clavulanic acid; AMP- Ampicillin; AZM- Azithromycin; C- Chloramphenicol; CN- Gentamicin; DA- Clindamycin; E- Erythromycin; FOX- Cefoxitin; IPM- Imipenem; KZ- Cefozolin; MY- Lincomycin; NOR- Norfloxacin; OX- Oxacillin; P- Penicillin; RD- Rifampicin; SXT- Sulfamethoxazole/trimethoprim; TE- Tetracycline; VA- Vancomycin

Table 20: Virulence and antibiotic resistance genes detected in each isolate

Isolate	Virulence genes				Antibiotic resistance genes					
	hla	*hld*	*lukS/F PV*	*sea*	*mecA*	*aac-aph*	*blaZ*	*ermC*	*msrA*	*tetK*
1	(-)	(+)	(-)	(+)	(+)	(-)	(+)	(-)	(-)	(+)
2	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(-)
3	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(-)	(-)	(+)
4	(-)	(+)	(-)	(+)	(+)	(-)	(+)	(-)	(-)	(+)
5	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
6	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
7	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
8	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
9	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
10	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(-)	(-)	(+)
11	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(-)	(-)	(+)
12	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
13	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
14	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
15	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
16	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
17	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
18	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
19	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
20	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
21	(-)	(+)	(-)	(+)	(+)	(+)	(-)	(+)	(-)	(+)
22	(-)	(+)	(-)	(+)	(+)	(+)	(-)	(+)	(-)	(+)
23	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
24	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
25	(-)	(+)	(-)	(+)	(+)	(+)	(-)	(+)	(-)	(+)
26	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
27	(-)	(+)	(-)	(+)	(+)	(+)	(-)	(+)	(-)	(+)

Table 20: continued…

Isolate	Virulence genes				Antibiotic resistance genes					
	hla	*hld*	*lukS/F PV*	*sea*	*mecA*	*aac-aph*	*blaZ*	*ermC*	*msrA*	*tetK*
28	(-)	(+)	(-)	(+)	(+)	(-)	(-)	(+)	(-)	(+)
29	(-)	(+)	(-)	(+)	(+)	(-)	(-)	(-)	(-)	(-)
30	(-)	(+)	(-)	(+)	(+)	(-)	(-)	(+)	(-)	(+)
31	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
32	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
33	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
34	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
35	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
36	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
37	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
38	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
39	(-)	(+)	(-)	(+)	(+)	(-)	(+)	(+)	(-)	(+)
40	(-)	(+)	(-)	(+)	(+)	(-)	(+)	(+)	(-)	(+)
41	(-)	(+)	(-)	(+)	(+)	(-)	(-)	(+)	(-)	(+)
42	(-)	(+)	(-)	(+)	(+)	(+)	(-)	(-)	(-)	(+)
43	(-)	(+)	(-)	(+)	(+)	(+)	(-)	(-)	(-)	(+)
44	(-)	(+)	(-)	(+)	(+)	(+)	(-)	(+)	(-)	(+)
45	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
46	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)
47	(-)	(-)	(-)	(-)	(+)	(-)	(+)	(+)	(-)	(-)
48	(-)	(-)	(-)	(-)	(+)	(-)	(+)	(+)	(-)	(+)
49	(-)	(-)	(-)	(-)	(+)	(-)	(+)	(+)	(+)	(+)
50	(-)	(-)	(-)	(-)	(+)	(-)	(+)	(+)	(+)	(-)
51	(-)	(-)	(-)	(-)	(+)	(-)	(+)	(+)	(+)	(+)
52	(-)	(-)	(-)	(-)	(+)	(-)	(+)	(+)	(+)	(-)
53	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(+)	(-)
54	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(+)	(-)	(-)

Table 20: Continued…

Isolate	Virulence genes				Antibiotic resistance genes					
	hla	*hld*	*lukS/F PV*	*sea*	*mecA*	*aac-aph*	*blaZ*	*ermC*	*msrA*	*tetK*
55	(-)	(-)	(-)	(-)	(+)	(-)	(+)	(-)	(+)	(+)
56	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(+)	(-)
57	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(+)	(+)
58	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(-)	(+)
59	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(-)	(+)	(+)
60	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(-)	(+)	(-)
61	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(-)	(+)
62	(-)	(-)	(-)	(-)	(+)	(+)	(-)	(-)	(+)	(+)
63	(-)	(-)	(-)	(-)	(+)	(+)	(-)	(-)	(+)	(-)
64	(-)	(-)	(-)	(-)	(+)	(+)	(-)	(-)	(+)	(+)
65	(-)	(-)	(-)	(-)	(+)	(-)	(+)	(+)	(-)	(-)
66	(-)	(-)	(-)	(-)	(+)	(-)	(+)	(-)	(-)	(+)
67	(-)	(-)	(-)	(-)	(+)	(-)	(+)	(-)	(-)	(-)
68	(-)	(-)	(-)	(-)	(+)	(-)	(+)	(-)	(-)	(+)
69	(-)	(-)	(-)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
70	(-)	(-)	(-)	(-)	(+)	(-)	(+)	(-)	(-)	(-)
71	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(-)	(-)
72	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(-)	(-)
73	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(-)	(-)
74	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(-)	(-)	(-)
75	(-)	(-)	(-)	(-)	(+)	(-)	(+)	(+)	(+)	(-)
76	(-)	(-)	(-)	(-)	(+)	(-)	(+)	(+)	(-)	(-)
77	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(+)	(-)	(-)
78	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(-)	(+)	(-)
79	(-)	(-)	(-)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
80	(-)	(-)	(-)	(-)	(+)	(-)	(+)	(+)	(-)	(-)