ANTIBIOGRAM AND VIRULENCE DETERMINANTS OF *PSEUDOMONAS* AND *LEGIONELLA* SPP. RECOVERED FROM TREATED WASTEWATER EFFLUENTS AND RECEIVING SURFACE WATER IN DURBAN

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

Signed:_____ **Name:** _____ **Date:**_____

PREFACE

The experimental work described in this dissertation was carried out in the School of Life Sciences, University of KwaZulu-Natal (Westville Campus), Durban, South Africa from February 2013 to December 2014, under the supervision of Professor A. O. Olaniran.

These studies represent the original work by 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 has been duly acknowledged in the text.

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Details of contributions 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 authors to the experimental work and writing of each publication).

Publications 1:

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CHAPTER 2: ANTIBIOGRAM AND VIRULENCE DETERMINANTS OF *PSEUDOMONAS* SPP. RECOVERED FROM TREATED WASTEWATER EFFLUENTS AND RECEIVING SURFACE WATER IN DURBAN

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1.1 Introduction

Water is South Africa's most essential resource, situated mainly in a semi-arid part of the world. The country's water resources are, in universal terms, rare and tremendously restricted and a key environmental problem facing the country is water pollution. Changes in climate conditions continue to put more strain on the water resources, and some communities solely rely on recycled water as an alternative to other water sources (Wen *et al.,* 2009). Many sources from industries, mining, effluents from municipalities, biocide runoffs, agriculture, urban areas and informal settlements (poor sanitation), and all these sources result in pollution, but don't result in treatment price increments, but they lead to the introduction of various pathogenic organisms to the water. The presence of such organisms may lead to waterborne disease outbreaks that may cause worse socio-economic implications (Mwabi *et al*., 2011). Universal needs, such as access to clean and pathogen-free water, good sanitation means form part of the most basic need human require on a day-to-day basis. If such needs are being me, this would reduce the level of health issues which are linked to unavailability of safe water. South Africa, as a developing country is also experiencing rapid, unavailability the essential sources such as access to safe water and good sanitation disposal services, are still the world's greatest concern. Highly populated areas seem to encounter such problems on a daily basis as majority of the residents are low-wage earners. Over half a billion of people residing in urban areas and more than 2 billion people residing in rural areas lack adequate sanitation facilities (UNEP, 2002). Regardless of the struggles for the past two decades, less attention has been paid to the sanitation division, and this had led to more attention being drifted to wastewater treatment as an option. Low priority has been given to such problems, thus more problems are being created. In developing countries approximately 5 % of its wastewater receives any sort of treatment before it is being discharged into the environment (UNEP, 2002), and 87% of the world's population use such water for their day-to-day activities from upgraded sources (WHO, 2004).

Wastewaters have different levels of contamination from different sources, such as those from the environmental pollutants and other contaminants, owed to their chemical and microbiological features (Bohdziewicz and Sroca, 2005). Different chemical quality variables such as chemical oxygen demand, salts and a wide range of metals are of great concern of in municipal wastewater. The organic nitrogen and phosphorus elimination scheme remains the widely examined chemical quality for municipal wastewater treatment. Surface waters can be contaminated by unnecessary nutrients in wastewater, and may lead to eutrophication. Environmental conditions that enhance the growth of toxin-producing bacterial lead to contamination of freshwater sources. A wide range of health related infections are caused by the toxins excreted by cyanobacteria, and these include gastroenteritis, nervous system disorders, liver damage and cause skin problems. Animals and humans can be greatly affected by the toxins excreted by cyanobacteria thus causing liver cancer (Chorus and Bartram, 1999).

Wastewater pathogens that most often cause disease include *Salmonella* spp., *Shigella* spp., pathogenic *Escherichia coli*, *Vibrio cholerae*, *Yersinia enterocolitica, Campylobacter jejuni*, Hepatitis A viruses, *Giardia* spp., *Cryptosporidium* spp., and *Entamoeba histolytica* (WHO, 1993). Worldwide distribution of such pathogens lead to higher rates of outbreaks especially in areas where access to safe water is of limited and sanitation services are of poor quality (UNEP, 2002). Most of these pathogens are distributed worldwide but outbreaks occur more frequently and endemicity is higher in areas where access to good quality water supplies and sanitation is limited (UNEP, 2002). In contrast, developing countries remain to experience an overwhelming rise of illness and death in several areas of the over populated worldwide. Approximately 2.1 million people die each year from diarrhoeal diseases and the communal of these deaths are amongst children in developing countries and 65% of these mortalities can be prohibited by water and sanitization interferences (WHO, 2002).

Use of inadequately treated water result in biological health risks associated with drinking water in developing parts of the world (Odjadjare *et al*., 2010) to irrigation reuse of treated wastewater (Blaise and Yongsi, 2010) and recreational water contact. Drinking water poses great health risk; but suck risks can be avoided by effective treatment of water, and adhering to safe drinking water standards (Zilbermana *et al.,* 2008). Water resources are being under stress as human population increases and standard of living of peoples continued to grow (Zilbermana *et al*., 2008). Increasing stresses for water to supply agriculture, industry and cities remain the major competition over the distribution of the limited freshwater resources (Zilbermana *et al.,* 2008). Eighty percent of the population in South Africa rely on surface water for their day-day activities (Venter, 2001). Microbiological pollution of South Africa's water resources is becoming an increasing hazard (Momba *et al.,* 2006). The main causes of contamination of water sources such as rivers, dams, groundwater as well as drinking water are: i) discharge of moderately treated sewage or sewage leakage ii) leakage of unmaintained septic tanks iii) inappropriate supervision of farm waste and iv) run-off of faecal matter during rainy periods (D'Agata *et al.,* 2004).

1.2 Current status of water quality in South Africa

South Africa is faced with a water supply disaster triggered by a mixture of low rainfall, high population increases and growing economy, whose demands for water do not conform to the supply of usable water supplies. More than 95% of the nation's freshwater resources had already been allocated, in 2005. The water quality of these resources has deteriorated due to growing pollution triggered by industry, mining and urbanization (Lashkaripour and Ghafoori 2011). Worsening issues are South Africa's invalid and insufficient water treatment and sewage treatment plant substructure and untrained operators (Mema, 2010) are of great concern for human health are the incidence of transport and fate of pollutants in the aquatic environment. The main difficulties are health-threatening microorganisms, lethal metals and

organic compounds. Pollution of groundwater by lethal and tenacious compounds can cause permanent contamination, persuading water operators long later when the innovative release to the environment has stopped.

Given the existing and expected upcoming growth rates of the population and predictable trends in socio-economic growth, South Africa's water resources are unlikely to withstand modern patterns of water use and waste discharge even with zero population development in South Africa, contaminants will remain to accumulate in freshwater schemes. Declining water quality can poorly disturb human health and the aquatic ecosystem and this has serious implications on various economic sectors such as industry and agriculture (Zilbermana *et al.,* 2008). South Africa is a water-scarce country with inadequate typical rainfall of about 450 mm/yr and unequally dispersed water resources. South Africa's dam storage capacity is 66% of this capacity with the dependable surface-water yield at 82% of its highest capacity under existing rainfall and evaporation conditions (FAO, 2003).

1.3 Microbial contaminants in wastewater

Wastewater treatment plants release significant quantities of pollution-indicator and pathogenic bacteria, resulting to the decline in quality of water sources (FAO, 2003; Momba and Mfenyana, 2006). South African General and Special Standards specify that treated sewage effluent ought to comply with a standard of zero faecal coliforms/100 ml. Failure to adequately treat and manage wastewater and excreta world-wide is linked to the major health and ecological properties (WHO, 2004). Faecal pathogens vary depending on the density and prevalence of health hazards in many communities. Raw sewage is reported to have the highest levels of pathogens worldwide (FAO, 2003). Table 1.1. displays the several classes of microbial pathogens which originate from untreated wastewater and their related diseases.

Table 1.1: Pathogens commonly found in Untreated Municipal Wastewater (DWAF, 2004)

Agent	**Disease**
Bacteria	
Campylobacter jejuni	Gastroenteritis, long term sequelae (*e.g*. arthritis)
Escherichia coli	Gastroenteritis
E. coli 0157:H7	Bloody diarrhoea, haemolytic uremic syndrome
Helicobacter pylori	Abdominal pain, peptic ulcers, gastric cancer
Legionella pneumophila	Legionnaire's disease
Leptospira (spp.)	Leptospirosis
Salmonella (various serotypes)	Salmonellosis, long term sequelae (*e.g*. arthritis)
Salmonella typhi	Typhoid fever
Shigella (3 serotypes)	Shigellosis (dysentery), long term sequelae (*e.g*. arthritis)
Vibrio cholerae	Cholera
Yersinia enterocolitica	Yersiniosis, long term sequelae (*e.g*. arthritis)
Protozoa	
Balantidium coli	Balantidiasis (dysentery)
Cryptosporidium parvum	Cryptosporidiosis, diarrhoea, fever
Cyclospora cayetanensis	Persistent diarrhoea
Entamoeba histolytica	Amoebiasis (amoebic dysentery)
Giardia lamblia	Giardiasis
Viruses	
Adenovirus (many types)	Respiratory disease, eye infections
Norovirus (several types)	Gastroenteritis
Enteroviruses (various types)	Gastroenteritis
Coxsackie A	Herpangina, aseptic meningitis, respiratory illness
Coxsackie B	Fever; paralysis; respiratory, heart and kidney disease
Norwalk virus	Gastroenteritis
Hepatitis A and E virus	Infectious hepatitis
Parvovirus (several types)	Gastroenteritis
Rotavirus (Groups)	Gastroenteritis

1.4 Waterborne disease outbreaks

An outbreak can be defined as an increase of cases of a particular infection above what would be normally expected (WHO, 2004). The possible human exposure to the pathogens accountable for triggering enteric disease is ever-present because of the closeness of humans to sources inadequately discharged waste. Exposure to pathogens can be elevated by different environmental conditions, thus resulting in various pathogen related infections. The prevalence of pathogens can be the main cause, thus become more significant because of an improved source of pathogen amount or improved virulence of the pathogens. Immunocompromised individuals are more at risk of diseases caused by such pathogens (Craun, 1991).

Treatment of water and waste is often non-existent or completely inadequate and until hygiene services are upgraded it will be impossible to influence significantly on the level of pathogen-related illnesses in developing countries. Insufficient treatment and delivery systems, have influences on source water and the emergence of resistant and virulent pathogens thus posing threat to human health in developed countries such as:

- Low resistance to pathogens, ensuing advanced susceptibility and risk of disease during systems failure
- Anthropogenic variations of water systems that have promoted eutrophication, variations in food chain assembly and unlimited growth of undesirable species,

  creating breeding sites for vector-borne diseases

- Changes in production methods (agricultural), with high-density animal processes carried out in areas near urban areas, leading to increased levels of transmission of pathogens from animals to humans (Craun, 1991).

1.5 Wastewater pathogens and their related diseases

Human and animal faeces are comprised of various pathogens which come in contact with water along certain paths. Excretion in water bodies is the major path for transferring pathogen which are carried by overland flow and/or subsurface water current. Human contamination and insufficiencies at water treatment plants have been associated with waterborne outbreaks on a large scale while most current waterborne outbreaks are associated with other water bodies such as swimming pools and rivers and dams (Okoh *et al.,* 2007; Putignani and Menichella, 2010).

1.5.1 *Salmonella*

Salmonella are rod shaped Gram negative bacteria comprising more than 2000 known serotypes that are members belonging to the family *Enterobacteriaceae*. These serotypes are virulent towards humans and can be the source of diverse symptoms from mild gastroenteritis to severe illness or death (Akinbowale *et al.,* 2007). There are considerable regional variations in the incidence of the serogroups (Atwill *et al.,* 2012). Majority of *Salmonella* originate from animals predominantly of poultry. The pathogenicity of *Salmonella* species differs in terms of serotype, density of organism as well as on host status (Zhang *et al.,* 2008). Numerous serogroups are the sources of infection like gastroenteritis with diarrhea in vulnerable persons (Atwill *et al.,* 2012).

1.5.1.1 Implication in Drinking Water

Salmonella is likely to contaminate surface water receiving domestic sewage discharge, abattoir wastes or farmyard wastes. Food contaminated with *Salmonella* can result in infection after 12-36 hrs of ingestion (Atwill *et al.,* 2012). *S. typhi* causes the world greatest waterborne infection epidemics (Lin *et al*., 2009). Humans and animal infected with

Salmonellae can be the main source of spread of contamination through water. Due to fecal contamination of water, waterborne diseases can arise more commonly by *Salmonella* spp: Environmental distribution of major diseases is frequently associated with the waterworks scheme of pipeline systems. Drainage or flooding of contaminated surface water can aid in the transfer of *Salmonella* into insecure well channels (Lin *et al*., 2009). Turbidity must be of low quantity in drinking water if proper chlorination is required. Polluted sediments are the major causes of various outbreaks in the distribution system (WHO, 2002). Developed nations practice of discarding of slaughterhouse waste in water bodies which is the major source of *Salmonella* infection (Lin *et al*., 2009).

1.5.1.2 Disease Caused by *Salmonella*

Salmonella infections in human have increased significantly in the last 20 years in the developed countries (Zhang *et al.,* 2008). In United States, about 2-4 million cases in Unite States of salmonellosis arise each year (Cabral, 2010), 70% of these are arising during travelling. Approximately 12.5 million people from poor countries are being affected annually by enteric fever (FAO, 2003). During 1977-1986, Chile experienced a substantial typhoid fever; which was caused by ingestion of contaminated vegetables that had been irrigated with contaminated wastewater (Ntengwe, 2005). In Pakistan during 1994, 100 cases of typhoid fever related with polluted drinking water were reported (Libralato *et al.,* 2012).

1.5.2 *Escherichia coli*

The excreta of humans and animals are composed of *E.coli*. *E. coli* are Gram negative, rod shaped, aerobic or facultative anaerobic, non- spore forming and has the ability to ferment sugars such as glucose and lactose. All strains are known to be catalase; nitrate and oxidase negative (Momba *et al*., 2006). There are six classes of E. coli strains which are known to be pathogenic, and majority of these strains are implicated in waterborne diseases (FDA 2002).

1.5.2.1 Implication in Drinking Water

Habitants of various environmental niches are coliform bacteria and *E. coli* also forms part of these habitants. *E. coli* strains are known to colonize watery surfaces such as plants and water. Dumping of waste inappropriately may cause *E. coli* strains to enter the human body through fluids thus causing infection. *E. coli* are known to be thermotolerant, thus the presence of fecal coliforms in water may be an indication of pollution either from human or animal sources (WHO 2002).

1.5.2.2 *Diseases Caused by E. coli*

The emergence of the enterohemorrhagic *E. coli* O157:H7 is a great threat to public health. In the Unites States more than 20,000 cases of infection and about 250 deaths are being reported annually (Nicolai, 2002). Children less than 5 years are greatly infected with *E. coli* strains thus leading to high epidemic rates (FAO, 2003; Mena and Gebra, 2009). Enteric *E. coli* pathogens have been previously isolated from patients with diarrhea. An outbreak of E. coli O157 has been reported in contaminated water leading to serious infections such as gastroenteritis. High morbidity and death rates among children in third world countries are linked to diarrheal diseases caused by *pathogenic E. coli*. More than 200 people from Kenya have been reported with diarrheal diseases caused by the pathogenic *E. coli* linked to the consumption of contaminated drinking water (Nicolai, 2002).

Pathogenic strains of *E coli* O157:H7 have been detected in environments which have been contaminated by these pathogens, and the presence of these strains in contaminated meat, drinking eater is still a major concern for public health, as they may pose serious implications on human health (Samie *et al*., 2009). Enteroinvasive strains of *E. coli* are capable of producing endotoxins which enable colonization and attachment in the small intestines (Nicolai, 2002). Enteroaggregative *E. coli* (EAggEC) are a group of *E. coli* characterized by

the capability to attach to cultured cell monolayers with an aggregative adhesion phenotype. Epidemiological studies on Enteroaggregative *E. coli* suggest that these stains are a major cause of prolonged diarrhea in children and may cause diarrhea in adults (Xi *et al*., 2009).

1.5.3 *Vibrio cholerae*

Vibrio species are Gram negative, motile, having monotrichous flagellum, comma shaped morphology, non-spore forming members of family *Vibrionaceae.* The members of this family are aerobes and facultative anaerobes. They are oxidase, catalase and nitrates positive. From the medical opinion, exceptional thought has been given to the individuality of those strains which are associated to cholera. Previously, *Vibrio cholerae* were classified on the basis of O1 antigens (WHO 1996), but later the *Vibrio* was divided into two i.e. 1.–classical" and 2. –El Tor" biotypes.

1.5.3.1 Implication in Drinking Water

Surface water is normal habitat for *V. cholerae* where it replicates and survives in association with other organisms. Cholera as an illness can multiply moreover by fecal contamination of food and drinking water by independent spread of the sequence of *V. cholerae.* There is increasing evidence that ecosystem plays a crucial role in regulation of its virulence genes. Environmental stress allows *Vibrio cholerae* become a pathogen by horizontal gene transfer. Dissemination of the bacteriophage which harbors the cholera toxin is influenced various environmental conditions (Boles *et al*., 2004). Consequently the isolation of *V. cholerae* O1 from drinking water has an alarming implication (WHO 2002). It is a well-established fact that cholera is a waterborne disease (WHO 2000); however food borne infection and person to person transfer has also been reported. Venter *et al.* (2001) and Obi *et al.* (2006) found that in several environmental conditions *V. cholerae* as well as *V. cholerae* serotype O1 is part of the indigenous flora of waters.

1.5.3.2 Diseases Caused by *Vibrio* spp.

V. cholerae cause infections through the consumption of water and food that has been contaminated by such species. *V.* cholerae has got a pilus that helps in the colonization of the small intestines thus producing the toxin. Enterotoxins produced by the serogroups O1 are the main causes of infectious diseases by causing an onset diarrhea. Stools that are watery and contain blood can also be caused by non- serogroups O1. This bacterium can also be the source of wound infections and bacteremia (Nicolai, 2002). Incidences of cholera have re-emerged as possible infectious diseases in the past and this has affected the world globally (Cabral, 2010). Ninety- four countries have been reported with high incidences in 1994 of cholera, this marks it as the higher number that has even been reported in only just 1 year (WHO 1995). Several African countries have faced the cholera epidemic in 2004, which resulted in more than 200 people being killed and more than 10000 being terminally ill. These occurrences witness that ancient opportunists like cholera are still a possible hazard to the public health worldwide predominantly in developing countries (WHO 2004).

1.5.4 *Shigella* spp.

Shigella are non-motile, non-spore-forming rods, Gram negative, strive both in aerobic and anaerobic conditions. Biochemically *Shigella* are capable of fermenting sugars with the production of acid with no gas formation, lactose negative, positive for catalase and nitrate tests , exclusive of *Sgigella dysetriae* serotype. Amongst *Shigella flexneri* the lysogenic bacteriophage is the main cause of antigenic specificity determination (Nicolai, 2002).

1.5.4.1 Implication in Drinking Water

Shigella are usually found in water polluted with human feces (Nicolai, 2002). Owing to its life-threatening pathogenicity levels, this bacterium is of crucial importance to public health.

Inadequately treated water may result in epidemics of bacillary dysentery that are linked with water supply. Such water-borne epidemics are often remarkable in the great numbers for people who are, and this disorder will continue until water supplied to communities is adequately treated (Ntengwe, 2005).

1.5.4.2 Disease Caused by *Shigella* spp.

An estimated 10% to 20% of diarrhea cases caused by *Shigella* have been reported annually for 0.3 million people in the US. Extremely high epidemic rates of *Shigella* spp. have been reported annually, with more than 150 million people being infected whist 0.65 million deaths (Nicolai, 2002). Children below the age of then are at high risk, and more prone to infection caused by *Shigella*. Ingestion of food that has been contaminated with this bacterium may develop shigellosis after 24-72 hrs (WHO 2000). Areas with poor sanitation services are more prone to *Shigella* outbreaks resulting in bloody diarrhea. In Africa, two major outbreaks have been reported occurring in Southern Africa and Central America. Rwandan immigrants in Zaïre were greatly affected by this bacterium which led to nearly 20,000 deaths in just 1 month only in 1994. In East and West African areas, *Shigella* epidemics were report during 1999 and 2003 (WHO, 2004). Bacteremia and dispersal are incomparable; signs and symptoms include bloody diarrhea, high fever, headache and abdominal pain (Nicolai, 2002). Antibiotics are regularly used in the course of the disease (Bole, 2004).

1.5.5 *Pseudomonas aeruginosa*

P. aeruginosa are Gram negative, rod shaped, motile with monotrichous flagella, capable of fermenting sugars, oxidase, catalase and citrate positive belonging to the family *Pseudomonadaceae*. *Pseudomonas* specie are capable of thriving in environmental conditions

of high temperatures. Production of a blue-green fluorescent color is the most obvious feature for the identification of *P. aeuginosa* (Igbinosa *et al.,* 2012).

1.5.5.1 Implication in Drinking Water

Pseudomonas are capable of growing in water with only traces of nutrient, e.g. tap water, this aids in its proliferation in hospital environments (Igbinosa *et al*., 2012). *P. aeruginosa* are capable of surviving both in inorganic and human milieus, thus it has been considered as a ubiquitous microorganism. In inorganic settings *P. aeruginosa* is commonly identified in aquatic tanks contaminated by bodily and human waste such as manure and descends inside the hospital. *Pseudomonas aeruginosa* are commonly found in moist areas such as feces, soil, water, sewage (Nikbin *et al*., 2012). In waters supplemented with biological material, *P. aeruginosa* proliferates and its manifestation designates the quality concerning cleanliness of the water distribution systems (Mema, 2010). The manifestation of *P. aeruginosa* in drinking water alters the odor and color of the water (WHO 2004). Furthermost infections triggered by *P. aeruginosa* are water related. Consumption of water of food contaminated with *Pseudomonas aeruginosa* can lead to serious (Nikbin *et al*., 2012).

1.5.5.2 Diseases Caused by *P. aeruginosa*

This microbe is most widespread in the hospital settings. Disease onset proliferation amongst indoor patients equivalently to the hospitalization period (Odjadjare *et al*., 2012). Hospital acquired infections are mainly caused by these species which are obtained from the research material and working utensils. Diseases caused by *P. aeruginosa* comprise rashes, pustules and ear infections particularly in swimming pools which are not disinfected regularly. In water delivered to hospital and in the production of pharmacological measures, *Pseudomonas aeruginosa* acquires contact to operation lesions and it can infect eye droplets thus causing serious infection (Okoh *et al*., 2007).

1.5.6 *Legionella*

In 1976, Legionnaires' disease was first described in Philadelphia. The disease is caused by the bacterium *Legionella pneumophila* belonging to the family Legionellaceae. The generic term "legionellosis" is now used to describe these bacterial infections, which can range in severity from a mild, febrile illness to a rapid and possibly lethal pneumonia. *Legionella* has been known as the causative agent for epidemics of Legionnaires' disease since 1947. Legionellosis arose due to human variation of the environment, since *Legionella* species originate from aquatic settings, and flourish in warm aquatic environments Wullings and van der Kooij 2006).

1.5.6.1 Implication in Drinking Water

Legionellae are capable of thriving in wide range of aquatic conditions, from low to high temperatures and pH ranges between 5.0 to 8.5. While proliferation is limited at temperatures between 20 and 43°C (Wullings and van der Kooij 2006). In a study investigating *Legionella* spp. in warm water systems bacterial colonies were counted from temperatures above 56°C. Temperature range from 55°C and 60°C seemed to be a critical temperature for survival in contaminated hot water systems (Darelid *et al*. 2002; Mathys *et al.*, 2008). Though epidemics caused by natural water were mostly related with temperatures higher than 30°C (Lemarchand *et al*., 2004). Paszko-Kolva et al. (1992) reported the possible link between clinical and environmental *L. pneumophila* isolates surviving up to 2 years under low nutrient settings. It was suggested that *L. pneumophila* discovered in cold water systems are survivors from warmer seasons when water temperatures were above 20°C (Wullings and van der Kooij 2006).

Although planktonic *Legionella* spp. have been reported to survive for extended time periods in the environment, their extraordinary ability to interact with other microorganisms is highly

beneficial for survival (Genkai-Kato *et al.,* 2012). They attach to surfaces in aquatic environments and readily form biofilms together with other microorganisms (Kwaik, 1998; Fields, 1996). Apart from providing a protective environment and increasing survival of community members, biofilms allow interaction with other microorganisms.

1.5.6.2 Diseases Caused by *Legionella*

Legionnaires' disease is frequently characterized by anorexia, malaise and lethargy; also, patients may develop a mild and unproductive cough. Nearly half of patients suffer from illnesses related to the nervous system, such as confusion, delirium and depression. These disorders may arise in the first week of the disease. Physical examination may reveal fine or coarse tremors of the extremities, hyperactive reflexes, absence of deep tendon reflexes, and signs of cerebral dysfunction. The clinical syndrome may be more subtle in immunocompromised patients (Nguyen *et al*., 1991).

1.6 Multidrug resistance in *Pseudomonas* and *Legionella* species

Changing population demographics and fluctuations in treatment technology are the major factors which results in the emergence of newly identified pathogens, with those related with the water systems (Lyczak *et al.,* 2000). Emerging waterborne pathogens belong to the ~~"n~~ewly recognized" group; suggesting that, though the etiologic agent was known for a long period, it was accepted only recently major causative agents for waterborne diseases (Sharma *et al*., 2003). *Pseudomonas* species are rated as the emerging waterborne pathogens (Momba, 2010). The *Pseudomonads* comprises species with environmental, economic and health-related importance (Sharma *et al*., 2003). The *Pseudomonads* are capable of adapting to various ecological conditions, such as soil and sewage (Goldberg, 2000). *Pseudomonas aeruginosa* from the *Pseudomonads* are regarded as opportunistic pathogens as they are

associated with various infections, particularly in patients who are immunocompromised (Pirnay *et al*., 2005). Though *Pseudomonads* are not usually known as waterborne pathogens, previous reports suggest that water systems are gradually becoming a preferred interface in the epidemiology of the pathogens. A number of waterborne infections are a result of the use of recreational waters implicated in various epidemics (Mena and Gebra, 2009) shower and pools (Pirnay *et al.,* 2005); thus making the pathogens of growing public health concern.

The occurrence of waterborne *Pseudomonas* pathogens is predominantly worrying to the public health sector for two reasons. Firstly, non-pathogenic bacteria may serve as reservoirs of genetic determinants, when transmitted to other bacterial strains, may result unique virulence abilities (Sharma *et al*., 2003). Secondly, incidence of multiple antibiotics resistant (MAR) *Pseudomonas* strains have been reported in recent reports. D'Agata (2004) detected an increase in the prevalence of MAR isolates denoting resistance to two classes of antibiotics.

There has been no new drugs that have been introduced for the treatment of pseudomonal infections, regardless of the increasing threat of MAR *Pseudomonas* species (Navon-Venezia *et al*., 2005; Alaoui *et al*., 2007). Thus restricting treatment possibilities for Pseudomonas related infections, therefore risking public health. The ability of *Pseudomonas* spp. to form biofilms which aids as a protective mechanism against harsh environmental conditions has led to their predominance as emerging waterborne pathogens (Szewzyk *et al*., 2000). Genetic diversity is achieved when these bacteria grow within a biofilm, thus gaining resistance against antibiotics and other disinfectants (Boles *et al*., 2004).

This clarifies as to why *Pseudomonas* species are gradually get fixed in the water system even after disinfection of water resources. Survival of *Pseudomonas* after chlorine disinfection was recently reported by Samie *et al*. (2009); while Xi *et al*. (2009) corroborated

by Huang *et al.* (2011) suggesting that stress-tolerant bacteria surviving chlorination are more resistant to antibiotics; whereas Shivrastava *et al*. (2004)reported that suboptimal chlorine treatment of drinking water was more selective for MAR *Pseudomonas aeruginosa*. Given the significant correlation between effluent quality and that of the receiving waters (Momba *et al.,* 2006), it would be safe to assume that the discharge of chlorinated wastewater effluents comprising substantial population of *Pseudomonas* species into receiving surface water bodies signify excessive threat to public health. This is the case as a majority of SA's population were reported to depend on these surface water bodies for their day-to-day activities (Venter *et al*., 2001; Mackintosh *et al*., 2003). Moreover, SA's population have the highest prevalence of immunocompromised individual, thus when they are exposed to water that has been previously contaminated with *Pseudomonas*, could lead to serious infections (Obi *et al.,* 2006). However substantial studies have been carried out on several pathogens isolated from wastewater effluents in SA, there is little information on the occurrence and antibiograms of *Pseudomonas* species isolated from municipal wastewater effluents in the Country. Survival strategies of *Pseudomonas* species in water systems, combined with their adaptable nature, it is very likely that these pathogens are in existent in wastewater effluents in SA even after disinfection processes. It is therefore of great importance that the prevalence of these pathogens in wastewater effluents destined to be discharged into SA's waters be monitored so as to protect public health.

Pseudomonas species are Gram negative motile rods and found in various ecological niches. Their capability to use diverse organic compounds as carbon and energy source as well as persistence in the absence of nutrients has been attributed to their genetic versatility which translates into enhanced metabolic activity with exceptional ability to adapt and colonize a wide variety of ecological niches (Samie *et al*., 2009). *Pseudomonas* spp. are so well adapted

in their environment that they survive extremes which includes temperatures ranging from 4 °C to 43 °C, and weak ion concentrations, among others.

L. pneumophila, a Gram-negative bacterium, is responsible for most legionellosis cases. Human infections occur via inhalation of infected aerosols, mainly from man-made aquatic systems (Palmer *et al.,* 1993; Diederen *et al*., 2008). Following the first large outbreak of legionellosis in Philadelphia in 1976, erythromycin was proposed as the drug of choice to treat legionellosis patients. More recently, fluoroquinolone compounds have been proposed as first-line drugs in legionellosis patients (Kuchta *et al*., 1983). However, mortality rates of 10%–15% are usually reported in legionellosis patients and death may occur despite fluoroquinolone therapy. On the other hand, Legionella species are present in most aquatic environments, where they interact with protozoa and where they may be exposed to residual concentrations of these antibiotics (Nguyen *et al*., 1991).

In *L. pneumophila*, in vitro selection of fluoroquinolone resistance has been reported. Resistance has been related to mutations affecting codons 83 and 87 of *gyr*A (*E. coli* numbering), although the last mutation resulted in resistance to only nalidixic acid, whereas MICs of fluoroquinolones remained unchanged (Foysal *et al*., 2011) However, no precise genetic reconstructions were performed to demonstrate their direct involvement in the resistance levels (Huang *et al*., 2011).

1.7 Mechanisms of antibiotic resistance

Three mechanisms are used to categorise resistance to antibiotics: (i) destruction of the antibiotics by bacterial enzymes (e.g. β-lactamases destroy a wide variety of β-lactam antibiotics or modifying enzymes inactivate chloramphenicol, aminoglycosides), (ii) modification of antibiotic targets (e.g. modification in gyrase or topoisomerase IV of quinolone resistance or target modification of vancomycin resistant enterococci) and (iii)

reduction of cell permeability or expulsion of the antibiotics by specific or non-specific efflux pumps systems (e.g. intrinsic resistance of *Pseudomonas aeruginosa*) (Mitscher, 2005; Walsh and Wright, 2005). Resistance to antibiotics by can be intrinsic or acquired. Some bacteria acquire resistance to certain antibiotics such as anaerobic bacteria to aminoglycosides, or Gram negative bacilli, especially *Pseudomonas* spp. to β-lactam antibiotics due to permeability of the outer membrane and efflux pumps (Barbosa and Levy, 2000; Poole, 2004). Antibiotic resistance attributed to the attainment of resistance, intrinsic mechanisms such as efflux pump systems which contribute majorly multiple resistance in bacteria (Alekshun and Levy, 2007). Two mechamisms can aid in the acquisition of resistance: (i) through mutations in the bacterial genome of the organism, and (ii) by means of acquisition of new DNAs from another bacterium through the horizontal gene transfer (Poole, 2004; Levy and Marshall, 2004; Andersson and Hughes, 2010). Low frequencies of bacteria enable the occurrence of spontaneous mutation (Chopra *et al*., 2003). Efficacies of antimicrobial treatments are greatly affected by mutation rates (Gerrish and García-Lerma, 2003). Evolution of antibiotic resistance by bacteria is enhanced by mutations, which play a significant role by selecting for existing resistance determinants or altering drug targets that reduced antibiotic affinities (Chopra *et al.,* 2003). Mutation is recognised as common mechanisms of resistance to synthetic antibiotics due to the lack of natural reservoirs of resistance genes (Woodford and Ellington, 2007).

1.8 Emergence of multiple resistant bacteria

The widespread use of antibiotics in human therapy, and in agriculture and aquaculture has led bacteria to be resistant to multiple antibiotics. Multiple antibiotic resistance by bacteria can be achieved through the two major mechanisms: (i) multiple antibiotic resistance genes accumulation (acquired mechanism), (ii) increased levels of expression of multidrug efflux pumps (intrinsic mechanism) (Alekshun and Levy, 2007; Nikaido, 2009). Multiple mutations

involve acquired mechanisms, typically in genes targeted by the antibiotics, and the transfer of numerous resistance genes through plasmids, bacteriophages, transposons, and integrons through horizontal gene transfer (Alekshun and Levy, 2007). Mutations play a significant role in the emergence of antibiotic resistance not only in acquired mechanisms by changing the targets of antibiotics but also in intrinsic mechanisms by decreasing uptake of drug through outer membrane (Alekshun and Levy, 2007). Dissemination of antibiotic resistance genes via horizontal genetic elements such as plasmids, transposons and integrons are the major mechanisms which play a significant role in multidrug resistance occurring in the environments (White *et al.,* 2001; Alekshun and Levy, 2007). Additional mechanism, such as multidrug efflux pumps (as intrinsic mechanism) also play a major rolein multiple antibiotic resistance in bacteria (Alekshun and Levy, 2007; Higgins, 2007; Nikaido, 2009). Multiple antibiotic resistance is most widespread among Gram negative and positive bacteria (White e*t al.*, 2001; Alekshun and Levy, 2007). Multidrug resistant bacteria continue to be prevalent throughout the world, compromising the treatments of critical infections (Alekshun and Levy, 2007; Nikaido, 2009).

1.9 Mobile genetic elements involved in horizontal transfer of antibiotic resistance genes

1.9.1 Plasmids

Plasmids are small DNA molecule within a cell that is physically separated from a chromosomal DNA and can replicate independently (Frost *et al.*, 2005). Such elements do not contain genes essential for vital cellular functions. Plasmids play a major role in horizontal gene transfer and essential vectors in genetic engineering (Smillie *et al*., 2010). Plasmids are comprised of numerous antibiotic resistance genes and are capable of effective transfer, in both conjugation and transformation mechanisms, among distantly related organisms (Nikaido, 2009; Smillie *et al*., 2010). Plasmids harbour genes encoding resistance to some

classes of antibiotics such as β-lactams, macrolides, aminoglycosides, tetracyclines, trimethoprim/sulfamethoxazole, phenicols and thus are mainly responsible for multiple resistance phenomena (Nikaido, 2009). Additionally, these vehicles contribute to the dissemination of other mobile genetic elements such as transposons or insertion sequences, integrons/gene cassettes (Cambray *et al*., 2010). In general, plasmids play as major driving forces for the horizontal dissemination of antibiotic resistance genes.

1.9.2. Transposons

Transposons are complex transposable elements, which change their position within a genome (Roberts *et al*., 2008).Two copies of insertion sequences are contained within a transposon structure with additional non-insertion function genes (Campbell *et al*., 1979). Transposons, unlike plasmids, are not capable of self-replication and therefore to be maintained, they must integrate into plasmids or chromosomal DNAs in the cell (Schwarz and Chaslus-Dancla, 2001). Transposons cannot attain target specificity, and therefore can excise and integrate into chromosomal DNAs (intracellular transposition) or conjugative plasmids (intercellular transposition) (Schwarz and Chaslus-Dancla, 2001). Transposons play a role in disseminating antibiotic resistance genes and possess mobilising capability (Frost *et al.*, 2005).

1.9.3 Insertion sequences

Insertion sequences (ISs) are small (often < 2.5 kb) segments of DNA that acts like a transposable element (Mahillon and Chandler, 1998). Insertion sequence, harbour only the gene encoding a transposase, responsible for the movement of the element, and two terminal inverted repeats (IRs) (Schwarz and Chaslus-Dancla, 2001). Dissemination of resistance genes and gene acquisition are controlled by ISs (Mahillon and Chandler, 1998).

1.9.3.1 Classes of integrons

Integrons are strongly associated with mobile genetic elements such as conjugative plasmids, transposons (termed mobile integrons or MIs), however, the discoveries of chromosomal integrons (CIs or super-integrons) in the genomes of hundreds of bacterial species have been required more studies on the roles of these elements contributed to host cells (Cambray *et al.,* 2010). To date, five different classes of integrin classes have been recognized based on the sequence of the encoded integrases, which showed 40%–58% identity.

1.9.3.2 Class 1 integrons

Class 1 integrons which are most prevalent and extensively characterised class for *Pseudomonas* spp. were first described by Stokes and Hall in 1989. Nearly all well-known antibiotic resistance gene cassettes originate within this class (Partridge *et al*., 2009). Generally, class 1 integrons are composed of two conserved segments (5'-CS and 3'-CS), which are separated by a variable region containing one or more gene cassettes (Rodríguez *et al.,* 2006). The 5'-CS region, the functional platform, contains the integrase gene (*intI1*), the integration site (*attI1*) and a promoter (Pc) region that allows expression of gene cassettes inserted at the *attI1* site in a suitable orientation. The 3'-CS region of class 1 integrons is comprised of a qacEΔ1 gene (confers resistance to quaternary ammonium compounds), a sul1 gene (confers resistance to sulphonamides) and two open reading frames (orf5 and orf6) of unknown function (Partridge *et al*., 2009).

1.9.3.3 Class 2 integrons

Class 2 integrons are strongly associated with Tn7 transposons and their derivatives (Cambray *et al*., 2010), *Legionella* spp. were belonged to this class of integrons. Forty-six percent similarty integrase genes of class 2 and class 1 integrons between to the sequence of

the class 1 integrase has been noted in a previous study by Hansson *et al*. (2002). Class 2 integrons are most prevalent to members of Enterobacteriaceae and clinical important pathogens worldwide, especially in *E. coli* (Pan *et al.,* 2006; Kim *et al.,* 2010). Class 2 integrons were also found in Enterobacteriaceae isolated from animals, aquatic habitats and environments associated to human impacts (Barlow *et al.,* 2008; Kadlec and Schwarz, 2008). Class 2 integrons usually harboured *ereA* (conferring resistance to erythromycin), *catB2* (chloramphenicol), *aadB* (gentamicin, tobramycin, and kanamycin), *dfr* (trimethoprim), sat (streptothrycin) and *estX (*encoding a putative esterase) of which the cassette array *dfrA1* - *sat1* - *aadA1* was most commonly found (Partridge *et al*., 2009).

1.9.3.4 Class 3, 4, 5 integrons

The first class 3 integron was isolated from a carbapenem-resistant *Serratia marcescens* strain (Arakawa *et al.,* 1995) and characterised (Collis *et al.,* 2002). The other two classes of mobile integrons (class 4 and 5) are associated with trimethoprim resistance phenotypes in *Vibrio* species (Cambray *et al*., 2010).

1.9.3.5 Gene cassettes and cassette arrays

Gene cassettes are small non-replicating mobilisable DNA element that comprises only a single gene and a recombination site (*attC*) (Labbate *et al.,* 2009). Majority of well-known gene cassettes do not have promoter, therefore, their expression depend on the promoter (Pc) of the integrons that they integrated into (Cambray *et al*., 2010). Insertion of one integron results multiple antibiotic resistance by bacteria (Partridge *et al*., 2009). Most cassette arrays described have two or three gene cassettes, which often confer resistance to different classes of antibiotics. Up to nine antibiotic resistance gene cassettes can be harboured on a single class 1 integron. To date, more than 130 different cassettes conferring resistance to almost every known antibiotic were identified (Cambray *et al*., 2010).

1.10 Scope of the current study

The release of bacterial pathogens into the environment after traditional wastewater processes may be due to the lack to set guidelines in South Africa. Wastewater treatment processes only eliminate between 50% and 90% of viruses present in wastewater (Okoh *et al.,* 2010), allowing for a significant release of bacterial pathogens into effluent that is being discharged. Due to their stability and persistence of water-borne pathogens, they subsequently become pollutants in environmental waters resulting in human exposure through pollution of drinking water sources and recreational waters, as well as foods. The performance of wastewater treatment systems is at present monitored largely by the use of bacterial indicator organisms. Considering that pathogens isolated from aquatic environments meet bacterial indicator standards, in some instances in connection with virus related outbreaks (Odjajare *et al*., 2012), the use of bacterial indicators has thus been considered an insufficient tool to monitor wastewater quality because bacterial and viral contaminations are not necessarily associated and linked with each other. Therefore it is of great importance that South African waters be monitored for the presence of pathogens in wastewater effluents in the interest of the environment as well as public health. Adaptation of bacterial pathogens to adverse environmental conditions aids in the acquisition of resistance genes. This study therefore aimed at evaluating the prevalence of *Pseudomonas* and *Legionella* spp. recovered from wastewater effluents and receiving surface water in Durban, and to determine the correlation between antibiograms, virulence determinants of *Pseudomonas* and *Legionella* spp. and their fingerprinting profiles using RAPD-PCR.

1.10.1 Hypothesis

It is hypothesized that treated effluents from wastewater treatment plants in Durban are a major source of pathogenic *Pseudomonas* and *Legionella* spp. in receiving water bodies. It is

further hypothesized that *Pseudomonas* and *Legionella* spp. from these effluents are multi-drug resistant and harbour resistance genes.

1.10.2 Objectives

The objectives of this study were:

1.10.2.1 To determine the prevalence and diversity of *Pseudomonas* and *Legionella* spp. recovered from treated wastewater effluent and the receiving surface water.

1.10.2.2 To evaluate the antibiotic resistance profiles of these emerging bacterial pathogens to the currently used antibiotics.

1.10.2.3 To profile the virulence determinants of these emerging bacterial pathogens.

1.10.2.4 To determine the level of relatedness of selected emerging bacterial pathogens.

1.10.3 Aims

The aims of this study were:

1.10.3.1 To confirm the identity of recovered emerging bacterial pathogens isolated from the treated wastewater effluent samples and receiving surface water using standard biochemical tests and PCR methods.

1.10.3.2 To determine the antimicrobial resistance profiles of these emerging bacterial pathogens using the antibiotic Kirby Bauer disk diffusion method

1.10.3.3 To identify specific resistance genes of the emerging bacterial pathogens using PCR methods

1.10.3.4 To profile virulence determinants of selected isolates using enzyme assays

1.10.3.5 To differentiate between species of the emerging bacterial pathogens using Random Amplified Polymorphic DNA (RAPD) analysis

CHAPTER 2

ANTIBIOGRAM AND VIRULENCE GENE SIGNATURES OF *PSEUDOMONAS* SPP. RECOVERED FROM TREATED WASTEWATER EFFLUENTS AND RECEIVING SURFACE WATER IN DURBAN

2.0 Abstract

Pseudomonas species are opportunistic pathogens implicated on various diseases such as cystic fibrosis and sickle cell anaemia. Since they are regarded as multidrug resistant (MDR) and extremely drug resistant (XDR) bacteria, *Pseudomonas* species signify a great threat to public health. This study investigated the prevalence of *Pseudomonas* spp. recovered from treated wastewater effluents and the receiving surface water. In addition the antibiogram and virulence factors of these isolates were determined to establish the potential dangers of these receiving surface waters to the users. The antibiograms of the *Pseudomonas* species show that all isolates (100%) were susceptible to gentamicin, ciprofloxacin, cefotaxime and cephalothin. Conversely, all isolates (100%) were resistant to penicillin, oxacillin ampicillin, vacomycin, sulphamethoxazole, , trimethoprim, erythromycin, nitrofurantoin and rifamycin. Varied resistances were observed against Chloramphenicol as isolates showed the following resistance regimes; 100% (*P. putida*), 68% (*P. aeruginosa*) and 67% against (other *Pseudomonas* spp.). Isolates showed varied susceptibilities to minocycline in the order 100% (*P. putida*), 60% (other *Pseudomonas* spp.) and 20% (*P. aeruginosa*). The antibiotic resistance pattern with respect to Nalidixic acid was 50% (*P. putida*), 94% (*P. aeruginosa)* and 92% (other *Pseudomonas* spp.). The Multiple antibiotic resistance index (MAR) for *Pseudomonas* spp. ranged between 0.55 - 0.83, with 80% of the isolates showing phenotypic resistance to beta-lactam antibiotics. The screening for antibiotic resistance genes revealed the absence of *blaOXA* and *blaampC* genes in all the *Pseudomonas* isolates. However, Integron conserved segment was detected in 13 % of *P. aeruginosa* isolates. Virulence factor determination revealed that 61% of the *Pseudomonas* spp. were capable of producing rhamnolipid as a biosurfactant, while 76% and 22% of the isolates were positive for elastase and protease, respectively. Results obtained from this study reveal that the majority of these *Pseudomonas* species could pose a threat to public health since they harbour some antibiotic resistance genes, which could be transferred to susceptible strains limiting the treatment options. Class1 integrons were detected as the most abundant type of integron in these *Pseudomonas* spp. isolates, with $bla_{\text{IMP-1}}$, $bla_{\text{VIM-1}}$ genes detected in MBL-positive isolates. Gene cassettes *dfrA1/AadA1a* and *aac6* were the two types of arrays detected in integrin positive isolates which harboured gene cassettes.

2.1 Introduction

Water is perhaps South Africa's most critical resource, since the country is located largely in a semi-arid part of the world. The country's water resources are, in global terms, scarce and extremely limited and a key environmental problem facing the country is water pollution (Lyczak *et al.,* 2000). Access to safe water and to sanitary means of excreta disposal are universal needs and, indeed, basic human rights (WHO, 2004; GDS, 2012). Safe water and sanitation are essential elements of human development and poverty alleviation and constitute an indispensable component of primary health care (Fatoki *et al.,* 2003).

As climate change continues to place stress on water resources, communities are increasingly looking towards recycled water as a supplementary source of water (Wen *et al*., 2009). Water pollution do not only result in an increase in treatment costs, but it may also introduce a wide range of infectious organisms to water resources such as *Pseudomonas* spp. which are opportunistic pathogens and a threat to human health (Goldberg, 2000; Okoh *et al.,* 2007). The presence of such organisms may lead to waterborne disease outbreaks with serious socio-economic implications. Surface water sources are of poor quality as they can contain pathogenic microorganisms and unsafe levels of other pollutants (Odjadjare *et al*., 2010; Okoh and Igbinosa, 2010). Sources of pollution in surface water include agricultural, domestic and industrial, resulting from the absence or inefficient wastewater treatment plants (WWTPs) (Xi *et al.,* 2009; Odjadjare *et al.,* 2010). Inefficient WWTPs intensify the risks of waterborne infections and negative impact to aquatic life (Jung *et al*., 2004). Since 1956, South Africa made it mandatory through the South African Water act that the effluent be treated to acceptable standards prior to return to the water course from where water was originally obtained (Shrivastava *et al*., 2004; Mema, 2010; Igbinosa *et al*., 2012).

Wastewater treatment facilities have become more cautious in ensuring the discharges of high quality wastewater effluents into receiving water bodies and to ensure a healthier environment (Iwane *et al.,* 2000). Due to massive worldwide increases in human population, water has been predicted to become one of the scarcest resources in the 21st century, and despite large advances in water and wastewater treatments, waterborne diseases still pose a major threat to public health, worldwide (Knezevic and Petrovic, 2008). Several questions have been raised on the capacity of current wastewater treatment systems to remove pathogens from wastewater with many waterborne diseases linked to supposedly treated water supplies (Lin *et al*., 2009). The release of untreated or inadequately treated municipal wastewater effluents may put public health at risk from drinking water contaminated with pathogenic bacteria, protozoans and several toxic substances (Paillard *et al*., 2005).

The detection, isolation and identification of the many different types of microbial pathogens associated with wastewater would be difficult, time consuming and hugely expensive if attempted on a regular basis (Sharma *et al.,* 2003; Li *et al*., 2009). To avoid these, indicator microorganisms are used to determine the relative risk of the possible presence of pathogenic microorganisms in a sample (Ashbolt *et al*., 2001; Okoh *et al.,* 2007). To function effectively as indicators, such microorganisms should be a member of the intestinal microflora of warm-blooded animals; should be present when pathogens are present, and absent in uncontaminated samples; it should be present in greater numbers than the pathogen(s); should be at least equally resistant as the pathogen to environmental factors and to disinfection in water and wastewater treatment plants; it should not multiply in the environment; it should be detectable by means of easy, rapid, and inexpensive methods, and the indicator organism should be non-pathogenic (Bitton, 2005).

Emerging waterborne pathogens constitute a major health hazard in both developed and developing nations (Lin *et al.,* 2004). Change in population demographics and changes in

treatment technology are the main factors leading to the emergence of new pathogens such as *Pseudomonas* spp., including those associated with water systems (Lachmayr *et al*., 2009). *Pseudomonas* species are rod-shaped, Gram negative aerobic bacteria, which can colonise a wide range of ecological niches. They can proliferate in environments with low and high nutrient levels such as sewage and the human body (Nseir *et al.,* 2011). *Pseudomonas* spp. have an exceptional ability to adapt to and colonise a wide variety of ecological environmental conditions. Infections by *Pseudomonas* spp. are often difficult to treat because of its virulence and the relatively limited choice of effective antimicrobial agents (Pitts *et al*., 2003; Alaoui *et al*., 2007; Igbinosa *et al.,* 2012).

The dissemination of resistance genes among environmental and commensal bacteria is most commonly caused by acquiring resistance through horizontal gene transfer (Davies, 1994; Davies and Davies, 2010). Horizontal gene transfer has a significant role in bacterial evolution, especially for the widespread distribution of antibiotic resistance genes. However, integrons, defective transposon derivatives, which are commonly harboured on plasmids and transposons, have emerged with multiple resistance features in environmental bacteria, due to the capability of mobilisation and expression of antibiotic resistance gene cassettes (Collis and Hall, 1992; Collis and Hall, 1995; Kang *et al*., 2005). There are five different classes of mobile integrons which have been identified to date, besides chromosomal integrons, which are usually not involved in resistance phenotypes (Agersø and Sandvang, 2005; Henriques *et al.,* 2006; Cambray *et al.,* 2010). They are distinguished by variations on the sequence of the encoded integrases (40%-58% identity). Classes 1 and 2 are most common in multiple resistant bacteria and they are of concern due to their capability of mobilisation to spread between intraspecies and interspecies (Partridge *et al.,* 2009). Many cassettes could be integrated into integrons causing multiple resistance. Most cassette arrays described have two or three gene cassettes, which often confer resistance to different classes of antibiotics. The

most number of antibiotic resistance gene cassettes carried by a single class 1 integron was reported of nine. To date, more than 130 different cassettes conferring resistance to almost every known antibiotic have been identified (Chen *et al.,* 2013).

In South Africa, studies on integrons and resistance gene transfer mechanisms have been conducted mainly in clinical isolates (Iversen *et al*., 2003; Lee *et al.,* 2006; Vo *et al*., 2010). Very limited studies have been published on non-human isolates such as food- borne pathogens (Chen *et al*., 2013; Aarts and Margolles, 2015), animal pathogens (Vo *et al*., 2010; Aarts and Margolles, 2015) and water-borne pathogens (Ribeiro *et al.,* 2013). Limited information exists on molecular antibiotic resistance and resistance transfer mechanisms of aquatic environmental bacteria in South Africa, Aquatic bacterial resistance and multiple resistance profiles from different environments, areas and/or countries are distinct by virtue of farming practices and health management (Peres *et al*., 2007), therefore, it is significant to understand resistance mechanisms and transferability of commensal bacteria from wastewater in South Africa to have better control of the spread of antibiotic resistance (Gad *et al.,* 2007; Tu *et al*., 2008).

Substantial amount of studies has been carried out on numerous pathogens isolated from wastewater effluents in SA, there is little on the predominance and antibiogram of *Pseudomonas* species isolated from wastewater effluents in the country. Survival mechanisms of *Pseudomonas* species in water systems, combined with their opportunistic features, it is very likely that these pathogens are present in wastewater effluents in South Africa even after disinfection processes. It is therefore of great importance that the presence of these pathogens in wastewater effluents meant to be discharged into South African waters be monitored so as to protect public health. This study therefore aimed at investigating the prevalence and antibiogram profiles of *Pseudomonas* species isolated from two wastewater treatment plants in Durban, South Africa.

2.2 Materials and Methods

2.2.1. Description of wastewater treatment plants used in this study and source of isolates

A of total 125 *Pseudomonas* species previously isolated from treated effluents of two wastewater treatment plants namely; Northern wastewater treatment (29°48′45.62″ S and 30° 59′ 45.62) and New Germany wastewater treatment plant (29°48′ 21.68″S and 30°53′ 50.44″E) were used in this study. The Northern wastewater treatment plants processes 70 megalitres per day (ML/day) of industrial and domestic wastewater; which is discharged into the Umgeni River after tertiary treatment by disinfection with chlorine at 3kg/day. The New Germany wastewater treatment plant treats mostly domestic wastewater but sometimes receive industrial wastewater as well. It has a maximum capacity of 7 ML but currently works at a capacity of 1 ML. Treated effluents are discharged into the Aller River after disinfection with chlorine at 0.5 to 0.8 kg/h and 1.0 to 1.2 kg/h for highly polluted influent.

2.2.2. Identification of presumptive *Pseudomonas* spp. isolates

The presumptive *Pseudomonas* spp. isolates recovered from treated effluents and receiving surface water were identified using biochemical tests and their identity further confirmed molecularly using PCR methods.

2.2.2.1. Biochemical confirmation of *Pseudomonas* spp. isolates

Identification of the presumptive *Pseudomonas* spp. isolates was performed by oxidase test, casein hydrolysis test, sugar fermentation in Triple Sugar Iron agar (TSI) and indole test. Specific media and reagents were prepared according to manufacturer's instructions and inoculated with a 24 h nutrient agar-grown culture of the isolates. Tubes and plates were incubated at 37°C for 24 to 48 h. Positively confirmed isolates were purified and further confirmed using Polymerase Chain Reaction.

2.2.2.2 Molecular identification of biochemically identified *Pseudomonas* spp. isolates

The purified isolates were grown on nutrient agar at 37°C for 24 h, and afterwards 5 bacterial colonies were picked and transferred into 100 µL nuclease-free water in 1.5 mL eppendorf tubes and standardized by vortexing. The tubes were then placed in a heating bath at 100 °C for 10 min, after which, the tubes were centrifuged at 11 000 rpm for 3 min at 25 °C and immediately placed on ice (Sambrook and Russell, 2001). Supernatant was transferred into a new tube and used directly as DNA template for PCR assay. Primers specific for *Pseudomonas* genus (Table 2.1) were used in a 25 µL PCR reaction. PCR conditions were as follows: 95 °C for 5 min, 94 °C for 15 s, 53 °C for 30 s and 72 °C for 45 s, this was repeated for 25 cycles with the exception of the 72 °C elongation step; and a final extension phase of 72 °C for 10 min. *Pseudomonas aeruginosa* reference strain ATCC 27853 was used as positive control while a reaction mixture containing nuclease-free water was used as a negative control (Widmer *et al*., 1998). The amplified PCR products were analysed by gel electrophoresis in 0.8 % (w/v) agarose gel at 60 V for 90 min in 1% TAE buffer. The products were visualized by UV transillumination (Syngene, UK) after staining in 0.1 mg/ml ethidium bromide for 15 min.

2.2.2.3 Species Specificity Screening of *Pseudomonas* isolates

All isolates confirmed to belong to the genus *Pseudomonas* were further screened and classified into three specific species of interest (*P. fluorescens, P. aeruginosa* and *P. putida*) selected based on the dominance of these species from the results obtained from the biochemical identification using the sets of primers listed in Table 2.1. The PCR conditions were as follows: *P. fluorescens* (2 min at 94 °C; 5 cycles consisting of 94 °C for 45 s, 55 °C for 1 min, 72 °C for 2 min; 35 cycles consisting of 92 °C for 45 s, 60 °C for 45 s, 72 °C for 2 min; final extension of 72 °C for 2 min; and final cooling at 4 °C); *P. aeruginosa* (95 °C for 1

min; 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 20 s; final extension at 68 °C for 40 s); *P. putida* (initial denaturation at 95 °C for 10 min, then 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 90 s and extension at 72 °C for 7 min) (Igbinosa *et al*., 2012). The amplification products were detected by electrophoresis in a 1.5 % (w/v) agarose gel at 60 V for 90 min in 1 % TAE buffer. The products were visualized by UV transillumination (Syngene, UK) after staining in 0.1 mg/ml ethidium bromide for 15 min.

Table 2.1: List of Primers used in this study

Target	Primer Sequences 5'–3'	Amplicon size (bp)	References
Pseudomonas spp.	GACGGGTGAGTAATGCCTA CACTGGTGTTCCTTCCTATA	617	Wagner *et al*. (2008)
P. aeruginosa	GGCGTGGGTGTGGAAGTC TGGTGGCGATCTTGAACTTCTT	199	Lutz and Lee (2011)
P. putida	TCACCTCCGAGGAAACCAGCTTG TCTGTTGTGAACGCCCTGTC	676	Hanning *et al.* (2009)
P. fluorescens	TGCATTCAAAACTGACTG AATCACACCGTGGTAACCG	850	Scarpellini *et al*. (2004)
bla_{TEM} gene	AGGAAGAGTATGATTCAACA CTCGTCGTTTGGTATGGC	535	Wang *et al.* (2006)
bla_{OXA} gene	TGAGCACCATAAGGCAACCA TTGGGCTAAATGGAAGCGTTT	311	Kuo *et al*. (2010)
$bla_{amp}C$ gene	GGTATGGCTGTGGGTGTTA TCCGAAACGGTTAGTTGAG	822	Yang *et al.* (2008)
intI1	CAGTGGACATAAGCCTGTTC CCCGAGGCATAGACTGTA	160	Mazel *et al.* (2000)
intI2	GTAGCAAACGAGTGACGAAATG CACGGATATGCGACAAAAAGGT	788	Mazel *et al*. (2000)
intI3	GCC TCC GGC AGC GAC TTT CAG ACG GAT CTG CCA AAC CTG ACT	979	Mazel *et al.* (2000)
5'-CS 3'-CS	GGCATCCAAGCAGCAAG AAGCAGACTTGACCTGA	Variable	Fonseca *et al*. (2005)
$bla_{\text{IMP-1}}$	GTTCCAAACATGGTTTGGTGGT TTTAACCCTTTAACCGCCTGCTCT	562	Wroblewska *et al.* (2007)
bla_{VIM-1}	AGTGGTGAGTATCCGACAG ATGAAAGTG CGTGGAGAC	261	Tsakris *et al.* (2000)

2.2.3. Antibiotic Resistance Profile Determination

Antibiotic resistance profiling of the isolates was performed using the Kirby- Bauer disk diffusion method described by Tao *et al*. (2010). Fresh culture were grown overnight in Mueller-Hinton broth and standardized to 0.5 McFarland by diluting with sterile Mueller-Hinton broth until a photometric reading of 0.08 to 0.1 was obtained on a spectrophotometer (Biochrom, Libra S12) at a wavelength of 625 nm. The standardized culture of the isolates were inoculated onto Mueller-Hinton agar using sterile swabs for confluence growth and allowed to dry for 10 min. Thereafter, appropriate antibiotic disks were placed at equidistance on the surface of the agar plates with a sterile forceps and the plates incubated at 37 °C for 18 to 24 h. The diameter of the zone of inhibition was measured to the nearest millimeter and recorded. Antibiotics were selected to represent some major classes of antibiotic and anti-pseudomonal antibiotics used as first line drug for pseudomonal infections. Antibiotics (Oxoid, UK) used in the study include penicillins (10 µg), gentamicin (10 µg), tetracycline (10 µg), streptomycin (10 µg), ciprofloxacin (5 µg), rifamycin (5 µg), trimethoprim (5 µg), sulphamethoxazole (25 µg), ampicillin (25 µg), chloramphenicol (30 µg), minocycline (30 µg), vacomycin (30 µg), cefotaxime (30 µg), nalidixic acid (30 µg), cephalothin (30 µg), erythromycin (15 µg), nitrofurantoin (300 µg), oxacillin (1 µg). *Pseudomonas* isolates were identified as resistant, intermediate or susceptible according to the National Committee for Clinical Laboratory Standard guidelines (CLSI, 2013). Multiple Antibiotic Resistance index was calculated as described by Blasco *et al*. (2008) as follows: MAR= a/b, where a = number of antibiotics to which the isolate was resistant; b = total number of antibiotics against which individual isolate was tested.

2.2.4. Molecular detection of antibiotic resistance and integrase genes

The DNA of the *Pseudomonas* isolates was extracted as previously described in section 2.2.2.2. following the method of Sambrook and Russell (2001). The set of primers used for the detection of antibiotic resistance genes are shown in Table 2.1. The PCR reaction was done in a total volume of 25 µL and the following conditions: bla_{TEM} gene (3 min at 93 °C, 40 cycles of 1 min at 93 °C, 1 min at 55 °C and 1 min at 72 °C and finally 7 min at 72 °C); bla_{OXA} gene and bla_{ampC} gene (94 °C for 5 min, 25 s of denaturation at 94 °C, 40 s of annealing at 53 °C and 50 s of extension at 72 °C for 30 cycles and a final cycle of 7 min at 72 °C. Integrons conserved segment were screened with the specific 5'-CS and 3'-CS primers (initial denaturation at 94 °C for 2 min, 20 s of denaturation at 94 °C, 30 s of annealing at 57 °C and 90 min of extension at 68 °C for a total of 30 cycles; 5 s were added to the extension time at each cycle). Integrase gene detection (*intI1, intI2, intI3*) was done in a 25 µL PCR mixture at the following conditions: 94 °C for 5 min, 30 cycles of 1 min of denaturation at 94 °C, 1 min of annealing at 59 °C, 1 min of extension at 72 °C and a final elongation at 8 min at 72 °C. bla_{IMP} and bla_{VIM}: initial denaturation at 94 °C for 5 min, then 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 40 s and extension at 72 °C for 50 s, followed by final extension at 72 °C for 5 min (Kouda *et al.,* 2007). Amplification products were analysed using 1.5% agarose gel electrophoresis in 1% TAE buffer at 60 V for 90 min (Igbinosa *et al*., 2012). The products were visualized by UV illumination (Syngene, UK) after staining in 0.1 mg/ml ethidium bromide for 15 min.

2.2.5. Molecular detection of gene cassettes

The DNA of the *Pseudomonas* isolates was extracted as previously described in section 2.2.2.2. following the method of Sambrook and Russell (2001). The set of primers 5'C and 3'C used (Table 2.1) for the detection of gene cassettes was done in a total volume of 50 µL

containing 3mM $MgCl_2$, 3 µl of total DNA as a template, 50 pmol of each primer, 1mM dNTPs and 1.6 U of *Taq* Polymerase at the following conditions: 95 °C for 5 min, 35 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 55 °C, 1 min of extension at 72 °C and a final elongation at 5 min at 72 °C (Fonseca *et al.,* 2005). The products were separated in a 2 % (w/v) agarose gel at 60 V for 90 min in 1% TAE buffer. The products were visualized by UV illumination (Syngene, UK) after staining in 0.1 mg/ml ethidium bromide for 15 min**.**

Amplicons corresponding to gene cassette regions were cleaved with *HaeIII* restriction enzyme. Briefly, each 20 µl of the restriction mixture contained 2 µl (20 U) of enzyme, 8 µl of PCR-amplified product, 1 µl of enzyme buffer and 9 µl of double-distilled water. As per manufacturer's guidelines, restriction mixtures were incubated at 37 °C for 1 h. (Fonseca *et al*., 2005). The products were separated in a 1.5 % (w/v) agarose gel at 60 V for 90 min in 1 % TAE buffer. The products were visualized by UV illumination (Syngene, UK) after staining in 0.5 mg/ml ethidium bromide for 15 min.

2.2.6. Screening for metallo-β-lactamase (MBL) production by the *Pseudomonas* spp. isolates

Imipenem disk diffusion method was employed as a screening test to select suspected MBL-*Pseudomonas* strains showing resistance to imipenem which were further confirmed by imipenem-EDTA combined disk method. Fresh culture of the isolates were grown overnight in Mueller-Hinton broth and standardized to 0.5 McFarland by diluting with sterile Mueller-Hinton broth until a photometric reading of 0.08 to 0.1 was obtained on a spectrophotometer (Biochrom, Libra S12) at 625 nm. The standardized culture of the isolates were inoculated onto Mueller-Hinton agar using sterile swabs for confluence growth and allowed to dry for 10 min. Two 10-µg imipenem disks were placed on the plates containing the test organism, and 750 mg of ethylenediaminetetraacetic acid (EDTA) (0.5 M, pH 8.0) solution was added to

one of the imipenem disk kept on Mueller Hinton agar plate. The inhibition zones of these disks were compared after 16 - 18 hrs of incubation at 37 °C. An increase in the inhibition zone of the imipenem and EDTA disk ≥7mm that of the imipenem disk alone, indicative of MBL positive isolate (Lee *et al.,* 2003).

2.2.7. Virulence determinants Assays

2.2.7.1. Elastase Assay

Elastase activity was measured using the elastin Congo red (ECR; Sigma) assay (Rust *et al*., 1994). Cells were grown in LB broth at 37 ºC for 16 h, centrifuged at 15 000 *g* at 4 ºC for 10 min and 0.5 mL supernatant was added to 1 mL of assay buffer (30 mM Tris buffer, pH 7.2) containing 10 mg of elastin Congo Red. The mixture was incubated at 37 ºC for 6 h. Insoluble ECR was removed by centrifugation and the absorption of the supernatant was measured at 495 nm. LB medium was used as a negative control. Isolates with an absorbance value ≥ 0.2 at 495 nm was considered positive for elastase.

2.2.7.2. Protease assay

Protease activity of *Pseudomonas* spp. isolates was determined using a method described by Schmidtchen *et al*. (2001). A 125-µl aliquot of 2% azocasein solution in Tris buffer (pH 7.8) was incubated with 75 µl of bacterial suspension at 37 °C for 45 min. The reaction was stopped by adding 600 µl of 10 % trichloroacetic acid. After incubation for 10 min at room temperature, the mixture was centrifuged for 5 min at 12,500 rpm, and 600 µl of the supernatant were transferred to a tube containing 500 µl of 1 M NaOH. The absorbance was measured at 440 nm. Isolates with an absorbance value ≥ 0.2 at 440 nm was considered positive for protease.

2.2.7.3. Screening for rhamnolipid production

Rhamnolipid production by *Pseudomonas* spp. was detected using M9-glutamate minimal medium agar plates. The agar was made up of 64g Na_2HPO_4-7H2O, 15 g KH_2PO_4, 2.5 g NaCl, 5.0 g NH_4Cl, 1M $MgSO_4$, 20 g solution of glucose, 1M $CaCl_2$, 15 g of agar, 0.2 g cetyltrimethylammonium bromide (CTAB) and 5 mg methylene blue l-1. The M9-glutamate minimal medium agar plates were inoculated with 2 ml of an overnight LB culture of *Pseudomonas* spp. strains. After an overnight incubation at 37 ºC, the diameter of the clear zone around the bacterial spot measured as evidence of rhamnolipid production (Senturk *et al*., 2012).

2.2.8 RAPD analysis for integron positive *Pseudomonas* spp.

The total reaction size was 25 µl, which contained 2µl of template DNA and 47.5 µM primers. Primer 272 (3'- AGCGGGCCAA- 5') was used (Mahenthiralingam *et al*., 1996). The cycling conditions were as follows: initial denaturation at 96 °C for min followed by 3 cycles of denaturation at 94 °C for 1 min, annealing at 36 °C for 2 min, extension at 72 °C for 2 min and 29 more cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 5 min. The RAPD-PCR products were loaded on a 1.5 % (w/vol) agarose gel. The products were visualized by UV transillumination (Syngene, UK) after staining in 0.1 mg/ml ethidium bromide for 15 min. Genotypic variation were analysed using the GelCompareII version 6.0 software package (Applied Maths) by Jacquard and Unweighted Pair Group Method with Arithmetic mean (UPGMA) cluster analysis to produce a dendogram.

2.3 Results

2.3.1. Identification of *Pseudomonas* spp. isolates

Test organisms showing purple colour after the addition of an oxidase reagent (3% H_2O_2) was deemed positive, zones of clearing around the growth area of the test organisms identifies the presence of the enzyme caseinase, tubes exhibiting alkaline slant and acidic butt with H_2S production on TSI slants, and yellow ring formed showing no colour change even after the addition of appropriate reagent (Kovac's reagent) indicated positive results for *Pseudomonas* spp. Of the presumptive isolates tested, a total of 125 isolates were biochemically positive for *Pseudomonas* spp. Of the 125 isolates biochemically confirmed, 100 isolates were identified to belong to the *Pseudomonas* genus based on the PCR assay. Similarly, distributions of *Pseudomonas* with respect to species were 84% for *P. aeruginosa*, 2% *P.putida,* 0% *f*or *P. fluorescens* and 14% for other *Pseudomonas* species (Figure 2.1)

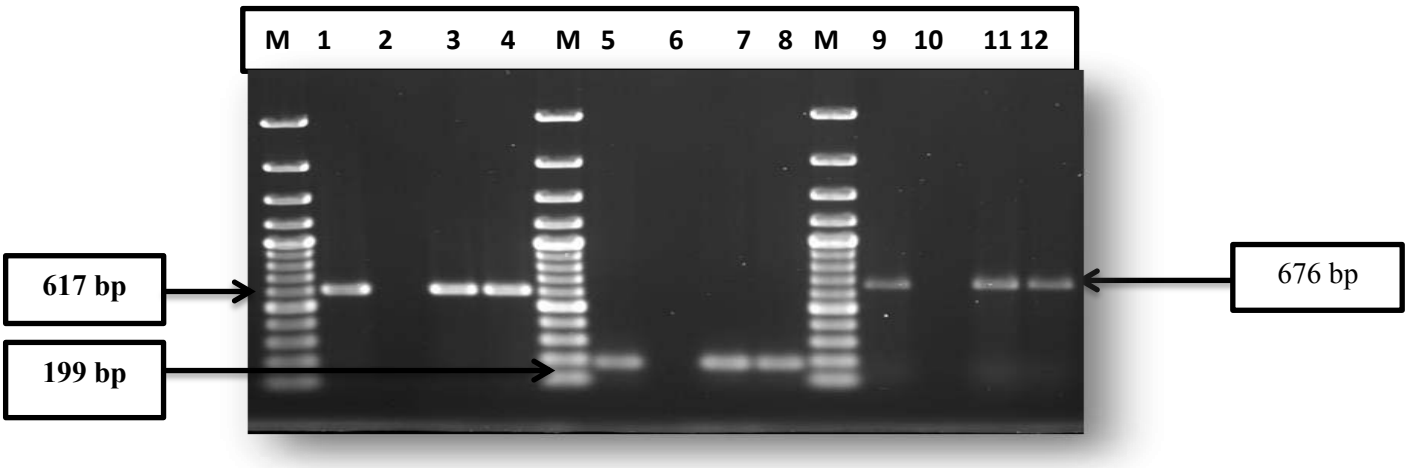

Figure 2.1: Electrophoresis gel showing the expected amplicon of *Pseudomonas* spp.- (617 bp) in lane 1; lane 2- negative control; lane 3 and 4 positive *Pseudomonas* spp. Lane 5 *P. aeruginosa* – (199 bp), lane 6- negative control; lane 7 and 8 positive *P. aeruginosa* isolates and lane 9 *P. putida*- (676 bp), 10- negative control; lane11 and 12 positive *P. putida* isolates. Lane M contains 100 bp plus DNA Ladder.

2.3.2. Antibiotic Resistance Profile of *Pseudomonas* spp. isolates

The antibiograms of the *Pseudomonas* species are as shown in Tables 2.2. All isolates (100%) were susceptible to ciprofloxacin, cefotaxime, cephalothin and gentamicin. Conversely, all (100 %) isolates were resistant to penicillin, ampicillin, oxacillin, sulphamethoxazole, vancomycin, trimethoprim, erythromycin, nitrofurantoin, streptomycin and rifamycin. Varied resistances were observed against Chloramphenicol as isolates showed the following resistance regimes; 100 % (*P. putida*), 68 % (*P. aeruginosa*) and 67 % (other *Pseudomonas* spp.) (Table 2.2). Isolates showed varied susceptibilities to minocycline with 100 %, 20% and 60 % of the *P. putida, P. aeruginosa* and other *Pseudomonas* spp., respectively found to be susceptible. The antibiotic resistance pattern with respect to nalidixic acid was 1 isolate (50 %) for *P. putida*, 76 (90 %) for *P. aeruginosa* and 13 (92 %) for other *Pseudomonas* spp. Twenty percent of the *P. aeruginosa* and 42 % of the other *Pseudomonas* spp. were found to be resistant to tetracycline while all the *P. putida* isolates were susceptible (Table 2.2). The tested isolates showed a high degree of multiple antibiotics resistances (MAR) being resistant to 10 and 15 antibiotics, distributed among 3 to 7 antibiotic classes (Table 2.3). The MAR index of the isolates ranger from 0.55 to 0.83. Isolates which had an MAR index of 0.83 were the most prevalent as 60 % of the isolates belonged to this phenotype.

Table 2.2: Antibiotic resistance profile of *Pseudomonas* spp. isolated from wastewater treatment plants and receiving surface waters

Antibiotics class	**Antibiotics**	***P. aeruginosa* (n=84)**			***P. putida* (n=2)**			**Other *Pseudomonas* species (n=14)**		
		S (%)	**I (%)**	**R (%)**	**S (%)**	**I (%)**	**R (%)**	**S (%)**	**I (%)**	**R (%)**
Penicillins	Penicillin Ampicillin Oxacillin	0 (0) 0 (0) 0 (0)	0 (0) 0 (0) 0 (0)	84 (100) 84 (100) 84 (100)	0 (0) 0 (0) 0 (0)	0 (0) 0 (0) 0 (0)	2 (100) 2 (100) 2 (100)	0 (0) 0 (0) 0 (0)	0 (0) 0 (0) 0 (0)	14 (100) 14 (100) 14 (100)
Cephems	Cefotaxime Cephalothin	84 (100) 84 (100)	0 (0) 0 (0)	0 (0) 0 (0)	2 (100) 2 (100)	0 (0) 0 (0)	0 (0) 0 (0)	14 (100) 14 (100)	0 (0) 0 (0)	0 (0) 0 (0)
Folate Pathway inhibitors	Sulphamethoxazole Trimethoprim	0 (0) 0 (0)	0 (0) 0 (0)	84 (100) 84 (100)	0 (0) 0 (0)	0 (0) 0 (0)	2 (100) 2 (100)	0 (0)	0 (0)	14 (100) 14 (100)
Ansamycins	Rifamycin	0 (0)	0 (0)	84 (100)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	14 (100)
Quinolones	Nalidixic acid	4 (5)	4 (5)	76 (90)	1 (50)	0 (0)	1 (50)	1 (8)	0 (0)	13(92)
Phenicols	Chloramphenicol	27 (32)	0 (0)	57 (68)	0 (0)	0 (0)	2 (100)	5 (33)	0 (0)	9 (67)
Tetracyclines	Tetracycline Minocycline Ciprofloxacin	17 (20) 17 (20) 84 (100)	8 (10) 0 (0) 0 (0)	59 (70) 67 (80) 0 (0)	2 (100) 2 (100) 2 (100)	0 (0) 0 (0) 0 (0)	0 (0) 0 (0) 0 (0)	6 (42) 8 (60) 100	0 (0) 6 (40) 0 (0)	8 (58) 0 (0) 0 (0)
Aminoglycosides	Gentamicin Streptomycin	84 (100) 0 (0)	0 (0) 0 (0)	0 (0) 84 (100)	2 (100) 0 (0)	0 (0) 0 (0)	0 (0) 2 (100)	14 (100) 0 (0)	0 (0) 0 (0)	0 (0) 14 (100)
Nitrofurantoins	Nitrofurantoin	0 (0)	0 (0)	84 (100)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	14 (100)
Microlides	Erythromycin	0 (0)	0 (0)	84 (100)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	14 (100)
Glycopeptides	Vancomycin	0 (0)	0 (0)	84 (100)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	14 (100)

Table 2.3: Distribution of the resistance phenotype and multiple antibiotic resistance index amongst *Pseudomonas* spp. isolates

Phenotype	Number of isolates (n=100)	Resistance profile	MAR index
A	60	TE, E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL, S	0.83
B	8	TE, E, AMP, NA, P, W, F, VA, KF, MH, RD, OX, RL, S	0.77
C	6	E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL, S	0.77
D	6	TE, E, AMP, NA, P, W, F, VA, KF, MH, RD, OX, RL, S	0.77
E	3	TE, E, AMP, NA, P, C, W, F, VA, KF, RD, OX, RL,S	0.77
F	1	TE, E, AMP, NA, P, C, W, F, VA, KF, RD, OX, RL, S	0.77
G	1	TE, E, AMP, P, C, W, F, VA, KF, MH, RD, OX, RL, S	0.77
H	2	TE, E, AMP, P, W, F, VA, KF, MH, RD, OX, RL, S	0.72
I	1	TE, E, AMP, NA, P, W, F, VA, KF, RD, OX, RL, S	0.72
J	1	E, AMP, NA, P, C, W, F, VA, KF, RD, OX, RL, S	0.72
K	1	E, AMP, NA, P, W, F, VA, KF, MH, RD, OX, RL, S	0.72
L	1	TE, E, AMP, NA, P, W, F, VA, KF, RD, OX, RL, S	0.72
M	1	E, AMP, P, W, F, VA, KF, MH, RD, OX, RL, S	0.66
N	1	E, AMP, NA, P, W, F, VA, KF, RD, OX, RL, S	0.66
O	1	TE, E, AMP, P, C, W, F, VA, KF,RD, OX, S	0.66
P	1	E, AMP, NA, P, W, F, VA, KF, RD, OX, RL, S	0.66
Q	1	E, AMP, NA, P, C, W, F, VA, KF, RD, OX, S	0.66
R	3	E, AMP, P, C, W, F, VA, KF, RD, OX, S	0.61
S	1	E, AMP, P, W, F, VA, KF, RD, OX, S	0.55

Legend: AMP—Ampicillin; KF—Cephalothin; C— Chloramphenicol; RD—Clindamycin; E—Erythromycin; MH—Minocycline; NA—Nalidixic Acid; F—Nitrofurantoin; OX—Oxacillin; P—Penicillin G; S—Streptomycin; TE—Tetracycline; VA—Vancomycin

2.3.3. Antibiotic resistance genes, integrase genes and gene cassette arrays

The screening for antibiotic resistance genes revealed the absence of *bla*OXA and *bla*ampC in all the *Pseudomonas* isolates, while the gene coding for *bla*TEM (Figure 2.2) was found in 30 % of the isolates. Fifty isolates were selected based on the different phenotypes and screened for the presence integrons. Twenty-two of the isolates were positive for class 1 integron, indicating a 44 % prevalence in the community. Three of the 22 isolates tested positive for the conserved-segment PCR with a variable region of class 1 integrons of 700 bp obtained in 2 of the isolates, while the other has two sizes at 700 and 500 bp (Table 2.4). Sequencing of the PCR product revealed the presence of *dfrA1/AadA1a*, which encodes

additional resistance to trimethoprim, in one of the isolates whilst the other harboured the *aac6* gene cassette which confers resistance towards gentamicin, tetracycline and chloramphenicol. The prevalence of $bla_{\text{IMP-1}}$ and $bla_{\text{VIM-1}}$ genes among the 5 metallo-β-lactamase producing *Pseudomonas* spp. isolates was 3 of 5 (60%) and 1of 5 (20%), respectively. In the MBL PCR assay, all the 3 strains that encode the $bla_{\text{IMP-1}}$ gene were (US 55, US 21 and DP 62) are *Pseudomonas aeruginosa*. Of the 3 strains (US 21) harbours both the $bla_{\text{IMP-1}}$ and $bla_{\text{VIM-1}}$ (Figure 2.3)

Amplicons obtained from the class 1 integron analysis having the same size and (RFLP) pattern are considered to harbour the same cassette array. The *Pseudomonas* spp., majority of types are 500 and 700 bp integron fragments. According to the integron cassette arrays, *Pseudomonas* spp. can be classified into two clusters. The results revealed that most of the strains possess variable gene cassette that is correlative with their individual phenotypes (Table 2.4). Among the 3 different-sized CS amplification products found, 2 different RFLP patterns were detected. Sequence analysis of the amplicons belonging to the individual RFLP patterns showed that different gene cassettes were found within the integrons (Table 2.4). The gene cassette array most frequently found associated with class 1 integrons, in *Pseudomonas* spp., was *dfrA1-aadA1* and *aac6.* Interestingly, two of the cassette arrays contained a linF gene. One of the linF genes was part of an *aadA2*-linF cassette and was found to be associated with a class 1 integron. Remarkably, only a small part of the resistance profile of the isolates could be explained by expression of the gene cassettes found within the integrons analysed.

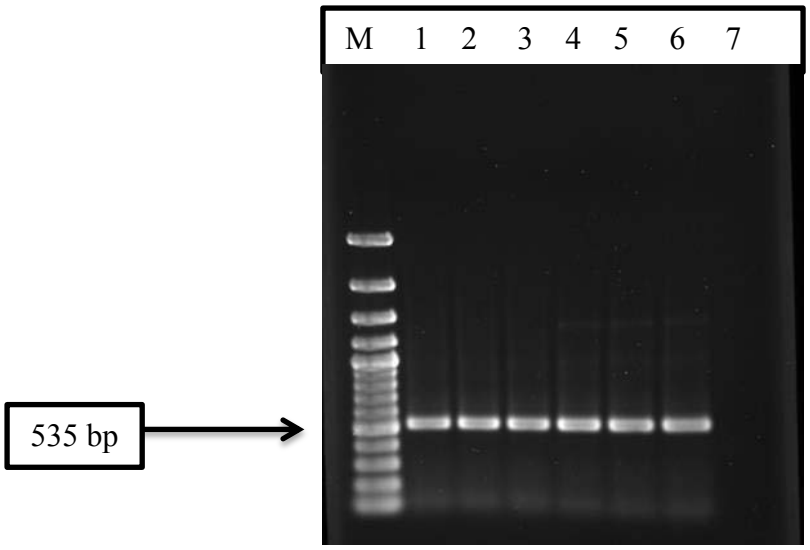

Figure 2.2: Agarose gel showing the expected amplicon size (535 bp) of *bla*TEM resistance gene in *Pseudomonas* spp. Lane M contains 100 bp plus DNA Ladder, lane 1 to 6 contains *Pseudomonas* isolates positive for *bla*TEM gene and lane 7 is the negative control.

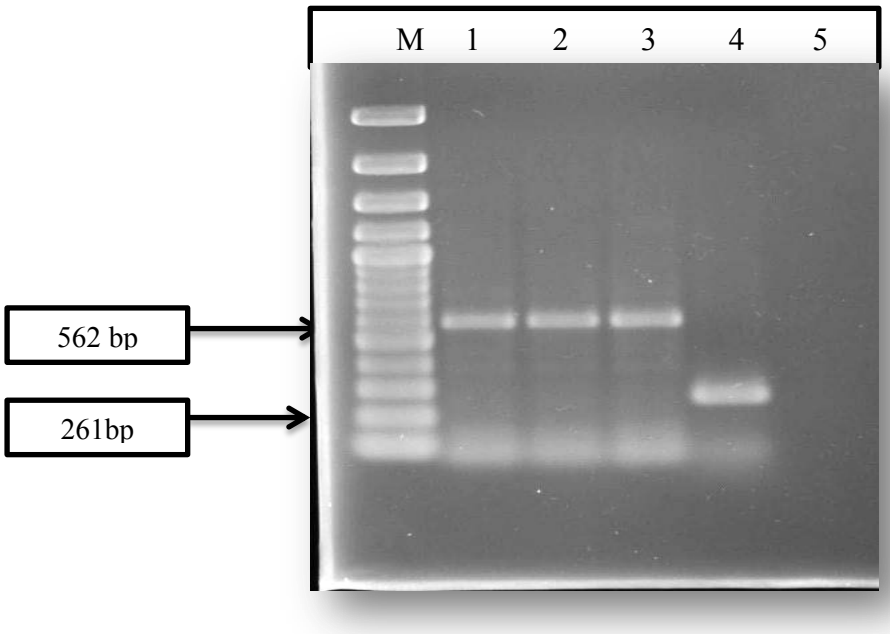

Figure 2.3: Agarose gel showing the expected amplicon size of bla_{IMP} (562 bp) and bla_{VIM} (261 bp) metallo-β-lactamase gene in *Pseudomonas* spp. Lane M contains 100 bp marker, lane 1 to 3 contains bla_{IMP} positive isolates and lane 4 contains bla_{VIM} positive isolate, lane 5 contains the negative control.

Table 2.4: Characterization of integrons and antibiotic resistance patterns of integron positive *Pseudomonas* spp. isolated from wastewater treatment plants

Strain designation	Species	Integrons	Size of the integrons variable part	RFLP pattern	Gene cassette	Antibiotic resistance pattern
BC 6	*Pseudomonas* spp.	*Intl1*	500 +700 bp	200, 300 +500 bp	*aac6*	E, AMP, NA, P, W, F, VA, KF, RD, OX, RL, S
BC 109	*P. aeruginosa*	*Intl1*	700 bp	300 + 500bp	*dfrA1-aadA1*	TE, E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL, S
DS 163	*P. aeruginosa*	*Intl1*	700 bp	300 + 500bp	*dfrA1-aadA1*	E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL, S

Antibiotics: AMP—Ampicillin; KF—Cephalothin; C— Chloramphenicol; RD—Clindamycin; E—Erythromycin; MH—Minocycline; NA—Nalidixic Acid; F—Nitrofurantoin; OX—Oxacillin; P—Penicillin G; S—Streptomycin; TE—Tetracycline; W—Trimethoprim; VA—Vancomycin

2.3.4. Metallo-β-lactamase (MBL) analysis and virulence determinant profiles of the *Pseudomonas* spp. isolates

All the 50 selected *Pseudomonas* spp. isolates, showed resistance against imipenem by disk diffusion method. However, Imipenem-EDTA disk method showed metallo-β-lactamase production in only 10 % (5/50) of the resistant imipenem resistant *Pseudomonas* spp. with an average of 7mm zone diameter between imipenem disk and imipenem plus EDTA disk for MBL positive isolates (Table 2.5). To further characterize the *Pseudomonas* spp. isolates, *in vitro* production of virulence factors that depend upon an active QS-circuitry was determined. A total of 38 (76 %) of these isolates produced elastase; LasB elastase the most potent elastase produced by *Pseudomonas* spp. is one of the major virulence factors controlled by QS. *Pseudomonas* spp. isolates displayed different levels of elastolytic activities since they demonstrate variable absorbance values at 495 nm (Table 2.5). A total of 11(22 %) of these isolates produced protease, is one of the major virulence factors controlled by QS. *Legionella* spp. isolates displayed different levels of proteolytic activities since they displayed varying absorbance values at 440 nm (Table 2.5). The blue halos formed on CTAB agar plates changed in colour and size with the intensity and incidence angle of the light source. The halo areas could be more reproducibly determined from the pictures taken with a UV transilluminator. Observed under the UV transilluminator, the circles formed around the point of inoculation had at least four distinguishable layers, an innermost light area, a bluest halo, a lighter blue zone outside the darker blue halo, and an outermost brown ring. Without the fixed underneath illumination, these layers were not always evident and would vary depending on the background and lighting condition.

Table 2.5: Metallo-β-lactamase activity and virulence determinant profiles of *Pseudomonas* spp. isolates

Bacterial strain	Species	β-lactamase activity [a] (IMP+EDTA disk-IMP disk	Rhamnolipid	Elastase [b](OD at 495 nm)	Protease [c] (OD at 440 nm)
BC 109	*P.aeruginosa*	- (4)	+	+ (0.233)	+ (0.235)
BC 129	*P.aeruginosa*	- (0)	-	+ (0.214)	- (0.173)
BC 140	*P.aeruginosa*	- (1)	+	+ (0.345)	- (0.145)
BC 17	*P.aeruginosa*	- (2)	+	+ (0.378)	- (0.125)
BC 172	*P.aeruginosa*	- (0)	+	+ (0.452)	- (0.055)
BC 70	*P.aeruginosa*	- (0)	+	- (0.147)	- (0.352)
BC 84	*P.aeruginosa*	+(11)	+	+ (0.452)	- (0.024)
DP 25	*P.aeruginosa*	- (3)	-	+ (0.214)	+ (0.235)
DP 45	*P.aeruginosa*	- (5)	-	+ (0.369)	- (0.178)
DP 57	*P.aeruginosa*	- (0)	+	+ (0.365)	- (0.123)
DP 58	*P.aeruginosa*	- (0)	+	+ (0.654)	- (0.045)
DP 59	*P.aeruginosa*	- (4)	+	+ (0.214)	- (0.178)
DS 161	*P.aeruginosa*	- (2)	-	- (0.014)	+ (0.248)
DS 163	*P.aeruginosa*	- (4)	+	+ (0.365)	- (0.002)
DS 32	*P.aeruginosa*	- (0)	+	+ (0.354)	- (0.154)
US 11	*P.aeruginosa*	- (0)	-	+ (0.521)	+ (0.654)
US 21	*P.aeruginosa*	+ (11)	+	+ (0.704)	+ (0.654)
US 4	*P.aeruginosa*	- (0)	-	+ (0.286)	+ (0.484)
US 55	*P.aeruginosa*	+ (10)	-	+ (0.753)	- (0.054)
US 64	*P.aeruginosa*	- (3)	+	- (0.590)	+ (0.245)
BC 129	*P.aeruginosa*	- (4)	-	+ (0.424)	- (0.025)
BC 169	*P.aeruginosa*	- (5)	-	+ (0.487)	- (0.025)
DP 21	*P.aeruginosa*	- (0)	-	- (0.154)	+ (0.254)
BC 38	*Pseudomonas* spp.	- (2)	-	- (0.015)	- (0.136)
DP 58	*P.aeruginosa*	- (0)	+	+ (0.245)	- (0.111)
DP 60	*P.aeruginosa*	- (2)	+	+ (0.687)	- (0.153)
DS 163	*P.aeruginosa*	- (2)	+	+ (0.884)	- (0.115)
DP 62	*P.aeruginosa*	+ (10)	+	+ (0.721)	- (0.125)
US 62	*P.aeruginosa*	- (4)	-	+ (0.684)	- (0.036)
US 9	*P.aeruginosa*	- (2)	+	+ (0.717)	+ (0.548)
DP 61	*P.aeruginosa*	- (3)	+	- (0.010)	+ (0.487)
DP 65	*P.aeruginosa*	- (2)	+	- (0.144)	- (0.125)
DS 59	*Pseudomonas* spp.	- (0)	+	+ (0.263)	- (0.024)
DP 65	*P.aeruginosa*	- (3)	-	+ (0.225)	- (0.021)
BC 27	*P.aeruginosa*	+ (7)	-	+(0.386)	- (0.120)
BC 123	*P.aeruginosa*	- (2)	-	+ (0.717)	- (0.195)
BC 103	*P.aeruginosa*	- (0)	+	+ (0.365)	- (0.012)
BC 28	*P.aeruginosa*	- (0)	-	+ (0.521)	- (0.163)
BC 166	*P.aeruginosa*	- (2)	-	+ (0.257)	- (0.137)
DS 21	*Pseudomonas* spp.	- (3)	-	+ (0.282)	- (0.145)
US 1	*Pseudomonas* spp.	- (1)	-	+ (0.214)	+ (0.543)
BC 100	*P.aeruginosa*	- (0)	+	+ (0.542)	-(0.093)
BC 6	*Pseudomonas* spp.	- (3)	+	- (0.002)	- (0.162)
DP 30	*Pseudomonas* spp.	- (4)	-	- (0.024)	- (0.147)
DS 2	*Pseudomonas* spp.	- (5)	+	- (0.125)	- (0.051)
N 6	*P.putida*	- (2)	+	+ (0.365)	- (0.173)
C 25	*Pseudomonas* spp.	- (2)	+	+ (0.452)	- (0.185)
C 3	*P.putida*	- (0)	+	+ (0.687)	- (0.116)
DSNTGW	*P.aeruginosa*	- (5)	+	- (0.024)	- (0.159)
BC 103	*P.aeruginosa*	- (0)	+	+ (0.452)	- (0.140)

a= Difference in inhibition zone of impenem and EDTA, and EDTA ≥ 7 mm is considered positive; b= OD valued ≥ 0.2 at 495 nm is considered positive; c= OD valued ≥ 0.2 at 440 nm is considered positive

2.3.5. RAPD fingerprinting of integron positive *Pseudomonas* spp. isolates

The results obtained in this study suggest that considerable genetic diversity exists among *Pseudomonas* spp. under investigation. Distinct profiles were obtained for the 22 strains tested, meaning that *Pseudomonas* spp. can be grouped into different profiles with respect to their phenotypic and genotypic characteristics (Figure 2.4). Polymorphisms based on fragment length were obtained as a means of differentiating *Pseudomonas* spp. isolates. The absence or presence of a band was also noted in determining variation among the strains. Fragments of different molecular weights were observed in the RAPD fingerprints produced after amplification with primer 272. Amplification of different intensities were observed and referred to as primary, secondary and tertiary amplification on visual analysis of the RAPD profiles. Primary amplification products refer to those products of high intensity and appear extremely bright on the gels. Secondary amplification products are those products that are not as bright as the primary amplification product but more intense that the tertiary amplification products, while the tertiary amplification products are the minor amplification products are of low intensity (Figure 2.4).

Primer 272 used in this study was capable of amplifying multiple polymorphic DNA fragments from all of the strains tested. Band patterns were reproducibly obtained under similar experimental conditions and comprised between 4 and 9 individual bands. The sizes of the amplified DNA varied from 1200 bp to 2 kb. Based on the numbers of band differences, two clusters (A and B) were identified as non MBL producing isolates, where cluster C was comprised 2 (25 %) which are MBL producing isolates, all carrying class 1 integrons. Cluster C of the MBL isolates comprised 1 (13 %) isolates carrying blaVIM genes, whereas cluster A and B did not carry any of the MBL genes (Figure 2.5).

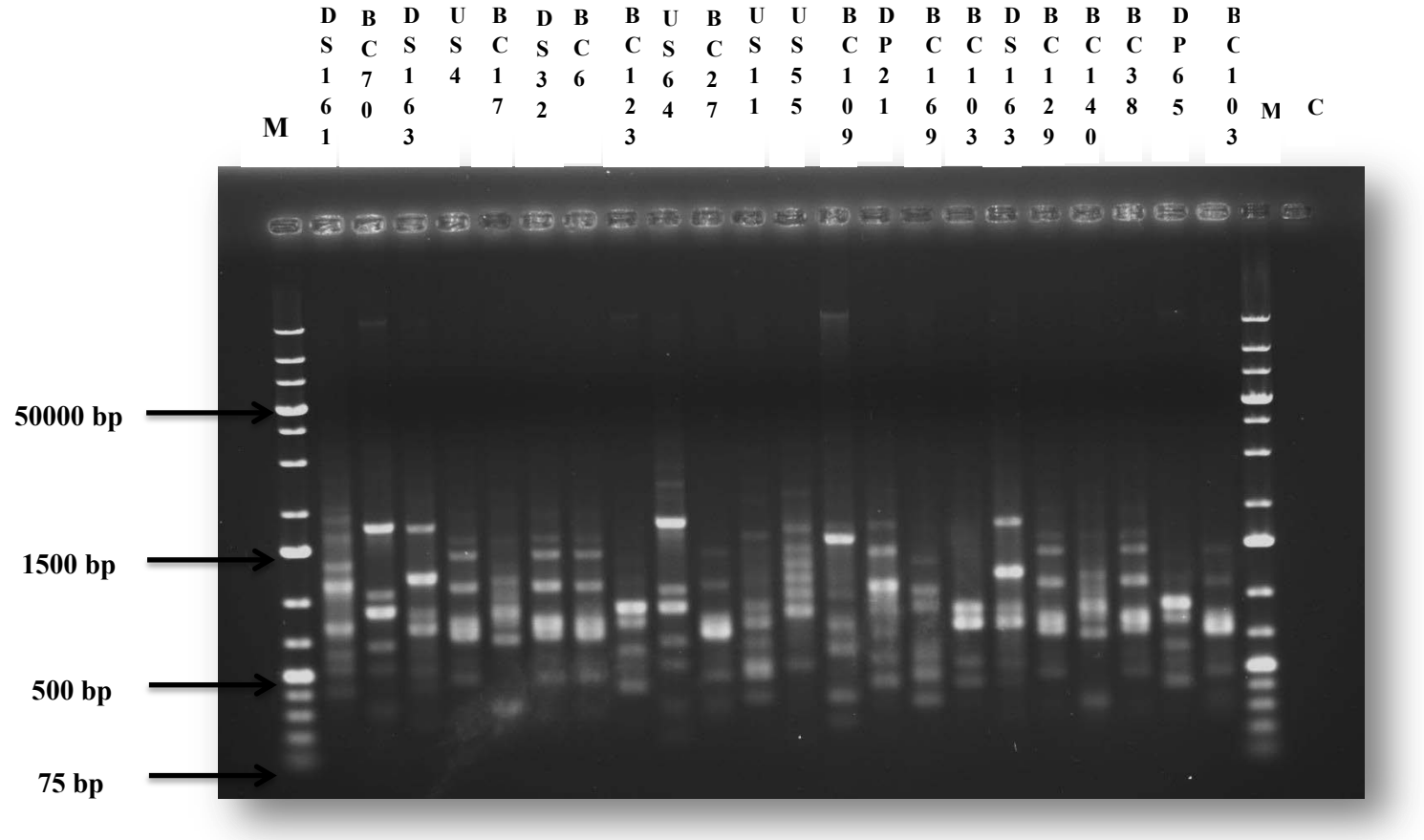

Figure 2.4: Integron positive *Pseudomonas* spp. isolate RAPD polymorphisms amplified by primer 272. Molecular size markers (1-kb Plus DNA ladder) were run in lanes M and lane C is the negative control.

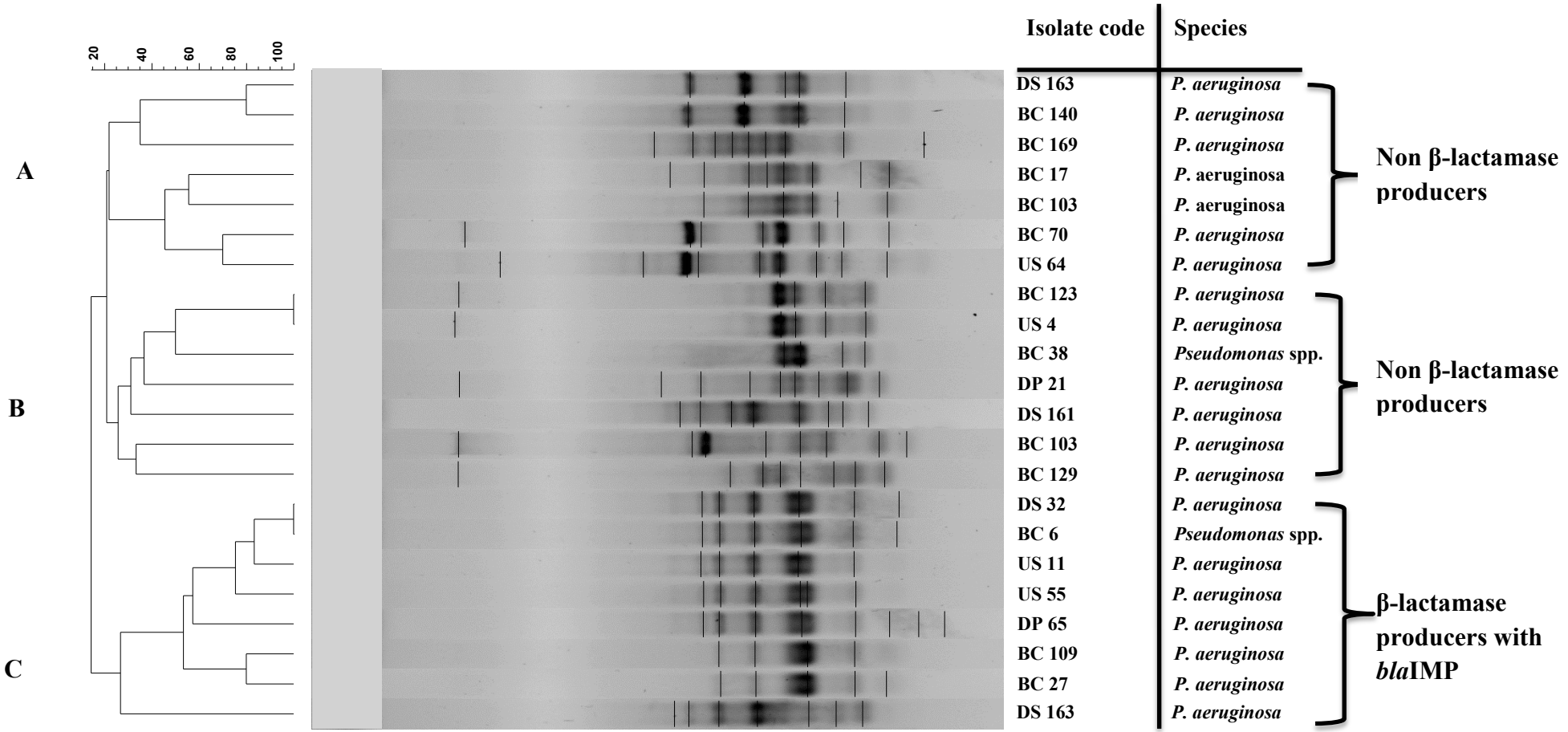

Figure 2.5: Dendrogram showing the cluster analysis of *Pseudomonas* spp. based on primer 272 PCR fingerprinting patterns using Jackard index and UPGMA clusterization. The scale at the top represents percentage similarity.

2.4 Discussion

The discharge of inadequately treated sewage water has a direct impact on the microbiological quality of surface waters and consequently the potable water derived from it. The inherent resistance of pathogens to water disinfection processes means that they may likely be present in the discharged effluent after treatment (Odjajare *et al.,* 2012). Such pathogens may harbour virulence and antibiotic resistance genes, thus posing a threat to the public. One hundred (100) strains of *Pseudomonas* (84% belonging to *P. aeruginosa*, 2% belonging to *P. putida* and 14% belonging to *Pseudomonas* spp.) recovered from treated effluent of wastewater treatment plants were characterised for their antimicrobial resistance profiles and virulence determinants in this study. Resistance to different classes of antibiotics shown by these *Pseudomonas* species isolated from treated wastewater effluent is an indication of the potential of these effluents as a reservoir for antibiotic resistant organisms (Momba *et al.,* 2006). Wastewater treatment process has also been identified as a potential vehicle for the selective enhancement and increase of multidrug resistant bacteria in the aquatic environment (Zhang *et al.,* 2009). Resistance to antimicrobial agents is an increasing public health threat as it limits therapeutic options and leads to increased mortality and morbidity. Given the increasing resistance rates in *Pseudomonas* spp., multidrug resistance can be expected to become more prevalent in treated wastewater effluents.

The tested isolates in this study exhibit slight intermediate sensitivity to the tetracyclines and minocycline with varying levels of sensitivity obtained for the *Pseudomonas* spp. Previous reports suggest that *Pseudomonas* species are frequently resistant to these antibiotics (Jombo *et al.,* 2008; Emannuel *et al.* 2011). However, Jombo *et al*. (2008) reported sensitivity of *P. aeruginosa* strains isolated from wastewater treatment plant in Jos, Nigeria to chloramphenicol; while Lateef *et al*. (2004) observed sensitivity to tetracyline in *Pseudomonas i*solates from pharmaceutical effluents. Sixty eight percent of the *Pseudomonas*

spp. in this study were resistant to chloramphenicol which may suggest increasing resistance to this antibiotic among *Pseudomonas* spp. The tested isolates in this study also exhibited high levels of resistance to the penicillins (100%), folate pathway inhibitors, ansamycins, nitrofurantoins, microlides and glycopeptides (Table 2.2). According to Pirnay *et al*. (2005), *Pseudomonas* species were naturally resistant to the penicillins, cephems and ansamycins because they have relatively impermeable membrane, inducible efflux systems and a chromosomally encoded inducible β-lactamase (Emmanuel *et al.,* 2011). The relatively high level of resistance to antimicrobial agents recorded in this study is a reflection of misuse or abuse of these agents in the environment. Multiple drug resistance is an extremely serious public health problem and it has been found associated with the outbreak of major epidemic throughout the world. Thus, the multiple – drug resistance shown by these pathogens are worrisome and of public health concern (Lateef, 2004). Encountering multiple antibiotic resistant bacteria in this study is therefore not a surprise but worrisome. Therefore, the rate of multiple antibiotic resistant pathogenic bacteria in this study characterizes a well- recognized phenomenon that is of a negative impact for public health, an observation that corroborates the findings of Adewoye and Adewoye, (2013).

Although Malekzadeh *et al*. (1995), reported resistance of *Pseudomonas* species isolates from wastewater to only single antibiotics, all the tested isolates in this study showed multiple antibiotic resistances (MARs) ranging from ten to fifteen antibiotics distributed among three to seven classes Consistent with the observation of this study, Paul *et al*. (1997) reported MAR *Pseudomonas* strains with resistance patterns varying between five and eight antibiotics; while Lateef *et al*. (2004) documented MAR *Pseudomonas* with resistance patterns of two to seven antibiotics. Two major intrinsic mechanisms were reported to confer bacterial resistance to multiple antimicrobial drug classes: mutations in outer membrane porins resulting in reduced permeability to antimicrobials; and over expression of multidrug

efflux pumps (Esiobu *et al*., 2002), which tend to pump out antibiotics before they (the antibiotics) have the opportunity of acting on their target (Navon-Venezia *et al*., 2005; Ashish *et al*., 2011). In addition, Navon-Venezia *et al*. (2005), observed that MAR bacterial strains may also arise due to unrelated mechanisms accumulating sequentially in an organism (Odjadjare *et al.,* 2012). The MAR indices were higher than the 0.2 limit in all the tested isolates (Table 2.3), suggesting that isolates in this study originated from high risks source(s) of contamination where antibiotics are often used (Odjadjare *et al*., 2012).

Li *et al.* (2009), reported the presence of bla_{TEM} in 17.3% *Pseudomonas* spp. isolated from effluent of wastewater treatment plant and 11% from the river downstream of the plant, however, bla_{OXA} gene was not detected. Similar trend was observed in the current study with only bla_{TEM} gene detected in 30% of the *Pseudomonas* isolates signifying wastewater as a reservoir for antibiotic resistance genes. The analysis of the integron variable region revealed that 19/22 (86 %) were devoid of gene cassettes, indicating a high occurrence of empty class 1 integrons among multidrug strains (Table 2.4). Several reports have shown the presence of aminoglycoside resistance genes associated with integrons found in gram-negative bacteria. The absence of class 2 and 3 integrons among the isolates tested in this study further confirmed the restricted distribution of these two genetic elements among bacterial populations (Lévesque *et al*., 1995; Gu *et al.,* 2007). In the current study, a small proportion of class 1 integrons carrying resistance gene cassettes was detected with sizes ranging from 500 to 700 bp. Genes conferring resistance to aminoglycosides and β-lactams are frequently found in integrons from *Pseudomonas* and Enterobacteriaceae, and the most common aminoglycoside resistance gene cassettes belong to *add* and *acc* families. The presence of *dfrA1-aadA1* gene cassettes in 2 of the 3 integron positive *Pseudomonas* spp. could account for the observed resistance to streptomycin, in addition to tetracycline resistance in these isolates (Table 2.4), whilst the presence of gene cassette *aac6* encoding gentamicin,

tetracycline, and chloramphenicol resistance could explain the observed resistance of this isolate to the antibiotics.

Integrons were significantly associated with resistance to certain antibiotics including streptomycin, trimethoprim, ampicillin, chloramphenicol, and tetracycline. However, resistance to only streptomycin, and trimethoprim, and to some extent streptomycin, could be directly related to the presence of resistance genes within the integron (Khosravi *et al*., 2012; Sunde and Sørum, 1999). The association of the other older antibiotics ampicillin, chloramphenicol, and tetracycline with the presence of an integron is likely to be due to genetic linkage between integrons and conjugative plasmids and transposons (Khosravi *et al*., 2012). Disturbingly, the widespread dissemination of the class 1 integron and associated gene cassettes in *Pseudomonas* spp. and other important pathogens would gravely complicate treatments of infections if not properly monitored (Pallecchi *et al.,* 2011). Hence, functional surveillance of antimicrobial resistance and appropriate. Effective measures geared towards curbing indiscriminate and unregulated use of antibiotics are urgently needed to prevent outbreaks of multidrug resistant bacteria in South Africa (Strateva and Yordanov, 2009; Li *et al*., 2010). Integrons, especially class I integrons, commonly contained antibiotic-resistance gene cassettes, including β-lactamase determinants (Weldhagen, 2004), and have been found to be closely related to MAR of bacteria, as they usually contain several antibiotic-resistance gene cassettes simultaneously (Mazel, 2006). However, the gene cassettes detected in this study mainly conferred resistance to aminoglycoside antibiotics, with no β-lactamase determinants detected.

Carbapenems are highly effective antibiotics against multidrug-resistant Gram-negative bacteria because of their stability against extended spectrum and AmpC-β-lactamases (Pitout *et al*., 2007; Lee *et al*., 2011). Resistance to carbapenems can be mediated by several mechanisms including decreased membrane permeability and increased efflux (D'Agata,

2004; Adel *at al*., 2010). However, production of metallo-β-lactamases has assumed increasing importance in recent years. Although prevalence of carbapenem resistance due to acquired MBLs is increasing, its overall prevalence is still low (Ho *et al*., 2002; Lutz and Lee, 2011). However, increasing use of carbapenems would provide the selective pressure for selection of these enzymes (Vettoretti *et al*., 2009). bla_{IMP} and bla_{VIM} genes were detected in 3 and 1 of the 5 metallo-β-lactamase-producing *Pseudomonas* aeruginosa isolates, respectively (Table 2.5), with one of the test strains (US 21) found to harbour both the bla_{IMP} and bla_{VIM}. In the absence of novel agents for the treatment of infections caused by multidrug-resistant gram negative bacteria in the near future, the uncontrolled spread of MBL producers may lead to treatment failures with increased morbidity and mortality. The early detection of MBL-producing *Pseudomonas* spp. may avoid the future spread of these multi drug-resistant isolates (Leung *et al.,* 2008; Cholley *et al.,* 2011). Thus, it is recommended that all IPM-non susceptible or resistant *Pseudomonas* spp. isolates be routinely screened for MBL production.

Three quorum sensing dependent virulence factors which play a major role in pathogenesis of *Pseudomonas* spp. namely elastase, protease and rhamnolipid production were determined in the *Pseudomonas* spp. isolates in this study. Results obtained confirmed the hypothesis that most pathogenic *Pseudomonas* spp. strains isolated from wastewater have increased expression of two virulence factors, elastase and protease, which are considered to be regulated by quorum sensing (QS). The fact that such results were observed in two-thirds of the isolates studied suggests that increased QS activity and QS-regulated virulence factor production may play a vital role in the pathogenicity of these *Pseudomonas* spp. strains. Elastase, a metalloproteinase secreted by a type II secretion system, has been shown to destroy respiratory epithelium tight junctions, leading to increased permeability disorders, increased interleukin-8 levels and a decreased host immune response (Aloush *et al*., 2006).

The observed production of elastase by two-thirds (Table 2.5) of the *Pseudomonas* spp. in this study corroborates the results of a previous study, where 60 % of *Pseudomonas* isolates were reported to produce extracellular virulence factors (Choy *et al.,* 2008). Protease production was detected in 22% of the *Pseudomonas* spp. which act as an exotoxin, and be an example of a virulence factor in bacterial pathogenesis (for example, exfoliative toxin). Bacterial exotoxic proteases destroy extracellular structures. Sixty percent (30/50) of *Pseudomonas* spp. isolates (Table 2.5) were confirmed for the production of anionic biosurfactant rhamnolipid which contributes to the establishment and maintenance of infection in cystic fibrosis. Elastase production by the *Pseudomonas* spp. in this study which contributes to the invasiveness of the organism were detected in 76% of the study isolates. These virulence factors contribute to the pathogenicity of *Pseudomonas* spp.

RAPD markers represent a convenient means of scanning and comparing the genome of individuals (Sing *et al*., 2006). The accuracy of RAPD markers in predicting genetic relationships among organisms has been demonstrated in molecular phylogenetic studies where groupings of individuals within several species on the basis of RAPDs have been shown to coincide with taxanomic systems based on morphological, genetic and agronomic criteria (Akanji *et al*., 2011). The degree of differentiation using RAPDs may be indicative of the ability of these DNA markers to provide more characters for diversity analysis at genetic levels rather than using indirect biochemical characters (Church *et al.*, 2006). The degree of variation in PCR products obtained by RAPD-PCR analysis reflects the sequence variation of RAPD priming sites among the strains tested.

Variable genetic diversity was observed within the 22 integron positive *Pseudomonas* spp. tested in this study, based on RAPD analysis. Distinct RAPD profiles were produced for all strains and were found to be extremely useful in differentiating *Pseudomonas* spp. strains. RAPD makers were also useful in examining relatedness as band patterns were consistent and

comprised between 4 and 9 bands of molecular weight ranging from 1200 bp to 2 kb (Figure 2.4). Clonality study showed that non-MBL producers harbouring class 1 integrons could be divided into two clusters, A and B, whilst the non-MBL producers were assigned into clusters C. Strains with the same antibiotic susceptibility demonstrated similar RAPD patterns. Thus, it could be suggested that isolates carrying MBL genes may be detected in different clusters from the non-MBL isolates (Figure 2.5). Similar to our findings, Pitout *et al*. (2007) also suggested three clusters for *Pseudomonas* spp. isolates: cluster 1 (*bla*VIM producing), cluster 2 (*bla*IMP producing) and cluster 3 (non- MBL). Diversity of RAPD types identified in this study suggests that the carriage of the *bla*VIM and *bla*IMP genes by integrons (Pitout *et al*., 2007) may facilitate the spread of these genes among genetically distinct *Pseudomonas* spp. strains. The current study revealed the detection of cassette arrays of class 1 integron (*dfrA1/AadA1a, aac6* and $bla_{\text{IMP-1,}}$ $bla_{\text{VIM-1,}}$) from *Pseudomonas* species isolates recovered from wastewater in South Africa, Durban.

2.5 Conclusion

This study demonstrated the prevalence of MAR Pseudomonas species in municipal effluents in Durban, South Africa. Since the emergence of MAR *Pseudomonas* species is of public health concern, our data support the need for regular and consistent monitoring of municipal sewage effluents with a view to preventing the dissemination of these pathogens into the environment. Our findings further suggest that MBL genotypes are not homogenous and as such, generalized criteria for interpretation of MBL phenotypic assays may not be possible. Thus, it is recommended that the phenotypic assays should be assessed and adopted based on the local situation. It is also suggested that PCR-RAPD can act as a cheaper and easier method for routine investigation of the clonality of isolates compared to other methods such as PFGE. Diversity of RAPD types identified in this study suggests that the carriage of the

blaVIM and blaIMP genes by integrons may facilitate the spread of blaVIM and blaIMP genes among genetically distinct *Pseudomonas* spp. This study highlights the resistance to imipenem due to IMP- and VIM-producing *Pseudomonas* spp. and their associated class 1 integrons. Horizontal dissemination of the class 1 integron-associated MBL genes may contribute to the further emergence of carbapenem resistance in other Gram-negative bacteria. Therefore, appropriate surveillance and control measures are essential to prevent the further spread of MBL-producing organisms in hospitals. Further studies should be carried out to give a better understanding of the impact of integrons on the dissemination of antimicrobial resistance in the environmental settings.

CHAPTER 3

ANTIBIOGRAM AND VIRULENCE GENE SIGNATURES OF *LEGIONELLA* SPP. RECOVERED FROM TREATED WASTEWATER EFFLUENTS AND RECEIVING SURFACE WATER IN DURBAN

3.0 Abstract

The occurrence and spread of multi-drug resistant bacteria is a pressing public health problem. This is particularly worrying among *Legionella* spp. which have been implicated in a wide range of human diseases. The purpose of this study was to profile antimicrobial resistance and virulence determinants of *Legionella* spp. recovered from treated wastewater effluent and receiving surface water. A total of 100 strains of *Legionella* belonging to three species (*L. pneumophila*, *L. micdadei* and Other *Legionella* spp.) previously isolated from treated effluent of two wastewater treatment plants and receiving surface water was identified and used in this study. All the isolates were screened for their antibiogram against a panel of 21 antibiotics by the Kirby-Bauer disc diffusion assay. All tested isolates showed complete sensitivity (100%) to cefotaxime, cephalothin, trimethoprim, nalidixic acid, ciprofloxacin, gentamicin, nitrofurantoin, ampicillin-sulbactam and ofloxacin. Conversely, all isolates were resistant to penicillin, ampicillin, oxacillin, sulphamethoxazole, rifamycin, chloramphenicol, tetracycline, streptomycin, erythromycin and clindamycin. The tested isolates showed a high degree of multiple antimicrobial resistance (MAR) to 9 and 11 antibiotics, distributed among 3 to 7 antibiotic classes, with the MAR index varying between 0.44 and 0.55. Fifty isolates were selected based on their different antibiotic resistance phenotypes and screened using PCR for the presence of integrons and associated resistance gene cassettes. Twenty-five of the isolates harboured class 2 integrons with 22 of the isolates also carrying gene cassettes. The *drfA1-sat1-aadA1* gene was most frequently found in the class 2 integrons (60 %), followed by bla_{OXA} and bla_{TEM} (60 % and 14 %, respectively). Of the 25 class 2 integron-positive isolates, only 3 isolates (14 %) lanked gene cassettes. The screening for antibiotic resistance genes revealed the absence of $bla_{\text{IMP-1}}$ and $bla_{\text{VIM-1}}$ genes in all the *Legionella* isolates. Virulene determinant screening revealed that 62% of the *Legionella* spp. were capable of producing rhamnolipid as a biosurfactant. Eight-eight percent and 56% of *Legionella* spp. were capable of producing elastase and protease, respectively. Results obtained from this study reveal the occurrence of multi-drug resistant *Legionella* spp. in treated wastewater and the receiving surface water, thus suggesting the potential danger of the end-users of these water resources for domestic use.

3.1 Introduction

South Africa is a water-scarce country, and the demands on this resource are growing as the economy expands and the population increases (Li *et al*., 2010). For the country to continue to develop economically, while meeting the wide-ranging needs for water, urgent steps must be taken to protect the quality of the resource (Kang *et al*., 2005; Li *et al*., 2009). It is well known that water sources are subjected to frequent dramatic changes in microbial and chemical qualities as a result of the variety of activities on the watershed (Lyczak *et al.,* 2000; Okoh *et al.,* 2007). These changes are caused by discharges of municipal raw waters or improperly treated effluent at a specific point-source into the receiving waters such as streams, rivers, lakes, ponds etc. (Costa *et al*., 2005; Mathys *et al.,* 2008; Khleifat *et al*., 2014). Point-source pollution problems not only increase treatment costs considerably, but also introduce a wide range of potentially infectious agents to waters that may be supplied to many rural and urban communities (Iwane *et al.,* 2001; Momba *et al*., 2006; Mema, 2010), thus resulting in incidences of waterborne diseases with far reaching socioeconomic implications (Palmer *et al*., 1993; Nguyen *et al*., 2006; Odjadjare *et al*., 2010).

Untreated and improperly treated wastewater is known to contain pathogens which can cause disease outbreak, hence the need for adequate treatment before discharge into receiving water bodies (Ashbolt *et al*., 2001; Emmanuel *et al*., 2011). The treatment of wastewater is carried out to minimize and eliminate potential health risks (Zhang *et al*., 2009). The major groups of pathogens that are of importance to wastewater are either bacteria, viruses, fungi or protozoa (FAO, 2003; Amaral *et al.,* 2004; Sheehan *et al*., 2005; Mwabi *et al*., 2011). Bacteria, which are the most common pathogens in water, gain entrance into water mostly through faecal contamination (Wen *et al*., 2009; Sharma, 2013; Khleifat *et al*., 2014). Pathogenic or potentially pathogenic bacteria are normally absent from a healthy intestine unless infection occurs (Sharma *et al*., 2003; Knezevic *et al*., 2008). In the case of an infection, large numbers

of pathogenic bacteria will be passed in the faeces. Some of the bacteria pathogens that are found in wastewater include *Salmonella, Shigella, Legionella* spp. pathogenic strain of *Escherichia coli*, *Pseudomonas* spp. *Yersinia, Campylobacter, Vibrio* (Bitton, 2005; Cabral, 2010; Okoh *et al.,* 2010).

Since there is a wide range of water-borne pathogens that are present in wastewater and owing to the inherent difficulties of analysis, thus making the direct determination of the numbers impractical on a routine basis, the microbiological analysis of water depends majorly on detecting the presence of indicator organisms (Rey *et al.,* 2002; Xi *et al*., 2009). Indicator organisms are present in large numbers in wastewater, although they may not necessarily be pathogenic (Doleans *et al*., 2004; Nguyen *et al*., 2006). Their detection suggests that human contamination of the water has occurred and that more dangerous organisms could be present (Murdoch, 2003; Gomez-Valero *et al.,* 2009). Most indicator organisms are present in faeces and sewage and can survive as long as pathogenic organisms (Doleans *et al*., 2004; Gomez-Valero *et al.,* 2009). The major factors that are considered in classifying an organism as an indicator organism includes its association with faecal contamination, its relationship with the pathogen concerned and efficient and simple testing procedures (Ohno *et al*., 2003; Krojgaard *et al*., 2011). Indicator organisms are therefore referred to as basic tools for the measurement of water quality since they provide evidences for the presence or absence of pathogenic organisms in water (Ohno *et al*., 2003; Plutzer and Torokne, 2012; Department of Health, 2013).

Since the discovery of the human pathogen *Legionella pneumophila* in 1976, about 50 species of *Legionella* have been described, and some of these pathogens are found in wastewater effluents (Leoni *et al*., 2005). About one-half of them have been associated with human Legionnaires' disease, which occurs after inhalation of aerosolized water contaminated with virulent *Legionella* strains (Catalan *et al*., 1997). Numerous cases of

legionellosis after exposure to contaminated water from the water distribution systems of hotels, hospitals, and cooling towers have been reported (Murdoch, 2003; Huang *et al*., 2010). The major reservoirs of *Legionella* spp. are freshwater environments such as lakes, rivers, groundwater, and hot springs, but they can also survive in seawater and water from wastewater treatment plants (Borella *et al*., 2005). Their presence in these different reservoirs demonstrates their ability to grow, or at least persist, under a wide range of different environmental conditions (e.g., temperature, pH) (Pallecchi *et al*., 2011). They can use a number of different strategies to survive in these different environments, including their use of free-living amoebae as hosts for intracellular replication, the protection of the cells in amoebal cysts (Leoni *et al*., 2001; Turetgen *et al*., 2005; Borella *et al*., 2005), their persistence in biofilms (Declerck*,* 2010; Zbikowska *et al*., 2013), and their ability to enter a viable but nonculturable state (Turetgen *et al*., 2005).

3.2. Materials and Methods

3.2.1. Description of wastewater treatment plants used in this study and sources of the isolates

A of total 118 *Legionella* species previously isolated from treated effluents of two wastewater treatment plants namely; Northern wastewater treatment (29°48′45.62″ S and 30° 59′ 45.62) and New Germany wastewater treatment plant (29°48′ 21.68″S and 30°53′ 50.44″E) were used in this study. The Northern wastewater treatment plants processes 70 megalitres per day (ML/day) of industrial and domestic wastewater; which is discharged into the Umgeni River after tertiary treatment by disinfection with chlorine at 3kg/day. The New Germany wastewater treatment plant treats mostly domestic wastewater but sometimes receive industrial wastewater as well. It has a maximum capacity of 7 ML but currently works at a

capacity of 1 ML. Treated effluents are discharged into the Aller River after disinfection with chlorine at 0.5 to 0.8 kg/h and 1.0 to 1.2 kg/h for highly polluted influent.

3.2.2 Identification of presumptive *Legionella* spp.

The presumptive *Legionella* spp. isolates recovered from treated effluents and receiving surface water were identified using biochemical tests and their identity further confirmed molecularly using PCR methods.

3.2.2.1. Biochemical identification of *Legionella* spp.

Identification of the presumptive *Legionella* spp. isolates was performed by oxidase test, catalase test, and gelatin liquefaction test. Specific media and reagents were prepared according to manufacturer's instructions and inoculated with a 24 h nutrient agar-grown culture of the isolates. Tubes and plates were incubated at 37 °C for 24 to 48 h. Positively confirmed isolates were purified and further confirmed using Polymerase Chain Reaction (PCR).

3.2.2.2. Molecular confirmation of biochemically identified *Legionella* spp. isolates

Isolates were inoculated in 1 mL of LB broth from a single colony and incubated at 37 °C with shaking (100 rpm) overnight. For PCR amplification, simple boiling method was used to extract DNA. Boiled cell lysate was prepared by suspending a single colony in 100 µL sterilised distilled H_2O and boiled for 10 min at 100 °C. Debris was removed by centrifugation at 15000 *g* for 5 min and 1-2 µL of the supernatant, containing DNA, was used in PCR reaction mix as DNA template (Yanez *et al*., 2003).

Primers specific for the genus *Legionella* were used in a 25 µL PCR reaction. The PCR mixture contained 2.5 U of *Taq* polymerase , 20 pM each primer (LEG 225 and LEG 858), 10

mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM (each) dNTPs. PCR conditions were as follows: initially denatured at 95 °C for 90 s and then subjected to 30 cycles of denaturation at 95 °C for 10 s, annealing at 64 °C for 1 min, and extension at 74°C for 1 min. A 1-µL portion of the first-step PCR products was added to 49 µL of the PCR mixture containing primers LEG 448 and LEG 858. The second-step (seminested) PCR was performed with an initial denaturation step at 95 °C for 90 s followed by 20 cycles of denaturation at 95°C for 30 s, annealing at 66°C for 1 min, and extension at 74 °C for 1 min. PCR-amplified DNA fragments (8 µL each) were separated in 2% agarose gels with 1% TAE buffer at 60 V. The products were visualized by UV transillumination (Syngene, UK) after staining in 0.1 mg/ml ethidium bromide for 15 min (Miyamoto *et al.,* 1997).

3.2.2.3. Species Specificity Screening of *Legionella* isolates

All isolates confirmed to belong to the genus *Legionella* were further screened and classified into three specific species of interest (*L. pneumophila*, *L. micdadei*, Other *Legionella* spp.) selected based on the dominance of these species from the results obtained from the biochemical identification using the sets of primers listed in Table 3.1. Multiplex PCR conditions were as follows: 94 °C for 5 min, 94 °C for 30 s, 54 °C for 40 s and 72 °C for 1 min, for 34 cycles and a final extension phase of 72 °C for 5 min was used. The amplification products were detected by electrophoresis in a 1.5% (w/v) agarose gel at 60 V for 90 min in 1% TAE buffer. The products were visualized by UV illumination (Syngene, UK) after staining in 0.1 mg/ml ethidium bromide for 15 min.

Table 3.1: List of Primers used in this study

Target	Primer Sequences 5'–3'	Amplicon size (bp)	References
LEG 225	AAGATTAGCCTGCGTCCGAT	654	Miyamoto et al. (1997)
LEG 448	GAGGGTTGATAGGTTAAGAGC	430	Miyamoto et al. (1997)
LEG 858	GTCAACTTATC GCGTTTGCT	654 or 430	Miyamoto et al. (1997)
L. pneumophila	CTAAAAGATTACGTGAATTG ATATGGTTTCTTGATAGCC	237	Zhou *et al*. (2011)
L. bozemanii	TGGTAAAATCCATGAGCA AACAGTGGGCATAATAGTA	307	Zhou *et al*. (2011)
L. longbeachae	TGGTGGACTACATGGCGTC ATACGATGGGCATAATCACAT	383	Zhou *et al*. (2011)
L. micdadei	ATTTAACCATTCGTCGTAAC TTGGGGAATGTTATTCGTG	431	Zhou *et al*. (2011)
bla_{TEM} gene	AGGAAGAGTATGATTCAACA CTCGTCGTTTGGTATGGC	535	Wang *et al.* (2006)
bla_{OXA} gene	TGAGCACCATAAGGCAACCA TTGGGCTAAATGGAAGCGTTT	311	Kuo *et al*. (2010)
$bla_{amp}C$ gene	GGTATGGCTGTGGGTGTTA TCCGAAACGGTTAGTTGAG	822	Yang *et al.* (2008)
intI1	CAGTGGACATAAGCCTGTTC CCCGAGGCATAGACTGTA	160	Mazel *et al.* (2000)
intI2	GTAGCAAACGAGTGACGAAATG CACGGATATGCGACAAAAAGGT	788	Mazel *et al*. (2000)
intI3	GCC TCC GGC AGC GAC TTT CAG ACG GAT CTG CCA AAC CTG ACT	979	Mazel *et al.* (2000)
5'-CS	GGCATCCAAGCAGCAAG	Variable	Fonseca *et al*. (2005)
3'-CS	AAGCAGACTTGACCTGA		
$bla_{\text{IMP-1}}$	GTTCCAAACATGGTTTGGTGGT TTTAACCCTTTAACCGCCTGCTCT	562	Wroblewska *et al.* (2007)
bla_{VIM-1}	AGTGGTGAGTATCCGACAG ATGAAAGTG CGTGGAGAC	261	Tsakris *et al.* (2000)

3.2.3. Antibiotic Resistance Profile Determination

Antibiotic resistance profiling of the isolates was performed using the disk diffusion method described by Tao *et al*. (2010). Fresh culture of the isolates were grown overnight in Mueller-Hinton broth and standardized to 0.5 McFarland by diluting with sterile Mueller-Hinton broth until a photometric reading of 0.08 to 0.1 was obtained on a spectrophotometer (Biochrom, Libra S12) at 625 nm. The standardized culture of the isolates were inoculated onto Mueller-Hinton agar using sterile swabs for confluence growth and allowed to dry for 10 min. Thereafter, appropriate antibiotic disks were placed at equidistance on the surface of the agar plates with a sterile forceps and plates incubated at 37 °C for 18 to 24 h. The diameter of the zone of inhibition was measured to the nearest millimeter and recorded. Antibiotics (Table 3.2) were selected to represent some major classes of antibiotics used as first line drug for Legionellae's infections. *Legionella* isolates were identified as susceptible, intermediate or resistant according to the National Committee for Clinical Laboratory Standard guidelines (CLSI, 2013).

Table 3.2: List of antibiotics (Oxoid, UK) used in the study

Antibiotics class	Antibiotics	Code	Concentration (µg)
Penicillins	Penicillin	P	10
	Ampicillin	AMP	5
	Oxacillin	OX	5
Cephems	Cefotaxime	CTX	30
	Cephalothin	KF	30
Folate Pathway inhibitors	Sulphamethoxazole	RL	25
	Trimethoprim	W	5
Ansamycins	Rifampicin	RD	5
Quinolones	Nalidixic acid	NA	30
Phenicols	Chloramphenicol	C	30
Tetracyclines	Tetracycline	T	10
	Minocycline	MH	30
	Ciprofloxacin	CIP	5
Aminoglycosides	Gentamicin	CN	10
	Streptomycin	S	10
Nitrofurantoins	Nitrofurantoin	F	300
Microlides	Erythromycin	E	15
Glycopeptides	Vancomycin	VA	30
Lincosamides	Clindamycin	DA	2
β-lactam	Ampicillin-sulbactam	SAM	20
Fluoroquinolones	Ofloxacin	OFX	30

Multiple Antibiotic Resistance index was calculated as described by Blasco *et al*. (2008) as follows: MAR= a/b, where a = number of antibiotics to which the isolate was resistant; b = total number of antibiotics against which individual isolate was tested.

3.2.4. Molecular detection of antibiotic resistance and integrase genes

The DNA of the *Legionella* isolates was extracted as previously described in section 3.2.2.2 following the method of Sambrook and Russell (2001). The PCR for the detection of antibiotic resistance genes was carried out in a 25 µL reaction volume for the different

primers indicated in table 3.1. The following conditions were used: *bla*$_{TEM}$ gene (3 min at 93 °C, 40 cycles of 1 min at 93 °C, 1 min at 55 °C and 1 min at 72 °C and finally 7 min at 72 °C); *bla*$_{OXA}$ gene and *bla*$_{ampC}$ gene (94 °C for 5 min, 30 cycles of 25 s of denaturation at 94 °C, 40 s of annealing at 53 °C and 50 s of extension at 72 °C and a final extension of 7 min at 72 °C (Igbinosa *et al*., 2012). Integrons conserved segment were screened with the specific 5'-CS and 3'-CS primers (initial denaturation at 94 °C for 2 min, 20 s of denaturation at 94 °C, 30 s of annealing at 57 °C and 90 min of extension at 68 °C for a total of 30 cycles; 5 s were added to the extension time at each cycle) (Fonseca *et al.,* 2005). Integrase gene detection (*intI1, intI2, intI3*) was done in a 25 µL PCR mixture at the following conditions: 94 °C for 5 min, 30 cycles of 1 min of denaturation at 94 °C, 1 min of annealing at 59 °C, 1 min of extension at 72 °C and a final elongation at 8 min at 72 °C (Mazel *et al.,* 2000). Amplification products were analysed using 1.5% agarose gel electrophoresis in 1% TAE buffer at 60 V for 90 min (Igbinosa *et al*., 2012). *bla*$_{IMP}$ gene and *bla*$_{VIM}$ gene (94 °C for 5 min, 30 cycles of 30 s of denaturation at 94 °C, 40 s of annealing at 52 °C and 50 s of extension at 72 °C and a final cycle of 5 min at 72 °C (Wroblewska *et al.* 2007). The products were visualized by UV illumination (Syngene, UK) after staining in 0.1 mg/ml ethidium bromide for 15 min.

3.2.5 Molecular detection of gene cassettes

The DNA of the *Legionella* isolates was extracted as previously described in section 2.5. following the method of Sambrook and Russell (2001). The PCR for the detection of gene cassettes was carried out in 50 µL reaction volume containing 3mM $MgCl_2$, 3 µl of total DNA as a template, 50 pmol of each primer, 1mM dNTPs and 1.6 U of *Taq* Polymerase using primer 5'C and 3'C (Table 3.1) with the following conditions: 95 °C for 5 min, 35 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 55 °C and 1 min of extension at 72 °C and a final extension of 5 min at 72 °C (Fonseca *et al.,* 2005). The products were separated in a

2% (w/v) agarose gel at 60 V for 90 min in 1% TAE buffer. The products were visualized by UV illumination (Syngene, UK) after staining in 0.5 mg/ml ethidium bromide for 15 min**.** Amplicons corresponding to gene cassette regions were cleaved with *HaeIII* restriction enzyme. Briefly, each 20 µl of the restriction mixture contained 2 µl (20 U) of enzyme, 8 µl of PCR-amplified product, 1 µl of enzyme buffer and 9 µl of double-distilled water. As per manufacturer's guidelines, restriction mixtures were incubated at 37 °C for 1 h (Fonseca *et al*., 2005). The products were separated in a 1.5% (w/v) agarose gel at 60 V for 90 min in 1% TAE buffer. The products were visualized by UV transiillumination (Syngene, UK) after staining in 0.5 mg/ml ethidium bromide for 15 min

3.2.6 Screening for metallo-β-lactamase (MBL) production by the *Legionella* spp. isolates

To identify MBL production in *Legionella* spp., IMP-EDTA disk synergy test was used as developed by Yong *et al*. (2002). To make 0.5 M EDTA solution 186.1g of disodium EDTA was dissolved in 1000 ml of distilled water and pH was adjusted to 8.0 by using NaOH. The mixture was then sterilised by autoclaving. EDTA imipenem disks were dried immediately in an incubator and stored at 4 °C or at -20 °C in an air tight vial without desiccant. Fresh culture of the isolates were grown overnight in Mueller-Hinton broth and standardized to 0.5 McFarland by diluting with sterile Mueller-Hinton broth until a photometric reading of 0.08 to 0.1 was obtained on a spectrophotometer (Biochrom, Libra S12) at 625 nm. The standardized culture of the isolates were inoculated onto Mueller-Hinton agar using sterile swabs for confluence growth and allowed to dry for 10 min. A 10-µg imipenem disk and an imipenem plus 750 µg EDTA were placed on Mueller Hinton agar. The inhibition zones of these disks were compared after 16 - 18 hrs of incubation at 37 °C. An increase in the inhibition zone of the imipenem and EDTA disk ≥7mm than that of the imipenem disk alone

is indicative of positive isolate. MBL index was determined as zone diameter of imipenem and EDTA disk minus zone diameter of imipenem disk alone,

3.2.7 Virulence determinants assays.

3.2.7.1. Elastase Assay

Elastase activity was measured using the elastin Congo red (ECR; Sigma) assay (Rust *et al*., 1994). Cells were grown in LB broth at 37 ºC for 16 h, centrifuged at 15 000 *g* at 4ºC for 10 min and 0.5 mL supernatant was added to 1 mL of assay buffer (30 mM Tris buffer, pH 7.2) containing 10 mg of elastin Congo Red. The mixture was incubated at 37 ºC for 6 h. Insoluble ECR was removed by centrifugation and the absorption of the supernatant was measured at 495 nm. LB medium was used as a negative control. Isolates with an absorbance value ≥ 0.2 at 495 nm are considered elastase positive

3.2.7.2 Protease assay

Protease activity of the *Legionella* spp. isolates was determined using a method described by Schmidtchen *et al*. (2001). A 125-µl aliquot of 2% azocasein solution in Tris buffer (pH 7.8) was incubated with 75 µl of bacterial suspension at 37 °C for 45 min. The reaction was stopped by adding 600 µl of 10 % trichloroacetic acid. After incubation for 10 min at room temperature, the mixture was centrifuged for 5 min at 12,500 rpm, and 600 µl of the supernatant were transferred to a tube containing 500 µl of 1 M NaOH. The absorbance was measured at 440 nm. Isolates with an absorbance value ≥ 0.2 at 440 nm are considered protease positive

3.2.7.3 Screening for rhamnolipid production

Rhamnolipid production by *Legionella* spp. was detected by using M9-glutamate minimal medium agar plates containing 64g Na_2HPO_4-7H2O, 15 g KH_2PO_4, 2.5 g NaCl, 5.0 g NH_4Cl,

1M $MgSO_4$, 20 g of glucose, 1M $CaCl_2$, 15 g of agar containing 0.2 g cetyltrimethylammonium bromide (CTAB) and 5 mg methylene blue l-1, were inoculated with 2 ml of an overnight LB culture of *Legionella* spp. strains. After an overnight incubation at 37 ºC, the diameter of the clear zone around the bacterial spot measured as evidence of rhamnolipid production (Senturk *et al*., 2012).

3.2.8 RAPD analysis of integron positive *Legionella* spp.

Genomic DNA was isolated from *Legionella* spp. and purified using the GeneJet Genomic purification Kit. DNA amplification for RAPD was performed on a thermocycler (Bio Rad, USA) using 10-mer primer OP-A3 (5'-AGTCAGCCAC-3'). The reaction mixture 10X buffer, 50 µM of $MgCl_2$, 10Mm of dNTPs, 10 µM of primer and 0.2 µM of *Taq* Polymerase. Amplification was carried out in a heated-lid automated DNA thermal cycler (Perkin-Elmer Applied Biosystems Inc., USA) for 40 cycles, each consisting of a denaturing step of 1 min at 94ºC, followed by annealing step of 1 min at 36 ºC and an extension step of 2 min at 72 ºC. The last cycle was followed by 5 min of long extension at 72 ºC (Khleifat *et al*., 2014). Amplification products were analysed using 1% agarose gel electrophoresis in 1% TAE buffer at 50 V for 240 min. The products were visualized by UV illumination (Syngene, UK) after staining in 0.1 mg/ml ethidium bromide for 15 min. Genotypic variation were analysed using the GelCompareII version 6.0 software package (Applied Maths) by Jackard and Unweighted Pair Group Method with Arithmetic mean (UPGMA) cluster analysis to produce a dendogram.

3.3. Results

3.3.1. Identification of *Legionella* spp. isolates

Of 118 presumptive *Legionella* spp. isolates subjected to biochemical tests, 100 were found to exhibit typical biochemical properties of *Legionella* spp. based on biochemical test (Table 3.3).

Table 3.3: Characteristics of *Legionella* species based on biochemical tests

Biochemical test	Possible identity	Percentage
Oxidase (+) **Catalase (+)** **Gelatin (-)**	*L. micdadei/ L. longbeachae*	38/ 118 (32%)
Oxidase (-) **Catalase(+)** **Gelatin (-)**	*L. bozemanni/ L. dumofi/ L. gormanii*	30/118 (25%)
Oxidase (+) **Catalase (+)** **Gelatin (+)**	*L. pneumophila*	51/118 (43%)

The first amplifications of the seminested PCR with primers LEG 225 and LEG 858 produced 654-bp DNA bands. The second amplifications of the seminested PCR with the primers LEG 448 and LEG 858 produced 430-bp DNA bands from all *Legionella* strains (Figure 3.1).

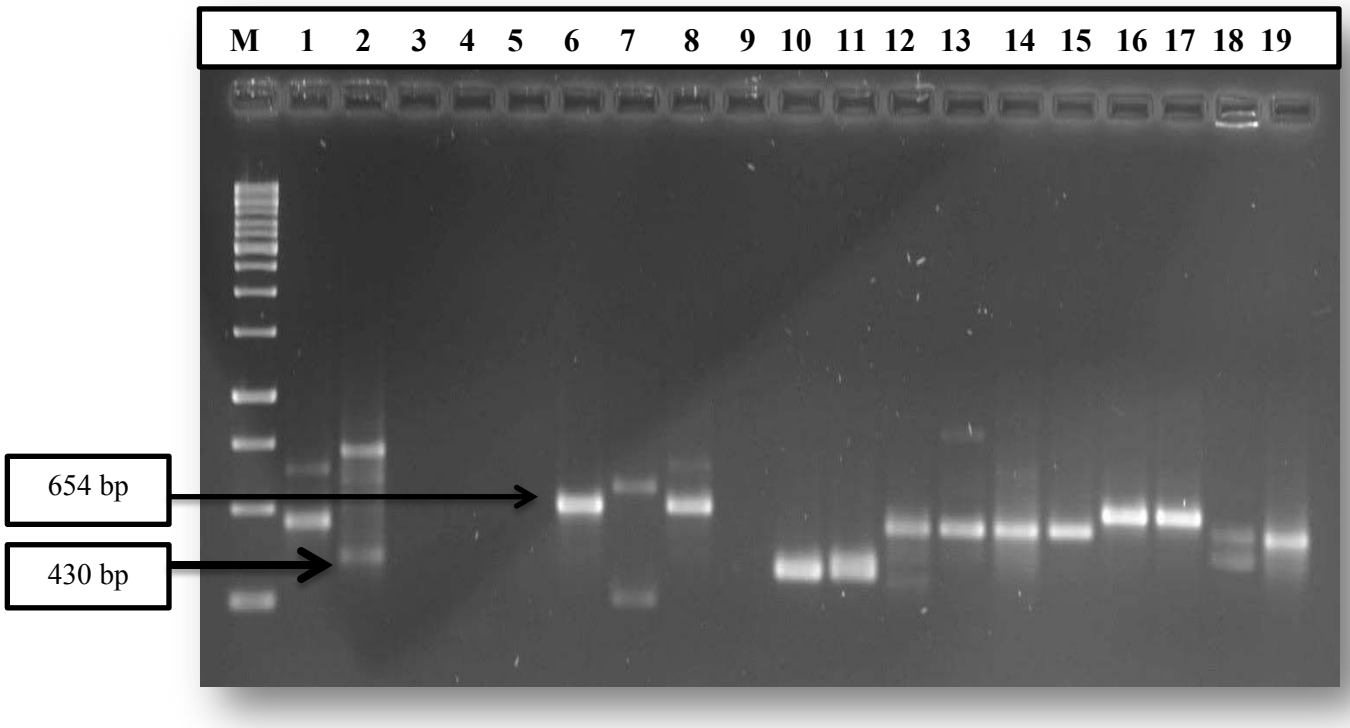

Figure 3.1: Agarose gel electrophoresis showing the typical first-step PCR products and the seminested PCR products. In lane M, a 1-kb DNA ladder was used as a DNA size marker.

Of the 118 isolates biochemically confirmed, 100 isolates were identified to belong to the *Legionella* genus. Similarly, distributions of *Legionella* with respect to species were 37% for *L. pneumophila*, 3% for *L. micdadei* and 60% for Other *Legionella* spp. (Table 3.3).

3.3.2 Antibiotic Resistance Profile of *Legionella* spp. isolates

A total of 100 strains of *Legionella* belonging to three species (*L. pneumophila*, *L. micdadei* and Other *Legionella* spp.) confirmed in this study. Isolates were subjected to antibiogram assay against a panel of 21 antibiotics (Table 3.4). All tested isolates showed sensitivity to cefotaxime, cephalothin, trimethoprim, nalidixic acid, ciprofloxacin, gentamicin, nitrofurantoin, ampicillin-sulbactam and ofloxacin. Conversely, all isolates were resistant to penicillin, ampicillin, oxacillin, sulphamethoxazole, rifamycin, chloramphenicol, tetracycline, streptomycin, erythromycin and clindamycin. The tested isolates showed a high degree of

multiple antimicrobial resistance (MAR) ranging between 9 and 11 antibiotics, distributed among 3 and 7antibiotic classes (Table 3.5). The MAR index varied from 0.44 to 0.55.

Table 3.4: Antibiotic resistance profile of *Legionella* spp. isolated from wastewater treatment plants and receiving surface waters

Antibiotics class	**Antibiotics**	***L. pneumophila* (n=37)**			***L. micdadei*(n=3)**			**Other *Legionella* species (n=60)**		
		S (%)	**I (%)**	**R (%)**	**S (%)**	**I (%)**	**R (%)**	**S (%)**	**I (%)**	**R (%)**
Penicillins	Penicillin Ampicillin Oxacillin	0 (0) 0 (0) 0 (0)	0 (0) 0 (0) 0 (0)	37 (100) 37 (100) 37 (100)	0 (0) 0 (0) 0 (0)	0 (0) 0 (0) 0 (0)	3 (100) 3 (100) 3 (100)	0 (0) 0 (0) 0 (0)	0 (0) 0 (0) 0 (0)	60 (100) 60 (100) 60 (100)
Cephems	Cefotaxime Cephalothin	37 (100) 37 (100)	0 (0) 0 (0)	0 (0) 0 (0)	3 (100) 3 (100)	0 (0) 0 (0)	0 (0) 0 (0)	60 (100) 60 (100)	0 (0) 0 (0)	0 (0) 0 (0)
Folate Pathway inhibitors	Sulphamethoxazole Trimethoprim	0 (0) 37 (100)	0 (0) 0 (0)	37 (100) 0 (0)	0 (0) 3 (100)	0 (0) 0 (0)	3 (100) 0 (0)	0 (0) 60 (100)	0 (0) 0 (0)	60 (100) 0 (0)
Ansamycins	Rifamycin	0 (0)	0 (0)	37 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	60 (100)
Quinolones	Nalidixic acid	37 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	60 (100)	0 (0)	0 (0)
Phenicols	Chloramphenicol	0 (0)	0 (0)	37 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	60 (100)
Tetracyclines	Tetracycline Minocycline Ciprofloxacin	0 (0) 11(30) 37 (100)	0 (0) 0 (0) 0 (0)	37 (100) 26 (70) 0 (0)	0 (0) 1 (40) 3 (100)	0 (0) 0 (0) 0 (0)	3 (100) 2 (60) 0 (0)	0 (0) 15 (25) 60 (100)	0 (0) 0 (0) 0 (0)	60 (100) 45 (75) 0 (0)
Aminoglycosides	Gentamicin Streptomycin	37 (100) 0 (0)	0 (0) 0 (0)	0 (0) 37 (100)	3 (100) 0 (0)	0 (0) 0 (0)	0 (0) 3 (100)	60 (100) 0 (0)	0 (0) 0 (0)	0 (0) 60 (100)
Nitrofurantoins	Nitrofurantoin	37 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	60 (100)	0 (0)	0 (0)
Microlides	Erythromycin	0 (0)	0 (0)	37 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	60 (100)
Glycopeptides	Vancomycin	7(20)	7(20)	23 (60)	1 (25)	0 (0)	2 (75)	24(40)	0 (0)	36 (60)
Lincosamides	Clindamycin	0 (0)	0 (0)	37(100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	60 (100)
β-lactam	Ampicillin-sulbactam	37 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	60 (100)	0 (0)	0 (0)
Fluoroquinolones	Ofloxacin	37 (100)	0 (0)	0 (0)	3 (100)	0 (0)	0 (0)	60 (100)	0 (0)	0 (0)

Table 3.5: Distribution of the resistance phenotypes and multiple antibiotic resistance amongst *Legionella* spp. isolates

Phenotype	Isolates (n=100)	Resistance profile	MAR index
A	46	AMP, MH, VA, OX, RD, RL, TE, E, P, S, DA	0.55
B	52	AMP, VA, OX, RD, RL, TE, E, P, S, DA	0.5
C	2	AMP, OX, RD, RL, TE, E, P, S, DA	0.44

Legend: AMP—Ampicillin; DA—Clindamycin; E—Erythromycin; MH—Minocycline; OX—Oxacillin; P—Penicillin G; R—Rifampicin; S—Streptomycin; RL—Sulphamethoxazole; TE—Tetracycline; VA—Vancomycin

3.3.3. Antibiotic resistance genes, integrase genes and gene cassette array

The screening for antibiotic resistance genes revealed the absence of bla_{ampC} in all the *Legionella* spp. isolates, while the gene coding for bla_{TEM} and bla_{OXA} (Figure 3.2 and Table 3.6) were detected in 14% and 60% of the isolates, respectively. The screening for antibiotic resistance genes revealed the absence of $bla_{\text{VIM-1}}$ and $bla_{\text{IMP-1}}$ genes in any of the *Legionella* isolates.

Fifty isolates were selected based on the different resistance phenotypes and screened for the presence of integrons. Twenty-five (50%) , of the 25 *intII*-PCR positive, 22 isolates tested positive for the conserved-segment PCR with a variable region of class 2 integrons of 300 bp (15/22), 400 bp (2/22), 700 bp (2/22), 900 bp (1/22) and 1.5 kb (2/22) (Table 3.6).

Sequencing of the PCR product revealed the presence of *drfA1-sat1-aadA1*, which encodes additional resistance to trimethoprim, and streptomycin in 17 isolates, and *sat2* and *aadA1*, encoding streptothricin acetyl-trasferase and aminoglycoside-adenyltransferase in 5 isolates,

respectively. Amplicons obtained from the class 2 integron analyses having the same size and (RFLP) pattern are considered to harbour the same cassette array. According to the integron cassette arrays, *Legionella* spp. can be classified into four clusters. The results revealed that most of the strains possess variable gene cassette that is correlative with their individual phenotypes (Table 3.6).

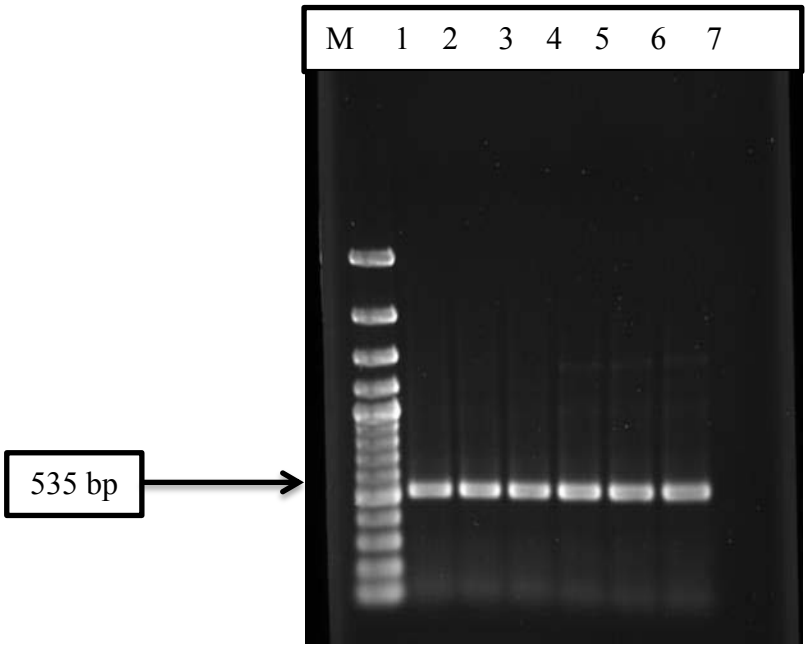

Figure 3.2: Agarose gel showing the expected amplicon size (535 bp) of bla_{TEM} resistance gene in *Legionella* spp. Lane M contains 100 bp plus DNA Ladder, lane 1 to 6 contains *Legionella* isolates positive for bla_{TEM} gene and lane 7 is the negative control.

Table 3.6: Characterization of integrons and antibiotic resistance patterns of integron positive *Legionella* spp. isolated from wastewater treatment plants and receiving surface water

Strain designation	Species	Integron	Size of the integrons variable part	Gene cassette	*bla*$_{OXA}$	*bla*$_{ampC}$	Antibiotic resistance pattern
L 1	*L. pneumophila*	*Int2*	300 bp	*drfA1-sat1-aadA1*	-	-	VA, OX, RD, CD, RL, TE, E, P, S
L 3	*Legionella* spp.	*Int2*	300 bp	*drfA1-sat1-aadA1*	-	-	MH, VA, OX, RD, CD, RL, TE, E, P, S
L 4	*L. pneumophila*	*Int2*	300 bp	*drfA1-sat1-aadA1*	-	-	MH, VA, OX, RD, CD, RL, TE, E, P, S
L 6	*Legionella* spp.	*Int2*	700 bp	*drfA1-sat1-aadA1*	-	-	MH, VA, OX, RD, CD, RL, TE, E, P, S
L 8	*L. pneumophila*	*Int2*	300 bp	*drfA1-sat1-aadA1*	+	-	MH, VA, OX, RD, CD, RL, TE, E, P, S
L 10	*L. pneumophila*	*Int2*	300 bp	*drfA1-sat1-aadA1*	+	-	VA, OX, RD, CD, RL, TE, E, P, S
L 11	*L. pneumophila*	*Int2*	300 bp	*drfA1-sat1-aadA1*	+	-	MH, VA, OX, RD, CD, RL, TE, E, P, S
L 15	*Legionella* spp.	*Int2*	300 bp	*drfA1-sat1-aadA1*	+	-	MH, VA, OX, RD, CD, RL, TE, E, P, S
L 16	*L. pneumophila*	*Int2*	300 bp	*drfA1-sat1-aadA1*	+	-	MH, VA, OX, RD, CD, RL, TE, E, P, S
L 17	*L. pneumophila*	*Int2*	300 bp	*drfA1-sat1-aadA1*	+	-	MH, VA, OX, RD, CD, RL, TE, E, P, S
L 21	*L. pneumophila*	*Int2*	300 bp	*drfA1-sat1-aadA1*	+	-	MH, VA, OX, RD, CD, RL, TE, E, P, S

Table 3.6: continued.

Strain designation	Species	Integrase	Size of the integrons variable part	Gene cassette	*bla*$_{OXA}$	*bla*$_{ampC}$	Antibiotic resistance pattern
L 22	*L. pneumophila*	*Int2*	300 bp	*drfA1-sat1-aadA1*	+	-	MH, VA, OX, RD, CD, RL, TE, E, P, S
L 23	*L. pneumophila*	*Int2*	900 bp	*sat1-aadA1*	+	-	VA, OX, RD, CD, RL, TE, E, P, S
L 25	*Legionella* spp.	*Int2*	400 bp	*drfA1-sat1-aadA1*	-	-	MH, VA, OX, RD, CD, RL, TE, E, P, S
L 27	*L. pneumophila*	*Int2*	300 bp	*drfA1-sat1-aadA1*	+	-	MH, VA, OX, RD, CD, RL, TE, E, P, S
L 28	*L. pneumophila*	*Int2*	700 bp	*sat1-aadA1*	+	-	VA, OX, RD, CD, RL, TE, E, P, S
L 33	*L. pneumophila*	*Int2*	300 bp	*drfA1-sat1-aadA1*	+	-	MH, VA, OX, RD, CD, RL, TE, E, P, S
L 35	*Legionella* spp.	*Int2*	400 bp	*sat1-aadA1*	-	-	VA, OX, RD, CD, RL, TE, E, P, S
L 36	*L. pneumophila*	*Int2*	300 bp	*drfA1-sat1-aadA1*	+	-	MH, VA, OX, RD, CD, RL, TE, E, P, S
L 40	*L. pneumophila*	*Int2*	300 bp	*drfA1-sat1-aadA1*	+	-	MH, VA, OX, RD, CD, RL, TE, E, P, S
L 42	*Legionella* spp.	*Int2*	1.5 bp	*sat1-aadA1*	-	-	MH, VA, OX, RD, CD, RL, TE, E, P, S
L 43	*L. micdadei*	*Int2*	1.5 kb	*sat1-aadA1*	-	-	OX, RD, CD, RL, TE, E, P, S
L 35	*L. pneumophila*	*Int2*	-	-	-	-	MH, VA, OX, RD, CD, RL, TE, E, P, S
L 41	*Legionella* spp	*Int2*	-	-	-	-	VA, OX, RD, CD, RL, TE, E, P, S
L 50	*Legionella* spp.	*Int2*	-	-	-	-	MH, VA, OX, RD, CD, RL, TE, E, P, S

Legend: AMP—Ampicillin; DA—Clindamycin; E—Erythromycin; MH—Minocycline; OX—Oxacillin; P—Penicillin G; R—Rifampicin; S—Streptomycin; RL—Sulphamethoxazole; TE—Tetracycline; VA—Vancomycin

3.3.4 Metallo-β-lactamase (MBL) activity and virulence determinants profile of the *Legionella* spp. isolates

All the selected 50 *Legionella* spp. isolates, showed resistance against imipenem by disk diffusion method, however, imipenem-EDTA disk method showed metallo-β-lactamase production in only 12% (6/50) of the imipenem resistant *Legionella* spp. with an average of 7 mm inhibition zone diameter between imipenem disk and imipenem plus EDTA disk for MBL positive isolates (Table 3.7). To further characterize the *Legionella* spp. isolates, *in vitro* production of virulence factors, elastase that depends upon an active QS-circuitry was determined. A total of 44 (88 %) of these isolates produced elastase; LasB elastase, the most potent elastase produced by *Legionella* spp. and is one of the major virulence factors controlled by QS. *Legionella* spp. isolates displayed different levels of elastolytic activities since they demonstrate variable absorbance values at 495 nm (Table 3.7). A total of 28 (56 %) of these isolates produced protease, one of the major virulence factors controlled by QS. *Legionella* spp. isolates displayed different levels of proteolytic activities since they displayed variable absorbance values at 440 nm (Table 3.7). Fifty isolates were screened for the production of rhamnolipid as a biosurfactant. The productive colonies of *Legionella* spp. indicating rhamnolipid production were surrounded by dark blue halos on the light blue agar plate. Bacterial colonies which produced dark blue halos against a light background were taken as biosurfactant producing strains.

Table 3.7: Metallo-β-lactamase activity and virulence determinant profiles of *Legionella* spp. isolates

Bacterial strain	Species	β-lactamase activity [a] (IMP + EDTA disk-IMPdisk)		Rhamnolipid	Elastase [b] (OD at 495 nm)	Protease [c] (OD at 440 nm)	
L1	*L. pneumophila*	-	(2)	-	+ (0.235)	-	(0.014)
L 2	*Legionella* spp.	-	(0)	-	+ (0.288)	-	(0.014)
L 3	*Legionella* spp.	-	(1)	-	+ (0.341)	-	(0.037)
L 4	*L. pneumophila*	-	(2)	-	+ (0.701)	-	(0.031)
L 5	*Legionella* spp.	-	(0)	-	+ (0.419)	+	(0.611)
L 6	*Legionella* spp.	-	(2)	+	+ (0.371)	+	(0.280)
L 7	*Legionella* spp.	-	(0)	+	- (0.162)	-	(0.018)
L 8	*L. pneumophila*	-	(4)	+	+ (0.324)	+	(0.085)
L 9	*Legionella* spp.	-	(2)	-	+ (0.340)	-	(0.013)
L 10	*L. pneumophila*	-	(2)	+	+ (0.261)	-	(0.018)
L 11	*L. pneumophila*	-	(0)	+	+ (0.282)	-	(0.013)
L 12	*L. micdadei*	-	(0)	-	+ (0.440)	+	(0.289)
L 13	*Legionella* spp.	+	(9)	+	+ (0.398)	+	(0.289)
L 14	*Legionella* spp.	-	(2)	-	+ (0.476)	+	(0.478)
L 15	*Legionella* spp.	-	(2)	-	+ (0.414)	+	(0.978)
L 16	*L. pneumophila*	-	(0)	+	+ (0.327)	-	(0.006)
L 17	*L. pneumophila*	-	(0)	+	+ (0.329)	-	(0.003)
L 18	*Legionella* spp.	-	(4)	-	+ (0.625)	+	(0.410)
L 19	*Legionella* spp.	-	(0)	+	+ (0.219)	+	(0.270)
L 20	*Legionella* spp.	-	(0)	+	+ (0.570)	+	(0.510)
L 21	*L. pneumophila*	-	(0)	+	+(0.463)	-	(0.005)
L 22	*L. pneumophila*	-	(0)	+	+ (0.247)	+	(0.215)
L 23	*L. pneumophila*	-	(0)	-	+ (0.205)	-	(0.003)
L 24	*L. pneumophila*	+	(7)	-	+ (0.350)	-	(0.006)
L 25	*Legionella* spp.	-	(2)	-	+ (0.173)	-	(0.014)
L 26	*Legionella* spp.	-	(2)	+	+ (0.273)	-	(0.019)
L 27	*L. pneumophila*	-	(0)	+	+ (0.447)	+	(0.211)
L 28	*L. pneumophila*	+	(7)	+	+ (0.418)	-	(0.054)
L 29	*L. pneumophila*	-	(0)	+	+ (0.327)	-	(0.009)
L 30	*Legionella* spp.	-	(2)	-	+ (0.411)	+	(0.901)
L 31	*Legionella* spp.	-	(3)	+	+ (0.549)	+	(0.661)
L 32	*L. pneumophila*	-	(2)	-	+ (0.298)	-	(0.002)
L 33	*L. pneumophila*	-	(3)	-	+ (0.390)	+	(0.275)
L 34	*Legionella* spp.	-	(0)	-	- (0.158)	+	(0.214)
L 35	*Legionella* spp.	-	(1)	+	- (0.195)	+	(0.253)
L 36	*L. pneumophila*	-	(0)	+	+ (0.209)	+	(0.536)
L 37	*Legionella* spp.	-	(2)	-	+ (0.564)	+	(0.281)
L 38	*Legionella* spp.	-	(2)	+	+ (0.213)	+	(0.276)
L 39	*Legionella* spp.	-	(0)	+	+ (0.252)	+	(0.319)
L 40	*L. pneumophila*	-	(2)	+	+ (0.306)	-	(0.027)
L 41	*L. pneumophila*	+	(7)	+	+ (0.409)	-	(0.014)
L 42	*Legionella* spp.	+	(12)	+	+ (0.209)	-	(0.013)
L 43	*L. micdadei*	-	(1)	+	+ (0.252)	-	(0.005)
L 44	*Legionella* spp.	-	(3)	+	+ (0.544)	-	(0.011)
L 45	*Legionella* spp.	-	(1)	+	+ (0.389)	+	(0.216)
L 46	*Legionella* spp.	-	(2)	+	+ (0.414)	+	(0.264)
L 47	*Legionella* spp.	+	(10)	+	+ (0.381)	-	(0.046)
L 48	*Legionella* spp.	-	(2)	+	+ (0.372)	-	(0.002)
L 49	*Legionella* spp.	-	(1)	+	+ (0.326)	+	(0.215)
L 50	*Legionella* spp.	-	(0)	-	- (0.159)	-	(0.051)

a= Difference in inhibition zone of impenem and EDTA, and EDTA ≥ 7 mm is considered positive

a= Difference in inhibition zone of impenem and EDTA, and EDTA ≥ 7 mm is considered positive; b= OD valued ≥ 0.2 at 495nm is considered positive; c= OD valued ≥ 0.2 at 440 nm is considered positive

3.3.5 RAPD fingerprinting of integron positive *Legionella* spp.

All 25 isolates which were integron positive were selected for RAPD-PCR analysis. OP-A3 RAPD primer was employed in this study for it has been successful in sub-grouping *Legionella* isolates. RAPD-PCR amplification of the selected *Legionella* isolates DNA showed a number of major bands (ranging from 250 bp to 2500 bp). Data interpretation criteria were based on differences in formation and/or position of the bands, The RAPD banding patterns generated by primer OP-A3 for the selected *Legionella* isolates are shown in Figure 3.3. Based on the numbers of band differences, two clusters (B and C) were identified as MBL producing isolates, where cluster C had isolates which are non-MBL producers. Cluster B was comprised 2 (25 %) which are MBL producing isolates, all carrying class 2 integrons. Cluster C of the MBL isolates comprised 1 (9%) of the MBL-producing isolates (Figure 3.4).

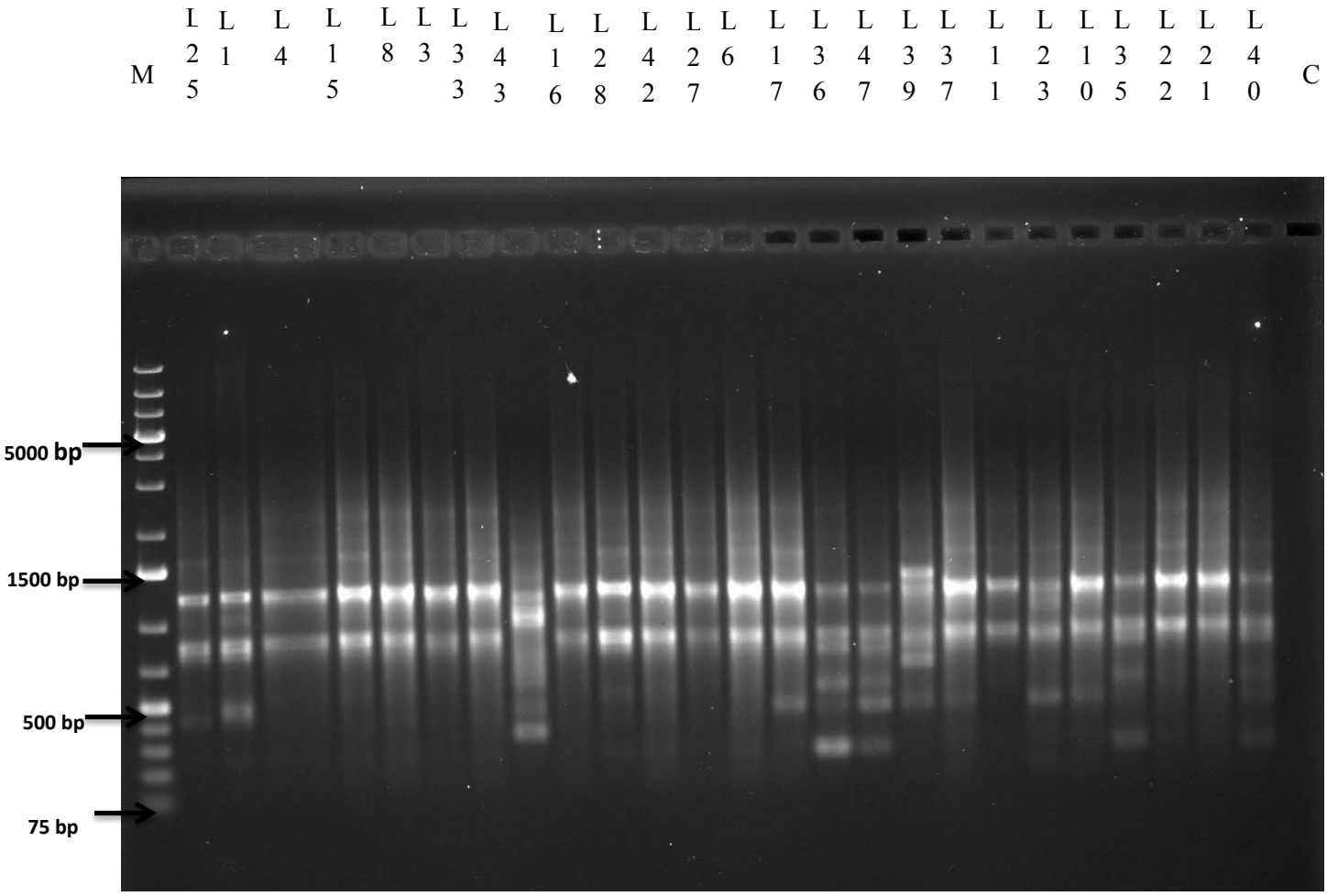

Figure 3.3: RAPD band patterns of *Legionella* isolates with primer OP-A3. Lanes M are molecular size markers (1 kb plus DNA ladder); lanes 1–25 are environmental isolates of *Legionella,* and lane C is the control.

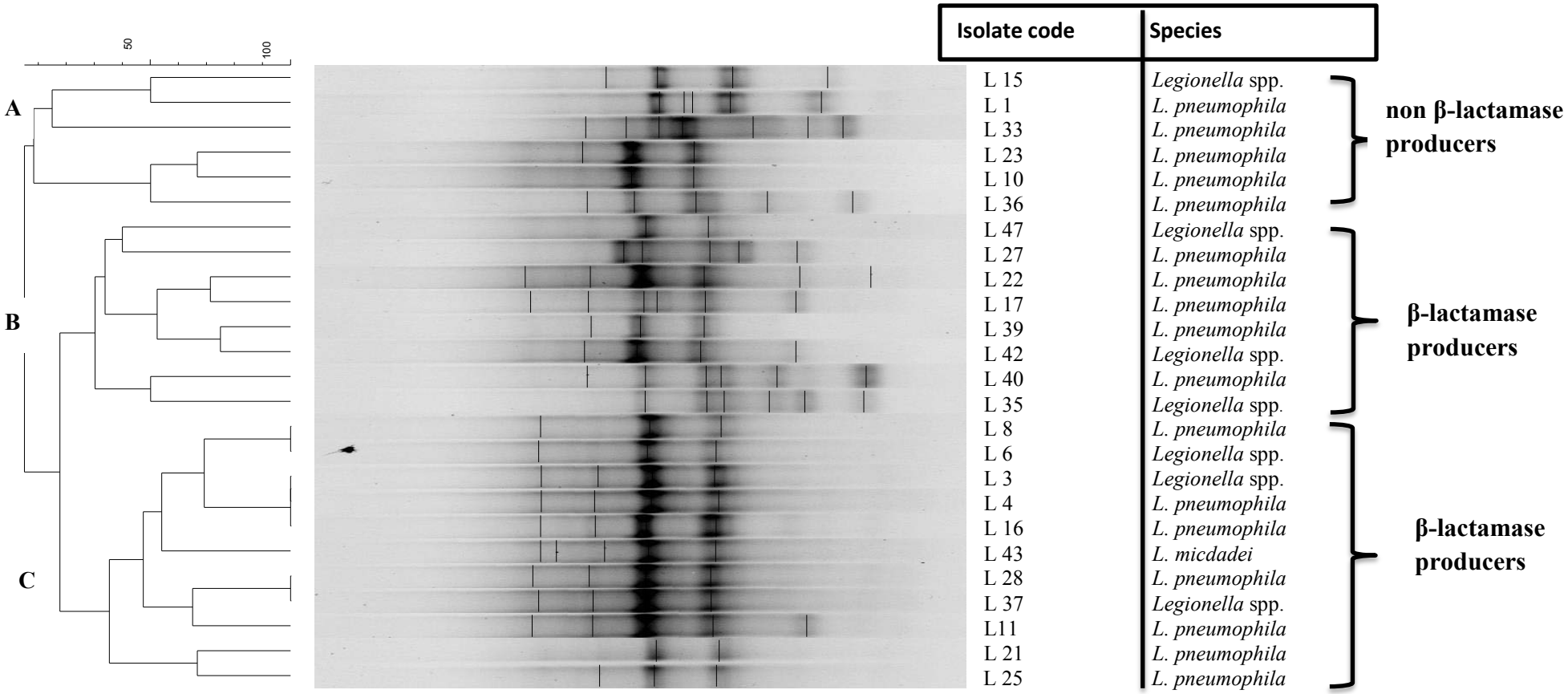

Figure 3.4: Dendrogram showing the cluster analysis of *Legionella* spp. based on primer OP-A3 PCR fingerprinting patterns using Jackard index and UPGMA clusterization. The scale at the top represent percentage similarity.

3.4 Discussion

The study of *Legionella* in treated wastewater requires special attention, especially when this water is to be used in spray irrigation, as *Legionella* is transmitted via the inhalation of aerosols and may consequently represent a health risk (Lin *et al*., 2009). In this study, the presumptively identified *Legionella* species recovered from treated effluent of wastewater treatment plants and receiving surface water were subjected to a range of biochemical tests and PCR for further confirmation, with results indicating that potentially pathogenic *Legionella* species have the ability to bypass conventional wastewater treatment processes. One hundred (100) strains of *Legionella spp.* (37 *L. pneumophila*, 3 *L. micdadei* and 60 Other *Legionella* spp.) were confirmed and characterised for their antimicrobial resistance profiles and virulence determinants in this study.

Resistance to β-lactams, quinolones, carbapenems and aminoglycosides are often detected in *Legionella* isolates and the rapid spread of antibiotic resistance genes among bacterial isolates is an increasing problem in infectious diseases. Results from this study show that all the tested isolates were susceptible to β-lactams. Recent studies have shown that resistance genes might have been carried by an integron which have not yet been detected for *Legionella* spp. Resistance genes bla_{OXA} and bla_{TEM} were found in 60% and 14% of the isolates, respectively. Many resistance genes exist as gene cassettes within integrons, which may themselves be located on transmissible plasmids and transposons (Perola *et al.,* 2005; Lin *et al*., 2009). Production of an integron- mediated β-lactamases from different molecular classes (carbenicillinases and extended-spectrum β-lactamases belonging to class A, class D oxacillinases and class B carbapenem hydrolysing enzymes) and synthesis of aminoglycoside modifying enzymes (phosphoryl-transferases, acetyltransferases and adenylyltransferases) are some of the resistance mechanisms in *Legionella* spp. (Henriques *et al*., 2006; Lin *et al*., 2009). The relatively high level of resistance to antimicrobial agents recorded in this study is a reflection of misuse or abuse of these agents in the environment. Multiple drug resistance is an extremely serious public health problem and it has been

found associated with the outbreak of major epidemic throughout the world. Thus, the multiple – drug resistance shown by these pathogens are worrisome and of public health concern (Lateef, 2004**;** Chaabna *et al.,* 2013). Consistent with the observation of this study, *Legionella* species have been reported to be highly sensitive to gentamicin (Kuete *et al*., 2010), Ciprofloxacin, Cefotaxime and Cephalothin (Atwill *et al.,* 2012). However, observation in this study is contrary to a previous report suggesting that cephems have lost their effectiveness against *Legionella* strains due to resistance (Akinbowale *et al.,* 2007). Previous studies on antibiotic resistant levels in wastewater treatment plants have reported contradictory results on whether or not wastewater treatment can increase the prevalence of antibiotic resistant bacteria (Alonso *et al*., 1999), and relatively little has been published on antibiotic resistance in *Legionella* spp. in the wastewater treatment process.

Our environmental *Legionella* isolates, recovered from wastewater presented multi-resistance to the drugs tested with the MAR index values higher than 0.5. All the tested isolates in this study showed multiple antibiotic resistances (MARs) ranging from nine to eleven antibiotics distributed among 3 to 7 classes. The MAR indices were higher than the 0.2 limit in all our tested isolates (Table 3.5), suggesting that isolates in this study originated from high risks source(s) of contamination where antibiotics are often used. Two major intrinsic mechanisms have been reported to confer bacterial resistance to multiple antimicrobial drug classes: mutations in outer membrane porins resulting in reduced permeability to antimicrobials; and over expression of multidrug efflux pumps (Esiobu *et al*., 2002; Kuete *et al*., 2010), which tend to pump out antibiotics before they (the antibiotics) have the opportunity of acting on their target (Navon-Venezia *et al*., 2005; Ashish *et al*., 2011). In fact, *Legionella* species are considered to be naturally resistant to aminopenicillins, amoxycillin plus clavulanate, first- and second-generation cephalosporins, cefotaxime, ceftriaxome, quinolones, trimethoprim, kanamycin, chloramphenicol, tetracyclines and nitrofurantoin (Lin *et al*., 2004; Blasco *et al*., 2008). In addition, Navon-Venezia *et al*.

(2005) observed that MAR bacterial strains may also arise due to unrelated mechanisms accumulating sequentially in an organism (Ruiz *et al.,* 2004).

Similar to previous report indicating the prevalence of class 2 integrons among wastewater bacterial isolates, class 2 integrons were the most prevalent among the *Legionella* spp. isolates tested in this study, with 25/50 (50%) of the isolates carrying class 2 integrons. However, analysis of the integron variable region revealed that 3/25 (6%) were lacking in gene cassettes, indicating a low occurrence of empty class 2 integrons among these strains. The absence of class 1 and class 3 integrons among these isolates established the restricted distribution of these two genetic elements among *Legionella* isolates. Genes causing resistance to aminoglycosides and β-lactams are often seen in integrons from the members of Enterobacteriaceae family. Of the 25 integron positive isolates, the variable regions of class 2 integrons of 2 strains contained two gene cassettes: *sat 2* and *aadA1*, encoding streptothricin acetyl-trasferase and aminoglycoside-adenyltransferase, respectively. Three isolates had three cassettes: *drfA1* responsible for resistance to trimethoprim, as well as *sat2* and *aadA1* (Table 3.6). This could be explained by the fact that the class 2 integrase gene (*intII*) contains an early stop codon resulting in a truncated form of the enzyme. The resultant integrase is therefore unable to excise existing cassettes or inserts new ones (Lévesque *et al*., 1995). To the best of our knowledge, this is the first report of the detection of class 2 integrons description of *drfA1-sat1-aadA1* gene cassette arrays in *Legionella* spp. from wastewater treatment plant in South Africa. The detection of *bla*TEM and *bla*OXA gene were detected in 14% and 60% of the isolates, respectively signify wastewater as a reservoir for antibiotic resistance genes.

Carbapenems are highly effective antibiotics against multidrug-resistant Gram-negative bacteria because of their stability against extended spectrum and AmpC-β-lactamases (Pitout *et al*., 2007; Lee *et al*., 2011). Resistance to carbapenems can be mediated by several mechanisms including decreased membrane permeability and increased efflux (Mazel *et al*., 2000; Adel *at al*., 2010). However, production of metallo-β-lactamases (MBLs) has assumed increasing importance in recent years (Lutz and Lee, 2011). Although

prevalence of carbapenem resistance due to acquired MBLs is increasing, its overall prevalence is still low (Ho *et al*., 2002; Lutz and Lee, 2011). However, increasing use of carbapenems could provide the selective pressure for selection of these enzymes (Vettoretti *et al*., 2009). None of the 6 isolates which are MBL-producers harboured the two metallo-β-lactamase genes (bla_{IMP} and bla_{VIM}) as they are only known to harbour bla_{FEZ} genes. The results support the notion that environmental microbiology laboratories must be able to distinguish MBL-producing environmental isolates from strains with other mechanisms responsible for carbapenem resistance. In the absence of novel agents for the treatment of infections caused by multidrug-resistant gram negative bacteria in the near future, the uncontrolled spread of MBL producers may lead to treatment failures with increased morbidity and mortality. The early detection of MBL-producing *Legionella* spp. may avoid the future spread of these multi drug-resistant isolates (Leung *et al.,* 2008).

Three quorum sensing (QS) dependent virulence factors thought to play a major role in the pathogenesis of *Legionella* spp.; elastase, protease and rhamnolipid production were screened for in these isolates. The present study confirmed the hypothesis that most pathogenic environmental strains isolated from wastewater have increased expression of two virulence factors, elastase and protease, which are considered to be regulated by (QS). The fact that such results were observed in two-thirds of the isolates studied suggests that increased QS activity and QS-regulated virulence factor production may play a role in the pathogenicity of these *Legionella* spp. strains. Elastase, a metalloproteinase secreted by a type II secretion system, has been shown to destroy respiratory epithelium tight junctions, leading to increased permeability disorders, increased interleukin-8 levels and a decreased host immune response (Aloush *et al*., 2006). Therefore, it is not surprising that most (two-thirds) of the *Legionella* spp. isolates in the present study showed increased levels of production of such a virulence factor. The remaining one-third of isolates had high levels of elastase (88%), this agrees with the results of a previous study, which revealed that 12% of *Legionella* isolates were defective in the production of extracellular virulence

factors (Choy *et al.,* 2008). Such defective isolates often have mutational defects in both lasR and rhlR. Although elastase deficient strains (those that had an OD reading of less than 0.02 at 495 nm) were found among these isolates, it does not invalidate the theory that QS-regulated virulence may play a role in *Legionella* spp. pathogenicity, but simply emphasises the fact that pathogenicity may be regulated by a wide range of mechanisms (Medina *et al.*, 2003; Kipnis *et al*., 2006). Sixty-two percent (31/50) of *Legionella* spp. isolates were confirmed for the production of anionic biosurfactant when isolated on Cethyl–trimethylammonium bromide (CTAB)-methylene blue agar plate, an indicator medium for production of anionic surfactants (Siegmund and Wagner, 1991). Extracellular anionic biofurfactants produced by these *Legionella* isolates developed an insoluble ion pairing complexation with the cationic tenside CTAB and the basic dye methylene blue, and produced dark blue halos against a light background on methylene blue agar plates. This method has been widely accepted for the screening of anionic biosurfactants producing microorganisms (Lequett and Greenberg, 2005).

The realization of type strain-specific patterns corresponding to the 25 integron positive *Legionella* species led to the evaluation of the intraspecies stability of RAPD profiles by testing all the 25 environmental isolates. By observing that multiple well-identified strains of a given species had a core of bands in common, we demonstrated that minimal profiles can be proposed for each species. This point reinforces the need for exchanges of rarely isolated bacterial species between microbiologists (Thangaraj *et al*., 2011; Sobral *et al.,* 2011). Analysis of RAPD profiles with the BioNumerics (Applied Maths) package confirmed the visual identification and allowed isolates of each species to be clearly clustered together. The standardization of the method provided a convenient way to compare patterns obtained from independent gel runs. This method of analysis has proved to be extremely useful for determining the most probable species assignment of a given isolate. Similar to previous studies (Qasem *et al.,* 2008; Lim *et al*., 2011) the DNA-typing methods used in the present study exhibited the ability to group the selected *Legionella* isolates, and were also able to discriminate differences in highly related isolates both visually

and with the aid of computer- assisted procedures. Using the fingerprint profile of RAPD-PCR to screen and subtype the isolates, the results revealed that those *Legionella* strains with the same antibiotic susceptibility showed similar polymorphism.

Clonality study showed that non-MBL producers harbouring class 1 integrons could be divided into two clusters, B and C, whilst the MBL producers were assigned into cluster A. Strains with the same antibiotic susceptibility patterns demonstrated similar RAPD patterns. Thus, it could be suggested that isolates carrying MBL genes may be detected in different clusters from the non-MBL isolates (Figure 3.4). Isolates found in cluster A harbour only bla_{OXA} gene which was found in 83% of the isolates. All the isolates from cluster A had gene cassettes. Fifty percent of the *Legionella* spp. isolates in cluster A produced rhamnolipid as a biosurfactant, with 83% and 33% producing elastase and protease, respectively. Isolates in this cluster (A) had the same resistance profile towards the tested antimicrobial agents. *Legionella* spp. isolates found in cluster B carried both bla_{TEM} and bla_{OXA} . Seventy-five of the isolates had gene cassettes, virulence determinants were produced in 80%, 88% and 50% for rhamnolipid, elastase and protease, respectively. Isolates had the same resistance pattern. Isolated found in cluster C harboured 45% and 9% for bla_{OXA} and bla_{TEM}, respectively. All the isolates were capable of producing elastase as a virulence factor, whilst 73% and 64% produced rhamnolipid and protease, respectively. Diversity of RAPD types identified in this study suggests that the carriage of the bla_{OXA} and bla_{TEM} genes by integrons (Pitout *et al*., 2007) may facilitate the spread of these genes among genetically distinct *Legionella* spp. strains.

3.5 Conclusion

This study shows that treated effluent from wastewater treatment plants are reservoirs of antibiotic resistant *Legionella* spp. and could lead to the contamination of the receiving surface waters with these strains. The isolates were susceptible to most third generation β lactams used whilst exhibiting multidrug

resistance to other antibiotics. The detection of resistance genes in the isolates means they are capable of causing infection in susceptible hosts. Thus, to prevent an outbreak of *Legionella* infection, urgent intervention by the regulatory authorities and workers of the treatment plants in South Africa; such as constant monitoring of treatment processes, infrastructural upgrade of wastewater treatment works, and provision of adequate sanitation and sewage disposal systems to rural communities on the banks of rivers is needed.

Multiple antimicrobial resistance was found in more than 80% of the isolates with, the MAR index values classifying the isolates as high-risk exposed-antibiotic sources. Cassette arrays of class 2 integron revealed the prevalence of genes that confer resistance to trimethoprim (*drfA1-sat1-aadA1*) and: *sat 2 and aadA1*, encoding streptothricin acetyl-trasferase and aminoglycoside-adenyltransferase, respectively, thus, providing more evidence to explain the high incidences of resistance to trimethoprim. In addition, the same amplicon patterns and identical gene cassette arrays of class 2 integrons found in *Legionella* spp. in the same wastewater treatment plant indicated possible transfer of antibiotic resistance genes via class 2 integrons among commensal flora of *Legionella* spp. These cassettes presumably have not yet received enough selective pressure or had sufficient evolutionary time to encourage their widespread dissemination. However, with the potential of integrons to capture and collect gene cassettes, it is likely that they will become more prevalent. Consequently, integrons will continue to threaten the usefulness of antibiotics as therapeutic agents. This study also provided data on the relation between RAPD genotypes profiles of the *Legionella* isolates from wastewater treatment plants and receiving surface. Diversity of RAPD types identified in this study suggests that the carriage of the bla_{VIM} and bla_{IMP} genes by integrons may facilitate the spread of bla_{OXA} and bla_{TEM} genes among genetically distinct *Legionella* spp. and their associated class 2 integrons.

CHARTER 4

GENERAL DISCUSSION AND CONCLUSION

4.1 General discussion

Untreated and improperly treated wastewater is known to contain pathogens which can cause disease outbreak, hence the need for adequate treatment before discharge into receiving water bodies. Treatment of wastewater is carried out to minimize and eliminate potential health risks. The major groups of pathogens that are of importance to wastewater are bacteria, viruses, fungi and protozoa (Cabello, 2006). In South Africa, studies on molecular antibiotic resistance are limited to clinical pathogens such as *Salmonella* spp., *Vibrio cholerae, Shigella* spp. or *Proteus* spp. (Nguyen *et al*., 2005; Vo *et al.,* 2010). To our knowledge, research on prevalence and characterisation of integrons and gene cassette arrays as well as the transferability of resistance genes by known mechanisms such as transformation and conjugation of *Pseudomonas* and *Legionella* spp. are still at its infancy. This project aimed to determine the extent of antibiotic resistance in *Pseudomonas* and *Legionella* spp. recovered from wastewater effluents of two wastewater treatment plants and receiving surface water in Durban, South Africa. Furthermore, molecular characterisation of antibiotic resistance (prevalence of class 1 and class 2 integrons, gene cassette arrays) and of *Pseudomonas* and *Legionella* spp. were investigated.

Resistance to β-lactams was approximately 52% among *Legionella* spp., however, the incidences of resistance observed against *Pseudomonas* spp. isolates were higher than 60%. These results implied that β-lactams are widely discharged into the wastewater treatment plants studied. In addition, the extremely high level of resistance found in these *Pseudomonas* isolates recovered from wastewater effluents as well as in other *Pseudomonas* spp. reported in many publications worldwide reflect the intrinsic resistance of this genus to β-lactams. The prevalence of β- lactamases (three principal classes including class C cephalosporinase, class D penicillinase, and class B metallo-β-lactamase) among *Pseudomonas* and

Legionella species in this study is a cause for concern for resistance to a wide variety of β-lactam antibiotics. Furthermore, ampicillin, tetracycline and amoxicillin are ranked as ~~–e~~ritically important" antibiotics for human medicine by WHO (Collignon *et al*., 2009). Therefore, the high incidences of resistance to these antibiotics in *Pseudomonas* and *Legionella* isolates not only implied the frequent use in human medicine but also reflected their lowered efficacy which suggested that such antibiotics need to be used prudently to retain their efficacy.

The dissemination of antibiotic resistance is of global concerns. Furthermore, treatments of human infections are compromised by the emergence of pathogens that are resistant to multiple antibiotics (Alekshun and Levy, 2007). Multiple antibiotic resistance in bacteria may result from one of the two mechanisms (Nikaido, 2009); firstly, bacteria may accumulate multiple genes, with each gene conferring resistance to a single antibiotic. This typically occurred on resistance plasmids which were assembled by transposons and integrons. Integrons are capable of capturing, rearranging and supplying a strong promoter for the expression of several antibiotic resistance genes (Cambray *et al*., 2010), and are often harboured on transposons or plasmids. Secondly, multiple resistance may be due to multidrug efflux pumps that extrude a wide range of drugs. Because the genome of *Pseudomonas* spp. has high plasticity, its high levels of multiple resistance were possibly due to accumulation of antibiotic resistance genes harboured on integrons. However, no specific resistance mechanism was reportedly associated with these two genera except the expression of inducible β- lactamases that confer resistance to a variety of β-lactam antibiotics (Janda and Abbott, 2010).

Among the 5 classes of mobile integrons (Cambray *et al*., 2010), class 1 and class 2 are recognised as the most widespread. This study focused on the prevalence of class 1 and class 2 integrons among the *Pseudomonas* and *Legionella* isolates and their possible roles in antibiotic resistance dissemination. High frequencies of occurrence (44%) of class 1 integron were observed in *Pseudomonas* isolates, of which *IntI*1 genes were detected in 13% (3 out of 22 isolates). However, class 2 integrons was detected in 50%

of the tested *Legionella* isolates (25 of 50 isolates),of which *IntII* genes were detected in 88% (22 out of 25 isolates).

Gene cassette variable regions of integrons were amplified with primers targeting the two conserved segments (5'-CS and 3'-CS) and were sequenced. Although there are currently over 130 gene cassettes described for observed resistance to almost every known class of antibiotics, cassettes encoding dihydrofolate reductases (*dfr* genes) and aminoglycoside- modifying enzymes (*aad*A genes) were the most abundant in this study.

Virulence factor determination revealed that 61% of the *Pseudomonas* and 62% of the *Legionella* spp. were capable of producing rhamnolipid as a biosurfactant, while 76 % and 56% of the *Pseudomonas* spp. were capable of producing elastase and protease, respectively of the 50 tested *Legionella* spp., 88 % and 22 % of the isolates were positive for elastase and protease production, respectively. Results obtained from this study reveal that the majority of these *Pseudomonas* and *Legionella* species could pose a threat to public health since they harbour some antibiotic resistance genes, which could be transferred to susceptible strains, limiting the treatment options. Analysis of *Pseudomonas* and *Legionella* genotypes using RAPD fingerprinting revealed that both genus's can be categorised into different clusters and phenotypes corresponding to their phenotypic resistance patterns. Both *Pseudomonas* and *Legionella* spp. were grouped into 3 clusters each, representing both genotypic and phenotypic relatedness. For *Pseudomonas* spp. cluster A and B comprised of non-β-lactamase producers whilst cluster C was comprised of β-lactamase producing isolates. For *Legionella* spp., cluster A comprised of non-β-lactamase producing isolates, and cluster B and C were β-lactamase producing isolates.

4.2. Potential for future development of this study

This study has no doubt formed the platform for more promising investigation in future. In order to improve the understanding of how resistance genes flow across bacterial strains, future experimental

studies to assess the integrons transfer using conjugation process could be performed. Other antibiotic resistance genes, and virulence transfer mechanisms such as conjugation should also be further investigated. Besides, Southern hybridisation can be done in future to determine the genetic localisation of the detected integrons. This could help to define whether integrons detection in this study are plasmid-borne or chromosomally-located.

In addition, PCR amplification of antibiotic resistance genes determinants such as genes encoding resistance towards β-lactamase for *Pseudomonas* spp. e.g. (*bla* $_{TEM}$, *bla* $_{SHV}$, *bla* $_{CTX}$, *bla* $_{OXA}$, *bla* $_{VEB}$, *bla* $_{DHA}$), and *bla* $_{FEZ-1}$ for *Legionella* spp., tetracycline (e.g. *tet*A, *tet*B, *tet*C), and chloramphenicol (e.g. *cml*A, *catB*2) could also be carried out. This could help in assessing the relationship between integrons and the specific resistance genes with the bacterial resistance phenotypes. Therefore, further studies are required for the screening of these *Pseudomonas* spp. and *Legionella* spp. isolates using other effective methods such as Southern blotting. Furthermore, the sample size could be increased to reflect the true prevalence of the three classes of integrons and their respective gene cassettes. Although, RAPD-PCR method employed in this study was able to provide information about the genotypic groups of the *Pseudomonas* and *Legionella* spp. isolates investigated, future studies should incorporate other DNA fingerprinting techniques such as Pulsed field gel electrophoresis and Amplified Fragment Length Polymorphisms to get more accurate picture of the level of genotypic relatedness of these isolates. More analysis of the correlation between the genotypic characterization of the isolates and their phenotypic patterns is also required.

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Appendix

Table I: Antimicrobial resistance patterns of the individual *Pseudomonas* spp. isolate used for generating the table 2.2

Isolates	S	TE	CTX	E	AMP	NA	CN	CIP	P	C	W	F	VA	KF	MH	RD	OX	RL
BC 103	R	S	S	R	R	S	S	S	R	S	R	R	R	R	I	R	R	I
BC 76	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 25	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
N6 21	R	S	S	R	R	R	S	S	R	R	R	R	R	R	S	R	R	S
BC 6	R	S	S	I	R	R	S	S	R	S	R	R	R	R	S	R	R	R
US 1	R	I	S	R	R	R	S	S	R	S	R	R	R	R	R	R	R	R
DP 27	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
DSNTGW	R	S	S	R	R	I	S	S	R	R	R	R	R	R	S	R	R	S
DS 2	R	S	S	R	R	S	S	S	R	S	R	R	R	R	S	R	R	S
DS 21	R	S	S	R	R	R	S	S	R	S	R	R	R	R	I	R	R	R
DP 58	R	I	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 40	R	I	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 123	R	I	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
DP 60	R	I	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
US 22	R	I	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
DS 163	R	I	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
DP 61	R	I	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
DP 1	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
US 64	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
US 21	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
DP 45	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
DS 156	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
US 24	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 78	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
DP 68	R	R	S	R	R	R	S	S	R	S	R	R	R	R	R	R	R	R
DP 45	R	R	S	R	R	R	S	S	R	S	R	R	R	R	R	R	R	R
DP 4	R	R	S	R	R	R	S	S	R	S	R	R	R	R	R	R	R	R

R-resistance S-susceptible I-intermediate

Table I: Continued

Isolates	S	TE	CTX	E	AMP	NA	CN	CIP	P	C	W	F	VA	KF	MH	RD	OX	RL
US 68	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 70	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
DP 58	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 103	R	I	S	R	R	S	S	S	R	S	R	R	R	R	R	R	R	R
BC 68	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 19	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 74	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 126	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 81	R	R	S	R	R	R	S	S	R	S	R	R	R	R	R	R	R	R
BC 83	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 27	R	R	S	R	R	S	S	S	R	R	R	R	R	R	R	R	R	R
BC 17	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 78	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 89	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 72	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 100	R	R	S	R	R	R	S	S	R	S	R	R	R	R	I	R	R	R
BC 67	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 75	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 20	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 129	R	R	S	R	R	R	S	S	R	S	R	R	R	R	R	R	R	R
BC 127	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 66	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 99	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
US 9	R	R	S	R	R	R	S	S	R	S	R	R	R	R	R	R	R	R
DS 161	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 169	R	R	S	R	R	R	S	S	R	S	R	R	R	R	R	R	R	R
BC 28	R	R	S	R	R	I	S	S	R	S	R	R	R	R	R	R	R	R
BC 172	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 26	**R**	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
US 2	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 84	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R

R-resistance S-susceptible I-intermediate

Table I: Continued

Isolates	S	TE	CTX	E	AMP	NA	CN	CIP	P	C	W	F	VA	KF	MH	RD	OX	RL
US 55	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 18	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
US 63	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
US 11	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
US 4	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
DP 25	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 82	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
US 62	R	R	S	R	R	R	S	S	R	S	R	R	R	R	R	R	R	R
US 58	R	R	S	R	R	R	S	S	R	S	R	R	R	R	R	R	R	R
BC 166	R	R	S	R	R	R	S	S	R	S	R	R	R	R	I	R	R	R
DP 62	R	R	S	R	R	R	S	S	R	S	R	R	R	R	R	R	R	R
DP 21	R	R	S	R	R	R	S	S	R	S	R	R	R	R	R	R	R	R
DS 32	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 25	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
US 21	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 79	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
DP 27	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
DP 61	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
DP 57	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 71	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
US 23	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 65	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
DP 61	R	R	S	R	R	R	S	S	R	R	R	R	R	R	I	R	R	R
DP 64	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
DP 59	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 127	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 24	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 38	R	R	S	R	R	R	S	S	R	S	R	R	R	R	R	R	R	R
BC 66	R	R	S	R	R	R	S	S	R	S	R	R	R	R	R	R	R	R
BC 129	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 21	R	R	S	R	R	R	S	S	R	S	R	R	R	R	R	R	R	R

R-resistance S-susceptible I-intermediate

Table I: Continued

Isolates	S	TE	CTX	E	AMP	NA	CN	CIP	P	C	W	F	VA	KF	MH	RD	OX	RL
DP 30	R	R	S	R	R	I	S	S	R	R	R	R	R	R	S	R	R	S
DP 65	R	R	S	R	R	R	S	S	R	R	R	R	R	R	I	R	R	R
DS 163	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
DS 59	R	R	S	R	R	R	S	S	R	R	R	R	R	R	I	R	R	R
DP 21	R	R	S	R	R	R	S	S	R	S	R	R	R	R	R	R	R	R
BC 109	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 123	R	R	S	R	R	I	S	S	R	S	R	R	R	R	R	R	R	R
BC 126	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
BC 140	R	R	S	R	R	R	S	S	R	R	R	R	R	R	R	R	R	R
C 3	R	I	S	R	R	S	S	S	R	R	R	R	R	R	S	R	R	S
C 25	R	I	S	R	R	S	S	S	R	R	R	R	R	R	S	R	R	S

R-resistance S-susceptible I-intermediate

Table II: Genotypic and phenotypic characterization of the individual *Pseudomonas* spp. recovered from treated effluents and receiving surface water used to generate the data presented in tables 2.2 and 2.4

Isolates	Antibiotic resistance profile	MI	P	RG			INT 1	GC (bp)	MBL	MBL-genes	
				blaOXA	blaTEM	blaampC				blaIMP	blaVim-1
BC 109	TE, E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.82	A	-		-	+	700	-	-	-
BC 129	TE, E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.82	A	-		-			-	-	-
BC 140	TE, E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.82	A	-		-	+		-	-	-
BC 17	TE, E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.82	A	-		-	+		-	-	-
BC 172	TE, E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.82	A	-		-			-	-	-
BC 70	TE, E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.82	A	-	+	-	+		-	-	-
BC 84	TE, E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.82	A	-		-			+	-	-
DP 25	TE, E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.82	A	-		-			-	-	-
DP 45	TE, E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.82	A	-		-			-	-	-
DP 57	TE, E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.82	A	-		-			-	-	-
DP 58	TE, E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.82	A	-	+	-			-	-	-
DP 59	TE, E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.82	A	-		-			-	-	-
DS 161	TE, E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.82	A	-		-	+		-	-	-
DS 163	TE, E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.82	A	-		-		700	-	-	-
DS 32	TE, E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.82	A	-		-	+		-	-	-
US 11	TE, E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.82	A	-		-	+		-	-	-
US 21	TE, E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.82	A	-		-			+	+	+
US 4	TE, E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.82	A	-	+	-	+		-	-	-
US 55	TE, E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.82	A	-		-	+		+	+	-
US 64	TE, E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.82	A	-		-	+		-	-	-
BC 129	TE, E, AMP, NA, P, W, F, VA, KF, MH, RD, OX, RL	0.76	B	-		-	+		-	-	-
BC 169	TE, E, AMP, NA, P, W, F, VA, KF, MH, RD, OX, RL	0.76	B	-		-	+		-	-	-
DP 21	TE, E, AMP, NA, P, W, F, VA, KF, MH, RD, OX, RL	0.76	B	-		-			-	-	-
BC 38	TE, E, AMP, NA, P, W, F, VA, KF, MH, RD, OX, RL	0.76	B	-		-	+		-	-	-
DP 58	E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.76	C	-		-			-	-	-

Table II: Continued

DP 60	E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.76	C	-		-			-	-	-
DS 163	E, AMP, NA, P, C, W, F, VA, KF, MH, RD, OX, RL	0.76	C	-		-	+		-	-	-
DP 62	TE, E, AMP, NA, P, W, F, VA, KF, MH, RD, OX, RL	0.76	D	-	+	-			+	+	-
US 62	TE, E, AMP, NA, P, W, F, VA, KF, MH, RD, OX, RL	0.76	D	-	+	-			-	-	-
US 9	TE, E, AMP, NA, P, W, F, VA, KF, MH, RD, OX, RL	0.76	D	-		-			-	-	-
DP 61	TE, E, AMP, NA, P, C, W, F, VA, KF, RD, OX, RL	0.76	E	-	+	-			-	-	-
DP 65	TE, E, AMP, NA, P, C, W, F, VA, KF, RD, OX, RL	0.76	E	-	+	-			-	-	-
DS 59	TE, E, AMP, NA, P, C, W, F, VA, KF, RD, OX, RL	0.76	F	-		-			-	-	-
DP 65	TE, E, AMP, NA, P, C, W, F, VA, KF, RD, OX, RL	0.76	F	-	+	-			-	-	-
BC 27	TE, E, AMP, P, C, W, F, VA, KF, MH, RD, OX, RL	0.76	G	-	+	-	+		+	-	-
BC 123	TE, E, AMP, P, W, F, VA, KF, MH, RD, OX, RL	0.71	H	-		-	+		-	-	-
BC 28	TE, E, AMP, P, W, F, VA, KF, MH, RD, OX, RL	0.71	H	-	+	-			-	-	-
BC 166	TE, E, AMP, NA, P, W, F, VA, KF, RD, OX, RL	0.71	I	-		-			-	-	-
DS 21	E, AMP, NA, P, C, W, F, VA, KF, RD, OX, RL	0.71	J	-		-			-	-	-
US 1	E, AMP, NA, P, W, F, VA, KF, MH, RD, OX, RL	0.71	K	-	+	-			-	-	-
BC 100	TE, E, AMP, NA, P, W, F, VA, KF, RD, OX, RL	0.65	L	-	+	-			-	-	-
BC 103	E, AMP, P, W, F, VA, KF, MH, RD, OX, RL	0.65	M	-		-	+		-	-	-
BC 6	E, AMP, NA, P, W, F, VA, KF, RD, OX, RL	0.65	N	-		-	+	500+700	-	-	-
DP 30	TE, E, AMP, P, C, W, F, VA, KF,RD, OX	0.65	O	-	+	-			-	-	-
DS 2	E, AMP, NA, P, W, F, VA, KF, RD, OX, RL	0.65	P	-	+	-			-	-	-
N 6	E, AMP, NA, P, C, W, F, VA, KF, RD, OX	0.65	Q	-		-			-	-	-
C 25	E, AMP, P, C, W, F, VA, KF, RD, OX	0.59	R	-		-			-	-	-
C 3	E, AMP, P, C, W, F, VA, KF, RD, OX	0.59	R	-		-			-	-	-
DSNTGW	E, AMP, P, C, W, F, VA, KF, RD, OX	0.59	R	-		-			-	-	-
BC 103	E, AMP, P, W, F, VA, KF, RD, OX	0.53	S	-		-	+		-	-	-

MI- MAR index; P- Phenotype; RG- Resistance genes, INT1- Integron 1, GC- Gene cassette, MBL- Metallo-β-lactamase

Table III: MBL activity for *Pseudomonas* spp. used to generate the result presented in table 2.5

Bacterial strain	Species	CDT IMP (mm)	CDT IMP + EDTA (mm)	MBL index	β-lactamase activity
BC 100	*P. aeruginosa*	26	26	0	-
BC 103	*P. aeruginosa*	25	25	0	-
BC 104	*P. aeruginosa*	25	30	5	-
BC 109	*P. aeruginosa*	32	28	4	-
BC 123	*P. aeruginosa*	35	33	2	-
BC 129	*P. aeruginosa*	26	30	4	-
BC 129	*P. aeruginosa*	30	30	0	-
BC 140	*P. aeruginosa*	30	31	1	-
BC 166	*P. aeruginosa*	30	28	2	-
BC 169	*P. aeruginosa*	21	26	5	-
BC 17	*P. aeruginosa*	25	27	2	-
BC 172	*P. aeruginosa*	30	30	0	-
BC 27	*P. aeruginosa*	28	35	7	+
BC 28	*P. aeruginosa*	30	30	0	-
BC 38	*Pseudomonas spp.*	27	29	2	-
BC 6	*Pseudomonas* spp.	29	32	3	-
BC 70	*P. aeruginosa*	33	33	0	-
BC 84	*P. aeruginosa*	27	38	11	+
C 25	*Pseudomonas* spp.	32	30	2	-
C 3	*P. putida*	30	30	0	-
DP 21	*P. aeruginosa*	30	30	0	-
DP 25	*P. aeruginosa*	29	32	3	-
DP 30	*Pseudomonas* spp.	28	32	4	-
DP 45	*P. aeruginosa*	30	35	5	-
DP 57	*P. aeruginosa*	32	32	0	-
DP 58	*P. aeruginosa*	30	30	0	-
DP 58	*P. aeruginosa*	32	32	0	-
DP 59	*P. aeruginosa*	35	31	4	-
DP 60	*P. aeruginosa*	30	32	2	-
DP 61	*P. aeruginosa*	30	33	3	-
DP 62	*P. aeruginosa*	25	35	10	+
DP 65	*P. aeruginosa*	30	32	2	-
DP 65	*P. aeruginosa*	32	35	3	-
DS 161	*P. aeruginosa*	30	32	2	-
DS 2	*Pseudomonas spp.*	30	25	5	-
DS 21	*Pseudomonas spp.*	30	33	3	-
DS 32	*P. aeruginosa*	30	30	0	-
DS 59	*Pseudomonas spp.*	30	30	0	-
DSNTGW	*Pseudomonas spp.*	30	35	5	-
N 6	*P.putida*	28	30	2	-
US 1	*P. aeruginosa*	27	28	1	-
US 11	*P. aeruginosa*	30	30	0	-
US 21	*P. aeruginosa*	27	38	11	+
US 4	*P. aeruginosa*	29	29	0	-
US 55	*P. aeruginosa*	30	40	10	+
US 62	*P. aeruginosa*	29	33	4	-
US 64	*P. aeruginosa*	35	32	3	-
US 9	*P. aeruginosa*	28	30	2	-

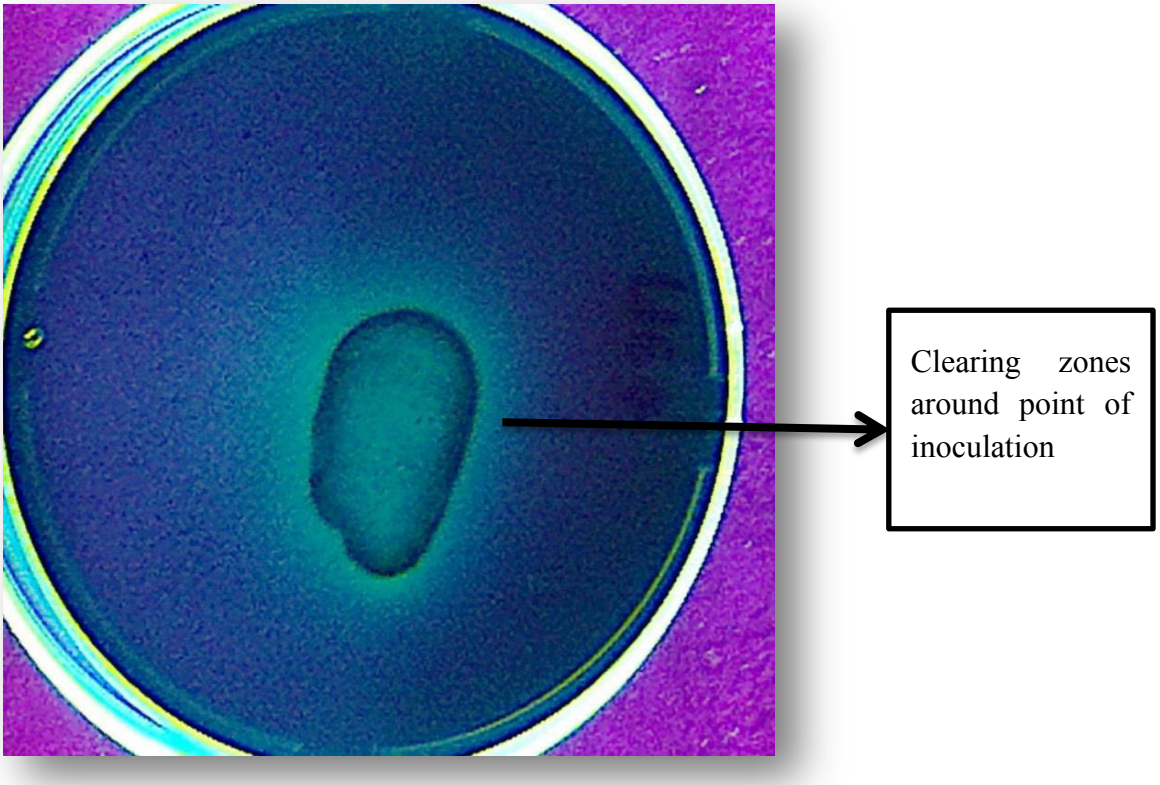

Figure I: Blue agar plates (CTAB agar plates) showing dark blue haloes indicating the production of anionic surfactants for *Pseudomonas* spp. Dark blue haloes were then detected UV transilluminator.

Table IV: Percentage score similarity generated from GelCompare software for *Pseudomonas* spp. band on the RAPD profiles obtained and presented in figures 2.4 and 2.5

Isolate code	Matrix
DS 163	100
BC 140	80.01 100.00
BC 169	40.00 30.00 100.00
BC 17	18.19 9.09 21.43 100.00
BC 103	22.23 11.11 25.00 55.56 100.00
BC 70	30.00 20.00 30.77 45.46 55.56 100.00
US 64	27.27 18.19 28.57 30.77 50.00 70.00 100.00
BC 123	11.11 12.50 0.00 18.19 10.00 30.00 7.69 100.00
US 4	25.00 12.50 7.69 18.19 10.00 18.19 7.69 100.00 100.00
BC 38	50.00 33.33 18.19 20.00 25.00 20.00 18.19 50.00 50.00 100.00
DP 21	40.00 30.00 20.00 30.77 36.37 41.67 28.57 40.00 40.00 30.00 100.00
DS 161	18.19 20.00 21.43 14.29 27.27 14.29 21.43 30.00 30.00 33.33 30.77 100.00
BC 103	18.19 20.00 13.33 14.29 7.69 33.33 13.33 30.00 30.00 9.09 30.77 23.08 100.00
BC 129	18.19 9.09 21.43 33.33 27.27 33.33 21.43 30.00 30.00 20.00 30.77 23.08 33.33 100.00
DS 32	10.00 11.11 15.39 27.27 20.00 7.69 15.39 10.00 10.00 11.11 25.00 27.27 16.67 16.67 100.00
BC 6	10.00 11.11 15.39 16.67 20.00 7.69 15.39 10.00 10.00 11.11 36.37 27.27 16.67 16.67 100.00 100.00
US 11	11.11 12.50 16.67 30.00 22.23 8.33 16.67 11.11 11.11 12.50 40.00 30.00 18.19 18.19 83.33 83.33 100.00
US 55	10.00 11.11 7.15 40.00 20.00 7.69 15.39 10.00 10.00 11.11 25.00 27.27 16.67 16.67 71.43 71.43 83.33 100.00
DP 65	8.33 9.09 13.33 33.33 16.67 14.29 21.43 0.00 8.33 9.09 21.43 23.08 14.29 23.08 55.56 55.56 62.50 55.56 100.00
BC 109	0.00 0.00 18.19 33.33 25.00 0.00 8.33 0.00 0.00 0.00 8.33 9.09 0.00 9.09 42.86 66.67 50.00 66.67 50.00 100.00
BC 27	0.00 0.00 7.69 44.45 22.23 8.33 16.67 0.00 0.00 0.00 7.69 8.33 0.00 18.19 37.50 57.15 42.86 57.15 62.50 80.01 100.00
DS 163	9.09 10.00 14.29 25.00 18.19 15.39 23.08 9.09 9.09 22.23 0.00 25.00 7.15 15.39 18.19 18.19 20.00 44.45 15.39 37.50 33.33 100.00

Table V: Antimicrobial resistance patterns of the individual *Legionella* spp. isolate used for generating the table 3.2

Isolate	F	MH	VA	KF	OX	RD	DA	SAM	RL	OFX	AMP	TE	E	CTX	NA	P	CIP	C	CN	W	S
L 1	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 10	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 100	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 101	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 102	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 103	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 104	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 105	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 106	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 107	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 108	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 109	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 110	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 111	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 112	S	S	I	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 113	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 12	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 12	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 13	S	I	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 14	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 15	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 16	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 17	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 18	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 19	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 2	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 20	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 23	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 24	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 25	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 28	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 3	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R

Table V: Continued

Isolate	F	MH	VA	KF	OX	RD	DA	SAM	RL	OFX	AMP	TE	E	CTX	NA	P	CIP	C	CN	W	S
L 30	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 34	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 35	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 36	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 37	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 38	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 39	S	I	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 4	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 41	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 43	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 46	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 47	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 48	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 49	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 5	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 50	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 51	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 52	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 53	S	S	I	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 54	S	I	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 57	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 58	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 6	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 64	S	I	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 66	S	I	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 67	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 69	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 70	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 71	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R

R-resistance S-susceptible I-intermediate

Table V: Continued

Isolate	F	MH	VA	KF	OX	RD	DA	SAM	RL	OFX	AMP	TE	E	CTX	NA	P	CIP	C	CN	W	S
L 72	S	I	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 73	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 74	S	I	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 75	S	I	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 76	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 77	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 78	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 79	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 80	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 81	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 82	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 83	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 84	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 85	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 86	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 87	S	I	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 88	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 89	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 9	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 90	S	I	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 91	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 92	S	I	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 93	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 94	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 95	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 96	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 97	S	R	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 98	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R
L 99	S	S	R	S	R	R	R	S	R	S	R	R	R	S	S	R	S	S	S	S	R

R-resistance S-susceptible I-intermediate

Table VI: Genotypic and phenotypic characterization of the individual *Legionella* spp. recovered from treated effluents and receiving surface water used to generate the data presented in table 3.4 and 3.5

Isolates	Antibiotic resistance profile	MI	P	RG			INT 2	GC	MBL	MBL-genes	
				blaOXA	blaTEM	blaampC				blaIMP	blaVim-1
L 1	VA, OX, RD, CD, RL, TE, E, P, S	0.4	B			-	+	+	-	-	-
L 2	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A		+	-			-	-	-
L 3	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A			-	+	+	-	-	-
L 4	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A			-	+	+	-	-	-
L 5	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A			-			-	-	-
L 6	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A			-	+	+	-	-	-
L 7	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A			-			-	-	-
L 8	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A	+		-	+	+	-	-	-
L 9	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A			-			-	-	-
L 10	VA, OX, RD, CD, RL, TE, E, P, S	0.4	B	+		-	+	+	-	-	-
L 11	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A	+		-	+	+	-	-	-
L 12	OX, RD, CD, RL, TE, E, P, S	0.35	C			-			-	-	-
L 13	VA, OX, RD, CD, RL, TE, E, P, S	0.4	B	+		-			+	-	-
L 14	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A	+		-			-	-	-
L 15	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A	+		-	+	+	-	-	-
L 16	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A	+		-	+	+	-	-	-
L 17	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A	+		-	+	+	-	-	-
L 18	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A	+		-			-	-	-
L 19	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A	+		-			-	-	-
L 20	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A	+		-			-	-	-
L 21	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A	+		-	+	+	-	-	-
L 22	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A	+		-	+	+	-	-	-
L 23	VA, OX, RD, CD, RL, TE, E, P, S	0.4	B	+		-	+	+	-	-	-
L 24	MH, VA, OX, RD, CD, RL, TE, E, P	0.45	A	+		-			+	-	-

Table VI: Continued

L 25	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A			-	+	+	-	-	-
L 26	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A		+	-			-	-	-
L 27	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A	+		-	+	+	-	-	-
L 28	VA, OX, RD, CD, RL, TE, E, P, S	0.4	B	+		-	+	+	+	-	-
L 29	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A	+		-			-	-	-
L 30	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A			-			-	-	-
L 31	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A	+		-			-	-	-
L 32	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A	+		-			-	-	-
L 33	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A	+		-	+	+	-	-	-
L 34	VA, OX, RD, CD, RL, TE, E, P, S	0.4	B			-			-	-	-
L 35	VA, OX, RD, CD, RL, TE, E, P, S	0.4	B			-	+		-	-	-
L 36	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A	+		-	+	+	-	-	-
L 37	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A			-	+		-	-	-
L 38	VA, OX, RD, CD, RL, TE, E, P, S	0.4	B			-			-	-	-
L 39	VA, OX, RD, CD, RL, TE, E, P, S	0.4	B	+		-	+		-	-	-
L 40	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A	+		-	+	+	-	-	-
L 41	VA, OX, RD, CD, RL, TE, E, P, S	0.4	B	+	+	-			+	-	-
L 42	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A		+	-	+	+	+	-	-
L 43	OX, RD, CD, RL, TE, E, P, S	0.35	C		+	-	+	+	-	-	-
L 44	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A	+		-			-	-	-
L 45	MH, VA, OX, RD, CD, RL, TE, E, P, S	0.45	A	+		-			-	-	-
L 46	VA, OX, RD, CD, RL, TE, E, P, S	0.4	B		+	-			-	-	-
L 47	VA, OX, RD, CD, RL, TE, E, P, S	0.4	B	+	+	-	+	+	+	-	-
L 48	VA, OX, RD, CD, RL, TE, E, P, S	0.4	B	+		-			-	-	-
L 49	VA, OX, RD, CD, RL, TE, E, P, S	0.4	B	+		-			-	-	-
L 50	VA, OX, RD, CD, RL, TE, E, P, S	0.4	B			-			-	-	-

MI- MAR index, P- Phenotype, RG- Resistance genes, INT2- Integron 2, GC- Gene cassette, MBL- Metallo-β-lactamase

Table VII: MBL activity for *Legionella* spp. used to generate the result presented in table 3.6

Bacterial strain	Species	CDT		MBL index	β-lactamase activity
		IMP (mm)	IMP + EDTA (mm)		
L 1	*L. pneumophila*	32	30	2	-
L 2	*Legionella* spp.	30	30	0	-
L 3	*Legionella* spp.	34	35	1	-
L 4	*L. pneumophila*	34	34	0	-
L 5	*Legionella* spp.	30	32	2	-
L 6	*Legionella* spp.	30	30	0	-
L 7	*Legionella* spp.	28	30	2	-
L 8	*L. pneumophila*	29	29	0	-
L 9	*Legionella* spp.	30	34	4	-
L 10	*L. pneumophila*	32	30	2	-
L 11	*L. pneumophila*	26	28	2	-
L 12	*L. micdadei*	30	30	0	-
L 13	*Legionella* spp.	27	36	9	+
L 14	*Legionella* spp.	32	30	2	-
L 15	*Legionella* spp.	32	30	2	-
L 16	*L. pneumophila*	35	33	2	-
L 17	*L. pneumophila*	30	30	0	-
L 18	*Legionella* spp.	30	30	0	-
L 19	*Legionella* spp.	39	35	4	-
L 20	*Legionella* spp.	30	30	0	-
L 21	*L. pneumophila*	30	30	0	-
L 22	*L. pneumophila*	30	30	0	-
L 23	*L. pneumophila*	35	35	0	-
L 24	*L.pneumophila*	37	30	7	+
L 25	*Legionella* spp.	32	34	2	-
L 26	*Legionella* spp.	30	30	0	-
L 27	*L. pneumophila*	30	30	0	-
L 28	*L. pneumophila*	27	34	7	+
L 29	*L.pneumophila*	30	30	0	-
L 30	*Legionella* spp.	30	32	2	-
L 31	*Legionella* spp.	32	35	3	-
L 32	*L. pneumophila*	30	35	5	-
L 33	*L. pneumophila*	35	32	3	-
L 34	*Legionella* spp.	40	40	0	-
L 35	*Legionella* spp.	36	35	1	-
L 36	*L. pneumophila*	30	30	0	-
L 37	*Legionella* spp.	32	30	2	-
L 38	*Legionella* spp.	34	32	2	-
L 39	*Legionella* spp.	30	30	0	-
L 40	*L. pneumophila*	30	32	2	-
L 41	*L.pneumophila*	28	35	7	+
L 42	*Legionella* spp.	23	35	12	+
L 43	*L.micdadei*	34	35	1	-
L 44	*Legionella* spp.	32	35	3	-
L 45	*Legionella* spp.	33	32	1	-
L 46	*Legionella* spp.	30	32	2	-
L 47	*Legionella* spp.	20	30	10	+
L 48	*Legionella* spp.	35	33	2	-
L 49	*Legionella* spp.	33	32	1	-
L 50	*Legionella* spp.	35	35	0	-

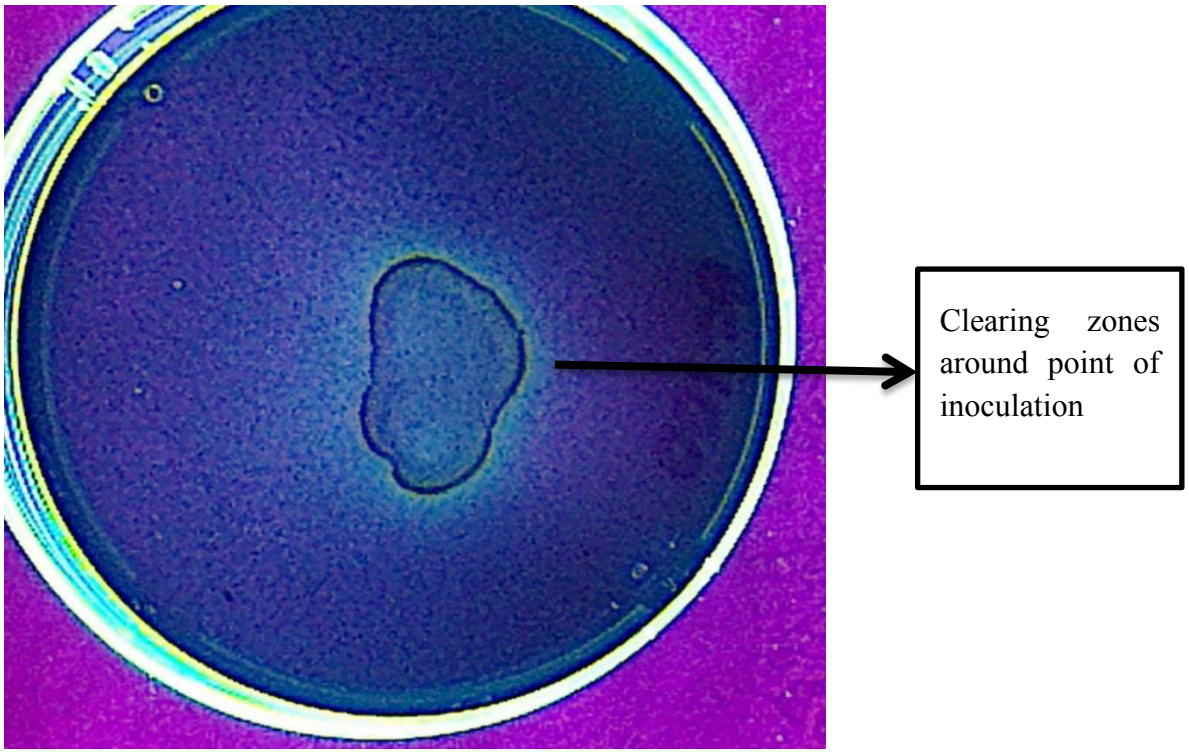

Figure II: Blue agar plates (CTAB agar plates) showing dark blue haloes under UV transilluminator indicating the production of anionic surfactants by *Legionella* spp.

Table VIII: Percentage score similarity generated from GelCompare software for *Legionella* spp. band on the RAPD profiles obtained and presented in figures 3.3 and 3.4

Isolate code	Matrix
L 25	100
L 1	50.00 100.00
L 4	10.00 20.0100.00
L 15	0.00 14.29 11.11 100.00
L 8	0.00 16.67 12.50 66.67 100.00
L 3	0.00 11.11 9.09 60.00 40.00 100.00
L 33	0.00 0.00 0.00 0.00 0.00 0.00 100.00
L 43	12.50 0.00 9.09 0.00 0.00 0.00 40.00 100.00
L 16	0.00 0.00 8.33 0.00 0.00 10.00 33.33 37.50 100.00
L 28	11.11 10.00 18.19 12.50 0.00 10.00 33.33 37.50 71.43 100.00
L 42	0.00 0.00 11.11 0.00 0.00 33.33 66.67 33.33 50.00 50.00 100.00
L 27	0.00 0.00 10.00 75.00 20.00 50.00 0.00 28.57 66.67 42.86 75.00 100.00
L 6	0.00 0.00 8.33 12.50 0.00 10.00 33.33 22.23 50.00 33.33 50.00 25.00100.00
L 17	0.00 10.00 8.33 0.00 0.00 10.00 33.33 22.23 33.33 20.00 28.57 11.11 50.00 100.00
L 36	0.00 0.00 0.00 0.00 0.00 0.00 25.00 0.00 0.00 12.50 0.00 0.00 28.57 28.57 100.00
L 47	0.00 0.00 0.00 0.00 0.00 0.00 25.00 33.33 12.50 12.50 0.00 0.00 28.57 12.50 100.00 100.00
L 39	14.29 0.00 0.00 0.00 0.00 0.00 50.00 12.50 0.00 11.11 0.00 0.00 0.00 0.00 40.00 75.0100.00
L 37	14.29 0.00 0.00 0.00 0.00 0.00 20.00 0.00 11.11 25.00 16.67 0.00 25.00 25.00 75.00 75.00 100.00 100.00
L 11	0.00 0.00 0.00 0.00 0.00 0.00 20.00 28.57 25.00 11.11 40.00 0.00 25.00 11.11 75.00 75.00 100.00 100.00 100.00
L 23	12.50 0.00 0.00 0.00 0.00 0.00 16.67 25.00 0.00 10.00 0.00 0.00 22.23 10.00 60.00 60.00 50.00 50.00 50.0100.00
L 10	0.00 0.00 10.00 0.00 0.00 12.50 50.00 28.57 42.86 66.67 75.00 14.29 42.86 25.00 16.67 75.00 33.33 33.33 60.00 28.57 100.00
L 35	0.00 0.00 0.00 0.00 0.00 12.50 50.00 28.57 25.00 42.86 40.00 0.00 42.86 25.00 40.00 75.00 60.00 60.00 100.00 50.00 100.00 100.00
L 22	0.00 0.00 9.09 0.00 0.00 11.11 40.00 25.00 22.23 37.50 33.33 0.00 22.23 37.50 14.29 33.33 50.00 50.00 50.00 25.00 80.01 50.0100.00
L 21	20.00 16.67 12.50 0.00 0.00 0.00 0.00 0.00 0.00 14.29 0.00 0.00 14.29 14.29 66.67 25.00 50.00 50.00 20.00 16.67 0.00 0.00 16.67 100.00
L 40	40.00 14.29 11.11 0.00 0.00 0.00 0.00 14.29 0.00 12.50 0.00 0.00 12.50 0.00 50.00 20.00 75.00 75.00 40.00 60.00 16.67 40.00 14.29 66.100.00