CHAPTER 1
LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

In the beginning there was the germ (Tierno, 2001), or single-celled micro-organisms that we now call germs. Without them this planet would not exist in the form which we know it today. They break down dead organic matter, live in some of the most uninhabitable places on the planet and even in and on our own bodies. We grow up with them being a part of us and some of them are an integral part of our bodies' natural defence system against other germs. However, there are those micro-organisms that can break through this defence and attack us causing illness and possibly death.

To become a scientist is to question, discover and unlock the secrets behind new micro-organisms that could result in human and animal death (thus termed pathogens). There are many such 'new' pathogens, and there are some that have been around for a long time, and are only now returning to the scientific spotlight due to the increasing effect they have on certain populations.

The job of a scientist is also to inform the medical community of any new diseases, to increase the knowledge base and to discover ways and methods of preventing transmission or treating the disease once it has been established in a human host.

Environmental sampling is a very important part of this research, because not all bacteria are transmitted from human to human, and a large number of them reside in our environment. As Tierno (2001) said in his book "The Secret Life of Bacteria", their numbers are so large any aliens looking down upon this planet would (perhaps correctly) assume them to be the dominant life form.

In this study two environmental pathogens were looked at, that have emerged in the last decades as important human pathogens; they are pathogenic *Leptospira* and *Burkholderia* species.

1.2 *BURKHOLDERIA*

1.2.1 Background and History

Burkholderia is a genus of proteobacteria, which consists of more than 45 known species, all of which occupy extremely diverse ecological niches ranging from soils to the respiratory tracts of humans and animals (Coenye and Vandamme, 2003; Payne *et al.,* 2005; LiPuma, 2007). Many *Burkholderia* species (spp.) have both symbiotic and pathogenic relationships with plants and are opportunistically pathogenic in humans. These species include the *Burkholderia cepacia* complex (Bcc), which is comprised of at least 17 different species that include: *B. gladioli*, *B. fungorum, B. mallei* (which causes glanders in horses and related animals), and *B. pseudomallei* (the causative agent of melioidosis) (Coenye and Vandamme, 2003; LiPuma, 2005; Sousa *et al*., 2010). Several other Bcc species have also been implicated in both cystic fibrosis (CF) patients and those who are immunocompromised; in fact the number of human infections caused by Bcc bacteria has increased markedly in only the last two decades (Eberl, 2006).

The first recorded case of *B. cepacia* as a human pathogen was a case of endocarditis in the 1950s. Since then it has been associated with many catheter related urinary tract infections, intravenous bacteraemias and wound infections (Stoyanova *et al*., 2007).

Burkholderia pseudomallei infection was first described in 1911 by a British pathologist, Captain Alfred Whitmore, and his assistant surgeon C.S. Krishnaswami through their work on patients at Rangoon General Hospital in Burma, (now Myanmar) (Whitmore, and Krishnaswami 1912, cited in Dance, 1991). They recorded a 'hitherto undescribed glanders-like illness' in the ill and neglected local population (particularly morphine addicts) (Cheng and Currie, 2005; White, 2003).

1.2.2 Morphology

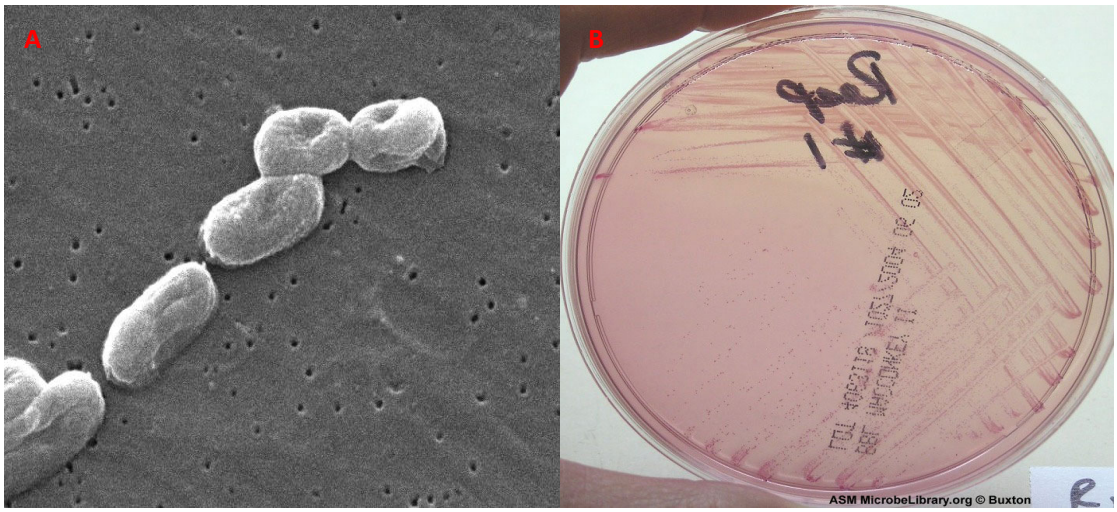

Figure 1.1 **(A) A scanning electron micrograph of *Burkholderia cepacia* and (B) a photograph of a *Burkholderia cepacia* culture on McConkey agar**. (Sources: Missouri University of Science and Technology: *Burkholderia cepacia*, ASM Microbe Library: MacConkey agar).

William Burkholder first described the Gram-negative, motile bacillus as a plant pathogen and cause of onion rot in 1950 (Burkholder, 1950, as cited in Jones *et al*., 2001). The general characteristics of *B. cepacia* include: a non-spore-forming, aerobic bacillus; motile with a respiratory metabolism that is typically catalase- and oxidase-positive; with the optimal temperature for growth being between 30 and 35°C (Govan *et al*., 1996).

All Bcc species show a large phenotypic variability, even within clinical isolates of the same strain. Even small changes in the carbon sources in agar can change the colonial morphology (Mahenthiralingam *et al*., 2008). The ability of Bcc species to change their metabolism and therefore their biochemical profile makes phenotypic identification very difficult (Mahenthiralingam *et al*., 2008).

Colony morphology for *B. pseudomallei* is typically dry, rough colonies on Ashdown's selective agar, but it can change *in vitro* between rough and smooth. This means that colony morphology demonstrates high variability within and between clinical samples (Chantratita *et al.,* 2005). There is also evidence of switching of *B. pseudomallei* colony morphotypes in response to stress *in vitro* and during experimental infection and human melioidosis. This is associated with complex phenotypic shifts associated with changes in the interactions with epithelial cells and macrophages *in vitro* as well as the persistence of infection in an animal model (Chantratita *et al.,* 2005).

1.2.3 Life Cycle

Burkholderia spp. are primarily soil saprophytes, and the members of the genus are found in a variety of ecological niches. They therefore inhabit humid areas, industrial zones, the rhizosphere of plants, and can be found in symbiotic relationships with a range of plants and mushrooms. *B. cepacia* and *B. gladioli* can become plant, animal and human pathogens, especially as nosocomial infections. *B. cepacia* can include in its transmission cycle the contamination of cosmetic and pharmaceutical solutions, water supplies, disinfectants, mouth wash, and anesthetics (Stoyanova *et al.*, 2007). It stands to reason that the *Burkholderia* spp. as a whole exhibit a similar ecology as described by Dance (2000) for melioidosis (Figure 1.2). Animals and humans acquire melioidosis from the environment. The organism is then excreted in sputum, pus, urine and faeces, from which entirely new environments may become contaminated. Under appropriate, and as yet poorly understood, circumstances, the organism increases in numbers, thereby creating a new endemic focus. Rarely, human-to-human, animal-to-human, and iatrogenic infections may occur.

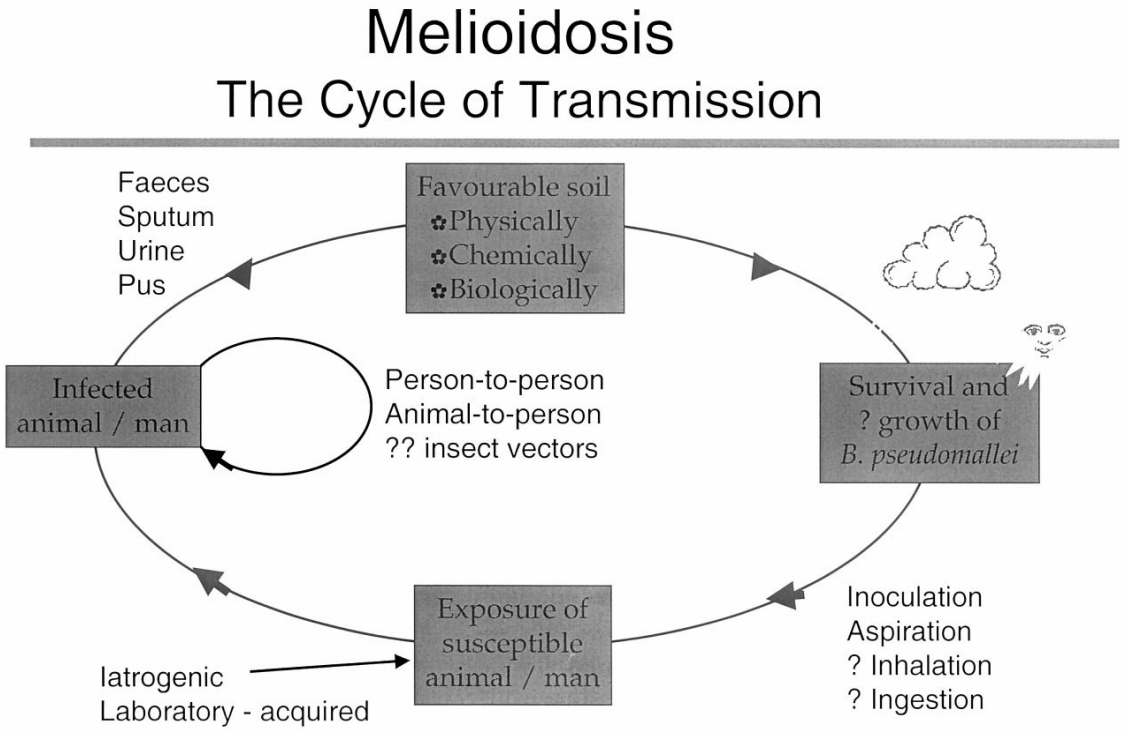

Figure 1.2 **Ecology of *B. pseudomallei* and the interactions between environmental *Burkholderia* spp. and human or animal hosts** (Source: Dance, 2000).

1.2.4 Epidemiology

Bcc organisms are distributed ubiquitously and are found most commonly on plant roots, within the rhizosphere and therefore in soil and moist environments (Gautam *et al*., 2011). As well as being important in causing disease in human hosts, they are also important plant pathogens (Gautam *et al*., 2011). Understanding the clinical epidemiology of Bcc outside of cystic fibrosis is difficult as they cause a smaller number of other infections. Epidemiological studies of *Burkholderia* spp. are further confounded by the difficulty in identifying these organisms and species misidentification is common (Mahenthiralingam *et al*., 2007). Although non-CF-related *Burkholderia* infections (other than melioidosis) are rare, *B. cepacia, B. cenocepacia* and *B. multivorans* are the most common isolates in these cases. Bcc infections in patients without compromised immune systems occur only sporadically, however there have been some cases of

pseudoepidemics and nosocomial infection caused by contaminated disinfectants and anaesthetics (Gautam *et al*., 2011).

The exact mode of infection with *B. pseudomallei* is unknown, most cases are, however, assumed to be acquired by inoculation. Sometimes cases can occur without any obvious signs of inoculation, as in tourists who have had no obvious contact with soil or water (Dance, 2002). However, it mainly affects people who have come into direct contact with infected environments and who also have an underlying predisposition. These are people with diabetes mellitus, renal disease, cirrhosis of the liver, thalassaemia, alcoholism or those whose immune systems are suppressed from either a disease or treatment. Melioidosis, however, does not seem to be associated with HIV. In Australia it has been linked with chronic lung disease as well as excessive consumption of kava (a plant of the western Pacific which is used for its sedative and anesthetic properties). Of these conditions diabetes is the most frequent with up to 50% of patients with melioidosis thus affected, and often there is evidence of poor blood glucose control before onset of infection (White, 2003).

Clinical disease can therefore result from two factors: failure of the hosts' immune system due to drugs or disease or from an especially large initial inoculum (i.e. from a near drowning or major trauma) (White, 2003).

1.2.5 Pathogenesis

Little is known about the mechanism of pathogenicity and the determinants of virulence of *B. cepacia* complex strains (Stoyanova *et al.*, 2007). A 22kDa pilin protein on the pilin fibers has been identified as a mucin binding facilitator to pilated strains of *B. cepacia.* Those isolates which exhibited the highest mucin-binding values tend to correlate to patients with severe illness, which led to speculation that binding variability may be associated with morbidity and mortality (Govan and Deretic, 1996). The lipopolysaccharide (LPS) of Bcc induces a strong host immune response which can contribute to cell damage sustained by the host (Leitão *et al.*, 2010).

In cystic fibrosis several virulence factors are suspected to play critical roles for the success of the pathogen (Leitão *et al.*, 2010). Extracellular lipase, metalloproteases and serine proteases are thought to be directly related to the interactions with epithelial cells. Extracellular lipases play a role in cellular invasion and their production is broadly distributed among Bcc spp., while metalloproteases and serine proteases play a role on the proteolysis of the extracellular matrix (Leitão *et al.*, 2010). Siderophore production (pyochelin, salicylic acid, cepabactin and ornibactin) also contribute to Bcc pathogenesis. Pyochelin plays a role in tissue injury as iron bound to it acts as a catalyst for hydroxyl radical formation which exposes the pulmonary artery, endothelial cells and pulmonary epithelial cells to superoxide and hydrogen peroxide (Leitão *et al.*, 2010).

After the organism is internalized by the cell it escapes from endocytic vacuoles into the infected cell's cytoplasm and forms membrane protrusions by inducing actin polymerization at one pole. These protrusions of actin facilitate the movement of the organism to different cells in the infected organism (White, 2003). *B. pseudomallei* has important type III secretion systems which play a vital role in its spread and in its intracellular survival (White, 2003). The role of exotoxins is as yet undetermined.

There are several different virulence factors proposed in the pathogenesis of *B. pseudomallei*, but the virulence of an individual strain is further complicated by its ability to change its surface determinants and colony morphology. The wrinkled type I morphotype is the most predominant, with six other distinct morphotypes observed (Adler *et al*, 2009). The differences evinced affect the intracellular survival of the bacteria in epithelial and macrophage cells and different lethalities and persistence were seen in mice. This suggests that morphotype switching of *B. pseudomallei in vivo* can provide a strong survival advantage (Adler *et al.*, 2009).

Bcc bacteria produce at least four different exopolysaccharides (EPS) that appear to be stable phenotypes. The EPS appear apparently affect the outcomes of human infections. Both shiny and mucoid variants of *B. cenocepacia* persist longer in animal models of infection than their nonmucoid isogenic variants (Zlosnik *et al.*, 2008). However, both mucoid and shiny variants persist longer in mouse models and interact more poorly with the innate immune systems than their nonmucoid isogenic counterparts.

1.2.6 Clinical disease

Burkholderia spp. are opportunistic pathogens for humans; they can cause life threatening pulmonary infections in patients who are on ventilators and in individuals with chronic granulomatous disease and cystic fibrosis (Stoyanova *et al.*, 2007). Endocarditis was the first report of *B. cepacia* as a human pathogen in the 1950s, and since then it has been recognized as a cause of numerous catheter associated urinary tract infections, intravenous bacteremias and wound infections. In 1970 it caused 'foot rot' in United States troops on swamp training exercises in Florida and in troops serving in Vietnam. The number of nosocomial infections increased from the 1980s due to disinfectants and intravenous solutions being contaminated (Stoyanova *et al.*, 2007).

Melioidosis presents as a febrile illness with a wide spectrum of disease severity that ranges from an acute fulminant septicaemia, to a chronic and debilitating local infection, as well as organ system involvement (White, 2003). Manifestations of these various forms can therefore be defined as acute, subacute and chronic (Brett *et al.*, 2000). There are however, no reliable pathognomonic features of acute or subacute melioidosis and other infections, including tuberculosis and typhoid fever are commonly confused with melioidosis (Inglis *et al.*, 2006). Pneumonia is the most common presentation of the disease and is involved in roughly half of all cases (Cheng and Currie, 2005). Other forms of infection include: pneumonia with septicaemia, septicaemia, cellulitis, fasciitis, skin abscess/ulcer, osteomyelitis, septic arthritis, prostatic abscess, cerebral abscess, meningoencephalitis, encephalomyelitis, suppurative parotitis,

conjunctival ulcer, hypopyon, and orbital cellulitis (Inglis *et al.*, 2006). The majority of severe infections occur in patients with a compromised immune system, for example: uncontrolled diabetes mellitus, chronic renal failure, liver cirrhosis or chronic lung disease (Inglis *et al.*, 2006). Thus due to its versatile nature and many manifestations melioidosis has been nicknamed 'the great mimicker' and this means that diagnosis and isolation is extremely important (Dance, 1991).

1.2.7 Diagnostics

Due to the serious implications of *B. cepacia* infection particularly in those patients with CF laboratory diagnosis should be carried out as accurately as possible. However, isolation and identification of *B. cepacia* is complicated and difficult (Van Pelt *et al.*, 1999). As such the diagnostic process is tedious for routine microbiological laboratories and therefore, and unfortunately, poor proficiency prevails worldwide (Gautam *et al.*, 2011). In these laboratories identification of putative isolates is performed using a combination of selective media, conventional biochemical analysis and/or commercial systems. The three most common media are *Pseudomonas cepacia* agar (PCA), Oxidation-fermentation bacitracin lactose agar (OFPBL), and more recently, *B. cepacia* selective agar (BCSA). BCSA has a superior performance and suppresses the growth of non-Bcc organisms. Routine methods may also be substantiated using molecular methods to verify species identification (Gautam *et al.*, 2011).

The gold standard of diagnosis of *B. pseudomallei* in clinical samples relies heavily on the isolation and identification of the organism from blood, sputum, cerebrospinal fluid or other clinical specimens and requires the use of selective media (Cheng and Currie., 2005; Dance, 1991; Inglis *et al.*, 2006). There have been a number of techniques employed to reduce the time taken to achieve a diagnosis; these include antigen detection from specimens, antibody detection, molecular techniques and rapid culture techniques (Cheng and Currie., 2005).

A definitive diagnosis can be defined as a culture of a Gram-negative bacillus from blood, other sterile fluid or sputum sample with the following features: all of the cultured bacteria are oxidase-positive, gentamicin-resistant, polymyxin (colistin)-resistant, plus one or more of *B. pseudomallei* agglutinating antibody-positive, *B. pseudomallei*-specific PCR-positive or *B. pseudomallei* 16S ribosomal deoxyribonucleic acid (DNA) sequence-positive (Inglis *et al.*, 2006).

A supportive**,** but not definitive diagnosis, is regarded as isolation of a Gram-negative bacillus from blood or other normally sterile fluid or sputum sample without any positive confirmatory tests in a clinical setting. This must be consistent with melioidosis symptoms along with the positive first line tests (oxidase positive etc); or a confirmatory bacterial identification panel (e.g. API, Vitek etc) but awaiting a definitive test. Diagnosis can also confirmed by a fourfold or greater rise in *B. pseudomallei* antibodies tested by indirect haemagglutination (IHA) or enzyme linked immunosorbent assay (ELISA) in a clinical setting consistent with what is expected for melioidosis. The third confirmation is a single, very high *B. pseudomallei* antibody titre in a clinical setting that is also consistent with melioidosis (Inglis *et al.*, 2006).

1.2.8 Treatment

Burkholderia are among the most antibiotic-resistant bacteria encountered and as such antibiotic treatment poses a challenge (Inglis *et al*., 2006; LiPuma, 2007). The treatment of cystic fibrosis patients infected with *B. cepacia* is therefore very difficult and treatment strategies usually use double or triple combinations of antibiotics to achieve bactericidal activity, which rarely result in the eradication of the pathogen, particularly in the case of chronic infection (Sousa *et al*., 2010). Usually, the effective antibiotics for *B. cepacia* are piperacillin, azlocillin, cephalosporins, ceftazidime, chloramphenicol, and trimethoprim-sulphamethoxazole (SXT) (Stoyanova *et al.*, 2007). *B. mallei* is sensitive to ceftazidime, imipenem, doxycycline and ciprofloxacin, while *B. gladioli* strains are sensitive to quinolones, aminoglycosides and imipenem. The therapy in all cases is prolonged and complex (Stoyanova *et al.*, 2007).

Inhalational immunotherapy can also be used to generate protective immunity to acute *Burkholderia* infection. A good example of this is found in mice that are unable to produce interleukin (IL) -12.and as such, are highly susceptible to *B. mallei*. Tumour necrosis factor alpha (TNFα) plays an important protective role in melioidosis as the antibody neutralization of TNFα increases susceptibility to infection (Goodyear *et al.*, 2009). Aerosolized antibiotics have emerged as important for the management of chronic lung infections, particularly in CF patients, with current therapies combining the use of two or three antibiotics (Leitão *et al.*, 2010).

Antibiotics that are routinely used to treat Gram-negative bacterial infection including gentamicin, quinolones, and third-generation cephalosporins have no reliable affect in melioidosis; it is therefore difficult to eliminate the infection in a clinical setting (Inglis *et al.*, 2006; White., 2003).

In adults the oral treatment of choice is a four-drug combination of chloramphenicol (40 mg/kg per day in four divided doses), doxycycline (4 mg/kg per day in two divided doses), trimethoprim-sulfamethoxazole (10 mg and 50 mg/kg per day, respectively, in two divided doses) (White, 2003).

In melioidosis presenting with severe, acute symptoms, including septicaemia, and with or without pneumonia, central nervous system infection and other invasive forms, treatment will be either ceftazidime (adult) (2-3 g or 40 mg/kg/dose every eight hours) given intravenously for 2-4 weeks with trimethoprim-sulphamethoxazole (10/50 mg/kg, up to 320/1600 g) every 12 hours. Alternatively meropenem (1 g or 25 mg/kg every eight hours intravenously) for around 2 weeks can be used (Inglis *et al.*, 2006).

During the eradication phase to completely remove all infection, prolonged antibiotic treatment is given, such as trimethoprim-sulphamethoxazole (8/40 mg/kg every 12 hours) for around 12-20 weeks, doxycycline (4 mg/kg/day) plus trimethoprim-sulphamethoxazole (8/40 mg/kg every 12

hours) for around 12-20 weeks, or chloramphenicol (40 mg/kg/day) plus doxycycline (4 mg/kg/day) (Inglis *et al.*, 2006).

1.2.9 Importance

Most *Burkholderia* spp. are not typically pathogenic for animals or healthy humans, with a few notable exceptions, namely *B. pseudomallei, B. mallei,* and *B. cepacia* (Coenye and Vandamme, 2003; LiPuma, 2007). At present, the knowledge on the natural diversity of members of this genus indicates that the range of interactions between these bacteria and their hosts is complex, diverse and often contradictory. In some species it is restricted to one host type and in others a wider host range is present (Coenye and Vandamme, 2003). *Burkholderia* spp. have the ability to colonise exterior surfaces as well as establish a facultative intracellular existence with eukaryotic cells (Levy *et al.,* 2009). Further studies should also be carried out to determine whether or not *Burkholderia* is present in the main water supply, which has important implications in infection control (Woo *et al*., 2003). The true worldwide impact of melioidosis as a public health problem remains unknown (Dance, 1991). This could possibly be due to either a lack of laboratory facilities within endemic areas or a lack of awareness of the disease or both (Wuthiekanun *et al*., 1990).

1.3. *LEPTOSPIRA*

1.3.1 Background and History

Leptospirosis is perhaps the most common zoonosis worldwide (Cachay and Vinetz, 2005). It is a zoonosis of ubiquitous distribution and causes a wide spectrum of diseas, ranging from subclinical infection to a severe syndrome which includes multi-organ failure and consequently a high mortality (Levett, 2001). The genus *Leptospira* consists of 16 named species and 4 unnamed genomospecies. Phyologenetic analysis has revealed three clades which represent species containing pathogenic serovars, non-pathogenic serovars and the intermediate serovars (Matthias *et al.,* 2008). Before 1989 *Leptospira* was divided into only two species, *L. interrogans,* which comprised all the pathogenic strains, and *L. biflexa,* which contained all the saprophytic environmental species (Levett, 2001). Howeve, both species have now been divided into many serovars and then genomospecies (Levett, 2001). The pathogenic serovars are *L. icterohaemorrhagiae, L. interrogans, L. alexandri, L. kirschneri, L. borgpetersenii, L. weillii, L. noguchii, L. santarosai*, *L.* genomospecies 1 and *L. kmetyi* (Brenner *et al*., 1999; Faine and Stallman 1982; Slack *et al.,* 2006; Slack *et al*., 2009; Yasuda *et al*., 1987). The intermediate serovars are composed of five species and include *L. broomii*, *L. inadai, L. fainei*, *L. licerasiae* and *L. wolffii* (Levett *et al*., 2006; Matthias *et al.,* 2008; Perolat *et al*., 1998; Slack *et al*., 2008).

1.3.2 Morphology

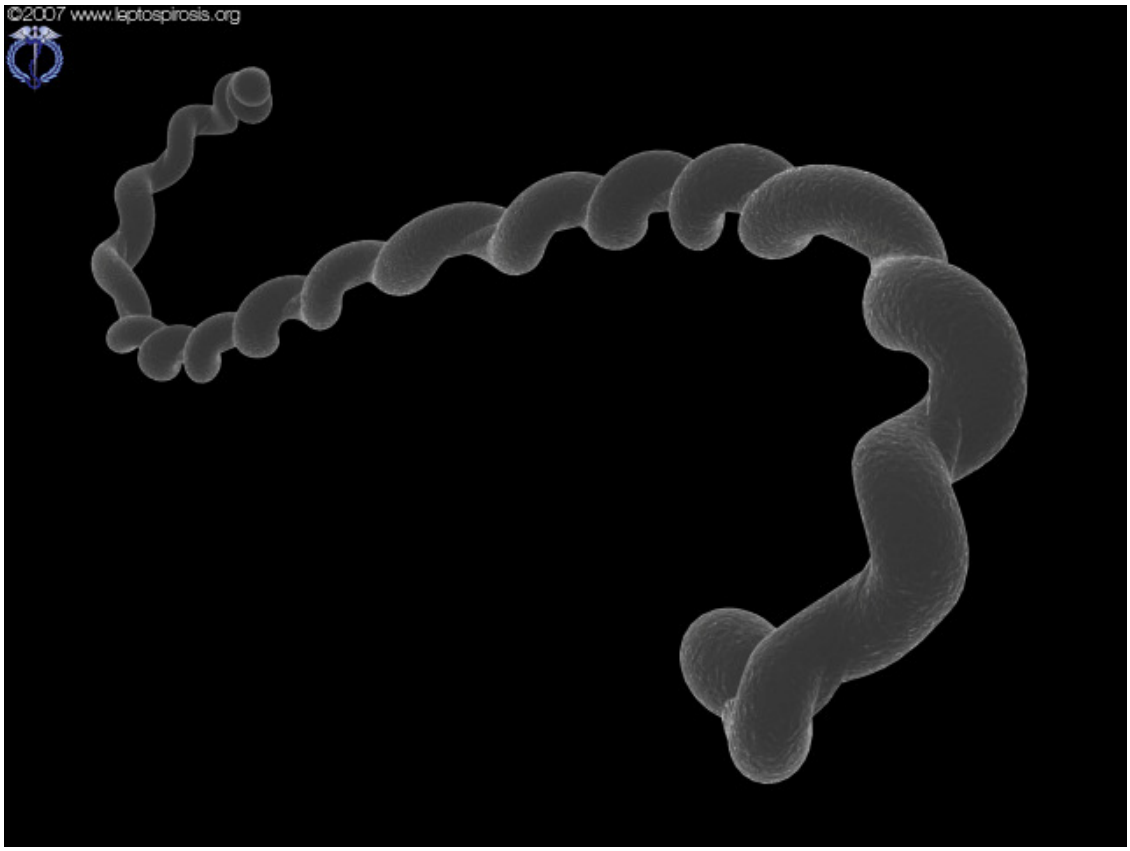

Figure 1.3 **Morphology of a leptospire.** This picture, generated in the 3DS Max drawing program, is based on direct measurements and cross-sectional profiles. (Source: The Leptospirosis Information Centre).

Leptospires are slow-growing, aerobic or micro-aerophilic, highly coiled, Gram-negative bacteria that form part of the broader group of spirochetes (Figure 1.3). They grow optimally at 38ºC and produce both oxidase and catalase (Levett, 2001). Their morphology is unique among spirochetes in that they have characteristic hooked ends and are tightly coiled (approximately 18 coils per cell) (Doern, 2000). At each end a periplasmic flagellum is attached sub-terminally and extends towards the cell's center without overlapping; these flagella lie inside the spirochetes

outer membrane and are integral to cell shape and motility. The structure of the proteins in these flagella is extremely complex (Plank and Dean, 2000; Levett, 2001). Leptospires are approximately 0.1 µm wide and 6-20 µm long, and their narrow helical form allows them to burrow into tissue and phagocytes, using translational and non-translational movement (Plank and Dean, 2000; Levett, 2001). Once inside tissues, the organisms have been seen to assume spherical or granular forms (Plank and Dean, 2000).

1.3.3 Life Cycle

Leptospirosis has a broad spectrum of host range, and many infected species of animals excrete leptospires in their urine, which leads to contamination of soil and water. Therefore, transmission is often via direct contact with the contaminated environment (Noubade *et al.,* 2002). The genome of the *Leptospira* genus is large when compared with the genomes of other spirochetes, reflecting the ability of leptospires to live in diverse environments, including freely in soil and water and in human hosts (Bharti *et al.,* 2003).

The saprophytic leptospires are present in the soil and water naturally; however, pathogenic serovars are normally maintained by persistent colonization of the renal tubules in carrier species, which can include rats, wild animals, livestock and pets. Carrier species normally remain unaffected and shed infectious leptospires during the course of their entire lives (Figure 1.4) (Bharti *et al.,* 2003).

Human infection results when exposure to the infected urine of carrier animals occurs, either directly or (most commonly) through exposure to contaminated soil and water (Bharti *et al.,* 2003).

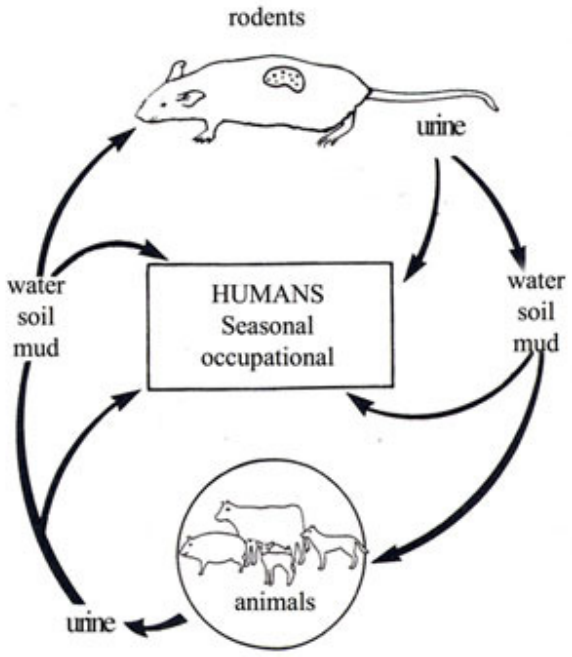

Figure 1.4 **The transmission cycles of pathogenic leptospirosis.** (Source: Pasteur Institute Research Project: Leptospirosis and Leptospires).

1.3.4 Epidemiology

Changing ways that humans interact with the environment allow for additional routes of exposure to leptospirosis, as human infection results from either direct exposure to the urine of infected animals or through exposure to contaminated soil and water supplies (Bharti *et* al., 2003; Levett, 2001; Plank and Dean, 2000). The prevalence of certain types of leptospires in a human population also depends on the reservoir animals and the serovars they carry, the local environmental conditions, human occupational and leisure activities, and agronomic and agricultural practices (Bharti *et al*., 2003; Levett, 2001). In more developed countries outdoor leisure activities provide more opportunities for infection (Levett, 2001; Plank and Dean, 2000). Communities in urban areas can also be at risk as infected rodent populations are a continuous source of transmission (Plank and Dean, 2000). The highest prevalence rates are in tropical developing countries, where leptospirosis is on the rise, associated with urban growth, decay and flooding (Figure 1.5) (Plank and Dean, 2000). Areas such as the the Caribbean, Latin America,

the Indian subcontinent, Southeast Asia, Oceania, and to a lesser extent Eastern Europe, seem to be the most significant foci of the disease; however, there has been little to no data collected in Africa (Figure 1.5) (Pappas *et al*., 2008). The combination of changing climates, human activity and construction may facilitate the movement of infectious leptospires from soil or the sewers into surface waters (Plank and Dean, 2000). In countries with large demographic shifts humans have altered their urban environments in ways which favour both endemic and epidemic leptospirosis (Plank and Dean, 2000).

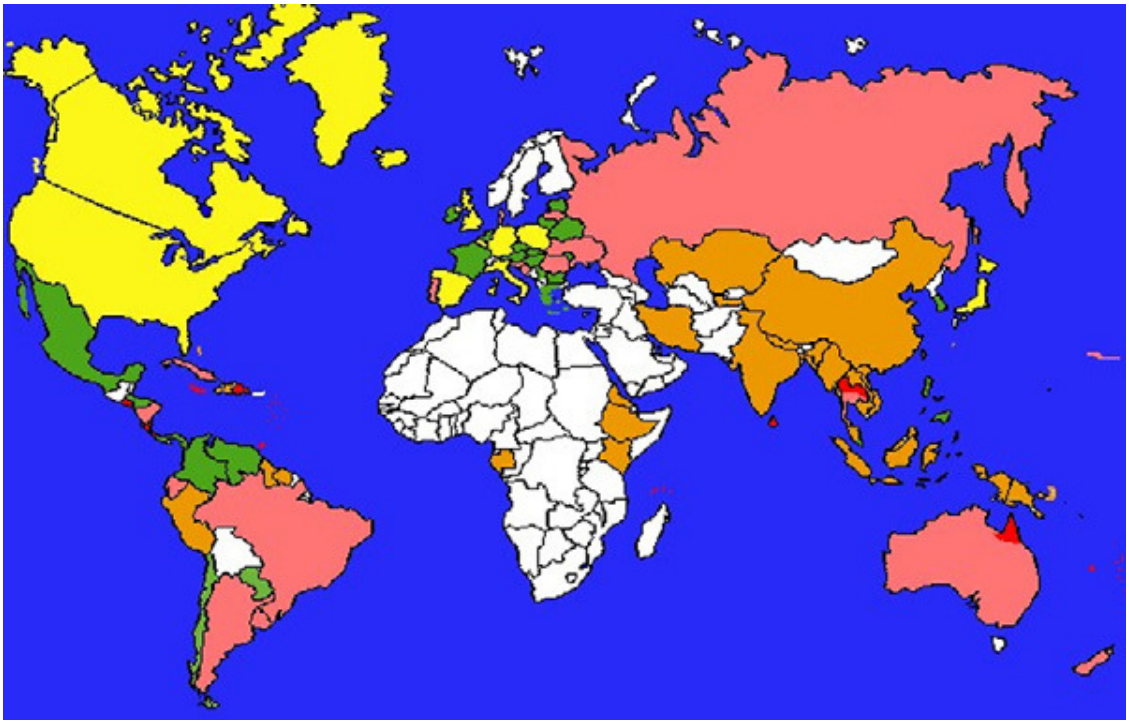

Figure 1.5 **Global annual incidence of human leptospirosis.** The colours reflect incidence in declining order of red, pink, green, yellow and gold, where gold reflects the areas with probable but not estimated high incidence. White reflects absence of data. (Source: Pappas *et al*., 2008).

1.3.5 Pathogenesis

The mechanism by which leptospires cause disease is poorly understood. There have been a number of presumed mechanisms suggested but their role in pathogenesis has yet to be elucidated (Bharti *et al.*, 2003; Levett, 2001; Plank and Dean, 2000). The highly variable nature of leptospirosis suggests that a large range of mechanisms are involved in contributing to both chronic and acute infection in host and carrier mammals alike (Bharti *et al.*, 2003).

The pathogenesis of leptospirosis can be divided into the direct effects of leptospire infection and the effects from host immune system response (Bharti *et al.*, 2003). An important mechanism of virulence is the ability of the leptospires to swim through the viscous medium of the host's blood stream; this motility is important in that it serves in the initial infection as well as to disseminate the organism throughout the body to the organs, where final infection and morbidity will occur (Bharti *et al.*, 2003). Some virulent strains of leptospires exhibit a chemotaxis towards haemoglobin and host tissues. Consistent with this are the virulence factors that assist; these include haemolysin, sphingomyelinase, and phospholipase (Bharti *et al.*, 2003).
Leptospires have also been shown to attach to mammalian cells, possibly due to a fibronectin-binding protein present on the surface of virulent leptospires that aids in initial attachment and binding at mucosal or cutaneous entry sites (Bharti *et al.*, 2003; Levett, 2001).

Virulent leptospires have also been shown to produce cell apoptosis, especially hepatocyte apoptosis, which limits the inflammatory response in the liver, allowing proliferation of leptospires in this organ (Levett, 2001; Plank and Dean, 2000). Leptospires also invade the sinusoids of the liver, the space of Disse, and the parenchymal cells. They are also able to invade the spaces between the parenchymal cells thereby disrupting the caniculi cells and causing the release of bile into systemic circulation without the concomitant release of liver enzymes (Plank and Dean, 2000).

Renal pathogenesis in leptospirosis may involve a leptospiral endotoxin that inhibits the sodium/potassium adenosine triphosphatase enzyme (ATPase) (Plank and Dean, 2000). In acutely infected animals both liver and kidney pathology seem to be related to the high numbers of organisms and the toxins they produce (Bharti *et al.*, 2003).
Pathology in pulmonary involvement is primarily due to haemorrhagic effects rather than inflammation (Bharti *et al.*, 2003).

1.3.6 Clinical disease

Leptospirosis is a disease of countless variations, with Weil's disease being the most severe presentation, which mimics the symptoms of many other diseases (Levett, 2001; Bharti *et al.*, 2003). This means that mechanisms of clinical presentations are often difficult to identify (Bharti *et al.*, 2003). Typical descriptions, however, include a biphasic (anicteric form) and fulminant disease in its icterohaemorrhagic form (Bharti *et al*., 2003).

A large proportion of infections are either subclinical or very mild (anicteric); a smaller proportion of infections have recognisable symptoms. These include a febrile illness of sudden onset, chills, headache, myalgia, abdominal pain, conjunctival suffusion, and less often a skin rash that is transient in nature, lasting only 24 hours or less (Figure 1.6) (Levett, 2001). Aseptic meningitis is found in less than 25% of all leptospirosis cases and petechial or purpuric lesions may also occur.

Figure 1.6 **(A) Conjunctival suffusion and (B) purpuric rash, symptoms of *Leptospira* infection**. (Source: Jansen and Schneider, 2011; The Leptospirosis Information Centre: Human Clinical Signs).

In icteric leptospirosis the clinical course is very rapidly progressive. Between 5 and 10% of all patients with leptospirosis have the icterohaemorrhagic form, which once developed has a high overall mortality rate of up to 15%, and a 54% mortality rate in severe cases with myocardial involvement (Bharti *et al*., 2003). The complications of severe leptospirosis emphasize its multisystemic nature. It causes thrombocytopenia and acute renal failure, myocarditis, as well as pulmonary symptoms ranging from cough, dyspnea and haemoptysis to adult respiratory distress syndrome with pulmonary haemorrhage severe enough to cause death.

Other manifestations and complications from *Leptospira* infection are: ocular (uveitis); infection in pregnancy resulting in foetal death; cerebrovascular accidents; rhabdomyolysis; thrombotic thrombocytopenic purpura; acute acalculous cholecystitis; erythema nodosum; aortic stenosis; Kawasaki syndrome; reactive arthritis; epididymitis; nerve palsy; male hypogonadism; and Guillain-Barré syndrome (Levett, 2001).

1.3.7 Diagnosis

At the moment there is no specific, sensitive, low-cost, fast or widely available test for leptospirosis (Plank and Dean, 2000). Because the clinical features of leptospirosis are so broad and similar to those of other infections (which include diseases such as influenza, aseptic meningitis, encephalitis, dengue fever, hepatitis, gastroenteritis, Ross River virus, Barmah Forest virus, dengue, *Rickettsia* spp. and *Borrelia* spp.), the accuracy of clinical diagnosis is low and confirmation requires laboratory tests (Effler *et al*., 2002; Slack *et al*., 2006; Wuthiekanun *et al*., 2007). Diagnosis of leptospirosis is further confused by the biphasic nature of the disease. The acute phase of the disease lasts for more or less a week, followed by an immune phase in which the body produces antibodies and the organisms are eliminated from the blood and tissue (Slack *et al*., 2006). Laboratory diagnosis can be made by either showing the presence of the organisms or by serological tests that show the presence of antibodies to the organisms (Bharti *et al*., 2003). In the acute phase diagnosis can be performed using different methods such as leptospire-specific ELISA or lateral flow enzyme immunosorbent assay; culture of the blood, cerebrospinal fluid, or urine; and amplification of leptospire-specific DNA using the polymerase chain reaction

(PCR). Each of these methodologies has its own inherent advantages and disadvantages (Slack *et al*., 2006).

In the immune phase diagnosis is performed using serological tests, the gold standard of which is the microscopic agglutination test (MAT) (Matthias *et al*., 2008; Slack *et al*., 2006). This serological typing of leptospiral isolates is traditionally a difficult and labor-intensive process which involves the use of cross-absorption agglutination reactions and large collections of rabbit antisera. There is also the need to use live leptospires with enough of a diversity of antigens to be able to detect the specific anti-leptospiral antibodies, and such antigens vary significantly from country to country and between regions within countries (Matthias *et al*., 2008).

Timely diagnosis of leptospirosis is essential as prompt and specific treatment, as early in the illness as possible, is important to ensure the correct patient management and therefore a good clinical outcome as well as to control of the spread of leptospirosis (Effler *et al*., 2002; Slack *et al*., 2006).

Molecular biology techniques have been developed that can be very sensitive and specific tools for the culture-independent diagnosis of microbial infections, with the capability of detecting as few as 10 organisms within a sample, as well as being less time consuming to perform than culture or some serological tests, (Luchini *et al*., 2008; Plank and Dean, 2000; Romero and Yasuda., 2006).

1.3.8 Treatment

For many years there has been some controversy as to the efficacy of antibiotic treatment or whether or not antibiotics should even be administered as most cases of leptospirosis resolve spontaneously (Bharti *et al*, 2003; Plank and Dean, 2000). The treatment of leptospirosis depends largely on the severity of the disease and the symptoms displayed; if the patient has only mild flu-like symptoms then they are treated symptomatically (Levett, 2001). Patients who present with more severe symptoms, however, (anicteric leptospirosis), require hospitalisation and close

observation. In cases where there is a particularly severe headache, lumbar puncture has been found to be very effective in producing dramatic improvement (Levett, 2001).

Doxycycline 100mg twice daily for seven days has been shown to reduce both the duration and severity of anicteric leptospirosis by an average of 2 days (Levett, 2001). In one study where intravenous penicillin was given at 6 MU/day for 7 days to patients with severe leptospirosis and jaundice but who were not on dialysis, it was found to halve the duration of the fever (Levett, 2001). However, any antibiotic treatment must be started as soon as possible and should not replace any other treatment (Levett, 2001).

1.3.9 Clinical Importance

The biodiversity of leptospires in the environment is affected by the geography, climate, biotic interactions and anthropogenic activities. Leptospirosis is presumed to be the most widespread zoonosis in the world, with incidences of infection significantly higher in countries with warm climates, primarily due to the improved survival of the leptospires in warm and humid environments (Levett, 2001). Leptospirosis is of significant importance to international public health, with more than half a million cases reported annually, due primarily to environmental exposure to pathogenic leptospires, with mortality rates of up to 25% in several outbreaks (Picardeau *et al*., 2008). Additionally, leptospirosis in production animals results in an important economic burden worldwide (Picardeau *et al*., 2008).

1.3.10 Human leptospirosis in South Africa

A number of case studies of human leptospirosis have been reported in South Africa, from all over the country and all reported before 1979 (Gear *et al*., 1958; Klopper 1969; Maze and Kirsch, 1981; Samson and Pillay, 1966; Thomson, 1964). In 1952 a deeply jaundiced fish hawker was admitted to Groote Schuur Hospital in Cape Town, and subsequently died (Thomson, 1964). Further post-mortem examination revealed severe myocarditis attributed to *L. interrogans* serovar icterohaemorrhagiae (Thomson, 1964). In 1953 there were two reported cases of Weil's disease in human patients in Cape Town (Gear *et al*., 1958). Several patients

were admitted to the Johannesburg Fever Hospital in 1957 with meningoencephalitus due to *L. canicola* (Gear *et al*., 1958). The age of these patients ranged from six to 25 and in all cases it was established that there was contact with animals, which was the likely source of infection (Gear *et al*., 1958). In 1964 a 21-year-old dock worker and a university student in Cape Town contracted *L. interrogans* serovar icterohaemorrhagiae; in 1965 and 1968 two separate infections also from Cape Town were reported, but none of these case reports stated how the patients had acquired the disease, only that they were all caused by *L. interrogans* serovar icterohaemorrhagiae (Klopper 1969). In 1965 in another part of Cape Town at the Somerset Hospital, a dock worker was admitted with jaundice, and the causal organism was established as *L. interrogans* serovar icterohaemorrhagiae, although the origin of infection was also not established (Samson and Pillay, 1966). Maze and Kirsch (1981) reported 14 cases from Groote Schuur Hospital in Cape Town, six confirmed and eight presumptive. The patient ages ranged from 22 to 62 years, with 13 male and one female (Maze and Kirsch, 1981). Six of these patients were classed in the high risk occupational index, with two being refuse collectors; two were dockworkers, one worked in an abattoir, and one was a fish trimmer Maze and Kirsch, 1981). After this time the case reports declined, possibly due to the fact that leptospirosis was no longer recognised as a rare disease.

1.4 OBJECTIVES OF THE STUDY

The leptospirosis truism is that wherever it is looked for it is found; in South Africa the RATZOOMAN project performed sampling in the Cato Crest area in Durban, KwaZulu-Natal. Human studies showed a 23% (50/219), prevalence of exposure to leptospirosis (Taylor *et al.,* 2008). There have been some studies on *Burkholderia* spp. in relation to plants in South Africa and water supplies in the Cape region of South Africa (Diergaardt *et al.,* 2003; Chen *et al.,* 2005; Guyon *et al.,* 2003). The possibility of melioidosis in Africa was first raised when Girard isolated a strain of *B. pseudomallei* (then *P. pseudomallei*) from the lymph node of a pig in Madagascar after several passages through tissue in guinea pigs (Dance, 1991). Since then the organism has been isolated in Chad, Niger, Burkina Faso, Uganda, Kenya, and Sierra Leone (Dance, 1991). There has been very little recent evidence of melioidosis in sub-Saharan Africa; since 1991 the only case reported was in a goat in the then Transvaal Province, South Africa; however confirmation of the identity of the isolate was not obtained (Dance, 2000).

Both *Leptospira* and *Burkholderia* species are therefore of great importance to public health worldwide. There has been at least one case of co-infection with *B. pseudomallei* and *L. interrogans,* due to these bacteria sharing a similar ecological niche (Lu and Tseng, 2005). However, little or no investigation has been performed on any clinically relevant *Burkholderia* spp., particularly *B. pseudomallei,* andon any links to *Leptospira* spp. in South Africa.

Specific objectives of this study were therefore:

1) To determine whether pathogenic *Burkholderia* spp. and *Leptospira* spp. are present in the environment in Johannesburg, Gauteng Province, South Africa, using culture and/or molecular techniques.
2) To determine the incidence of pathogenic *Leptospira* spp. in febrile patients suspected of having leptospirosis, using molecular and serological techniques.
3) To determine the prevalence of *Burkholderia* spp. and pathogenic *Leptospira* spp. in rodent populations from Johannesburg, Gauteng Provice, South Africa, using molecular techniques.

CHAPTER 2

MATERIALS AND METHODS

2.1 SAMPLE COLLECTION

2.1.1 Environmental Samples

A total of 156 environmental samples was taken (Table 2.1), at different seasons over a two year period (2010-2011) in Bruma, Rhodes Park, Gilloolys Farm, Modderfontein Dam No 4, and Alexandra (Figure 2.1). These sites were chosen as they form part of the Jukskei River catchment, and they are in different ecological areas along the catchment.

In Bruma the river flows through a suburban area where the sides of the river have been heavily concreted, just before it flows into a dam renowned for its heavily polluted waters. Rhodes Park is a large recreational area in the middle of a suburb; the water in its lake comes from ground water as well as various pipes flowing into the lake (the respective source of these pipes is unknown). Gilloolys Dam is located in Gilloolys Farm, also a larger recreational facility next to a main highway. This dam is fed by the same stream as Bruma Lake but has larger reserves of fish and is a popular bird watching and hiking area. Modderfontein Dam no 4 started as an effluent waste facility for the AECI explosives factory; this waste was high in chemicals such as nitrates, sulphates, phosphates, ammonia and fluoride. In 2007 (after five years of effluent waste being diverted by a canal), the dam was rehabilitated to the point where fish species could be introduced and it has now become part of a nature conservation area and has been rejoined with the natural water course. The effluent contaminants remaining in the dam soil do not readily dissolve into the water; however any seepage is being monitored constantly by AECI. Alexandra has been an established township on the banks of the Jukskei River since 1912. However, a population explosion has led to various informal settlements being built on the flood plain of the Jukskei River itself as well as several of its tributaries. This then led to overloading of facilities, poor access for maintenance and poor sanitation for the informal clusters. This overcrowding of

the river bank has subsequently led to significant pollution of the river, and thus the inhabitants are in danger from the health hazard it has become.

In 2010, totals of 27 soil and 27 water samples were collected from three locations in Alexandra, Johannesburg (Figure 2.1). The locations included sites outside Alexandra at Pretoria Road (UJ9), inside Alexandra at the informal settlement (DWJ44), and at Marlboro Road Bridge (DWJ12). Three water and three soil samples were collected from each location on three different dates in autumn, winter and spring respectively.

In 2011, 79 soil and 77 water samples were collected from various locations along the Jukskei River (Figure 2.1). In autumn, a total of six samples was collected from Alexandra informal settlement (one soil), the Jukskei River near Lombardy East (one soil and one water sample), and in Dobsonville, Soweto (two soil and one water sample). In winter a total of 64 samples was taken further upstream along the Jukskei River at Rhodes Park (a tributary joining the main Jukskei River just before DWJ06) and Bruma Lake (DWJ06). On two occasions eight water and eight soil samples were collected from each location respectively.
In spring, a total of 32 samples was collected from Gilloolys Farm (DWJ07) and Modderfontein Dam No 4 (DWJ03). Eight water and eight soil samples were collected from each location respectively.

All the samples taken were used to culture for *Burkholderia* species as well as to extract for DNA (deoxyribonucleic acid) for *Leptospira* and *Burkholderia* PCR (polymerase chain reaction).

Table 2.1 Environmental samples collected in the Juskskei River catchment during 2010 and 2011.

Location	Collection date	Number of Water samples	Number of Soil samples
Pretoria Road (UJ9)	Autumn 2010 Winter 2010 Spring 2010	3 3 3	3 3 3
Alexandra (DWJ44) Informal Settlement	Autumn 2010 Winter 2010 Spring 2010	3 3 3	3 3 3
Alexandra Extension 7	Autumn 2011	0	1
Alexandra-Marlboro bridge (DWJ12)	Autumn 2010 Winter 2010 Spring 2010	3 3 3	3 3 3
Dobsonville	Autumn 2011	1	2
Lombardy East	Autumn 2011	1	1
Rhodes Park	Autumn 2011 Winter 2011	8 8	8 8
Bruma Lake (DWJ06)	Autumn 2011 Winter 2011	8 8	8 8
Gilloolys Farm (DWJ07)	Spring 2011	8	8
Modderfontein Dam no 4 (DWJ03)	Spring 2011	8	8
TOTAL		77	79

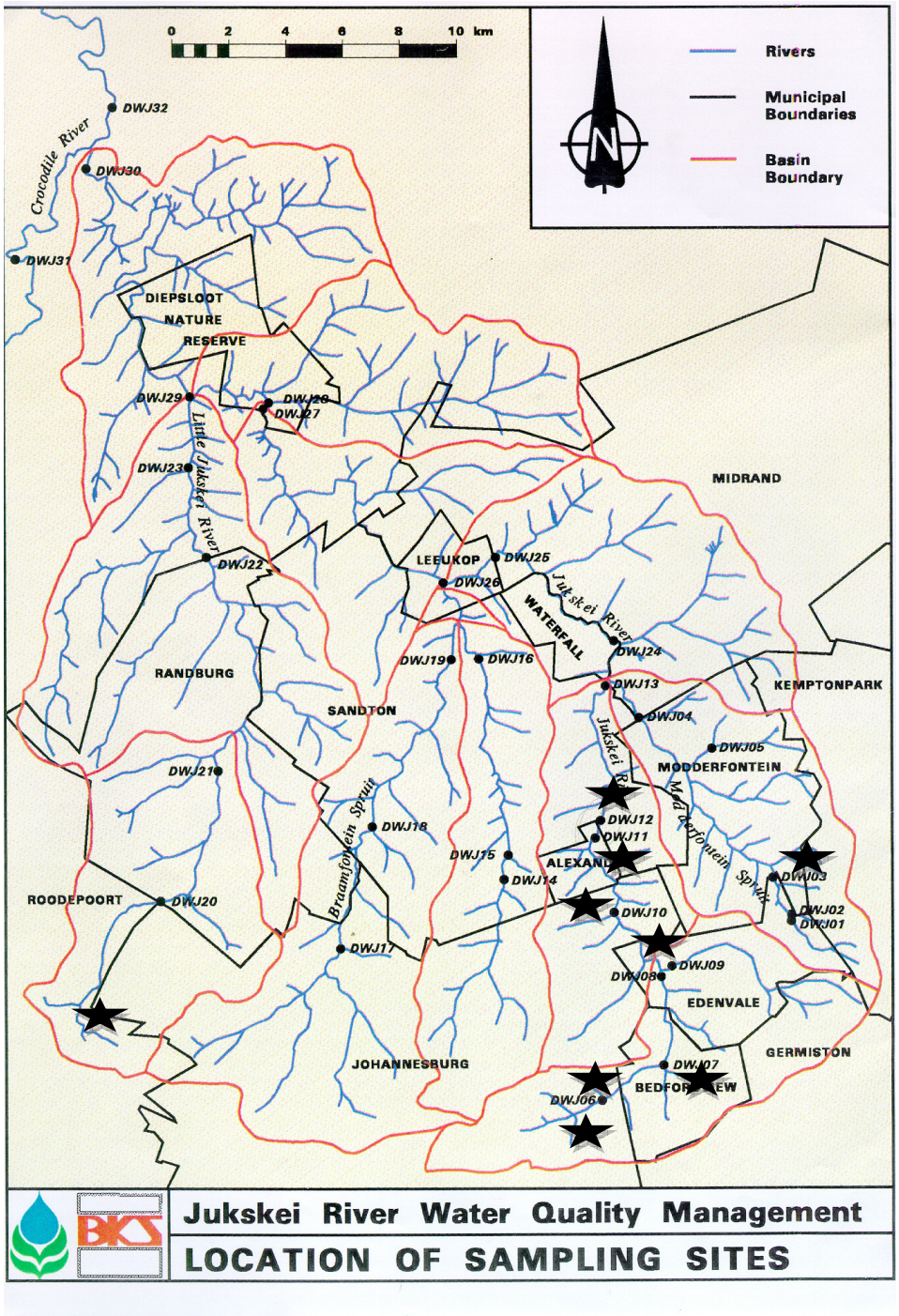

Figure 2.1 **The drainage basin of the Jukskei River in Gauteng showing the respective sampling sites (black stars).** Source: Greater Johannesburg state of environmental health: Jukskei River Catchment.

2.1.2 Human Samples

A total of 332 human serum samples wase submitted to the Special Bacterial Pathogens Reference Unit, National Institute for Communicable Diseases for routine testing with the *Leptospira* immunoglobulin M (IgM) ELISA. The sample collection time frame was from the beginning of 2010 until the middle of 2011. Ethical approval (clearance certificate: M110102, Appendix 1) for the use of residual patient samples was obtained from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, Johannesburg. Of the 332 serum samples, DNA was extracted from 247 samples with a sufficient volume ZR Genomic DNA^{TM}-Tissue MiniPrep kit (Zymo Research). These samples were subjected to nested *Leptospira* 16S rDNA PCR amplification to determine if an active infection.

2.1.3 Rodent Samples

Totals of 193 rat serum and 193 kidney samples were collected from rodents caught in City of Johannesburg (Region A) (Figure 2.2), and Alexandra informal settlement (Region E) (Figure 2.2), from January of 2010 until June of 2011. Samples were subjected to both *Leptospira* and *Burkholderia* PCR. Ethics approval (clearance certificate: 2011/01/01) was obtained from the University of the Witwatersrand Animal Ethics Screening Committee (Appendix 2).

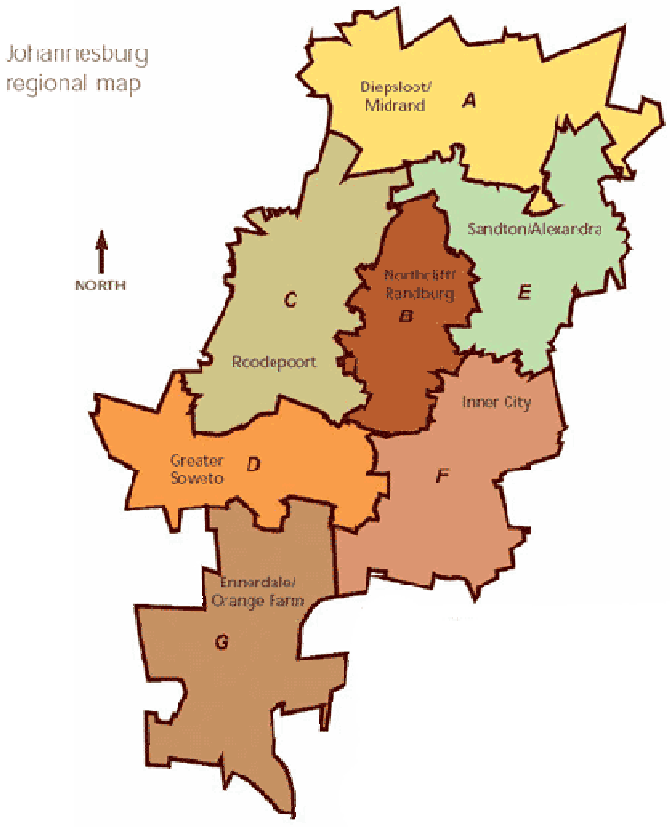

Figure 2.2 **Map illustrating the regions of the City of Johannesburg.** (Source The official website for the City of Johannesburg: Regions.).

2.2 CULTURE OF *BURKHOLDERIA* SPECIES

2.2.1 Media Selection

Ashdown's selective media (Appendix 3) was used for culturing of *Burkholderia* spp. as it was developed specifically for isolating *B. pseudomallei* (Ashdown, 1979). It is a selective media which contains gentamicin and crystal violet that selects for Gram-negative, non-lactose fermenting bacteria that are resistant to these components, and specifically environmental bacteria that are resistant to this antibiotic.

2.2.2 Processing of Environmental Samples:

Serial dilutions were used to process the environmental samples as it was determined this was the best method, along with using selective agar, to dilute out undesirable organisms. Soil samples were mixed with sterile distilled water at a ratio of 10 g of soil to 15 ml of sterile distilled water; this mixture was vortexed thoroughly and allowed to stand overnight at room temperature before serial dilutions of the supernatant were made. Similarly water samples (10 ml) were allowed to stand at room temperature to sediment overnight before processing.

Ten-fold serial dilutions were prepared by serially transferring 500 µl of supernatant into three 10-ml tubes filled with 4.5 ml of sterile water. Dilutions were mixed thoroughly by inverting tubes back and forth a few times after every dilution step. Ten microlitres (10 µl) of the supernatant and each dilution factor (1:10, 1:100 and 1:1000) were then streaked onto separate Ashdown's agar plates and incubated at 37^0C for 48 hours.

A previously-isolated *B. pseudomallei* isolate sent to the laboratory in 2009 and originating from from a patient in Madagascar, was used as a control strain. Each batch of soil and water samples was inoculated with this control strain. This was done to ensure that there were no factors in the soil or water that would inhibit the growth of *Burkholderia*.

2.2.3 Colony Identification

Following incubation 624 agar plates in total were inspected for culture growth. As growth on the Ashdown's agar was limited due to its selective nature, only a few colonies were observed per plate. The colour and morphology of the colonies were noted, as well as the serial dilution at which they were grown, and then a Gram stain was performed. Suspicious Gram-negative colonies were freshly streaked onto 5% horse blood agar (Diagnostic Media Products) for pure growth and incubated at 37^0C for 48 hours. Cultures were subsequently analysed with the biochemical oxidase and catalase tests; if they were positive for both tests then they were analysed further using the analytical profile index (API®) 20NE (bioMérieux). Cultures identified as *Burkholderia* spp. were subjected to DNA extraction using the QIAamp® DNA mini

kit (QIAGEN)(section 2.3.2) and *Burkholderia* spp. *RecA* PCR amplification (section 2.4.1) to verify the genus identification obtained with the API® 20 NE.

2.2.3.1 Gram Stain and Microscopy

A small portion of the culture being tested was picked up with a loop and mixed into a small drop of sterile water on a glass slide along with Gram-negative (*Escherichia coli* ATCC 25922) and Gram-positive (*Staphylococcus aureus* ATCC 25923) controls. The slide was then passed over a flame for approximately 30 seconds (s) to fix the sample. A solution of primary stain (crystal violet, Diagnostic Media Products (DMP)) was added for a minute then gently rinsed, then a solution of mordant (iodine, DMP) for a minute which was also gently rinsed under a tap. A decolouriser in the form of an alcohol solution was then used for 30 s and a final solution of counterstain in the form of safranin (DMP) was added and then gently rinsed off. The slide was then placed under the microscope and viewed with the 100x oil immersion objective of an Olympus BX41 light microscope. Any Gram-negative rod-shaped bacteria that were viewed were subjected to further biochemical tests.

2.2.3.2 Oxidase test

The oxidase test was performed to determine whether or not the bacterial culture being tested could produce cytochrome c, which enables use of oxygen for energy production. The test was performed by dissolving 0.15 g of the oxidase reagent powder (Merck) into 5ml of sterile water to form a 3% (w/v) solution. A drop of oxidase reagent was placed onto filter paper together with a colony picked up from the culture media with a sterile swab. *Pseudomonas aeruginosa* ATCC 27853 was used as a positive control strain, while *E. coli* ATCC 25922 was used a negative control strain.

2.2.3.3 Catalase test

The catalase test was performed to determine whether or not the bacterial colonies being tested have the catalase enzyme. This enzyme turns hydrogen peroxide into water and oxygen, thus forming visible bubbling around the colony being tested. It was performed by picking up a small

amount of colony off the media with a sterilised wire or plastic loop and placed onto a clean glass slide. A drop of 3% (v/v) hydrogen peroxide (DMP) solution was then added to the colony and if bubbles were produced it was considered positive. *S. aureus* ATCC 25923 was used as a positive control strain. Any cultures that were positive for both of these biochemical tests were then subjected to further biochemical testing using the API® 20NE system.

2.2.3.4 API® 20NE

A total of 167 cultures that were Gram-negative, and oxidase- and catalase-positive was identified using the API® 20NE (bioMérieux) system. This is a standardised system to detect non-fastidious, non-enteric Gram-negative bacterial rods. The test strip itself contains approximately 20 tubules each one with a different dehydrated substrate; once these tubules are inoculated with the API®-bacterial colony suspension they are rehydrated and change colour after incubation, according to whether or not the bacteria inoculated can utilize the substrate in metabolism. The strip was prepared according to manufacturer's instructions; first the incubation chamber was prepared by filling the bottom with approximately 5 ml (millilitres) of water to create a humid atmosphere. The specimen number, where it was sampled and the dilution factor was recorded on the side of this chamber and the strip removed from its foil packaging and placed into the incubation chamber. An ampule of the API® NaCl 0.85% medium (bioMérieux) was opened and with a sterile pipette 1-4 identical colonies were picked up and placed into the ampule until a 0.5% McFarland standard suspension (DMP) was obtained. Tests NO_3 to PNPG were inoculated by distributing the suspension into the tubules but not filling them. The strip was tilted slightly in order to avoid bubble formation. An ampule of API® AUX Medium (supplied with kit) was opened and the remaining saline suspension was added and homogenised well with the pipette. The remaining tubules were then filled to the top, with care to leave a flat or slightly convex meniscus. Mineral oil was added to tubes GLU, ADH and URE to create an anaerobic environment within these tubules. The chambers were closed and the strip incubated at 27°C for 48 hours.

After the incubation period the strip was read as per the table provided by the manufacturer. The spontaneous readings were interpreted first then several reagents were added to the non-

spontaneous tubules to obtain a reading; these two tests were NO_3 and TRP. Nit 1 (DMP) and Nit 2 (DMP) were added to NO_3, a positive result was obtained when the cupule turned deep red; when Kovacs (or James) reagent (DMP) was added to TRP the positive result was a pinkish red; Figure 2.3 shows strips with all positive and all negative results.

A

B

Figure 2.3 **API® 20NE strips illustrating positive (A) and negative (B) reactions** Source: Regnum Prokaryotae.

A numerical profile was generated to obtain the identification profile; on the test sheet the tests are separated into groups of three, with a number one, two or four indicated for each. By adding the values that corresponded to positive reactions a within each group a seven digit value was obtained. These numerical profiles were entered into the APIweb database™ identification software (bioMérieux), which interpreted the values and gave an identification based on the biochemical profile of the organism.

2.3 DNA EXTRACTION

2.3.1 DNA Extraction from Environmental Samples

Purification of nucleic acids is not as easy from soil and water environments as with other sample types (Picard *et al*., 1992). Soil and water samples were extracted using the ZR Soil Microbe DNA kit™ (Zymo Research), as per manufacturer's instructions.

The base of the Zymo-Spin™ IV-µHRC Spin Filters was snapped off**,** inserted into a collection tube and centrifuged in a Spectrafuge 24D benchtop micro centrifuge (Labnet) at exactly 9 300 revolutions per minute (rpm) for 1 min. Approximately 0.25 grams of soil or 250 µl of water (sediment) were added to a ZR Bashing Bead™ lysis tube to which 750 µl lysis solution was also added. The tube was secured in a Finevortex (FinePCR) mixer fitted with a 2 ml tube holder assembly and vortexed at maximum speed for 5 min before centrifugation at 10 400 rpm for 1 min. This supernatant (up to 400 µl) was transferred to a Zymo-Spin™ IV spin filter in a collection tube and centrifuged at 8 700 rpm for 1 min. Soil DNA binding buffer (1,200 µl) was added to the filtrate in the collection tube. Eight hundred microlitres (800 µl) of this mixture was transferred to a Zymo-Spin™ IC column in a collection tube and centrifuged at 10400 rpm for 1 min. The flow through was discarded and this step repeated with rest of the soil binding filtrate mixture. Two hundred microliters (200 µl) of DNA pre-wash buffer, was added to the Zymo-Spin™ IC column in a new collection tube and centrifuged at 10 400 rpm for 1 min. Five hundred microlitres (500 µl) of the Soil DNA wash buffer was added to the Zymo-Spin™ IC column and centrifuged at 10 400 rpm 1 min. The Zymo-Spin™ IC column was transferred to a clean 1.5ml micro-centrifuge tube (Merck) and 100 µl of DNA elution buffer was added directly to the column matrix and centrifuged at 10 400 rpm for 30 seconds to elute the DNA. This eluted DNA was transferred directly to the resin of the prepared Zymo-Spin™ IV-µHRC Spin Filter in a clean 1.5 ml micro-centrifuge tube and centrifuged at exactly 9 300 rpm for 1 min. The DNA was stored at minus -20ºC until use.

2.3.2 DNA Extraction from Bacterial Cultures

Genomic DNA was extracted from bacterial cultures of using the QIAamp® DNA mini kit (QIAGEN) as per manufacturer's instructions, using the protocol for isolation of genomic DNA from bacterial cultures.

The bacterial cultures were removed from culture plate with an inoculation loop and suspended in 180 µl of buffer ATL by vigorous stirring in an Eppendorf safelock micro-centrifuge tube (Merck). 20 µl of proteinase K was added and the mixtures were pulse-vortexed for approximately 10 s and placed in a Hagar heating block at 56ºC for half an hour, before brief centrifugation to remove droplets from tube edges and lid. Two hundred microlitres (200 µl) of absolute ethanol (98%) was added to the mixtures, briefly vortexed and centrifuged. The mixtures were then transferred to the spin columns included with the QIAamp® DNA mini kit and centrifuged at 8 000 rpm for 60 s. The flow through in the collection tubes were disposed of in a beaker containing 10 % (v/v) formalin and the spin columns were placed into a clean collection tube. Five hundred microlitres (500 µl) of buffer AW1 was pipetted into the spin column, and the tubes were then centrifuged at 8 000 rpm for 60s before discarding the flow through as above and placing the spin column into new collection tubes. Five hundred microlitres (500 µl) of buffer AW2 was pipetted into the spin column, before centrifugation at 13 000 rpm for 3 min. The flow-through was discarded and the spin columns were placed back into the collections tubes and centrifuged at 13 000 rpm for 60 s to eliminate buffer AW2 carry over.

The collection tubes were discarded and the filters were placed into Eppendorf tube. Elution occurred in two identical steps, where 50 µl buffer AE (elution buffer) was added to the spin column, allowed to stand at room temperature for 2 min and centrifuged at 8 000 rpm for 1 min. This step was repeated once more before the final volume of 100 µl DNA was obtained. The extracted DNA was stored at -20 ºC until use.

2.3.3 DNA Extraction from Human and Rodent Samples

Total DNA was extracted from human and rodent samples using the ZR Genomic DNA™ Tissue MiniPrep kit (Zymo Research) as per the manufacturer's instructions, using the Solid Tissue and Whole Blood, Serum and Plasma protocols.

A hundred microlitres (100 µl) of serum was added to a microcentrifuge tube together with 95 µl of 2x digestion buffer, and 5 µl of proteinase K. Then the tubes were vortexed and incubated at 55°C for 20 min. Alternatively, 25 mg of kidney tissue was placed into a microcentrifuge tube with 95 µl of sterile water, 95 µl of 2x digestion buffer and 10 µl proteinase K, after which the tubes were vortexed and incubated at 55°C for 1-3 hours.

Seven hundred microlitres (700 µl) of genomic lysis buffer was added to the tube and mixed thoroughly by vortexing. The mixture was transferred to a Zymo-Spin™ IIC column in a collection tube and centrifuged at 10 400 rpm for 1 min. Two hundred microlitres (200 µl) of DNA pre-wash buffer was added to the spin column in a new collection tube and centrifuged at 10 400 rpm for 1 min. Four hundred microlitres (400 µl) of g-DNA wash buffer was added to the spin column and centrifuged at 10 400 rpm for one minute. The spin column was transferred to a clean micro centrifuge tube and 100 µl DNA elution buffer was added to the spin column and incubated for 5 min at room temperature, then centrifuged at top speed (approximately 13 300 rpm) for 30 seconds to elute the DNA. The extracted DNA was stored at -20 ºC until use.

2.3.4 DNA Quantification

DNA concentrations of extracted samples were measured on a NanoPhotometer ™ P-Class P300 (Implen GmbH). The NanoPhotometer ™ P-Class submicroliter cell was inserted into the cell holder with the cell windows facing the light beam (directed from right to left as indicated on the machine with small arrows). The machine was selected for double stranded DNA mode and a lid of factor 5 was used (as there was a large amount of DNA). The machine was blanked first using a 2 µl drop of elution buffer (from the kit used to extract the DNA) was placed directly onto the centre of the measuring window and the lid placed on top. The blank button was pressed to create a blank measurement. The lid was removed and the residue cleaned using a slightly damp

tissue. A sample was then placed on the measuring window (2 µl) and the lid placed on top as before, measurement of the sample was then taken by pressing start. A printout was obtained from the machine with each sample and their respective concentrations. Once the amount of DNA in a sample was determined in ng/µl, further dilution and testing of the sample could be performed.

2.4 PCR AMPLIFICATION

Polymerase chain reaction (PCR) has been used as a specific and sensitive method to detect microorganisms in aquatic and soil environments, and is particularly useful in detecting fastidious organisms that are too difficult or dangerous to culture in vitro. It can also be used to study the diversity of organisms in these complex environments, from which only a small proportion of indigenous bacteria can be isolated in vitro.

Table 2.2 List of primers used in this study

Primer Name	Sequence (5'-3')	Target Gene	Position	Amplicon Size (bp)	Reference
BUR1	GATCGA(**A/G**)AAGCAGTTCGGCAA	*Burkholderia* spp.	72-92[a]	869	Payne *et al.,* 2004
BUR2	TTGTCCTTGCCCTG(**A/G**)CCGAT	*RecA* gene	819-938[a]		
Sabp 75F	GCGCCG(**A/C**)TCAAT(**G/C**)(**G/T**)(**G/T**)TTCG	*B. pseudomallei*	771-789[b]	ca.501	Merritt *et al*., 2006
Sabp 554R	GGCCCA(**A/G**)TGCAG(**C/T**)TA(**A/G**)GTCTCGT	*lpxO* gene	289-311[b]		
Lepto16s11f	GGCGGCGCGTCTTAAACATGC	*Leptospira* spp.	38-58[c]	ca.1327	Matthias *et al.,*
Lepto16s1388r	TGTGTACAAGGTCCGGGAAC	16S rDNA gene	1364-1345[c]		2008
Lepat 1	GAGTCTGGGATAACTTT	Pathogenic/Opportunistic	123-139[c]	ca. 332	Murgia *et al.,* 1997
Lepat 2	TCACATCG(**C/T**)TGCTTATTTT	*Leptospira* spp.	434-455[c]		
		16S rDNA gene			

[a]Relative to *B. cenocepacia* J2315 *RecA* gene sequence

[b]Relative to *B. pseudomallei 1106a LpxO gene*

[c]Relative to *L. interrogans* serovar Copenhageni 16S rDNA gene sequence

2.4.1 *Burkholderia* spp. *RecA* Gene

Genus-specific amplification of the *Burkholderia* spp. *RecA* gene (partial) was performed as described by Payne *et al* (2004) (Table 2.2). The reaction mixture for this PCR was 25 µl in total, with each reaction containing 1 U AmpliTaq Gold® DNA polymerase (Applied Biosystems), 1.5 mM $MgCl_2$, 1x PCR buffer II, 250 µM of each deoxynucleoside triphosphate (dNTP), 10 ρmol of each primer (BUR1 and BUR2) respectively, ultra high quality (UHQ) water, and 10 to 50 ng of DNA template. Thermal cycling was carried out in a Veriti® Thermal Cycler (Applied Biosystems) using the following program: enzyme activation at 95°C for 10 min, 30 cycles of denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, extension at 72°C for 45 s, and one cycle of elongation at 72°C for 5 min. A positive (*B. cepacia* ATCC 25416 DNA) and non-template (water) control was included with each run to verify the results and check for contaminants. Amplicons were analysed on a 1.2% (w/v) agarose gel as described in section 2.6.

2.4.2 *Burkholderia pseudomallei lpxO* Gene

The PCR amplification was taken and optimized from the original paper as described by Merritt *et al* (2006) (Table 2.2), with modifications. These modifications were decided on after a round of Taguchi optimization (as described in section 2.5), as well as changing the magnesium concentration so that an optimal amplified DNA band was seen, with low signal to noise ratio. The reaction mixture was 20 µl in total, with each 20 µl containing 0.5 U AmpliTaq Gold® DNA polymerase (Applied Biosystems), 1.75 mM $MgCl_2$, 1x PCR Buffer II, 200 µM of each dNTP, 0.01% (w/v) bovine serum albumin (BSA), 10 ρmol of each primer (Sabp 76F and Sabp 554R) , and 10 to 50 ng of DNA template. Thermal cycling was carried out in a Veriti® Thermal Cycler (Applied Biosystems) using the following programme: enzyme activation at 95°C for 10 min, 30 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 12 s, extension at 72°C for 45 s, and one cycle of elongation 72°C for 7 min. A positive *B. pseudomallei* (isolated from a patient from Madagascar, 2009) and non-template (water) control was included with each run to verify results and check for contaminants. This positive control was also

used in the optimisation runs. Amplicons were analysed on a 2.5% (w/v) agarose gel as described in section 2.6.

2.4.3 *Leptospira* 16S rDNA Gene

A nested PCR (n-PCR) directed at the *Leptospira* spp. 16S rDNA gene was used to detect the presence of *Leptospira* DNA in the environmental, human and rodent samples. The first round of amplification was performed with primers as described by Matthias *et al* (2008) (Table 2.2). The first round PCR reaction mixture was optimized using Taguchi optimization (section 2.5). This mixture was 50 µl in total, containing 1U GoTaq® Hot Start polymerase (Promega), 1.5 mM $MgCl_2$, 1x GoTaq® Flexi Buffer, 200 µM of each dNTP, 10 ρmol of each primer (Lepat 16S11f and Lepat 16S1388r), UHQ water, and 5 µl of DNA template. Thermal cycling was carried out in a Veriti® Thermal Cycler (Applied Biosystems) using the following programme: enzyme activation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 20 s, primer annealing at 54°C for 20 s, extension at 72°C for 90 s, and one cycle of elongation 72°C for 5 min. Amplicons were analysed on a 1.2% (w/v) agarose gel as described in section 2.6.

The second round of amplification was performed with pathogenic/opportunistic *Leptospira*-specific primers as described by Murgia *et al* (1997), with the following modifications. The reaction mixture of the second round PCR was 50 µl in total, containing 1 U of GoTaq® Hot Start polymerase (Promega), 2.5 mM $MgCl_2$, 1x GoTaq® Green Buffer, 200 µM of each dNTP, 25 ρmol of each primer (Lepat1 and Lepat2), UHQ water, and 1 µl of template DNA from the previous reaction round. Thermal cycling was carried out in a Veriti® Thermal Cycler (Applied Biosystems) using the following program: enzyme activation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 20 s, primer annealing at 47.5°C for 20 s, and extension at 72°C for 45 s and one cycle of elongation at 72°C for 6 min. Amplicons were analysed on a 1.2% (w/v) agarose gel as described in section 2.6.The n-PCR was validated using both culture and known human serum positive samples, A positive *L. interrogans* culture 9obtained from Onderstepoort Veterinary Institute) and non-template (water) control was included with each run to verify results and check for contaminants.

2.5 PCR OPTIMIZATION USING TAGUCHI TECHNIQUE

The *Burkholderia lpxO* PCR was changed from the original and optimization was performed using Taguchi square orthogonal array technique (Cobb and Clarkson, 1994). This technique is performed by combining various concentrations of PCR reagents and components in an orthogonal array; which will simultaneously reveal the effects and interactions of each specific component using as few reactions as possible (Table 2.3).

Table 2.3 The components which make up a Taguchi square, optimized with at least three different concentrations.

Variable	A	B	C
DNA (ng)	20	40	60
MgCl2 (mM)	1.5	2.0	2.5
Primers (ρmol)	5	10	20

Tube Number	DNA	MgCl	Primers
1	A	A	A
2	A	B	B
3	A	C	C
4	B	A	B
5	B	B	C
6	B	C	A
7	C	A	C
8	C	B	A
9	C	C	B

2.6 AGAROSE GEL ELECTROPHORESIS

Amplicons were analysed on a 1.2%-2.5% (w/v) Seakem® LE agarose (Lonza) gel (Appendix 4) using 1xTAE buffer (Appendix 5). Five microlitres (5 µl) of each sample was mixed with 1 µl of loading buffer and pipetted into the respective separate well in the gel. Two microlitres (2 µl) of the HyperLadder™ I (Bioline) or O'GeneRuler™ 50 bp DNA Ladder (Fermentas) were used as a molecular weight markers in order to verify the size of the amplicons. The electrophoresis equipment was closed by placing the lid on and the electrodes connected, making sure to connect the positive (red) and the negative (black) correctly, so the DNA migrated in the right direction. The gel was run at 100V

and the progress was observed by the movement of the blue dye through the gel; once the dye neared the end of the gel (15-30 min later), the power was turned off and the electrodes were disconnected. The tray and gel were carefully removed together and placed into the Syngene Gel Doc System for visualization.

2.7 VISUALISATION AND DOCUMENTATION

The agarose gels were supplemented with ethidium bromide (0.5 µg/ml) so that it could be visualized using an ultraviolet (UV) light. Agarose gels were viewed using the G:BOX HR gel documentation system (SYNGENE) and documented using the GeneSnap software.

2.8 *LEPTOSPIRA* IgM ELISA

A commercially-available *Leptospira* IgM ELISA (PanBio) was used to serologically detect acute leptospiral infections in patient serum samples (Winslow *et al.*, 1997). This ELISA works on the principle that any *Leptospira* IgM antibodies present in patient serum will bind to the *Leptospira* antigen attached to the polystyrene surface of the microwells. Residual serum is removed from these microwells by washing with 1% (diluted) buffer (provided in the kit). The peroxidase-conjugated anti-human IgM is thereafter added to the wells and the plate is re-incubated allowing for the bound antigen-antibody complexes to bind to the conjugate. Wells are washed again and a colourless substrate system, tetramethylbenzidine/hydrogen peroxide (TMB Chromogen) is added. The substrate is hydrolyzed and the chromogen turns blue. The TMB turns yellow once the reaction is stopped using phosphoric acid. Colour development indicates the presence of IgM antibodies to *Leptospira* in the serum sample.

The ELISA was performed according to manufacturer's instructions. The reagents were allowed to reach room temperature (20-25°C) before starting the test to ensure that no condensation formed in the wells that could compromise the test results. The required number of microwells (in strips of 8) were removed from the sealed foil pack and inserted into a strip holder. Specimens were batched, since there are 2 controls (reactive and non-reactive), 3 calibrators and 1 external quality control (commercially purchased

positive control from Serion, Institut Virion) included with every test run to ensure that the test is functioning correctly.

Separate micro-tubes were used to dilute the samples, controls, and calibrators. For the reactive control, non-reactive control external quality control, and the patient serum; 10 µl of serum was initially diluted in 90 µl of sample diluent, followed by a second dilution where 20 µl of the initial dilution is added to 180 µl of sample diluent. The calibrator was prepared by diluting 10 µl in 1 000 µl of sample diluent. All dilutions were well homogenized before being applied to microwells.

A hundred microlitres (100 µl) of each of these dilutions was pipetted into the allocated microwells. The ELISA microwells were covered to prevent desiccation and incubated at 37 °C for 30 min, after which the plate was removed and placed into the BioTek® automated washer (Analytical & Diagnostic Products). The washer aspirates all the wells and adds a 1x (v/v) wash solution to fill the well (350 µl), and repeats this process until the microwells have been washed six times. Horseradish peroxidase (HRP) conjugated anti-human IgM (100 µl) was pipetted into all the wells; the plate was incubated for 30 min and washed as previously described. One hundred microlitres (100 µl) of TMB chromogen was pipetted into each well and incubated at room temperature for 10 min, timing the incubation period from the addition of the TMB to the first well. After the 10 min 100 µl of 1M phosphoric acid was added to each microwell to stop the reactions.

Microwells that contained sera positive for *Leptospira* IgM antibodies were visualized by a deep turquoise blue colour of the TMB that turned yellow once the 1M phosphoric acid was added. Negative results appeared as very light yellow or clear. The absorbance factors were read by the BioTek® ELx800 plate reader by the computer protocol KC4, at a wavelength of 450 nm with a reference filter of 600 – 650 nm. Absorbance readings were divided by the cut off value to produce index values. The cut off value was calculated by the KC4 program using the average absorbance of the calibrator controls and the cutoff factor. The interpretation of the test results can be viewed in Table 2.3.

Table 2.4 The interpretation of test results of the Panbio *Leptospira* IgM ELISA.

Result	Index	Interpretation
Negative	≤ 0.9	No detectable IgM antibody present
Equivocal	0.9-1.1	When the sample was equivocal a second sample was requested, if a repeat of the test was also equivocal
Positive	≥ 1.1	This was the presence of detectable IgM at a 1:100 dilution

2.9 DATA ANALYSIS

Data analysis was performed using a standard Fishers t-test with the help of the statistical computer programme PATHSNAP Statistical Package by P. W. Strike. The data was organised into contingency tables so as to analyse the difference between two kinds of categorical data. The p-value (predictive value) obtained from this test is then used to determine whether or not the difference between these data was significant. A chi-square value was also calculated manually in MS Excel to confirm this.

CHAPTER 3

RESULTS

3.1 ENVIRONMENTAL SAMPLES

3.1.1 *Burkholderia* Culture

All the environmental samples (both soil and water) that were taken were subjected to serial dilution and growth on Ashdown's selective agar. The ten-fold serial dilutions of soil and water samples were plated onto Ashdown's selective media, which was developed specifically for isolating *Burkholderia pseudomallei*. As this is a selective media, which contains gentamycin and crystal violet, it selects for bacteria that are resistant to these components, and specifically environmental bacteria that are resistant to this antibiotic. A total of 624 Ashdown's agar plates was used for culturing of the environmental samples. Each plate that bore a colony was recorded and then the cultures were subjected to microscopy and biochemical assays.

After being identified using oxidase and catalase tests as well as the Gram stain and microscopy, the suspicious cultures were put through an API® 20 NE (bioMérieux) test using the manufacturer's instructions. After incubation for 48 hours and then scoring, the results were put entered into the API®web™ identification software which evaluated the scores for each respective culture and gave the closest species match to the value of a percent (% identification) (Appendix 6). Of the 167 APIs performed, there were 27 different species of bacteria that were isolated in total. Of these 27, five species were isolated more often than others; these were: *Comamonas acidovorans, Comamonas testosterone, Pseudomonas flourescens, Alcaligenes faecalis,* and *Burkholderia cepacia* (Figure 3.1).

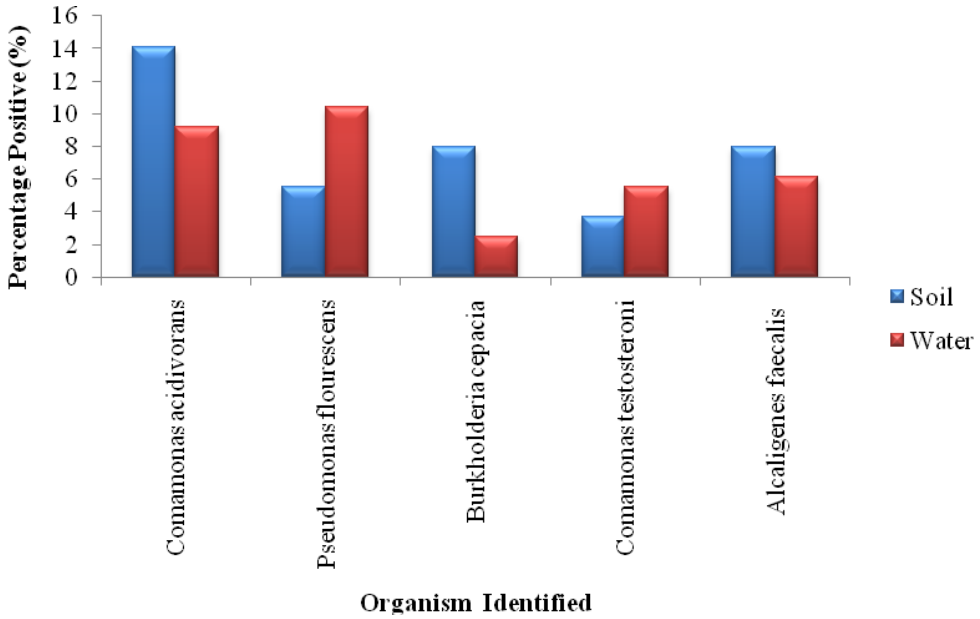

Figure 3.1 **The percentage of five most frequent organisms isolated in soil and water samples collected in the Jukskei River catchment during 2010 and 2011.**

More *Burkholderia* species (identified as *Burkholderia cepacia* by API) were isolated from soil (76.5%; 13/17) than from water (23.5%; 4/17) using culture. This result is statistically not significant (p = 0.13, Fishers two-sided test; chi-square statistic 3.619, 1 degree of freedom). The highest rate of organism isolation was from Rhodes Park, which had 64.7% (11/17) of the total number of *Burkholderia* spp**.** isolated (Figure 3.2); of these 52.9% (9/17) were isolated from soil and 11.8% (2/17) from water. Soil samples collected from Marlboro, Dobsonville, Alexandra and Modderfontein as well as water samples from Pretoria Road and Modderfontein yielded one *Burkholderia* spp. isolate (5.8%, 1/17) respectively.

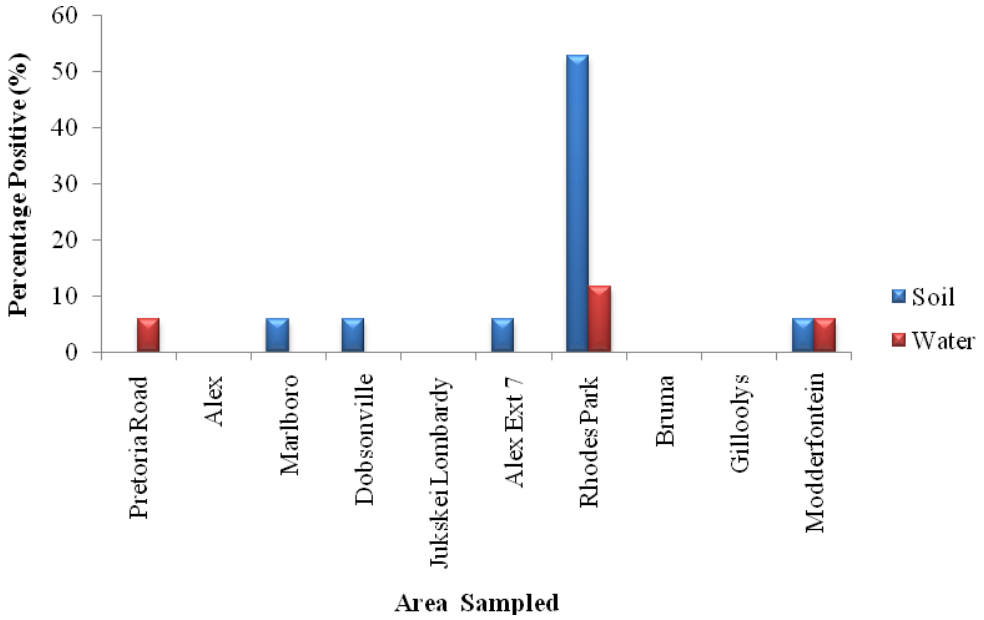

Figure 3.2 **The percentages of *Burkholderia* organisms isolated by culture from soil and water samples collected in the Jukskei River catchment during 2010 and 2011.**

3.1.2 PCR

3.1.2.1 *Burkholderia* PCR

Burkholderia RecA PCR was performed on the environmental samples (n=156) as well as a confirmatory test on the *Burkholderia cepacia* culture isolates. There were 36 positive and unconfirmed positive samples that were not initially presumptive for *Burkholderia cepacia* by the API®web™ but were the next organism chosen by the program as a possibility. Thirty-six (36) culture isolates were extracted for DNA and PCRs were performed to confirm that they were *Burkholderia* spp. Of the 36, 26 were confirmed to be *Burkholderia* spp. by PCR, an example of PCR positive samples with amplicon sizes of ca. 869 bp is shown in Figure 3.3.

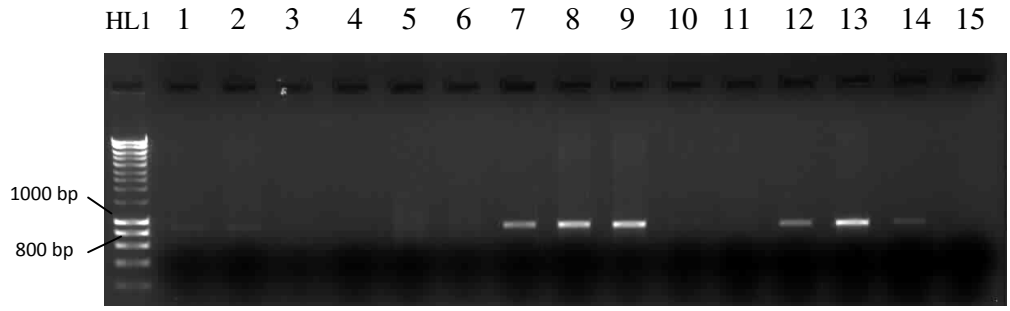

Figure 3.3 **Agarose gel analysis showing the results of a *Burkholderia* spp. *RecA* PCR on cultured isolates from the API positives.** Lanes (L to R): HyperLadder^{TM} I (Bioline), C4, C5, C6, C7, C8, C9, C10, C11, C12, C13, C14, C15, positive control *B. cepacia* ATCC 25416, positive control *B.pseudomallei* Madagascar, non-template control. Refer to Appendix 7 for sample information

The *Burkholderia* spp. *RecA* PCR positive cultures and environmental samples were also subjected to the *B. pseudomallei LpxO* PCR. Of the 26 *RecA* PCR positives subjected to the *LpxO* PCR, none of the samples yielded an amplicon of the expected size (501 bp).

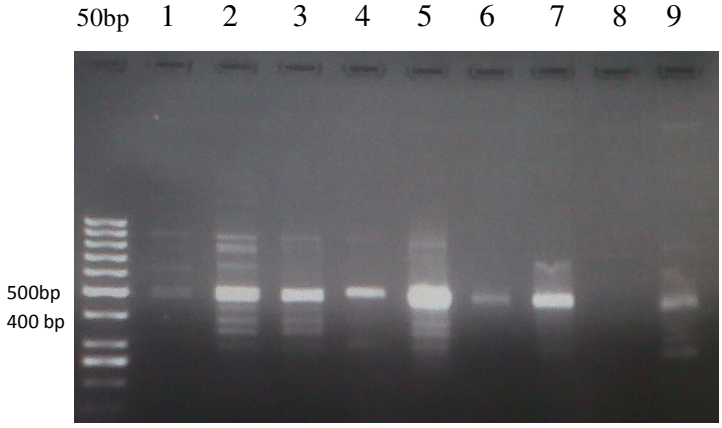

Figure 3.4 **Taguchi optimization performed on *Burkholderia LpxO* PCR using a control strain of *Burkholderia pseudomallei*.** Lanes (L to R): O'Generuler 50 bp DNA ladder (Fermentas), Tube 1, Tube 2, Tube 3, Tube 4, Tube 5, Tube 6, Tube 7, Tube 8, Tube 9. All tubes were mixed according to Table 2.3.

Taguchi optimization of the *lpxO* PCR helped identify a good reaction mixture (Tube 4, Figure 3.4) but there was still some non-specific binding. Thus the reaction mixture from tube four was used and the magnesium concentration changed to 1.75 mM, and a better binding was achieved. The soil, water and culture samples that were positive in the *RecA* PCR for *Burkholderia* spp. were run and all were negative for *Burkholderia pseudomallei*.

Of the soil and water samples processed there were significantly more *Burkholderia* spp. detected from soil than from water (p=0.015, Fishers one-sided test, chi-squared value of 11.4, one degree of freedom) (Figure 3.5). There were 42 positive *RecA* PCRs in total, an example of which can be seen in Figure 3.6. The most PCR-positive soil samples came from Bruma and Rhodes Park (16.7%, 7/42); the next highest was Modderfontein with 11.9% (5/42), Alexandra at 7.1% (3/42), Marlboro Road and Dobsonville at 4.8% (2/42) and Jukskei at Lombardy and Alexandra Extension 7 both at 2.4% (1/42). The most frequently positive water samples were also from Bruma Lake with 14.3% (6/42), Modderfontein (9.5%; 4/42) and Rhodes Park (4.8%; 2/42).

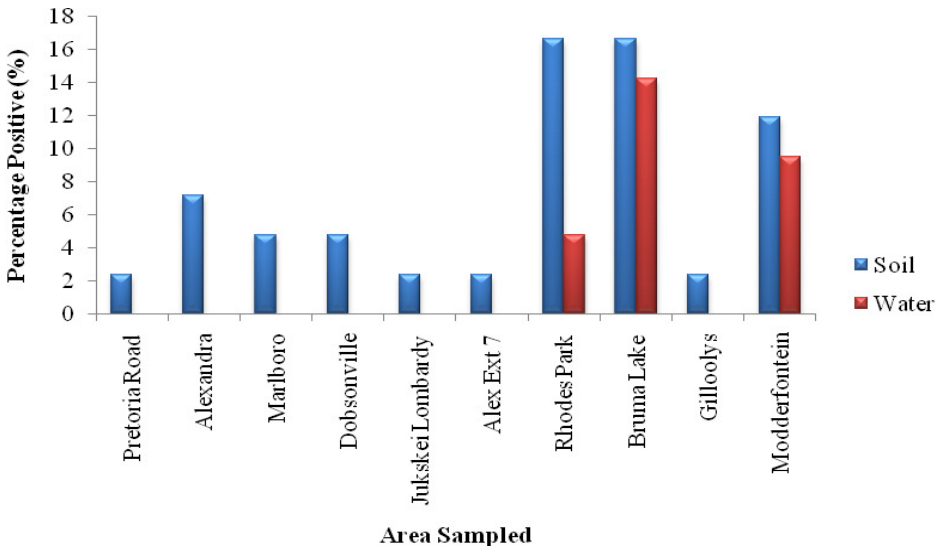

Figure 3.5 **The percentages of *Burkholderia* species detected with *Burkholderia* spp. *RecA* PCR amplification from water and soil samples collected from Jukskei River catchment during 2010 and 2011.**

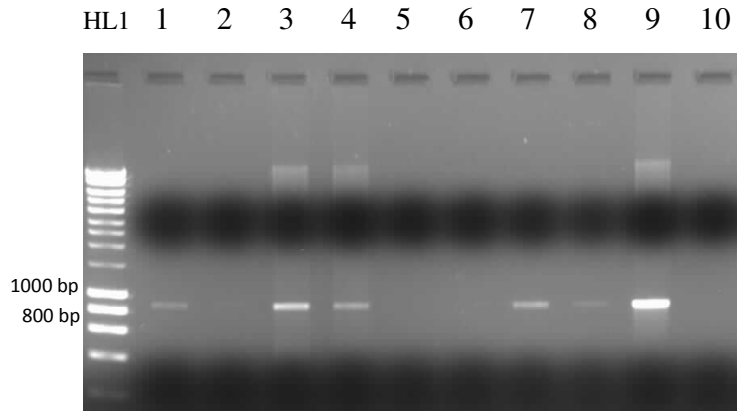

Figure 3.6 Agarose gel analysis of *Burkholderia* spp. *RecA* PCR soil samples from the Modderfontein Dam no.4. Lanes (L to R): HyperLadder^{TM} I (Bioline); S72; S73; S74; S75; S76; S77; S78; S79; positive control *B. cepacia* ATCC 25416; non-template control. Refer to Appendix 7 for sample information.

When comparing culture to PCR method for *Burkholderia* spp. (where the total number of positives from both PCR and culture was 59), overall significantly more positive results were found on PCR ($p = 0.009$, Fisher two-sided test, chi-square value of 8.4, one degree of freedom) (Figure 3.7). In total there were more isolates with culture and PCR found at Rhodes Park, Bruma and Modderfontein with Bruma having the highest percent at 22 % (13/59). The culture results at Rhodes Park were higher in percentage than PCR, 18.6% (11/59) and 15.3% (9/59) respectively, while Modderfontein the PCR percentage was higher than culture at 15.3 (9/59) and 3.9% (2/59) respectively. Alexandra only had organisms identified in PCR at 5.1% (3/59) while Alexandra Extension Seven the percentage positives of PCR and culture were equal to each other at 1.7% (1/59). Marlboro and Dobsonville had equal percentage positives for PCR and culture at 3.9% (2/59) (PCR) and 1.7% (1/59) (culture) respectively. Samples taken from Gilloolys and Jukskei near Lombardy on revealed PCR positives at 1.7% (1/59) each, Pretoria Road had an equal number of isolates in culture and PCR at 1.7% (1/59).

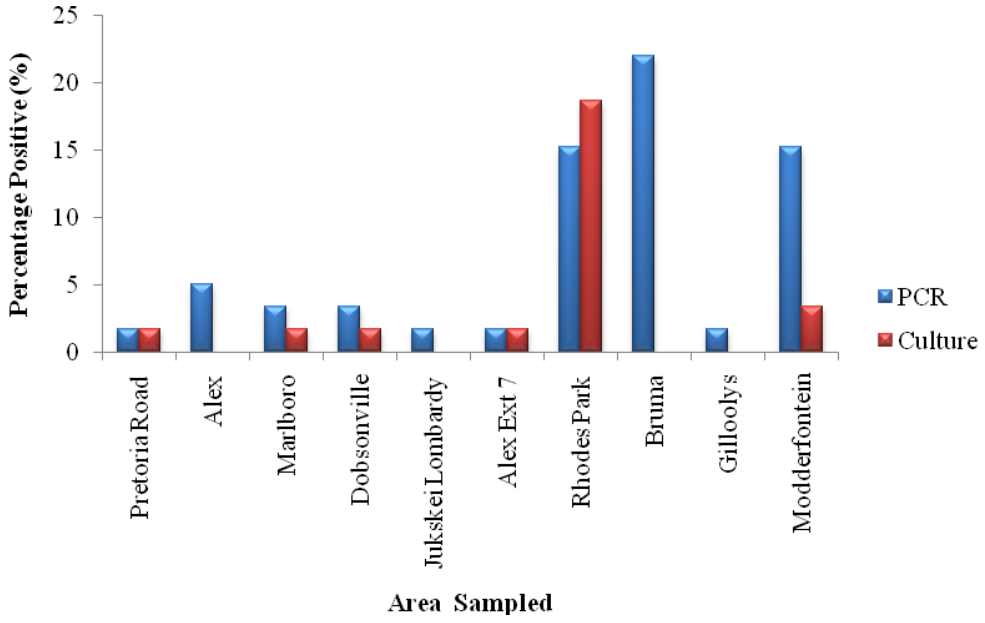

Figure 3.7 The percentage of *Burkholderia* culture and PCR positive samples.

3.1.2.2 *Leptospira* 16S rDNA PCR

Nested-PCR was performed to amplify the *Leptospira* spp. 16S r DNA. The first round PCR was developed to bind to DNA sequences belonging to both pathogenic and non-pathogenic leptospires, and the second round PCR (binding within the product from the first) is specific for pathogenic leptospires only. This makes the PCR more sensitive and specific, as well as producing results from only small amounts of DNA that a single round PCR may not be able to amplify. The first round PCR was optimized using a Taguchi square optimization and the second PCR round PCR was performed according to previous Taguchi optimization as performed by Dr J. Rossouw. The results for the Taguchi optimization can be seen in Figure 3.8 it was decided to use the mixture from Tube 1 as all the tubes had good products and Tube 1 had the least of all reagents in. The reactions in the individual tubes (Tube 1-6) were set up according to Table 2.3 with the concentration of DNA staying the same at 5 ng.

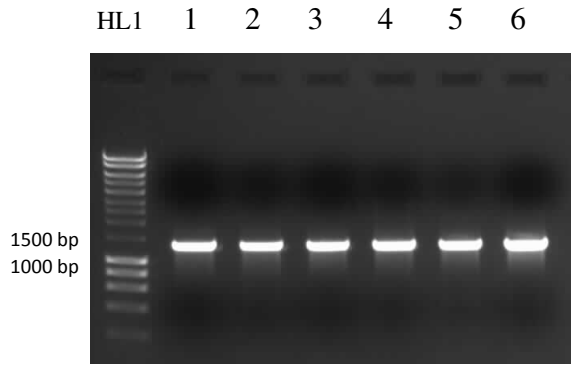

Figure 3.8 Agarose gel analysis of the products of the first round *Leptospira* 16S rDNA Taguchi optimization. Lanes (L to R): HyperLadder^{TM} I (Bioline), Tube 1, Tube 2, Tube 3, Tube 4, Tube 5, Tube 6. All tubes were mixed according to Table 2.3.

Of all the 79 soil and 77 water samples which were extracted for DNA and run on the first round PCR 21 of the samples yielded an amplicon of the expected size (ca. 1327 bp). Leptospires were found mostly in Rhodes Park and Bruma and most of them in soil at 23.8% (5/21); there was a large percentage of *Leptospira* spp. found in the water at Bruma Lake at 14.3% (3/21) (Figure 3.9). The only other area where leptospires were isolated by PCR in water was Modderfontein Dam at 4.8% (1/21). The next highest soil percentage positives were found at Dobsonville, Gilloolys and Modderfontein Dam at 9.5% (2/21) each. The lowest percentage positive for soil was Alexandra Extension seven with 4.8% (1/21). When a Fishers two-sided test was performed comparing pooled soil and water data there was a significant difference between the two at P = 0.0018 (chi-square value of 7.5 and one degree of freedom).

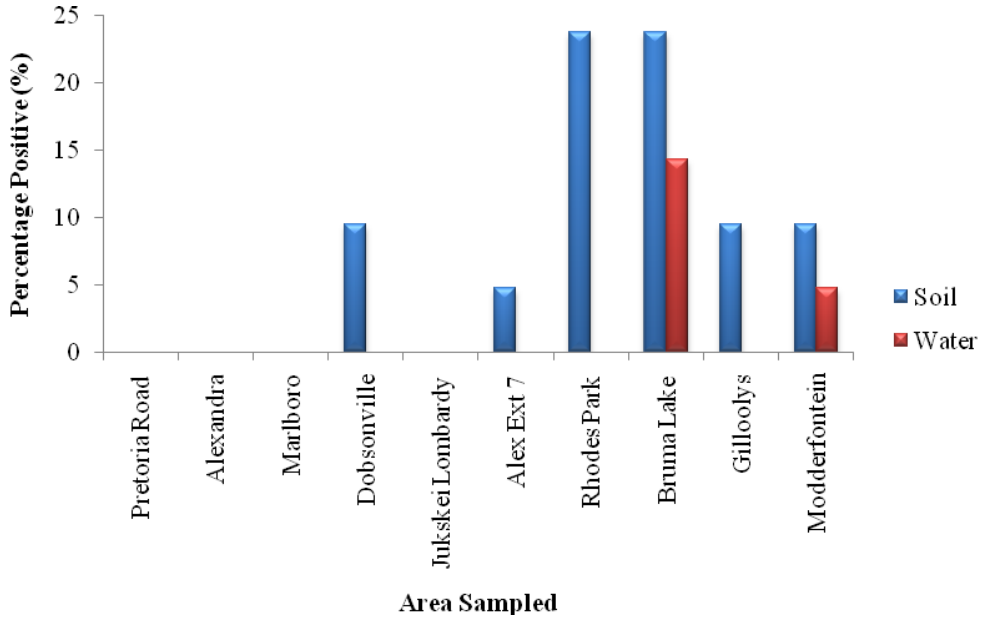

Figure 3.9 Percentage of total leptospires found in soil and water by site.

An example of a positive gel analysis for the first round PCR is seen in Figure 3.10.

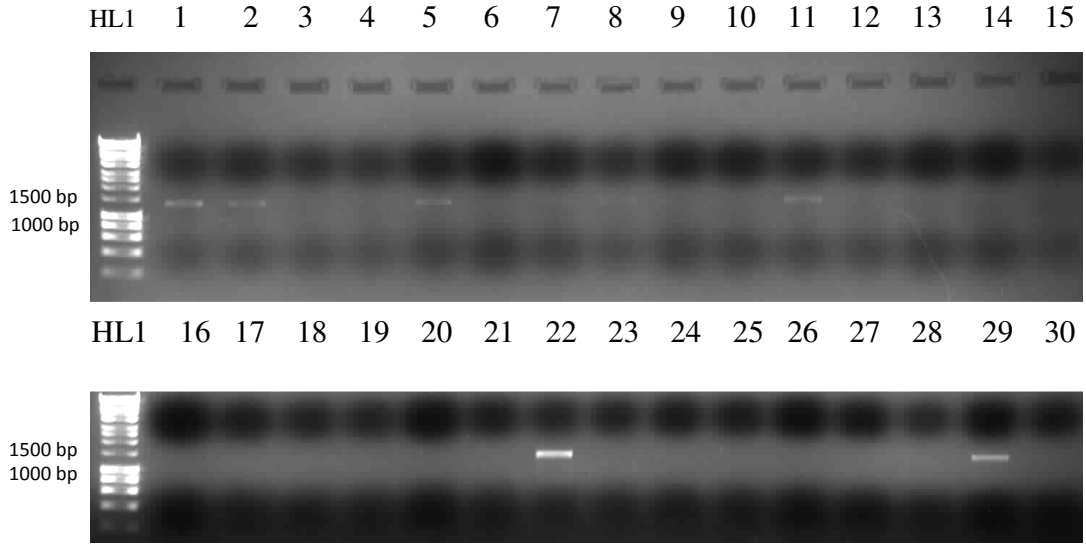

Figure 3.10 Agarose gel analysis showing the first round *Leptospira* 16S results. Lanes (L to R): HyperLadder™ I (Bioline), S36, S37, S38, S39, S40, S41, S42, S43, S44, S45, S46, S47, S48, S49, S50, HyperLadder™ I (Bioline), S51, S52, S53, S54, S55, S56, S57, S58, S59, S60, S61, S62, S63, *Leptospira* positive control, non-template control. Refer to Appendix 7 for sample information.

All the first round PCR products were then put through the second round PCR; an example of a positive second round PCR is seen in Figure 3.12. A total of nine samples yielded an amplicon of the expected size (ca. 332 bp). Only two samples had both first and second round amplicons of the expected sizes.

There were no pathogenic leptospires found in any water samples (Figure 3.11). The percentage of pathogenic leptospires found in Rhodes Park soil was the highest at 33.3% (3/9), with the next highest percentages being from Bruma and Modderfontein at 22.2% (2/9) each. The lowest percentage positives were from Gilloolys and Dobsonville at 11.1% (1/9). The number of pathogenic leptospires found in soil when compared with water was highly significant (p = 0.0002, one-sided Fishers exact test, chi-square value of 13.9, one degree of freedom).

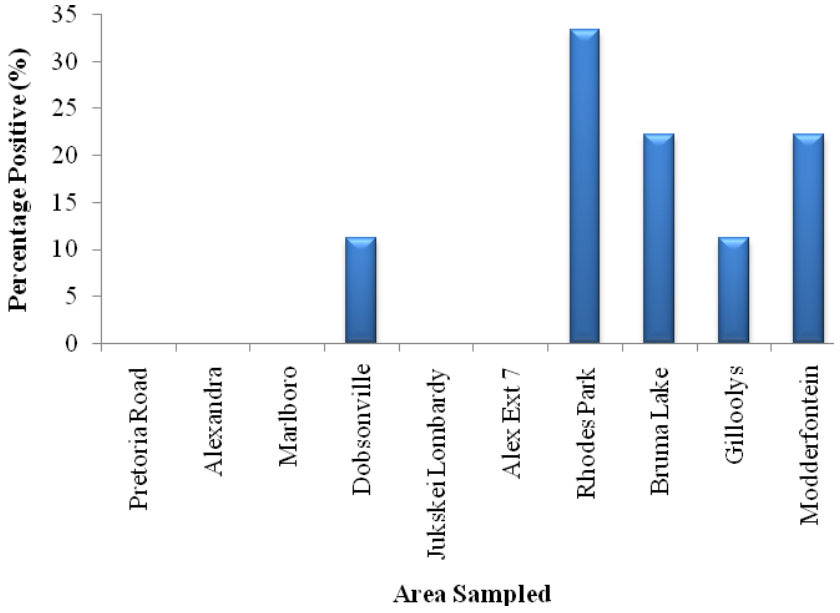

Figure 3.11 Percentage of pathogenic leptospires isolated in soil samples.

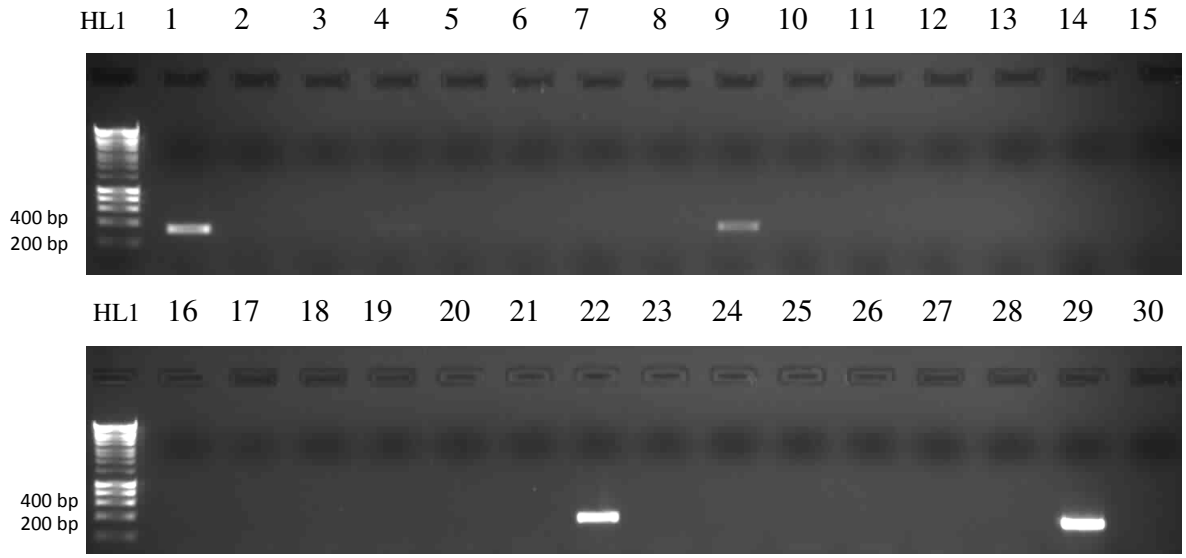

Figure 3.12 Agarose gel analysis showing the results from a second round *Leptospira* 16S rDNA. Lanes (L to R): HyperLadder™ I (Bioline), S36, S37, S38, S39, S40, S41, S42, S43, S44, S45, S46, S47, S48, S49, S50, HyperLadder™ I (Bioline), S51, S52, S53, S54, S55, S56, S57, S58, S59, S60, S61, S62, S63, *Leptospira* positive control, non-template control. Refer to Appendix 7 for sample information.

When comparing the PCR results for total (including pathogenic) leptospires found in the areas sampled (Figure 3.13), the highest percentage of *Leptospira* spp. were found in Rhodes Park and Bruma Lake 28.6% (8/28) and 25% (7/28) respectively, and then 14.3% (4/28) for Modderfontein and 10.7% (3/28) for Gilloolys and Dobsonville each. The highest values of total leptospires were found at Rhodes Park and Burma Lake (17.9%; 5/28 each) with Rhodes park having the highest positive percentage for pathogenic leptospires at 10.7% (3/28). Bruma Lake had a positive percentage of pathogenic leptospires of 7.1% (2/28), with Modderfontein having an number of pathogenic and total leptospires at 7.1% (2/28). Dobsonville had low pathogenic percentage of 3.6% (2/28) and a higher total percentage of 7.14% (7/28). The lowest percentage of all areas sampled was Alexandra Extension seven and Gilloolys with only pathogenic leptospires at 3.6% (1/28). A Fishers two-sided test performed on this data comparing pathogenic and non-pathogenic leptospires revealed that $p = 0.02$ (chi-squared value of 8.44 and one degree of freedom) which is significant but not as significant as the previous data sets.

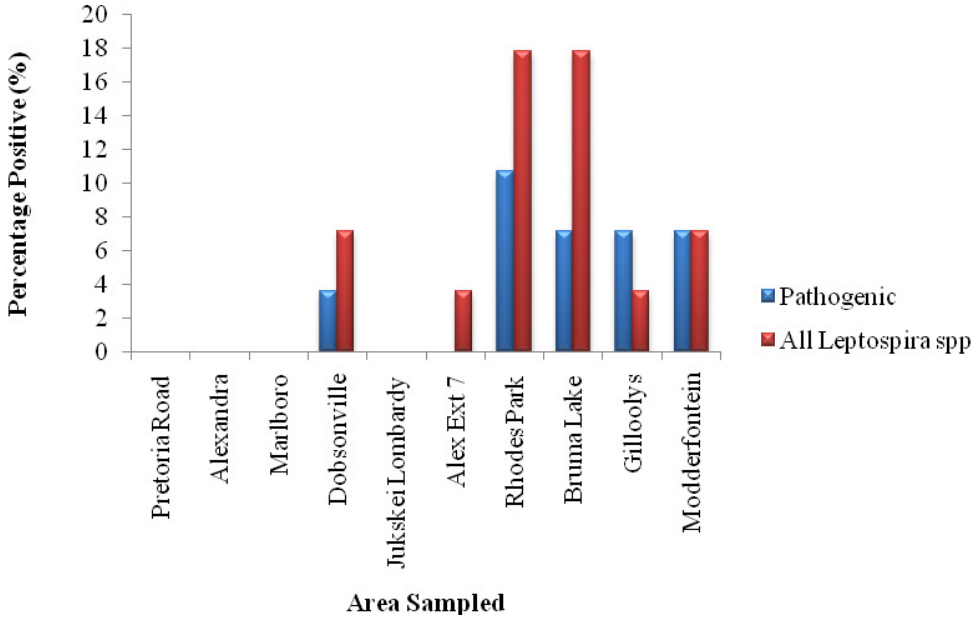

Figure 3.13 Percentage of pathogenic of the total amount of leptospires found in soil samples.

3.2 HUMAN AND ANIMAL SAMPLES

3.2.1 Animal

Of the 193 serum and 193 kidney samples that were selected for processing from 193 rodents, only 141 serum and 107 kidney samples had DNA extracted; this was due to there being insufficient amount of sample before processing. There were therefore a total of 248 samples extracted for DNA. All of these samples were subjected to *RecA* PCR and first and second round *Leptospira* 16S rDNA. Of all 248 samples, none of them was positive for *Burkholderia* spp.; therefore an *lpxO* PCR was not performed. Of all of the 248 samples, none came up positive in either first or second round PCR for non-pathogenic or pathogenic leptospires.

3.2.2 Human

There were 247 samples extracted for DNA of the 332 samples selected for processing; this was also due to there being insufficient amount of serum in some of the samples. Of the 247 samples tested with the *Leptospira* 16S rDNA n-PCR, none of the samples yielded amplicons of the expected sizes for either the first or second round (1327 bp and 332 bp, respectively). This means that they were negative for pathogenic leptospires.

3.3 *LEPTOSPIRA* IgM ELISA

Three hundred and thirty two (332) samples were processed in the Special Bacterial Pathogens Reference Unit in the time period chosen (January 2010 to June 2011). Of these samples there were 26 samples that were positive for *Leptospira* IgM antibodies. In 2010, there were 13 positives from 224 samples which is 5.8%; in 2011 there were 13 positive samples from 94 patient samples which is approximately 12% (Figure 3.14). The negative results were therefore 94.2% (211/224) for 2010 and 88% (13/95) for 2011. A Fishers two-sided performed revealed a p value of 0.13 (chi-square value of 3.9 and one degree of freedom).

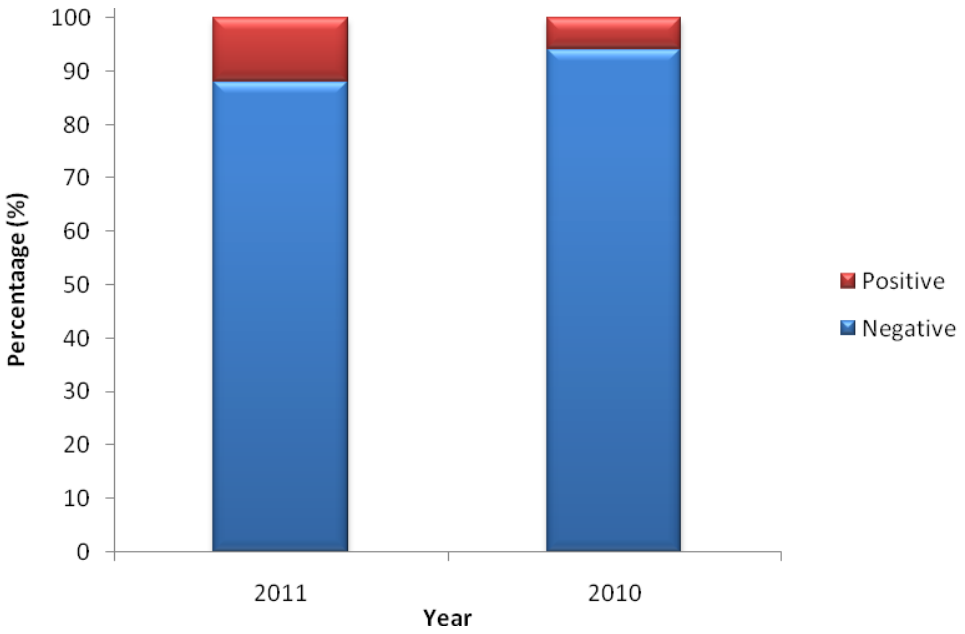

Figure 3.14 Shows the percentage positive and negative samples for the *Leptospira* IgM ELISA for 2010 and 2011.

CHAPTER 4

DISCUSSION AND CONCLUSION

In this study the prevalence of *Burkholderia* spp. in the environment as well as in human and animal hosts was investigated. This was done by performing environmental sampling for culture and molecular testing, and by using retrospective human and rat samples for molecular testing. When the environmental samples were processed using a selective culture technique there were several other environmental bacteria frequently isolated, aside from *B. cepacia*, that are also potentially pathogenic to humans, these were: *C. acidivorans*, *P. fluorescens*, *C. testosteroni* and *A. faecalis*.

C. acidovorans is a ubiquitous organism in soil and water that has been isolated from inhalation equipment and has been the cause of nosocomial bacteraemia associated with the use of an indwelling pressure-monitoring device, and corneal ulcerations (Horowitz *et al*., 1990). It has also been described as causing endocarditis, keratitis in immunocompromised patients, bacteraemia in a patient with AIDS, and bacteraemia in an elderly patient that was associated with tropical fish. (Horowitz *et al*., 1990; Lair *et al.*, 1996; Lee *et al*., 2008; Smith and Gradon, 2003). However, despite all these cases of documented infection it is still considered an unusual human pathogen that is rarely recovered from clinical specimens (Lee *et al*., 2008; Smith and Gradon, 2003).

P. fluorescens is a member of the fluorescent pseudomonad group, which unlike their better known counterpart *P. aeruginosa* are generally considered to be of low virulence and therefore of little clinical significance (Hsueh *et al*., 1998). Despite this *P. fluorescens* has been isolated in cases of granulomatous pneumonitis, urinary tract and wound infections, donated blood, from bacteraemia in oncology patients and in bacteraemia caused by a contaminated saline flush (Gershman *et al*., 2008; Gibb *et al*., 1995; Hsueh *et al*., 1998; Redding and McWalter, 1980; Von Graevenitz and Weinstein, 1971). There is some evidence that when *P. fluorescens* does cause infection in humans, treatment with ostensibly appropriate antibiotics may be ineffective; also, nosocomial infection by this bacterium needs to be closely monitored (Redding and McWalter, 1980).

C. testosteroni is like much of the other organisms isolated: a Gram-negative, non-glucose-fermenting, aerobic, motile, non-spore forming bacillus (Abraham and Simon, 2007; Reddy *et al*., 2009). It is also ubiquitously found in soil, water and on plants, and it has even been isolated from the water of hospital oxygen humidifier reservoirs (Abraham and Simon, 2007; Tsui *et al*., 2011). It is not a well-recognised human pathogen, although several cases of infection have been documented; it has caused bacteraemia in a number of patients including several with cancer and one AIDS, as well as a catheter-related bacteraemia from a haemodialysis patient (Abraham and Simon, 2007; Lair *et al*., 1996; Nsair *et al*., 2011). It has been implicated in post-operative opthalmitis in an elderly patient with diabetes, in endocarditis, and in a case of meningitis in a homeless man (Cooper *et al*., 2005; Jin *et al*., 2008; Reddy *et al*., 2009). The full pathogenic potential of this organism has not yet been fully recognised but it is considered to be an opportunistic organism in most infection cases (Abraham and Simon, 2007).

There was not much information about the pathogenicity of *A. faecalis* as it is usually considered a harmless saprophyte normally found in the intestinal tract of humans (Sherman *et al*., 1960). Human infection has been noted on a few occasions with a variable clinical picture, depending on which organ is infected (Sherman *et al*., 1960). Some of these clinical cases have been meningitis and bacteraemia in newborn infants, children with diarrhoea, bilateral conjunctivitis in a two-week-old infant and in one case the organism was isolated from an adult patient with bacteraemia but no noticeable foci of infection (Sherman *et al*., 1960; Wilson *et al*., 1975). In this case the authors concluded that the organism must have been a contaminant, notwithstanding the use of sterile techniques to culture it from the patient's blood as it did not re-occur in following samples and its origin remained unidentified (Wilson *et al*., 1975). Recent papers and case studies on *A. faecalis* are few and far between; perhaps it is seldom important when it is isolated (as in the case of Wilson *et al*., 1975), or it is more difficult to isolate than previously thought.

Burkholderia spp. are also renowned for being opportunistic pathogens for humans and have been known to cause life-threatening pulmonary infections in patients who are on ventilators and in individuals with chronic granulomatous disease and cystic fibrosis (Stoyanova *et al.*, 2007). Endocarditis was the first report of *B. cepacia* as a human pathogen in the 1950s, and since then it has caused numerous catheter-associated urinary tract infections, bacteraemias and wound infections. In 1970, it caused 'foot rot' in United States troops on swamp training exercises in Florida and in troops serving in Vietnam. The number of nosocomial infections increased from the 1980s due to disinfectants and intravenous solutions being contaminated (Stoyanova *et al.*, 2007).

These organisms seem to predominantly affect patients with compromised immune systems; in the case of *C. acidovorans* it was isolated from patients that were elderly and from a patient with AIDS. *P. fluorescens* was isolated from patients with cancer, and *C. testosteroni* was also isolated from respective patients with AIDS, and a kidney failure undergoing dialysis. *A. faecalis* was found in newborns and babies whose immune systems are still developing. Bcc infections in patients without compromised immune systems occur only sporadically (Gautam *et al*., 2011).

South Africa ranks among the top five countries with the highest HIV prevalence in the world, with approximately 17.5% of the population infected (Barton-Knott, 2009). It has the highest number of people infected globally estimated at around 5.3 million as of 2007 (Barton-Knott, 2009). If environmental bacteria are opportunistic pathogens and selectively infect those members of the population with immune systems that are compromised, it seems fair to conclude that perhaps there are more human infections than previously thought. If *Burkholderia* spp. produce a broad range of symptoms (as with their environmental counterparts) then perhaps they have been overlooked in the diagnosis of disease. If Bcc infects patients with compromised airways (as with patients who have cystic fibrosis) then they may pose a risk to patients already infected with tuberculosis, and there have been cases in India where *B. pseudomallei* has mimicked, and thus been misdiagnosed as, tuberculosis (Vidyalakshmi *et al*., 2008). However, the relative growth and competition rates between Bcc bacteria and *Mycobacterium tuberculosis* still need to be studied.

More *Burkholderia* spp. were isolated by culture from soil samples (76.47%) than water samples (23.53%); of these, soil samples 52.9% were isolated from Rhodes Park. This could indicate there is a higher prevalence of the organism in soil from Rhodes Park than anywhere else; it could also indicate that the organism survives best in this particular soil than from any other area sampled. As *Burkholderia* is a known saprophyte this is not particularly surprising; however, the area in which it was found shows that, at least with regards to culture isolation, it prefers the conditions at Rhodes Park.

The time of year the samples were taken may also have something to do with this result, although the cold does not seem to affect the organisms found at Rhodes Park, perhaps it affected the amount of organism isolated from samples taken in other areas. It has been thought that some bacteria are viable but not culturable (VBNC); bacteria in this state are found normally in substrate-limited habitats such as soil (McDougald *et al*., 1997). The ability to distinguish between Bcc organisms in this state and in a 'normal' growing state could be helpful in determining whether or not these environmental organisms are of any potential risk to human populations in the area (Miller *et al*., 2002). This may mean, therefore, that there is more substrate that *Burkholderia* can use at Rhodes Park than at any of the other areas sampled so the organisms stay in a more culturable state. Further research needs to be performed on what exactly this substrate may be.

Another reason could be that the numbers of desired bacterium may have been low and may have been impossible to detect with culture as they were outgrown by other more numerous or fast growing organisms in the samples (Miller *et al*., 2002). The bacteria could also be non-culturable due to the selective agents in the media; however this possibility was ruled out as the control strain grew on all the media that was made.

Culture also has a long processing time and if the bacteria are not viable it can produce other contaminating bacteria, as well as waste of a large volume of material used to process the samples if there is no growth obtained at all. There is also a risk of contamination of plates; to reduce this risk working in a sterile environment, using sterile methods and extensive operator training, is important.

There were also no isolates of *B. pseudomallei* detected either by culture or PCR methods. This could signify that in the areas sampled around Johannesburg at least, there is no *B. pseudomallei* present in the environment; therefore the risk of human infection is low to nil. However, more areas need to be sampled elsewhere in South Africa before it is confirmed that there is no *B. pseudomallei* present at all in this country.

Of all the environmental samples tested with the *RecA* PCR there were a significantly higher number of positives identified from soil than water, ($p = 0.015$). The most PCR positive samples were identified from Rhodes Park and Bruma Lake, with the next highest found in the Modderfontein Dam soil samples. In the water samples taken, Bruma Lake and Modderfontein Dam yielded the highest rate of PCR positive isolates. This means, therefore, that there are more *Burkholderia* spp. organisms found in soil than in water samples. Rhodes Park, Bruma Lake and Modderfontein are the top three areas from which to *Burkholderia* spp. were isolated. As discussed previously this could be due to more VBNC organisms found in these areas, as well as the presence or absence of a specific substrate with which the organism being isolated uses in various areas. There were more target organisms isolated from soil than water, confirming that *Burkholderia* spp. are rhizospheric organisms are more likely to be found in the soil around the roots of trees and other plants. However, as it is also found in water, waterborne sources of infection should not be discounted.

When the culture and PCR positivity rates were compared, there was a significant difference between them ($p=0.009$). There are several possible explanations for this, such as the presence VBNC organisms, as well as media selectivity and other bacteria out-competing it for resources on an agar plate and outpacing the Bcc organisms in growth, as well as the amount of organism present in the soil. This could also be due to the increased sensitivity and specificity of the PCR technique as it only amplifies target DNA and not any other superfluous DNA that may be extracted from these samples. This means in the case of DNA extracted from environmental samples PCR is more specific and more likely to produce a positive result.

There have been other studies which have looked for *Burkholderia* spp. in the environment. In Italy a study done by Bevivino *et al.* (2002), isolating *Burkholderia* spp. from the rhizosphere of maize plants in northern Italy showed that among these environmental isolates there was a prevalence of *B. cepacia* genomovar III and *B. ambifaria*. They also found that there are differences in the pathogenic potentials among *Burkholderia* spp. and that determinants of virulence are not solely confined to clinical specimens but are spread out among the different species that make up the Bcc. In the United Kingdom the diversity of *Burkholderia* isolates from the rhizospheres of woodland plants was studied (Richardson *et al*., 2002). The authors concluded that although the species they isolated were related to each other, they were not related to other plant or human strains (Richardson *et al*., 2002). It has also been suggested in a further study that only complex communities of *B*. *cepacia* and closely related strains of species coexist at high population levels, and that species composition and abundance may dramatically vary between individual plants (Ramette *et al*., 2005).

There was no *Burkholderia* organism detected in human and animal populations in Johannesburg. This does not mean, however, that the organism is not prevalent in these populations. A negative result in the human and animal samples perhaps indicates that while the bacteria can move from the natural environment into a human host in other ways, unlike leptospires they may not be carried by the rat population as reservoir or paratenic hosts into humans. While *Burkholderia* spp. are very clearly present in the natural environment in Johannesburg, they are unlikely to cause many infections in the people living close to these environments. Occupational or recreational exposure to the environment probably poses a higher risk, particularly for immunocompromised people, but this study was not designed to assess these aspects.

Further studies should be done in populations that are immunocompromised and in patients hospitalised for cystic fibrosis. As it is confirmed to be found in the environment, the rate of transmission to human hosts in South Africa needs to be determined; it is perhaps present, but under-diagnosed or dismissed as a contaminant in cases of infection.

This study also sought to show the prevalence of pathogenic leptospires in the environment as well as in human and animal hosts. This was done mainly through molecular analysis of environmental, and animal samples, as well as serological and molecular analysis of human samples. From these methods it was found that there were some positive leptospire samples in the environment, mostly in soil and only detected with PCR. More *Leptospira* spp. overall were found than specifically pathogenic species, which suggests a predominance of saprophytic (non-pathogenic) species. The relative chemical compositions of the respective soil and water bodies may play an important role in this, although where one species of *Leptospira* is found it may be possible for others to survive, and pathogenic leptospires have been known to survive for long periods of time especially in slightly alkaline waters (Trueba *et al*., 2004). In laboratory culture however, saprophytic (non-pathogenic) leptospires can outgrow pathogenic ones (Ganoza *et al*., 2006). There may also have been something in the environmental samples (soil and water) that inhibited the growth of certain *Leptospira* spp.; this could have been a chemical, pH or temperature factor.

A negative result for all *Leptospira* species was found in all animal samples with the PCR; as we were looking for current infection this may not be totally unexpected. However, it was anticipated that there would be some positive results in the rat kidney samples, as pathogenic leptospires has been known to use the rat as a reservoir host, colonising the kidneys and causing seronegative infection (Bharti *et al*., 2003; Levett, 2001). A previous study in Cato Crest in Durban found that out of 221 rodents sampled, 10% (22) were positive for leptospirosis antibodies (Taylor *et al*., 2008). However a much earlier study performed in Johannesburg in 1946 found no leptospirosis in the local rat population (Buchanan, 1946). This may indicate that the rats in Johannesburg may have different populations of pathogenic leptospires and, therefore, their immune systems react differently and clear the infection rather than colonising the kidneys. More likely, however, is that there is a different ecology present in Johannesburg that is unsuitable for maintaining the pathogenic organism, or the detection technique is not as sensitive as it needs to be. A serological test (such as an ELISA) may have better indicated the level of current or previous infection in these rat populations, as the presence of pathogenic

leptospires in the environments sampled does indicate that there are potential sources of these leptospires.

The samples that were tested were submitted for routine laboratory serology testing for *Leptospira* IgM antibodies using an ELISA, which is a good method for detecting antibodies for a specific disease. Out of 332 serum samples in total, only 7.8% (26/332) were positive; in 2010 there were fewer positive samples than in 2011 at 5.8% (13/224) compared to 12% (13/95). This could be due to the samples being used only in the first six months as opposed to 2010, where it was a full year. Also it was over the summer months, when there is more outdoor activity by the population in general. It is harder to find leptospires by some laboratory detection methods (culture, microscopy etc) after 5-7 days of infection as the immune system has cleared the leptospires from the areas of infection (Faine, 1994). Ideally, blood that is taken for serological testing should be taken as soon as possible in the illness and followed by a second specimen 5-7 days later. The serum collected needs to be kept cold during transport and frozen as soon as it is received; unfortunately this is not always the case. This may also create false negative results as the antibodies that were in the serum have been too degraded to cause a reaction.

The serological test may be negative in the early stages but will show up positive in later testing (Faine, 1994). Therefore a negative result in the human samples does not mean that there is no infection in the population; it may be that the samples were taken at the wrong stage of the disease, i.e. before or after a high enough infectious load has occurred, especially in serum samples, and after the infection has been cleared away by the immune response. This is an important limitation, therefore, as follow-up samples do not always occur, and the first round ELISA may not produce a positive reaction. A follow-up sample will be positive, as by then infection has progressed far enough to produce detectable amounts of antibodies. In addition, many patients would have received antibiotics effective against leptospires. The sample size may have been too small, and also the specific samples chosen may have been incorrect; also, repeated freezing and thawing of the respective samples may have degraded any DNA or antibodies present. This means that while the diagnostic *Leptospira* ELISA is useful in establishing whether

or not the patient has a current infection, presence of IgM indicates an immune system that is reacting to an infection and that the infectious organisms may already be cleared. If a PCR was to be performed on serum it needs to be drawn in the very early stages of infection in order to detect the organism itself. A different serological test to determine levels of IgG antibody in the serum may also be useful to establish if there were any previous infections. Not all patients can form IgG, which is why paired serum to show a rise in IgM titre is more useful.

There were no positive human samples detected by PCR. The number of positive samples from the routine ELISA shows that the level of active infection in the samples tested should be at higher prevalence than indicated with retrospective PCR. Previous case reports and studies in South Africa have also shown that this should be the case and (as this study has shown) there is a reservoir of pathogenic leptospires in the environment (Buchanan, 1946; Gear *et al*., 1958; Klopper 1969; Maze and Kirsch, 1981; Samson, 1966; Taylor *et al*., 2008; Thomson, 1964).

Therefore, although there was also no *Leptospira* organism detected by PCR in any of the human and animal samples tested, this may be due to limitations within the DNA extraction and processing of these samples, as well as how the samples were stored and treated before being processed. The timing of when the samples were taken is also important; too far along the immune system reaction and the organism will be cleared from the blood in both human and rat subjects. An ELISA will better detect or confirm the infection in cases such as these, and in humans some ELISA results were positive and showed a definite presence of *Leptospira* infection in the population in South Africa (not just Johannesburg, as these samples are sent to the laboratory from all over the country).

This study attempted to find two medically important organisms in the environment. Aside from some studies performed with *Burkholderia cepacia* as plant pathogens and no real case studies in humans for Bcc infection, this is the first study of its kind in South Africa to sample the environment to look for both *Leptospira* and *Burkholderia* organisms. More studies need to be performed on how the human population is affected by *Burkholderia,* especially in immunocompromised patients and the children and adults diagnosed with cystic fibrosis or chronic granulomatous disease. A greater understanding of the species-related differences in pathogenicity of *Burkholderia* and of the risks posed by different strains found in the environment implies a more in-depth study not only of the biological mechanisms involved in the pathogenicity of *Burkholderia,* but also of the eco-physiology of each species belonging to the genus (Bevivino *et al*., 2002). Leptospirosis continues to be an important human pathogen and has caused disease in patients all over South Africa. More studies need to be performed to determine which species and serovars are infecting humans populations and how this organism will affect patients with compromised immune systems, and how often it is incorrectly or under-diagnosed.

In conclusion, the results that were obtained showed a definite presence of leptospirosis and *Burkholderia* organism in the environment. The objectives of the study were therefore met and a better understanding of where these organisms are found achieved. The human and animal incidence of *Burkholderia* is still at question as there were none isolated from any of these samples, due to various factors mentioned; a better understanding of how and when to obtain these samples needs to be discerned. In this study the samples used for leptospire isolation yielded negative results, despite evidence of leptospirosis in humans and animals, as demonstrated by previous studies and the diagnostic ELISA results. These organisms do pose a threat to the population in Gauteng in general and particularly to those of the population that are immunocompromised.

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APPENDIX 1

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL) CLEARANCE CERTIFICATE

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Mrs Adrienne Saif

CLEARANCE CERTIFICATE	**M110102**
PROJECT	The Detection of Burkholderia spp. and Pathogenic Leptospira spp. in South Africa
INVESTIGATORS	Mrs Adrienne Saif.
DEPARTMENT	Division of Virology & Communicable Diseases
DATE CONSIDERED	2802/2011
DECISION OF THE COMMITTEE*	Approved unconditioally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 22/07/2011 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Professor John Frean

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

APPENDIX 2

ANIMAL ETHICS SCREENING COMMITTEE CLEARANCE CERTIFICATE

AESC3

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2011/01/01

APPLICANT: Ms A Saif

SCHOOL: Pathology
DEPARTMENT: NICD
LOCATION: Sandringham

PROJECT TITLE: The detection of Burkholderia spp and pathogenic Leptospira spp in the RSA

Number and Species

Blood samples

Approval was given for to the use of animals for the project described above at an AESC meeting held on 20110125. This approval remains valid until 20130124.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

None

Signed: _____ (Chairperson, AESC) Date: 07/02/2011

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 08/02/2011

cc: Supervisor: Dr J Rossouw
Director: CAS

Works 2000/lain0015/AESCCert.wps

APPENDIX 3

ASHDOWN'S SELECTIVE AGAR RECIPE

Media:

Ashdown's (per 500ml)
Trypticase Soy Agar 20g
4% Glycerol: 20ml
Crystal Violet 2.5mg
Neutral Red 25mg
Gentamicin 200µl (Concentration of 1g/100µl)
Distilled water 500ml

Method

The ingredients for the Ashdown's selective agar were placed into two 1L bottles to make up to 500 ml each (leaving room for the media to expand when heated in the autoclave so it did not boil over). They were then shaken well so all the solid material dissolved and autoclaved on liquid cycle for approximately 20 minutes (min) at 121ºC per litre of media autoclaved.

After the autoclaving was completed it was then placed into a shaking water bath at 50ºC for approximately half an hour until the media reached 50ºC. It was poured into Petri dishes, only enough so that the bottom was covered, and allowed to set.
All Petri dishes were placed into plastic bags and then into a 37ºC incubator overnight, to control for contaminating organisms. After they had been in the incubator; the plastic bags were sealed properly with the BARD sterile sealer and refrigerated until they were used.

APPENDIX 4

AGAROSE GEL RECIPE

Agarose Gel:

Preparing the 2.5 % and 1.2% agarose gel

Measure 0.875g and 0.42g respectively of Agarose powder and added it to a 500ml flask

Add 35ml of 1xTAE buffer to the flask

Melt the Agarose in a microwave until the solution becomes clear.

Let the solution cool to about 50-55°C, swirling the flask occasionally to cool evenly.

2µl of Ethidium Bromide are added to the solution.

The ends of the casting tray are sealed with rubber ends and the melted Agarose solution is poured in and the combs added.

This is cooled until solid and it appears slightly milky white.

The combs are gently removed as are the rubber ends and the gel is placed in the electrophoresis chamber with the 1xTAE buffer. More buffer is added if necessary until it is approximately 2-3mm above surface of gel.

APPENDIX 5

TAE BUFFER RECIPE

50x TAE (Tris-acetic-EDTA) buffer

Ingredients

242 g Tris base (40mM)
57.1 ml Glacial acetic acid (20mM)
100 ml 0.5M EDTA (pH~8) (1mM)
186.1 g $Na_2EDTAx2H_20$ to 100 ml sterile water and adjust pH to 8 using10 M NaOH to 1000 ml of sterile water

Method

The 186.1 g of $Na_2EDTAx2H_20$ was dissolved in 700 ml of sterile distilled water to make up 0.5 M of EDTA. The pH was adjusted to ~8.0 by adding 10 M of NaOH until the solution reached the target pH. Sterile water was added to make up a final volume of 1000 ml.

Two hundred and fourty two grams (242 g) of Tris base was dissolved in 700 ml water.

Glacial acetic acid, 57.1 ml was added to the Tris solution and followed with 100 ml of 0.5 M EDTA (ph~8.0) solution.

The pH was checked and adjusted to 7.9 using glacial acetic acid.

Sterile water was added to make up a final volume of 1000 ml of 50x TAE buffer.

APPENDIX 6

THE RESULTS OF THE API TESTS FOR THE TOP FIVE PATHOGENIC ORGANISMS ISOLATED

Date Collected	Organism Isolated	ID %	Comment	Area Isolated	Soil/ Water
06/05/2010	*Comamonas acidovorans*	99.8	Very good Identification	Pretoria Road	Soil
	Comamonas acidovorans	99.9	Excellent Identification	Alexandra	Soil
	Burkholderia cepacia	99.2	Very good Identification	Pretoria Road	Water
	Pseudomonas flourescens	99.7	Very good Identification	Pretoria Road	Soil
	Pseudomonas flourescens	99.9	Very good Identification	Pretoria Road	Water
06/08/2010	*Comamonas testosteroni*	86.5	Acceptable Identification	Pretoria Road	Water
	Comamonas testosteroni	34.1	Low Discrimination	Pretoria Road	Water
	Comamonas testosteroni	72.7	Low Discrimination	Alexandra	Soil
	Comamonas testosteroni	42.8	Low Discrimination	Marlboro Road	Water
	Pseudomonas flourescens	99.9	Very good Identification	Pretoria Road	Water
	Pseudomonas flourescens	99.9	Very good Identification	Pretoria Road	Water
	Pseudomonas flourescens	99.9	Excellent Identification	Alexandra	Water
	Pseudomonas flourescens	52.7	Good Identification to the Genus	Marlboro Road	Water
	Pseudomonas flourescens	86.5	Acceptable Identification	Marlboro Road	Water
	Pseudomonas flourescens	88.0	Low Discrimination	Marlboro Road	Water
	Pseudomonas flourescens	78.5	Good Identification to the Genus	Marlboro Road	Water
	Alcaligenes faecalis	59.3	Low Discrimination	Pretoria Road	**Soil**
	Alcaligenes faecalis	48.0	Low Discrimination	Marlboro Road	Water

	Comamonas acidovorans	99.4	Very good Identification	Marlboro Road	Water
06/10/2010	*Comamonas testosteroni*	86.6	Acceptable Identification	Pretoria Road	Soil
	Comamonas testosteroni	98.2	Good Identification	Pretoria Road	Water
	Comamonas testosteroni	65.7	Low Discrimination	Marlboro Road	Soil
	Comamonas testosteroni	45.4	Low Discrimination	Marlboro Road	Soil
	Comamonas testosteroni	34.1	Low Discrimination	Marlboro Road	Water
	Comamonas testosteroni	42.8	Low Discrimination	Marlboro Road	Water
	Comamonas testosteroni	76.1	Low Discrimination	Marlboro Road	Water
	Comamonas testosteroni	90.1	Good Identification	Marlboro Road	Water
	Burkholderia cepacia	94.0	Low Discrimination	Marlboro Road	Soil
	Alcaligenes faecalis	48.0	Low Discrimination	Marlboro Road	Soil
	Alcaligenes faecalis	48.0	Low Discrimination	Marlboro Road	Water
	Alcaligenes faecalis	48.0	Low Discrimination	Marlboro Road	Soil
	Alcaligenes faecalis	36.8	Low Discrimination	Marlboro Road	Water
	Alcaligenes faecalis	36.8	Low Discrimination	Marlboro Road	Water
	Pseudomonas flourescens	97.2	Good Identification	Pretoria Road	Water
	Pseudomonas flourescens	97.2	Good Identification	Alexandra	Water
	Pseudomonas flourescens	97.2	Good Identification	Alexandra	Water
	Pseudomonas flourescens	82.4	Low Discrimination	Marlboro Road	Soil
	Pseudomonas flourescens	80.0	Low Discrimination	Marlboro Road	Water
	Pseudomonas flourescens	80.0	Low Discrimination	Marlboro Road	Water
	Pseudomonas flourescens	84.8	Low Discrimination	Marlboro Road	Water

	Pseudomonas flourescens	84.8	Low Discrimination	Marlboro Road	Water
	Pseudomonas flourescens	86.5	Low Discrimination	Marlboro Road	Soil
	Pseudomonas flourescens	99.9	Excellent Identification	Marlboro Road	Soil
	Pseudomonas flourescens	99.9	Excellent Identification	Marlboro Road	Soil
	Pseudomonas flourescens	99.9	Excellent Identification	Marlboro Road	Soil
	Pseudomonas flourescens	99.1	Very Good Identification	Marlboro Road	Soil
	Pseudomonas flourescens	90.3	Low Discrimination	Marlboro Road	Water
13/03/2011	*Comamonas testosteroni*	91.7	Good Identification	Dobsonville	Soil
	Comamonas testosteroni	85.7	Low Discrimination	Dobsonville	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Jukskei	Water
	Comamonas acidovorans	99.9	Excellent Identification	Jukskei	Water
	Alcaligenes faecalis	59.3	Low Discrimination	Dobsonville	Soil
	Alcaligenes faecalis	49.4	Low Discrimination	Jukskei	Soil
	Alcaligenes faecalis	49.4	Low Discrimination	Jukskei	Water
	Alcaligenes faecalis	49.4	Low Discrimination	Jukskei	Water
	Burkholderia cepacia	99.9	Very Good Identification	Dobsonville	Soil
	Burkholderia cepacia	99.5	Good Identification	Alex Ext 7	Soil
29/05/2011	*Comamonas acidovorans*	99.4	Very Good Identification	Rhodes Park	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Rhodes Park	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Rhodes Park	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Rhodes Park	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Rhodes Park	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Rhodes Park	Water

	Comamonas acidovorans	99.4	Very Good Identification	Rhodes Park	Water
	Comamonas acidovorans	99.4	Very Good Identification	Rhodes Park	Water
	Comamonas acidovorans	99.4	Very Good Identification	Rhodes Park	Water
	Comamonas acidovorans	99.4	Very Good Identification	Rhodes Park	Water
	Comamonas acidovorans	99.4	Very Good Identification	Rhodes Park	Water
	Comamonas acidovorans	99.4	Very Good Identification	Rhodes Park	Water
	Comamonas acidovorans	99.4	Very Good Identification	Rhodes Park	Water
	Comamonas acidovorans	99.4	Very Good Identification	Bruma	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Bruma	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Bruma	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Bruma	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Bruma	Soil
	Comamonas acidovorans	89.8	Low Discrimination	Bruma	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Bruma	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Bruma	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Bruma	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Bruma	Soil
	Comamonas acidovorans	93.9	Good Identification	Bruma	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Bruma	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Bruma	Soil
	Pseudomonas flourescens	82.3	Excellent Identification to the Genus	Bruma	Soil
	Alcaligenes faecalis	47.0	Low Discrimination	Rhodes Park	Soil

	Alcaligenes faecalis	71.4	Identification Not Valid	Rhodes Park	Water
	Alcaligenes faecalis	49.4	Low Discrimination	Rhodes Park	Water
	Alcaligenes faecalis	47.0	Low Discrimination	Bruma	Soil
	Alcaligenes faecalis	47.0	Low Discrimination	Bruma	Soil
	Comamonas testosteroni	88.0	Acceptable Identification	Rhodes Park	Water
	Burkholderia cepacia	98.8	Good Identification	Rhodes Park	Soil
	Burkholderia cepacia	98.3	Good Identification	Rhodes Park	Soil
	Burkholderia cepacia	98.3	Good Identification	Rhodes Park	Soil
	Burkholderia cepacia	99.5	Very Good Identification	Rhodes Park	Soil
	Burkholderia cepacia	99.5	Very Good Identification	Rhodes Park	Soil
	Burkholderia cepacia	99.5	Very Good Identification	Rhodes Park	Soil
	Burkholderia cepacia	99.5	Very Good Identification	Rhodes Park	Soil
	Burkholderia cepacia	53.2	Low Discrimination	Rhodes Park	Soil
10/07/2011	*Alcaligenes faecalis*	82.2	Low Discrimination	Rhodes Park	Soil
	Alcaligenes faecalis	99.4	Very Good Identification	Bruma	Soil
	Alcaligenes faecalis	71.4	Identification Not Valid	Bruma	Water
	Pseudomonas flourescens	99.9	Excellent Identification	Bruma	Water
	Pseudomonas flourescens	99.9	Excellent Identification	Rhodes Park	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Rhodes Park	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Rhodes Park	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Bruma	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Bruma	Soil
	Comamonas acidovorans	99.4	Very Good	Bruma	Soil

			Identification		
	Burkholderia cepacia	99.8	Good Identification	Rhodes Park	Soil
	Burkholderia cepacia	98.3	Good Identification	Rhodes Park	Water
	Burkholderia cepacia	98.3	Good Identification	Rhodes Park	Water
28/08/2011	*Alcaligenes faecalis*	47.0	Low Discrimination	Gilloolys	Soil
	Alcaligenes faecalis	47.0	Low Discrimination	Gilloolys	Soil
	Alcaligenes faecalis	47.0	Low Discrimination	Gilloolys	Soil
	Alcaligenes faecalis	34.2	Identification Not Valid	Modderfontein	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Modderfontein	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Modderfontein	Soil
	Comamonas acidovorans	99.4	Very Good Identification	Modderfontein	Water
	Burkholderia cepacia	99.7	Very Good Identification	Modderfontein	Soil
	Burkholderia cepacia	98.2	Good Identification	Modderfontein	Water

APPENDIX 7

SAMPLE INFORMATION

Figure No	Sample Name in Figure	Full Sample Information
Figure 3.3	C4	Culture 4 Marlboro 1:100 3.2
	C5	Culture 5 Marlboro 1:100 3.1
	C6	Culture 6 Marlboro 1:1000 3.2
	C7	Culture 7 Marlboro 1:1000 3.1
	C8	Culture 8 Marlboro 1:100 2
	C9	Culture 9 Marlboro 1:10 2
	C10	Culture 10 Marlboro 1:1000 2.1
	C11	Culture 11 Marlboro 1:1000 2.2
	C12	Culture 12 Marlboro 1:1000 2.3
	C13	Culture 13 Marlboro 1:10 3
	C14	Culture 14 Marlboro 1:10 1.2
	C15	Culture 15 Marlboro N 1.1
Figure 3.6	S72	Modderfontein 1
	S73	Modderfontein 2
	S74	Modderfontein 3
	S75	Modderfontein 4
	S76	Modderfontein 5
	S77	Modderfontein 6
	S78	Modderfontein 7
	S79	Modderfontein 8
Figure 3.10	S36	Rhodes Park 5 29/05/2011
	S37	Rhodes Park 6 29/05/2011
	S38	Rhodes Park 7 29/05/2011
	S39	Rhodes Park 8 29/05/2011
	S40	Bruma 1 29/05/2011
	S41	Bruma 2 29/05/2011
	S42	Bruma 3 29/05/2011
	S43	Bruma 4 29/05/2011
	S44	Bruma 5 29/05/2011
	S45	Bruma 6 29/05/2011
	S46	Bruma 7 29/05/2011
	S47	Bruma 8 29/05/2011
	S48	Rhodes Park 1 10/07/2011
	S49	Rhodes Park 2 10/07/2011
	S50	Rhodes Park 3 10/07/2011
	S51	Rhodes Park 4 10/07/2011
	S52	Rhodes Park 5 10/07/2011
	S53	Rhodes Park 6 10/07/2011
	S54	Rhodes Park 7 10/07/2011

	S55	Rhodes Park 8 10/07/2011
	S56	Bruma 1 10/07/2011
	S57	Bruma 2 10/07/2011
	S58	Bruma 3 10/07/2011
	S59	Bruma 4 10/07/2011
	S60	Bruma 5 10/07/2011
	S61	Bruma 6 10/07/2011
	S62	Bruma 7 10/07/2011
	S63	Bruma 8 10/07/2011
Figure 3.12	S36	Rhodes Park 5 29/05/2011
	S37	Rhodes Park 6 29/05/2011
	S38	Rhodes Park 7 29/05/2011
	S39	Rhodes Park 8 29/05/2011
	S40	Bruma 1 29/05/2011
	S41	Bruma 2 29/05/2011
	S42	Bruma 3 29/05/2011
	S43	Bruma 4 29/05/2011
	S44	Bruma 5 29/05/2011
	S45	Bruma 6 29/05/2011
	S46	Bruma 7 29/05/2011
	S47	Bruma 8 29/05/2011
	S48	Rhodes Park 1 10/07/2011
	S49	Rhodes Park 2 10/07/2011
	S50	Rhodes Park 3 10/07/2011
	S51	Rhodes Park 4 10/07/2011
	S52	Rhodes Park 5 10/07/2011
	S53	Rhodes Park 6 10/07/2011
	S54	Rhodes Park 7 10/07/2011
	S55	Rhodes Park 8 10/07/2011
	S56	Bruma 1 10/07/2011
	S57	Bruma 2 10/07/2011
	S58	Bruma 3 10/07/2011
	S59	Bruma 4 10/07/2011
	S60	Bruma 5 10/07/2011
	S61	Bruma 6 10/07/2011
	S62	Bruma 7 10/07/2011
	S63	Bruma 8 10/07/2011