The Relevance of Bacteriophage Therapy in the Era of Antibiotic Resistant Bacteria

By

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Certificate of Authorship

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Sandra Patricia Porteous Morales

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Abstract

Against a backdrop of rising antibiotic resistance and a dwindling pipeline of new antibacterial drugs, this thesis set out to examine the potential of bacteriophage (phage) therapy as an alternative or complementary means of treating bacterial infection. Phage therapy is used as a frontline antibacterial therapy in the former Soviet bloc countries but remains an unexplored technology in Western science. To investigate the reasons behind this and other aspects of phage therapy, this thesis undertook the development of bacteriophage-based products against two important human bacterial pathogens, methicillin-resistant *Staphylococcus aureus* (MRSA) and multi-drug resistant *Pseudomonas aeruginosa* (MRPA).

To develop phage products in today's scientific and regulatory framework it was necessary to return to basic principles. The first stage in this process involved the assembly of reference collections of the target bacteria. Once these were available, bacteriophages were isolated from a range of environmental sources, and their spectrum of activity and physical characteristics evaluated. Bacteriophages with the appropriate reactivity profile were then tested for stability, morphology, and further analysed using molecular biological techniques. Phages with therapeutic potential were then combined into mixtures or "cocktails" and their activity evaluated against clinical isolates from different geographical regions. Lastly, a commercial phage product was used in one compassionate case study involving a hospital patient to treat a refractory *P. aeruginosa* urinary tract infection.

Fifty-two MRPA and fifty-eight MRSA phages were isolated over a period of 18 months. Selected phage therapeutic candidates were shown to be physically stable and genetically different from each other. They also showed a broad spectrum of activity against the targeted pathogens and this resulted in the production of three prototypes cocktails for each target pathogen. The MRPA cocktails achieved a reactivity of 62%-90% against clinical isolates from four geographical areas while the MRSA cocktails achieved a reactivity of 61%-96% in two geographical areas. In the clinic, a compassionate phage therapy treatment

was well tolerated, produced no adverse side-effects, and in combination with antibiotics, resulted in the complete eradication of a refractory *P. aeruginosa* urinary tract infection.

This thesis has demonstrated, for the first time in Australia, that it is possible to develop stable, fully characterised, broad-spectrum bacteriophage-based products with the potential to treat human infections caused by MRSA and MRPA. It also showed the value of phage therapy in the clinic by eradicating a chronic *P. aeruginosa* infection. Furthermore, though not presented in the main body of this thesis, two of the bacteriophage prototypes developed here (one MRSA and one MRPA) were recently shown to be effective in treating bacterial infections in two separate animal models.

Phage therapy has the potential to play a major role in addressing the serious problems caused by the ever-widening antibiotic resistance crisis. No doubt, there will be production and regulatory hurdles to overcome and an urgent requirement to train a new generation of microbiologists and clinicians skilled at developing and administering these powerful antibacterials. However, it is now obvious that the old paradigm of depending on a constant stream of novel antibiotics is no longer valid and alternative technologies such as this must be fully explored.

Posters, oral presentations and peer reviewed publications in chronological order

Poster Presentations

1. **Morales, S.P.,** *et al.* (2007) Bacteriophage Therapy: An alternative to conventional antibiotic treatment for resistant infections; Proceedings of The Australian Society for Microbiology Conference, Adelaide.
2. **Morales, S.P** *et al*. (2007) Bacteriophage Therapy in Aquaculture: Isolation of *Streptococcus iniae* phages for the treatment of farmed Barramundi fish in Australia: Proceedings of the 17th Evergreen International Phage Biology Meeting, ed. C. Loc-Carrillo, Olympia.
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6. Pabary, R., Singh, C., **Morales, S.**, *et al* (2012) Anti-Pseudomonal bacteriophage cocktail reduces inflammatory responses in the murine Lung. *Thorax* 67 (S103): A50-A51.

7. Drilling, A., **Morales, S.,** *et al* (2013) Developing a novel treatment for sinonasal *Staphylococcus aureus* biofilms: The enemy of my enemy is my friend. Proceedings of The Australian Society for Microbiology Conference, Adelaide.

Oral Presentations

1. **Morales, S.P**., *et al*. (2009) In vitro and in vivo efficacy of phage therapy cocktails for the treatment of multidrug-resistant *Pseudomonas aeruginosa*; Proceedings of The Australian Society for Microbiology Conference, ed. J. Sofronidis, Perth.
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3. Harper, D. R. and **Morales, S** (2012). Bacteriophage therapy: practicability and clinical need meet in the multidrug-resistance era. **Future** *Microbiology* 7:797-799.
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AWARDS

Becton Dickinson ASM Student Award winner in 2011 for the work done on the subject: "**Development of bacteriophage cocktails for the management of nosocomial and community- acquired MRSA**".

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1 INTRODUCTION

1.1 The Historical Impact of Bacterial Infectious Diseases

For most of recorded history mankind has been at the mercy of infectious diseases. Wave after wave of epidemic and pandemic disease (or pestilence as it was known in early times) have regularly decimated human populations and their associated livestock, causing massive social, religious, and economic dislocation that can be traced back to the 6th-8th century (Soltysiak, 2006). The history of infectious diseases has been well covered in the literature (Nelson and Masters-Williams, 2007) but the sheer scale of these epidemics still beggars belief. The most famous example is the "Black Death" of the middle ages, the pandemic of bubonic and pneumonic plague caused by ***Yersinia pestis,*** which raged from 1346 to 1352 and killed an estimated 40% of the population of Europe, and 75 million worldwide (out of a total global population of 400 million). So profound was the effect of this single bacterial pathogen that it took Europe nearly 150 years to recover. Nor was this the only culprit. Other bacterial infections to take their toll over the years have included diphtheria (***Corynebacterium diptheriae***), typhoid (**Salmonella Typhi**), childbirth fever (Staphylococcal and Streptococcal infections), tuberculosis (***Mycobacterium tuberculosis***) and cholera (***Vibrio cholerae***). Tuberculosis (TB) was so prevalent in the 19th Century that it killed one quarter of the adult population in Europe.

In reading the historical accounts of these pandemic diseases one is struck by how helpless the human race was in the face of infection, and how, over thousands of years, in the struggle between humans and bacterial pathogens the balance remained clearly in favour of the microorganisms. Then little more than a century and a half ago this balance began to shift the other way. The development of the germ theory of disease by Pasteur in 1860-64 (Pasteur et al., 1878) antiseptic surgery by Lister in 1867 (Lister, 1867) and the introduction of widespread vaccination from 1879 (Plotkin and Plotkin, 2011) onwards had a profound effect on the incidence of infectious diseases. In the late 1930s, the discovery of antibiotics

would further help the fight against bacterial infectious diseases and the development of modern medicine.

1.2 The Discovery of Antibiotics: their clinical impact and the problem of resistance

In 1928, British bacteriologist, Alexander Fleming, discovered an antibacterial substance after noticing that a mould, which had accidentally fallen into a bacterial culture in his laboratory, had, apparently, killed the bacteria (Fleming, 1929). Fleming named the substance penicillin and, after giving it to laboratory mice, he observed that it killed bacteria without harming healthy mammalian cells. Unfortunately, although it was an important discovery, he made little further progress. It was left to two other scientists, Howard Florey and Ernst Chain (Chain et al., 1940) to develop a form of penicillin that could be used to fight bacterial infections in humans. By 1945, spurred on by the demands of World War II, the production of penicillin "the miracle drug", highly effective against a wide range of pathogenic bacteria, had become a straightforward process (Neushul, 1993). Through the years, the antibiotic arsenal continued to expand and, by the 1950s, aminoglycosides such as streptomycin (Waksman and Woodruff, 1940), tetracyclines (Duggar, 1948), cephalosporins (Burton and Abraham, 1951), macrolides such as erythromycin (Haight and Finland, 1952) and polypeptides such as bacitracin (Newton et al., 1951) had been discovered. Close to the end of the 20th century more than 100 different antibiotics had been commercialised (Chu et al., 1996). Their mechanisms of action most commonly target bacterial protein and cell wall synthesis as well as metabolic pathways (Levy, 1998). A summary of the main classes of antibiotics and their mechanisms of action is presented in Table 1-1

Table 1-1. Main classes of antibiotics and their mechanism of action

Antibiotic Class	Mechanism of action (Reference)	Example
Aminoglycosides	Bind to the bacterial 30S ribosomal subunit (Forge and Schacht, 2000)	Amikacin, gentamicin
Carbapenems	Inhibit cell wall synthesis (Birnbaum et al., 1985)	Imipenem, meropenem
Cephalosporins (Also subdivided into five generations)	Prevent the development of bacterial cell wall by disrupting the synthesis of peptidoglycan (Burton and Abraham, 1951)	Cefoxitin, ceftriaxone
Glycopeptides	Inhibit peptidoglycan synthesis (Finch and Eliopoulos, 2005)	Vancomycin
Lincosamides	Inhibit protein synthesis by binding to the 50S subunit of bacterial DNA (Rezanka et al., 2007)	Clindamycin, lincomycin
Lipopeptides	Bind to the membrane and thus inhibits synthesis of protein, DNA and RNA (Raja et al., 2003)	Daptomycin
Macrolides	Inhibit protein bisynthesis by inhibiting translocation of petidyl tRNA (Shiomi et al., 2003)	Erythromycin, azithromycin
Penicillins	Disrupt the synthesis of peptidoglycan (Fleming, 1929)	Amoxicillin, methicillin
Polypeptides	Inhibit peptidoglycan production. They are very toxic (Kirby and Roberts, 1961)	Bacitracin,colistin, polymyxin B
Quinolones	Inhibit DNA gyrase synthesis and thus replication (Emmerson and Jones, 2003)	Ciprofloxacin, nalidixin acod
Sulfonamides	Inhibit folate synthesis. Without folate, bacterial cell cannot divide. (Betina, 1985)	Trimethoprim
Tetrcyclines	Inhibit the binding of aminoacyl-tRNA to the mRNA ribosome complex (Chopra and Roberts, 2001)	Tetracycline

Undoubtedly, the discovery of antibiotics has had an immeasurable impact on the development of modern medicine by greatly reducing illness and death from bacterial infectious diseases. Childhood diseases such as diphtheria vanished almost overnight, TB sanitaria closed down. The mortality of plague, for example, was reduced from thirteen million alone in India in 1903 to 232,438 cases in 1950 (Pollitzer, 1951). However, the honeymoon did not last long. Between 1940 and 2004 several hundred new infectious diseases have emerged, many of which are zoonotic and bacterial. More than half of these diseases have emerged since the 1970s, with the 1980s being the decade with most new infections (Jones et al, 2008).

For antibiotics the warning signs were there from the very outset. The first small cloud in the horizon appeared with the early identification of antibiotic-resistant bacteria by Fleming himself, who reported that bacteria could develop resistance against his “miracle drug” (Fleming, 1946, Demerec, 1949). Regrettably, and perhaps understandably, given the excitement caused by the results of penicillin, the warnings of resistance were ignored and penicillin was used indiscriminately (Nichols, 1965). For example, it is now known that 14% of hospital isolates of *Staphylococcus aureus* were resistant to penicillin by 1946 and by the 1970s the resistance in most clinical and community acquired isolates was of over 85% (Salgado et al., 2003).

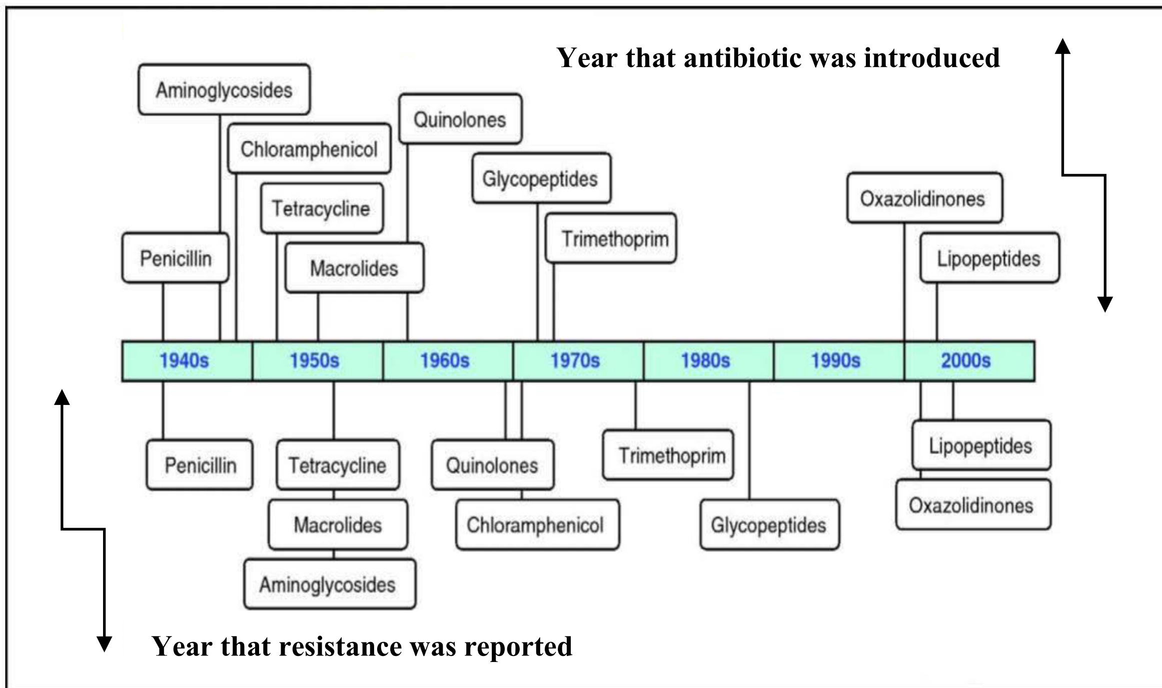
Unfortunately, the problem of antibiotic resistance was not confined to penicillin alone. Streptomycin, the first of the aminoglycoside class of antibiotics, discovered by Schatz and Waksman in 1943, was hailed as yet another wonder drug because of its success in the treatment of tuberculosis. Even so the first randomised trial of streptomycin in 1946, for treatment of pulmonary tuberculosis, described the development of resistance to the new drug (Crofton and Mitchison, 1948). As new classes of antibiotics such as the quinolones and glycopeptides began to emerge, bacterial resistance was also reported shortly after introduction. Similarly, resistance to lipopeptides such as daptomycin, the latest antibiotic class developed, has already been reported (Green et al., 2006, Mangili et al., 2005, Hayden et al., 2005, Sabol et al., 2005). A summary highlighting the dates of introduction of

antibiotics versus the date when antibiotic resistance was first described is presented in Figure 1-1.

Bacteria have existed on planet Earth for over 3.5 billion years (Müller, 1977). They have, therefore, adapted and survived changes in the environment before the existence of mankind. Thus, their ability to overcome environmental antimicrobials should have been anticipated. In fact, there is evidence that antibiotic resistance mechanisms were present in bacteria even before the indiscriminate use of present-day antibiotics (Smith, 1967) and has been determined to be as old as 2,000 years (Dancer et al., 1997) and even 10,000 years (Song et al., 2005).

Regrettably, and despite the warnings, the ready availability and effectiveness of antibiotics inevitably meant that, during the years, they have been not only overused but also misused. For example, the extensive use of cephalosporins has enhanced the proliferation of *Enterococcus faecalis*, an intestinal bacterium which has inbuilt resistance to these particular drugs. In healthy individuals, the immune system is able to safeguard the host growth against the drug resistant *E. faecalis*, preventing it from producing any illness (Edmond et al., 1996).

Figure 1-1. **Timeline of the introduction of antibiotics in the clinical practice vs. the first report of antimicrobial resistance to the particular antibiotic (sourced from Hougberg et al, 2010)**



However, in patients with compromised immunity, the bacteria can spread to the heart valves and other organs and establish fatal systemic disease (Collignon, 2000, Levy, 1998). Thus, the liberal use of antibiotics over the last six decades has exerted a major selective pressure for emergence and persistence of these acquired resistance mechanisms.

The exponential rise of antibiotic resistance in bacterial pathogens causing disease in humans has aroused global concern (Hawkey and Munday, 2004, Infectious Diseases Society of America, 2004, Kristinsson and Monnet, 2008, Levy, 2005, Talbot et al., 2006, Lepape and Monnet, 2009). In the United States the problem was first critically discussed by the American Society for Microbiology (1994). A report by Britain's House of Lords on antimicrobial resistance and a report from the US Institute of Medicine on emerging infections followed soon afterwards (Wise et al., 1998). In 1996 Hiroshi Nakajima, the Director-General of the World Health Organization (WHO) warned: "**We are standing on the brink of a global crisis in infectious diseases. No country is safe from them. No country can any longer afford to ignore their threat**" (WHO 1996). Again in 2000 the WHO alerted that the world was running the risk of stepping back into a pre-antibiotic era and bacterial infections easily treated since the advent of antibiotics were again the cause of numerous deaths (World Health Organization, 2000). In an effort to address the problem, the WHO launched a global strategy for the containment of antimicrobial resistance, including antibiotics resistance. The scheme urged interventions to minimize the emergence of resistance and decelerate its spread.

For example, the WHO estimated that as much as 50% in tonnage of all antibiotics sold in North America and Europe at the time were used unnecessarily in farmed animals. Overtime, around 40% of antibiotics developed for human use have also been used in farmed animals, not only for treatment but also as a prophylactic (preventive) measure against infection (Harrison and Lederberg, 1998). Furthermore, in many developed and developing countries antibiotics are used in animals for growth promotion (Wegener et al., 1999, Wegener, 2003). Although, it is not clear how antibiotics promote growth (Dibner and Richards, 2005), this continuing exposure has played a fundamental role in the escalating numbers of resistant bacteria in farmed animals (Wegener et al., 1999, Singer et

al., 2003). In agriculture, antibiotics are also used in high concentrations to control and prevent infections. In both interventions, the outcome was the same, the extensive use of antibiotics increased the numbers of resistant bacteria that entered the food chain and colonized the human intestinal tract causing infections (Levy, 1998). However, recently Scandinavian countries were able to demonstrate that if the use of antibiotics in farming as growth promoters was reduced, the prevalence of antibiotic resistant bacteria could also be greatly reduced (Bengtsson and Wierup, 2006).

As a consequence of these findings the regular use of antibiotic supplements in food-producing animals and poultry, for either prophylactic or growth promotion purposes, has been dramatically curtailed or prohibited. Other similar interventions were logically organized by fields, in which systems and behaviours were identified as contributors to resistance and where changes were expected to have a positive effect reverting or minimising the development of antibiotic resistant bacteria. These included consumers and prescribers, veterinarians, hospital managers, the pharmaceutical industry and governments (World Health Organization, 2001).

Some 10 years after the introduction of the program, very little evidence exists to show that these interventions have helped decelerate the crisis. The European Antimicrobial Resistance Surveillance System (EARSS) concluded, in one of its annual reports, that "the loss of effective antimicrobial therapy increasingly threatened the delivery of crucial health services in hospitals and in the community" (2009). This conclusion was reinforced by The Antimicrobial Availability Task Force (AATF) of the Infectious Diseases Society of America (IDSA) (Boucher et al., 2009) and the European Centre for Disease Prevention and Control (ECDC) in conjunction with the European Medicine Agency (EMEA) (ECDC/EMEA Joint Technical Report, 2009).

The multiple combination of inherent and acquired mechanisms that bacteria possess has helped further the emergence of three classes of antibiotic resistant bacteria, particularly Gram-negative bacteria, as defined by Souli and colleagues (Souli et al., 2008). The multidrug-resistant (MDR) bacteria or bacteria resistant to two or three classes of

antibiotics, the extensively drug resistant (XDR) bacteria or bacteria resistant to all but one or two classes of antibiotics and the pandrug-resistant (PDR) bacteria or bacteria resistant to all available classes of antibiotics. The increased number of reports of MDRs (Abbo et al., 2005, Bartoloni, 2006, Cox et al., 2004), XDR (Souli et al., 2008) and, in particular, the recent reports of extensive dissemination of PDR strains (Falagas and Karageorgopoulos, 2008, Yong et al., 2009, Tsioutis et al., 2010) in hospitals and in the community are the major concern for modern medicine.

Of special interest are the "ESKAPE" pathogens: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumanii, Pseudomonas aeruginosa* and *Enterobacter* species. The acronym was suggested in reference to these pathogens in particular because of their increased ability to "escape" antibiotics and the morbidity and mortality associated with them (Boucher et al., 2009). For example, mortality rates for deaths involving MRSA increased over 15-fold during the period 1993 to 2002 in the United Kingdom (Crowcroft et al., 2004). It was further suggested that the acronym could be expanded to include *Clostridium difficile,* and the Enterobacteriaceae family. The inclusion of *C. difficile* and the Enterobacteriaceae family in a new "ESCAPE" list of pathogens would also acknowledge the seriousness of *C. difficile* as one of the leading nosocomial infections and would include not only *K. pneumoniae* and *Enterobacter* sp. but also other important pathogens such as *Escherichia coli* that had also shown increased levels of antibiotic resistance (Peterson, 2009). Two of these pathogens, *S. aureus* and *P. aeruginosa,* are of particular interest not only because of their high levels of antibiotic resistance to antibiotics but also because of the clinical burden of infection that they represent.

1.2.1 *Staphylococcus aureus* and antibiotic resistance

Staphylococcus aureus can be considered part of human normal flora. It is estimated that about 30% of the population carries the bacterium asymptomatically (Kluytmans et al., 1997, Graham et al., 2006). Human colonization by this micro-organism has been observed to be temporary or persistent (Sanford et al., 1994). Unfortunately, *S. aureus* can also become a human pathogen and be responsible for a variety of hospital and community-

acquired infections. Its ability to cause human disease was first reported early in the 1880s (Ogston, 1881) and today, *S. aureus* continues to be one of the most problematic bacterial species around the world (Boucher et al., 2009). Pathogenic strains can cause bacteraemia, infections of the skin (commonly in post-operative wound infections), pulmonary infections, endocarditis and osteomyelitis among other chronic infections (Diekema et al., 2001, Ma, 2005, Jarmrat et al., 2005). In the majority of the cases, the bacterium can start an infection process when there is a breakage in the skin barrier or mucous membranes and even enter the bloodstream (Lowy, 1998).

The virulence factors responsible for the extensive damage capability of *S. aureus* are wide-ranging including surface proteins, production of small colony variants, production of a large number of enzymes such as proteases, lipases, and elastases that expedite the invasion and destruction of the host tissues (Gordon and Lowy, 2008). Once *S. aureus* adheres to the tissue cells, it is able to multiply and persist by forming biofilms, a complex cell association, that assist the bacteria to escape host defences and antimicrobials (O'Toole et al., 2000, Donlan and Costerton, 2002). Generally, severe infections require treatment with oral antibiotics and sometimes hospitalization and treatment with intravenous antibiotics.

Staphylococcus aureus has an exceptional ability to adapt under antibiotic pressure. Less than a decade after the introduction of penicillin, strains resistant to the antibiotic were reported (Barber and Rozwadowska-Dowzenko, 1948) and, within six years, 25% of the strains isolated in hospitals were resistant (Chambers, 2001). In 1959, in order to continue the battle against the pathogen, methicillin -a semi-synthetic type of penicillin- known at the time as celbenin was introduced. Following the penicillin trend, within a year the first methicillin-resistant *S. aureus* (MRSA) isolates were reported (Jevons, 1961, Barber, 1961). Nowadays, these strains are reported to be resistant also to all other antibiotics of the beta-lactam family and rather too commonly also show resistance to other classes of antibiotics (Blanc et al., 2007). Since its emergence, MRSA has been responsible for a number of nosocomial outbreaks initially at the endemic level (Aires de Sousa et al., 1998, Denis et al., 2004, Johnson et al., 2005) but gradually it has become a pandemic pathogen (Oliveira, 2002, Harris, 2010).

In the United States, it was estimated that about 94,360 invasive MRSA infections occurred in 2005 (Klevens et al., 2007). Unfortunately, 18,650 of these cases resulted in death. Currently, the incidence of MRSA infections is higher than the incidence of AIDS, tuberculosis and viral hepatitis combined (Boucher, 2010). In Australia, the percentage of MRSA strains isolated significantly increased from 10.3% in 2000 to 16% in 2006 (Coombs et al., 2009a) and it was found that the incidence varied greatly among States from 0.6 per 100,000 population per year (0.6/100,000) in Tasmania to 13.3/100,000 in the Northern Territories (Ferguson, 2007). Furthermore, an estimated 2,000 people per year acquired MRSA in their bloodstream and a third of these MRSA infected patients died within 30 days (Collignon et al., 2005). In Europe, the MRSA incidence rate is measured by the European Antimicrobial Resistance Surveillance System (EARSS) as a median of bacteraemias per 100,000 patient days reported in each country. The latest incidence rate was reported to be around 4.8 per 100,000 patient days, up from 3.5 in 2007 (European Antimicrobial Resistance Surveillance System, 2009). The increase in incidences and in the percentage of MRSA strains reported in hospitals is of great concern, since the development of resistance constantly challenges the capacity to treat *S. aureus* infections. However, there have been contradictory results regarding the clinical importance of infections caused by MRSA isolates versus methicillin-susceptible *S. aureus* (MSSA) isolates.

In earlier studies, researchers found no evidence of increased virulence in MRSA isolates (Hershow et al., 1992). On the other hand, recent studies showed that patients in the intensive care unit (ICU) carrying MRSA were more likely to develop an infection than those patients carrying MSSA or non-carriers (Honda et al., 2010). Studies also showed an increase in morbidity and mortality associated with infections caused by MRSA strains (Blot et al., 2002, Cosgrove et al., 2003, Waness, 2010). Presently, the argument on the increased virulence of isolates harbouring methicillin-resistance continues. This is because, ultimately, morbidity and mortality rates are determined not only by the ability of the pathogen to evade antibiotics, but also by the condition of the patient's immune system; conditions and co-morbidities that could complicate the outcome.

Conversely, the associated costs in the treatment of MRSA infections as a result of longer hospital stays and limited treatment options are unquestionable (Gordon and Lowy, 2008, Eber et al., 2010, Lodise and Mckinnon, 2007, Engemann et al., 2003, Watters et al., 2004). Noskin and colleagues (2005) estimated infections caused by *S. aureus* cost the US Hospitals US$9.5 billion in excess charges. A more recent study looked at the clinical and economic impact of MRSA in surgical site infections (SSIs). The group found that SSIs due to MRSA resulted in more than three weeks of additional hospitalization and over $60,000 in extra costs per patient (Anderson et al., 2009). The latest report in Ireland estimated MRSA cost the Irish health system €23 million extra a year (Smyth et al., 2010) and, in the UK, every case cost the National Health System (NHS) an extra £9,000 (Devlin, 2008). The associated higher costs prompted the introduction of surveillance programs in an effort to reduce and control the problem. Some of these programs showed positive results with a significant 70% reduction of both MRSA and overall *S. aureus* infections during hospitalization and at 30 days post hospitalization (Robicsek et al., 2008). In Europe, for example, reported incidence rates were lower for the countries that implemented rigorous control measures (European Antimicrobial Resistance Surveillance System, 2009).

Initially, MRSA strains were believed to be confined to the hospital setting (HA-MRSA or EMRSA). Therefore, the discovery of MRSA strains in the community was considered of particular importance. The first outbreak of CA-MRSA was reported among drug users (CDC, 1981). In Australia, the first community-acquired MRSA (CA-MRSA) infections were reported in 1993 from aboriginal communities (Udo et al., 1993). Since then, CA-MRSA has been reported worldwide and caused outbreaks among relatively healthy subjects who reported no contact with Hospitals (Mulvey, 2005, Buck, 2005, DeLeo et al., 2010, Diederen and Kluytmans, 2006, Tristan et al., 2007). Contrary to HA-MRSA, CA-MRSA was reported to infect younger people, causing skin and soft tissue infections and other infections such as the serious necrotizing pneumonia (Zinderman et al., 2004, Herold et al., 1998, Garnier et al., 2006, Boubaker et al., 2004, Stryjewski and Chambers, 2008).

The ability for CA-MRSA to affect younger, relatively healthy people suggested that the bacteria had an inherent greater virulence when compared with its predecessor HA-MRSA.

Better adaptation conditions, superior ability to escape the host immune system and unique toxin production were some of factors reported as possible reasons for the phenomenon. The production of panton-valentine leukocidin (PVL), a toxin produced by two genes, has been the most studied and generally it has been attributed to the increased virulence. When PVL is produced it attacks and destroys human white blood cells (Vandenesch et al., 2003). Interestingly, PVL is rarely observed in HA-MRSA or MSSA isolates (Johnsson et al., 2004). However, the precise mechanisms and virulent factors are still under investigation (DeLeo et al., 2010).

The capacity to resist methicillin, in both HA-MRSA and CA-MRSA is determined by the presence of a gene, the *mec*A gene, The gene encodes for a variant of a penicillin binding protein 2A that reduces the affinity for β-lactam antibiotics (Gordon and Lowy, 2008). The *mec*A gene is found on a part of the genome named the staphylococcal cassette chromosome (SCC). The wide geographic distribution of the MRSA isolates forced the development of a nomenclature system that could be easily standardized and the presence of this cassette has been exploited to distinguish between isolates from around the globe. Until now, eight main variants of SCC*mec*, type I to type VIII, have been identified (Deurenberg and Stobberingh, 2008). Enright and colleagues (2002) proposed the system that harmonised MRSA nomenclature across the world and permitted the comparison of MRSA between laboratories. The system utilizes a combination of the SSC*mec* types together with seven housekeeping genes sequence types (STs) that are identified by using a multilocus sequence typing (MLST). Following this classification, it was determined that in general HA-MRSA belong to SCC*mec* type I, II, III and VI while CA-MRSA belong to types IV, V, VII and VIII. This classification afforded the formation of reference collections but, more importantly, allowed for the maintenance of epidemiological surveillance of the pathogens.

The high prevalence of MRSA is a clinical concern as it has forced the use of antibiotics from the last effective line of defence such as vancomycin, linezoid and daptomycin. The administration of these antibiotics is generally restricted to those patients that have already failed treatment with other antibiotics and for those with a high risk of mortality. This is

mainly because of the toxicity that they exhibit. However, even without widespread use, isolates with intermediate and full resistance to vancomycin and daptomycin have been reported (Mangili et al., 2005, Hayden et al., 2005, Tiwari and Sen, 2006). This trend has shown that *S. aureus* is able to develop resistance to virtually any antibiotic; it is almost not a question of "if" but "when" the resistance would be acquired. Sadly, after the discovery of the first penicillin resistant isolate, little attention was paid to the suggestion of Barber and Rozwadowska-Dowzenko (1948) that antibiotics should be used "sporadically rather than massively" in order to avoid selective pressure. For the future, there is then a clear need for new antibacterial agents with completely novel mechanisms of action, compared with those of antibiotics, in order to provide adjunct support for antibiotic treatments and, hopefully, making it even more feasible for antibiotics to remain effective for longer, by minimising their use in the clinical setting.

1.2.2 ***Pseudomonas aeruginosa*** **and antibiotic resistance**

On the other hand, *P. aeruginosa* is a Gram-negative rod widely spread in nature, inhabiting soil, water, plants, and animals. *Pseudomonas aeruginosa* has negligible nutritional requirements and is able to endure a wide range of physical conditions. In the laboratory, *P. aeruginosa* often generates a characteristic sweet fruity smell and clinical isolates generally produce pyocyanin, a blue-green pigment that can be detected by growing the organism on cetrimide agar. *P. aeruginosa* can also become a human opportunistic pathogen and, consequently, its presence represents a major concern when found in patients with compromised immune systems such as those under chemotherapy therapy and in patients with AIDS (Mendelson et al., 1994, Domingo et al., 1998, Shepp et al., 1994).

The pathogenicity of *P. aeruginosa* involves multiple factors and is very complex as the bacterium is both invasive and toxigenic. Generally, an infection in humans occurs following the rupture of a physical barrier, namely the skin or mucous membranes. The infection process was described as a three-stage process. Firstly, bacterial attachment occurs and this is followed by rapid colonization. Secondly, the colonization causes a local infection and, thirdly, it spreads into the bloodstream causing systemic disease or

bacteraemia (Pollack, 1984). The secretion of extracellular proteases increases the virulence of the bacterium as this complements it stability to adhere and invade tissues (Pollack, 1984). This extracellular mechanism is of particular importance in patients who suffer respiratory tract infection and cystic fibrosis, because it is associated with a high morbidity and mortality (Tramper-Stranders et al., 2005). *Pseudomonas aeruginosa* has many other toxic factors, like pyocyanin, a type III secretion system (T3SS), lipases and phospholipases (Sadikot et al., 2005).

Pseudomonas aeruginosa is also a common cause of nosocomial infections such as pneumonia (Hamer, 2000), urinary tract infections (UTIs) (Goldman et al., 2008), skin and wound infections (James et al., 2008, Keen III et al., 2010), endocarditis (Venkatesan et al., 2005), as well as infections of the central nervous system (Chuang et al., 1999), ears (Englendera et al., 1990), eyes (Spencer, 1953), bones and joints (Miskew et al., 1983), and the gastrointestinal tract (Spach et al., 1993). Some of them are associated with very high mortality rates. However, bacteraemia-associated infections are perhaps the most serious sequelae caused by the pathogen with reported mortality rates of up to 21.0% during hospitalization (Micek et al., 2005). A more recent study with 106 patients suffering bacteremia showed a 30-day mortality rate of 26.4% (Cheong et al., 2008). When the strains were antibiotic resistance the mortality rate was found to be 67% (Tacconelli et al., 2002, Gasink et al., 2006). Furthermore, the presence of co-morbidities was also associated with an increased risk of death (Parkins et al., 2010)

In the United States, *P. aeruginosa* is the fourth most commonly isolated nosocomial pathogen, responsible for 10.1% of all hospital-acquired infections (Sadikot et al., 2005). In Europe, 17% of the isolates were often found to be multi-resistant, that is resistant to three or more antibiotics, according to the European Antimicrobial Resistance Surveillance System (2009). Furthermore, one of the most dominant phenotypes (6% incidence) was reported to be resistant to all five classes of antibiotics tested: piperacillin, ceftazidime, carbapenems, fluoroquinolones and aminoglycosides (European Antimicrobial Resistance Surveillance System, 2009). *Pseudomonas aeruginosa* infections also resulted in much

longer hospital stays and associated higher cost (Harris et al., 1999, Tacconelli et al., 2002, Gasink et al., 2006).

The ability of *P. aeruginosa* to escape antibiotics is intrinsic (Jalal and Wretlind, 1998) and, in many clinical cases, it was shown that resistance appeared even before exposure to the antibiotic had occurred (Martínez et al., 2009). The bacterium has several mechanisms to overcome antibiotics and these are usually combined, thus providing for extensive protection. Resistance mechanisms include β-lactamases, aminoglycoside-modifying enzymes, topoisomerase mutations, decreased permeability and the activities of efflux pumps (Bonomo and Szabo, 2006). *Pseudomonas aeruginosa* also has the ability to escape the immune system, consequently increasing survival and eventually the development of biofilms (Cigana et al., 2009). Biofilm development, moreover, appears to add to the pathogen's persistence in the human body and the establishment of chronic infections (James et al., 2008, Donlan and Costerton, 2002). The persistence of biofilms is of particular importance for people with cystic fibrosis (CF) causing progressive respiratory failure and high mortality (Treerat et al., 2008).

All these characteristics and the high levels of antibiotic-resistance that it has exhibited in the clinical setting have won *P. aeruginosa* a place in the list of "ESKAPE" pathogens, mainly because of the difficulties that this brings for treatment as discussed. The repeated incidence of both *S. aureus and P. aeruginosa* infection in the human population has been steadily increasing through the past 30 years and thus new alternative treatments are desperately required (Infections Diseases Society of America, 2010).

1.2.3 Other pathogens and antibiotic resistance

Further to the "ESKAPE" or "ESCAPE" pathogens, *M. tuberculosis* continues to be a major public health concern. Tuberculosis (TB) is currently ranked as the second leading cause of death worldwide (WHO, 2013) with the prevalence of multidrug-resistant (MDR) TB (Abubakar et al., 2013) and extensively drug-resistant (XDR) TB in the rise (Lynch, 2013). The increase in antibiotic resistant TB has been theorised as a consequence of the antibiotic selective pressure exerted by the current 6-months regimen of 4 first-line drug

used for the treatment of drug-susceptible TB and the 20-months for MDR-TB (Llamas-González and Flores-Valdez, 2013). Although several vaccines have entered Phase I and Phase II trials, new antibiotics are just starting to emerge from the pipeline (WHO, 2013).

More recently, the appearance of a pan-drug resistant (PDR) strain carrying a beta-lactamase (New Delhi metallo-beta-lactamase or NDM-1) has become the centre of attention in the medical and scientific community (Bonomo, 2011). The NDM-1 strain was initially characterised from a *K. pneumoniae* strain that was isolated from a patient in Sweden (Yong et al., 2009). The patient reported to have received medical care in New Delhi, India. Later papers have reported on the broad dissemination of this MLB gene in India, Pakistan, Bangladesh and the United Kingdom (Kumarasamy et al., 2010), Australia (Poirel et al., 2010) and Canada (Tijet et al., 2011).

The clinical importance of this strain relied on the fact that the gene encoding this metallo-beta-lactamase (MLB) is located in a very mobile genetic element (Arya and Agarwal, 2010) and the pattern of proliferation is proving to be more complex than any of the other beta-lactamases studied (Kumarasamy et al., 2010). Of particular concern is that NDM-1 enzymes were also already found in *Escherichia coli* strains (Pitout, 2010). A rapid and global spread of NDM-1 among other bacterial populations is expected to have severe consequences on the treatment of hospital and community-acquired infections as the only treatment existing is colistin and tigecycline, two antibiotics abandoned by the clinicians a long time ago, because of the toxic side-effects they had on patients (Wolinsky and Hines, 1962, Li et al., 2006).

Compounding the extremely difficult situation is the slim pipeline of new antibacterial drugs under development to treat multi-resistant bacteria (Boucher, 2010, Page and Heim, 2009, ECDC/EMEA Joint Technical Report, 2009). Many pharmaceutical companies have reduced their investment in antimicrobial research and have instead entered the market of “blockbuster” medicines for other conditions such as cholesterol lowering levels, depression or erectile dysfunction (Clarke, 2003, Nelson, 2003, Projan, 2003, Payne et al., 2007). The main reason for this marketing change is not only the higher cost associated

with the development of a new antibiotic, but also the perception of pharmaceutical companies that profits may be lower in comparison (Charles and Grayson, 2004). Pharmaceutical companies believe that as the use of antibiotics becomes more strictly controlled and there are calls to reduce their use (Friedman and Whitney, 2008), the cost associated with their development for a potentially smaller market is a high-risk investment. In contrast, “blockbuster” medicines are developed to treat conditions that require daily regular dosage from a relatively young age and generally, use over extended periods of time or even for a lifetime. More importantly, these types of drugs are not affected by the emergence of resistance, thus providing more secure and larger profits.

Currently there are only five major pharmaceutical companies still involved in the development of novel antibacterials (Boucher et al., 2009). Although smaller biotechnology companies are stepping forward to address this gap, their success is uncertain, mainly because of the cost associated with clinical trials (Barrett, 2005). This situation has prompted the IDSA to issue a “call to arms”, outlined in a historic letter to the Presidents of the United States and the European Union in November 2009 (Infectious Diseases Society of America, 2009). In this letter, the authors called for a global commitment “to address the emerging disaster caused by the confluence of increasing bacterial resistance and a stagnant antibacterial drug pipeline”. The core initiative in the letter was to commit governments, pharmaceutical companies, universities, research entities, intellectual property managers, policy makers and medical personnel towards the establishment of a long-lasting, maintainable research and development program that offered incentives to companies to research antibacterial drugs. The IDSA group is aiming for ten new drugs by 2020 (Infections Diseases Society of America, 2010).

In response, the United States and European Union leaders reached a decision to establish a Transatlantic Task Force (TTF) for dealing with pressing problems of antimicrobial resistance. The TTF would be focused on finding practical solutions for the resistance problem, developing guidelines for the appropriate therapeutic use of antimicrobial drugs in both the medical and veterinary field, the prevention of nosocomial and community-

acquired, drug-resistant infections and in suggesting plans for expanding the pipeline for new antimicrobial drugs.

Antibiotics are undoubtedly one of the greatest success stories in medicine, resulting in dramatic reductions in mortality rates. However, it is now evident that the emergence of antibiotic resistance poses a significant problem to world health. There is an urgent need to find antibacterials with novel mechanisms of action to ensure an ongoing supply of safe and effective antimicrobial therapies into the future (Hougberg et al., 2010). Given the ability of bacteria to evolve and acquire resistance to antibiotics with such ease and speed, can we continue to rely on the existing paradigm of 20 year development timelines for a new antibiotic, followed by rapid appearance of resistance? An ideal solution would be to find some form of antibacterial agent with the ability to evolve in parallel with their targets. This implies that the solution must then involve a biological (as an alternative to a chemical) mechanism of action. Already other biological therapies such as prebiotics and probiotics (Johannsen and Prescott, 2009, Amalaradjou and Bhunia, 2012), maggot therapy (Hunter et al., 2009) and honey (Langemo et al., 2009) have been revisited and investigated for the treatment of gastrointestinal and wound infections. One other proposed alternative is that of bacteriophage therapy.

1.3 Bacteriophage Therapy

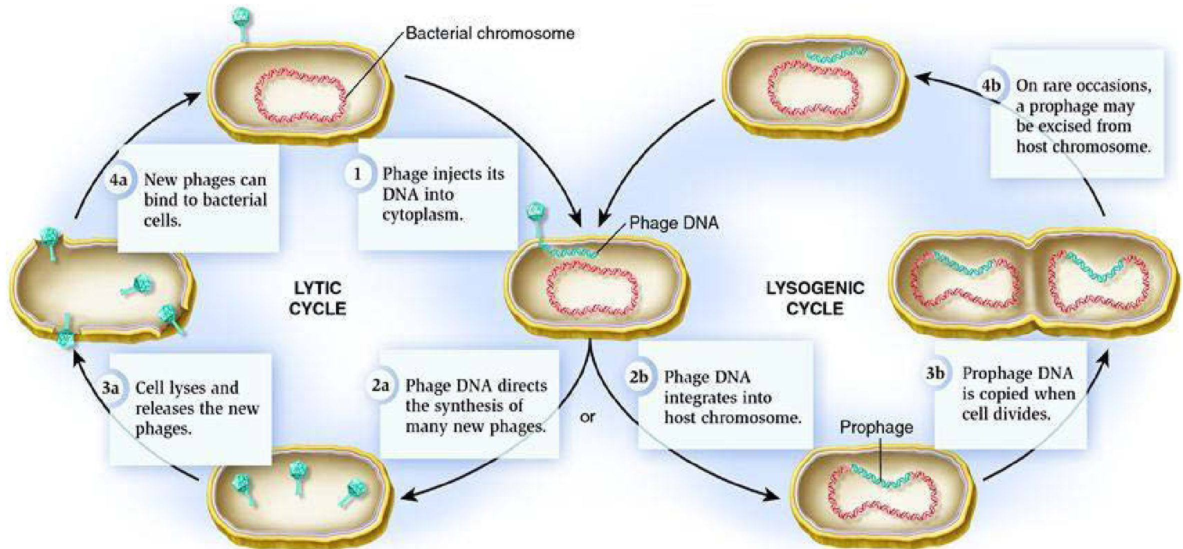
The exponential rise of antibiotic resistance together with the reduced pipeline of antibiotics (Gould, 2009), has created the perfect storm and focused attention on another little-known and long forgotten biological method of controlling bacteria, that of bacteriophage (phage) therapy. Since the mechanism by which bacteriophages interact with their host is completely different from that of antibiotics it has been proposed that phage therapy may provide a useful alternative or complementary strategy to antibiotic use (Barrow and Soothill, 1997, Sulakvelidze et al., 2001, Summers, 2001, Merril et al., 2003, Bradbury, 2004).

1.3.1 Bacteriophages, bacteriophage cycle and mechanisms of resistance

Bacteriophages (commonly known as “phages”) are naturally occurring viruses that infect and kill bacteria. The interaction of the phage with the bacterium is highly specific, relying on the recognition of particular receptors on the bacterial cell surface. Bacteriophages are also considered to be the most abundant life form on Earth, with an estimated number of 10^{30} to 10^{32} (Ashelford et al., 2003, Breitbart et al., 2003, Wommack and Colwell, 2000, Suttle, 1994). A bacteriophage kills the bacterial cell by attaching to the surface of the bacterium, replicating inside, and eventually destroying its host (Kutter and Sulakvelidze, 2005). When infecting the bacterium, the bacteriophage can undertake two different pathways, namely, a lytic cycle or a lysogenic cycle.

During a lytic cycle, the bacteriophage attaches to a bacterial cell and injects the viral nucleic acid into the cell (Figure 1-2). The virus then takes control over the host cell’s mechanisms to produce viral components. These components are assembled into new viruses that destroy the host cell, by rupturing its plasma membrane (lysis). New viruses are released so that they can spread and infect other cells. These viruses that do cause bacterial lysis are referred to as virulent or obligatory lytic phages (Waldor and Mekalanos, 1996). Obligatory lytic phages are characterised by the absence of integrase genes in the genomes, which prevents their insertion in the bacterial chromosome. This characteristic prevents them from entering a lysogenic cycle and coexisting with the host bacterium (Karam, 1994).

Figure 1-2. Bacteriophage lytic and lysogenic cycle



In a lytic cycle, a bacteriophage attaches to a bacterial cell and injects its viral nucleic acid. The phage then takes control over the host and produces viral components. The components are then assembled into new viruses. The new phages exit the host cell by lysing the membrane. New phages are then released and ready to infect other cells. In a lysogenic cycle, the viral nucleic acid becomes part of the host DNA and it does not immediately take control of the metabolic activities of the host or destroys the cell. The viral nucleic acid then coexists with the host DNA as a prophage until exposure to stressful environmental factors triggers the prophage to enter the lytic cycle.

(Image sourced from http://biologyforums.com/index.php?action=gallery;sa=view;id=550, Biology blogs online)

On the other hand, during a lysogenic cycle the virus also attaches to a bacterial cell and injects the nucleic acid into the cell. However, the nucleic acid becomes part of the host cell's chromosome and it does not immediately take control over the metabolic activities or destroys the host cell (Figure 1-2). The actual replication is delayed and the virus exists as a prophage until exposure to certain environmental factors, such as radiation and chemicals, initiate the lytic cycle. Those viruses that do not cause an immediate infection are referred to as temperate or latent (Waldor and Mekalanos, 1996).

Temperate bacteriophages have been shown to play a role in the pathogenesis of bacterial infections by modifying the virulence and evolution of a bacterial host (Fortier and Sekulovic, 2013, Canchaya et al., 2003). Genomic analysis studies have proposed that horizontal gene transfer (HGT) by specialised transduction (transfer of a specific DNA from one bacterium to another when a prophage excises from a bacterial chromosome taking adjacent bacterial genes) influences directly the genetic selection of each strain (Qiu et al., 2009). For example, in *S. aureus* bacteria, temperate bacteriophages carry the Panton-Valentine leukocidin (PVL), the immune evasion cluster (IEC) and enterotoxin A virulence genes that impact the ability of the bacterium to colonize and cause disease (McCarthy et al., 2012). HGT also is also a key factor in the evolution and emergence of new methicillin-resistant clones (Xia and Wolz, 2013). Increased pathogenesis due to other phage-encoded toxins have been described in other pathogens, including *C. diphtheria* (Holmes and Barksdale, 1969), *E. coli* (Allison et al., 2003), *C. difficile* (Brouwer et al., 2013) , *V. cholera* (Faruque and Mekalanos, 2012) and Salmonella Typhimurium (Ehrbar and Hardt, 2005).

Although obligatory lytic bacteriophages can also mediate transduction, this transduction process is generalised and not specialised. Generalised transduction happens due to the low errors that occur during the packaging of the bacteriophage DNA at the replication stage and results in random bacterial DNA fragments packed within a virus capsid without the virus DNA (Ackermann and DuBow, 1987). Given the greater biological ability of temperate phages to modify their hosts, potentially increasing their virulence, it is critical that only strictly obligatory phages are considered as therapeutic candidates in order to

minimize the risks for transferring novel genetic material into a receiver bacterial cell (Jiang and Paul, 1998, Moons et al., 2013). The use of temperate phages for therapy would not only increase such risks but could also be detrimental because the integration of the phage could create bacterial cells resistant or immune to the therapy, theoretically reducing the efficacy of the treatment (Lwoff, 1953).

In addition, it is critical to understand the co-evolutionary processes between bacteria and bacteriophages for the development of bacteriophage-based therapies. The coevolution of bacteriophages and bacteria has been demonstrated with the development of comparative genomic studies (Brussow et al., 2004, Chan and Botstein, 1972). One of the most remarkable characteristics revealed from genome comparative analyses is that phages are persistently mosaic. Recurrent recombination of phages with superinfecting phages (Chan and Botstein, 1972) or temperate phages as well as the random acquisition of bacterial chromosomal genes supports their diversification (Hatfull and Hendrix, 2011). However, the uninterrupted dynamics between the phage and bacteria has resulted in a “phage-host arms-race” (Stern and Sorek, 2011). In this race, bacteria have developed a variety of mechanisms to prevent and counterattack bacteriophage infection (Labrie et al., 2010), although it seems that such adaptation and surviving strategies result in reduced bacterial virulence or general fitness (Loc-Carrillo et al., 2005).

Given that adsorption is the first step in infection process, predictably the first barrier of resistance for bacteria against bacteriophage infection is the modification the receptors. For example, it has been demonstrated that if *S. aureus* produces less protein A, bacteriophage adsorption increases ((Nordstrom et al., 1974) while *P. aeruginosa* tends to produce an excess of alginates (Castillo and Bartell, 1974) that reduces bacteriophage adsorption. A second barrier is the presence of restriction-modification systems to protect bacterial cells against viral infection by cleaving the foreign DNA once it has been injected into the cells (Loenen et al., 2014). A third barrier involves the use of clustered, regularly interspaced short palindromic repeats (CRISPRs) ((Barrangou et al., 2007). It has been proposed that these short spacers in association with *cas* genes (CRISPR-associated proteins) are adaptive immunity systems that may confer resistance to bacteriophages by providing an RNA

interference-like mechanism (Sorek et al., 2008) or by targeting the viral DNA (Marraffini and Sontheimer, 2008). Although three main types of these systems have been described (Haft et al., 2005), a classification system that does not rely on a single criterion and flexible enough to allow for updates has been proposed (Makarova et al., 2011).

Despite these bacterial mechanisms of defence, it is estimated that 10^{23} bacteriophage infections occur per second (Suttle, 2007). As mentioned before, bacteria and bacteriophages are in a constant race for survival and so, not surprisingly, bacteriophages have also acquired reversing systems to overcome the host resistance mechanisms as reviewed by Samson and colleagues (2013). Phages are capable of adapting to new receptor-binding proteins by either one or a combination of mutations in the genes that encode for them. Similarly, to destroy bacterial extracellular material produced to prevent adsorption, phages have evolved to produce enzymes to degrade this material. Phages can also evade DNA cleaving by restriction endonucleases and CRISPR-cas systems by modifying the restriction sites and single nucleotide substitutions respectively (Samson et al., 2013).

The described bacterial resistance mechanisms, and the strategies developed by phages to overcome these, demonstrate a continuous co-evolutionary state of bacteria-phage. Selective pressure of phages over bacteria may result in the development of phage resistant strains. In response, bacteriophages will also mutate to ensure that the phage population persists. It is the ability of bacteriophages to adapt and evolve what makes them an interesting tool for the potential management of antibiotic resistant strains. However, strategies will need to be adopted in order to outwit bacteria (as discussed in chapter 5) and ensure the development of safe-effective therapy.

1.3.2 Bacteriophage history

Bacteriophages were discovered independently by two scientists between 1915 and 1917, more than 20 years prior to the isolation of Penicillin. In 1915, Frederick Twort reported an "ultracosmic virus that somehow killed bacteria in solution" (cited by (Adams, 1959). Two years later, Felix d'Hérelle, a French-Canadian scientist, while working with *Shigella* sp., responsible for dysentery infections, noticed a lytic agent that was filterable, but not visible by microscopy. It was grown and passed on from one bacterial culture to another culture. The effect observed could not be attributed to any other species identified (d'Herelle, 1924). d'Hérelle named this agent a "bacteriophage", not in the sense of "to eat", but in that of "developing at the expense of" bacteria.

Highly excited by the efficiency of the bacteriophages against *Shigella* sp., d'Hérelle continued his studies on phages and was the first person to realise their potential as therapeutic agents. In 1919, with the support of the paediatrician Victor Hutinel at the Hospital (Hôpital des Enfants-Malades) in Paris, d'Hérelle, for the first time in history, used phages to treat a 12-year-old boy with severe dysentery; within 5 days of treatment the boy was completely cured (Summers, 1999). By translating his research findings into clinical application d'Hérelle had started a new form of antimicrobial therapy, bacteriophage therapy, the practical application of these very powerful lytic viruses to treat bacterial infections.

After d'Hérelle's first successful use of phage therapy, other scientists around the world became interested in the new phenomenon and its potential as a therapeutic method. By 1925 a total of 150 papers had been published (Summers, 1999). Bacteriophages were used in the treatment of carbuncles, furunculosis and abscesses on approximately a thousand cases with more than 90% success (Lampert et al., 1935). Treatment of dental (Schultz, 1932), urinary tract (Larkum, 1926), and cholera infections (Pollitzer, 1954), among other conditions, was also described. As s consequence of this, despite little or no understanding of the biology of bacteriophages. It would be another 20 years before d'Herelle's "invisible

antagonistic microbe" revealed itself through the invention of the electron microscope in the early 1940s.

Nevertheless scientists in Europe and the United States began to produce their own phage treatments on a large scale for the treatment of cholera, typhoid fevers and bubonic plague (Kutter and Sulakvelidze, 2005). In 1932 in India alone, 191,000 vials of bacteriophages were used for the treatment of cholera. The multiple disease conditions for which bacteriophages were used in the early days of their discovery, and during its application in the Former Soviet Union (FSU) have been recently summarised (Chanishvili, 2012a). However, despite initial success during the two decades after its discovery, bacteriophage therapy also experienced many setbacks.

From his first publication, scientists questioned the accuracy regarding the description of the phenomenon that d'Hérelle had described. Three key contradictory hypotheses against bacteriophage therapy were proposed and heatedly debated. The first theory credited the lytic process to an inert agent carried by the bacteria, thus explaining the ability to transfer the lytic ability from culture to culture (Bordet and Ciuca, 1921). In other words, in contrast to d'Hérelle's opinion that bacteriophages were small cells, for Bordet and colleagues they were modified bacterial proteins. Bordet and colleagues were probably working with a bacterial culture infected with a prophage. However, at the time, very little was known about phage biology and the lytic and lysogenic cycle that phages were capable of undertaking. The lysogenic phase of phages was not recognized until the early 1950s (Lwoff, 1953) and remained, until then, one of the main theories against the viral nature of phage.

The second theory also supported the idea that phages were an inert substance. American biologists conducted numerous studies and mounted arguments showing that the lysis observed were due to an enzyme capable of causing bacterial autolysis and, when it was released to the external environment, it lysed the sensitive bacterium (Bronfenbrenner and Sulkin, 1941). The third theory stated that the lytic activity was a cascade reaction and that the solution simply stimulated the immune system, facilitating the healing process

(Ackermann and DuBow, 1998). These three interpretations of the lytic phenomenon were very similar and reasonable, considering the level of knowledge and scientific tools available between the 1920s and the 1940s.

Around the same time, Australian virologist, Sir MacFarlane Burnet, was one of d'Hérelle's few allies. Burnet became very interested in bacteriophages and, in contrast to other colleagues, he believed, as d'Hérelle did, that bacteriophages were viruses that infected bacteria. However, Burnet and d'Hérelle disagreed on two fundamental issues of bacteriophage biology. First, Burnet went further than d'Hérelle by proposing that they could also exist as a stable non-infectious particle in the bacterial host (Burnet and McKie, 1929). Secondly he proved that there were many different "species" of bacteriophage (Burnet, 1928, Burnet, 1927, Burnet, 1930). d'Hérelle instead insisted that there was only a single, highly variable, species of virus. Disappointingly, Burnet's research did not translate into therapeutic use, but it definitely changed the course of phage research towards its molecular applications (reviewed by (Neeraja, 2006).

Nevertheless, d'Hérelle continued with his work and focused his investigations primarily on getting better understanding of the basic biology of phages and their therapeutic properties (d'Hérelle, 1924). His patient and methodical studies of the subject continued and, importantly, drew positive international attention. In 1923, bacteriophages were again successfully used in the treatment of bacillary dysentery. In 1926, he was successful in treating patients suffering from bubonic plague (d'Herelle, 1930). Then, in 1927, he went to India at the request of the British government to attempt to eradicate the cholera epidemics. To confirm the effectiveness of the treatment, d'Hérelle chose two villages remote from any medical facility. The results showed 8% of deaths in the village treated with phage preparations against 62% in the village left untreated (d'Hérelle, 1930).

The different theories presented at the time, however, continued to cause bitter debate, confusion and scientific disagreement that encouraged constant criticism of the work presented. The high specificity required between phage and target bacteria for successful treatment was still poorly understood. The problem was compounded by the fact that

doctors often used mismatched phages with corresponding lack of success. Companies manufacturing the phages further complicated the issue by making exaggerated claims and supplying poor quality products (Summers, 2001). It was hardly surprising that negative reports began to appear in the literature questioning the effectiveness of phage therapy.

In the early thirties, two respected physicians, Stanhope Bayne-Jones and Monroe Eaton, were commissioned by the Council on Pharmacy and Chemistry of the American Medical Association to prepare a comprehensive review about phage therapy from the available literature. More than 100 papers on bacteriophage therapy were reviewed by the duo and, in 1934, they published a detailed report concluding that the bacteriophage was not a virus capable of parasitizing bacteria and that the material, in fact, appeared to be inanimate, possibly an enzyme (cited by Sulakvelidze & Kutter, 2005). Nowadays, thanks to the development of transmission electron microscopy in the late 1930s (Pfankuch and Kausche, 1940) and the progress in the field of molecular biology, it is evident that the entire report was incorrect; possibly misguided by the high numbers of non-active phage preparations in the market. However, the damage caused to bacteriophage therapy by this report had immeasurable consequences for its development in the West.

By the early 1940s, mired in controversy and, with the widespread availability of penicillin and other broad-spectrum antibiotics, phage therapy fell into decline and eventually vanished from the Western scientific radar, except as a bacteriological typing tool and as a platform for molecular biology. By contrast, in Georgia, the Eliava Institute - the largest institute for the research and production of bacteriophage preparations (that d'Hérelle helped to build in collaboration with Georgi Eliava) - continued to expand and perfect phage therapy into an everyday tool for antibacterial treatment (Sulakvelidze et al 2001; Häusler, 2006). During the years of the Cold War and behind the Iron Curtain, the Institute employed about 12,000 researchers and produced up to two tons per day of phage products against a dozen bacterial pathogens, products that were distributed all around the former Soviet Union. Even today, phage products continue to be sold over the counter in both Georgia and Russia (Häusler, 2006).

Another site that specialised in the development of bacteriophage for therapeutic use was in the Hirszfeld Institute of Immunology in Wroclaw, Poland. The Polish group published one of the first comprehensive reports about the efficacy of phage therapy in English (Slopek et al., 1987). The reported percentage of success was greater than 90% after the treatment of 550 patients that were infected with and without antibiotic resistant strains. Only patients who had failed antibiotic treatment and other clinically available treatments were treated with bacteriophages at the institute. Importantly, the report addressed and discussed safety issues about phage therapy with only minimum side effects reported in three patients. The side effects were manifested as allergic type symptoms, when applied on the skin, of two patients and mild gastroenteritis in one patient. This reinforced the concept that bacteriophage therapy was a natural therapy with very minor side effects. However, the criticism remained that the treatments were not standardised or included separate controls. The present position and experience of the institute has been reviewed comprehensively elsewhere (Gorski et al., 2009, Kutter et al., 2010).

The worsening antibiotic resistance crisis has reignited interest in bacteriophage therapy in Western countries and initial studies have confirmed many of the original scientific observations (Bull et al., 2002, Barrow, 1997, Smith and Huggins, 1982). In addition to the traditional use of the whole bacteriophage, with the current better understanding of their biology, the therapeutic potential of their component parts has also been studied. This has resulted in the proposal of different therapeutic approaches (Fischetti et al., 2006, Lu and Koeris, 2011) including the use of phage lysins (Fischetti, 2005), phage endolysins (Borysowski et al., 2006) and more recently peptidoglycan hydrolases (Rodriguez-Rubio et al., 2012).

Despite the historical controversies it would appear from the literature that phage therapy would be an ideal remedy to counteract today's escalating antibiotic resistance crisis. Lytic phages are reported to be self-amplifying, self-limiting, highly specific, do not harm the body's normal bacterial flora and in the past have been shown to cause no serious side-effects. Most importantly they are **unaffected** by antibiotic resistance and work equally well against antibiotic-resistant and susceptible bacteria.

But herein lies the essential paradox of phage therapy: given the problems of antibiotic resistance, and with its almost century-long history, why is phage therapy not already a front-line treatment in Western countries like Australia? How much of this history is fact as opposed to myth? What scientific, clinical, practical or economic or historical reasons have prevented this? What relevance or value does phage therapy have in today's era of failing antibiotics and increasing resistance? Could bacteriophages be used to treat human bacterial infections?

1.4 Project aims

This thesis set out to investigate the development of phage therapeutics against two of the most important ESKAPE pathogens described here earlier, namely *Staphylococcus aureus and Pseudomonas aeruginosa,* for which there are presently very few or no new antibiotics available (Page and Heim, 2009, DeLeo et al., 2010). By starting from basic first principles it was hoped that many of the questions raised could be addressed.

The first step in the development of phage therapeutics was the selection of the target bacteria to be used for the isolation process of novel bacteriophages. Clinically relevant isolates were collected and reference bacterial collections of both *S. aureus* and *P. aeruginosa* isolates were then established. These bacterial reference collections were used as host indicator bacteria for bacteriophage isolation.

The second step in the process was the isolation of lytic bacteriophages. To do this, water samples were collected from several locations where the target pathogens were more likely to be present. Bacteriophages were then isolated using three classic methods described in the literature. These methods were tested and compared for their potential to recover phages from the environment. The identified bacteriophages were purified. Purified bacteriophages were propagated on their corresponding host bacteria. Subsequently, bacteriophage master stocks were prepared and titrated. The prepared purified stocks were stored at 4°C for further testing and bacteriophage characterisation.

Once the potential therapeutic phages were purified and propagated, the third step was the evaluation of their spectrum of activity against the established reference collection. The relatively narrow spectrum of activity of bacteriophages (when compared with that of antibiotics) is seen as a disadvantage of bacteriophage therapy. Thus, the selection of bacteriophages with a broad spectrum of activity was sought.

As a fourth step, bacteriophages that were identified as having therapeutic potential, based on their spectrum of activity against a reference collection, were tested against a broader representative collection of clinical isolates. The aim was to confirm that the selected bacteriophages were capable of infecting bacteria commonly found within a community and that their activity was not limited to a reference collection.

In the past, unstable bacteriophage products were blamed for the lack of clinical outcomes. Thus, the fifth step concentrated on the biological strengths and limitations of the selected bacteriophages. Their ability to remain active at different pH levels and at different temperatures over prolonged periods of time was evaluated. Selected bacteriophages were also investigated for their efficacy to diminish the growth of their indicator bacteria at different concentrations or multiplicity of infection (MOI). Their adsorption rates and burst sizes were also determined. The *in vitro* study of the effect of these parameters was important as they could provide relevant information about the performance of the selected bacteriophages at a therapeutic level.

The use of therapeutic phages as a mixture or “cocktail” has been the classical approach to therapy in Former Soviet Union (FSU) countries where the therapy has been most widely used. However, the selection criteria for the preparation of these therapeutic mixes have not been described. Therefore, the sixth step in this study investigated three different formulation approaches. The therapeutic mixes developed were evaluated for their ability to minimise the appearance of phage-resistant cells and to display a broader spectrum of activity, in comparison with the individual bacteriophages.

After completing the biological characterisation of these phage therapeutic prototypes the seventh and final stage of development concentrated on the morphological and molecular

characterisation of the selected bacteriophages. The investigation of their morphological properties by transmission electron microscopy (TEM) was carried out and was an essential step for the classification of the novel identified phages within a family. Additionally, pulsed field gel electrophoresis and restriction digest laboratory protocols were developed to investigate the genetic profiles of the isolated bacteriophages.

Finally, this thesis describes, to the best of our knowledge, the first use of human phage therapy in Australia. Bacteriophage therapy was used to treat a patient suffering from a refractory urinary tract infection caused by *P. aeruginosa* in Sydney. Informed consent was obtained from the patient, and ethical approval obtained from the Western Sydney Human Research Ethics Committee to perform the phage treatment at Westmead Hospital.

At the theoretical level the concept of using the natural predators of bacteria, the bacteriophages, to eradicate the multi-resistant and pan-resistant pathogens of today is highly attractive. However, for the reasons given earlier, the practise and application of phage therapy disappeared from Western medicine almost 60 years ago, taking with it much of the scientific knowledge and clinical expertise gained to that point. Of course we are fortunate that our Georgian, Russian, and Polish colleagues continued with the science but even so much of the literature is difficult to access and the methods empirical in nature.

In summary, to understand the possibilities of re-introducing bacteriophage therapy as a practical therapeutic tool in modern mainstream medicine, it was essential to built on the understanding of how to select and develop therapeutic phage preparations. This project aimed not only to generate the first characterised library of therapeutic bacteriophages against *S. aureus* and *P. aeruginosa* in Australia but also to determine the necessary steps and aspects involved in the selection, development and standardisation of therapeutic bacteriophage-based products. Throughout the process, the advantages and limitations of this biological therapy were rigorously interrogated to identify and attempt establishing procedures that could preclude the mistakes made in the early days of the therapy. This thesis also intended to examine the realistic potential and relevance of bacteriophage therapy as a modern treatment in the era of antibiotic resistant bacteria, particularly in

Australia where there has been a lack of exploratory studies targeted to investigate both the development of bacteriophage preparations and their potential as therapeutics tools.

2 GENERAL MATERIALS AND METHODS

2.1 Bacterial Strains

2.1.1 Bacterial stock cultures

Staphylococcus aureus and *P. aeruginosa* strains were sub-cultured on nutrient agar (Amyl-media AA 130) and incubated under micro-aerobic conditions at 37°C for 18-24h. Cultures were received from the hospitals on blood agar plates. The lists of *S. aureus* reference strains and *P. aeruginosa* clinical isolates are provided in Tables 2-1 and 2-2 respectively. The list of clinical *S. aureus* and *P. aeruginosa* clinical isolates are provided in Appendix 1 and 2 respectively. For long-term storage, the cultures were re-streaked on nutrient agar plates (section 2.2.2) and incubated under aerobic conditions overnight at 37°C. The fresh culture was then transferred onto protect preserved storage beads (Oxoid AUCOO26) following the manufacturer's instructions and stored at -80°C. When required, a bead from the frozen stock was removed and sub-cultured on 3m1 nutrient broth and incubated under aerobic conditions overnight at 37°C. The overnight culture was streaked on a nutrient agar plate and incubated under aerobic conditions overnight at 37°C. The plates were then stored under aerobic conditions at 4°C and cells harvested as and when required. This subculture procedure was repeated at monthly intervals in order to maintain a viable bacterial stock for preparing fresh cultures.

Table 2-1. Methicillin-resistant *Staphylococcus aureus* reference strains

Strain	SCC*mec*	ST	Clone	Source
SPS# 544	-	-	Japan- Vancomycin Resitant	ATCC 700968™
SPS# 545	IV	8	USA 300 CA-MRSA, PVL +	ATCC BAA-1556™
SPS# 546	IV	-	USA 300 EMRSA PVL +	ATCC BAA-1680™
SPS# 547	II	-	USA 600, PVL -	ATCC BAA-1685™
SPS# 548	V	-	Non USA, PVL +	ATCC BAA-1688™
SPS#621	I	250	Archaic	Westmead Hospital
SPS#622	I	~1	-	Westmead Hospital
SPS#623	II	5	NY/Japan EMRSA	Westmead Hospital
SPS#624	II	36	UK EMRSA-16	Westmead Hospital
SPS#625	IIIA	239	AUS-3 EMRSA	Westmead Hospital
SPS#626	III	239	AUS-2 EMRSA	Westmead Hospital
SPS#627	IIIB	239	Brazilian	Westmead Hospital
SPS#628	IV	1	WA-MRSA-1	Westmead Hospital
SPS#629	IV	59	WA MRSA 15	Westmead Hospital
SPS#630	IV	78	WA MRSA-2	Westmead Hospital
SPS#631	V	573	WA MRSA-10	Westmead Hospital
SPS#632	V	5	WA MRSA-11	Westmead Hospital
SPS#633	Novel	1	WA MRSA-40	Westmead Hospital
SPS#634	Novel	78	WA MRSA-42	Westmead Hospital
SPS#745*	IV	22	UK EMRSA-15	Westmead Hospital
SPS#746*	IV	93	Queensland CA-MRSA	Westmead Hospital

Molecular characteristics of reference strains used for testing representing some of the major clones in the clinical setting around the world. EMRSA: Nosocomial methicillin resistant S. aureus.

*CA-MRSA: community-acquired methicillin resistant S. aureus. Molecular elements include: SCCmec (Staphylococcal cassette chromosome mec) and ST (sequence type or alleic profile). *Reference strains were obtained after the initial selection of the S. aureus phages (chapter 3) was made but both isolates were considered in further reference tests (chapter 5).*

Table 2-2. ***Pseudomonas aeruginosa*** **reference strains**

Strain	Isolated from	AMK	GEN	TOB	IPM	CAZ	SXT	CIP
SPS#266*	Human	R	R	R	R	R	-	R
SPS#267*	Human	R	R	R	R	S	R	S
SPS#268	Human	I	I	S	S	S	R	S
SPS#269*	Human	I	I	S	R	R	R	R
SPS#270	Human sputum	S	S	S	R	I	R	S
SPS#271	Human skin	S	S	S	R	S	R	S
SPS#272*	Human calf wound	S	R	R	S	S	R	R
SPS#273	Human blood	S	S	S	I	S	R	S
SPS#274	Human ankle wound	S	S	S	S	S	R	S
SPS#275	Human sputum	S	S	S	S	S	R	S
SPS# 276 (PA01)	ATCC reference	S	S	S	S	S	R	S

*Antibiotic testing: R= Resistance, I= Intermediate resistance, S= Sensitive. Abbreviations of antibiotics used for testing: Amikacin (AMK) Gentamicin (GEN), Trobamycin (TOB), Imipenem (IPM), Ceftazidime (CAZ), Ticarcillin/Clavunalate (TIM), Trimethoprim /Sulfamethoxazole, (SXT), Ciprofloxacin (CIP). Strains were considered multi-drug resistant if they showed resistance to 3 or more of the antibiotics used. *Multi-drug resistant P. aeruginosa strains.*

2.2 Growth Media

2.2.1 Nutrient broth (NB) for *S. aureus* and *P. aeruginosa* bacteria

Nutrient broth (Amyl-media AM 130) was prepared in accordance with the manufacturer's instructions. It was sterilised by autoclaving at 121°C for 15 min. Medium was stored at room temperature for a maximum of two weeks.

2.2.2 Nutrient Agar (NA) for *S. aureus* and *P. aeruginosa* bacteria

Nutrient agar was prepared by mixing nutrient broth (Amyl-media AM 130) with 1% of the agar base (Amyl media KM 250). The medium was sterilised by autoclaving at 121°C for 15min, on liquid cycle. When the agar had cooled to 50°C in a waterbath, the final medium was poured into sterile Petri dishes (15-20ml per plate) and allowed to set. Once set, the plates were allowed to dry for a minimum of 24h and stored at room temperature for a maximum of one week.

2.2.3 Blood agar base for *S. aureus* and *P. aeruginosa* bacteria

Blood agar base was prepared by mixing blood agar base (Amyl-media AM 17) in accordance with manufacturer's instructions. The base was sterilised by autoclaving at 121°C for 15min.When the agar had cooled to 45°C in a waterbath, sterile defibrinated horse blood (Oxoid I-lB 100) was aseptically added to the medium (5%v/v) and gently mixed. The final medium was poured into sterile Petri dishes (15-20ml per plate) and allowed to set. Once dried, the plates were stored at 4°C for a maximum of 2 weeks.

2.3 Media for Bacteriophage Isolation and Propagation

2.3.1 Nutrient semi-solid agar

Nutrient semi-solid agar (NSA) was prepared by mixing Nutrient Broth (Amyl-media AA 130) as per the manufacturer's instructions with the selected agar base (Amyl media RM250) producing a 0.4% (w/v) agar consistency. The medium was sterilised by autoclaving at 121°C for 20min. Once set, the medium was stored at room temperature and

melted by microwaving at 320 watts for 4-6min as required. A variation of the NSA was prepared for a modified isolation method. The 3x NSA was made up with three times the amount of nutrient broth and three times the amount of agar per 100ml of water in comparison to the normal NSA.

2.3.2 Salt magnesium (SM) buffer

Salt magnesium buffer was prepared by adding 5.8g of NaC1 (Sigma-aldrich Chemicals 7647-14-5); 2.0g of $MgSO_4.7H_20$ (Sigma-aldrich Chemicals 7487-88-9); 5ml of 2% (w/v) gelatin (Sigma: 9000-70-8) and 50ml of 1.0M Tris-HC1 pH 7.3-7.5 (Sigma-aldrich T-5941) to 1L distilled water. The buffer was dispensed in 80ml amounts and sterilised by autoclaving at 121°C for 20min.

2.4 Enumeration of *S. aureus* and *P. aeruginosa* in Pure Culture

To calculate the number of colony forming units (CFU) on an overnight culture, 100µl of bacterial suspension were added to 900µl of sterile water in an Eppendorf tube and mixed well to give a dilution factor of 10^{-1}. This ten-fold serial dilution was diluted down to 10^{-8} dilution factor. One-hundred microliters from each 10^{-6}, 10^{-7} to 10^{-8} dilutions were spread onto nutrient agar plates in duplicate. The plates were incubated at 37°C under aerobic conditions and the number of colonies counted at 24h. The number of colony forming units (CFU) per millilitre was calculated from the number of colonies and the total dilution factor. The optical density (OD) of the overnight bacterial culture was taken at 600nm wavelength for future reference.

2.5 Microbiological Confirmatory Tests

Bacterial isolates received were identified and/or molecularly characterised previously at the hospitals. The reference collection of *S. aureus* isolates was molecularly characterised by multilocus sequence typing (MLST) and/or the presence of Staphylococal cassette chromosome *mec* (SCC*mec) while* the *P. aeruginosa* collection was identified and their

antibiotic profiles determined using a VITEK system. At arrival, the isolates identities were reconfirmed by standard microbiological techniques.

2.5.1 Confirmatory tests for *Staphylococcus aureus*

2.5.1.1 *Gram stain*

A 5µl inoculum of the organism was aseptically removed from the culture plate and emulsified with a drop of sterile saline onto a glass slide and heat fixed. Gram stain reagents (BDH Laboratory supplies 351936R) were used for the test. The slide was flooded with crystal violet dye and left on for 45s. This was then rinsed out with deionised water, and the excess discarded. Lugol solution was applied for 45s to fix the crystal violet to the wall left on and then washed out. Decolourisation was carried out for 15s to 30s and then washed out. Safranin was used as a counterstain and applied for 30s, rinsed and then blot dried. Retention of the crystal violet after decolourisation produced a purple stain in Gram-positive cells. Typical *S. aureus* strains were Gram-positive cocci in pairs, tetrads and grape-like clusters.

2.5.1.2 *Catalase test*

A small quantity of the organism from a fresh culture was aseptically placed on a sterile Petri dish and one drop of Catalase reagent (Sigma Aldrich 7722-84-1) added to the sample. Catalase is an enzyme that breaks up hydrogen peroxide into water and oxygen in the cell before it can do any cell damage. The reaction, if positive, produces free oxygen and water from hydrogen peroxide and this is identified by the production of bubbles. *S. aureus* is catalase positive.

2.5.1.3 *Coagulase test*

Staphytect Plus (Oxoid DR0850), a latex slide agglutination test, was used to confirm the presence of a clumping factor in *S. aureus* strains according to manufacturer’s instructions. An identifying characteristic of *S. aureus* is its ability to produce free and bound coagulase (or clumping factor).

2.5.1.4 *Methicillin- resistant Staphylococcus aureus (MRSA) selective agar*

Bacterial isolates were screened for methicillin resistance by growth on mueller-Hinton agar (Amyl-media AM 102). Media was prepared in accordance with the manufacturer's instructions and supplemented with 4%NaCl (Sigma-aldrich Chemicals S7653) and 6µg of oxacillin (Sigma-aldrich Chemicals 28221) per millilitre. Plates inoculated with 1µl of a bacterial suspension (10^8cells/ml, 0.5 McFarland standard) were incubated for 24h at 35°C and examined for the presence of growth. A duplicate 10µl sample was inoculated onto Brilliance™ MRSA plates (Oxoid PO5196A).

2.5.2 Confirmatory tests *Pseudomonas aeruginosa*

2.5.2.1 *Gram stain*

A 5µl inoculum of the organism was aseptically removed from the culture plate and emulsified with a drop of sterile saline onto a glass slide and heat fixed. Gram stain reagents (BDH Laboratory supplies 351936R) were used for the test. The slide was flooded with crystal violet dye and left on for 45s. This was then rinsed out with deionised water, and the excess discarded. Lugol solution was applied for 45s to fix the crystal violet to the wall left on and then washed out. Decolourisation was carried out for 15s to 30s and then washed out. Safranin was used as a counterstain and applied for 15s, rinsed and then blot dried. The Gram-stain for *Pseudomonas aeruginosa* showed a Gram-negative rod measuring about 0.5 to 0.8µm by 1.5 to 3.0µm.

2.5.2.2 *Oxidase test*

A small quantity of the organism from a fresh culture was placed on an oxidase strip (Oxoid MB 0266A) and the oxidase reaction recorded after 20s as described by the manufacturer. The dye is normally colourless in the reduced state, but is rapidly oxidised to a purple colour by 'oxidase-positive' species (which contain a c-type cytochrome). *P. aeruginosa* is characterised by a positive oxidase test.

2.5.2.3 *Cetrimide selective agar (CSA)*

Cetrimide selective agar (Amyl-media: AM 27) was prepared in accordance with the manufacturer's instructions. It was sterilised by autoclaving at 121°C for 15min. Once the medium had cooled to 50°C in a waterbath, the medium was poured into sterile Petri dishes (15-20ml per plate) and allowed to set. The plates were then dried for a minimum of 24h and stored at room temperature for a maximum of one week. Inoculated plates were examined for the presence of characteristic blue, blue-green, or yellow-green pigment. *Pseudomonas aeruginosa* typically produces both pyocyanin and fluorescein.

2.6 Bacteriophage Isolation, Purification and Propagation

2.6.1 Preparation of bacterial lawns

One bead of the required *S. aureus* or *P. aeruginosa* strain was sub-cultured in 3ml nutrient agar broth (section 2.2.1) under aerobic conditions at 37°C for 16-18h. The bacterial suspension (200µ1 for *S. aureus* and 100µl for *P. aeruginosa*) was gently mixed with 3ml nutrient semi-solid agar (section 2.3.1) tempered at 45ºC. This was then poured onto pre-dried nutrient agar plates (section 2.2.2) and plates gently rotated to produce an even lawn. The overlays were left to set at room temperature for 20-60min before use.

2.6.2 Isolation of bacteriophage from solid samples

A 10% (w/v) solution of the solid sample was made in SM buffer and then left with vigorous shaking overnight at 4°C. The sample was then left at room temperature for 2h to allow the solid matter to sediment. The supernatant was then transferred on to a sterile 50m1 tube and centrifuged at 10000rpm for 10min. The supernatant was then filtered through a 0.22µm pore size filter (33mm-Millex Millipore PES membrane) and stored at 4˚C until needed for testing. Bacterial lawn plates were made using a suspension of a host strain (section 2.1.1).

The filtered supernatants were use for bacteriophage isolation as follows:

2.6.2.1 *Direct plating method:*

In the direct plating method also known as the pour plate method (Adams, 1959), 100µl of the filtered pre-concentrated water sample (Amicon-15 centrifugal filters, Millipore, Cat N˚UFC901008 at 10000rpm x 15min) was mixed with either 200µl or 100µl of the bacterial suspension of interest (section 2.6.1) and incubated at 37ºC for 10min. The mix was then added to 3m1 of nutrient semi-solid media (section 2.3.1) tempered at 45ºC and poured onto pre-dried nutrient agar plates (section 2.2.2).

2.6.2.2 *Spot test method:*

In this method, 200µl or 100µl of the bacterial suspension of interest (section 2.6.1) were gently mixed with 3ml of nutrient semi-solid agar (section 2.3.1) tempered at 45°C. This was then poured onto pre-dried nutrient agar plates (section 2.2.2). Plates were dried at room temperature for 20-60min. Twenty microliters of the filtered solution in duplicate was spotted on top of the lawns and allowed to dry with the lids slightly open for 20min. Plates were incubated under aerobic conditions at 37°C and inspected for phage plaques after 24h. When a plaque was observed, it was picked by using a sterile 200µl pipette-tip and resuspended in 300µl of SM buffer (section 2.3.2) with 80µl of chloroform. The selected plaque was left overnight at 4°C to allow the phage to resuspend in the buffer and the suspension was used to purify the phage as described in (section 2.6.2.1). Bacteriophage suspensions were diluted to obtain 10-50 plaques maximum per plate to reduce count errors. After a minimum of five passages, the suspension was considered to contain a single bacteriophage clone (section 2.6.4).

2.6.2.3 *Enrichment method*

In the enrichment method, filtered water samples were grown with the bacterial isolate of interest. Nine milliliters of the sample of interest was mixed with 1ml of 10-times strength nutrient broth and 100µl of the overnight culture in a 50ml sterile tube. Control tubes were set up with sterile filtered water. Tubes were incubated overnight at 37˚C and then refiltered and tested for bacteriophages by method 2.6.2.1 when the turbidity of the tube

was not visually different to that of the control (suggesting no phage activity) or by method 2.6.2.2 when the test tube was less turbid than the control tube. Any plaques observed were picked and purified as described in section 2.6.4.

2.6.2.4 *Modified direct plating method:*

Using a modified pour plate method, 2ml of filtered sample tempered at 45°C were mixed with either 200µl or 100µl of the bacterial suspension of interest (section 2.6.1) and incubated at 37°C for 10min. The mixture was then added to 1m1 of modified nutrient semi-solid medium (3x NSA) (section 2.3.1) tempered at 50°C, and the mix was poured onto pre-dried nutrient agar plates (section 2.2.2).

2.6.3 Isolation of bacteriophage from sewage samples

Sewage samples were obtained from Westmead Hospital and other small privately owned sewage treatment plants located in New South Wales. Samples were centrifuged (4000rpm x10min) and filtered through a 0.22µm (33mm Millipore PES membrane filters) to eliminate present bacteria. Samples were tested for the presence of bacteriophage as with solid samples, described above (section 2.6.2).

2.6.4 Bacteriophage purification

Samples processed for bacteriophage isolation that produced clear lysis on a bacterial lawn from the spot method (section 2.6.2.1) were cored using a 1ml pipette-tip and suspended in 300µl SM buffer (section 2.3.2). Following the direct plating method (section 2.6.2.1), individual plaques formed were removed as plugs and placed in individual Eppendorf tubes containing 300µl SM buffer. Individual plugs were then passaged at least five times in order to obtain a suspension of 'pure' bacteriophage, which could be used for propagation (2.6.5).

2.6.5 Bacteriophage propagation

The propagating strain was sub-cultured in 3m1 nutrient broth (section 2.2.1) under aerobic conditions at 37°C for 16-24h. Two hundred microliters an adequate dilution of the

bacteriophage suspension was mixed with 200µl or 100µl (section 2.6.1) of the corresponding propagating strain suspension and incubated for 10min at 37°C in order to allow bacteriophage to adsorb and replicate. Each suspension was then aliquoted to 3ml of nutrient semi solid agar (section 2.3.1), tempered to 45°C in the waterbath and applied to the surface of nutrient agar plates (section 2.2.2). The overlays were left to set at room temperature for 20min then incubated under aerobic conditions at 37°C for 24h. Salt magnesium buffer (section 2.3.2) was added to the surface of each plate (5ml) and the bacteriophage allowed to elute onto the buffer overnight at 4°C. The final harvested bacteriophage was filtered (33mm Millipore 0.22µm PES membrane syringe filters) into a 5ml gamma-sterilized dark tube and stored at 4°C.

2.6.6 Bacteriophage titration

2.6.6.1 *Spot test method*

To 900µ1 of SM buffer (section 2.3.2) in a sterile Eppendorf tube, 100µl of bacteriophage suspension were added and mixed well to give a dilution factor of 10^{-1}. This ten-fold serial dilution was diluted down to 10^{-8} dilution factor. Two drops of 10µl from each dilution (starting with the highest dilution) were spotted on to a bacterial lawn plate of the appropriate *S. aureus* or *P. aeruginosa* strain (section 2.6.1) or a full plate prepared. The spots were applied onto the plate from left to right, ending with the undiluted bacteriophage suspension to the right side of the plate. Once the spots were dried, the plates were incubated under aerobic conditions at 37°C for 24h. The lysis produced and the number of plaques formed was recorded. The number of plaque forming units (PFU) per millilitre in the bacteriophage suspension was calculated from the total dilution.

2.6.6.2 *The double layer agar method*

Ten-fold serial dilution of a bacteriophage suspension was diluted down to the required dilution factor. Then 100 µl of the required phage dilution was mixed with either 200µl or 100µl of the bacterial suspension of interest (section 2.6.1) and incubated at 37°C for 10min. The mix was then added to 3m1 of nutrient semi-solid media (section 2.3.1) tempered at 45°C and poured onto pre-dried nutrient agar plates (section 2.2.2). The plate

was dried and then incubated under aerobic conditions at 37°C for 24h. The number of plaque forming units (PFU) per millilitre in the bacteriophage suspension was calculated from the total dilution.

2.6.7 Lytic spectrum of bacteriophage

Ten microliters of each plaque purified bacteriophage isolate was spotted in duplicate onto bacterial lawns (section 2.6.1) of the reference and clinical isolates (Table 2-1 to 2-4). Experiments when possible were repeated a minimum of three times. Positive reactions (+) were recorded when a minimum of 10 individual plaques (IP) was observed. The presence of IP was noted and their clarity scored as turbid (+), clearing with hazy-partial background (++) and complete clearing (+++). If a confluent spot was observed its intensity was also scored as turbid (+), clearing with hazy-partial background (++) and complete clearing (+++). Negative reactions were recorded as (-).

2.6.8 Preparation of bacteriophage for electron microscopy examination

The purified and propagated bacteriophage suspensions (2.6.5) were centrifuged and washed three times with ammonium acetate 0.1M (pH 7.0) at 22000*g* for 60min. If necessary, due to excessive breakage of tails or amorphous structures, bacteriophage preparations were fixed with 2% paraformaldehyde (Sigma-aldrich P6148) for 30min on the bench prior to centrifugation. For staining, a 15µl drop of the washed phage suspension and the stain uranyl acetate 2% (pH 4.0) were deposited separately over parafilm with a Pasteur pipette. A 0.3% pioloform coated 300-mesh grid was first dropped on top of the phage suspension for 1-10min. The grid was lifted and the excess liquid gently withdrawn with filter paper. The grid loaded with the sample was then dropped on top of the stain for 1-5min. Any excess stain was withdrawn with filter paper. The grid was immediately examined with a JEOL IOOCX Transmission Electron Microscope operating at an acceleration voltage of 100kV.

3 ISOLATION OF *STAPHYLOCOCCUS AUREUS* AND *PSEUDOMONAS AERUGINOSA* BACTERIOPHAGES

3.1 Introduction

The emergence of drug resistant bacterial pathogens has renewed the interest in bacteriophage therapy in the West (section 1.3). The selection of potential therapeutic phages involves a series of sequential steps. The first critical step is the selection of the target bacteria to be used during the isolation process. Care must be taken to ensure the bacteria represent the population that the isolated phages should be able to infect. Once the target bacteria have been selected the isolation of specific bacteriophages can commence. This isolation step starts with the collection of environmental samples from places where the particular bacteria of interest are known to reside. As demonstrated previously, (Chibani-Chennoufi et al., 2004, Vinod et al., 2005, Carey-Smith et al., 2006, Moineau et al., 1996, Loc-Carrillo et al., Unknown, Atterbury et al., 2003), the likelihood of bacteriophage isolation increases if the bacterial host is present in the environmental sample. Liquid or solid test samples are then processed for bacteriophage isolation, purification and propagation.

Staphylococcus aureus and *P. aeruginosa* bacteriophages were previously isolated from different types of environmental samples (Budzik et al., 2004, Son et al., 2010, Flaherty et al., 2005, Jensen et al., 1998a, Synnott et al., 2009, Garcia et al., 2009). This section describes the three first steps in the development of therapeutic bacteriophage libraries against *S. aureus* and *P. aeruginosa*. The establishment of the target bacteria, the isolation of virulent bacteriophages from water samples collected in Australia and the testing for bacteriophage lytic host range. This collection of bacteriophages will represent the first set of potential therapeutic bacteriophages isolated in Australia with the potential to treat infections caused by these pathogens.

3.2 Bacterial Identification

For the isolation of novel bacteriophages, a reference bacterial collection of both *S. aureus* and *P. aeruginosa* isolates was established on the basis of their antibiotics profiles and/or their known genotypic characteristics (Table 1-4). All isolates received from the hospitals were confirmed upon arrival as per section 2.5.

3.2.1 *Staphylococcus aureus* identification

Each *S. aureus* strain was identified as a Gram-positive, cluster-forming coccus, which were catalase and coagulase positive (section 2.5.1). All strains were confirmed as methicillin-resistant *Staphylococcus aureus* (MRSA) after positive growth was observed in the screening Mueller-Hinton agar (2.2.5) (Hindler and Warner, 1987) and the commercial already made Brilliance™ MRSA Chromogenic medium (Oxoid PO1162). Community-acquired MRSA were identified by the presence of the PVL gene (Johnsson et al., 2004) at the microbiology unit of the Royal North Shore hospital.

3.2.2 *Pseudomonas aeruginosa* identification

Pseudomonas aeruginosa strains were identified as Gram-negative rods with no particular arrangement, were oxidase positive and catalase positive. All the strains showed positive growth on the selective cetrimide medium (2.2.4). The strains appeared blue-green in colour, flat, large, and oval shape with a characteristic fruity smell. Susceptibility to antibiotics was performed at the microbiology unit of the Westmead hospital by using the VITEK system (Canton et al., 2001).

3.3 Selection of Bacterial Isolates for Bacteriophage Isolation

3.3.1 *Staphylococcus aureus* indicator bacteria

For bacteriophage isolation, a rotational system was established to ensure all bacterial isolates from the reference collection (Table 2-1) and from the clinical isolates collection (Appendix 1) were used as indicator bacteria at least once. Bacterial isolates were

processed in numerical order in groups of ten against every environmental sample received (section 2.6).

3.3.2 *Pseudomonas aeruginosa* indicator bacteria

Primarily, a collection of ten *P. aeruginosa* was used as indicator bacteria for the isolation of specific phages (Table 2-2). Strains were identified as multi-drug resistant *Pseudomonas aeruginosa* (MRPA), if resistance to three or more classes of antibiotics was observed (Falagas et al., 2006, Souli et al., 2008). These ten isolates were selected for bacteriophage isolation not only because of the decreased sensitivity to antibiotics but also because they were pathogens that caused a number of clinical outbreaks and complications in patients with different conditions at the intensive care unit from 2005-2007 (Iredell - personal communications). Thus, from a clinical point of view, it was useful to target these particular isolates. However, a rotational system was also established to use all isolates available (Appendix 2) as indicator bacteria at least once during the isolation process. In addition, *P. aeruginosa* PA01 (ATCC™ Number15442**)** was also added to the reference collection as an indicator bacteria (Table 2-3).

3.4 Bacteriophage Isolation

3.4.1 Samples for bacteriophage isolation

Sewage samples from Westmead Hospital in Sydney and other small private sewage treatment plants of several facilities, located in New South Wales, were obtained to investigate the presence of bacteriophages. A total of 150 samples were received and processed for bacteriophages from June 2006 to June 2008. Liquid and solid samples were processed upon arrival, as described before (section 2.6.3.), and investigated for bacteriophages within a week.

3.4.2 Bacteriophage isolation protocols

Bacteriophages were isolated from soil and sewage samples as described in sections 2.6.2 and 2.6.3 respectively. Bacteriophages were plaque purified, amplified and stored as described in section 2.6.4 and 2.6.5.

3.5 Bacteriophage Lytic Host Range

The lytic activity of isolated bacteriophages was determined against the reference strains (Tables 2-1 and 2-3). Lytic host range was tested as described in section 2.6.7.

3.6 Results

3.6.1 *Staphylococcus aureus* bacteriophages

Fifty-eight phages with lytic activity against *S. aureus* isolates were isolated from 180 sewage samples collected between June 2006 and June 2009. Phages were designated as Sa 1 to Sa 58 as the purification (section 2.6.4) was completed.

During the isolation process, 20 *S. aureus* strains, out of 120 strains in both collections (Table 2-1 and Appendix 1), were useful for the isolation of bacteriophages. From the reference collection (Table 2-1) isolates SPS# 621 (*mec* I), SPS# 624 (*mec* II), SPS#626 (*mec* III), SPS# 631 (*mec* V) and SPS# 548 (*mec* V- non USA type) were positive for bacteriophage isolation. From the collection of clinical isolates (Appendix 1), six human clinical isolates recovered from the nose (SPS # 25, 287, 295, 306, 309, and 316), four from wounds (SPS #289, 297, 308, 320), two from sputum (SPS # 304, 317), two from groin samples (SPS# 293 and SPS#310) one from a eye infection (SPS#296) were positive for bacteriophage isolation. The list of each isolated phage with its corresponding indicator strain and plaque morphology is present

Table 3-1. List of *Staphylococcus aureus* bacteriophages isolated.

Phage	strain #	Plaque Description
Sa 1	279	2mm FA
Sa 2	279	1-2mm FA
Sa 3	60	1-2mm FA
Sa 4	60	1-2mm FA
Sa 5	25	2mm FA
Sa 6	25	<1mm PA
Sa 7	25	1-2mm FA
Sa 8	60	<1-1mm PA-FA
Sa 9	316	1-2 mm PA-FA
Sa 10	25	1mm PA-FA
Sa 11	60	1-2mm PA-FA
Sa 12	316	2mm FA
Sa 13	25	1mm FA
Sa 14	25	1mm FA
Sa 15	25	2mm FA
Sa 16	25	1mm FA
Sa 17	25	1mm FA
Sa 18	316	2mm FA
Sa 19	316	1-2mm FA
Sa 20	25	2mm FA
Sa 21	25	1-2mm PA
Sa 22	631	2mm FA
Sa 23	624	1-2mm FA
Sa 24	624	1-2mm FA
Sa 25	631	1-2mm PA-FA
Sa 26	25	1mm FA
Sa 27	621	<1-1mm PA-FA
Sa 28	626	2mm FA
Sa 29	624	1mm FA

Phage	strain #	Plaque Description
Sa 30	621	1-2mm FA
Sa 31	548	1-2mm FA
Sa 32	293	1mm FA
Sa 33	293	2mm FA
Sa 34	317	2mm FA
Sa 35	289	1mm FA
Sa 36	296	1mm FA
Sa 37	316	1-2mm FA
Sa 38	287	1-2mm FA
Sa 39	308	1mm FA
Sa 40	310	1-2mm FA
Sa 41	308	2-3mm FA
Sa 42	308	2mm FA
Sa 43	297	1mm FA
Sa 44	320	1mm FA
Sa 45	287	Pinprick FA
Sa 46	297	1mm PA-FA
Sa 47	316	1mm FA
Sa 48	293	1mm FA
Sa 49	293	1mm FA
Sa 50	309	<1mm FA
Sa 51	310	1-2mm FA
Sa 52	309	<1mm FA
Sa 53	296	<1-1mm FA
Sa 54	295	1-2mm FA
Sa 55	308	2mm PA-FA
Sa 56	304	2mm FA
Sa 57	317	1-2mm FA
Sa 58	306	1mm FA

Bacteriophages plaque sizes (mm) and clarity of the plaque scored as FA (full activity or complete clearing) or PA (partial activity only with turbid or opaque zones) are described against the indicator bacteria

All *S. aureus* bacteriophages isolated were spot tested in duplicate (2.6.7), a minimum of three times, against the reference strains (Table 2-1) to assess their host spectrum. The results are presented in Table 3.2.

Due to the large number of phages effective against the reference collection, their "ratio of infection" was analysed to select those with the broadest spectrum of activity against the reference collection. The "ratio of infection" was defined as the number of isolates the phage infected over the total number of isolates tested. Bacteriophages with a ratio of infection ≥ 0.7 were further considered (Table 3-3).

The patterns of activity of 12 bacteriophages isolated with SPS#25 were also analysed. When tested against the 19 isolates in the reference collection, 12 phages produced 14 different phage patterns as shown in Table 3-4.

Table 3-2. **Host range of *Staphylococcus aureus* bacteriophages isolated against the reference collection (Table 2-1).**

	544	545	546	547	548	621	622	623	624	625	626	627	628	629	630	631	632	633	634
Sa 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sa 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sa 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sa 4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sa 5	+	+	++	+	+	+	+	+	+	++	+	+	+	+	+	+	+	++	+
Sa 6	+	+	++	+	+	++	+	+	+++	+++	+	+	+	+	+	+	+	++	+
Sa 7	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Sa 8	-	-	-	-	-	+	-	-	+++	-	-	-	-	-	-	+++	-	-	-
Sa 9	+++	+	+	+	+++	+	+++	+	+++	+++	+	+++	+	+++	+++	+	+++	+	+++
Sa 10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sa 11	-	+	-	-	-	+	-	-	+++	-	-	-	-	-	-	+++	-	+	-
Sa 12	+++	-		-	+++	+++	+	+	+++	+++	+++	+++	-	+++	-	+++	+++	+++	+++
Sa 13	-	+	+	-	+	-	-	+	-	+	-	+	-	-	-	-	-	-	-
Sa 14	+	+	+++	+	+	+	+++	+++	+	+++	+++	-	+	+++	+++	+	+	-	+
Sa 15	+	+	-	-	+	-	+	-	-	+	-	-	-	+	+	-	+	+	+
Sa 16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sa 17	-	+	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-
Sa 18	++	++	-	+++	++	-	+++	-	+++	++	++	-	++	+	+++	-	+++	+++	++
Sa19	+	-	-	+++	+	+++	+	+	+++	-	-	-	-	-	-	+++	+++	+++	-
Sa20	-	-	-	-	-	+++	+	+	+++	+	+	-	-	-	-	+++	+++	+++	-
Sa21	-	-	-	-	-	+++	+	+	+++	+	+	-	+	-	-	+++	+++	+++	-
Sa22	++	+	++	+++	+	+++	+	+	+++	+	+	-	+	-	-	+++	+++	+++	-

continued Table 3-2.

	544	545	546	547	548	621	622	623	624	625	626	627	628	629	630	631	632	633	634
Sa23	-	-	-	-	+++	+++	+++	+++	+++	+++	+	+	+++	+	+	+++	+++	+++	+
Sa24	-	-	-	-	-	+++	+++	+++	+++	+++	+	+	+++	+++	+++	+++	+++	+	+
Sa25	++	++	++	+++	++	IP +	IP +	IP+++	IP+++	IP +	IP +	-	-	-	-	IP +	IP+++	IP +	-
Sa26	-	-	-	-	-	+++	+++	+++	+++	IP+++	+	-	+	-	-	+++	+++	+++	-
Sa27	-	-	-	-	-	+++	+	+	+++	+	+	-	+	-	-	+++	+++	+++	-
Sa28	-	-	-	-	-	+++	+	+	+++	+	+	-	+	-	-	+++	+++	+++	-
Sa29	-	+	++	+	-	+++	+	+	+++	+	+	-	+++	-	-	+++	+++	+++	-
Sa30	+	+	-	-	-	+++	+	+	+++	+++	+	-	+	-	-	+++	+++	+++	-
Sa31	+	+++	+	+	+++	+	+++	+	+++	+++	+	+	+++	+	+	+++	+++	+++	+
Sa32	+++	+++	+++	+++	+	+++	+	+++	+++	+	+++	+	+++	+	+	+++	+++	+++	+
Sa33	-	+++	+	+++	-	+++	+++	+++	+++	+	+	-	+	-	-	+++	+++	+++	-
Sa34	+++	+++	+++	+++	+	+++	+++	+	+++	+	+	+++	+++	+	+	+++	+++	+++	+
Sa35	-	+++	+	+++	-	+++	+	+	+++	+	+	-	+	-	-	+++	+++	+++	-
Sa36	+++	IP+++	+	+++	-	+++	+	+++	+++	+	+	-	-	-	-	+++	+++	+++	-
Sa37	-	IP+++	-	+++	-	+++	+++	+++	+++	+	+	-	-	-	-	+++	+++	+++	-
Sa38	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+++	+++	+++
Sa39	-	+++	+	+++	-	+++	+++	+++	+++	+	+	-	+	-	-	+++	+++	+++	-
Sa40	-	IP+++	-	+++	-	+++	+	+	+++	+	+	-	-	-	-	+++	+++	+++	-
Sa41	+	+++	+++	+++	+	+++	+++	+	+++	+	+	+++	+++	+++	+	+++	+++	+++	+
Sa42	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Sa43	+	+++	-	+++	+	+++	+++	+++	+++	+	+	+	+++	+++	+	+++	+++	+++	+++
Sa44	-	+++	-	+++	++	+++	+++	+++	+++	+++	+++	-	+	-	-	+++	+++	+++	-

continued Table 3-2.

	544	545	546	547	548	621	622	623	624	625	626	627	628	629	630	631	632	633	634
Sa45	+	+++	+	+++	-	+++	+++	+++	+++	+++	+	-	+	-	-	+++	+++	+++	-
Sa46	-	+	-	+++	-	+++	+	+	+++	-	-	-	-	-	-	+++	+++	+++	-
Sa47	-	+	+	+++	-	+++	+	+	+++	+	+	-	-	-	-	+++	+++	+++	-
Sa48	+	+	+	+++	-	+++	+	+	+++	+	+	-	+	-	-	+++	+++	+++	-
Sa49	-	+	-	+++	-	+++	+	+	+++	+	+	-	+	-	-	+++	+++	+++	-
Sa50	+	+	+	+++	+	+++	+++	+++	+++	+++	+	+	+++	+	+	+++	+++	+++	+
Sa51	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Sa52	-	IP +	-	IP+++	-	IP+++	IP+++	IP+++	IP+++	IP +	IP +	-	-	-	-	IP+++	IP+++	IP+++	-
Sa53	+	+++	+	+++	++	+++	+++	+++	+++	+++	+	-	+	-	-	+++	+++	+++	-
Sa54	-	-	++	+++	+++	+++	+	++	+++	+	+	+++	++	-	-	+++	+++	+++	-
Sa55	+	+	+	+++	-	+++	+	+	+++	+	+	-	+	-	-	+++	+++	+++	-
Sa56	+	+	+	+++	-	+++	+	+	+++	+	+	-	+++	-	-	+++	+++	+++	-
Sa57	+	+	+	+++	-	+++	+	+	+++	+++	+	-	+	-	-	+++	+++	+++	-
Sa58	+++	+++	+++	+++	+	+++	+	+++	+++	+	+++	+	+++	+	+	+++	+++	+++	+

Reference strains are presented from left to right and phages from top to bottom. Positive Positive reactions (+) were recorded when a minimum of 10 individual plaques (IP) was observed. The clarity of the IP or the spot was scored as turbid (+), clearing with hazy-partial background (++) and complete clearing (+++). Negative reactions were recorded as (-).

Table 3-3. **List of selected *S. aureus* bacteriophages based on their "ratio of infection"(ROI). The ROI is defined as the number of isolates the phage infected over the total number of isolates tested.**

	544	545	546	547	548	621	622	623	624	625	626	627	628	629	630	631	632	633	634	ROI	ROI (≥++)
Sa 5	+	+	++	+	+	+	+	+	+	++	+	+	+	+	+	+	+	++	+	1.0	0.3
Sa 6	+	+	++	+	+	++	+	+	+++	+++	+	+	+	+	+	+	+	++	+	1.0	0.3
Sa 9	+++	+	+	+	+++	+	+++	+	+++	+++	+	+++	+	+++	+++	+	+++	+	+++	1.0	0.5
Sa 31	+	+++	+	+	+++	+	+++	+	+++	+++	+	+	+++	+	+	+++	+++	+++	+	1.0	0.5
Sa 32	+++	+++	+++	+++	+	+++	+	+++	+++	+	+++	+	+++	+	+	+++	+++	+++	+	1.0	0.7
Sa 34	+++	+++	+++	+++	+	+++	+++	+	+++	+	+	+++	+++	+	+	+++	+++	+++	+	1.0	0.7
Sa 38	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+++	+++	+++	1.0	0.9
Sa 41	+	+++	+++	+++	+	+++	+++	+	+++	+	+	+++	+++	+++	+	+++	+++	+++	+	1.0	0.9
Sa 42	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	1.0	0.9
Sa 50	+	+	+	+++	+	+++	+++	+++	+++	+++	+	+	+++	+	+	+++	+++	+++	+	1.0	0.5
Sa 51	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	1.0	1.0
Sa 58	+++	+++	+++	+++	+	+++	+	+++	+++	+	+++	+	+++	+	+	+++	+++	+++	+	1.0	0.6
Sa 14	+	+	+++	+	+	+	+++	+++	+	+++	+++	-	+	+++	+++	+	+	-	+	0.9	0.4
Sa 43	+	+++	-	+++	+	+++	+++	+++	+++	+	+	+	+++	+++	+	+++	+++	+++	+++	0.9	0.7
Sa 22	++	+	++	+++	+	+++	+	+	+++	+	+	-	+	-	-	+++	+++	+++	-	0.8	0.4
Sa 23	-	-	-	-	+++	+++	+++	+++	+++	+++	+	+	+++	+	+	+++	+++	+++	+	0.8	0.5
Sa 53	+	+++	+	+++	++	+++	+++	+++	+++	+++	+	-	+	-	-	+++	+++	+++	-	0.8	0.7
Sa 12	+++	-		-	+++	+++	+++	+++	+++	+++	+++	+++	-	+++	-	+++	+++	+++	+++	0.7	0.7
Sa 18	++	++	-	+++	++	-	+++	-	+++	++	++	-	++	+	+++	-	+++	+++	++	0.7	0.7
Sa 24	-	-	-	-	-	+++	+++	+++	+++	+++	+	+	+++	+++	+++	+++	+++	+	+	0.7	0.5
Sa 25	++	++	++	+++	++	+	+	IP+++	IP+++	+	+	-	-	-	-	+	IP+++	I+	-	0.7	0.4

continued Table 3-3.

	544	545	546	547	548	621	622	623	624	625	626	627	628	629	630	631	632	633	634	ROI	ROI (≥++)
Sa 29	-	+	++	+	-	+++	+	+	+++	+	+	-	+++	-	-	+++	+++	+++	-	0.7	0.4
Sa 33	-	+++	+	+++	-	+++	+++	+++	+++	+	+	-	+	-	-	+++	+++	+++	-	0.7	0.5
Sa 35	-	+++	+	+++	-	+++	+	+	+++	+	+	-	+	-	-	+++	+++	+++	-	0.7	0.4
Sa 36	+++	IP++ +	+	+++	-	+++	+	+++	+++	+	+	-	-	-	-	+++	+++	+++	-	0.7	0.5
Sa 37	-	IP++ +	-	+++	-	+++	+++	+++	+++	+	+	-	-	-	-	+++	+++	+++	-	0.7	0.5
Sa 39	-	+++	+	+++	-	+++	+++	+++	+++	+	+	-	+	-	-	+++	+++	+++	-	0.7	0.5
Sa 44	-	+++	-	+++	++	+++	+++	+++	+++	+++	+++	-	+	-	-	+++	+++	+++	-	0.7	0.7
Sa 48	+	+	+	+++	-	+++	+	+	+++	+	+	-	+	-	-	+++	+++	+++	-	0.7	0.3
Sa 54	-	-	++	+++	+++	+++	+	++	+++	+	+	+++	++	+++	-	+++	+++	+++	-	0.7	0.7
Sa 55	+	+	+	+++	-	+++	+	+	+++	+	+	-	+	-	-	+++	+++	+++	-	0.7	0.3
Sa 56	+	+	+	+++	-	+++	+	+	+++	+	+	-	+++	-	-	+++	+++	+++	-	0.7	0.4
Sa 57	+	+	+	+++	-	+++	+	+	+++	+++	+	-	+	-	-	+++	+++	+++	-	0.7	0.4

Reference strains are presented from left to right and phages from top to bottom Calculated ROI highlighted in green includes all positive reactions while the ROI (≥ ++) includes reactions that were ≥ ++. ROIs of S. aureus bacteriophages selected for further characterization are highlighted in orange.

Table 3-4. Host range patterns of bacteriophages isolated with SPS# 25 against the reference collection

	544	545	546	547	548	621	622	623	624	625	626	627	628	629	630	631	632	633	634
Sa 5	+	+	++	+	+	+	+	+	+	++	+	+	+	+	+	+	+	++	+
Sa 6	+	+	++	+	+	++	+	+	+++	+++	+	+	+	+	+	+	+	++	+
Sa 7	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Sa 10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sa 13	-	+	+	-	+	-	-	+	-	+	-	+	-	-	-	-	-	-	-
Sa 14	+	+	+++	+	+	+	+++	+++	+	+++	+++	-	+	+++	+++	+	+	-	+
Sa 15	+	+	-	-	+	-	+	-	-	+	-	-	-	+	+	-	+	+	+
Sa 16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sa 17	-	+	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-
Sa20	-	-	-	-	-	+++	+	+	+++	+	+	-	-	-	-	+++	+++	+++	-
Sa21	-	-	-	-	-	+++	+	+	+++	+	+	-	+	-	-	+++	+++	+++	-
Sa26	-	-	-	-	-	+++	+++	+++	+++	IP+++	+	-	+	-	-	+++	+++	+++	-
	1	3	8	13	9	4	5	11	6	12	14	4	10	2	2	14	5	7	2

From left to right reference strain SPS# and from top to bottom isolated bacteriophage Sa. The numbers at the end of each column represent The bacteriophage pattern assigned to the particular reference isolate when tested for sensitivity against the 12 bacteriophages isolated using the clinical strain SPS#25 only as indicator bacteria. Fourteen different phage patterns were identified.

3.6.2 *Pseudomonas aeruginosa* bacteriophages

Fifty-two phages with lytic activity against *P. aeruginosa* isolates were isolated from 150 sewage and soil samples collected between June 2006 and June 2008. Phages were designated as Pa 1 to Pa 52 as the purification process (section 2.6.4) was completed.

In total, ten *P. aeruginosa* strains were useful during the bacteriophage isolation process. From the reference collection (Table 2-3) only one strain SPS# 273, isolated from human blood, resistant to Trimethoprim / Sulfamethoxazole (STX) and with intermediate resistant to imipenem was positive for bacteriophage isolation. From the collection of clinical isolates (Appendix 2), SPS# 110, 264, 277, 278, 284, 323, 433, 439 and 445 were positive for bacteriophage isolation. The list of isolated phages with their corresponding indicator strains and plaque morphology is presented in Table 3-3.

All *P. aeruginosa* bacteriophages isolated were spot tested in duplicate (2.6.7) a minimum of three times, against the reference strains (Table 2-3) to assess their lytic spectrum of activity. The results are presented in Table 3-4.

Table 3-5. **List of *P. aeruginosa* bacteriophages isolated.**

Phage	strain #	Plaque Description
Pa 1	433	3-4mm FA
Pa 2	433	2mm PA-FA
Pa 3	439	3mm FA
Pa 4	439	2mm FA centre, PA halo
Pa 5	439	1mm FA
Pa 6	273	2mm FA, PA halo
Pa 7	273	1mm FA
Pa 8	433	4-5 mm FA
Pa 9	433	1-2mm FA
Pa 10	323	1mm FA
Pa 11	323	1mm FA
Pa 12	278	2mm FA
Pa 13	278	2mm FA, double halo
Pa 14	278	1-2mm FA irregular edges
Pa 15	278	3mm FA, PA halo
Pa 16	277	1-2mm FA
Pa 17	277	2mm FA
Pa 18	277	2mm FA, small halo
Pa 19	323	3mm FA
Pa 20	439	1mm PA-FA
Pa 21	433	4-5mm FA
Pa 22	439	3-4mm FA
Pa 23	323	1-2mm FA
Pa 24	273	2mm FA irregular edge, PA halo
Pa 25	439	2-3mm FA
Pa 26	433	4-5mm FA

Phage	strain #	Plaque Description
Pa 27	433	1mm PA-FA
Pa 28	439	2mm FA
Pa 29	278	2mm PA
Pa 30	323	< 1-1mm FA
Pa 31	323	1mm FA
Pa 32	323	2mm FA centre, PA halo
Pa 33	433	5mm FA
Pa 34	278	3-4mm PA
Pa 35	273	1mm FA
Pa 36	433	4mm FA
Pa 37	439	2mm FA
Pa 38	433	3-4mm FA centre, PA halo
Pa 39	323	1mm FA, PA halo
Pa 40	273	1mm FA, PA halo
Pa 41	110	2-3mm PA
Pa 42	264	1-2mm PA
Pa 43	284	3-4mm FA centre, multiple rings
Pa 44	284	2-3mm FA, PA halo
Pa 45	323	1mm FA, PA halo
Pa 46	323	1mm FA centre, double halo
Pa 47	323	1mm FA, PA halo
Pa 48	323	2mm FA, PA halo
Pa 49	433	2-3mm FA centre, irregular halo
Pa 50	445	2-3mm FA, PA halo
Pa 51	445	1-2mm FA
Pa 52	445	3mm PA

Bacteriophage plaque sizes and clarity of the plaque scored as FA (full activity or complete clearing) or PA (partial activity with turbid or opaque plaques) are described as observed against the indicator bacteria.

Table 3-6. **Host range of *P. aeruginosa* bacteriophages isolated against the reference collection (Table 2-3).**

	266	267	268	269	270	271	272	273	274	275	276
Pa 1	+++	+++	+++	+	+++	+++	++	+++	++	+++	+++
Pa 2	+++	++	+	-	++	+++	+	+++	-	+	-
Pa 3	+++	+	+++	-	+++	+++	++	+++	-	+++	+++
Pa 4	-	-	+	-	++	+++	+	+++	-	+	-
Pa 5	-	-	-	-	-	-	-	+++ IP	-	-	+++ IP
Pa 6	+++	+++	+++	++	++	+++	++	+++	+++	++	-
Pa 7	-	-	-	-	-	-	+	+	-	-	-
Pa 8	++	+	++	-	-	-	-	-	-	+++	-
Pa 9	+++	++	+	-	+	++	+	+++	+	+	-
Pa 10	+++	+++	+++	-	++	+++	++	++	++	++	+++
Pa 11	-	-	-	-	-	+++	+	++	+++	-	+++
Pa 12	-	-	-	-	-	-	+	-	-	-	-
Pa 13	-	-	-	-	-	-	-	-	-	-	-
Pa 14	-	-	-	-	-	-	+	++	-	-	-
Pa 15	-	-	-	-	-	-	-	-	-	-	-
Pa 16	-	-	-	-	-	-	+	-	-	-	-
Pa 17	-	-	-	-	-	-	+	++	+	-	-
Pa 18	-	-	-	-	-	-	+	-	-	-	-
Pa 19	-	-	-	-	-	-	-	-	-	-	+++
Pa 20	-	-	-	-	+	-	-	++	-	-	+
Pa 21	++	+	++	+	-	-	++	-	-	+++	-
Pa 22	-	-	-	-	-	-	++	-	-	-	-

continued Table 3-6.

	266	267	268	269	270	271	272	273	274	275	276
Pa 23	-	-	-	-	-	-	-	-	-	-	++
Pa 24	+++	+++	+++	-	+++	+++	++	+++	-	+++	+++
Pa 25	+++	+++	+++	+++	+++	+	-	+++	-	+++	+++
Pa 26	+++	+++	+++	-	+++	++	-	+++	-	+++	+++
Pa 27	-	-	+++	-	+++	-	-	+++	-	+++	+++
Pa 28	-	-	+++	-	+++	-	-	+++	-	+++	+++
Pa 29	-	-	-	-	-	+++	+	+	+	-	-
Pa 30	-	-	-	-	-	+++	+	+++	+++	-	+++
Pa 31	-	-	-	-	-	-	-	-	-	-	-
Pa 32	+++	+++	+++	-	+++	++	-	+++	++	+++	++
Pa 33	+	-	-	-	++	+++	++	++	+++	-	-
Pa 34	++	-	-	-	-	+++	+++	-	+++	-	-
Pa 35	+++	+++	+++	-	+++	+++	-	+++	-	+++	+++
Pa 36	-	-	-	-	+	-	-	-	-	-	-
Pa 37	+++	+++	+++	-	+++	+++	+++	+++	-	+++	+++
Pa 38	-	-	-	-	-	++	-	+++	-	-	-
Pa 39	-	-	+++	-	+++	-	-	+++	-	+++	+++
Pa 40	-	-	+++	-	+++	+++	++	+++	-	+++	+++
Pa 41	-	-	-	-	-	-	-	-	-	-	-
Pa 42	-	-	-	-	-	+	+	-	-	-	-
Pa 43	-	-	-	+	-	+++	-	+++	+++	-	-
Pa 44	-	-	-	-	-	+++ IP	-	+	-	-	-

continued Table 3-6.

	266	267	268	269	270	271	272	273	274	275	276
Pa 45	+++	+++	+++	-	+++	-	-	+++	-	+++	+++
Pa 46	-	-	-	-	+	-	-	++	-	-	-
Pa 47	+++	+	+++	-	+++	+++	++	+++	-	+++	+++
Pa 48	-	-	+++	-	+++	-	-	++	-	+++	+++
Pa 49	-	-	+++	-	++	++	+	+++	-	+++	+++
Pa 50	-	++ IP	+	+++ IP	+	+	+	+++	+	+	+
Pa 51	-	-	-	-	-	-	-	-	-	-	-
Pa 52	+++ IP	+	+	+++	-	++	+	+++	+	+	+

Reference strains are presented from left to right and phages from top to bottom. Positive reactions (+) were recorded when a minimum of 10 individual plaques (IP) was observed. The clarity of the IP or the spot was scored as turbid (+), clearing with hazy-partial background (++) and complete clearing (+++). Negative reactions were recorded as (-).

Table 3-7. List of selected *P. aeruginosa* bacteriophages based on their "ratio of infection" (ROI). The ROI was defined as the number of isolates the phage infected over the number of isolates tested.

	266	267	268	269	270	271	272	273	274	275	276	ROI	ROI (≥++)
Pa 1	+++	+++	+++	+	+++	+++	+++	+++	++	+++	+++	1.0	0.9
Pa 6	+++	+++	+++	++	++	+++	++	+++	+++	++	-	0.9	0.9
Pa 10	+++	+++	+++	-	++	+++	++	++	++	++	+++	0.9	0.9
Pa 50	-	++	+	+++	+	+	+	+++	+	+	+	0.9	0.3
Pa 52	+++	+	+	+++	-	++	+	+++	+	+	+	0.9	0.4
Pa 3	+++	+	+++	-	+++	+++	++	+++	-	+++	+++	0.8	0.7
Pa 9	+++	++	+	-	+	++	+	+++	+	+	-	0.8	0.4
Pa 24	+++	+++	+++	-	+++	+++	++	+++	-	+++	+++	0.8	0.8
Pa 25	+++	+++	+++	+++	+++	+	-	+++	-	+++	+++	0.8	0.7
Pa 32	+++	+++	+++	-	+++	++	-	+++	++	+++	++	0.8	0.8
Pa 37	+++	+++	+++	-	+++	+++	+++	+++	-	+++	+++	0.8	0.8
Pa 47	+++	+	+++	-	+++	+++	++	+++	-	+++	+++	0.8	0.7
Pa 2	+++	++	+	-	++	+++	+	+++	-	+	-	0.7	0.5
Pa 26	+++	+++	+++	+++	+++	+++	-	+++	-	+++	+++	0.7	0.7
Pa 35	+++	+++	+++	+++	+++	+++	-	+++	-	+++	+++	0.7	0.7
Pa 51	-	-	-	-	-	-	-	-	-	-	-	0.0	0.0

Reference strains are presented from left to right and phages from top to bottom. Calculated ROI highlighted in green includes all positive reactions while the ROI (≥ ++) includes reactions that were ≥ ++ only. ROIs of P. aeruginosa phages selected for further characterization are highlighted in orange (except for Pa 35 and Pa 47 as the patterns were identical to Pa 26 and Pa 37 respectively)

3.7 Discussion

The presence of a bacteriophage in an environmental sample is generally associated with the presence of its bacterial host. Therefore, for bacteriophage isolation, sample selection is critical. *Staphylococcus aureus* and *P. aeruginosa* bacteria are associated with horizontal transmission systems (Willemsen et al., 2008, Sheagran, 1984, Sista et al., 2004, Bonten and Weinstein, 1996, Gosbell, 2005). Therefore, people in enclosed environments, like hospitals, may generally be more prone to colonisation by these pathogens as a consequence of crowded conditions and probable exposure to people who were colonized previously. Thus, water samples for this study were collected from human sewage treatment plants and other private sources from several locations around New South Wales, including hospitals, nursing homes and rehabilitation centres, where the target pathogens were more likely to be present.

The isolation of bacteriophages from the selected water samples was demonstrated and resulted in a total of 58 *S. aureus* and 52 *P. aeruginosa* bacteriophages isolated. However, a negative outcome in the recovery of *S. aureus* phages was observed when the pH of the water sample was measured to be either below 6.0 or above 8.0 (Sigma-aldrich, pH strips Cat N° P4786, data not shown). Although, water samples were collected crude and prior to any chemical treatment, the samples at the sampling places were likely to have been in contact with other substances like detergents, soaps, shampoos and even antiviral and antibacterial solutions. It is likely that such substances had the ability to acidify or alkalinize the samples, thus affecting the stability of phages. The results suggested that *S. aureus* phages might be significantly affected by pH changes. On the contrary, the isolation of *P. aeruginosa* phages was not noticeably affected by pH changes in these samples, with pH values found to be within 5 and 8.5. The sensitivity of bacteriophages to pH changes is generally one of the parameters assessed while selecting for therapeutic phages. This feature will be studied and presented more in detail for the selected bacteriophages isolated in the next section (section 4.2.3).

The relatively narrow spectrum of activity of bacteriophages (when compared with that of antibiotics) is seen both as an advantage and disadvantage of bacteriophage therapy. It is an advantage because when compared to antibiotics, bacteriophages are very specific in their mode of action and against the target isolate (section 1.3). This means that during therapy the normal flora is left intact. This specificity is highly advantageous particularly when treating gastro-intestinal infections or skin infections where opportunistic pathogens can overgrowth and predominate in the absence of normal flora. A disadvantage is that the particular bacterial isolate must be identified and "matched" to the therapeutic bacteriophage prior to administration of the treatment. This is because the process of identifying the bacterial isolate and "matching" it to the corresponding phage is perceived to be a lengthy and inconvenient as, not surprisingly, it may delay treatment. A way to minimise this difficulty is to select for bacteriophages with the ability to infect a large number of strains within a species, thus naturally broadening this relatively narrow spectrum. Therefore, for the isolation of bacteriophages, it is essential to have a collection of bacterial isolates that represent the local diversity of clones and, if possible, a collection of characterised isolates identified as successful clones worldwide within the species of interest.

For the isolation of *S. aureus* bacteriophages a collection of 21 different isolates was initially considered (Table 2-1). The isolates were selected as indicator bacteria following their molecular differences (SCC*mec* type and/or MLST types) as discussed previously in section 1.2.1 (Oliveira and Lencastre, 2002). The geographical area where the strains were originally first isolated or were most predominant was also considered. Two isolates in particular, SPS# 623 and 627, known worldwide as the New York/Japan and the Brazilian isolates respectively, were specially considered as they were part of the five major clones regarded as successful epidemic clones and responsible for the greater part of HA-MRSA infections around the world (Gordon and Lowy, 2008).

The initial assumption was that the phage isolation process could not only be more targeted against the most virulent or most successful epidemic clones around the world but also simplified by using a reference collection of well-characterised isolates. Thus, at the end of

the process all major clones of interest should have a bacteriophage capable of infecting the specific clones and conceivably multiple clones. However, although highly desirable, not all those particular strains were useful in the initial detection of bacteriophages. From the reference collection, only bacterial isolates SPS# 548, 621, 624, 626 and 631 were positive for bacteriophage isolation. However, the results obtained (Table 3-2) highlighted the fact that although the other strains in that collection were not able to "detect" bacteriophages in a particular water sample processed, the bacteriophages isolated from the same samples using clinical isolates (Appendix 1) were still able to infect them. For example, Sa 21 was isolated using the modified direct plating method (section 2.6.2.4) from water sample W145 and SPS#25, an MRSA clinical strain from unknown origin isolated at the Royal North Shore Hospital in Sydney. The same water sample was processed following the same isolation method against SPS# 544 to 548 and SPS# 621, 623, 627 and 631 but all failed to show bacteriophage plaques. However, after purification of Sa 21 was completed and the lytic host range test was performed, it was observed that strains SPS# 621, 623 and 631 were sensitive to the phage. These results could suggest two hypotheses.

First, that the SPS#25 strain was carrying a lysogenic phage. In order to prove that the phage activity observed was not due to the presence a lysogenic phage in the strain SPS#25, the strain was subjected to phage induction with mitomycin (10µg/ml^{-1}) in triplicate as described before (Tenreiro et al., 1993). If the strain was lysogenic, the test would induce the lysogenic phage into a lytic cycle and the phage would then be recovered in the supernatant. The three supernatants recovered from the test were tested against the reference collection. The results showed all the reference strains were negative for phage activity (data not shown) and thus, the isolated phages infecting the reference strains could have only come from the environmental sample.

Second, that the absence or presence of unknown chemical compounds in the environmental samples made not possible or interfered with particular bacteriophage attachment sites in the reference strains, and these sites are absent or are unaffected by such compounds in SPS#25. Thus, after the purification process when full elimination of any original substances in the sample had occurred and an enhanced environment was provided,

the phage could then infect the reference strains. In the future, chemical comparative studies of water samples could be performed in order to study further how the lack or presence of certain substances can affect the process of bacteriophage isolation. This knowledge could potentially simplify the number of samples to be screened and help in the development of more targeted isolation protocols if such substances are added or neutralized to improve phage infection.

It would be logical to suspect that using a strain repeatedly during the isolation process would pose the danger of isolating bacteriophages with a similar host range. However, this was not entirely the case for the 12 bacteriophages isolated with SPS#25. When tested against the 19 isolates in the reference collection, the 12 phages produced 14 different phage patterns as shown in Table 3-4. This showed that although it is possible to select for similar phages, the great majority could also be very different from each other as demonstrated in this case. On the other hand, it also showed the risks of isolating bacteriophages such as Sa 10 and Sa 16 that were totally negative against all the isolates in the reference collection (Table 3-4). It is important to highlight that similar observations to the one just described above were made when using SPS# 60 and SPS# 316.

Although with the observations made here it is unfeasible to grade strains as “good” or “bad” for isolation, the observations were helpful to highlight and begin to comprehend the complexity and biases involved in isolation protocols, the specificity of bacteriophages and some of the variables that can potentially affect the process of bacteriophage infection. Unfortunately, further research in relation to the nature and basis behind the observations, while worthy of note, were not within the scope of the project.

Bacteriophage isolation protocols, as many other scientific protocols, are biased towards the type of bacteriophage that may prove to be superior against the target of interest. Consequently, for phage isolation, enrichment methods were previously recommended when strain-specific phages were required (Carlson, 2005). These enrichment protocols, in theory, produce large numbers of phages and are also flexible as they can be used not only to isolate phages against the targeted isolate but also have the potential to isolate phages

against the endogenous bacteria present in the analysed sample. However, a major disadvantage is that once the overnight enrichments are performed, each sample must be plated (section 2.6.2.1) or spot tested (section 2.6.2.2). This process can be tedious, lengthy and much more expensive as additional lab materials are required.

In this study, a modified direct plating method (section 2.6.2.4) was preferred over the direct plating, spot testing or enrichment method (section 2.6.2.1-2.6.2.3). The direct plating or spot test method use 100µl and 10-20µl of an enriched sample respectively (Carlson, 2005). Therefore, the success of isolation depends on the presence of high concentrations of bacteriophages in the sample. However, this study found that in 2ml of a sample as little as one single plaque could be present and so by using the direct plating or the spot test alone such phage could have been missed. The enrichment method was attempted when using strains SPS# 548, 627 and 634 as indicator bacteria, for which several methods were tried before an obligatory lytic phage capable of infecting them was found. However, by using the enrichment method, only in one occasion a sample produced a bacteriophage (Sa 31) that was not picked when performing the modified direct plating method. Thus, the results showed that the modified direct plating method developed here was able to recover phages from the environment in a more efficient manner than the classic recommended methods. The modified method was also significantly simpler, faster and more economic as plaque purification can be started directly from the isolation plate without the need of additional steps. This is contrary to enrichment protocols where the enriched sample must undergo an extra "identification step" to confirm if the enrichment was successful and bacteriophages were indeed present in the sample.

Nonetheless, different factors must be considered when using a modified direct plating method. When using this method special care must be taken to ensure the samples contain no substances capable of affecting bacterial growth or the phage itself, as this will have a direct effect on phage production. Also the selection of centrifugal filters for concentration or bacterial sterilization of the sample must be carefully considered, as phages in low numbers may be lost during these steps by attachment to the membranes. Filters with membranes made of polyethersulfone (PES) were used in this study as they had high-dirt

loading capacity and low protein binding and were found to bind less bacteriophage than Polyvinylidene difluoride (PVDF) ($p < 0.05$, data not shown).

The plaque morphology and appearance for each *S. aureus* and *P. aeruginosa* phage isolated is presented in Table 3-1 and 3-5 correspondingly. The clearness of the plaque described is generally specific to the phage-host pair (Adams, 1959). When there are no insensitive bacterial cells within a culture, the resulting phage plaques on a plate are clear (+++ full activity). On the other hand, if a small percentage of bacterial cells are phage resistant, the resulting plaques would be clear but scattered colonies or slight fuzziness will be observed (++ full activity). In the presence of large numbers of phage resistant bacteria, the plaques will appear turbid (+ partial activity) or in some cases totally overgrown and almost invisible to the naked eye (- No activity). However, the presence of turbid plaques may also reflect the lysogenization of bacteria (Bronson and Levine, 1971)

Though the presence of a turbid phage plaque has been considered to be presumptive identification of a temperate phage *in vitro*, the production of a turbid plaque in a single isolate should not immediately disqualify a phage. Equally, a clear plaque in a particular host-phage pair does not immediately suggest that the phage is not capable of establishing lysogens in another bacterial strain (Bronson and Levine, 1971). As discussed earlier (Section 1.3.1), the use of temperate phages for therapy is undesirable. However, before a phage is excluded due to potential lysogenic ability, further analysis of its activity against a larger group of bacterial isolates may be performed. This would help to determine if the phage has the ability to produce consistently clear plaques with the absence of visible potential lysogens or whether the presence of turbid plaques is regular suggesting strongly low-virulence or lysogenic characteristics. Therefore, none of the isolated bacteriophages was disqualified from the evaluation process based on the appearance of the plaque morphology only.

As described before, bacteriophages may have a very narrow or broad-spectrum of activity within the species that they infect. However, there have been reports of phage cross-activity between genus with coliphages infecting *Salmonella* sp. and other Enterobacteria (Mojica-a

and Garcia, 1976, Goodridge et al., 2003). Thus, host range characterisation of isolated *S. aureus* and *P. aeruginosa* bacteriophages is an essential phase required in order to exploit their potential therapeutic applications. The method of choice usually employed to assess the spectrum of activity of a phage against a particular set of indicator bacteria and to distinguish bacteriophages from each other is the host range test.

The host range of a bacteriophage refers to the number of bacterial hosts that a phage can successfully attach to and multiply within a specific population (Adams, 1959). The host range of all bacteriophages isolated was tested as described in section 2.6.7 against the reference collections (Tables 2-1 and 2-2) and the results are presented in Table 3-2 for the *S. aureus* phages and Table 3-6 for *P. aeruginosa* phages.

It is frequently accepted that bacteriophages have a rather narrow host range of activity (Goodridge and Abedon, 2003). However, in this study, each of *S. aureus* reference isolate tested (Table 2.1) was susceptible to at least five different bacteriophages (Table 3-2). Only six (Sa 1, Sa 2, Sa 3, Sa 4, Sa 10, Sa 16) out of 58 bacteriophages were not effective against any isolates in the reference collection. Thirty (52%) of the isolated *S. aureus* phages were able to infect 50% or more of the reference collection, with 12 of those phages: Sa 5, Sa 6, Sa 9, Sa 31, Sa 32, Sa 34, Sa38, Sa 41, Sa 42, Sa 50, Sa 51 and Sa 58 able to infect all the isolates. On the other hand, the host range for the *P. aeruginosa* phages appeared to be a little bit more limited with 27 (52%) Pa phages able to infect 50% or more of the *P. aeruginosa* reference strains but only one phage (Pa 1) able to infect all the isolates in the reference collection.

Commonly, differences in phage patterns after the host range test is performed are used to separate bacteriophages from each other and to select those considered worthy to be further investigated (Adams, 1959). However, due to the large number of phages effective against the reference collections and the innumerable number of phage patterns observed, the use of this method was not helpful in the selection of phages for further characterization. Instead, a system to separate the phages by analysing their “ratio of infection” against the reference collection was made. The “ratio of infection” was defined as the number of

isolates the phage infected over the total number of isolates tested. A system then was established in which if the phage infected all the strains, irrespective of the potency of that infection (*i.e.* partial or full activity), the ratio of infection was 1.0. Bacteriophages with a ratio of infection ≥0.7 were further considered.

This analysis reduced the number of *S. aureus* bacteriophages from 58 to 33 (Table 3-3). Bacteriophages Sa 5, Sa 6, Sa 9, Sa 31, Sa 32, Sa 34, Sa 38, Sa 41, Sa 42, Sa 50, Sa 51 and Sa 58 showed all a ratio of infection of 1.0. Bacteriophages Sa 14 and Sa 43 followed with 0.9 and Sa 22, Sa 23 and Sa 53 were 0.8. Bacteriophages Sa 12, Sa 18, Sa 24, Sa 25, Sa 29, Sa 33, Sa 35, Sa 36, Sa 37, Sa 39, Sa 44, Sa 48 and Sa 54-57 were the last ones in the list with a ratio of infection of 0.7. For the *P. aeruginosa* bacteriophages the reduction on numbers of bacteriophages was more noticeable with 15 bacteriophages only showing a ratio of infection ≥0.7 (Table 3-7). Bacteriophage Pa 1 was the only *P. aeruginosa* phage that showed a ratio of 1.0. Bacteriophages Pa 6, Pa 10, Pa 50 and Pa 52 were 0.9 while Pa 3, Pa 9, Pa 24, Pa 25, Pa 32, Pa 37 and Pa 47 were 0.8. Pa 2, Pa 26 and Pa 35 were the last ones to be added with a ratio of infection of 0.7.

As the number of *S. aureus* bacteriophages was still rather high to be taken through for further characterization, it was decided to repeat the exercise and re-calculate the ratio of infection of these phages but considering only those activities that were clear (+++ or ++) and ignoring those that were turbid (+) (Table 3-2 and 3-6). The same principle was applied again to the list of *P. aeruginosa* phages (Table 3-3 and 3-7). *Staphylococcus aureus* phages Sa 12, Sa 18, Sa 32, Sa 34, Sa 38, Sa 41, Sa 42, Sa 43, Sa 44, Sa 51, Sa 53 and Sa 54 (Table 3-3) and *P. aeruginosa* phages Pa 1, Pa 3, Pa 6, Pa 10, Pa 24, Pa 25, Pa 26, Pa 32 and Pa 37 (Table 3-7) showed not only the broadest host range against the panel of strains tested (Table 3-2 and 3-6), but also a higher ratio of infection when considering clear (+++ or ++) phage reactions only and thus, these phages where selected for further characterization. Bacteriophages Pa 35 and Pa 47 showed the same phage patterns than Pa 26 and Pa 3 respectively but smaller plaques in size and were therefore excluded.

In addition, Pa 51 was also selected for further characterization despite the fact it was not able to infect any of the isolates in the reference collection (Table 3-6). Pa 51 was the first and most efficient phage isolated against a mucoid multi-drug resistant *P. aeruginosa* isolate (SPS #445) that was added to the reference of clinical isolates later in the process. At the time, the isolate was responsible of a chronic respiratory infection in a 56 years old female patient and no effective antibiotic treatments were available for it. Consequently, it was considered important to continue with the characterization of the only phage capable of infecting that particular pathogen.

4 CHARACTERIZATION OF *STAPHYLOCOCCUS AUREUS* AND *PSEUDOMONAS AERUGINOSA* BACTERIOPHAGES

4.1 Introduction

Bacteriophages that are believed to have therapeutic potential, based on their spectrum of activity against a reference collection, must undergo further biological characterization. The first practical step to follow in the selection process is to evaluate the activity of the bacteriophages of interest against a broader representative collection of clinical isolates. This would confirm that selected bacteriophages are capable of infecting bacteria commonly found within a community and that their activity is not limited to a reference collection. Further characterization can then be aimed to obtain additional details on the innate qualities of potential therapeutic bacteriophages and to identify their strengths and limitations at a number of levels.

The production of bacteriophages has been theorized to be both a process dependent on the concentration of bacteria (Payne & Jansen 2001) as well as entirely randomized process in which phage relies entirely on chance encounters with their hosts (Kasman et al., 2002). One could argue that both theories are linked as the higher the concentration of bacteria, the higher the chance for a phage to find its host. Based on these theories, mathematical models have been drawn suggesting that the outcome of a treatment with bacteriophages is thus dependent on several factors such as the dose, adsorption rates and burst size of the phage used for treatment (Payne & Jansen, 2003; Weld *et al.* 2004). The study of the kinetics of a phage in an *in vitro* model is therefore important as this may help to predict its behaviour *in vivo*.

Furthermore, some environmental factors such as temperature, pH conditions and bacteria were reported to influence bacteriophage concentration in its environment (Ackermann and DuBow, 1987). In fact, the continued existence of bacteriophages in nature is determined by environmental conditions, though such effects were generally considered to be an

indirect consequence of any effects on environmental bacteria (Weinbauer, 2004). Thus, the *in vitro* study of the effect of these parameters during the development of a bacteriophage product is important as they could become particularly relevant for its performance at therapeutic level (Gill and Hyman, 2010b). For example, if oral administration is the most suitable method of delivery for a particular infection, it is then desirable to select for phages that can naturally resist low pH environments like the one found in the human gastrointestinal tract. Here, a number of *S. aureus* and *P. aeruginosa* bacteriophages isolated in Australia were selected (Table 3-6 and 3-7) in order to investigate their general properties and main biological characteristics that may be important in their subsequent application as therapeutic drugs.

4.2 Characterization of Bacteriophage

4.2.1 Lytic activity of selected bacteriophage against clinical isolates

The lytic activity of isolated bacteriophages was determined against the collection of clinical isolates (Tables 2-2 and 2-4) as described in section 2.6.7.

4.2.2 Temperature stability

Three milliliters of each bacteriophage suspension was aliquoted in three separated sterile-capped tubes and one tube placed at 4°C, 25˚C (room temperature) and 37˚C. The tubes were incubated for 10-12 months at the test temperature. Aliquots as required were taken monthly from each sample and the stability of the bacteriophages titres tested (section 2.6.6.1).

4.2.3 pH stability

To test for pH stability, SM buffer (section 2.3.2) was altered by addition 1M HCl in order to set a pH value of 3.0. For the test, 3 x 100µl of a bacteriophage suspension was added to 900µl of pH 3.0. In parallel, a second set of tubes was tested for pH stability if protected with sodium bicarbonate. For the test 10µl of the phage suspension was pre-mixed in a tube with 1ml of 1% sodium bicarbonate before adding 100µl to 900 µl of the modified SM

buffer at pH 3.0. The bacteriophage suspensions were then incubated at 37°C for 60min and subsequently serially diluted in SM buffer (pH 7.5) to evaluate the titre in triplicate as described in section 2.6.6.1

4.2.4 One step growth curves

An overnight culture of each indicator strain was harvested by centrifugation, resuspended in 1ml of nutrient broth and duplicate aliquots set up. The corresponding phage was added to each tube at an MOI of 0.005 and allowed to adsorb for 10min at 37˚C. Each mixture was then centrifuged for 5min at 8000rpm and the pelleted cells resuspended in 30ml of Nutrient broth. Tubes were incubated at 37˚C. Five-hundred microliters samples were taken at 15min intervals for 90min for bacteriophage counts.

The supernatants recovered above were used for calculation of bacteriophage adsorption as previously described (Sanders and Klaenhammer, 1980). Briefly, the supernatant collected after 10min of incubation of a phage with its propagating strain was assayed immediately for residual phage by the spot test method (section 2.6.6.1). Phage adsorption was calculated as a percent of adsorption = [(control phage titre - residual phage titre)/control titre] x 100.

4.2.5 Effect of bacteriophage concentration on *S. aureus* and *P. aeruginosa*

An overnight culture of the indicator bacteria was set up as described in section 2.6.1 and incubated under aerobic conditions at 37°C for 16-18h. To study the effect of different initial concentrations of the selected bacteriophage in the growth of bacteria and subsequent production of bacteriophage, four 50ml tubes with 30ml of nutrient broth were inoculated with the overnight culture of interest. One tube remained as the control and the other three were inoculated with the required volume of bacteriophage stocks to achieve a multiplicity of infection (MOI) of 0.1, 1 and 10. The control group was added the same amount of SM buffer required to achieve the MOI of 10. The tubes were incubated at 37˚C for 24 hours and 1ml samples were taken for OD readings every 4h for the first 16h and then at 24h. One-millilitre samples were additionally taken and centrifuged to remove bacteria. Phage

samples were diluted and titrated and the effect of each phage disrupting the growth of its indicator bacteria at different concentrations during the test period observed and compared.

4.3 Results

4.3.1 Characterization of *S. aureus* (Sa) bacteriophages

4.3.1.1 *Lytic activity of Sa phages against clinical isolates*

On the basis of spot test to check the host range, the 12 *S. aureus* bacteriophages were found to infect all the clinical isolates tested. Forty-seven (49.5%) of the strains tested were highly susceptible to all 12 Sa phages and another eleven (11.6%) strains were highly susceptible to 11 of the Sa phages (Table 4-1). When only those phages with the clearer reactions were considered, bacteriophages Sa 18, Sa 32, Sa 53 and Sa 54 showed the lowest spectrum of activity within the group with a ratio of infection (ROI) of 0.66, 0.7, 0.77 and 0.79 respectively. All the other selected Sa phages showed an ROI above 0.90.

Thirty-three different phage patterns were identified in the 95 clinical isolates tested. However, with the exception of those isolates that were sensitive to all 12 Sa phages, none of the isolates showed the same phage patterns observed against the reference collection (Table 3-6). Following the phage patterns showed some of the isolates were separated in groups:

Group 1: isolates SPS# 1, 4, 5 and 6
Group 2: isolates SPS # 42, 44 and 412
Group 3: isolates SPS # 2, 45, 46, 59, 61, 337
Group 4: isolate SPS# 346, 347, 351, 352
Group 5: isolates SPS# 355 and 356.
Group 6: isolates SPS# 327 and 328
Group 7: isolates SPS# 7, 58, 287 to 300, 304, 306 to 310, 313, 314, 316 to 321, 324, 325, 326, 329-334, 339, 342-345, 348-350.

Isolates SPS # 3, 25, 43, 47, 54, 56, 57, 60, 286, 301, 302, 303, 305, 311, 312, 315, 335, 336, 338, 340, 341, 353, 354, 357, 358 and 413 all showed unique phage patterns. The results are summarised in Table 4.1

Table 4-1. **Host range of selected *S. aureus* bacteriophages (Sa) against the clinical collection (Appendix 1)**

Phage	Sa 12	Sa 18	Sa 32	Sa 34	Sa 38	Sa 41	Sa 42	Sa 43	Sa 44	Sa 51	Sa 53	Sa 54
SPS#286	+++	-	-	+++	++	++	+++	++	++	++	-	-
SPS#287	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
SPS#288	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP
SPS#289	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
SPS#290	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP
SPS#291	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP
SPS#292	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP
SPS#293	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP
SPS#294	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
SPS#295	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
SPS#296	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
SPS#297	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
SPS#298	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP
SPS#299	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP
SPS#300	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP
SPS#301	+++	+++	+++	++	+++	++	+++	+++	+++	+++	+++	++
SPS#302	-	-	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
SPS#303	+++	-	+++ IP	+++	+++	+++	+++	+++	+++	+++	+	+
SPS#304	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
SPS#305	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
SPS#306	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
SPS#307	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP

continued Table 4-1.

Phage	Sa 12	Sa 18	Sa 32	Sa 34	Sa 38	Sa 41	Sa 42	Sa 43	Sa 44	Sa 51	Sa 53	Sa 54
SPS#308	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
SPS#309	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
SPS#310	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
SPS#311	+++	-	+++ IP	+++	+++	+++	+++	+++	+++	+++	++	++
SPS#312	+++	-	-	+++	++	+++	+++	++	++	+++	-	-
SPS#313	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP
SPS#314	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP
SPS#315	++	-	+	+++	++	+++	+++	++	+++	+++	+	+
SPS#316	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
SPS#317	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
SPS#318	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
SPS#319	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP
SPS#320	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
SPS#321	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP
SPS#324	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP
SPS#325	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP
SPS#326	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP
SPS#327	+++	-	-	+++	++	++	+++	+	++	++	-	-
SPS#328	+++	-	-	+++	++	++	+++	+	++	++	-	-
SPS#329	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP
SPS#330	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP
SPS#331	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP

continued Table 4-1.

Phage	Sa 12	Sa 18	Sa 32	Sa 34	Sa 38	Sa 41	Sa 42	Sa 43	Sa 44	Sa 51	Sa 53	Sa 54
SPS#332	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP
SPS#333	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP
SPS#334	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP
SPS#335	+++	++	++	+++	+++	+++	+++	+++	+++	+++	++	+
SPS#336	++	++	+	++	++	++	++	++	++	++	+	+
SPS#337	+++	-	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP
SPS#338	+++	+++	ND	+++	+++	+++	+++	+++	+++	+++	ND	ND
SPS#339	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++IP	+++IP
SPS#340	++	-	-	++	++	++	++	+	++	++	-	-
SPS#341	+++	-	++ IP	+++	+++	+++	+++	++	+++	+++	+++IP	+++IP
SPS#342	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++ IP	+++ IP
SPS#343	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++ IP	+++ IP
SPS#344	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++ IP	+++ IP
SPS#345	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++ IP	+++ IP
SPS#346	++	++	++ IP	++	++	++	++	++	++	++	++	++
SPS#347	++	++	++ IP	++	++	++	++	++	++	++	++	++
SPS#348	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++ IP	+++ IP
SPS#349	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++ IP	+++ IP
SPS#350	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++ IP	+++ IP
SPS#351	++	++	++ IP	++	++	++	++	++	++	++	++ IP	++ IP
SPS#352	++	++	++ IP	++	++	++	++	++	++	++	++ IP	++ IP
SPS#353	+++	-	-	+++	+++	+++	++	+	++	+++	-	-

continued Table 4-1.

Phage	Sa 12	Sa 18	Sa 32	Sa 34	Sa 38	Sa 41	Sa 42	Sa 43	Sa 44	Sa 51	Sa 53	Sa 54
SPS#354	+++	-	+	++	++	++	++	++	++	++	+	-
SPS#355	+++	-	+	++	++	++	++	++	++	++	+	++ IP
SPS#356	+++	-	+	++	++	++	++	++	++	++	+	++ IP
SPS#357	+++	-	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++ IP	++ IP
SPS#358	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++ IP	++ IP
SPS#1	+++	++	+++ IP	+++	+++	+++	+++	+++	+++	+++	++ IP	++ IP
SPS#2	+++	-	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++ IP	+++ IP
SPS#3	++	+	++	++	++	++	++	++	++	++	++ IP	++ IP
SPS#4	+++	++	+++ IP	+++	+++	+++	+++	+++	+++	+++	++ IP	++ IP
SPS#5	+++	++	+++ IP	+++	+++	+++	+++	+++	+++	+++	++ IP	++ IP
SPS#6	+++	++	+++ IP	+++	+++	+++	+++	+++	+++	+++	++ IP	++ IP
SPS#7	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++ IP	+++ IP
SPS#25	++	++	+++ IP	+++	+++	+++	+++	+++	+++	++	+++	++
SPS#42	+++	++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++ IP	+++ IP
SPS#43	+++	-	+++ IP	+++	++	++	+++	+++	+++	+++	++ IP	++ IP
SPS#44	+++	++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++ IP	+++ IP
SPS#45	+++	-	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++ IP	+++ IP
SPS#46	+++	-	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++	+++ IP
SPS#47	+++	-	-	+++	++	++	++	+	++	++	-	-
SPS#54	+++	-	-	+++	+	++	+++	-	+	++	-	-
SPS#55	+++	-	++	++	++	++	++	++	++	++	++ IP	++ IP
SPS#56	+++	-	++	++	++	+++	+++	++	++	++	++ IP	++ IP

continued Table 4-1.

Phage	Sa 12	Sa 18	Sa 32	Sa 34	Sa 38	Sa 41	Sa 42	Sa 43	Sa 44	Sa 51	Sa 53	Sa 54
SPS#57	+++	-	-	+++	++	+++	+++	++	++	++	-	-
SPS#58	+++	+++	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++ IP	+++ IP
SPS#59	+++	-	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++ IP	+++ IP
SPS#60	++	-	++	++	++	++	++	++	++	++	++ IP	++ IP
SPS#61	+++	-	+++ IP	+++	+++	+++	+++	+++	+++	+++	+++ IP	+++ IP
SPS#412	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
SPS#413	++	-	-	++	+	+++	+++	+++	++	++	-	-
ROI	0.94	0.67	0.841	0.95	0.95	0.95	0.95	0.93	0.94	0.94	0.83	0.83
ROI >++	0.94	0.66	0.70	0.95	0.93	0.95	0.95	0.88	0.93	0.94	0.77	0.79

Reference strains are presented from top to bottom and phages from left to right. Positive reactions (+) were recorded when a minimum of 10 individual plaques (IP) was observed. The clarity of the IP or the spot was scored as turbid (+), clearing with hazy-partial background (++) and complete clearing (+++). Negative reactions were recorded as (-).The Calculated Ratio of infection (ROI) is presented for each bacteriophage.

4.3.1.2 *Temperature stability of Sa phages*

Sa bacteriophages were very stable at 4°C storage during the twelve-month period tested with a total average reduction in their concentration of only 0.24-Log. Sa 38 and S 53 were the most sensitive phages to cold storage with a 0.57-Log and 0.59 Log drops after the year. However, the drop in titre was not found to be statistically significant ($p>0.05$) when compared to the starting point.

The stability of the Sa phages was more affected by storage at 25°C during the twelve-month period tested. The total average reduction in their concentration was of 4.6-Log, over 10 times more the concentration lost that when stored at 4°C. Phages Sa 5 and Sa 38 showed the biggest loss in titre with a 7.5-Log and a 7.0-Log drop correspondingly by the end of the twelve-month period. Phage Sa 42 followed closely with a 6.0-Log drop while Sa 18, Sa 34 and Sa 41 showed a 5.0-Log drop. Phage Sa 12 and Sa 43 both dropped 4.3-Log and Sa 32, 44, 53 and Sa 54 dropped 3.5-Log.

The loss in phage concentration was more obvious when the storage was performed at 37°C. In average, all Sa phages lost 1.7-Log in the first two months. However, by the third month their concentration dropped sharply with a further average loss of 4.0-Log. Phage Sa 34 was the most sensitive to the test temperature with a 7.3-Log drop. Phages Sa 12, Sa 41, Sa 42 and Sa 51 were also severely affected with an average loss of 6.4-Log in their concentration. Sa 54 was the only phage that showed viable particles after 6 months of storage at the test temperature.

4.3.1.3 *pH stability of Sa phages*

Exposure of Sa bacteriophages to a pH value of 7.5 for 60min at 37˚C had no effect on their ability to infect their target bacteria when compared to the control stocks generally stored in SM buffer pH 7.5 and 4ºC (>0.05). On the contrary, when exposed to a pH value of 3.0 for 60min at 37˚C, all Sa phages were inactivated with <1.0 PFU/ml detected after 60min at 37˚C ($p < 0.05$). However, bacteriophage samples pre-mixed with 1% sodium bicarbonate were protected from inactivation and no statistically significant differences were observed when compared with the control at pH 7.5 ($p>0.05$). The results are summarised in Table 4-2.

4.3.1.4 *One-step growth curves of Sa phages*

The latent period and burst size was determined for each selected Sa phage as exemplified in Fig 4-1. Bacteriophage single-step growth curves performed showed Sa 18 and Sa 44 had the smallest burst size with an average burst of 55 ± 3 and 60 ± 3 PFU/cell respectively. Sa 38 and 41 showed the largest burst size with 461 ± 23 and 400 ± 20 PFU/cell and were followed closely by Sa 53 with a burst size of 371 ± 19 PFU/cell. Sa 12 and Sa 51 had an average burst size of 300 ± 15 PFUs/cell while Sa 34, Sa 42 and Sa 43 showed an average burst size of 275 ± 14, 280 ± 14 and 233 ± 12 PFU/cell. Sa 32 and Sa 54 showed smaller burst sizes with 131 ± 7 and 170 ± 9. The latent period for all Sa phages varied between 10 to 25min.

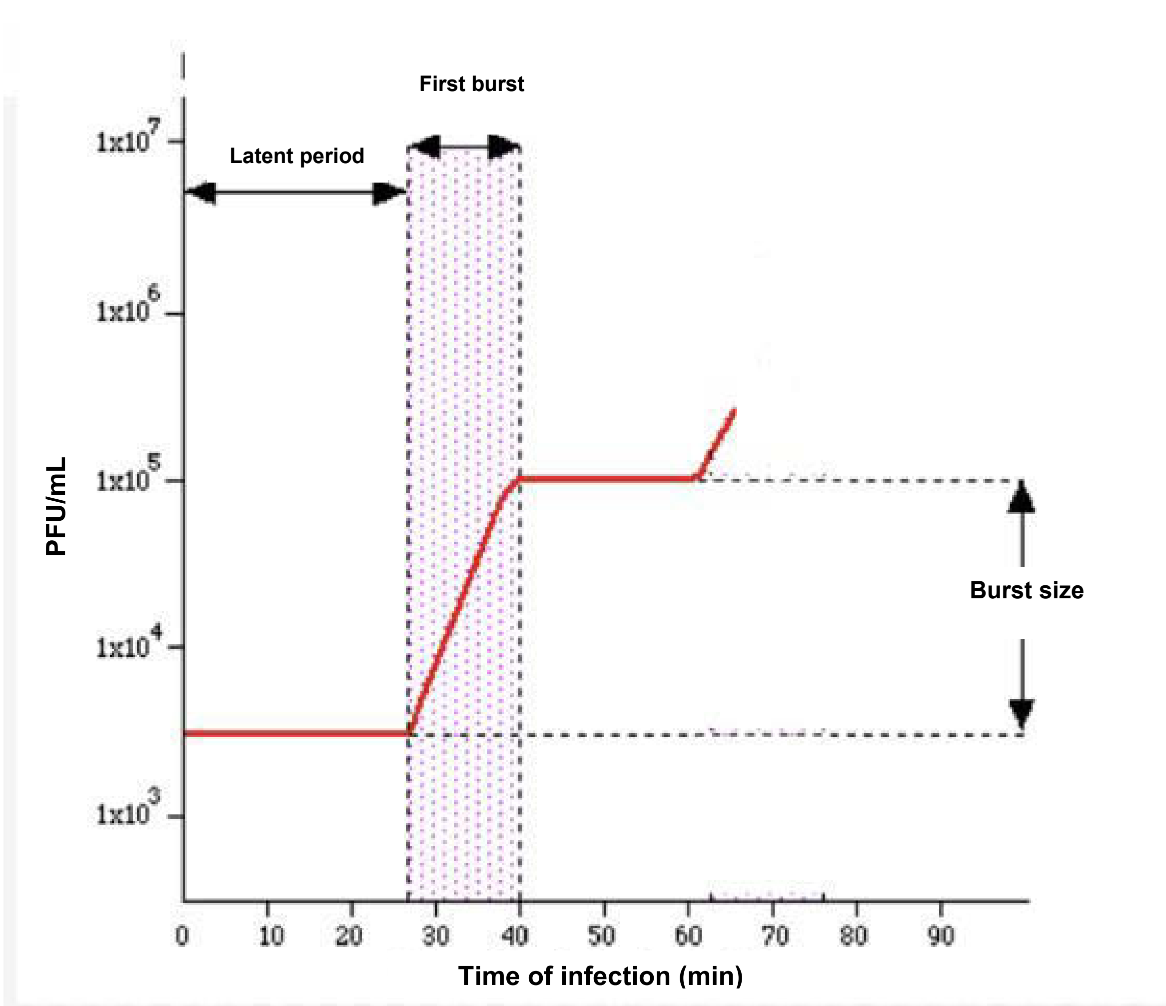
The percentage of adsorption achieved within 10min by each bacteriophage to its indicator bacteria was also measured. Bacteriophage Sa 54 and Sa 18 showed the smallest percentage of adsorption with 73.3% and 83.3% respectively. All the other Sa phages showed percentages of adsorption of over 92%. The results are summarised in Table 4-3.

Table 4-2. pH stability of Sa bacteriophages

Phage	Indicator strain (SPS#)	Test samples at 37ºC (PFU/ml)			Control at 4ºC (PFU/ml)
		pH 3.0	pH 3 +$NaHCO_3$	pH 7.5	pH 7.5
Sa 12	**316**	<1	7.00E+09	1.20E+10	1.60E+10
Sa 18	**316**	<1	4.00E+07	1.00E+08	1.50E+08
Sa 32	**293**	<1	3.30E+03	5.90E+03	6.10E+03
Sa 34	**317**	<1	3.00E+09	1.10E+10	1.40E+10
Sa 38	**287**	<1	1.30E+07	2.70E+07	2.90E+07
Sa 41	**308**	<1	5.30E+09	8.70E+09	8.60E+09
Sa 42	**308**	<1	1.50E+09	5.00E+09	5.00E+09
Sa 43	**297**	<1	4.20E+05	6.60E+05	6.30E+05
Sa 44	**320**	<1	1.20E+07	2.40E+07	2.90E+07
Sa 51	**310**	<1	1.00E+08	6.00E+08	6.60E+08
Sa 53	**296**	<1	3.50E+04	4.70E+04	4.90E+04
Sa 54	**295**	<1	2.60E+03	2.70E+03	2.80E+03

Bacteriophages at pH 3.0, pH 3.0 protected with sodium bicarbonate (NaHCO3) and pH 7.5 were incubated at 37°C for 1h and then serially diluted 10-fold in SM buffer before plating out for enumeration of viable phage. The value given is the mean of three separated experiments and titrations. Bacteriophage stocks stored at pH 7.5 and 4ºC were used as co

Figure 4-1. **One-step growth curve of bacteriophage**



The phage growth parameters are indicated in the figure and correspond to latent period and burst size. The latent period is measured in minutes and the burst size in plaque forming units (PFU) per cell for each infection.

Table 4-3. One-step growth curve of Sa phages

Phage	Indicator strain (SPS#)	% of Adsorption (after 10min)	Latent Period range (min)	Burst size (±SD)
Sa 12	**316**	99.5	15-25	300 ± 15
Sa 18	**316**	83.3	<10-15	55 ± 3
Sa 32	**293**	99.5	15-25	131 ± 7
Sa 34	**317**	96.3	15-25	275 ± 14
Sa 38	**287**	99.7	15-25	461 ± 23
Sa 41	**308**	97.0	<10-15	400 ± 20
Sa 42	**308**	92.0	<10-15	233 ± 12
Sa 43	**297**	99.5	15-25	280 ± 14
Sa 44	**320**	99.5	15-25	60 ± 3
Sa 51	**310**	98.8	15-25	300 ± 15
Sa 53	**296**	99.9	<10-15	371 ± 19
Sa 54	**295**	73.3	<10-15	170 ± 9

The ability of each phage to absorb to a bacterial cell (% of adsorption), its latent period and burst size was measured in a single step growth curve. (±SD) indicates standard deviations.

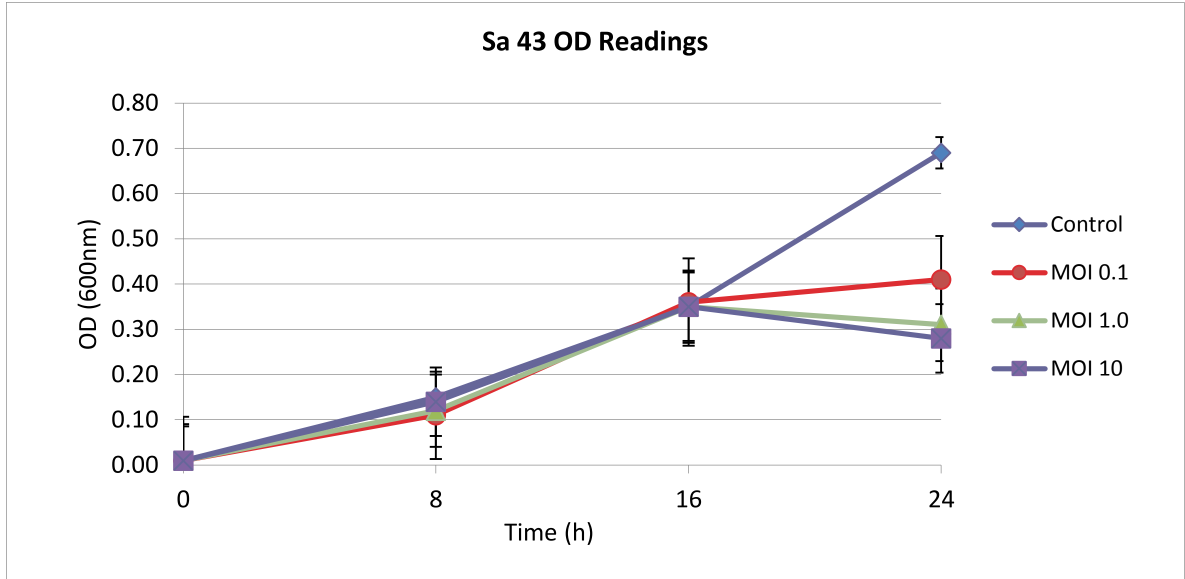
4.3.1.5 *Effect of bacteriophage concentration on the growth of S. aureus bacteria*

Bacteriophages were investigated for their efficacy to diminish the growth of their indicator bacteria when the infection was carried out in a broth culture. The efficacy of the phages was evaluated at three different multiplicities of infection (MOIs). That is the ratio of bacteriophage to bacteria. The initial MOIs tested were approximately 0.1, 1 and 10.

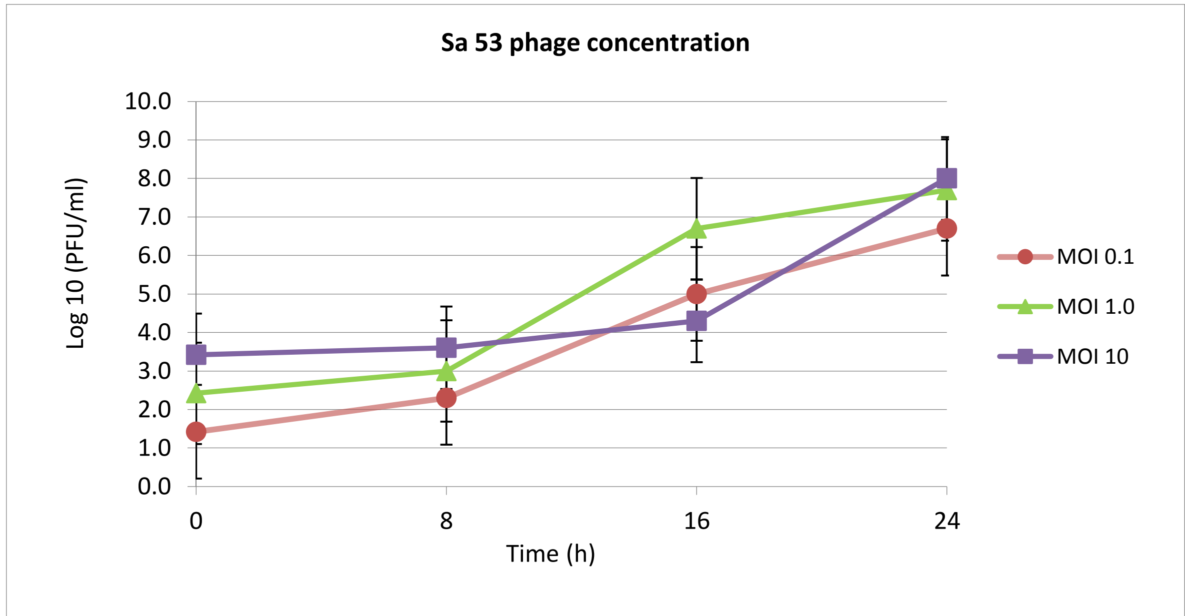
In general, the relationship between the host and bacteriophages at the different MOI showed bacteriophages to amplify immediately after infection for those for which amplification was observed. Eight hours post inoculation, bacterial growth was seen to be slower than the control group and after 24h no bacterial growth was detected. However, some bacteriophages were not capable of inhibiting bacterial growth despite their obvious amplification. According to the growth patterns of the infected bacteria and phage amplification, the bacteriophages tested could be separated in four different groups.

The first group with Sa 34, Sa 43, Sa 53 and Sa 54 showed a dose dependent bacterial growth reduction when compared with the control, meaning that the higher the number of phages at the beginning of the infection, the larger the reduction of bacterial growth (*i.e.* Sa 43, Figure 4-2a). However, bacteriophage amplification was observed for all different MOIs and after 24h their concentration had increased over 3 to 5-Log_{10} irrespective of the initial numbers (*i.e* Sa 53, Figure 4-2b).

Figure 4-2. **Effect of *S. aureus* bacteriophage concentration on *S. aureus* bacteria (group 1)**



a. Effect of bacteriophage Sa 43 on bacterial growth. *When infected at an MOI of 0.1, 1.0 and 10. Bacterial growth of the indicator strain SPS#297 showed a reduction that appeared to be dose dependent when compared to the control.*



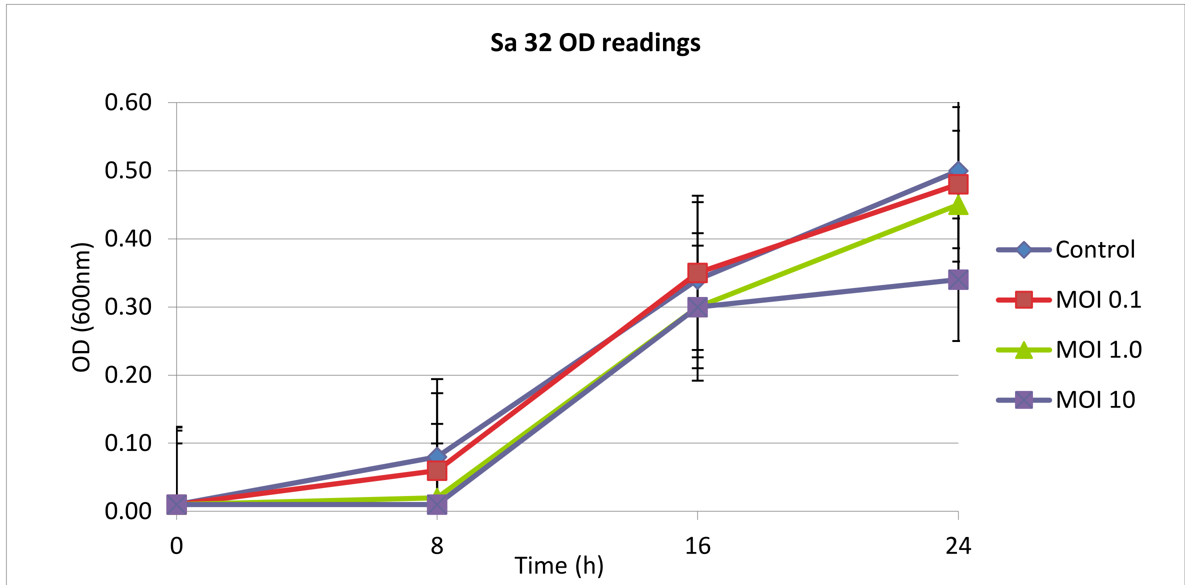
b. Bacteriophage Sa 53 growth *Amplification of phage Sa 53 in indicator strain SPS#296 was observed for all three different MOIs. After 24 hours, phage concentration increased 3 to 5-Log*$_{10}$ *irrespective of the initial MOI.*

Similarly, the second group with Sa 18 and Sa 32 showed, as for the first group, a dose dependent bacterial growth reduction in comparison to the control (*i.e.* Sa 32, Fig 4-3a) but differed with the first group in that bacteriophage amplification was only seen for the highest MOI correspondingly. The remaining two MOIs for each bacteriophage infection showed a decrease or no increase in bacteriophage numbers (*i.e.* Sa 32, Fig 4-3b), which could suggest lysogenization in the case of the lower MOIs (0.1 and 1.) and the potential induction of lysogenic phages when a high MOI of 10 was used.

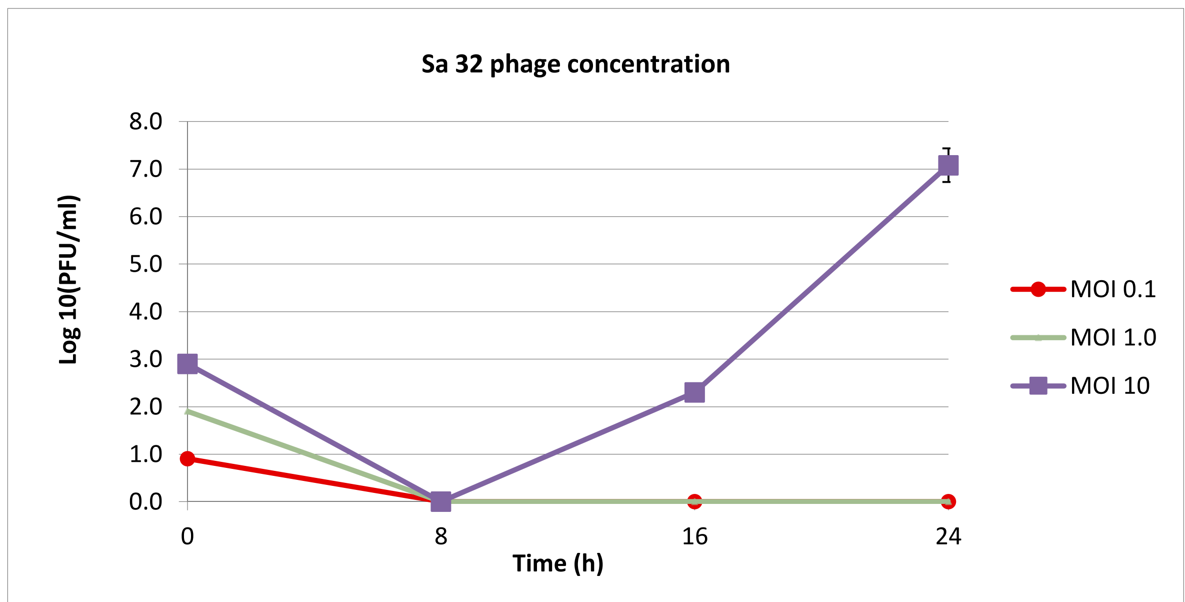
In the third group with Sa 12, Sa 38, Sa 44 and Sa 51, bacteriophages were capable of inhibiting *S. aureus* growth significantly after 24 hours (*i.e.* Sa 44, Fig 4-4a). Bacteriophage numbers increased for all three MOIs, however, the biggest increase in numbers was seen for the MOI of 0.1 (*i.e.* Fig, Sa 51).

The fourth and last group with Sa 41, Sa 42 showed the same ability than the third group to inhibit bacterial growth and phage amplification but differed from it in that bacterial re-growth was seen for the MOI of 10 after 16 hours (*i.e.* Sa 41, Fig 4-5). Bacteriophage numbers increased for all three MOIs, as observed for group 3, with the largest concentration increase observed again for the MOI of 0.1.

Figure 4-3. Effect of *S. aureus* bacteriophage concentration on *S. aureus* bacteria (group 2)

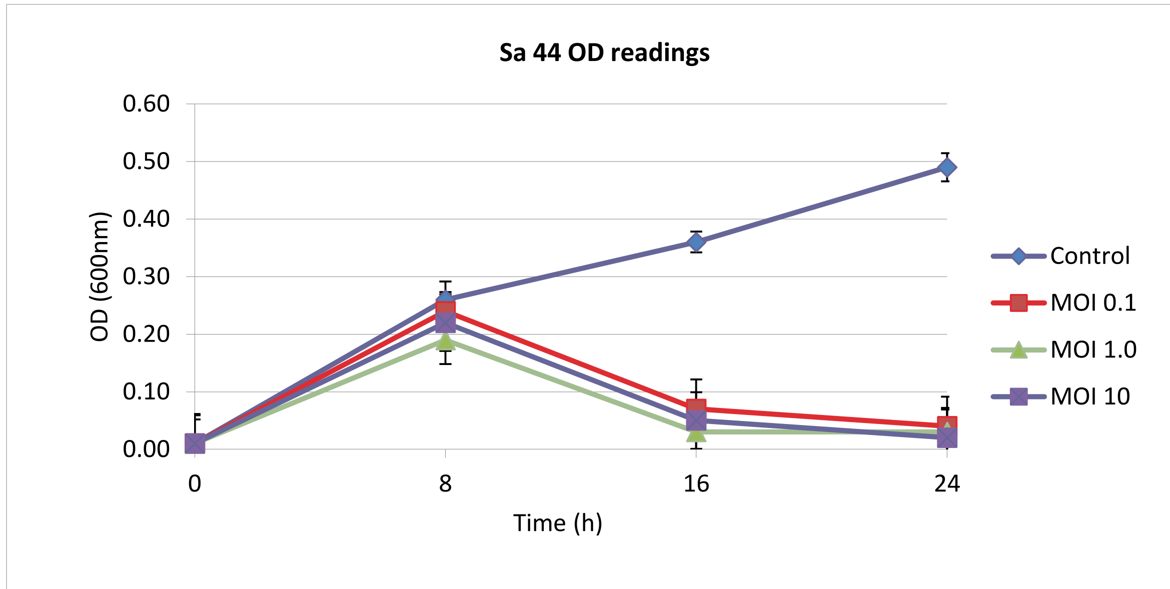


a. Effect of bacteriophage Sa 32 on bacterial growth. *Bacteriophage Sa 44 was capable of inhibiting growth of the indicator strain SPS#293 in what appeared to be a dose dependant process. However, after 24 hours, only the MOI of 10 showed a growth reduction that was significant in comparison to the control (p <0.05).*

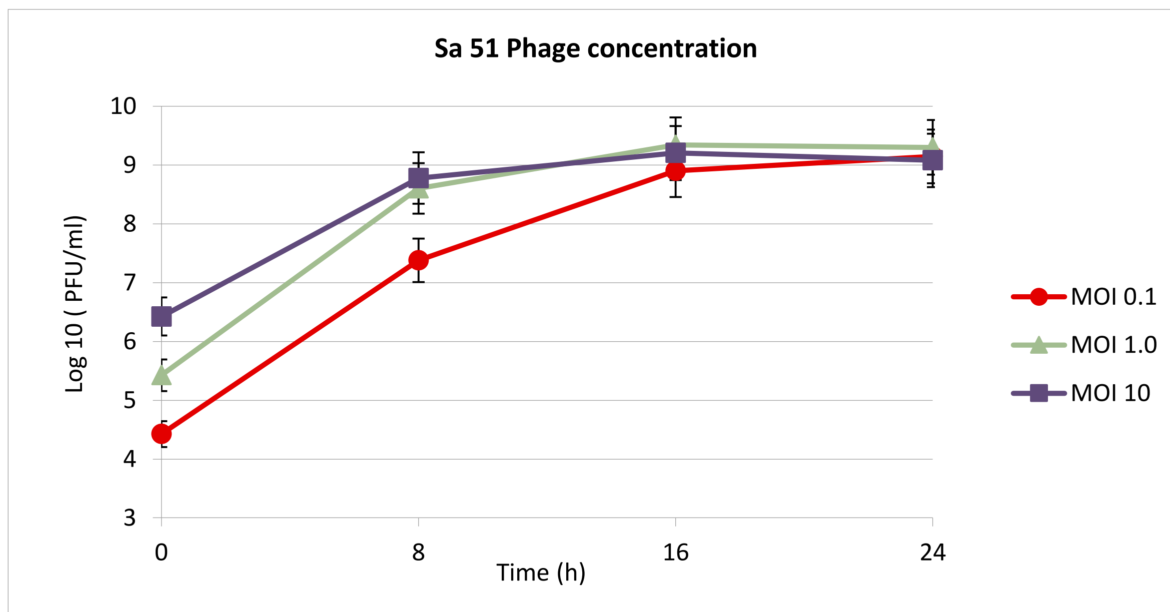


b. Bacteriophage Sa 32 growth *Amplification of bacteriophage Sa 53 in indicator strain SPS#293 was observed only for the MOI of 10 while the MOI of 0.1 and 1.0 showed a reduction in phage numbers after 8 hours and no further amplification.*

Figure 4-4. **Effect of *S. aureus* bacteriophage concentration on *S. aureus* bacteria (group 3)**

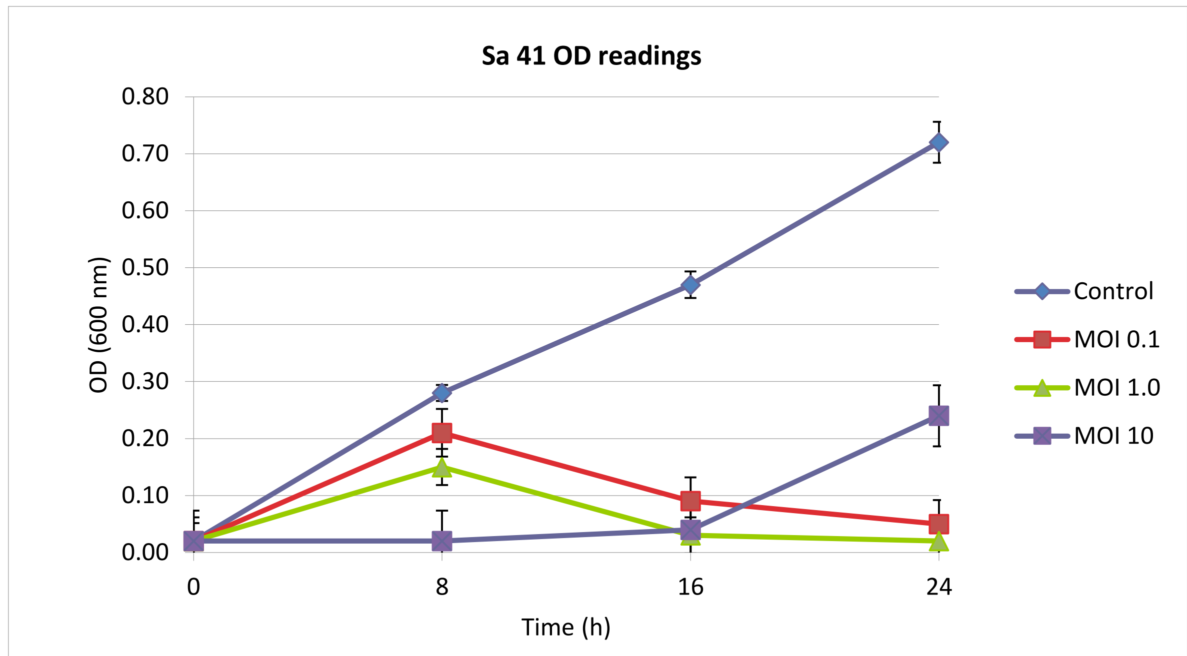


a. Effect of bacteriophage Sa 44 on bacterial growth. *Sa 44 was capable of inhibiting S. aureus growth significantly in comparison to the control ($p<0.05$). After 16 hours, all three MOIs prevented bacterial growth equally ($p>0.05$).*



b. Bacteriophage Sa 51 growth. *Bacteriophage numbers increased for all three MOIs, however, the biggest increase was seen for the MOI of 0.1. After 24 hours, bacteriophage concentration was equal for the three MOIs ($p>0.05$).*

Figure 4-5. Effect of *S. aureus* bacteriophage concentration on *S. aureus* bacteria (group 4)



a. Effect of bacteriophage Sa 41 on bacterial growth. *Bacteriophage Sa 41 was capable of inhibiting S. aureus growth significantly after 24 hours in what appeared to be a dose dependant process for the first 8 hours. After 16 hours, bacterial growth was equally reduced for all infected cultures. However, bacterial regrowth was observed for the MOI of 10 after 24 hours,*

4.3.2 Characterization of *P. aeruginosa* (Pa) bacteriophages

4.3.2.1 *Lytic activity of Pa phages against clinical*

The ten *P. aeruginosa* bacteriophages selected were found to infect 77 (96.3%) of the clinical isolates tested. However, three (3.7%) of the clinical strains were not sensitive to any of the phages and only three strains (SPS# 123, 439, 498) were sensitive to all ten phages (Table 4-4). Considering the ratio of infection (ROI) values, the bacteriophages could be separated in three distinctive groups, those with a low (0.1-0.59), medium (0.6-0.74) or high ROI (0.75-1) value.

Pa 3, Pa 32, Pa 51 showed the lowest spectrum of activity with a ROI of 0.18 and were closely followed by Pa 37 with a ROI value of 0.36. In the second group, those with a medium ROI value were Pa 6 and Pa 10 with ROI values of 0.69 and 0.64 respectively. The phages Pa 1, Pa 24, Pa 25 and Pa 26 showed the broadest lytic spectrum with ROI values above 0.80 (Table 4-4).

The phage spot testing of the 10 selected phages against the 80 clinical isolates revealed 53 different phage patterns. However, none of the patterns matched any of the 10 phage patterns identified against the reference collection (Table 3-7). Forty isolates showed unique phage patterns and the rest were grouped in 13 groups.

Group 1: isolates SPS# 445, 461 and 467
Group 2: isolates SPS # 411, 455 and 495
Group 3: isolates SPS # 111,112 and 116
Group 4: isolate SPS# 472, 488, 491, 492 and 502
Group 5: isolates SPS# 473, 474, 476, 494, 497 and 513
Group 6: isolates SPS# 478, 479 and 485
Group 7: isolates SPS# 466 and 508
Group 8: isolates SPS# 481 and 462
Group 9: isolates SPS# 487, 501, 503
Group 10: isolates SPS# 480, 484, 489 and 500
Group 11: isolates SPS# 475 and 482

Group 12: isolates SPS# 123 and439

Group 13: isolates SPS# 463 and 464

Isolates SPS # 113, 115, 118, 119, 285, 322, 432, 433, 438, 440, 446, 456, 457, 458, 459, 460, 465, 468, 469, 470, 471, 483, 486, 490, 493, 496, 498, 499, 504, 505, 506, 507, 509, 510, 511 and 512 all showed unique phage patterns. The results are summarised in Table 4-4.

Table 4-4. Host range of *P. aeruginosa* phages against clinical isolates (Appendix 2)

SPS#\Phage	Pa 1	Pa3	Pa6	Pa10	Pa 24	Pa 25	Pa 26	Pa 32	Pa 37	Pa 51
111	-	-	-	-	+++	+++	-	-	+++	-
112	-	-	-	-	+++	+++	-	-	+++	-
113	-	-	-	++	+++	+++	+++	+	+++	-
114	++	++	-	-	+++	+++	++	-	++	-
115	-	+	++	+	+++	+++	+	-	+++	++
116	-	-	-	-	+++	+++	-	-	+++	-
117	++	+	+++	+	+++	+++	+	-	+++	+
118	+++	-	+++	+++	+++	+++	++	-	+++	++
119	-	-	-	-	+++	+	++	+++	+++	-
123	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
285	-	-	+	-	++	-	-	-	-	-
322	+	-	+++	+++	+++	+++	+++	-	+++	-
323	++	+++	+++	+++	+++	+++	++	+++	+++	++
411	-	-	-	-	-	-	-	-	-	-
432	+++	-	-	-	+++	+++	+++	++	+++	-
433	+++	+++	+++	-	+++	+++	+++	+++	+++	-
438	+++	+++	+++	+++	+++	+++	+++	++	+++	-
439	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
440	+++	+++	+++	+	+++	+++	+++	+++	+++	-
445	-	-	-	-	-	-	-	-	-	+++
446	+++	+++	-	-	+	+++	+++	+	+++	-
455	-	-	-	-	-	-	-	-	-	-

continued Table 4-4.

SPS#\Phage	Pa 1	Pa3	Pa6	Pa10	Pa 24	Pa 25	Pa 26	Pa 32	Pa 37	Pa 51
456	+++	-	+	+++	+++	+++	++	-	-	-
457	++	-	+	+	++	+++	+	-	-	-
458	-	-	+	-	++	+++	+++	-	+	+++
459	++	-	++	++	++	+++	+++	-	-	-
460	++	-	++	++	++	+++	++	-	-	-
461	-	-	-	-	-	-	-	-	-	+++
462	+++	+++	-	-	+++	+++	+++	+++	+++	++
463	++	-	+++	+++	+++	+++	+	-	-	-
464	++	-	+++	+++	+++	+++	+	-	-	-
465	++	-	+++	++	+++	+++	+	-	-	-
466	+++	-	+++	++	+++	+++	+	-	-	-
467	-	-	-	-	-	-	-	-	-	+++
468	+++	+++	-	+++	+++	+++	+++	+++	+++	+++
469	+++	+	+++	+++	+++	+++	+	+	-	-
470	+++	+	+++	+++	+++	+++	+++	-	-	-
471	+++	+	+++	+++	+++	+++	+++	-	-	-
472	+++	-	+++	+++	+++	+++	+++	-	-	-
473	+++	-	+++	+++	+++	+++	++	-	-	-
474	+++	-	+++	+++	+++	+++	++	-	-	-
475	+	-	+++	++	+++	+++	+++	-	+++	-
476	+++	-	+++	+++	+++	+++	++	-	-	-
477	+	-	-	-	-	-	-	-	-	-

continued Table 4-4.

SPS#\Phage	Pa 1	Pa3	Pa6	Pa10	Pa 24	Pa 25	Pa 26	Pa 32	Pa 37	Pa 51
478	+++	-	+++	++	+++	+++	++	-	-	-
479	+++	-	+++	++	+++	+++	++	-	-	-
480	++	-	+++	++	+++	+++	++	-	-	-
481	+++	-	++	++	+++	+++	++	++	-	-
482	+	-	+++	++	+++	+++	+++	-	+++	-
483	++	-	+++	++	+	+++	+++	-	-	-
484	++	-	+++	++	+++	+++	++	-	-	-
485	+++	-	+++	++	+++	+++	++	-	-	-
486	++	+	++	-	+++	+++	-	-	-	-
487	++	-	+++	+++	+++	+++	++	-	-	-
488	+++	-	+++	+++	+++	+++	+++	-	-	-
489	++	-	+++	++	+++	+++	++	-	-	-
490	+++	-	+++	++	+++	+++	+++	-	-	-
491	+++	-	+++	+++	+++	+++	+++	-	-	-
492	+++	-	+++	+++	+++	+++	+++	-	-	-
493	+++	-	+++	+++	+++	+++	+	-	-	-
494	+++	-	+++	+++	+++	+++	++	-	-	-
495	-	-	-	-	-	-	-	-	-	-
496	++	-	+++	++	+++	+++	-	-	-	-
497	+++	-	+++	+++	+++	+++	++	-	-	-
498	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
499	-	-	+++	-	++	++	-	-	++	+

continued Table 4-4.

SPS#\Phage	Pa 1	Pa3	Pa6	Pa10	Pa 24	Pa 25	Pa 26	Pa 32	Pa 37	Pa 51
500	++	-	+++	++	+++	+++	++	-	-	-
501	++	-	+++	+++	+++	+++	++	-	-	-
502	+++	-	+++	+++	+++	+++	+++	-	-	-
503	++	-	+++	+++	+++	+++	++	-	-	-
504	+++	+++	+	+++	+++	+++	+++	+++	+++	-
505	+++	+++	-	++	+++	+++	+++	-	+++	+
506	+++	-	-	-	+++	+++	-	-	++	-
507	++	-	+++	-	++	++	+	-	+++	+++
508	+++	-	+++	++	+++	+++	+	-	-	-
509	++	-	+++	++	++	+++	++	-	-	-
510	-	-	++	-	-	-	-	-	-	++
511	++	++	-	-	++	++	+++	+++	++	
512	-	-	+++	+	+++	-	++	-	-	-
513	+++	-	+++	+++	+++	+++	++	-	-	-
ROI	0.79	0.25	0.75	0.70	0.90	0.88	0.80	0.21	0.38	0.21
ROI ≥ ++	0.74	0.18	0.66	0.64	0.88	0.86	0.69	0.18	0.36	0.18

Reference strains are presented from top to bottom and phages from left to right. Positive reactions (+) were recorded when a minimum of 10 individual plaques (IP) was observed. The clarity of the IP or the spot was scored as turbid (+), clearing with hazy-partial background (++) and complete clearing (+++). Negative reactions were recorded as (-).

4.3.2.2 *Temperature stability of Pa phages*

All Pa bacteriophages were exceedingly stable when stored at 4°C during the twelve-month period. The tested phages showed a minor average reduction in their concentration of 0.8-Log only. This reduction was not found to be statistically significant ($p>0.05$) when compared to their starting point. Pa 51 and Pa 32 were the most sensitive phages to cold storage with a total drop of 2.5-Log and 1.5-Log respectively. The decline in their concentrations was found to be statistically significant ($p<0.05$) when compared to their starting point. On the other hand, the concentrations of the Pa phages showed an average reduction of 3.0-Log when stored at 25°C during the same test period. This drop in concentration was nearly 10 times more than the concentration lost when stored at 4°C. Phages Pa 26, Pa 32 and Pa 51 showed the biggest loss in titre with a 5.8, 8.0 and 3.0-Log drop respectively by the end of the twelve-month period.

At 37°C, the loss in phage concentration was variable among the phages. Pa 1 showed a 1.6-Log drop during the first three months. Then, between the third and fifth month, a further 1.5-Log drop a month was observed with a rapid and final 4.3-Log drop by the sixth month. Pa 3 showed a gradual 0.4-Log average loss for the first three months followed by a 1-Log drop on month four and an average 2.5-Log drop until month 7 by when the phage became totally inactivate. Pa 6, Pa 10 and Pa 32 were the most stable phages with a total 4.0-Log, 3.0-Log and 4.9-Log drop within the twelve-month period. Pa 10 and Pa 32 were interesting in that after a 2.8-Log drop in the fourth month and 4.1-Log drop in the fifth month correspondingly, their titres stabilised and not major drops were observed again. Pa 51 showed a 2-Log decrease in concentration every month and by the third month the concentration fell below the detection limit while Pa 26 showed a 1.0-Log reduction after the first month but for the second month it had dropped 6-Log in its concentration. By the third month Pa 26 had not viable particles. Pa 37 showed a gradual 0.3-Log decrease for the first five months and this was followed by a sharp 3.0-Log decrease with the phage totally inactivated by the ninth month.

4.3.2.3 *pH stability of Pa phages*

Exposure of Pa bacteriophages to a pH value of 7.5 for 60min at 37˚C had no effect on their ability to infect their target bacteria when compared to the control stocks generally stored in SM buffer pH 7.5 and 4ºC (>0.05). When exposed to a pH value of 3.0 for 60min at 37˚C, phage Pa 10 was completely inactivated ($p<0.05$). Bacteriophage Pa 1 and Pa 32 showed the biggest drop in concentration with a 5-Log reduction observed after exposure to the acidic conditions ($p<0.05$). Pa 51 followed closely with a 3-Log reduction ($p<0.05$). Pa 3, Pa 6, Pa 24, Pa 25, Pa 26 and Pa 37 showed a reduction that was not greater than 2-Log in comparison to the bacteriophages incubated at pH 7.5 and 37ºC. However, all bacteriophage samples affected by the low pH incubation were protected from inactivation when pre-mixed with 1% sodium bicarbonate and no statistically significant differences were observed when compared to the control at pH 7.5 ($p>0.05$). The results are summarised in Table 4-5.

4.3.2.4 *One-step growth curves of Pa phages*

The latent period and burst size was determined for each Pa phage selected as previously exemplified in Fig 4-1. Bacteriophage single-step growth curves performed showed Pa 24 and Pa 26 had the smallest burst size with an average burst of 58 ± 3 and 45 ± 2 PFU/cell respectively. Pa 37 followed closely with a burst size of 120 ± 6 while Pa 10 showed the largest burst size with 476±24 PFU/cell. Bacteriophages Pa 1, Pa 3, Pa 6, Pa 25, Pa 32 and Pa 51 showed burst sizes ranging between 200 ± 10 to 289 ± 15 and latent periods ranging from less than 15min up to 60min. Interestingly, Pa 24 with the smallest burst size also had the shortest latent period and Pa 10 with the largest burst size had one of the longest latent periods. The results are summarised in Table 4-6.

The percentage of adsorption achieved within 10min by each bacteriophage to its indicator bacteria was also measured. Bacteriophage Pa 6, Pa 10 and Pa 51 showed the smallest percentage of adsorption with 37.5%, 58.3% and 58% respectively. Pa 32 showed a medium percentage of adsorption with 82.2% while the remaining Pa phages showed percentages of adsorption of over 98%. Results are summarised in Table 4-6.

Table 4-5. pH stability of *P. aeruginosa* phages

Phage	Test samples at 37ºC (PFU/ml)			Control at 4ºC (PFU/ml)
	pH 3.0	pH 3 +NaHCO3	pH 7.5	pH 7.5
Pa 1	1.20E+04	4.00E+09	3.40E+09	3.70E+09
Pa 3	1.20E+08	1.20E+10	2.00E+10	2.40E+10
Pa 6	1.40E+09	4.00E+10	1.00E+11	1.30E+11
Pa 10	<10	8.00E+09	1.40E+10	1.50E+10
Pa 24	3.00E+09	1.00E+10	8.50E+09	8.20E+09
Pa 25	1.00E+9	2.00E+11	4.00E+11	4.60E+11
Pa 26	3.20E+07	6.00E+09	8.00E+09	8.30E+09
Pa 32	8.00E+03	6.00E+08	4.00E+08	4.40E+08
Pa 37	1.30E+09	2.20E+11	3.20E+11	3.10E+11
Pa 51	2.00E+03	4.00E+06	8.00E+06	8.10E+06

Bacteriophages at pH 3.0, pH 3.0 protected with sodium bicarbonate (NaHCO3) and pH 7.5 were incubated at 37°C for 1h and then serially diluted 10-fold in SM buffer before plating out for enumeration of viable phage. The value given is the mean of three separated experiments and titrations. Bacteriophage stocks stored at pH 7.5 and 4ºC were used as controls.

Table 4-6. One-step growth curve of Pa phages

Phage	% of Adsorption (after 10min)	Latent Period range (min)	Burst size (±SD)
Pa 1	99.9	45-55	225 ± 25
Pa 3	99.3	15-25	289 ± 15
Pa 6	37.5	60-70	283 ± 14
Pa 10	58.3	60-70	476 ± 24
Pa 24	98.40	<10-15	58 ± 3
Pa 25	99.9	15-25	200 ± 20
Pa 26	99.9	15-25	45 ± 2
Pa 32	82.2	60-70	225 ± 11
Pa 37	99.9	30-40	120 ± 6
Pa 51	58.00	60-70	200 ± 10

The ability of each phage to absorb to a bacterial cell (% of adsorption), its latent period and burst size was measured in a single step growth curve. (±SD) indicates standard deviations.

4.3.2.5 *Effect of bacteriophage concentration on P. aeruginosa bacteria*

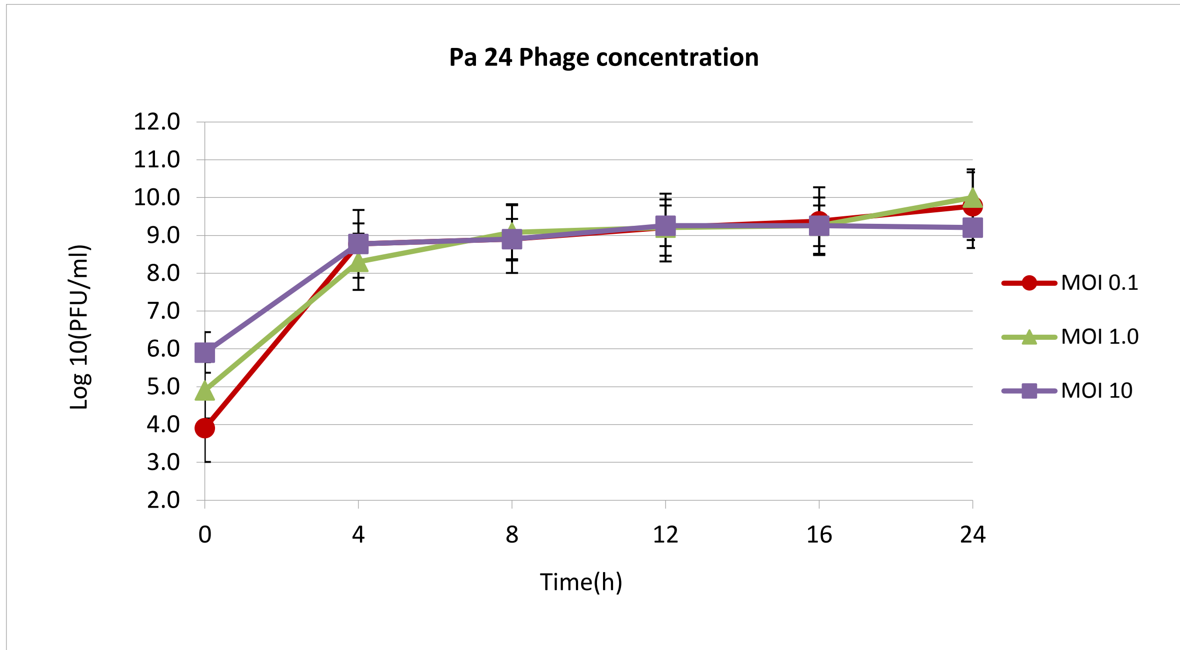
Bacteriophages were investigated for their efficacy to prevent the growth of their indicator bacteria when the infection was carried out in a broth culture. The efficacy of the phages was evaluated at three different multiplicities of infections (MOIs). That is the ratio of bacteriophage to bacteria. The initial MOIs tested were approximately 0.1, 1 and 10.

Bacteriophage multiplication was evident in all the infections performed. Pa 1, 3, 24, 25, 26, 32, 37 and 51 showed very similar growth patterns. For these phages, the highest increase in their numbers was observed during the first 4h after the beginning of the infection process For bacteriophages Pa 3, Pa 24, Pa 26, Pa 32 and Pa 37 all three MOI's tested reached concentrations that were not different from each other after 4h ($p>0.05$) and remained relatively constant with 1-2 Log_{10} increases observed after 16h (*i.e.* Pa 24, Fig 4-6).

In contrast, for Pa 1 and Pa 51, the MOI of 1.0 showed a higher phage concentration than the MOI of 10 after 4 h ($p<0.05$). However, the final bacteriophage concentrations at 24h for all three MOI's were comparable ($p>0.05$) (*i.e.* Pa 51, Figure 4-7). For bacteriophage Pa 6 the peek increase in their titre for all three MOIs was observed after 8 h post-inoculation while Pa 10 showed a constant titre increase for over the 24h period. For both phages the final titre for the three MOI's used were comparable ($p>0.05$).

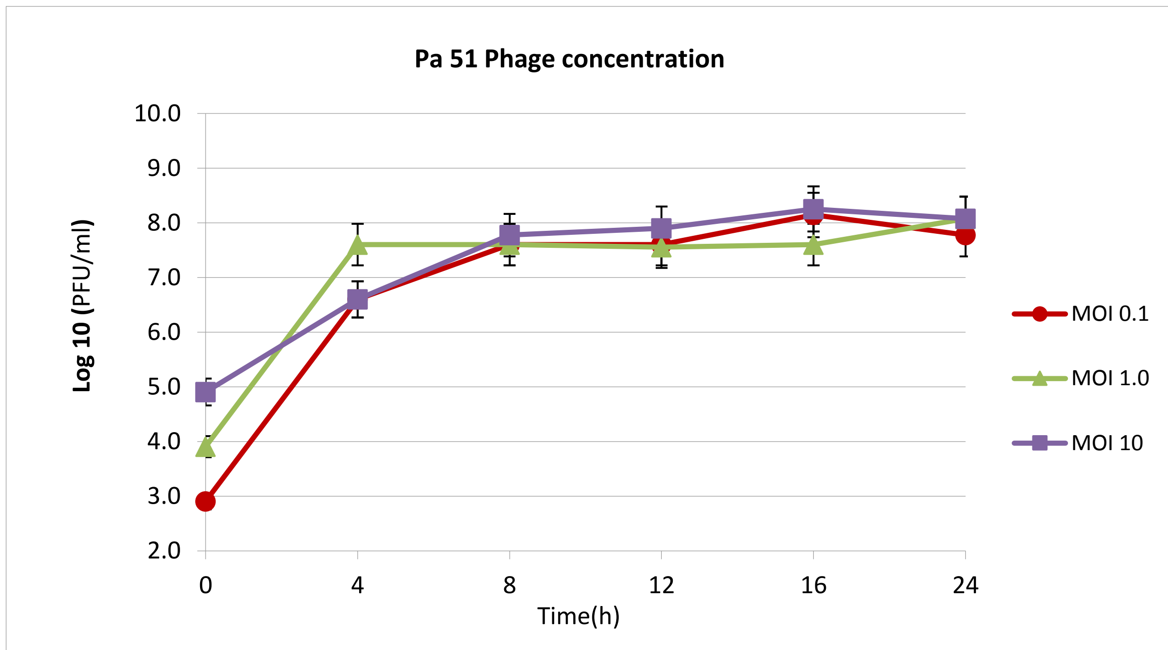
Similarly, indicator bacteria infected with phages Pa 1, Pa 3, Pa 24, Pa 25, Pa 26, Pa 37 and Pa 51 showed alike growth patterns. The growth of these bacteria, in comparison to the uninfected control, was halted by the bacteriophages for 16 h irrespective of the initial MOI used. However, after 16 h, re-growth was observed in all test tubes (*i.e.* Pa 51, Fig 4-8). Nonetheless, after 24h, the difference in growth between the control tubes and the phage-infected test tubes was statistically significant for all infections performed ($p<0.05$).

Figure 4-6. ***P. aeruginosa*** **bacteriophage Pa 24 growth**



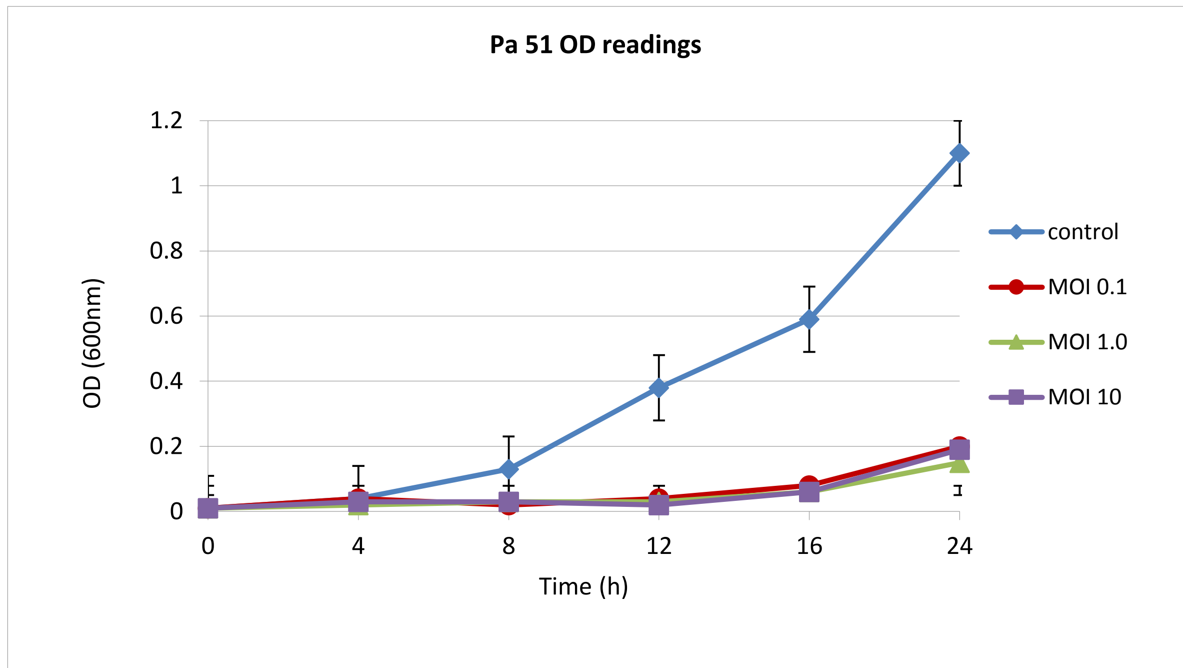
The highest increase in Pa 24 concentration was observed during the first 4h after the beginning of the infection process. All three MOI's tested reached concentrations that were not different from each other after 4h ($p>0.05$) and remained relatively constant with 1-2 Log_{10} increases observed after 16h.

Figure 4-7. ***P. aeruginosa*** **bacteriophage Pa 51 growth**



The highest increase in Pa 51 concentration was observed during the first 4h after the beginning of the infection process. The MOI of 1.0 showed a higher phage concentration than the MOI of 10 after 4h ($p<0.05$). However, after this, phage concentration for the MOI 1.0 remained relatively constant while the MOI of 0.1 and 10 showed a 1-2 Log_{10} increase over the next 16h. The final bacteriophage concentrations at 24h for all three MOI's were comparable ($p>0.05$).

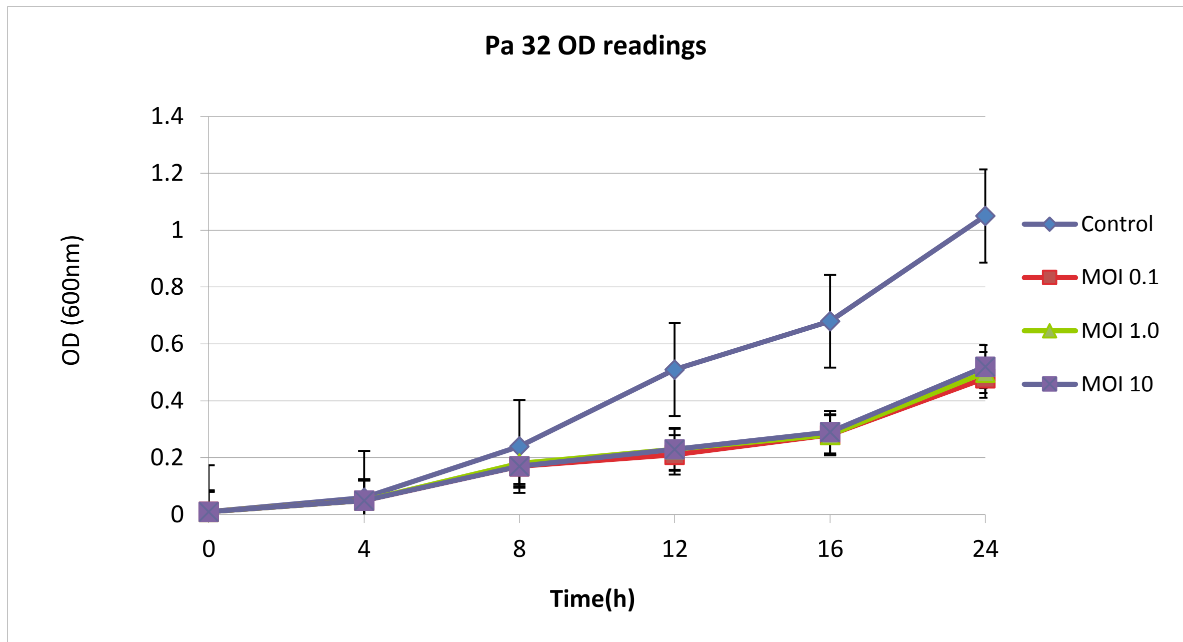
Figure 4-8. Effect of bacteriophage Pa 51 on bacterial growth



Pa 51 was capable of inhibiting P. aeruginosa growth significantly in comparison to the control for 16h regardless of the initial MOI (p<0.05). However, after 16h, bacterial overgrowth was observed for all three MOIs. Nonetheless, after 24h, the difference in growth between the control tubes and the phage-infected test tubes was statistically significant for all infections performed (p<0.05).

The infections carried out with bacteriophages Pa 6, Pa 10 and Pa 32 showed no bacterial growth reduction, when compared to the control, during the first 8 h ($p>0.05$) Then between 8 h and 16 h, the OD of the infected tubes remained relatively unchanged while the control tube continued to grow. Finally, after 16h, bacterial growth in the infected test tubes was resumed and the OD readings of all test tubes were comparable irrespective of the initial MOI used ($p>0.05$) (*i*.e. Pa 32, Fig 4-9). However, as for the first group, the difference in growth between the control tubes and the phage-infected test tubes was statistically significant for all infections performed after 24h ($p<0.05$).

Figure 4-9. **Effect of bacteriophage Pa 32 on bacterial growth**



Bacteriophage Pa 32 infection showed no bacterial growth reduction during the first 8h in comparison to the control ($p>0.05$). Then between 8h and 16h, the OD of the infected tubes remained relatively unchanged, while the control tube continued to grow. After 16h, bacterial growth in the infected tubes was resumed and the OD readings of all test tubes were comparable irrespective of the initial MOI used ($p>0.05$). However, after 24h, the difference in growth between the control tube and the phage-infected test tubes was statistically significant for all infections performed ($p<0.05$).

4.4 Discussion

Frequently, bacterial reference collections used of bacteriophage isolation are relatively small in numbers, mostly for practical reasons (Atterbury et al., 2003, Sillankorva et al., 2008, Uchiyama et al., 2008). In this study, in addition of size and as discussed earlier (section 3.7), special care was taken to ensure the reference collections used for bacteriophage isolation comprised the most relevant bacterial clones from a clinical point of view in Australia. That is those isolates that are more frequently found responsible of causing disease in hospitals and in the community. Furthermore, previous knowledge obtained about the small genome variation of each isolate by sequenced-based methods such as multi-locus sequence typing (MLST) was also considered (section 1.2.1).

However, even when sequenced-based methods such as MLST are used, small differences between bacterial isolates that are closely related may not be detected (Harris et al., 2010, Johnson et al., 2007). Thus, the possibility of small undetectable genome variations between reference strains and clinical isolates must be considered during the isolation process of bacteriophages, as such differences could result in an increase or a significant reduction of the overall activity of a potential therapeutic phage product. This would suggest that after the efficiency of a bacteriophage is determined against a collection of bacteria that represent the most prevalent isolates affecting the community, the testing of its activity against a collection of clinical isolates is essential.

The importance of this additional step was confirmed in this study. As predicted, the results showed an increase or a reduction in the initial ratio of infection (ROI) calculated for most of the selected bacteriophages. Bacteriophages Sa 12, Sa 34 and Sa 44 showed an average increase of 0.34 while bacteriophages Sa 32, Sa 38, Sa 41, Sa 42, Sa 53 and Sa 54 showed smaller increases in their ROI values of 0.1, 0.03, 0.05, 0.05, 0.17 and 0.19 correspondingly. However, bacteriophages Sa 18 and Sa 51 showed small reductions of 0.04 and 0.06. In comparison, the changes in the ROI values of the *P. aeruginosa* phages showed more significant reductions. Bacteriophages Pa 1, Pa 3, Pa 6, Pa 10, Pa 32 and Pa

37 showed a reduction of 20% or more in their ROI values while Pa 24 and Pa 51 both showed an increase in their value of 0.08 and 0.18 respectively.

Furthermore, the results obtained after the testing of the clinical isolates with the selected phages (Table 4-1 and 4-4) revealed different phage infection patterns to those observed against the reference collection (Table 3.3 and 3-7). Thirty-three different *S. aureus* phage patterns were identified in the clinical isolates tested and only 47 of the strains tested showed similar patterns to those used in the reference collection. For the *P. aeruginosa* phages, the number of phage patterns reached 53 out of 80 clinical isolates tested. Although the selected phages would not meet all the criteria to be used in a bacterial typing tool –as bacteriophages used in typing schemes generally tend to have a much narrower spectrum of activity (Saragea and Maximescu, 1966), the large number of different phage patterns observed, highlighted the diversity of isolates that can be found in hospitals and communities in Australia.

In composing the reference collections, the most predominant isolates in the clinical environment were considered first, therefore, these results of multiple phage patterns may not be totally unexpected. For instance, the *S. aureus* reference library contained the current top four most predominant types of HA-MRSA and CA-MRSA isolates in Australia but not some other much less predominant types previously reported. An epidemiological and typing study performed during the first semester of 2009 in Western Australia reported an additional four clones of HA-MRSA and four of CA-MRSA circulating in the community, albeit at very low levels (Coombs et al., 2009b). Furthermore, from 2003 to 2009 a total of 48 novel clones were identified in the same area (Coombs et al., 2009b).

The larger number of phage patterns observed for the *P. aeruginosa* isolates may be better explained by the natural strain diversity, which has been identified to be in some cases even down to a particular hospital, including their antibiotic resistance patterns (Prashanth et al., 2010). Furthermore, the population structure from different patient populations, such as those suffering from cystic fibrosis (CF), was demonstrated to be not only highly diverse but also very host-specific (Van Mansfeld et al., 2010). Similar results on the specificity of

P. aeruginosa isolates in CF patients were also reported in Australian studies (O'Carroll et al., 2004).

Thus, this diversity may be partly responsible for the lack of a standardised *P. aeruginosa* molecular typing system, such as that developed for MRSA isolates, and explains why the latest *P. aeruginosa* studies have been focusing on local diversity (de Vrankrijker et al., 2011, Van Mansfeld et al., 2010, Silva et al., 2011, Kouda et al., 2009). Recently, and most commonly, the selection of *P. aeruginosa* isolates for studies is based on the results of their antibiograms, as performed in this study, as their susceptibility to particular antibiotics provides unique key data in regards to the development and distribution of resistance patterns that can be directly connected to clinical outcomes (Cuttelod et al., 2010).

The clear diversity of bacterial clones, as well as the complexity of identifying these rapidly to a molecular level, suggests, therefore, that the inclusion of clinical isolates for bacteriophage testing is an essential step during the development of a phage-based product. This testing must be included with an aim to ensure that bacteriophages with the ability to infect both predominant and not so predominant isolates are selected for therapeutic use. This study showed that the majority of the selected bacteriophages were highly effective (as demonstrated by their high ROI values) not only against the reference collection but also against the clinical isolates tested and thus, that the isolation of bacteriophages with broad spectrum of activity for future product development was possible.

Additionally to the isolation of bacteriophages with a broad spectrum of activity, in order to establish bacteriophage therapy as a means of controlling infections caused by *S. aureus* and *P. aeruginosa*, it is vital to comprehend the dynamics of potential therapeutic bacteriophages. Thus, *in vitro* testing was done to establish the natural strengths and weaknesses for each of the selected bacteriophages such as temperature and pH stability, ability to adsorb to the target bacteria, burst size and effect on bacteria growth.

The results of the temperature stability testing showed that the *S. aureus* bacteriophages were very stable at 4°C storage during the twelve-month period tested with a total average reduction in their concentration of only 0.24-Log ($p>0.05$). Their stability was more

affected by storage at 25°C with an average reduction 10 times greater than the concentration lost when the phages were stored at 4°C ($p<0.05$). At 37°C, all *S. aureus* phages lost in average 1.7-Log in the first two months and only one phage showed viable particles after 6 months of storage at the test temperature. Similarly, all but two *P. aeruginosa* bacteriophages (Pa 32 and Pa 51) were highly stable when stored at 4°C during the twelve-month period ($p>0.05$). At 25°C, an average reduction 10 times greater than the concentration lost when the phages were stored at 4°C was also measured ($p<0.05$) while at 37°C their sensitivities varied with the last phage fully inactivated by the ninth month (section 4.3.2.2).

Temperature stability studies with a view to predicting product stability during storage conditions are a requirement for regulatory agencies during the development of biological products. This is because biological products naturally change in storage. However, as long as the characteristics of the product remain within the original specifications, the product will be considered stable, and the period of time that the product remains stable at the suggested storage conditions is denoted to as the shelf-life (European Medicines Agency, 2003). The data collected to date suggest that prospective products in liquid form will need to be stored at 4°C to preserve their titres and a shelf-life of over 10-12 months can be expected.

The pH stability testing results showed that exposure of either the *S. aureus* or *P. aeruginosa* bacteriophages to a pH value of 7.5 for 60 min at 37˚C had no effect on their ability to infect their target bacteria when compared to the control stocks stored in SM buffer pH 7.5 and 4ºC ($p>0.05$). However, all *S. aureus* phages were inactivated when exposed to a pH value of 3.0 for 60min at 37˚C ($p<0.05$) while the *P. aeruginosa* exhibited different levels of sensitivity (section 4.3.2.3). All bacteriophage samples affected by the low pH incubation were protected from inactivation when pre-mixed with 1% sodium bicarbonate and no statistically significant differences were observed when compared to the control at pH 7.5 ($p>0.05$).

The stability of bacteriophages at neutral pH was well described in the initial periods of phage research (Kerby et al., 1949, Hook et al., 1946) and the results obtained in this, and recent studies (Kłak et al., 2010), have confirmed the earlier observations. This suggested that for long-term storage, in addition to storing bacteriophages at 4ºC, a neutral pH storage matrix should be used. Additionally, for the selection of potential therapeutic bacteriophages, their ability to tolerate low pH conditions was investigated. The effect of low pH conditions on the viability of bacteriophages is particularly important if a bacteriophage product is to be delivered orally. In this study, the low pH tolerance of all *S. aureus* and some *P. aeruginosa* phages to acidic conditions implied that the neutralisation of gastric acid could be essential for bacteriophage survival *in vivo*.

The importance of neutralising stomach acids before bacteriophage delivery by administering aluminium and magnesium hydroxide or calcium and magnesium carbonate has been identified before (Dabrowska et al., 2005). This study showed that the addition of sodium bicarbonate, prior to the exposure of bacteriophages to acid conditions, helped to protect sensitive bacteriophages from the effects of low pH conditions. However, when possible, bacteriophages that are naturally resistant to low pH conditions should be preferred for therapeutic purposes, in order to increase their probabilities of survival and preserve the therapeutic dose delivered.

In general, the life cycle of a lytic bacteriophage consists of an adsorption period, bacteriophage attachment and nucleic acid injection, followed by an eclipse period where phage proteins are synthetized and a last stage in which the bacteriophages develop and a new progeny is released (Hyman and Abedon, 2009). The period of time needed between the adsorption of a phage to a bacterium and the release of the new progeny is known as latent period, and the number of new bacteriophages produced for each bacteriophage-infected bacterium is known as the burst size. In this study, the percentage of adsorption, latent period and burst size of each selected bacteriophage were measured. These three parameters were considered critical to investigate if each of the potential therapeutic bacteriophages identified could replicate actively and efficiently once it met its target bacteria.

All bacteriophages were found to adsorb very efficiently to their host cell with *P. aeruginosa* phage Pa 6, Pa10 and Pa 51 showing the lowest percentage of adsorption.

One-step growth curves analysis showed the *S. aureus* phages have very short latent periods of 10min to 25min while the latent periods for the *P. aeruginosa* phages varied greatly. Four phages showed a short latent period of 15min to 25min, four showed a long latent period of 60min to 70min and two showed "medium" latent periods of 30min to 40min and 45min to 55min (Table 4-6). Interestingly, those *P. aeruginosa* phages with the longest latent periods also showed, in general, the largest burst sizes. Bacteriophage one-step growth curves performed also showed *S. aureus* bacteriophages had burst sizes of 55 ± 3 PFU/cell up to 461 ± 23 PFU/cell. The calculated burst sizes for the *P. aeruginosa* phages were similar from 45 ± 2 PFU/cell up to 476 ± 24 PFU/cell. From the one-step growth studies, all selected *S. aureus* and *P. aeruginosa* bacteriophages exhibited bacteriophage amplification, demonstrating their ability to infect the targeted host.

All the selected bacteriophages showed active replication on their corresponding indicator strains as demonstrated not only by the calculated burst sizes (Table 4-3 and 4-6) but also by the increases in phage concentration observed when the infection was carried out in a broth culture and at multiplicities of infection (ration of phage to bacteria) of 0.1, 1.0 and 10 (sections 4.3.1.5 and 4.3.2.5). The results showed that some infection processes appeared to be dose dependent (Fig. 4-5) while others were equally effective in reducing the target bacteria over a 24h period regardless of the initial phage concentration (Fig 4-4a, 4-8). However, some bacteriophages (Sa 41 and 42) were not capable of inhibiting bacterial growth totally despite their obvious amplification or failed to amplify on their bacterial host (Sa 18 and Sa 32).

The analysis of growth kinetics suggested that the majority of the bacteriophages were capable of infecting and multiplying on their host bacteria. Of the *S. aureus* bacteriophages tested, when compared to the rest of the selected phages, Sa 18, Sa 32, Sa 41 and 42 appeared to be the least suitable for therapeutic use due to their inability to amplify and

inhibit bacterial growth, perhaps due to potential lysogenization in the corresponding indicator strains. The *P. aeruginosa* phages amplified successfully on their host bacteria although all showed slight bacterial regrowth after 16h, suggesting a potential risk to develop bacterial resistance. Traditionally, in order to address the issues of resistance, bacteriophage mixes or "cocktails" were developed. The information about the different characteristics observed for each bacteriophage examined in this study (spectrum of activity and physical characteristics), will be combined and used to formulate prototype phage cocktails. The development of prototype cocktails may determine the ability of a set of bacteriophages to work synergistically, it may expand their spectrum of activity and determine their ability to minimise the appearance of phage-resistant cells.

5 DEVELOPMENT OF BACTERIOPHAGE COCKTAILS

5.1 Introduction

One of the great advantages of bacteriophages is their exquisite specificity for a particular bacterial species, sub-species, serovar, or even an individual isolate. However, this advantage can also be a major disadvantage, in that a particular bacteriophage may be too specific. This limitation has traditionally been overcome by combining phages with overlapping reactivities into a phage "cocktail", resulting in a broad-spectrum antibacterial agent. Phage cocktails are thus combinations of phages that, jointly, display a broader spectrum of activity in comparison with their individual phage components. Several groups have evaluated the use of phage cocktails to decrease loads of various bacterial pathogens and settings with different degrees of success (Tanji et al., 2005, Denou et al., 2009, Fu et al., 2010). Therefore, the development of bacteriophage cocktails can be used as a strategy to overcome any issues of narrow host range due to the high specificity of bacteriophages.

Furthermore, the development of phage cocktails can also facilitate the use and acceptance of phage therapy in a busy clinical setting. The formulation of phage cocktails with a broad range of activity would help to minimise the time required between the diagnosis of an infection and commencement of treatment. These broad-spectrum preparations also provide the opportunity to reformulate and develop a patient-specific cocktail, if desired. The question then arises whether a phage combination, developed in one country against the dominant isolates of a particular bacterial species, would prove too specific in another geographical location, thus minimising the cost-effectiveness of a potential treatment

Another major concern with regard to the use of therapeutic phages is the appearance of phage-resistant bacteria (Brussow, 2005). The understanding that the co-evolution of bacteria and bacteriophage leads to a continuous battle between the phage and its corresponding phage-resistant bacterium is the basis for such concern (Lenski, 1984). This issue of resistance may also be addressed with the use of cocktails. The use of cocktails had

been shown to reduce the frequency of bacteriophage-resistant bacteria (O'Flynn et al., 2004) and has for a long-time been the practice in the Republic of Georgia where bacteriophage therapy is an over the counter approved medicine (reviewed by.Kutter, De Vos et al. 2010).

In this study, therapeutic phage cocktails were developed against *P. aeruginosa* and *S. aureus* strains isolated in Australia and their activities tested against clinical isolates across a number of widely differing geographical locations. The ability of these cocktails to minimize the appearance of phage-resistant cells was also evaluated.

5.2 Formulation of Bacteriophage Cocktails

The formulation of the phage cocktails was made following the results on the phage specificity observed and the ratio of infection calculated (section 4.3) for each *S. aureus* and *P. aeruginosa* selected bacteriophage (Table 3-6 and Table 3-7). As a general rule, it was decided that the number of phages per cocktail should not be higher than six, preferably three to four. There are several practical and economic reasons for this. During the production of a bacteriophage product, it is almost certain that regulatory agencies would require for each phage component to be grown separately in order to maintain all growth variables under tight control. This would mean the establishment of a continuous inspection system that will be constrained with increasing GMP requirements for each bacteriophage going from Phase I to commercialization. Thus, the larger the number of components in the cocktail the more complicated the production process could become. This could be both time-consuming and economically unsustainable.

For the formulation of the *P. aeruginosa* cocktails, three different approaches were considered for comparison. For the first approach, the most traditional method in which bacteriophages with the broadest host range are combined was used to formulate the first cocktail. For the second approach those bacteriophages with a narrow but complementary range of activity were combined. The third approach used was a combination of the two methods in which phages with broad and narrow spectrum of activity were combined. The following three cocktails were thus formulated:

- MediPhage - Pa prototype cocktail E: Pa 1, Pa 24, Pa 25 and Pa 26
- MediPhage - Pa prototype cocktail F: Pa 3, Pa 6, Pa 10, Pa 32 and Pa 37
- MediPhage - Pa prototype cocktail G: Pa 1, Pa 6, Pa 25 and Pa 51

For the formulation of the *S. aureus* bacteriophages only the most traditional method was used due to the relatively broad spectrum of activity that all the selected phages showed. However, one combination included one of the phages with a broad spectrum of activity and the phage that showed the narrowest host range against the collection of clinical isolates. In the selection process, it was also ensured that the indicator bacteria used to grow the phages within a cocktail were isolated from different sources. Three prototype cocktails were then formulated:

- MediPhage – Sa prototype cocktail 1: Sa 34, Sa 44 and Sa 51
- MediPhage – Sa prototype cocktail 2: Sa 12 and Sa 18
- MediPhage – Sa prototype cocktail 3: Sa 38, Sa 42 and Sa 54

5.3 Host Range of *S. aureus* (Sa) Bacteriophage Cocktails Against Local and Foreign Isolates

5.3.1 Lytic activity of Sa bacteriophage cocktails against reference collection

The spectrum of activity and titre of the bacteriophage cocktails formulated was determined against the reference collection (Table 2-1) as described in section 2.6.6.1.

5.3.2 Lytic activity of Sa bacteriophage cocktails against clinical isolates collected in Australia

The spectrum of activity and efficiency of plating of the bacteriophage cocktails formulated was determined against the collection of clinical isolates (Appendix 1) as described in section 2.6.6.1.

5.3.3 Lytic activity of Sa bacteriophage cocktails against clinical isolates collected in the United Kingdom

Kits of the *S. aureus* bacteriophage cocktails 1, 2 and 3 were prepared and sent over to the UK for testing in an effort to obtain a third party evaluation of the activity of the prototype mixes. Each bacteriophage kit consisted of five O-ring vials and each vial was filled with 200µl neat cocktail (vial 5) and its corresponding dilutions 10^{-1} (vial 4), 10^{-2} (vial 3), 10^{-3} (vial 2) and 10^{-4} (vial 1). Bacteriophage dilutions were prepared in SM buffer and stored at 4˚C until used. A collection of 109 *S. aureus* isolates were tested against the three formulated cocktails and their sensitivity tested as per protocol 2.6.6.1.

5.4 Host Range of *P. aeruginosa* (Pa) Bacteriophage Cocktails Against Local and Foreign Isolates

5.4.1 Lytic activity of Pa bacteriophage cocktails against reference collection

The spectrum of activity of the bacteriophage cocktails formulated was determined against the reference collection (Table 2-3) as described in section 2.6.6.1.

5.4.2 Lytic activity of Pa bacteriophage cocktails against clinical isolates collected in Australia

The spectrum of activity of the bacteriophage cocktails formulated was determined against the collection of 85 clinical isolates collected in Australia. Ten microliters of each bacteriophage cocktail ($Log_{10}8$ PFU/ml) was spotted neat in triplicate onto bacterial lawns (section 2.6.1) of each test isolates. The activity of the cocktail was noted and their clarity scored as turbid (1), clearing with hazy-partial background (2) and complete clearing (3). Negative reactions were recorded as (0).

5.4.3 Lytic activity of Pa bacteriophage cocktails against clinical isolates collected in the United Kingdom[1]*

Kits of the *P. aeruginosa* bacteriophage cocktails E, F and G were prepared and sent over to the UK for testing in an effort to obtain a third party evaluation of the activity of the prototype mixes. Each bacteriophage kit consisted of five O-ring vials and each vial was filled with 200µl neat phage (vial 5) and its corresponding dilutions 10^{-2} (vial 4), 10^{-3} (vial 3), 10^{-4} (vial 2) and 10^{-5} (vial 1). Bacteriophage dilutions were prepared in SM buffer and kits stored at 4˚C until used.

A collection of 142 *P. aeruginosa* clinical strains isolated from patients suffering from cystic fibrosis and chronic wound infections was tested against the three formulated cocktails and their sensitivity tested as per protocol 2.6.6.1.

5.4.4 Lytic activity of Pa bacteriophage cocktails against clinical isolates collected in the United States

A collection of 189 *P. aeruginosa* strains isolated from patients with cystic fibrosis (CF) was tested against the formulated cocktails and their sensitivity tested. Ten microliters of each bacteriophage cocktail ($Log_{10}8$ PFU/ml) was spotted neat in triplicate onto bacterial lawns (section 2.6.1) of each test isolates. The activity of the cocktail was noted and their clarity scored as turbid (1), clearing with hazy-partial background (2) and complete clearing (3). Negative reactions were recorded as (0).

5.4.5 Lytic activity of Pa bacteriophage cocktails against clinical isolates collected in Guatemala City[2]

Bacteriophage cocktail kits of *P. aeruginosa* cocktails E and F were prepared and sent over to Guatemala City in an effort to obtain a third party evaluation of the activity of the prototype mixes. Each bacteriophage kit consisted of five O-ring vials and each vial was

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[1] Bacteriophage kits and testing protocol were prepared by Sandra Morales and the testing kindly performed by Chris Orton at a private microbiology Lab in the UK.

[2] Bacteriophage kits and testing protocol were prepared by Sandra Morales and the testing kindly performed by Sergio Perez at a private microbiology Laboratory in Guatemala city.

filled with 200µl neat phage (vial 5) and its corresponding dilutions 10^{-2} (vial 4), 10^{-3} (vial 3), 10^{-4} (vial 2) and 10^{-5} (vial 1). A collection of 150 *P. aeruginosa* strains isolated from patients with several medical conditions was then tested against the formulated cocktails and their sensitivity against the formulated cocktails tested as per protocol 2.6.6.1.

5.5 Bacteriophage Resistant Cells

5.5.1 Frequency of emergence of bacteriophage-insensitive mutants: individual phage vs phage cocktails

A proportion of bacteria within a population may possess an innate ability to resist infection by bacteriophages. These bacteria are referred to as Bacteriophage-insensitive mutants (BIM). The frequency of emergence of BIM was previously described by O'Flynn and colleagues (O'Flynn *et al*, 2004) and it was here studied for the selected bacteriophages. Overnight cultures from every indicator strain were grown in triplicate and their concentration confirmed by the standard plate count technique. A known concentration of bacteria was then infected with the corresponding phage and phage cocktail at an MOI of 10 in triplicate. Test samples were then plated and incubated overnight at 37°C. The BIM frequency was determined as the number of surviving colonies divided by the original bacterial titre. Standard deviations were determined and the results compared using the comparing means t-test analysis (AnalystSoft, StatPlus:mac - statistical analysis program for Mac OS.Version 2009).

5.5.2 Susceptibility of presumptive phage-resistant cells to individual bacteriophages within a cocktail

Overnight cultures of the indicator strains used to grow each selected bacteriophage were prepared as per protocol section 2.1.1. Each well of a 24-well plate was then set up with 2ml of NB and a known concentration of each indicator strain with and without its corresponding selected bacteriophage at an MOI of 10. Inoculated wells were left to grow for 24h at 37˚C and the difference in bacterial optical density (turbidity at 600nm) between the phage-infected cultures infected to the control well was compared. Phage infected wells that showed turbidity were collected, centrifuged (6,000*g* x 5min) to remove

bacteriophages and the pelleted bacterial cells resuspended in 200µl of NB. Three consecutive passages of each strain were done on NA plates to eliminate any residual bacteriophages. A lawn of each presumptive phage-resistant cell recovered was prepared (section 2.6.1) and its susceptibility to the initial phage and the phages present within a formulated cocktail tested (section 2.6.7).

5.6 Results

5.6.1 Host range of *S. aureus* (Sa) bacteriophage cocktails against local and foreign Isolates

5.6.1.1 *Lytic activity of Sa bacteriophage cocktails against reference collection*

The spectrum of activity for each of the Sa bacteriophage cocktails formulated was determined against the reference collection (Table 2-1). The three bacteriophage cocktails were effective against all the reference strains. The ratio of infection (ROI) for bacteriophage cocktails CSa 1, CSa 2 and CSa 3, when considering the clear activities only, was 0.95, 0.90 and 0.90 respectively.

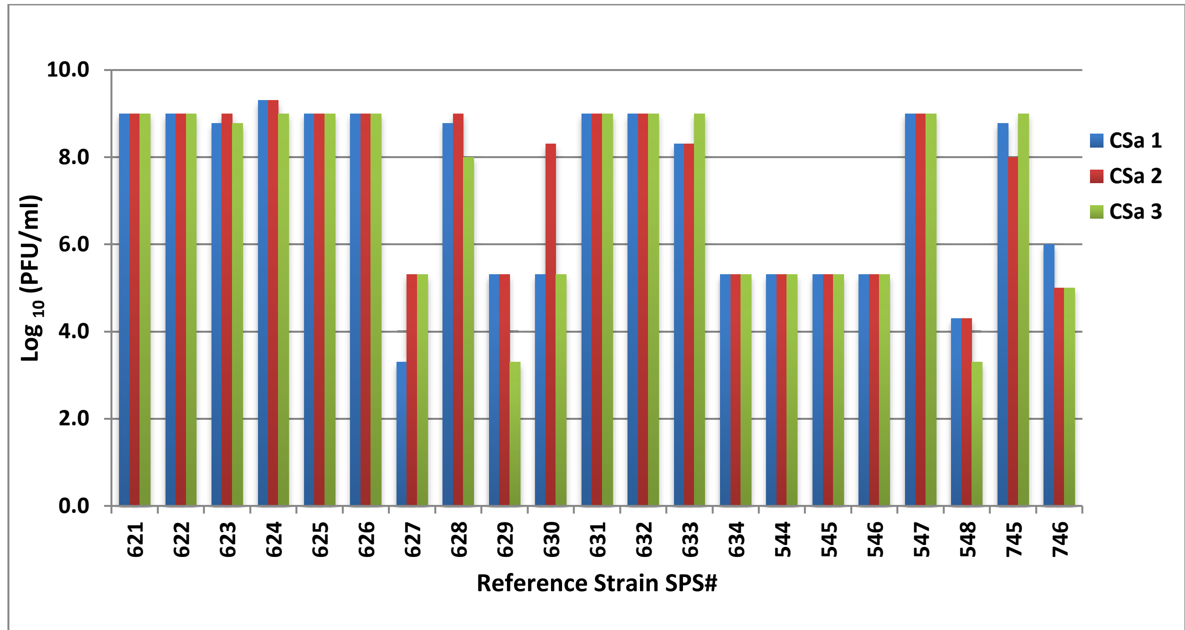
The visualisation of bacteriophage plaque forming units (PFU) against a particular isolate demonstrates the ability of a particular phage or phage cocktail to infect a bacterial isolate. The higher the efficiency of a phage or phages against an isolate, the higher the PFU counts. For bacteriophage cocktails CSa 1 and CSa 2, the highest calculated concentration was observed against the reference strain SPS#624 with a titre of $2x10^9$ PFU/ml. For Sa 3, the highest concentration observed was also $1x10^9$ PFU/ml, although this value was not exclusively obtained with SPS#624 (Figure 5-1).

5.6.1.2 *Lytic activity of Sa bacteriophage cocktails against Australian clinical isolates*

The spectrum of activity for each of the Sa bacteriophage cocktails formulated was determined against the *S. aureus* clinical isolates (Appendix 1). The three bacteriophage cocktails combined were effective against all the isolates tested. The ratio of infection (ROI) for bacteriophage cocktails CSa 1, CSa 2 and CSa 3, when considering the clear activities only, was 0.94, 0.61 and 0.92 respectively (Table 5-2). Bacteriophage cocktail

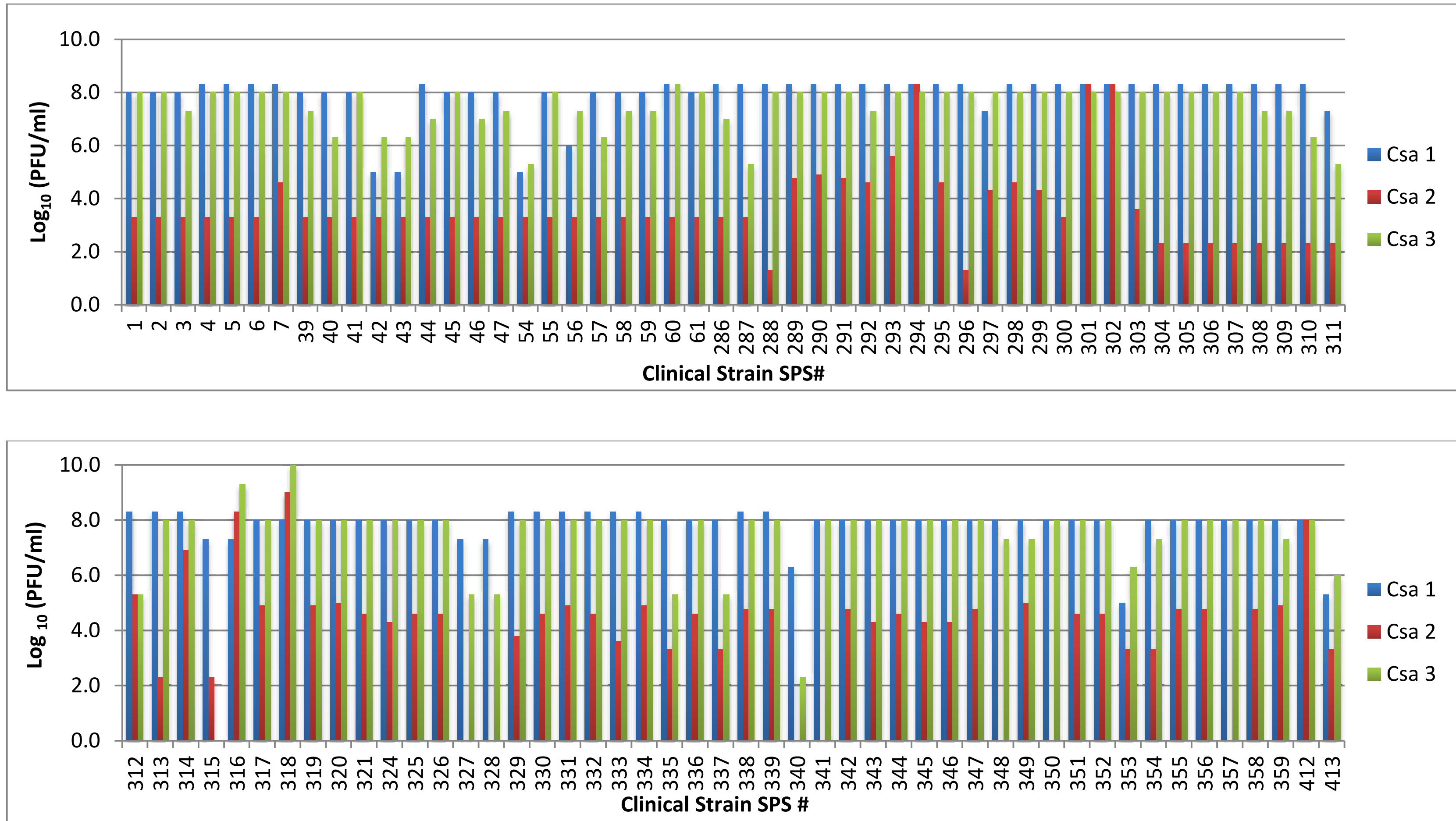
CSa 2 not only showed the lowest spectrum of activity but fewer infective units when compared with the reference strain (section 5.6.1.1). Bacteriophage cocktail CSa 1 showed the highest efficiency of plating followed closely by cocktail CSa 3. The results are summarised in Figure 5-2.

Figure 5-1. **Lytic activity of *S. aureus* (Sa) cocktails 1, 2 and 3 against the reference collection**



Bacteriophage cocktails CSa 1, CSa 2 and CSa 3 (Log$_{10}$ 8 PFU/ml) were 10-fold diluted to 10^{-6} and used to spot onto the 21 S. aureus reference strains. The calculated titre of the cocktails against each isolate is presented (Log$_{10}$ PFU/ml).

Figure 5-2. **Efficacy of *S. aureus* bacteriophage cocktails against clinical strains isolated in Australia**



Bacteriophage cocktails CSa 1, CSa 2 and CSa 3 (Log_{10} 8 PFU/ml) were 10-fold diluted to 10^{-6} and used to spot onto the 19 S. aureus reference strains. The calculated titre of the cocktails against each isolate is presented (Log_{10} PFU/ml).

5.6.1.3 *Lytic activity of SA bacteriophage against clinical isolates collected in the United Kingdom*

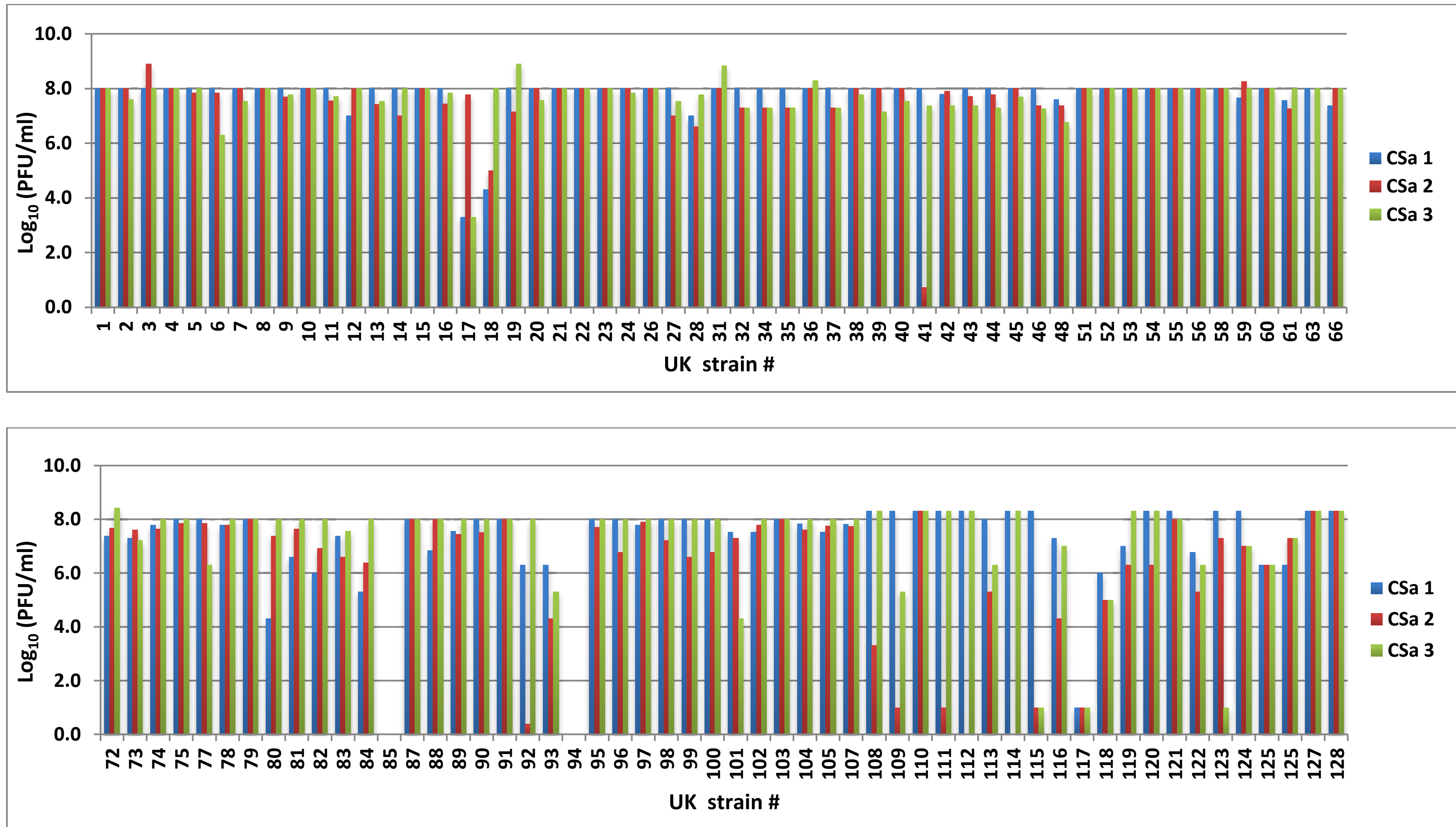
The spectrum of activity for each of the Sa bacteriophage cocktails formulated was determined against 108 *S. aureus* clinical isolates collected in the UK. The antibiotic profiles and site of isolation of the strains are presented in Table 5.3. A non - *S. aureus* clinical strain (TCS #85) was added to the collection. The three bacteriophage cocktails combined were effective against 107 of *S. aureus* isolates tested. None of the Sa cocktails were active against the non - *S. aureus* strain. The ratio of infection (ROI) for bacteriophage cocktails CSa 1, CSa 2 and CSa 3, when considering the clear activities only, was 0.96, 0.92 and 0.96 respectively. The results are summarised in Figure 5-3.

5.6.2 Host range of *P. aeruginosa* (Pa) bacteriophage cocktails against local and foreign isolates

5.6.2.1 *Lytic activity of Pa bacteriophage cocktails against reference collection*

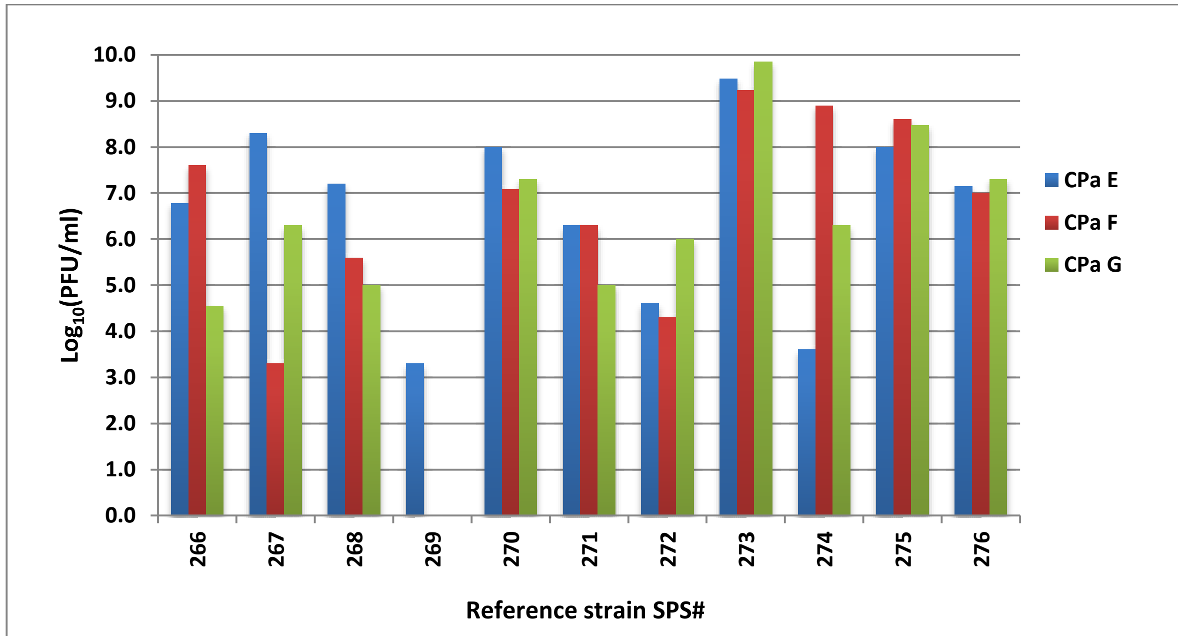
The spectrum of activity for each of the Pa bacteriophage cocktails formulated was determined against the reference collection (Table 2-3). Bacteriophage cocktail CPa E was effective against all the reference strains while bacteriophage cocktail CPa F and CPa G failed to infect the reference strain SPS#269. However, these results were expected as the individual bacteriophages within the cocktail, with the exception of Pa 25, showed low or no activity against the isolate. The ratio of infection (ROI) for the three CPa bacteriophage cocktails when considering the clear activities was 1.0, 0.91 and 0.91 for cocktail CPa E, F and G respectively. For the three CPa bacteriophage cocktails the highest concentration was reached with the reference strain SPS# 273 with a titre of 3 $x10^9$, 1.7 $x10^9$ and 7 $x10^9$ PFU/ml for cocktail CPa E, CPa F and CPa G correspondingly (Figure 5-4).

Figure 5-3. Efficacy of *S. aureus* bacteriophage cocktails against clinical strains isolated in the United Kingdom



Bacteriophage cocktails CSa 1, CSa 2 and CSa 3 (Log_{10} 8 PFU/ml) were 10-fold diluted to 10^{-5} and used to spot onto the 108 S. aureus strains isolated in the United Kingdom. The calculated titre of the cocktails against each isolate is presented (Log_{10} PFU/ml).

Figure 5-4. **Lytic activity of *P. aeruginosa* (Pa) cocktails E, F and G against the reference collection**



Bacteriophage suspensions (Log$_{10}$ 8 PFU/ml) were 10-fold diluted to 10^{-6} and used to spot onto the 11 P. aeruginosa reference strains. The calculated titre of the cocktails against each isolate is presented (Log$_{10}$ PFU/ml).

5.6.2.2 *Lytic activity of Pa bacteriophage cocktails against clinical isolates collected in Australia*

The spectrum of activity for each of the Pa bacteriophage cocktails formulated was determined against the *P. aeruginosa* clinical collection (Appendix 2). The three bacteriophage cocktails combined were effective against 97% of the isolates tested. Four isolates only (SPS# 411, 455, 477 and 495) were not sensitive to any of the cocktails developed. However, the results were not surprising as the testing showed the strains were not sensitive to the individual phages within the cocktail. The ratio of infection (ROI) for bacteriophage cocktails CPa E, CPa F and CPa G, when considering the clear activities only, was 0.81, 0.64 and 0.85 respectively. The results are summarised in Figure 5-5

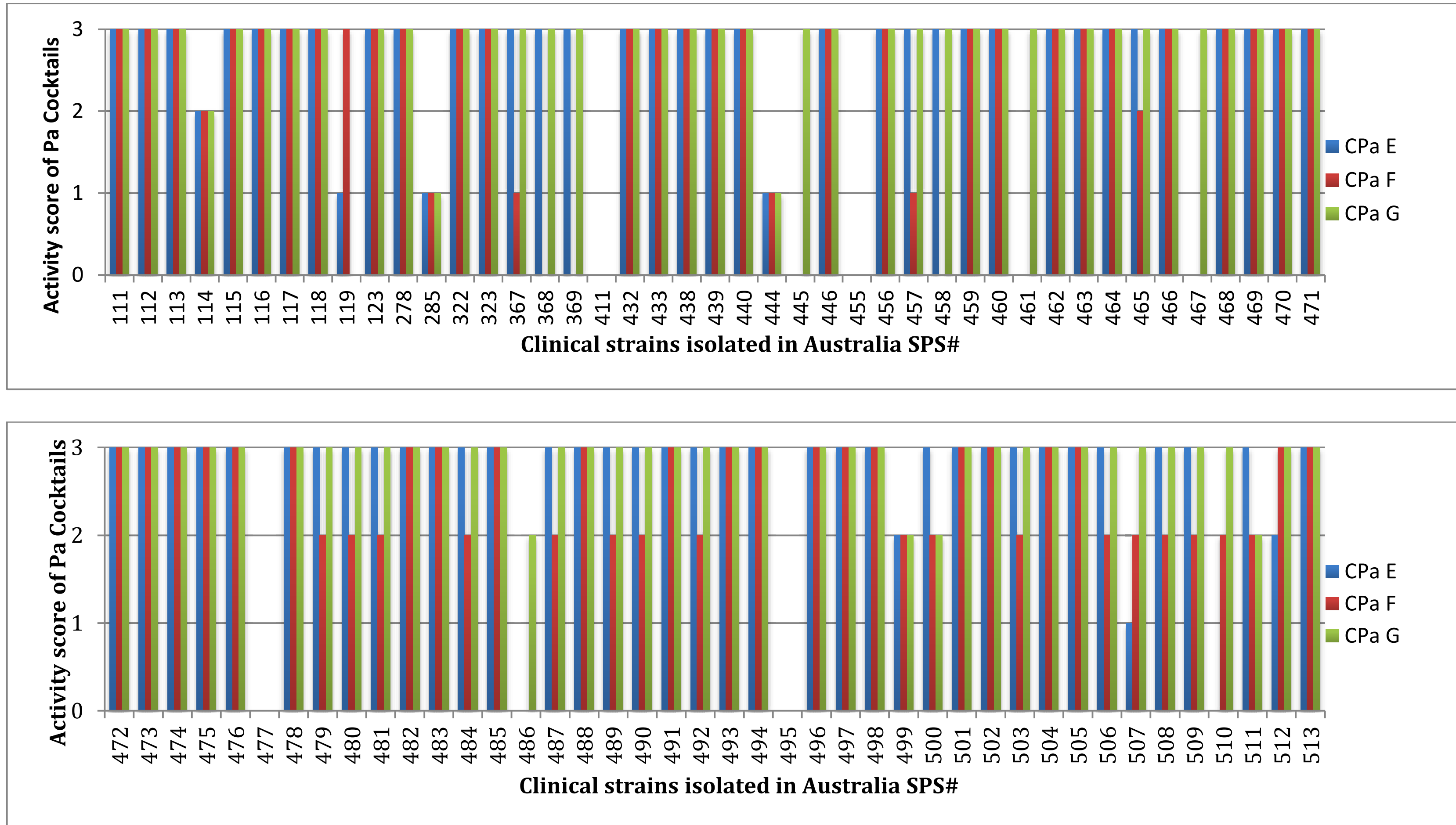
5.6.2.3 *Lytic activity of Pa bacteriophage cocktails against clinical isolates collected in the United Kingdom*

The spectrum of activity for each of the Pa bacteriophage cocktails formulated was determined against *P. aeruginosa* clinical isolates from the UK. The three bacteriophage cocktails combined were effective against 90% of the isolates tested. The ratio of infection (ROI) for bacteriophage cocktails CPa E, CPa F and CPa G, when considering the clear activities only, was 0.74, 0.90 and 0.87 respectively. However, the isolates that were sensitive to the CPa cocktails showed, in the majority of the cases, higher average efficiency of plating than in the reference strain. The results are summarised in Figure 5-6.

5.6.2.4 *Lytic activity of Pa bacteriophage cocktails against clinical isolates from the United States*

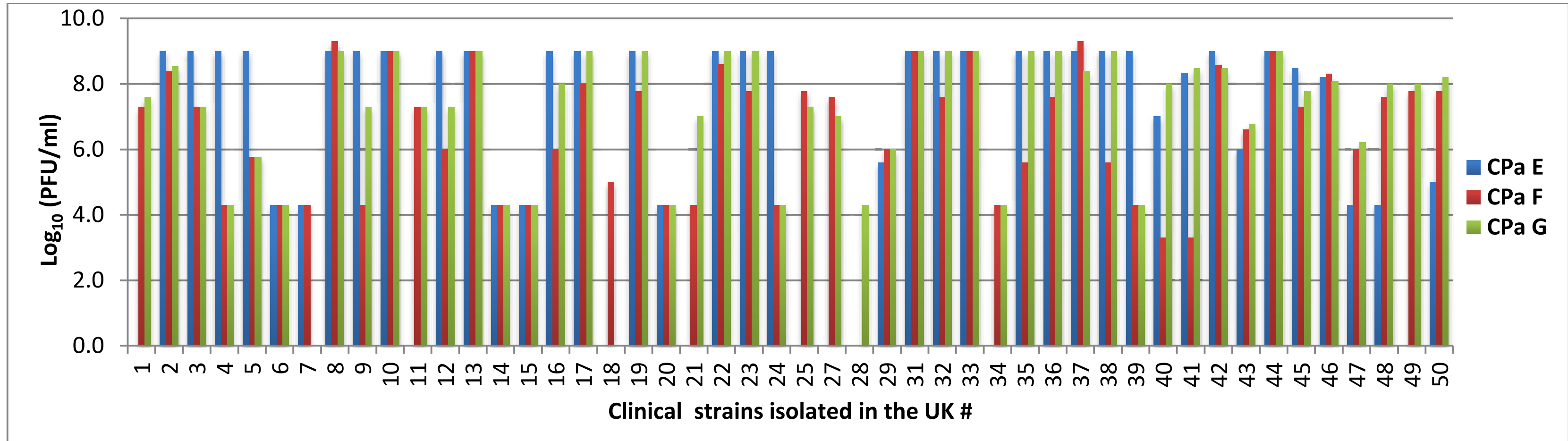
The spectrum of activity for each of the Pa bacteriophage cocktails formulated was determined against 189 *P. aeruginosa* clinical strains isolated from patients suffering from cystic fibrosis in the United States. The three bacteriophage cocktails combined were effective against 86% of the isolates tested. The ratio of infection (ROI) for bacteriophage cocktails CPa E, CPa F and CPa G, when considering the clear activities only was 0.75, 0.73 and 0.82 respectively. The results are summarised in Figure 5-7

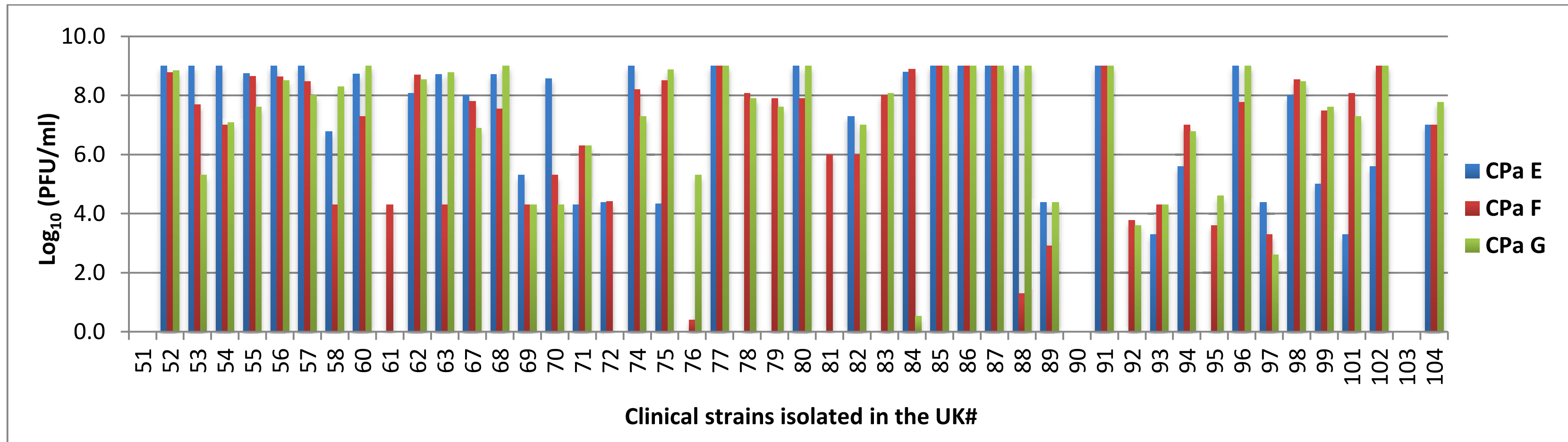
Figure 5-5. Lytic activity of Pa cocktails against the clinical isolates collected in Australia



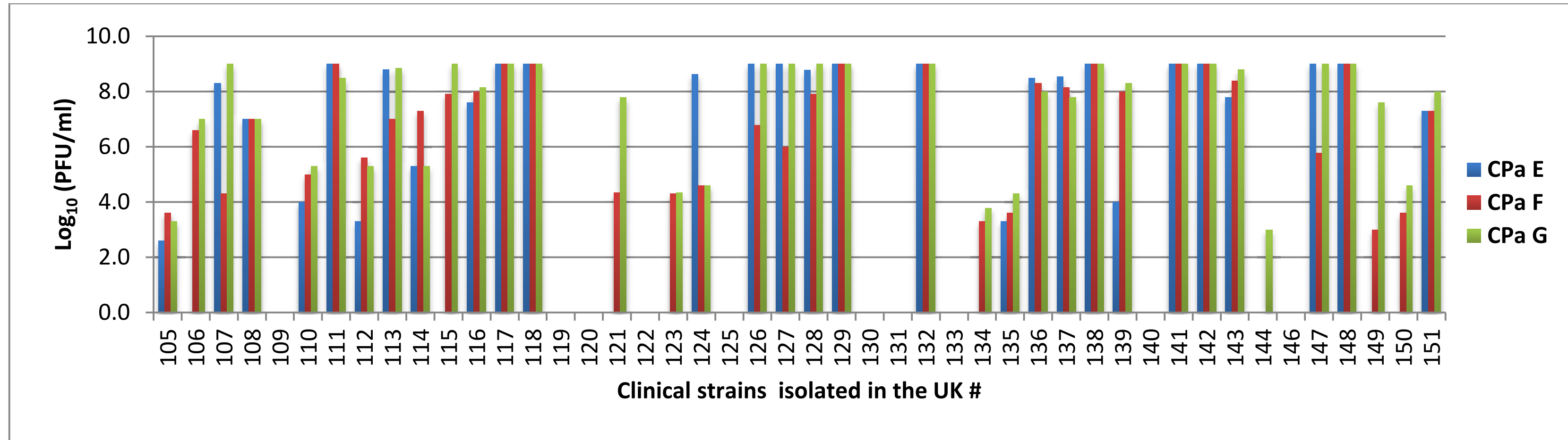
Bacteriophage suspensions (Log_{10}8 PFU/ml) were used to spot onto the 85 P. aeruginosa clinical strains. Spots were performed in triplicate. The clarity of the spot was scored as turbid (1), clearing with hazy-partial background (2) and complete clearing (3). Negative reactions were recorded as (0).

Figure 5-6. **Lytic activity of *P. aeruginosa* (Pa) cocktails E, F and G against clinical isolates collected in the United Kingdom**



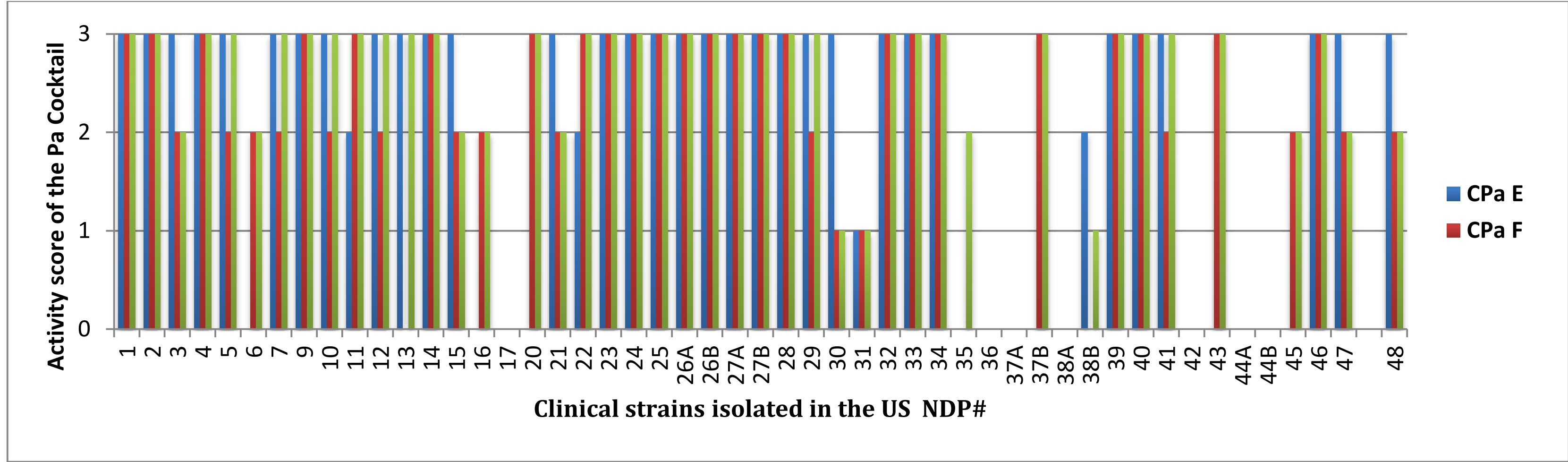


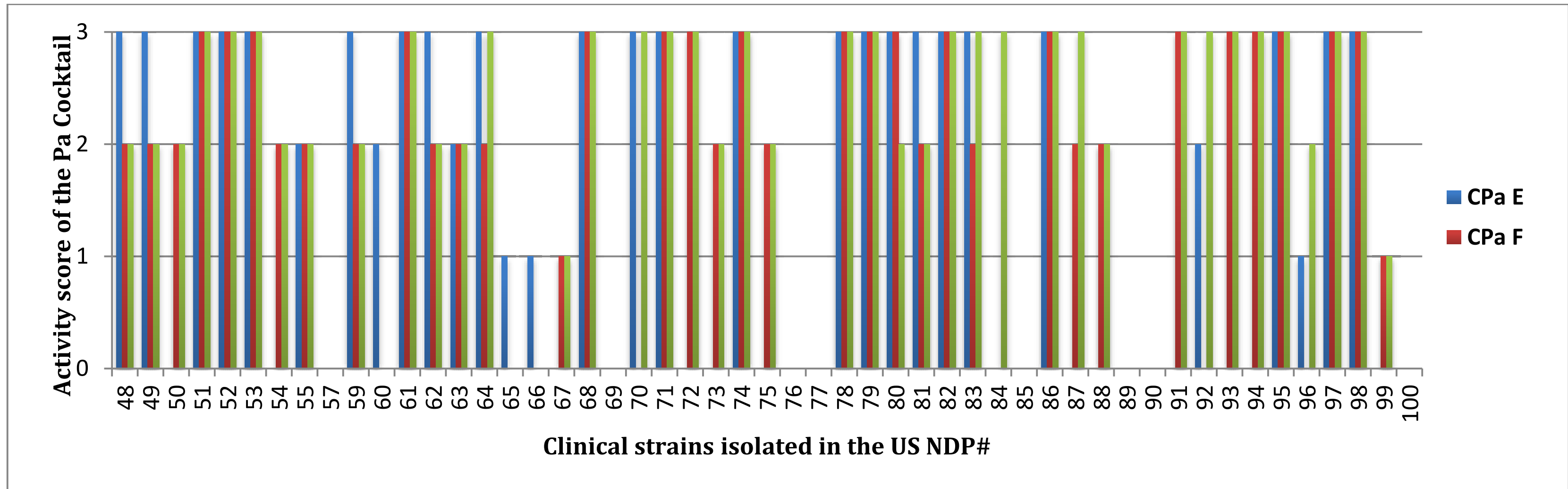
continued Figure 5-6

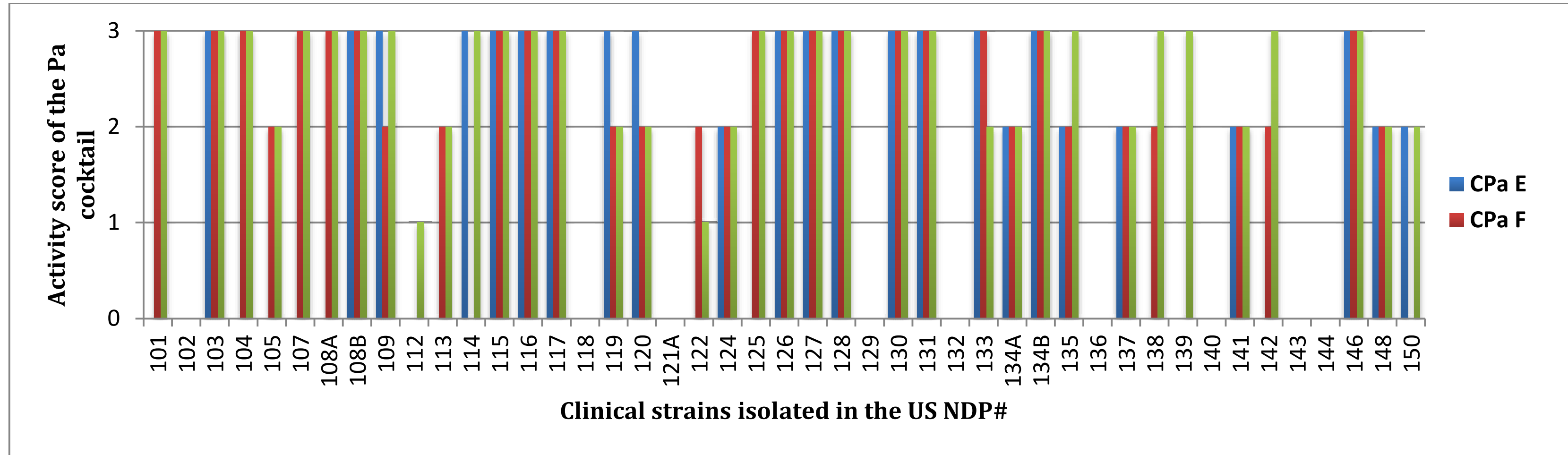


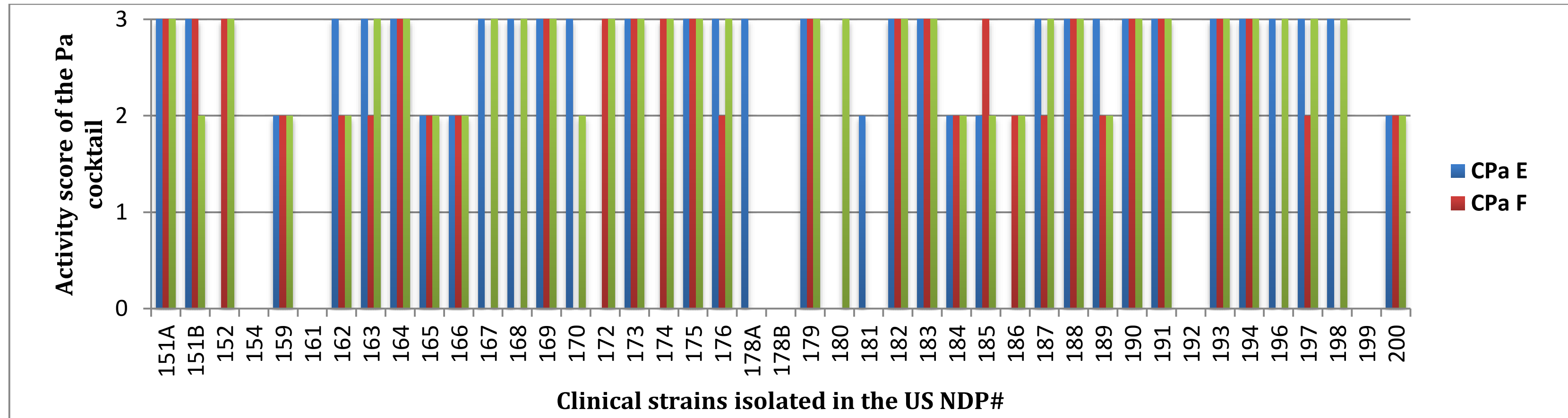
Bacteriophage suspensions ($Log_{10}8$ PFU/ml) were 10-fold diluted to 10^{-5} and used to spot onto 142 P. aeruginosa clinical strains. The calculated titre of the cocktails against each isolate is presented (Log_{10} PFU/ml).

Figure 5-7. Lytic activity of *P. aeruginosa* (Pa) cocktails E, F and G against clinical isolates from the United States









Bacteriophage suspensions (Log$_{10}$8 PFU/ml) were used to spot onto 189 P. aeruginosa clinical strains. Spots were performed in triplicate. The clarity of the spot was scored as turbid (1), clearing with hazy-partial background (2) and complete clearing (3). Negative reactions were recorded as (0).

5.6.2.5 *Lytic activity of Pa bacteriophage cocktails against clinical isolates collected in the Guatemala City*

The spectrum of activity for each of the Pa bacteriophage cocktails E and F formulated was determined against *P. aeruginosa* clinical strains isolated in Guatemala. The two bacteriophage cocktails combined were effective against 86% of the isolates tested. The ratio of infection (ROI) for bacteriophage cocktails E and F was 0.85 and 0.62 respectively. The results are summarised in Figure 5-8.

5.6.3 Comparison of the calculated ratio of infection (ROI) of the developed phage cocktails against the reference and clinical strains tested

5.6.3.1 *Calculated ratio of infection (ROI) of S. aureus phage cocktail CSa 1, CSa 2 and CSa 3 against the reference and clinical strains tested*

A comparison of the calculated ROI for each *S. aureus* phage cocktail when tested against the *S. aureus* reference collection (Table 2-1), the clinical isolates collected in Australia (Appendix 1) and in the United Kingdom is presented in Table 5-1

5.6.3.2 *Calculated ratio of infection (ROI) of P. aeruginosa phage cocktails CPa E, CPa F and CPa G. against the reference and clinical strains tested*

A comparison of the calculated ROI for each *S. aureus* phage cocktail when tested against the *S. aureus* reference collection (Table 2-3), the clinical isolates collected in Australia (Appendix 2) and in the United Kingdom are presented in Table 5-2.

Table 5-1. ***S. aureus*** **phage cocktails calculated ratio of infection (ROI) against the reference and clinical strains tested.**

	Reference collection	**AU clinical isolates**	**UK clinical isolates**
CSa 1 (ROI>++)	0.95	0.94	0.96
CSa 2 (ROI>++)	0.90	0.61	0.92
CSa 3 (ROI>++)	0.90	0.92	0.96

The ratio of infection (ROI) was defined as the number of isolates the phage cocktail infected over the total number of isolates tested. To calculate the ROI for the cocktails, only those positive infections, which showed full activity (ROI>++) as previously calculated (Table 3-6) were used.

Table 5-2. ***P. aeruginosa*** **phage cocktails calculated ratio of infection (ROI) against the reference and clinical strains tested.**

	Reference collection	AU clinical isolates	UK clinical isolates	US clinical isolates	Guatemala Clinical isolates
CPa E (ROI>++)	1.00	0.81	0.74	0.75	0.85
CPa F (ROI>++)	0.91	0.64	0.90	0.73	0.62
CPa G (ROI>++)	0.91	0.85	0.87	0.82	-

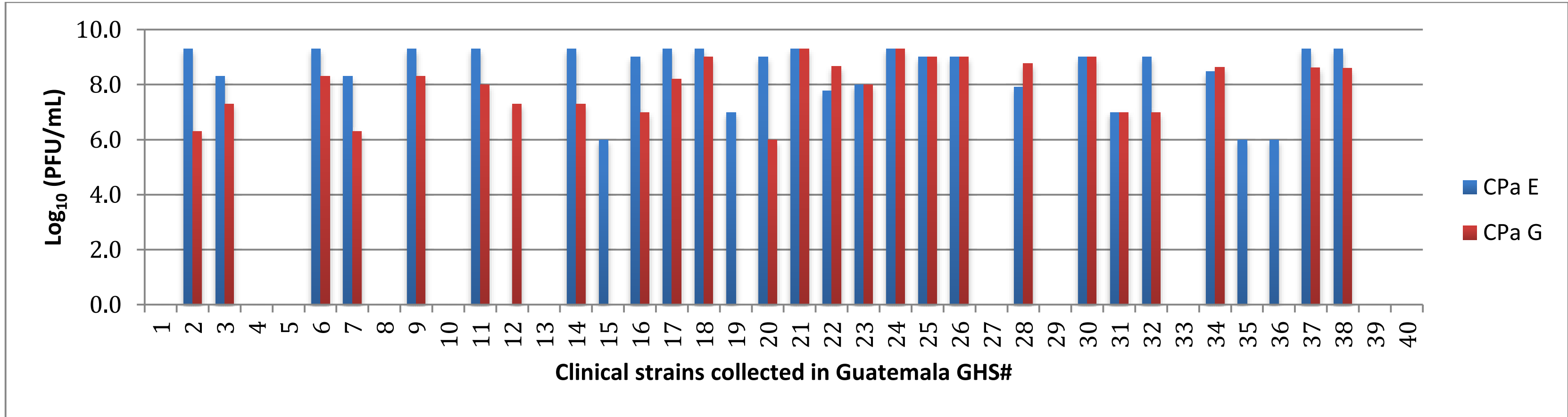
The ratio of infection (ROI) was defined as the number of isolates the phage cocktail infected over the total number of isolates tested. To calculate the ROI for the cocktails, only those positive infections that showed full activity (ROI>++) as previously calculated (Table 3-7) were used. (-) Phage cocktail not tested.

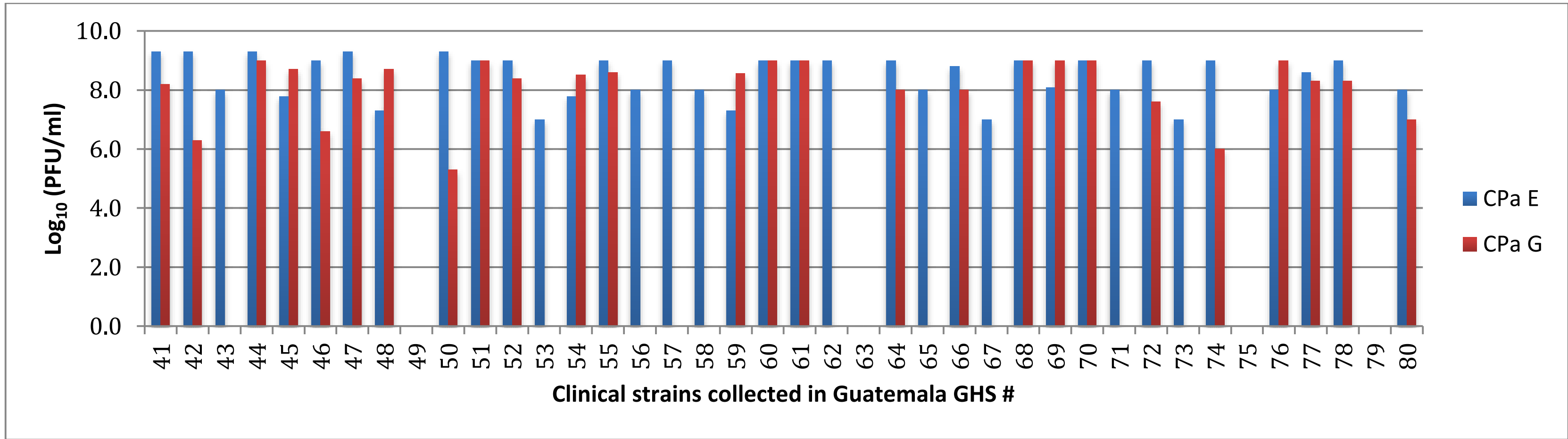
5.6.4 Bacteriophage resistant bacteria

5.6.4.1 *Frequency of emergence of bacteriophage-insensitive mutant bacteria: individual Sa phage vs Sa phage cocktails*

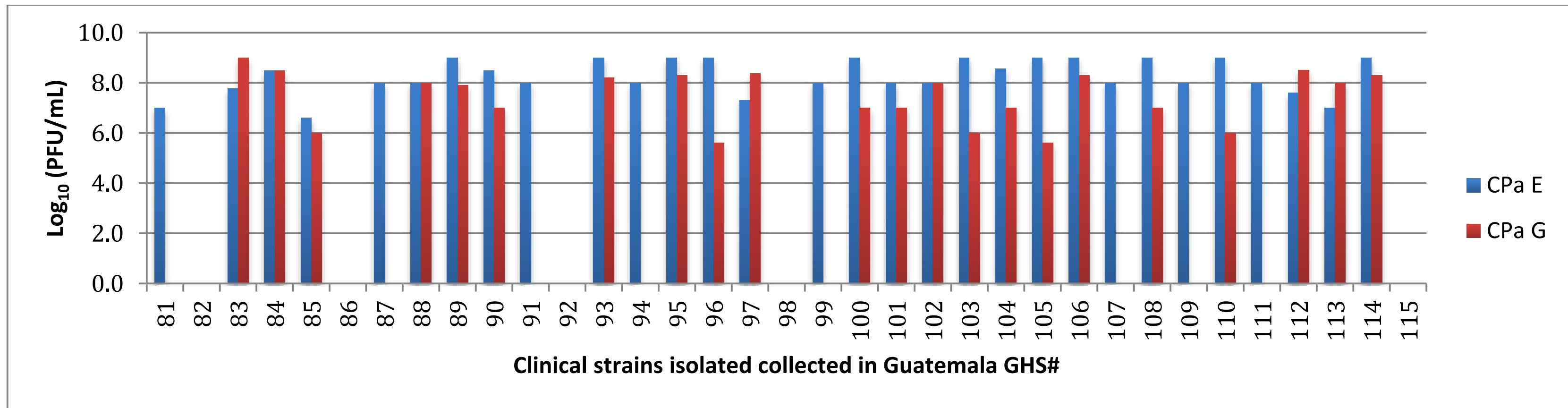
The development of BIM bacteria when infected with an individual *S. aureus* phage or a *S. aureus* bacteriophage cocktail was compared. In general, the frequency of development of BIM bacteria when infected with the bacteriophages was remarkably low with less than 10^{-7} CFU per infection observed. However, the appearance of BIM bacteria was lower when the bacteriophage cocktails were used. Infections performed using Sa cocktail 1 completely inhibited the appearance of BIM bacteria while Sa cocktails, 2 and 3, greatly reduced their appearance. These differences though were statistically significant only for bacteriophage Sa 12 and Sa 18, which were part of cocktail 2. The results are summarised in Table 5-3

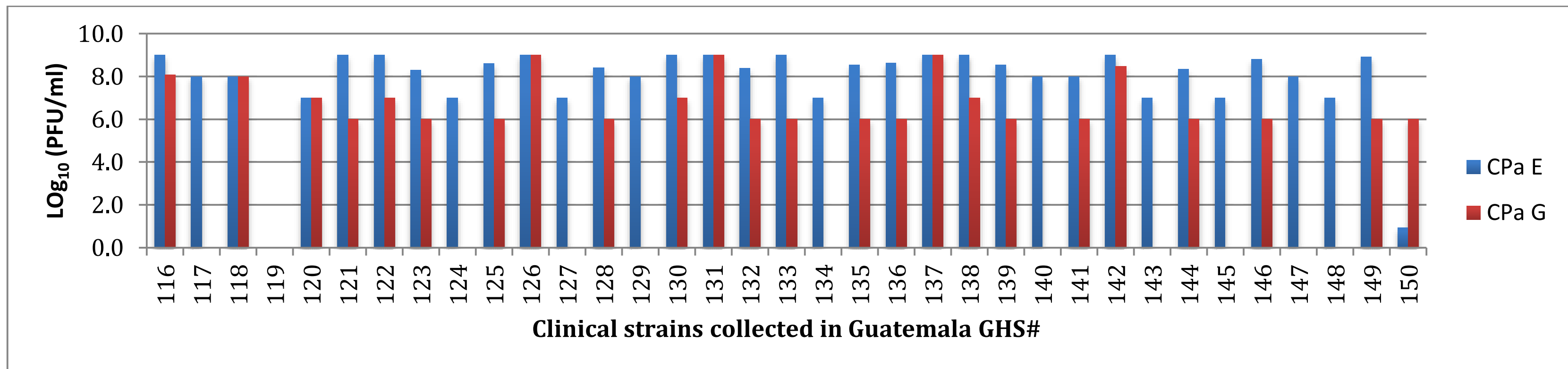
Figure 5-8. **Lytic activity of *P. aeruginosa* (Pa) cocktails E, F and G against clinical isolates collected in Guatemala**





Continued Figure 5-8





Bacteriophage suspensions (Log_{10} 8 PFU/ml) were 10-fold diluted to 10^{-5} and used to spot onto 150 P. aeruginosa clinical strains. The calculated titre of the cocktails against each isolate is presented (Log_{10} PFU/ml).

Table 5-3. ***Staphylococcus aureus*** **and frequency of emergence of bacteriophage-insensitive mutants (BIM).**

Phage (s)	Indicator strain	BIM ± (Mean SD)		*P value*
Sa 34	317	2.566E-09	±3.9E-09	>0.05
Cocktail 1	317	0	0.00E+00	
Sa 44	320	0.000E+00	±1.89E-09	>0.05
Cocktail 1	320	0	0.00E+00	
Sa 51	310	2.500E-09	±4.69E-09	>0.05
Cocktail 1	310	0	0.00E+00	
Sa 5	25	7.230E-07	±2.41E-07	>0.05
Cocktail 2	25	6.94118E-07	±1.80E-07	
Sa 12	316	1.867E-07	±4.58E-08	0.007*
Cocktail 2	316	7.32143E-08	±2.0448E-08	
Sa 18	316	3.244E-07	±8.84E-08	0.01*
Cocktail 2	316	7.32143E-08	±2.0448E-08	
Sa 38	287	1.156E-07	±3.88E-08	>0.05
Cocktail 3	287	5.9375E-08	±2.62E-08	
Sa 42	308	1.958E-07	±1.31E-07	>0.05
Cocktail 3	308	7.03125E-08	±2.86E-08	
Sa 43	296	4.15E-07	±9.00E-07	>0.05
Cocktail 3	296	2.30769E-08	±2.50E-08	

*Comparison of the ability of individual Sa phages and Sa phage cocktails to inhibit the appearance of BIMs. BIMs are defined as the proportion of bacteria within a given population that possess an innate ability to resist bacteriophage infection. The BIM frequency was determined as the number of surviving colonies after infection with an individual phage or a phage cocktail divided by the titre of the control indicator bacteria. Standard deviations (SD) were determined and the results compared using the comparing means t-test analysis ($p<0.05$). *Results were statistically significant.*

5.6.4.2 *Frequency of emergence of bacteriophage-insensitive mutant bacteria: individual Pa phage vs Pa phage cocktails*

The development of BIM bacteria when infected with an individual *P. aeruginosa* phage or a *P. aeruginosa* bacteriophage cocktail was compared. In the majority of the samples tested, the frequency of development of BIM bacteria when infected with a Pa bacteriophage cocktail was lower than that of bacteria infected with an individual Pa phage. These differences though were statistically significant only for bacteriophage Pa 24 when compared with bacteriophage Pa cocktail E and Pa 3 and Pa 10 when compared to Pa cocktail F. The results are summarised in Table 5-4.

Table 5-4. ***Pseudomonas aeruginosa*** **and frequency of emergence of bacteriophage bacteriophage-insensitive mutants (BIM).**

Phage (s)	**Indicator Strain**	**BIM±(Mean SD)**			***P (<0.05)***
Pa 1	433	2.58E-05	±	2.83905E-05	*>0.05*
Cocktail E	433	1.00E-06	±	3.46944E-07	
Pa 24	273	1.95E-06	±	4.5794E-07	0.003*
Cocktail E	273	5.00E-08	±	1.04083E-07	
Pa 25	439	6.40E-07	±	8.16074E-07	>0.05
Cocktail E	439	1.90E-07	±	7.55304E-08	
Pa 26	439	1.67E-06	±	8.16074E-07	>0.05
Cocktail E	439	1.90E-07	±	7.55304E-08	
Pa 3	439	3.08E-06	±	1.12173E-06	0.01*
Cocktail F	439	1.11E-07	±	2.10027E-07	
Pa 6	273	1.30E-06	±	9.28614E-07	>0.05
Cocktail F	273	0.00E+00	±	4.33013E-08	
Pa 10	323	4.92E-06	±	1.7936E-06	0.02*
Cocktail F	323	5.00E-07	±	4.5613E-07	
Pa 32	323	2.24E-06	±	2.49353E-06	>0.05
Cocktail F	323	5.00E-07	±	4.5613E-07	
Pa 37	439	2.04E-06	±	2.21279E-06	>0.05
Cocktail F	439	1.11E-07	±	2.10027E-07	
Pa 1	433	2.58E-05	±	2.83905E-05	>0.05
Cocktail G	433	5.13E-05	±	3.41049E-05	
Pa 6	273	1.30E-06	±	9.28614E-07	>0.05
Cocktail G	273	2.12E-06	±	6.87484E-07	
Pa 25	439	6.40E-07	±	8.16074E-07	>0.05
Cocktail G	439	1.60E-07	±	4.85936E-08	
Pa 51	445	1.78E-06	±	1.47485E-07	0.00006*
Cocktail G	445	7.89E-06	±	5.80252E-07	

*Comparison of the ability of individual Pa phages and Pa phage cocktails to inhibit the appearance of BIM bacteria. BIMs are defined as the proportion of bacteria within a given population that possess an innate ability to resist bacteriophage infection The BIM frequency was determined as the number of surviving colonies after infection with an individual phage or a phage cocktail divided by the titre of the control indicator bacteria. Standard deviations (SD) were determined and the results compared using the comparing means t-test analysis (p<0.05). *Results were statistically significant.*

5.6.5 Susceptibility of presumptive phage-resistant cells to individual bacteriophages within a cocktail

5.6.5.1 *Susceptibility of presumptive phage-resistant cells to individual S. aureus (Sa) bacteriophages present within a cocktail*

Indicator bacteria were infected with their corresponding Sa bacteriophage and attempts to recover phage-resistant cells were made. However, phage- resistant cells were not recovered after infection with the individual Sa bacteriophages present in Sa cocktails 1, 2 and 3 (section 5.2). Although slight turbidity was observed in the wells of the infected cultures, attempts to propagate the few recovered cells after centrifugation and three passages were unsuccessful due to the continuous presence of bacteriophages. The presence of bacteriophages in the recovered cultures meant that the formation of bacterial lawns to evaluate their susceptibility to the bacteriophages present in the formulated cocktails was not possible.

5.6.5.2 *Susceptibility of presumptive phage-resistant cells to individual P. aeruginosa (Pa) bacteriophages present within a cocktail*

Indicator bacteria were infected with their corresponding CPa bacteriophage and presumptive phage-resistant cells recovered. Susceptibility to their corresponding phages and other phages present within a formulated cocktail were then tested. For the Pa bacteriophage cocktail CPa E (Pa 1, Pa 24, Pa 25 and Pa 26) phage-resistant cells were observed after infection with Pa 1 and Pa 26. Bacterial cells with lower sensitivity were observed after infection with Pa 25. However, these phage-resistant cells remained sensitive to the other phages present in the cocktail. The results are summarised in Table 5-5

Table 5-5. **Susceptibility of presumptive phage-resistant cells to individual *P. aeruginosa* (Pa) bacteriophages present within cocktail E.**

Presumptive phage-resistant strain	Infected with:	Pa 1	Pa 24	Pa 25	Pa 26
R-433	Pa 1	-	+++	+++	+++
R-273	Pa 24	+++	+++	+++	+++
R-439	Pa 25	-	+++	+	-
R-433	Pa 26	+++	-	+++	-

Phage indicator strains were infected with their corresponding phages and presumptive phage-resistant cells recovered. The susceptibility of these cells to other bacteriophages present in Pa cocktail E was then tested and scored as non-susceptible (-), turbid or slightly susceptible (+), clear with hazy background or partially susceptible (++) and complete clearing or completely susceptible strain (+++).

For the Pa bacteriophage cocktail CPa F (Pa 3, Pa 6, Pa 10, Pa 32 and Pa 37) phage-resistant cells were observed only after infection with Pa 6 and Pa 10. Interestingly, phage resistant cells after infection with phage Pa 6 were also resistant to Pa 10 and vice versa, suggesting that these two phages could share a similar mechanism of infection. Bacterial cells with lower sensitivity were also observed after infection with Pa 32. However, all the phage-resistant bacteria recovered remained sensitive to at least three other phages present in the cocktail. The results are summarised in Table 5-6.

For the Pa bacteriophage cocktail G (Pa 1, Pa 6, Pa 25 and Pa 51) phage-resistant cells were observed for all four bacteriophages tested. Bacterial cells with lower sensitivity were again recovered after infection with Pa 25. However, all the phage-resistant bacteria recovered remained sensitive to at least one of the other phages present in the cocktail; except for the phage-resistant strain SPS#445 which was initially sensitive just to phage Pa 51. The results are summarised in Table 5-7**.**

Table 5-6. **Susceptibility of presumptive phage-resistant cells to individual *P. aeruginosa* (Pa) bacteriophages present within cocktail F.**

Presumptive phage-resistant strain	Infected with:	Pa 3	Pa 6	Pa 10	Pa 32	Pa 37
R-439	Pa 3	+++	+++	+++	+++	+++
R-273	Pa 6	+++	-	-	+++	+++
R-323	Pa 10	+++	-	-	+++	+++
R-323	Pa 32	+++	+++	+	+	+++
R-439	Pa 37	+++	+++	+++	+++	+++

Phage indicator strains were infected with their corresponding phages and presumptive phage-resistant cells recovered. The susceptibility of these cells to other bacteriophages present in Pa cocktail F was then tested and scored as non-susceptible (-), turbid or slightly susceptible (+), clear with hazy background or partially susceptible (++) and complete clearing or completely susceptible strain (+++).

Table 5-7. Susceptibility of presumptive phage-resistant cells to individual *P. aeruginosa* (Pa) bacteriophages present within cocktail G.

Presumptive phage-resistant strain	Infected with	Pa 1	Pa 6	Pa 25	Pa 51
R-433	Pa 1	-	+++	+++	ND
R-273	Pa 6	+++	-	+++	ND
R-439	Pa 25	-	+++	+	++
R-445	Pa 51	ND	ND	ND	-

Phage indicator strains were infected with their corresponding phages and presumptive phage-resistant cells recovered. The susceptibility of these cells to other bacteriophages present in Pa cocktail F was then tested and scored as non-susceptible (-), turbid or slightly susceptible (+), clear with hazy background or partially susceptible (++) and complete clearing or completely susceptible strain (+++). ND means not tested.

5.7 Discussion

The use of therapeutic phages as a mixture or "cocktail" has been the classical approach to therapy in general. This is indeed the situation in those countries such as the Republic of Georgia and Russia where the use of bacteriophage cocktails for human treatment, not only against a single pathogen but against up to five different pathogens is part of their current medical program (Chanishvili *et al* 2001(Parfitt, 2005, Weber-Dabrowska et al., 2000b, Kutter et al., 2010, Abendon et al., 2011). The logic behind the use of phage cocktails instead of a single phage approach is the anticipated ability of the cocktails to extend the spectrum of activity of a product against a wider population of bacteria as well as its ability to prevent or reduce the appearance of bacteriophage resistant cells (BRC). This BRC is also referred to as bacteriophage-insensitive mutants (BIMs).

In this study, phage cocktails against both local and overseas collections of isolates of *S. aureus* and *P. aeruginosa* were developed and their activities compared with the activities seen against the individual phages that composed the cocktails. The activity of the *S. aureus* phage cocktails against the Australian collection of clinical isolates showed a significant reduction in the number of phage patterns when compared with the number of phage patterns observed with the single bacteriophages. The individual bacteriophages against the reference collection produced 33 different phage patterns divided into seven groups and 26 individual patterns (Table 4-1). However, the testing with the three cocktails reduced the number of different patterns in the clinical collection to 10 in the clinical isolates. Similarly, the activity of the *P. aeruginosa* phage cocktails against the Australian collection of clinical isolates also showed a significant reduction in the number of phage patterns observed when the single bacteriophages were used. The number of phage patterns was reduced from 49 (13 groups and 36 individual patterns) to 14 patterns. For both systems, while the number of patterns was reduced, the spectrum of activity was contrastingly increased (Figure 5-1 and 5-4). In a clinical setting, availability of a broad spectrum of activity cocktail against a determined specific pathogen may allow for the initiation of an empirical treatment until a more specific bacteria-phage match can be performed. These results support the hypothesis that the use of bacteriophage cocktails could be useful and/or practical to overcome the

negative medical perception that bacteriophage therapy treatment may not be as effective as antibiotics due to their host limitations.

Furthermore, three different approaches were taken to develop the cocktails tested. The first method combined 3-4 phages, which showed the broadest spectrum of activity against the local reference and clinical collection (CSa 1 and CPa E). This is the described classical method. The second method combined five (CPa F) phages, which individually showed the lowest spectrum of activity against the local reference and clinical collection while the third method used a combination of phages with a broad and a narrow spectrum of activity (CPa G). Because of the relatively broad spectrum of activity of all the selected *S. aureus* phages (Table 3-6 and 4-1), the formulation of the cocktails was done following the most traditional method only. However, cocktail CSa 2 included one of the phages with a broad spectrum of activity and the phage that showed the narrowest host range against the collection of clinical isolates.

The results showed that Cocktail CSa 1 showed the highest ratio of infection (ROI) against the reference collection and both the local and overseas collection of clinical isolates while the activity of CSa 3 was only slightly lower (Table 5-1). Cocktail CSa 2, which was expected to show reduced activity, given the addition of the phage with a narrow spectrum of activity, showed that its activity against the collection of UK isolates was comparable to that activity showed against the reference collection. This could be explained by the fact that the two phages present in the cocktail, despite the reduced spectrum of activity of Sa 18, were highly effective against the two most predominant epidemic clones of MRSA strains in the UK (UK-MRSA 15 and UK MRSA 16), and may not necessary represent the combination present in the cocktail but the distribution of the bacterial population present in the environment. Interestingly, cocktail CSa 2 showed a reduced ratio of infection against the Australian clinical isolates tested. This outcome could be the effect of the phage formulation where the reduced spectrum of activity struggled to deal with a much variable population of MRSA isolates present in Australia (Coombs *et al,* 2006, Chua *et al,* 2011). Since the clinical isolates tested in the UK or in Australia were not molecularly characterised this observation cannot be confirmed. However, the activities of cocktails,

CSa1 and CSa 3, were also similar despite the differences in formulation (Table 5-1), but both formulations had phages with a broad spectrum. This observation would support the above theory. The results also suggested that the combination of phages with broad and narrow activities may function in a synergistic manner, or did not interfere with each other.

Similarly, the results showed that Cocktails CPa E and CPa G, showed a high ROI against the reference collection as well as both the local and overseas collection of clinical isolates (Table 5-2). However, the individual testing of the each of the bacteriophages against the collection of US isolates (data not shown) showed that the positive reactions were mostly due to the activity of Pa 51. Cocktail CPa F was expected to show reduced activity due to the multiple additions of phages with a narrow spectrum of activity. This reduction was seen against the clinical collection tested in Australia and Guatemala while the formulation proved to be very comparable to CPa E and CPa G against the clinical isolates from the UK. This observation again may not necessary represent the combination present in the cocktail, but the distribution of the bacterial population present in the environment.

Another reason for the development and use of bacteriophage cocktails is their potential effect in preventing or delaying the emergence of BIMs (Tanji *et al,* 2004, O'Flynn *et al*, 2004, Sulakvelidze and Kutter, 2005). In this study, the ability of individual bacteriophages to prevent or minimise the development of BIMs, when compared with the formulated bacteriophage cocktails, was demonstrated. For the *S. aureus* individual bacteriophages and the three cocktails, the frequency of development was significantly lower (10^{-7} to 10^{-9} CFU) when compared with those reported for *E.coli* cocktails (O'Flynn *et al*, 2004). The formulated cocktail CSa 1 completely inhibited the bacterial growth, suggesting the absence of BIMs in the host strains while CSa 2 and CSa 3 greatly reduced bacterial survival but failed inhibit growth completely, suggesting the presence of BIMs. The low survival of the indicator bacteria observed when cocktail CSa2 and CSa 3 were tested could perhaps be due to the lower percentage of absorption (Table 4-3) of individual phages within the cocktails (in particular Sa 42 and Sa 18), resulting feasibly in a less efficient infection process than that observed for cocktail CSa1 in solid media. No studies reporting the frequency of BIMs for *S. aureus* phages have been reported previously. The efficacy of

the bacteriophage cocktails against the indicator strains in liquid media, used to grow the individual phages within the cocktail, made it impossible and thus unnecessary to assess the susceptibility of recovered presumptive phage-resistant cells.

For the three formulated *P. aeruginosa* cocktails their effectiveness reducing the frequency of BIMs was also shown, although the reductions in comparison to the frequencies calculated for the individual bacteriophages were not always statistically significant (Table 5-2). Presumptive phage-resistant strains were recovered and tested for susceptibility to their corresponding phages and other phages present within a formulated cocktail. The results showed that all recovered presumptive phage strains had indeed lower sensitivity against their corresponding phages tested, but remained sensitive to at least one phage within the cocktail formulated. This would suggest that the cell receptors for infection are complementary within the formulations. However, phage resistant cells after infection with phage Pa 6 were also resistant to Pa 10 and vice-versa. This would suggest that these two phages could share a similar mechanism of infection or that lysogenization may have occurred rendering the strains resistant to infection by the other phage.

The potential success of the technology in relation to the reduction of bacterial resistance demands that the elected bacteriophages do not have similar mechanisms of action in order to ensure that phage resistant mutants that may become resistant to one phage remain sensitive to other phages within the cocktail. Targeted bacteria may acquire resistance against phage through specific mechanisms in order to protect themselves against a variety of processes including the incursion of alien DNA and lysogenic conversion (Brüssow et al., 2004, Canchaya et al., 2004). Bacterial cells most commonly become refractory to a phage by activating a restriction-modification system composed of restriction endonucleases released at different stages to avoid intracellular biosynthesis of the phage (Kruger and Bickle, 1983). Therefore, a much more detailed study of the bacterial receptors involved in the attachment of bacteriophages to bacterial cells should be a critical part of commercial bacteriophage preparations, if long-term effectiveness is desired. For example a cocktail containing phages that use the bacterial wall as their receptor could be

hypothetically less successful than a cocktail designed with phages known to use independent receptors (Fu *et al* 2010).

The data collected during this study confirmed that as performed over the last decades of research in the Eastern European countries, the development of bacteriophage formulations based on bacteriophages with a broad spectrum of activity is effective against a wide range of bacterial isolates. Although the likelihood of phages interfering with each other upon co-infection is a concern (Abedon and Thomas-Abedon, 2010), this study demonstrated not only a continuous increase in the spectrum of activity but also the inhibition and/or reduction of phage-resistant cells upon infection with all three types of formulated cocktails. However, the results collected also suggest that in the future, commercial preparations may benefit from developing formulations that include phages with both a broad and a narrow spectrum of activity. This approach may be more useful to overcome the worldwide geographical barriers by providing coverage of a wider bacterial population, which includes predominant and rare isolates. This geographical barrier has not been challenged before because of the limited use of the technology.

6 TRANSMISSION ELECTRON MICROSCOPY OF BACTERIOPHAGES

6.1 Introduction

Transmission electron microscopy (TEM) is a basic tool in Microbiology and one of significant importance in virology since its introduction in the late 1930s when the first transmission electron microscopes were made available (Von Borries et al., 1938). The first images of a bacteriophage were observed in 1940 (Pfankuch and Kausche). This event was of enormous importance in the history of bacteriophage therapy as it finally confirmed the existence of bacteriophages and silenced those sceptics who doubted Felix d'Hérelle research and his pioneering bacterial treatment since its discovery (section 1.3.2.3).

The emergence of TEM and negative staining (Brenner and Horne, 1959) was promptly identified as an important technique for the morphological characterization of viruses, with the first system of morphological classification proposed in 1943 (Ruska). Currently, the international committee on taxonomy of viruses (ICTV) classifies bacteriophages into one order, 17 families, and three "unclassified" groups according to their shape, nucleic acid type and other morphological characteristics (Ackermann, 2006, Fauquet et al., 2005, Ackermann et al., 1992, Ackermann, 2005). A summary of the families with their main characteristics is presented in Table 6-1 (Ackermann, 2006)

Table 6-1. Summary of taxonomic phage families by morphotypes (Adapted from Ackermann, 2007)

Shape	Nucleic acid	Virus group	Details	Schematic Diagram
Tailed	DNA, 2, L	*Myoviridae*	Tail contractile	
	DNA, 2, L	*Siphoviridae*	Long, non-contractile tail	
	DNA, 2, L	*Podoviridae*	Short tail	
Polyhedral	DNA, 1, C	*Microviridae*	C Conspicuous capsomers	
	DNA, 2, C, S	*Corticoviridae*	Complex capsid, lipids	
	DNA, 2, L	*Tectiviridae*	Inner lipid vesicle Pseudotail	
	DNA, 2, L	SHI, group[#]	Inner lipid vesicle	
	DNA, 2, C	STV1, group[#]	Turret-shaped, protrusions	
	RNA, 1, L	*Leviviridae*	Poliovirus-like	
	RNA, 2, L, seg	*Cystociridae*	Envelope, lipids	
Filamentous	DNA, 1, C	*Inoviridae*	Long filaments or short rods	
	DNA, 2, L	*Lipothrixviridae*	Envelope, lipids	
	DNA, 2, L	*Rudiviridae*	TMV-like	
Pleomorphic	DNA, 2, C, S	*Plasmaviridae*	Envelope, lipids, no capsid	
	DNA, 2, C, S	*Fuselloviridae*	Envelope, lipids, no capsid, and lemon shaped	
	DNA, 2, L, S	*Salterprovirus*	Envelope, lipids, no capsid, lemon shaped	
	DNA, 2, C, S	*Guttaviridae*	Droplet-shape	
	DNA, 2, L	*Ampullaviridae*[#]	Bottle-shaped	
	DNA, 2, C	*Bicaudaviridae*	Two-tailed, growth cycle	
	DNA, 2, L	*Globuloviridae*	Paramyxovirus-like	

C=circular, L=Linear, S=superhelical, Seg=Segmented, 1=single stranded, 2=double stranded, [#]=awaiting classification.

Since 1959, over 5500 bacteriophages have been characterised by TEM (Ackermann, 2006). Ninety six percent of the bacteriophages identified to date consist of tailed bacteriophages, of which 61.7% are designated as part of the *Siphoviridae* family, 24.5% as *Myoviridae* family and 13.9% assigned to the *Podoviridae* family. These three families form together the *Caudovirales* order (Sharp, 2001). Phages belonging to the *Myoviridae* family have a long contractile tail, *Siphoviridae* have a long, non-contractile tail and *Podoviridae* have a short tail (Ackermann, 2005; Sharp, 2001).

Characterization by TEM allows detailed analysis and the measurement of bacteriophage structures such as head, collar, sheath and tails when present (Ackermann, 2005). Hence, in this work, the investigation of such morphological properties by TEM is essential for the classification of phages within a family. The analysis of morphological characteristics may also offer clues on the expected behaviour of any novel phage and this is very important for the selection of phages for therapeutic or industrial applications.

6.2 Transmission electron microscopy of *S. aureus* (Sa) and *P. aeruginosa* (Pa) bacteriophages

Staphylococcus aureus phages Sa 12 , Sa 18, Sa 34, Sa 38, Sa 42, Sa 44, Sa 51, Sa 53, Sa54 and *P. aeruginosa* phages Pa 1, Pa 3, Pa 6, Pa 10, Pa 24, Pa 25, Pa 26, Pa 32, Pa 37 and Pa 51 were prepared and observed by TEM as described in section 2.6.8. For each phage, an average of twenty bacteriophage particles was measured on the screen at their maximum magnification by using the Image J64 software. The software was also used to calculate the median and standard deviation.

6.3 Results

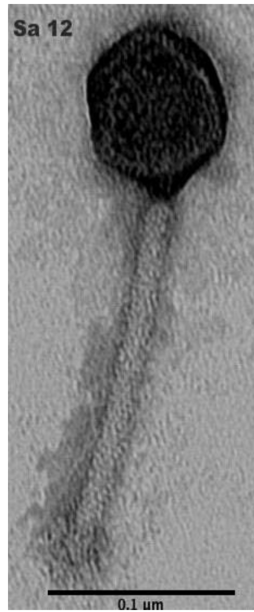
6.3.1 TEM of *S. aureus* (Sa) phages

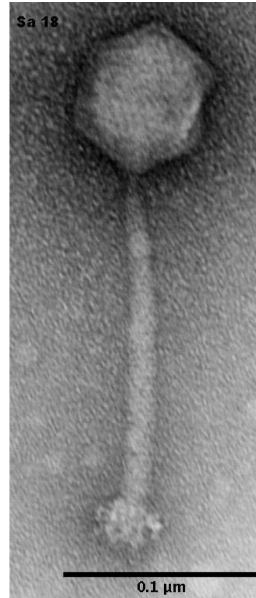
Selected bacteriophages were examined under the electron microscope. The electron micrographs of phages Sa 12 (Fig 6-1a), Sa 18 (Fig 6-1b), Sa 34 (Fig 6-1c), Sa 38 (Fig 6-1d), Sa 42 (Fig 6-2a), Sa 51 (Fig 6-2b) and Sa 54 (Fig 6-2c), showed shapes typical of the

Myoviridae family and very similar sizes. These phages showed an icosahedral head with average diameter of 74.8 ± 3.2nm, a tail length of 182.7 ± 9.1nm and a tail diameter of 14.5 ± 1.5nm. Phage Sa 12 (fig 6-1a) had a longer tail and Sa 34 (Fig 6-1c) showed a slightly longer head (82 ± 4nm) in comparison to the other *Myoviridae* phages observed.

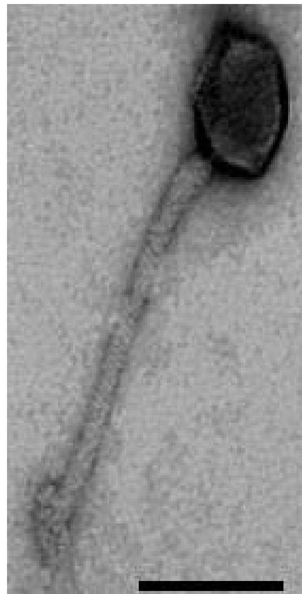
Bacteriophage Sa 38 also showed the presence of capsomers not observed in the other Sa Phages (Fig 6-3). In addition, Sa 34 showed in its tails tuft-like structures and once contracted the tails measured a group media average of 113 ± 10nm in length and 21 ± 1nm in width and a bare core of 60 ± 3 in length and 7 ± 2 width (Fig 6-4).

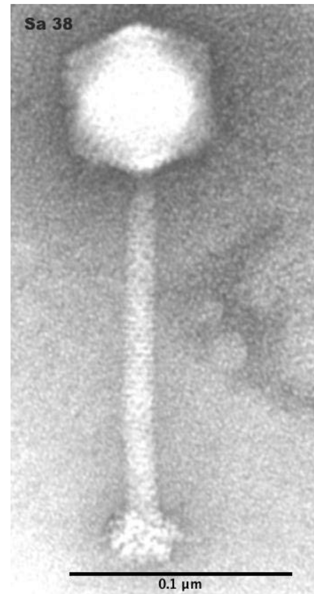
Figure 6-1. (a-d) TEM image of *S. aureus* bacteriophages belonging to the Myoviridae family.





6-4a) Sa 12 **6-4b) Sa 18**

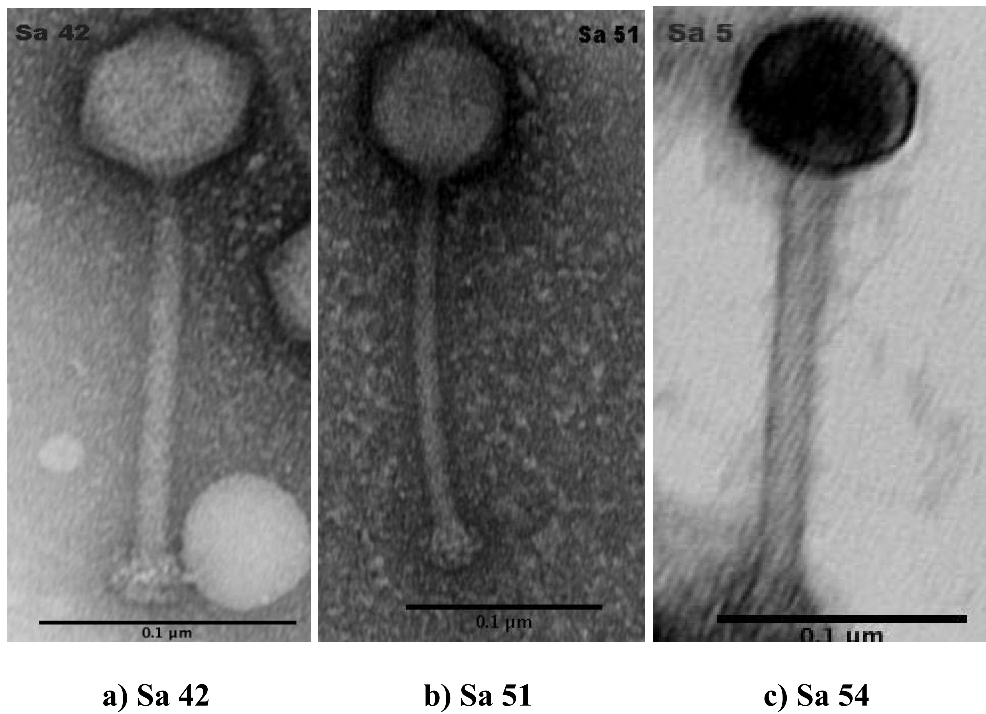




6-4c) Sa 34 **6-4d) Sa 38**

Sa bacteriophages showed an icosahedral head with a group media of 74.8 ± 3.2nm, a tail media length of 182.7 ± 9.1nm and a tail media diameter of 14.5 ± 1.5nm. Scale bar 100nm.

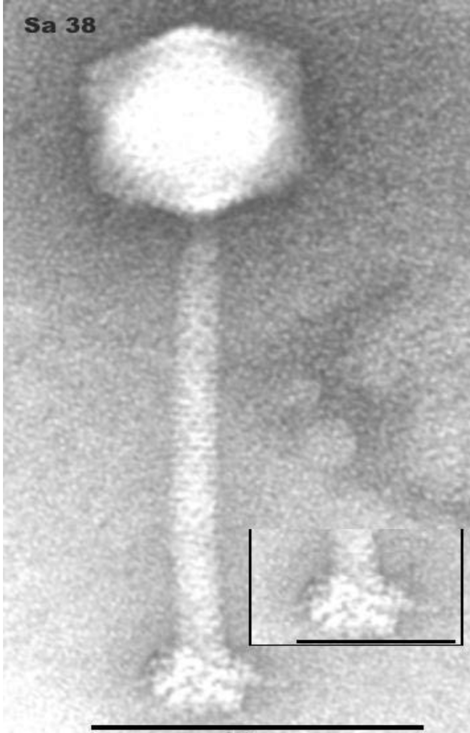
Figure 6-2. (a-c) TEM images of *S. aureus* bacteriophages belonging to the Myoviridae family.



a) Sa 42 **b) Sa 51** **c) Sa 54**

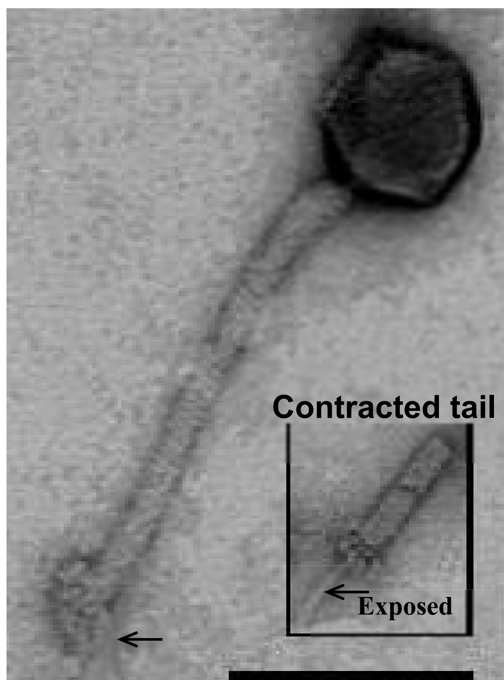
Sa bacteriohages showed an icosahedral head with a group media of 74.8 ± 3.2nm, a tail media length of 182.7 ± 9.1nm and a tail media diameter of 14.5 ± 1.5nm. Scale bar 100nm.

Figure 6-3. TEM image of *S. aureus* phage Sa 38.



The phage showed capsomers in the head and tufts-like structures in the tail (Scale bar 100nm).

Figure 6-4. TEM image of *S. aureus* Sa 34.



Uncontracted phage Sa 34 showed tufts-like structures (arrow) and when the tail was contracted (small box) it exposed its core (Scale bar 100nm).

Bacteriophages Sa 41, 44 and Sa 53 were also identified as part of the *Myoviridae* family but their sizes could not be determined. The protocol used for the purification of phage samples prior to their analysis in the electron microscope proved to be very effective for most of *S. aureus* phages. However, the protocol was not successful for phages Sa 41, 44 and Sa 53. A thick surrounding substance or matrix-like layer was observed persistently attached to the phage particles making it difficult to perform measurements, although the general characteristic of a *Myoviridae* phage were observed. Other purification protocols and staining techniques were attempted but the affixed matrix was still observed. However, this matrix-like layer, possibly produced by the host bacteria and secreted during the propagation of the phages, did not seem to inactivate the phages as demonstrated by the phage titers calculated (>10^8 PFU/ml, data not shown) after purification.

A summary with the morphological characteristics of the bacteriophages observed is presented in Table 6.3.

Table 6-2. Morphological characteristics of *S. aureus* (Sa) bacteriophages as observed by electron microscopy.

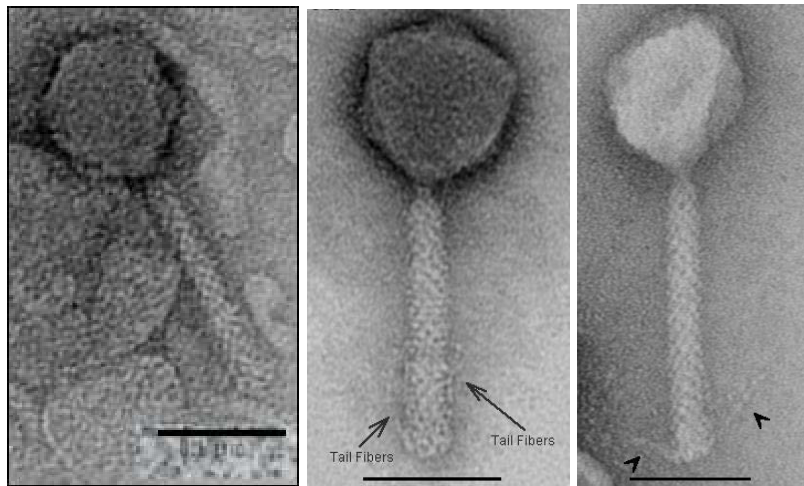
	Family name	Morphotypes (Ackermann, 1996)	Head Dimensions (nm)	Tail Dimensions (nm)		Electron Micrograph
				Diameter	Length	
Sa 12	Myoviridae	A1	75 ± 3	16 ± 2	197 ± 9	6-1a
Sa 18	Myoviridae	A1	76 ± 3	16 ± 1	176 ± 7	6-1b
Sa 34	Myoviridae	A1	82 ± 4	14 ± 1	187 ± 17	6-1c
Sa 38	Myoviridae	A1	75 ± 4	15 ± 1	176 ± 8	6-1d
Sa 41	Myoviridae	A1	ND	ND	ND	-
Sa 42	Myoviridae	A1	72 ± 3	11 ± 2	173 ± 5	6-2a
Sa 44	Myoviridae	A1	ND	ND	ND	-
Sa 51	Myoviridae	A1	73 ± 4	13 ± 2	184 ± 10	6-2b
Sa 53	Myoviridae	A1	ND	ND	ND	-
Sa 54	Myoviridae	A1	71 ± 2	17 ± 2	186 ± 8	6-2c

± indicates standard deviations. ND= Not determined

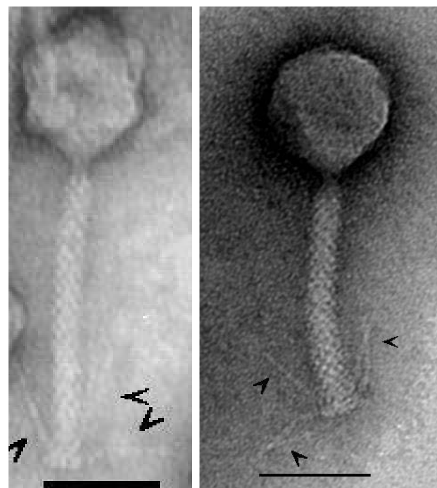
6.3.2 TEM of *P. aeruginosa*(Pa) phages

Selected bacteriophage infecting *P. aeruginosa* reference strains (Table 2-3 to 2-4) were examined under the electron microscope. The electron micrographs showed typical bacteriophage belonging to the *Myoviridae, Siphoviridae* and *Podoviridae* family. Five out of ten of the examined phages belonged to the *Myoviridae* family: Pa 1, Pa 3, Pa 24, Pa 25 and Pa 37 (Figure 6-5a-e), three to the *Siphoviridae* family: Pa 6, Pa 10 and Pa 26 (Figure 6-6a-c) and two to the *Podoviridae* family: Pa 32 and Pa 51 (Fig 6-7a-b). A summary with the morphological characteristics of the bacteriophage observed is presented in Table 6.2.

Figure 6-5. TEM images of *P. aeruginosa* phages belonging to the Myoviridae family.



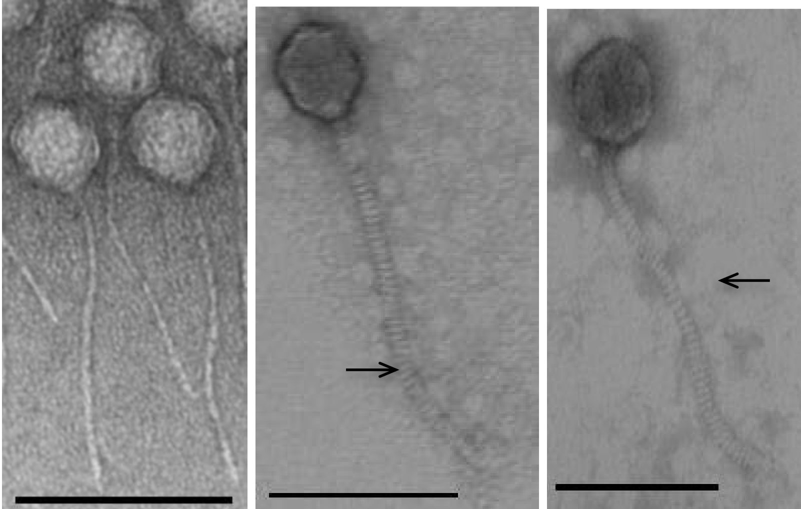
6-5a) Pa 1 **6-5b) Pa 3** **6-5c) Pa 24**



6-5d) Pa 25 **6-5e) Pa 37**

Bacteriophages Pa 1, Pa 3, Pa 24, Pa 25 and Pa 37 showed an icosahedral head with a group median value of 55 ± 5nm in diameter, a tail length of 115 ± 10.0nm and a tail diameter of 10 ± 1.0nm. Arrows in the figures show tail fibers observed in the phages. Scale bar 50nm.

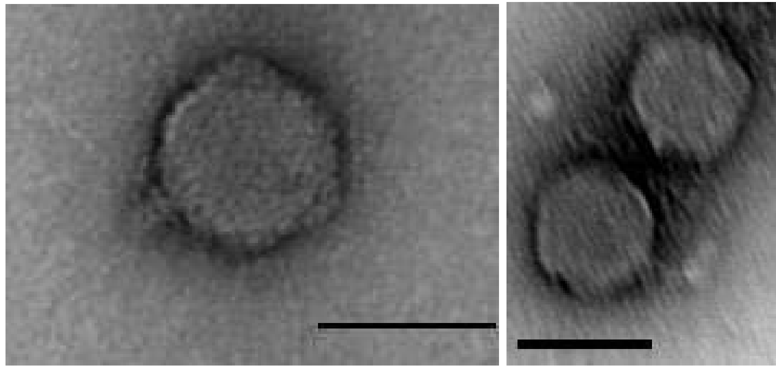
Figure 6-6. TEM images of *P. aeruginosa* phages belonging to the Siphoviridae family.



6-6a) Pa 6 6-6b) Pa 10 6-6c) Pa 26

Bacteriophages Pa 6, Pa 10 and Pa 26 showed long-flexible not contractible tails. Pa 10 and 26 also showed ridges across the tail (arrows). Scale bar 100nm.

Figure 6-7. **TEM images of the P.aeruginosa phages belonging to the Podoviridae family.**



6-7a) Pa 32 **6-7b) Pa 51**

Bacteriophages Pa 32 and Pa 51 showed icosahedral heads and short tails. These morphological characteristics classified these phages as part of the morphotype C1 in the Podoviridae family. Scale bar 50nm

Table 6-3. **Morphological characteristics of *P. aeruginosa* bacteriophages as observed by electron microscopy.**

Phage	Family name	Morphotypes (Ackermann, 2001)	Head Dimensions (nm)	Tail Dimensions (nm)		Other Characteristics	Electron Micrograph
				Diameter	Length		
Pa 1	Myoviridae	A1	59 ± 2	10 ± 1	95 ± 2	---	6.5a
Pa 3	Myoviridae	A1	63 ± 4	8.5 ± 1	116 ± 4	Tail Fibres	6.5b
Pa 6	Siphoviridae	B1	40 ± 2	4 ± 1	125 ± 5	---	6.6a
Pa 10	Siphoviridae	B1	50 ± 3	9 ± 1	200 ± 10	Ridges in the tail	6.6b
Pa 24	Myoviridae	A1	60 ± 2	10 ± 1	100 ± 2	Tail Fibres	6.5c
Pa 25	Myoviridae	A1	60 ± 2	10 ± 1	125 ± 2	Tail Fibres	6.5d
Pa 26	Siphoviridae	B1	50 ± 3	9 ± 1	200 ± 10	Ridges in the tail	6.6c
Pa 32	Podoviridae	C1	53 ± 2	---	14 ± 2	---	6.7a
Pa 37	Myoviridae	A1	60 ± 5	12 ± 2	120 ± 2	Tail Fibres	6.5e
Pa 51	Podoviridae	C1	48 ± 2	---	12 ± 1	---	6.7b

± indicates standard deviations. ND= Not determined

The *P. aeruginosa* bacteriophages isolated appeared to be very similar in size and shape. These phages showed an icosahedral head with average diameter of 55 ± 5nm, a tail length of 115 ± 10.0nm and a tail diameter of 10 ± 1.0nm. Pa 1 (fig 6-5a) had a slightly shorter tail and Pa 3 (Fig 6-5b) showed a slightly longer head (63 ± 4nm) than the other phages observed. Pa 3 (Fig 6-5b), Pa 24 (Fig 6-5c), Pa 25 (Fig 6-5d) and Pa 37 (Fig 6-5e) showed also typical tail fibres not seen in Pa 1 (Fig 6-5a). Bacteriophage Pa 25 appeared slightly different to Pa 37 in that it had a longer tail (125nm) with a thinner tail diameter (10nm).

According to these characteristics, these five phages were assigned to morphotype A1, *Myoviridae* family.

Bacteriophages Pa 6, Pa 10 and Pa 26 showed long flexible tails under the electron microscope. Pa 6 (Fig 6-6a) showed a shorter tail (125 ± 5nm) and a smaller head (40 ± 2nm) than Pa 10 (Fig 6-6b) and Pa 26 (Fig 6-6c). Pa 10 and Pa 26 appeared to be identical in the electron micrograph and slightly bigger than Pa 3 with a head size of 50±5nm and a longer tail of 200±10nm. Pa 10 and Pa 26 also shared clear ridges in the tails. According to these characteristics, these three phages were therefore assigned to morphotype B1, *Siphoviridae* family.

Bacteriophage Pa 32 (Fig 6-7a) showed icosahedral heads (53 ± 2nm) and short tails (14 ± 2). Pa 51 (Fig 6-7b) showed the same shape but a slightly smaller head (48 ± 2nm) than Pa 32. These morphological characteristics made these phages part of the morphotype C1 in the *Podoviridae* family.

Some other interesting features were observed during the analysis. In figure 6-8 bacteriophage Pa 1 with empty heads appeared to be attached to bacterial debris with their sheath remaining contracted. Figure 6-9 shows Pa 32 at two different states, before and after the nucleic acid was expelled from the head. Figure 6-10 shows some knob-like structures observed at the end of the tails in Phage Pa 10.

Figure 6-8. Bacteriophage Pa 1 with contracted and tails and empty heads. Scale bar 100nm

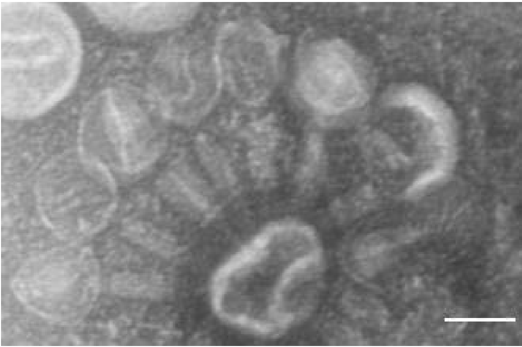


Figure 6-9. Bacteriophage Pa 32 before (right) and after (left) injection of DNA. Scale bar 50nm

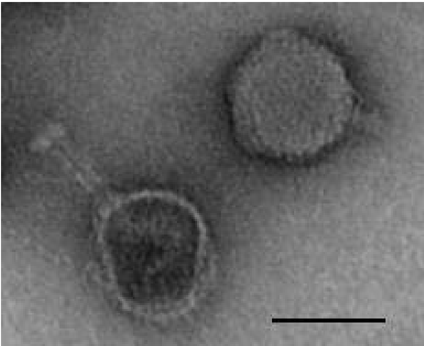
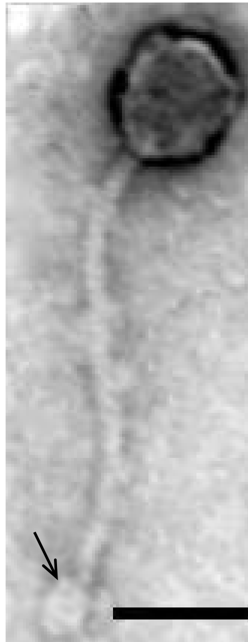


Figure 6-10. Phage Pa 10 with knob-like structure (indicated by the arrow) observed in the tail (Scale bar 50nm)



6.4 Discussion

6.4.1 TEM of *S. aureus* (Sa) phages

Until recently, the *S. aureus* phages most studied were part of the international typing set (Bradley, 1963, Brown et al., 1972). This set was characterised by either icosahedral or elongated heads, with flexible tails, and a baseplate (Lee and Stewart, 1985).The latest review of bacteriophage taxonomy (Ackermann, 2006) reported a total of 215 *Staphylococcus* phages. Of these 215 phages infecting *Staphylococcus* sp., 13% belonged to the *Myoviridae* family, 85% to the *Siphoviridae* family and 2% to the *Podoviridae* family. In this study, all *S. aureus* bacteriophages, were tailed and belonged to the *Myoviridae* family. Phages showed icosahedral heads with an average diameter of 74.8 ± 3.2nm, a contractile tail 182.7 ± 9.1nm in length and 14.5 ± 1.5nm in diameter.

Bacteriophages with identical features to those identified here and capable of infecting not only *Staphylococcus* sp but also *Bacillus* sp. were isolated (Pohjanpelto and Nyholm, 1965) and compared previously (Jarvis et al., 1993). The comparison of these phages by the authors demonstrated than despite the common morphology each phage was unique when other factors were considered. The authors also remarked that this type of phage infects *Staphylococcus* sp. rather frequently (Ackermann and DuBow, 1987, Jarvis et al., 1993). The results obtained appeared to confirm those observations, and suggested that in order to differentiate phages with identical in morphology other factors such as spectrum of activity, burst size and genome size must be considered.

The morphological characterization of these particular *S. aureus* phages by electron microscopy did not facilitate differentiation within the group and showed only small size differences in head and tail sizes. However, other differences such as their place of isolation, host and lytic spectrum of activity (section 4.2.1) suggested them to be different from each other. Further differentiation was achieved by restriction digests (section 7.2.3). In addition, the temperature (section 4.2.2) and pH stability (section 4.2.3) of these phages has showed them to be different and potentially useful as a therapeutic tool.

6.4.2 TEM of *P. aeruginosa* (Pa) phages

To date, five hundred phages that infect the *Pseudomonas* genus have been described (Ackermann, 2006). Tailed phages represented more than 97% of these bacteriophages and less than 3% of them were described with polyhedral, filamentous or pleomorphic morphology (PFP phages) (Table 6.1). In this study, all bacteriophages observed were tailed and not PFP phages were observed. Fifty percent of the phages observed belonged of the *Myoviridae* family (possessing icosahedral heads and contractile tails), 30% to the *Siphoviridae* family (non-contractile flexible tails) and 20% to the *Podoviridae* family (short tails) (n=10). These percentages were similar to those results reported previously were 48% belonged to the *Siphoviridae* family, 28% to the *Myoviridae* family and 24% to the *Podoviridae* family (Ackermann, 2006).

Most of the *P. aeruginosa* bacteriophages within the corresponding family examined appeared to be indistinguishable from each other. In the phages observed within the *Myoviridae* family, as their structure appeared identical, the measurements of all the bacteriophage studied (n=5) were used to obtain mean values for the group as a whole. The results showed a median head size of 60.4 ± 3nm, a tail of 111.2 ± 2.4nm in length and 10.1 ± 1.2nm in width. In the *Siphoviridae* family, only Pa 6 appeared to be slightly different from Pa 10 and Pa 26, which showed identical shape and measurements in the electron micrograph. The tails consisted of flexible tubes and frequently showed some knob-like features at the end (Fig 6-8). In addition, sub-units that gave the appearance of ridges were also observed (Fig 6-3b and 6-3c). Comparable structures were previously described in phages infecting *P. aeruginosa* (Morgan and Stanisich, 1975).

Similar lytic *Siphoviridae* bacteriophages infecting human isolates of *P. aeruginosa* were recently isolated and reported in Novi Sad (Serbia) (Knezevic et al., 2009). Although the size of the head and the length of the tail of those phages were about 7 ±nm bigger, the selection of phages with such similar structures in a completely different geographical area is certainly a point to note. In the future, comparisons of the spectrum of activity of phages isolated in separate geographical areas, which share very similar morphological characteristics could be considered A study at this level could reveal useful information to

be considered for the development of phage cocktails with broad-spectrum of activity as discussed in section 5.4.

Pa 32 and Pa 51 electron micrograph showed an icosahedral head with short tails suggesting these phages belonged to the *Podoviridae* family. Similarly, to what it was observed in the *Myoviridae* family, phages Pa 32 and Pa 51 appeared to be indistinguishable from each other. However, the electron photographs showed Pa 32 to be larger than Pa 51, although, the size of the heads for both phages were 10nm smaller than those reported previously (Ackermann and DuBow, 1987).

In general, bacteriophage characterization by electron microscopy offered a few clues at perceiving some differences such as variations in head and tail size (*i.e*. Pa 3, Pa 6) in all of the bacteriophages observed. However, the selected *P. aeruginosa* bacteriophage examined within this study had a number of similar characteristics that made it difficult to distinguish easily between each other. Nevertheless, differences such as their lytic activity (section 4.2.1), place of isolation and host highlighted them to be different from each other. Further differentiation was achieved by restriction digests (section 7.2.3). In addition, the broad spectrum of some of these bacteriophages along with their temperature (section 4.2.2) and pH stability (section 4.2.3) has showed them to be potentially useful biological agents at reducing the number of *Pseudomonas aeruginosa* in a clinical setting.

6.4.3 TEM general discussion

Previously it was suggested that the relatively high number of tailed phages in the environment may only be reflecting the interest and investigation of specific bacteria that attracted more attention because of their industrial applications (Brussow, 2001). Furthermore, the seeming large numbers of phages belonging to the *Caudovirales* order that infect *P. aeruginosa* and *S. aureus* could be due to a the high profile conditions and special interest on these particular bacteria (Ackermann and Kropinski, 2007). It could also be highlighting the bias conditions used in research protocols. Factors such as the media, temperature, time, etc., are biased towards the selection of phages, which are rapid to

propagate, and with relatively broad spectrum of activity: two of the major sought-after characteristics in therapeutic bacteriophages.

The taxonomy of bacterial viruses, including the establishment of orders and families has been an evolving process since its first proposal in 1943. Phages were initially divided into six morphotypes considering morphology and DNA homology (Bradley, 1967). Later the division was expanded by the addition of new types based on the type of tail and shape of the heads (Ackermann and Eisenstark, 1974). However, no one distinctive condition was found to be completely satisfactory for the sub-classification of phages (Ackermann et al., 1992). Thus, the number of phage families has been continuously restructured, from thirteen in 1992 (Ackermann, 1992) to the more recently classification by the ICTV which accepts one order, 17 families, and three "floating" groups (Ackermann, 2006). This classification in combination with Bradley's morphotypes was used for the identification of the phages isolated in this project. All identified phages fitted a family and morphotype previously described. However, with the rediscovery of bacteriophages not only as therapeutics but also as an important player in the biosphere (Comeau et al., 2008), many "phage taxonomists" believed the ICTV classification must be updated (Nelson, 2004). The belief is that a new system that takes into consideration the recent information generated with the boom in genomics and proteomics should be implemented. As a result, such a new techniques were already suggested to reorganize phage families (Rohwer and Edwards, 2002).

These new developed techniques were recently used to attempt merging classical physicochemical characteristics to proteomic data from previously isolated phages (Lavigne et al., 2008, Lavigne et al., 2009). The groups studied all published genomes of *Podoviridae* phages and 102 *Myoviridae* phages. The results suggested that the ICTV classification for the *Podoviridae* group was mostly accurate as the phages had common distinctive characteristics (Lavigne et al., 2008). However, based on the results, the group suggested the establishment of three new subfamilies and eight independent genera in the *Myoviridae* group (Lavigne et al., 2009). At the current rate of bacteriophage genome sequencing (Ackermann and Kropinski, 2007), it is reasonable to think that the number of

sequence similarities to be considered for the classification of phages is only likely to increase and even more systems likely to be suggested.

In the future, if new classification systems based on the genomics and/or proteomics of bacteriophages were accepted by the ICTV, the results obtained in this work may need to be revisited, as phage genomes would need to be sequenced and screened against all the databases available in order to classify them within families accurately. Although such type of systems will be very useful to identify the differences in bacteriophages with identical morphology, the process itself may not be necessarily practical for phage selection in early research stages. Hence, the current system with the specific protocol used may still prove very useful as it provides enough complementary information to separate rapidly those phages that behave similarly during their selection for therapeutic use if the morphology appears identical.

7 MOLECULAR CHARACTERIZATION OF BACTERIOPHAGES

7.1 Introduction

Bacteriophages are generally found in a diversity of environments, including the ocean (Suttle, 2007), soil (Germida and Casida, 1981, Summer et al., 2006), sewage waters (Tanji et al., 2005, Synnott et al., 2009), humans (Chibani-Chennoufi et al., 2004, Hitch et al., 2004), animals (Callaway et al., 2008, Park et al., 2000, Vinod et al., 2006) and food (Yoon et al., 2002, Atterbury et al., 2003).

The biodiversity of phages in the environment (Angly et al., 2006, Suttle, 1994) as well as their effect on marine carbon cycling (Paul et al., 2002) were, until recently, unknown to investigators. This could be due, largely, to the fact that the common methods used for isolation would detect less than 1% of bacteriophages present in any water sample examined (Chen et al., 2001). Advantageously, the development of the field of metagenomics (the study of a marine virus community without the need to cultivate it) has enabled scientists to acquire a better understanding of their extensive occurrence.

To date, it is estimated that the biosphere consists of approximately $10^{31\text{-}32}$ bacteriophages (section 1.3.1). However, just over 5,500 phages have been characterised by electron microscopy (Ackermann, 2006) and only about 500 have been sequenced (Comeau et al., 2008). Thus, the collection of molecular data of any newly isolated phages has become an important element not only for increasing the knowledge and understanding of bacteriophage ecology but also, in this case, for the safe development and application of bacteriophage therapy.

The field of metagenomics has proved to be a very valuable tool in the new surge to understand bacteriophage ecology and their influence in the marine environment (Suttle, 2007). However, this technology is not adequate for the characterization of individual phages to be used for therapy. For this task, more straightforward methods are generally

exploited. The use of pulsed-field gel electrophoresis (PFGE) (Lingohr et al., 2008) and restriction digests (Huang et al., 1982) allows for their initial characterization and, more importantly, helps to identify and distinguish those with similar genomes. Genome sequencing can then be performed to determine the presence of undesirable genes such as integration, toxic or antibiotic resistance-like genes. In this study, PFGE and restriction digest laboratory protocols were developed to assist with the characterization of genomic DNA of the selected bacteriophages (Table 3-6 and 3-7). Furthermore, it was hoped that the data generated would prove useful in helping to design a much-required system in order to quality control the integrity of each bacteriophage during the manufacturing process in the future.

7.2 Molecular characterization of bacteriophage

7.2.1 DNA extraction of phage DNA

For the isolation of bacteriophage DNA a combination of two methods reported previously (Santos, 1991, Su et al., 1998) were combined and modified as follows. Twenty milliliters of the bacteriophage stock of interest produced as per section 2.6.5 were divided into two 50ml Falcon tubes. DNase (6µg/ml) and RNase (3µg/ml) were added to each tube and incubated for 1h at 37°C. After this, filter sterilized $ZnCl_2$ was added to each tube to a final concentration of 40mM and incubated again for 5min at 37°C. The solutions were then aliquoted in 12×2ml Eppendorf tubes (~1725µl/tube) and centrifuged at 18,000*g* for 5min at room temperature to pellet phage particles. Supernatants were drained with a pipette and each pellet resuspended in 1ml of TENS containing 6µl of a 20mg/ml proteinase K stock. All tubes were incubated at 65°C for 15min to lyse phages. Solutions were pooled into 4×15ml falcon tubes (3ml per tube) and chilled 3M potassium acetate was added to a final concentration of 0.4M. Samples were mixed thoroughly by inversion and left on ice for 45min. Samples were again aliquoted into 2ml Eppendorf tubes (~1700µl/tube), centrifuged for 5min at 18,000*g* and the supernatant collected into fresh Eppendorf tubes. This step was repeated two more times. Supernatants were then divided equally into 16 tubes (~800µl each) and an equal volume of cold isopropanol added. Tubes were mixed by inversion 20

times, left in ice for 15min and then centrifuged at 18,000*g* for 5min. The supernatants were discarded and 1ml of 70% ethanol was added, gently, into each tube. Supernatants were fully drained and the tubes left, with the lid open in a biohazard hood with the airflow for 30-45min to allow the ethanol to evaporate. Each pellet was then completely dissolved in 25µl TE manually first, then centrifuged at 1,400*g* for 10min and then left on the bench for 30min to allow for total resuspension before pooling all tubes together. DNA samples were kept frozen at -20°C until analysis by restriction digest.

7.2.2 Restriction enzyme digestion of bacteriophages

Phage DNA was obtained from high-titer phage preparations as described above (section 7.2.1). Each enzymatic treatment of DNA was performed in triplicate as recommended by the manufacturers. In an effort to determine a detailed profile, the following panel of restriction enzymes with different recognition sequences was used: *Hind*III*, Pst*I*, Bam*HI, *Eco*RI (Sigma-aldrich); *Eco*RV*, Hinc*II*, Hpa*I*, RSa*I, *Bg*II (Invitrogen); *Cla*I*, Ava*I*, Dra*I*, Apa*I and *Sma*I (Promega). DNA electrophoresis was carried out in 0.8-1.2% agarose gels using standard TAE buffer (Bio-Rad #161-0743) and a horizontal gel unit (Bio-Rad Sub-cell GT basic, 40w, 200V). Gels were run at 90V from 2 to 3.5h. A 2.5kb molecular ruler (Bio-Rad 170-8205) and Amplisize molecular ruler (Bio-Rad 170-8200) were used as the molecular markers. Gels were stained in 0.4µg/ml ethidium bromide and DNA analysed with UV transillumination (Bio-Rad, Gel-Doc XR system).

7.2.3 Pulsed-field gel electrophoresis (PFGE) of bacteriophage DNA

Four hundred microliters of a bacteriophage suspension in SM buffer (section 2.6.5) were mixed with an equal volume of 2% molten agarose (Ultra Pure DNA Grade Agarose: BioRad #162-0137) in TE buffer (10 mM Tris.Cl; 1 mM EDTA at pH 8.0) and quickly dispensed into plug moulds (BioRad). The plugs were allowed to set at room temperature and then transferred into 5mL Falcon tubes containing 2ml of lysis buffer (100mM EDTA pH 8.0, 1% (w/v) N-lauroyl sarcosine; 0.2% (w/v) SDS; 0.5mg/ml Proteinase K). The plugs in the lysis buffer were incubated in a waterbath at 55°C for 18h. The lysis solution was then discarded, 2ml of wash buffer (20 mM Tris:HCl; 50mM EDTA at pH 8.0) added and

left for 1h at room temperature. Three more 30min washes with 2ml of wash buffer were subsequently performed at room temperature. Finally, the wash buffer was discarded and 2ml TE buffer were added to the plugs and stored at 4˚C. DNA-agarose plug slices were loaded in a well in 1% agarose gel (Bio-Rad PFGE grade high melting point agarose) prepared in 0.5X TBE running buffer (50mM Tris-base, 50mM boric acid, and 1mM EDTA, pH 8.5). DNA plugs were run by PFGE in three separate experiments using the CHEF-DR III system (Bio-Rad laboratories) in 2.4L 0.5X TBE buffer (50mM Tris-base, 50mM boric acid, and 1mM EDTA, pH 8.5) for 18h at 14°C, with ramping times of 2s to 4s at 6 V/cm. Agarose gels were stained with ethidium bromide (0.4µg/100ml) for 45min and de-stained with distilled water for 15min. Gels were observed with a transilluminator (Bio-Rad, Gel-Doc XR system). A 5kb ladder, 4.9–120kb, concatemers of pBR328 (Bio-Rad laboratories 170-3624) or lambda (λ) ladder, 0.05–1 Mb, concatemers of phage λcl857Sam7 (Bio-Rad laboratories 170-3635) were used as the molecular markers.

7.3 Results

7.3.1 DNA extraction of bacteriophages

The combination of the two, phage-DNA, extraction methods reported previously was highly efficient for the DNA extraction of the selected bacteriophages. The combined method exploited two changes: first, the inclusion of proteinase K, absent in the protocol reported by Santos (1991): second, the replacement of two extractions with phenol/chloroform/isoamyl alcohol, present in the protocol reported by Su *et al.* (1998), with three washes of 3M potassium acetate. The implementation of these two changes meant that the ratio of adsorption at 260nm and 280nm, used to measure the purity of DNA, was consistently) calculated over 1.75 (data not shown).

Furthermore, by exploiting the peak density function of the Quantity One® software package (Bio-Rad v. 4.2.1) for the imaging and analysing of 1-D electrophoresis gels, the amount of DNA required for optimal restriction enzyme digestion for each bacteriophage was calculated. Earlier, after trial and error, it was determined that 8µl of *P. aeruginosa* phage Pa25 extracted DNA was the required volume for optimal digestion. This

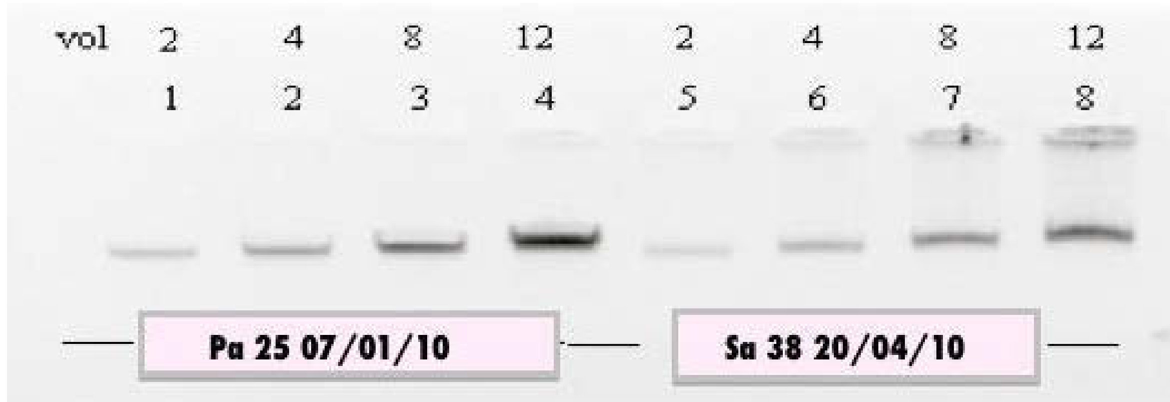
information was exploited to develop a calibration curve measuring the peak intensity of four different volumes (2µl, 4µl, 8µl and 12µl) of Pa 25 extracted DNA and the peak intensity of 3µl of the 2.5kb marker (Bio-Rad 170-8205). The five samples were loaded onto a 0.8% agarose gel stained with 8µl of ethidium bromide (10mg/ml). The gel was run at 90V and imaged after 30min to calculate the DNA peak density of each sample run (Quantity One® software package, Bio-Rad v. 4.2.1). Knowing that one band of the 2.5kb marker was equivalent to 20ng of DNA, and using the peak intensity function of the software it was then possible to develop a calibration curve (Fig 7-1). This curve was used to calculate the required volume of subsequent extracted phage DNA samples, where the unknown variable "x" from the linear regression was the optimal volume for optimal digestion. An example is described in Figure 7-1.

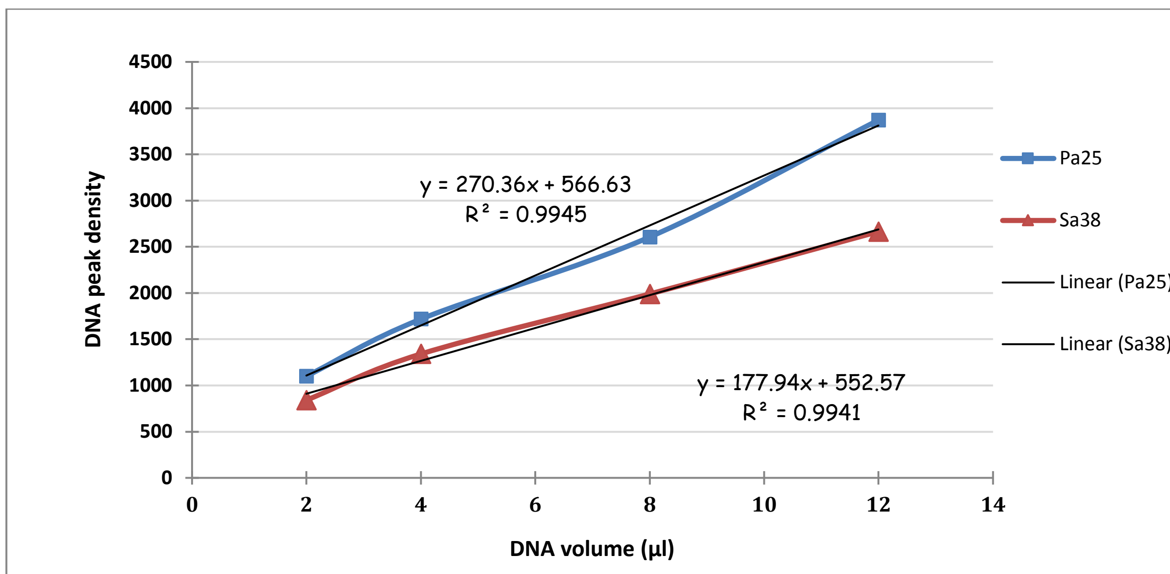
7.3.2 Molecular characterization of *S. aureus* (Sa) bacteriophages

7.3.2.1 *Restriction enzyme digestion of Sa bacteriophages*

Bacteriophages Sa 12, 34, 38, 41, 42, 44, 51, 53 and 54 were digested with several restriction enzymes (section 7.2.2). All the selected phages were resistant to cleavage by *ApaI, BamHI*, *BgII* and *ClaI*. In addition Sa 12, 34, 41, 44 and 51 were resistant to digestion by *SmaI*. Digestion of all the Sa phages with *AvaI* and Sa 38, 42, 53 and 54 with *SmaI* resulted in digests containing less than or equal to four DNA fragments. On the other hand, the DNA of all Sa phages when digested with *DraI* and *RSaI* were cut too frequently (CTF), making difficult the accurate identification of bands in the gel. Restriction digests with *EcoRI, EcoRV, HindIII, HincII, HpaI and PstI* produced DNA patterns of over nine fragments per reaction, with the exception of Sa 38 digested with *PstI* that produced only five fragments. The number of restriction fragments produced from each of the selected phages by the different enzymes is presented in Table 7-1.

Figure 7-1. Comparison of DNA extracted: Pa 25 v Sa 38





Pa 25 extracted DNA was used to construct a calibration curve, in which 8µl of Pa 25 DNA were established to be the required volume for optimal digestion. The regression line of the DNA peak density measured for 2µl, 4µl, 8µl and 12µl of an extracted DNA sample was then used to calculate the required volume (x) for optimal restriction enzyme digestion. In this example 28 µl of Sa 38 phage DNA were needed for optimal digestion.

Table 7-1. Number of restriction fragments produced by *S. aureus* (Sa) phages

Enzyme	Sa 12	Sa 34	Sa 38	Sa 41	Sa 42	Sa 44	Sa 51	Sa 53	Sa 54
ApaI	0	0	0	0	0	0	0	0	0
Ava I	4	3	2	2	2	3	3	2	2
BamHI	0	0	0	0	0	0	0	0	0
Bgl II	0	0	0	0	0	0	0	0	0
Cla I	0	0	0	0	0	0	0	0	0
DraI	CTF	CTF	CTF	CTF	CTF	CTF	CTF	CTF	CTF
EcoRI	13	10	10	9	9	10	10	15	19
EcoRV	15	10	11	13	12	10	9	21	24
HincII	28	28	28	29	28	29	29	29	30
HindIII	19	22	22	21	21	22	22	21	21
HpaI	27	27	26	28	28	26	26	28	28
PstI	11	11	5	11	9	11	11	11	12
Rsa I	CTF	CTF	CTF	CTF	CTF	CTF	CTF	CTF	CTF
SmaI	0	0	2	0	2	0	0	2	2

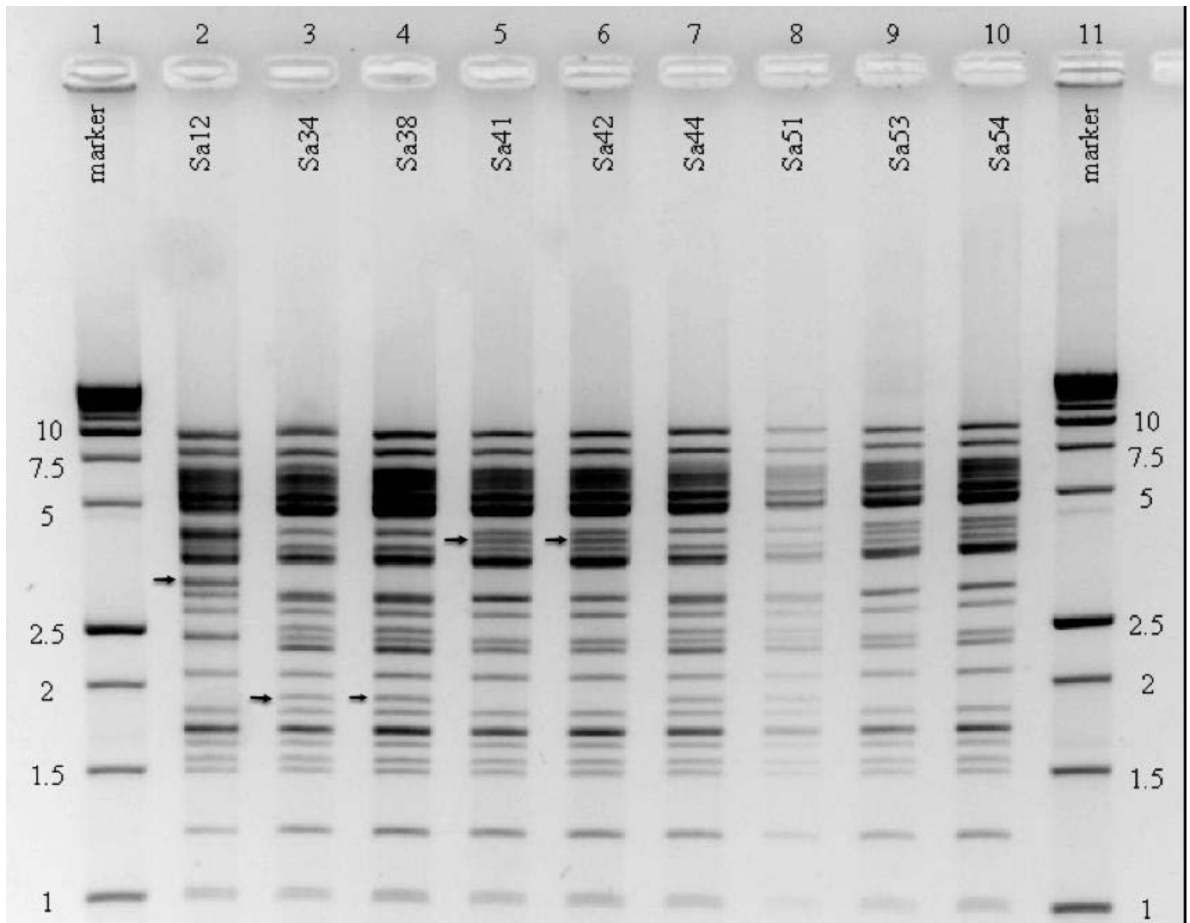
Each bacteriophage was treated with the enzymes listed and the number of bands observed recorded. CTF= the phage genome was cut too frequently by the enzyme.

Differences in the restriction patterns generated with some, but not all, of the enzymes allowed for the designation of unique identification patterns for each of the selected phages. The restriction digest with *Sma*I divided the phages in two groups. Group 1 with phages Sa 12, Sa 34, Sa 41, Sa 44 and Sa 51 and group 2 included Sa 38, Sa 42, Sa 53 and Sa 54. Restriction digest with *Pst*I helped to separate Sa 38 from the other phages included in group 2. However, the restriction digests with *Hind*III and *Hinc*II were the most useful in detecting the discrete DNA differences among the phages.

The restriction digest of Sa phages with *Hind*III showed Sa 12 was different not only from the phages in group 1, but also from those in group 2. It also showed phage Sa 34 was different from Sa 41 and Sa 44 and that Sa 41 was different from Sa 44 (Figure 7-2).

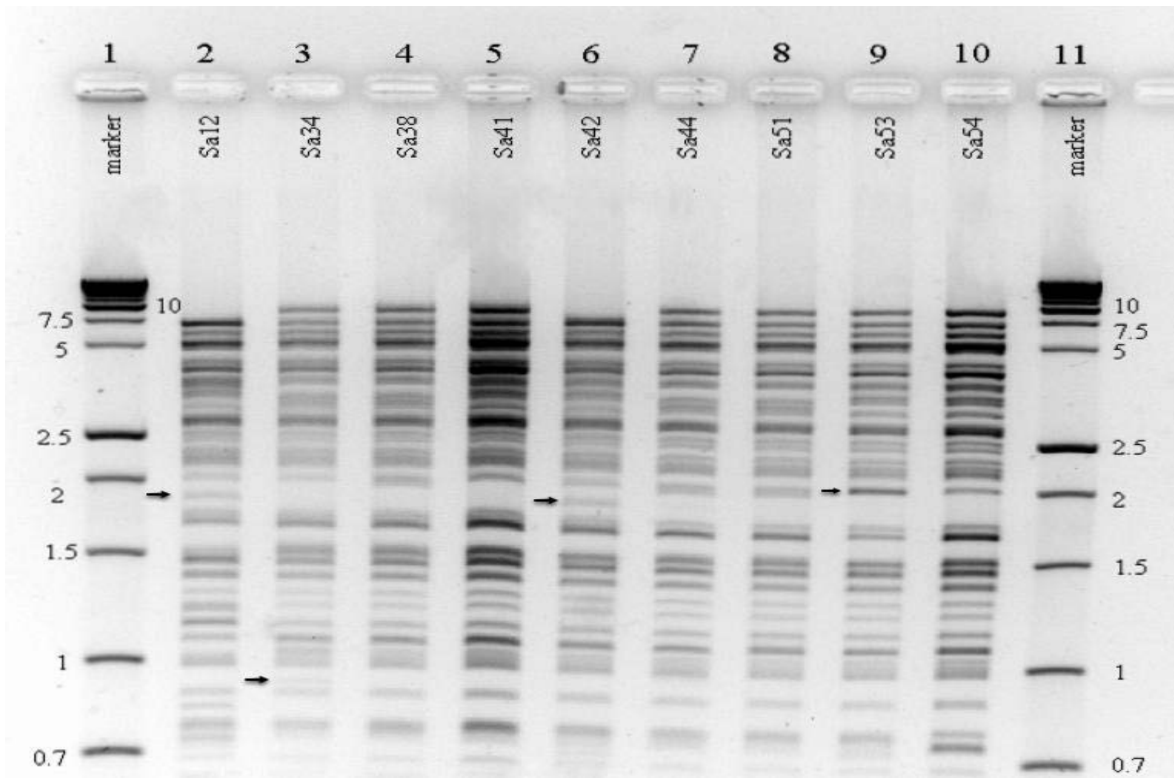
The restriction digest of Sa phages with *Hind*III also showed that Sa 12 was different from the other phages and aided in the separation of Sa 34 and Sa 42 from the other phages within their corresponding group (Figure 7-3). However, both enzymes failed to show any differences between Sa 44 and Sa 51 in group 1 and Sa 53 and Sa 54 in group 2.

Figure 7-2. Restriction digest of Sa bacteriophages with *Hind*III



Genome digestion of Sa bacteriophages with HindIII. Lanes 1 and 11 represent the 2.5Kb marker. Lanes 1 to 10 show bacteriophages Sa 12, Sa 34, Sa 38, Sa 41, Sa 42, Sa 44, Sa 51, Sa 53 and Sa 54 correspondingly. The minor differences identified are highlighted with the arrow (→). The digested phage DNA was separated electrophoretically in a 1.2% agarose gel at 90V for 3 hours.

Figure 7-3. Restriction digest of Sa bacteriophages with *Hinc*II



*Genome digestion of Sa bacteriophages with Hinc*II. *Lane 1 and 11 represent the 2.5Kb marker. Lanes 1 to 10 show bacteriophages Sa 12, Sa 34, Sa 38, Sa 41, Sa 42, Sa 44, Sa 51, Sa 53 and Sa 54 correspondingly. The minor differences identified are highlighted with the arrow (→). The digested phage DNA was separated electrophoretically in a 1.2% agarose gel at 93V for 2.5 hours*

7.3.2.2 *Pulsed-field gel electrophoresis (PFGE) of Sa bacteriophages*

Pulsed-field gels were performed to investigate the genomes of bacteriophages Sa 12, 34, 38, 41, 42, 44, 51, 53 and 54. Employing a lambda (λ) ladder of 0.05–1Mb as the marker, the genome size for each of the Sa phages was estimated. The genomes of the bacteriophages studied were comparable in size with an average media of 147.4 ± 1.8kb calculated. The results are summarised in Figure 7-4.

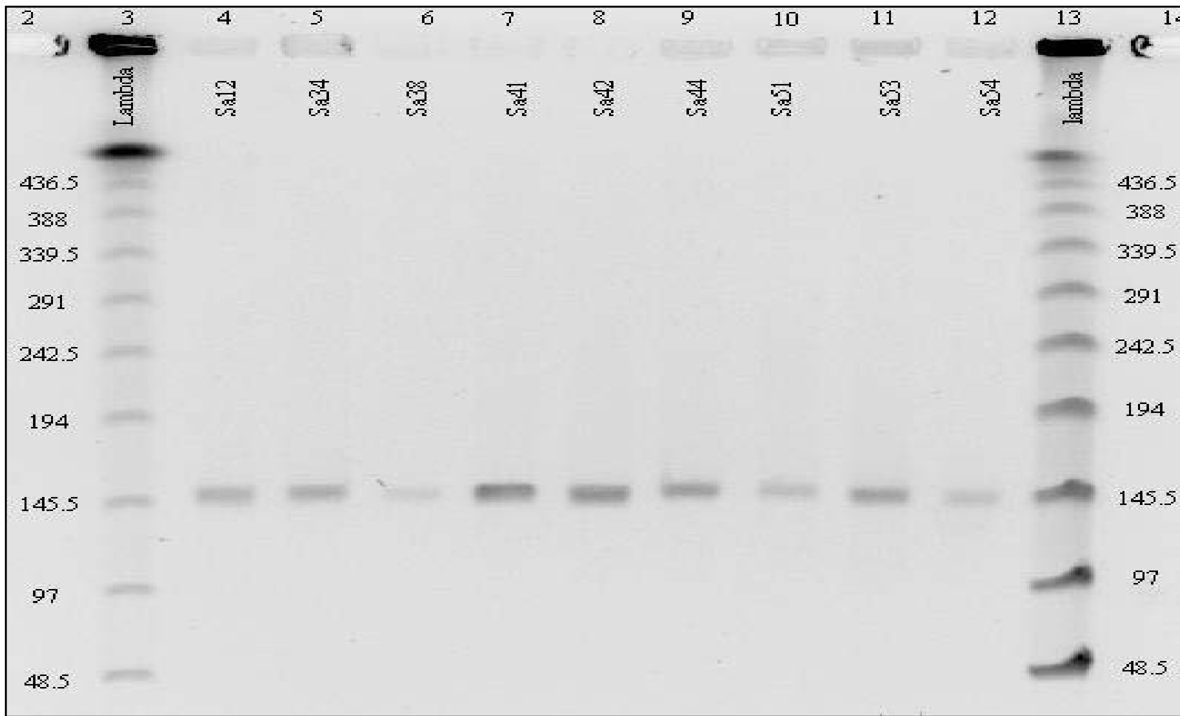
7.3.3 Molecular characterization of *P. aeruginosa* (Pa) bacteriophages

7.3.3.1 *Restriction enzyme digestion of Pa bacteriophages*

Bacteriophages Pa 1, 3, 6, 10, 24, 25, 26, 32, 37 and 51 were digested with several restriction enzymes (section 7.2.2). The DNA of *P. aeruginosa* phage Pa 6 and Pa 32 were the most resistant to digestion by the restriction enzymes used. Pa 6 was sensitive only to digestion by *Eco*RI, *Hinc*II, *Hind*III and *Hpa*I, while Pa 32 was sensitive only to digestion by *Ava*I, *Cla*I*, Hinc*II and *RSa*I. All *P. aeruginosa* phages were resistant to *Pst*I except for Pa10 (16 bands) and Pa 26 (21 bands) and also resistant to *Bam*HI, except for Pa 26 (13 bands) and Pa 51 (6 bands). The number of restriction fragments produced from each of the selected phages by the different enzymes is presented in Table 7-2.

The restriction patterns observed for each of the *P. aeruginosa* phages with the different enzymes were unique and showed them to be different from each other as demonstrated by the digests observed with *Cla*I (Figure 7-5) and *Hpa*I (Figure 7-6).

Figure 7-4. **PFGE of *S. aureus* (Sa) bacteriophages**



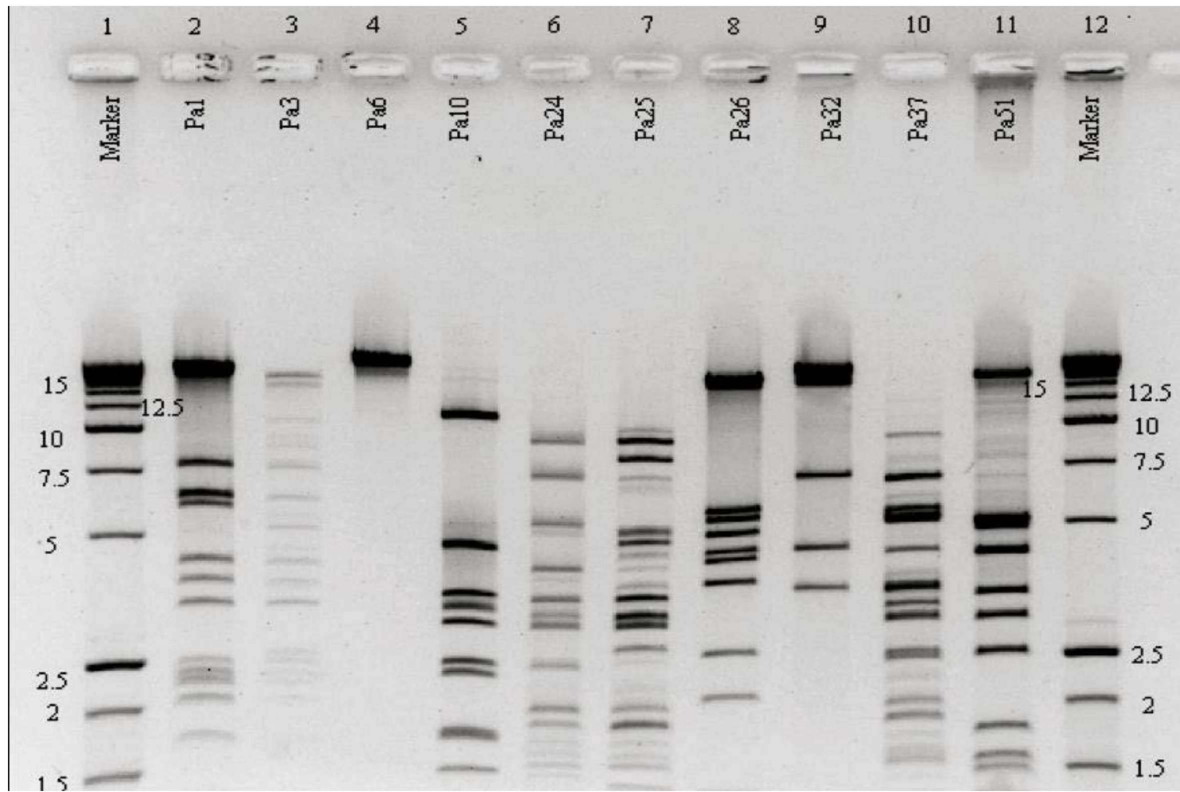
The genome size of Sa bacteriophages was determined by PFGE. Lanes 3 and 13: lambda (λ) ladder 0.05–1 Mb; lane 4: Sa 12 (147 ± 2kb); lane 5: Sa 34 (149 ± 4kb); lane 6: Sa 38 (147 ± 1kb); lane 7: Sa 41 (149 ± 2kb); lane 8: Sa 42(147 ± 2kb); lane 9: Sa 44 (147 ± 1kb); lane 10: Sa 51(148 ± 1kb); lane 11: Sa 53: (146 ± 1kb) and Lane 12: Sa 54 (145 ± 2kb). The symbol, ±, indicates standard deviations of the calculated molecular weight for each bacteriophage in three separated experiments.

Table 7-2. Number of restriction fragments produced by *P. aeruginosa* (Pa) phages.

Enzyme	Pa 1	Pa 3	Pa 6	Pa 10	Pa 24	Pa 25	Pa 26	Pa 32	Pa 37	Pa 51
ApaI	12	14	0	ND	12	ND	0	0	0	ND
Ava I	>16	24	0	14	ND	2	14	CTF	0	14
BamHI	0	0	0	0	0	0	>13	0	0	6
Bgl II	0	0	0	8	0	ND	12	0	ND	2
Cla I	13	12	0	10	15	>20	12	4	>15	10
DraI	3	0	0	ND	5	ND	2	0	ND	ND
EcoRI	22	7	8	7	7	13	15	0	11	10
EcoRV	11	ND	0	8	10	13	10	0	13	20
HincII	11	22	10	15	6	11	15	16	12	10
HindIII	11	15	11	2	0	7	13	0	8	8
HpaI	9	9	5	0	4	7	9	0	5	9
PstI	0	0	0	16	0	0	21	0	0	0
Rsa I	14	12	0	CTF	CTF	ND	CTF	CTF	CTF	CTF
SmaI	*14*	13	0	CTF	6	ND	0	0	0	ND

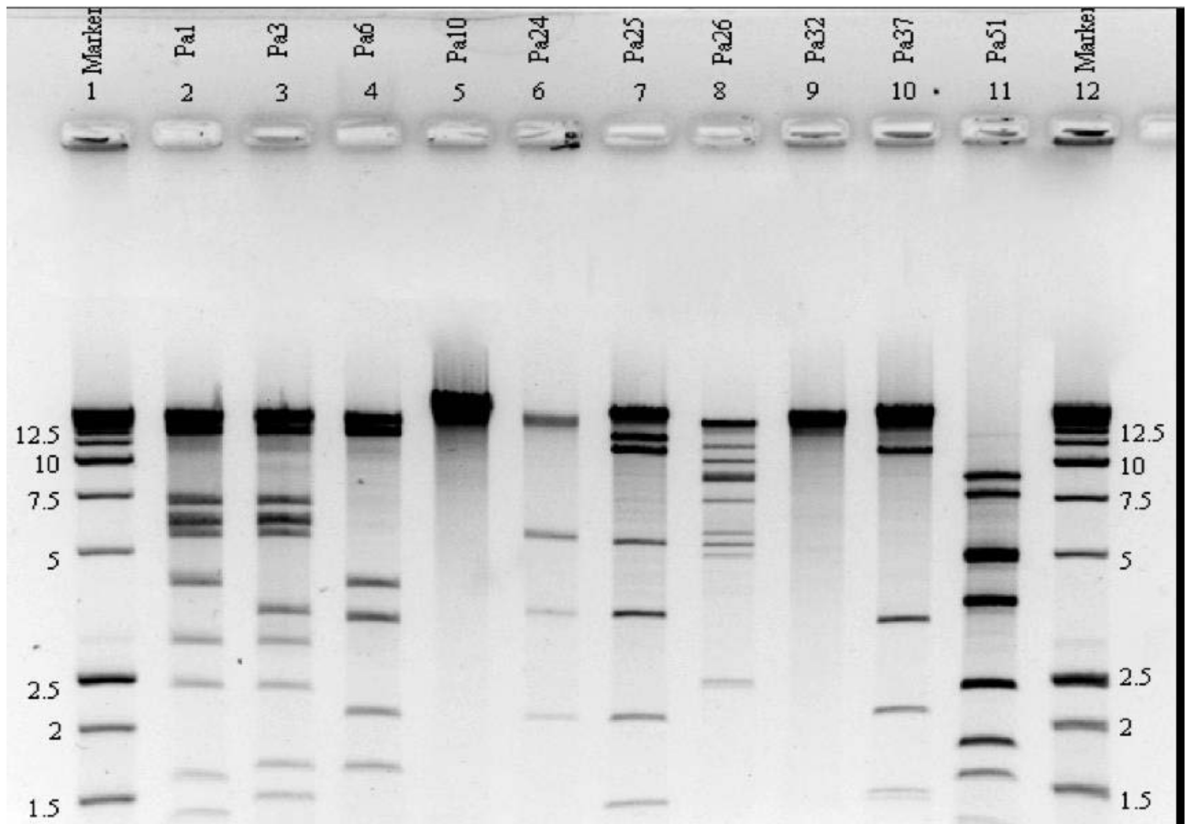
Each bacteriophage was treated with the listed enzymes and the number of bands observed recorded. CTF signifies that the phage genome was cut too frequently by the enzyme. ND: The activity of the enzyme was not determined. The symbol, >, signifies greater than

Figure 7-5. Restriction digest of Pa bacteriophages with ClaI



*Genome digestion of Pa bacteriophages with Cla*I. *Lanes 1 and 12 represent the 2.5Kb marker combined with the amplisize molecular ruler (5—2000bp) . Lanes 2 to 11 show bacteriophages Pa 1, Pa 3, Pa 6, Pa 10, Pa 24, Pa 25, Pa 26, Pa 32, Pa 37 and Pa 51 correspondingly. Each bacteriophage showed a unique restriction pattern. The digested phage DNA was separated electrophoretically in a 1.2% agarose gel at 90v for 3 hours.*

Figure 7-6. Restriction digest of Pa bacteriophages with HpaI



*Genome digestion of Pa bacteriophages with Hpa*I. *Lanes 1 and 12 represent the 2.5Kb marker combined with the amplisize molecular ruler (5—2000bp) . Lanes 2 to 11 show bacteriophages Pa 1, Pa 3, Pa 6, Pa 10, Pa 24, Pa 25, Pa 26, Pa 32, Pa 37 and Pa 51 correspondingly. Pa 10 and Pa 32 were not susceptible to the enzyme. However, other enzymes showed these two phages were different from each other. The digested phage DNA was separated electrophoretically in a 1.2% agarose gel at 90v for 3 hours.*

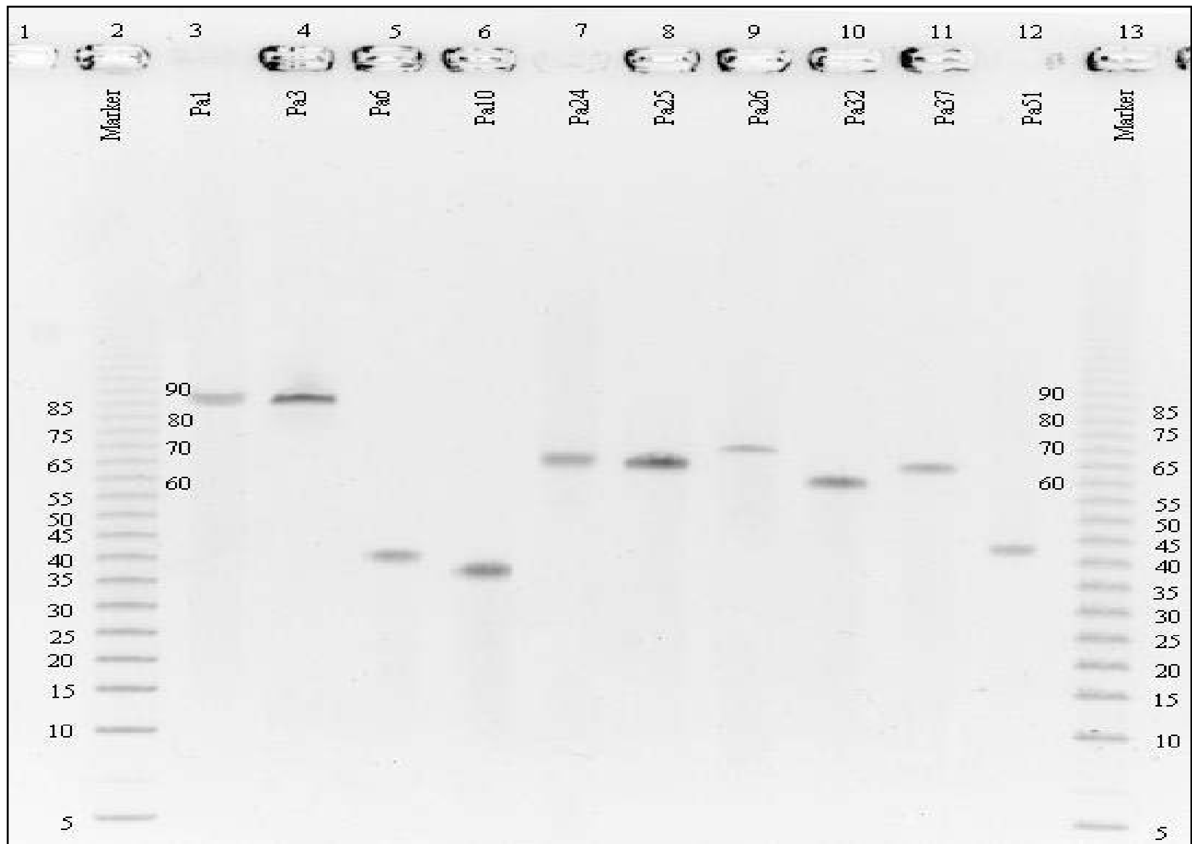
7.3.3.2 *Pulsed-field gel electrophoresis (PFGE) of Pa bacteriophages*

Pulsed-field gels were performed to investigate bacteriophage genome and DNA fragments obtained from restriction digest employing a 5kb ladder, 4.9–120kb, concatemers of pBR328 (Bio-Rad laboratories 170-3624) as the marker. The results showed Pa 51 and Pa 32 had the smallest genomes with a molecular weight of 42 ± 0.5 kb and 59 ± 0.1 kb while Pa 1 and Pa 3 showed the largest with 85 ± 0.2 and 85 ± 1kb respectively. Pa 6 and Pa 10 showed similar sizes with a molecular weight of 38 ± 2 and 36 ± 0.4. The genome sizes of Pa 24, Pa 25, Pa 26 and Pa 37 were also comparable with 65 ± 0.2, 64 ± 0.2, 69 ± 0.3 and 63 ± 0 kb correspondingly. The results are summarised in Figure 7-7.

7.3.4 Analytical testing system prototype of *S. aureus* (Sa) bacteriophages

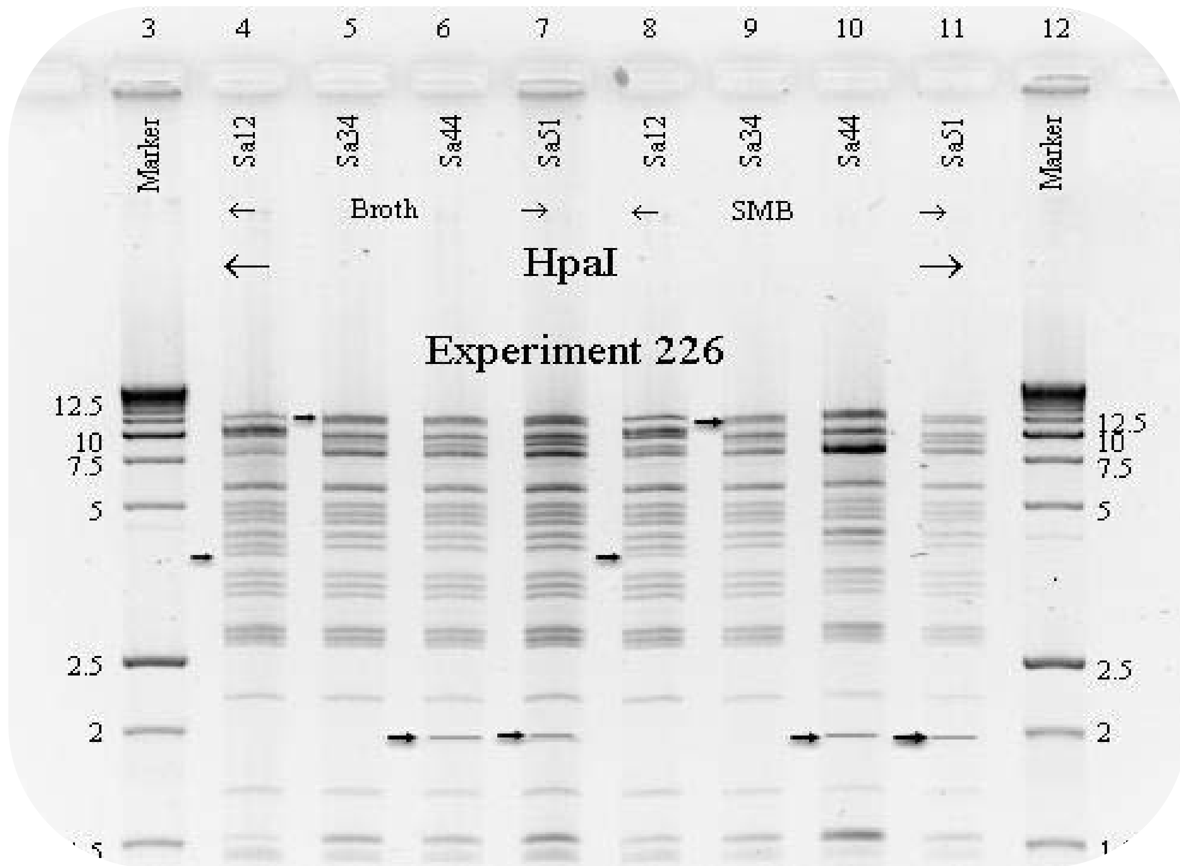
The restriction digest patterns obtained during the course of this study were tested for their adequateness to support a much-required analytical system to control the integrity of each bacteriophage during the manufacturing process in the future. The feasibility and reproducibility of such system was demonstrated for selected bacteriophages by producing, consecutively and over time, three small 30ml batches of specific bacteriophage lysates. The genomic DNA was then recovered and the restriction enzyme digests performed as described previously. The results showed that the phage restrictions patterns remained unchanged (*i.e.* Fig. 7-8).

Figure 7-7. PFGE of *P. aeruginosa* (Pa) bacteriophages



The genome size of Pa bacteriophages was calculated by PFGE. Lanes 2 and 13: 5 kb ladder, lane 3: Pa 1 (85 ± 0.2kb); lane 4: Pa 3 (85 ± 1kb); lane 5: Pa 6 (38 ± 2kb); lane 6: Pa 10 (36 ± 0.4kb); lane 7: Pa 24 (65 ± 0.2kb); lane 8: Pa 25 (64 ± 0.2kb); lane 9: Pa 26 (69 ± 0.3kb); lane 10: Pa 32: (59 ± 0.1kb), Lane 11: Pa 37 (63 ± 0kb) and lane 12: Pa 51 (42 ± 0.5kb). ± indicates standard deviations of the calculated molecular weight for each bacteriophage in three separated experiments.

Figure 7-8. Bacteriophage restriction patterns variations over time



Bacteriophages Sa 12, Sa 34, Sa 44 and Sa 51 were grown separately with their corresponding hosts and their genomic changes determined as any changes in the restriction patterns. Lanes 1 and 10 represent the 2.5Kb marker combined with the amplisize molecular ruler (5—2000bp). Lanes 2 and 6, Lanes 3 and 7, Lanes 4 and 8 and Lanes 5 and 9 show respectively the restriction pattern of Sa 12, Sa 34, Sa 44 and Sa 51 before and after 3 cycles of propagation. Restriction patterns remained unchanged for all bacteriophages tested.

7.4 Discussion

The isolation of genomic DNA by the developed method proved to be successful for both *S. aureus* and *P. aeruginosa* bacteriophages. All the isolated bacteriophages were found to display different banding patterns with numerous restrictions enzymes. This confirmed that all the bacteriophages carried double-stranded DNA and were genetically different. The *S. aureus* genome sizes were approximately 147.4 ± 1.8kb and, although they appeared to be very closely related to each other, small differences were identified by using a combination of restriction enzymes. These small differences could also be confirmed by their host range differences (Chapter 3).

Schicklmaier and colleagues (1999) reported similar results of closely related restriction patterns of temperate phages released from Salmonella *sp.* strains. The authors compared temperate phage released from strains sampled in limited geographical areas against those released from strains sampled worldwide and were able to show that temperate phages released from strains collected in limited areas were significantly less heterogeneous than phages released from the worldwide sampled collection (Schicklmaier, et al 1999). In this study, the collection of samples for bacteriophage isolation was limited to the state of New South Wales (NSW), Australia. Similarly to the observation from Schicklmaier et al (1999), it is possible to speculate that the low variability of the *Hind*III (Figure 7-2) and *Hpa*I (Figure 7-3) restriction patterns of the Sa phages isolated could reflect a low diversity of the bacteriophage population in itself, although clearly the bacteriophages in this study were isolated from the environment and not from bacterial strains. This would suggest that, in the future, it might be important to expand the geographical area for sample collection and isolation in order to expand the diversity of therapeutic bacteriophages.

Bacteriophage genomes of *S. aureus* have been reported to be as small as 15kb (Kwan et al., 2005) or as large as 139kb (Gu et al., 2012, Kim and Myung, 2012) and were previously classified within three size classes based on their genome sizes: Class I <20kb, Class II ~40kb and Class III >125kb (Kwan et al., 2005). A comparative genomic analysis found the size ranges of 11 Twort-like phages was 127-140kb (Łobocka et al., 2012). Given

the genome sizes calculated by PFGE, the *S. aureus* bacteriophages isolated in this study would belong to the third class and would seem to be the larger than previously reported in the literature. However, the actual genomic sizes would only be determined when the phages are sequenced and annotated.

The closest relatives belonging to this class III include bacteriophage K (O'Flaherty et al., 2005), bacteriophage ISP (Vandersteegen et al., 2011), bacteriophage Sb-1 (Kvachadze et al., 2011), and G1 (Kwan et al., 2005). All of these bacteriophages were reported as phages belonging to the *Myoviridae* family and studied for their therapeutic potential, as were the bacteriophages isolated in this study. Bacteriophages belonging to this class were shown, also, to lack toxic or virulence-associated proteins supporting their therapeutic use (Vandersteegen et al., 2011). Despite the phenotypic and morphological similarities identified among those bacteriophages previously reported and the bacteriophages identified in this study, full genome sequencing should be completed to confirm the absence of undesirable genes prior to their introduction into the clinic.

Bacteriophage genomes of *P. aeruginosa* have been reported to be as small as 23kb (Kumari et al., 2009) or, unusually, as large as 280kb (Mesyanzhinov et al., 2002). The bacteriophages isolated in this study showed a range from 36 ± 0.4kb to 85 ± 0.2kb. Based on the molecular weight only, bacteriophages Pa 24, Pa 25 and Pa 37 could be part of the PB1-like virus genus, which is generally recognised for its broad spectrum of activity and virulent (lytic) activity while Pa 51 could be part of the ΦKMV-like virus genus (Ceyssens and Lavigne, 2010), a reported therapeutic genus (Merabishvili et al., 2009). Pa 6 and Pa 10 could potentially be part of the D3112-like virus genus, which is of particular concern as D3112-like phages are well-described temperate bacteriophages (Heo et al., 2007, Roncero et al., 1990, Wang et al., 2004). However, a previous study has also reported the existence of a bacteriophage that resembles phage D3112 but did not encode in its genome a *c*-repressor gene, which is known to be critical in maintaining the lysogenic state, suggesting the phage may be strictly lytic (Kim et al., 2012). Bacteriophage Pa 1 showed a relatively larger size and based on its molecular weight could be classified as part of the PAMx31 group recently described in Mexico (Sepulveda-Robles et al., 2012). No genus could be assigned to Pa 26 and Pa 32

given the genome sizes and could suggest them to be novel bacteriophages. However, only full genome sequencing would confirm the lytic virulent nature and assumed classifications of these phages by ensuring a high identity of their nucleotide sequences to representatives of their specific genus.

Previous morphological assessments failed to show significant differences among the *P. aeruginosa* phages Pa 1, Pa3, Pa24, Pa25 and Pa 37 classified as members of the *Myoviridae* family (Chapter 6). However, the differences in the restriction patterns showed them to be different. Likewise, Pa 10 and Pa 26 belonging to the *Siphoviridae* family and Pa 32 and Pa 51 members of the *Podoviridae*, which appeared to be indistinguishable by TEM were also shown to be different from each other. This kind of phenotypic similarities with molecular differences have been reported before (Lynch et al., 2012, Ceyssens et al., 2011) and the ability to appreciate this rapidly may become more relevant as collections of therapeutic bacteriophages continue to expand in the future. To date, there is no evidence to suggest that a particular genotype will infect a particular isolate. Thus, this would imply that the biological activity of specific bacteriophages might continue to drive the earlier selection process by formulating relevant reference collections as concluded in this study (Chapter 3 and 5). However, and in contrast to the workflow set up in this study, prior to the deeper analysis of the individual phage strengths and limitations (Chapter 4), earlier sequencing may be recommended in order to discard from the process those bacteriophages that, albeit showing a broad-spectrum of activity, could potentially carry undesirable genes.

In the future, to achieve commercial production of bacteriophage-based products, a combination of phenotypic and molecular characteristics will be required in order to control the production process and demonstrate “Chemistry, Manufacturing and Controls” (CMC) for biological products. CMC is the description for drugs and an important and comprehensive section in files that support clinical studies and marketing applications. One of the main steps for demonstrating the safe production of a biological product is the ability to manufacture without product alteration. Regulatory agencies will be concerned about any variation throughout the process with no documented consequences. Basically, the most critical element for a regulatory agency to accept a biological product is the ability of the

company to anticipate that any possible manufacturing changes will not represent meaningful changes in clinical safety or product efficacy. Therefore, potential manufacturers of bacteriophage-based products would need to document any manufacturing variations and evaluate the resulting product to the pre-existing product.

The commercial production of bacteriophages to the CMC level may be challenging, as it seems to be affected by two compounding factors. First is the genetic stability of the bacteriophages per se over time and, second, how any changes can be monitored cost-effectively during production. The genetic stability of bacteriophages is affected by a variety of factors, including the change of host (Arber, 1965, Arber, 1966), biological synthesis errors (Hall et al., 2011, Scanlan et al., 2011) , environmental pressures (Rigvava et al., 2013, Yue et al., 2012) and genome modifications due to the potential recombination of an infecting phage with residing prophages in the strains used for propagation (Schmieger, 1999).

Reasonably, establishing the classical approach of maintaining a consistent host would minimize, although not completely eliminate, these risks. In particular, those in relation to host-induced modifications and error rate of DNA synthesis, which depend on host restriction endonucleases, the DNA polymerase and sigma factors that can be either phage or phage-host encoded. In addition, it will also be important to identify and characterize potential prophages in the bacterial host in an attempt to pre-empt potential phage recombinants with different efficacy and characteristics when the phage is propagated (Schmieger, 1999). If the phage-host-pair is characterised and maintained, reducing the risks for frequency of genetic changes, the restriction pattern should also remain unchanged overtime. Thus, once an exclusive restriction pattern is determined, this pattern may be adequate as the basis of an analytical test for a bacteriophage preparation during production. This qualified test could potentially allow for a rapid and consistent identification of the individual phage components prior to generating a mix or cocktail. The feasibility and reproducibility of such a test was demonstrated in this study for selected bacteriophages suggesting (but not proving) a low frequency of genome changes in each qualified pair that might be sufficient to verify the identity of the phages.

There is no doubt that the full genome sequencing of potential therapeutic bacteriophages will be a necessity in the future to exclude any bacteriophages carrying undesirable genes as well as to confirm that the original bacteriophages have not been modified overtime. As the sequencing technology progresses in both speed and costs, it will be far-seeing to consider the use of full genome sequences as part of the analytical testing system package, specially as the sensitivity for low concentration contaminants is much higher than that of PFGE or restriction digests combined. However, until then, the use of restriction patterns appears to be a simple, reproducible, economic and fast way to identify, discriminate and quality control potential therapeutic bacteriophages from research to product release in the near future.

8 CASE STUDY: BACTERIOPHAGE THERAPY FOR A REFRACTORY *PSEUDOMONAS AERUGINOSA* INFECTION[3]

8.1 Introduction

Pseudomonas aeruginosa is an opportunistic pathogen and responsible for a variety of human infections (section 1.5.2). The ability of *P. aeruginosa* to develop biofilms seems to add to the pathogen's persistence in the human body and the establishment of chronic infections (James et al., 2008, Donlan and Costerton, 2002, Cigana et al., 2009). The development of biofilm-associated infections that are refractory to antibiotic therapy is of special concern, particularly in immunocompromised patients, since, in these patients, the possibilities for treatment are greatly reduced.

Bacteriophage therapy has been reported for its potential to deliver effective targeted therapy (Heo et al., 2009, Biswas et al., 2002, Weber-Dabrowska et al., 2000a), including its specific use for the treatment of *P. aeruginosa* (Debarbieux et al., Rhoads et al., 2009b, Azeredo and Sutherland, 2008). This case study presents the compassionate use of bacteriophage therapy to treat a patient suffering from a refractory urinary tract infection caused by *P. aeruginosa* in Sydney, Australia. The patient in question had had multiple admissions to the hospital and treatment with intravenous antibiotics as well as many months of outpatient ciprofloxacin and gentamycin therapy. These periods of antibiotic treatment alleviated the symptoms but failed to eradicate the infection. Eventually, the pathogen developed resistance to the ciprofloxacin and the intensive and prolonged use of gentamicin caused the patient to suffer from damage to the middle ear. After clinical assessment, the patient was facing the possibility of radical surgery to remove the bladder

[3] This work was published in the *Journal of Medical Microbiology* 60 (11): 1697-1700.

as the only means to eliminate the symptoms and prevent the pathogen from spreading to other cavities or even going systemic.

Under such circumstances, informed consent was obtained from the patient, and ethical approval obtained from the Western Sydney Human Research Ethics Committee to perform phage treatment. The problem isolate was screened against existing bacteriophage libraries and a "patient-specific" bacteriophage cocktail was prepared in collaboration with colleagues at the Eliava Institute of Bacteriophage, Microbiology and Virology (EIBMV), in Tbilisi, Georgia .The treatment was provided at Westmead Hospital during December 2007.

8.2 Case Study

8.2.1 Patient's clinical history [4]

A 67-year-old woman presented with recurrent urinary tract infections (UTIs) and blood in her urine. The identified microorganism causing the infection was *Pseudomonas aeruginosa*. Antibiotic treatments were generally effective, but symptomatic culture-positive relapses occurred within days to weeks of ceasing therapy. Multiple gentamicin courses for symptomatic UTIs throughout 2006 and 2007 resulted in postural unsteadiness. Furthermore, the patient later suffered from an adenocarcinoma that required pelvic irradiation. This then forced the placement of ureteric stents in December 2006. The introduction of stents appeared to have complicated the condition of the patient and there was evidence of biofilm formation.

In April 2007, a week of ceftazidime therapy and bilateral stent replacement was followed by oral norfloxacin for 30 days. Symptomatic relapse with ciprofloxacin-sensitive *P. aeruginosa* occurred a week after ceasing the antibiotic. Stents were changed for a second time on October 2007, with five days of meropenem commencing 24 hours before stent change. Once again quinolones (norfloxacin and ciprofloxacin) were usually helpful, but

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[4] Patient's clinical history was kindly provided by Dr. Jon Iredell

relapses within days to weeks of ceasing therapy were observed, always at >10^5 cfu/ml and in association with pyuria and haematuria. *P. aeruginosa* was cultured consistently, and *Enterococcus* spp. was co-cultured on a few occasions. *P. aeruginosa* (sensitive to aminoglycosides and imipenem) was resistant to norfloxacin and ciprofloxacin and to anti-pseudomonal penicillin/ β-lactamase inhibitor combinations (timentin and tazocin) by late October 2007. Following the failure of the multiple antibiotic treatments, the patient was facing radical surgery.

In mid-December 2007, the patient was admitted for bacteriophage therapy treatment to eradicate her *Pseudomonas* infection. Prior to commencing treatment, the patient's clinical examination showed normal haematological and biochemical indices and only a mild pelvic tenderness was reported.

8.2.2 Therapy approval

Prior to the administration of the therapy, the required ethical approvals were obtained. The first approval required was that of the patient. Informed consent was sought, as the patient was mentally and physically in the position to make an informed decision regarding the use of phage treatment. Consequently, written approval from the patient and her family was obtained.

Furthermore, the Therapeutics Goods Administration (TGA) office was consulted with regard to the use of unregistered bacteriophage medicine in Australia. In Australia, there is a Special Access Scheme (SAS) for the access to unapproved products (TGA, 2009). Application of SAS to an unapproved product varies according to the health status of the person and, as a result, the patients are divided in two different categories:

- **Category A:** "persons who are seriously ill with a condition from which death is reasonably likely to occur within a matter of months, or from which premature death is reasonably likely to occur in the absence of early treatment"
- **Category B:** "all other patients"

Legislation calls for the assessment on the category that best describes the condition of the patient to be made by the practitioner in charge. After consideration by the physicians, the patient was classified as a Category A patient. Since the patient was classified as a Category A Special Access Scheme, there was no need to seek prior approval, from the TGA, for the use of the unapproved bacteriophage product. In this category, the treating registered physician is considered to be the approving authority providing he/she is prepared to prescribe the product in question and has obtained written approval from the patient. However, as a formality, a Category A form SAS was completed in order to notify TGA about the treatment.

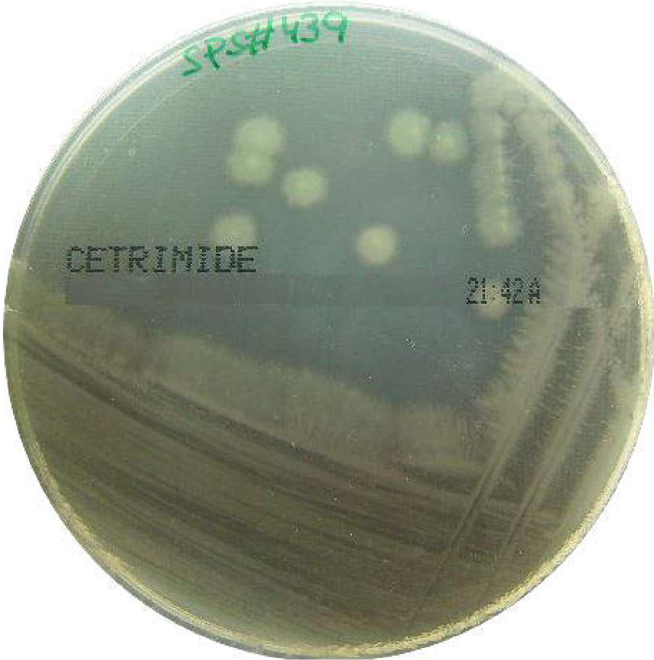
In addition, however, a formal treatment request was submitted to the Westmead Hospital Human Research Ethics Committee. The evidence submitted included the details of a long-term chronic infection, several replacements of stents made in an effort to eliminate the infection and multiple failed intravenous and oral antibiotic treatments applied. Considering that the patient had no other option than radical surgery available, the committee approved the treatment with bacteriophages as proposed (section 8.2.3). Additionally, on day 3 after commencing treatment, the committee was consulted again to obtain approval for major protocol change. The change involved a two-day delay on the introduction of antibiotics from day 4 to day 6. This change presented to the committee was also approved. Finally, the bacteriophage product was imported into the country under import permit 06016557 granted to Special Phage Services Pty Ltd by the Australian Quarantine and Inspections Services (AQIS).

8.2.3 Production of patient-specific bacteriophage cocktail

Two *P. aeruginosa* isolates recovered from the urine of the patient were received from Westmead Hospital and confirmed as *P. aeruginosa* isolates, as described before (section 2.5.2). Colonies were grown in nutrient broth overnight at 37°C and streaked in cetrimide agar for ID confirmation and colony morphology description. Two distinct colony variants were identified when plated on cetrimide plates (section 2.5.2.3). The first isolate showed small, 2mm compact, flat colonies with regular edges (Fig 8-1a) while the second one showed large ~3-4 mm flat colonies with irregular edges (Fig 8-1b).

Figure 8-1. *P. aeruginosa* isolates recovered from the patient's urine.

a) Isolate 1 *2 mm, compact, round flat colonies*



b) Isolate 2 *3-4 mm flat colonies with irregular edges*

The isolates were then tested against the existing *P. aeruginosa* phages at the time, Pa 1 to Pa 24 (Table 8-1) and were also tested against a commercial bacteriophage product, Pyophage, manufactured in Tbilisi, Georgia by Eliava Institute of Bacteriophage, Microbiology and Virology (EIBMV). The results showed that both isolates were highly sensitive to bacteriophages Pa 1, Pa 3, Pa 5 and Pa 24 while the Pyophage product was effective only against isolate 1 (Table 8-1). The phage sensitivity patterns observed, together with the phenotypic differences described, meant that the bacteriophage treatment should be developed to target the two strains identified.

The results obtained suggested that a combination of the bacteriophages Pa 1, Pa 3, Pa 5 and Pa 24 would be effective against the two isolates in question. However, after careful consideration of the regulatory requirements for the production and use of a local experimental phage cocktail versus the use of an already approved commercial product under the Special Access Scheme, it was decided to use the commercial Pyophage product. However, because the product was not effective against the two isolates, the isolates were sent to Tbilisi, Georgia. In Tbilisi, Sandra Morales in collaboration with the scientists at the EIBMV, developed a patient specific-phage cocktail (batch serial No 051007) to target both isolates.

The patient-specific phage cocktail produced had five main phage components (anti-*Proteus*, anti-*Staphylococcus*, anti-*Streptococcus,* anti-*Pseudomonas aeruginosa* and anti-*Escherichia coli* phages). The inclusion of five different components in the production was due to manufacturing regulations in the country. Bacteriophages used in the production were isolated from the Mtkvari River between 1995 and 2007 and the indicator bacterial strains used for phage propagation were collected from hospitals in Tbilisi and other Georgian regions between 2005 and 2007. For the production of the anti-*Pseudomonas* component, the isolate 2 from the patient was used.

For bacteriophage production, a 2 L flask with 1 L of Luria broth (for *Escherichia coli*), Nutrient broth (for *Staphylococcus* sp., *Proteus* sp., *Pseudomonas aeruginosa*) or Brain-heart infusion broth (for *Streptococcus* sp.) was inoculated with 500µl of the corresponding

bacteriophage component (10^6 PFU/ml) and 1ml of its corresponding bacterial strain in exponential growth. The flasks were incubated overnight in aerobic conditions at 37˚C. After overnight incubation, each bacteriophage component was filtered through a series of 0.8μm to 0.22μm filters. The five individual filtered components were then aseptically combined and dispensed in 5ml glass ampoules. The ampoules were incubated for two weeks at 37˚C to ensure the sterility of the final product.

The activity of the specific-phage product was tested against the two isolates on arrival in Sydney as per section 2.6.6. The phage cocktail proved to be highly active against the two isolates with a final titre of 2 x10^6 PFU/ml.

Table 8-1. **Bacteriophage activity against the *Pseudomonas aeruginosa* strains recovered from the infected urine.**

	Isolate1	Isolate 2
Pa 1	+++	+++
Pa 2	++	-
Pa 3	+++	+++
Pa 4	+++	++
Pa 5	+++	+++
Pa 6	+++	-
Pa 7	++	-
Pa 8	+++	++
Pa 9	++	+
Pa 10	+++	-
Pa 11	+++	-
Pa 12	++	++
Pa 13	+	-

	Isolate 1	Isolate 2
Pa 14	+++	-
Pa 15	-	-
Pa 16	++	++
Pa 17	+++	-
Pa 18	+++IP	+++IP
Pa 19	+++	-
Pa 20	+++	-
Pa 21	+++	++
Pa 22	+++	++
Pa 23	-	-
Pa 24	+++	+++
Pa Cocktail	+++	+++
Pyophage	+++	-

Positive reactions (+) were recorded when a minimum of 10 individual plaques (IP) were observed. The clarity of the IP or the spot was scored as turbid (+), clearing with hazy-partial background (++) and complete clearing (+++). Negative reactions were recorded as (-).

8.2.4 Bacteriophage therapy: administration protocol [5]

The patient was hospitalised and left under observation 24h prior to commencing treatment. On day 1, a catheter was placed and 20ml (~2 x 10^7 PFU) of the bacteriophage cocktail were instilled directly into the bladder every 12 hours for ten days, with the catheter clamped for 30min after each instillation. Urine was collected before treatment and at 4h intervals after the morning dose during the first two days, and then each morning prior to bacteriophage and/or antibiotic treatment dosing. The catheter was changed on day 3 and day 10. The original protocol called for meropenem (1g twice daily) and colistin (polymixin E, 100mg twice daily) to be commenced on day 4. However, the introduction of antibiotic treatment was in fact delayed until day 6. The reasons for this change will be discussed in detail in the results section. The patient was discharged home on intravenous meropenem alone after day 10. On day 20, both stents were removed while still on meropenem. In total, the patient received ten days of intravesical bacteriophage therapy through a Foley catheter twice daily and included antibiotic treatment. The phage administration protocol and the sample collection routine for monitoring the treatment are outlined in Table 8-2.

[5] The clinical protocol designed in collaboration with Dr. Alan Dublanchet and Special Phage Services Pty Ltd. The Bacteriophage treatment was administered to the patient by Dr. Ahmad Khawaldeh.

Table 8-2. Phage administration and sample collection protocol

Date	Time	Intervention	Therapy applied	Sample collection
Day 0	am	Patient admission	No	No
Day 1	07:50	Urine collection	No	Urine collection.
	08:00	Catheterisation	Phage only	No
	12:00	None	No	Yes
	16:00	None	No	Yes
	21:00	None	Phage only	Yes
Day 2	09:00	Medical Examination	Phage only	Yes (prior to treatment)
	12:00	None	No	Yes
	16:00	None	No	Yes
	21:00	None	Phage only	No
Day 3	08:00	Medical Examination	No	No
	08:30	Catheter Replaced	No	Yes
	9:00	None	Phage only	Yes (prior to treatment)
	16:00	None	No	No
	21:00	None	Phage only	No
Day 4	09:00	Medical Examination	Phage only	Yes (prior to treatment)
	12:00	None	No	Yes
	16:00	None	No	Yes
	21:00	None	Phage only	No
Day 5	09:00	Medical Examination	Phage only	Yes (prior to treatment)
	12:00	None	No	No
	16:00	None	No	No
	21:00	None	Phage only	No
Day 6 To Day 9	09:00	Medical Examination	Phage+ Antibiotic	Yes (prior to treatment)
	12:00	None	No	Yes
	16:00	None	No	No
	21:00	None	Phage+ Antibiotic	No
Day 10	08:00	Medical Examination	Phage+ Antibiotic	Yes (prior to treatment)
	08:30	Remove catheter	No	No
	12;30	None	No	Yes
	16:30	None	No	Yes
Day 21	9:00	Stents changed	Antibiotic Only	Yes

8.3 Laboratory Materials and Methods

8.3.1 Enumeration of bacteria in urine samples [6]

8.3.1.1 *Direct plating method*

In order to estimate total viable numbers of bacteria in the urine samples collected, serial dilutions of 200µl of each urine sample were inoculated onto blood agar plates (Oxoid Ltd, Hampshire, England) and incubated aerobically at 37˚C for 16 hours for bacterial colony counts.

8.3.1.2 *Multiplex- Poymerase chain reaction (PCR)*

Two hundred microliter aliquots of urine were also extracted (GenElute Mammalian DNA Extraction kit; Sigma-Aldrich, Castle Hill, New South Wales, Australia) and eluted into 200µl of RNase-DNase Free water (Gibco, **Invitrogen Australia Pty. Ltd,** Mount Waverley, Victoria, Australia). Control specimens (*S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P aeruginosa* ATCC 27853, *E. faecalis* ATCC 29212, *S. pneumoniae* ATCC 49615) suspended at ~10^3 CFU/ml in sterile 0.9% w/vol NaCl were extracted by the same protocol. Oligonucleotide primers (targeting the 16S rDNA of *Enterobacteriaceae*, *Pseudomonas* sp., *Enterococcus* sp., *Streptococcus* sp. and *Staphylococcus* sp.) were supplied as a lyophilised, prepacked, gene-disc assay (Bacterial Count, AusDiagnostics Pty Ltd., Alexandria, New South Wales, Australia) (Stanley and Szewczuk, 2005).

First round MT-PCR amplifications were performed in a 20µl reaction volume, containing MT-PCR Step 1 mastermix (Quantace, Alexandria, New South Wales, Australia), 10 U MangoTaq (Bioline, Alexandria, New South Wales, Australia) and 8µl DNA template in a RotorGene RG6000 cycler (Corbett Research): 95ºC 5min hot start; 10 cycles of 95ºC 10 sec, 60ºC 20 sec; 72ºC 20 sec as previously described (Szewczuk et al., 2010). Four microliters of each first-round amplification product were diluted 1:75 as per manufacturer's instructions and a 20µl aliquot added to the corresponding six positions on the gene-disc, before second-stage amplification (95ºC 1min hot start; 30 cycles of 95ºC

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[6] Enumeration of bacteria was performed by Lee Thomas and Belinda Dillon at Westmead Hospital.

1sec, 60ºC 10sec and 72ºC 10sec). Fluorescence was detected after each extension step and melt curves generated from 72ºC to 95ºC in 0.5ºC intervals. Quantitation of the amplification signal was normalised against an internal artificial control and standard curves were generated with the controls (above) at 10^1 to 10^7 cfu/ml. Melt temperatures were consistent in triplicate runs and reproducibility was high (standard curves for all targets showed an r^2=0.9922-0.9997). The detection limit of the assay was tested by inoculating serial dilutions of bacteria into PBS (data not shown).

8.3.2 Enumeration of bacteriophage in urine [7]

8.3.2.1 *Overlaid plates preparation*

Target *P. aeruginosa* strains were inoculated onto nutrient agar (NA) plates using a 10µl loop and incubated under aerobic conditions, at 37˚C for 18h to obtain individual colonies. Three individual colonies were then inoculated into 3ml of nutrient broth (Amyl-media) and incubated aerobically at 37˚C for 4h, to obtain ~10^6cfu/ml in logarithmic growth phase. One hundred microliters of the bacterial suspension was gently mixed with 3ml 0.4% nutrient agar (Amyl-media) at 50˚C and poured as an overlay onto 1% NA plates. Overlaid agar plates were left to solidify at room temperature for 20min.

8.3.2.2 *Urine sample preparation*

The Urine was diluted 1:1 in 0.9% w/vol phosphate-buffered saline (PBS), filtered through a 0.2µm filter and evaluated for bacteriophage plaque counts (PFU) immediately after sampling as per section 2.6.6. One-hundred microliters of filtered urine were serially diluted in a modified Tris buffer (5.8g/L of NaCl, 2.0g/L of MgSO4.7H2O, 5ml of 2% (w/v) gelatin in 50ml of 1.0M Tris-HCl; pH 7.0) and 10µl drops of the dilutions were applied onto the overlaid plates with *P. aeruginosa* isolates and allowed to adsorb into the overlay agar plate. Plaques were counted after 18h aerobic incubation at 37ºC. All assays were performed in triplicate.

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[7] All bacteriophage counts were performed by Sandra Morales at Special Phage Services Pty Ltd.

Prior to commencement of the phage treatment, the stability of the phages in a urine sample was determined. A urine sample from a healthy volunteer was collected first thing in the morning. Ten milliliters of the sample were filtered (0.22µm) and the rest left unfiltered. Four samples were then spiked with 500µl of Pyophage and two different buffers as summarised in Table 8.3. Five-hundred microliters of Pyophage were also added to 2ml of SM buffer. The bacteriophage concentration in each sample was then measured daily for five days as per section 2.6.6. All assays were performed in triplicate.

Furthermore, to establish the frequency of resistance to bacteriophages after the therapeutic intervention, three colonies on each blood agar plate, recovered during the evaluation of viable CFU, were collected and re-streaked onto NA plates for bacteriophage testing as described above.

Table 8-3. Stability of bacteriophage in urine samples.

Sample #	Urine: volume / type	Buffer: volume / type	Bacteriophage
1	2ml Unfiltered	None	500µl Pyophage
2	2ml Filtered	None	500µl Pyophage
3	1ml Filtered	1ml PBS	500µl Pyophage
4	1ml Filtered	1ml SM	500µl Pyophage
5	None	2ml SM	500µl Pyophage

8.4 Results

8.4.1 Clinical observations

Patient manifested less pain and the practitioner reported less pelvic tenderness. No discomfort or side effects were reported. The laboratory reported less turbidity in the urine 24h after the first phage application, with the filtration process becoming easier as the treatment progressed. The initial clinical observations encouraged the practitioner to delay the introduction of antibiotics. Approval to delay the introduction of antibiotic until day 6 was then sought by the physician in charge and granted by the ethical committee.

8.4.2 Phage stability in urine samples

The testing of 500 µl of the Pyophage product, resuspended in filtered and unfiltered urine samples, showed that the concentration of bacteriophages remained steady and there was no statistically significant drop in their concentration after five days ($p>0.001$). The test also showed that there was no difference among the different samples (#1 to 4) when compared with the phage control sample (#5) in the SM buffer ($p>0.01$), with only a slight reduction of bacteriophage concentration in the unfiltered sample 1 that was not buffered. Calculated bacteriophage concentrations are presented in Table 8-4.

Table 8-4. Bacteriophage counts (PFU/ml) in urine samples with or without buffer for five days.

Time	Sample 1 +/- SE (PFU/ml)		Sample 2 +/- SE (PFU/ml)		Sample 3+/- SE (PFU/ml)	
day 1	4.80E+05	1.15E+04	4.70E+05	2.40E+04	5.10E+05	6.67E+03
day 2	4.70E+05	1.76E+04	4.70E+05	1.76E+04	5.05E+05	2.31E+04
day 3	4.80E+05	2.31E+04	4.60E+05	1.15E+04	4.90E+05	3.53E+04
day 4	3.20E+05	1.15E+04	4.50E+05	1.33E+04	5.00E+05	3.71E+04
day 5	3.30E+05	6.67E+03	4.10E+05	2.40E+04	4.85E+05	2.91E+04
Average at day 5	**4.16E+05**	**2.85E+03**	**4.52E+05**	**2.61E+03**	**4.98E+05**	**5.48E+03**

Time	Sample 4+/- SE (PFU/ml)		Sample 5+/- SE (PFU/ml)	
day 1	5.20E+05	3.06E+04	5.10E+05	4.67E+04
day 2	5.15E+05	4.81E+04	5.10E+05	4.06E+04
day 3	5.00E+05	5.29E+04	5.00E+05	3.53E+04
day 4	5.20E+05	3.46E+04	5.20E+05	5.03E+04
day 5	4.90E+05	3.71E+04	5.10E+05	2.91E+04
Average at day 5	**5.09E+05**	**4.22E+03**	**5.10E+05**	**3.82E+03**

Bacteriophage plaque forming units (PFU/ml) in spiked urine samples from a healthy volunteer and control samples were performed daily for five days. Triplicate counts were performed and the average of the three measurements ± its standard error (SE) determined. Sample 1: unfiltered urine with Pyophage. Sample 2: filtered urine sample with Pyophage. Sample 3: filtered urine diluted 1:1 in PBS with phage. Sample 4: filtered urine diluted 1:1 in SM buffer with Pyophage. Sample 5: SM buffer with Pyophage.

8.4.3 Bacteria and bacteriophage evaluation

Initial viable *P. aeruginosa* counts were $3x10^6$ CFU/ml (Figure 8-2; Table 8-5). Bacterial counts declined steadily before antibiotics were commenced on day 6. Two phenotypically different *P. aeruginosa* isolates were identified in cultures on days 1 - 6, However, DNA fingerprinting, using PFGE, and *XbaI* restriction enzyme showed these two isolates to be identical (data not shown). The two strains were susceptible to colistin and meropenem and to the bacteriophage cocktail applied. After seven days of treatment, no viable bacteria were recovered. Bacteriophage counts in urine rose after 24 hours and remained high until bacterial numbers subsided (Figure 8-2). Bacteriophage numbers expanded from $3.9x10^3$ pfu/ml 4h after initial dose to 1.6×10^6 PFU/ml 20h later (Table 8-5). Bacteriophage counts remained high until after disappearance of the target organism and then diminished sharply despite continued administration for several days (Figure 8-2).

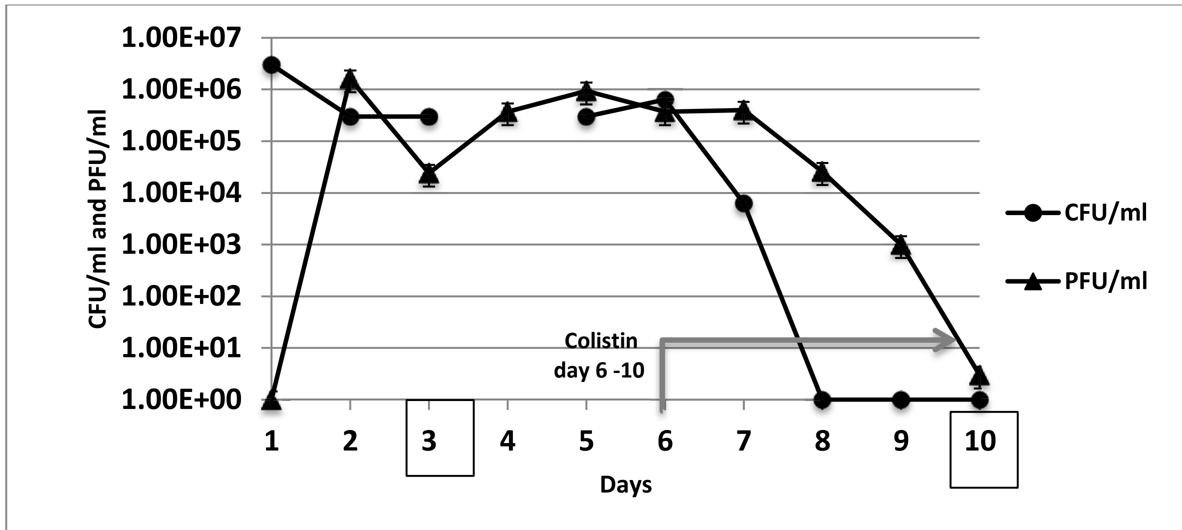
Cultures and MT-PCR detected both *P. aeruginosa* phenotypes (Figure 8-2 and Table 8-5). The two methods also detected the presence of an *E. faecalis* strain in the urine that was first detected on day 1 (data not shown). However, *E. faecalis* numbers were unaffected, and testing of an *E. faecalis* strain isolated from urine was shown to be non-susceptible to the bacteriophage cocktail *in vitro* (not shown). No antibiotic- or bacteriophage-resistant *P. aeruginosa* were recovered after treatment.

Table 8-5. ***P. aeruginosa*** **bacteriuria and phage counts throughout the phage therapy treatment applied.**

Treatment (Days)	Direct plating Estimated viable CFU/ml	MT-PCR Estimated Total CFU/ml ±SD	Phage counts PFU/ ml±SD
Day 1 (Pre-phage dose)	$3.00 \times 10^{6*}$	$4.27 \times 10^5 \pm 1.42 \times 10^5$	0
Day 1 (+4h)	$1.60 \times 10^{5*}$	$4.92 \times 10^5 \pm 1.78 \times 10^4$	$3.9 \times 10^3 \pm 1.53 \times 10^2$
Day 1 (+8h)	$2.40 \times 10^{5*}$	$5.70 \times 10^4 \pm 1.03 \times 10^4$	$2.3 \times 10^4 \pm 1.45 \times 10^2$
Day 1 (+12h)	N.D	$1.53 \times 10^4 \pm 2.27 \times 10^3$	$5.30 \times 10^4 \pm 1.50 \times 10^3$
Day 2 (Pre-phage dose)	$3.00 \times 10^{5*}$	$8.37 \times 10^4 \pm 2.20 \times 10^4$	$1.60 \times 10^6 \pm 1.01 \times 10^6$
Day 2 (+4h)	$1.12 \times 10^{5*}$	$3.58 \times 10^5 \pm 1.68 \times 10^5$	$1.4 \times 10^6 \pm 2.31 \times 10^5$
Day 2 (+8h)	$3.00 \times 10^{5*}$	$3.03 \times 10^4 \pm 4.43 \times 10^4$	$9.3 \times 10^5 \pm 2.52 \times 10^4$
Day 2 (+12h)	$3.00 \times 10^{5*}$	$7.55 \times 10^4 \pm 5.81 \times 10^3$	$9.3 \times 10^5 \pm 1.93 \times 10^4$
Day 3(Pre-phage dose)	3×10^5*	$3.87 \times 10^4 \pm 6.17 \times 10^4$	$2.30 \times 10^4 \pm 3.53 \times 10^3$
Day 4 (Pre-phage dose)	N.D	$2.66 \times 10^4 \pm 8.14 \times 10^2$	$3.70 \times 10^5 \pm 2.67 \times 10^4$
Day 5 (Pre-phage dose)	3×10^5*	$2.25 \times 10^5 \pm 3.02 \times 10^4$	$9.30 \times 10^5 \pm 2.67 \times 10^5$
Day 6 (Pre-phage dose)	6.4×10^4	$1.94 \times 10^5 \pm 1.48 \times 10^5$	$3.70 \times 10^5 \pm 5.81 \times 10^4$
Day 7 (Pre-phage dose)	6.3×10^3	$6.75 \times 10^4 \pm 3.35 \times 10^4$	$4.00 \times 10^5 \pm 5.77 \times 10^1$
Day 8 (Pre-phage dose)	0	$8.39 \times 10^4 \pm 4.29 \times 10^4$	$2.60 \times 10^4 \pm 8.82 \times 10^0$
Day 9 (Pre-phage dose)	0	$2.57 \times 10^3 \pm 6.73 \times 10^2$	$1.00 \times 10^3 \pm 8.82 \times 10^1$
Day 10 (Pre-phage dose)	0	$1.43 \times 10^3 \pm 5.64 \times 10^2$	$3.00 \times 10^0 \pm 3.33 \times 10^{-2}$
Day 21 (Pre-phage dose)	0	0	0

*All samples were taken prior to bacteriophage dose administration. Counts by direct plating were set up as a single set of dilutions only. All other samples were set up in triplicate. *Two P. aeruginosa colony types present day 1-5 only; Antibiotics were present from day 6 after phage pre-dose sampling. N.D signifies Not done.*

Figure 8-2. Logarithmic plot of early morning urine. Viable *P. aeruginosa* (CFU/ml) and bacteriophage counts (PFU/ml).



Bacteriophage product was administered every morning from day 1 to day 10, and samples collected in the morning prior to phage dosing. Antibiotic administration was commenced on day 6 (arrow) after taken the morning sample. Catheter changes and removals in day 3 and 10 (boxed) are indicated. Both bacterial and phage counts on day 21 were below the detection limits (≤10cfu/ml and ≤2pfu/ml) and were therefore not included in the graph.

8.5 Discussion

Two distinct *P. aeruginosa* colony variants were isolated and identified in the patient (Fig 8-1). The isolates showed the same antibiotic profile and DNA fingerprinting, using PFGE and *Xba*I restriction enzyme. However, the two isolates showed different phage patterns when tested for bacteriophage sensitivity (Table 8-1). The presence of different phenotypes in samples from patients has been previously reported. In general, the mucoid phenotype is one of the most studied, particularly those mucoid strains affecting CF patients (Ciofu et al., 2001, Pugashetti et al., 1982, Derry, 2005). However, a phenotypic state, known as "small colony variants" or SCVs, has also been described (Häußler et al., 1999). These small colony variants are characterised by slow growth on agar plates and an enhanced ability to survive antibiotic treatments (Häußler 2004) and it is believed that antibiotic treatment causes selection pressure that could favour the occurrence of SCVs (Spiers et al., 2000). None of the two isolates in this study showed a mucoid phenotype and although isolate 1 could be considered to be a SCV if compared to isolate 2, no differences in growth were observed between the two isolates when incubated overnight at 37°C in nutrient or cetrimide agar.

The data collected in this study showed that these subpopulations also had *in vitro* differential phage susceptibility, as demonstrated by the different phage patterns observed (Table 8-1). Zierdt and Schmidt (1964) identified differences in phage susceptibility of *P. aeruginosa* isolates from the same patient and speculated that such differences were due to differing lysogenic state of the isolates. Similarly, different phage susceptibility profiles could be explained by the loss of a specific bacteriophage receptor either of protein or lipopolysaccharide (LPS) nature (Rakhuba et al., 2010). Such small genotypic changes may not be necessarily detected by PFGE. However, the fact that the isolate was eradicated with the combined phage-antibiotic treatment would also suggest that *in vivo* the development of distinct phenotypes did not confer extra-protection at least against bacteriophage infection, as it is believed to do it against antibiotic treatment.

One of the most proclaimed advantages of bacteriophages when compared with antibiotics is that bacteriophages replicate on-site and disappear from the system once the pathogen has been eliminated (Sulakvelidze et al., 2001). In this case study, bacteriophage numbers increased very rapidly *in vivo* and remained high throughout the treatment until the disappearance of the *P. aeruginosa* isolates on day 8 (Figure 8-2). Phage numbers then diminished sharply in the absence of the target organism despite continued administration for several days (Figure 8-2). This would suggest that the bacteriophage infection followed a self-sustaining and self-limiting model, suggesting that intermittent or limited dosing schedules could have also been effective.

Antibiotics alone had previously failed on many occasions in the described situation, but the use of a patient-specific bacteriophage cocktail, delivered directly over 10 days as an adjunct therapy, effectively eradicated a refractory *P. aeruginosa* infection associated with a significant biofilm. The treatment was well-tolerated, easily delivered via bladder instillation and was not associated with persistence of bacteriophage nor with development of resistance in the target pathogens. This experience suggests that 7-10 days of bacteriophage therapy might be adequate in a similar context. It could be argued that the drop in *P. aeruginosa* CFU after day 6 was due only to the administration of antibiotics. However, by this time, there had already been a 1.5-Log drop in CFU. It must emphasised that the sample was taken prior to the commencement of the antibiotic treatment.

Furthermore, no short-term or long-term secondary effects were reported during the use of the bacteriophage treatment while the use of gentamicin had caused the patient to suffer from a permanent balance disorder related to her ear. This outcome forced the clinicians to stop the treatment. The associated side-effects with the use of antibiotics such as intestinal disorders, allergies, and secondary infections are well-known but commonly endured because the benefits of treatment generally compensate for any toxic effects (Dancer, 2004).

The use of phage therapy for the treatment of 31 patients suffering from suppurative skin infections reported a few minor side-effects, such as mild skin irritations, in six patients

(Cisło et al., 1987). All signs of skin irritation disappeared after the treatment was discontinued. The researchers suggested that the side-effects could have been attributed to the release of endotoxins from bacteria destroyed *in situ* by the phages. Such effects have also been observed when antibiotics were used (Holzheimer, 2001, Lepper et al., 2002). In this case study, there was no evidence of side-effects due to endotoxin release.

Throughout the treatment, three colonies recovered from every blood agar plate used for bacterial counts were tested against the Pyophage product. No phage resistant bacteria were isolated. Furthermore, ten days after treatment was finished no bacteria and no phage were recovered. In contrast, treatment with ciprofloxacin resulted in the emergence of ciprofloxacin resistant bacteria, as did the treatment with meropenem at least on one occasion. The low or, in this case, the absence of development of resistance to bacteriophage treatment could be attributed to the use of "bacteriophage cocktails" as discussed before (section 5.4).

Medical examination of the patient post-treatment revealed an unhealed bladder ulceration, which appeared to be due to her radiotherapy. However, the urine remained sterile even after the elimination of all antibiotics for 12 months. This showed that even though the patient was at very high risk of re-infection or relapse, the bladder had not been reinfected. This once again demonstrated the eradication of the *P. aeruginosa* microflora.

The problem of antibiotic resistance continues to grow at an exponential rate (section 1.1) and there are, every year, fewer new anti-*Pseudomonas* drugs in the pipeline (Page and Heim, 2009). In this case, antibiotics alone had failed to eradicate the *P. aeruginosa* isolate causing the infection in this patient and, thus, it is unlikely that it would have done so during the course of this treatment. However, the additive effort of combined phage-antibiotic treatment was successful in eradicating the bacteria permanently. The advantages of such a combined phage-antibiotic treatment had been reported since the beginning of the antibiotic era (Himmelweit, 1945). In conclusion, while it cannot be suggested on the basis of this case study that bacteriophage alone would have been curative, the greater than ten-fold fall in *P. aeruginosa* concentration in the urine that appeared to precede the

administration of antibiotics strongly suggests an action *in vivo* to mimic that observed *in vitro*.

While all major international health bodies are warning of an impending health crisis with fewer alternatives available for the treatment of bacterial infections around the world, this case study showed the potential of bacteriophages for the treatment of a refractory *P. aeruginosa* infection. This case study provided clinical evidence for the potential use of bacteriophage in the treatment of bacterial infections and should encourage the development of further research in Australia, including the design of double blind clinical trials to prove their potential at the appropriate scientific standards.

Lastly, in Australia Sir MacFarlane Burnet was an early and avid proponent of bacteriophage therapy with over 30 publications in the subject between 1924 and 1937 (section 1.3). However, no reports of an actual clinical application were found and, as such, this case report represents the first use of human phage therapy in Australia.

9 SYNOPSIS AND GENERAL DISCUSSION

9.1 Synopsis

It is now generally accepted that the world is entering a serious health crisis in the form of antibiotic resistance. Numerous international health bodies led by the World Health Organisation (WHO) have warned of serious consequences if uncontrolled use of antibiotics is not curtailed, and if new or alternative antibacterial agents are not found as a matter of urgency (Boucher et al., 2009, Carlet et al., 2012). The situation is so serious that Dr. Gro Bruntland, former head of the WHO, issued a call to arms in 2000 concluding with the statement "Our grand-parents lived during an age without antibiotics. So could many of our grand-children" (World Health Organization, 2000). Set against a backdrop of raising antibiotic resistance, the emergence of multi-drug resistant (MDR) bacteria, "superbugs", and an almost exhausted pipeline of new antibacterial drugs, this thesis set out to investigate the potential of bacteriophage (phage) therapy as an alternative and effective means of treating antibiotic resistant infections.

Phage therapy was first successfully used in Paris in 1919 to treat patients with dysentery. However, despite widespread interest in the first half of the 20th century, today only a few countries of the Former Soviet bloc (Russia, Georgia and Poland) use phage therapy on a routine basis. Developing phage therapy products today possess numerous problems. Most of the historical literature is difficult to access, being published mainly in Russian and Georgian. There is a marked absence of detail in terms of dosage levels, pharmacodynamics and production quality standards. Much of the treatment described is empirical in nature and understandably; there are few clinical trials of the modern "double blind" type. To investigate the use of phage therapy in a Western setting, it was therefore necessary to return to first principles and develop the products *de novo*. Two of the most important human pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa,* for which there are very few or no new antibiotics available (Page and Heim, 2009, DeLeo et al., 2010), were chosen for this investigation. Phages specific for these pathogens were

isolated and progressed to a series of stages or steps to produce prototype therapeutic phage products.

The first step in the development of phage therapeutics was the selection of the target bacteria to be used for the isolation process of novel bacteriophages. Clinically relevant isolates were collected directly from two teaching hospitals in Sydney (Royal North Shore Hospital and Westmead Hospital) and their identities confirmed at arrival. Reference bacterial collections of both *S. aureus* and *P. aeruginosa* isolates were then established on the basis of their antibiotic profiles and/or their known genotypic characteristics. The *S. aureus* library contained distinct bacterial clones known to be clinically relevant not only in Australia but also around the world, creating a "universal" collection.

Previous studies have reported the use of strains isolated from distinct samples or patients (within the same area) for the isolation of bacteriophages against *S. aureus* (Synnott et al., 2009, O'Flaherty et al., 2005, Capparelli et al., 2007, Hsieh et al., 2011). However, the establishment of this type of "universal" collection was the first of its kind described in the literature for the isolation and testing of *S. aureus* human therapeutic bacteriophages. Similarly, *P. aeruginosa* laboratory strains have been used for the isolation of lytic bacteriophages (Jensen et al., 1998b) and attempts to develop collections that include clinically relevant isolates have also been made (Li et al., 2010, Alemayehu et al., 2012). However, the use of bacterial susceptibility to particular antibiotics, which provides key information with regard to the development and distribution of antibiotic resistance patterns that can be directly connected to clinical outcomes (Cuttelod et al., 2010) had not been reported.

Traditionally, the isolation of bacteriophages has been considered the first critical step for phage therapy (Gill and Hyman, 2010a). The idea being that phage therapy has been generally effective, particularly in animal models, once a bacteriophage is matched to a particular pathogen. However, in this study it was reasoned that in order for bacteriophage therapy to overcome one of its main criticisms, that of its narrow spectrum of activity and thus its likelihood of becoming a practical and valid technique in a busy clinical setting, the

establishment of well-characterised collections of reference isolates was in fact the first phase in the research and development process.

Although not all the reference strains were necessarily used to isolate novel bacteriophages (section 3.7), they were definitely essential for determining their spectrum of activity, thus ensuring, that during the selection process, significant progress was made towards developing a "universal" product. It is clear that the rapid explosion of microbiological and molecular techniques now allows for the characterisation of bacterial isolates to intricate levels that were not available to early phage therapists and, consequently, the development of reference collections, like those established here, was not even feasible. In future, therefore, one can assume that the full array of modern bacterial identification technologies will be brought to bear on the targeted development of bacteriophage-based products. These same technologies will also play a critical role in monitoring the emergence of new or potential bacteriophage resistant populations in the clinical environment.

After establishing the reference collections, the second step in the process was the isolation of *S. aureus* and *P. aeruginosa* bacteriophages. To do this, water samples were collected from human sewage treatment plants and other private sources from several locations around New South Wales, including hospitals, nursing homes and rehabilitation centres, where the target pathogens were more likely to be present. In Australia, there have been reports on the isolation of bacteriophages against *Streptococcus* sp. (Powell and Davidson, 1985), *Vibrio harveyi* (Crothers-Stomps et al., 2010), *Bacteroides ruminicola* sub sp. *brevis* (klieve et al., 1991), *Enterococcus* sp. (Fard et al., 2010), *Tsukamurella* sp. (Petrovski et al., 2011b), *Actinobacteria* (Petrovski et al., 2011a) and *Lactococcus lactis* (Eduardo et al., 2012). This survey was the first of its kind to investigate the presence of *S. aureus* and *P. aeruginosa* bacteriophages in Australia, specifically in the New South Wales area.

Fifty-eight bacteriophages targeting *S. aureus* and 52 targeting *P. aeruginosa* were isolated using a modified direct plating method (section 2.6.2.4), which was developed for this work. Enrichment methods were previously recommended for phage isolation when strain-specific phages were required (Carlson, 2005). The results showed that the modified direct

plating method developed here was able to recover phages from the environment in a more efficient manner than the classic recommended methods (section 2.6.2). The modified method was also significantly simpler, faster and more economic as plaque purification can be started directly from the isolation plate without the need of additional steps (section 3.7).

In the third stage those phages meeting the initial selection profile were purified and propagated, and their activity spectrum evaluated against the established reference collections. When compared with antibiotics, bacteriophages have a relatively narrow activity spectrum (even down to the subspecies level) and this is often quoted as a potential disadvantage of bacteriophage therapy (Hyman and Abedon, 2010). However this study showed that the isolation of bacteriophages with broader range of activity was possible, as demonstrated by the ability of individual bacteriophages to infect a large percentage of the isolates in the reference collections. Thirty of the isolated *S. aureus* phages were able to infect 50% or more of the isolates in the reference collection, with 12 of those phages able to infect all the isolates. On the other hand, the host range for the *P. aeruginosa* phages appeared to be, when compared with the *S. aureus* phages, a little bit more limited with 27 phages also able to hit 50% or more of the *P. aeruginosa* reference strains but only one phage (Pa 1) infected all the isolates in the reference collection (section 3.7).

This activity profiling was expanded in the fourth step to confirm that the selected bacteriophages were capable of infecting bacteria, not only in the reference collections, but also commonly found within the wider community. Selected bacteriophages (Table 3-3 and 3-7) were tested against a broader representative collection of local clinical isolates. The results showed that eleven out of twelve selected *S. aureus* phages showed a percentage of activity higher than 80%. Five of these showed a percentage of activity of 95% (n=99) (Table 4-1). Six out of the ten selected *P. aeruginosa* phages showed a percentage of activity higher than 70%. Three of these showed a percentage of activity of 80% (n=80) (Table 4-4). Two *S. aureus* bacteriophages capable of infecting 87% and 53% (n=15) of bovine mastitis *S. aureus* strains (Synnott et al., 2009), one phage capable of infecting 100% (n=34) human clinical isolates (Vandersteegen et al., 2011) and one phage isolated in Taiwan capable of infecting 80% (n=205) (Hsieh et al., 2011) were previously reported.

Thirteen *P. aeruginosa* phages infecting 42% (n=19) clinical isolates from China (Li et al., 2010), two infecting 70% and 69% (n=200) clinical isolates from the Republic of Georgia (Karumidze et al., 2012) and two infecting 73% and 76% (n=32) isolates from various specimens in Serbia (Knezevic et al., 2009) were reported. This suggests that the *S. aureus* and *P. aeruginosa* bacteriophages described in this study may have the broadest spectrum of activity reported. The results and the earlier reports also suggest that the isolation of broad-spectrum bacteriophages is possible and that current concerns about bacteriophages having a narrow spectrum of activity against the targeted species may be unwarranted.

In the past, disappointing clinical outcomes were often attributed to poor stability of the available bacteriophage products or preparations (Straub and Applebaum, 1933). It is now generally accepted that the chances of attaining therapeutic cure are greatly enhanced if phages are pre-selected for stability across a range of physico-chemical conditions, while remaining capable of adsorbing to and multiplying efficiently within the target bacteria. In other words, to be effective therapeutic phages must maintain a high level of viability from date of manufacture until their application (Gill and Hyman, 2010a). This fifth stage, therefore, concentrated on the stability characteristics of the selected bacteriophages, and in particular their ability to remain active at different pH levels and at different temperatures over prolonged periods of time.

The pH stability testing results showed that exposure of either the *S. aureus* or *P. aeruginosa* bacteriophages to a pH value of 7.5 for 60min at 37˚C had no effect on their ability to infect their target bacteria when compared with the control stocks generally stored in SM buffer pH 7.5 and 4ºC (p>0.05). However, all *S. aureus* phages were inactivated when exposed to a pH value of 3.0 for 60min at 37˚C (p <0.05) whereas the *P. aeruginosa* phages exhibited different levels of sensitivity (section 4.3.2.3). All bacteriophage samples affected by the low pH incubation were protected from inactivation when pre-mixed with 1% sodium bicarbonate and no statistically significant differences were observed when compared with the control at pH 7.5 (p>0.05).

Inactivation of potential therapeutic *S. aureus* and *P. aeruginosa* phages under pronounced acid conditions have been reported before (Vandersteegen et al., 2011, Knezevic et al., 2011, Ahiwale et al., 2012). This can potentially affect their viability, if delivered orally, due to the acidic conditions of the stomach. However, the study showed that the addition of a protecting amphoteric compound, such as sodium bicarbonate, protected all bacteriophages from the low pH effects. This suggested that the neutralization of gastric acid or an adequate protective delivery method would be essential for bacteriophage survival *in vivo*. The importance of neutralizing stomach acids before bacteriophage delivery by administering medications, such as aluminium and magnesium hydroxide or calcium and magnesium carbonate has long been known and applied by the Georgian and Polish groups (Dabrowska et al., 2005) and microencapsulation of phages has been proposed for oral delivery in animals (Ma et al., 2008). In the future bacteriophages naturally resistant to low pH conditions will be one of the preferred selection criteria. However, research programs to develop optimal oral bacteriophage delivery systems are still needed.

Storage temperature is a critical aspect that determines bacteriophage survival. The results of the temperature stability testing showed that the *S. aureus* bacteriophages were very stable at 4°C storage during the twelve-month period tested with a total average reduction in their concentration of only 0.24-Log ($p>0.05$). Their stability was more affected by storage at 25°C with an average reduction 10 times greater than the concentration lost when the phages were stored at 4°C ($p<0.05$). At 37°C, all *S. aureus* phages lost in average 1.7-Log in the first two months and only one phage showed viable particles after 6 months of storage at the test temperature. Similarly, all but two *P. aeruginosa* bacteriophages (Pa 32 and Pa 51) were remarkably stable when stored at 4°C during the twelve-month period ($p>0.05$). At 25°C, an average reduction 10 times greater than the concentration lost when the phages were stored at 4°C was also measured ($p<0.05$) while at 37°C their sensitivities varied with the last phage fully inactivated by the ninth month (section 4.3.2.2).

Fortier and Moineau (2009) investigated phage temperature stability and survival under various storage conditions and found that very few long-term temperature stability studies have been reported. Phage temperature stability tests for over a 2 year period were reported earlier for one *S. aureus* and one *P. aeruginosa* phage (Clark, 1962). As observed in this study, both bacteriophages remained stable at 4°C but lost activity at room temperature (26°C). A 12-18 year term study of 25 *S. aureus* lyophilized phages showed that only a 1-log drop in titre was seen when stored at -20°C. (Zierdt, 1988). However, freeze-drying as a storage method had been previously reported to be less effective than phage in liquid form after a 2-year period (Clark, 1962) and was therefore not attempted here. Recently, another *S. aureus* phage was reported to retain its titre in liquid form for over 36 months at 4°C (Hsieh et al., 2011) while no recent reports of long-term stability studies on *P. aeruginosa* phages were found. Temperature stability studies with a view to predicting product stability during storage conditions are a requirement for regulatory agencies during the development of biological products. The rising interest in bacteriophages as therapeutic drugs will inevitably result in additional investigations into optimum temperature stability and storage methods. However, the data collected to date suggest that prospective products in liquid form will need to be stored at 4°C to preserve their titres.

This finding could carry serious commercial implications, if a potential commercial product is to be delivered in liquid form. It means that the storage and shipping of the product must be done maintaining a cold chain, which can be expected to be significantly more expensive than shipping and storage at room temperature. The findings also meant that, if the product needs to be maintained at 4°C, the number of outlets (*i.e* hospitals, pharmacies) capable of storing and dispensing the product, particularly in developing countries, might be greatly reduced too. Further research aspects to solve this problem could compare the testing of novel phage matrices with the one tested in this study, in order to improve stabilisation at room temperature. Additionally, future isolation programs could also consider the inclusion of samples found naturally at higher temperatures.

To select bacteriophage with therapeutic potential it was essential to study their growth kinetics and their host interactions *in vitro* in order to project their potential efficacy *in vivo*. All selected *S. aureus* and *P. aeruginosa* bacteriophages exhibited bacteriophage amplification (section 4.3), demonstrating their ability to infect the targeted host. However, each phage showed individual differences in its ability to infect its host at the three different multiplicity of infection (MOI) ratios tested. Some infection processes appeared to be dose dependent (Fig. 4-5) while others were equally effective in reducing the target bacteria over a 24h period regardless of the initial phage concentration (Fig 4-4a, 4-8). A similar dose dependant process was recently reported for a *S. aureus* phage (Vandersteegen et al., 2011), although the infection process was followed for only 400 minutes. These observations were useful to further exclude certain bacteriophages from the development process, as significant reduction of bacterial growth and prevention of bacterial regrowth are essential qualities for all prospective therapeutic phages.

However, it is their ability to adsorb to and multiply efficiently within their host bacteria that is possibly their most powerful weapon. All bacteriophages were shown to adsorb very efficiently to their host cell with *P. aeruginosa* phage Pa 6, Pa 10 and Pa 51 showing the lowest percentage of adsorption to their host cell. This indicated that very few collisions between the phage and the target cell were required for adsorption to occur. One-step growth-curve analyses showed the *S. aureus* phages have very short latent periods of 10min to 25min while the latent periods for the *P. aeruginosa* phages varied greatly. Four phages showed a short latent period of 15 min to 25 min, four showed a long latent period of 60 min to 70 and two showed “medium” latent periods of 30 min to 40 min and 45 min to 55 min (Table 4-6).

The long latent periods found in this study appeared to be common (O'Callaghan et al., 1969, Garbe et al., 2011, Monson et al., 2011) although phages with short (Ahiwale et al., 2012) and medium (Fu et al., 2010) latent periods have also been reported. Interestingly, those *P. aeruginosa* phages with the longest latent periods also showed, in general, the largest burst sizes. Bacteriophage single-step growth curves performed also showed *S. aureus* bacteriophages had burst sizes of 55 ± 3 PFU/cell up to 461 ± 23 PFU/cell. The

calculated burst sizes for the *P. aeruginosa* phages were similar from 45 ± 2 PFU/cell up to 476 ± 24 PFU/cell. Similar burst sizes had been described for both bacteriophage types (Rees and Fry, 1981, Matsuzaki et al., 2003, Schrader et al., 1997, Shaffer et al., 1999), although bacteriophages with smaller burst sizes to those found in this study have also been reported (Garbe et al., 2011, Garcia et al., 2009).

To be therapeutically useful, a drug must be distributed directly to the site of action and remain in high concentration for adequate periods of time. Small molecule drugs such as antibiotics naturally drop in concentration and therefore multiple doses are required to eliminate the infection. The ability of bacteriophages to replicate in-situ, increasing and maintaining the adequate therapeutic concentration over time, is a distinct advantage when compared with antibiotics. The evidence collected to date, and reviewed in detail elsewhere (Abedon and Thomas-Abedon, 2010), has suggested that the success of bacteriophage therapy in achieving bacterial clearance depends on the production of high phage densities close to the target bacteria. This means that production of new phages must be sufficiently large enough to ensure that the therapeutic concentration remains unaffected by the body's natural clearance mechanisms and/or other non-specific and specific immune responses. Thus, the analysis of growth kinetics, as performed in this study, is an important step to be considered during the selection of bacteriophages in order to demonstrate that those with therapeutic potential can replicate actively and efficiently once a target bacterium is attacked.

The use of therapeutic phages as a mix or "cocktail" has been the classical approach to therapy in former Soviet Bloc countries where the therapy has been most widely used (Abedon et al., 2011, Kutter et al., 2010). The use of phage mixes or "cocktails" has been preferred over the use of a single phage approach for two main reasons. Firstly, bacteriophage mixes ideally should have a broader spectrum of activity against a larger population of bacteria. Secondly, the development and use of bacteriophage cocktails is helpful in preventing or delaying the emergence of bacteriophage insensitive mutants (BIMs) (O'Flynn *et al*, 2004, Sulakvelidze and Kutter, 2005). However, the criteria to select and mix individual therapeutic bacteriophages, for inclusion in therapeutic mixes to

ensure the broadest spectrum of activity and minimum development of phage resistant bacteria, has not been described. Therefore, the sixth step in this study investigated three different formulation approaches, which resulted in the production of three bacteriophage prototypes for each target pathogen.

For the first approach, the most traditional method in which bacteriophages with the broadest host range are combined was used to formulate the first cocktail. For the second approach those bacteriophages with a narrow but complementary spectrum of activity, were combined. The third approach used was a combination of the first two methods in which phages with broad and narrow spectrum of activity were combined. These therapeutic mixes were evaluated for their ability to display a broader spectrum of activity and to minimise the appearance of phage-resistant cells, in comparison with the individual bacteriophages. The results showed that the cocktails developed using the third approach, a combination of phages with a broad and a narrow spectrum of activities, were slightly more efficient against the bacterial collections tested than those developed using the first classic approach. The addition of phages with a narrow spectrum of activity appeared to increase the percentage of activity of the cocktails by providing coverage against isolates that would have been otherwise missed by the phages with a broad spectrum of activity. This was particularly true for the *P. aeruginosa* mixes (section 5-7). Phage mixes developed following the third approach performed in a synergistic manner and did not appear to interfere with each other while cocktails developed using the second approach, a combination of phages with a narrow but complementary spectrum of activity, showed at times a reduced spectrum of activity suggesting a non-complementary and/or antagonistic activity.

Despite the differences observed with regard to the spectrum of activity, the inhibition and/or reduction of phage-resistant cells was demonstrated for the three different types of formulated cocktails. The formulated *S. aureus* cocktails completely inhibited or greatly reduced the appearance of BIMs (Table 5-3). For the three formulated *P. aeruginosa* cocktails their effectiveness in reducing the frequency of BIMs was also shown, although the reductions, when compared with the frequencies calculated for the individual

bacteriophages, were not always statistically significant (Table 5-4). No studies reporting the frequency of BIMs for *S. aureus* or *P. aeruginosa* phages were found in the literature.

As demonstrated here, potential BIMs might pre-exist among the populations of target bacteria. The existence of these innate phage resistant strains may be the result of phage pressure in the environment, bacterial cells that lack the appropriate receptor or are lysogenic to the particular phage. This clearly suggests that the frequency of the mutations may be subjected to changes according to the bacteriophage-host pair selected. It is difficult to determine how much emphasis should be placed on the frequency of BIMs in the host-strains, as this may not be an accurate representation of the isolates encountered in the clinic. At the same time, in the clinic, it would be impractical to attempt to measure the frequency of mutation of a cocktail against a particular isolate prior to treatment. Thus, until more *in vitro* data is correlated with *in vivo* data, the assumption that the developed cocktail must show a low frequency of mutation against the host bacteria will remain an important step in the development of bacteriophage mixes.

The results obtained in this study have confirmed that the development and use of bacteriophage mixes should be preferred over individual bacteriophages for clinical treatment. It also suggested that developing phage mixes which include phages with both a broad and a narrow spectrum of activity might be a more advantageous approach in order to expand their efficacy against a wider bacterial population as well as to minimise the development of phage resistance bacteria.

After completing the biological and physico-chemical characterisation of these phage therapeutic prototypes, the seventh and final stage of development concentrated on the morphological and molecular characterisation of the individual bacteriophages. The investigation of their morphological properties by transmission electron microscopy (TEM) was carried out and was an essential step for the classification of the novel identified phages within a family, which then offered clues about their expected therapeutic behaviour. All bacteriophages with therapeutic potential reported to date belong to the

Caudovirales order, which is composed of three families: the *Myoviridae* family, the *Siphoviridae* family and the *Podoviridae* family (section 6.4).

All *S. aureus* bacteriophages isolated were tailed and belonged to the *Myoviridae* family. Phages showed icosahedral heads with an average diameter of 74.8±3.2 nm, a contractile tail 182.7±9.1 nm in length and 14.5±1.5 nm in diameter. Bacteriophages with identical features to those identified here and capable of infecting not only *Staphylococcus* sp but also *Bacillus* sp. were isolated (Pohjanpelto and Nyholm, 1965) and compared previously (Jarvis et al., 1993). Of the *P. aeruginosa* bacteriophages observed, 50% belonged to the *Myoviridae* family (possessing icosahedral heads and contractile tails), 30% to the *Siphoviridae* family (non-contractile flexible tails) and 20% to the *Podoviridae* family (short tails) (n=10). Comparable morphologies were previously described in phages infecting *P. aeruginosa* (Morgan and Stanisich, 1975, Knezevic et al., 2009). The morphological characterization of the *S. aureus* and the *P. aeruginosa* bacteriophages by electron microscopy did not facilitate differentiation within the group and showed only small size differences in head and tail sizes. However, it did confirm their classification within the Order *Caudovirales*. Other differences such as their place of isolation, host and lytic spectrum of activity suggested that they were different from each other.

Additionally, pulsed field gel electrophoresis and restriction digest laboratory protocols were developed to assist with the characterisation of genomic DNA of the selected bacteriophages. The isolation of genomic DNA by the methodology developed was useful to confirm that all *S. aureus* and *P. aeruginosa* bacteriophages had unique profiles and were genetically different from each other. Given the genome sizes calculated by PFGE, the *S. aureus* bacteriophages isolated in this investigation would seem to be the largest reported in the literature to date, however the actual genomic sizes can only be confirmed once the phages are sequenced and fully annotated. The closest phage relatives include bacteriophage K (O'Flaherty et al., 2005), bacteriophage ISP (Vandersteegen et al., 2011), bacteriophage Sb-1 (Kvachadze et al., 2011), and G1 (Kwan et al., 2005). All of these bacteriophages were reported as phages belonging to the Family *Myoviridae* and studied for their therapeutic potential, as were the bacteriophages isolated in this study.

The *P. aeruginosa* bacteriophages isolated showed a size range from 36 ± 0.4 kb to 85 ± 0.2 kb. Based on the molecular weight only, three bacteriophages Pa 24, Pa 25 and Pa 37 could be part of the PB1-like virus genus, which is generally recognised for its broad spectrum of activity and virulent (lytic) activity while Pa 51 could be part of the ΦKMV-like virus genus (Ceyssens and Lavigne, 2010), a reported therapeutic genus (Merabishvili et al., 2009). Pa 6 and Pa 10 could potentially be part of the D3112-like virus genus. This is of particular concern as D3112-like phages are well-described temperate bacteriophages (Heo et al., 2007, Roncero et al., 1990, Wang et al., 2004). However, a previous study has also reported the existence of a bacteriophage that resembles phage D3112 but did not encode in its genome a *c*-repressor gene, which is known to be critical in maintaining the lysogenic state, suggesting the phage may be strictly lytic (Kim et al., 2012). Bacteriophage Pa 1 showed a relatively larger size and based on its molecular weight could be classified as part of the PAMx31 group recently described in Mexico (Sepulveda-Robles et al., 2012). No genus could be assigned to Pa 26 and Pa 32 given their genome sizes, suggesting that they may be novel bacteriophages. However, despite the phenotypic and morphological similarities identified among the *S. aureus* and *P. aeruginosa* bacteriophages, previously reported, and the bacteriophages identified in this study, only full genome sequencing would confirm these assumed classifications and novelty by ensuring a high identity of their nucleotide sequences to representatives of their specific genus.

Morphological assessments failed to show significant differences among the *S. aureus* and the *P. aeruginosa* phages. However, the differences in the restriction patterns showed all the selected phages were different (Fig 7-4 and 7-5). Once an exclusive restriction pattern is determined, this pattern may be adequate as the basis of an analytical test for a bacteriophage preparation during production. This qualified test could potentially allow for a rapid and consistent identification of the individual phage components prior to generating a mix or cocktail. The feasibility and reproducibility of such a test was demonstrated in this study (section 7.3.4) for selected bacteriophages suggesting (but not proving) a low frequency of genome changes in each qualified pair that might be sufficient to verify the identity of the phages.

There is no doubt that the full genome sequencing of potential therapeutic bacteriophages will be a necessity in the future to exclude any bacteriophages carrying undesirable genes as well as for confirming that the original phages are not modified overtime. As sequencing technology progresses in both speed and costs, it is possible to contemplate the use of full genome sequences as part of the analytical testing system package. In this study, the use of restriction patterns was a simple, reproducible, economic and speedy way to identify and discriminate the isolated phages. The system was also useful to quality control (*i.e* to confirm the absence of major genomes changes) batches of some of the therapeutic bacteriophages produced consecutively over time. However, although the patterns remained unchanged, small point mutations or phage insertions or recombination in high molecular levels, for example, may not be efficiently observed by restriction digest.

To date, there is no evidence to suggest that a particular phage genotype will infect a particular isolate. Thus, the biological activity of specific bacteriophages might continue to drive the earlier selection process by formulating relevant reference collections as concluded in this study (Chapter 3 and 5). Therefore, from the establishment of relevant reference collections to the design and characterization of appropriate prototype cocktails, the initial steps identified during the course of this project will continue to be relevant. However, and in contrast to the workflow set up in this study, prior to the deeper analysis of the individual phage strengths and limitations (Chapter 4), earlier sequencing may be recommended in order to discard from the process those bacteriophages that, despite showing a broad-spectrum of activity, could potentially carry undesirable genes.

Finally, this thesis describes, to the best of our knowledge, the first use of human phage therapy in Australia. Bacteriophage therapy was used to treat a Sydney hospital patient suffering from a refractory urinary tract infection caused by *P. aeruginosa* (section 8.2.1). The combined phage/antibiotic treatment regimen was designed in collaboration with French colleagues (Table 8-2), the therapy approved prior to administration (section 8.2.2) and the treatment provided at Westmead Hospital.

Immediately after and during treatment, the patient manifested less pain and the attending physician reported less pelvic tenderness. No discomfort or side effects were reported. Bacterial counts declined steadily before antibiotics were commenced on day 6 and after seven days of treatment, no viable bacteria were recovered. Bacteriophage counts in urine rose after 24 hours and remained high until bacterial numbers subsided (Figure 8-2). Four hours after initial dose to 20h later, bacteriophage numbers increased up to 3-Logs (Table 8-5). Bacteriophage counts remained high until after disappearance of the target organism and then diminished sharply despite continued administration for several days (Figure 8-2).

This data suggested that the bacteriophage infection followed a self-sustaining and self-limiting model. This ability of bacteriophages to replicate on-site and disappear from the system once the pathogen has been eliminated is one of the most valuable advantages of bacteriophages when compared with antibiotics (Sulakvelidze et al., 2001). Furthermore, no phage resistant bacteria were isolated suggesting the benefits of using a bacteriophage cocktail. Antibiotics alone had failed to eradicate the *P. aeruginosa* isolate causing the infection in this patient. However, the additive effort of combined phage-antibiotic treatment was successful in eradicating the bacteria permanently. The advantages of a combined phage-antibiotic treatment have been reported since the beginning of the antibiotic era (Himmelweit, 1945).

Although on the basis of this case study it cannot be suggested that bacteriophage alone would have been curative, the greater than ten-fold fall in *P. aeruginosa* concentration in the urine that appeared to precede the administration of antibiotics strongly suggests an action *in vivo* to mimic that observed *in vitro*. The use of phage therapy to treat bladder infections has been reported previously (Michon, 1936, Chanishvili, 2012b, Larkum, 1926). This case study provided clinical evidence for the potential use of bacteriophage in the treatment of bacterial infections in a modern clinical setting and should encourage the development of further research in Australia, including the design of double blind clinical trials to prove their potential at the appropriate scientific standardss.

9.2 General Discussion

9.2.1 From *in vitro* to *in vivo* efficacy

Once the bacteriophage selection process was identified and the prototype products developed were tested *in vitro*, their potential for controlling or eliminating bacterial infections caused by *S. aureus* and *P. aeruginosa* bacteria was evaluated *in vivo*. This work was achieved by working in collaboration with a variety of groups both locally and overseas and thus, the protocols used were not described in detail or included in the main body of this study. However, the results obtained are presented as evidence of the value of the phage selection process here described in identifying bacteriophages with therapeutic potential.

The *S. aureus* prototype cocktail 1 was examined for its ability to eradicate *S. aureus in vivo* in a previously validated chronic rhinosinusitis (CRS) model in sheep (Le et al., 2008)[8]. This type of chronic infection can be challenging to treat with antibiotics and usually leads to the patient requiring surgery (Uren et al., 2008). Delivery of the phage treatment was achieved by direct instillation of the samples into both sheep sinus cavities. Phage treatment was administered once daily, over a period of five days at a final concentration of 10^8 plaque forming units (PFU).

The collected data showed in the phage treated group a reduction in biofilm formation that was statistically significant ($p<0.001$) in comparison with the control. Histological analysis of sinus tissue collected from the phage only treated group did not show any gross changes. The results showed that the bacteriophage treatment was effective and that a single daily dose of phage treatment at a final concentration of 10^8 PFU for five consecutive days did not cause damage to the sinus tissue (Drilling et al., 2014). The use of bacteriophages for

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[8] This work was done in collaboration with the University of Adelaide and Flinders University. Acknowledgment for the work performed is given to A. Drilling, Prof. Wormald, Prof. Speck and Dr. A. Smithyman, S. Morales prepared the bacteriophage product, methodology and protocol for phage administration for the study and helped with some sample processing in one of the stages. Acknowledgment for financial support for product development and travel expenses is given to Special Phage Services.

the treatment of sinusitis was seemingly a major indication for phage therapy at the Pasteur Institute in Paris back in 1975 (Abedon et al., 2011). Currently, sinusitis treatment is available to patients in Poland at the Institute of Immunology and Experimental Therapy (IITD) and in the Republic of Georgia through the Phage Therapy Centre. However, no formal or recent reports were found in peer-reviewed literature describing a treatment regimen or its efficacy.

Similarly, *P. aeruginosa* cocktail E was used in an acute lung model in mice to investigate the potential of the cocktail in combating *P. aeruginosa* infections in patients suffering from cystic fibrosis[9] (Pabary et al., 2012). Cystic fibrosis (CF) disease results from specific mutations in the gene that is known as the CF trans-membrane conductance regulator gene (CFTR). Deficiencies in CFTR result in reduced chloride secretion into the airways and increased sodium adsorption from the airways. This results in dehydration of the airway mucous causing reduced mucociliary clearance, increased mucous retention and finally augmented vulnerability to chronic infection (George et al., 2009).

In the in vivo study, adult BALB/c mice were inoculated intranasally with 50µl of PBS containing either $2.5x10^8$ or $12.5x10^9$ colony forming units per ml (CFU/ml) of a clinical lab strain and the lab strain PA01 correspondingly. Immediately after, the mice were intranasally administered 20µl of bacteriophage cocktail (treated, n=21) or SM buffer (controls, n=21). Twelve mice were sacrificed at 24h after infection and the others at 48h. Bronchoalveolar lavage fluid (BALF) were collected, serially diluted and cultured overnight at 37^oC. The remaining samples were centrifuged; cell pellets resuspended and inflammatory cell counts determined using a haemocytometer.

All mice treated with bacteriophage (n=6) had cleared infection at 24h compared with none

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[9] This work was done in collaboration with Imperial College London. Acknowledgment for the work performed is given to R.Pabary, C. Singh, A. Bush, K. Alshafi, D. Bilton, E. Alton, J. Davies and Dr A Smithyman. S. Morales prepared the bacteriophage product and participated in the development of the methodology and protocol for phage administration for the study. Acknowledgment for financial support for the development of the product and the cost of the study is given to Special Phage Services.

of the controls (n=6) (median [range] CFU/ml= 0 [0–0] vs. 1305 [190–4700], $p<0.01$). The inflammatory cell counts were not different. At the 48h time point, most mice had cleared the infection, with no phage-related differences. However, treated mice demonstrated significantly fewer inflammatory cells in BALF compared with controls (median [range]= 4.50 [2.84–5.86] × 104/ml vs. 9.12 [6.93–13.86], $p<0.01$ for the clinical strain; median [range]= 6.04 [5.56–10.60] × 104/ml vs. 9.72 [8.56–15.28], $p<0.01$ for the laboratory strain).

This study showed the potential of bacteriophages in preventing lung infection. The results were in accordance to similar studies reported previously in the literature (Morello et al., 2011, Alemayehu et al., 2012, Debarbieux et al., 2010). However, the direct effect of bacteriophage treatment in reducing total inflammatory cell counts has not been previously reported. Further work is currently underway to investigate the effect of bacteriophage therapy in a chronic respiratory model, which is more representative of the real case scenario of a CF patient. Additional experiments are also underway to measure any possible inflammatory response to bacteriophages administered to the lung in the absence of bacteria.

Animal studies have strongly supported the efficacy and safety of bacteriophage therapy against a variety of bacterial pathogens. The results obtained in these two animal studies added to the body of evidence regarding the potential of bacteriophage therapy to control and prevent bacterial infections. The data generated also appeared to confirm the traditional claims of safety, which have long been associated with the application of phages.

The successful treatment of the Sydney hospital patient for a highly refractory P. aeruginosa bladder infection was notable for three reasons; this was the first recorded use of bacteriophage therapy to treat a patient in Australia; it provided further evidence to support the self-amplifying and self-limiting model of phage action; and thirdly it added further support to observations noting the complementary effect of combined phage/antibiotic treatment.

9.2.2 Prospects and challenges for phage therapy in the future

Despite the growing interest in phage therapy, and recent reports highlighting the potential of bacteriophages to treat infections caused by MRSA and *P. aeruginosa* bacteria, the introduction and acceptance of the therapy in modern regulated Western environments is yet to be accomplished. However, the evidence found in the literature and the work performed here strongly suggest that phage therapy could provide an alternative, or at least a complementary means of treating the increasing number of antibiotic resistant infections. There is no doubt that bacteriophages have the ability to reduce or eliminate bacterial infections and that their mechanism of action is self-amplifying, self-limiting and highly specific, thus reducing the harm to the body's normal bacterial flora. Most importantly, bacteriophages perform equally well against antibiotic-resistant and susceptible bacteria.

Technically, this work also demonstrated that bacteriophage-based products can be developed to meet the demands of a busy clinical setting, and strategies to select phages with maximum therapeutic potential can be identified. However, the isolation of lytic bacteriophages within a well-controlled procedure is only the first phase for the eventual implementation of bacteriophage therapy, and several technical and procedural obstacles remain. One thing is clear however, and that is in order to accomplish the introduction of bacteriophages as therapeutic drugs, controlled clinical trials must be performed to confirm the safety and efficacy of the technology.

The majority of work performed with bacteriophages in humans has been focussed in countries like the Republic of Georgia, Russia and Poland. Unfortunately, in the 1990s, during the early stages of the phage therapy revival, published reports often did not include adequate controls (Chanishvili, 2012b). This served only to increase Western scepticism to an antibacterial technology, which they assumed, erroneously as it turned out, to be of Russian origin with a distinctly dubious pedigree. Acceptance of phage therapy would be greatly enhanced if these countries, with their many years of practical clinical experience, were able to carry out much needed double-blind, clinical trials. Their experience could potentially help guide the development and approval of schemes in other parts of the world. In particular, the involvement of trained clinical personnel from these experienced countries

could be fundamental in passing on and training interested physicians in other countries who will need to learn how to apply and deliver phages effectively.

Most recently, in Wroclaw, the Institute of Immunology and Experimental Therapy (IITD) was granted authorisation to treat people with phages in cases where all other standard antimicrobial therapies had failed. The authorised treatments may be considered case studies only and not controlled cases, but their approval under European legislation is an important step in recognising the value of the therapy. In the past, prior to the accession of Poland to the European Community the Institute reported success in the treatment of several infections caused by antibiotic resistant *Staphylococcus* sp. and *Pseudomonas* sp. The range of recovery for 1123 patients was of 64% to 100% depending on the etiologic factor for which they were treated. The scientists reported bacteriophage treatment was most effective in the treatment of purulent meningitis and furunculosis. High efficacy was also reported in the treatment of septicaemia, otitis media, osteomyelitis, infections of burns, purulent mastitis and chronic suppurative fistulas (Weber-Dabrowska et al., 2000a). Between January 2008 and December 2010, the Institute has treated over 153 patients suffering from genital and urinary tract infections, soft tissue infections, orthopaedic infections and respiratory tract infections that were untreatable with antibiotics (Międzybrodzki et al., 2012).

To date, some progress has been made towards demonstrating the safety of the therapy following modern regulatory standards. A clinical trial was set up to study the safety of coliphages in fifteen healthy adult volunteers (Bruttin and Brussow, 2005). The volunteers received two different doses of the coliphage T4 and placebo mixed with drinking water. Faecal analysis, 24h after the dose was given, detected the coliphage in the volunteers exposed to the phage. As predicted from animal studies (Dubos et al., 1943, Smith and Huggins, 1982) two weeks after the administration of the phage, there was no phage detectable in any of the volunteers. More significant, the counts of normal *Escherichia coli* did not decrease and no adverse symptoms associated with phage application were reported.

In a follow-up study, 15 healthy adults from Bangladesh received not a single phage but a phage cocktail composed of 9 *E. coli* phages at two different concentrations 3x107 and $3x10^9$ plaque forming units (Sarkar et al., 2012). The result showed no adverse events by self-report or clinical examination. The laboratory tests for liver, kidney, and haematology function were also reported as normal. Importantly, no impact of oral phage was seen on the faecal microbiota composition from stool. The phages used in this study were first extensively studied *in vivo* in a murine model and comparative phage genomic analysis used for guidance in the development and design of an appropriate mix (Denou et al., 2009). These series of studies represented not only a significant step in modern phage therapy regarding its safety, but also suggested the potential of phage therapy in the treatment of diarrhoeal diseases in humans.

The positive results in these two safety studies in adults encouraged the sponsor, Nestle Research Center, to run two small safety trials in healthy children of decreasing age in Bangladesh (Brussow, 2012). These were followed by randomized, double blind placebo-controlled studies to evaluate the effect of an orally delivered *E. coli* specific phage cocktail in the control of enterotoxigenic *E. coli* (ETEC) and enteropathogenic *E. coli* (EPEC) induced diarrhea in male children aged 4-60 months. The study arms included the T4 phage cocktail developed by the sponsor as well as a phage cocktail produce by Microgen, a Russian company that produces and sells phage therapy products over-the-counter in pharmacies but is yet to published data on the clinical efficacy of its product. The trial was conducted at the International Center for Diarrheal Disease Research in Dhaka/Bangladesh (ICDDR,B). Details of the study can be found in the clinical trials register (www.clinicaltrials.gov), identifier NCT00937274. Currently there are no study results published despite the study been reported as terminated. Nestle is the only multinational company involved in phage therapy studies at this time.

In the United States, a phase I trial was undertaken to investigate the safety of bacteriophages when used for the treatment of chronic wounds (Rhoads et al., 2009a). Thirty-nine patients were treated for 12 weeks with either a placebo or bacteriophages targeted against *P. aeruginosa*, *S. aureus* and *E. coli*. The study reported no adverse side

effects attributed to the product. However, there was no significant difference between the phage treated and the control groups for the variables measured. The efficacy of the bacteriophage preparation will be evaluated in a phase II efficacy study.

The first controlled clinical trial of a therapeutic bacteriophage preparation to evaluate the efficacy of a bacteriophage product was performed in England (Wright et al., 2009). The trial dealt with refractory ear infections caused by *P. aeruginosa.* Twelve patients were treated with a single dose of bacteriophage, twelve received a placebo and both groups were monitored for 42 days. In general, clinical symptoms improved for the test group in comparison with the placebo group and *P. aeruginosa* counts were lower for the phage treated group. These encouraging developments continue to indicate that phage therapy has the potential to be a practical alternative to conventional antibiotic therapy. However, much work still needs to be done in order to overcome negative reports and scepticism in the West. In particular the lack of products manufactured under current good manufacturing practices (cGMP) is clearly a technical and procedural obstacle that remains. Presently, no human study has reported the use of a cocktail produced under cGMP standards.

There is a general agreement that, for small clinical trials run under the umbrella of local ethics committees, phage therapy products have to be well characterised, sterile, free of endotoxin and incapable of causing cytotoxic reactions. To explore the reproducibility of this relatively simple manufacturing protocol, two prototype cocktails designed in this study, one *S. aureus* and one *P. aeruginosa* prototype cocktail, were produced following the reported protocol (Merabishvili et al., 2009). After production, the *S. aureus* Sa prototype cocktail was sent to an accredited bacteriology laboratory (AMS Laboratories, Sydney) for sterility, endotoxin and cytotoxicity testing. The results showed the produced batch was sterile (test passed Pharmacopoeia methods), endotoxin levels found were of 0.78 EU/ml and no cytotoxic effects were detected (Morales et al., 2011). However, although this manufacturing and purification protocol has been accepted for some local safety and ethics committees to perform some pre-clinical and small clinical studies, products manufactured under cGMP standards are still required.

The challenge of manufacturing bacteriophage products under cGMP is one of the main points that require consideration. Current GMP standards were developed to provide a regulated system that could guarantee appropriate design, monitoring, and control of manufacturing procedures and the facilities where such products are produced (World Health Organization, 2011). The implementation of cGMP regulations protects the identity, potency, quality, and purity of drug products by requiring that manufacturers of a therapeutic product to control manufacturing procedures adequately.

As noted earlier, official guidelines for the manufacture of phage-based products do not exist yet, in particular for the testing and release of the final product. Discussions between the manufacturers and the regulators will need to address the issues of absent guidelines for the required steps along the way, some of which may be unique to this technology. For example, how and how often will the collection of reference isolates be updated for host range testing? Monitoring centres currently under development, such as the Global Microbial Identifier (GMI), which attempts to focus on the use of genome sequencing techniques for microbiological identification and epidemiological surveillance in a global system, may facilitate this process of overseeing the appearance of new strains and thus, allow for product reformulation as required. A comparable scheme has been established and used over the years for the annual adjustment of seasonal flu-vaccines and therefore, it will not be unreasonable from the regulators to expect a similar structure for the development of phage-based products. The organization of such scheme, however, is likely to take time and demand significant financial investment.

What types of matrices will be used which are both safe for human administration and efficient in prolonging product stability? Bacteriophages have been traditionally stored in a salt-magnesium buffer (SMB) (section 2.3.2), which was shown to keep the viruses stable over time at 4°C. However, it is very unlikely that the regulators will accept this matrix for human use because of the presence of porcine gelatin and Tris-HCl buffer. Therefore, there is an urgent need to develop and test novel bacteriophage storage matrices. What analytical test will be used to test for product potency? Traditional bacteriophage titrations (section 2.6.6) can only account for active particles. The presence and the quantity of inactive

and/or defective phage particles in a product has not been addressed until now, despite the fact that phage particles are recognised for their capacity to induce an immune response (Gorski et al., 2012). At the present time there is no easy method (see below) to distinguish between active and inactive particles. However, as the development of phage therapeutic products becomes more sophisticated, regulators may require more detailed information on the exact proportion of active versus inactive components.

In this study, TEM was used for the classification of the novel bacteriophages within a family as an initial characterization requirement for phages to be used for therapy. However, in the future, this technique could also be used as part of the quality control process to release batches of bacteriophage-based products. This is because TEM is possibly the only technique available that could discriminate between defective (inactive) viral particles from entire (and presumably active) bacteriophages. TEM is a rapid and economical technique that could reasonably play a role in the quality control process of bacteriophage-based products but its usefulness is dependent on skilful technicians with the required expertise. This could make the procedure difficult to harmonise and the establishment of standard operating procedures very challenging.

How will strains to be used in the manufacturing process be qualified (host strains vs surrogate strains)? Surrogate host bacteria that do not possess antibiotic resistance or toxin genes, or that are not known, as pathogenic strains may be the preferred option for large-scale bacteriophage production. This is not only because their introduction would minimise greatly the health and safety risks associated with growing large batches of phages in pathogenic bacteria, but also because it will consequently reduce the expenses linked to performing such manufacturing processes. The difficulty with this approach may be how best to ascertain that those phages isolated and tested against the target human pathogenic strains, will also grow efficiently in the surrogate host. Similarly, that once the phage is grown in the surrogate strain, the resulting phage continues to be effective against the pathogenic bacteria.

The use of surrogate strains has been tested for companies producing phage-based products for the reduction of pathogenic bacteria in food. Micreos Food Safety (formerly known as EBI food Safety) uses *Listeria innocua,* a strain that lacks a 10-kb virulence locus, as a surrogate strain for the production of its product Listex™-P100. Listex™-P100 targets *L. monocytogenes,* a strain that causes a disease called listeriosis and tends to contaminate cheese, fish and other ready-to-eat products. However, although this approach seems to be working for this one food product, it has never been tested for the production of human therapeutic bacteriophages. The major restriction will be the acquisition of suitable surrogate strains for the particular product of interest. In this study, for example, bacteriophages against *P. aeruginosa* were isolated. This species is known to be a human opportunistic pathogen that is intrinsically resistant to antibiotics, but not a specific bacterial pathogen of humans. From this perspective, all strains can be potentially qualified as either pathogenic or surrogate strains. The criteria for qualifying a surrogate strain in this case will need to be discussed and agreed between the regulators and the manufacturing companies.

How will the safety profile of selected bacteriophages be defined and monitored (i.e harmonised list of potential risky genes)? Genome sequencing of potential therapeutic bacteriophages will be essential in the future to exclude any bacteriophages carrying undesirable genes. However, current databases, such as the NCBI database, which are used to probe phage genomes against bacterial genomes with the aim to detect known genes of potential concern and suspected transduced sequences, are public. This means that the content of the databases depends on its contributors to help keep the database as comprehensive, current and accurate as possible. Therefore, the addition (or deletion) of novel genes, as well as their designations over time, are difficult to control within a GMP system and could potentially result in false positives. Again, the criteria for monitoring genome sequences will need to be discussed and agreed between the regulators and the manufacturing companies.

In addition to the issues in relation to the production and quality control of bacteriophage-based products to cGMP standards, the implementation of the therapy faces other technical

challenges, many of which have been reviewed in detail before:

1) Bacteriophage distribution: Bacteriophages are known to cross tissue barriers after administration, including the blood-brain barrier (Dubos et al., 1943), when administered orally, anally, intraperitoneally, intramuscularly and intranasally (Górski et al., 2006). However, their exact mechanism of action to do so is not yet fully understood and very few studies have examined phage circulation, for example, after oral administration (Weber-Dabrowska et al., 1987), even though bacteriophages are currently used over the counter in liquid form for oral consumption in FSU countries. Therefore, further studies are required to understand how phage circulation occurs in the human body, in particular into the bloodstream, in order to maximise therapy protocols.

   Natural vs genetically engineered phage or phage components: due to their history and their size bacteriophage genomes have been extensively studied. The knowledge collected over the years has even allowed the "creation of life" by synthetically reconstructing a phage of 5000bp in the laboratory (Smith et al., 2003). The researchers, at the J. Craig Venter Institute, created the synthetic genome of the phage and implanted it into a cell. The virus then became "biologically active", meaning it reproduced itself. This potentially opens a window of opportunity to manipulate and genetically engineer bacteriophages. More recently, molecular biology approaches have been utilized to produce phages without the ability to lyse bacteria, which were effective at minimising the release of endotoxin due to their inability to cause bacterial lysis (Paul et al., 2011). The approaches for designing and enhancing phage therapeutics through genetic engineering are vast and therefore this approach may well represent an important component of future advancement in therapeutic efficacy (Goodridge, 2010). However, there may be objections for their use both from the regulators and the general public who are still uneasy about genetically modified products (GMO's), particularly in a country like Australia.

Similarly, specific component of the phage, such as their lysins (phage-encoded bacteria cell-wall hydrolytic enzymes,) have also been suggested as promising antibacterials (Fenton et al., 2010). The ability of these enzymes to destroy bonds in the peptidoglycan layer of gram-positive bacteria makes them a very attractive target for both the detection and decontamination of pathogenic bacteria (Courchesne et al., 2009). As with natural bacteriophages, the advantage of lysins over antibiotics relies on their specificity, novel mechanism of action and apparent low probability of bacteria developing resistance (Fischetti, 2008). A potential disadvantage when compared to whole phages is their inability to adapt to potential mutant or lysine resistant strains. However, like for whole bacteriophage particles, apparent problems with the use of lysins include their immnunogenicity, the initiation of pro-inflammatory responses and development of resistant bacteria, even if those occur at very low rates (Borysowski et al., 2006) and therefore their safety and efficacy must be demonstrated in clinical trials

2) Bacteriophage delivery routes: one of the main factors affecting the potential of any drug to reach therapeutic concentrations at the site of the infection is their delivery method. Bacteriophage preparations have been delivered in a variety of ways including orally, rectally, topically, intraperitoneally, intranasally and by irrigation, baths, aerosols, drops and nebulisers (Kutter et al., 2010, Golshahi et al., 2011, Sulakvelidze et al., 2001, Międzybrodzki et al., 2012). Each delivery method in itself can include a variety of carriers such as bandages, wet compresses tampons, creams, capsules, liquid, tablets and dried powders (Sulakvelidze et al., 2001). However, modern knowledge about the choice, preparation and applicability of each of these potential delivery routes for different medical conditions appear to be very limited. Comparative studies on these delivery methods may be required during clinical trials to determine which method delivers, more efficiently, the required therapeutic dose for each particular condition. This task may be less daunting if collaborative training programs for Western clinicians could be set up with their more highly experienced FSU colleagues. Many of these clinicians have years of practical experience in bacteriophage administration and could easily pass on this experience to a new generation of doctors, nurses, and scientists in

the West. The collaboration efforts that resulted in the bacteriophage preparation and protocol in the treatment of the bladder infection patient are a good example of this approach (section 8).

3) Bacteriophages and the immune response: as reviewed by Górski and colleagues (2012), a growing body of data has shown that bacteriophages have the potential to interact with some mammalian cells, mainly with cells of the reticulo-endothelial system. This means that bacteriophages have the capacity to induce immune responses and, thus, trigger the generation of specific antibodies. Furthermore, phages can have nonspecific effects on diverse functions of immune cells involved in both innate and adaptive immune responses (Kurzępa et al., 2009). The clinical evidence to date would suggest that short-term phage treatments are safe and do not induce an antibody response, at least not one that deters the efficacy of the treatment (Sulakvelidze et al., 2001). In principle, the development of antibodies during the treatment of acute infections may not be a significant problem because the activity of the phage clearing the infection is in theory faster than the production of antibodies in the host. Nonetheless, the potential development of antibodies could obstruct the effectiveness of bacteriophage treatment *in vivo.* As the application of phage therapy expands and treatments are used repeatedly and more frequently, for example, for the treatment of chronic infections such as those suffered by cystic fibrosis patients, the understanding of the effects of antibody development must be well understood. Currently, data about the long-term use of bacteriophages and their interactions with the immune system is limited and so research in this particular field during clinical trials will be indispensable to prove the long-term safety and efficacy of the therapy.

4) Off-the-Shelf or Individualised Phage Products: ("Prêt-à-Porter or Sur-mesure?"): One of the most discussed issues is whether modern bacteriophage-based products should be developed and registered as off-the-shelf cocktails (Prêt-à-Porter) or as personalised or individualised treatments (Sur-mesure) (Pirnay et al., 2011). The clinical experience in countries where bacteriophage therapy has been used continuously for almost 100 years

has shown that over time off-the-shelf cocktails become less effective and, like a flu vaccine, every year they must be reformulated to target the appearance of emerging resistance patterns. This reformulation has been achieved by introducing bacteriophages that are biologically enhanced in the laboratory to infect efficiently the new target bacteria or by isolating new bacteriophages from the environment. Furthermore, in the Republic of Georgia, for example, an off-the-shelf treatment may be administered to patients without initial "matching" in order to contain an infection, and then, if needed, a personalised treatment is immediately developed, again by adapting existing phages or isolation of novel phages. This sur-mesure approach, however, has not involved or taken into consideration the analyses of the characteristic of bacteriophages further than the sensitivity of the isolate to the phage. Therefore, not surprisingly, this approach will be in conflict with current modern regulatory systems where the full biological and safety profile of each therapeutic phage will be expected.

However, if the regulators demand that each bacteriophage mix is put through the full regulatory process from pre-clinical to phase IV trials prior to their approval for use, the reintroduction of the technology will be endangered by making the cost and resources required to do so financially forbidding. Unquestionably, as presented in this study, the biological characteristics of each phage must be well understood prior to their introduction to the clinic but, at the same time, the inability to reformulate phage mixes rapidly will ultimately jeopardise the usefulness of the therapy. Initially, companies may be obligated to manufacture products following the off-the-shelf approach and to demonstrate the safety and the efficacy of the technology first, before a commitment can be made to allow for a system where banks of characterised bacteriophages are set up, and those bacteriophages are allowed to be used for product reformulation or as authorised for modern bacteriophage-based products used to prevent and treat infections in plants (OmniLytics, 2004).

As discussed earlier (section 1.2.3), many pharmaceutical companies have reduced their investment in antimicrobial research. Therefore, in the case of bacteriophage therapy, rather small biotechnology companies have taken on the task of trying to commercialize human

products (Henein, 2013). For such companies, the investment and risks are high and potential investors would want to see any acquired intellectual property (IP) properly protected. Although novel mixes of bacteriophages can lawfully be patented, a patent in this particular field offers limited protection only (Pirnay et al., 2012). The long-history, publications and use of bacteriophage for almost 100 years, means that many phages and their novel use have already been publicly disclosed and thus, their 'prior art' requirement is difficult to achieve. The difficulty in guaranteeing IP is a current deterrent for traditional investor. Phage therapy companies would need to develop creative ways to formulate, deliver or manufacture products in order to obtain more solid patents and/or develop a comprehensive system to protect their assets from potential competitors before starting the costly registration process.

The registration of bacteriophage products through standard regulatory pathways must be achieved for bacteriophage-based products to become part of mainstream medicine. This can only be achieved by actively engaging industry with the regulatory authorities, such as the Therapeutic Goods Administration (TGA) body in Australia. The absence of exact guidelines for the registration of bacteriophage-based products, discussed at length here, has driven suggestions for modifications to the current regulatory paths, specifically changes to the European regulatory process (Verbeken et al., 2012). However, given the current knowledge and legislation of biological products such as flu-vaccines and the lack of controlled efficacy evidence that the regulators would like to see first, there is a very small probability for this suggestions to be considered (Harper and Morales, 2012). Companies will need to suggest alternative methodologies to include standardised protocols that can control, cost-effectively, the unique and different challenges that bacteriophages have to offer. Fortunately, with advances in molecular techniques and using extensive current knowledge about phage biology, companies now have the upper hand in making the second wave of phage therapy a successful one.

9.3 Conclusion

As described in the introduction we are moving from the golden age of antibiotics into a far more dangerous and unpredictable time in the treatment of infectious diseases – the Era of Resistance. Through overuse, misuse, complacency, and greed we have squandered the initiative provided by the discovery of antibiotics, even though some degree of resistance was always inevitable. Bacteria have demonstrated their ability to change and adapt very rapidly and thus, at this critical point, it is crucial to make use of every possible approach in defeating these emerging "Superbugs".

The relevance of bacteriophage therapy in the era of antibiotic resistant bacteria is unquestionable and its revival is very timely. Bacteriophages offer a safe, fast evolving, intelligent method of keeping ahead of the resistant bacteria. The first wave of phage therapy from 1920 to 1940 was mired in controversy, mainly because the life cycles and biology of phages were very poorly understood. With the benefit of hindsight it is now clear that the discovery of bacteriophages came 20 years too early to be fully appreciated. The scientific framework necessary to fully appreciate their potential simply did not exist.

In the current renaissance of phage therapy, however, the biology of phages is understood in exceptional detail – they are one of the best understood biological entities, to the point that they can be created from scratch. If, in this revival period, the technology is moved forward on a solid basis of impeccable science and modern regulated clinical trials so that there is no controversy, these "friendly" viruses can be applied to their full potential and to our benefit.

We can look to a future where banks of highly potent phage-based products will be held in specialised phage therapy clinics and, with time, in hospitals across the country. Where a patient suffering from a bacterial infection will have the responsible pathogen identified and subjected to both an antibiogram and a "phagebiogram". This will give the attending clinician the option of treating the infection with antibiotic alone, phage alone or a combination of the two. However, it is unlikely that phage therapy will be strategically used as a stand-alone treatment; to do so would simply repeat the mistakes of the past with

antibiotics. Bacteriophages will most likely be used before or in combination with antibiotics, perhaps strengthening antibiotic stewardship programs in health care facilities by helping to reduce the overuse of conventional antibiotics and thus, reducing the selective pressure on bacterial pathogens to evolve new antibiotic resistance mechanisms.

Since their development, antibiotics have proven to be remarkable and effective drugs. They will continue to be the foundation of our response to bacterial infection but we can no longer ignore the widespread and rapidly growing problem of antibiotic resistance. Neither can we ignore the dwindling pipeline of new antibiotics. As shown in this thesis, phage therapy has considerable potential as an alternative or complementary means of treating antibiotic resistant infections. However, one must resist the temptation to regard bacteriophages as “magic bullets”. There are and there will always be limitations to their use. For this reason, increasing expertise and proficiency in the selection and development of phage products must accompany clinical training programs aimed at optimising the use of these very specific antibacterials.

Appendix 1: *Staphylococcus aureus* clinical isolates reference

Species	Code	Site of Isolation	Source	PN	CXT	ER1	CLE	AMP	AUG	CEP	NOR	CX3	MPM	TOB	KAN	CZ1	CR2	TAZ	PIP	CP2
CA-MRSA	SPS#286	Wound	RNSH	R	R	R	R	R	S	S	S	S	S	S	S	R	S	S	R	R
CA-MRSA	SPS#287	Nose	RNSH	R	R	R	R	R	R	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#288	Nose	RNSH	R	R	S	S	R	R	S	R	R	S	S	S	R	R	S	R	R
C-MRSA	SPS#289	Wound	RNSH	R	R	R	R	R	-	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#290	Urine	RNSH	R	R	S	S	R	R	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#291	Wound	RNSH	R	R	S	S	R	S	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#292	Nose	RNSH	R	R	S	S	R	S	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#293	Groin	RNSH	R	R	R	R	R	S	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#294	Nose	RNSH	S	R	S	S	R	S	S	R	S	S	S	S	R	R	S	R	R
CA-MRSA	SPS#295	Nose	RNSH	R	R	R	R	R	S	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#296	Eye	RNSH	R	R	R	R	R	S	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#297	Wound	RNSH	R	R	R	R	R	S	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#298	Nose	RNSH	R	R	S	S	R	S	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#299	Wound	RNSH	R	R	R	R	R	R	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#300	Nose	RNSH	R	R	R	R	R	S	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#301	Wound	RNSH	S	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
CA-MRSA	SPS#302	Nose	RNSH	R	R	R	R	R	R	S	S	R	S	S	S	R	R	R	R	S
CA-MRSA	SPS#303	Wound	RNSH	R	R	R	R	R	R	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#304	Sputum	RNSH	S	R	R	R	R	S	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#305	Wound	RNSH	S	R	S	S	R	S	S	R	R	S	R	R	R	R	S	R	R
CA-MRSA	SPS#306	Nose	RNSH	R	S	S	S	R	S	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#307	Nose	RNSH	R	R	R	R	R	S	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#308	Wound	RNSH	R	R	R	R	R	R	R	R	R	S	S	S	R	R	R	R	R
CA-MRSA	SPS#309	Nose	RNSH	R	R	S	S	R	S	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#310	Groin	RNSH	R	R	S	S	R	S	S	R	R	S	S	S	R	R	S	R	R

Species	Code	Site of Isolation	Source	PN	CXT	ER1	CLE	AMP	AUG	CEP	NOR	CX3	MPM	TOB	KAN	CZ1	CR2	TAZ	PIP	CP2
CA-MRSA	SPS#311	Wound	RNSH	S	R	R	R	R	S	S	S	S	S	S	S	R	R	S	R	S
CA-MRSA	SPS#312	Wound	RNSH	S	R	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S
CA-MRSA	SPS#313	Nose	RNSH	R	R	R	R	R	R	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#314	Nose	RNSH	R	R	S	S	R	S	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#315	Wound	RNSH	R	R	S	S	R	S	S	R	S	S	S	S	R	R	S	R	R
CA-MRSA	SPS#316	Nose	RNSH	R	R	R	R	R	R	R	R	R	S	S	S	R	R	R	R	R
CA-MRSA	SPS#317	Sputum	RNSH	R	R	R	R	R	R	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#318	Joint	RNSH	R	R	R	R	R	R	R	R	R	S	S	S	R	R	R	R	R
CA-MRSA	SPS#319	Nose	RNSH	R	R	S	S	R	R	R	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#320	Wound	RNSH	S	S	S	S	R	S	S	R	S	S	S	S	R	R	S	R	R
CA-MRSA	SPS#321	Ear	RNSH	R	R	S	S	R	S	S	R	S	S	S	S	R	R	S	R	R
CA-MRSA	SPS#324	Wound	RNSH	S	S	R	R	R	S	S	R	S	S	S	S	R	R	S	R	R
CA-MRSA	SPS#325	Wound	RNSH	R	R	R	R	R	S	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#326	Wound	RNSH	R	R	S	S	R	S	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#327	Axilla	RNSH	R	R	R	R	R	S	S	S	S	S	S	S	R	S	S	R	S
CA-MRSA	SPS#328	Wound	RNSH	S	R	S	S	R	S	S	S	S	S	S	S	R	S	S	S	S
CA-MRSA	SPS#329	Wound	RNSH	R	R	R	R	R	S	S	R	S	S	S	S	R	R	S	R	R
CA-MRSA	SPS#330	Wound	RNSH	R	S	R	R	R	S	S	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#331	Wound	RNSH	R	R	R	R	R	R	R	R	R	S	S	S	R	R	S	R	R
CA-MRSA	SPS#332	Urine	RNSH	R	R	S	S	R	R	R	R	R	S	S	S	R	R	R	R	R
CA-MRSA	SPS#333	Wound	RNSH	R	R	R	R	R	S	S	R	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#334	Wound	RNSH	R	R	S	S	S	S	S	S	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#335	Nose	RNSH	R	R	R	S	R	S	S	S	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#336	Nose	RNSH	R	R	S	S	R	S	S	R	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#337	Wound	RNSH	R	R	R	S	R	S	S	R	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#338	Wound	RNSH	R	R	R	R	R	S	S	R	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#339	Wound	RNSH	R	R	S	S	R	S	S	S	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#340	Nose	RNSH	S	R	S	S	S	S	S	S	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#341	Unknown	RNSH	R	R	S	S	R	S	S	R	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#342	Nose	RNSH	R	R	S	S	R	S	S	R	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#343	Wound	RNSH	R	R	S	S	R	S	S	R	ND	ND	ND	ND	ND	ND	ND	ND	ND

Species	Code	Site of Isolation	Source	PN	CXT	ER1	CLE	AMP	AUG	CEP	NOR	CX3	MPM	TOB	KAN	CZ1	CR2	TAZ	PIP	CP2
CA-MRSA	SPS#344	Sputum	RNSH	R	R	R	R	R	S	S	R	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#345	Wound	RNSH	S	R	S	S	R	S	S	R	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#346	Wound	RNSH	R	R	R	R	R	R	R	R	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#347	Wound	RNSH	R	R	R	R	R	S	S	R	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#348	Nose	RNSH	R	R	R	R	R	R	R	R	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#349	Faeces	RNSH	R	R	R	R	R	R	S	R	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#350	Wound	RNSH	R	R	S	S	R	R	S	R	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#351	Wound	RNSH	R	R	S	S	R	S	S	R	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#352	Wound	RNSH	R	R	R	R	R	S	S	R	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#353	Eye	RNSH	R	R	R	R	R	S	S	R	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#354	Wound	RNSH	S	R	S	S	R	S	S	S	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#355	Nose	RNSH	R	R	R	R	R	R	S	R	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#356	Wound	RNSH	R	R	R	R	R	R	R	R	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#357	Wound	RNSH	S	R	R	R	S	S	S	S	ND	ND	ND	ND	ND	ND	ND	ND	ND
CA-MRSA	SPS#358	Wound	RNSH	R	R	S	S	R	R	R	R	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA No.2	SPS#1	Unknown	RNSH	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA No.4	SPS#2	Unknown	RNSH	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA No.5	SPS#3	Unknown	RNSH	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA No.6	SPS#4	Unknown	RNSH	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA No.7	SPS#5	Unknown	RNSH	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA No.11	SPS#6	Unknown	RNSH	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA No.13	SPS#7	Unknown	RNSH	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA	SPS#25	Unknown	RNSH	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA	SPS#42	Unknown	RNSH	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA	SPS#43	Unknown	RNSH	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA	SPS#44	Unknown	RNSH	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA	SPS#45	Unknown	RNSH	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA	SPS#46	Unknown	RNSH	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Species	Code	Site of Isolation	Source	PN	CXT	ER1	CLE	AMP	AUG	CEP	NOR	CX3	MPM	TOB	KAN	CZ1	CR2	TAZ	PIP	CP2
MRSA	SPS#47	Unknown	RNSH	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA	SPS#54	Unknown	RNSH	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA	SPS#55	Unknown	RNSH	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA	SPS#56	Unknown	RNSH	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA	SPS#57	Unknown	RNSH	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA	SPS#58	Unknown	RNSH	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA	SPS#59	Unknown	RNSH	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA	SPS#60	Unknown	RNSH	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA	SPS#61	Unknown	RNSH	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA	SPS#412	Sputum	West-mead	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MRSA	SPS#413	Sputum	West-mead	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Antibiotics abbreviations: PN: Penicillin; CXT=Ceftriaxone; ER1=Erythromycin; CLE: Clindamycin; AMP: Ampicillin; AUG: Augmentin (Amoxicillin-clavulanic acid); CEP: Cephalexin; NOR: Norfloxacin; CX3; Cefoxitin; MPM: Meropenem; TOB: Tobromycin; KAN: Kanamycin CZ1: Cefazolin; CR2: Ceftriaxone; TAZ: Ceftazidime; PIP: Piperacillin; CP2: Cefipime. R: Resistant to antibiotic; S: susceptible to antibiotics; ND: not tested.

Appendix 2 *Pseudomonas aeruginosa* clinical isolates reference

Species	Code	Site of Isolation	Source
P. aeruginosa	SPS#111	Wound	RNSH (Sydney, Australia)
P. aeruginosa	SPS#112	Wound	RNSH (Sydney, Australia)
P. aeruginosa	SPS#113	Wound	RNSH (Sydney, Australia)
P. aeruginosa	SPS#114	Wound	RNSH (Sydney, Australia)
P. aeruginosa	SPS#115	Wound	RNSH (Sydney, Australia)
P. aeruginosa	SPS#116	Wound	RNSH (Sydney, Australia)
P. aeruginosa	SPS#117	Wound	RNSH (Sydney, Australia)
P. aeruginosa	SPS#118	Wound	RNSH (Sydney, Australia)
P. aeruginosa	SPS#119	Wound	RNSH (Sydney, Australia)
P. aeruginosa	SPS#123	Human	University of New South Wales (Sydney, Australia)
P. aeruginosa	SPS#285	Lung infection	Royal Melbourne Hospital (Melbourne, Australia)
P. aeruginosa	SPS#322	Human Clinical	Westmead Hospital (Sydney, Australia)
P. aeruginosa	SPS#323	Human Clinical	Westmead Hospital (Sydney, Australia)
P. putida	SPS#411	Human Clinical	Westmead Hospital (Sydney, Australia)
P. aeruginosa	SPS#432	Human Clinical	Westmead Hospital (Sydney, Australia)
P. aeruginosa	SPS#433	Human Clinical	Westmead Hospital (Sydney, Australia)
P. aeruginosa	SPS#438	Bladder	Westmead Hospital (Sydney, Australia)
P. aeruginosa	SPS#439	Bladder	Westmead Hospital (Sydney, Australia)
P. aeruginosa	SPS#440	Bladder	Westmead Hospital (Sydney, Australia)
P. aeruginosa	SPS#445	Lung infection	Westmead Hospital (Sydney, Australia)
P. aeruginosa	SPS#446	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#455	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#456	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#457	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#458	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#459	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#460	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#461	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#462	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#463	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#464	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#465	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#466	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#467	Human clinical	RNSH (Sydney, Australia)

Species	Code	Site of Isolation	Source
P. aeruginosa	SPS#468	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#469	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#470	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#471	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#472	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#473	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#474	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#475	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#476	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#477	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#478	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#479	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#480	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#481	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#482	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#483	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#484	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#485	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#486	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#487	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#488	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#489	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#490	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#491	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#492	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#493	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#494	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#495	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#496	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#497	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#498	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#499	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#500	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#501	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#502	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#503	Human clinical	RNSH (Sydney, Australia)

Species	Code	Site of Isolation	Source
P. aeruginosa	SPS#504	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#505	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#506	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#507	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#508	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#509	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#510	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#511	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#512	Human clinical	RNSH (Sydney, Australia)
P. aeruginosa	SPS#513	Human clinical	RNSH (Sydney, Australia)

*****RNSH: Royal North Short Hospital*

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