

**Isolation and Characterisation of Bacteriophages
That Infect Capsulated *Streptococcus pneumoniae***

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Abstract

Isolation and Characterisation of Bacteriophages That Infect Capsulated *Streptococcus pneumoniae*

S. pneumoniae (*pneumococcus*) is a major cause of pneumonia, sepsis, and meningitis, responsible for over 1.2 million deaths per year. Rates of antibiotic resistance are rising, with over one third of isolates in the US and parts of Europe showing a reduced susceptibility to penicillin. Although pneumococcal conjugate vaccines have resulted in a decline in invasive disease caused by the pneumococcal serotypes included in the vaccine, non-vaccine serotypes have been shown to cause replacement disease. To address these important clinical challenges and provide cross serotype protection against pneumococcal infection, alternative therapies are urgently needed such as the exploitation of bacteriophages, which can specifically target and kill pneumococci. Bacteriophages are being developed to treat a range of bacterial pathogens due to their ability to kill pathogens which are resistant to conventional antibiotics, and due to their specificity and ability to access and replicate in difficult micro-environments within the human body. Although the previously isolated pneumococcal lytic phages; Dp-1 and Cp-1 showed promise as a treatment for pneumococcal infection, they could only infect non-capsulated strains, which are attenuated and non-invasive in the human clinical setting. This project describes the isolation and characterisation of a new lytic phage SP-QS1, which can infect and significantly reduce the load of capsulated pneumococcal strains that cause human invasive disease. SP-QS1 is a distinctive new siphovirus with prolated-head, non-contractile tail and tail fibres. The interaction between SP-QS1 and *S. pneumoniae* in both *in vitro* and in *in vivo* assays demonstrated that it is able to significantly reduce the amount of *S. pneumoniae* in two mouse models of invasive pneumonia; the intranasal and the intravenous model of infections. The genomic sequencing of SP-QS1 revealed that genes with recognisable homologies are often ordered according to the following; genes involved in phage packaging, structural proteins, replication and genes associated with cell lysis. Interestingly, SP-QS1 genome does not encode CRISPR sequences, proteins with trans-membrane domains or regulatory elements. In addition, because the phage genome does not encode integrase genes, it appears to be a genuine lytic phage. Genetic characterisation of SP-QS1 genome illustrated that this phage encodes for glycosyltransferase, and it is suggested to be responsible for capsule degradation. SP-QS1 shows promise to control and treat the infections caused by *S. pneumoniae*.

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Abbreviations

AOM	Aom Acute Otitis Media
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
CFU	Colony Forming Units
Cp-1	Complutense Phage
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CsCl	Caesium Chloride
DCs	Dendritic Cells
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide Triphosphate
Dp-1	Diplophage 1
dsDNA	Double-Stranded DNA
EDTA	Ethylenediaminetetraacetic Acid
End	Endonuclease
fct	Filtered, Choline, Tris-Hcl
G-C	Guanine-Cytosine
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
ICTV	International Committee On Viruses Taxonomy
INI	Intranasal Infection
IPD	Invasive Pneumococcal Disease
IVI	Intravenous Infection
MICs	Minimum Inhibitory Concentrations
mL	Millilitres
MOI	Multiplicity Of Infection
mRNA	Messenger RNA
MSR	Modified Spurr's Resin
MTase:	Methyltransferase
mtDNA	Mitochondrial DNA
NCBI	National Center For Biotechnology Information
ng	Nanogram
NLRP2	Nod Like Receptor 2

Abbreviations

NP	Nasopharyngeal
NPS	Nasopharyngeal Swab
NVTs	Non-Vaccine Serotypes
OD	Optical Density
OM	Otitis Media
ORF	Open Reading Frame
p.f.u.	Plaque Forming Unit
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PCV	Pneumococcal Polysaccharideconjugate Vaccine
PEG	Polyethylene Glycol
PFGE	Pulsed-Field Gel Electrophoresis
Ply	Pneumolysin
PMN	Polymorphnuclear Leukocyte
PO	Propylene Oxide
pol	Polymerase
Pol I	Polymerase I
PPS	Pneumococcal Polysaccharide Vaccine
pri	Primase
RNA	Ribonucleic Acid
RNase e	Ribonucleas
RNR	Ribonucleotide Reductase
RT	Respriatory Tract
RTI	Respiratory Tract Infection
SP-QS1	<i>Streptococcus pneumoniae</i>, Al-Qunfidah, Siphovirus, And Number 1
β	Beta
TBE	Tris/Borate/Edta
TE	Tris/EDTA
TEM	Transmission Electron Microscopy/Microscope
Tris	Trishydroxymethylaminomethane
tRNA	Transfer RNA
UK	United Kingdom
USA	United States Of America

Abbreviations

VAT	Vaccine Associated Serotypes
VBS	Viruses to Bacteria Ratio
WHO	World Health Organisation
α	Alpha
μg	Microgram

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1.1 General characteristics of *S. pneumoniae*

S. pneumoniae is a facultative gram positive capsulated oval or spherical bacterium. It is the most common bacterial respiratory pathogen, causing community-acquired pneumonia in the United Kingdom with a mortality rate of over 20% in patients with concurrent septicaemia (Kadioglu et al., 2008). Pneumococcal disease is the main cause of child mortality in the developed world, and according to the World Health Organisation (WHO), two million children under the age of five die every year from pneumococcal infection (Spier, 2008). Invasive infection can develop following mucosal infection, with meningitis suspected to typically follow otitis media or blood infections. The inner ear and haematogenous infection are the main routes that allow the bacterium to enter the body, facilitating the onset of meningitis. It has been reported that meningitis is associated with acute otitis media (AOM), especially in those people with a cochlear implant (Wei et al., 2006).

Nasopharynx is the normal niche for *S. pneumoniae*; the pneumococcus colonises these sites in the first month of life. This colonisation is normally seen as an asymptomatic carriage and is not usually followed by invasive infection, due to the protection afforded by the innate immune system. However, a disturbance of homeostasis between host and pathogen, such as that caused by viral infection, malnutrition or local damage of the mucosa can be followed by invasive infection (Plotkowski *et al.*, 1986; Hament *et al.*, 1999).

S. pneumoniae infections can be treated with β -lactams (penicillin G, amoxicillin and cephalosporins), respiratory fluoroquinolones, macrolides and vancomycin. Despite the continuing development of new classes of antibiotics, pneumococci have shown resistance to most of them. The first reports of resistance were made in Australia and New Guinea in the 1930s against penicillin (Austrian and Gold, 1964). Highly penicillin

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resistant pneumococci were reported in 1977 in South Africa, where resistance against cephalosporins was also reported (Appelbaum et al., 1977). Many years after this publication, the rapid spread of penicillin resistant pneumococci was reported in all over the world. Previous pneumococcal spread arose mostly due to the outbreak of a few multi-resistant clones of serotypes 6B, 9V, 14, 19A, 19F and 23F (Kroon et al., 2000). An example of this, one study reported that a total of 1817 *S. pneumoniae* strains were isolated during winter of 2002–2003 from patients with community-acquired respiratory tract infections in different US medical centers. These isolates were screened for antibiotic susceptibility using MICs of 27 antimicrobial agents by assaying the broth microdilution method described by the NCCLS. The study showed a high rates of resistance for some antibiotics, such as penicillin (34.2%), trimethoprim-sulfamethoxazole (31.9%), and erythromycin (29.5%) (Doern et al., 2005).

The pneumococcal cells are arranged in pairs or in small chains. They grow on blood agar or blood serum under microaerophilic conditions. The presence of CO₂ enhances the growth of this organism by stimulating its metabolism as CO₂ can be converted into organic constituents by some microorganisms (Ruben and Kamen, 1940). It has been found that CO₂ can be reduced to methane during aliphatic alcohol fermentation and can be also converted into formic acid and succinic acid. Colonies on blood agar medium are surrounded by α -haemolysis where blood cells are lysed incompletely (Barnard, 1996).

The colony appearance can vary between strains; this has been demonstrated with some pneumococci which produce an interchangeable colony morphology that is opaque or transparent, depending on the teichoic acid content and the presence of capsular polysaccharide (Weiser et al. 2006). Transparent variants were found to express a higher content of teichoic acid and a lower content of capsular polysaccharide and vice

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versa in opaque variants, with less teichoic acid and more capsular polysaccharide (Kim et al., 1999). Certain strains have been shown to be able to transform their cells from capsulated to non-capsulated and vice versa, depending on the biological activity of pneumococci as they reduce the amount of capsule during colonization and increase it during host invasion (Kim and Weiser, 1998; Magee and Yother, 2001; Bender *et al.*, 2003).

1.2- Pneumococcal Vaccines

There are two vaccines licensed in the UK that are currently used to immunise people against *S. pneumoniae*; the polysaccharide-protein conjugate vaccine and the polysaccharide only vaccine.

1.2.1- The pneumococcal conjugate vaccine (PCV)

The conjugate vaccine (PCV-7) was licensed in 2000 in the United States (Whitney et al., 2003). In September 2006, it was licensed and implemented in the childhood vaccination schedule in the UK (Koshy E et al., 2010). PCV is comprised of a linkage of a polysaccharide and protein carrier (Poland, 1999) giving the opportunity to induce T cell-dependent responses leading to B cell memory induction and consequently improved B cell responses. It provides good protection in childhood but only against 7 invasive serotypes (4, 6B, 9V, 14, 18C, 19F and 23F); at the present time 91 serotypes of pneumococci have been described. Moreover, the link between polysaccharides and carrier proteins is very restricted, as a high number of carrier antigens can impair the antibody response to the polysaccharide (Di John *et al.*, 1989; Peeters *et al.*, 1991).

Studies revealed that PCV-7 provides good protection against pneumococcal infection in children < 2 years. It reduced the nasopharyngeal carriage in this population group (Esposito *et al.*, 2003; Clarke *et al.*, 2004; Veenhoven *et al.*, 2004; Zissis *et al.*, 2004;

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De Schutter *et al.*, 2006), however, PCV-7 has low efficacy as the majority of pneumococci have been found associated with serotype 1 which responsible for severe pneumococcal infection in children > 2 years (Camou *et al.*, 2003; De Schutter *et al.*, 2006; Hansen *et al.*, 2006). The majority of positive culture-based cases are attributed to serotype 1 in the United States and the UK, whereas serotype 14 was isolated in 30% of positive cases in Taiwan. Not only serotype 1 was reported to cause invasive pneumococcal infection in the US, but also serotype 3 and serotype 19 (Rees *et al.*, 1997; Byington *et al.*, 2002; Hsieh *et al.*, 2004). Serotypes 3 and 19 are not included in PCV-7. Serotype replacement is a big challenge in pneumococcal vaccination. It has been reported that the use of PCV-7 resulted in an increase in the emergence of new strains such as serotype 15 and serotype 33 (Black *et al.*, 2004; Byington *et al.*, 2005; Gonzalez *et al.*, 2006). Studies on the second generation of this vaccine (PCV-9) in Africa showed that the high incidence of pneumococcal infection among children with broad infecting serotypes made the subsequent generation more vulnerable. Furthermore, the serotype-specific protection for PCV-7 becomes lower than that in the developed countries (Pavia *et al.*, 2009). Thus, the use of PCV-7 elevates the concern of increased prevalence of pneumococcal infections with non-vaccine serotype (NVTs) and reduces the value of vaccination (Weinberger *et al.*, 2011).

Recently, PCV-7 is no longer used and is substituted with PCV-9 which includes two more capsular antigens (serotypes 1 and 5) and latterly PCV-13. PCV-13 was licensed in 2010 by the Food and Drug Administration (FDA) which includes 4 additional capsular antigens serotypes (3, 6A, 7F, and 19A).

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1.2.2 The pneumococcal polysaccharide vaccine (23-valent vaccine)

The polysaccharide vaccine is T-independent antigen vaccine which contains antigens for 23 pneumococcal serotypes. These serotypes are serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F. It provides protection against 85-90% of pneumococcal diseases affecting adults and children (Bruyn *et al.*, 1992), however it is not effective in children under 2 years of age, the group with the highest rate of pneumococcal infection. That is because this group of people response poorly to T-independent antigens. T-independent antigens are required to stimulate the response of B-lymphocytes but not T-lymphocytes, that results in an immune response which is neither long-lasting nor characterised by anamnestic response (Douglas *et al.*, 1983; Koskela *et al.*, 1986; Stein, 1992). Moreover, Polysaccharide vaccine is not effective against AOM infections because it has a little effect on nasopharyngeal carriage which is potentially high, particularly with otitis-born children (Rosén *et al.*, 1984; Faden *et al.*, 1991). The inefficiency of this vaccine derives from the inability of polysaccharide to induce T cell-dependent immune responses. This has led to the absence of memory associated with B cell responses, resulting in a relatively short period of protection (Poland, 1999).

The pneumococcal polysaccharide vaccine was licensed in the UK in 1989 and is currently used to vaccinate adults at high risk (Conaty *et al.*, 2004). In 2000, this vaccine was recommended for administration to people over 65 in the UK (Fedson and Liss, 2004).

Worldwide, PCV13 is used to immunise children with three doses recommended to be given at 6 weeks of infant age with a minimum of 4 weeks between subsequent doses (Russell F. *et al.*, 2011), whereas, the polysaccharide vaccine (23-valente vaccine) is

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used to immunise adults over 65 years and adults at high risk aged 19-64 years (CDC., 2010, 2012).

Neither the polysaccharide vaccine nor the conjugate vaccine gives protection against all pneumococcal serotypes. Therefore, there is an urgent need to investigate other strategies to control pneumococcal diseases.

1.3- Pneumococcal colonisation and clearance

Nasal carriage of pneumococci starts from early colonisation in children, as the nose is considered to be a reservoir of *S. pneumoniae*. Direct contact with the secretion of a colonised person causes the spread of nasal carriage from person to person. An understanding of pneumococcal colonisation enables the determination of host factors that can have an effect on pneumococcal colonisation and clearance. Pneumococcal carriage decreases dramatically after childhood, which might suggest a non-specific immune response during prior infection. This decrease of nasal carriage correlates with the presence of antibodies generated against pneumococcal surface protein A and capsule polysaccharides (McCool *et al.*, 2002).

Mucus secretions are the first line of defence that pneumococci encounter in the nasal cavity. However, the expression of the pneumococcal capsule plays an important role in diminishing entrapment in the mucus (Nelson *et al.*, 2007). Furthermore, the repulsion between capsule and mucus components might enable *S. pneumoniae* to adhere to epithelial cells. However, this adherence might potentially be inhibited during capsule expression (Cundell *et al.*, 1995; Kamerling, 2000) or alternatively might be inhibited by N-acetylglucosamine- β -(1, 3)-galactose (Andersson *et al.*, 1983).

Some adhesins are involved in pneumococcal attachment to host cells. One such adhesin is phosphorylcholine, which exploits its similarity to that possessed by platelets

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and attaches to the host cell via the platelet activating factor. The other is the choline binding protein A, which binds to human secretory components (Hammerschmidt et al., 1997).

Pneumococcal clearance may be avoided by the action of pneumococcal exoglycosidases (neuraminidase, β -N-acetylglucosaminidase and β -galactosidase). These enzymes change the structure of human glycol-conjugates by removing the terminal sugars, which are otherwise known to be involved in clearing of pneumococci from the nasal carriage (King et al., 2006).

1.4- Pneumococcal interaction with host immune response

Once the pneumococci bypass the mucus and adhere to the host's epithelial cells, they encounter other immune responses. Within the first three days, neutrophil proliferate and attack the bacteria leading to an inflammatory response (van Rossum *et al.*, 2005; Nelson *et al.*, 2007). The production of specific immunoglobulins with activated complement followed by phagocytosis generates host-mediate killing (McCool and Weiser, 2004). Studies have shown that CD4⁺ T cells are more important in the clearance of pneumococci than the humeral response. Interestingly, it was discovered that mice, which lack the Toll-like receptor 2 (TLR2) had prolonged pneumococcal carriage (Malley *et al.*, 2005). Some studies have shown that the CD4⁺ T cells, generated by an early host response, infiltrate to the site of infection. This infiltration is suggested to be induced by bacterial pneumolysin (PLY) as migratory CD4⁺ T cells are correlated with the presence of PLY in vitro. Moreover, CD4⁺ T cell chemotaxis was not stimulated when pneumolysin deficient pneumococci were used in the experiment (Kadioglu *et al.*, 2000; Kadioglu *et al.*, 2004). CD4⁺ T cells participate in antibody-independent immune responses, as mice which lack these cells fail to survive during the

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vaccination challenge, whereas, antibody-deficient mice survived. Furthermore, CD4⁺ T cells play a key role in pathogen clearance from the nasal cavity (van Rossum *et al.*, 2005). Recently, it has been found that natural CD4⁺ T cells occur in higher quantities in children and it is postulated that they might modulate nasopharyngeal carriage (Zhang *et al.*, 2007). Recently, it been found that released pneumolysin synergises with TLR agonists to induce the production of cytokines by dendritic cells and macrophages, however, in contrast with previous studies, it does not do that alone. Moreover, cytokine production including IFN- γ and IL-17A by splenocytes is promoted by PLY. PLY is also essential for pneumococci to promote the production of IFN- γ and IL-17A by natural killer cells and $\gamma\delta$ T cells, respectively in lungs following infection. Furthermore, activation of node-like receptor protein 3(NLRP3) is required for both PLY and IL-1 β and also required for protection against pneumococcal infection (McNeela *et al.*, 2010).

1.5- Pneumococcal virulence factors

Several antigens of pneumococci are implicated in pneumococcal infection. Some of these factors are responsible for colonisation and adherence, while others are responsible for interaction with the host cells. These factors are:

1.5.1- Capsule

The capsule is the most important factor determining invasion. It is thought to help in the colonisation of the pathogen by enabling the bacteria to resist phagocytosis, i.e. it interferes with the binding of complement C3 to the cell wall, which results in bacterial survival and proliferation inside the infected tissue with consequential tissue damage (Tonnaer *et al.*, 2006). The capsular polysaccharide determines the virulence of the pneumococci. Species that express more capsular polysaccharide and less teichoic acid

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are potent inducers of inflammation, while those expressing more teichoic acid and less capsular polysaccharide have increased ability to adhere to epithelial cells (Long et al., 2003).

1.5.2- Pneumolysin (PLY)

Pneumolysin is a 52 kDa protein which exhibits cytolytic activity against cells with cholesterol-containing membranes (Boulnois et al., 1991). It belongs to the family of cholesterol-dependant cytolysin which includes streptolysin O and perfringolysin O expressed by *Streptococcus pyogenes* and *Clostridium perfringens*, respectively. The toxin is an exogenous product and requires another protein to be released from pneumococci, autolysin which breaks the peptide cross-linking of the peptidoglycan in cell wall, this breakage in peptidoglycan results in the formation of holes in the cell wall facilitating the release of pneumolysin (Whatmore et al., 1999).

PLY is thought to interact with Toll-like receptor 4 (TLR4) as the activation of TLR4 was observed by a pneumococcal mutant that produced PLY in the absence of haemolytic activity (Malley et al., 2003). However, the interaction of TLR4 with PLY is controversial as some studies confirmed that pneumolysin does not activate TLR4 (Tweten, 2005). As described in (section 1.4), PLY has not been found to induce the production of cytokines by dendritic cells (DCs) and macrophages alone but it does that in a synergy with TLR agonists. It has also been found that protection against *S. pneumoniae* requires the production of IL-1 β by PLY which is NLRP3-dependant (McNeela et al., 2010).

Due to increasing levels of pneumococcal resistance to antibiotics, the serotype replacement against vaccines makes these vaccines not able to give a thorough protection against all *S. pneumoniae* serotypes for all different human ages, different

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strategies of therapy are required to control and treat the infections of this bacterium such as phage therapy.

1.6 General Characteristic of Bacteriophages

Bacteriophages are a group of viruses which are able to infect bacteria. They consist of heads with or without a tail. The head is a protein capsid that takes one of three shapes: icosahedral, filamentous or prolate-shaped; this may be surrounded by an envelope (Ackermann, 2006). There are 14 recognised families of phages, comprising different morphologies and different genetic material (Maniloff and Ackermann, 1998). The genetic material of phages is either single or double stranded DNA or RNA located in the head. However, most bacteriophages have double stranded DNA and belong to one of three families *Podoviridae*, *Myoviridae* and *Siphoviridae* (figure 1.1).

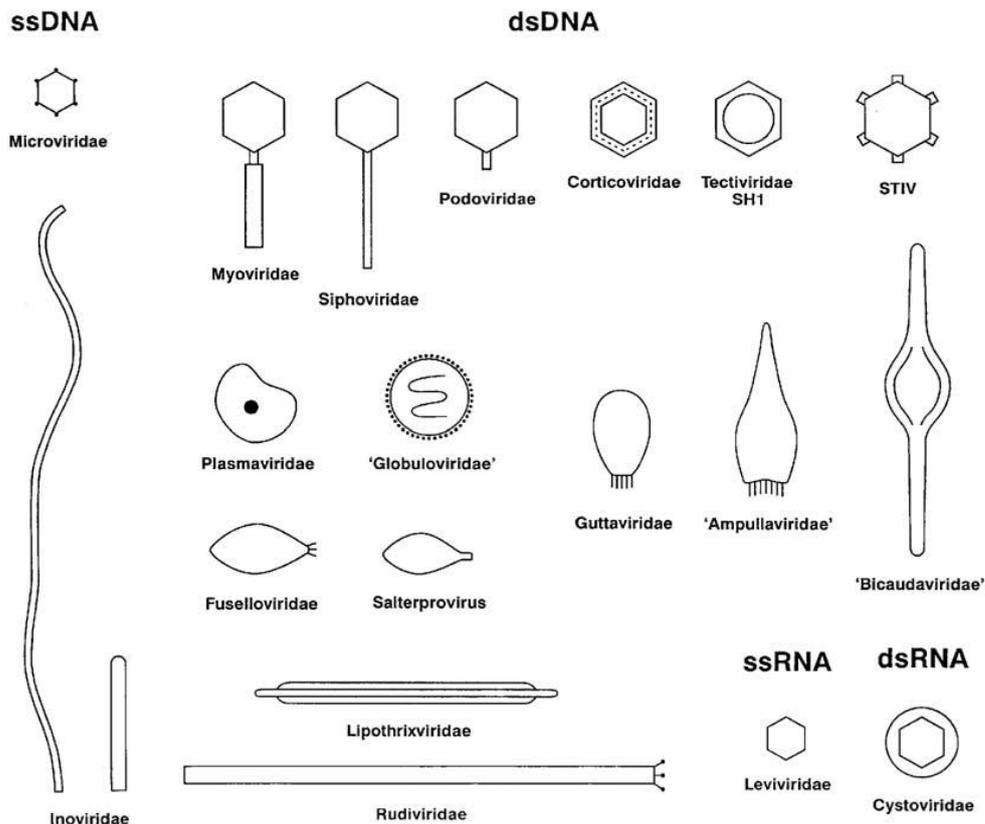


Figure 1.1: Different morphologies of bacteriophages (Ackermann, 2003).

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1.6.1 Phage Abundance and Distribution

Bacteriophages are found where their host cells are found, and they are therefore distributed in a variety of environments. Viruses are the most abundant microorganisms on Earth (Bergh et al., 1989). In aquatic environments, viruses are 10-20 times higher in number than bacteria, with abundances ranging from 10^4 to 10^8 viruses ml⁻¹ (Weinbauer, 2004a). It has been reported that the abundance of viruses decreases gradually from coastal to offshore water (Frank and Moebus, 1987). Cyanophages have the highest viral number of 9.6×10^8 viruses/ml⁻¹ in an aquatic environment (Hennes and Suttle, 1995). The abundance of viruses is higher in fresh water than marine system (Maranger and Bird, 1995). The number of viruses varies in oxic and anoxic water layers, it has been reported that in the eutrophic Plußsee viral abundance is significantly higher in anoxic than oxic water layers, however, viral abundance is more higher in O₂/H₂S interface (Weinbauer and Höfle, 1998). Furthermore, studies have shown that the number of viruses increases during and after the period of phytoplankton bloom and that attributed to the high abundance of bacteria after that incident (Bratbak *et al.*, 1990; Hennes and Simon, 1995).

Viruses are also abundant in soil ranging from 10^8 to 10^9 viruses per gram dry weight of soil, this range of abundance is slightly higher in wetter forest soil, and is lower in managed agricultural soil (Williamson et al., 2005). The depth of soil has an influence on viral abundance. It has been reported that the viral number decreases with sediment depth in marine system, however, the abundance of benthic viruses is 10-1000 times higher than that in water that overlying on this sediment (Steward et al., 1996). The sediment of sea water is suggested to be the reservoir of *Synechococcus* phages as well as the phages of algae *Heterosigma akashiwo* (Suttle, 2000). The number of tailed

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phages on earth is estimated at 10³¹, with a variable population in microbial communities (Jarling et al., 2004).

The ratio between viruses and bacteria was used to indicate the relationship between them (Wommack and Colwell, 2000). Viruses to bacteria ratio (VBR) in marine pelagic water is 5-10 whereas it is low as about 0.01-1.2 in organic matter collected from sediment traps and this low value might be attributed to strong viral decay during storage (Haral *et al.*, 1996; Taylor *et al.*, 2003) VBR is low in lake and river water reporting ratio of 1-4 (Maranger and Bird, 1996). Although, the number of viruses in anoxic layer is more than that for oxic water layer, VBR is lower in anoxic lakes and that might refer to the organic matter which resulted in more bacterial productivity (Maranger and Bird, 1996). Generally, VBR in Limnetic system is typically higher than in marine pelagic system. There are some factors were suggested to be causing this variability in VBR between these types of water such as the increase dependence of freshwater bacteria on allochthonous material in association with the contribution of cyanobacteria (Maranger and Bird, 1995). The viral and bacterial ration in soil is reported as low as 0.04 for all tested rhizosphere soil samples (Ashelford et al., 2003).

The distribution of bacteriophages such as those infecting picophytoplankton *M. pusilla* and cyanophages was reported (Moebus and Nattkemper, 1983; Zhong *et al.*, 2002). There are some reports have shown the geographical restricted distribution of bacteriophages in ocean (Wichels et al., 2002). The lack of information on the distribution of viral species resulted in very limited knowledge on their affiliation as ubiquitous or endemic species. The use of approaches such as denaturing gradient gel electrophoresis (DGGE), sequencing and Quantitative PCR (qPCR) or (real time PCR) facilitated the quantifying the abundance of phage and certainly increase the knowledge of viral biogeography (Gruber *et al.*, 2001; Weinbauer, 2004a). In addition to these

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approaches, some other techniques such as monoclonal antibodies, DNA analysis, enzyme-based detection of bacteriophages in their situ, specific primers or probes to detect phage genomes within their hosts (Blasco *et al.*, 1998; Chibani Azaïez *et al.*, 1998; Fuller *et al.*, 1998; Puig *et al.*, 2000).

1.6.2 Classification of Phage

Félix d'Hérelle, who gave bacteriophage this name, described bacteriophages as one bacteriophage with many races, the Bacteriophagum intestinale (d'Hérelle, 1918). In 1943, the first phage classification was proposed by Ernst Ruska who distinguished three morphological types of phages (Ruska, 1943). In 1948, Holmes subordinated phages within the order Virales (Holmes, 1948). Viral order included one genus and 46 species based on host range. In 1962, phages were classified based on their nucleic acid type (DNA or RNA), capsid shape, presence or absence of an envelope, and number of capsomers by Lwoff, Horne, and Tournier (Lwoff *et al.*, 1962). They placed tailed phages in an individual order and named as Urovirales.

In 1971, the International Committee on Taxonomy of Viruses (ICTV) classified bacteriophages into six groups based on their morphology using electron microscope, T4, λ , ϕ X174, MS2, T7 and fd. Currently, The ICTV classifies phages into 14 families (Figure 1) and 37 genera with different virions (Fauquet *et al.*, 2005). These virions have binary, cubic or helical symmetry. Phages with binary symmetry are tailed, giving them a unique morphology amongst viruses as a whole. This group of phages are named as *Caudovirales* which possess double stranded DNA. This group of bacteriophages includes *Myoviridae*, *Siphoviridae* and *Podoviridae*. (Ackermann, 2009).

The “phonetic” classification used by the ICTV was not satisfied by taxonomist (Lawrence *et al.*, 2002), thus suggested the use of another criterion to phage

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classification such as the type of genetic material, however, genetic evolution during phage infection enabled the horizontal gene transfer among them, tailed phages particularly created an in this such approach as appears as a net of entities representing a complex with criss-cross pattern of relationship (Hendrix et al., 1999). The type of genetic material is not suitable for distinguishing tailed phages as all have dsDNA genomes (Ackermann, 2009).

An alternative approach to phage classification is to use the phage proteomic tree. In this approach, phage genomes in a tree- and distance generating computer programs are compared together to generate phage taxonomy (Rohwer and Edwards, 2002). However, the use of this approach resulted in inconsistency classification as two different phages were placed in the same clade such as the tectivirus PRD1 grouped with ϕ 29 – a member of the *Podoviridae*; and, phages λ (*Siphoviridae*) and P22 (*Podoviridae*) (Ackermann, 2009).

A Step-Wise procedure is the common used approach for phage classification. In this approach the phage morphology is considered first as it classify tailed phages into *Myoviridae*, *Siphoviridae* and *Podoviridae*. Then tailed phages will be investigated depending on any desirable properties such as the molecular weight of the DNA or any other criterion could be applied in the lab (Ackermann, 2009).

1.6.3 Phage Life Cycles

Phages attach to host cells via different receptors, such as lipopolysaccharide, teichoic acid, polysaccharide, and cell components like surface proteins, flagella and pilli. Once attached, phage genetic material is injected into the cell and the phage then enters one of two life cycles; lytic and temperate (Figure 1.2). The lytic cycle leads to the production of new phage progenies and cell lysis. This lysis is manipulated by different

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mechanisms according to phage type; phages with double-stranded nucleic acid have a precise time-programmed holin-endolysin- mechanism. Holin is a protein that forms holes in the cell wall as well as activating the endolysin enzyme which causes cell lysis. In contrast, phages with single stranded DNA or RNA induce the production of a single protein called murine hydrolase. This protein causes cell lysis. Filamentous phages have the ability to produce new progeny which can be released through cell walls without causing lysis (Young et al., 2000).

The lytic cycles mentioned previously are the most common. However phage infection has been further classified into six “lower-level” categories. These include the following; (1) lytic infection, which leads to cell lysis and phage release, (2) chronic infection which allows the virions to be released without any impact on host cells, (3) lysogeny infection, which leads to phage genome incorporation into host cell chromosomes and replication within it but with no virions released, (4) pseudo-lysogeny infection, which involves no phage genome replication, but the phage enhances the ability of other phages to replicate and to be released from host cells, (5) phage restriction infection which is considered as anti-phage immunity (involving the loss of phages but not of the bacteria) and (6) phage abortive infections, which involve loss of both phages and their host cells (Abedon, 2008).

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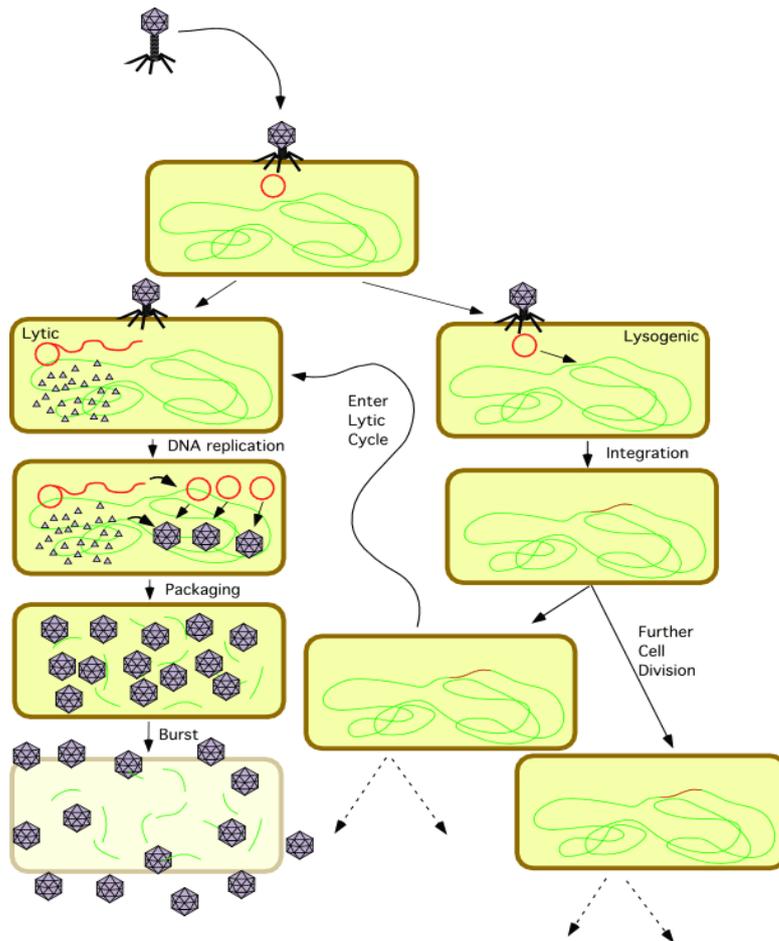


Figure 1.2: Bacteriophage life cycles.

(http://inst.bact.wisc.edu/inst/images/book_3/chapter_9/9-1.gif).

1.6.4 Latent period and burst size

The latent period describes the period from when the phage has entered the host cell to the time of releasing new phage progeny. It includes the eclipse period which describes the time when the phage DNA takes over the host biosynthetic machinery and phage specific proteins have been made. The number of phage progeny released from each individual cell is called the burst size (Ackermann, 1998); (Weinbauer, 2004b). All different periods of phage infection are as shown in the Figure 1.3.

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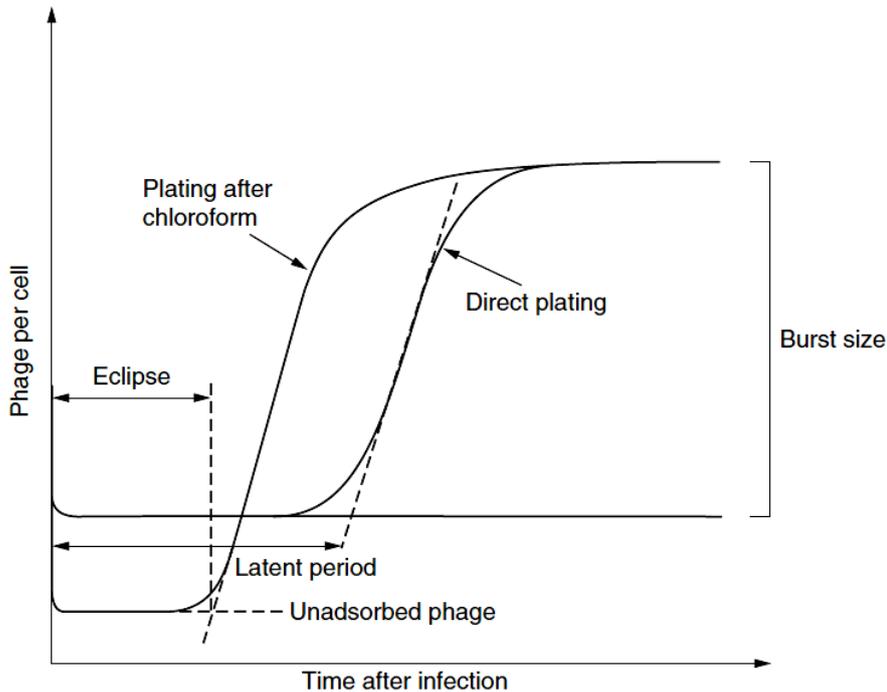


Figure 1.3: different periods of phage life cycle (Gachechiladze K. 2005)

Both the latent period and burst size determine the phage proliferation rate. Many factors play a role in determining the length of the latent period, including the species of phage, type of infected cells and their physiological condition, culture media and temperature (Ackermann and DuBow, 1987 ; Weinbauer, 2004a).

The number of phage progeny (burst size) varies depending on the bacterial strains and other environmental factors. These factors are the amount of nutrients in the culture media, which leads to efficient bacterial growth and consequently a high proliferation rate of phage progeny (Weinbauer, 2004b). The size of host cells can effect the number of phage progeny. It has been found that large cells produce a larger number of phages compared with smaller ones (Weinbauer and Peduzzi, 1994).

1.6.5 Lysogeny

The extent lysogeny of among bacterial strains is variable as some bacteria do not carry prophages and some other bacteria contain multiple prophages in their chromosome

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such as *E. Coli* 0157:H7, this bacterial strain contains 18 prophage genome elements which represent 16% of the total genome count (Ohnishi et al., 2001). It has been found that many prophages from pathogenic bacteria encode virulence factors such as phages gamma and C1 in the chromosome of *Corynebacterium diphtheriae* and *Clostridium botulinum*, respectively (Freeman, 1951; Barksdale and Arden, 1974). This property of lysogenic phages allowed researchers to identify specific prophages in a large set of related bacterial strain using molecular techniques such as microarray and PCR (Canchaya et al., 2003).

Prophage carriage in pneumococci is relatively high. Some studies have shown that 58.3% of pneumococci are lysogenic while others have indicated 42% lysogeny among tested *S. pneumoniae* strains. These findings rely on plaque formation using mitomycin C induced lysates (Bernheimer, 1979). Recent studies have used PCR-based assays to assess prophage carriage. These studies have been based on the hybridisation of pneumococcal phage DNA with autolysin encoding gene (*lytA*). The PCR-based assays were undertaken on 791 pneumococcal isolates obtained from The Rockefeller University collection. The study has shown that 76% of these isolates are lysogenic strains (Ramirez et al., 1999). Romero et al. (2009) reported prophage carriage in 108 clinical samples, using both mitomycin C induction and PCR-based assay. Their observation showed that prophage carriage in tested isolates using the aforementioned techniques was 40% and 50%, respectively.

There are different induction agents which might lead to the induction of lysogenic phages. However, some antibiotics, such as mitomycin C, and UV radiation are the most common and powerful agents that can be used (Ackermann and DuBow, 1987).

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1.7 Phage Therapy

Phage therapy is a strategy that has been suggested for treating bacterial infection in both man and animals (Jado et al., 2003). Bacteriophages are extremely specific to infect and kill their host bacterial species. This is in contrast to antibiotics which effect the human bacterial microflora (Bartlett, 2002). Moreover, phage therapy eliminates the incidence of secondary infection that might occur due to the clearance of normal flora; a problem that arises with use of antibiotics (Carl et al, 2006).

The discovery of bacteriophages dates to observations by Ernest Hankin in 1896. He noticed that an undefined substance gave a protection against *Vibrio cholera* in India. The same phenomenon was noticed in Russia when observing activity against *Bacillus subtilis*. Two decades later, Fredrick Twort reported the same observation and attributed the phenomenon to a virus. However, he did not pursue his work on them due to financial reasons (Sulakvelidze *et al.*, 2001). Following this, Felix Hubert d’Hérelle observed bacteriophages in 1910 in Mexico; he used bacterium-free filtrate and *Shigella* for his immunisation study to observe clear areas on bacterial lawn. These areas were called taches, taches vierges and finally plaques. He also identified *Shigella* phages in France during Maisons-Laffitte outbreak in 1916. D’Herelle was the first one who proposed the name “bacteriophage” based on the Greek “bacteria” and “phagein”, meaning eat. *Shigella* phage was used to treat *Shigella dysentery* in rabbits (Summers, 1999).

The first trial of phage therapy was carried out by d’Herelle in association with paediatrician Victor-Henri Hutinel. They ingested the *Shigella* phage preparation with some of his interns, to confirm the safety of the preparation. The same phage was then given to a 12 year old boy with severe *Shigella dysentery*. The patient was cured completely after a couple of days. The phage treatment regime was repeated again with

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another patient who recovered after one day with only a single dose (Summers, 1999).

Richard Bruynoghe and Joseph Maisin published the results of the first phage therapy trial in 1921; they treated opened lesions with *Streptococcus* phage by direct injection in and around the wound (Sulakvelidze *et al.*, 2001).

Phages have also been used commercially as antibacterial therapeutic agents in many places in Europe and the United States (Larkum N., 1932; Merril *et al.*, 2003). Phage suspension was used to treat 550 cases of bacterial infections in Poland between 1981 and 1986. These infections were caused by different bacteria (*Streptococcus*, *Klebsiella*, *Escherichia*, *Proteus* and *Pseudomonas*) that were found to be unresponsive to antibiotic therapy. The results showed that 92.4% of treated patients were cured, and the remaining 6.9% experienced an improvement in their condition. In contrast, 0.7% of cases showed that phage therapy was ineffective (Slopek *et al.*, 1987).

However, following antibiotic discovery the use of bacteriophages was discontinued in many countries. Despite this some western European countries and the Soviet Union continued their work on phages. Two institutes in Europe are commercially using phages as therapeutic agents: Eliava Institute of Bacteriophage, Microbiology and Virology (EIBMV) and Hirszfield Institute of Immunology and Experimental Therapy (HIET). They were founded in Georgia in 1923 and in Poland in 1952, respectively.

1.8 Pneumococcal Phages

The isolation of lytic and temperate pneumococcal phages has been reported previously (McDonnell *et al.*, 1975; Ronda *et al.*, 1981; Diaz *et al.*, 1992; Gindreau *et al.*, 2000). Recently, four pneumococcal phages have been isolated and characterised. These include temperate phages; MM1 and EJ-1 and lytic phages; Cp-1 and Dp-1. These phages were isolated from different strains of *S. pneumoniae* (López and García, 2004;

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López, 2004), all details of their isolation will be discussed later. Pneumococcal phages have also been isolated from the nasopharynx, where pneumococci typically colonise (McDonnell et al., 1975). Therefore, swabs of throat and sputum samples might represent an ideal localisation for lytic pneumococcal phages.

1.8.1- Temperate pneumococcal phages

The phage EJ-1 belongs to *Myoviridae* morphotype phages which possess a 42-kb linear DNA. The first induction of this phage was in 1992 by Diaz et al. based on the pneumococcal strain 101/87. This phage, in contrast with other pneumococcal phages, does not have a terminal protein linked to the DNA. It has the gene encoding choline-dependent lytic enzyme (N-acetylmuramyl-L-alanine amidase), which is similar to pneumococcal autolysin. The insertion region of this phage has been found near to the lytic enzyme gene. Increasingly, phage EJ-1 was not shown to have any lytic activity on the pneumococcal strain studied (wild-type R6 (Rockefeller University), M31 (AlytA), CP1000 (lacking DpnI and DpnII), and 101/87 (Diaz et al., 1992).

The phage MM1 was induced by Gindreau et al in 2000 from antibiotic-resistant Spanish/American 23F *S. pneumoniae*, it is Siphovirus with 40-kb double-stranded genome. MM1 has a DNA-protein complex that is capable of becoming integrated into the pneumococcal chromosome (Gindreau et al., 2000). The lysogeny with MM1 reduces the speed of pneumococcal growth; however, it causes significant improvement in cell adhesion to plastic and human cells making pneumococci much more virulent. Furthermore, the choice of lysogenic or lytic pathways can be influenced by the growth stage of the bacteria. The search for lysogeny may play a role in transparency selection. In addition, it was found that the lytic cycle of MM1 might be favoured over the lysogenic in the opaque-phase phenotype (Loeffler and Fischetti, 2006).

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Temperate phage HB-3 is a siphovirus induced from pneumococcal strain HB-3 in 1990 (Romero et al., 1990a). The genome of HB-3 shares the same property of phage MM1 as DNA of both of them linked with protein used for integration into pneumococcal chromosome (López and García, 2004). This phage has not been sequenced, therefore, the genetic characterisation unknown. However, the phage has been found codes for amidase which shares many biochemical and immunological properties with that one of its host (Romero et al., 1990b).

Phage VO1 is a temperate bacteriophage of the type 19A multidrug-resistant epidemic 8249 strain of *S. pneumoniae*. There is a similarity between this phage and phage MM1 in their specific integration site, protein composition, restriction patterns, and molecular dissection of the lytic system. In contrast with other temperate pneumococcal phages, the LytA amidase coded by VO1 is significantly different from that of the host bacterium. In addition, the genome has a gene putatively coding for a C5 methyltransferase. Despite of high similarity between this phage and MM1 and HB-3, the viability in genes those encode for LytA amidase and C5 methyltransferase suggests a horizontal transfer and lysogenic state co-evolution (Obregon et al., 2003b).

1.8.2 Lytic pneumococcal phages

Two lytic phages of *S. pneumoniae* have been isolated previously: Diplophage 1 (Dp-1) and complutense phage (Cp-1).

1.8.2.1 Diplophage Dp-1

Dp-1 was isolated from the throat swab of a patient with upper respiratory infection in 1974; belonging to the *Siphoviridae* family, it consists of a polyhedral head, tail and base plate (McDonnell *et al.*, 1975; Varea *et al.*, 2004). Phage morphology is shown in Figure 1.4. The isolation and storage of this phage presents some difficulties, such as

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low titre and poor stability during storage. It also requires murein hydrolase activity to be released from infected cells (Ronda-Lain et al., 1977) .

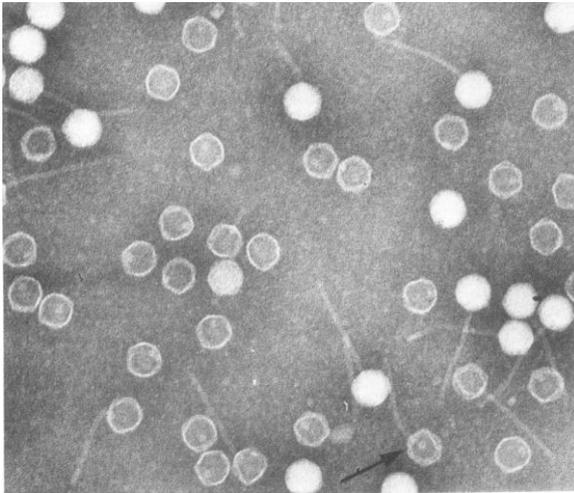


Figure 1.4: Dp-1 morphology under E-microscope (Lopez et al., 1977).

Unlike other pneumococcal phages, Dp-1 lytic enzyme (Pal) has a different N-terminal domain. The C-terminal domain of Pal contains a choline binding module (ChBM) which is homologous to the same domain of lytic enzyme produced by the host bacteria. ChBM attaches to the choline residues of the bacterial envelope. When choline has been replaced by ethanolamine (EA) or monoethylamino ethylamine (MEA) and dioethylamino ethylamine (DEA), the pneumococci show complete resistance to the phage (McDonnell *et al.*, 1975; Varea *et al.*, 2004). The use of Dp-1 to treat the infection of pneumococci is not appropriate as this phage is not able to infect invasive capsulated *S. pneumoniae* (Bernheimer and Tiraby, 1976) Thus, lytic enzyme which encoded by this phage was suggested to be used alternatively (Sheehan et al., 1997).

The Pal amidase (pal-1) has been shown to have the ability to kill 15 clinical strains (serotypes 1, 2, 3, 4, 5, 6, 9v, 10, 14, 15, 18, 19F, 23F and 24) and two mutants (R36A and Lyt 4-4) of *S. pneumoniae* in vitro. It also prevents and minimises nasopharyngeal

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carriage in mice (Loeffler et al., 2001). Pal-1 led to the effective protection and treatment of murein mice with bacteraemia (Loeffler and Fischetti, 2003). However, using whole phages as therapeutical agent seems much preferable than using lytic enzymes because phages propagate and release to invade new cells. Thus one or two doses will be adequate for treatment, whereas using lytic enzymes as treatment requires several doses and that over the permissible limits that indicated by human and animal acts.

1.8.2.2 Complutense phage Cp1

Cp-1 is a podovirus, which has an irregular hexagonal flat-ended end head, with a size of 60 by 45 nm, a short tail 20 nm long and 15 nm thick and a fibrous head (Figure 1.5). The first isolation of this phage was from a throat swab of healthy children in 1981; it produced very broad clearance compared with previously isolated pneumococcal phages; however, its production was low due to the low burst size (Ronda et al., 1981).

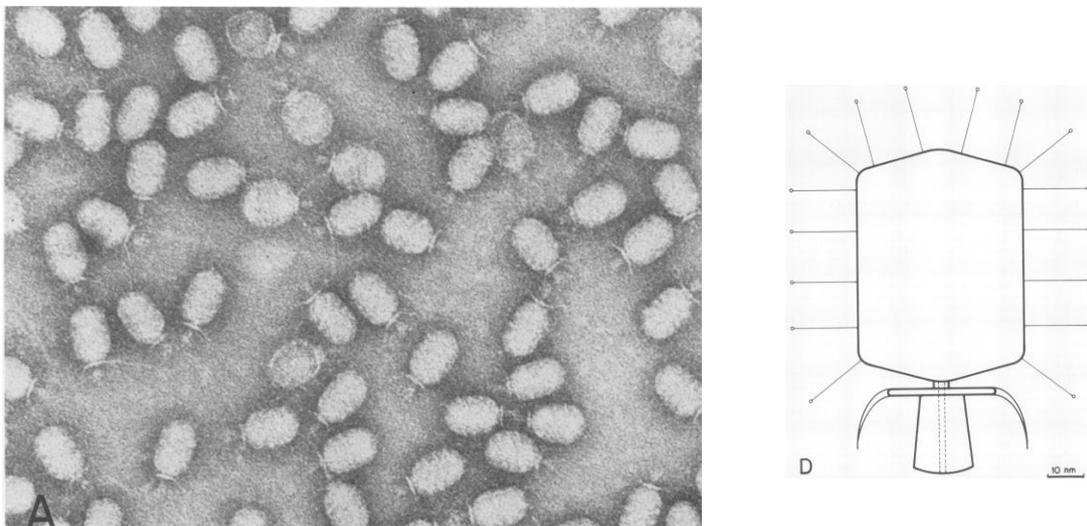


Figure 1.5: Electron micrograph and schematic drawing show the morphology of phage Cp-1 (Ronda et al., 1981).

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Genetic material of phage Cp-1 incorporates a double stranded DNA of about 19 kb, the terminal protein of this bacteriophage is linked to linear DNA of the 5' end through a threonine residue (Martín et al., 1995). The complete genomic sequence of Cp-1 revealed the similarity between this phage and *Bacillus* phage (ϕ 29); what is more, they have a similarity in their requirement for a protein-priming mechanism for initiation of their replication (Salas, 1991). This phage has the same problem of phage Dp-1, it lacks consistent stability and the ability to infect capsulated pneumococci, therefore, phage lytic enzyme Cpl-1 was used as alternative therapeutical agent to control and treat the infection of pneumococci.

Phage lytic enzyme Cpl-1 activity was determined using animal models. This enzyme has two terminal domains, the N-terminal domain which contains catalytic activity against the major bond of peptidoglycan, and the choline-terminal domain which binds specifically to a certain subjects (mainly carbohydrates) (Lopez *et al.*, 1997; Navarre *et al.*, 1999). Grandgirard et al (2008) demonstrated the ability of Cp1-1 to kill *S. pneumoniae* (serotype 3) in Wistar rats with meningitis. They discovered that a single dose of Cpl-1 injected intracisternally reduced the bacterial count to below the detection limit 30 minutes after injection. They also discovered that a dose of 30 U of Cpl-1 for 30 seconds and 10 minutes decreased the colony forming units from 10^7 to 10^3 in both incubation periods. Mice with bacteraemia were protected by an intravenous dose of Cpl-1 (2 μ g), the viable count of *S. pneumoniae* was decreased within 15 minutes from log of $10^{4.70}$ cfu/ml⁻¹ to undetectable levels ($<\log_{10} 2.00$ cfu/ml⁻¹) (Loeffler et al., 2003).

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1.9 General comparison of pneumococcal phage genomes

The lytic pneumococcal phages Cp-1 and Dp-1, and two temperate phages MM-1 and EJ-1 have been sequenced previously. Some genes from the genome of these phages have been functionally characterised and others have been described as hypothetical. The genomic length of phages Cp-1, Dp-1, MM1 and EJ-1 are 19,343 bp, 56,506 bp, 40,248 and 42,935 bp respectively (Martin *et al.*, 1996; Pelletier *et al.*, 2000; Obregon *et al.*, 2003a; Romero *et al.*, 2004). The genomic map of the four pneumococcal phages is shown in Figure 1.6. Temperate phage VO1 has been sequenced and characterized (Obregon *et al.*, 2003b), however, data is not accessible.

1.9.1 Lytic pneumococcal phage genomes

Phage Dp-1 genome has an organisation that falls into four clusters: replication, packaging, structural and lytic. That arrangement is similar to the genome organisation of lactic acid bacteria lytic phages (Martín *et al.*, 1995; Brussow, 2001). Two remarkable features in the genome of this phage are that eleven ORFs are responsible for the transcription of leftward and five genes (orf1 to orf5) which encode proteins involved in the synthesis of 6-pyruvoyltetrahydropterin; these include GTP cyclohydrolase and synthase (orf 5 and orf 3 respectively). The organisation of all mentioned genes appears to be implicated on the forming the genome structural unit (Garcia *et al.*, 1979).

The genome of Cp-1 is a double stranded DNA with a terminal protein covalently linked to its 5' end and a 236-bp perfect inverted repeat (Martín *et al.*, 1995). Two stages are involved in the transcription of this phage genome; the first is the early expression of those genes that are involved in DNA replication; while the other genes are those encoded for the structural components of the phage particle, morphogenesis, and host

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lysis. The first promoter is located in orf1 and is followed by two promoters located between orf2 and orf3; these promoters direct the transcription of early genes (Martín *et al.*, 1995; Martin *et al.*, 1996). Although, phage Cp-1 has a similarity with phage ϕ 29 having a short RNA that is regulating a particular stage in its replication, there is no sequence similarity between them. Phage Cp-1 shares a similarity with *Bacillus* phage GA-1 and B103 in terms of sequence. However, the location of promoter is different, in *Bacillus* and *Pseudomonas* phages, the promoter is located at the far-left end of their genomes; whereas, it is located at the right end in phage Cp-1 (Martin *et al.*, 1996; López and García, 2004). Phage Cp-1 holds a terminal protein-priming that is used as the initiation for replication. This protein is directed by the third free amine terminus from the 3'-end of the template molecule. A stepwise sliding back mechanism accounts for the maintenance of Cp-1 DNA ends (Meijer *et al.*, 2001).

1.9.2 Temperate pneumococcal phage genomes

Temperate phage MM1 is similar to lytic phage Cp-1 in the peculiarity of containing covalently linked protein. Phage MM1 has circularly permuted and terminally redundant DNA. The genome of this phage is organised into five major clusters starting with the gene encoding for the integrase (Romero *et al.*, 1990c; Obregon *et al.*, 2003a). The first five leftmost genes of the genome are the lysogenic cluster, which encodes protein C1. This protein is a repressor for the elongation of those transcripts that are mediated by promoters PR and PL (Obregón *et al.*, 2003). The phage MM1 genome has only one of inverted clusters of genes in its DNA which represents the lysogeny cluster (Obregon *et al.*, 2003a).

Bacteriophage EJ-1 was described as the first myovirus that could infect a low G+C content Gram-positive bacterium. Bacteriophage EJ-1 genomic organisation falls into

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this cluster; lysogeny, DNA replication, regulation, packaging, and head morphogenesis protein clusters are those similar to siphovirus infecting lactic acid bacteria. It has been found that those genes that are encoded for the putative tail protein (orf53 to orf64) are similar to those in the genome of *Bacillus subtilis* myovirus PBSX. Orf 56 is the only Orf encoded for tail protein similar to the that one produced by Gram-negative bacteria (Romero et al., 2004).

EJ-1 shares a similarity in several genes which belong to lysogeny, replication and regulation clusters with *S. pyogenes* SF370.3 defective prophage (Romero *et al.*, 2004). One more substantial property of the genome of this phage is that the gene encoding excisionase is remote from the genes coding integrase; this is in contrast with other temperate phages for both Gram-positive and Gram-negative bacteria (Bruttin et al., 1997).

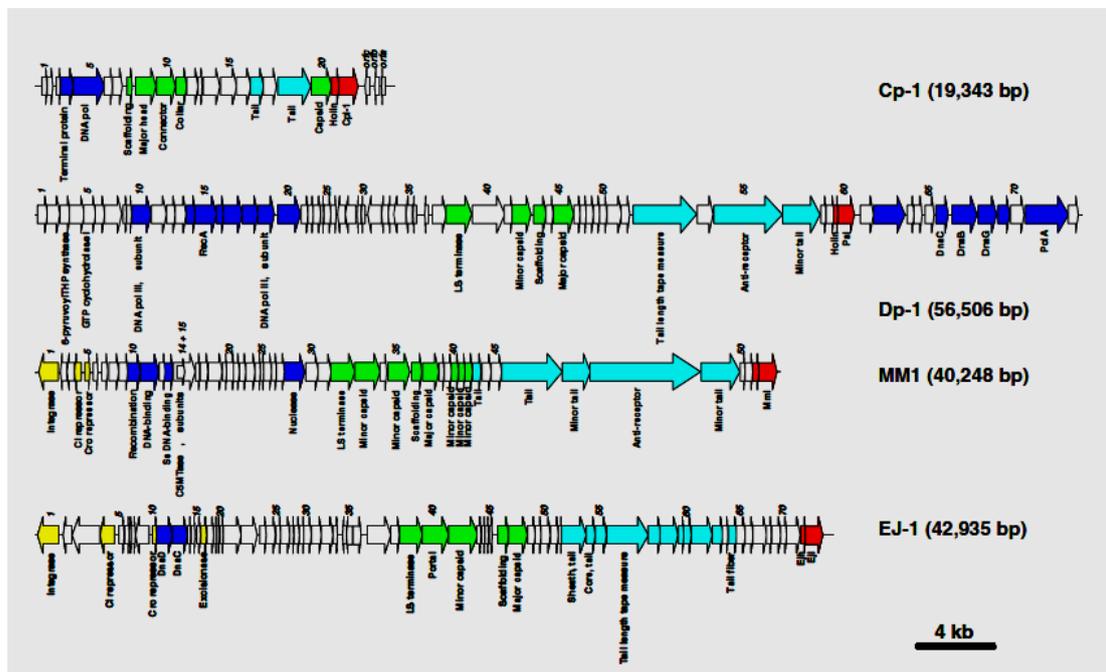


Figure 1.6: Schematic representation of the genomes of lytic pneumococcal phage Cp-1, Dp-1 and temperate MM1, and EJ-1 (López and García, 2004).

Yellow, dark blue, green, light blue and red arrows correspond to genes involved in lysogeny regulation, DNA replication, packaging and head-assembly, tail morphogenesis, and lysis of the host cell, respectively.

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1.10 Thesis aims and outlines

This study aims to address the isolation and characterisation of new lytic *S. pneumoniae* bacteriophages, which can infect capsulated strains. Pneumococcal lytic phages (whole phages and their lytic enzymes) are useful for the treatment of pneumococcal infections, due to their ability to kill bacteria *in vitro* (both in liquid and solid media) (McDonnell *et al.*, 1975; Grandgirard *et al.*, 2008). In this study lytic pneumococcal phages will be isolated and whole phages will be used as therapeutic agents in mice models, which have not been done before. The project will lead to an understanding of the interaction between pneumococcal phages and the treated animal. Prior to animal treatment, isolated phages will be characterised based on their physiological properties, such as their burst size and latent periods. One of the aims of this project will be to establish morphology with an electron microscope and genome size by using pulsed field gel electrophoresis.

Chapter 2 focuses on the different materials and methods used in this study, explaining the strains of pneumococcal used in the isolation and the sources of isolated phages. It also highlights the preparations which were assayed, based on the collected samples (phage sources), and the different techniques involved in the isolation. All the procedures which enable us to image isolated phages can assist us in explaining the interaction between phages and their host cells *in vitro* and *in vivo*, and those involving the genetic characterisation of the phage genome are identified in this chapter. **Chapter 3** aims to isolate lytic phages and to induce temperate phages and characterise them. Therefore, this chapter explains in detail the processes of the isolation of lytic pneumococcal phages. It shows the different biological characteristics of isolated

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phages; including their behaviour on bacterial lawns, propagation, purification, morphology, classification and the different stages of infection.

Chapter 4 aims to detect the interaction between isolated phages SP-QS1 and their host cells *in vitro*. This chapter is crucial to indicate the ability of SP-QS1 to decrease the viable count of pneumococci facilitating further animal experiment. The growth curve of infected different pneumococcal serotypes with bacteriophage SP-QS1 is presented in this chapter. Furthermore, the phage SP-QS1 growth curve is constructed to demonstrate the ability of this phage to propagate and to determine the final phage titre.

Chapter 5 concentrates on the most important part of the thesis, which is the behaviour of both *S. pneumoniae* serotype 2 (D39) and phage SP-QS1 towards each other in the mouse model. The chapter aims to indicate the ability of SP-QS1 to cause pneumococcal killing and reducing its viable count *in vivo*. Mouse survival was suspected in this particular experiment. Two different mouse models of infection were involved in this chapter; intranasal infection and sepsis infection. The survival rate of the infected mice treated with this phage was measured and is reported here as well.

Chapter 6 presents the genomic characterisation of phage SP-QS1. This chapter aims to characterise phage genome, elucidate the type of phage and predict an important genes such as gene encoded for toxin and proteins which involved in capsule degradation. The phage DNA was extracted and following this, the genome of this phage was sequenced using new generation sequencing-454 pyrosequencing technique. Genes were predicted for the sequenced DNA and annotated to detect their functional proteins. Data collected from annotation was used to build phylogenetic trees of genes of interest to deduce the evolutionary history of this phage.

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Finally, **Chapter 7** provides a conclusion to the thesis overall, and this is presented in view of the possible prospects for future related work.

Chapter 2: Materials and methods

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2.1 Bacteria

Seven strains of *S. pneumoniae* were used in this project: D39 and its mutants R36 and R6, serotype 3, serotype 4, 6B 35A, 23F and 19F. These serotypes are all virulent strains that can cause invasive infections, with the exception of serotype D39 and its mutant R36 (non-capsulated), which are laboratory strains. All used strains were provided from our laboratory beads collections stocks.

2.2 Culture media

2.2.1 Blood agar

To prepare blood agar, 39 g of Columbian agar base was added to one litre of distilled water. The mixture was sterilised by autoclaving for 15 minutes at 121°C. The medium was cooled to 50°C and then 5% horse blood was added and then shaken to obtain homogeneous mixture. The medium was poured out into plates which were left until the medium turned solid and then inverted and stored at 4°C.

2.2.2 Brain heart infusion broth (BHIB) and solid medium

The amount indicated by the manufacturer (37 g) was added to one litre of distilled water. The pH of the broth was adjusted to 7.4 and then the medium autoclaved at 121°C for 15 minutes. The same instructions of broth preparation were followed to prepare solid medium with the addition of 1.5% agar before autoclaving. The medium was poured out into plates which were left until the medium turned solid and then inverted and stored at 4°C.

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2.2.3 Modified BHI broth and solid medium

Modified BHI media was prepared using the same instruction for normal BHI. In modified BHI, the broth was filtered (ftc) and the pH was adjusted to 7.4 by Tris-HCl. Then the broth was autoclaved and supplemented 5 ng of choline chloride. 1.5% agar was added to modified BHI base before autoclaving. The medium was poured out into plates which were left until the medium turned solid and then inverted and stored at 4°C.

2.2.4 Soft top agar

The top sloppy agar was prepared using modified BHI broth and 0.4% agar and supplemented with 0.4% glycine.

2.2.5 C medium

C medium consists of 0.5% protease, 1.5% yeast extract, 10 mM K_2HPO_4 , 0.4 mM $MgSO_4$ and 17 mM NaCl. Agar was added to this media in both base layer (1.5%) and top layer (0.4%).

2.2.6 K-Cat medium and Na-Cat medium

K-Cat medium is a modified Cat medium in which sodium is replaced with potassium. The broth is a combination of 0.1% yeast extract, 1% casitone, 0.5% tryptone, 0.5 % NaCl, 10 ml 20% glucose and 30 ml 0.5M K_2HPO_4 . Na-Cat medium consists of the same components but contains 30 ml 0.5 M Na_2HPO_4 instead of K_2HPO_4 . Agar was added a different percentage (0.4% and 0.6%).

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2.2.7 SM medium

SM medium was prepared from SM buffer with the addition of 5 ml of 2% gelatine and 1.5% agar in the base and 0.4% of agar in the sloppy top agar.

2.2.8 LEM broth

LEM broth contains 10 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl and 10 ml 1M MgSO₄.

2.3 Solutions

All solutions were prepared according to the ingredients listed below.

2.3.1 SM buffer (pH 7.5)

Ingredients	Amounts per liter
NaCl	5.8 g
MgSO ₄	2 g
0.5 M Tris-HCl	5 ml
Water to	1000 ml

2.3.2 Tris-acetate (TAE) buffer (50x)

Ingredients	Amounts per liter
Tris	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH8.0)	100 ml
Water to	1000 ml

2.3.3 TE buffer

Ingredients	Amounts per liter
1M Tris-Cl [pH 8.0]	10 ml
0.5 M EDTA	2 ml
Water to	1000 ml
Water to	1000 ml

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2.3.4 Phosphate buffer saline (PBS)

Ingredients	Amounts per liter
NaCl	8 g
Na ₂ HPO ₄	1.44 ml
KHPO ₄	0.24 g
KCl	0.2 g
Water to	1000 ml

2.3.5 Lysis buffer

Ingredients	Amounts per liter
1 M Tris-Cl	20 ml
10% SDS	20 ml
0.5 M EDTA	40 ml
Water to	1000 ml

2.3.6 Sputum liquefying buffer

100 mM NaCl, 8 mM MgSO₄.7H₂O and 50 mM Tris-HCl pH 7.5.

2.3.7 Sodium dodecyl sulphate (SDS) (10% wt/vol)

100 g of SDS was added to one liter of distilled water.

2.4 Bacterial methods

2.4.1 Culturing and confirmation of *S. pneumoniae*

For all pneumococcal strains, bacteria were grown from beads collection stock in 10 ml of brain Heart Infusion (BHI) broth and incubated overnight at 37°C. The following day, they were centrifuged at 1750 × g for 15 minutes. The pellets were re-suspended in 1 ml of BHI-serum broth (20% of foetal calf serum). 700 µl from the re-suspended pellets for each serotype was added to 10 ml of BHI-serum broth, which was then incubated at 37°C. One ml of this suspension was immediately used to measure absorbance using a Jenway® 6305

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UV/Vis spectrophotometer (Bibby Scientific Limited). The initial absorbance of the culture was about 0.2. The absorbance of the culture was measured every hour at a wavelength of 500. Incubation was continued until growth reached an exponential phase, obtained when an absorbance of ~ 1.6 nm was achieved. When this absorbance rate had been obtained, the growth suspensions were aliquoted into 500 μ l and were stored at -80°C .

To confirm the identity of *S. pneumoniae*, several biochemical tests were performed. These included growing on blood agar, gram staining, the catalase test and the Optochin sensitivity test. All these tests are described in appendix 1.

2.4.2 Animal passage pneumococci preparation

An aliquot of non-passaged pneumococci stock stored at -80°C was thawed and then streaked for isolation on a blood agar plate. Plates were incubated in a CO_2 gas jar overnight at 37°C . A sweep of colonies was inoculated into 10 ml BHI broth in a Universal Tube with a tight cap. The culture tube was incubated statically overnight at 37°C . After incubation, the tube was centrifuged at $1500 \times g$ for 15 minutes and the supernatant discarded using a sterile Pasteur pipette. The pellet was re-suspended in 5 ml sterile PBS (to give an OD_{500} of 1.4-1.6) and was kept at room temperature. The viability of the culture was confirmed as not being less than cfu of $1 \times 10^7/\text{ml}^{-1}$.

100 μ l of pneumococci suspension was injected intraperitoneally into two MF1 mice (Harlan) aged ~ 9 weeks using a 0.5 ml fine insulin syringe. After 22-28 hours, it is expected that the animals should be at least 2+ Starey (wild type only). If these disease signs are not reached within this time, this process should be repeated again. The mice were anaesthetised with 5% (v/v) inhaled anaesthetic (isoflurane) at a rate of 1.6-1.8 L

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O₂/minute in an anaesthetic box. A cardiac puncture blood sample was extracted using a syringe with a 23G needle. Without allowing recovery from anaesthesia, the animal was killed by dislocating its neck. Death was confirmed by the absence of eye and joint reflexes. 50 µl of blood from the cardiac puncture was inoculated into 10 ml of BHI broth in a Universal Tube with a tight cap. Inoculated culture tubes were incubated statically for 16-20 hours at 37°C. After incubation, the cloudy suspension was removed with minimal disturbance to the loose sediment of red blood cells. The suspension was centrifuged at 1500 × g for 15 minutes and the supernatant discarded using a sterile Pasteur pipette. The pellet was re-suspended in 1 ml BHI serum broth, which consists of 80% (v/v) BHI broth and 20% (v/v) filtered foetal calf serum. 700 µl of the re-suspended pellet was added to 10 ml of fresh BHI serum broth. The OD₅₀₀ of the suspension should be around 0.70 and was checked to see if this was the case. The culture tube was incubated statically at 37°C for five hours until the OD₅₀₀ of the culture reached 1.6. 500 µl of this culture was aliquoted into sterile cryotubes, labelled and saved at -80°C. Once aliquots had been stored at -80°C for 24 hours, a single aliquot was thawed and tested for viable count, which should not be less than 1 × 10⁷ cfu/ml⁻¹. Streaking pneumococci on blood agar was used to confirm their growth. Optochin discs were used in the whole study to confirm that the existing bacterium was *S. pneumoniae*.

2.4.3 Standard Manual and Automated Growth Curve

The growth curve allows the determination of when pneumococci will reach the exponential phase. It was carried out based on readings of the absorption assayed during the preparation of aliquots. These were recorded and used to determine the growth curves for

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each serotype. An automated growth curve was carried out using the Carioca Flash 2041 micro-plate (96-well Nucleon U-bottomed) reader (Thermo Scientific). From overnight growth, the culture was centrifuged at $1500 \times g$ for 15 minutes. The pellet was re-suspended in 1 ml BHI-serum. From this suspension, 10 μ l was added to 10 ml of BHI-serum in a Universal Tube. 100 μ l of this mixture was placed in a well. All serotypes were placed in a row with five duplicates in a column. In the first column, a blank was added (100 μ l of BHI serum broth). The plate then was placed into the plate reader and the machine was set up to measure the absorption (OD_{500}) every 30 minutes for 20 hours. After 20 hours, the data was collected and analysed to plot the growth curve for each strain.

2.4.4 Quantification of *S. pneumoniae*

This process is necessary to enumerate colony-forming units in stored aliquots using the Miles-Misra assay (Miles and Misra, 1938). Shortly after 24 hours of storage at -80°C , serial dilutions (ten-fold) from thawed aliquots of all tested serotypes were prepared to a dilution of 10^{-6} . Dilutions were duplicated for each serotype aliquot. The blood agar plate was divided into six sections. Three drops of 20 μ l of each dilution were spotted over each section. The previous process was assessed on two plates and repeated for all dilutions and all serotypes. All plates were incubated in CO_2 gas jar at 37°C overnight. The following day, the dilution containing the optimum numbers of colonies (~ 200 colonies) was counted and the viable count was calculated using the following formula:

$$\text{CFU per ml} = \text{Mean number of colonies in sector} \times \text{Dilution} \times 1000/60.$$

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2.4.5 Establishment of a confluent lawn of bacteria

Bacterial lawns are essential for further experimental processes as both spot tests and plaque assays depend on a good quality confluent lawn of culture. Lawns which are too thin can affect the appearance of putative plaques. In thin lawns, plaques become hard to from natural break, whilst thick lawns are a consequence of bacterial overgrowth on plaques, so plaques may be overgrown. 0.5% blood agar plates were prepared as a base layer for the bacterial lawn. From overnight growth of re-suspended pellets, 700 µl was added to 10 ml of BHI-serum broth, which was then incubated at 37°C for five hours. At the end of the incubation period, 500 µl of exponential growth was added to 3 ml of 0.4% sloppy agar (3.7 g BHI broth base and 0.4 g of agar). The mixture was then poured and distributed all over the plate and left on the bench to set. The same procedure was applied using a BHI agar base (1.5% agar) with BHI sloppy agar (0.6% agar). Plates were incubated in CO₂ jar overnight.

2.5 Phage Methods

2.5.1 Spot tests

Spot tests were the initial tests used to isolate phages from the samples in this study. The spot test procedure was performed as described by Armon and Kott (1993). Briefly, confluent lawns of host serotypes were made from 500 µl of exponential cells and 100 µl of catalase (10^3 to 2×10^3 units per plate) on the base agar plates. Once lawns were made and the agar set, 10 to 20 µl of processed samples was spotted on the top of pneumococcal lawns. Spotted plates were left on the bench to dry and then incubated overnight at 30°C or at 37°C using aerobic and an-aerobic conditions. Plates were observed the following day for

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the presence of zones of killing, which indicates phage lytic activity. The cleared patches were scraped and put in an Eppendorf tube containing 1 ml of SM buffer and stored overnight at 4°C. The Eppendorf was centrifuged at $600 \times g$ for 10 minutes the following day to precipitate host bacteria and agar. The supernatant was collected and filtered using a 0.22 μm pore size filter. The filtrate was then used to repeat the spot assay and plaque assay.

2.5.2 Plaque Assays

A plaque assay is the confirmatory test used to determine the presence of phages after their initial inhibition in the spot assays (Adams, 1952). It is used to obtain single plaques to distinguish phages from each other, as each single plaque should be originated from a single phage. This assay is also used to obtain highly purified stock phages following the phage being made clonal.

To isolate phages from collected samples, culture was grown as described in (2.1.3). From this culture, 500 μl of exponential cells were first dispensed in Bijou tubes in addition to 100 μl of catalase (10^3 to 2×10^3 units per tube) to neutralise the amount of H_2O_2 produced. 20 μl of the putative phage containing sample was added to bacteria cells and catalase in addition to 3 ml of sloppy agar. The tube containing the mixture was rolled between the palms of the hands for 20 seconds to distribute the cells and phages in agar and then poured on the top of the base agar. When the agar set, the plate was incubated overnight at 30°C or at 37°C using aerobic and an-aerobic conditions. Plates were observed the following day for the presence of single plaques.

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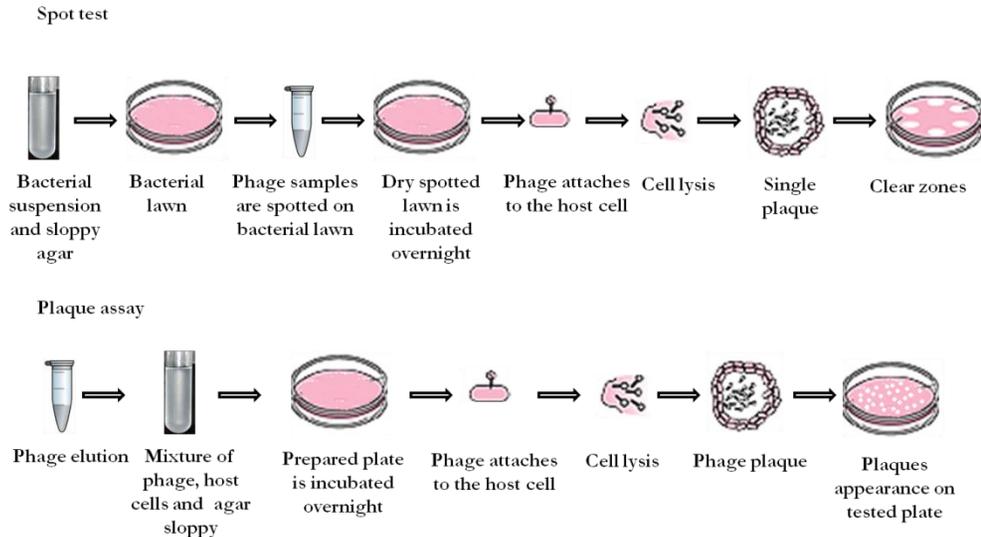


Figure 2.1: Spot test and plaque assay.

After obtaining single plaques, a single plaque was transferred individually in a bijou tube with 1 ml of BHI and incubated for two hours at 37°C. Serial ten-fold dilutions from this phage elution were made. A volume of 1 ml of each dilution was used a subsequent plaque assay in the same technique as that described above, however, plates this time were incubated overnight at 30°C. These processes were repeated several times to ensure that the phage was clonal. Isolated phages were saved at 4°C for further processes.

2.5.3 Temperate pneumococcal phage induction

5 ml of fresh BHI broth growth media was inoculated with 50 µl of an overnight culture of *S. pneumoniae* strains. The initial absorbance was measured at 500 nm and incubated at 37°C for 30 min. Mitomycin C or norfloxacin was added to a final concentration of 0.1 µg/ml. The absorbance was measured at 500 nm each hour for 6–8 hours until a decrease of the optical density was observed. The culture was centrifuged at 3000 × g for 12 minutes at 4°C. The supernatant was neutralized to pH 7.0 with 0.1 M NaOH. The supernatants were

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filtered through a membrane filter of 0.22 µm pore size. The sterile supernatant was stored at 4°C.

2.5.4 Lytic pneumococcal phage isolation

Different environmental samples (258 samples) were used as sources of the pneumococcal phage isolation. These samples include volunteer and clinical samples and tissues of mice that had been infected with invasive *S. pneumoniae*. These samples are shown in Table 2.1. All collected samples were tested by spot test and plaque assay techniques.

2.5.4.1 Sample Collection for phage isolation

a) Samples from healthy volunteer

Despite the fact that pneumococci are cleared from the nasal carriage of most adults, some people still have pneumococci in their nasal cavity. Samples were collected from healthy children and adults. As the geographical distribution of pneumococci varies around the world, 42 samples were collected from the UK and 12 from Saudi Arabia. Screened volunteers included healthy toddlers, infant and adults. Some adults had handled *S. pneumoniae* in their laboratories for at least one year.

Volunteer sampling involved two different samples; throat and nasal swabs. Throat swabs were collected using wooden sterile swabs and wooden tongue depressor. The wooden depressor was used to push the tongue down and then swab was inserted in volunteer's mouth all the way down to the throat. Throat was swabbed from different areas and pulled out and repacked into its tube. Nasal swabs were collected using flexible metal swabs that enable the movement through the nasal cavity without causing any damaging in attached tissues. Nasal swab was inserted throw the nasal cavity until reaching the nasopharynx and

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rotated gently for few seconds, and then pulled out and repacked. Collected swabs were stored in the refrigerator for further experiments.

B) Clinical samples

Clinical samples were collected from patients who were suspected to either carry pneumococci, such as chronic pulmonary disease patients or patients suffering from chest infections. A total of 19 samples were collected from Leicester Royal Infirmary (UK). A total of 153 samples were collected from four different hospitals in the cities of Taif, Jeddah, Abha and Al-Qunfidah in Saudi Arabia (Figure 2.2). Saudi Arabia has a high prevalence of pneumonia despite the obligate vaccination (Memish *et al.*, 2000), and in addition, the ease of seeking ethical permission for clinical sample collection provided an opportunity to collect more samples.



Figure 2.2: Map of Saudi Arabia, highlighting the collection sites of clinical samples.

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c) Tissues samples from infected mice with invasive pneumococci

Mice were infected with invasive *S. pneumoniae* serotype D39 in a final concentration of 1×10^6 cfu/ml⁻¹ and left for 48 hours. Lungs and spleens of these infected mice were collected at the end of the infection period and used for pneumococcal phage isolation.

Table 2.1: Number and collection sites of collected samples

Site of collection	Throat/nasal swab	Sputum	Pulmonary fluid	Mouse lung	Mouse spleen
UK clinical	—	19	—	16	16
UK volunteer	42	—	—	—	—
Saudi clinical	45	94	14	—	—
Saudi volunteer	12	—	—	—	—
Total	99	113	14	16	16
	258				

2.5.4.2 Sample preparation

Collected samples were processed using different procedures according to their type to make them suitable for further tests.

Swabs and 0.5 ml of sputum samples were added to 1 ml of SM buffer immediately or after overnight incubation with exponential phase growth of different pneumococcal strains. Sputum was also processed using a different procedure as described by Tejedor et al (1982). In brief, 1 ml of sputum was suspended into 7 ml of sputum manipulation buffer. The sample was centrifuged at $10,000 \times g$ for 30 minutes to remove bacteria and insoluble

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material. The clear supernatant was then spun at $100,000 \times g$ for 8 to 12 hours to pellet the phage particles. The top fluid overlying the small pellet was carefully removed and the pellet resuspended in 0.5 ml of SM buffer supplemented by 0.01% gelatine. Phages were assayed either by spot tests or plaque assays using dilutions of the resuspended pellet onto lawns of *S. pneumoniae* serotypes.

All samples were cultured on blood agar plates to isolate *S. pneumoniae*. Swabs were all incubated with BHI broth overnight to increase the chance of pneumococcal recovery. Plates were incubated in CO₂ gas jar at 37°C overnight.

The lungs and spleens of infected mice were collected at the end of the infection period and used for pneumococcal phage isolation. Tissue samples were collected in a universal tube containing 10 ml of SM buffer and were homogenised using an Ultra-Turrax T8b homogeniser (Sigma Aldrich). All homogenised samples were stored in the refrigerator overnight to allow the release of phages from tissues. They were centrifuged ($1750 \times g$ for 15 minutes) the following day and the supernatants filtered with 0.22 µm pore size filters. The filtrate was tested for phage isolation using both spot tests and plaque assays.

Recovery of *S. pneumoniae* was carried out by streaking all collected samples on a blood agar plate and incubating them in CO₂ gas jar at 37°C overnight.

2.5.4.3 Optimisation of appropriate phage isolation conditions

The isolation of bacteriophages requires optimisation, including cultivation conditions, the type of culture media and the micronutrients that are essential for bacteriophage isolation. It is worth mentioning that due to the ability of *S. pneumoniae* to produce hydrogen dioxide (H₂O₂) in culture media, catalase (10^3 to 2×10^3 units per plate) was added to the sloppy

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agar layer to neutralise the amount of bacterial produced hydrogen dioxide. All media described in section 2.2 were used in the optimisation to indicate the best growth conditions and appropriate media for the isolation. The optimisation also includes the addition of different chemicals such as 5 M magnesium chloride and 1 M calcium chloride to both media bases and soft top agar. Two different incubation conditions were used in the isolation: micro-aerophilic (using CO₂ gas jar) and aerobic atmosphere. The incubation temperature varied from 30°C to 37°C to maximise the chance of phage isolation.

2.5.5 Phage amplification

2.5.5.1 Plate amplification of phages

This assay is useful and appropriate for phages which can produce single plaques on bacterial lawns. In this type of propagation, the plates which developed plaques were either flooded with 3 ml of SM buffer or scraped in universal tubes containing 5 ml of the SM buffer. Both flooded and scraped plates were stored at 4°C overnight. Plates and tubes were soaked and shaken, respectively, at regular intervals during their storage. Phage elution was collected by Pasture pipette from flooded plates and then centrifuged at $1750 \times g$ for 15 minutes. Phages were harvested from scraped plaques by centrifugation under the same conditions mentioned above. This process is assumed to give a higher phage titre each cycle. Serial dilutions (tenfold) were made from phage elution and the same procedure was repeated until the titre of 10^{10} pfu/ ml⁻¹ of the phage was obtained. Phage stock was stored at 4°C for further processes.

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2.5.5.2 Liquid amplification of phages

This type of propagation is the only available method to propagate phages that do not form single plaques on plates. The zone of killing caused by phages from the spot test was placed into 100 ml of exponential phase growth culture of pneumococci. After three hours of incubation, 200 μ l of pre-warmed broth media (BHI, C-medium or K-cat medium) was added to the same culture. Following overnight incubation, the culture was centrifuged at $1750 \times g$ for 15 minutes. The supernatant was filtered with 0.22 μ m pore size filters and stored at 4°C for further processes, such as spot testing and PEG purification.

2.5.6 Phage concentration using polyethylene glycol (PEG)

This purification protocol was used for both liquid culture and single plaque propagated phages. In this assay, purified phages (2×10^7 pfu/ml⁻¹) were added to 5 ml of overnight culture of pneumococci in a bottle containing 500 ml of BHI (fct). The mixture was incubated at 37°C overnight. The bottle was centrifuged at $3300 \times g$ for 15 minutes the following day and supernatant collected in a new bottle. Sodium chloride was added in a final concentration of 1M and placed on ice for one hour. The debris was separated by centrifugation at $3300 \times g$ for 15 minutes. Polyethylene glycol was added at a final concentration of 10% to the supernatant and incubated at 4°C overnight when the PEG had completely dissolved. The PEG-phage complex was harvested after centrifugation at $3300 \times g$ for 20 minutes. The supernatant was discarded and the tube inverted to allow residual solution and PEG to drain from the pellet. The pellet was gently resuspended in 1 ml SM buffer and saved at 4°C until needed. The pellet is sometimes difficult to resuspend, and

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then was therefore refrigerated overnight with SM buffer and then resuspended using 1 ml Gilson pipette.

2.5.7 Phage purification

2.5.7.1 Phage particle purification by isopycnic centrifugation in a caesium chloride gradient (CsCl)

This protocol is used to make highly pure phage preparations. After obtaining the PEG precipitated lysate, a gradient technique was set up using three different concentrations of caesium chloride: 1.3 g/ml (40.41 g of CsCl in 100 ml of phage buffer), 1.5 g/ml (67.48 g of CsCl in 100 ml of phage buffer) and 1.7 g/ml (94.29 g of CsCl in 100 ml of phage buffer). 3 ml of each concentration was added to a 12 ml soft ultra-clear tube, starting with the highest CsCl concentration at the bottom. 2 ml of phage lysate was added at the top of these gradients and centrifuged in TH641 rotor for 20 hours at $114688 \times g$ at 4°C. The phage band was collected by needle and syringe and dialysed three times using one litre SM buffer for eight hours at 4°C for each. The phage preparation was stored at 4°C.

2.5.7.2 Phage particle purification by high performance liquid chromatography (HPLC)

High performance liquid chromatography an approach commonly used for protein purification was used to purify phage particles. Phages have charged residues which can bind to stationary phases in the presence of an appropriate solvent. Two different concentrations of SM buffer (100 mM and 2 M) were used as solvents. The low salt solvent was used to enhance the attachment of phage SP-QS1 to the column, and the salt concentrated buffer was used to liberate this attached phage to form the stationary phase.

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Phage lysate was injected into the HPLC apparatus at a final concentration of 10^{-7} for two hours. Different fractions were collected at different retention times according to the peaks shown on the graph. Purified phage elution was filtered with 0.22 μm pore size filter and assayed for plaque assays to determine the phage titre.

2.5.8 Transmission electron microscopy

Phages were concentrated from the lysate using PEG precipitate and then purified by isopycnic centrifugation in a caesium chloride gradient. Following these preparations, phages were examined under a TEM microscope using the negative staining procedure.

For negative staining, EM grids were treated using an Emitech K100X glow discharger (EM Technologies Ltd) to make them hydrophobic. Each grid was handled with tweezers in an inverted position. Each sample was handled with different tweezers to avoid cross-contamination. 3-5 μl of the phage specimen was added to the grid – shiny side up - and then left for five minutes. The excess sample was removed using Whatman filter paper. The grids were washed twice using distilled deionised water. Uranyl acetate (1% w/v) dye was used to stain the grid for 45 seconds and the excess of the stain was then immediately removed with filter paper. The grid was then ready to be viewed on the JEOL 1400 TEM with an accelerating voltage of 80 KB. Images were captured using Olympus soft imaging system on a Megaview III digital camera with iTEM software (Olympus).

2.5.9 One-step growth curve

Prior to the application of one-step growth, phage SP-QS1 titre was determined using plaque assay. One-step growth was then carried out using a phage multiplicity of infection (MIO) of 0.1. MOI is calculated using the following:

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MOI= the number of phages/the number of bacterial cells

The one-step growth technique described by Ellis and Delbrück (1939) was used with some modifications. In short, 9 ml of exponential phase culture cells was added to 1 ml of phage lysate in universal tubes. 1 ml of Phosphate-Buffered Saline (PBS) was added to the controls. The mixture was incubated at 37°C for 15 minutes. The mixture was then centrifuged at $1750 \times g$ for 10 minutes. The pellets were resuspended into 10 ml of pre-warmed (fct) BHI-serum medium. The suspension was dispensed into ten Bijou tubes and incubated at 37°C. One tube was assayed for plaque assays for 10 minutes, 20 minutes, 30 minutes, 40 minutes, one hour, two hours and three hours. PFUs were calculated each time and the results compared. The phage growth curve was drawn using GraphPad prism5 software.

2.5.10 Phage in vitro impact on infected host cells and their growth curve

This experiment was carried out manually using phage SP-QS1 with different pneumococcal serotypes, including D39, serotype 3 and 4, 23F, 6B and 19F. To detect the optimal ratio of phages and pneumococci for rapid bacterial clearance, different MOIs were examined. In universal tubes, 9 ml of exponential phase growth of pneumococci at a final concentration of 1×10^6 cfu/ml⁻¹ was mixed with 1 ml of phage SP-QS1 lysate at a final concentrations of 1×10^6 , 1×10^7 and 1×10^8 pfu/ml⁻¹ (MOI of 0.1, 1 and 10, respectively) for 15 minutes at 37°C. The only serotype that was examined using these three different MOI was D39, whereas the rest of serotypes were tested with MOI of 10 only. 1 ml of PBS was added to the controls.

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Pneumococcal cultures were centrifuged at $1750 \times g$ for 10 minutes. The pellets were resuspended into 10 ml of pre-warmed (fct) BHI-serum medium. The suspensions were incubated at 37°C for five hours. The optical density, colony-forming units and phage-forming units were measured every hour until the end of the incubation period. Processed plates for the cfu count were incubated overnight at 37°C in a CO_2 gas jar while those processed for pfu count were aerobically incubated overnight at 30°C . Data were collected the following day and analysed using the Excel and Prism programmes.

To confirm the results obtained from this experiment, the interaction of phage SP-QS1 with each pneumococcal serotype was tested in triplicate and repeated twice.

2.5.11 Pneumococcal phage interaction with *S. pneumoniae* in vivo

The ability of phage SP-QS1 to clear *S. pneumoniae* was tested to maximise the possibility of using it for phage therapy. Two different in vivo experiments were assayed to detect the behaviour of the phage toward pneumococci, including intranasal and sepsis infections.

2.5.11.1 Intranasal model of infection

Phage stock was used to run five individual experiments. 25 female MF1 mice (Harlan) with the age of 9 weeks were challenged with $50 \mu\text{l}$ pneumococci via the intranasal route at a final concentration of 1×10^6 (cfu/ $50 \mu\text{l}^{-1}$). Mice were divided into two groups: control and treated mice. Ten control mice received $50 \mu\text{l}$ of BHI post-dose after one and five hours intranasally, while ten phage-treated mice received $50 \mu\text{l}$ of phage lysate instead (pfu/ $50 \mu\text{l}^{-1}$). Both BHI and phage lysate were administrated twice; after one hour and five hours from the beginning of bacterial challenge. The final concentration of used phage was 1×10^7

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(pfu/50 μl^{-1}) in all experiments, giving MOI of 10. The rest of mice (n: 5) were injected with phage lysate only.

The plaque-forming units and bacterial colony forming units were determined before and after their injection in mice. Three mice from each group (controls and treated mice) were culled at time zero and their lungs and nasopharynx collected, homogenised and processed for the colony-forming unit count. All mice involved in the experiment were assayed using the pain scoring scheme (Morton, 1985) regularly to determine the severity of infection over time. The score sheet used is shown in Appendix 2.

After 24 hours, five control mice and five treated mice were culled and their lungs and nasopharynx collected. Cardiac puncture blood was also extracted from all the mice. The remaining mice were subjected for tail bleed to determine the presence of bacteria in their blood and were then left for a further 24 hours. Blood was collected from mouse tail in 0.5 ml Eppendorf tubes containing 1 μl heparin to avoid clotting. Nasopharynx and lung samples were homogenised and these samples in addition to the blood samples were assessed for colony-forming unit count to determine the viable count of *S. pneumoniae*. The results of this part of the experiment were compared with the results of the pain score and the 48 hour results.

After 48 hours from the onset of infection, the remaining control and tested mice were culled and their blood, lungs and nasopharynx collected and processed for viable counts. All data from this experiment was analysed with t-test to calculate both standard deviation and P- value using GraphPad prism5 software.

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2.5.11.2 Sepsis model of infection

A fresh stock of phage was used to run this experiment. Twelve female MF1 mice (Harlan) were challenged with 50 µl pneumococci via the intravenous route at a final concentration of 1×10^6 (cfu/50 µl). Six control mice received 50 µl of BHI post-dose after one, three, and six hours intravenously, while six phage-treated mice received 50 µl of phage lysate (phage/50 µl) instead. This led to a MOI of 10, which should be consistent with the intranasal model of infection.

The phage and bacterial titres were determined before and immediately after (approximately 20 minutes) their injection in mice. All mice involved in the experiment were assayed for pain scoring regularly to determine the severity of infection over time.

All tested mice (controls and treated mice) were assayed for tail bleeding six hours after the beginning of the bacterial infection. Blood samples were collected in 0.5 ml Eppendorf tubes containing 1 µl heparin to avoid clotting and then processed for the bacterial titre determination. The same process was repeated again after 24 hours to evaluate the severity of infection after this time of incubation.

After 48 hours, the remaining mice were assayed for cardiac puncture and then culled to collect their nasopharynx and lungs samples. The viable count of the bacteria in the collected tissues and blood was tested. All data from this experiment was analysed with t-test to calculate standard deviation and P value using GraphPad prism5 software.

2.5.11.3 Survival experiment

This experiment was carried out to determine the effect of using phage SP-QS1 to prolong the period of mouse resistance to pneumococcal infection. The procedure used in this

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experiment is similar to that used for the intranasal model of infection (2.10.2). However, in this procedure mice were checked and scored without taking any samples. To briefly summarise, female MF1 mice (Harlan) were challenged with 50 µl pneumococci via the intranasal route in a final concentration of 1×10^6 (phage/50 µl). Mice were divided into two groups: control and treated mice. Ten control mice received 50 µl of BHI post dose twice after one and five hours intranasally, while ten phage-treated mice received 50 µl of phage lysate (phage/50 µl) instead. Mice were checked and scored regularly for 48 hours. Data was processed and analysed in GraphPad prism5 software using Kaplan-Meier survival analysis.

2.6 Genetic characterisation of the phage SP-QS1 genome

2.6.1 Assaying phage DNA by pulse field gel electrophoresis (PFGE)

PFGE is used to determine the size of phage genomes. DNase and RNase (Sigma) were added to phage lysate at a final concentration of 14 µg/ml and 30 µg/ml respectively, and incubated at 37°C for one hour or preferably overnight. 2% low melting point agarose (SeaPlaque® GTG® Agarose) was prepared in $0.5 \times$ TBE. An equal volume of 40 µl of agarose and processed phage lysate were mixed in a 1.5 ml Eppendorf and transferred to the gel mould, which was then refrigerated for at least 30 minutes or kept for two hours at room temperature. Each agarose plug was placed in an Eppendorf tube containing 1 ml of lysis buffer. The Eppendorfs were left in a water bath at 55°C overnight. Plugs were washed three times in the following day with $1 \times$ TE buffer. 200 ml of 1% pulse field certified agarose was prepared in $0.5 \times$ TBE. Once the gel was set, marker and sample plugs were inserted into wells. Bio-Red CHEF of the PFGE was rinsed with ultrapure water

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and then with $0.5 \times$ TBE. Two to three litres of $0.5 \times$ TBE was added to the tank and the gel correctly positioned. The bands were separated at 6 volts/cm at 14°C for 17 hours. The gel then was examined using the transilluminator (EuroSciCon) to visualise the DNA bands (Lingohr *et al.*, 2008).

2.6.2 Phage DNA Extraction

High quality and quantity DNA is needed for molecular analysis. In order to achieve this, the PEG purified phage lysate must be completely free from bacterial chromosomal DNA. Removing chromosomal DNA was achieved by the adding of DNase (14 mg/ ml) (Sigma) and RNase (30 mg/ ml) (Sigma) to phage lysate overnight at 4°C . To extract the DNA, an equal volume of phenol was added to the processed phage lysate for two minutes. The mixture was centrifuged for 10 minutes at $21000 \times g$ and the aqueous layer was then transferred to another tube, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) was added to this and given two minutes before being centrifuged for 10 minutes at $21000 \times g$. The aqueous layer was extracted with an equal volume of chloroform:isoamyl alcohol (24:1 v/v) and left to set for two minutes and then centrifuged for 10 minutes at $21000 \times g$. After centrifugation, the aqueous layer was added to 0.4 volumes of 7.5 M ammonium acetate and two volumes of isopropanol and then left on ice for one hour. It was then centrifuged at $21000 \times g$ for 20 minutes at 4°C . The resulting DNA pellet was briefly air-dried and washed once with $500 \mu\text{l}$ of 70% (v/v) ethanol and centrifuged at $21000 \times g$ for 20 minutes to recover the DNA pellet. The pellet was left to air-dry and was then resuspended in 20-50 μl of elution buffer (10 mM Tris-HCL, pH 8.5), depending on the size of resulting pellet. A NanoDrop ND-1000 spectrophotometer (Thermo Scientific) was

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used to quantify the phage DNA. Phage DNA concentration was measured initially using a NanoDrop ND-1000 and then confirmed using Qubit® 20 (Invitrogen), as NanoDrop ND-1000 gave a measurement approximately 10 times higher.

2.6.3 DNA sequencing and annotation

The phage genome was sequenced using 454-pyrosequencing. The concentration of DNA required for sequencing was 30 µg/ ml. Sequenced DNA was annotated using Artemis (Rutherford et al. 2000).

Genmark.hmm 20 (Besemer and Borodovsky, 1999), GLIMMER 3.02 (NCBI) (Salzberg et al. 1998) and GeneNote (Shmueli *et al.*, 2003) were used to predict ORFs. The putative functions of predicted ORFs were assigned using Blastp (Altschul et al. 1990). The circular genome of the phage SP-QS1 was yielded using Q script language version 6 (<http://www.star.le.ac.uk/~rw>). The organization of genes was designed using Gliffy software (<http://www.gliffy.com/gliffy/>).

The existence of transfer RNA was identified by scanning the phage SP-QS1 genome using tRNAscan-SE 1.21 (Lowe TM and Eddy SR, 1997). The terminators were detected using transtermHP programme (Kingsford C.L *et al.*, 2007). The direct repeat sequences or spacers of CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats) were obtained from the CRISPR database (Rousseau *et al.*, 2009).

2.6.4 Genome characterisation

Blastp was used to generate file comparison and the genomes of Streptococcus phage SP-QS1 and *Enterococcus* phage SAP-6 and BC-611 were visually compared using ATC (Carver *et al.*, 2005). Each set of phage genes from the two phages were compared to

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determine their similarity using blastp (the amino acid sequences). Results with an e-value of less than 10^{-4} were removed. Shared and similar genes were the top hits in the blastp search that could identify one another. The percentage identity value between ORFs was obtained from the alignment of two ORFs' amino acid sequences in ClustalX (1.83) by calculating the percent identity matrix (PIM). Both default parameters (gap opening and gap extension) - pairwise and multiple options - were involved in the alignment (Thompson et al. 2002). Pairwise and multiple comparisons were set to 2 and 0.1, respectively.

2.6.5 Phylogenetic trees

The genes of interest were selected after their alignment using Clustal X 1.83 (Thompson et al. 2002). These genes were terminase large subunit, minor capsid and DNA polymerase. They were combined with the top 25 blastp hits and then submitted to the MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (Tamura et al. 2007). A phylogenetic tree was constructed using the neighbour-joining method as well as the number of differences model. The tree was constructed to be unrooted, and the parameters used to test the phylogeny were bootstraps from 100 replicates with a random seed.

Chapter 3: The isolation of pneumococcal phages

Chapter 3. The isolation of pneumococcal phages

3.1 Introduction

Although two lytic pneumococcal phages have been isolated, neither of them have shown the ability to infect invasive capsulated *S. pneumoniae* (McDonnell *et al.*, 1975). The host cells for these lytic pneumococcal phages were R36 and R6 which are non-capsulated strains. The resistance of pneumococci to these phages was attributed to the role of the capsule in protecting capsulated strains of *S. pneumoniae* (Bernheimer and Tiraby, 1976).

Four temperate pneumococcal phages have been previously induced: EJ-1, MM1, HB-3 and VO1 (Romero *et al.*, 1990c; Diaz *et al.*, 1992; Gindreau *et al.*, 2000; Obregon *et al.*, 2003b). The Temperate phage HB-3 is a siphovirus which were induced from the pneumococcal strain HB-3 in 1990. The phage HB-3 similar to the phage MM1 in that they both have a protein linked to their genomes which are used for the integration into pneumococcal chromosome. This phage has been found codes for amidase which shares many biochemical and immunological properties with that one of its host (Romero *et al.*, 1990c). The phage EJ-1 belongs to the *Myoviridae* family phages which possess a 42-kb linear DNA. The first induction of this phage was in 1992 by Diaz *et al.* from the pneumococcal strain 101/87 (Diaz *et al.*, 1992). The phage MM1 was induced from antibiotic-resistant Spanish/American 23F *S. pneumoniae*, it is a siphovirus with 40-kb DNA (Gindreau *et al.*, 2000). Phage VO1 is a temperate bacteriophage of the type 19A multidrug-resistant epidemic 8249 strain of *S. pneumoniae*. There is a similarity between this phage and phage MM1 in their specific integration site, protein composition, restriction patterns, and in the way which lysis their hosts (Obregon *et al.*, 2003b). Two phages have been sequenced with accessible data; MM1 and EJ-1 (Obregon *et al.*, 2003a; Romero *et al.*, 2004). VO1 has been sequenced but the data is

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not accessible. None of the phages described in this paragraph has shown the ability to infect capsulated strains of pneumococci.

The objective of this study was to isolate lytic phages which have wide host range and can infect capsulated pneumococci. To facilitate the isolation of lytic phages, several temperate phages were induced to understand the dynamic of lytic phages.

In this project, 258 different samples (described in section 2.2) were screened for the isolation of pneumococcal phages. These samples were obtained from the UK and Saudi Arabia and came from 54 healthy volunteers and 172 patients with respiratory conditions. The remaining samples (32 samples) were lung and spleen tissues from mice infected with invasive pneumococcal strain D39. Phage isolation was undertaken using capsulated *S. pneumoniae* strains as host cells including; serotype 2 (strain D39), the invasive serotypes 3 (strain A66), 4 (strain TIGR4), 23F, 19F and 6B. In addition, two non-capsulated mutants derived from strain D39; R36 (Avery *et al.*, 1944) and R6 (Tomasz A and Hotchkiss RD, 1964) were also used for phage isolation. The capsulated strains were selected as they represent a diverse array of pneumococcal strains, the laboratory strain (D39) which the most strain studied and have appropriate mouse model of infection, the most invasive strains (serotype 4 and 6B), strain with thick capsule and other capsulated strains which are invasive but not as common as the previous strains (23F and 19F). These strains were used to increase the probability of isolating appropriate phages which can infect the capsulated strains. All these strains contain prophages in their genomes except strain D39 (Ramirez *et al.*, 1999).

All these strains were previously serotyped and identified and confirmed as strains of *S. pneumoniae* using their colony appearance on blood agar (α hemolysis), gram staining

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and Optochin sensitivity. These confirmatory tests were routinely checked consistently throughout the study.

3.2 Results and discussion

3.2.1 Optimising growth parameters

The results of bacterial culture and characterisation show that all the bacteria strains were as expected gram positive cocci, capsulated and sensitive to Optochin discs. The period spent reaching exponential phase growth for these strains vary from five hours, for the wild serotype D39, to nine hours for the mutant strain R36. Using the same bacterial culture procedure for both wild and mutant strains resulted in similar viable counts, of approximately 1×10^8 (cfu/ml⁻¹). Growth curves are shown in Appendix 3.

When all serotypes were confirmed as pneumococci and their viability checked, the data from growth curve was used to identify the optimum time required to obtain exponential phase. The exponential phase growth is essential to form confluent growth. Most capsulated strains reach this phase within 5-6 hours. Confluent lawns preparation are substantial for phage isolation as both sparse and dense lawns can affect the formation of plaques, plaques are either not formed or covered by bacterial growth on sparse and dense lawns, respectively. The preparation of confluent pneumococcal lawns showed that there is no difference between using 0.5% blood agar supplemented plates and non-supplemented as *S. pneumoniae* was able to give confluent growth on all agar plates.

3.2.2 The induction of temperate pneumococcal phages

Phage induction from *S. pneumoniae* serotypes showed that there were several phages induced from prophage serotypes 6B and 23F. One phage was induced from serotype 6B using mitomycin C (6BT-1), while two were induced from serotype 23F using

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mitomycin C and norfloxacin (23FT-1 and 23FT-2, respectively). These phages were able to show zones of lysis on the pneumococcal lawn of strains D39, 19F and 35A.

The temperate phages have different morphologies to the previously isolated ones, with each of them being distinctive. Lysogenic phage MM1 is a siphovirus with an icosahedral head and long tail and tail fibres (Gindreau *et al.*, 2000). The morphology of temperate phage EJ-1 has an icosahedral head, sheathed contractile tail separated from the head by a nick, and a basal plate with spikes (Diaz *et al.*, 1992). These morphologies are not similar to any of the newly induced phages. In contrast, temperate phage 6B-1 appears to have icosahedral head, sheathed contractile tail and spikes (Figure 3.1 A). Despite temperate phages 23FT-1 and 23FT-2 (Figure 3.1 B and C) are both podoviruses with an icosahedral head and short tail; they were induced using different antibiotics, mitomycin C and norfolxacin, respectively.

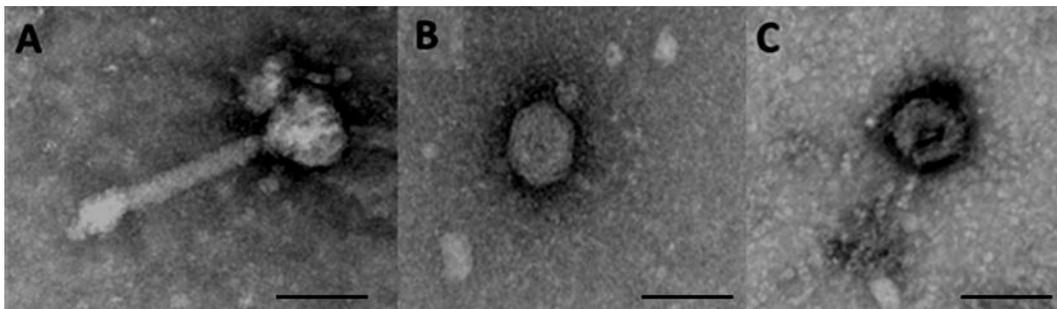


Figure 3.1: Pneumococcal temperate phages.

A is phage 6BT-1 which was induced using serotype 6B; B and C are phages 23FT1 and 23FT2, respectively, which were induced using serotype 23F. Scale bar is 500 nm.

3.2.3 The isolation of lytic pneumococcal phages

All collected samples were screened initially with spot tests. The screening with spot tests showed that 25 samples were able to form lytic activity on pneumococcal lawns. The rest of samples were screened with phage enrichment by incubating them with pneumococci overnight and then tested with spot tests. Phage enrichment was assayed

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to increase the chance of the isolation by increasing the titre of expected phages. However, none of them showed any zone of lysis or plaques in spot tests.

19 sputum samples and 6 throat swabs from clinical samples spots cleared lawns of pneumococcal strains D39, 6B, 23F and 19F, however, none of these samples showed individual plaques. The zones of lysis on pneumococcal lawns decreased gradually corresponding to phage lysate dilutions and then disappeared (Figure 3.2), this phenomenon was also observed when induced phages were tested with spot tests and plaque assays. Phages isolation from mouse tissue following invasive pneumococcal disease showed no zone of lysis on pneumococcal lawn indicating that might there was apparent not to be phages existing in their processed organs. Alternatively, they might present in low numbers which not enable them to cause lysis or the grow conditions were might not have identified in a way which allow them to grow. Because these phage were not able to grow on plates, liquid propagation was undertaken to propagate them and then were visualised by TEM.

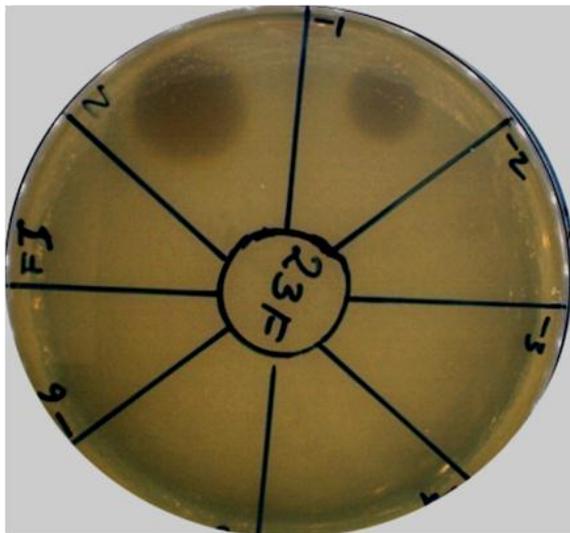


Figure 3.2: The appearance of non-plaquing phages on pneumococcal lawn.

The letter n is neat and 23F is one of tested strains.

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The disability of obtaining individual plaques was obtained every time using all media types used in the study. The same result was obtained consistently with all the pneumococcal strains tested. Furthermore, the addition of magnesium chloride and sodium chloride to the base media, and/or to sloppy agar, had no effect on obtaining single plaques. Three phages were visualised by TEM after phage liquid propagation and were named as phages 6BL-1 and 6BL-2 (6B: is the strain used for the isolation, L: lytic, 1 and 2: phage number) and 23FL-1 (23F: is a pneumococcal strain, L: lytic, 1: phage number). 6BL-1 is belonging to *Siphoviridae* family with an icosahedral head and long tail and tail fibres (Figure 3.3 A). 6BL-2 being a member of *Myoviridae* with an icosahedral head, contrail sheathed tail separated from tail fibres with unsheathed tail (Figure 3.3 B). 23FL-1 belonging to the *Siphoviridae* with a circular head, short thin tail and tail fibres (Figure 3.3 C). Although, these phages are appearing as lytic, they could not be further propagated and used for further analysis. The phages described in this paragraph could be temperate phage; however, my induction showed that induced phages have different morphologies from these phages.

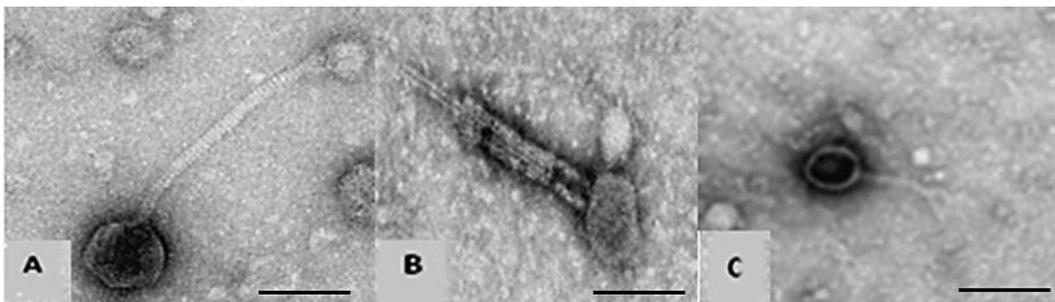


Figure 3.3: Different lytic phages have been isolated from clinical samples.

A and B are lytic phages belonging to *Siphoviridae* and *Myoviridae*, respectively. They were isolated using serotype 6B as a host cell (6BL-1 and 2); C is a lytic phage belonging to *Podoviridae* which was isolated using serotypes 23F (23FL-1) as a host cell. Scale bar is 500 nm.

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All samples that showed a zone of lysis in spot test were assayed for clearing pneumococcal growth in liquid culture. The addition of these samples to exponential phase growth of pneumococci showed no reduction in bacterial growth ODs.

There are several reasons to explain why these isolated phages did not propagate on pneumococcal lawns. One possible explanation is that all samples which gave a zone of lysis were clinical samples; therefore they might contain traces of antibiotics which probably caused that lysis. Another explanation is that all tested serotypes carry prophages except serotype D39 (Romero *et al.*, 2009). Therefore, it is possible that prophages may provide superinfection exclusion or immunity against additional related phage entrance into the bacterial cells or phage replication after penetration (Chesney and Scott, 1975; Hershey and Dove, 1983). Another reason could be that the initial phage infection during spot tests occurred when pneumococcal cells transformed from being capsulated to non-capsulated (Kim and Weiser, 1998). Finally, the disability in the production of individual plaques by described phages might be related to phage traits such as low burst size. Phages which have low burst size could make small plaques but were being covered by bacterial growth (Abedon and Yin, 2009; Kaur *et al.*, 2012).

The formation of individual plaques was only obtained using the modified BHI media base and the plaque assay technique. Modified BHI media contained a combination of filtered brain heart infusion (fct) and 5 ng choline chloride, with the pH adjusted to 7.4 using Tris-HCl. The base of this media was prepared in the same way as for the broth with the addition of 1% agar. The top sloppy agar (0.4% agar) was supplemented with 0.4% glycine. Several attempts to isolate lytic phage were tried and eventually plaques were obtained after this modified media was used with another assay procedure, in this

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new assay, the zones of the lysis areas which were obtained from spot tests were bunched and incubated at 37°C for 1 to 2 hours in 1 ml (fct) BHI broth. Following this procedure, the plaque assays were undertaken which resulted in distinctive plaques. The plaques were obtained from a clinical throat swab sample from admitted child with a respiratory tract infection at general hospital in the city of AL-Qunfidah, Saudi Arabia. The distinctive isolated lytic phage which gave these plaques was found to belongs to *Siphoviridae* and possess a prolate head of size ~ 45 by 112 nm, a non-contractile tail of size ~ 140 nm ending with ~20 nm tail fibres (Figure 3.4). *Siphoviridae* are double-stranded DNA (dsDNA) viruses of the order *Caudovirales* (tailed phages). The lytic phage was named as phage SP-QS1 (SP: *S. pneumoniae*, Q: The city of Qunfidah where the samples were collected from, S: siphovirus, 1: Phage number).

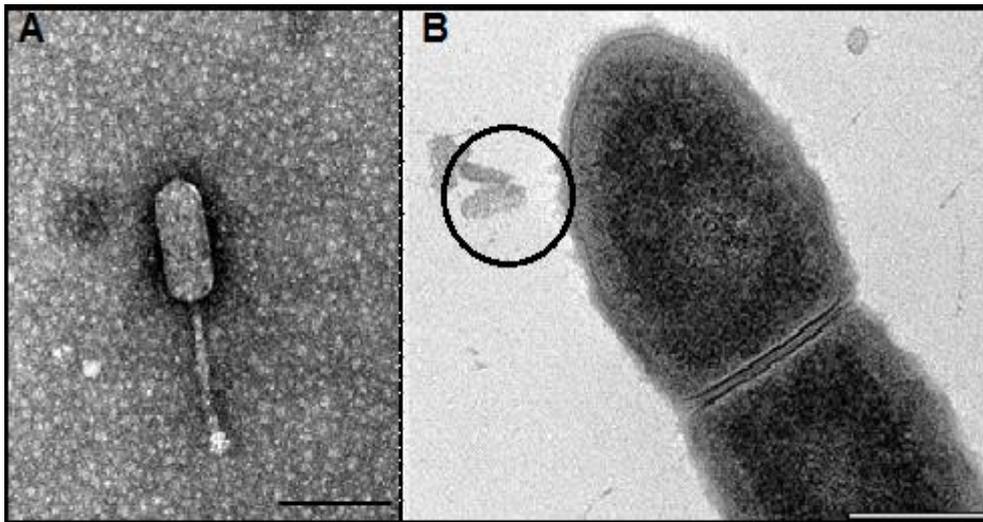


Figure 3.4: Phage SP-QS1.

A) TEM image shows the distinctive prolate-head, long tail and tail fibre. Scale bar is 500 nm. B) Microtome image of phage SP-QS1 attached to serotype 2.

SP-QS1 was able to clear the pneumococcal growth in high titre lysate and the cell lysis decreased unit single plaques formed (Figure 3.5).

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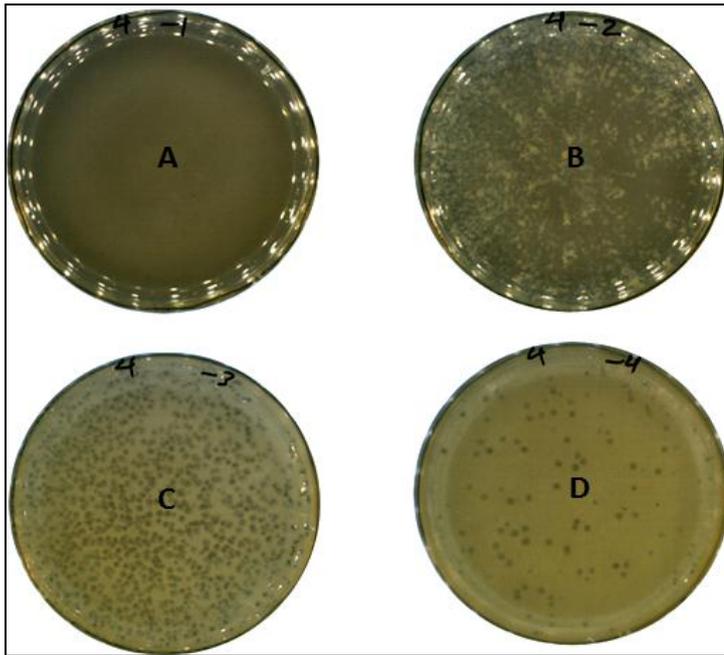


Figure 3.5: Phage SP-QS1 plaque assay. A and B: confluent lysis; C and D: phage plaques which decreased corresponding with phage dilutions.

All induced temperate and isolated lytic phages obtained in the study are shown below in Table 1.

Table 3.1: Temperate and lytic phages studied in this project

Phage name	Phage type	Classification	Host cells	Plaque -purified
6BT-1	Temperate	<i>Podoviridae</i>	Serotype 6B	No
23FT-1	Temperate	<i>Podoviridae</i>	Serotype 23F	No
23FT-2	Temperate	<i>Siphoviridae</i>	Serotype 23F	No
6BL-1	Lytic	<i>Siphoviridae</i>	Serotype 6B	No
6BL-2	Lytic	<i>Siphoviridae</i>	Serotype 6B	No
23FL-1	Lytic	<i>Siphoviridae</i>	Serotype 23F	No
SP-QS1	Lytic	<i>Siphoviridae</i>	Serotype D39	Yes

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3.2.4 Phage amplification

Phage amplification resulted in obtaining a highly purified phage when a single phage clonal assay was used. This technique enabled the obtainment of a high phage titre over the purification. Every step of amplification using single plaque resulted in more concentrated phage, which was sometimes tenfold higher than in the previous cycle. Despite the ability to propagating phages using liquid amplification method, however, the titration was very poor and the purification of non-plaquiring phages was not possible, in addition to that the titration of phage particles became difficult and more challenging.

3.2.5 Phage purification

In order to determine best way to make stock of phage SP-QS1, two phage purification techniques were used to obtain highly purified phages with high titre.

3.2.5.1 Phage purification using caesium chloride gradients

The purification of the SP-QS1 using a CsCl gradient resulted in the formation of a very thick band (Figure 3.6). The band contains the phage particles which migrate depending on their size and density through the gradient. Due to the phage purity which was resulted from clonal assay, only one band was obtained by CsCl gradient purification which set between densities 1.3 and 1.5 g/ml. The phage band was dialysed for three rounds using SM buffer and then stored at 4°C.

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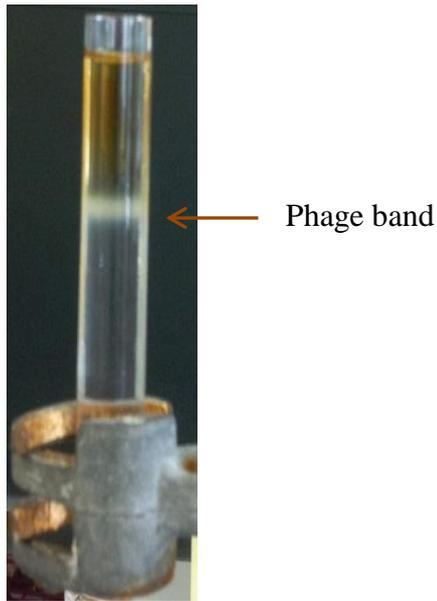


Figure 3.6: SP-QS1 purification using caesium chloride gradients.

The arrow indicates a phage band which is present in CsCl gradient between 1.3 and 1.4 g/ml.

3.2.5.2 Phage purification using high performance liquid chromatography (HPLC)

A HPLC approach for phage purification was attempted to obtain highly purified phage particles and then to test their ability to infect their host cells. This kind of purification is very important when SP-QS1 is used as therapeutical agent to treat human pneumococcal infections. The phage was retrieved in the second fraction of the purification cycles as shown in Figure 3.7. Despite the success of phage purification with this approach, the efficiency of this technique was too low as the titre of the phage decreased following purification. The titre of tested new lytic phage was 10^7 (pfu/ml⁻¹) prior to HPLC and the phage titre was 10^3 after purification, which meant that only 0.0001% of the phage remained while the rest of the phage particles were washed off.

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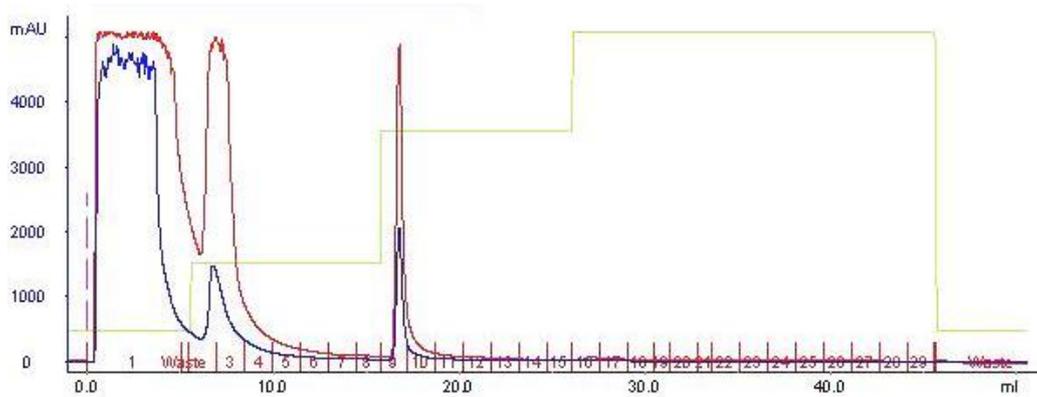


Figure 3.7: SP-QS1 purification using a HPLC approach.

The second fraction started from 3 to 8 on the scale.

It has been determined that using CsCl to purify phage SP-QS1 gave highly purified phage particles with a higher titre than using HPLC.

3.2.7 Host range of phage SP-QS1

Spot tests were used to determine if phage SP-QS1 can infect all *S. pneumoniae* serotypes. Zones of lysis were observed after overnight incubation at 30°C in an aerobic environment. Serotypes 3, serotype 4, and 6B were susceptible to phage, whereas serotype 23F and 19F were not susceptible. Other phages were excluded from this experiment as they are not able to form plaques on pneumococcal lawns.

3.2.8 One-step growth curve of phage SP-QS1

This part of the study explained the biological parameters of phage SP-QS1, which describe the lytic life cycle of this phage. Data collected during this phase facilitated understanding of the other observations made regarding the life cycle.

Three replicates of two individual experiments to obtain one-step phage growth curves were used and the average data was analysed to obtain growth parameters including latent period and the burst size of phage SP-QS1. The experiment was assayed with multiplicity of infection (phage/ bacterial cell ratio) of 10 to insure that each cell would

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be infected with one phage. The life cycle of phage SP-QS1 is shown in Figure 3.8. This experiment could not be conducted with other phages as they did not propagate well in a liquid medium.

The latent period for phage SP-QS1 was found to be approximately 90 minutes on strain D39. Although, the previous pneumococcal phages have long latent periods during their life cycle, phage SP-QS1 has the longest latent period among these phages. The latent periods for lytic phages Dp1 and Cp-1 are around 30 and 50 minutes, respectively (McDonnell *et al.*, 1975; Ronda *et al.*, 1981). The burst size of this phage is 4.66×10^3 phage/ml⁻¹.

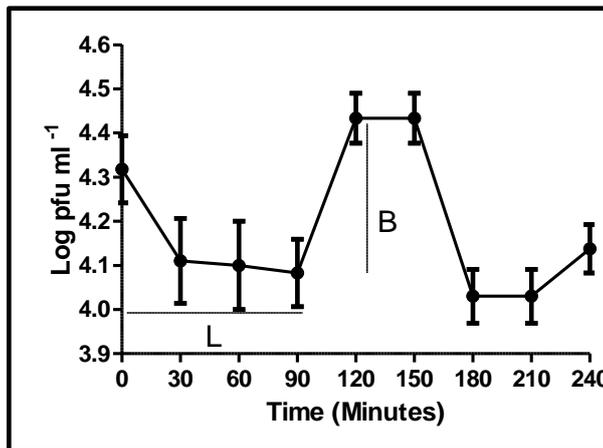


Figure 3.8: Phage SP-QS1 one-step growth experiment using MOI of 0.1 on strain D39.

L indicates latent period and B indicates burst size. The experiment was repeated twice with three replicates, and data are shown as mean \pm SD.

3.3 Conclusion

There is an increase in the emergence of pneumococcal strains which are resistant to different antibiotics of choice, this corresponds with lack of overall protection of vaccination for humans of all ages (Koskela *et al.*, 1986; Di John *et al.*, 1989; Peeters *et al.*, 1991). Previously, it has been thought that no phage could invade capsulated pneumococci as the capsule works as a protective tool against the previously isolated

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lytic pneumococcal phages (Bernheimer and Tiraby, 1976). In addition, these previously isolated lytic phages are known to be unstable during storage. Therefore, an objective of this study was the isolation of a new bacteriophage capable of infecting capsulated *S. pneumoniae* with a potential application in the control and treatment of infection caused by this pathogen.

In order to contextualise lytic phages, several temperate phages were induced with mitomycin C and norfloxacin. The induction resulted in the obtaining of three phages which were found to belong to *Myoviridae* (6BT-1) and *Podoviridae* (23FT-1 and 23FT-2) using strains 6B and 23F, respectively.

To isolate lytic phages, 258 samples were analysed and screened. The screening of collected samples with extensive effort using spot test and plaque assays resulted in the isolation of one detective lytic phage SP-QS1. This phage showed its ability to form zones of lysis and individual plaques on pneumococcal lawns.

Phage SP-QS1 was isolated from a clinical throat swab specimen from a child with aspiration respiratory infection. This phage has a distinctive morphology with a prolate head, long tail and tail fibres. Phage SP-QS1 has a relatively broad host range and its growth curve shows that it has a long latent period of around 90 minutes. The burst size of this phage was 4.66×10^3 phage/ml⁻¹.

The ability of phage SP-QS1 to form individual plaques enables obtaining highly purified phage with high titre which enables the detection of phage activity against invasive pneumococcal strains in both *in vitro* and *in vivo*.

Chapter 4: *In vitro* interaction of pneumococcal phage SP-QS1

Chapter 4. *In vitro* interaction of pneumococcal phage SP-QS1

4.1 Introduction

As an obligate parasite, bacteriophages can infect, replicate within and lyse their hosts. This interaction between phages and their hosts was confirmed for both long-tailed phages such as myovirus and siphovirus with *S. aureus* and *E. Coli*, respectively (O'Flaherty *et al.*, 2004; Berkane *et al.*, 2006). The ability of a phages to kill host bacteria can be exploited in phage therapy to clear pathogenic bacteria that cause food spoilage, infectious diseases in humans and agricultural diseases in plants and animals (Khalil *et al.*, 2012). Some advantages of phage therapy such as host specificity of bacteriophages and the lack of toxicity and immunogenicity associated with lytic phages are potential advantages that phage therapy has over antibiotics (Alisky *et al.*, 1998).

The interaction between phages and their hosts begins with phage recognition of specific receptors on the bacterial cell wall. These receptors are different from one bacterium to another; for example, choline has been indicated as a receptor for pneumococcal phage Dp1 (McDonnell *et al.*, 1975) and lipopolysaccharides or outer membrane porin protein of *E. Coli* are the binding receptors of lambda phage (Bartual *et al.*, 2010). Generally, two phage proteins are involved in receptor recognition, phage tail tip protein and phage tail fibre protein (Skurnik and Strauch, 2006). The full dynamic of phages and their host depends on parameters such as phage adsorption rate, burst size, latent period, maximum bacterial growth and phage density. It has been found that phages with high adsorption rates burst size would reduce the bacterial population more than phages with low adsorption rates (Levin and Bull, 2004).

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Host density has an impact on phage density following community-wide host lysis. The use of more phages in a culture with sensitive bacteria increases the chance of infecting and lyse all bacteria. Phage-bacterial ratio or multiplicity of infection (MOI) has an impact on the burst size and lysis timing as different MOI resulted in different outcomes (Abedon, 2006).

In order to exploit phage specific doses which can be used for *in vivo* application, this chapter designed to detect the effect of phage SP-QS1 on *S. pneumoniae* strains D39, 3, 4, 23F, 6B and 19F in liquid culture. It was also established to detect the time that is required by SP-QS1 to clear pneumococci. The data in this chapter presented the influence of phage infection on optical density and bacterial viable count with more attention on the latter. The reduction in both parameters is encouraging the application of interaction between phage SP-QS1 and pneumococci in mouse model of infection. Phage MOI and the number of phage administrations and their timing in phage/host cell *in vivo* interaction rely on the outcomes of this chapter.

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4.2 Results and discussion

4.2.1 Effect of phage SP-QS1 on strain D39

In order to detect the effect of SP-QS1 on D39 cells, OD₅₀₀ and viable counts were monitored at an MOI of 0.1, 1 and 10 (Figure 4.1A-C) and (Figure 4.2A-B). The culture turbidity increased from approximately 0.5 to approximately 0.6 in the first 30 minutes in both controls and phage-treated cultures using MOI of 0.1, and then it decreased in phage-treated cultures to 0.58 which recorded a total reduction of 30% while the OD₅₀₀ of controls continued to increase (Figure 4.1A). A bacterial challenge with phage using this MOI resulted in a gradual reduction in the viable counts to reach the lowest value after 90 minutes (0.7 logs lower than the control). This reduction continued steadily for half an hour and then bacterial viable count gradually increased. Its ultimate value was 0.29 logs lower than that for the control after five hours of incubation (Figure 4.2 A).

When MOI of 1 was used, the culture OD₅₀₀ decreased to 0.46 after two hours from the beginning of incubation in phage-treated culture, that recorded a reduction of 43% compared to the control OD₅₀₀ (Figure 4.1B). On the other hand, the total viable count of strain D39 using this MOI decreased dramatically to reach a log₁₀ of 4.08 in the first two hours, while the control had a log₁₀ of 5.82. This reduction represents more than one log difference between controls and phage-treated cultures. The culture viable count continued to decrease for another hour until the difference between the two tested cultures was 1.4 logs. However, this decrease did not continue further and the viable counts of phage-treated cultures increased sharply over the next two and a half hours. The viability of strain D39 in phage-treated culture ended with a value very close to that for the control: log₁₀ of 5.20 and 5.55, respectively (Figure 4.2 B).

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When MIO of 10 used in the study, the reduction in OD₅₀₀ of phage-treated culture obtained after two hours from the start point of the phage treatment. The culture OD₅₀₀ decreased to 0.18 and that registered a difference of 78% compared with the controls (OD₅₀₀ of 0.81) as shown in figure 4.1C. Following that, it was then a gradual increase in cultural OD₅₀₀ until phage-treated cultures reached the same bacterial OD₅₀₀ as controls at the end of incubation period. However, using MOI of 10 registered the clearest effect of phage-bacterial interaction in the study. Viable plate count of strain D39 in phage-treated culture dropped within the first half of the incubation period (180 minutes) and remained steady for one hour. Interestingly, the viability of this strain continued to decrease for the next hours until became undetectable at the end of incubation period (Figure 4.2C). Although no growth was detected when the phage-treated culture was assayed for viable count, the OD₅₀₀ of phage treated cultures increased at the same time point recording an OD₅₀₀ more than the controls.

The increase in bacterial OD₅₀₀ in phage-treated cultures (Figure 4.1C) might be attributed to cells starving during stationary phase. When infected cells reached stationary phase, bacteria cannot be infected in this period and a resubmission of cell growth is required to release phage progeny (Chibani-Chennoufi *et al.*, 2004). The pH of culture decreases in stationary phase, which can cause a reduction in the ionic exchange between bacteria and the environment, decreasing the cell energy and prevents enzymatic activation or sometimes causes enzymatic inactivation (Harold, 1972; Russell and Dombrowski, 1980). It has been reported that pneumococci can propagate phage in low pH (Holtje and Tomasz, 1975; Ronda-Lain *et al.*, 1977). Therefore, the addition of new culture media is recommended to increase the pH of the culture and allow for the activation of murein hydrolase enzyme in infected

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pneumococci.(McDonnell *et al.*, 1975; Lopez *et al.*, 1977) Thus, it has been hypothesized that non-infected pneumococci continue replicating slowly during stationary phase and infected pneumococci are unable to release detained phage progeny to infect new cells. This might explain the increase in optical density of phage-treated culture with no viable bacteria detected (Figure 4.2 C).

The growth of phage SP-QS1 was created and found to be correspondent with the MOI ratios. A higher MOI resulted in higher phage titre. The phage titre increased after phage adsorption by about 0.4, 0.7 and 4 logs using MOIs of 0.1, 1 and 10 respectively. The phage growth curve is shown in Figure 4.3.

The influence of phage SP-QS1 on strain D39 growth optical density and pneumococcal viable count was confirmed as discussed above; however, it is necessary to detect an influence of this phage on recently passaged D39 using the same procedure.

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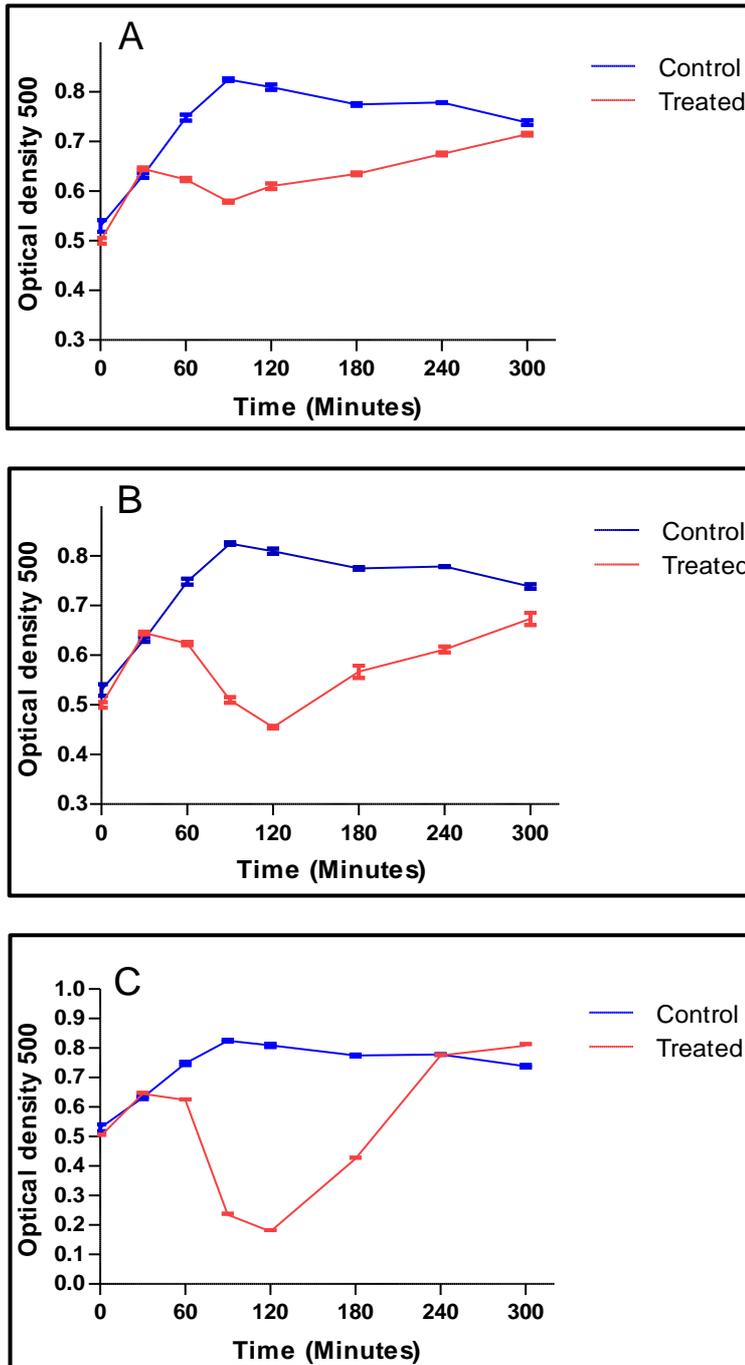


Figure 4.1: The effect of SP-QS1 on strain D39 OD₅₀₀ *in vitro* following phage infection.

Phage was administered at MOI of 0.1, 1, and 10 from A-C, respectively. The culture OD₅₀₀ decreased as MOI increased (data presented as mean of two experiments with three triplicates for each +/- SD).

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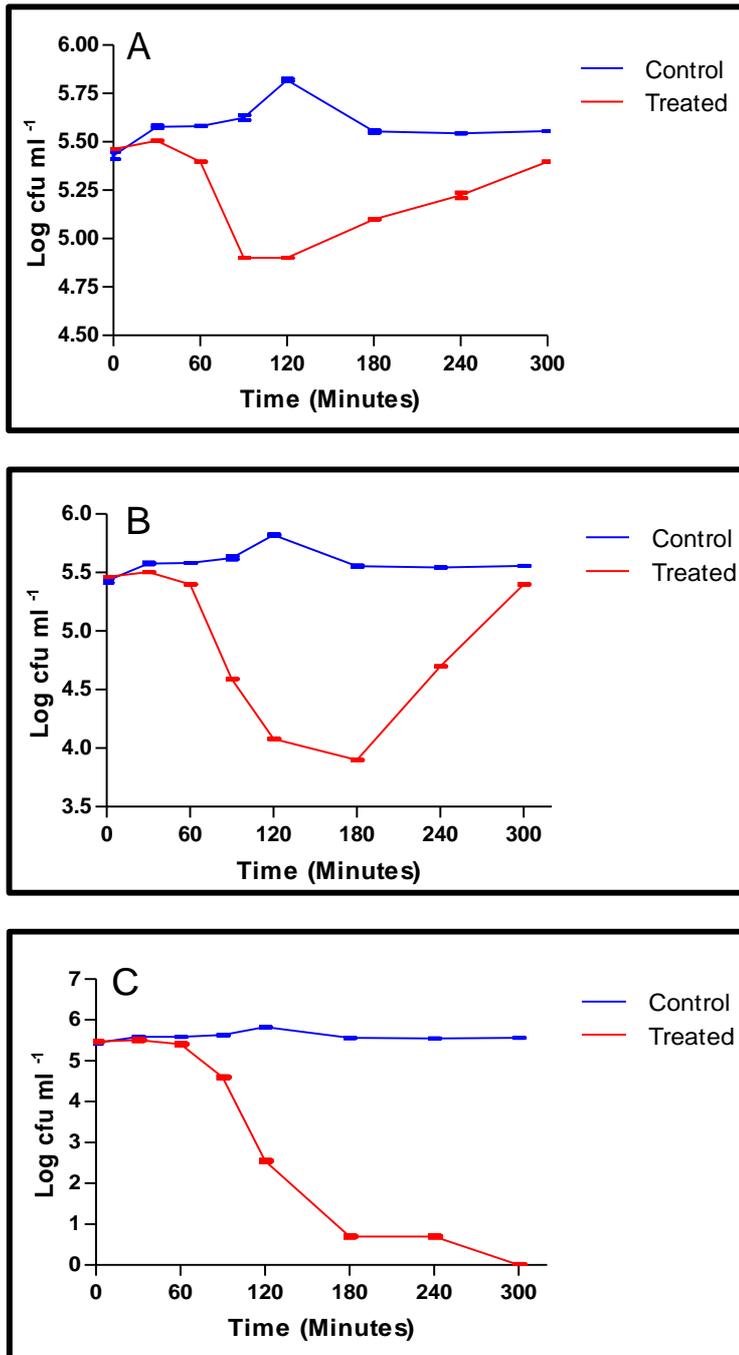


Figure 4.2: The effect of SP-QS1 on strain D39 viable counts *in vitro* following phage infection.

Phage was administered at MOI of 0.1, 1, and 10 from A-C, respectively. Pneumococcal viable counts decreased as MOI increased (data presented as mean of two experiments with three triplicates for each +/- SD).

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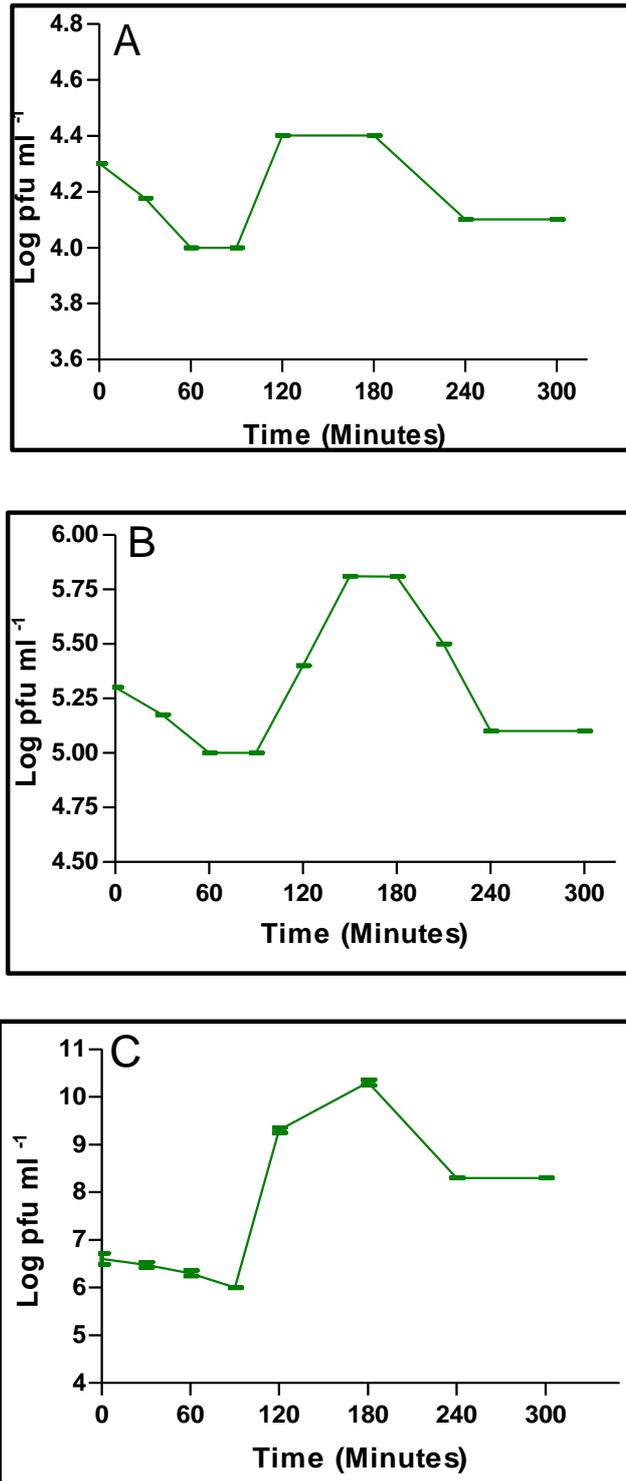


Figure 4.3: Phage SP-QS1 growth curve using strain D39 as the pneumococcal host.

Phage was administered to host cells at MOI of 0.1, 1 and 10, A-C, respectively (data presented as mean of two experiments with three triplicates for each +/- SD).

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4.2.2 Effect of phage SP-QS1 on passaged strain D39

In order to determine if phage SP-QS1 be effective at different type of strain D39 this is passaged serotype D39. Passaged D39 is an invasive pneumococcus that was used to infect mice recently to confirm its ability to cause severe pneumonia in mouse model. Severe infection should be observed on tested mice after 22-28 hours.

Passaged D39 would be different from laboratory strain because of its recent contact with mouse immune system which might cause several biological differences from normal strain. Thus, applying this experiment on this pathogenic strain is crucial as it indicates whether the phage SP-QS1 is able to interact with this particular wild type pneumococcus in the same manner as it does with the laboratory strain. It was applied to support the *in vivo* phage administration to pneumococcal strain D39. The bacterial/phage ratio used with this strain was an MOI of 10 as it gave the highest reduction in pneumococcal viable counts with the laboratory strain as described earlier.

The OD₅₀₀ of phage-treated culture decreased sharply in the first hour to an OD₅₀₀ of 0.18. Following this reduction, it increased dramatically to approximately 0.65 after four hours and then continued to increase until the reported OD₅₀₀ of 0.70, that was exactly the same as the OD₅₀₀ of control at the end of incubation period (Figure 4.4A). The result of this part of the study corresponded with that of the laboratory D39 strain under the same conditions (Figure 4.1C).

In contrast to OD₅₀₀, viable pneumococcal count of phage-treated culture showed a relative similarity for both the control and phage-treated samples in the first two hours, before gradually decreasing to reach the lowest value of an approximately 5-logs-difference with controls. However, it started to increase one hour later, as shown in Figure 4.4B. This is in contrast to the same experiment with the laboratory strain D39, in which the viable bacterial count decreased after one hour and continued to decrease

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until it reached a non-detectable value after five hours. This gave an indication that pathogenic pneumococci have a greater survival rate than the laboratory strain.

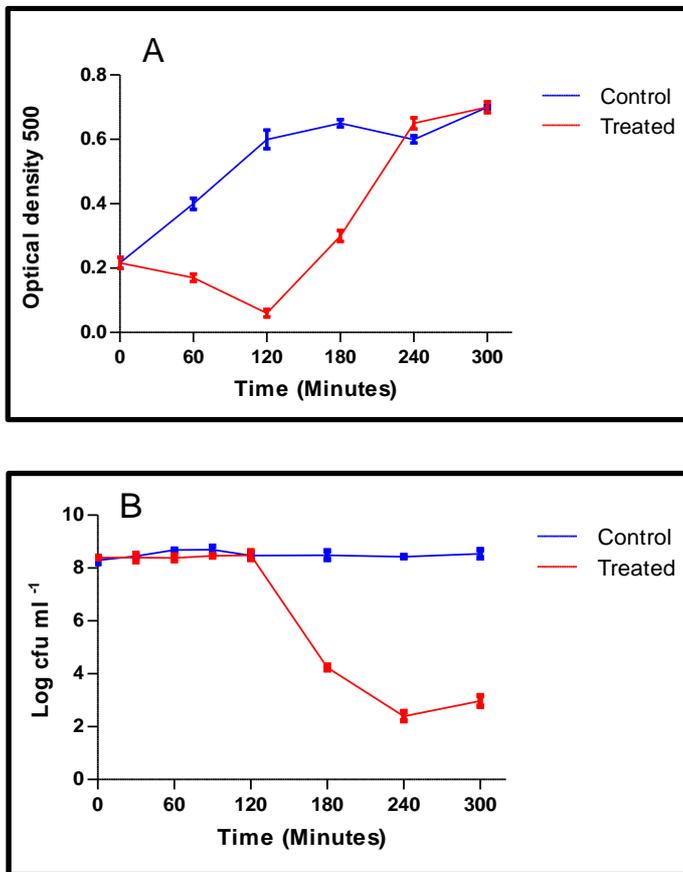


Figure 4.4: The effect of SP-QS1 on passaged strain D39 OD₅₀₀ (A) and viable counts (B) *in vitro*.

Phage was administered at MOI of 10. (A) Optical density (500 nm) decreased in the first two hours and then increased again. (B) Pneumococcal viable counts decreased after two hours and reached its lowest value after four hours before increasing again (data presented as mean of two experiments with three triplicates for each +/- SD).

The interaction between SP-QS1 and passaged D39 showed a slightly different phage growth curve compared to the laboratory D39. The titre of obtained phage using passaged D39 as host was approximately two logs lower than that obtained when laboratory D39 was used under the same conditions. The percentage of free phages was

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14% using passaged D39, while it was only 10% using laboratory D39. The phage growth curve is shown in Figure 4.5.

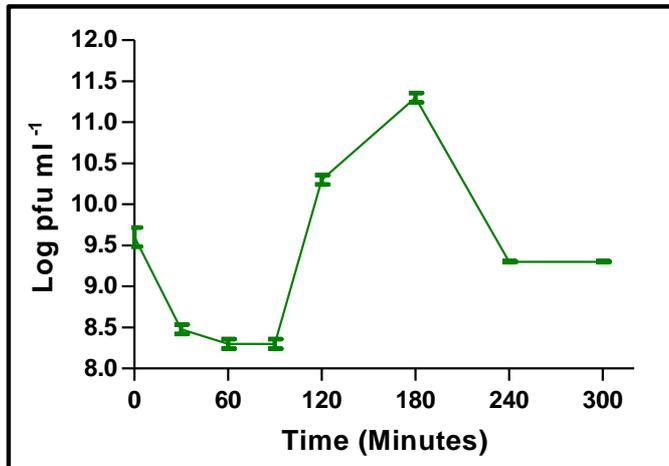


Figure 4.5: Phage SP-QS1 growth curve using passaged D39 as host.

Phage was administered at MOI of 10 and Data presented as mean of two experiments with three triplicates for each +/- SD.

4.2.3 Effect of phage SP-QS1 on strain serotype 3

Strain 3 is sensitive to phage SP-QS1 when tested with a spot test during host range screening. In this part of the study, the interaction between this strain of pneumococci was examined using the same procedure that was assayed on strain D39. However, only an MOI of 10 was assayed with this strain. One important feature of this strain is the presence of a huge capsule around the cell wall (Abdelnour et al., 2009), which might affect phage behaviour during the interaction.

It was found that phage infection did not change bacterial growth OD₅₀₀ in the first three hours. However, the OD₅₀₀ of phage-treated culture decreased gradually during the last two hours of the incubation period, as shown in Figure 4.6A. The influence of phage infection on growth OD₅₀₀ of strain 3 occurred two hours later compared to the influence of this phage on the growth OD₅₀₀ of strain D39 under the same conditions.

The viable counts of strain 3 in phage-treated culture supported the opinion that capsule

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thickness might play a role in delaying the interaction between phage SP-QS1 and pneumococci with a huge capsule. The number of viable bacteria is similar to that of the control during the first three hours of the incubation period. This was followed by a continuous decrease until no growth was detected after five hours of incubation, as shown in Figure 4.6B.

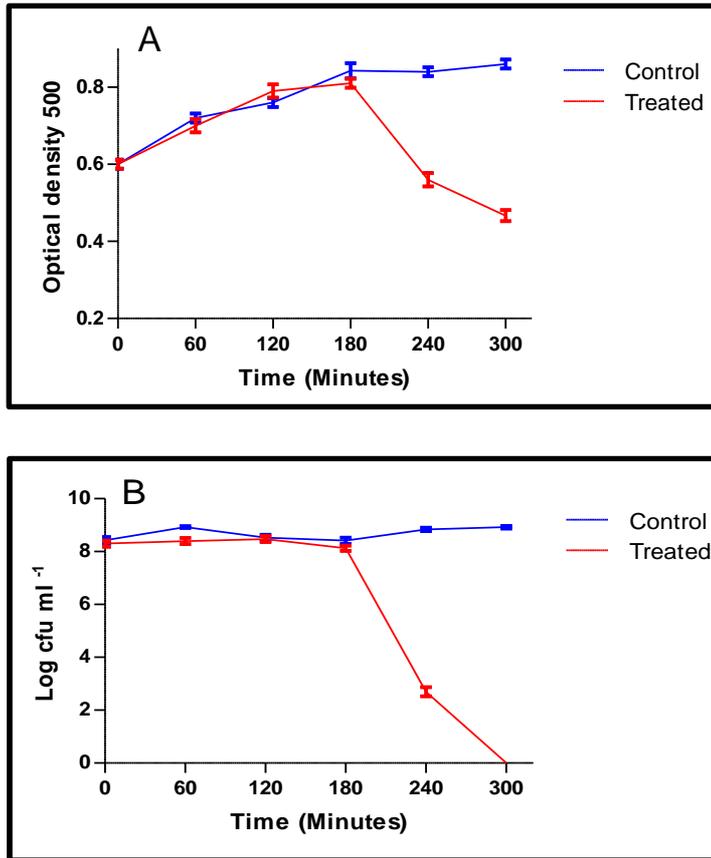


Figure 4.6: The effect of SP-QS1 on strain 3 OD₅₀₀ (A) and viable counts (B) *in vitro*

Phage was administered at MOI of 10. (A) Optical density (500nm) decreased after 3 hours and continued decreasing to the end of incubation. (B) Pneumococcal viable counts decreased sharply after 3 hours and reached undetectable value after 5 hours (data presented as mean of two experiments with three triplicates for each +/- SD).

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The phage growth curve using this strain appeared to give a lower phage titre compared to that obtained using strain D39 as host. The titre increased from around log 9.5 to approximately log 12.5, a difference of about three logs, which is one log lower than phage titre using strain D39. However, the phage titre remained consistent for one hour when it reached the peak and then sharply decreased at the end of incubation period, as shown in Figure 4.7

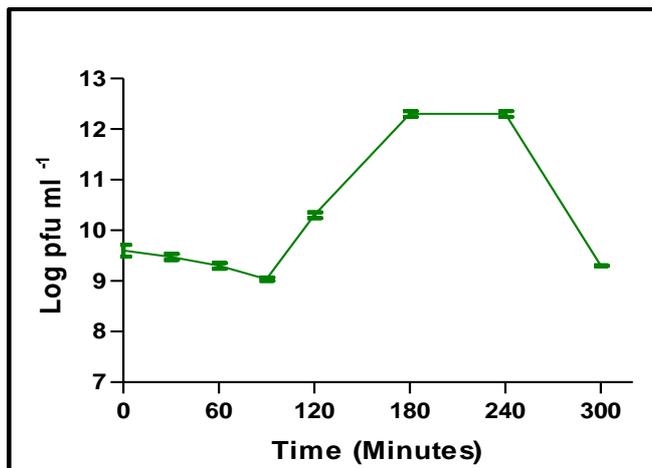


Figure 4.7: Phage growth curve using strain 3.

Phage was administered at MOI of 10 and Data presented as mean of two experiments with three triplicates for each +/- SD.

4.2.4 Effect of phage SP-QS1 on strain 4

The interaction between phage SP-QS1 and strain 4 (Tiger 4) showed similar outcomes to those obtained with strain 3; however, the reduction in bacterial OD₅₀₀ was slightly greater. Phage-treated culture with SP-QS1 underwent the same growth as the control, but was slightly higher for the first three hours; the culture of phage-treated optical density then decreased sharply. This decrease in pneumococci began two and a half hours after the beginning of infection. The lowest bacterial OD₅₀₀ was approximately 0.3nm and was observed four hours from the beginning of the infection (Figure 4.8A).

In contrast to the results obtained with cultural OD₅₀₀ reduction, the viable plate count

Chapter 4. *In vitro* interaction of pneumococcal phage SP-QS1

of strain 4 began to decrease two hours after the beginning of the infection, which continued decreasing gradually until it reached a non-detectable value after five hours (Figure 4.8B). The combination of viable count results with that of culture OD₅₀₀ showing that while some pneumococcal cells were not lysed, they were no longer viable.

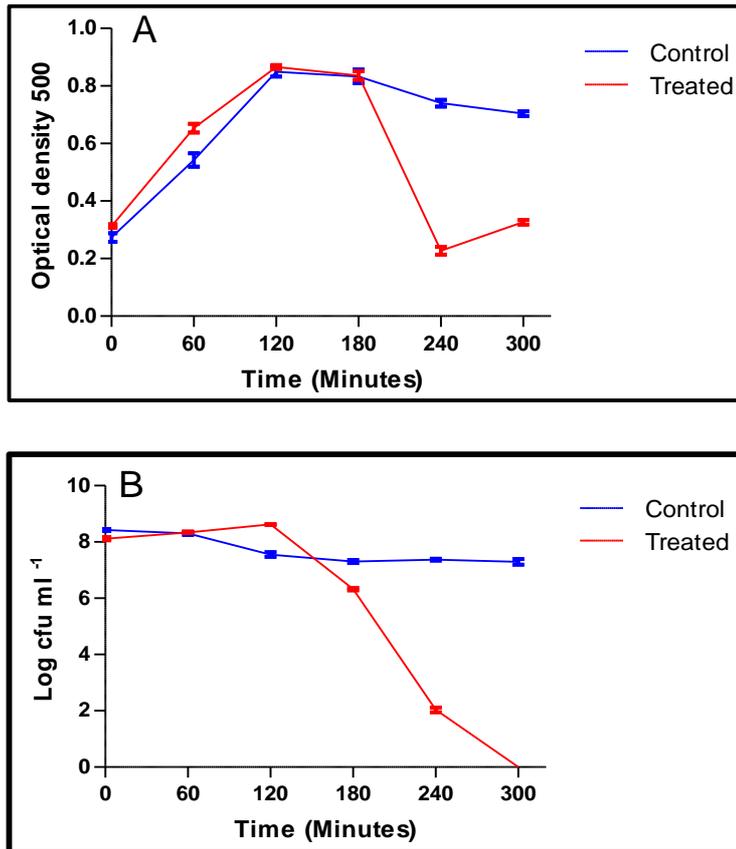


Figure 4.8: The effect of SP-QS1 on strain 4 OD₅₀₀ (A) and viable count (B) *in vitro*. Phage was administered at MOI of 10. (A) Optical density (500nm) increased in the first three hours and then decreased sharply an hour later, then slightly increased. (B) Pneumococcal viable counts decreased after two hours and continued decreasing until they reached undetectable values at the end of the incubation (data presented as mean of two experiments with three triplicates for each +/- SD).

The growth curve of phage SP-QS1 using this strain showed a fast burst of phage progenies one hour after the infection. The release of new phages occurred one hour

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earlier than the phage release using strain D39 as host. Phage titre was maintained at log of 12.5 for one hour and then decreased to approximately log of 9.0 at the end of the experiment, as shown in Figure 4.9.

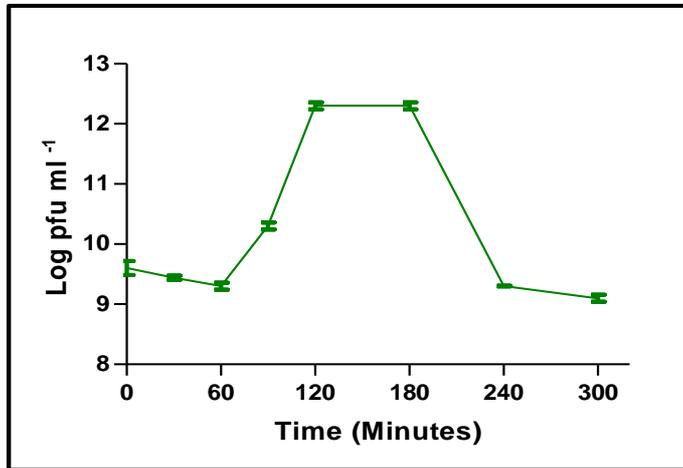


Figure 4.9: Phage SP-QS1 growth curve using strain 4 as host.

Phage was administered at MOI of 10 and Data presented as mean of two experiments with three triplicates for each +/- SD.

4.2.5 Effect of phage SP-QS1 on strain 6B

The interaction between phage SP-QS1 and strain 6B showed different behavior compared to other phage-infected strains. The OD₅₀₀ of phage-treated culture decreased after two hours from the beginning of the infection and remained stable for three hours, and then decreased again. However, the decrease in phage-treated culture was about 0.39 by the end of the incubation period (five hours) compared to the starting OD₅₀₀ (Figure 4.10A).

The colony-forming unit of phage-treated culture of strain 6B with phage SP-QS1 showed a decrease that was not observed with other strains. The viability of strain 6 remained constant in the first hour, then dropped from a log of 8.2 to a log of 6.5 one hour later. The viable counts then decreased in the next hour to log of 6.1. A reduction

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of a three logs was obtained four hours from the beginning of the phage-host interaction, making a difference of five logs compared to starting viable count. However, the viable count increased again by approximately two logs at the end of incubation period (Figure 4.10B).

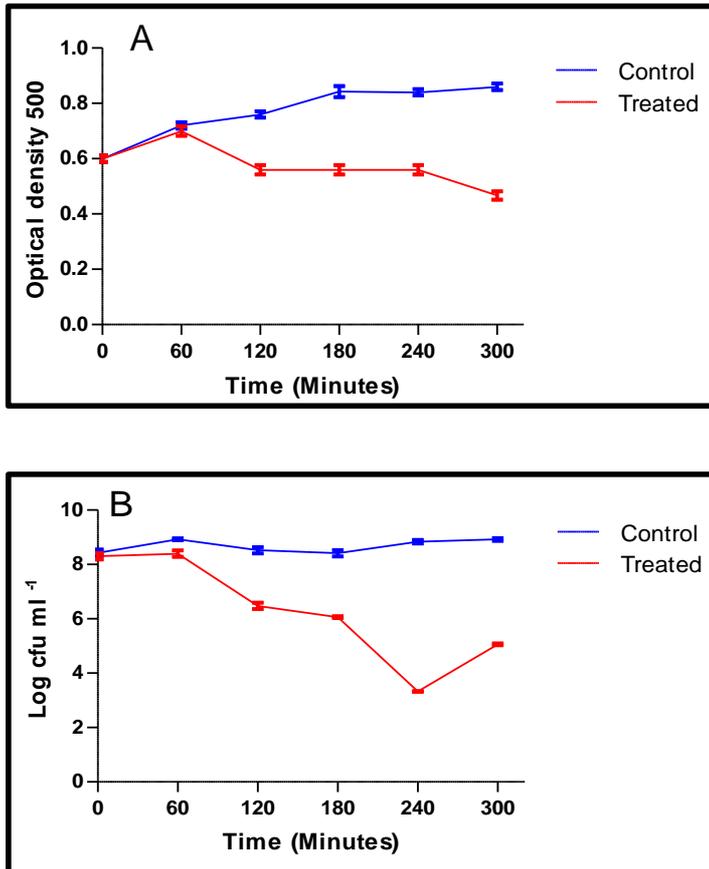


Figure 4.10: The effect of SP-QS1 on strain 6B OD₅₀₀ (A) and viable count (B) *in vitro*.

Phage was administered at MOI of 10. (A) Optical density slowly decreased after slight increase in the first hour. (B) Pneumococcal viable counts decreased in ~ 2 logs after one hour and decreased for another 3 logs after 2 more hours and increased ~ 2.5 logs again (data presented as mean of two experiments with three triplicates for each +/- SD).

The phage growth curve of SP-QS1 showed that the latent period of the phage titre was about an hour. The increase in phage number started after 90 minutes, reporting a phage

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titre log of 10.3. The burst period lasted for 1.5 hours. Phage titre then decreased by about one log and remained at that value for a further hour (Figure 4.11).

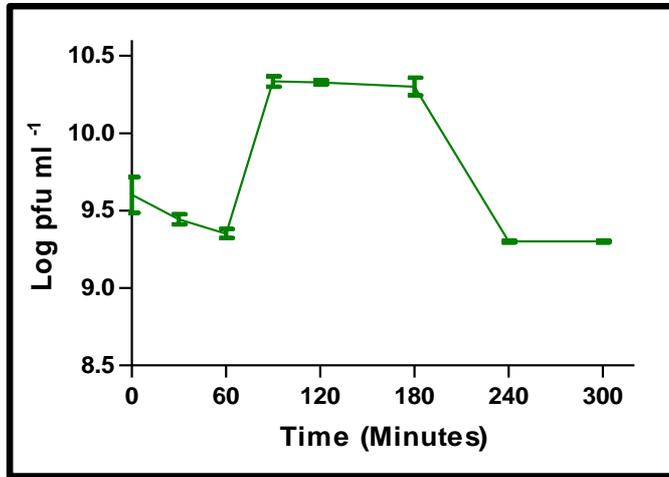


Figure 4.11: Phage SP-QS1 growth curve using strain 6B as host.

Phage was administered at MOI of 10 and Data presented as mean of two experiments with three triplicates for each +/- SD.

4.2.6 Effect of phage SP-QS1 on strain 23F and 19F

In order to detect the interaction of phage SP-QS1 with non-susceptible strains, 23F and 19F were selected as host range screening experiment showed that phage SP-QS1 was not able to infect them. That was expected because 19F was considered as a variant on the Spanish strain 23F (Coffey et al., 1996). The procedure used to assay strain D39 was undertaken on these strains. The results clearly demonstrate that the growth of strain 23F was not affected by phage SP-QS1 administration. Bacterial OD₅₀₀ of phage-treated culture was relatively similar to that of the control (Figure 4.12A).

The total bacterial count of strain 23F in both phage-treated and control cultures was 8.89, which decreased after one hour to reach 8.2. Two hours later, the viable count of this serotype in phage-treated culture increased slightly more than that of the control;

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however, it ended with a value of about 8.46 (3×10^8 bacteria/ml⁻¹) in both cultures as shown in Figure 4.12B.

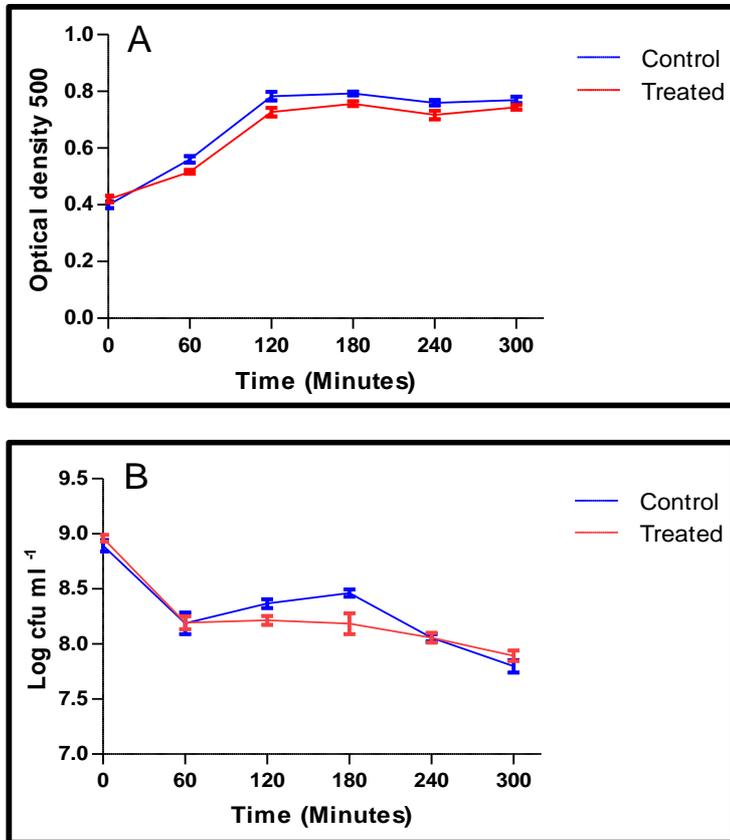


Figure 4.12: The effect of SP-QS1 on strain 23F OD₅₀₀ (A) and viable counts (B) *in vitro*.

Phage was administered at MOI of 10. (A) Optical density (500 nm) ended with the same value for the phage-treated and control cultures. (B) Pneumococcal viable counts of both cultures decreased in the first hour in about one log, then phage-treated cfu log slightly for two hours later, and finally decreased (data presented as mean of two experiments with three triplicates for each +/- SD).

The phage growth curve showed that following a small reduction in phage titre in the first 30 minutes, there was no change in phage titre at all points during the incubation. (Figure 4.13). This initial reduction might indicate phage adsorption, but not phage replication, as no phages were obtained.

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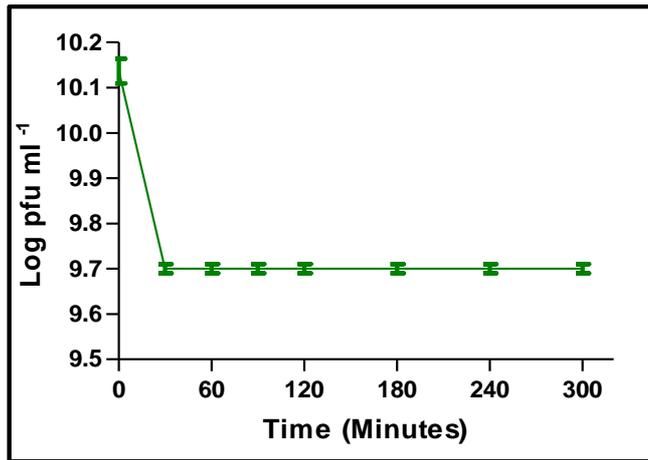


Figure 4.13: Phage growth curve using strain 23F as host.

Phage was administered at MOI of 10 and Data presented as mean of two experiments with three triplicates for each +/- SD.

The optical density of 19F of both phage-treated and control cultures was set at 0.6. In the first hour, the OD₅₀₀ of both cultures increased to 0.83nm, and then decreased slightly to 0.7 at the end of the incubation period, as shown in Figure 4.14 A.

The two cultures in this experiment (phage-treated and control) started with the similar viable count of log₁₀ 8.25. One hour after infection, the viable count of phage-treated culture was relatively lower than that of the control. However, it increased gradually to half a log₁₀ higher than the control at approximately the mid-point of the incubation period. It then started to decrease slightly to a registered log of 8.46, the same as the control five hours following the start of phage administration (Figure 4.14B).

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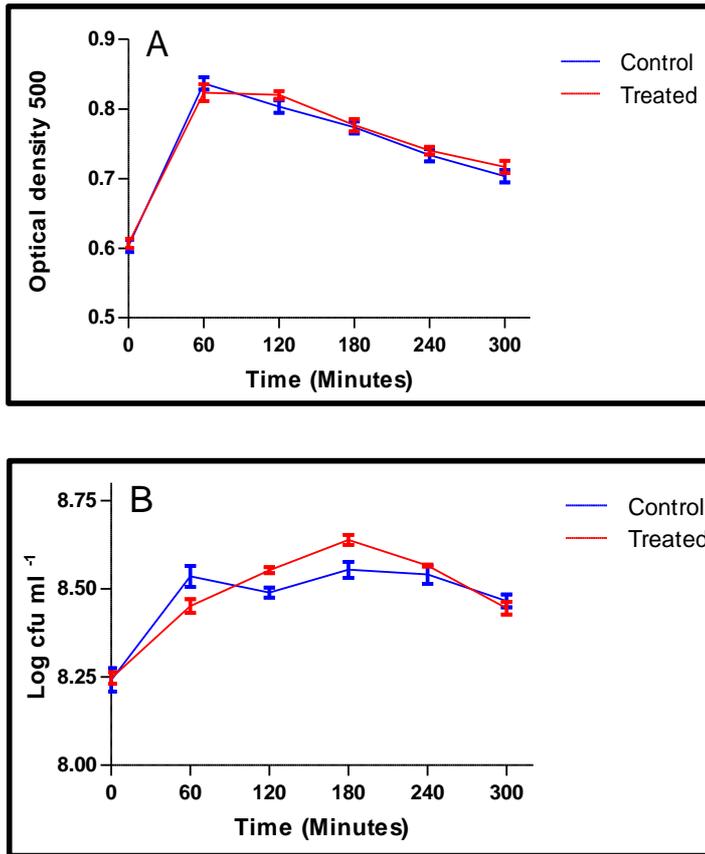


Figure 4.14: The effect of SP-QS1 on strain 19F OD₅₀₀ (A) and viable counts (B) *in vitro*.

Phage was administered at MOI of 10. (A) Optical densities (500nm) were similar in both cultures (+/- SD). (B) Pneumococcal viable counts increased gradually in the first three hours and then decreased to end with the same value as the control after 5 hours (data presented as mean of two experiments with three triplicates for each +/- SD).

The phage growth curve indicated that no phage progeny was produced as no phage adsorption was observed. The phage titre remained steady at the same value from the beginning of phage administration to the end of incubation (Figure 4.15).

Chapter 4. *In vitro* interaction of pneumococcal phage SP-QS1

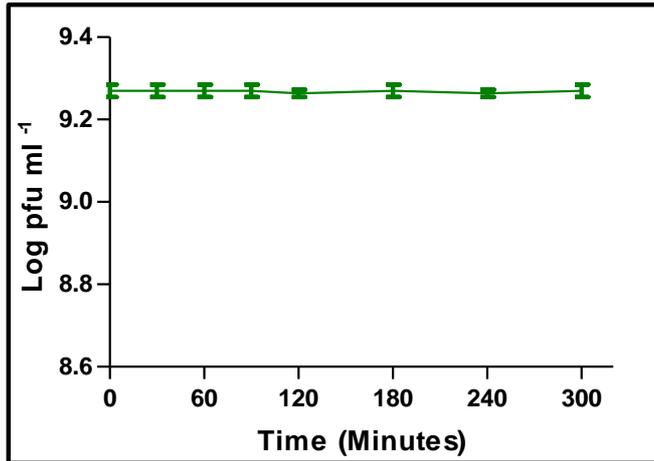


Figure 4.15: Phage growth curve using strain 19F as host.

Phage was administered at MOI of 10 and Data presented as mean of two experiments with three triplicates for each +/- SD.

4.3 Conclusion

Prior to considering phage SP-QS1 application *in vivo* as a therapeutic agent, the ability of this phage to lyse *S. pneumoniae in vitro* should be confirmed. Therefore, the impact of phage SP-QS1 on growth culture optical density and viable counts of *S. pneumoniae* was confirmed with strains D39 (laboratory and passaged strain), 3, 4 and 6B, but not with strains 23F and 19F.

The MOI that gave the clearest results in term of drop in bacterial viable counts was MOI of 10. The reduction in both OD₅₀₀ and the viable counts was the highest when this bacterial/phage ratio was used. This most likely indicates that more phages are required to cause more bacterial damage as the burst size of this phage is relatively low. Moreover, the phage titre using an MOI of 10 resulted in production of high phage titre, which increased by four logs compared to the increase of 0.5 and 0.75 logs with MOIs of 0.1 and 1, respectively.

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A reduction in both optical densities and colony-forming units was obtained with all strains that were infected by phage SP-QS1 using spot tests. In general, the decrease in the optical densities of phage-treated cultures was variable; the reduction was observed early with laboratory D39 and half an hour later with passaged D39. The decrease in OD₅₀₀ was of less than two hours' duration before it increased again. The optical densities of phage-treated cultures of serotypes 3 and 4 were similar; it increased in the first three hours, in line with controls, and then decreased gradually. Fast reduction in the OD₅₀₀ was observed with 6B, which continued to decrease slowly to the end of incubation period, marking a difference of only 0.2 compared to the control. On the other hand, no reduction in the OD₅₀₀ of phage-treated cultures of strains 23F and 19F was obtained. Therefore, it seems that the phage cannot penetrate these strains.

Colony-forming units of phage-treated cultures showed a substantial decrease with strain D39 (laboratory strain) and with serotypes 3 and 4, where the viable pneumococcal count was non-detectable after five hours of incubation. This decrease was comparatively lower with strains 6B and passaged D39. This reduction in bacterial viability was correlated with OD₅₀₀ reduction with all the above strains.

It has been noted in this study that the capsule may be played an important role in delaying the effect of phage SP-QS1 on pneumococcal strains. The reduction took place earlier in strains D39, 4 and 6B, but occurred about half an hour later with passaged D39 and strain 3. These two hosts share one feature, namely the presence of a thick capsule, which might eliminate phage penetration and as a result, showed late viable count reduction and fewer phage titres. Serotype 3 is known to have a thick mucoid capsule (Abdelnour *et al.*, 2009a). The thick capsule is produced to enable pneumococci to resist phagocytosis (Hathaway *et al.*, 2012b). Passaged D39 was derived from mouse

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blood with a severe pneumococcal infection; the pneumococci clearly produced a thick capsule, particularly in the late stage of infection. Furthermore, it was found that the percentage of free phages was relatively higher when passaged D39 was used rather than a laboratory strain. This suggests that the large content of the capsule might delay phage adsorption to host cells and then the infection with phage SP-QS1.

In summary, SP-QS1 was shown to be able to lyse variant pneumococcal strains such as D39, strains 3, 4 and 6B. This property of the phage would enable *in vivo* interaction with pneumococci using an appropriate mouse model of infection.

Chapter 5: *In vivo* interaction of phage SP-QS1

Chapter 5. *In vivo* interaction of phage SP-QS1

5.1 Introduction

The emergence of antibiotic resistance has become a major problem in recent years (Richard Wise *et al.*, 1998). Many formerly well-controlled diseases such as those caused by *S. pneumoniae* have once more become a serious threat to public health in a variety of contexts (Alanis, 2005; Goldberg *et al.*, 2006). Pneumococcal resistance to antibiotic combined with lack of thorough vaccine protection against pneumococcal infection has led to the impetus to uncover alternative antimicrobial therapies, such as phage therapy (Fedson, 1999; Hanlon, 2007). Some advantages of phage therapy such as host specificity of bacteriophages and the lack of toxicity and immunogenicity associated with lytic phages are potential advantages that phage therapy has over antibiotics (Alisky *et al.*, 1998). However, the development and adoption of phage therapy remains slow, largely because most of regulatory procedure and developmental steps associated with antibiotic therapies cannot easily be transferred to use with phage therapies. For example, pharmacokinetics (*in vitro* interaction) and pharmacodynamics (*in vivo* interaction) are not relevant to antibiotic therapy but are critical in phage therapy (Payne and Jansen, 2000; Merril *et al.*, 2003; Weld *et al.*, 2004). Although animal studies are limited, phage-host *in vivo* treatment has been successfully proven with a variety of phages and some of these phages are now commercially available, such as *Enterococcus faecium* phage ENB6 in the USA which decreases the colonisation of this bacterium to rescue immune-compromised patients (Smith and Huggins, 1982; Biswas *et al.*, 2002).

Phage growth depends on the physiological state of the bacterial host, which is strongly affected by environmental conditions. Therefore, the outcome of phage-bacteria *in vivo* interactions are unpredictable in light of the physical and chemical conditions that will

Chapter 5. *In vivo* interaction of phage SP-QS1

exist *in vivo* (Kudva *et al.*, 1999). The variation of outcomes between *in vitro* and *in vivo* might refer to the physiological differences in the growth conditions between the two environments, phage-host specificity and the possibility of phage inactivation during the preparation, phage-lysate contamination and phage immune response (Weld *et al.*, 2004).

There are ten mouse strains are used for pneumococcal research which showed their susceptibility for different model of infections such as pneumonia, otitis media and sepsis, these strains are BALB/C, DBA/2, MF1, CBA/Ca, AKR, C57BL/6, NIH, FVB/n, CSH/He and C3H/HeJ (Kadioglu and Andrew, 2005). MF1 strain was used for phage SP-QS1 as this strain is susceptible for all model of pneumococcal infection; survival rate of this strain is around 48 hours and cheaper than other strains.

As phage SP-QS1 has the ability to infect and kill *S. pneumoniae* serotypes D39, 3, 4 and 6B under *in vitro* conditions. This chapter aims to detect the ability of phage SP-QS1 to infect and kill serotype D39 (the original host) under *in vivo* conditions. Strain D39 was selected for the *in vivo* interaction because there is mouse model of infection appropriate for this strain in our laboratory. Two mouse models of infections were assayed; an intranasal model of invasive pneumonia and a sepsis model of infection. The intranasal model of invasive pneumonia was used to indicate the ability of phage to clear pneumococcal carriage. Sepsis model of infection was used to identify the capability of SP-QS1 to lyse and eradicate *S. pneumoniae* in severe pneumococcal infection.

Chapter 5. In vivo interaction of phage SP-QS1

5.2 Results and discussion

5.2.1 Phage SP-QS1 and *S. pneumoniae* interaction in intranasal model of invasive pneumonia

The phage SP-QS1 was introduced intranasally so it could interact with *S. pneumoniae* serotype D39 as was described in materials and methods (section 2.5.11.1). In brief, female MF1 mice (Harlan) were challenged with 50 μ l pneumococci via the intranasal route at a final concentration of 1×10^6 cfu/ml⁻¹ in 50 μ l. The mice were divided into two groups; the control and phage-treated mice. Control mice received 50 μ l of BHI post-dose after one and five hours intranasally, while phage-treated mice received 50 μ l of phage lysate instead at a concentration of 1×10^7 pfu/ml⁻¹ in 50 μ l giving a MOI of 10. The plaque-forming unit and bacterial colony forming units were determined before and after their injection into mice. Four individual experiments were assayed using the same procedure, and the number of tested mice in each experiment was 26 in the first experiment and 25 in the remaining experiments. Six mice in first experiment and five mice in the rest of experiments were culled at time zero and their lungs and nasopharynx collected, homogenised and processed for viable plate counts. The remaining twenty mice in each experiment were used as controls and phage-treated mice, ten mice for each group.

All the mice involved in the experiment were assayed for pain scoring regularly to determine the severity of infection over time. After 24 and 48 hours, control mice and phage-treated mice were culled and their lungs and nasopharynx collected. Cardiac puncture blood was also extracted from all the mice. Organ samples were homogenised and processed with blood samples for the colony-forming unit count.

Chapter 5. *In vivo* interaction of phage SP-QS1

The pneumococcal count showed no significant decrease in all the samples collected from phage-treated mice after 24 hours from the beginning of the study. The pneumococcal viable counts in the nasopharyngeal and lungs were similar in both, registering 0.5 log₁₀ difference compared to the control mice samples. However, the difference in viable count was slightly higher, at about 0.7 in blood (Figure 5.1).

The major reduction in bacterial viable count was observed 48 hours after phage administration. As shown in figure 5.2, a significant decrease was obtained. The reduction in pneumococcal viable count was more considerable corresponding with the severity of the pneumococcal infection. The reduction detected in phage-treated mouse nasopharynx and lungs was of the order of 1 and 2.5 difference, respectively compared with the mice control samples. A significant bacterial reduction (p value of 0.0002) was reported in blood samples, with a 5 log₁₀ of reduction as a result of phage treatment.

The sequential decrease in pneumococcal viable count, combined with the development of pneumococcal infection, supports the hypothesis described in chapter 4 which suggest the capsule degradation by phage SP-QS1. That was suggested because the capsule becomes thicker during colonisation, which would be expected to delay the penetration of the phage (Weinberger *et al.*, 2009). When pneumococci migrate to the lungs this decreases the capsule layer allowing attachment to epithelial cells and then the infection of bacterial host (Bättig *et al.*, 2006; Hathaway *et al.*, 2012a). In this stage, phage attachment may be becomes high because of the decrease in capsule thickness. A consequence of this is that the phage propagates more quickly during immigration into blood stream. Therefore, a high titre of phage in blood is expected and then the reduction in pneumococcal viable count becomes observed more than that one in both lung and nasopharynx.

Chapter 5. *In vivo* interaction of phage SP-QS1

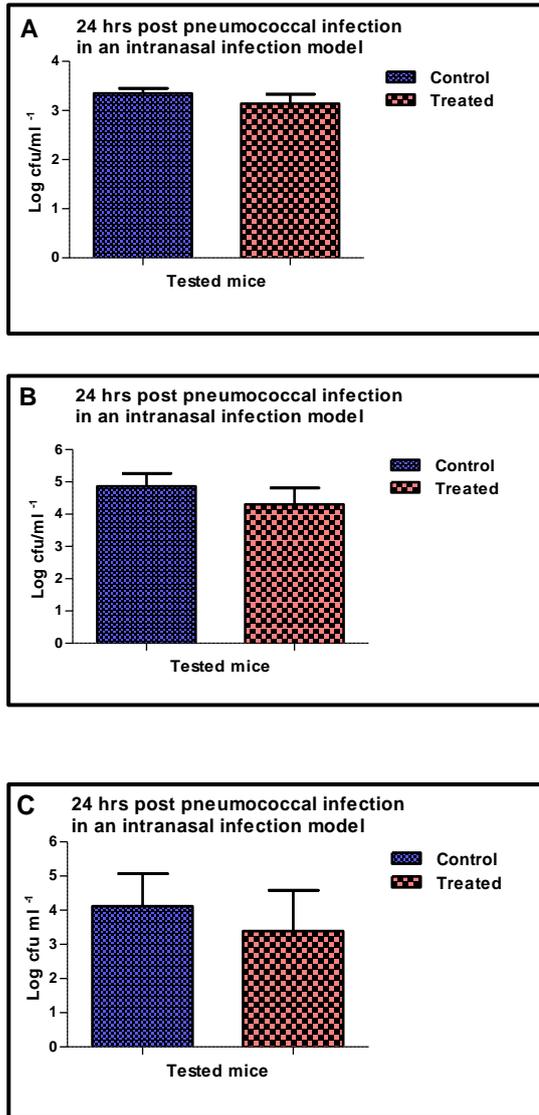


Figure 5.1: Serotype 2 (D39) cfu counts in (A) nasopharyngeal, (B) lungs and (C) blood at 24 hours post pneumococcal infection in an intranasal infection model.

Phage were administered intranasally at an MOI of 10 at 1hour and 6 hours post pneumococcal infection (data presented are mean of 10 mice per group +/- SD).

Chapter 5. *In vivo* interaction of phage SP-QS1

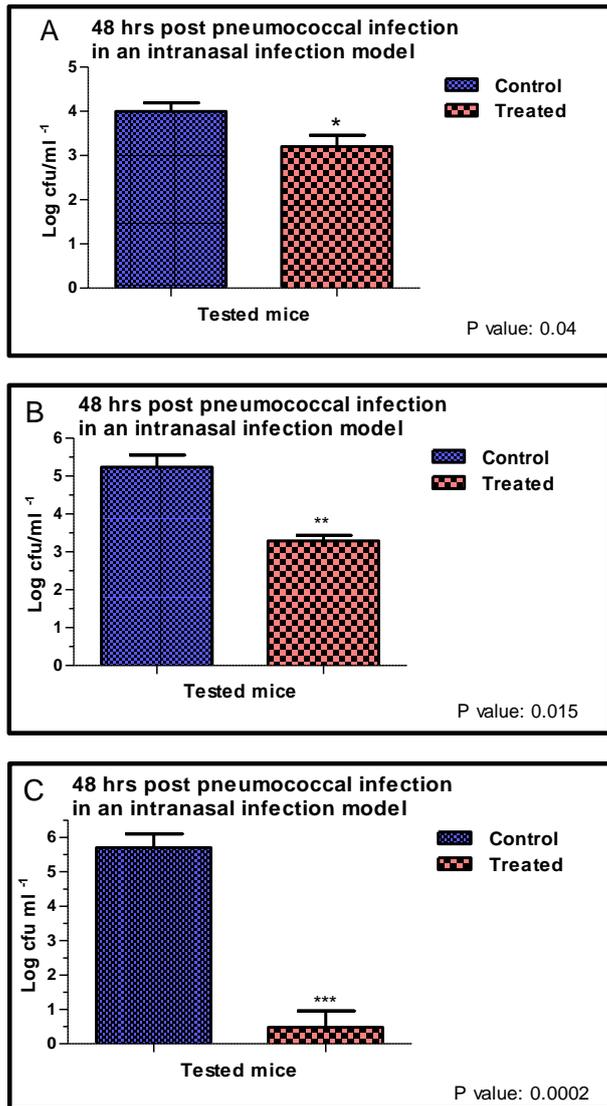


Figure 5.2: Serotype 2 (D39) cfu counts in (A) nasopharyngeal, (B) lungs and (C) blood at 48 hours post pneumococcal infection in an intranasal infection model.

Phage were administered intranasally at an MOI of 10 at 1 hour and 6 hours post pneumococcal infection (data presented are mean of 10 mice per group +/- SD).

As compared to control values, the percentage reduction in pneumococcal viable count in phage-treated mice compared to the control mice was 31.7%, 82% and 99% in the nasopharyngeal, lungs and blood tissues, respectively, as shown in Figure 5.3.

Chapter 5. *In vivo* interaction of phage SP-QS1

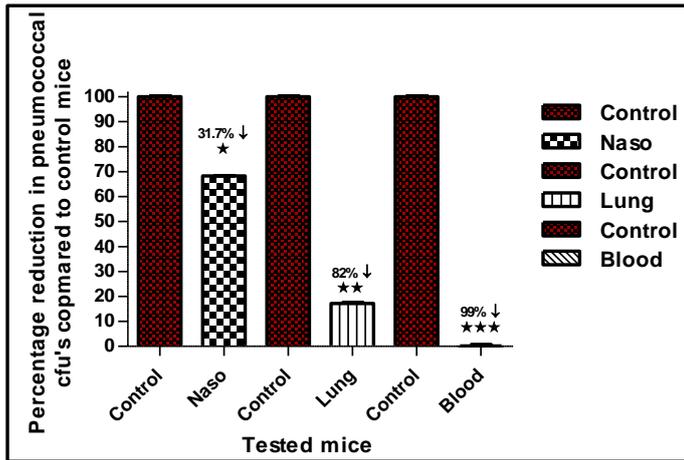


Figure 5.3: Percentage cfu differences in tissue and blood in intranasal phage SP-QS1 treated mice compared to equivalent tissue samples in control mice.

It is been shown that the percentage of pneumococcal c.f.u's reduction in phage-treated mice compared to control mice was 31.7%, 82% and 99% in nasopharynx, lungs and blood respectively (data presented are mean of 10 mice per group +/- SD).

The efficacy of the phage treatment was also demonstrated by the pain score for the mice in the intranasal infection model, which showed a significant reduction in hourly mean pain scores in the phage treated group as compared to the control group, with control mice exhibiting maximal pain scores (moribund) within 48 hours (Figure 5.4).

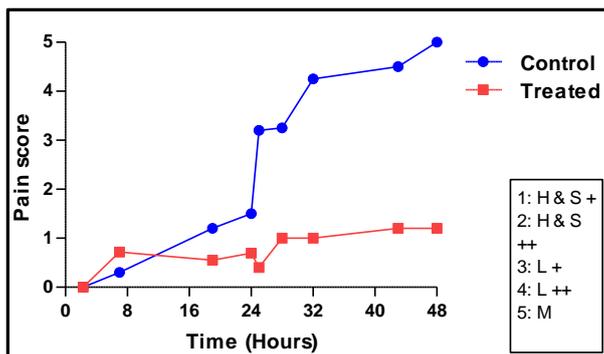


Figure 5.4: Pain score in control and intranasal phage treated mice.

Pain score-1 is hunched and starry coat +, pain score-2 is hunched and starry coat ++, pain score-3 is lethargic +, pain score-4 is lethargic ++, and pain score-5 is moribund (data presented are mean of 10 mice per group +/- SD).

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5.2.2 Phage SP-QS1-pneumococcal interaction in a sepsis model of infection

A sepsis model of infection was assayed exactly as described in materials and methods (section 2.5.11.2). In brief, a fresh stock of phages was used to run this experiment. Twelve female MF1 mice (Harlan) were intravenously challenged with 50µl pneumococci via the dorsal tail route in a final concentration of 1×10^6 cfu/ml⁻¹ in 50 µl. Six control mice received 50 µl of BHI post-dose, three times, after one, three hours and six hours intravenously; while six phage-treated mice received 50 µl of phage lysate in a final concentration of 1×10^7 pfu/ml⁻¹ in 50 µl. This led to a MOI of 10, which should be consistent with the intranasal model of infection.

The phage and bacterial titres were determined before and after the mice were injected. All the mice involved in the experiment were assayed for pain, scoring regularly to determine the severity of infection over time. Blood samples were collected from all the mice tested (controls and treated mice) 6, 24 and 48 hours after the beginning of the bacterial infection, blood viable counts determined.

After 6 hours of phage-pneumococcal interaction, the pneumococcal viable counts in the blood of both phage-treated mice and control mice were relatively similar. This result was expected as both phage and pneumococci were proliferating coincidentally. However, as the infection with *S. pneumoniae* was continued, a reduction in pneumococcal viability was observed in phage-treated mice 24 hours from the beginning of the bacterial challenge. At this time point, phage activity gave only less than one log₁₀ difference between controls and phage-treated mice. The major reduction in pneumococcal viability was obtained 48 hours after the beginning of the phage/bacterial interaction, the viability of pneumococci in phage-treated mouse significantly dropped by about 5 logs (p value of 0.0003). This experiment yielded a

Chapter 5. *In vivo* interaction of phage SP-QS1

significant conclusion in that the phage's ability to infect and kill *S. pneumoniae* increases with a longer duration of phage/bacterial challenge in blood. It was found that the percentage reduction in pneumococcal viable counts in phage-treated mice compared to control mice was 34% and 96.67% after 24 hours and 48 hours respectively (Figure 5.5 and Figure 5.6).

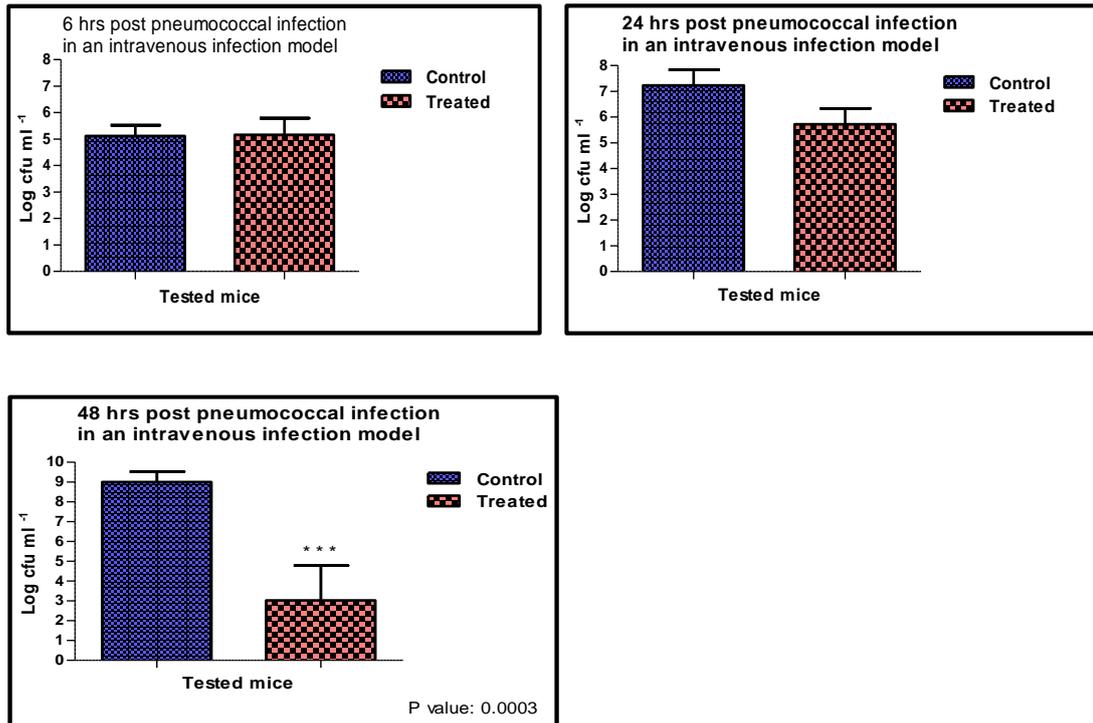


Figure 5.5: Serotype 2 (D39) cfu counts in blood at 6, 24 and 48 hours post pneumococcal infection in sepsis infection model. Phage was administered intravenously at an MOI of 10 at 1 hour and 6 hours post pneumococcal infection. It is been shown that pneumococcal log₁₀ cfu decreased dramatically with longer phage/bacterial interaction (data presented are mean of 6 mice per group +/- SD).

Chapter 5. *In vivo* interaction of phage SP-QS1

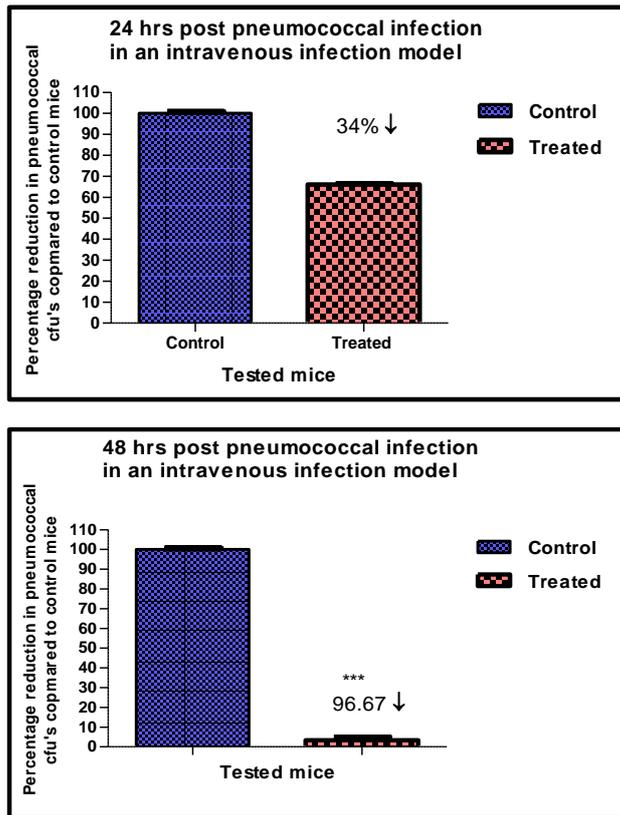


Figure 5.6: Percentage cfu differences in tissue and blood in intravenous phage SP-QS1 treated mice compared to equivalent tissue samples in control mice.

After 48 hours of phage/bacterial interaction, the percentage reduction in pneumococcal viable counts in phage-treated mice compared to control mice was 96.67% whereas was only 34% after 24 hours (data presented are mean of 6 mice per group +/- SD).

The efficacy of the phage treatment was also demonstrated by the pain score of mice in the intravenous infection model. This study showed a significant reduction in hourly mean pain scores in the phage treated group as compared to the control group, over the first 40 hours. Control mice exhibited maximal pain scores (moribund) within 48 hours, whereas phage-treated mice exhibited one plus lethargic at the same time point. The infection started to develop in phage-treated mice 10 hours later than in the control mice as shown in Figure 5.7.

Chapter 5. *In vivo* interaction of phage SP-QS1

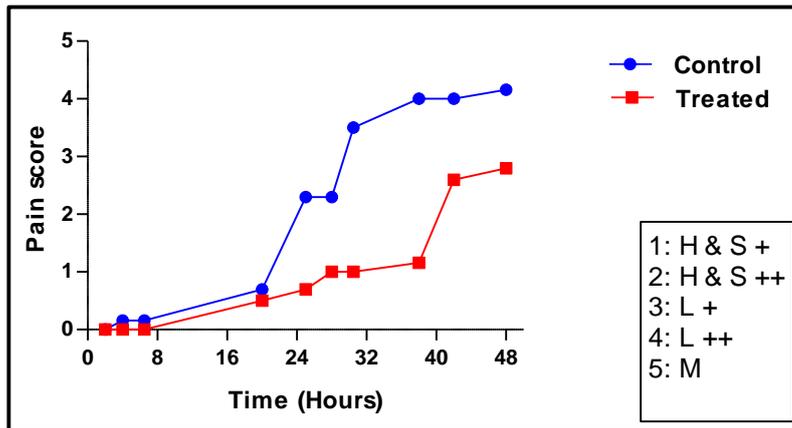


Figure 5.7: Pain score in control and intravenous phage treated mice.

where pain score-1 is hunched and starry coat +, pain score-2 is hunched and starry coat ++, pain score-3 is lethargic +, pain score-4 is lethargic ++ and pain score-5 is moribund (data presented are mean of 10 mice per group).

5.2.3 Influence of phage treatment on mice Survival after pneumococcal infection

Survival of infected mice with serotype D39 using phage SP-QS1 was tested as described in 2.13.3. To briefly summarise, female MF1 mice (Harlan) were challenged with 50 μ l pneumococci via the intranasal route in a final concentration of 1×10^6 cfu/ml⁻¹ in 50 μ l. Mice were divided into two groups: control and phage-treated mice. Ten control mice received 50 μ l of a BHI post infection dose, twice, after one and five hours intranasally, while ten phage-treated mice received 50 μ l of phage lysate in a final concentration of 1×10^7 pfu/ml⁻¹ in 50 μ l instead. Mice were checked and scored regularly throughout period of study.

A survival experiment was assayed with intravenous infection models. The same phage and bacterial conditions used in intranasal administration were used for intravenous administration; however, Pneumococcus was administered intravenously. Phage lysate and BHI were injected into phage-treated mice and control mice respectively three times, after one, three and six hours following the beginning of bacterial administration.

Chapter 5. *In vivo* interaction of phage SP-QS1

Four survival experiments were assayed with intranasal infection model and one was assayed with the intravenous infection model.

In intranasal infection model, the survival of phage-treated mice was 100% whereas only 25% of control mice survived as shown in Figure 5.8. The survival of phage-treated mice indicates that this phage provided protection against invasive pneumonia.

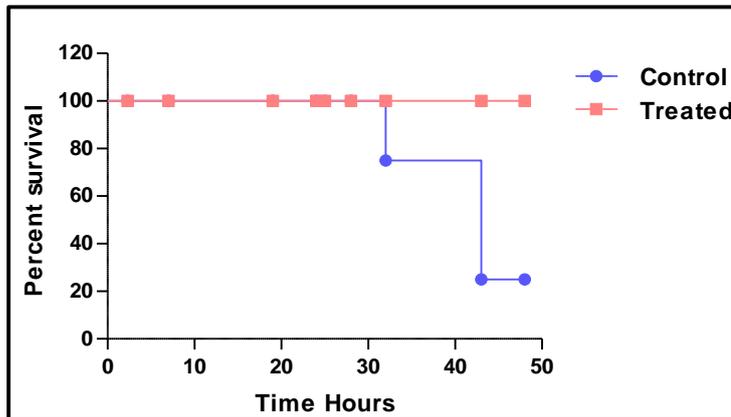


Figure 5.8: Animal survival study in model of invasive pneumonia.

Kaplan-Meier survival chart showing the number of mice still alive in different hours. The survival of phage-treated mice was 100%, compared to 25% survival in control mice (data presented are mean of 10 mice per group).

The survival of mice treated with phage SP-QS1 in sepsis infection model illustrates that all phage-treated mice survived for the first 42 hours, following this there was a sharp drop in their number reporting a 30% survival portion at the end of the observation period, whereas control mice lost about 10% of their population 25 hours after the commencement of the bacterial challenge. Five hours later, the survival proportion in control mice decreased to 40% and after 12 hours it finished with only 20% of the control mice. This is slightly lower than the survival rate for phage-treated mice however the latter survived 12 hours later than control mice without displaying any mice reduction (Figure 5.9).

Chapter 5. *In vivo* interaction of phage SP-QS1

To conclude the survival in sepsis model of infection, there was a significant difference between controls and phage treated mice till about 40 hours post-challenge, then no significant difference despite there were hardly being any pneumococci left in blood. This is suggested to be related to the inflammatory response during the infection. Pneumococci were lysed by SP-QS1 causing a release of high loads of pneumolysin into the blood stream, causing inflammation. Developing of inflammation associated with cytokine storm which could be the reason why these mice die (Kadioglu *et al.*, 2008).

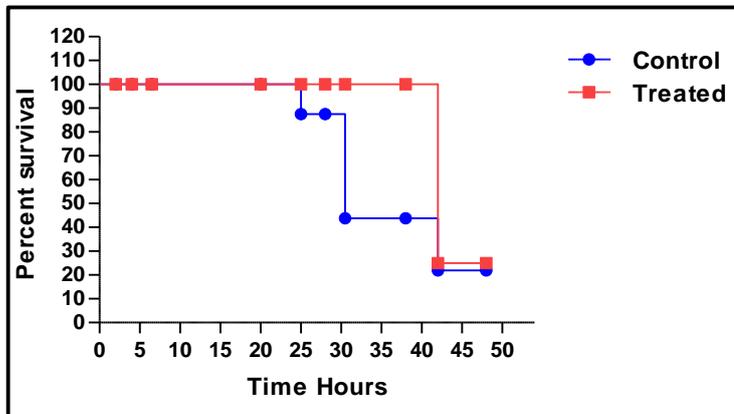


Figure 5.9: Animal survival study in sepsis infection model.

The curves report the number of mice still alive in different hours. Data refer to 6 phage treated mice and 6 control mice from the beginning of pneumococcal infection to their natural death. The survival proportion in phage-treated mice was only ~30% which was similar to the control mice, however, the survival rate of 100% in phage-treated mice was observed for 12 hours later than control mice which started to show severe mice morbidity after 30 hours from the beginning of the study.

Chapter 5. *In vivo* interaction of phage SP-QS1

5.3 Conclusion

The interaction between phage SP-QS1 and serotype 2 *S. pneumoniae* in the mouse model showed a significant efficacy for bacterial reduction. Two mouse models of infection were studied in this chapter, the intranasal infection model and intravenous infection model (sepsis).

The ability of phage SP-QS1 to kill pneumococci and eliminate the development of severe illness in phage-treated mice was obtained in an intranasal infection model. The reduction in pneumococcal viable count was observed after 48 hours of the interaction. The percentage of pneumococcal reduction was 31.7%, 82% and 99% in nasopharynx, lungs (p value: 0.015) and blood (p value: 0.0002) respectively. This observation was confirmed with a pain score study, which showed a significant reduction in hourly mean pain scores in the phage treated group as compared to the control group; with control mice exhibiting maximal pain scores (moribund) by 48 hours.

The reduction in pneumococcal viable count was observed in the sepsis infection model. Six hours after the pneumococcal administration intravenously, no bacterial viability reduction was obtained in phage-treated mice blood and that might resemble the greatest challenge between bacterial and phage proliferation. When the infection extended for 24 hours, one and half \log_{10} of reduction was obtained and a percentage reduction of 34% recorded as compared to control mice. The major reduction was obtained after 48 hours as pneumococcal viable count decreased in about 6 logs recording a percentage reduction of 96.67% (p value 0.0003) compared to control mice. The pain score study showed that despite the worsening condition of phage-treated mice at the end of observation period, a healthy condition prevailed in this group survived for 40 hours before the mice started developing a severe condition. The worse condition

Chapter 5. *In vivo* interaction of phage SP-QS1

might be attributed to the immune response and not to the attack of pneumococci as the bacterial viable counts were decreased by the end of treatment period.

Survival studies in intranasal infection resulted in good protection as 100% of phage-treated mice survived, whereas only 25% of control mice survived. The survival study in the intravenous infection model resulted in a survival rate of 100% in the first 40 hours of the phage bacterial interaction; however, it decreased subsequently, eventually reporting the same survival rate as control. This might be due to a severe immune response, which developed during the infection as a result of bacterial damage. However, pneumococci sepsis infection is rarely developed directly and it almost follows respiratory infection. Thus phage SP-QS1 could be nebulised for inhalation therapy to treat those people with pneumonia and could also be used as a treatment against pneumococcal carriage.

Chapter 6: Genetic characterisation of phage SP-QS1

Chapter 6. Genetic characterisation of phage SP-QS1

6.1 Introduction

Phage genomes are characterised based on their size into three groups; Large size phage genomes between 30–50kbp (corresponding to nearly 50% of all phages), small size phage genomes which measure less than 10kbp (about 20% of total), and a third group containing genomes size between 100–200 kbp (6% of the total) (Hatfull, 2008).

The small size and simplicity of isolation of bacteriophages enabled the first complete phage genome to be sequenced, and that was 5,386 bp single-stranded DNA (ssDNA) phage Φ X174 in 1977 (Sanger F et al., 1977). The 48,502 bp genome of the lambda phage was the first double-stranded DNA temperate phage to be sequenced (Sanger et al., 1982). The 39,936 bp genome of the phage T7 was the first double-stranded DNA lytic phage to be sequenced (Dunn JJ and Studier FW, 1983). Mycobacteriophage L5 was the first sequenced dsDNA tailed-phage genome of a virus infecting a non-*Escherichia coli* host (Hatfull and Sarkis, 1993). NCBI has reported that there are about 500 completely sequenced phage genomes ranging from 2,435 bp for *Leuconostoc* phage to 316,674 bp for *Pseudomonas* phage 201phi2–1. In addition to these known sequenced phages, phage sequencing data has been derived from random cultures from various sources such as faeces, sea water and marine sediments (Rohwer *et al.*, 2000; Breitbart *et al.*, 2002; Breitbart *et al.*, 2003; Breitbart *et al.*, 2004).

Sequencing data has shown that all dsDNA of bacteriophages are mosaics with access to a large scale of genomic pool, however, there is no uniform of all phages (Hendrix et al., 1999). It was thought that bacteriophages have undergone lateral gene transfer and their origins placed at the level of the last universal common ancestors of eubacteria, archaea and eukarya, if this hypothesis true, the vertical evolution of phages would be postulated (Brüssow and Desiere, 2001).

Chapter 6. Genetic characterisation of phage SP-QS1

There are two pneumococcal lytic phages Dp-1 and Cp-1 which have genomic sizes of 58,063 and 19,343 bp respectively. Neither of the phage genomes have any gene encoded for capsule degradation enzyme such as glycosyl hydrolase (Martín *et al.*, 1996; Sabri *et al.*, 2011). Phage Cp-1 genome contains a terminal protein, which is covalently linked to its 5' end and a 236-bp-long perfect inverted repeat (Martín *et al.*, 1995).

Four temperate pneumococcal phages have been previously induced: EJ-1, MM1, HB-3 and VO1. All these phages are not able to infect capsulated pneumococci (Romero *et al.*, 1990c; Diaz *et al.*, 1992; Gindreau *et al.*, 2000; Obregon *et al.*, 2003b).

Generally, siphoviruses have been characterised according to head morphology into two classes; phages with isomeric heads and phages with prolate heads. The isomeric head group contains the majority of siphophages which are well described by complete genomic sequences. A few prolate-headed phages have been characterised at the molecular details such as *Lactobacillus* phage C2 and mycobacterium phages Che9c, corndog, and brujita (Lubbers *et al.*, 1995; Hatfull *et al.*, 2010).

SP-QS1 is the first prolate siphophage genome sequenced. It is also the first phage known to infect the capsulated strains of *S. pneumoniae*. Thus this chapter aims to characterise the traits of the genome of this phage such as the presence of toxins or recognizable integrase as that might facilitate the use of this phage to treat pneumococcal infection in human. The genome was also sequenced to determine the life cycle and the biology of this phage. Sequencing data can help to explain the ability of SP-QS1 to infect capsulated pneumococci and the other phage cannot. Analysing data from genome sequencing enables to determine the evolutionary relationship of this phage with the others which retained matches with high blast scores.

Chapter 6. Genetic characterisation of phage SP-QS1

The annotation of this novel genome illustrates the homology of phage SP-QS1 to those of *Enterococcus* phage SAP6 (acc. no JF731128.1) and BC-611 (acc. no AB712291.1). Genes involved in phage packaging are homologous to those of *Enterococcus* phage SAP6 as well as phage portal protein, phage tail and tail fibres from structural protein. The rest of structural proteins including phage head, major tail and minor capsid are homologous to those for *Enterococcus* phage BC-611. Phage replication proteins primase and helicase are homologous to those for *Enterococcus* phage SAP6 whereas DNA polymerase is similar to those for *Enterococcus* phage BC-611. SP-QS1 protease is homologous to those for *Enterococcus* phage SAP6.

Phylogentic analysis of phage SP-QS1 has shown unresolved relationship to other phages based on DNA polymerase data analysis, however, the terminase gene and the capsid gene phylogeny studies suggest that SP-SQ1 is most closely related to the *Enterococcus* phages SAP6 and BC-611.

6.2 Results and discussion

6.2.1 Genome Properties

The genome sequence of phage SP-QS1 is 58, 063 base pairs. The appropriate size was initially predicted by pulse field gel electrophoresis (Figure 6.1). The guanine-cytosine (G-C) content average of the phage genome is 40%; similar to the content of the host serotype D39 39.71% (Tettelin H et al., 2001). The completed SP-QS1 genome sequence was deposited in the NCBI database under the accession number (HE962497).

Chapter 6. Genetic characterisation of phage SP-QS1

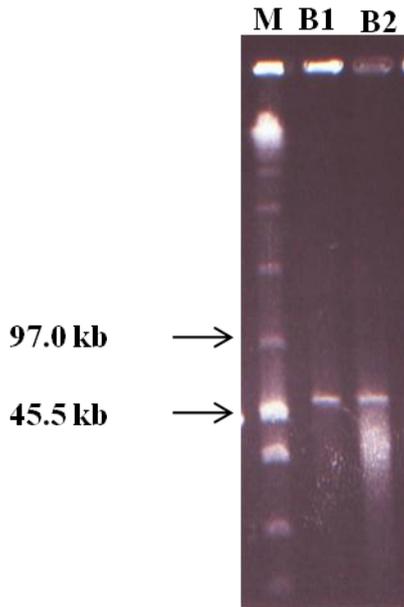


Figure 6.1: Phage SP-QS1 Genome Size - PFGE Image.

The First and second SP-QS1 genomic bands are a concentrate and diluted phage lysate, respectively.

6.2.2 Open Reading Frames (ORFs) Prediction

The number of ORFs predicted using GeneMark was 97 and by GLIMMER were 100. A combined analysis therefore suggested 103 putative ORFs. Blastp searches (accessed on 30 May 2012) were performed on putative ORFs and all predicted functions recorded in Table 6.1 (all hits scoring less than 10^{-4} were considered non-significant and so, excluded). 29 ORFs were found to have putative functions. The remaining ORFs which retained matches with high blast scores were hypothetical proteins as shown in appendix 4. The majority of predicted ORFs are homologous to the *Enterococcus* phage SAP6 and BC-611. A list of ORFs and related protein predictions are shown in (Figure 6.2).

Chapter 6. Genetic characterisation of phage SP-QS1

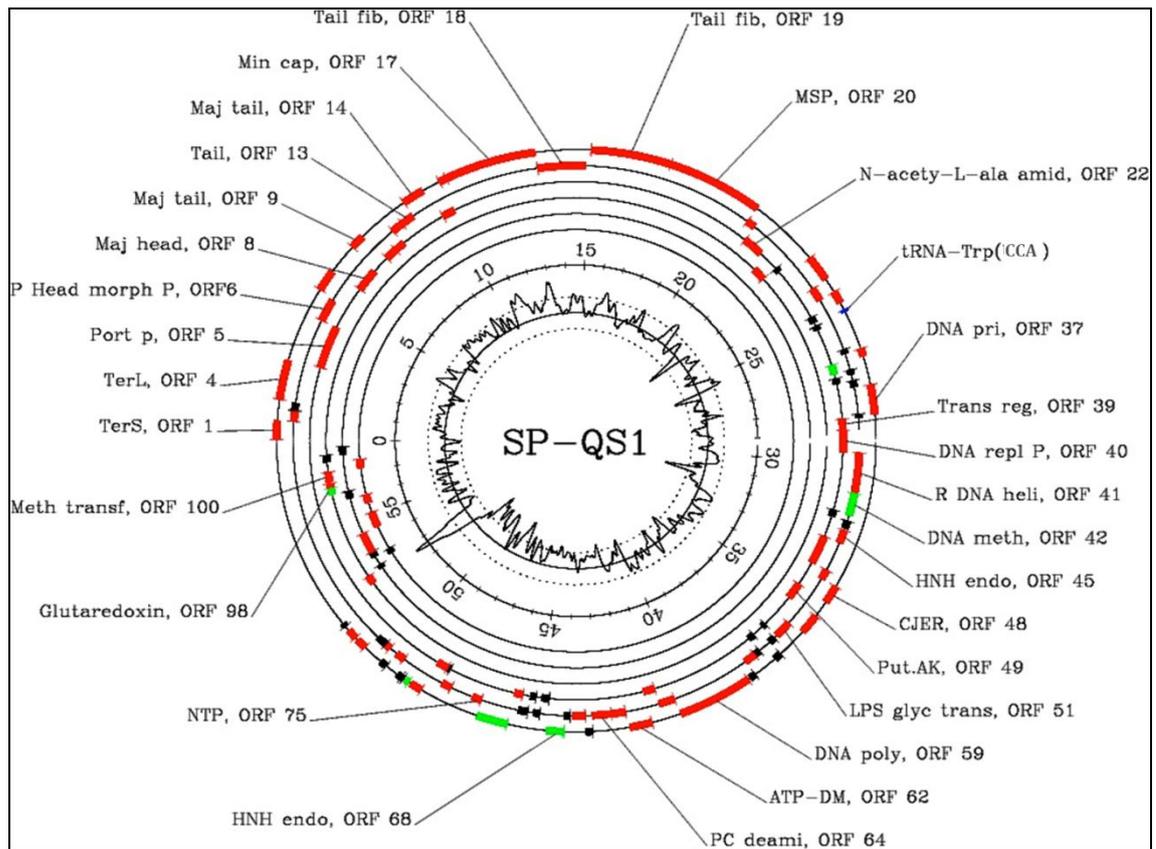


Figure 6.2: Organisation of the Genome of Phage SP-QS1.

The circles from outside to inside represent the following: 1 to 6 the six reading frames, 7 is the scale bar (in kilo bases), and 8 is GC content (smoothed with a sigma $_200$ bp; Gaussian; range from 29 to 59%, with a mean of 40%). In addition, given labels indicate ORF numbers and gene designations where a putative homologue has been identified. The following colour scheme has been used: red, ORFs encoding proteins exhibiting similarity to other phage proteins, green, ORFs bacterial proteins; blue, eukaryotic genes, and black is unknown.

6.2.3 Transfer RNA (tRNA) and Pseudogenes

Streptococcus phage SP-QS1 encodes for one transfer RNA (at position 24820 bp to 24891 bp) within the genome. It encodes for a tryptophan CCA anticodon. The gene encoding for tryptophan has been found in the DNA of strain *S. pneumoniae* D39 (NCBI). It has been reported that phage tRNA is required by their hosts and may increase the fitness of these hosts (Miller et al., 2003).

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Scanning genome sequences with CRISPRFinder and transtermHP showed that the phage did not encode CRISPR sequences, proteins with trans-membrane domains, or regulatory elements. Furthermore, the phage genome did not encode any integrase genes and so appears to be a genuine lytic phage. Finally, the phage did not encode any putative toxins.

Table 6.1: Phage SP-QS1 Predicted ORFs and closest matches.

ORF	Strand	Start	End	Length	Predicted function	E-value	Closest match
1	+	40	639	600	Phage terminase small subunit	2.00E-112	<i>Enterococcus</i> phage SAP6
4	+	1327	2598	1272	Phage terminase large subunit	0	<i>Enterococcus</i> phage SAP6
5	+	2655	4190	1536	Phage portal protein	0	<i>Enterococcus</i> phage SAP6
6	+	4202	4957	756	Phage head morphogenesis protein	1.00E-145	<i>Enterococcus</i> phage BC-611
8	+	5793	6599	807	Major head protein	1.00E-148	<i>Enterococcus</i> phage BC-611
9	+	6754	7194	441	Major tail protein	8.00E-74	<i>Enterococcus</i> phage BC-611
13	+	8429	8863	435	Phage tail protein	6.00E-80	<i>Enterococcus</i> phage SAP6
14	+	8884	9573	690	Major tail protein	8.00E-130	<i>Enterococcus</i> phage SAP6
17	+	10405	13290	2886	Minor capsid protein	0	<i>Enterococcus</i> phage BC-611

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18	+	13304	14863	1560	Phage tail fibre protein	2.00E-176	<i>Enterococcus</i> phage SAP6
19	+	15040	17538	2499	Phage tail fibre protein	0	<i>Enterococcus</i> phage SAP6
20	+	17551	20577	3027	Phage minor structural protein	0	<i>Enterococcus</i> phage SAP6
22	+	20925	21638	714	N-acetylmuramoyl-L-alanine amidase	3.00E-136	<i>Enterococcus</i> phage SAP6
37	+	27352	28299	948	DNA primase	0	<i>Enterococcus</i> phage SAP6
39	+	28374	28727	354	Transcriptional regulator	2.00E-57	<i>Enterococcus</i> phage BC-611
40	+	28776	29552	777	DNA replication protein	3.00E-151	<i>Enterococcus</i> phage SAP6
41	+	29564	30928	1365	Replicative DNA helicase	0	<i>Enterococcus</i> phage SAP6
42	+	30941	31693	753	DNA methylase	7.00E-73	<i>Enterococcus gallinarum</i> EG2
45	+	32228	32668	441	HNH endonuclease	8.00E-78	<i>Enterococcus</i> phage BC-611
48	+	34021	34590	570	Crossover junction endodeoxyribonuclease RuvC	2.00E-104	<i>Enterococcus</i> phage SAP6
49	+	34587	35156	570	Putative adenylate kinase	1.00E-103	<i>Enterococcus</i> phage BC-611

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51	+	35873	36436	564	Glycosyltransferase	2.00E-103	<i>Enterococcus</i> phage BC-611
59	+	38062	40413	2352	DNA polymerase I	0	<i>Enterococcus</i> phage BC-611
62	+	41359	42054	696	ATP-dependent metalloprotease	2.00E-122	<i>Enterococcus</i> phage SAP6
64	+	42575	43207	633	Putative cytidine deaminase	2.00E-12	<i>Enterococcus</i> phage phiEF24C
68	+	44116	44658	543	HNH endonuclease family protein	2.00E-33	<i>Streptococcus</i> <i>agalactiae</i> 2603V/R
75	+	46907	47212	306	Nucleoside triphosphate pyrophosphohydrolase	2.00E-46	<i>Enterococcus</i> phage BC-611
98	-	56236	56496	261	glutaredoxin	2.00E-9	<i>Actinomyces</i> <i>sp.</i> oral taxon 848 str. F0332
100	-	56728	57105	378	methyltransferase	1.00E-40	<i>Enterococcus</i> phage SAP6

6.2.4 Genome architecture

The organisation of the phage SP-QS1 genome roughly follows the architecture typically seen in other siphoviruses including the well described coli-phage lambda, Sfi21 and Sfi11 (*S. thermophilus* phages), L5 (*M. tuberculosis* phage) C31

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(*Streptococcus coelicolor* phage) and the Vibrio-phages VP16 C and VP16T (Seguritan et al., 2003). Genes with recognisable homologies have been categorised in terms of the following; (1) those genes (ORFs 1 and 4) involved in phage packaging (terminase), (2) structural proteins (portal proteins – tail fibre proteins), (3) replication (primase - polymerase) (4) genes associated with cell lysis (protease) as shown in Figure 6.3. In terms of genomic architecture, the main difference between SP-QS1 and previously described phages is the position of the protease. Here, the SP-QS1 protease is positioned after the DNA polymerase, in most siphoviruses, the protease is next to the portal protein.

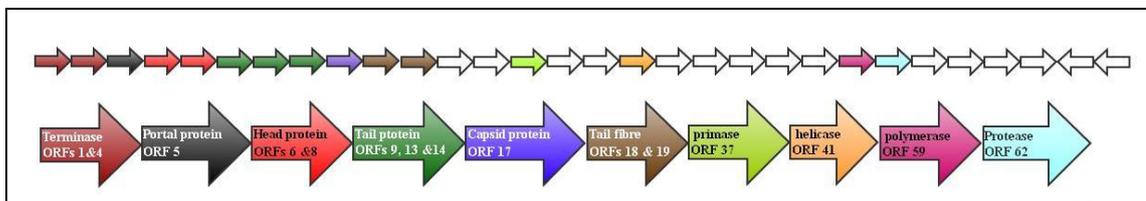


Figure 6.3: Schematic of keys siphovirus genes which are for phage SP-QS1.

ORFs are colour coded based on the Blastp E-values and are labelled based on the Blastp hits shown in table 6.1. In this chart, ORFs represent Blastp E-values ($\geq 1e-4$) only. ORFs employed to predict encoded putative function.

6.2.5 Glycosyltransferase and capsule degradation

The capsule is the key to pneumococcal virulence. The previously isolated phages are unable to bypass this pneumococcal capsule (Bernheimer and Tiraby, 1976). However, phage SP-QS1 can infect capsulated *S. pneumoniae*. One gene might be responsible for capsule degradation, enabling such phage to infect and lyse invasive capsulated pneumococci. This gene is the gene encoding for glycosyltransferase.

The ORF 51 in SP-QS1 is predicted to encode for the glycosyltransferase. Glycosyltransferase is an enzyme which catalyses the transfer of sugar moieties from donor molecule to specific receptor molecules (Coutinho et al., 2003). There are two

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major structural types of glycosyltransferase; GT-A, which is a metal ion dependant, and GT-B, which comprises of two Rossmann folds separated by a fork that forms the substrate-binding site. The enzyme is also classified as either an inverting, or a retaining enzyme, depending on the anomeric carbon atom. For example, where the anomeric carbon atom has the same configuration in both donor and product, the enzyme is classified as retaining. Conversely, with a different configuration, the enzyme is classed as an inverting enzyme (Davies, 2001).

Glycosylated structural proteins are found in many viruses and occupy a number of roles such as; mimicking host glycans to evade the immune system, the folding and assembly of viral particles, aiding the stabilisation of viral particles and functioning as receptors to bind host cells (Vigerust and Shepherd, 2007). In bacteriophages, glycosyltransferase acts to modify phage DNA so as to protect it from restriction modification (Gram H and Ruger W., 1986). Furthermore, this enzyme is involved in the biosynthesis of the bacterial outer core, which functions as a receptor for specific phages such as the *Yersinia enterocolitica* serotype O:3 phage ϕ R1-37 (Skurnik *et al.*, 1995; Leipold *et al.*, 2007). Therefore, this enzyme may play two roles during SP-QS1 infection, firstly in the modification of phage receptors; possibly preventing further phage infections following takeover of the cellular machinery and secondly, to protect SP-QS1 DNA from pneumococcal restriction modification systems. Finally, because the function of glycosyltransferase is similar to that of glycosyl hydrolase which has been found encoded by phages of those infected capsulated bacteria (Mushtaq *et al.*, 2005; Scorpio *et al.*, 2007), phage SP-QS1 might use glycosyltransferase to degrade the capsule of *S. pneumoniae*.

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6.2.6 Phage structural proteins

Five structural genes encoding for structural proteins; head, tail, capsid and tail fibres. Head proteins locate in ORFs 6 and 8 which were predicted as phage head morphogenesis protein and major head protein, respectively. A top hit for the two proteins in blastp analysis was the *Enterococcus* phage BC-611. Phage head genes were found next to the gene which encodes for portal protein. Phage tail proteins are located in ORFs 9, 13 and 14 which were predicted as major tail protein, phage tail protein and major tail protein, respectively. Data analysis revealed that ORF 9 has similarity to *Enterococcus* phage BC-611 while ORFs 13 and 14 have similarity to *Enterococcus* phage SAP6. ORF 17 is the only ORF that was predicted to encodes for minor capsid protein and was found similar to that protein encoded by *Enterococcus* phage BC-611, furthermore, the phylogenetic tree of capsid gene suggest that SP-SQ1 is most closely related to the *Enterococcus* phages SAP6 and BC-611 (Figure 6.4). Phage tail fibre protein and phage minor structural protein are located in ORFs 18 and 19 and ORF 20, respectively. A top hit matching of all these proteins was the *Enterococcus* phage SAP6.

6.2.7 Predicted Nucleic Acid – Metabolism, Modification and replication

The prediction of SP-QS1 genome indicate the encoding of 15 proteins involved in phage DNA metabolism and modification. Here only three of these proteins described in details due to their importance; endonuclease, methyltransferase, and DNA polymerase. Endonuclease is essential enzyme in DNA replication and repair. Methyltransferase plays an important role in the protection of phage DNA from host restriction modification and DNA polymerase is the key factor in the DNA replication.

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6.2.7.1 HNH Endonuclease

There are two H-N-H endonucleases predicted genes in SP-QS1 which can be found in two different ORFs; 45 and 68. These enzymes are homologous with the protein encoded by *Enterococcus* phage BC-611 and *S. agalactiae* 2603V/R, respectively.

Homing endonucleases are encoded by introns and inteins, these make a site-specific double strand break in the intronless and inteinless alleles. This results in both intron, and intein, duplication via recombinogenic ends which engage in a gene conversion process to produce stratification (Belfort and Roberts, 1997). Homing endonucleases are distinct from restriction enzymes due to the default occurrence of a functional motif within the active centre of restriction enzymes (Aggarwal, 1995; Pingoud and Jeltsch, 1997). Furthermore, recognition sequences span 12-40 bp in homing endonuclease whereas in restriction enzymes they span only 3-8 bp (Lambowitz AM and Belfort M, 1993; Roberts and Macelis, 1997). Both enzymes are able to act alone or with additional protein subunits however, accessory protein subunits are relatively different since restriction enzymes (type I and II) require modification and/or specificity in order to initiate action (Bickle T.A. Nucleases. 2nd edn *et al.*, 1993; Shibata *et al.*, 1995; Zimmerly *et al.*, 1995). On a final note, homing endonucleases have been found in eukaryote, archaea and bacteria whereas, restriction enzymes have been found in archaea, bacteria and some eukaryotic viruses (Aggarwal, 1995; Pingoud and Jeltsch, 1997).

Homing endonucleases are classified into four families characterised by the following sequence motifs - LAGLIDADG, GIY-YIG, H-N-H and His-Cys box - based on structure, DNA recognition and genomic location (Belfort and Perlman, 1995). It is been found that H-N-H endonucleases encode sequences spanning 30-33 amino acids.

The structure of this enzyme includes two pairs of conserved histidines surrounding a

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conserved asparagine to form a zinc finger-like domain (Gorbalenya, 1994; Shub *et al.*, 1994). H-N-H endonuclease has been found in phages including T-even phage H-N-H enzyme I-TevIII, which produces a double-strand cut. The enzyme has a property enabling it to generate 5' rather than 3' extensions (Eddy and Gold, 1991). It has also been found in *Bacillus subtilis* phage enzymes I-HmuI and I-HmuII, which can cleave one DNA strand on both intron-containing, and intronless, targets (Goodrich-Blair and Shub, 1996).

Both H-N-H endonuclease sequences of *S. pneumoniae* phage were aligned with the top 12 homologies as a result of blastp using the ClustalW program. This was further manipulated by Bioedit v7.1.3 software. It appears from the alignment that histidine/asparagin content is relatively high. When comparing aligned sequences it seems clear that overall, homology is relatively low. Indeed, this gene sequences (ORF 68) are extremely homology to those of *S. agalactiae* prophage and *Enterococcus* phage EFRM31 (as shown in appendix 4). The alignment other gene's sequences was not applicable due to the limitation of high hits obtained from blastp.

SP-QS1 H-N-H endonuclease is suggested to be responsible to make a site-specific double strand break in the intronless and inteinless alleles making them ready for duplication.

6.2.7.2 Methyltransferase

The genome of SP-QS1 encodes two methyltransferase genes predicted to encode methyltransferase located at two different ORFs; 42 and 100. These were found to be homologous with methyltransferase encoded by *Enterococcus gallinarum* EG2 and *Enterococcus* phage SAP-6, respectively. This enzyme is thought to protect SP-QS1 from bacterial restriction thereby allowing the phage to propagate.

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Blastp searches resulted in limited hits regarding predicted homology with ORF 100, which encode methyltransferase. Therefore, only the first methylase encoded by ORF 42 sequences were used in alignment with the top 12 hits. These were obtained from blastpsearches using ClustalW. The alignment revealed an approximately 50 % homology to all aligned sequences. It was noted that methylase sequences of SP-QS1 was placed in alignment between methylase sequences of *E. gallinarum* EG2 and *ActinoBacillus pleuropneumoniae* 4074 prophage, all sharing a high degree of homology compared with other organisms/genes (as shown in appendix 5).

Methyltransferase is an enzyme catalyst that transfers a methyl group from a donor S-adenosylmethionine to the 5-carbon position of the cytosine ring (Schmitt et al., 1997). Four methylation processes can take place within the nucleus: the first is referred to as de novo methylation, where unmethylated cytosines, predominately within the CpG dinucleotide, become methylated. The second, maintenance methylation, concerns maintenance of hemimethylated, newly synthesised strands, following replication (Bestor and Verdine, 1994). The third process, passive demethylation, is where maintenance methylation activity decreases by 50% during each round of DNA replication. The fourth, active demethylation, is where enzymatic processes are applied to decrease methylation levels in the absence of DNA replication (Weiss *et al.*, 1996; Frémont *et al.*, 1997).

Bacterial methyltransferase is used for restriction modification to protect bacteria against phage DNA by digesting it via restriction enzymes (Arber W and Dussoix D, 1962; Dussoix D and Arber W, 1962). As a consequence, phages respond by adopting strategies to eliminate restriction sites (Weinbauer, 2004a). Methyltransferase is encoded by lytic phages such as bacteriophages T2 and T4 (Schlagaman and Hattman, 1989). It has been discovered that bacterial restriction-associated MTase, possess a

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distributive mechanism activated via phage processivity during methylation. This interferes with biological functions involved in restriction modification (Yang Z et al., 2003).

Methyltransferase in SP-QS1 is suggested to protect phage genome from restriction modification that is might be generated by pneumococci.

6.2.7.3 DNA Polymerase I (Pol I)

The annotation of SP-QS1 genome revealed that this genome encodes for DNA polymerase I (Pol I). The gene encoded for Pol I located within ORF 59. A top hit homologous obtained from blastp analysis was the *Enterococcus* phage BC-611, however, as described in the introduction (section 6.1), the phylogenetic tree indicates that DNA polymerase is not a reliable marker as relationships between the phages included in the analysis were unresolved.

DNA polymerase is an enzyme used for replication and transcription in order to allow micro-organisms to replicate and survive. This enzyme has the ability to produce corresponding biopolymers with high levels of accuracy, integrity and processivity in turn, facilitating its wider application throughout numerous molecular and biological systems (Henry and Romesberg, 2005). DNA polymerase also functions to protect against DNA damage (Lange et al., 2011). Based on amino acid sequence comparisons, DNA polymerase can be divided into seven different families; A, B, C, D, X, Y and RT. Replicative bacteriophage DNA polymerase belong to type A and B however, DNA repair involves all families associated with DNA polymerase. Furthermore, these families are unrelated based on sequence comparisons. Nevertheless, similarities do exist between families A and B in terms of biochemical and structural features (Filée J et al., 2002 ; Harada F et al., 2005).

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Remarkably, despite considerable diversity in relation to bacterial climates, Pol I sequences have been conserved over time (Gutman et al., 1993). Three Thousand base pairs are involved in the gene encoded for Pol I, which can encode 1000 amino acids residues in a simple polypeptide chain. Regardless of evolution, Pol I retain 35 % of amino acid identity, with 50 % homology. This property enables the substitution of deleted endogenous Pol I activity units, exchanging one organism for another originating from different bacteria (Suzuki et al., 1996). *E. Coli* Pol I is the prototype for a DNA polymerase which has the same structure as other polymerases; a large cleft surrounded by fingers, a palm and a thumb (Ollis et al., 1985). Interestingly, Pol I has been linked to mitochondrial DNA polymerase- γ which features in some bacteriophages including T3 and T4 (Filée J et al., 2002). Searches using PSI-BLAST for *E. Coli* Pol I Sequences, retrieved an homology with bacteria, followed by the DNA polymerases of several gram positive and gram negative bacteriophages and also N-terminal domains for novel eukaryotic DNA polymerase/helicase (Harris et al., 1996).

Because DNA polymerase I, has been found in bacteria, this enzyme could be transferred from one strain to another. However, SP-QS1 DNA polymerase is homological to DNA polymerase in *Enterococcus* phage BC-611 whereas its host, *S. pneumoniae* D39, contains DNA polymerase III (holoenzyme). The latter is different from Pol I in light of features such as multi-subunit structure, the need for TP to bind tightly with DNA, the rapidity of DNA synthesis, and the ability to remain bound to DNA during repeated polymerization. This renders the enzyme remarkable and highly processive (Kelman Z and O'Donnell M, 1995).

SP-QS1 DNA polymerase phylogenetic analysis showed that an unresolved relationship with those phages examined in the disrobed analysis (Figure 6.4). Previous results are supported by findings relating to sequence alignment measured using ClustalW. These

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results indicated that SP-QS1 DNA sequences have a low homology with the sequences employed in alignment. The alignment process suggests that DNA polymerase SP-QS1 have undergone different mutations namely, point mutations, which are represented by mismatches, and indel mutations, represented by gaps (as illustrated in appendix 6).

As DNA polymerase responsible for DNA replication in all micro-organisms thus, SP-QS1 polymerase I is suggested to play the same role leading to the production of new copies of its DNA.

6.3 Phylogenetic relationship of key genes within the genome of SP-QS1

To determine the affiliation of SP-QS1 with known phages, three genes were chosen on the basis of their known utility for resolving phage evolutionary relationships; the DNA polymerase gene, the capsid gene and the terminase gene. All genes were combined with the top 25 blastp hits and then analysed using both maximum parsimony and neighbourhood joining algorithms using 1000 bootstraps. Because the algorithm used did not affect tree topology the neighbourhood joining trees are presented here.

Data analysis based on the phylogenetic tree indicates that DNA polymerase is not a reliable marker as relationships between the phages included in the analysis were unresolved. The phylogenetic tree of DNA polymerase showed that phage SP-QS1 was poorly formed a clade with prophage of *Bacillus atrophaeus* 1942. The terminase gene and the capsid gene suggest that SP-SQ1 is most closely related to the *Enterococcus* phages SAP6 and BC-611 and they formed a tight clade together. These data suggest SP-SQ1 has an interesting evolutionary history and indeed, may possibly result from previous co- infections with *Enterococcus*. However it is pertinent to state that *Enterococcus* has a different niche to *S. pneumoniae* and thus, it is unlikely that the phage isolated in this project was associated with an *Enterococcus* species, This is

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because the *Enterococcus* bacteria inhabits the intestine whereas, *S. pneumoniae* reside in the nasopharynx. Phylogenetic trees are shown in Figure 6.4.

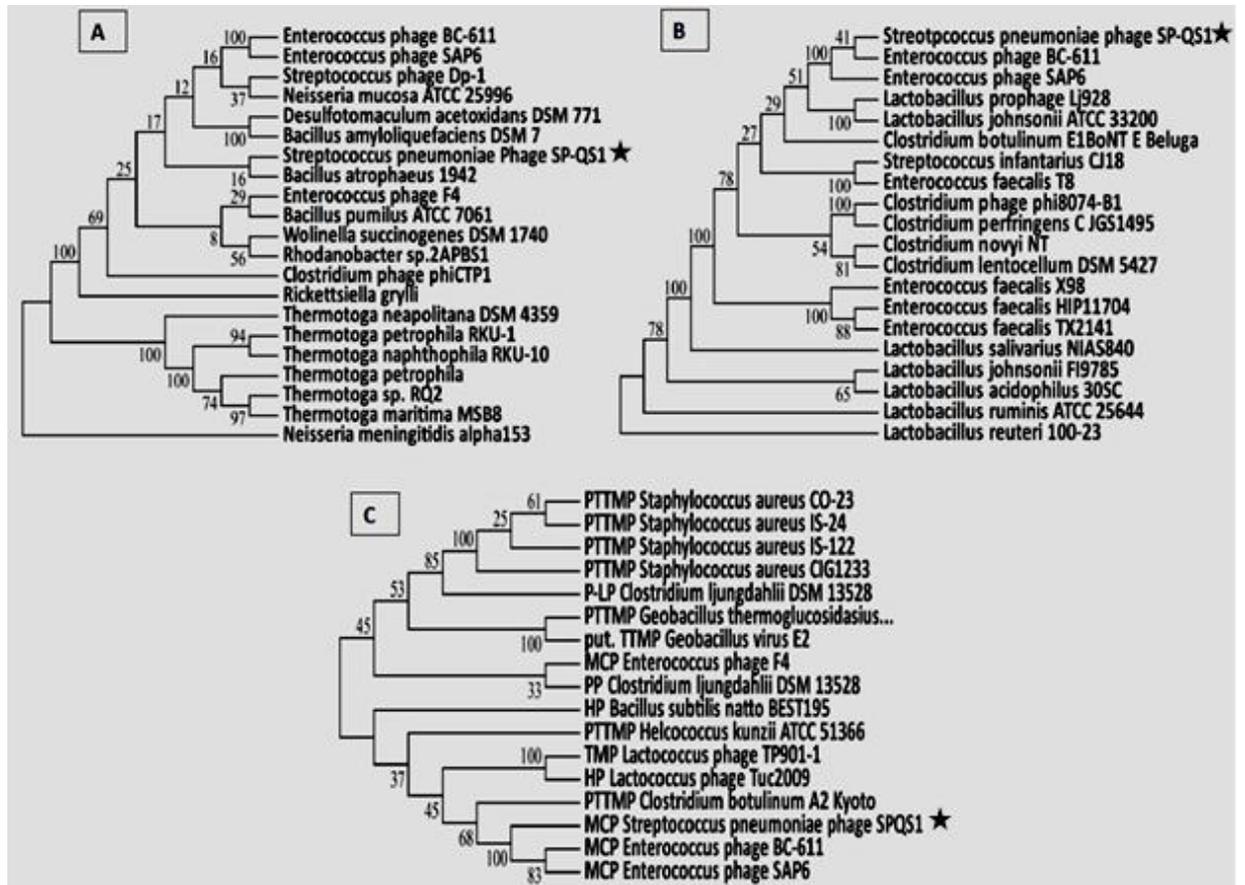


Figure 6.4: Phylogenetic Trees with Three Encoding Genes.

Phylogenetic analyses were based on the amino acid sequences of the A: DNA polymerase, B: Terminase and C: Capsid proteins. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the p-distance method based on their amino acid sequences.

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6.4 Conclusion

SP-QS1 is the first has the ability to infect capsulated *S. pneumoniae*. It is also the first prolate-head phage genome sequenced. The genome contains 58, 063 base pairs with an overall G-C content of 40%. Furthermore, the genome has 103 predicted ORFs. Nevertheless, only 29 ORFs have been found to predict the assignment of putative functions. Despite high numbers regarding ORFs within the genome of SP-QS1, the number of ORFs with the ability to assign putative function remains low. This finding appears consistent with other phages such as *Proteobacteria* phage Φ JL001 and *Vibrio harveyi* phage VHS1, they have 17/91 and 24/125 functional ORFs, respectively (Lohr *et al.*, 2005; Khemayan *et al.*, 2012).

SP-QS1 genes with recognisable homologies are often ordered according to the following; (1) genes involved in phage packaging, (2) structural proteins, (3) replication and (4) genes associated with cell lysis. Interestingly, SP-QS1 genome does not encode CRISPR sequences, proteins with trans-membrane domains or regulatory elements. In addition, because the phage genome does not encode integrase genes, it appears to be a genuine lytic phage. Also, in omitting to encode putative toxins, the latter two traits mean that it is well suited for therapeutic use in humans.

The annotation of this novel genome illustrates the homology of phage SP-QS1 to those of *Enterococcus* phage SAP6. However, phage SP-QS1 encodes certain proteins which make this phage distinguished from *Enterococcus* phages SAP6 and BC-611, including DNA methylase and glutaredoxin. These two proteins facilitate SP-QS1 genome protection and replication via host cells. There are also a number of genes encoded for N-acetylmuramoyl-L-alanine amidase, methylase and glycosyltransferase making it distinct from previous pneumococcal lytic phages Cp-1 and Dp-1. Glycosyltransferase has been suggested to degrade pneumococcal capsule and associate with N-

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acetylmuramoyl-L-alanine amidase to facilitate phage infection. Moreover, the SP-QS1 genome involves genes encoded for tryptophan as tRNA, located at 24820 bp and 24891 bp. Tryptophan is required by their hosts and may increase the fitness of these hosts (Miller et al., 2003).

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Chapter .7 Conclusion and future work

7.1 The respiratory invasive pathogen; *S. pneumoniae*

S. pneumoniae can be found in the upper human respiratory tract, and particularly the nasopharynx (Hament *et al.*, 1999). It constitutes a major global public health problem, and can cause pneumonia, otitis media, sinusitis and meningitis, mainly in children and the elderly (WHO, 2003). Pneumococcal disease is the main cause of child mortality in the developed world, and, according to the World Health Organization, two million children under the age of five die every year from pneumococcal infection (Spier, 2008). Despite the availability of pneumococcal conjugate vaccine (PCV-7 and -13) and pneumococcal polysaccharide vaccine (23-valent vaccine), the protection resulting from their use is insufficient to control the infections by pneumococci, which has more than 90 serotypes (Rosén *et al.*, 1984; Faden *et al.*, 1991; Camou *et al.*, 2003; Hansen *et al.*, 2006). Moreover, the Capsular gene replacement ability of this bacterium after vaccination has created a significant challenge for bacteriologists in developing an appropriate vaccine which can provide thorough protection against pneumococcal infections (Filippo *et al.*, 2010; Weinberger *et al.*, 2011). In addition, the prevalence of antibiotic resistance of bacteria has increased, and *S. pneumoniae* is no exception shows a multidrug resistance to a variety of different antibiotics (Whitney *et al.*, 2000; Adam, 2002). Due to the pneumococcal antibiotic resistance and to incompetent protection of pneumococcal vaccines, another strategy should be suggested to control and treat *S. pneumoniae* infections, such as phage therapy.

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7.2 *S. pneumoniae* and phage therapy

7.2.1 Capsulated *S. pneumoniae* and the application of Dp-1 and Cp-1

The first isolated lytic pneumococcal phage was a siphophage Dp-1 from the throat swab of a patient with an upper respiratory infection in 1974; the isolation was carried out using non-capsulated pneumococcus R36A as host (McDonnell *et al.*, 1975). Podovirus Cp-1 was isolated in 1981 from the throat swabs of healthy children, using non-capsulated pneumococcus serotype R6 (Ronda *et al.*, 1981). Both phages showed their ability to clear their hosts efficiently, but not any strains of capsulated *S. pneumoniae*, as reported by Bernheimer and Tiraby in 1976; these researchers concluded that the infection by these pneumococcal phages is inhibited by pneumococcal capsules (Bernheimer and Tiraby, 1976). This case of phage disability to infect capsulated *S. pneumoniae* motivated this project and my PhD studies.

In this study, it has been noticed that pneumococcal capsule has an influence on phage SP-QS1 and *S. pneumoniae* interactions. In chapter 4, it was detected that the reduction in pneumococcal viable counts took place earlier in strains with thinner capsule such as D39, 4 and 6B comparing with passaged D39 and strain 3. These last two hosts share one feature, namely the presence of a thick capsule, which might eliminate phage penetration and as a result, showed late viable count reduction and fewer phage titres (Abdelnour *et al.*, 2009b; Hathaway *et al.*, 2012a). In chapter 5, it was indicated that phage treatment of infected mice resulted in more reduction in pneumococcal viable count in lungs than in nasopharynx. It is known that the capsule becomes thicker during colonisation, which would be expected to delay the penetration of the phage (Weinberger *et al.*, 2009).

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7.2.2 Novel phage SP-QS1 isolation and its activity against capsulated *S. pneumoniae*

258 different samples were screened from throat and nasopharyngeal swabs, sputum and pulmonary fluid (collected from volunteers and clinical samples from the UK and from different hospitals in four cities in Saudi Arabia), using capsulated pneumococci D39, ST3, ST4, 35A, 23F and 19F as hosts. Several techniques and assays were used for the screening of collected samples which initially resulted in the isolation of several putative lytic phages; however, none of them propagated well and formed individual plaques. After significant effort, the isolation of a novel phage SP-QS1 was obtained. The phage was isolated from the throat swab of a child with an upper respiratory tract infection using serotype D39 as host cells. SP-QS1 showed its ability to clear capsulated *S. pneumoniae* lawns of serotypes D39, 3, 4 and 6B, but not 23F and 19F.

The application of phage against pneumococci *in vitro*, using an MOI of 10, resulted in a substantial decrease in bacterial colony-forming units with serotypes D39 (laboratory strain) and 3 and 4, where the log of cfu was non-detectable after five hours of incubation. This decrease was comparatively slightly lower with serotypes 6B and passaged D39. The viability of *S. pneumoniae* was decreased in about 5 logs when they were treated with SP-QS1, compared with the control. This decrease was obtained at different times with different tested serotypes, and this was suggested to be related to the thickness of the bacterial capsule. It was reported that when serotypes possess a big capsule, such as serotype 3 and passaged D39, the decrease in bacterial viable counts takes longer than with those with thinner capsules. This indicates that SP-QS1 degrades the pneumococcal capsule prior to infection. This suggestion was supported later, when

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the genome of this phage was sequenced and genes were predicted and annotated, which will be described later.

The reduction of pneumococcal colony-forming units using phage SP-QS1 was also confirmed using *in vivo* mouse models of infections; intranasal and intravenous infection models. The treatment of *S. pneumoniae* with SP-QS1, using an MOI of 10 in both models of infections, resulted in the reduction in bacterial colony-forming units in about 6 logs in blood samples after 48 hours. Despite the ability of this phage to clear capsulated pneumococci *in vivo*, the survival experiments have not resulted in thorough mouse survival, and this was attributed to mouse hyper immune response, as the colony-forming units always decreased at the end of phage treatment.

7.3 Genomic characterisation of phage SP-QS1

The genome of SP-QS1 has been found to contain 58,063 base pairs with an overall G-C content of 42%. 29 of 103 ORFs from these have been predicted to assign putative functions. SP-QS1 genes with recognisable homologies are roughly ordered according to (1) genes involved in phage packaging, (2) structural proteins, (3) replication and (4) genes associated with cell lysis. Interestingly, SP-QS1 genome does not encode any CRISPR sequences, proteins with transmembrane domains or regulatory elements. The phage genome also does not encode any integrase genes, and so appears to be a genuine lytic phage. Furthermore, it does not encode any putative toxins. These two traits mean that it is well suited for therapeutic use in humans.

The genome of SP-QS1 contains genes encoded for proteins which were found to be essential for causing the infection. These proteins are glycosyltransferase and N-acetylmuramoyl-L-alanine amidase, the former is suggested to be responsible for capsule degradation and the latter is responsible for cell wall penetration. Other proteins

Chapter .7 Conclusion and future work

encoded by the genome SP-QS1 are methylase and glutaredoxin, which were found to be involved in phage infection. These proteins were reported to play a role in phage DNA protection from bacterial restriction modification and DNA replication by host cells.

Phage releasing from infected cells requires the presence of lytic enzymes such as holin-endolysin- mechanism or murine hydrolase. Researchers exploit this character of lytic enzymes to lyse bacteria without using bacteriophages “lyse from without” (Young *et al.*, 2000; Nelson *et al.*, 2001). Several lytic enzyme were isolated, purified and undertaken to lyse bacterial hosts in both *in vitro* and *in vivo* such as Cp1-1 and Pal enzymes of pneumococcal phages Cp-1 and Dp-1, respectively (Varea *et al.*, 2004; Grandgirard *et al.*, 2008). Furthermore, some of these lytic enzymes were found to lyse wide range of *Streptococci* such as phage C₁ lytic enzyme which clears its host without causing any effect on Indigenous microorganisms analysed (Nelson *et al.*, 2001). Therefore, Phage SP-QS1 lytic enzyme glycosyltransferase and cell wall degrading enzyme N-acetylmuramoyl-L-alanine amidase could be isolated, purified and used to lyse capsulated *S. pneumoniae* and other Streptococci in both *in vitro* and *in vivo*.

7.4 Future prospects

My work has shown that phage SP-QS1 can infect the capsulated *S. pneumoniae* in intranasal and intravenous mouse model of infections, so it might consider as a part of useful set of next generation antimicrobial.

This phage could be tested with regard to the pneumococcal host range, which may be extended, as phage SP-QS1 has demonstrated its ability to infect four out of six capsulated serotypes. It can be also used to screen other *Streptococcus species* and other respiratory pathogens such as *Enterococcus*, *Klebsiella* and *Haemophilus*. The *in vitro*

Chapter .7 Conclusion and future work

and *in vivo* interaction of phage SP-QS1 could be assayed using other pneumococcal serotypes and other respiratory pathogens. Using the same procedures, other phages could be isolated in order to increase the chance of the host range prior to making multivalent phage lysate, so as to increase the opportunity for pneumococcal phage therapy.

Another application of this phage is in the study of the proteins encoded in the genome. Proteomic studies will enable the discovery of the structures and functions of particular key genes. With the view to that exploitation, some proteins are clearly worthy investigating such as N-acetylmuramoyl-L-alanine amidase and glycosyltransferase. It is important to test recombinant enzymes on different pneumococcal serotypes in order to determine its ability to clear pneumococci *in vitro* and *in vivo*.

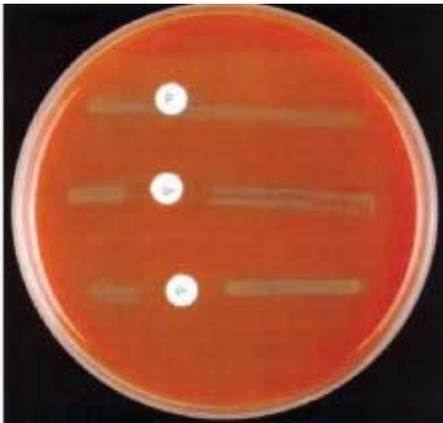
Appendix

Appendix 1: Pneumococcal confirmatory tests

1- Culturing pneumococci on blood agar for colony appearance and Optochin sensitivity:

Blood agar is prepared as described in materials and methods (section 2.2.1), then the test assayed as the following:

- a) Touch the suspect α -hemolytic colony with a sterile bacteriological loop and streak for isolation onto a blood agar plate in a straight line. Several strains can be tested on the same plate at once, streaked in parallel lines and properly labeled.
- b) Aseptically place an optochin disk with a diameter of 6 mm on the streak of inoculum, near the end where the wire loop was first placed. Because the inoculum is streaked in a straight line, three to four colonies may be tested on the same plate (Figure 16).
- c) Incubate the plates in a CO₂-incubator or candle-jar at 35°C for 18–24 hours.
- d) Read, record, and interpret the results. The optochin sensitivity showed below.



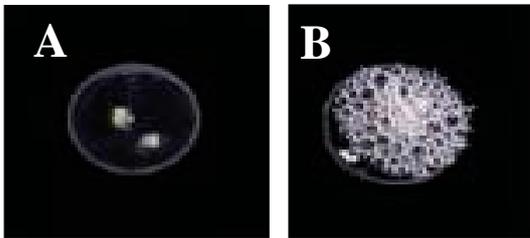
The colony appearance and optochin susceptibility test for *S. pneumoniae* uses optochin disks; this laboratory manual presents guidelines for interpretation of the optochin susceptibility test based on a 6-mm, 5- μ g optochin disk. The strain in the top streak grew up to the disk: it is resistant to optochin and therefore is not a pneumococcus. The strains in the centre and lower streaks are susceptible to optochin and appear to be pneumococci (obtained from:

http://www.who.int/csr/resources/publications/drugresist/WHO_CDS_CSR_RMD_2003_6/en/, accessed on 30 March 2013).

Appendix

2- Catalase test.

- a) Pick a colony from an 18-24 hr culture and place it on a clean glass slide. Avoid carry-over of blood agar which can cause false positives.
- b) Put one drop of 3% H₂O₂ over the organism on the slide. Do not reverse the order of the procedure as false positive results may occur. Do not mix.
- c) Observe for immediate bubbling (gas liberation) and record the result.
- d) Discard the slide into a discard container. Positive and negative catalase is shown below



Catalase test; (A) positive and (B) negative (obtained from http://www.who.int/csr/resources/publications/drugresist/WHO_CDS_CSR_RMD_2003_6/en/, accessed on 30 March 2013).

3- Gram Staining

A drop of distilled water is first placed on each slide. Small part of bacterial colony is transferred into that drop of water, mixed and left to dry. Then gram staining procedure is assayed as the following:

- a) Cover with crystal violet for one minute.
- b) Gently rinse off the stain with water and shake off the excess.
- c) Cover with gram's iodine for one minute.
- d) Pour off the Gram's iodine.
- e) Run 95% ethyl alcohol down the slide until the solvent runs clear (10-20 seconds).
- f) Rinse with water to stop the action of the alcohol.
- g) Cover with safranin for 20 seconds. Dry slide examined under light microscope.

Appendix

Appendix 2: Animal experiment score sheet.

Division of Biomedical Services: Study Score Sheet

Cage ID:	SIDE 1
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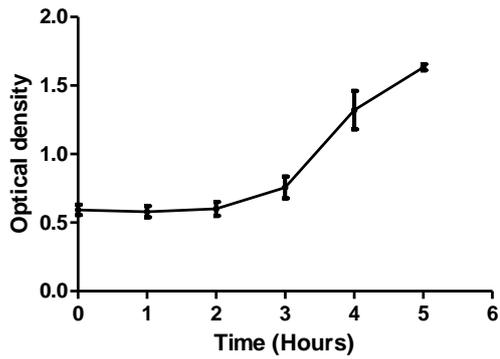
PPL #: 60/4327	19b# 1	USER#	Tel#
----------------	--------	-------	------

Animal Ref: nem	Date of procedure	Date: Time:						
Code#	Condition	-/+/**	-/+/**	-/+/**	-/+/**	-/+/**	-/+/**	-/+/**
1	NORMAL							
2	HUNCHED							
3	STAREY/PILOERECT							
4*	LETHARGIC							
5*	MORIBUND							
6	DEAD							
<i>Assessor Please initial</i>								
Animal Ref: rem	Date of procedure	Date: Time:						
Code#	Condition	-/+/**	-/+/**	-/+/**	-/+/**	-/+/**	-/+/**	-/+/**
1	NORMAL							
2	HUNCHED							
3	STAREY/PILOERECT							
4*	LETHARGIC							
5*	MORIBUND							
6	DEAD							
<i>Assessor Please initial</i>								
Animal Ref: lem	Date of procedure	Date: Time:						
Code#	Condition	-/+/**	-/+/**	-/+/**	-/+/**	-/+/**	-/+/**	-/+/**
1	NORMAL							
2	HUNCHED							
3	STAREY/PILOERECT							
4*	LETHARGIC							
5*	MORIBUND							
6	DEAD							
<i>Assessor Please initial</i>								
Animal Ref: bem	Date of procedure	Date: Time:						
Code#	Condition	-/+/**	-/+/**	-/+/**	-/+/**	-/+/**	-/+/**	-/+/**
1	NORMAL							
2	HUNCHED							
3	STAREY/PILOERECT							
4*	LETHARGIC							
5*	MORIBUND							
6	DEAD							
<i>Assessor Please initial</i>								
Animal Ref: 2rem	Date of procedure	Date: Time:						
Code#	Condition	-/+/**	-/+/**	-/+/**	-/+/**	-/+/**	-/+/**	-/+/**
1	NORMAL							
2	HUNCHED							
3	STAREY/PILOERECT							
4*	LETHARGIC							
5*	MORIBUND							
6	DEAD							
<i>Assessor Please initial</i>								

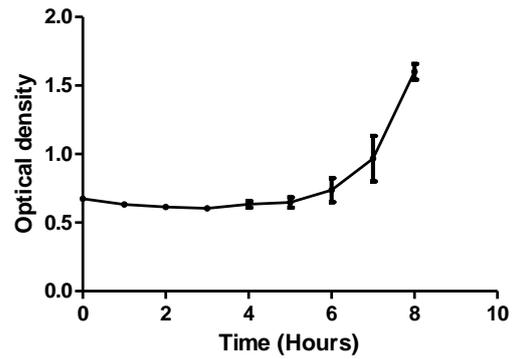
<p>Score Criteria</p> <p>—= No Visible adverse affects + = Adverse affects visible ++ = Adverse affects critical stage</p> <p>* Code # 4 ++ = NOTIFY USER TO CULL IMMEDIATELY * Code # 5 = +/** CULL IMMEDIATELY</p>

Chapter .7 Conclusion and future work

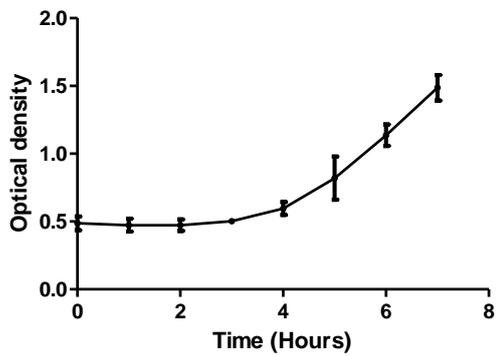
Serotype D39 Manual growth curve



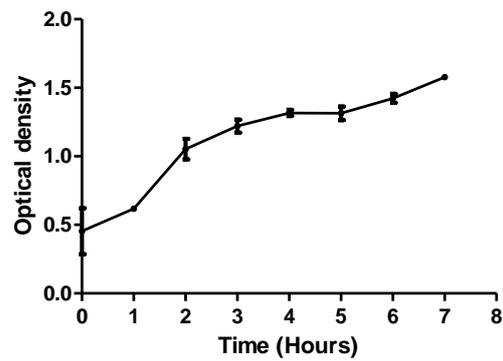
Serotype 2 (R36) manual growth curve



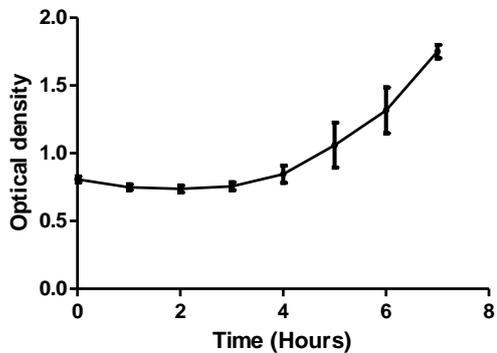
Serotype 3 manual growth curve



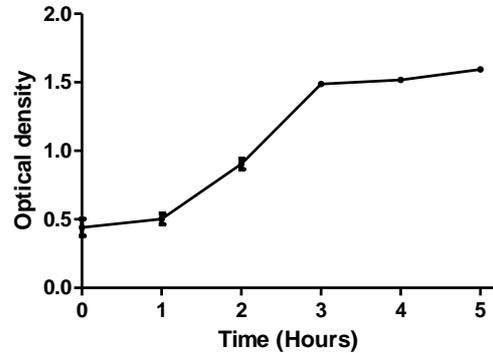
Serotype 4 manual growth curve



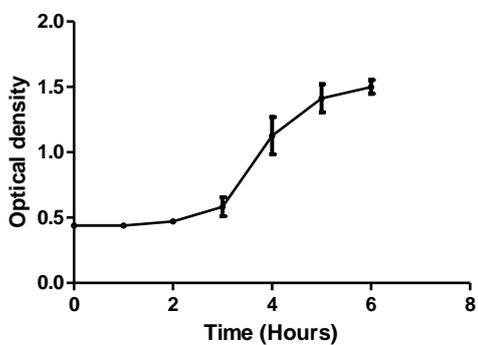
Serotype 35A manual growth curve



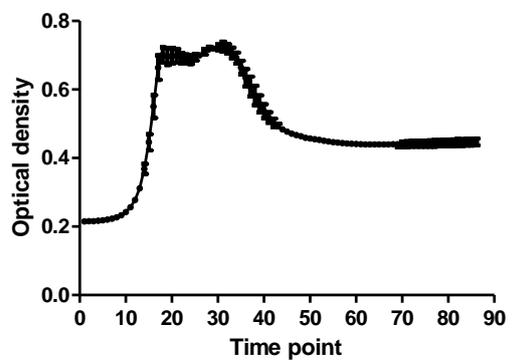
Serotype 23F manual growth curve



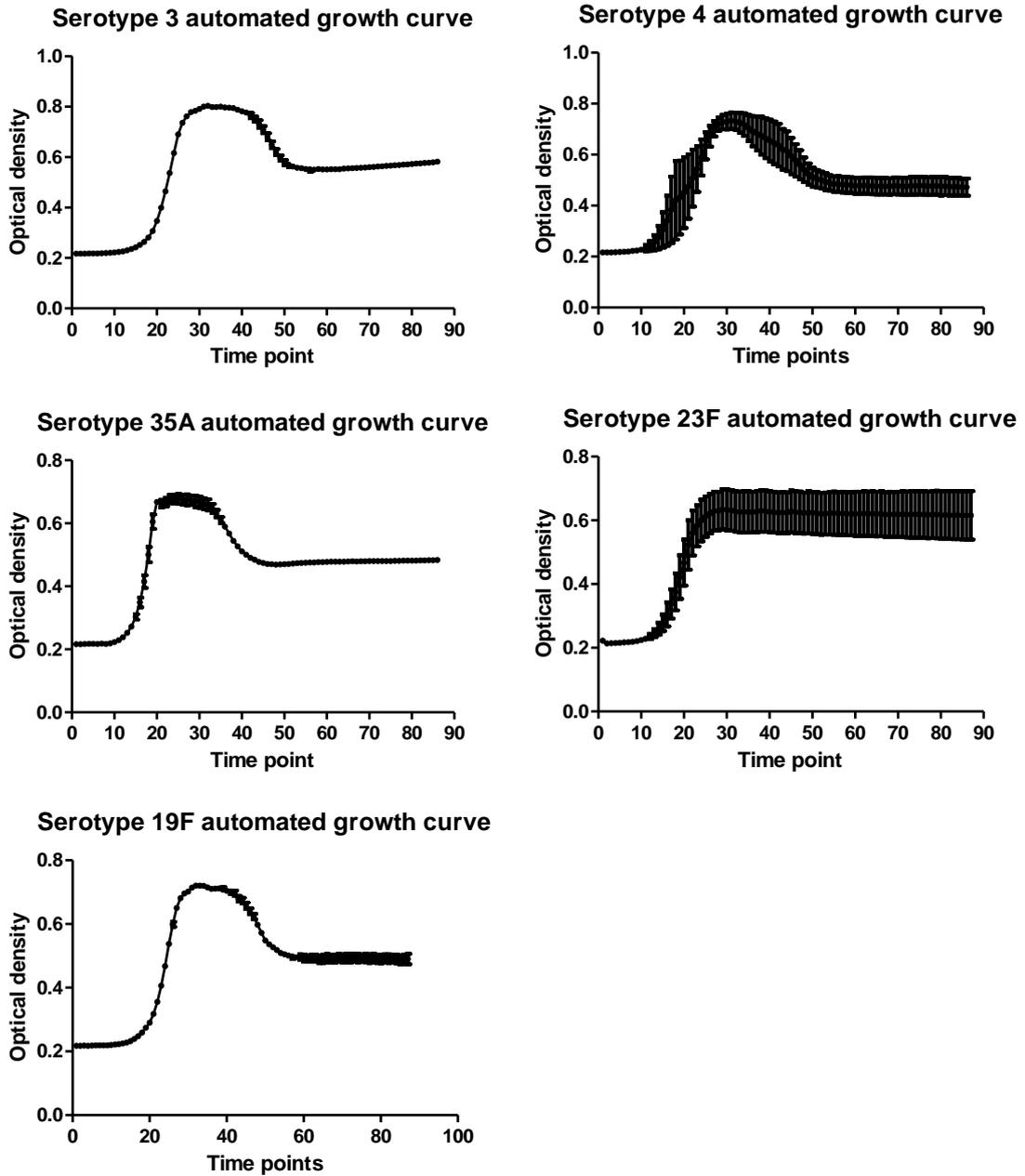
Serotype 19F manual growth curve



Serotype D39 automated growth curve



Appendix



Appendix 3: Manual and automated pneumococcal growth curves.

Appendix

Appendix 2: Hypothetical proteins and unknown predicted ORFs of phage SP-QS1 genome.

ORF	Strand	Start	End	Length	COMMENTS
2	+	653	997	345	Hypothetical protein [<i>Enterococcus</i> phage BC-611]
3	+	1016	1264	249	Hypothetical protein BFZC1_08870 [<i>Lysinibacillus fusiformis</i> ZC1]
7	+	5068	5739	672	Hypothetical protein [<i>Enterococcus</i> phage SAP6]
10	+	7254	7658	405	Hypothetical protein [<i>Enterococcus</i> phage BC-611]
11	+	7674	8051	378	Hypothetical protein [<i>Enterococcus</i> phage BC-611]
12	+	8036	8416	381	Hypothetical protein [<i>Enterococcus</i> phage SAP6]
15	+	9717	10157	441	Hypothetical protein [<i>Enterococcus</i> phage BC-611]
16	+	10168	10392	225	Hypothetical protein [<i>Enterococcus</i> phage BC-611]
21	+	20591	20878	288	Hypothetical protein EFAP1_gp05 [<i>Enterococcus</i> phage EFAP-1]
23	-	21922	22395	474	Hypothetical protein [<i>Enterococcus</i> phage BC-611]
24	+	22419	22562	144	Unkown
25	+	22861	23685	825	Hypothetical protein [<i>Enterococcus</i> phage SAP6]
26	+	23738	24172	435	Hypothetical protein [<i>Enterococcus</i> phage BC-611]
27	+	24175	24561	387	Hypothetical protein [<i>Enterococcus</i> phage BC-611]
28	+	24561	24761	201	Unkown
29	+	24906	25046	141	Unkown
30	+	24921	25046	126	Unkown
31	+	26021	26167	147	Hypothetical protein GME_15700 [<i>Halomonas</i> sp. TD01]
32	+	26167	26412	246	Hypothetical protein EFP_gp156 [<i>Enterococcus</i> phage phiEF24C]
33	+	26412	26744	333	conserved Hypothetical protein [<i>Enterococcus faecalis</i> TX0630]
34	+	26744	26896	153	Unkown
35	+	26910	27098	189	Unkown
36	+	27110	27322	213	Unkown
38	+	28253	28372	120	Unkown
43	+	31659	31883	225	Unkown
44	+	31895	32155	261	pseudouridine synthase [<i>Grosmannia clavigera</i> kw1407]
46	+	32661	33689	1029	Hypothetical protein [<i>Enterococcus</i> phage SAP6]
47	+	33689	34021	333	Hypothetical protein [<i>Enterococcus</i> phage BC-611]
50	+	35149	35781	633	Hypothetical protein [<i>Enterococcus</i> phage SAP6]
52	+	36438	36554	117	Unkown

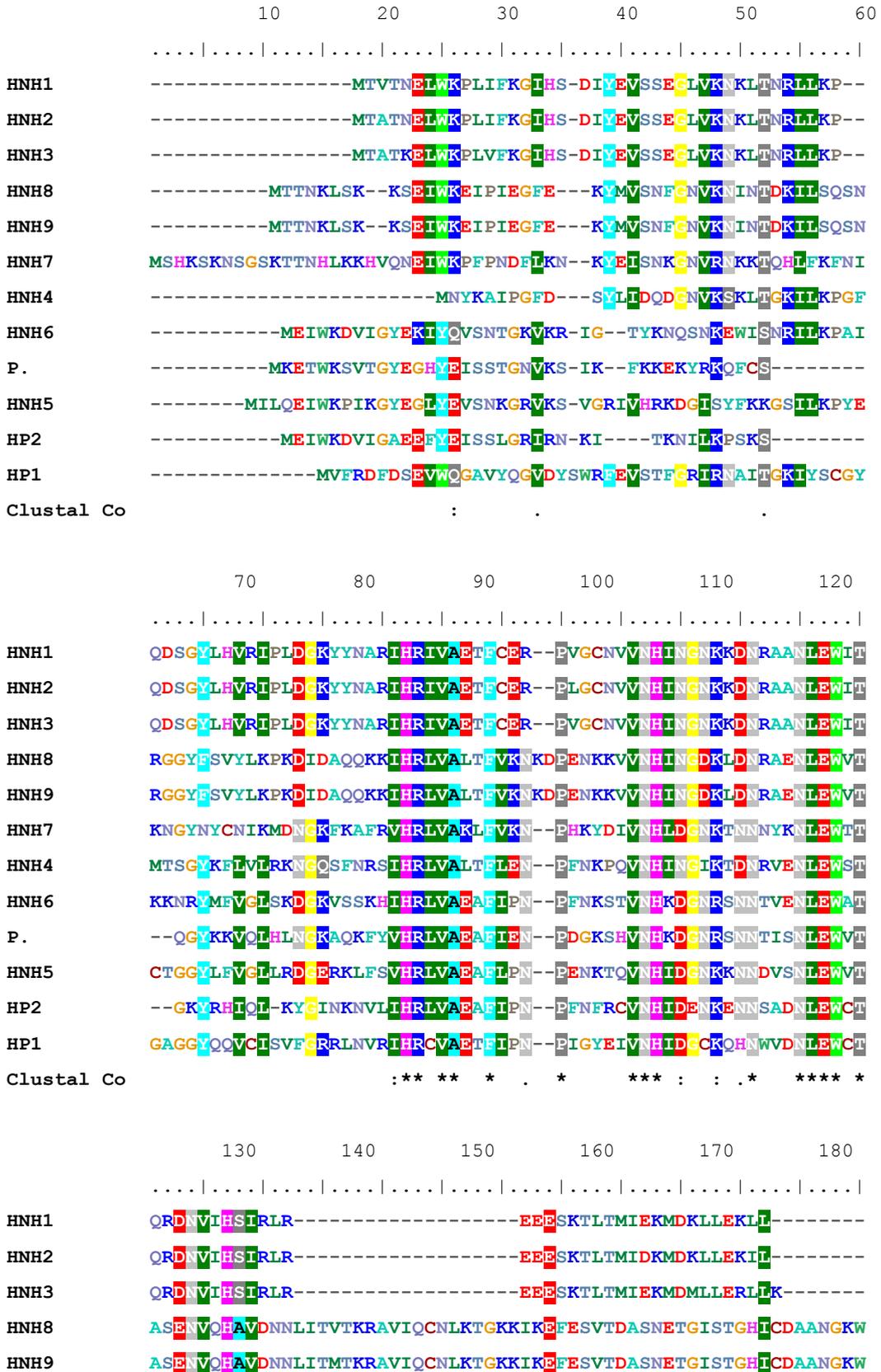
Appendix

53	+	36566	36760	195	Unkown
54	+	36775	36999	225	Unkown
55	+	36996	37205	210	Unkown
56	+	37208	37420	213	Unkown
57	+	37421	37798	378	Hypothetical protein [<i>Enterococcus</i> phage BC-611]
58	+	37798	37983	186	Unkown
60	+	40448	40969	522	Hypothetical protein [<i>Enterococcus</i> phage BC-611]
61	+	40971	41366	396	Hypothetical protein [<i>Enterococcus</i> phage SAP6]
63	+	42113	42574	462	Hypothetical protein [<i>Enterococcus</i> phage BC-611]
65	+	43219	43425	207	Unkown
66	+	43439	43849	411	Hypothetical protein [<i>Enterococcus</i> phage SAP6]
67	+	43928	44116	189	Hypothetical protein [<i>Cricetulus griseus</i>]
69	+	44673	44930	258	Unkown
70	+	44930	45145	216	Unkown
71	+	45135	45326	192	Hypothetical protein [<i>Enterococcus casseliflavus</i> EC30]EC10]
72	+	45329	45637	309	Unkown
73	+	45609	45896	288	Hypothetical protein [<i>Enterococcus</i> phage BC-611]
74	+	45898	46860	963	Hypothetical protein HMPREF0877_0104 [<i>Weissella paramesenteroides</i> ATCC 33313]
76	+	47978	48301	324	Hypothetical protein [<i>Enterococcus</i> phage BC-611]
77	+	48294	48398	105	Unkown
78	+	48402	48788	387	Hypothetical protein [<i>Enterococcus</i> phage BC-611]
79	+	48781	49155	375	Hypothetical protein [<i>Enterococcus</i> phage SAP6]
80	+	49234	49371	138	Hypothetical protein GVAMD_0344 [<i>Gardnerella vaginalis</i> AMD]
81	+	49435	49662	228	Unkown
82	+	49510	49662	153	Hypothetical protein Sterm_3583 [<i>Sebaldella termitidis</i> ATCC 33386]
83	+	49796	50089	294	Hypothetical protein EFP_gp116 [<i>Enterococcus</i> phage phiEF24C]
84	+	50104	50313	210	Unkown
85	+	50387	50557	171	Hypothetical protein EFP_gp130 [<i>Enterococcus</i> phage phiEF24C]
86	+	50582	50902	321	Hypothetical protein HMPREF0994_04907 [<i>Lachnospiraceae bacterium 3_1_57FAA_CT1</i>]
87	+	50992	51303	312	Hypothetical protein EFP_gp140 [<i>Enterococcus</i> phage phiEF24C]
88	+	51412	51714	303	Hypothetical protein [<i>Enterococcus</i> phage BC-611]
89	+	51889	52002	114	Unkown

Appendix

90	-	52498	52818	320	Hypothetical protein
91	-	52818	52958	141	Unkown
92	-	53135	53347	213	Hypothetical protein [<i>Methylococcus</i> SolV]
93	-	53349	53567	219	Hypothetical protein [Desulfovibrio sp. 3_1_syn3]
94	-	53568	54401	834	Hypothetical protein [<i>Enterococcus</i> phage SAP6]
95	-	54437	55102	666	Hypothetical protein [<i>Enterococcus</i> phage BC-611]
96	-	55547	55912	366	Hypothetical protein [<i>Enterococcus</i> phage BC-611]
97	-	55932	56234	303	Hypothetical protein (Transcriptional activator domain protein) [<i>Oscillochloris trichoides</i> DG6]
99	-	56521	56658	138	Hypothetical protein [<i>Enterococcus</i> phage phiFL4A]
101	-	57107	57493	387	Hypothetical protein [<i>Enterococcus</i> phage SAP6]
102	-	57493	57750	258	Unkown
103	-	57750	58064	315	Hypothetical protein HMPREF9473_03471 [<i>Clostridium hathewayi</i> WAL-18680]

Appendix



Appendix

HNH7 I S G N N Q H A A D N N L T S V T K R R I S Q Y -- V G E V L Y T Q Y D S L S E A S E A T G I H M S R I V E V C K G T R
 HNH4 R S E N I K H M Y D T ----- G L K K Y K P L H Y K G K S G A N H N R S L K V I C K ---
 HNH6 Y L E N N I H S T K - V L K R D T K N --- S S D S R P V L Q F D K Q G - N F I K E Y P S M R E A Q R Q -----
 P . N S E N M K H A Y K Y L L P K D Y A K D L K S K T S K P V E Q I D Q H T L Q V L R K F K S T K A A A R F -----
 HNH5 E K E N V N H A I K H G L L K N R G E D S P N S K L T K T D I E N I R R M Y A T K Q Y T Q Y Q L A E I Y G -----
 HP2 Y Q Y N C K Y G K G -- A L K R N S K ----- I I Q Y D M C E N A I K I -----
 HP1 R Q E N Y F H A V D L E L I D Y D V P ----- A Q L G Y L S H L G A Y T G S C N G M S K I T -----

Clustal Co * :

190 200 210 220 230 240

.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

HNH1 -----
 HNH2 -----
 HNH3 -----
 HNH8 K Q A G G F A W K Y S K K D S A K V D I D M S K -- F K Q L V D F P N Y L I N N E G Q V Y S L A R K R F M K P I H R G D
 HNH9 K Q A G G Y A W K Y S K K D S A K I D I D M S K -- F K Q L V D F P N Y L I N N E G Q V Y S L A R K R F M K P I H R G D
 HNH7 E E Y E G Y V F K Y T D I N P N E K V I D P E K E G F K N I K T F P N Y W I N S K G Q I Y S K P F K K F L K L N K H K T
 HNH4 --- N N G C V Y G S F A E A E R I L N L S Y ---
 HNH6 -----
 P . -----
 HNH5 -----
 HP2 -----
 HP1 -----

Clustal Co

250 260 270 280 290 300

.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

HNH1 ----- S P E A K K E F M E E F V N G -----
 HNH2 ----- S P E A K K E F M E E L F N G -----
 HNH3 ----- S P E A K N E F M E E Y V E W L K G -----
 HNH8 S G M N I S L S E ----- G D K Q K T M L V H K L V A S Y F L K K N N D D H N H V H H K D G N R Q N N N V S N L E W
 HNH9 S G M N I S L S E ----- G D K Q K T M L V H K L V A S Y F L K K N N D D H N H V R H K D G N R Q N N N V S N L E W
 HNH7 G S L Q I Q L T K K N P E G K G Q I K T I L V H N L V A I Y F L K K P E G D F N C V T H K D G D K S N N N I K N L K W
 HNH4 ----- G S V S R S I K N N K P I N G L L F E K Q -----
 HNH6 ----- T G I D A I D K V C N H V K Y R K T A G G Y --- K W E Y K D N Y N K S V E T I E T
 P . ----- I G I S P V H I A N V C K G K R R T S K G F --- I W R Y A -----
 HNH5 ----- V G Q S T I W K I V N N E R W R D D S Y S H E K Q K Y S H A K I T E E Q A K E I K S
 HP2 -----
 HP1 ----- E D D V R E I R M N Y I P R G S G V R C N R K E L A N Q Y G V S A N L I S K I V S G Q I W

Clustal Co

310 320 330 340 350 360

.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

Appendix

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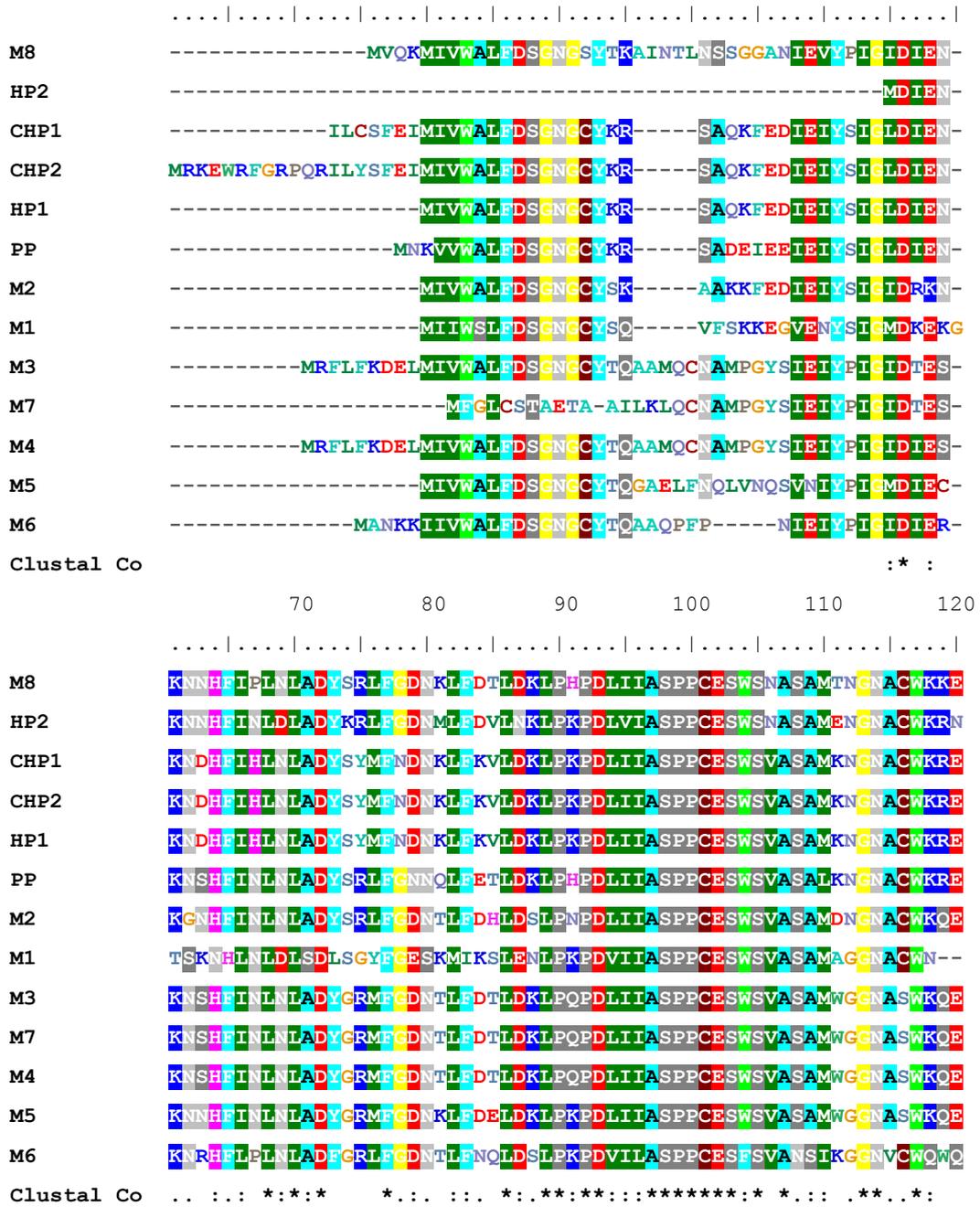
HNH1 -----
HNH2 -----
HNH3 -----
HNH8  CYLGGMDKHKPE SHFSHDYYDEKTAIPLTERKKAVKTIKSSGSKTSKSI GSKT NKSAGSK
HNH9  CYLGGMDKHKPE SHFSHDYYDEKTAIPLTERKKAVKTIKSSGSKTSKSI GSKT NKSAGSK
HNH7  KYVAGIQPN-----FDI-----
HNH4  -----
HNH6  TSDDGRE-----
P.    -----
HNH5  KYLTGKYKQLADDYGLSIAVSRIINGKSWKNVG-----
HP2   -----
HP1   THV-----
Clustal Co

          370      380      390      400      410      420
...|...|...|...|...|...|...|...|...|...|...|...|
HNH1 -----
HNH2 -----
HNH3 -----
HNH8  TASKLGSKTAIKSGSKTT SKISTKSGSKTAIKSGSKTSKSI----GSKTSKTSKSIKYE
HNH9  TASKLGSKTAIKSGSKTT SKISTKSGSKTAIKSGSKTAS KISTKSGSKTSKTSKSIKYE
HNH7  -----
HNH4  -----
HNH6  -----
P.    -----
HNH5  -----
HP2   -----
HP1   -----
Clustal Co

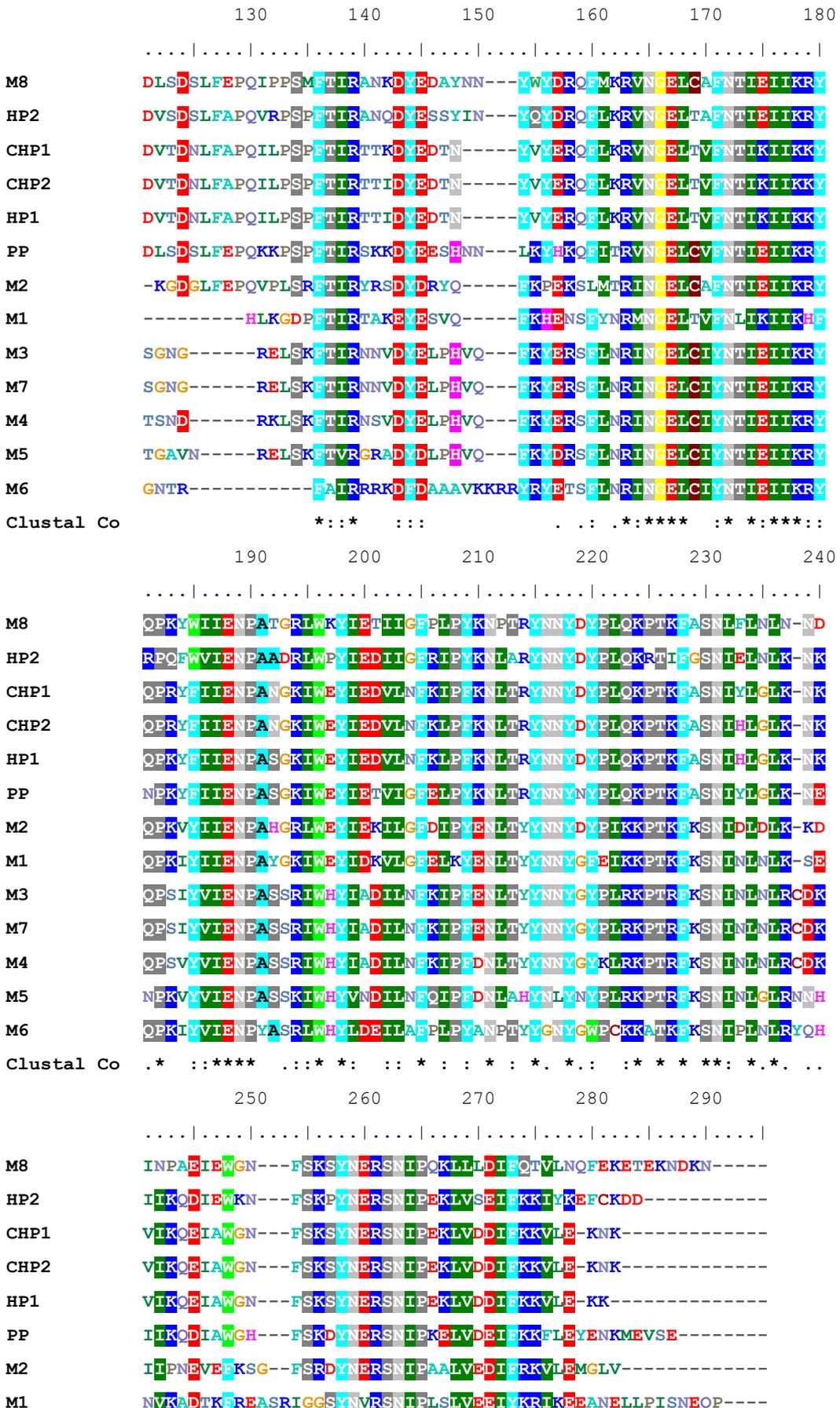
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Appendix 1: Alignment of SP-QS1 H-N-H Endonuclease with Homological Sequences obtained using a BlastP Search using the ClustalW program and then manipulated by Bioedit v7.1.3 software. SP-QS1 clusters appear to be similar to that one of end of *S. agalactiae* prophage and *Enterococcus* phage EFRM31. HNH 1-7 are the H-N-H endonuclease of; *S. pneumoniae* phage SP-QS1, *S. agalactiae* 2603V/R, *Enterococcus* phage EFRM31, *Streptococcus* phage PH15, *Enterococcus* phage BC-611, *Lactobacillus rhamnosus* HN001, *Streptococcus* phage StB20. HP 1 and 2 are homing nuclease of *Lactococcus amylovorus* GRL 1112 and *Bacillus sp.* 3_1_33FAA. P is a putative endodeoxyribonuclease *Lactococcus* phage A.

Appendix



Appendix



Appendix

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M3      SIKADKDWREF--SKSYNERSNIEPIQLILDIVANVEKTLKSNIQSLSQDGLFL-
M7      SIKADKDWREF--SKSYNERSNIEPIQLILDIVANVEKTLKSNIQSLSQDGLFL-
M4      SIKADKDWGEF--SKSYNERSNIEPIPLILDIVANVEKALKSNIQSLSQDGLFYG
M5      KSKPQQQWEDF--SKSYNERSNIEPLDLIVDIYKAVNQYLTNPICVPSERLLF--
M6      YISG-KTMFDC--LHSYNARSATPLPLIHDIYRRIIEVLGGNDASF-----
Clustal Co      ** ** * *: :* . :

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Appendix 2: Alignment of SP-QS1 Methylase with homological Sequences using the ClustalW program and then manipulated by Bioedit v7.1.3 software. The SP-QS1 sequences appear homology to those of *Enterococcus gallinarum* EG2 and *ActinoBacillus pleuropneumoniae* 4074 prophage. M1-8 are methylase of phage SP-QS1, *E. gallinarum* EG2, *A. pleuropneumoniae* 4074, *A. pleuropneumoniae* D13039, *Haemophilus influenzae* PittII, *Haemophilus somnus* 2336, *A. pleuropneumoniae* CVJ13261, *S. pneumoniae* SP18-BS74, respectively. HP1 and 2 are hypothetical proteins of *E. faecalis* TX0411 and *Streptococcus* phage PH10. CHP1 and 2 are conserved hypothetical protein for *E. Faecalis* TX0470 and *E faecalis* TX2141. PP is a phage protein of *Lactococcus lactis* KF147.

```

          10          20          30          40          50          60
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
Poly10   MARLFLFDGTALAYRAYYALDRSLSTSTGIPTNATYGVARMLVRFIKDHIIVGKDYVAVA
Poly11   MARLFLFDGTALAYRAYYALDRSLSTSTGIPTNATYGVARMLVRFIKDHIIVGKDYVAVA
Poly12   MARLFLFDGTALAYRAYYALDRSLSTSTGIPTNAVYGVARMLVKFIKEHIIPEKDYAAVA
Poly4    -----
Poly5    -----
Poly8    -----
Poly6    -----
Poly7    -----
Poly3    -----
Poly13   -----
Poly1    -----
Poly2    -----
Poly9    -----
Clustal Co
          70          80          90          100         110         120
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
Poly10   FDKKAATFRHKLLETYKAQRPKTPDLLIQQLPYIKKLVEALGMKVLEVEGYEADDIIATL
Poly11   FDKKAATFRHKLLETYKAQRPKTPDLLIQQLPYIKKLVEALGMKVLEVEGYEADDIIATL
Poly12   FDKKAATFRHKLLEAYKAQRPKTPDLLVQQLPYIKRLIEALGFVKVLELEGYEADDIIATL
Poly4    -----MELNFDGLMKTMEKA

```

Appendix

Poly5 -----MELTVD-LQPTMIAE
Poly8 -----MEFFKG-----VSFE
Poly6 -----MEIKPLKLN-----VNG
Poly7 -----MEGLRLN-----LNA
Poly3 -----
Poly13 -----MK
Poly1 -----MK
Poly2 -----MK
Poly9 -----
Clustal Co

 130 140 150 160 170 180
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
Poly10 AVKGLPLFDEIFIVTGDKDMLQLVNEKIKVWRIVKGISDLELYDAQVKVEKYGVEPQQIP
Poly11 AVKGLPLFDEIFIVTGDKDMLQLVNEKIKVWRIVKGISDLELYDAQVKVEKYGVEPQQIP
Poly12 AVKGCTFFDEIFIITGDKDMLQLVNEKIKVWRIVKGISDLELYDSKKVKERYGVEPHQIP
Poly4 ALSERGAAERVKEAQQKKARVKAATSWEDRDK-----
Poly5 KQNEQEAMLRAKEAQEKYIEATREPEWEDAWQI-----
Poly8 TGTKGDALARVSEAQKKELKNYEPTWEVWLTGY-----
Poly6 HKAAP-----AADVAKRKQAANA VEPIEDAWRR-----
Poly7 LKPAAPKSDAVEATAKRRKAKAKTAEPIEESWRK-----
Poly3 -----
Poly13 ISVDLPNEDKAKAAIKKVKGKKPLLTMEEAWAK-----
Poly1 ISVDLPNEDKAKAAIKKVKGKKPLLTMEEAWAK-----
Poly2 ISVDLPNEDKAKAAIKKVKGKKPLLTMEEAWAK-----
Poly9 -----
Clustal Co

 190 200 210 220 230 240
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
Poly10 DLLALTGDEIDNIPGVTGIGEKTA VQLLEKYKDLEDILNHVRELPQVKRKALLRDR ENAI
Poly11 DLLALTGDEIDNIPGVTGIGEKTA VQLLEKYKDLEDILNHVRELPQVKRKALLRDR ENAI
Poly12 DLLALTGDEIDNIPGVTGIGEKTA VQLLGKYRNLEDILEHARELPQVRKALLRDR EVAI
Poly4 -----QIFGMSKFKSG-LDRORAERV
Poly5 -----KVFSKKAITENPRREKAI AV
Poly8 -----PSTTGKHKNGIFQTKITPKDKDRLLDV
Poly6 -----IYASKLSDSDRDKLDEV
Poly7 -----IFAMKLSDADRKR VTEV
Poly3 -----
Poly13 -----IFSMKNSAKDKERLKL V
Poly1 -----IFSMKNSAKDKERLNL V
Poly2 -----IFSMKNSAKDKERLKL V
Poly9 -----MALFNFRKGI DASSAI
Clustal Co

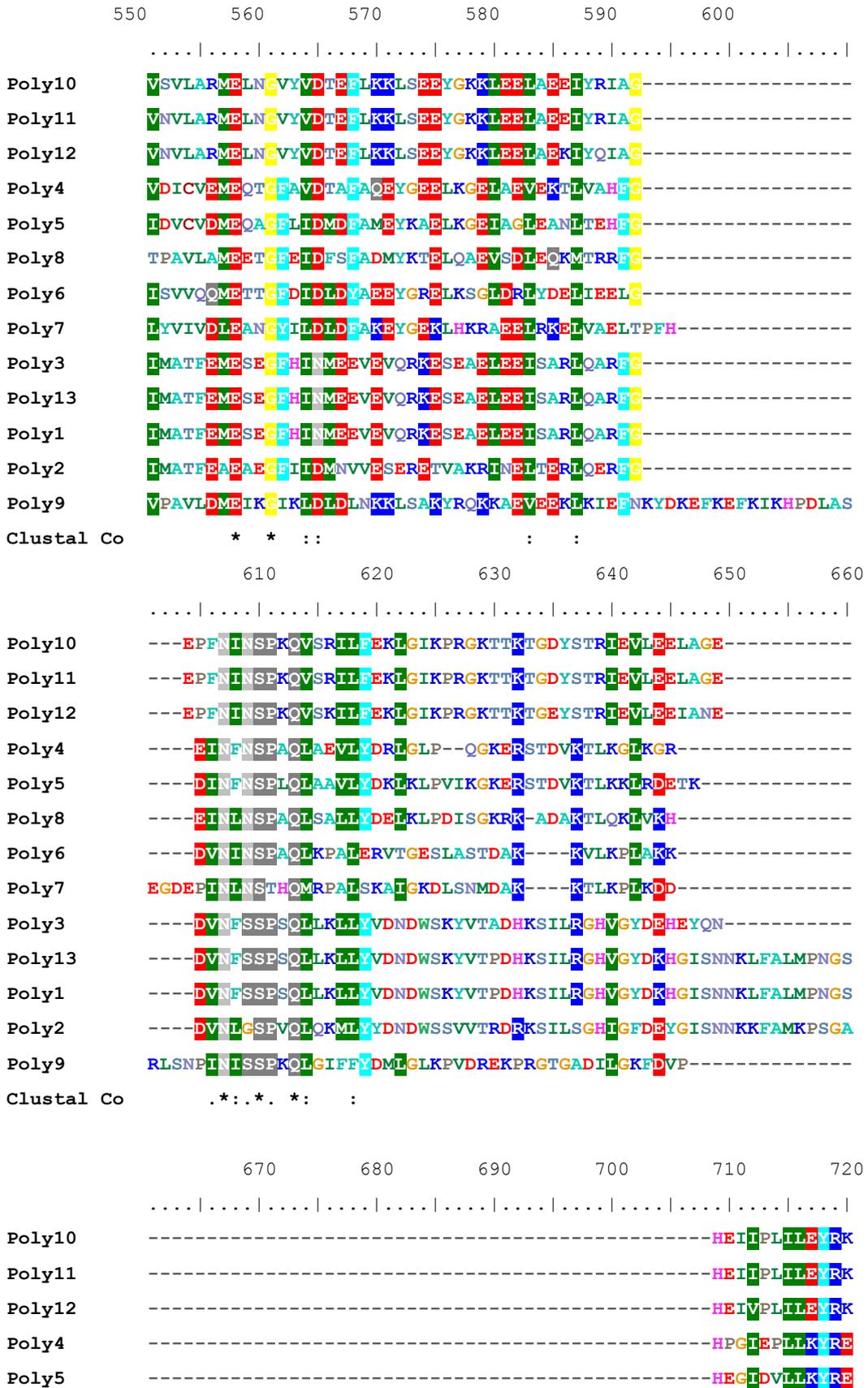
Appendix

```
                250      260      270      280      290      300
...|...|...|...|...|...|...|...|...|...|...|...|...|
Poly10  LSKKLAILETNVPIEINWEEELRYQGYDREKLLPLLKELEFASIMKEIQLYEESEEVGYRT
Poly11  LSKKLAILETNVPIEINWEEELRYQGYDREKLLPLLKELEFASIMKEIQLYEESEEVGYRT
Poly12  LSKKLATLVTNAPVEVDWEEEMKYRQGYDKRKLPLLKELEFASIMKEIQLYEEAEEPTGYEI
Poly4    KAAVENGVLG-----MDVPP-EKITGKLVSEKFRLELELAEKNEKITAKLLKSKEDNYRL
Poly5    KKACEAGEIR-----LDISS-KKLSCAAVFRLWRELOQQDTARKINELIANTPANYRL
Poly8    KTAIEACQLG-----MGVESIKKFSKAHALNLYKQLAEARKAEIIKGYLANMPENYHT
Poly6    KAAMEDCRLARDPDCVNKAGKPKKFSKAEALRLWKIVQAQNREDKLRKRVETEPENYWL
Poly7    KAAMDACKLARDPADCVNKAGNPKAFSKAEALRLWKILOEAQREETLRQMVENTPNNYEL
Poly3    -----
Poly13  KEYLEECKVSR-----EEDKLNKNEFSKAEALRIYQKVQEMEREAKFDEIRKKEWEKQPI
Poly1    KEYLEECKVSR-----EEDKLNKNEFSKAEALRIYQKVQEMEREALLESIRNKDLSLYPI
Poly2    KEYLEECKVSR-----EEDKLNKNEFSKAEALRIYQKVQEMEREALLESIRNKDLSLYPI
Poly9    RRTKK-----RKVSTPVRGGNNLVTTIELIRKTVNEKLGGMDDLAC
Clustal Co
```

```
                310      320      330      340      350      360
...|...|...|...|...|...|...|...|...|...|...|...|...|
Poly10  VKDLVEFEKLEKLEKRE---SPSFAIDLETSS---LDPFD-CDIVGISVSEK--PKEA
Poly11  VKDLVEFEKLEKLEKRE---SPSFAIDLETSS---LDPFD-CDIVGISVSEK--PKEA
Poly12  VKDHKTFEDLEKLEKRE---VPSFAIDLETSS---LDPFN-CEIVGISVSEK--PKTA
Poly4    IQTEDQFKALADLRH---EPAFADFDTETTC---VDVYQ-DVIVGVSLTLPRAGYHV
Poly5    VQTEEQLDQLVTDLQQ---EDEIADVDTETTC---LDVYN-DVIVGLSFTLPKADYHV
Poly8    VNTHEGMEWVLSLFEDSYALGAEVALDTEETC---VEWWDRTVGLSILTFEGDVEENF
Poly6    ITDEKRLAEFVALLEN---EEEIADVDTETTC---VDVWN-DYIVGHVITAIKADVHA
Poly7    ITTEERFQALIDALNN---EETIADVDTETTC---VDVYT-DVIVGLSFTLPNADWIV
Poly3    -NNIVELASWIDNALECD--NEYIAMDFFETVCDNGGDMYREEISGFSLTYRKGEIING
Poly13  INNIVELASWIDNALECD--NEYIAMDFFETVCDNGGDMYREEISGFSLTYRKGEIING
Poly1    INDRFTLIEWNKAVSCD--DEYIAMDFFETVCDNGGDNKYAEDISGFSLTYRKGEVING
Poly2    INDRFTLIEWNRAVSCD--DEYIAMDFFETVCDNGGDNKYAEDISGFSLTYRKGEVING
Poly9    VTSEELANYIKHFKD---NCGGLDTEETC-----LNVVDDKIAGVNLVTPGEKGI
Clustal Co      :      :      . * * * .      ..
```

```
                370      380      390      400      410      420
...|...|...|...|...|...|...|...|...|...|...|...|...|
Poly10  YYIPLHHRNAQNLD-----EKEVLLKKLKEILEDPGAKIVGQNLKEDYKVLNVKVEVPVP
Poly11  YYIPLHHRNAQNLD-----EKEVLLKKLKEILEDPGAKIVGQNLKEDYKVLNVKVEVPVP
Poly12  YYIPLHHRNAQNLD-----ETLVLSKLLKEILEDPSKIVGQNLKEDYKVLNVKVEISPVY
Poly4    YIPVAHNNAGPQIR-----RSYVNLGLKRYLTDPTVGKVLHNAKEDIHMLLRHMRMRG
Poly5    YIPVAHKVAGQQLS-----RRAVLTALKPILTDVNI GKVLHNSKVDIHMLIRHGLRLRG
Poly8    YIEYGHSTSDHEQLT-----RGYVVKKLLKPHLEKKGTKLVLHNSKEDSHMLLKDITIRN
Poly6    YIPTRHKTILPQLS-----RDLVNETLRPFYEDPTVGKLAHNAKEDIHMLDRECIKLRG
Poly7    YIPVEH-VDCEQLS-----RAYVLEGLAPVFNDESIGKVLHNAFEDIAMLRRHCFDLKG
```


Appendix



Appendix

Poly8 -----ESDLKLLLEYRD
Poly6 -----YPVVKKLLEYKD
Poly7 -----YEIITAKLLEYKN
Poly3 -----
Poly13 VILDPIVNAEGK-----VVPKNDRNKLQANAKAMKKIAKAVDEVQDILLDYKD
Poly1 VILDPMVNAEGK-----VVPKNDRNKLQANAKAMKKIAKAVDEVQDILLDYKD
Poly2 IILDPIVKKVNAGFDERGEAVYKEVAVAKDDRNKLQANSKALKKIANVAPEIQDILLELKD
Poly9 -----VAKLLEYRE

Clustal Co

730 740 750 760 770 780
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

Poly10 IQKLIKSTYIDALEKMNPKTGRIHASFNQGTATGRLSSS-----DPNLQNLPTKS
Poly11 IQKLIKSTYIDALEKMNPKTGRIHASFNQGTATGRLSSS-----DPNLQNLPTKS
Poly12 IQKLIKSTYIDALEKLVNPKTGRIHASFHQGTATGRLSSS-----DPNLQNLPTKS
Poly4 LTKLLGTYVEALEPHQLKA-DGRIHGSFNQVATVTGRFASR-----EPNLQNLPPR
Poly5 LTKLLGTYVEALEPQLIKK-DGLLHGSFNQVATVTGRFASR-----EPNLQNLPPR
Poly8 LNKLLTTYVEPLEEKVNKNTCKLHSSFNQSATVTSRFASK-----DPNLQNLPEK
Poly6 SEKLYSTYINALEELIDOKTKLYTNFNPNCAKTRFSSG-----GTGVNLQNLQEKK
Poly7 IVKLSGTYIDTLEPKONPTTERWHSRFNPMGTVTGRFSSGKDEEDKTOQGFNVQNPPE

Poly3 -----

Poly13 LTKHLTAFVVKIDTFIAP-DCKLHGQFNQFCTVTGRFSAS-----NPNLQQLQEKK
Poly1 LTKHLTAFVVKIDTFIAP-DCKLHGQFNQFCTVTGRFSAS-----NPNLQQLQEKK
Poly2 LTKYMSSLEPDTDFIQP-DCKLHGDFNQVCTVTLRYSAS-----NPNLQQLVSKP
Poly9 LDKLLGTYIDKLEPTVLSKRTCKIHCTFNQYCTVTGRFSSD-----SPNLQNLIPSHN

Clustal Co

790 800 810 820 830 840
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

Poly10 EEGKEIRKATVPQDPNWWIVSADYSQIELRILAHLSGDENLLKAFEEGIDVHTLTASRIF
Poly11 EEGKEIRKATVPQDPNWWIVSADYSQIELRILAHLSGDENLLRAFEEGIDVHTLTASRIF
Poly12 EEGKEIRKATVPQDPDWWIVSADYSQIELRILAHLSGDENLVKAFEEGIDVHTLTASRIY
Poly4 -----ARRLIVAPKGWLLLCIDFSQIEPRILAHMSGDPHFREPYLKGEDLYSTLAARVF
Poly5 -----ARRLIVAPEDSLILCVDFSQIEPRVLAHMSGDPHLOEPPYLKQODLYSTLASRVF
Poly8 -----ARKLIVAPKGYLIFCIDYSQIEPRTLAHMSGDAGLQYPYLNNIDLYASTASKIF
Poly6 -----ARKLFVAPEGYAILCGDWSQOEYRCLAYFTQDPKLVDNMFRCDLYASTAAEVF
Poly7 -----ARPMFGPPPKLLVCADEKAQEIROYVILSCEPVLINAFLEERDPYATMASNFY
Poly3 -----
Poly13 -----ARKMFEAPEGSLILGADFSQOEPRLIAHSSCCLELNIYKECRDLYSEMASAIF
Poly1 -----ARKMFEAPEGSLILGADFSQOEPRLIAHSSCCQOELNIYNECRDLYSEMASAIF
Poly2 -----ARAMMVEDDSTIVCAEFSQOELRLLCHISGDEAIAKAVYASGIDFYSSVASMIF
Poly9 -----KDIRKMFIPDDGNILICDYSQOEPRLIAHMSGDEKMIKAYKNCKDIYATIASYVY

Clustal Co

Appendix

```

      850      860      870      880      890      900
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
Poly10  NVKPEEVT-----EEMRRAGKMNFSIIYGVTPYGLSVRLGVPVKEAEKMIIV
Poly11  NVKPEEVT-----EEMRRAGKMNFSIIYGVTPYGLSVRLGVPVKEAEKMIIV
Poly12  NVKPEEVT-----EEMRRAGKMNFSIIYGVTPYGLSVRLGVPVKEAEKMIIV
Poly4   KVELSECGDGS-----KMRKMMKIQLLAVMYGTSMTLSEQLGISVVEAHRFIE
Poly5   KVSVEECGDDS-----KMRKMMKVGLLAVMYGTSMTLADQLGITVEEANQFIE
Poly8   KLEYEACLEADGETYKKAAGLPKHERKMMKVEGLLAVMYGITVPSLAESLGISVPEAQKEMD
Poly6   GKEIEEC-----GDGSVYRQAKVIMLAVAYGGGANMLKDATIGVEKHEAOKFLD
Poly7   KRPEHEEVYKNAD-----GSDTRERKMKVAVLALTYGMSDYSLADMLCVGKKEATEFEKE
Poly3   -----
Poly13  NKEIEECLDGS-----IYRKNTKMIVLAIMYCMGAYSLADILRIDAQEAOKMID
Poly1   NKEIEECLDGS-----IYRKNTKMIVLAIMYCMGAYSLADILRIDAQEAOKMID
Poly2   DKPEYEDVRDGT-----KERSQTKMIVLAILYCMGAGSLADILKTIKPEAQNEMD
Poly9   KVEYEECLEFNPDGTN-TIGKQRRSNLKGVVLGIIYGRGVNSLAEKLNLTKEAQSIIID

```

Clustal Co

```

      910      920      930      940      950      960
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
Poly10  NYFVLYPKVRDYIQRVVSEAKEKGYVRTLFGKRKRDIPQLMAR-----
Poly11  NYFVLYPKVRDYIQRVVSEAKEKGYVRTLFGKRKRDIPQLMAR-----
Poly12  SYFTLYPKVRSYIQQVVAEAKKGYVRTLFGKRKRDIPQLMAR-----
Poly4   GFYDEYPDVYAFIKDTWEFVKNEYVATLYGRKRREF--PGHREQAIVYDQLAAKI-----
Poly5   DFEATYPDVYAFICATHEFAKONEYVETLYGRKRREF--PGHREKAIVYDKLATEI-----
Poly8   DFYSSYPEMTAWMAKQVAHADETCYVETMQGRKRREF--IGHTVIAKHVHALHAKV-----
Poly6   SFFERFPVVEVVEVSNQKFKKGFVWMDHLQKRREF--LPDAKD-----
Poly7   ELFQSMFKLSAWLKENEEFVRKNGYVWADLNARKRREF--LPDAKLPKRNIPYKQWV-----
Poly3   -----
Poly13  DFFVYYPEVEETWIEGNKKTVVKQRYVETLFGMRRREFKHENFDILKKNWN-----
Poly1   DFFVYYPEVEETWIEGNKKTVVKQRYVETLFGMRRREFKHENFDILKKNWN-----
Poly2   TFLAKYPQVEKWIENKKTARSQRYVETIFGTRRRREFKKNENFSILDKQWV-----
Poly9   EFTLFPKVKQFTEDSIRMARELGYVTTLSRRRRRLPDINLPRYEFKYKDKSTSDNFNPF

```

Clustal Co

```

      970      980      990      1000     1010     1020
.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
Poly10  -----DRNTQAEGER
Poly11  -----DRNTQAEGER
Poly12  -----DKNTQSEGER
Poly4   -----CKALGTERVPLDFWS--NKGIPKDLKRKFGAVKGEVERVRR
Poly5   -----CEILGTKAVPYDYWQYKKELPVILRRQFRAVKGEVERVRR
Poly8   -----VNILGRE--PENIWR--EPLPRNLKKQYWEVVKDYQVAVR
Poly6   -----RSAKGHYSVAVFT
Poly7   -----DPKYDDA-----RKHNSRINRAIR

```


Appendix

```
Poly11 -----  
Poly12 -----  
Poly4 -----  
Poly5 -----  
Poly8 -----  
Poly6 RIVGEGWQKGAV-----  
Poly7 -----NKEAV-----  
Poly3 -----  
Poly13 -----  
Poly1 -----  
Poly2 CGDIVSYVREGSEDIGSVYVNGNYLRDLNDDEWKVYSKELK  
Poly9 -----  
Clustal Co
```

Appendix 3: SP-QS1 DNA polymerase Alignment together with the Top 12 Hits attained via Blastp using the ClustalW program and then manipulated by Bioedit v7.1.3 software. The alignment process indicated low homology between the sequences of SO-QS1 Poly I, and other poly sequence micro-organisms. Poly 1-13 are the DNA polymerase of the following; *Enterococcus* phage BC-611, *Enterococcus* phage SAP6, *Enterococcus* phage F4, *Pelotomaculum thermopropionicum* SI, *Desulfuromonas acetoxidans* DSM 771, *Bacillus pumilus* ATCC 7061, *Bacillus amyloliquefaciens* DSM 7, *Bacillus atrophaeus* 1942, *Corynebacterium* phage phiCTP1, *Thermotoga sp.* RQ2, *Thermotoga maritima* MSB8, *Thermotoga neapolitana* DSM 4359 and *Streptococcus pneumoniae* Phage SP-QS1 respectively.

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