The role of pneumolysin sequence variants and strain background in the virulence of *Streptococcus pneumoniae*

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By

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Statement of Originality

This accompanying thesis submitted for the degree of PhD entitled **"The role of pneumolysin sequence variants and strain background in the virulence of *Streptococcus pneumoniae*"** is based on work conducted by the author in the Department of Infection, Immunity and Inflammation of University of Leicester during the period between September 2014 and September 2018.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of this work has been submitted for another degree in this or any other University.

Signed……... Date……

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Abstract

Streptococcus pneumoniae is a major cause of pneumonia, bacteraemia, meningitis and otitis media. Pneumolysin (Ply) is the key toxin of the bacterium, and it has many adverse effects on immune cell functions. Ply variants have been described among clinical pneumococcal. These variants were reported to differences in cytotoxic activity but the impact of these differences on virulence is hard to ascertain because they are being made in different strain background.

To study the significant of polymorphisms of Ply function in a single genetic background, unmarked mutations were introduced into the *ply* in the *S. pneumoniae* type 2 D39 strain background using pORI280 to replace the natural copy of *ply* gene. The selected Ply variants were expressed and purified using affinity chromatography and gel filtration to determine haemolytic and complement activation activities. The virulence of the D39 recombinant carrying variants of *ply* were tested in a mouse model of pneumococcal pneumonia.

The results showed that of eight naturally occurring single nucleotide polymorphisms (SNP), all Ply alleles reduced the haemolytic activity of Ply in different degrees compared to the D39 Ply excepting the Ply allele 9 which increased haemolytic activity. *In vivo*, D39 expressing Ply alleles 2, 8, 11, 12 or 18 are significantly attenuated whereas Ply alleles 9 and 15 were not attenuated in virulence in a pneumonia model of infection. The numbers of CFU for streptococcus carrying *ply* alleles 2, 8, 11, 12 and 18 were significantly lower in the lung and spleen tissues, compared with wild type D39. Furthermore, pneumococcal bacteraemia in mice infected with D39 expressing Ply alleles 2, 8, 11, 12 or18 were significantly attenuated compared with wild type D39.

In conclusion the Ply variations and the genetic background of the *S. pneumoniae* strain carrying them have an important impacts on pneumococcal virulence.

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Abbreviations

Ab	Absorbance
Ala	Alanine (A)
Asn	Asparagine (N)
Asp	Aspartic acid (D)
Arg	Arginine (R)
BAB	Blood agar base
BHI	Brain heart infusion
BSA	Bovine serum albumin
CFU	Colony forming unit
CSP	Competence stimulating peptide
dH_2O	Distilled water
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ELISA	Enzyme linked immunosorbent assay
Glu	Glutamic acid (E)
Gln	Glutamine (Q)
HA	Haemolytic activity
His-tag	Histidine-taq
HU	Haemolytic unit
IgG	Immunoglobulin G
IPT	Invasive pneumococcal disease
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
Kb	Kilobase
kDa	Kilodalton
LA	Luria-Bertani agar
LB	Luria-Bertani broth
Ile	Isoleucine (I)
Lys	Lysine (K)
Met	Methionine (M)
mg	Milligram

ml	Millilitre
µg	Microgram
µl	Microliter
µM	Micromolar
OD	Optical density
OM	Otitis media
PAGE	polyacrylamide gel
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PCV	Pneumococcal conjugate vaccines
Ply	Pneumolysin
PNACL	Protein nucleic acid chemistry laboratory
RBCs	Red blood cells
rpm	Round per minute
SDS	Sodium dodecyl sulphate
TAE	Tris acetic acid EDTA
UV	Ultraviolet
v/v	volume per volume
w/v	weight per volume
WT	Wild type
X-gal	5-Bromo-4-chloro-3-indolyl ß-D-galactopyranoside
x g	Gravity force

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Chapter 1: Introduction

1.1 *Streptococcus pneumoniae*

The pneumococcus was discovered early in 1881 by Louis Pasteur in France and George M. Sternberg in the United States (Pasteur, 1881, Sternberg, 1881). The pneumococcus is a major worldwide cause of mortality and morbidity especially among the elderly and young children (Henriques-Normark and Tuomanen 2013). It is the main cause of pneumonia, bacteraemia, otitis media, meningitis, endophthalmitis, conjunctivitis and keratitis. The defined pneumococcal agent *Streptococcus pneumoniae* is a Gram positive, catalase negative, non-spore forming bacterium, and is a non-motile and fermentative. It gets its energy by fermentation of host polysaccharide (Marion *et al.* 2012). Pneumococci α-haemolytic activity on blood agar, and they are sensitive to ethylhydrocupreine HCl (optochin) (Pikis *et al.* 2001). The pneumococcal serotypes can be determined by using a Quelling reaction test (Selva *et al.* 2012). Based on its polysaccharide capsule structure, there more than 95 serotypes of this bacterium (Joseph and Rao 2011, Calix *et al.* 2012, Katoh *et al.* 2017). Some serotypes are associated with invasiveness and the others with host colonisation but the basis of this is not known. A key virulence factor of *S. pneumoniae* is pneumolysin (Ply) (van Pee *et al.* 2016). This protein is the topic of this thesis.

1.2 Treatment of pneumococcus diseases

Pneumococcal diseases are treated with group of β-lactam antibiotics (Tateda *et al.* 1999), including ampicillin, penicillin, ceftriaxone, cephalosporin C and aztreonam compounds (Normark and Normark 2002). The β-lactam antibiotics inhibit penicillin-binding proteins PBPs) of the peptidoglycan of the pneumococcal cell wall (Charpentier and Tuomanen 2000). However, pneumococcal resistance to penicillin has been mentioned since the 1940s (Davies et al. 2010). This resistance is unlike the β-lactamase-acquired resistance of most other bacteria, and it is likely due to the modification of target penicillin-binding protein structures, which affects their binding of the β-lactam (Barcus *et al.* 1995). Other antibiotics used for pneumococcal infection treatment include macrolides and fluoroquinolones.(van der Poll and Opal 2009).

1.3 Pneumococcal diseases

1.3.1 Pneumonia

Pneumonia is an acute inflammation of lung tissues, caused by a variety of bacteria, including *S. pneumoniae, Staphylococcus, Pseudomonas, Haemophilus, Chlamydia and Mycoplasma* (Couriel 2002, Brook 2011). It commonly affects people under five years and the elderly (Cillóniz *et al.* 2018). The symptoms of pneumonia include fever, dry coughing and bloody mucus (Hoare and Lim 2006). The breathing can be shallow and rapid, accompanied by shivering and sweating and chest pain. In pneumonia the alveoli become larger due to filling with fluid resulting in reduction of oxygen absorption (Rudan *et al.* 2008). Bronchopneumonia can occur in people of any age, and it increases the risk of heart failure (Eurich *et al.* 2017). The World Health Organisation (WHO) has reported that about 920,000 children under 5 years of age have been killed by pneumonia and the most common cause is *S. pneumoniae* (Wang *et al.* 2018).

1.3.2 Meningitis

S. pneumoniae can infect the membranes that cover the brain and spinal cord (meninges) and cause meningitis (Jauneikaite *et al.* 2014). Meningitis is a very serious disease that can affect babies, young children, teenagers and adults. If not treated quickly, meningitis can progress to septicaemia and damage to the nerves or the brain resulting in death (Hoffman and Weber 2009). The symptoms of meningitis include headache with high fever and drowsiness (Sáez-Llorens and McCracken Jr 2003).

1.3.3 Septicaemia

Lung infection with *S. pneumoniae* is the most common source for septicaemia due to the bacteria entering the bloodstream and spreading through the body (Naveau and Houssiau 2005). Sepsis symptoms include temperatures more than 38°C or less than 36°C and heart rate of more than 90 beats/min (Lever and Mackenzie 2007). The initial response of the infected host is release of the inflammatory mediators, leading to an imbalance between oxygen consumption and oxygen delivery (Garcia-Vidal *et al.* 2010)0. Finally sepsis leads to organ dysfunction and septic shock and death (Nguyen *et al.* 2006).

1.3.4 Otitis media (Ear infections)

Otitis media or an infection of the middle ear can be bacterial or viral and usually affects babies and young children (Cohen *et al.* 2010). One of the bacterial sources of otitis media infection is the pneumococcus colonising the nasopharynx and transferring to the ear causing the otitis inflammation (Morris and Pichichero 2017). A recent study showed that *S. pneumoniae* is the most common cause of acute otitis media (Chi *et al.* 2018). The symptoms of otitis media include fever, pulling sensation on the ear, draining of fluid from the outer ear and earache (Bergenfelz and Hakansson 2017).

1.4 Pneumococcal vaccines

There are more than 90 different pneumococcal serotypes, which have been classified based on the chemical structure of their capsular polysaccharides (Mika *et al.* 2017). Currently there are two types of pneumococcal vaccine; pneumococcal polysaccharide vaccine (PPV-23) and pneumococcal conjugate vaccine (PCV) (Tomczyk *et al.* 2014). The pneumococcal vaccine polyvalent is Pneumovax 23 or Pnu-Immune (Schenkein *et al.* 2008). It was used first time in the United States in 1945 and it covers 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 (Hajj Hussein *et al.* 2015). It is used to vaccinate elderly people and people between 2 and 64 years (Fedson 2014). Children under 2 years old are not vaccinated with PPV-23 because this vaccine did not elicit a protective immune response in this group (Daniels *et al.* 2016).

One of the types of pneumococcal vaccine is PCV-7 which includes polysaccharides of seven pneumococcal serotypes 4, 6B, 9V, 14, 18C, 19F and 23F conjugated with nontoxic diphtheria carrier protein (CRM197) (Principi and Esposito 2018). This vaccine was licensed for vaccination infants and young children in 2000 in the United States (Kobayashi *et al.* 2015). This vaccine has been shown to be highly effective in immunisation of young children, protecting against the most frequent invasive pneumococcal serotypes (Galanis *et al.* 2016), because it can produce B-cells memory and IgG by inducing a T-cell dependent immune response (Choi *et al.* 2011). In 2010 conjugated capsule polysaccharides were added to the PCV7 to update to PCV13, including pneumococcal serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F (Díez-Domingo *et al.* 2011, Del Amo *et al.* 2014).

1.5 Pneumococcal virulence factors

The pneumococcus has many virulence factors involved in colonisation and invasive diseases (Weiser 2010).Understanding the roles of these factors can increase understanding the pathogenesis of pneumococcal infections. Figure 1-1 shows a schematic representing of certain pneumococcal virulence factors each of which is explained in following sections adapted from (Kadioglu et al. 2008). The pneumococcal cell wall and capsular polysaccharides are shown in Figure 1-2.

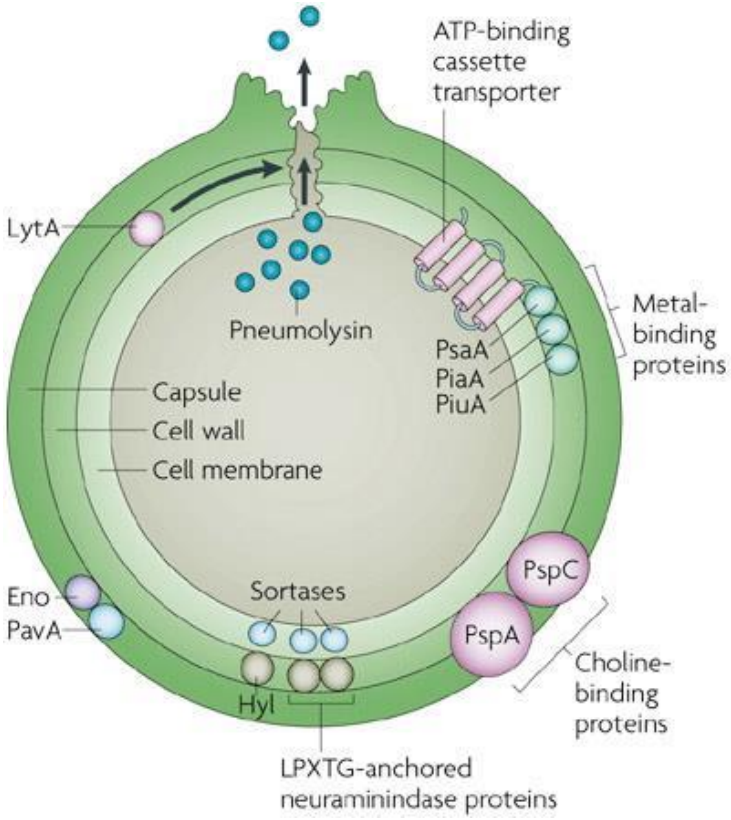

Figure 1-1: Shows a schematic representing of certain pneumococcal virulence factors.
Pneumococcal virulence factors including the capsule, cell wall, autolysin A (LytA), neuraminidases, Hyaluronidase (Hyl), Pneumococcal surface proteins A and C (PspA & PspC), Pneumococcal adhesion and virulence A (PavA) and pneumolysin (Ply) (Kadioglu et al. 2008).

1.5.1 Capsule

The polysaccharide capsule expressed on the outer cell surface is one of the pneumococcal virulence factors. Its thickness is approximately 400 nm and it has an anti-phagocytic role (Musher et al. 1986). The pneumococcus capsule prevents complement C3b-mediated opsonisation of bacterial cells (Mitchell and Mitchell 2010). There are more than 90 pneumococcal serotypes based on the antigenic type of capsular polysaccharide (Bai and Vidal 2017). The capsule consists of polymers containing 2-8 repeating oligosaccharides (AlonsoDe Velasco et al., 1995). The genes responsible for the capsular synthesis are arranged in cassettes comprising all the genetic material necessary for the synthesis capsular substances (Garica et al. 1997).

1.5.2 Cell wall

The pneumococcal cell wall is composed of peptidoglycan and teichoic acid, which are covalently linked (Hoffmann *et al.* 2007, Maestro and Sanz 2016). The peptidoglycan is common to all pneumococcal serotypes, and is composed of N-acetylglucosamine and N-acetylmuramic residues (Bui *et al.* 2012). Teichoic acid (TA) containing 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose,D-glucose,ribitol-phosphate and units of N-acetylgalactosamine (GalNAc) (Thomas *et al.* 1992, Vialle *et al.* 2005, Seo *et al.* 2008). The phosphorylcholine (P-Cho) groups present in the teichoic acid (TA) of the pneumococcal cell wall, may help the bacteria to adhere to host tissue by binding of the P-Cho to the G-protein-coupled platelet-activating factor receptor of host cell (Cundell *et al.* 1995). The choline kinase contributes in the production of this decorated teichoic acid, and a study showed that a choline binding knockout in *S. pneumoniae* reduced the colonization of mice compared to the wild type strain (Kharat *et al.* 2008). There are variety of other surface proteins in the pneumococcal cell wall including choline-binding proteins (CBPs) and also murine hydrolases, which have important roles in remodelling the cell wall during bacterial growth and cell division (Zimmerman and Ibrahim 2017). The pneumococcal cell walls can also activate the complement system inducing host inflammation (Maestro and Sanz 2016).

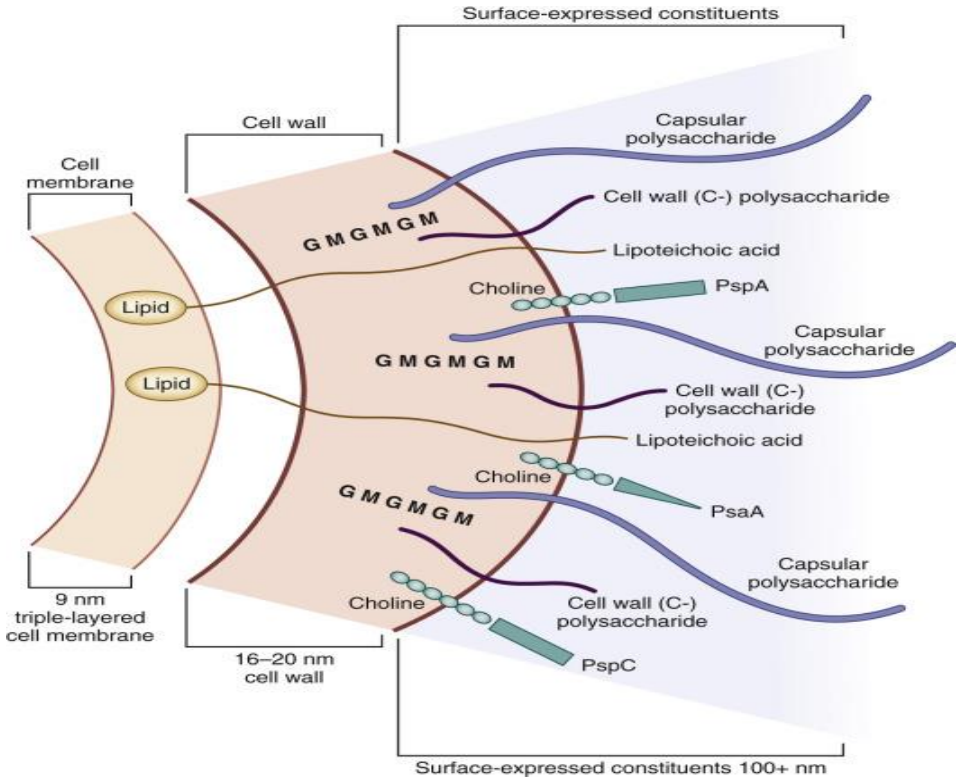

Figure 1-2: Schematic shows the pneumococcal cell wall and capsular polysaccharides.

1.5.3 Autolysin A (LytA)

The autolysin is an N-acetylmuramoyl 1-alanine amidase (LytA) that is a choline-binding protein (Kietzman *et al.* 2016). It is an intracellular enzyme, but some is exported and attached to the cell wall (Mellroth *et al.* 2012). LytA promotes bacterial lysis by breakdown of the cell wall leading to release of Ply (Martner *et al.* 2008). Deletion of the LytA gene gives a decrease in pneumococcal virulence in mice comparing to the wild type pneumococcus (Canvin *et al.* 1995, Hirst *et al.* 2008).

1.5.4 Neuraminidases

Neuraminidases (sialidases) are present in the pneumococcal population in three types; Nan A (115 kDa), Nan B (75 kDa) and Nan C (82 kDa), which are present in 100%, 96% and 51% of pneumococcal strains (Banerjee *et al.* 2010, Brittan *et al.* 2012). Neuraminidases consists of an N-terminal signal sequence, lectin binding domain and a catalytic domain (Parker *et al.* 2009). Nan A contains a C-terminal LPXTGX amino acid sequence, which is connected covalently to peptidoglycan of the cell wall (Walther *et al.* 2016). Sailidases have the ability to cleave terminal N-acetyl neuraminic acid (sialic acid) from glycoproteins that exist on host cells and this may lead to adhesion of pneumococci to host tissues (Brittan *et al.* 2012, Chao *et al.* 2015).

1.5.5 Hyaluronidase (Hyl)

Hyaluronidase is an enzyme composed of an N-terminal catalytic domain and a C-terminal supportive ß-domain which linked by short peptide bond (Akhtar and Bhakuni 2003). Hyl cleaves hyaluronic acid of the host tissues into disaccharides, which may be a source of carbohydrates for *S. pneumoniae* (Marion *et al.* 2012). Furthermore, Hyl can degrade chondroitin sulfates which are polysaccharides present in host tissues and this helps the pneumococcus to penetrate the host cells (Jedrzejas 2001).

1.5.6 Pneumococcal surface proteins C and A (PspA & PspC)

The pneumococcal surface protein C (PspC) is composed of alpha-helical coiled-coil, proline rich (PR), and choline-binding domains (Balachandran *et al.* 2002). PspC has the ability to bind to human immunoglobulin A and to complement C3. PspC can protect *S. pneumoniae* against complement pathway attack (Ricci *et al.* 2013). The

pneumococcal surface protein A (PspA) is a surface exposed protein, and it is approximately 84 kDa (Tu *et al.* 1999). PspA is positively charged, which helps to stabilise the capsular negative charge. It consists of an N-terminal signal sequence, a proline-rich domain, a choline-binding domain and the hydrophobic c-terminal domain (Yother and Briles 1992). PspA is present almost in all pneumococcal invasive strains. It binds the human lactoferrin and interferes with complement deposition that is present on the bacterial surface when inside the host (Baril *et al.* 2006).

1.5.7 Pneumococcal adhesion and virulence A (PavA)

PavA is a 62 kDa pneumococcal fibronectin binding protein of pneumococci. It presents in the outer cell surface and binds to immobilized human fibronectin, thereby it helps pneumococcal adhesion to the host tissues (Holmes *et al.* 2001). PavA directly and indirectly impacts on pneumococcal pathogenesis. It directly impacts, via fibronectin binding adherence to host cells, while also having indirect impact via host cell interactions and inflammatory responses (Pracht *et al.* 2005). Fibronectin is present as soluble and less soluble forms. The soluble form is present in the amniotic fluid, plasma and CSF, while the less soluble localised in the membrane and extracellular matrix of host cells (Van der Flier *et al.* 1995). A *pavA*-knockout pneumococcus strain was cleared rapidly from mouse nasopharynx, as leaving such pavA bacteria unable to translocate from the airway epithelial cell to the bloodstream following an intranasal challenge (Kadioglu *et al.* 2010). NOT Clear...Explain

1.5.8 Pneumolysin (Ply)

The lytic activity of Ply on host cells was mentioned for the first time in 1914 by Rufus Cole. He observed haemolytic activity of pneumococci on guinea pig, rabbit, sheep and human blood cells (Cole 1914, Taylor 2013). Ply is a member of the cholesterol-dependent cytolysin family (CDC) (Lawrence *et al.* 2015). CDCs have the same basic structure, consisting of 4 domains (Bourdeau *et al.* 2009). Domains 2 and 3 are arranged adjacently and on top of them is domain 1, while domain 4 is at the carboxy terminal end and at the base of the structure (Figure 1-3). Ply is a 53-kDa protein and consists of 473 amino acids.(Jefferies *et al.* 2007). Ply is a key virulence factor, produced by most pathogenic strains of *S. pneumoniae* (Jefferies *et al.* 2010). It has lytic function which creates pores in target host cells (Mitchell and Mitchell 2010, Harvey *et al.* 2014).

Unlike other CDCs Ply does not have a N-terminal secretion signal for transport to the extracellular environment (Price *et al.* 2012). Ply is released as a soluble monomer and makes large oligomeric rings, result in pore formation in host cells (Tilley *et al.* 2005). There are studies that are suggesting that Ply release during the stationary phase is mediated by autolysin activities (Tilley *et al.* 2005). However, experiments in mutated pneumococci negative for autolysin showed that Ply was released in the stationary phase, but the mechanism is still unknown (Hakenbeck et al. 2009). It is thought that Ply release may be due to cell death (Taylor 2013). Ply has many effects in addition to its lytic activity, it slows ciliary movement of the respiratory epithelium thereby facilitating bacterial access to the circulation and also activating the complement system (Tilley *et al.* 2005, Jefferies *et al.* 2007).

The classical complement pathway activation of happens when antibodies bind to the target pathogen. Ply has an important role to protect the pneumococcus from the complement attack helping bacterial spreading to other tissues. A study that reported that the ability of Ply to activate the complement system is due to sequence homology with the Fc region of IgG (Rossjohn *et al.* 1998). The mechanism by which the Ply activates the complement system was the subject of debate as previous researchers suggested that Ply can activate the classical pathway of complement in the absence of antibodies (Jefferies *et al.* 2007) but the most recent study reported that Ply fails to activate the classical pathway (Yuste *et al.* 2005, Ali *et al.* 2013). It was found however, that the lectin pathway recognition molecules recognize Ply and consequently activate complement via the lectin pathway (Malley *et al.* 2003, Ali *et al.* 2013).

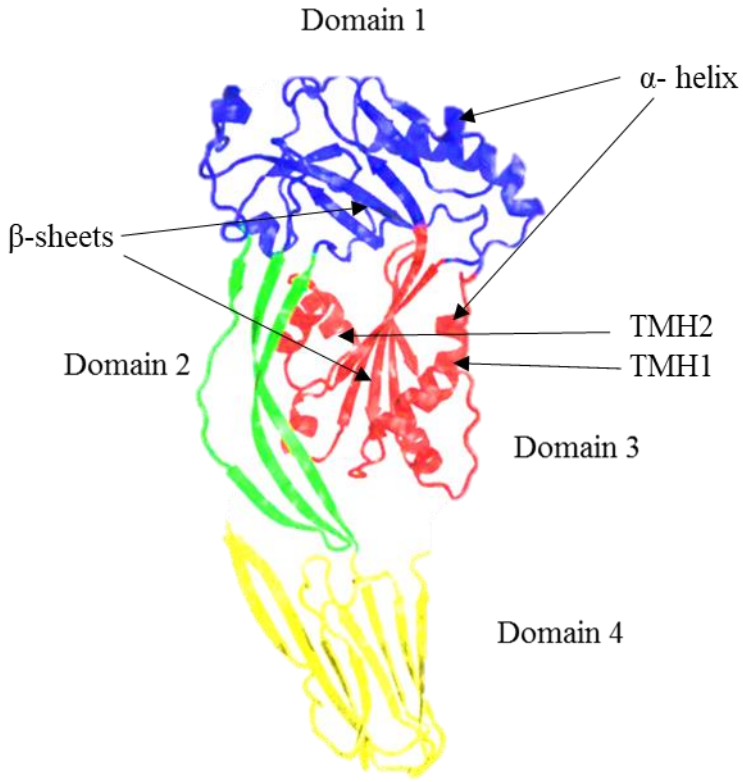

Figure 1-3: Diagram showing the four domains of pneumolysin (Ply).
The domain1 blue, domain2 green, domain3 red and domain4 yellow color (Marshall et al. 2015).

Figure 1-4: Pneumolysin amino acid residues showing the sequence of each domain and the important regions of protein.
The amino acid residues which are highlighted, as blue, green, red and yallow as domain 1, 2, 3, and 4 respectively. The bold and underlined letters (160-186) and the italic and underlined letter (257-280) represent the transmembrane hairpin structures TMH1 and TMH2 respectively. The black colour letters (427-437) represent the cholesterol binding Trp-rich loop (undecapeptide region) (Mitchell and Dalziel 2014, Lawrence *et al.* 2015).

1.6 Lytic mechanism of CDCs

The CDC family of toxins are so-called because of the release on cholesterol for pore forming. Its lytic mechanism starts with binding to cholesterol of the cell membrane (Lukoyanova *et al.* 2016). However other studies reported that some members of CDC family bind to the human complement regulatory protein (CD59) instead of binding to cholesterol (Tilley and Saibil 2006, Taylor 2013). New CDC family members include *Streptococcus intermedius*, *Streptococcus mitis* and *Gardnerella vaginalis*, which produce intermedilysin, lectinolysin and vaginolysin respectively (Taylor 2013). The lytic mechanism for all CDC members basically has the same steps. Pore-formation mechanism has been investigated by different techniques including cryo-electron microscopy (cryo-EM), electron microscopy, kinetic assays, and atomic force microscopy (Czajkowsky *et al.* 2004, Dunstone and Tweten 2012, van Pee *et al.* 2017a). The steps of pneumolysin pore-forming was studied by using cryo-electron microscopy (van Pee *et al.* 2017a). Figure 1-4 as shown is the first step the Ply binding to host cell membrane via the undecapeptide region (427-437 amino acid sequence) of domain 4 resulting in oligomeric ring formation (van Pee et al. 2016). In the second step, domain 2 rotates about 90° and causing the vertical collapse which leads to moving domains 1 and 3 down close to the cell membrane (Taylor 2013). Next step the two helix bundles in domain 3 refold into two amphiphilic β-hairpins structures (HP1 and HP2) (Leung *et al.* 2014). The oligomeric non-covalent assemblly leads to prepore formation and following that two changes synchronize in domains 2 and 3 (prepore collapse and β-hairpins structure formation respectively) (van Pee *et al.* 2017b). Following that the hairpins insert into the cell membrane forming the β-barrel structure resulting in a large pore formation and cell lysis (Lukoyanova *et al.* 2016, van Pee *et al.* 2017a).

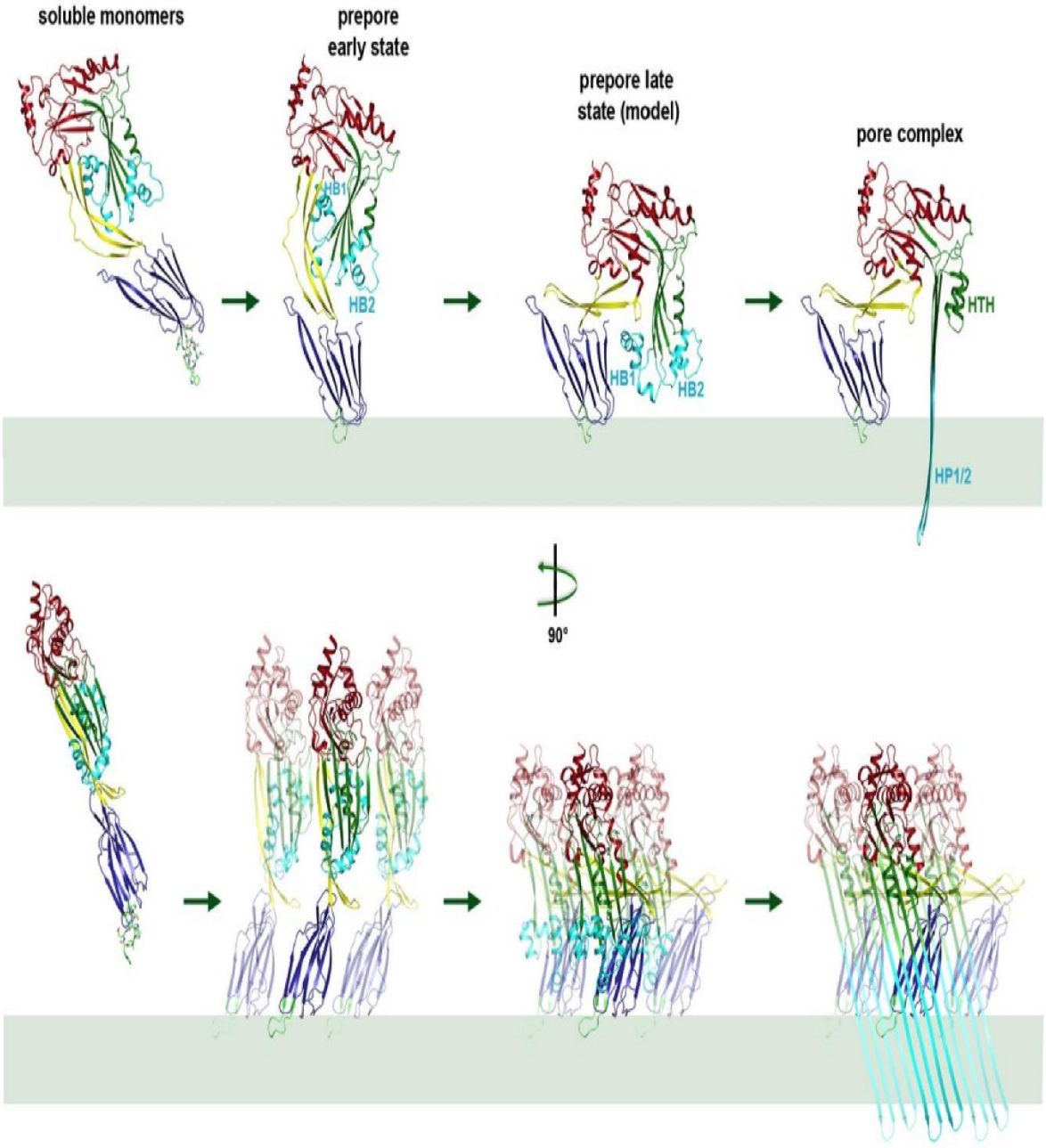

Figure 1-5: Stepwise mechanism of pneumolysin pore formation.
Stepwise schematic diagram shows the mechanism of pneumolysin pore formation. Domain 4 (blue color) binding to the host cell membrane via the undecapeptide region (427-437 amino acid sequence). The oligomeric non-covalent assemblly leads to prepore formation and following that two changes synchronize in domains 2 and 3 (prepore collapse and β-hairpins structures respectively), thereby forming a whole trimeric pore in the membrane cell (van Pee et al. 2017b). Domain 1 is shown in red here, domain 2 in yellow, domain 3 in green and domain 4 in blue, in contrast to colour of domains of Figures 1-2 and 1-3.

1.7 Role of pneumolysin in pathogenesis

The role of pneumolysin in the pneumococcus diseases has been studied since the 1980s, when the Paton and his colleagues immunized mice with a partially inactivated pneumolysin. They noticed moderate protection after subsequent challenge of the mice with virulent pneumococci (Paton *et al.* 1983). Following the sequencing of the *ply* gene for first time (Walker *et al.* 1987), the modified Ply i.e. *ply* point mutations or construction of isogenic mutant pneumococci deficient in *ply* gene were tested *in vivo* and *in vitro*. The modified Ply W433F or D385N showed protection against pneumococcal diseases (Paton *et al.* 1986, Berry *et al.* 1989). Previous studies used pneumolysin deficient mutants in murine models of infection and showed reduction of colonisation in the nasopharynx and increased the clearance of bacterial from the lung (Hirst *et al.* 2004). Other studies using modified *ply* gene products showed different types of mutations resulted in alteration of Ply activities like reduction of the haemolytic activity or deficiency in the complement activation (Hill *et al.* 1994, Baba *et al.* 2002). The Ply-deficient mutant and wild type pneumococci were used in a murine model of bacteraemia. The Ply-negative mutant caused bacteraemia later than the wild type. Also the number of wild type bacteria present when the animals died was about 10^{10} CFU/ml of blood, while the number of Ply-negative mutant cells was constant for several days at 10^{7} CFU/ml (Mitchell and Dalziel 2014). Ply also has a key role in hearing loss during meningitis infection (Winter *et al.* 1997).

Oher studies used Ply-negative mutant in models of ocular infection and showed that the absence of Ply reduced the virulence of pneumococci in ocular keratitis (Norcross *et al.* 2011). The pneumolysin-negative mutant was given intranasally to mice and the mutant virulence was reduced significantly when comparing with wild type pneumococci such as D39 (Berry *et al.* 1989). The pneumococcus lacking pneumolysin was tested in the bacteraemia model and resulted in chronic bacteraemia, while the wild type pneumococci caused death of the mice after 28 hours post infection (Hirst *et al.* 2004). Another study showed that pneumococci expressing pneumolysin were able to breach endothelial cells, while pneumococci lacking pneumolysin were unable to breach endothelial cells (Zysk *et al.* 2001).

Pneumolysin has been shown to induce the presence by inflammatory cells at infection sites. A previous study showed that after infection with isogenic mutant pneumococci deficient in the *ply* gene the influx of leukocytes was significantly delayed (Jounblat *et al.* 2003). The presence of *ply* increased the transmission of pneumococcus throughout the lower respiratory tract thereby the pneumolysin is essential for virulence (Kadioglu *et al.* 2000). The biological features of purified pneumolysin were studied *in vitro* by interaction of purified pneumolysin with cells of tissues (Rubins *et al.* 1993). The impact of this interaction resulted in many activities, for example, the activation of the complement system, inhibition of ciliary beat of respiratory mucosa, inhibition of polymorphonuclear cell respiration and separation of epithelial cell tight junctions (Hirst *et al.* 2000, Kadioglu *et al.* 2002).

The activation of the classical pathway induced by the assembly of antibodies on the pathogen results in formation of the membrane attack complex (MAC) and then cytolysis of the target pathogen. Ply plays an important role against the complement attack thereby it protects the pneumococcus and helping it spread throughout the tissues by depletion of complement and induce the inflammation in the host cells (Mitchell *et al.* 1991). The ability of Ply to activate complement thought to be due to structural homology to the Fc region of IgG (Mitchell and Dalziel 2014).

A recent study showed that circulating Ply can cause cardiac injury and increase the level of troponin protein in the blood. This protein is considered to be a biomarker of cardiac injury (Alhamdi *et al.* 2015). The cardiac injury was detected by the level of troponin protein in the mice blood post-pneumococcal infection. The troponin level in the mice infected with wild type D39 after 12 hours of infection was higher than 24 hours post-infection, while troponin protein was not detected in the mice infected with pneumolysin deficient isogenic D39 (Alhamdi *et al.* 2015). Another contribution of pneumolysin to pathogenesis, is that it is reported to have the ability to induce DNA double strand breaks and result in cell apoptosis (Rai *et al.* 2016).

1.8 Pneumolysin as a vaccine

Streptococcus pneumoniae has more than 90 serotypes based on the capsular polysaccharide. The current pneumococcal vaccine only covered 23 polysaccharide serotypes of the total pneumococcal strains (Mitchell and Dalziel 2014). 23-valent polysaccharide pneumococcal vaccine (PPV) shows protection against pneumococcal pneumonia and it reduces the severity of pneumonia disease in elderly people. However there are studies which reported that PPV provides incomplete protection especially for people with underlying high-risk conditions (Ada and Isaacs 2003). Currently the highly immunogenic vaccine against pneumococcal infection in the infant is the 7-valent conjugate pneumococcal vaccine (CPV) contains serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F (Bechini *et al.* 2009). Due to the emergence of non-vaccine serotypes which contribute to invasive pneumococcal diseases, it is necessary to address or develop non-capsular pneumococcal vaccines, from highly immunogenic antigens across all pneumococcal serotypes and which naturally stimulate development of antibodies in the children two years old (Rapola *et al.* 2000, Holmlund *et al.* 2006). The potential of a genetically conserved protein-based vaccine which could overcome the limitations of pneumococcal vaccines.

Pneumolysin is an important experimental protein-subunit vaccine. Recent studies reported that administration of the toxoid Ply versions in animal models of disease significantly reduced pneumococcal colonization (Kaur *et al.* 2014). The Ply version used has the deletion of the alanine amino acid at position 146, it showed reduction in pore formation and lower stimulation of the inflammatory effects *in vivo* compared with the wild type version of Ply (Lea-Ann S. Kirkham *et al.* 2006).

Other pneumococcal protein was studied and evaluated as possible vaccine candidates such as the pneumococcal surface protein A (PspA), pneumococcal histidine triad proteins (PhtD) and pneumococcal choline-binding protein A (PcpA) (Moffitt and Malley 2016). However recent studies strongly recommended the Ply as protein-subunit vaccine due to Ply being a highly conserved protein across all pneumococcal strains compared with other protein candidates. Also the immunization with Ply toxoid can be assist in the sera by neutralization of Ply lytic activity (Walker *et al.* 1987). Another study has reported that the phase 1 trial of detoxified Ply showed increases in the level of IgG antibody and neutralization capacity in humans (Kamtchoua *et al.* 2013).

1.9 Pneumolysin natural variation

The Ply amino acid sequence was believed to be conserved across the serotypes of *S. pneumoniae,* (Feldman *et al.* 1990) (Mitchell *et al.* 1990) but recent studies showed that Ply has approximately 20 amino acid variations (Jefferies *et al.* 2007, Jefferies *et al.* 2010, Yun *et al.* 2015). In these studies *ply* genes were sequenced in more than 300 pneumococcal isolates from patients with invasive and non-invasive strains. The cell lysates of these isolations were used to determine the haemolytic activity of each Ply allele (Jefferies *et al.* 2007, Jefferies *et al.* 2010). Figure 1-6 illustrates the locations of variants across the amino acid sequence of pneumolysin as well as showing the haemolytic activity of each variant. It was hypothesised that these sequence variants will change pneumococcal virulence but the impact may differ between strains.

The wild type D39 (serotype 2) represents the reference strain and *ply* allele 1. The variants of each *ply* allele are listed in Table 2.2. The *ply* allele 2 has the mutation encoding D380N, it has full haemolytic activity. Allele 3 has 5 mutations (encoding T172I, K224R, A265S, V270 and K271 deletion), and has showed reduction of haemolytic activity. Allele 4 has the mutation encoding D380N, and the extra insertion of 8 amino acids at position 415 (but the study did not mention the nature or name of theses amino acids) this allele showed low lytic activity (Jefferies *et al.* 2007). Allele 5 has same allele 3 mutations as well as encoding the mutation (Y150H). This allele showed no haemolytic activity. Allele 6 also similar to allele 3 in DNA sequence and reduced haemolytic activity, but it has the extra mutation encoding S167F. Allele 7 has same level of haemolytic activity to D39 Ply, it has the mutations encoding N14D and K224R. Allele 8 showed low haemolytic activity, it has the mutation encoding A273D. Allele 9 also has full haemolytic activity and it has the mutation encoding I267M (Jefferies *et al.* 2007).

Allele 10 has the mutations encoding T154M, A265S, V270 and K271 deletion. It showed high haemolytic activity. Allele 11 has the mutation encoding D380N as well as the mutation encoding Q136K, it showed high haemolytic activity. Allele 12 also has two mutations encoding D380N and Q402E, according to the study was archived by Jefferies at al., in 2007, this allele did not show specific haemolytic activity due to it was not recognized in ELISA assay using the ant-Ply 7. Allele 13 has the mutations encoding E260D and D380N, it also showed reduced haemolytic activity (Jefferies *et al.* 2007).

Allele 14 has the mutation encoding D380N as well as insertion of 871 base pairs at the position 142 of Ply DNA sequencing, it is non-haemolytic (Jefferies *et al.* 2007). Allele 15 has the mutation encoding E260D, this variant showed low haemolytic activity. Allele 16 has four mutations encoding K224R, P296S, P394A and V468I, it also reduced the haemolytic activity. Allele 17 also has four mutations encoding N14D, K224R, D323N and H386N, it showed reduced lysis activity. Allele 18 has the mutation encoding V439I, this variant also showed low haemolytic activity. The haemolytic activity of all the previous alleles were determined in the bacterial cell lysates (Jefferies *et al.* 2007, J. M. Jefferies *et al.* 2010). Allele 19 has the mutations encoding D366N and V327I. Allele 20 has the mutations encoding K224R and H386Y, These alleles were discovered by Yun at al. in 2015 but they did not determine the haemolytic activity of bacteria carrying these variants (Yun et al. 2015).

Figure 1-6: Pneumolysin allelic variations.

The blue, red and yellow squares represent the variation of each Ply allele in domains 1, 3 and 4 respectively.

Allele	Amino acid and its position																								
	14	136	142	150	154	167	172	224	260	265	267	270	271	273	296	323	366	372	380	386	394	402	415	439	468
1	N	Q		Y	T	S	T	K	E	A	I	V	K	A	P	D	D	V	D	H	P	Q		V	V
2																			N						
3							I	R		S		DEL	DEL												
4																			N				INS		
5				H			I	R		S		DEL	DEL												
6						F	I	R		S		DEL	DEL												
7	D							R																	
8														D											
9											M														
10					M					S		DEL	DEL						N						
11		K																	N						
12																			N			E			
13									D										N						
14			INS																N						
15									D																
16								R							S						A				I
17	D							R								N				N					
18																								I	
19																	N	I							
20								R												Y					

*Del represents amino acid deletion and IN indicates amino acid insertion.

1.10 Aims of this study

Pneumolysin is a key virulence factor of *S. pneumoniae*, it has an important role in human pathogenesis. Recent studies reported that pneumolysin has twenty variations in its amino acid sequence (Lock *et al.* 1996, Jefferies *et al.* 2007, Jefferies *et al.* 2010, Yun *et al.* 2015), some of them haemolytic in different degrees and the others non-haemolytic. Ply variations present in strains associated with invasive and non-invasive diseases. These studies have reported that Ply activities may vary based on the pneumococcal genetic background. The aims of this study are

- To determine the impact of the Ply amino acid sequence variation in a single genetic background. The variants of Ply 2, Ply 8, Ply 11, Ply 12, Ply 15 and Ply 18 were chosen because some of these variants have reported in the previous study that have one variant and reduced the Ply haemolytic activity as well as some of variants located in the important sequence regions in the Ply.
- To determine the impact of these variations on type 2 D39 virulence. The D39 strain was used because it is transformable strain, and it is well studied by others and the group of Professor Peter Andrew in the murine models of pneumococcal infection and *in vitro*.

Chapter 2: Materials and methods

2.1 Chemicals materials

Unless otherwise stated all chemicals used in this study were supplied by Oxiod (UK), Sigma-Aldrich (UK), Fisher Scientific Ltd. (UK) and New England Biolabs Ltd. (UK). All culture media and materials requiring sterilisation were autoclaved for 20 minutes at 121°C, 15 psi (pound per square inch).

2.2 Bacterial Strains and Media

Streptococcus pneumoniae D39 (serotype 2), used in this study was obtained from the beads stock (S17) of Professor Peter W. Andrew, Department of Infection, Immunity and Inflammation, University of Leicester (UK). All bacterial strains and plasmids used and constructed in this study are listed in Table 2-1.

Approximately 4 g of Blood Agar Base (BAB) was dissolved in 100 ml of distilled water and then autoclaved, after cooling the medium was supplemented with 5% (v/v) of defibrinated horse blood and appropriate antibiotic, and medium was poured into plates.

Brain Heart Infusion medium (BHI) was prepared by adding 3.7 g to 100 ml of distilled water and then autoclaved and kept at room temperature until use. 40 g of Tryptic Soya Agar (TSA) was dissolved in 100 ml of distilled water, after autoclaving 5% (v/v) of defibrinated horse blood and appropriate antibiotics were added to the medium and poured into plates. The media BAB, BHI and TSA were used to culture *S. pneumoniae* D39 strain (serotype 2). Luria Bertani Broth (LB) was prepared by adding 20g of media to 100 ml of distilled water and autoclaved. Luria Bertani Agar (LA) was prepared by dissolving 35g of medium in 100 ml of distilled water and after autoclaving media were supplemented with appropriate antibiotic and then poured into plates and kept until use.

Table 2-1: Strains and plasmids used in this study

Bacterial strain or plasmid	**Description**	**source**
Bacteria		
S. pneumoniae		
S. pneumoniae D39	Virulent strain, serotype 2, capsulated	Peter W Andrew
S. pneumoniae Δ*ply* D39	D39; Δ*ply:* Spec.^{R}	This study
D39 allele 2	N380D (GAT changed into AAT)	This study
D39 allele 8	D273A (GCT changed into GAT)	This study
D39 allele 9	I267M (ATA changed into ATG)	This study
D39 allele 11	Q136K (CAA changed into AAA) N380D (GAT changed into AAT)	This study
D39 allele 12	Q402E (CAG changed into GAG) N380D (GAT changed into AAT)	This study
D39 allele 15	E260 D (GAG changed into GAT)	This study
D39 allele 18	V439 (GTT changed into ATT)	This study
Complement D39 allele 8	A273D (GAT changed into GCT)	This study
Complement Δ*ply* D39	Insert *ply* into D39; Δ*ply:* Spec ^{R}	This study
E. coli Strains		
DH5α	Plasmid propagation	Invitrogen, UK
One Shot Top10	Plasmid propagation	Invitrogen, UK
EC1000	Plasmid propagation	Leenhouts*a*l.,1996
BL21 (DE3)	Protein expression	AgilentTech,USA
Plasmid		
pDL278	Shuttle vector, amplification of Spec.^{R} (spectinomycin)	Yesilkaya, 1999
pCEP	Δ*ply* D39 complementation; Kan^{R}	Guiral *et al*.2006
pORI280	Erm^{R}, RepA gene replacement vector, constitutive lacZ, 5.3 kb	Leenhouts*a*l.,1996
pLEICES-93	Six His-Tag Amp^{R} for protein expression	PROTEX, UK

2.3 Identification of *S. pneumoniae*

2.3.1 Optochin test

Streptococcus pneumoniae strains are sensitive to the Optochin disks (ethylhydrocupreine hydrochloride) (Sigma-Aldrich). Bacteria was streaked onto blood agar plate, and an Optochin disk was placed onto the surface and incubated overnight in a candle jar at 37°C. Inhibition zones as approximately 14 mm or more around the disk confirms the presence of *S. pneumoniae*.

2.3.2 Quellung reaction

Pneumococcal serotype was tested by using a Quelling reaction. It is based on the binding of certain antibodies to the polysaccharide, which makes up the pneumococcus capsule. A smear from an overnight culture was prepared onto a slide by mixing one pneumococcal colony with a drop of distilled water and, dried near a Bunsen burner. The smear was covered by 1:1 of anti-serum (Statens Serum Institut, Denmark) and 1% w/v methylene blue mixture. The slide was put in Petri dish and incubated at 37°C for one hour. The slide was observed under the microscope and a positive result was swelling around the bacterial cell.

2.4 DNA extraction from *S. pneumoniae*

DNA of *S. pneumoniae* was extracted according to the method of Saito and Miura, (1963). Briefly, an overnight 10 ml culture was spun down at 2000 g for 10 minutes (Saito and Miura, 1963). The supernatant was discarded, and the pellet was re-suspended in 400 µl of Tris-ethylenediamine tetraacetic acid (TE buffer) (1 M Tris-HCl, 0.5M EDTA and dH_2O, pH 8.0) containing 25% (w/v) sucrose. After that, 60 µl of 0.5 M EDTA, 40 µL of 10% (w/v) sodium dodecyl sulphate (SDS 1 g in 10 ml dH_2O) and 2 µL of 12.5 mg/mL proteinase K were added to the bacterial suspension and the mixture was incubated at 37˚C for 1-2 hours. Following centrifugation (Microfuge, Sigma) for 5 minutes at 13000 x g, the supernatant was added to an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1 v/v/v) (Invitrogen, UK), then mixed gently until emulsion was formed. The mixture was centrifuged at 13000 x g for 10 minutes and the upper aqueous phase was carefully removed to a fresh tube containing (24:1 v/v) chloroform and iso-amylalcohol.

The mixture was centrifuged and 500 µl of the upper layer was mixed with 2.5 mL of absolute ethanol. After that, 25 µl of 3 M sodium acetate, pH 5.2 was added and the mixture was spun down for 5 minutes. The ethanol was discarded and 500 µl of 70% (v/v) ethanol was added and mixture was centrifuged as before. The pellet was left to dry after discarding the ethanol, and the DNA was then rehydrated by adding 250 µl of TE buffer. NanoDrop™ Spectrophotometer (Thermo Scientific, UK) was used at A260 to determine the DNA concentration.

2.5 Primer design

Primers were designed using A plasmid Editor (ApE) software system to construct the point mutations or knock out *ply* using gene splicing by the overlap extension (SOE) method (Horton *et al.* 1990, Iannelli and Pozzi 2004). All primer sequences used in this study are shown in Table 2-2.

Table 2-2: Primers used in this study underlined letters indicate restriction sites, and the bold letters indicate *aadA* gene homology sequences.

Primer's name	Primer's Sequence 5'-3'	Reference
ply-NcoI-F	CATGCCATGGGAGGTAGAAGATGGCAAATA	This study
ply-BamHI-R	ACGGGATCCCTAGTCATTTTCTACCTTATC	This study
A2-D380N-F	GCCCAATATTATATTACTTGGAATGAATTATCCTATGATC	This study
A2-D380D-R	CCTTGATGATCATAGGATAATTCATTCCAAGTAATATAATA	This study
A8-A273D-F	TCAAGGTAGATCCTCAGACAGAGTG	This study
A8-A273D-R	CACTCTGTCTGAGGATCTACCTTGA	This study
A9-M267I-F	TTTGAAGCTTTGATGAAAGGAGTC	This study
A9-M267I-R	GACTCCTTTCATCAAAGCTTCAAA	This study
A11-Q136K-F	AAGTGGCATAAAGATTATGGTCA	This study
A11-Q136K-R	TGACCATAATCTTTATGCCACTT	This study
A12-Q402E-F	GGACAGAAATGGGGAGGATTTGACGGCTCAC	This study
A12-Q402E-F	GTGAGCCGTCAAATCCTCCCCATTTCTGTCC	This study
A15-E260D-F	GAGTGATGAAGTAGATGCTGCTTTTG	This study
A15-E260D R	GCTTCAAAAGCAGCATCTACTTCATC	This study
A18-V439I-F	GGTGGCGTACGATTTATGAAAAAAC	This study
A18-V439I-R	TCGGTTTTTTCATAAATCGTACGCC	This study
Spec-F	ATCGATTTTCGTTCGTGAATACATGTTAT	This study
Spec-R	GTTATGCAAGGGTTTATTGTTTTCTA	This study
ply-Left/F	TATTGTCAAGGTTATTGGCGACA	This study
ply-Left/R	**TATTCACGAACGAAAATCGAT**ATTTACTGCTTTATTTGCCATCT	This study
ply-Right/F	**AACAATAAACCCTTGCATAAC**GATAAGGTAGAAAATGACTAGGA	This study
ply-Right/R	CACCTTTTGTTGACAGTCTACTCCAGACATATCATA	This study
pLEICS-01-F	TACTTCCAATCCATGGCAAATAAAGCAGTAAATGA	This study
pLEICS-01-R	TATCCACCTTTACTGTCACTAGTCATTTTCTACCT	This study

2.6 Polymerase Chain Reaction (PCR)

PCR amplification was performed using two enzymes. First, PrimeSTAR HS premix (1.25 U/25 µl PrimeSTAR HS DNA Polymerase, 2X dNTP mixture 0.4 mM each, 2X PrimeSTAR buffer including 2mM Mg^{2+}) (Clontech, USA) (Horton *et al.* 1990). The PCR mixture typically consisted of 25 µl PrimeSTAR HS premix, 2 µl templates (20 ng/ µl), 2 µl of each forward and reverse primer (1pmol/reaction), and 21 µl of DNA-free water. The second enzyme was, HotStarTaq *Plus* Master Mix (HotStarTaq *Plus* DNA Polymerase, PCR Buffre, 3 mm $MgCl_2$, and 400 µM dNTP) (Qiagen, UK). The PCR mixture consisted of 10 µl 2X HotStarTaq, 2 µl of specific primer (1pmol/reaction), 2 µl template (20ng/ µl) and 6 µl of DNA-free water. Both of these enzymes were used for DNA amplification and mutant confirmation. The thermo cycler conditions of PrimeSTAR HS premix and HotStarTaq *Plus* Master Mix are described in Tables 2-3 and 2-4 respectively.

Table 2-3: PCR conditions of PrimeSTAR HS premix conditions.

PrimeSTAR HS premix		
Type of cycle	Temp/time	No. of cycle
denaturation	98˚C/10 min	30
annealing	55˚C/5 sec	
extension	72˚C/45 sec/1000bp	

Table 2-4: PCR conditions of HotStarTaq *Plus* Master Mix.

HotStarTaq Plus Master Mix		
Type of cycle	Temp/time	No. of cycle
Initial activation	95˚C/5 min	1
denaturation	95˚C/45 sec	35
annealing	55˚C/45 sec	
extension	72˚C/1min/1000bp	
final extension	72˚C/10 min	1

2.7 Agarose gel electrophoresis

One gram of agarose powder (Bioline, UK) was added to 100 ml of 1X Tris-acetate-EDTA (TAE) (40 mM Tris-acetate, 1mM EDTA, pH 7.7) (Meyers *et al.* 1976). The suspension was heated in a microwave oven, and then cooled to 55-60˚C. Ethidium bromide was added to the suspension to 0.2 µg/ml final concentration. The gel was poured into a casting assembly and left to solidify. Approximately 5 µl of DNA samples were mixed with 5 µl of 6X loading dye (New England Biolabs (NEB), UK) and then put into the well. DNA was visualised under a UV light (Sambrook *et al.* 1989).

2.8 PCR Clean up System (Quick Protocol)

The PCR product was cleaned using a QIAquick DNA purification kit (Promega, UK). Membrane binding solution (4.5 M guanidine isothiocyanate) was added to the PCR product at 1:1(v/v). Then the mixture was transferred to a SV Minicolumn (Promega) and centrifuged for 1 minute at 16000 g and the flow-through was discarded. Then, 700 µl of membrane washing solution (10mM potassium acetate pH 5.0, 16.7µM EDTA pH 8.0 and 80% v/v ethanol) was added, and column was centrifuged for 1 minute at 16000 g, and then another 500 µl of membrane washing solution was added. SV Minicolumn was centrifuged for 5 minutes at 16000 g, and flow-through was discarded. After that, the column was transferred to a new 1.5 ml eppendorf tube and 30-50 µl of nuclease-free water was added to the bottom of the column and left for 1-2 minutes at room temperature. Next, the tube was centrifuged at 16000 x g for 2 min, and the eluted DNA was stored at 4 ˚C or - 20 ˚C.

2.9 Gene Splicing by Overlap Extension (SOEing product)

The two-steps of a SOEing PCR protocol (Horton 1995, Pearce *et al.* 2002) were used to delete *ply*. First step the spectinomycin resistance gene *aadA* (1158 bp) was amplified from the plasmid pDL278 (Hasan Yesilkaya *et al.* 2000). The left flank (600 bp) and right flank (600 bp) of the *ply* gene was amplified individually using primers (Spec-F/Spec-R, *ply*-Left/F *ply*-Left/R and *ply*-Right/F *ply*-Right/R respectively). In the second step, the amplified *aadA* gene was fused with upstream and downstream *ply* fragments using primers (*ply*-Left/F and *ply*-Right/R). All PCR steps were done using PrimeSTAR HS DNA Polymerase (Clontech, USA) as described above (Section 2.5). The SOEing

product was then transformed into D39 and allelic replacement between the *ply* gene and *aadA* gene was achieved by homologous recombination.

2.10 Genetic Transformation of Pneumococcus Cells

The DNA insertion into the genome of *S. pneumonaie* was achieved by two steps (Lerman and Tolmach 1957), preparing the competent cells and transformation of DNA fragments into *S. pneumonaie*.

2.10.1 Competent Pneumococcus Cells

A frozen stock of *S. pneumonaie* D39 bacteria was grown in 10 ml of BHI until the OD_{600} nm reached 0.05. The bacterial culture was then diluted 1:10 in autoclaved growth solution (BHI 100ml, 20% glucose 1 ml, 4% (w/v) BSA 4% 4ml and 1% $CaCl_2$ 1ml) (Steinmoen et al., 2002). The culture was incubated at 37˚C for 45-60 min, then divided into 200µl aliquots with 50% v/v autoclaved glycerol and stored at -80˚C.

2.10.2 Transformation of DNA into *S. pneumonaie*

An aliquot of 200 µl of competent pneumococcus cell was thawed on ice. Then 2 µl of 50 ng/ml of competent stimulating peptide-1 (CSP-1) (Cheng *et al.* 1997, Alloing *et al.* 1998, Bricker and Camilli 1999) was added to the culture. The culture was incubated at 37˚C for 14 min, and then ~ 1µg of DNA was added to the mixture and incubated at 37˚C for 45 min. The reaction mixture was then plated on Blood Agar Base (BAB) or Tryptic Soya Agar (TSA) plates supplemented with appropriate antibiotic (spectinomycin 100 µg/ml or erythromycin 0.5 µg/ml). Plates were incubated overnight at 37˚C in a 5% v/v CO_2 incubator (Avery *et al.* 2017).

2.11 Plasmid extraction using a Mini-prep kit

A QIAprep spin Mini-prep kit (Qiagen) was used to purify plasmids from *E. coli*. A broth of 10 µl of LB with appropriate antibiotic was inoculated with the *E. coli* culture and incubated with shaking, at 37˚C for 14-16 hour. The overnight culture was centrifuged at 4000 g for 10 min (Sorvall cells T, Thermo Scientific). Then the bacterial pellet was suspended in 250 µl of Buffer P1 mixed with RNase solution, and then 250 µl of buffer

P2 was added and the tube was inverted carefully several times. After adding 350 µl of buffer N3, the tube was inverted to prevent DNA precipitation. After centrifugation of the mixture at 13000 g (Microfuge, Sigma) for 10 min, the supernatant was added to a spin column and centrifugation was repeated at 13000 g for 30-60 sec. After discarding the supernatant, 500 µl of buffer PE was added to wash the DNA and then the tube was centrifuged at 13000 g (Microfuge, Sigma) for 1 min. The tube was washed again with 750 µl buffer PE and centrifuged for 1 min and the supernatant was discarded. The spin column was transferred into a 1.5 ml sterile eppendrof tube. The plasmid was eluted with 50 µl of buffer EB (10mM Tris-Cl, pH 8.5) and incubated at room temperature for 1 min before centrifugation at 13000 rpm (Microfuge, Sigma) for 1 min. The extracted plasmid was stored in -20 ˚C until use.

2.12 Restriction Digestion

The digestion mixture normally contained 1 µl (10 U/ µl) of restriction enzyme, 1 µg of DNA, 5 µl of enzyme buffer (X10) and made up to 50 µl with nuclease-free water. The reactions usually were incubated at 37 ˚C for 2-4 hour, depending on the restriction enzyme (Lu *et al.* 2000).

2.13 DNA Ligase

DNA ligase was used to join DNA fragments with the appropriate plasmid (Pusch *et al.* 1998). The standard reaction consisted of 2 µl T4 DNA ligase (NEB, UK), 1:3 ratio vector and inserted DNA respectively, 3 µl of 10X reaction buffer and with nuclease-free water up to 20 µl. The reaction was incubated at 8-10 ˚C overnight, and then heated for 10 min at 65 ˚C.

2.14 Transformation of plasmid into *E. coli*

Plasmids were transferred (Froger and Hall 2007) by using chemically competent *E. coli* cells, one Shot® TOP 10 (Invitrogen, UK) for propagation and into BL21 (DE3) (Agilent Technologies, USA) for expression. A falcon polypropylene round bottom tube was pre-chilled on ice. One vial of TOP 10 competent cell was thawed on ice, and then an aliquot was put into a pre-chilled falcon tube. An aliquot of 2-5 µl of plasmid was added to the reaction, and the mixture incubated on ice for a half hour (Sambrook *et al.* 1989). The

reaction was then incubated at 42˚C for 45 second in a water bath, and then transferred to the ice for 2 minutes. An aliquot of 300 µl of fresh LB was added to mixture, and then incubated for 1 hour in a shaking incubator at 37 ˚C (New Brunswick Scientific). After that, 150 µl of incubated reaction was plated on LA plates, which contained the required antibiotic (kanamycin 50µg/ml or erythromycin 100µg/ml), and then these plates were incubated overnight at 37 ˚C.

2.15 Unmarked point mutation with pORI280 plasmid

To construct unmarked strains expressing point mutations in the *ply* gene, the pORI280 plasmid (Figure 2-1) (Leenhouts *et al.* 1996, Kloosterman *et al.* 2006) was used in this study with *S. pneumoniae* D39 was used as the background strain. Plasmid pORI280 cannot replicate in *S. pneumoniae* due to it requiring the replication gene *repA* to be supplied in *trans* from the propagation host (*E. coli*) (Leenhouts *et al.* 1996). It has an erythromycin resistance gene and β-galactosidase gene. The pORI280 plasmid and mutated *ply* alleles constructed by PCR as in 2.15.1 were digested using the enzymes *Nco*I and *Bam*HI, and then ligated using the T4 DNA ligase (NEB) see sections 2.9 & 2.10. The recombinant plasmid was transformed into the *E. coli* competent cells one Shot® TOP 10 (Invitrogen UK) as described previously in section 2.11. The pORI280-*ply* plasmid was extracted from TOP 10 cells using QIAprep spin Miniprep kit (Qiagen) as described in section 2.8. After that the pORI280-*ply* plasmid was transformed into D39 (section 2.7), the bacterial culture was plated on tryptic soy agar (TSA) supplemented with 0.5 µg/ml of erythromycin, and X-Gal (40 µg/ml of 5-bromo-4-chloro-indolyl-β-D-galactopyranoside). The successful integration of the pORI280- *ply* plasmid with genome of D39 results in blue colonies that are resistant to erythromycin. The integration of the plasmid with the genome of D39 was by a single cross-over event that produces a WT *ply* gene and mutant *ply* gene at this point of process. After that during the non-selective culturing the plasmid was removed with WT *ply* gene or mutant *ply* gene and the blue colonies were changed to white colonies.

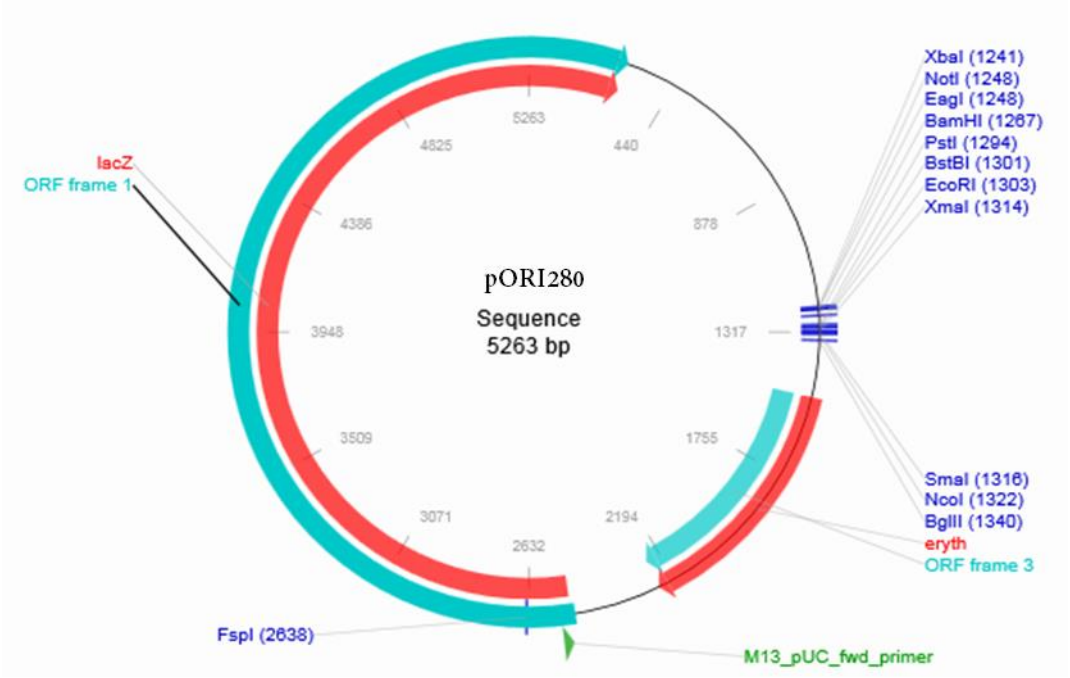

Figure 2-1: Genetic map of plasmid pORI280 used to engineer and deliver ply gene variants.
The plasmid pORI280 contains the restriction enzyme sites, erythromycin resistance gene (EmR), origin of replication (ORI+) and β-galactosidase gene of *E.coli* (lacZ) (Leenhouts et al. 1996, Kloosterman et al. 2006).

2.15.1 Construction of point mutation in *ply* of D39

To construct *ply* alleles using two steps of PCR and a set of primers as listed in Table 2.2, genomic DNA of D39 was used as a template to amplify the mutated *ply* with PrimeSTAR HS premix (Section 2.5). In the first PCR step, two pairs of primers were constructed for each point mutation, the primers *ply-NcoI*-F/AX-R and *ply-BamHI*-R/AX-F (where X represents the allele code) were used to amplify the up and downstream flanking regions of each point mutation. Following that in the second step the up and downstream flanking regions of each mutation were combined up flank (833 bp) and down flank (609 bp) of allele 8 respectively. Allele 2 has the mutation D380N, located in domain 4. To construct this allele the aspartic acid (D) (GAT) was changed into asparagine (N) (AAT) using primers A2-D380N-F and A2-D380N-R. Allele 8 in domain 3 also has one mutation, A273D, the amino acid alanine (GCT) was changed into aspartate (GAT) with the internal primers A8-A273D-F and A8-A273D-R. Allele 9 has the change I267M, which presents in domain 3. The Isoleucine (ATA) was changed into methionine ATG using the primers A9-I267M-F and A9- I267M-R. Allele 11 have two mutations; Q136K in domain 1 and mutation D380N, as in allele 2. The mutation Q136K was constructed by using allele 2 as DNA template and changing the glutamine (CAA) into lysine (AAA), using the primers A11-Q136K-F and A11- Q136K-R. Allele 12 includes the allele 2 mutation and the Q402E mutation, therefore allele 2 was used as a template. Glutamine (CAG) was changed into glutamic acid GAG at position 402 of the D39 Ply sequences, using primers A12-Q402E-F and A12-Q402E-R to amplify the Q402E mutation. Allele 15 is located in domain 3. It has one mutation Q136K, and this mutation was constructed by changing glutamic acid GAG into aspartic acid GAT using the primers A15-Q136K-F and A15-Q136K-R. Allele 18 has the mutation V439I and it located in domain 4. The amino acid valine (GTT) was changed into isoleucine (ATT) using the primers A18-V439I-F and A18-V439I-R. The intention was to determine the impact of these variations on the activity of the *ply*, and the impact of the variation on the background D39 (Robert A Lock *et al.* 1996, Lea-Ann S Kirkham *et al.* 2006, Jefferies *et al.* 2007, Price *et al.* 2012).

2.15.2 Confirmation of successful mutation

2.15.2.1 Colony PCR

To confirm if a constructed mutation was successfully transformed on the plasmid into *E. coli* or inserted into the target location of the pneumococcal genome, colony PCR was used as pre-confirmation method with a HotStarTaq plus Master Mix enzyme (section 2.5). A transformed colony was used as a template and appropriate primers *ply*1-F and *ply*1400bp-R were used to amplify mutant *ply*, whereas the primers Spec-F, Spec-R, *ply*-Left/F and *ply*-Right/R were used to amplify the inserted spectinomycin resistance cassette Table 2-5.

Table 2-5: Primers used for confirmation of pneumococcal mutations.

Primer's name	Primer's Sequence 5'-3'	Reference
*ply*1-F	ATGGCAAATAAAGCAGTAAATGACTTTATA	This study
*ply*1400bp-R	CTAGTCATTTTCTACCTTATCCTCTACCT	This study
Spec-F	ATCGATTTTCGTTCGTGAATACATGTTAT	This study
Spec-R	GTTATGCAAGGGTTTATTGTTTTCTA	This study
ply-Left/F	TATTGTCAAGGTTATTGGCGACA	This study
ply-Right/R	TCCAGACATATCATAGTTCAAGT	This study

2.15.2.2 **DNA sequencing**

Genomic DNA of a transformed cell was extracted and used as a template to amplify mutated *ply* using PCR, and HotStarTaq plus Master Mix enzyme with appropriate primers Table 2-6. PCR product was purified (section 2.5.2) and then sequenced by the Protein Nucleic Acid Chemistry Laboratory (PNACL), University of Leicester.

Table 2-6: Primers used for sequencing of pneumolysin mutations.

Primer's name	Primer's Sequence 5'-3'	Reference
*ply*1-F	ATGGCAAATAAAGCAGTAAATGACTTTATA	This study
*ply*400bp-F	TGGCATCAAGATTATGGTCAGGTCA	This study
*ply*700bp-F	TGGTCTATATTTCGAGTGTTGCTTATG	This study
*ply*400bp-R	ACTTAGCCAACAAATCGTTTACCGC	This study
*ply*1400bp-R	CTAGTCATTTTCTACCTTATCCTCTACCT	This study

2.16 Pneumococcal cell lysis

S. pneumoniae overnight culture was centrifuged at 13000 g for 5 minutes and the supernatant was removed (De *et al.* 2000, Kadioglu *et al.* 2004). The pellet was re-suspended with 1 ml of Phosphate Buffered Saline (PBS) (136 mM NaCl, 2.68 mM KCl; 10, 14 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4). The bacterial suspension was given 15 seconds of sonication pulses every 45 seconds, and the process was repeated 10 times for each sample, which was kept on ice all the time. The sonication amplitude used was about 7 microns. The samples were centrifuged at 15000 g for 2 minutes, and then the supernatant was stored at -80C until use.

2.17 Determination of protein concentration

The Bradford assay kit (Bio-Rad, UK) was used to determine protein concentration (Bradford 1976). An aliquot of 10µl of tested solution protein were added to the wells of a microplate in triplicate. Then 10µl of serial dilutions of bovine serum albumin BSA (0, 50, 100, 200, 400, 600 and 800 µg/ml) were put in the other triplicate rows. An aliquot of 300µl of the Coomassie reagent were added to each well, and then the microplate was mixed for 30-60 seconds on a shaking platform. After incubating the plate for 10 minutes

at room temperature, the absorbance at 595nm was measured by using a microplate reader. The average of each triplicate samples was subtracted from the blank average and the standard curve used to determine the concentration of the test samples.

2.18 Haemolytic assay

Five millilitres of horse blood (Oxoide) were centrifuged at 3000 g for 10 minutes and the supernatant was removed without disturbing the pellet (Owen *et al.* 1994). The red blood cells were diluted to 4% v/v in phosphate buffered saline (PBS). After that, 50 µl of PBS were added to each well of a round-bottomed 96-well plate, followed by adding 50 µL/well of the diluted erythrocytes. An aliquot of 50 µL of serially diluted cell extract or pneumolysin solution were added to the plate and plate was incubated at 37˚C for half an hour. Wells that did not receive Ply received 50 µL of PBS and were considered as the negative control. The plate was centrifuged at 1200 g for 5 minutes and then 100 µL of the supernatant were moved to an ELISA plate and the absorbance was measured at 450 nm, the percentage was calculated using the following equation:

%Haemolytic activity = Ply allele haemolytic activity / Ply D39 haemolytic activity (100%)

2.19 Inhibition of Ply haemolysis with cholesterol

The inhibition of lytic activity of Ply alleles was tested with cholesterol. An aliquot of 20 mM cholesterol was serially diluted in ethanol across 96-well microtiter plate. Following that, 45 µl of pneumococcal cell lysate of each Ply alleles were added to another microtiter plate, then 5 µl of each cholesterol dilution was added to the corresponding wells of Ply alleles, and the plat was incubated at room temperature for 5-10 minutes, then 50 µl of 2% v/v sheep blood RBCs was added to each well. Following the incubation of the plate at room temperature for 30 minutes, the plate was centrifuged at 1750 g at 4°C, and then 50 µl of supernatant of each well was transferred into the corresponding wells of a flat-bottom microtiter plate and the absorbance was measured at 410 nm.

2.20 Bacterial Growth

Pneumococcal growth was achieved using Brain Heart Infusion broth (BHI), and monitored using a Multiskan TM GO Microplate Spectrophotometer (Thermo Scientific, UK). Bacteria were cultured at 37 °C until the OD600 was reached to 0.5, then 5 µl of the culture and 195 µl of BHI were added to a flat bottom 96-well microplate. The plate was incubated in the spectrophotometer (Thermo Scientific, UK) for 24 hours at 37°C, and the bacterial growth was estmated each hour at OD500nm.

2.21 Determination of colony forming units (CFU)

To determine bacterial viable counts, the method of Miles and Misra was used (Miles *et al.* 1938). An aliquot of 180 µl of Phosphate Buffered Saline (PBS) was put into a 96 well microplate, and then 20 µl of bacterial culture was 10-fold serially diluted up to 10^6. Blood agar plates were labelled in 6 sections and 60 µl of each dilution was dropped in appropriate section of the plate. The plates were incubated overnight at 37C° in a candle jar; and colonies were counted next day.

2.22 Genetic complementation of pneumococcal mutations

Mutations of *ply* were complemented with an intact *ply* gene to exclude the possibility of a polar effect. Genetic complementation of Δ*ply* D39 was done using pCEP plasmid (Guiral *et al.* 2006). The pCEP plasmid cannot replicate in *S. pneumoniae*, and is aproximatly 9.5 kb in size. It has about 2 kb of DNA sequences that are homologuous to the transcriptionally silent region (downsterm of the *amiA* operon) in the genome of pneumococcus (Younis 2015). Unmarked mutations were complemented using the pORI280 plasmid (section 2.12). The intact *ply* was amplified using PCR, and HotStarTaq plus Master Mix enzyme with *ply-NcoI*-F and *ply-BamHI*-R primers, then PCR product was purified (section 2.5.2). Plasmids and amplified *ply* were double digested with *Nco*I and *Bam*HI (section 2.9), and then the digested *ply* was ligated to the appropriate plasmid using T4 DNA ligase (section 2.10). Recombinant plasmid was transformed into *E. coli* and then into the pneumococcal D39 strain (section 2.11 and 2.7 respectively).

2.23 Determination of Ply allele concentration by ELISA

The concentration of Ply variants in the pneumococcal cell lysates were determined using ELISA, as described previously (Cima-Cabal *et al.* 2001). Nunc Maxisorb ELISA plates were coated with 2.5 µg/ml of mutant *ply* alleles diluted with coating buffer buffer (0·3 M $NaHCO_3$, 0·2 M Na_2CO_3 at pH 9·6) and incubated overnight in 4˚C. Pure wild type pneumolysin (kindly provided from Prof Russell Wallis, University of Leicester) from 2000 pg /ml to 31.25 pg/ml was used to provide a standard curve. Next day the plates were washed three times with 250µl of washing buffer (PBS + 0.1% v/v Tween 20). Plates were blocked with 200 µl/well of blocking buffer (1% w/v BSA in PBS) and incubated at room temperature for 1-2 hours, then washed again three times with washing buffer. Mouse anti-Ply antibody (monoclonal anti Ply-7) (Abcam) was diluted with washing buffer 1/2500, then 100µl/well was added and incubated at room temperature for one hour and the washing was repeated three times with washing buffer. The anti-mouse IgG conjugated with alkaline phosphatase (Sigma) was diluted 1/5000, and 100µl/well was added to each well and incubated at room temperature for one hour. Plates were washed three times with 250µl of washing buffer, then 100 µl/well of alkaline phosphate substrate (Sigma) was added and incubated at room temperature for 10-15 min. Absorbance was determined at wavelength 405 nm.

2.24 C3 deposition assay

The deposition of C3 on the pneumococcal cell lysates was measured by ELISA assay. Nunc Maxisorb ELISA plates were coated with 1 µg/well of mannan or bacterial lysate carrying Ply variations (100µl of 10µl/ml solution) diluted with coating buffer (0.3M $NaHCO_3$, 0.2M Na_2CO_3 at pH 9.6) and incubated overnight in 4˚C. Next day the plate was blocked with 280 µl/well of blocking buffer 1% w/v BSA in Tris Buffer Saline (TBS/ 2mM $CaCl_2$ and 1mM Mg^{2+}) and incubated at room temperature for 1-2 hours. Normal human serum and unimmunized with pneumococcal vaccine was serially diluted with (TBS) and 100 µl/well were added to the plate to introduce complement and then incubated at 37˚C for one hour, following that plate was washed three times with washing buffer (TBS, 5mM $CaCl_2$ and 0.025% Tween 20, pH 7.4). The rabbit anti-human C3c (Dako) was diluted 1/5000 with TBS buffer, and 100µl/well was added and incubated at room temperature for one hour, then plate washed again three times with washing buffer.

The anti-rabbit IgG (whole molecule alkaline phosphate produced goat) was diluted 1/5000 with LP buffer (1mm Mg and 2mM $CaCl_2$), and 100µl/well was added and incubated at room temperature for one hour, after that 100 µl/well of alkaline phosphate substrate (Sigma) was added and incubated at room temperature for 10-15 min. Absorbance was determined at wavelength 405 nm.

2.25 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

Recombinant Ply was analysed on 4-20% Mini-PROTEAN® TGX Stain-Free ™ Gels (BIO-RAD, USA) (Wang *et al.* 2006). An aliquot of 10 µl of Ply samples were mixed with 10X SDS loading buffer (6.25 ml 0.5 M Tris-HCl pH 6.8, 10 mM DTT, 1 g SDS, 50 mg bromophenol blue, 10 ml glycerol and 6.25 ml dH_2O), then the samples were heated for 5 min at 95˚C. After adding 1X SDS running buffer (Tris base 15.1 g, glycine 94 g, 50 ml 10% (w/v) SDS and dH_2O up to 1000 ml), the gel was put in the tank, then the marker Color Prestained Protein Standard, Broad Range (NEW ENGLAND, BioLabs, UK) and the protein samples were loaded into the wells. Electrophoresis was applied at 150 mV, after which the gel was stained with Coomassie Blue solution (0.4 g of 0.1% (w/v) coomassie brilliant blue, 40% (v/v) methanol, 10% (v/v) acetic acid and dH_2O up to a final volume of 400 ml) for 1 hour with 15 rpm shaking. Coomassie Blue stain was removed by treating the gel with the destining solution (25% (v/v) isopropanol, 10% (v/v) acetic acid and dH_2O up to 400 ml) for 1 hour with 15 rpm shaking, then rinsed with dH_2O and visualised under the BIO-RAD UV machine.

2.26 Western blotting

Western blotting assay was used to analyze recombinant Ply samples (Towbin *et al.* 1979, Mitchell *et al.* 1989). Samples were run on SDS-PAGE gel (section 2.21). After running the gel, filter paper, sponges and nitrocellulose membrane (Roche Applied, USA) were put in transfer buffer (Tris 5.9 g, Glycine 2.9 g, methanol 100 ml, 3 ml of 10% (w/v) SDS, dH_2O 900 ml, pH 8.3). Nitrocellulose membrane was placed in the positive side and the gel was placed on the negative side of the blotter. The gel sandwich was placed in the blotting cassette and arranged from positive side to negative side as follows; soaked sponge, soaked filter paper, nitrocellulose membrane, gel, soaked filter paper and soaked

sponge. After putting the cassette and ice bucket in the blotting tank, the cold transfer buffer was added to the tank, then blotting was run at 230 mA for one and half hours. The membrane was blocked for 3 hours at room temperature with blocking buffer (5% (w/v) skimmed milk (Sigma) in 50 ml PBS), After washing the membrane four times with washing buffer (0.05 % (v/v) Tween 20 in PBS), the membrane was incubated at room temperature for two hours with mouse anti-Ply-7 (Abcam) (1:2500 dilution in 5% (w/v) skimmed milk). The membrane was washed four times with washing buffer and then incubated for two hours with the anti-mouse IgG conjugated with Horse Radish Peroxidase (Sigma) (1/5000 dilution in 5% (w/v) skimmed milk). To detect the antibody complex on the membrane, 3 ml of solution I and II of the ECL reagents (Amersham) were mixed together and added to the membrane. After this step, the membrane was visualized immediately using a BIO-RAD UV machine.

2.27 Expression and purification of recombinant Ply

2.27.1 Cloning of recombinant Ply

The plasmid pLEICS-01 Figure 2-2, containing an ampicillin resistant gene and hexa-histidine tagged fragment was used for protein expression. Recombinant *ply* was amplified using PCR (section 2.5), and primers were described in Table 2-7, then the PCR product was cleaned using Wizard® SV Gel and PCR Clean-Up kit (section 2.5.3). Cloning of recombinant *ply* into pLEICS-01 was performed by the protein expression laboratory (PROTEX), University of Leicester. Recombinant plasmids were sequenced using primers T7 promoter/F and pLEICS-01/R, and the full-length *ply* was sequenced using primers described in Table 2-6.

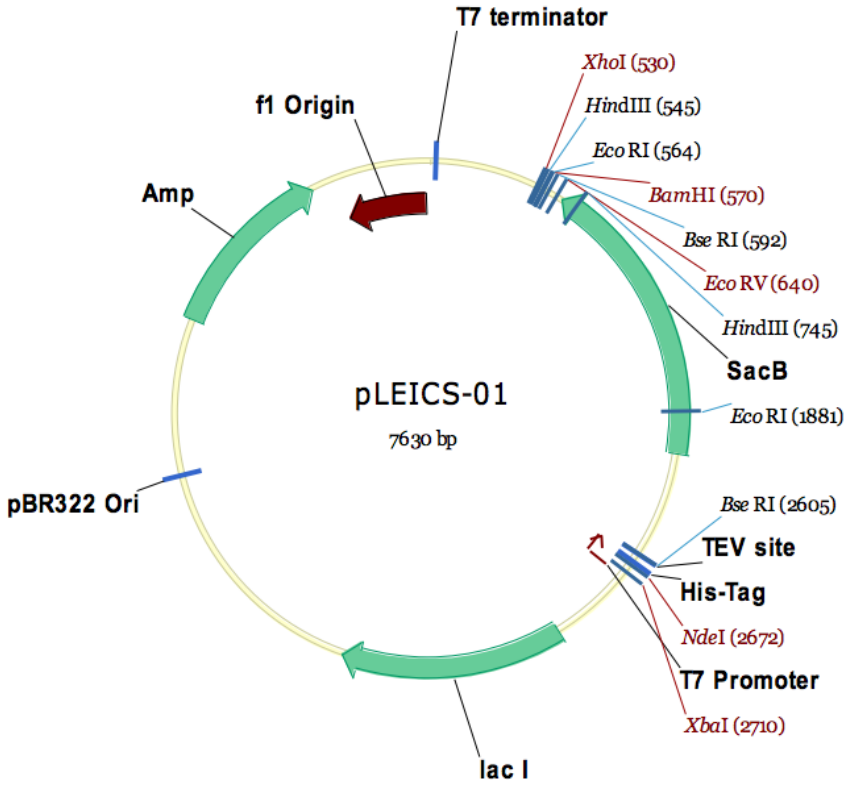

Figure 2-2: The map of pLEICS-01 plasmid.
Plasmid pLEICS-01 containing 6 His-Tag sequence and ampicillin cassette.

2.27.2 Pneumolysin expression

Plasmid pLEICS-01-*ply* was transformed into *E coli* BL21 (DE3) (section 2.11) for protein expression analysis (Mitchell *et al.* 1989). BL21 (DE3) cells were inoculated in 1000 ml of LB supplemented with 100 µg/ml of ampicillin, then incubated with shaking at 220 rpm and at 37°C until the OD600 was reached 0.6-0.8. Bacterial culture was then induced using 1mM IPTG and incubated overnight at 18°C in shaking at 220 rpm. Next day bacterial cells were centrifuged at 8000 g for 20 min at 4°C, then the pellet was suspended with lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 20 mM imidazole and 1% v/v Tween 20 at pH 7.5) containing protease inhibitor tablet. Sonication was done on the ice 4-5 times using 7 amplitudes for 15 second and 45 second for rest intervals to avoid protein denaturation. Cell lysates were centrifuged at 20000 g for 30 min at 4°C, then supernatant was filtered through 0.45 µm filter (Fisher Scientific, UK).

2.27.3 Protein purification

Protein purification was performed by two steps (Faraj 2017), the first being affinity chromatography using a 1 ml nickel-Sepharose fast-protein liquid chromatography (FPLC) column (GE healthcare, UK). After washing the column with 5 ml of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 20 mM imidazole and 1% v/v Tween 20 at pH 7.5), the protein sample was loaded onto the column. The column was then washed again with 20 ml of lysis buffer to remove unbound proteins. After that protein was eluted with elution buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 500 mM imidazole). Fractions containing Ply were identified by running on a 14% SDS-PAGE.
Second step of protein purification, was gel filtration, Superdex 200 16/60 HiLoad column was equilibrated with 20 mM Tris-HCl pH 7.5 buffer. The protein sample was loaded into the AKTA purifier loop (GE Healthcare Life Sciences, UK) and the fractions were collected and Ply proteins identified by 14% SDS-PAGE (section 2.21) also, Ply samples identify were confirmed using western blot and antibody probing (section 2.21). Centrifugal concentrator tubes (Amicon Ultra-15 Centrifugal Filter Units, Ireland) were used to consternate the Ply fractions, then protein concentration was determined using the Bradford assay (section 2.15). Purified protein was sequenced by the Protein Nucleic Acid Chemistry Laboratory (PNACL), University of Leicester. Ply aliquots were snap frozen in liquid nitrogen and stored in -80°C until use.

Table 2-7: Primers used for expression and purification of Ply alleles.

Primer name	Primer Sequence 5'-3'	Reference
A12.Q402A/F	ACAGAAATGGG**GC**GGATTTGACG	This study
A12.Q402A /R	CGTCAAATCC**GC**CCCATTTCTGT	This study
A12.Q402D/F	CAGAAATGGG**GAT**GATTTGACGGCT	This study
A12.Q402D/R	GCCGTCAAATC**ATC**CCCATTTCTGT	This study
A18.V439L/F	GGTGGCGTAC**GC**TTTATGAAAAAACC	This study
A18.V439L/R:	GTTTTTTCATAAA**G**CGTACGCCACCATT	This study
A18.V439A/F	GGTGGCGTACGG**C**TTATGAAAAAAC	This study
A18.V439A/R	CGGTTTTTTCATAA**G**CCGTACGCCA	This study
A8-A273D-F	TCAAGGTAGATCCTCAGACAGAGTG	This study
A8-A273D-R	CACTCTGTCTGAGGATCTACCTTGA	This study
A12-Q402E-F	GGACAGAAATGGG*GAGGATTTGACGGCTCAC	This study
A12-Q402E-F	GTGAGCCGTCAAATCCTCCCCATTTCTGTCC	This study
A15-E260D-F	GAGTGATGAAGTAGATGCTGCTTTTG	This study
A15-E260D R	GCTTCAAAAGCAGCATCTACTTCATC	This study
A18-V439I-F	GGTGGCGTACGATTTATGAAAAAAC	This study
A18-V439I-R	TCGGTTTTTTCATAAATCGTACGCC	This study
pLEICS-01-F	TACTTCCAATCCATGGCAAATAAAGCAGTAA ATGA	This study
pLEICS-01-R	TATCCACCTTTACTGTCACTAGTCATTTTCTAC CT	This study

2.28 *In vivo* virulence studies

All animal experiments were carried out under the Animals for Scientific Procedures Act, in accordance with the appropriate project and personal licenses. All experiments were approved by the University of Leicester Animal Welfare and Ethical Review Board (AWERB). CD-1 mice were bred and supplied from the Division of Biomedical Sciences, University of Leicester.

2.28.1 Preparation of inocula

Pneumococcal inoculums were prepared by culturing *S. pneumoniae* on blood agar plates and incubated overnight at 37°C in a 5% v/v CO_2 incubator. Next day a sweep of colonies was inoculated with 10 ml of BHI broth and incubated overnight at 37˚C until OD500 had reached 1.4-1.6. Bacterial cultures were then centrifuged at 3000 rpm for 15 min and following discarding the supernatant the pellets were re-suspended with 1 ml of 80% (v/v) BHI and 20 %(v/v) sterilized foetal calf serum. An aliquot of 700 µl of re-suspended culture was added to 10 ml of BHI-serum broth and the OD500 was adjusted to 0.7 and the cultures were incubated until the OD500 had reached 1.6. Then the cultures were divided into 500 µl aliquots and stored at -80°C until use. The prepared pneumococcal inoculums were thawed at room temperature, then centrifuged 13000 g for 2 min. The supernatants were discarded, and the pellets were re-suspended with 500 µl of PBS. Three different concentrations of pneumococcal inoculums were intranasal administered, (1 x10^6 CFU/mouse, 5x 10^6/ CFU/mouse and 1x10^7/ CFU/mouse) to set up pneumonia infection.

2.28.2 Optimization of acute pneumonia infection with CD-1 mice

To optimize and set up pilot pneumonia model infection, available mice were 8 to 9 weeks of age CD-1 female mice were used in this study. The experiment was run using three groups of mice (10 mice /group) and three different doses (1 x 10^6, 5 x 10^6 and 1 x 10^7). The administration of infection doses was kindly done by Dr Sarah Glenn. Following anaesthetization of the mice with 2.5% (v/v) Isoflurane (Isocare, UK) over oxygen (1.4 to 1.6 liters/min), 50 µl of PBS containing the appropriate dose of *S. pneumoniae* were administered thorough the mouse nasal passage. After infection of the mice with three doses, the viable count of inoculum doses was confirmed on blood agar plates (section

2.21). Pneumococcal disease signs of infected mice were recorded (Starry coat, hunched and lethargic) for seven days (survival times) (Morton and Griffiths 1985, Gaspar *et al.* 2014). Approximately 20 µl of tail blood was collected at 24 hours post infection, then viable counts of *S. pneumoniae* in the blood were confirmed using Miles and Misra method (Miles et al., 1938) and blood agar plates. Data were analyzed using the Mann-Whitney U test, in the GraphPad Prism7.

2.28.3 Model of acute pneumonia infection

CD-1 mice were female and used at 6-9 weeks of age. Mice were randomised into groups of ten. They were lightly anaesthetised with 3% Isoflurane over 1.8L/min oxygen. Mice were intranasally infected with 50µl of D39 expressing Ply alleles 2, 8, 9, 11, 12, 15 and 18 as well as wild type D39 was used as positive control, the procedures was described previously in section 2.28.2.

2.28.4 Mice Immunization

The mutations Ply D205R and Ply N339R were constructed previously by Professor Russel Wallis group, these two mutations were showed non-haemolytic activity with the sheep RBCs (Marshall *et al.* 2015). To construct these two mutations the DNA template was kindly provided by Professor Russel Wallis. The expression and purification of Ply mutations were done as explained previously (Section 2.24). The female CD-1 outbred mice (Charles River, UK), 10 to 11 weeks of age were immunized with Ply D205R and Ply N339R, this experiment was done in collaboration with Dr. Sarah Glenn and Brindha Gap-Gaupool. Experiment was run by using four groups of mice (10 mice/group). Ply mutations were conjugated with alum (Fischer) as adjuvant and PBS, then group1, 2, 3 and 4 were immunized with 20µg/mouse of Ply D205R, Ply N339R, PBS alone and alum alone respectively, groups 3 and 4 were used as experiment control. Mice immunization was performed three times and 10 days interval between each time, venous blood was collected form mice by tail bleeding before each immunization. Blood was kept at room temperature for 10 min for clotting, then centrifuged at 2000 g and 4°C for 10 min and the serum was stored at -80°C until use. Two weeks post last immunization, intranasal infection was used to challenge the mice with $1x10^6$ CFU/ mouse of D39 strain in 50µl of PBS (section 2.30.2). Mice were observed regularly for survival time (96 hours), humanly culled when they became lethargic.

2.28.5 Determination of anti-Ply in mice serum by ELISA assay

To determine the concentration of anti-Ply IgG in the mice serum, Nunc Maxisorb ELISA plates were coated with 5 µg/ml of wilt type Ply diluted with PBS and incubated overnight in 4˚C. Following washing the plate with washing buffer (PBS, 0.5% (v/v) Tween 20) 200 µl /well of blocking buffer (PBS, 0.5% (v/v) Tween 20, 5% (w/v) milk (Sigma), 5% Sheep Blood Serum (Fisher)) was added then plate incubated for 1 hour at room temperature. Dilutions of mice serum 1:50 and 1:500 in blocking buffer were added to the plate and incubated at room temperature for 2 hours after that plate was washed with washing buffer. The anti-mouse IgG conjugated with alkaline phosphatase (Sigma) was diluted 1/5000, and 100 µl/well was added to each well and incubated at room temperature for 2 hours. Anti-Ply4 (kindly provided by Prof Russell Wallis) was serially diluted from 1000 ng/ml to 31.25 ng/ml and used instead of mice serum as primary antibody to provide a standard curve.100 µl/well of alkaline phosphate substrate (Sigma) was added and incubated at room temperature for 10-15 min. Absorbance was determined at wavelength 405 nm.

2.28.6 Ply neutralization with IgG

Following the mice immunization with Ply D205R and Ply N339R, the ability of anti-Ply IgG in the mice sera was assessed for neutralization of the haemolytic activity of Ply D39. The mice serum was serially diluted 1:1 in PBS in the 96-well bottom plate with a final volume of 25 µl, and then 25 µl of 1.1 µg/ml of Ply D39 was added to each well. Following the incubation at 37°C for one hour, the 96-well plate was centrifuged at 1000 g for 5 min, and then from each well 50 µl of the supernatant was transferred to the corresponding well of 96-well flat bottom plate. The absorbance of RBCs lysis was measured at 450 nm.

2.28.7 Pneumococcal viable count in lung and spleen tissues

The chest of culled mouse was opened by using sterile tools and the lung and spleen were collected and put in the pre-weighed universal tube containing 10 and 5 ml of cold PBS respectively and then kept on ice box. The weight of lung or spleen tissue was determined by weighting the tube containing the tissue and PBS. The tissue was homogenized using Ultra-Turrax T8 homogenizer (IKA-Werke), and after that the pneumococcal viable count per mg of lung or spleen tissue was determined (section 2.21). Animal Blood collection.

2.28.8 Tail bleeding

Followed 24-48 hours of mice infection the venous blood was collected form mice by tail bleeding. Mice were kept at 37°C incubator for 20 min to induce the vasodilatation, the mouse was put in the plastic cylinder, then the venous blood was collected using a 0.5 ml insulin syringe and the blood was added to the serial Eppendorf tube containing 1 µl of heparin sodium (5 units/ µl). The bacterial count in the blood samples was determined using the Miles and Misra method as described previously (section 2.21).

2.29 Statistical analysis

Data were statistically analysed using Graphpad Prism version 7 (Graphpad, California, USA). One or two-way analysis of variance (ANOVA) was used to compare the groups of studies. EC50 or IC50 were used to determine the 50% of haemolytic activity or the inhibition of lytic activity. The Mann Whitney test was used for in vivo studies. Significance was defined as (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$).

Chapter 3: Results

Section A

3.1 Gene Splicing by Overlap Extension

The *ply* gene of D39 was replaced with a spectinomycin resistance cassette (*aadA*) using gene splicing by overlap extension PCR (SOEing) (Horton 1995). This method does not require use of plasmids or a propagation step in the bacterial host (Horton *et al.* 1990). Because the pneumococcus is naturally competent, the SOEing method is an ideal strategy to insert mutations into this bacterium (Seitz and Blokesch 2014). The reason behind insertion of the *aadA* gene (aminoglycoside-3-adenylyltransferase) was to generate an isogenic *ply* negative mutant strain to study the impact of the pneumolysin (Ply) variation on the virulence of *S. pneumoniae* D39.

The steps of construction of the mutant are illustrated in Figure 3-1, which shows the orientations of up and downstream flanks, further *aadA* gene position within a fusion fragment, and the replacement of *ply* with *aadA* by homologous recombination event.
In the first PCR the upstream, downstream regions of *ply* were amplified using the genomic of D39 as a template and primers *ply*-Left/F with *ply*-Left/R and *ply*-Right/F with *ply*-Right/F, respectively, whereas *aadA* was amplified from the plasmid pDL278 (Yesilkaya *et al.* 2000) using primers Spec-F and Spec-R Table 2-2. The PCR products were then analysed by agarose gel electrophoresis. The upstream and downstream flanks of the *ply* generated single bands of approximately 600bp (Figure 3-2, lanes 1 and 2), whereas *aadA* was approximately 1158bp (Figure 3-2 lane 3). The PCR products were purified using a nucleotide cleaning up kit (section 2.7 in Materials and Methods).

In the second PCR step, the purified amplicons of the *aadA* gene, upstream and downstream flanks were used as a template to join the three DNA fragments together using primers *ply*-Left/F and *ply*-Right/F (Table 2-2). The agarose gel electrophoresis shows the successful ligation of the three bands (up and down stream of the *ply* gene with *aadA* gene) to give the expected single band of approximately 2358bp, in contrast to the WT *ply* gene when it incorporated with up and down stream flanks the band size is approximately 2616bp Figure

3-3 C, lane 1, and lane 2) respectively. The Figure 3-3 A and B illustrates the position and size of *aadA* gene or WT *ply* gene after fused with up and down flanks of *ply*.

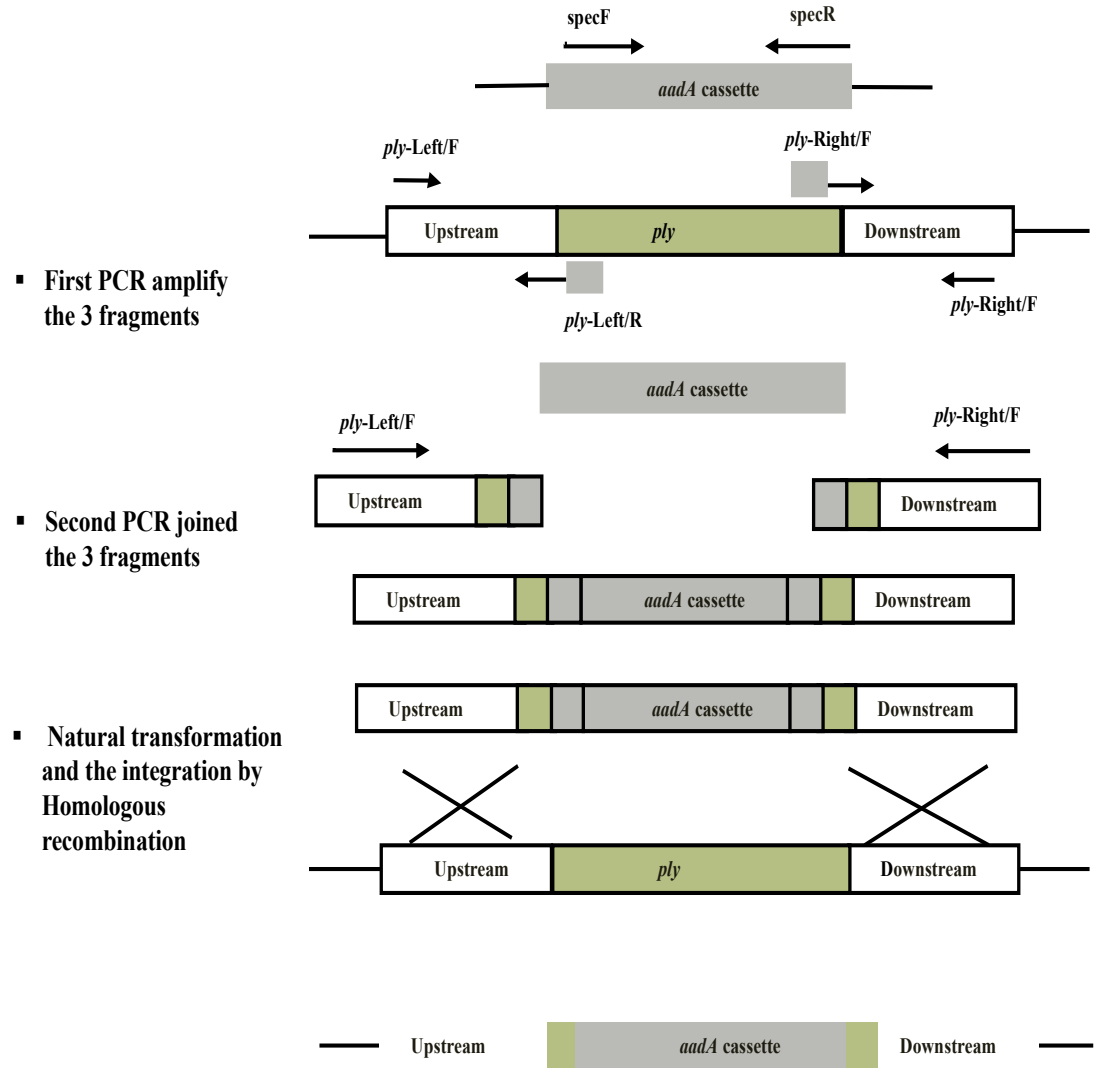

Figure 3-1: Schematic showing SOEing PCR steps.
PCR steps for allelic replacement mutagenesis and ply deletion by homologous recombination, based on Song et al (2005).

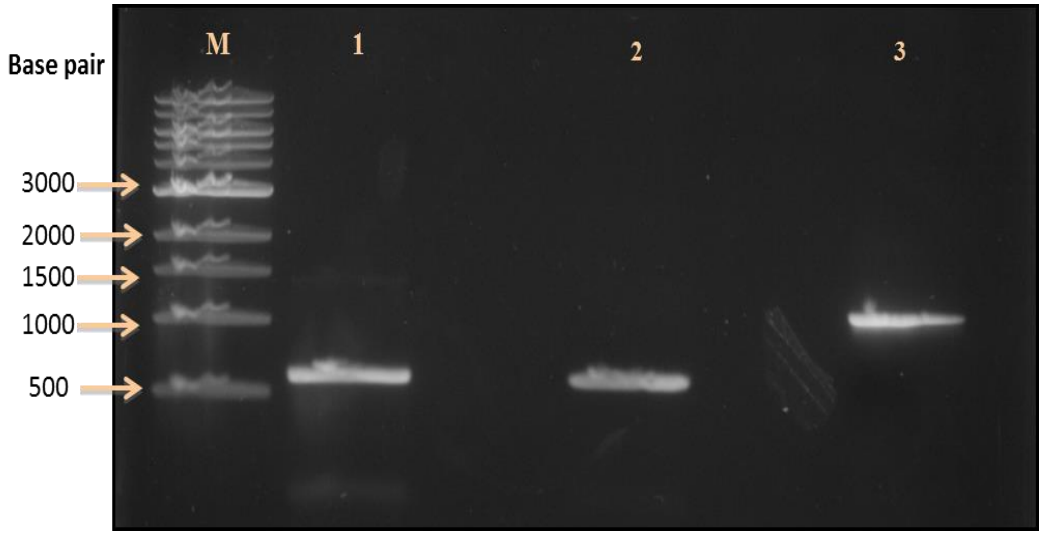

Figure 3-2: Agarose gel analysis showing the amplified up and down stream bands of ply gene.

Agarose gel analysis shows the up and down stream bands of ply approximately 600bp (lanes 1 and 2, respectively), and the spectinomycin resistance gene (aadA), the band size is 1158bp (lane 3). M: 1kb DNA ladder size markers (New England Biolabs, UK).

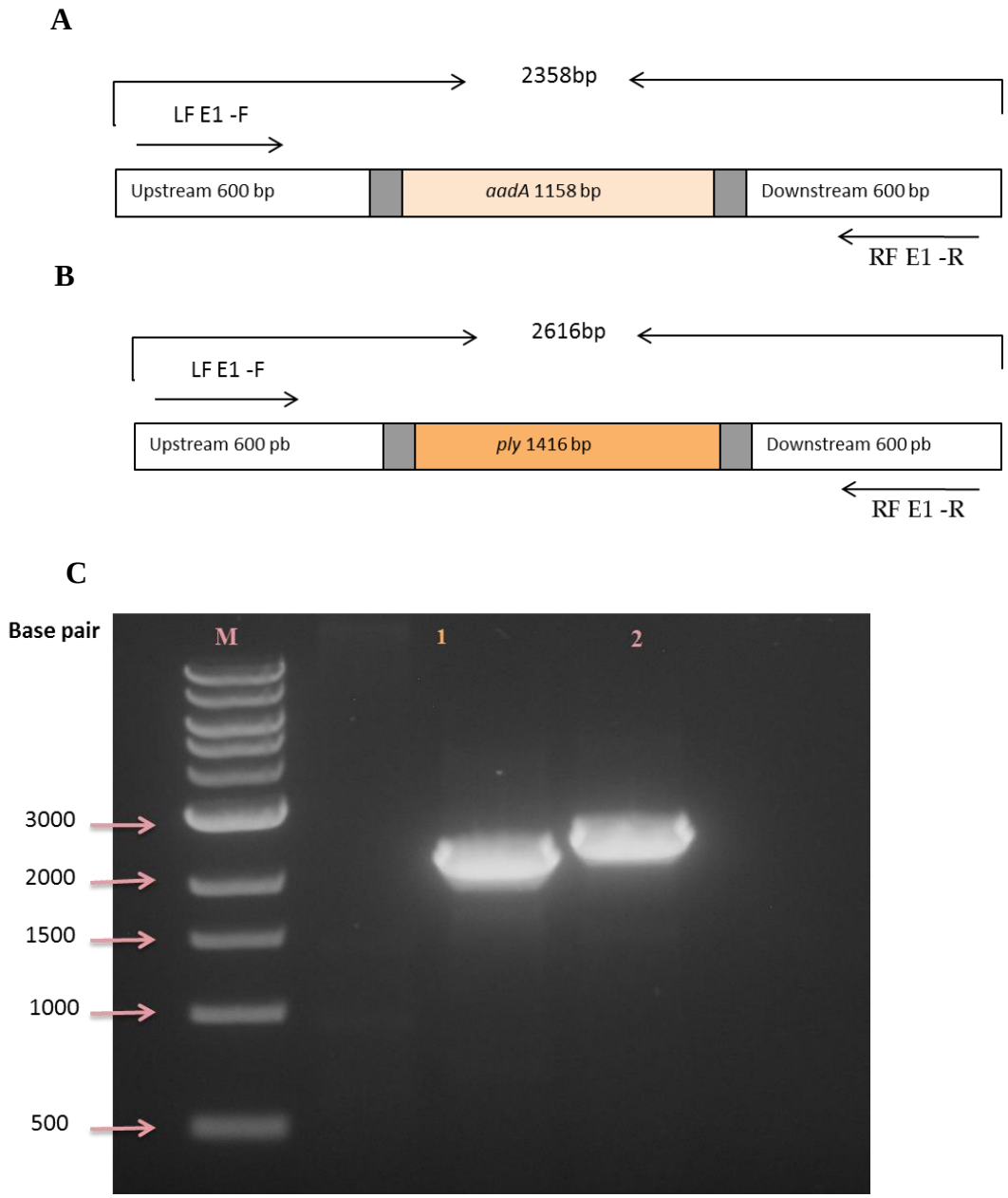

Figure 3-3: SOEing ligation process including agarose gel analysis.
(A) Schematic shows the position and size of aadA gene, up and down flanks of ply within fused SOEing product. (B) Schematic represents the position and size of WT ply, up and down flanks were fused using SOEing method. (C) Agarose gel electrophoresis shows the successful fusion of the three DNA fragments upstream and downstream flanks of ply with the aadA (2358bp) (lane 1), and the up and down flanks with WT ply (2616bp) (lane 2). M: 1kb DNA ladder size markers (New England Biolabs, UK).

3.2 Confirmation of *ply* deletion

The successful replacement of *ply* with the *aadA* cassette was confirmed by using PCR (section 2.5), and the primers listed in Table 2-2. The confirmation was performed by amplifying the up and down flanks of *ply*, which had incorporated the *aadA* cassette, using the primers *ply*-Left/F, *ply*-Right/R, and the extracted DNA from transformed a pneumococcus colony was used as a template. The PCR confirmation strategy is illustrated in Figure 3-4. It consists of three PCR steps; first step the *aadA* was amplified alone using the primers Spec-F and Spec-R, the expected band size of *aadA* gene is (1158bp). In the second PCR step to confirm that the inserted cassette is in the target region of D39 genome, the spectinomycin resistance cassette was amplified with the left flanking region or right flanking region of *ply* using primers *ply*-Left/F and Spec-R or Spec-F and *ply*-Right/R and the expected size band is (1758bp) as we can see this illustration in Figure 3-4. The third PCR step was the amplification of whole mutated region (upstream, *aadA* and downstream region of *ply*) or D39 *ply* performed using primers *ply*-Left/F and *ply*-Right/R. The expected band size of whole mutated region is 2358bp, while the expected band size of D39 *ply* incorporated with the upstream is 600bp and downstream is flanking region 600bp is 2616bp as illustrated in Figure 3-4. Agarose gel electrophoresis shows the *aadA* cassette band of approximately 1158bp, the upstream region with *aadA* cassette and the band size is 1758bp, and the band size of the whole mutated region is approximately 2358bp, while the WT *ply* is about (2616bp) Figure 3-5, lanes1, 2, 3 and 4 respectively). Stocks of Δ*ply* D39 strain were made and frozen within the lab 227 culture collection until use.

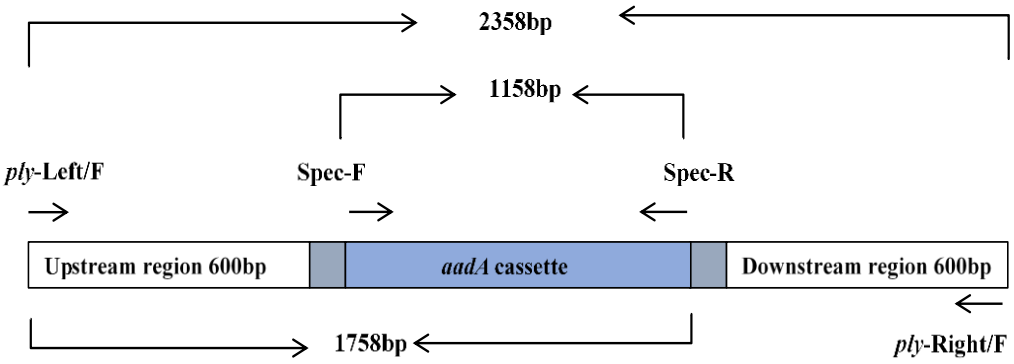

Figure 3-4: Schematic showing the PCR confirmation strategy of *ply* replacement with spectinomycin resistance cassette (*aadA*).
This Figure illustrates the primers that used to amplify whole mutation or aadA cassette alone as well as the up or downstream which incorporated with *aadA* cassette to confirm the successful *ply* deletion.

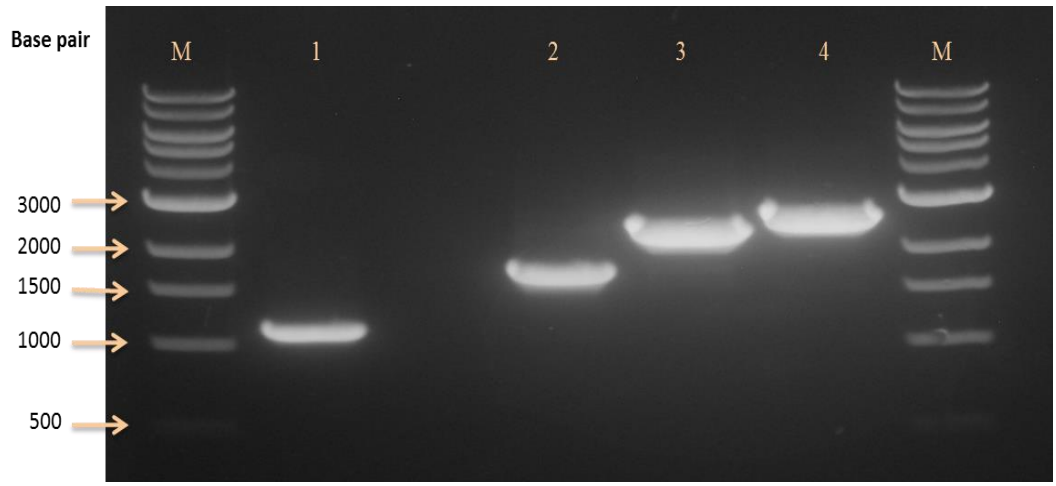

Figure 3-5: Agarose gel electrophoresis analysis showing the successful replacement of *ply* with *aadA* cassette.
The confirmation was performed by PCR using the genomic DNA of knocked out *ply* D39 as a template. Lane 1 shows spectinomycin resistance cassette and the band size is (1158bp). Lane 2 shows upstream flanking region incorporated with *aadA* cassette, band size is (1758bp). Lane 3 confirm the successful deletion of *ply*, and insertion of *aadA* cassette into the right place of genomic D39, the amplified band represents the upstream and downstream flanking regions incorporated with *aadA* cassette, the expected band size for whole mutation (2358bp). Lane 4 represents a control sample, it shows amplified band of *ply* D39 incorporated with the upstream and downstream flanking regions, the expected band size (2616bp). M: 1kb DNA ladder size markers (New England Biolabs, UK).

The Mouse anti-Ply antibody (monoclonal anti Ply-7) (Abcam) was used to detect the Ply concentration in the cell lysates of D39 and Δ*ply* D39. Pure D39 Ply (kindly provided from Prof Russell Wallis, University of Leicester) from 2000 pg /ml to 31.25 pg/ml was used to provide a standard curve, the amount of Ply concentration was quantified using GraphPad Prism 7. In Figure 3-6 an ELISA assay was used to determine the Ply concentration in the cell lysates of D39 and Δ*ply* D39 using monoclonal mouse anti-Ply-7 antibody and anti-mouse IgG conjugated with alkaline phosphatase, and the absorbance determined at 405 nm. Data was average of three independent experiments. A significant difference was seen when comparing the Ply concentration in the cell lysate of Δ*ply* D39 to the wild type D39 cell lysate using one-way ANOVA.

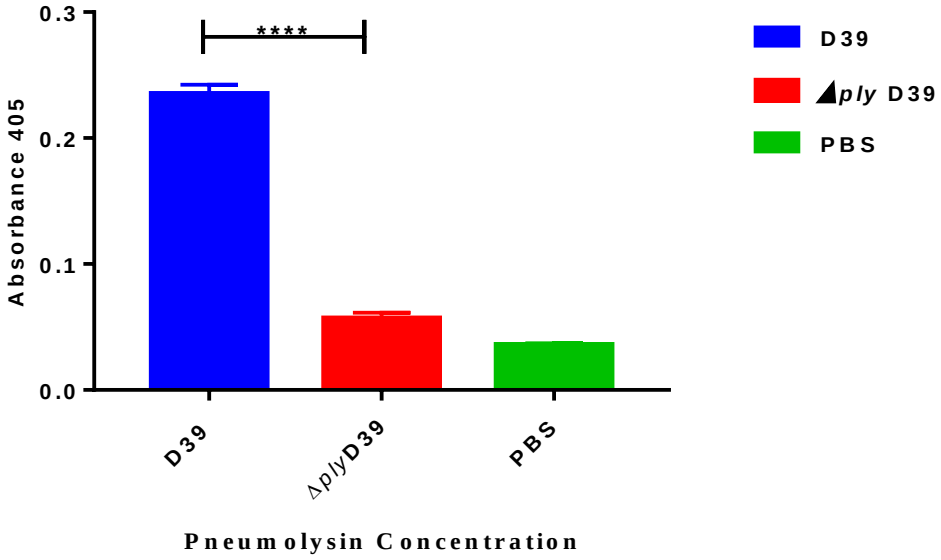

Figure 3-6: ELISA assay was used to determine the Ply concentration in the cell lysates of D39 and Δ*ply* D39.
The Ply concentration was determine using monoclonal mouse anti-Ply-7 antibody and anti-mouse IgG conjugated with alkaline phosphatase, and the absorbance determined at 405 nm. Data was average of three independent experiments, significant difference was seen where comparing the Ply concentration in the cell lysate of Δ*ply* D39 to the wild type D39 cell lysate using one-way ANOVA, Dunnett's multiple comparisons test was done using GraphPad Prism 7 (**** P<0.0001).

The haemolytic activity was tested in Δ*ply* D39 to confirm the inactivation of Ply activity. The cell lysate of pneumococcal wild type D39 strain was used as a positive control, while phosphate buffered saline (PBS) was the negative control. The absorbance of diffusible haemoglobin was measured at 450 nm. The cell lysate of Δ*ply* D39 had no detectable haemolytic activity (0 HU/mg), which proves the successful disruption of the *ply* in D39. In contrast the wild type D39 strain had the specific haemolytic activity of 2.38 x 10^7 HU/mg Figure 3-7.

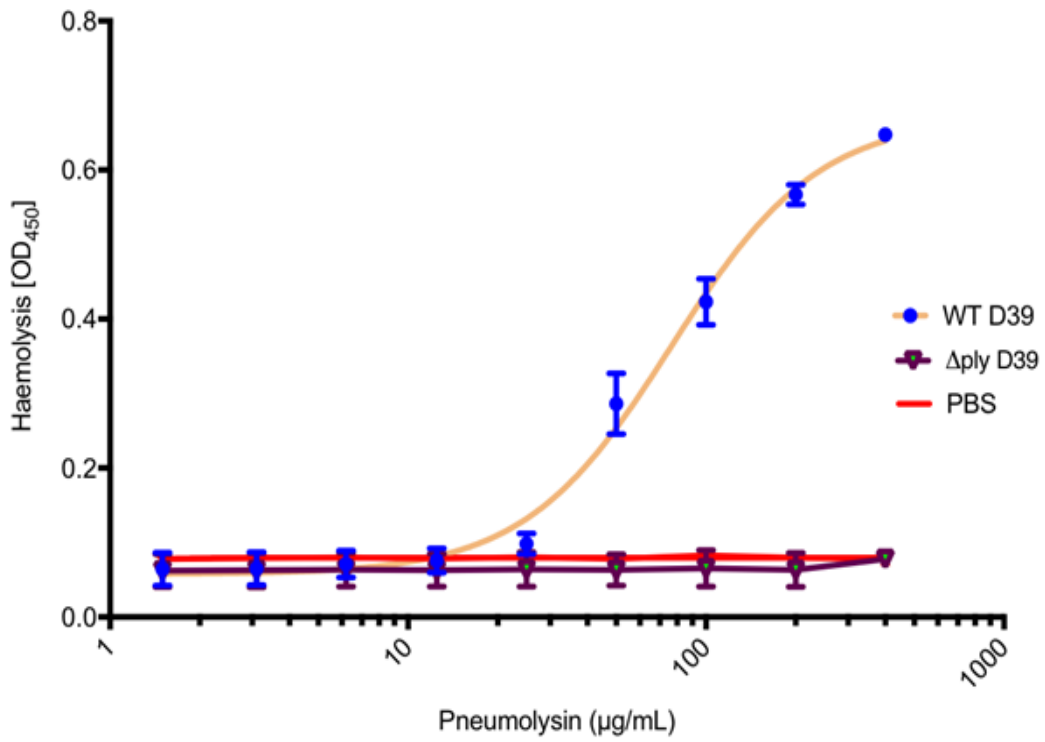

Figure 3-7: Assay of haemolytic activity of *S. pneumoniae*, WT D39 and Δply D39 strain.
The haemolytic activity was tested using 2% RBCs of sheep blood. The absorbance of haemoglobin lysis was measured at 450 nm, PBS was used as a negative control. The specific haemolytic activity of wild type D39 is 2.38 x 107 HU/mg, while Δply D39 has 0 HU/mg. Graph shows the significant difference in the haemolytic activity of Δply D39 is seen comparing with wild type D39 strain using one way ANOVA, Dunnett's multiple comparisons test was done using GraphPad Prism 7 (**** P<0.0001).

Section B

3.3 Strategy for making unmarked point mutations in *ply* gene

Previous studies were mentioned that there are variations in Ply amino acid sequence among *S. pneumoniae* strains (Kirkham *et al.* 2006, Jefferies *et al.* 2007, Jefferies *et al.* 2010, Yun *et al.* 2015). Unmarked point mutations were constructed in the *ply* gene of the D39 strain to generate the Ply variations, and then the mutated *ply* genes were inserted into D39 as a background strain. The pORI280 plasmid was used to construct these unmarked point mutations (Leenhouts *et al.* 1996, Kloosterman *et al.* 2006). The two-steps pORI280 plasmid strategy used to construct a single nucleotide substitution in *ply* is illustrated in Figure 3-8. Step one, the successful integration of recombinant pORI280 plasmid into the *ply* gene in the genome of D39 occurred via a single homologous recombination cross-over event (e.g. flank A of mutated *ply* integrate with flank A of *ply* D39), and that resulted in blue colonies. Step two, non-selective culturing was performed to the positive transformant (blue colonies) on TSA supplemented with X-gal and without erythromycin. The second recombination event was occurred by repeating the homologous integration via flank A or B, and this is resulting ether D39 *ply* or mutated *ply* and white colonies.

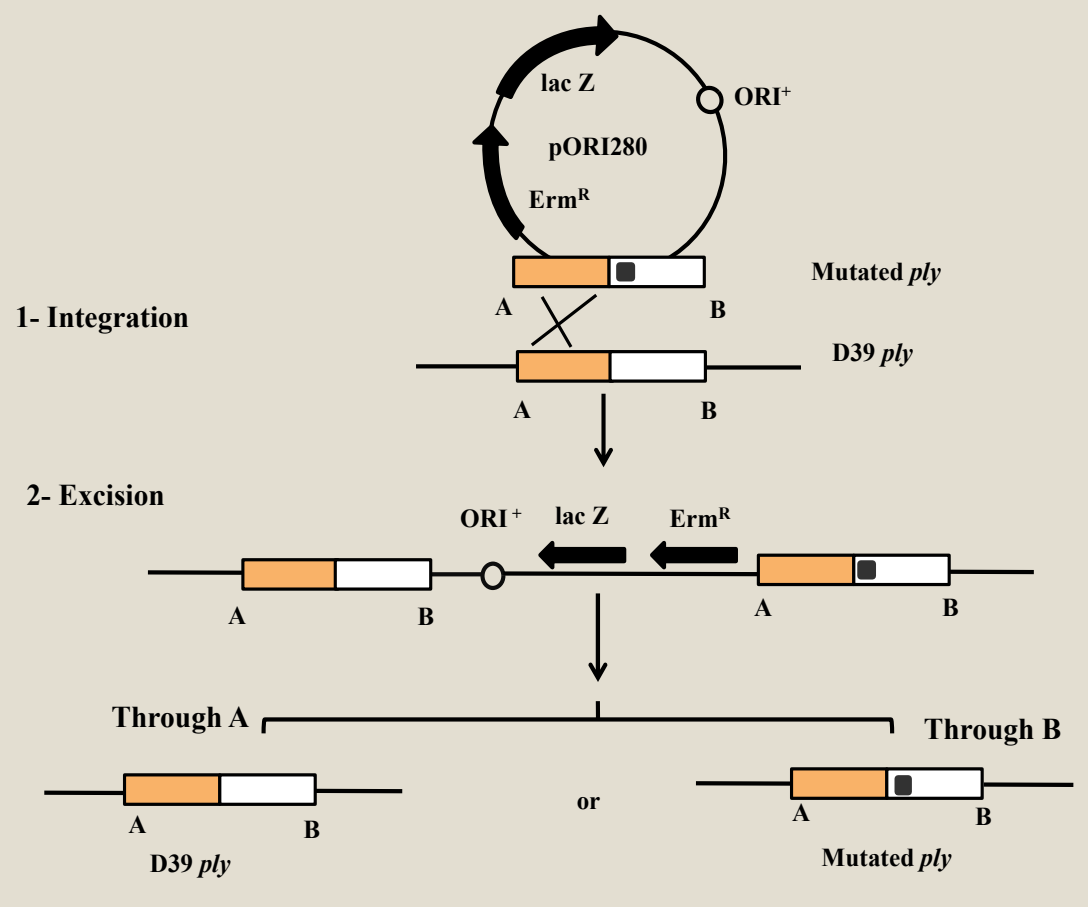

Figure 3-8: Schematic showing the strategy for construction of unmarked point mutation with the use of pORI280 plasmid.
The strategy contains the left and right flank sequences of mutated *ply* (A & B, respectively), the black square represents the point mutation. Plasmid integration into the genome of D39 occurred via a single cross-over event ether with the A or B flank (for example the flank (A) of mutant *ply* integrated with flank (A) of WT *ply*, the orange colour). Then following non-selective culturing with no antibiotic was performed on TSA with X-gal, the plasmid underwent excision by a second recombination step via the A or B flank, and this resulting ether in a mutated *ply* or restoration of the D39 *ply* (diagram constructed from Kloosterman *et al*. (2006) and Leehouts *et al*. (1996)).

3.4 Construction of alleles of *ply* in D39

The Ply variations were constructed in the *ply* gene of the D39 strain, and then the constructed Ply variants were inserted into the D39 strain as a background bacterium. Ply alleles 2, 8, 9, 15 and 18, each one containing single allelic variation (D380N, A273D, I267M, Q136K and V439I respectively), while alleles 11 and 12 have variants of allele 2 as well as Q136K and Q402E respectively. The constructed Ply alleles were amplified with PrimeSTAR HS premix using PCR (section 2.12.1). The genomic DNA of D39 strain was used as a template, and all the primers were used in this study listed in Table 2-2. The PCR was carried out in the first step to amplify the mutated left and right overlapping fragments of *ply* D39 sequence to generate the up and downstream flanks of Ply alleles.

Ply allele 2 contains the variant D380N was constructed first, then used as a template to construct Ply alleles 11 D380N/Q136K and 12 D380N/Q402E. Agarose gel electrophoresis analysis lanes 1-6 showing the amplification of mutated *ply* D39 to generate the upstream (1140bp) and downstream (279bp) flanks of *ply* allele 2, upstream (408bp) and downstream (1009b p) flanks of *ply* allele 11 and upstream (1206bp) and downstream (21bp) flanks of *ply* allele 12 respectively Figure 3-9. Following amplification of up and downstream fragments of each allele *ply* using the appropriate primers listed Table 2-2.

In Figure 3-10 agarose gel analysis lanes 1-8 showing the amplification of mutated *ply* D39 to generate the left (819bp) and right (598bp) flanks of *ply* allele 8, left (801bp) and right (616bp) flanks of *ply* allele 9, left (780bp) and right (637bp) flanks of *ply* allele 15 and left (1317bp) and right (100bp) flanks of *ply* allele 18, all these bands are the expected size of up and downstream regions of each constructed Ply allele.

In the second PCR the upstream and downstream fragments, which containing the putative mutant for each allele were combined to amplify the appropriate mutated *ply* allele using PrimeSTAR HS premix and the external primers *ply-NcoI*-F and *ply-BamHI*-R Table 2-2. Agarose gel electrophoresis analysis shows the expected size of amplified mutated *ply* alleles (1417bp). In Figure 3-11 lanes 1-7 representing the constructed *ply* alleles 2, 8, 9, 11, 12, 15 and 18 respectively and the lane 8 represents the D39 *ply* as a positive control.

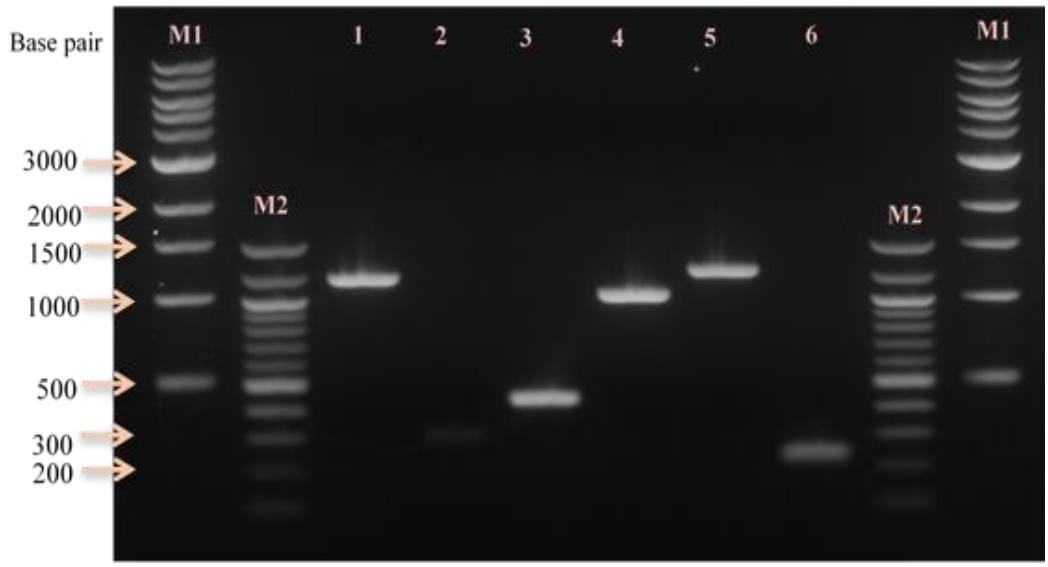

Figure 3-9: Agarose gel electrophoresis analysis showing the amplification of mutated upstream and downstream flanks of *ply* D39 to generate alleles 2, 11 (Q136K) and 12 (Q402E) respectively.

Lanes 1 and 2 show the expected size of left (1140bp) and right (279bp) flanks to generate allele 2, lanes 3 and 4 represent the expected size of left (408bp) and right (1009bp) regions to generate allele 11, while lanes 5 and 6 show the expected size of left (1206bp) and right (211bp) flanks to generate allele 12 (Q402E).

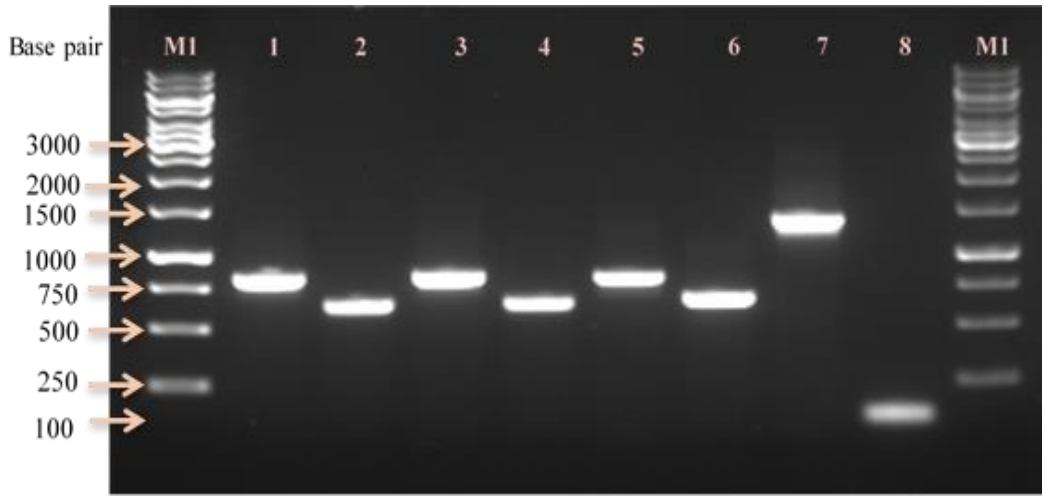

Figure 3-10: Agarose gel analysis showing the amplification of mutated upstream and downstream flanks of *ply* D39 to generate alleles 8, 9, 15 and 18.

Lanes 1-2 show the expected size of left (819bp) and right (598bp) flanks to generate allele 8, Lanes 3 and 4 represent the expected size of left (801bp) and right (616bp) flanks to generate allele 9, while Lanes 5 and 6 show the expected size of left (780bp) and right (637bp) flanks to generate allele 15 and Lanes 7 and 8 show the expected size of up (1317bp) and down (100bp) flanks to generate allele 18 M: 1kb DNA ladder size markers (New England Biolabs, UK).

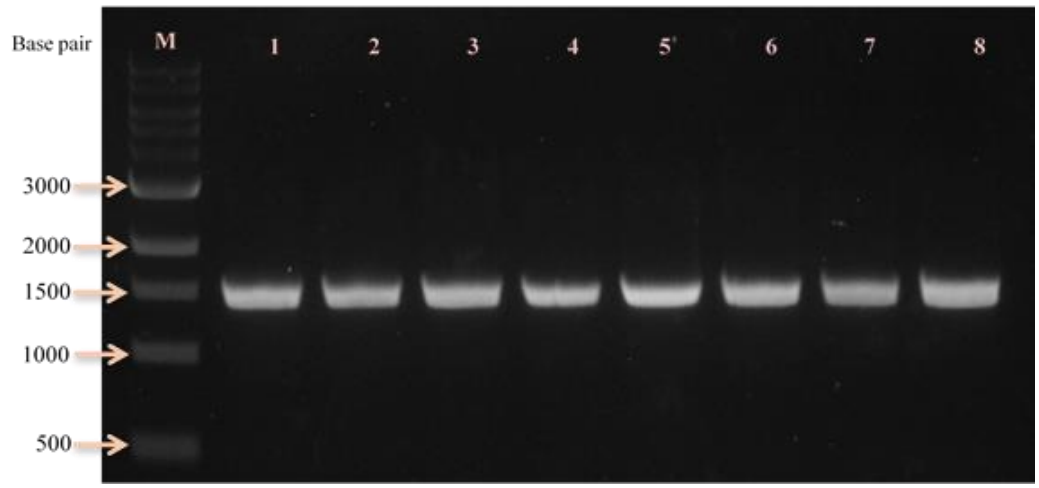

Figure 3-11: Agarose gel electrophoresis analysis showing each amplified *ply* allele. The *ply* alleles were amplified using the primers *ply*-NcoI-F *ply*-BamHI-R (Table 2.2) and the upstream and downstream flanks, which containing the putative mutant of each allele, were combined to amplify the full length of mutated Ply allele. Lanes 1-7 representing alleles 2, 8, 9, 11, 12, 15 and 18 respectively. Lane 8 shows the wilt type D39 *ply* as a control sample. The *ply* alleles incorporated with NcoI and BamHI restriction sites and the expected size of each amplified band is (1420bp). M: 1kb DNA ladder size markers (New England Biolabs, UK).

The pORI280 plasmid and each mutated *ply* allele were digested with *NcoI* and *BamHI* (section 2.9). In Figure 3-12 agarose gel electrophoresis analysis shows in lane 1 the digested and linearized pORI280 plasmid and the band size is 5263bp, while lane 2 shows the undigested plasmid. The digested DNA fragments were purified to remove any salts or enzymes from the samples. Following that each constructed *ply* allele was ligated with the digested pORI280 by using T4 DNA ligase (section 2.10).

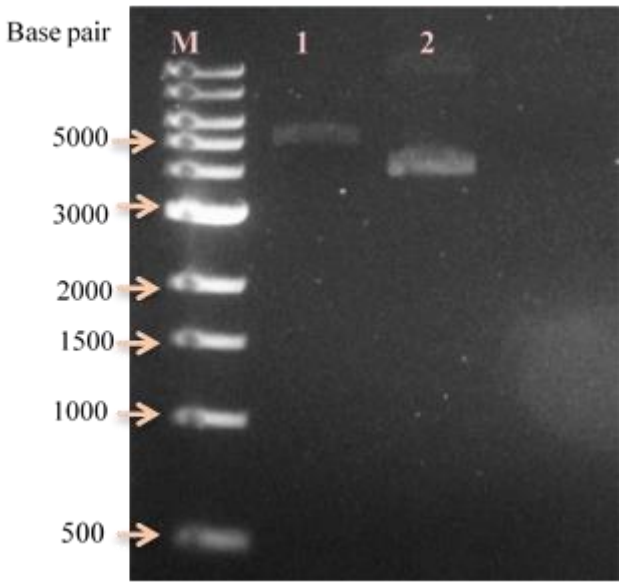

Figure 3-12: Agarose gel electrophoresis showing the digested pORI280 plasmid. The plasmid was digested with *NcoI* and *BamHI* in lane 1 and the expected band size is 5263bp. Lane 2 shows the undigested pORI280 plasmid. M: 1kb DNA ladder size markers (New England Biolabs, UK).

Each constructed allele was inserted between the *NcoI* and *BamHI* sites of pORI280 plasmid to form the recombinant plasmids pEM2, pEM8, pEM9, pEM11, pEM12, pEM15 and pEM18, which are containing *ply* alleles 2, 8, 9, 11, 12, 15 and 18 respectively. Following that each recombinant plasmid was transformed into *E. coli* cells, one Shot® TOP 10, for propagation (Section 2.11) and then each plasmid was extracted using the QIAprep spin Mini-prep kit (Qiagen) (Section 2.11), and then each mutated *ply* allele was amplified from the extracted plasmid using the primers *ply-NcoI*-F *ply-BamHI*-R (Table 2.2), the purpose of this step to confirm that each recombinant plasmid is carrying the mutated *ply* allele. Agarose gel electrophoresis analysis shows the successful amplification of each mutated *ply* allele from the recombinant plasmid, lanes 2-8 representing the amplification of mutated *ply* alleles 2, 8, 9, 11, 12, 15 and 18 respectively and the lane 1 represents the *ply* D39 as a positive control Figure 3-13.

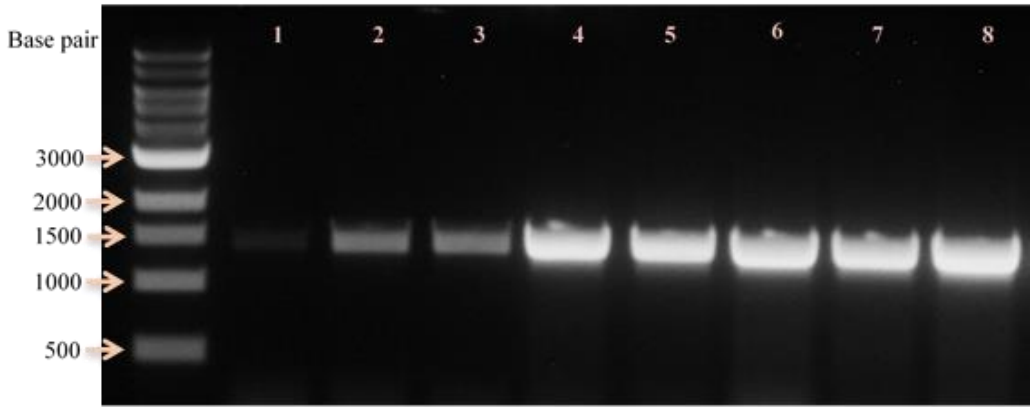

Figure 3-13: Agarose gel electrophoresis analysis showing the amplification of each mutated *ply* allele from the appropriate recombinant plasmid.
The *ply* alleles were amplified using the primers *ply-NcoI*-F *ply-BamHI*-R (Table 2.2). Lane 1 shows the D39 *ply* as a control sample, while Lanes 2-8 representing the constructed *ply* alleles 2, 8, 9, 11, 12, 15 and 18 respectively were amplified from recombinant plasmids pEM2, pEM8, pEM9, pEM11, pEM12, pEM15, and pEM18 respectively, the expected size of each amplified band is (1420bp). M: 1kb DNA ladder size markers (New England Biolabs, UK).

The recombinant plasmids were transformed into D39, the successful transformation resulting in blue colonies. The plasmid was removed from blue colonies by non-selective culturing was done on TSA plates supplemented with X-gal and without antibiotic. Following excision of plasmid from pneumococcal DNA, the erythromycin resistance gene and *lacZ* reporter are removed, and this process resulted in white colonies. Erythromycin sensitivity testing was done for white colonies and then the final confirmation step was achieved by DNA sequencing of each mutated *ply* allele.

3.5 Confirmation of unmarked point mutation

The successful transformation of each recombinant plasmid into D39 was confirmed using the erythromycin sensitivity test, and the DNA sequencing of mutated *ply* allele, which performed by the Protein Nucleic Acid Chemistry Laboratory (PNACL), University of Leicester. Following the successful transformation of each recombinant plasmid pEM2, pEM8, pEM9, pEM11, pEM12, pEM15 and pEM18 into D39.The mutated *ply* alleles were amplified from the genomic DNA of white colonies by PCR, as previously described in section 2.5. To eliminate the possibility of any extra mutations were happened during the construction of *ply* alleles, the full-length of mutated *ply* alleles were sequenced by PNACL using the primers listed in Table 2-5 and then sent for DNA sequencing.
The DNA sequencing results were confirmed the successful construction of *ply* alleles 2, 8, 9, 11, 12, 15 and 18 respectively.

Figure 3-14, illustrates the successful replacement of aspartic acid (GAT) into asparagine (AAT) at position 380 amino acid sequences of *ply* D39 to generate *ply* allele 2. Figure 3-15, shows the successful replacement of alanine (GCT) into aspartic acid (GAT) at position 273 amino acid sequences of ply D39 to generate *ply* allele 8. Figure 3-16 shows the successful replacement of isoleucine (ATA) into methionine (ATG) at position 276 amino acid sequences of *ply* D39 to generate *ply* allele 9. Figure 3-17 illustrates the successful replacement of isoleucine (ATA) into methionine (ATG) at position 276 amino acid sequences of *ply* D39 to generate *ply* allele 11.

Figure 3-18 represents the successful replacement of glutamine (CAG) into glutamic acid (GAG) and aspartic acid (GAT) into asparagine (AAT) at positions 402 and 380 of amino acid sequences of *ply* D39 to generate *ply* allele 12. Figure 3-19 shows the successful replacement of glutamic acid (GAG) into aspartic acid (GAT) at positions 260 of amino acid sequences of *ply* D39 to generate *ply* allele 15. Figure 3-20 illustrates the successful replacement of valine (GTT) into isoleucine (ATT) at positions 439 of amino acid sequences of *ply* D39 to generate *ply* allele 18.

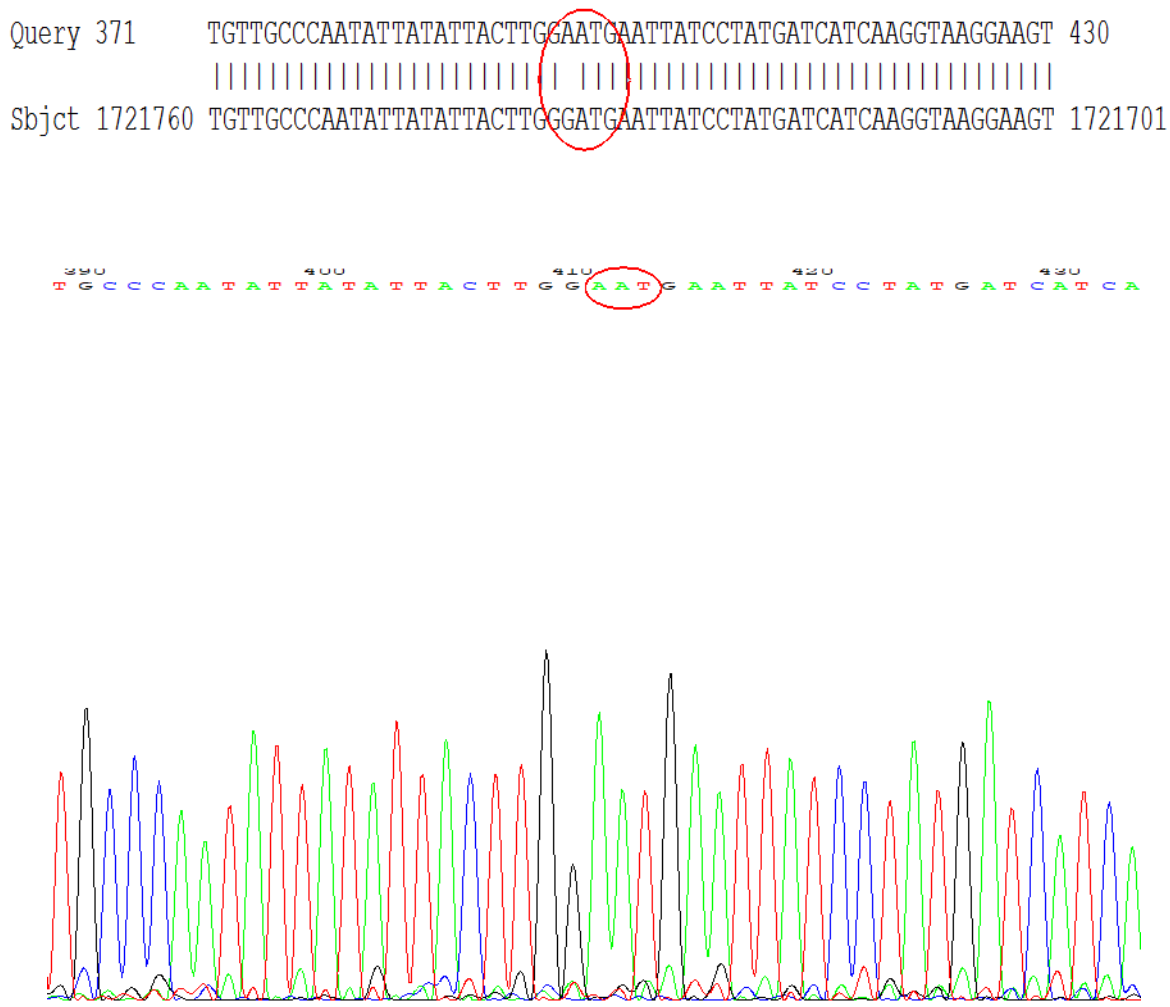

Figure 3-14: DNA sequencing of constructed *ply* allele 2.
DNA sequencing illustrates the successful replacement of aspartic acid (GAT) into asparagine (AAT) at position 380 amino acid sequences of *ply* D39 to generate *ply* allele 2.

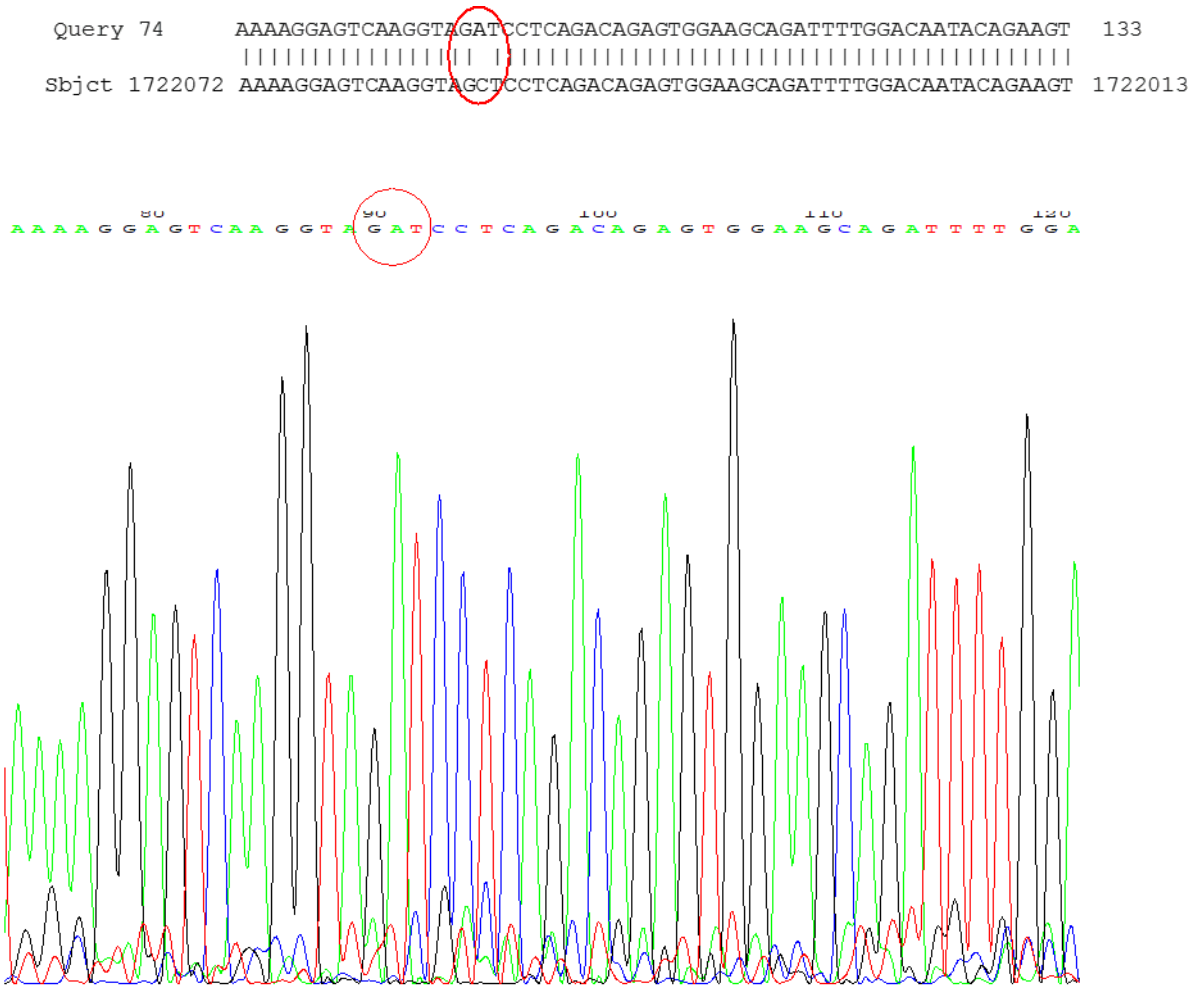

Figure 3-15: DNA sequencing of constructed *ply* allele 8.
DNA sequencing illustrates the successful replacement of alanine (GCT) into aspartic acid (GAT) at position 273 amino acid sequences of *ply* D39 to generate *ply* allele 8.

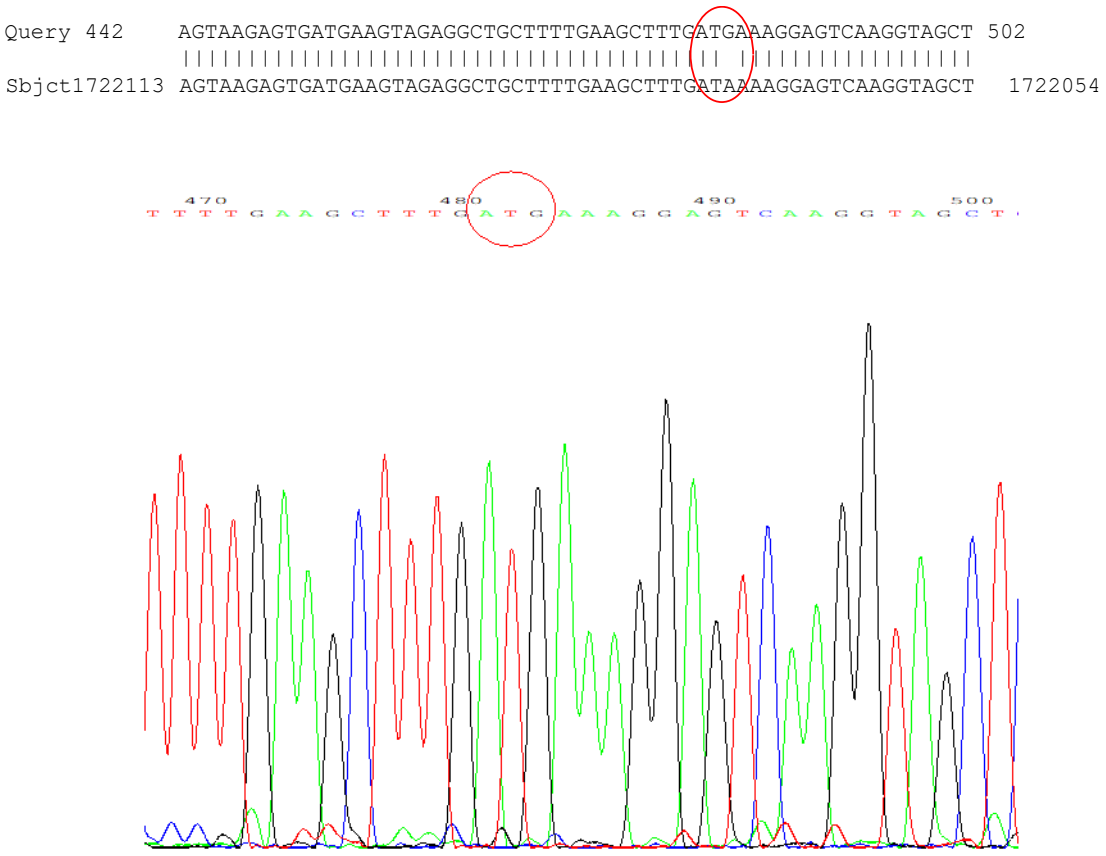

Figure 3-16: DNA sequencing of constructed *ply* allele 9.
DNA sequencing illustrates the successful replacement of isoleucine (ATA) into methionine (ATG) at position 276 amino acid sequences of *ply* D39 to generate *ply* allele 9.

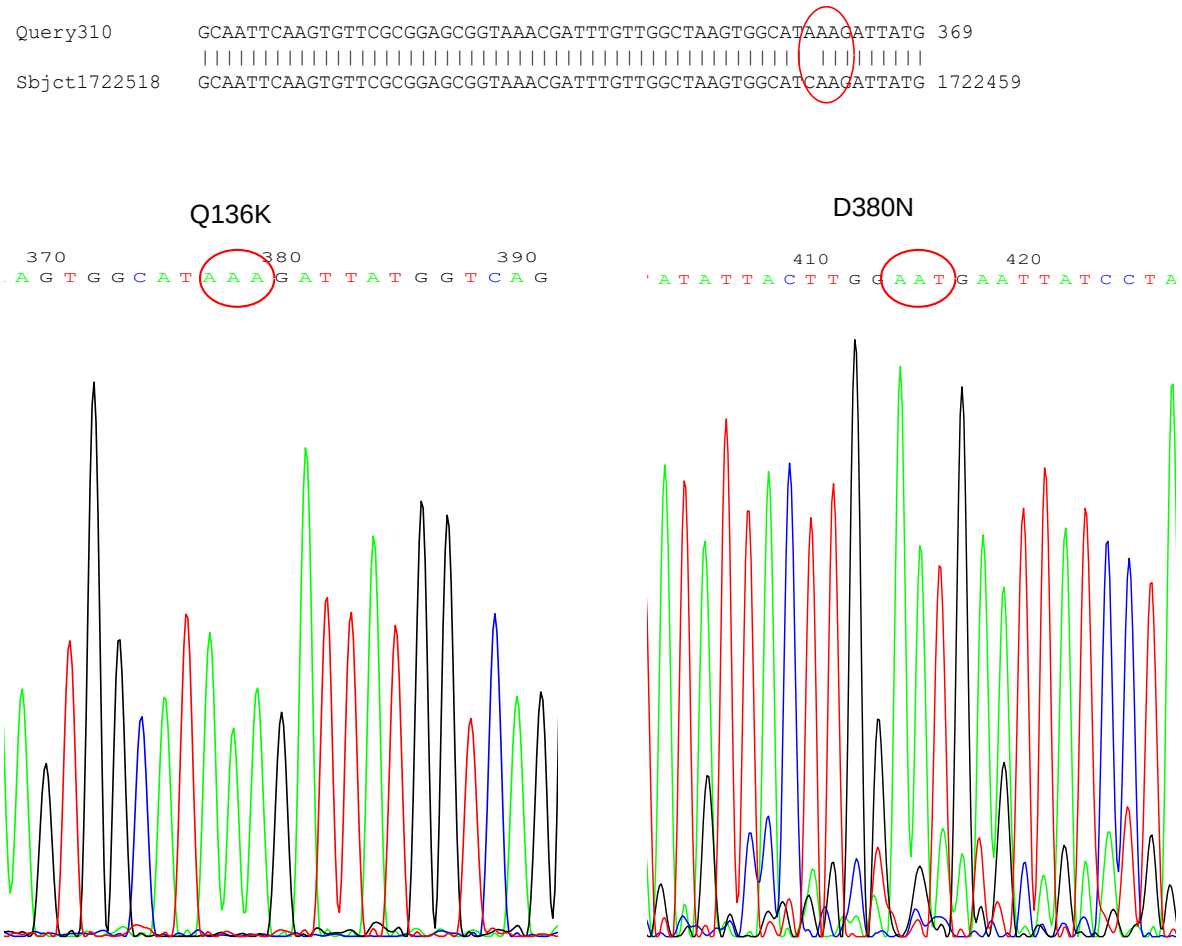

Figure 3-17: DNA sequencing of constructed *ply* allele 11.
DNA sequencing illustrates the successful replacement of glutamine (CAA) into lysine (AAA) and aspartic acid (GAT) into asparagine (AAT) at positions 136 and 380 amino acid sequences of *ply* D39 respectively to generate *ply* allele 11.

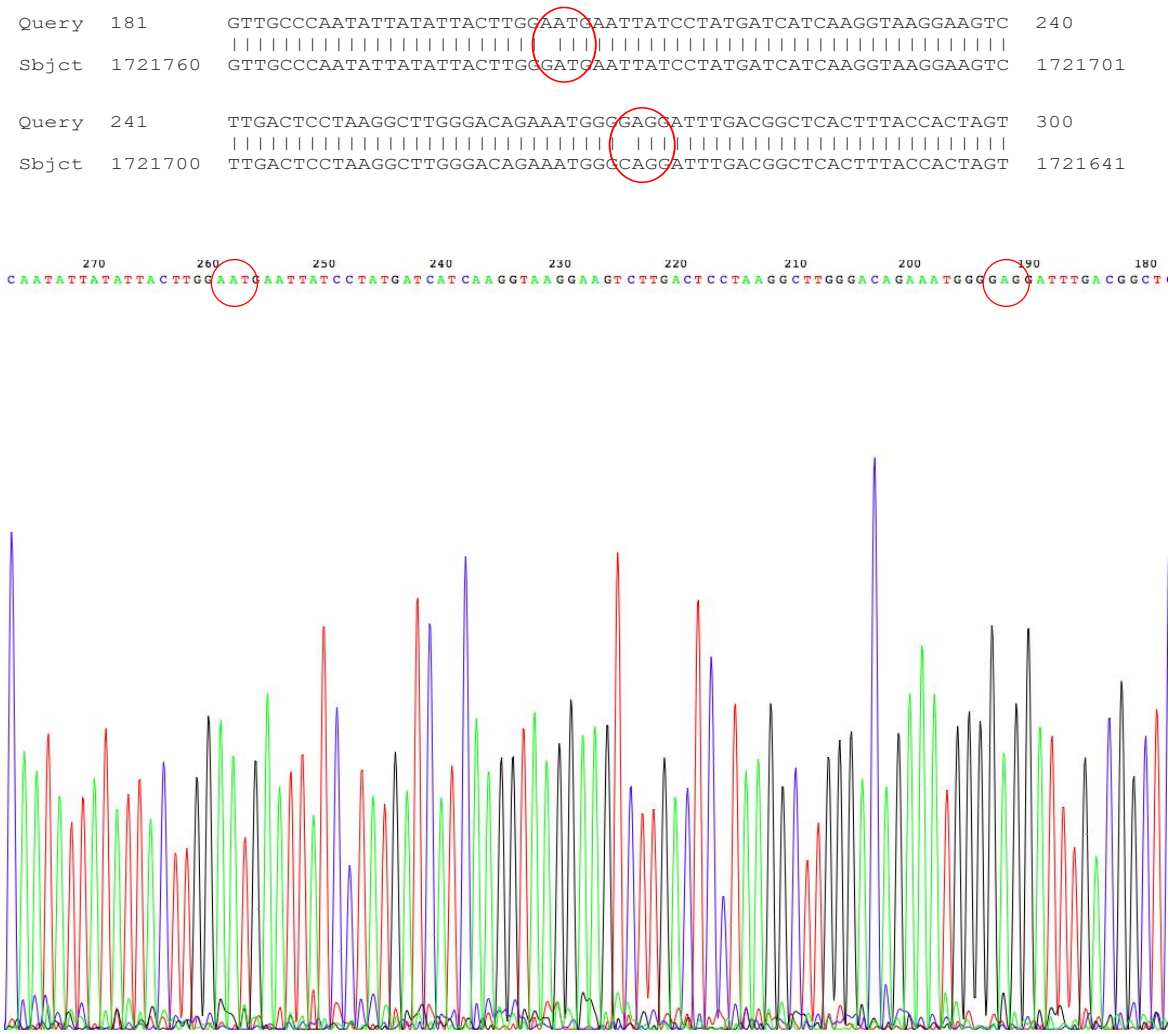

Figure 3-18: DNA sequencing of constructed *ply* allele 12.
DNA sequencing illustrates the successful replacement of glutamine (CAG) into glutamic acid (GAG) and aspartic acid (GAT) into asparagine (AAT) at positions 402 and 380 of amino acid sequences of *ply* D39 to generate *ply* allele 12.

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Query 319       GAGTAAGAGTGATGAAGTAGATGCTGCTTTTGAAGCTTTGATAAAAGGAGTCAAGGTAGC   378
                |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct 1722114   GAGTAAGAGTGATGAAGTAGAGGCTGCTTTTGAAGCTTTGATAAAAGGAGTCAAGGTAGC   1722055
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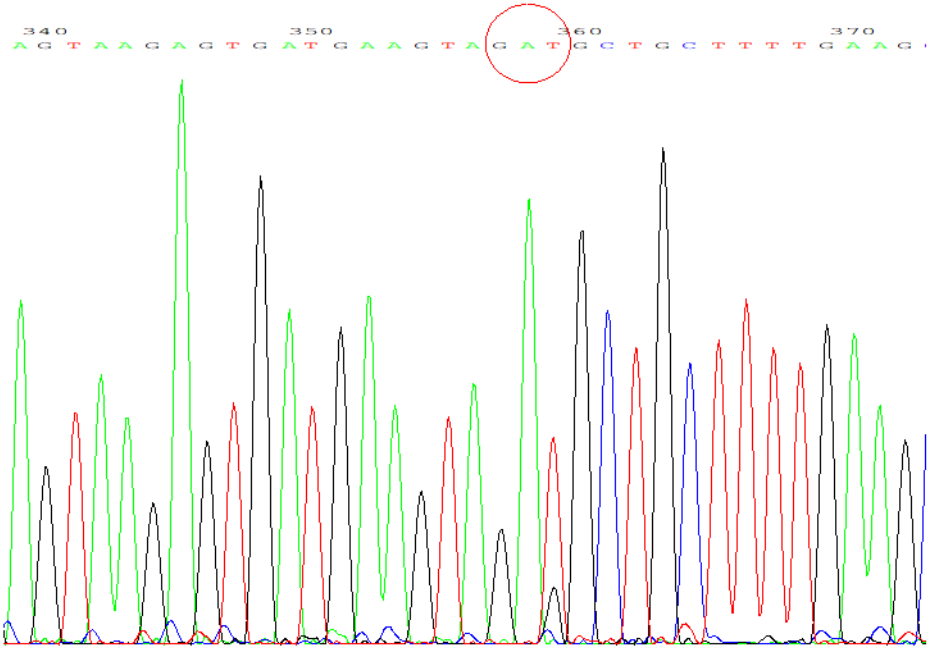


Figure 3-19: DNA sequencing of constructed *ply* allele 15.
DNA sequencing illustrates the successful replacement of glutamic acid (GAG) into aspartic acid (GAT) at positions 260 of amino acid sequences of *ply* D39 to generate *ply* allele 15.

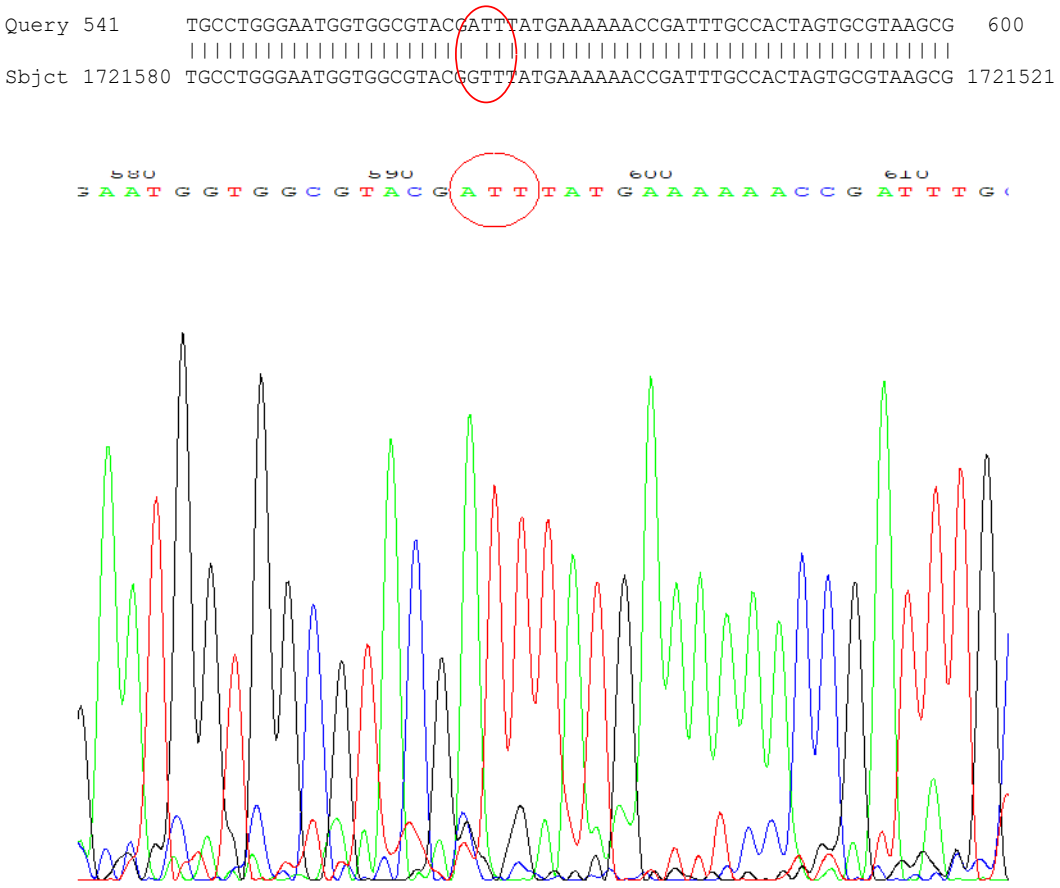


Figure 3-20: DNA sequencing of constructed *ply* allele 18.
DNA sequencing illustrates the successful replacement of valine (GTT) into isoleucine (ATT) at positions 439 amino acid sequences of *ply* D39 to generate *ply* allele 18.

3.6 Determination of Ply allele concentration by ELISA

The concentration of Ply variants in the pneumococcal cell lysate was determined using an ELISA assay. An aliquot of 2.5 µg/ml of pneumococcal cell lysate was coated in the Nunc Maxisorp plate, and then the Mouse anti-Ply antibody (monoclonal anti Ply-7) (Abcam) was applied to detect the Ply allele concentration. Pure Ply (kindly provided from Prof Russell Wallis, University of Leicester) from 2000 pg /ml to 31.25 pg/ml was used to provide a standard curve. Figure 3-21 shows the ELISA assay used to determine the Ply variants concentration in cell lysate using monoclonal mouse anti-Ply-7 antibody and anti-mouse IgG conjugated with alkaline phosphatase. The data shows the Δ*ply* D39 was significantly lower than the Ply D39 ($p<0.0001$), the concentration of alleles 2 and allele 11 were significantly lower compared with Ply D39 ($p<0.0003$). Moreover, no significant difference was seen in the Ply concentration of alleles 15 and 18. In Table 2-1 the codon usage table shows the frequency of occurrence codons of wild type Ply and the Ply variants 2, 8, 9, 11, 12, 15 and 18.

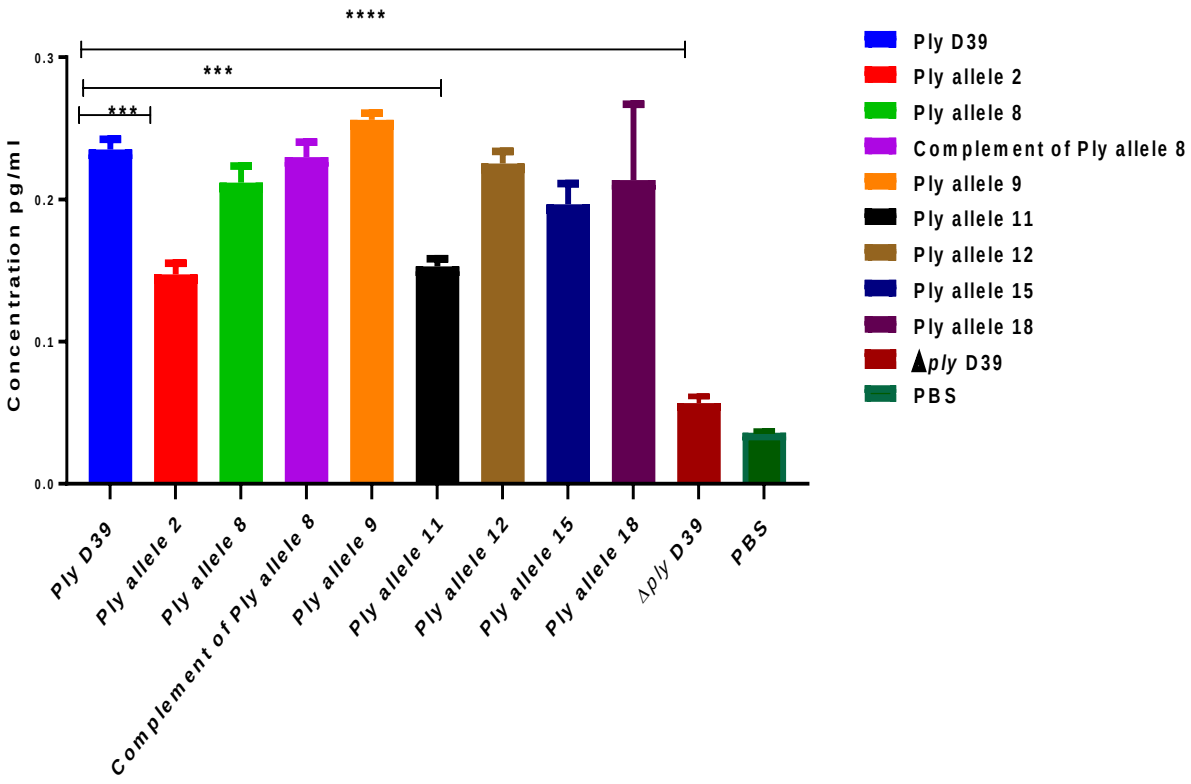


Figure 3-21: ELISA assay was used to determine the Ply variant proteins concentrations in the cell lysate.
Ply variant proteins concentrations in the cell lysate were determined using monoclonal mouse anti-Ply-7 antibody and anti-mouse IgG conjugated with alkaline phosphatase. The adsorbance was determined at 405 nm. Data was average of three independent experminets, one-way ANOVA, Dunnett's multiple comparisons test was done using GraphPad Prism 7 (*** P <0.0003 and **** P<0.0001).

Table 3-1: Codon usage table of *S. pneumoniae* D39 shows the frequency of occurrence codons of wild type Ply and the Ply variants 2, 8, 9, 11, 12, 15 and 18.

Ply allele	D39 WT Ply	Expression level	Ply variant	Expression level
Ply 2 (D380D)	GAT	37.1	AAT	29.5
Ply 8 (A273D)	GCT	30.5	GAT	37.1
Ply 9 (I276M)	AAT	7.0	ATG	23.9
Ply 11 (Q136K & D380D)	CAA GAT	26.7 37.1	AAA AAT	43.3 29.5
Ply 12 (D380D & Q402E)	GAT CAG	37.1 13.6	AAT GAC	29.5 21,0
Ply 15 (E260D)	GAG	21.0	GAT	37.1
Ply 18 (V439I)	GTT	27.8	ATT	40.1

3.7 Haemolytic activity of Ply alleles in pneumococcal crude extract

The haemolytic activitie of each Ply allele was determined using 2% v/v sheep blood RBCs, and compared with lysis activity of D39 Ply (Reference strain, 100%). The results showed reduced lysis by all alleles, except allele 9, shown in Figure 3-22.The EC50 of Ply alleles was determined using GraphPad Prism 7 and the results of EC50 and the specific haemolytic activity of each Ply variant are given in Table 3-2. The specific haemolytic activity of Ply allele 9 was (3.01 x 10^7 HU/mg), it is more than lytic activity of Ply D39 (2.38 x10^7 HU/mg), while the Ply alleles, 2, 8, 11, 12, 15 and 18 were reduced the specific lysis activity (1.80 x 10^7 HU/mg, 1.70 x 10^7 HU/mg , 1.96 x 10^7 HU/mg, 2.13 x 10^7 HU/mg, 1.50 x 10^7 HU/mg and 7.75 x 10^6HU/mg respectively) compared with specific lytic activity of Ply D39. Pure Ply D39 was used as positive control, whereas the Δ*ply* D39 was used as negative control. The natural Ply variants, which constructed in *ply* D39, have a significant impact on Ply lysis activity. Allele 12 has mutant right in Trp-rich loop predicated cholesterol binding region.

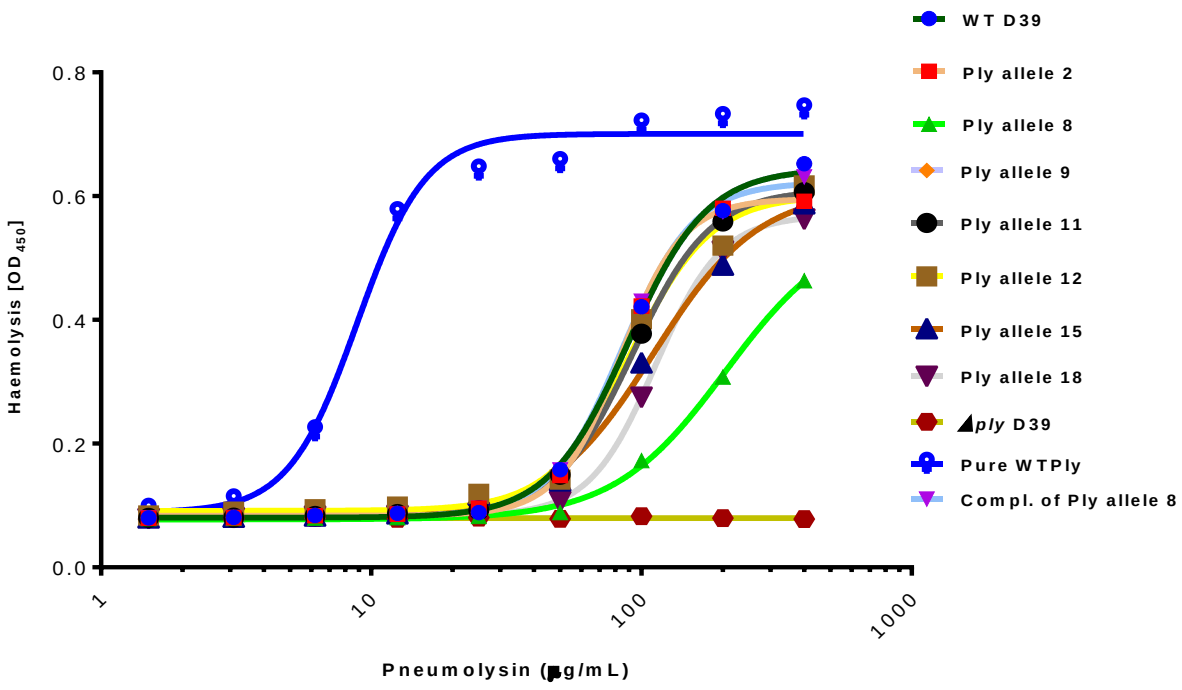


Figure 3-22: Haemolytic activity of Ply variants in pneumococcal crude extract. Haemolytic assay showing the lysis activity of constructed Ply allales in D39. The cell lysate of each Ply allele with 2% v/v sheep blood RBCs were used to determine the haemolytic activiity of each allele. Pure Ply D39 was used as positive control, whereas Δ*ply* D39 as negivte control. The absorbance of lysis RBCs was measured at 450 nM. The data was avarage of four indepentent experiments, and the EC50 was determined using GraphPad Prism 7.

Table 3-2: Illustrate EC50 and the specific haemolytic activivty of each allele/mg of protein. The EC50 of Ply alleles were calculated using GraphPad Prism 7.

Allele Ply	**EC50 (µg/ml)**	**Specific haemolytic activity/mg protein**	**Haemolytic activity (% of D39 Ply)**
D39 Ply	83.49	2.38×10^7	100%
Allele 2	110.9	1.80×10^7	75%
Allele 8	204.6	1.70×10^7	22.3%
Complement of allele 8	84.73	2.31×10^7	100%
Allele 9	90.5	3.01×10^7	126%
Allele 11	89.1	1.96×10^6	85.2%
Allele 12	91.45	2.13×10^7	89%
Allele 15	92.92	1.50×10^6	63%
Allele 18	112.6	7.75×10^6	32%
Δ*ply* D39	Not detectable	0	0%

3.8 Inhibition of Ply haemolysis with cholesterol

To determine the haemolytic activity of cell lysate Ply alleles after incubation with 2-fold serial dilutions of 20mM cholesterol and 2% v/v sheep blood RBCs. The result was showed that cholesterol inhibits the Ply alleles in deferent degrees. Figure 3-23 shows the ability of Ply alleles to lyse RBCs after cholesterol. 50% lysis inhibition (IC50) of each Ply allele due to cholesterol was calculated using GraphPad Prism 7. The IC50 of Ply alleles 2, 8, 9, 11, 12, 15, 18 and D39 Ply are (3.848, 6.696, 0.0005, 10.26, 12.38, 6.974, 7.215 and 2.691 mM respectively).

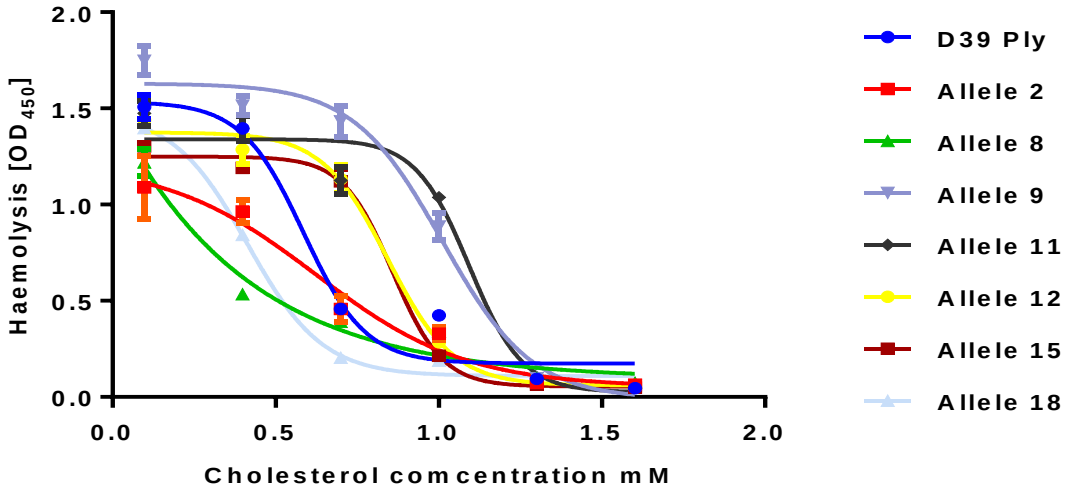


Figure 3-23: Inhibition of Ply haemolysis with cholesterol.
Inhibition of haemolytic activity of Ply alleles by cholesterol. Graph illustrates the haemolytic activity of each Ply alleles after incubation with 2-fold serial dilution of cholesterol. The IC50 of Ply allele 2, 8, 9, 11, 12, 15, 18 and D39 Ply are (3.848, 6.696, 0.0005, 10.26, 12.38, 6.974, 7.215 and 2.691 mM respectively). IC50 was determined using GraphPad Prism 7.

3.9 Growth of *Streptococcus pneumoniae* in BHI

Following insertion of the constructed *ply* alleles into D39 background, growth of pneumococcal D39 was studied in BHI to find out whether the constructed *ply* alleles affected growth (Figure 3-24). The OD600 was determined hourly over 24 hours period by using Multiskan^{TM} GO Microplate Spectrophotometer (Thermo Scientific, UK). The data shows no significant differences were seen between the growths of D39 expressing Ply alleles 2, 8, 9, 11, 12 15, 18, complement Ply 8 or Ply of wild type D39.

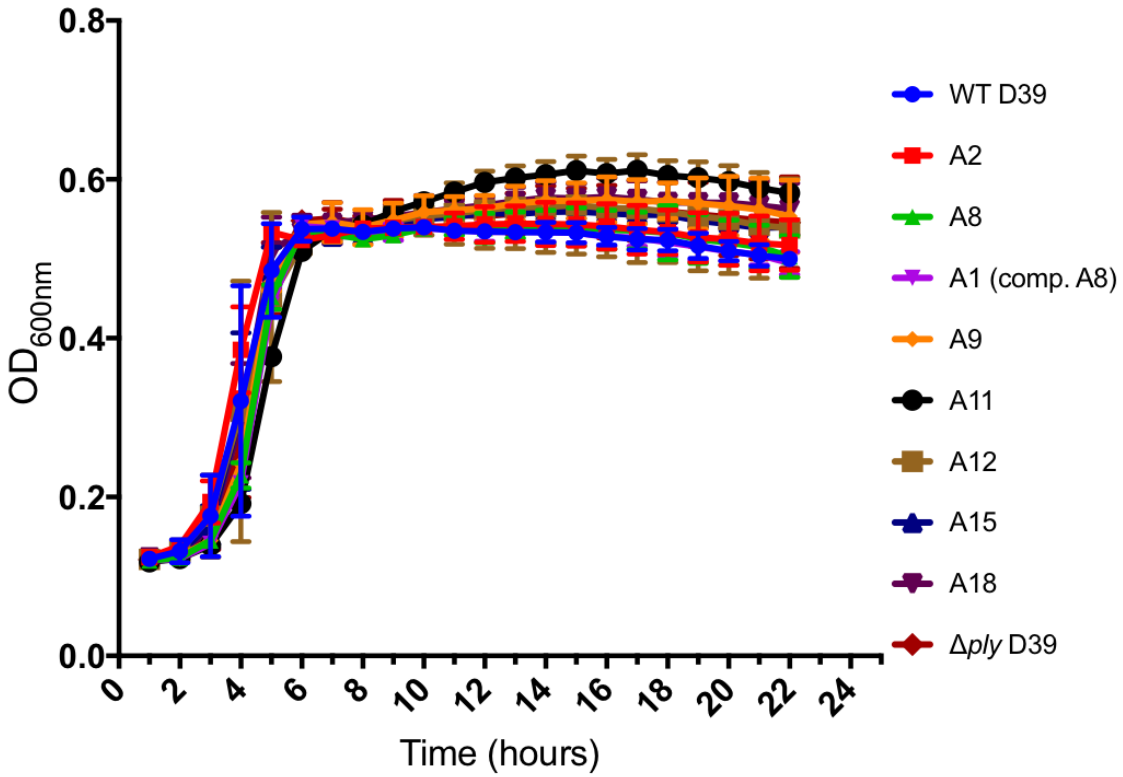
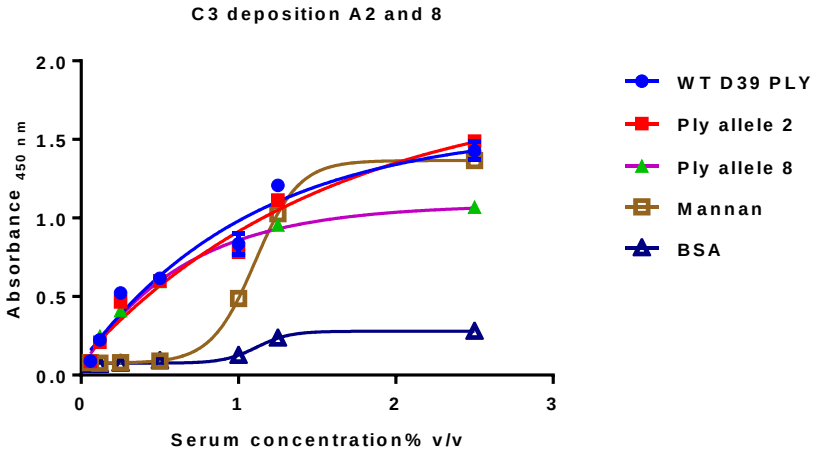


Figure 3-24: Growth curve in BHI of D39 expressing Ply variations.
No significant differences were observed when comparing the growth of D39 containing Ply alleles with the wild type D39. Data was avarage of three independent experiments, One-way ANOVA, Dunnett's multiple comparisons test was done using GraphPad Prism 7 (Dako).

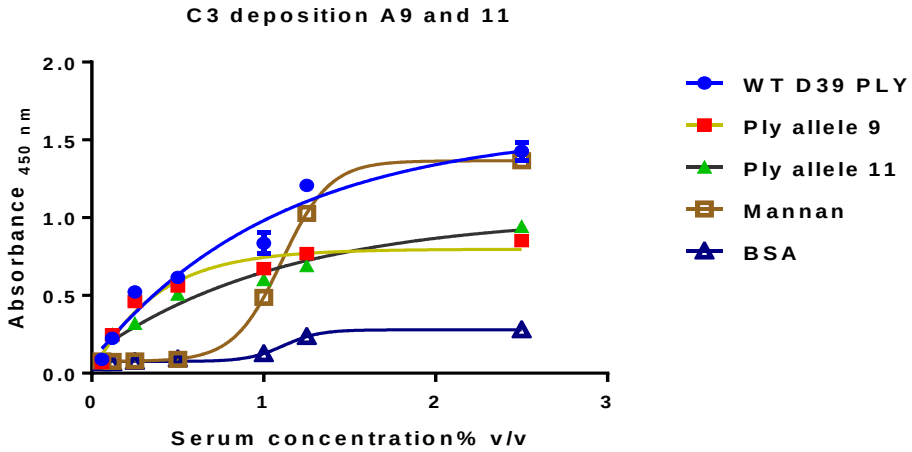
3.10 C3 deposition assay

C3 accumulation assay was achieved to measure the ability of Ply alleles in the pneumococcal cell lysates for activation of C3 deposition on the surfaces of the cell lysate. Pneumococcal cell lysate was incubated with 2% (v/v) normal human serum (NHS) and then rabbit anti-human C3c (Dako) was used to detect the C3 deposition. Figure 3-25 A, B, C and D showing the results of C3 deposition assays for Ply alleles 2, 8, 9, 11, 12, 15 and 18 respectively. Mannan and PBS were used as positive and negative controls respectively. No significant difference was observed when comparing the C3 deposition of Ply alleles to the wild type. Furthermore, Ply alleles 9 and 11 were lower in C3 deposition but not significantly compared with wild type D39 ($p>0.05$).

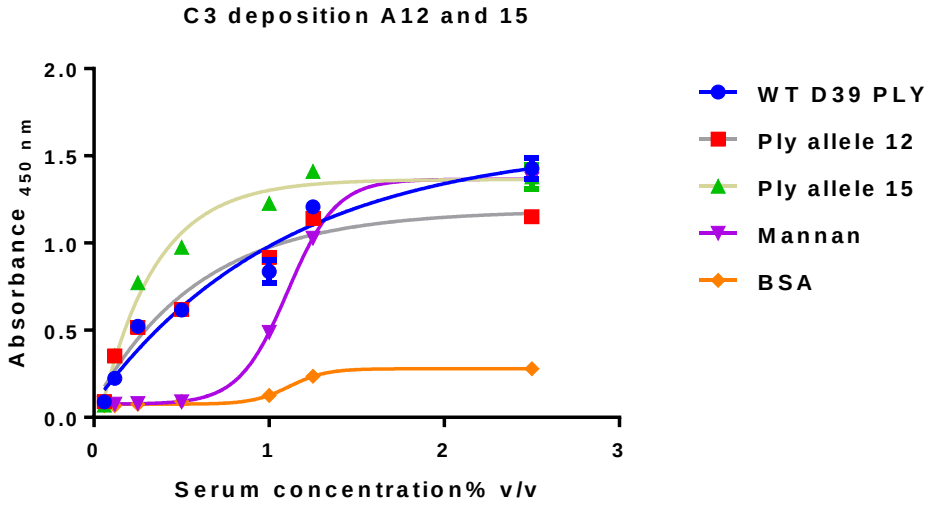
A



B



C



D

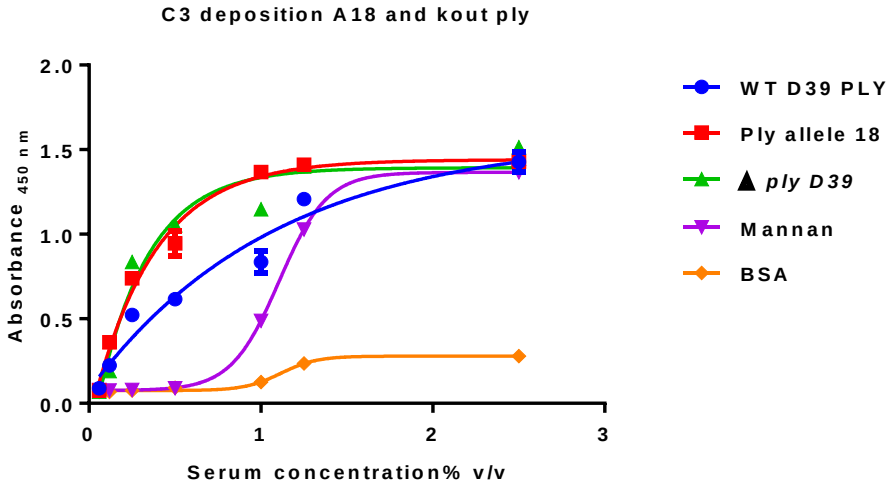


Figure 3-25: C3 deposition assays showing the ability of Ply alleles in the pneumococcal cell lysates for activation of C3 deposition.
(A, B, C and D) C3 deposition assays showing the ability of Ply alleles in the pneumococcal cell lysates for activation of C3 deposition. Following incubation the pneumococcal cell lysates with 2% normal human serum C3 accumulation was detected by using rabbit anti-humanC3c (Dako). Only Ply alleles 9 and 11 were lower but not significantly compared with wild type D39 ($p>0.05$).

Section C

3.11 Expression and purification of Ply alleles

The recombinant pLEICE-01 plasmid containing mutated *ply* gene was transformed into *E. coli* BL21 (DE3), the Ply experssion was induced with 1mM IPTG. Following that Ply purification was perforemd by two steps; firstly using affinity chromatography on a 1 ml nickel-Sepharose column to remove most of impurities. The fractions containing Ply were identified by running on a 14% SDS-PAGE and staining with Coomassie blue. Figure 3-26 SDS-PAGE (A,B and C) showing the Ply8, Ply Q402A and Ply Q402D fractions after affinity chromatography purification on the nickel-Sepharose column, lanes 1-5 in Figure A shows the fractions of Ply8. Lanes 1-4 and 5-8 in Figure B and C representing the fractions of Ply Q402A and Ply Q402D respectively. Figure 3-27 SDS-PAGE, (C lanes 1-3 and D lanes 1-4) showing the fractions of Ply V439A and Ply allele 12 respectively following affinity chromatography purification on the nickel-Sepharose column. Whereas Figure 3-28 SDS-PAGE, (E lanes 1-5 and F lanes 1-5) shows the fractions of Ply allele 15 and Ply allele 18 respectively following affinity chromatography purification on the nickel-Sepharose column. Following chromatography purification the purified Ply alleles were analysed on SDS gels PAGE as a single band with a molecular mass approximately 53 kDa. Result of SDS gel analysis shows a single band for the Ply alleles, while lanes 1-7 represent the single band of Ply alleles 8, 12, 15, 18, Q402A, Q402D and V439A respectively (Figure 3-29).

In the second step the gel filtration on a Superdex 200 16/60 column was used to remove the non-Ply impurities. Following the affinity chromatography the fractions containing each Ply allele were pooled and then loaded onto a Superdex 200 16/60 column. Ply alleles were eluted with 20 mM Tris-HCl pH 7.5 as a single band with a molecular mass of 53 kDa, based on the elution of known standards, showing the Ply is monomeric in solution (Gilbert et al., 1998, Solovyova et al., 2004). The chromatography elution profile of Ply alleles 8, 12, 15, 18, Q402A, Q402D and V439A were given respectively in the following Figure 3-30, Figure 3-31, Figure 3-34, Figure 3-35, Figure 3-32, Figure 3-33 and Figure 3-36. Following gel filtration the fractions containing Ply were pooled then concentrated using high recovery centrifugal filters (Tullagreen, Ireland). The purified Ply alleles were divided into 50 µl aliquots and snap frozen in liquid nitrogen and stored at -80°C.

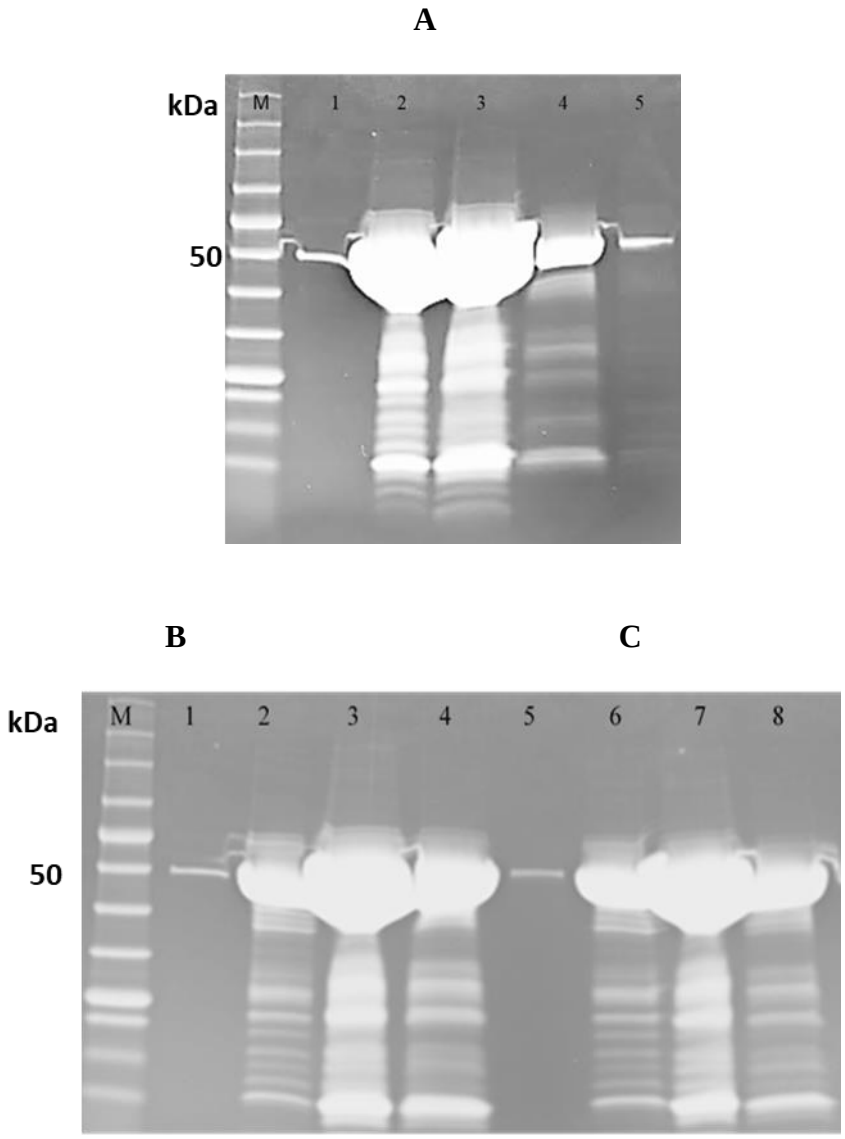


Figure 3-26: SDS-PAGE of Ply8, Ply Q402A and Ply Q402D fractions after affinity chromatography purification.
SDS-PAGE (A,B and C) showing the Ply8, Ply Q402A and Ply Q402D fractions after affinity chromatography purification on the nickel-Sepharose column, lanes 1-5 in Figure A shows the fractions of Ply8. Lanes 1-4 and 5-8 in Figure B and C representing the fractions of Ply Q402A and Ply Q402D respectively. M represent the marker colour Prestained Protein Standard, Broad Range (New England, Biolabs, UK).

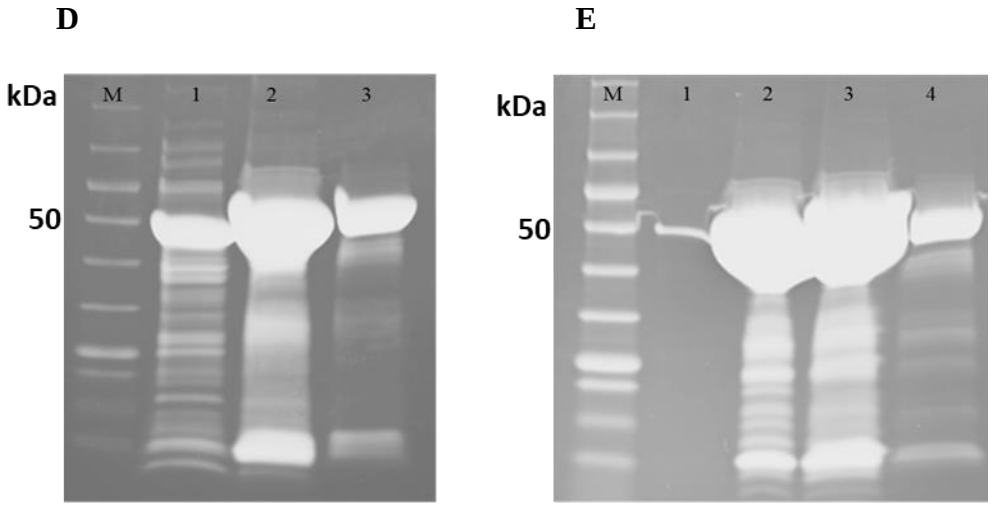


Figure 3-27: SDS-PAGE of Ply V439A and Ply allele 12 fractions after affinity chromatography purification.
SDS-PAGE, (C lanes 1-3 and D lanes 1-4) showing the fractions of Ply V439A and Ply allele 12 respectively following affinity chromatography purification on the nickel-Sepharose column. M represent the marker colour Prestained Protein Standard, Broad Range (New England, Biolabs, UK).

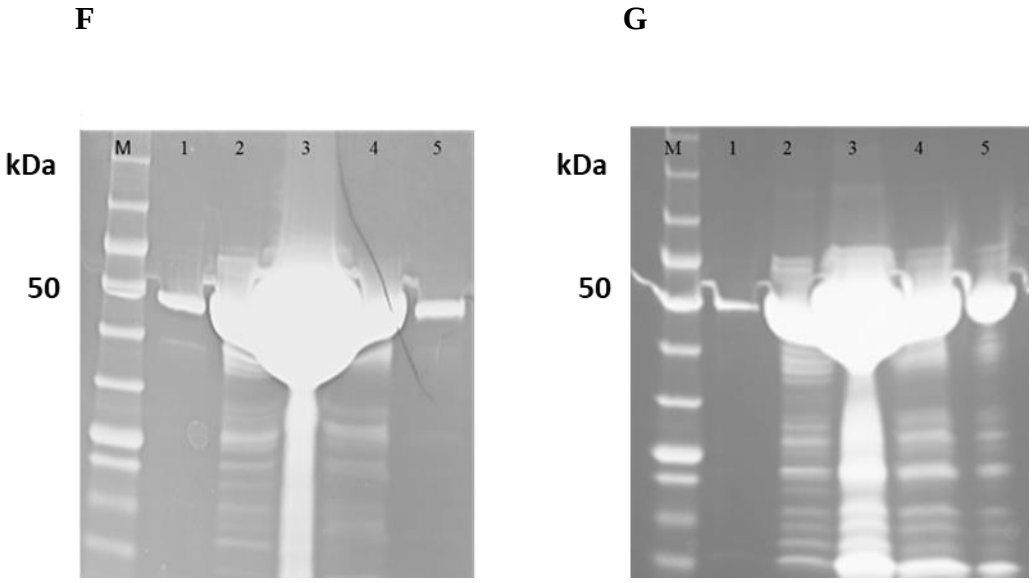


Figure 3-28: SDS-PAGE of Ply allele 15 and Ply allele 18 fractions after affinity chromatography purification.
SDS-PAGE, (E lanes 1-5 and F lanes 1-5) showing the fractions of Ply allele 15 and Ply allele 18 respectively following affinity chromatography purification on the nickel-Sepharose column. M represent the marker colour Prestained Protein Standard, Broad Range (New England, Biolabs, UK).

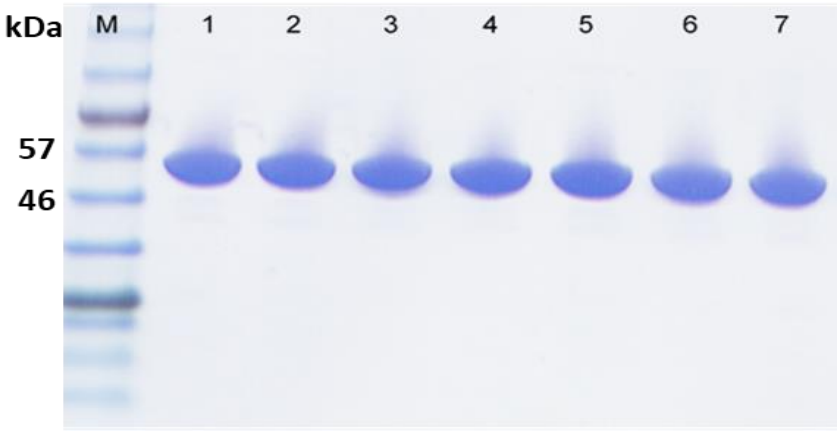


Figure 3-29: SDS-PAGE showing a single band each the beak of Ply proteins after affinity chromatography purification.
Lanes 1-7 represent the Ply alleles 8, 12, 15, 18, Q402A, Q402D and V439A respectively. The SDS gels were stained with Coomassie blue. M represents the marker colour Prestained Protein Standard, Broad Range (New England, Biolabs, UK).

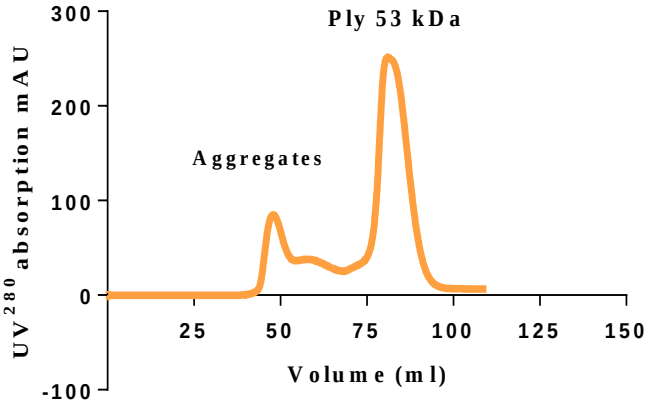


Figure 3-30: Chromatography showing the elution profile of Ply alleles 8 from the Superdex 200 16/60 column.

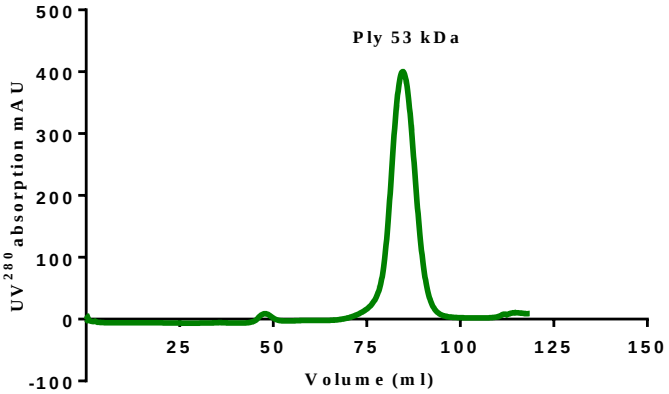


Figure 3-31: **Chromatography showing the elution profile of Ply alleles 12 from the Superdex 200 16/60 column.**

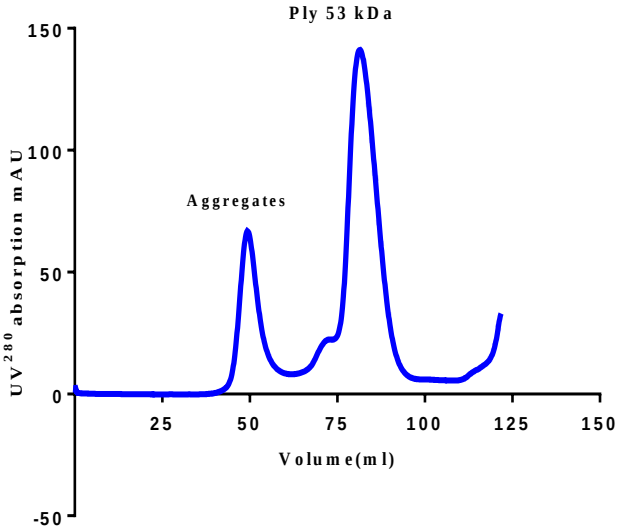


Figure 3-32: Chromatography showing the elution profile of Ply alleles 15 from the Superdex 200 16/60 column.

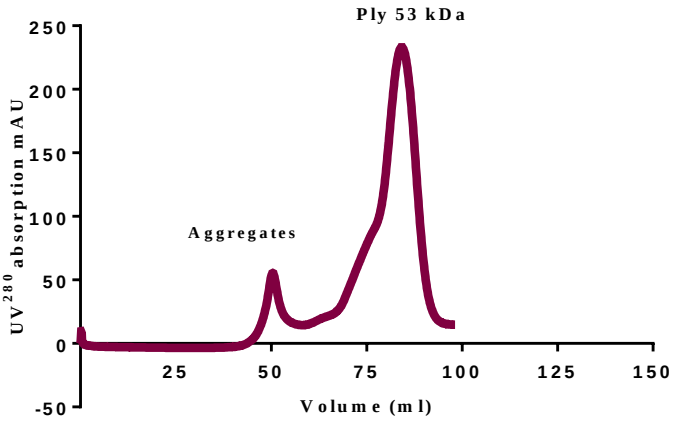


Figure 3-33: Chromatography showing the elution profile of Ply alleles 18 from the Superdex 200 16/60 column.

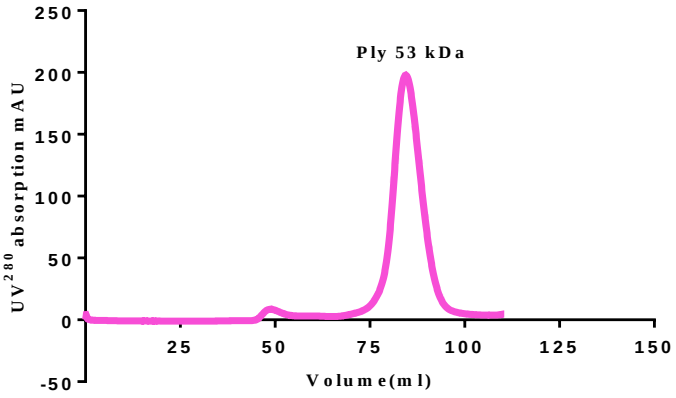


Figure 3-34: Chromatography showing the elution profile of Ply Q402A from the Superdex 200 16/60 column.

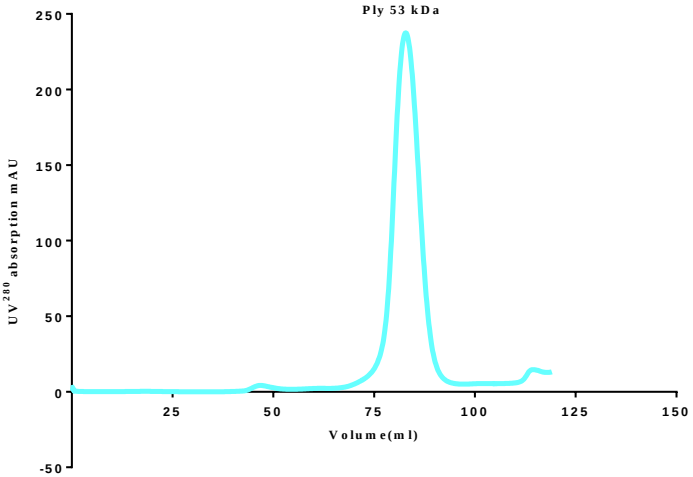


Figure 3-35: Chromatography showing the elution profile of Ply Q402D from the Superdex 200 16/60 column.

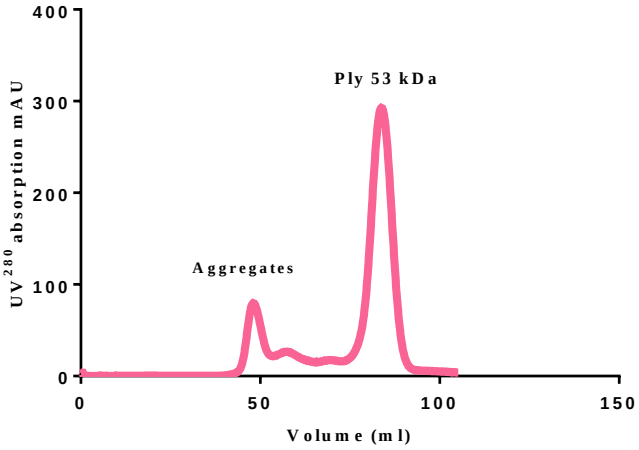


Figure 3-36: Chromatography showing the elution profile of PlyV439A from the Superdex 200 16/60 column.

3.12 Haemolytic activity of pure Ply alleles

Following the purification of Ply encoded by *ply* alleles 8, 12, 15, 18, Q402A, Q402D and V439A by affinity chromatography and gel filtration, and the lytic activity of each Ply allele was tested using 2% v/v sheep blood RBCs. The haemolytic activitie of each pure Ply alleles were compared with lysis activity of D39 Ply (Reference strain, 100%) using 2% sheep blood RBCs. The EC50 of pure Ply alleles were determined using GraphPad Prism 7 and the result was illustrated in. The results were showed reducing the haemoglobin lysis in Ply alleles 15, 8, and 12 and the specific haemolytic activity are (8×10^4, 1.6×10^5 and 3.2×10^5 respectively), and the percentage of the haemolytic activity for each allele compared with haemolytic activity of D39 Ply were explained in Table 3-3

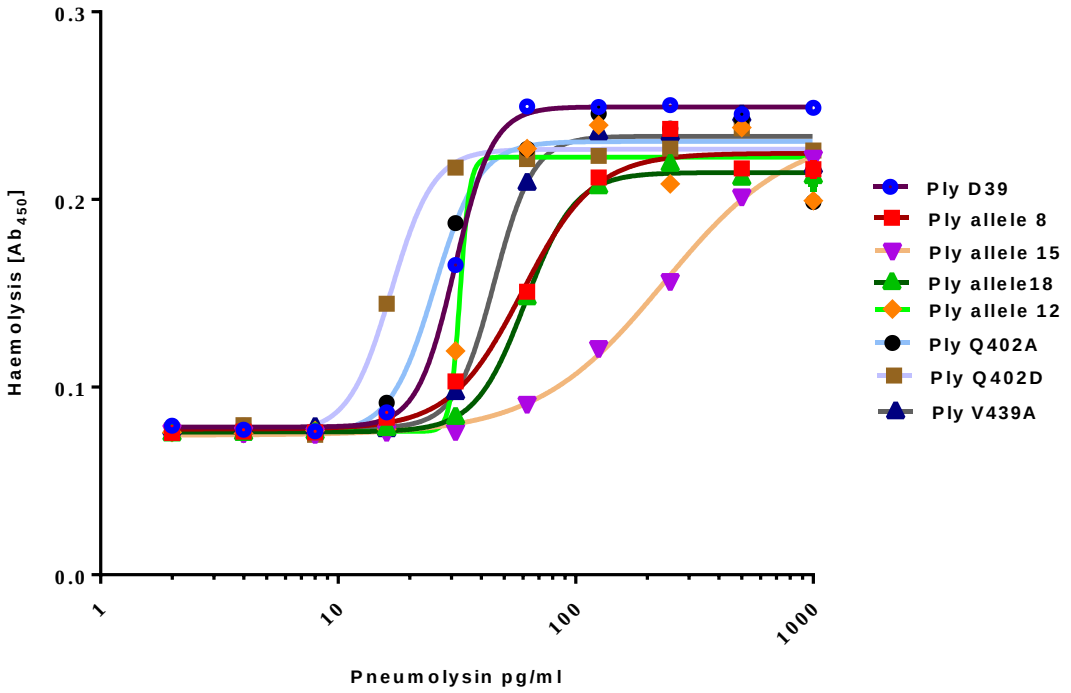


Figure 3-37: Haemolytic activity of purified Ply alleles.
The lytic activity of purified Ply alleles were tested using 2% v/v sheep blood RBCs, and compared with lysis activity of D39 Ply (Reference strain, 100%). The absorbance of lysis RBCs was measured at 450 nM. The data was average of three independent experiments. The EC50 of Ply alleles and D39 Ply were determined using GraphPad Prism 7.

Table 3-3: Illustrate the specific haemolytic activity of pure Ply alleles and the reference strain (D39 Ply), and the percentage of this activity of each pure Ply allele, as well as the 50% of haemolytic activity of D39 Ply and Ply alleles, which are calculated using GraphPad Prism 7.

Pure Ply	EC50 (µg/ml)	H.A.% pure Ply	Specific haemolytic activity/mg Ply
D39 Ply	31.02	100%	6.4×10^5
Allele 8	59.84	25%	1.6×10^5
Allele 12	32.59	51%	3.2×10^5
Allele 15	232.6	12.5%	8.0×10^4
Allele 18	61.39	100%	6.4×10^5
Q402A	25.45	100%	6.4×10^5
Q402D	16.72	187%	1.2×10^6
V439A	45.44	100%	6.4×10^5

3.13 Western blotting

Following changing the glutamine (Q) at the position of 402 of Ply amino acid sequence into alanine (A) or aspartic acid (D), as well as changing the valine (V) at position 439 to alanine (A). The binding of anti-Ply 7 with constructed Ply alleles was tested by using a western blot assay. The western blotting assay shows that the constructed Ply alleles were recognised by the anti-Ply 7 and single band of each Ply allele was detected. In the Figure 3-38, Lanes 1-5 represent the Ply alleles 12, Q402A, Q402D, V439A and wild type Ply. The result showed thick bands of alleles Q402A, Q402D and V439A looking approximately same the band of Ply D39, but recognition by the antibodies of allele 12 is looking reduced compare to wild type Ply D39, seems poorly detected by Anti-Ply 7 which binds at 400-407 amino acid sequence of Ply. It was mentioned previously by (Jefferies et al. 2007) that allele 12 it has haemolytic activity, but it cannot be detected by the monoclonal anti-Ply 7. In conclusion the amino acid type has impact on the binding of anti-Ply 7 with constructed Ply.

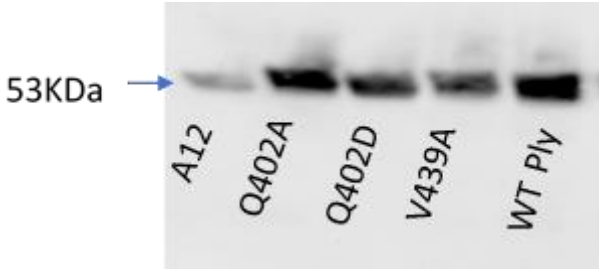


Figure 3-38: Western blot assay of purified Ply variants after affinity chromatography purification.
Western blot assay showing a single band of equal amounts of pure Ply variants after affinity chromatography purification. Lanes 1-5 represent the Ply alleles 12, Q402A, Q402D, V439A and wild type Ply. The Ply size is around 53 kDa.

3.14 C3 deposition on purified Ply

The purified Ply alleles were tested for activation of C3 deposition by incubating the Ply alleles with 2% of normal human serum for one hour at 37°C. The decreasing the amount of residual complement over the time indicates that Ply induces the C3 deposition in the serum, which detected by using rabbit anti-human C3c (Dako). No significant differences were seen when comparing the C3 deposition of pure Ply alleles 8, 18, 12, and 15 to the pure Ply of wild type D39 Figure 3-39.

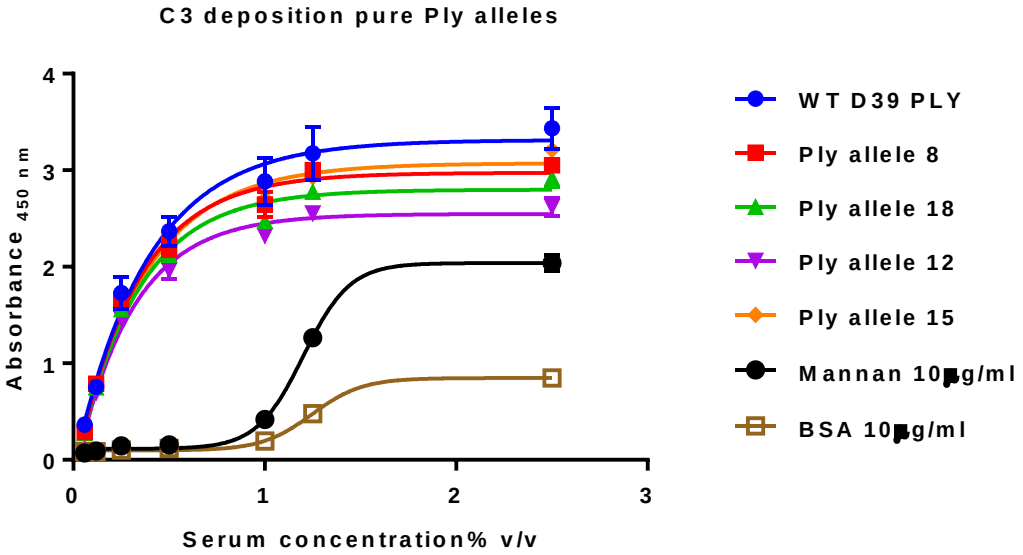


Figure 3-39: C3 deposition assays of purified Ply variants.
C3 deposition assays showing the ability of pure Ply alleles for activation of C3 deposition. Following incubation the Ply alleles with 2% normal human serum C3 accumulation was detected by using rabbit anti-human C3c (Dako). No significant differences were seen when comparing the C3 deposition of pure Ply alleles to the pure Ply of wild type D39 ($p>0.05$).

Section D

3.15 *In vivo* virulence studies

The *in vitro* results of D39 expressing different Ply alleles showed reduced haemolytic activity of Ply alleles 8, 18 and 15 to different degrees, compared to the Ply of wild type D39. The virulence of the D39 recombinants carrying variants of *ply* were tested in a mouse model of pneumococcal pneumonia that develops after intranasal infection.

3.15.1 The impact of Ply variations in D39 on pneumococcal virulence

Mice were infected intranasally with approximately $2x10^6$ CFU/ mouse of D39 expressing Ply alleles 2, 8, 9, 11, 12, 15, 18 or wild type D39. For logistical reasons, the infection experiments were done in two batches. Firstly, the infections were with D39 expressing Ply alleles 8, 15, 18 and wild type D39. In the second set of experiments mice were infected with D39 expressing Ply alleles 2, 9, 11 or 12 and the wild type D39 infection was repeated. The survival times of infected mice with D39 expressing Ply alleles 8, 15, 18 or wild type D39 are shown in Figure 3-40 A and B. At 33 hours post-infection 2 mice of the group infected with wild type D39 died and then 5 mice died at 45 hours post-infection of the same group. The median survival time of mice infected with wild type D39 (42.2 h ± 6.34, n=7). The median survival times of mice infected with D39 expressing Ply allele 8 (144 ± 00.0, n= 9) or 18 (144 ± 00.0 n=10), were significantly ($p<0.0001$) different from the wild type D39 infected group. In contrast, the median survival time of Ply allele 15 infected mice (52 ± 00.0, n= 6), was not significantly different from the wild type D39 ($p>0.05$). Whereas 6 mice out of 10 died by 52 hours post-infection with D39 carrying Ply allele 15. In contrast, all of the mice infected with D39 expressing Ply allele 8 or 18 survived until the end point of experiment (144 h), except one mouse of the group infected with Ply allele 8 died after 77 hours infection.

The survival times of infected mice with D39 expressing Ply alleles 2, 9, 11, 12 or Ply D39 are shown in Figure 3-44 A and B. At 48 hours post-infection 3 mice of the group infected with wild type D39 died and 3 more mice died at 54 hours post-infection of the same group. One mouse of the group infected with D39 expressing Ply allele 9 died at 48 hours post-infection, and then 3 mice of this group died by 78 hours as well as other one died at 100 hours post-infection. All of the mice infected with D39 expressing Ply allele 2, 11 or 12 survived until the end point of experiment (144 h). The median survival time of mice infected with wild type D39

(54 h ± 13.6, n=7). The median survival times of mice infected with D39 expressing Ply allele 2 (144 ± 00.0, n= 10), 11 (144 ± 00.0 n=10) or (144 ± 00.0 n=10), were significantly different from the wild type D39 infected group ($p<0.0001$ for both of them). In contrast, the median survival time of Ply allele 9 infected mice (70 ± 10.4, n= 10), was not significantly different from the wild type D39 ($p>0.05$).

3.15.2 Viable counts of pneumococci in the blood and tissues of infected mice

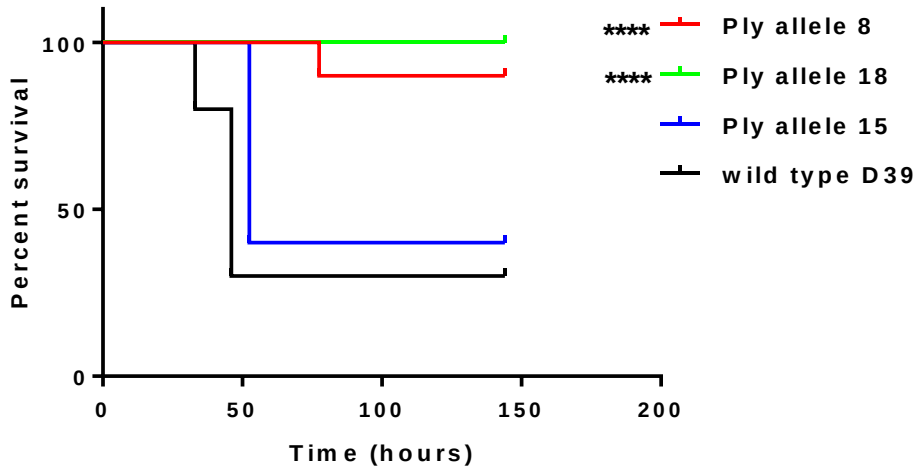
Because of the differences in virulence between wild type Ply and the Ply allele pneumococcus survival of mice infected with D39 containing the Ply variants was determined. To determine the extent of pneumococcal dissemination in the mice infected with D39 expressing different Ply alleles, tail vein blood was collected from each mouse at 24 hours post-infection. Figure 3-41 shows the bacterial load in the Ply alleles 8 and 18 were not detected and significantly ($p<0.0001$) different at 24 hours post-infection compared to wild type D39 Ply (Log_{10} 2.24 ± 0.7 CFU/ml, n=10). However, the bacterial load in the Ply 15 infected group (Log_{10} 1.22 ± 0.6 CFU/ml, n=10) was not significant from wild type D39 ($p>0.05$). Figure 3-45 shows the bacterial load in the mice infected with Ply alleles 2, 11 or 12 were not detected and significantly ($p<0.0001$) different at 24 hours post-infection compared to wild type D39 (Log_{10} 3.32 ± 0.6 CFU/ml, n=10). Moreover, the bacterial load in the Ply 9 infected group (Log_{10} 1.39 ± 0.6 CFU/ml, n=10) was significantly lower than wild type cohort ($p>0.01$).

The pneumococcal growth in the homogenised lung and spleen tissues were determined after lethargic mice were culled, and also for the mice culled at the end of the experiment. The number of colony forming units per mg of lung tissues was determined. Figure 3-42 shows the bacterial numbers in the lungs of mice infected with D39 carrying Ply alleles 8 or 18 were not detected and significantly ($p<0.0001$) different at 24 hours post-infection compared with the colony count of wild type D39 (Log_{10} 3.7 CFU/mg ± 1.0, n=10). No difference was observed in the bacterial load in the mice were infected with D39 expressing Ply allele 15 (Log_{10} 2.7 CFU/mg ± 0.9, n=10) ($p>0.05$). Figure 3-43 illustrates the bacterial load in the lung tissues of mice infected with D39 carrying Ply allele 2, 11 or 12 were also not detected and significantly ($p<0.0001$) different at 24 hours post-infection compared with the colony count of wild type D39 (Log_{10} 3.6 CFU/mg ± 0.9, n=10) and D39 expressing Ply allele 9 (Log_{10} 2.6 CFU/mg ± 0.8, n=10) ($p<0.05$). In contrast, D39 expressing Ply allele 9 (Log_{10} 2.6 CFU/mg ± 0.8, n=10) was not different compared to wild type D39 ($p>0.05$).

The bacterial load in the spleens of mice infected with D39 expressing Ply alleles 2, 8, 9, 11, 12, 15, 18 or wild type of D39 also was determined. Figure 3-43 shows the bacterial numbers in the spleens of D39 expressing Ply allele 8 or 18 were not detected and significantly ($p<0.0001$) different compared with the colony count of wild type D39 (Log_{10} 3.8 CFU/mg ± 0.8, n=10) and Ply allele 15 (Log_{10} 2.1 CFU/mg ± 0.6, n=10). In contrast the bacterial load in the mice infected with Ply allele 15 D39 (Log_{10} 2.1 CFU/mg ± 0.6, n=10) was not significantly lower than the bacterial load in the wild type D39 ($p>0.05$).

Bacterial numbers in the spleens of mice infected with D39 expressing Ply alleles 2, 9, 11, 12 or wild type of D39 also was determined. Figure 3-47 shows the bacterial load in the spleen tissues of D39 expressing Ply allele 2, 11 or 12 also were not detected and significantly ($p<0.0001$) different compared with the colony count of wild type D39 (Log_{10} 3.6 CFU/mg ± 0.9, n=10). In contrast, the bacterial load in the mice cohort infected with Ply allele 9 D39 (Log_{10} 1.8 CFU/mg ± 0.5, n=10) was not significantly different from the bacterial load in the wild type D39 ($p>0.05$).

A



B

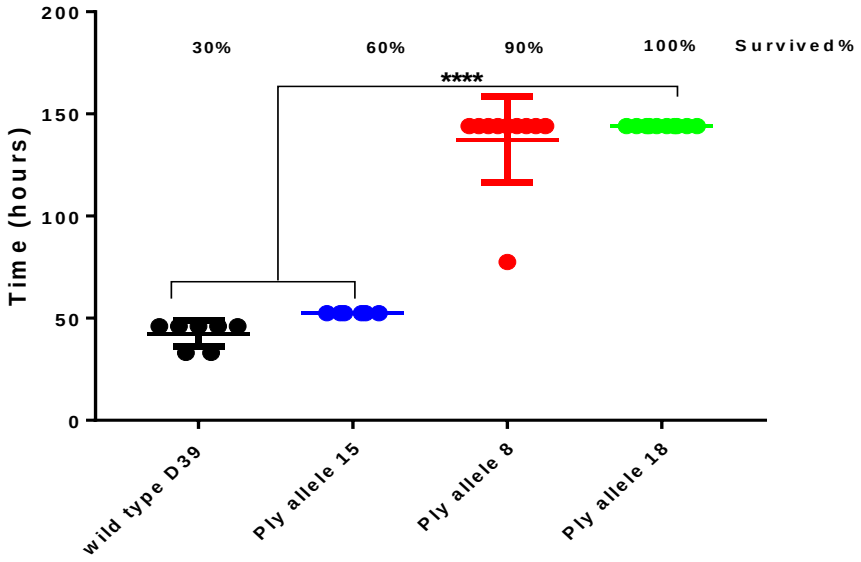


Figure 3-40: Survival times and percent of mice infected intranasally with D39 expressing Ply variants 8, 15, 18 or wild type D39.
(A): Survival times of mice infected intranasally with D39 expressing Ply variants 8, 15, 18 or wild type D39 approximately 2 x 10^6 pneumococci/ mouse in 50 µl PBS. Each line represents 10 mice. **(B):** Percent of mice surviving to the end of the experiment (144h), the horizontal lines represent the median survival time for each group. Each dot represents the survival time of individual mouse. Mice infected with D39 expressing Ply alleles 8 and 18 show significant differences in survival times comparing with wild type D39 (****$p<0.0001$). A Mann Whitney test was used for data analysis.

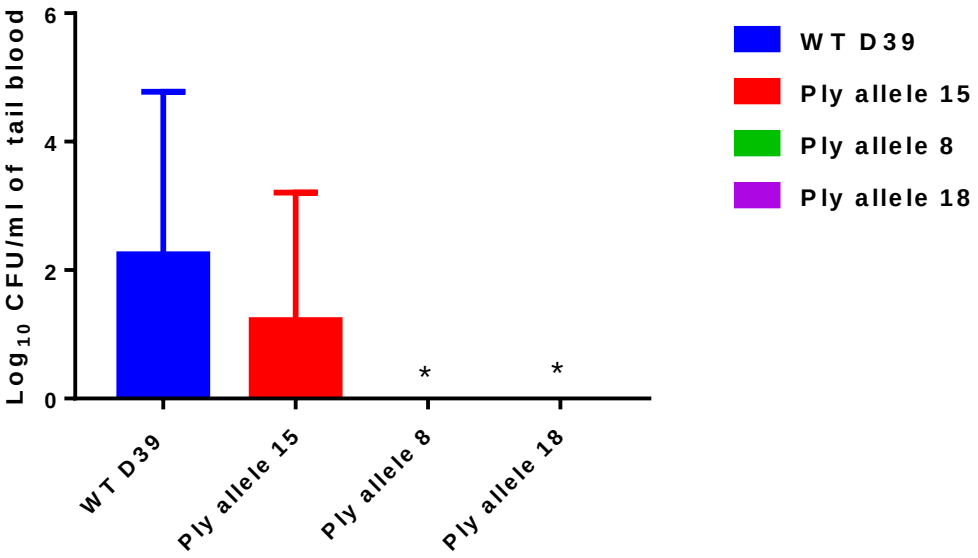


Figure 3-41: Progression of pneumococcal bacteraemia in mice infected with D39 expressing Ply variants 8, 15, 18 or wild type D39.
Progression of pneumococcal bacteraemia in mice infected intranasally with approximately $2x10^6$ CFU/mouse of D39 expressing Ply alleles 8, 15, 18 or wild type D39 at 24 hours post-infection. Each column is the mean of data from ten mice. There were significant differences in bacterial counts of mice infected with D39 expressing Ply allele 8 or 18 compared to wild type D39, using one way ANOVA test (****p<0.0001).

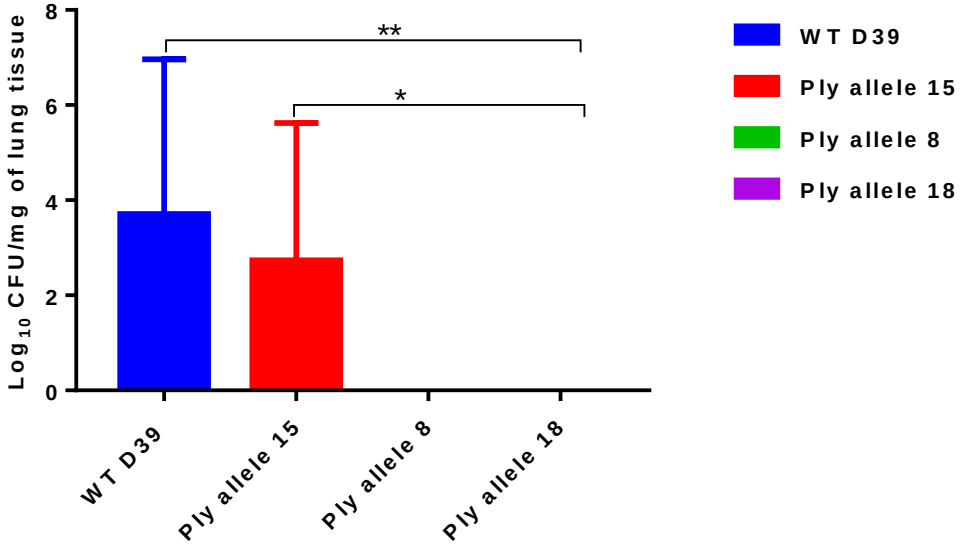


Figure 3-42: Pneumococcal counts in the lung of mice infected with D39 expressing Ply variants 15, 8, 18 or Ply wild type D39.
Pneumococcal counts in the homogenized lung tissues of mice infected with D39 expressing Ply alleles 15, 8, 18 or Ply D39 wild type were determined. Each column represents the mean data of ten mice and the standard error of the mean. Ply alleles 8 and 18 were significantly lower than the counts of wild type D39 using one way ANOVA (*p<0.05, **p<0.01).

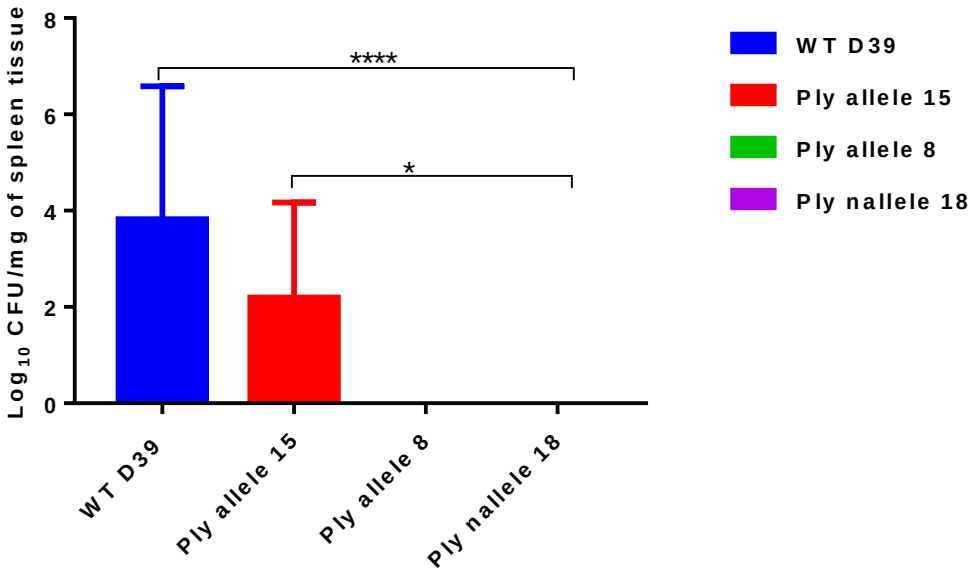
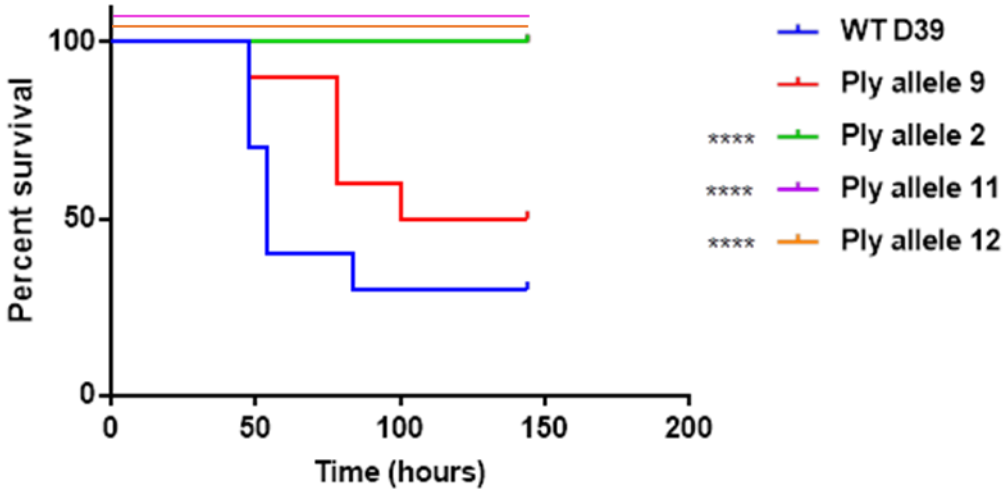


Figure 3-43: Pneumococcal counts in the spleen of mice infected with D39 expressing Ply variants 15, 8, 18 or Ply wild type D39.
Pneumococcal counts in the homogenized spleen tissues of mice infected with D39 expressing Ply variants 15, 8, 18 or Ply D39 wild type were determined. Each column represents the mean data of ten mice and the standard error of mean. Ply alleles 8, 15 and 18 were significantly lower than the counts of wild type D39 using one way ANOVA (*$p<0.05$, ****$p<0.0001$).

A



B

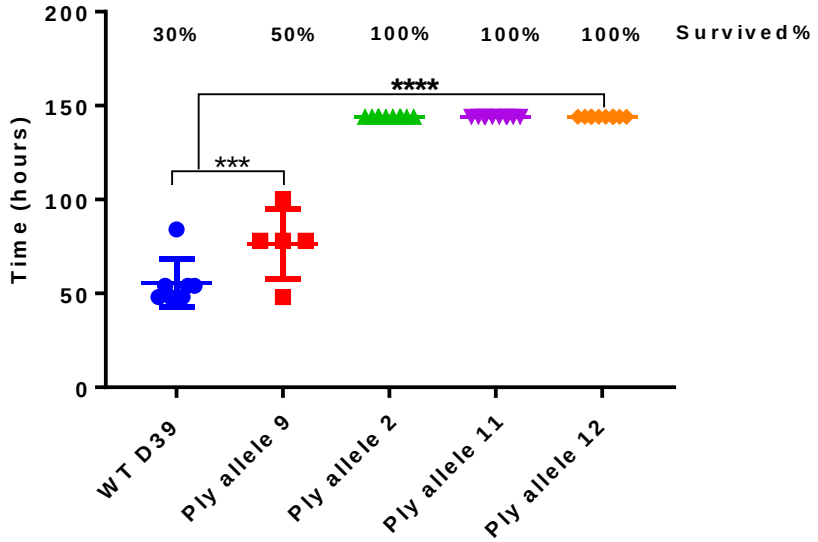


Figure 3-44: Survival times and percent of mice infected intranasally with D39 expressing Ply variants 2, 9, 11, 12 or wild type D39.
(A): Survival times of mice infected intranasally with D39 expressing Ply alleles 2, 9, 11, 12 or wild type D39, approximately 2 x 10^6 pneumococci/ mouse in 50 µl PBS. Each line represents 10 mice. (**B**): Percent of mice surviving to the end of the experiment (144h), the horizontal lines represent the median survival time for each group. Each dot in each group represent the survival time of individual mouse. Mice were infected with D39 expressing Ply variants 2, 11 or 12 show significant differences in survival times comparing with wild type D39. Mann Whitney test was used for data analysis (****p<0.0001).

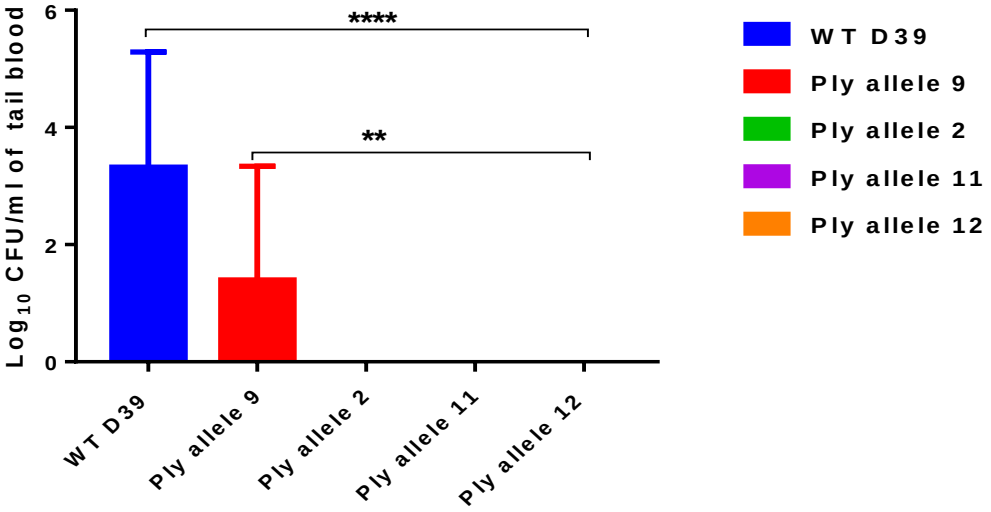


Figure 3-45: Progression of pneumococcal bacteraemia in mice infected with D39 expressing Ply variants 9, 2, 11, 12 or wild type D39.
Progression of pneumococcal bacteraemia in mice infected intranasally with approximately $2x10^6$ CFU/mouse of D39 expressing Ply variants 9, 2, 11, 12 or Ply D39 wild type at 24 hours post-infection. Each column is the mean of data from ten mice. There were significant differences in bacterial counts of mice infected with D39 expressing Ply allele 2, 11, 12 and 9 compared to wild type D39, using one way ANOVA test (**p<0.01,****p<0.0001). In contrast, there was no differences between mice infected with D39 ply allele 9 or wild type D39 (p>0.05).

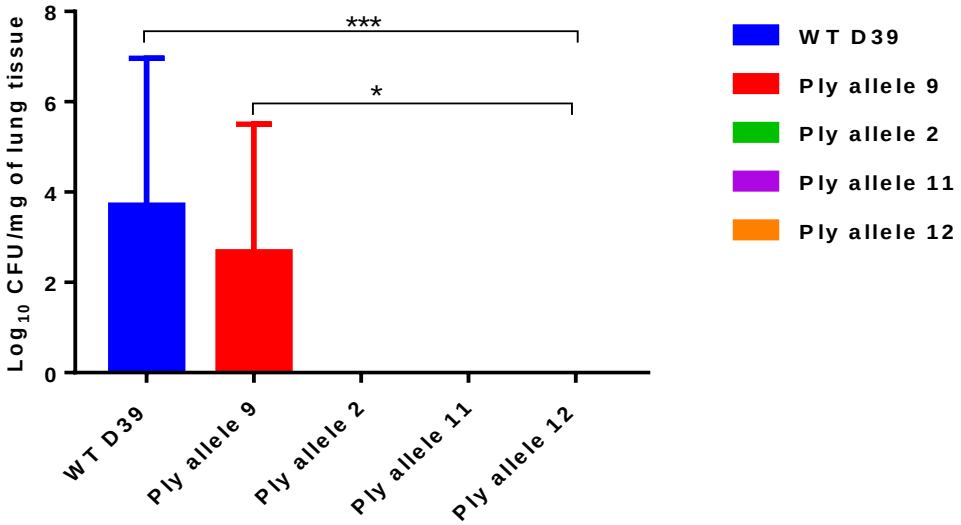


Figure 3-46: Pneumococcal counts in the lung of mice infected with D39 expressing Ply variants 9, 2, 11, 12 or Ply wild type D39.
Pneumococcal load in the homogenised lung tissues of mice infected with D39 expressing Ply variants 9, 2, 11, 12 or Ply D39 wild type were determined. Each column represents the mean data of ten mice and the standard error of the mean. Ply alleles 2, 11, 12 and 9 were significantly lower than the counts of wild type D39 using one way ANOVA (*p<0.05, ***p<0.001).

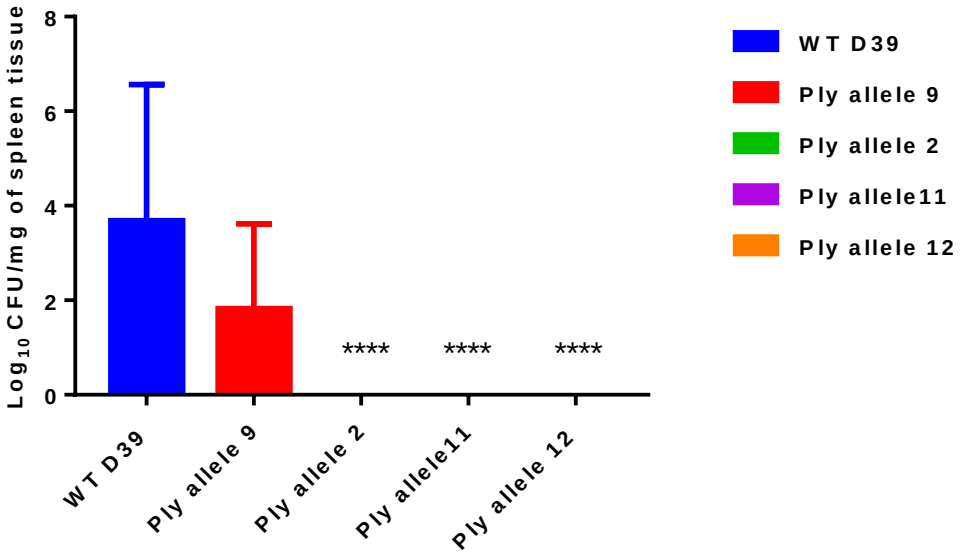


Figure 3-47: Pneumococcal counts in the spleen of mice infected with D39 expressing Ply variants 9, 2, 11, 12 or Ply wild type D39.

Pneumococcal counts in the homogenized spleen tissues of mice infected with D39 expressing Ply variants 9, 2, 11, 12 or Ply D39 wild type were determined. Each column represents the mean data of ten mice and the standard error of mean. Ply alleles 2,11 and 12 were significantly lower than the counts of wild type D39 using one way ANOVA (****$p<0.0001$).

Section E

3.16 Mice immunization with purified Ply D205R and Ply N339R

Pneumococcal diseases such as pneumoniae and bacteraemia kill ~1million people each year. The prevalence of pneumococcal diseases may be attributed to the rise of antibiotic resistance pneumococcal isolates and the limitations of current pneumococcal vaccines including the 23-valent Pneumococcal polysaccharide vaccine (PPV23) and the 13-valent Pneumococcal conjugate vaccine (PCV13); PPV23 is not immunogenic in children under two years old whereas PVC13 provides limited coverage as it only protects against 13 of the 90 pneumococcal polysaccharides. Therefore, new pneumococcal vaccines must be developed and due to its conserve nature and high immunogenicity, pneumolysin (Ply) is a potential vaccine candidate. However, Ply is highly toxic and must be detoxified before it is used as a vaccine. The Ply mutations D205R and N339R had diminished Ply activity (Marshall *et al.* 2015) and so the recombinant Ply D205R and Ply N339R were expressed and purified using the procedures previously described in Section 2.27. The procedures of mice immunisation with Ply D205R or Ply N339R and acute pneumonia infection were previously described in sections 2.28.4 and 2.28.3 respectively.

3.16.1 Determination of anti-Ply level in mouse serum after immunisation with Ply D205R and Ply N339R

Mice immunization was done three times with 20µg/ml of ether Ply D205R or Ply N339R, with alum (Fischer) as an adjuvant. Alum and PBS were used as a control. (This experiment was done in calibration with Dr. Sarah Glenn and Brindha Gap-Gaupool). Venous blood was collected from mice by tail bleeding 24 h before the first and second immunisation and also after the third immunisation. ELISA was used to determine the concentration of anti-Ply IgG in the mouse serum using wild type Ply as the antigen. The ELISA results show that antibody in mice sera before immunization there was no detectable. After the first immunisation the antibody concentration was significantly higher ($p<0.0001$) in the mice immunised with Ply D205R compared to mice immunised with Ply N339R or alum or PBS, while the immunisation was not significantly higher in the group Ply N339R ($p>0.05$) compared with alum or PBS. Following the second immunisation there is no significant differences ($p>0.05$) in the antibody concentration between Ply D205R and Ply N339R groups, whereas the immunisation of Ply D205R and Ply N339R were significantly higher ($p<0.0001$) compared with control groups.

After the third immunisation there was no significant differences in the antibody concentrations between the alum and PBS groups ($p>0.05$), while the Ply D205R and Ply N339R groups were significantly higher ($p<0.0001$) compared with control groups. After three immunisations, there is no significant difference of antibody concentration in the groups given Ply D205R and Ply N339R ($p>0.05$) (Figure 3-48).

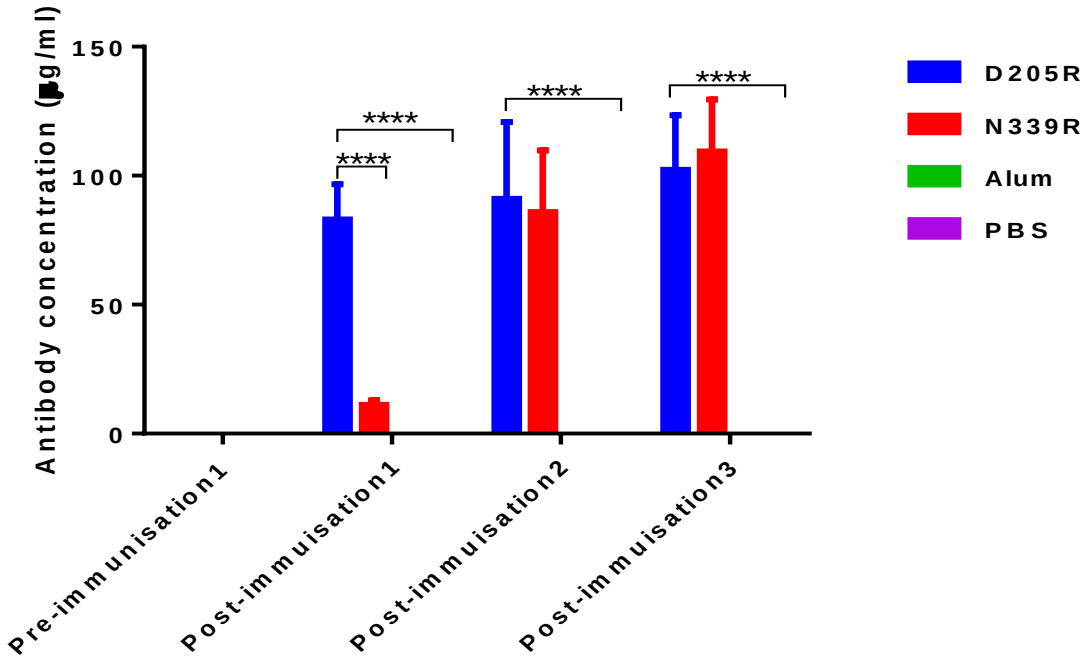


Figure 3-48: Determination of anti-Ply level in mouse serum after immunisation with Ply D205R and Ply N339R.
Anti-Ply IgG concentration in mouse serum after three immunizations with Ply D205R or Ply N339R, alum or PBS as determined by ELISA. Results show there is no detectable anti-Ply antibody in the mouse sera 24 h before immunization. After the first immunization antibody concentration was significantly higher in the group was immunized with Ply D205R compared to Ply N339R or alum or PBS. Data are the average of three independent experiments, two-way ANOVA followed by Dunnett's multiple comparisons test was done using GraphPad Prism 7 (**** $P<0.0001$).

3.16.2 Survival of immunised mice following infection with *S. pneumoniae* D39

Following immunisation of the mice three times with 20µg/ml of ether with Ply D205R or Ply N339R, Two weeks post the final immunisation mice were infected intranasally with approximately $2x10^6$of wild type D39/ mouse. Figure 3-49 shows the mice immunised with Ply D205R or Ply N339R did not show significant difference in survival compared to the immunised mice with PBS and alum ($p>0.05$). The median survival times of mice immunised with Ply D205R or Ply N339R and then infected with wild type D39 (63.3 ± 10.5, n= 10), (62.0 ± 11.1, n=10) respectively. No significant differences could be seen between both immunised groups ($p>0.05$). Moreover, the median survival times of mice immunised with alum or PBS and then infected with wild type D39 (82.0 ± 5.8, n= 10), (66.6 ± 10.5, n=10) respectively. No significant differences were seen between the mice given either control. Also no significant differences were seen between the mice immunised with controls or with Ply D205R or Ply N339R ($p>0.05$).

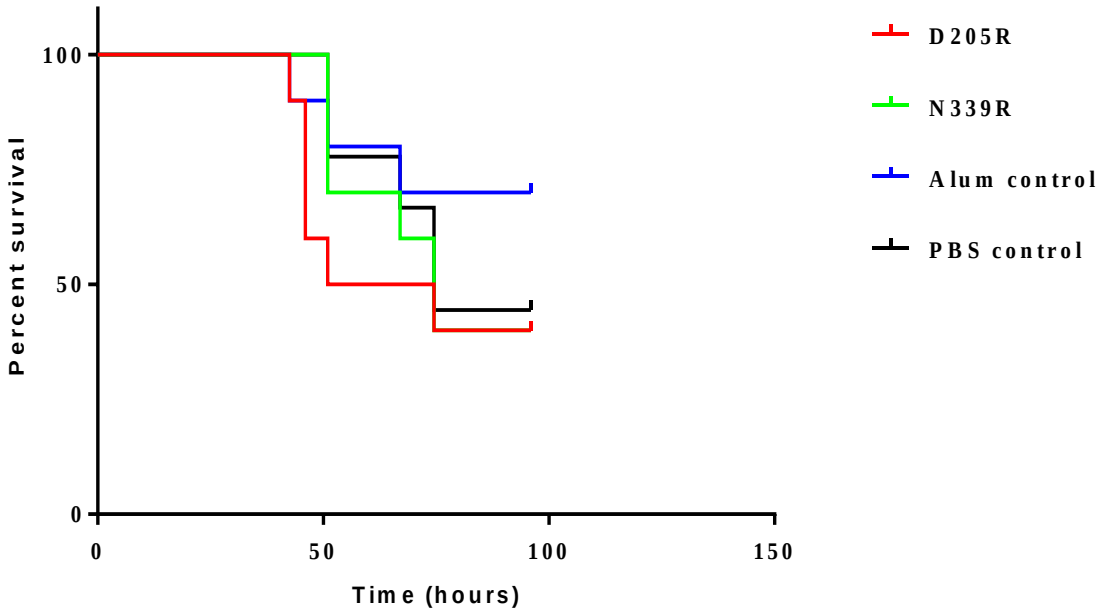


Figure 3-49: Survival of immunised mice following infection with wild type D39. Percent survival times of mice infected intranasally with approximately 2 x 10^6 pneumococci/ mouse in 50 µl PBS two weeks post immunisation three times with 20µg/ml of ether with Ply D205R or Ply N339R conjugated with alum as adjuvant, only alum or PBS were used as control. No significant difference ($p>0.05$) were seen comparing the survival time of immunised mice with Ply D205R or Ply N339R or controls using Mann Whitney test.

3.16.3 Neutralization assay

A haemolytic assay was used to test Ply neutralising ability of antibodies in mice sera following the immunization with Ply D205R or Ply N339R. Following incubation of the mouse serum with wild type Ply of D39 for one hour at 37°C, the absorbance was measured at 405 nm. Sera of mice immunised with Ply N339R had higher neutralising ($p<0.05$) activity compared with neutralising activity of Ply D205R, Ply N339R also had significantly ($p<0.001$) higher neutralising activity compared with PBS control. In contrast no significant differences were observed between the neutralising activity of sera mice immunised with Ply D205R and PBS control ($p>0.05$).

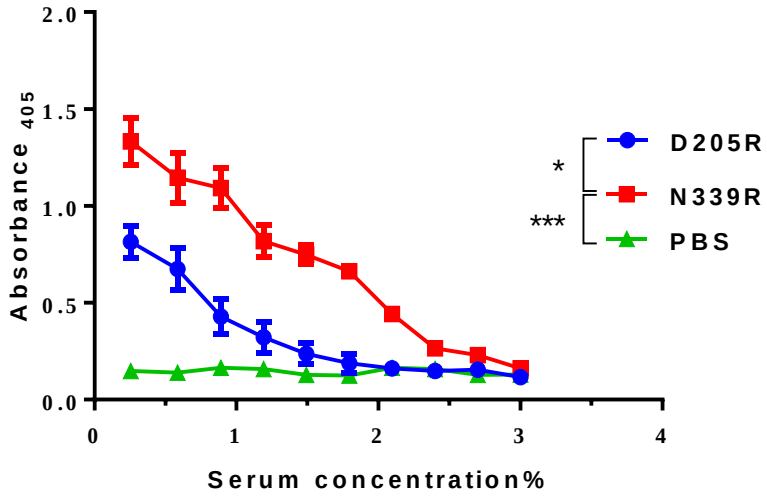


Figure 3-50: Neutralization assay.
Neutralization assay to measure the inhibition of ctytotoxic activity of Ply wild type D39 with IgG in mice sera following the immunization the mice three times with Ply D205R or Ply N339R, PBS was used as a negative control. The mice serum was serially diluted 1:1 in PBS and incubated with1.1 µg/ml of Ply D39 for one hour at 37°C. The absorbance was measured at 405 nm. The data was average of three individual reading. Mice sera immunised with Ply N339R significantly had higher neutralising activity compared with neutralising activity of Ply D205R or PBS (*$p<0.05$) (***$p<0.001$). No significant differences were observed between the neutralising activity of sera mice immunised with Ply D205R and PBS control. One-way ANOVA followed by Dunnett's multiple comparisons test was done using GraphPad Prism.

Chapter 4: Discussion

4.1 Discussion

The amino acid sequence of pneumolysin was thought to be totally conserved (Francis *et al.* 2009), but recently twenty Ply alleles were reported and the variation of DNA sequence was found to be 3.3% (Jefferies *et al.* 2007, J. M. Jefferies *et al.* 2010, Yun *et al.* 2015). *S. pneumoniae* has a natural competence ability to take up the foreign DNA and incorporate this DNA into its genome by recombination, therefore this competence ability could be one reason for pneumococcal protein variation (Johnsborg and Havarstein 2009). Ply variations may be resulting from horizontal transfer of DNA fragments from commensal organisms that occupy the same niche as the pneumococcus. However the main reason for effect of these Ply variations on the virulence is still unknown (Jefferies *et al.* 2007).

Naturally occurring pneumolysin variants are expressed in different genetic pneumococcal backgrounds, which are associated with invasive and non-invasive disease (Jefferies *et al.* 2007, J. M. Jefferies *et al.* 2010). Six of these variants are reported to be non-haemolytic, whereas others have different degrees of lytic activity. The reason for differential virulence of pneumococcal strains expressing different versions of Ply alleles is unclear, because we do not know if the pneumolysin variations causes the diversity in the virulence of strains or the genetic backgrounds of these strains affected the pneumococcal virulence. Therefore, the impact of these Ply variants needed to be tested in single genetic background.

In order to study the role of Ply variations in a single genetic background, type 2 D39 strain was used because it is transformable strain, and it is well studied by others and the group of Professor Peter Andrew in the murine models of pneumococcal infection and *in vitro*.

The naturally occurring Ply variations reported by previous studies (Jefferies *et al.* 2007, Jefferies *et al.* 2010, Yun *et al.* 2015) were successfully introduced into the *ply* gene of D39 by using pORI280 plasmid. This plasmid allows insertion of a recombinant *ply* gene in its native location by homologous recombination and pneumolysin gene expression from its own promoter. In addition, pORI280 cannot replicate in *S. pneumoniae* it requirs the replication gene *repA* (Leenhouts *et al.* 1996, Kloosterman *et al.* 2006). In the pORI280 system, *lacZ* expression was used to visualize the recombination event by blue/white screening. Furthermore, following use of this system there is no antibiotic cassette left in the chromosome of D39, because the antibiotic cassettes is removed with the plasmid during the non-selective

culturing. An antibiotic cassette can cause polar effects on the expression of downstream genes, if it is left in the bacterial genome (Kloosterman *et al.* 2006).

Ply alleles were expressed from different pneumococcal strains associated with invasive phenotypes and others with colonisation phenotypes. The position of the variant in the Ply amino acid sequence may play an important role in the pneumococcal virulence. Ply has four domains and the amino acid sequence of each domain is shown in Figure 4-1 (Mitchell and Dalziel 2014, Lawrence *et al.* 2015). Domain 1 contains the residues 1-21, 58-147, 198-243 and 319-342, domain 2 contains the residues 22-57, 343-359, and domain 3 includes the residues 148-197, 244-318. Furthermore, domain 4 includes the residues 360-370. Domains 1-3 form the N-terminal part of Ply structure, while domain 4 forms the C-terminal region of Ply structure (Mitchell and Dalziel 2014). Figure 4-2 shows that domain 1 has three α-helices and one β-sheet, and domain 2 has also one β-sheet. In addition, it also shows that domain 3 consists of two α-helical bundles, which surround the 5-stranded β-sheets. Whereas domain 4 is a β-sandwich and it contains the Trp-rich loop (427-437), which is also called the undecapeptide region(Lawrence *et al.* 2015).

The assessment of each Ply allele included the haemolytic activity of each pure Ply allele as well as the lytic activity of these Ply alleles in the D39 crude lysate, and the effect of these variants on pneumococcal virulence. Figure 4.2 shows the location of each variant in the Ply domains. Some of the Ply alleles were expressed in *E. coli* and then purified by affinity chromatography and gel filtration.

Ply variant 2 was not purified, and the haemolytic activity of D39 cell lysate expressing Ply allele 2 was reduced to about 25% compared to the lytic activity of cell lysate of wild type D39. A previous study reported that the lysates of Ply allele 2 and wild type D39 had similar level of haemolytic activity(Jefferies *et al.* 2007). The reduction of the lytic activity of allele 2 may be due to the contribution of genetic background in the Ply activity, or it could be the type of amino acid change affected the biological activity of Ply. The virulence of D39 expressing Ply allele 2 was tested in pneumonia model that developed after intranasal infection. The mice infected with D39 carrying Ply allele 2 showed significant survival time compared to mice infected with wild type D39. This result is different from the previous study because they discovered this allele in the clinical isolates of *S. pneumoniae*. The significant reduction of virulence of D39 carrying Ply allele 2, may be due to the location of this variant being located

in the bottom of domain 4 affecting the biological activity of Ply as the binding of Ply is mediated by this domain. The reason why this allele has impact on virulence in an animal infection model but no significant effect in humans could be linked to strain background, and differences due to the infection process in mouse and human. Previously it was reported that Ply allele 2 and wild type D39 are the most common alleles distributed across isolated pneumococcal strains (Jefferies *et al.* 2007).

Ply variant 8 A273D expressed in *E. coli* and purified by affinity chromatography and gel filtration. The haemolytic activity of pure Ply 8 was more than its activity in the crude lysate of D39 carrying Ply 8. This indicates that the activity of Ply 8 was affected by the components of the crude lysate. This may be due to the presence of other pneumococcal proteins or the activity of Ply itself being affected by the sonication and breakdown of the pneumococcal cell wall because it is thought that Ply localized in the pneumococcal cell wall (Price and Camilli 2009).The haemolytic activity of cell lysate of D39 carrying Ply allele 8 was reduced about 80% relative to the wild type. This is approximately similar to the finding of Jefferies et al (2007) who reported that this allele has lower haemolytic activity. The virulence of D39 expressing Ply allele 8 also investigated in pneumonia model and the virulence was significantly lower compared to the wild type D39. The impact of the variant of Ply allele 8 on the toxicity of Ply and on the virulence of D39 *in vivo*, could be due to its location into the transmembrane hairpins structure 2 TMH2 (257-280), which plays a key role to form pore by the insertion into the cell membrane and form a β-barrel. Also the type of amino acid due to mutation at this sensitive position may affects the biological activity of Ply. The non-polar, small and the hydrophobic amino acid alanine was replaced with the hydrophilic, polar and negatively charged aspartate amino acid. All these differences in the amino acid features may affect the contribution of Ply in the virulence of D39 *in vivo*. Also reduction in virulence with the strain carrying allele 8 could be largely due to impact of mutation on cytotoxic activity of Ply rather than its role in complement activation as reported in Berry et al., (1995) (Berry *et al.* 1995).

Ply allele 9 also was not purified and its haemolytic activity in the lysate of D39 surprisingly increased compared to the wild type D39, while the lytic activity of Ply allele 9 in the previous study was about 76% of haemolytic activity of wild type D39. This variant also located into the transmembrane hairpins structure 2 TMH2 (257-280). D39 expressing Ply allele 9 was tested in murine model of infection and it did not attenuate the pneumococcal virulence. Ply

allele 9 located in domain 3 and the substitution of isoleucine to methionine amino acid also in the TMH2 structure. In addition, both of these amino acids are hydrophobic and approximately have the same size.

Ply allele 11 also was not purified, and the haemolytic activity of D39 lysate carrying Ply allele 11 reduced and it was about 85% relative to the wild type D39, whereas the lytic activity of this allele in the previous study was reported to be 144% compared to the wild type D39. The virulence of D39 expressing Ply allele 11 was tested in the mouse pneumonia model. The mice infected with D39 carrying Ply allele 11 showed significant survival time compared to mice infected with wild type D39. This allele has 2 variants, one has the replacement of Q136K and located in domain 1, and the other has D380N located in domain 4. Ply allele 11 shares Ply allele 2 with the variant D380N. Both strains expressing Ply allele 2 and Ply allele 11 were attenuated in virulence, very likely due to the presence of D380N replacement.

Ply allele 12 was purified and the lytic activity of pure Ply allele 12 was reduced about 50% compared to the haemolytic activity of pure Ply of D39. Previous study reported that Ply allele 12 was haemolytic but they did not mention the specific lytic activity of this allele and reported that Ply allele 12 is not recognised by anti-Ply 7 in the ELISA assay (Lea-Ann S. Kirkham *et al.* 2006). On the other hand, in my study the anti-Ply 7 antibody recognised Ply allele 12 in the ELISA and western blot assays. D39 expressing Ply allele 12 was also tested in murine model of infection and it was found to be significantly attenuated in virulence compared to the wild type D39. Ply allele 12 has 2 variants D380N and Q402E, and both of these variants located in domain 4. Ply allele 12 also shares Ply allele 2 with the variant D380N.

Ply allele 15 was also expressed in *E. coli* and was purified by affinity chromatography and gel filtration. The haemolytic activity of pure Ply15 was found to be reduced 12.5% of that of wild type Ply. On the other hand, the haemolytic activity of Ply allele 15 in the D39 cell lysate was about 63% compared to the lytic activity of crude lysate of wild type D39. This result disagree with the lytic activity that was reported in the previous study because they reported that lytic activity of Ply allele 15 is very low, about 8% compared to the wild type D39. The virulence of D39 expressing Ply allele 15 was tested in pneumonia model that developed after intranasal infection. The sickness score of mice infected with D39 carrying Ply allele 15 were not different compared to mice infected with wild type D39. The variant of Ply allele 15 is located in domain 3 and also in the transmembrane hairpins structure 2 TMH2 (257-280). Ply allele 15 seems to

have the same the virulence attribute as Ply allele 9, both of which located in the transmembrane hairpin structure, which is a crucial position and play an important role in the pore formation.

Ply allele 18 was purified and the lytic activity of pure Ply18 was same as the wild type Ply allele. In contrast, the haemolytic activity of Ply allele 18 in the D39 cell lysate was lower, about 32%, compared to the lytic activity of crude lysate of wild type D39. The result of haemolytic activity in cell lysate of Ply allele 18 in D39 was consistent with the previous study (Jefferies *et al.* 2007). The virulence of D39 expressing Ply allele 18 was also investigated in pneumonia model. The virulence of strain expressing Ply allele 18 was significantly lower compared to the wild type D39. The variant V439I of Ply allele 18 was located in the bottom domain 4 and was very close to the Trp-rich loop (undecapeptide region 427-437 amino acid sequence). So the SNP that replaces the valine with isoleucine at position 439 may play a key role to reduce the virulence activity of D39 expressing this allele.

Figure 4-1: Location of important pneumolysin amino acid residues.
Ply amino acid residues, which are highlighted, as blue, green, red and yellow as domain 1, 2, 3, and 4 respectively. The bold and underlined letters (160-186) and the italic and underlined letter (257-280) represent the transmembrane hairpins structures TMH1 and TMH2 respectively. The black colour letters (427-437) represent the Trp-rich loop (undecapeptide region). The letters in purple colour represent the location of natural Ply variations (Mitchell and Dalziel 2014, Lawrence *et al.* 2015).

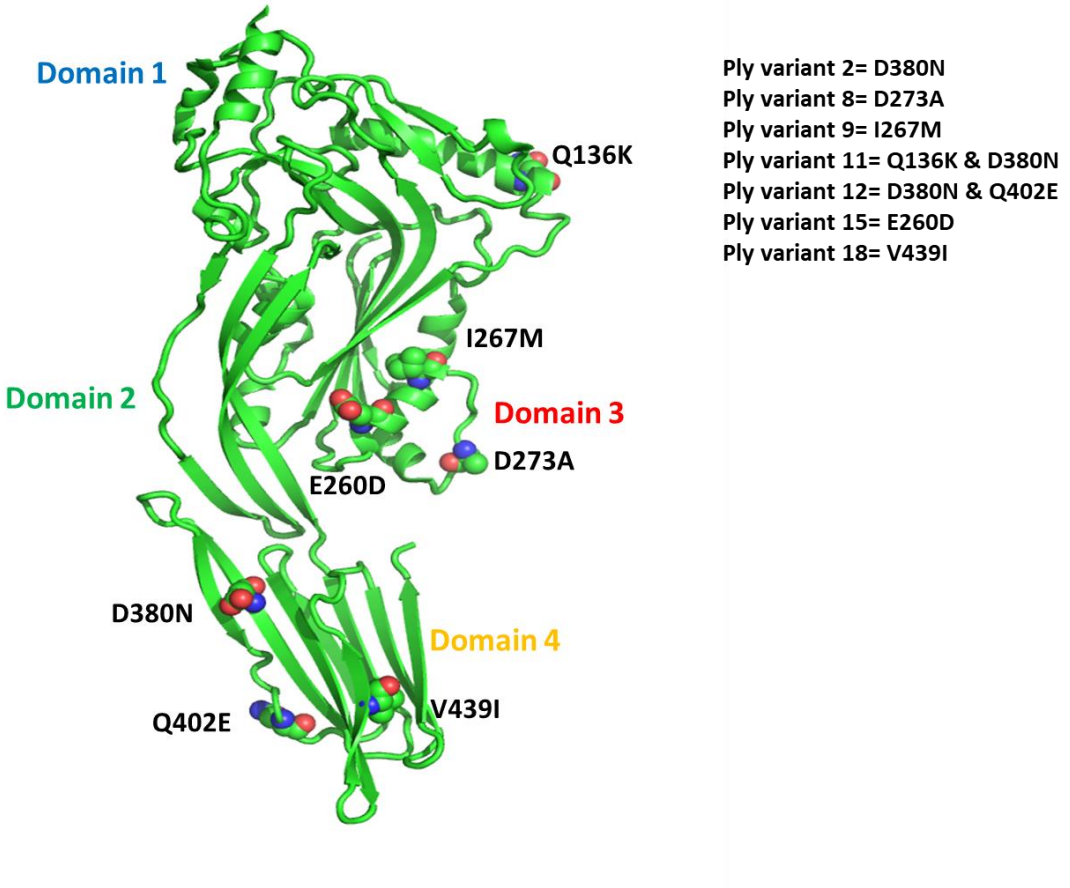


Figure 4-2: 3D structure of pneumolysin.
The structure shows the locations of constructed variants into the Ply of D39. Structure kindly provided by Professor Russell Wallis, University of Leicester.

To test the impact of specific amino acids at specific positions in the Ply amino acid sequence the variants of Ply allele 12 (Q402E) and Ply allele 18 (V439I) were chosen. For this, Q402E was changed into Q402A or to Q402D and V439I was changed into V439A. All these positions located in the domain four of Ply, and this domain plays an important role by binding to the membrane of the host cell. In the position 402 of Ply amino acid sequence the acidic residue glutamine (Q), was changed first to the aspartic acid (D), which is small and negatively charged amino acid, and then the glutamine was replaced with the alanine. Alanine is neutrally charged amino acid and smaller than valine. The impact of these amino acids on the activity of pure Ply was tested in the haemolytic activity assay and C3 deposition assay. This changes do not reduce the lytic activity of pure Ply, nor do they affect the complement activation pathway. Previous study reported that Ply allele 12 (Q402E) was not recognised by anti-Ply 7, however it was detected in our study by this antibody.

Western blotting assay was used in order to test the impact of the amino acids A and D in position 402 of Ply, to the binding of Ply to anti-Ply 7, which recognize the amino acids at positions 401-407 in Ply (Jefferies et al., 2007). The binding of anti-Ply7 to Ply (Q402A) and Ply (Q402D) likely was more effective than the binding of anti-Ply with Ply (Q402E) as shown in Figure 3.38. This result indicates that the glutamic acid (E) slightly affects the interaction of Ply with antibody Ply7.

4.1.1 Pneumolysin allele distributions

The distribution of Ply variants across the pneumococcal strains was analysed in collaboration with Professor Marco Oggioni and Dr. Roxana Zamudio Zea by using Nucleotide BLAST program. The analysis covered studies from different geographical areas included Massachusetts pneumonia study (USA) which includes 617 pneumococcal strain collection (Croucher *et al.* 2015). Southampton pneumonia study (UK) included 515 pneumococcal strain collection (Gladstone *et al.* 2017), and Netherlands meningitis study which included 627 pneumococcal strain collection (Lees *et al.* 2017). The grey colour represents the Ply alleles that are no covered in the Jefferies studies (Jefferies *et al.* 2007, Jefferies *et al.* 2010, Yun *et al.* 2015).

The distributions of Ply alleles across the pneumococcal serotypes in in the UK is shown in Figure 4.3. Generally the high frequency Ply alleles in the pneumococcal strains were Ply 1 (red) and Ply 2 (blue). There are certain strains that contain more than one Ply allele, like serotypes 1, 6A, 6B,6C, 10A, 15A, 15C, 19A, 19F, 20, 21, 22F, 23F,33F, 34, 35B, 35F and NT (non-typeable). In contrast other strains have only one allele for instance, the serotypes 12F, 23A, 31and 6C contain only Ply allele 1, and serotypes 4, 7c, 9N, 9V, 14, 15B, 16F, 18C, 24F, 3, 33D and 38, contain the Ply allele 2. In the previous Southampton study, the researchers detected four variants Ply5, Ply7, Ply8 and Ply9, (Jefferies *et al.* 2007, Jefferies *et al.* 2010, Yun *et al.* 2015), which are not present in the Massachusetts set. . However the Southampton carriage isolate collection does not have strains with all pneumolysin alleles published earlier by the same group, with types 3, 4, 6, 10, 12 and 13 not being presents.

The distributions of Ply alleles across the pneumococcal serotypes in in the USA is illustrated in figure 4.4. In this figure we can see that the high frequency Ply alleles are Ply 1 (red) and Ply 2 (blue) in the pneumococcal strains and this is consistent with distribution of pneumococcal strains in the UK. There are certain strains that contain more than one Ply allele, like serotypes 6, 6A, 6B, 6C, 7F, 9A, 9N, 9V, 10A, 11A, 15, 15B/C, 18C, 19F, 23A, 23B, 23F, 34, 35B, 35F and NT. In contrast another strains have only one allele for instance, serotypes 2, 10, 22F, 31, 37, pool D and pool G contain the Ply allele 1 (red), and the serotypes 3, 7, 7C, 9N, 11, 11A, 14, 15F, 16F, 17F, 18, 21, 23, 25A, 33, 33F, 38 and pool I contain Ply allele 2 (blue). The most prevalent alleles in all three data sets were Ply1 (21-37%) and Ply2 (49-58%). There were minor differences between the data sets.

The distribution of Ply alleles in the pneumococcal sequence types (ST) representing three different regions (UK, USA and Netherlands) are shown in Figure 4.5, Figure 4.6 and Figure 4.7 respectively. In the Netherlands strains allele Ply21 was found to be in multiple sequence type 191 strains, also present to a lower extent in the other two collections in the ST191 serotype 7F strains. The alleles present in the non-typeable strains were only analysed in detail in the American strains, and 10/11 of NT strains were found to carry *ply* alleles with frameshifts or stop codons encoding for incomplete/truncated proteins. With respect to the haemolytic activities of Ply alleles, these data confirm that Ply1 and Ply2 which have high haemolytic activity are the most prevalent variants, but for the other variants there appears to be no correlation.

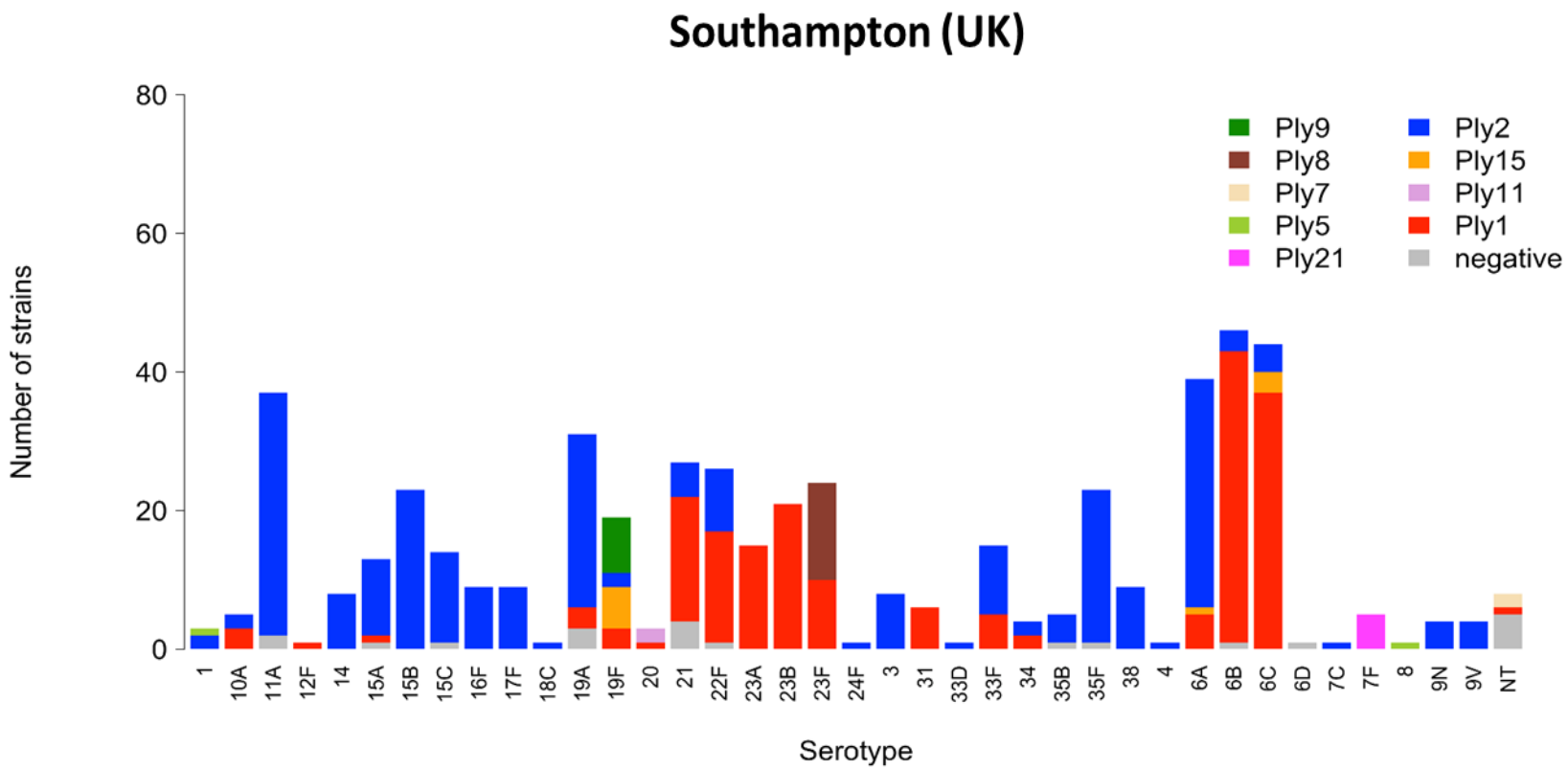


Figure 4.3: Distributions of Ply alleles across the pneumococcal serotypes in in the UK.
The figure shows the high frequency Ply alleles are Ply 1 (red) and Ply 2 (blue). There are strains contains more than one Ply allele, like serotypes 1, 6A, 6B,6C, 10A, 15A, 15C, 19A, 19F, 20, 21, 22F, 23F,33F, 34, 35B, 35F and NT. In contrast another strains have only one allele for instance, the serotypes 12F, 23A, 31and 6C contain only Ply allele 1, and serotypes 4, 7c, 9N, 9V, 14, 15B, 16F, 18C, 24F, 3, 33D and 38, contain the Ply allele 2. The grey colour representing the Ply alleles that no covered in the Jefferies studies.

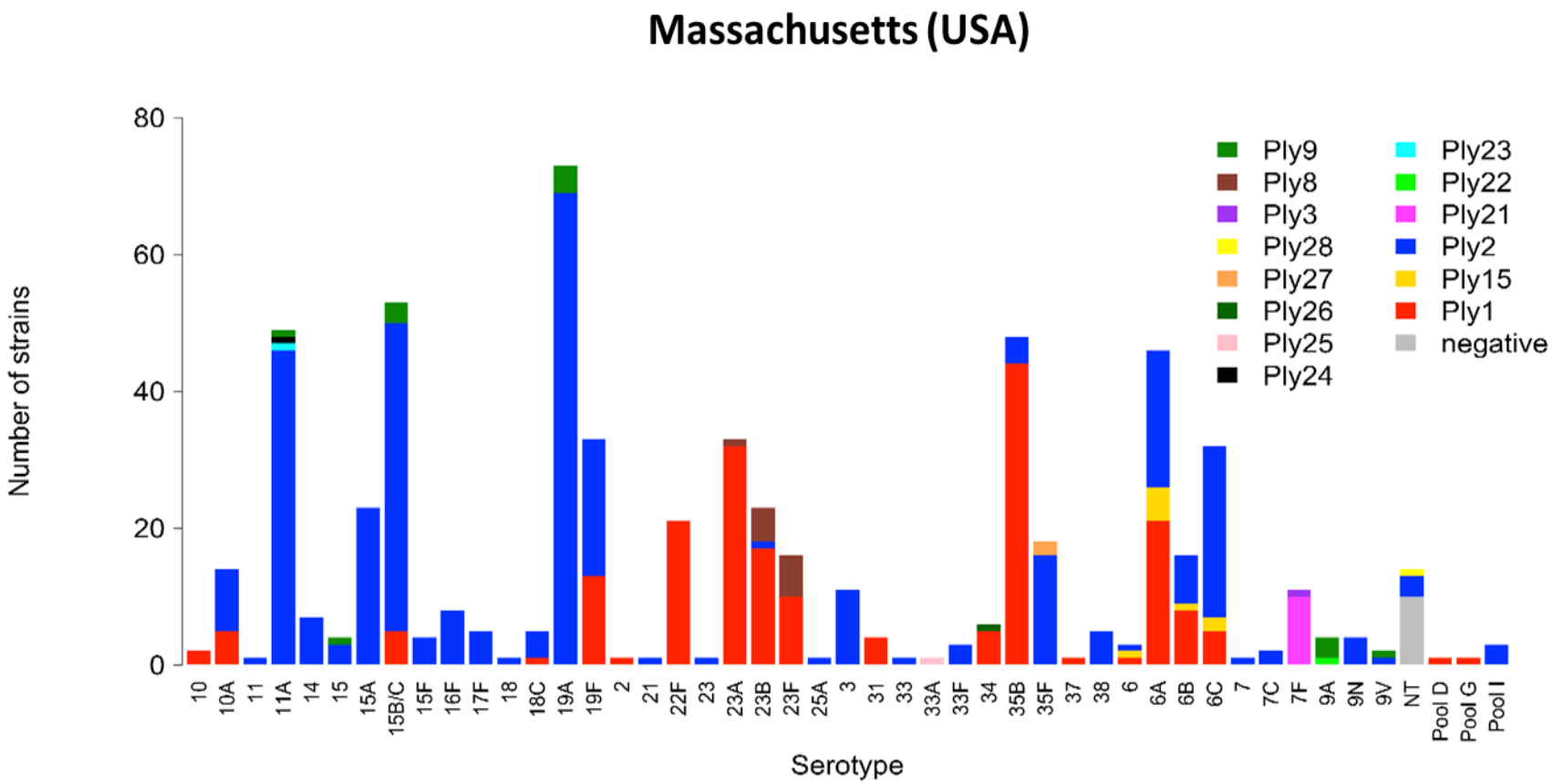


Figure 4.4: Distributions of Ply alleles across the pneumococcal serotypes in in the USA.
In this figure we can see the high frequency Ply alleles are Ply 1 (red) and Ply 2 (blue). There are strains contains more than one Ply allele, like serotypes 6, 6A, 6B, 6C, 7F, 9A, 9N, 9V, 10A, 11A, 15, 15B/C, 18C, 19F, 23A, 23B, 23F, 34, 35B, 35F and NT. In contrast another strains have only one allele for instance, serotypes 2, 10, 22F, 31, 37, pool d and pool G contain the Ply allele1 (red), and the serotypes 3, 7, 7C, 9N, 11, 11A, 14, 15F, 16F, 17F, 18, 21, 23, 25A, 33, 33F, 38 and pool I contain Ply allele 2 (blue). The grey colour representing the Ply alleles that no covered in the Jefferies studies.

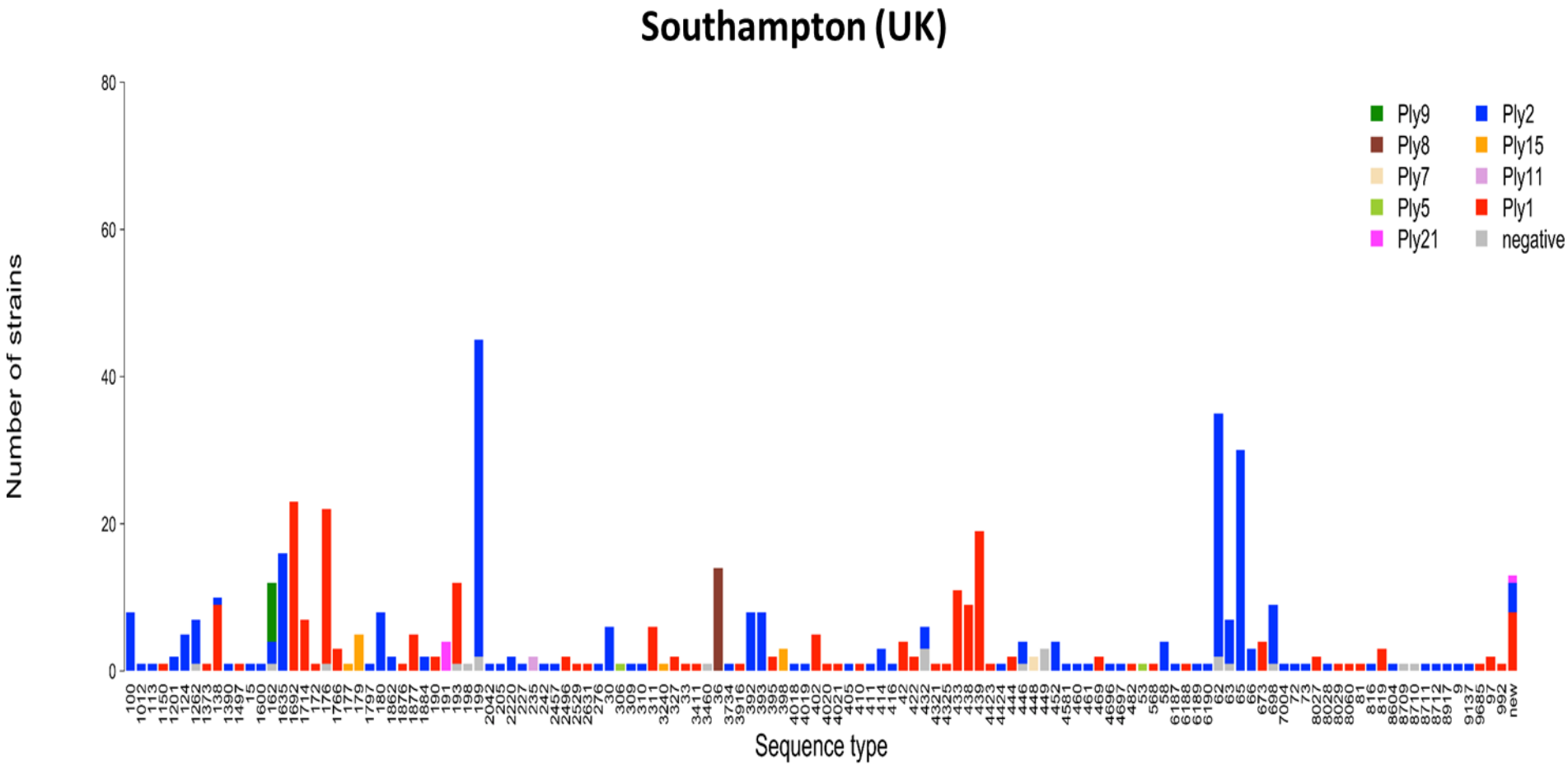


Figure 4.5: Distribution of Ply alleles in the sequence type of pneumococcal strains collections in the UK.
This figure shows the distribution of Ply alleles in the sequence type of pneumococcal strains collections in the UK. As we can see most of the STs contain specific Ply allele and only few of STs include different Ply alleles. It is clear that Ply alleles 1 and 2 are the more frequency alleles.

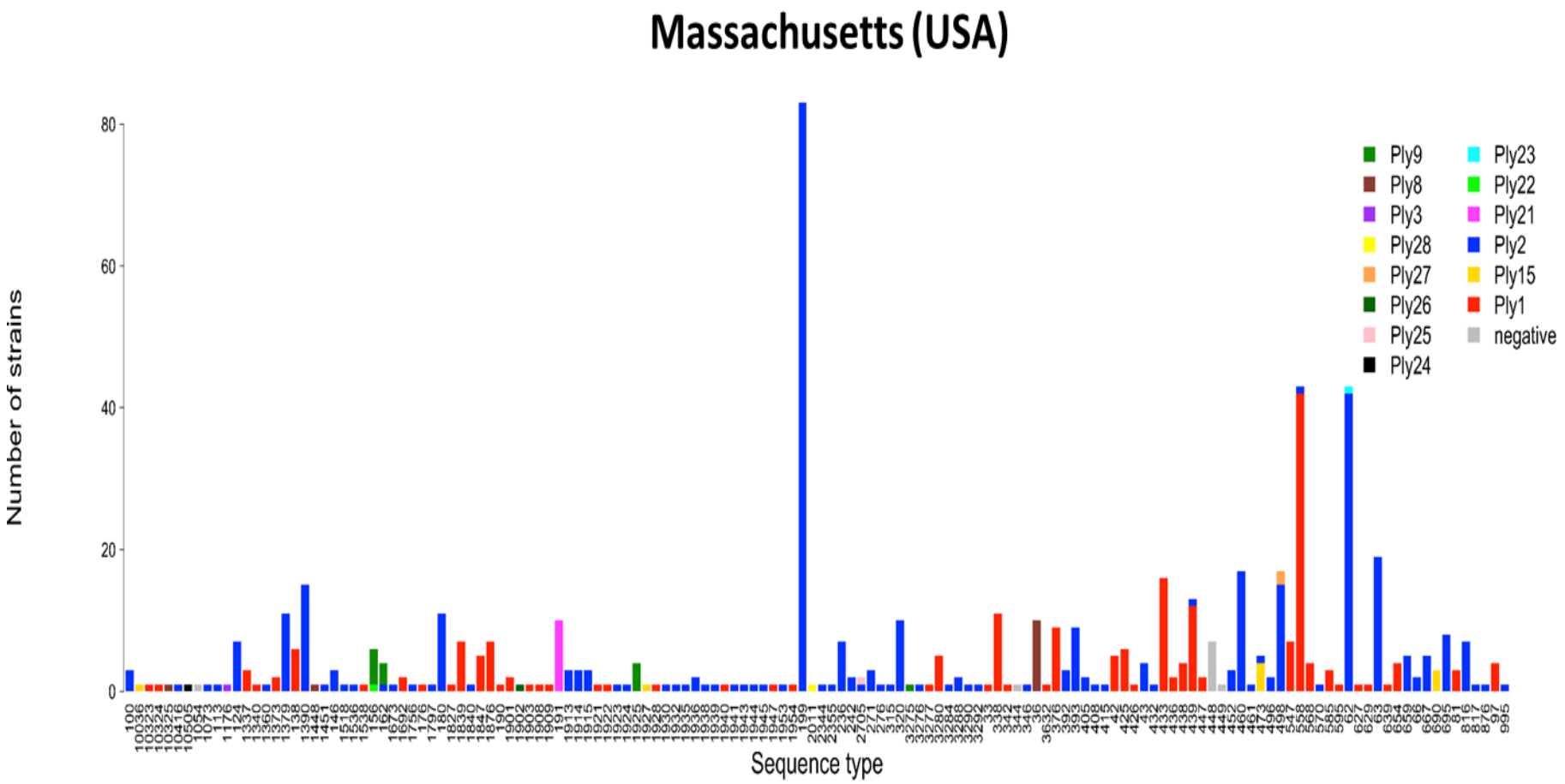


Figure 4.6: Distribution of Ply alleles in the sequence type of pneumococcal strains collections in the USA.
This figure shows the distribution of Ply alleles in the sequence type of pneumococcal strains collections in the USA. As we can see most of the STs contain specific Ply allele and only few of STs include different Ply alleles. The more frequency Ply alleles 1 and 2, but in the USA the Ply allele 2 more than Ply allele.

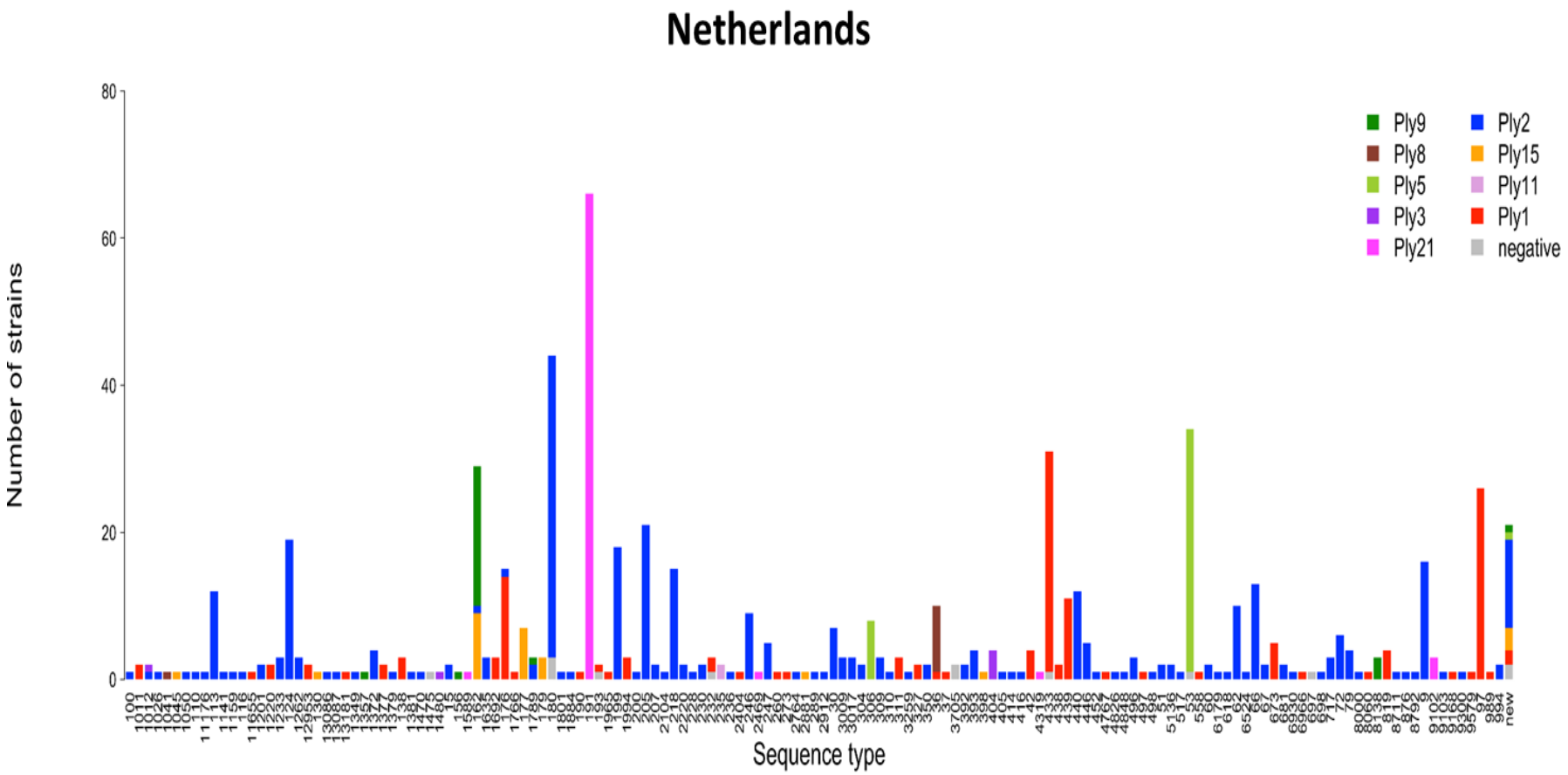


Figure 4.7: Distribution of Ply alleles in the sequence type of pneumococcal strains collections in the Netherlands.
This figure shows the distribution of Ply alleles in the sequence type of pneumococcal strains collections in the Netherlands. In this figure we can see the more frequency Ply allele is Ply 2. Also most of STs have only one Ply allele and few of STs include different Ply alleles.

4.2 Final Remark

Pneumolysin is a key virulence factor of *S. pneumoniae*, which causes serious human diseases like pneumonia, bacteraemia and meningitis. Recently twenty Ply variants were determined from more than 300 clinical isolates of *S. pneumoniae* (Jefferies *et al.* 2007, Jefferies *et al.* 2010, Yun *et al.* 2015). In this study the virulence of the Ply variants in a single genetic background was investigated. D39 expressing Ply alleles 2, 8, 11, 12 and 18 had significant impact on the pneumococcal virulence in a mouse model of pneumococcal infection. This result indicates that Ply plays an important role in the pneumococcal diseases.

4.3 Future Work

The level of pneumolysin expression *in vitro* differs from its expression *in vivo*. The pneumococcal strains express different versions of Ply and the impact of this on the pneumococcal virulence could be variable (Mitchell and Dalziel 2014). In this study the role of specific Ply variations in D39 was determined in the pneumococcal infection, and these Ply variants significantly reduced the pneumococcal virulence. We need more work to understand the mechanism of this role and how it affects the pneumolysin activity. Based on my findings some future experiments can be suggested. Some Ply alleles in D39 significantly reduced the pneumococcal virulence, therefore, we need to transfer these Ply alleles into other pneumococcal strain background, especially the strains isolated from infected people with pneumococcal pneumonia or meningitis. For instance, the transformation of these alleles into one of the strains belonging to serotype 1 or 8, which express the Ply allele 5, non-haemolytic and contains six variations and associated with invasive disease, can reveal strain background specific impacts of these alleles (Jefferies *et al.* 2007). In this study I attempted to construct Ply allele 5, unfortunately, following the cloning the DNA sequencing was showed there are extra mutations, and due to the limited time, I could not repeat the cloning. Therefore, my suggestion is that Ply allele 5 could be amplified directly from the genomic DNA that express this allele and then can be inserted into D39 background, or vice versa, i.e. insert the Ply of D39 into Ply allele 5 background.

On the other hand, Ply allele 14, is non-haemolytic allele and discovered in serotype 1, and this allele isolated from patients infected with pneumonia. This allele shares Ply allele 2 with the variation D380N, as well as it has insertion of 871 base pairs at the amino acid position 142 of pneumolysin, and the insertion of 871 base pairs encodes IS1515 (Jefferies *et al.* 2007). The DNA sequence of *ply* gene (2290 base pair) contains the IS1515 has been deposited in the EMBL/GenBank under accession number EF490446 (Garnier *et al.* 2007). Therefore, to construct Ply allele 14, the insertion of 871 base pare could be inserted into the D39 expressing Ply allele 2.

Moreover, D380N is present in the Ply alleles 2, 11 and 12. D39 expressing these alleles had a significant impact on pneumococcal virulence in a mouse model of pneumococcal infection. The D380N had a crucial role on pnemolysin potency, however it is interesting to investigate the impact of other substitutions as well Q136K and E260D of Ply alleles 11 and 12, respectively. I need to construct the alleles with Q136K or E260D substitutions in the *ply* of D39 and then, they can be tested for their contribution to the virulence of D39.

There is another DNA manipulation in the Ply allele 2 could be done by changing D380N to D380E and the impact of this amino acid replacement can be tested *in vitro* and *in vivo*. The inflammatory response in the lungs of survived and dead mice can be quantified by using qRT-PCR. The level of mRNA expression of C3, C1q, tumour necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), TLR-4, IL-6 and IL10 can be determined. Moreover, the expression levels of cytokine or chemokine in the lungs of survived and dead mice can be evaluated.

Appendix

Ply allele 2

Alignments

Streptococcus pneumoniae D39, complete genome
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Range 1: 1721458 to 1722873

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Ply allele 8

Alignments

Streptococcus pneumoniae D39, complete genome
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Sbjct  1721973  CGAGTTGTAACAGGCAAGGTGGATATGGTAGAGGACTTGATTCAAGAAGGCAGTCGCTTT  1721914
Query  961      ACAGCAGATCATCCAGGCTTGCCGATTTCCTATACAACTTCTTTTTTACGTGACAATGTA  1020
Sbjct  1721913  ACAGCAGATCATCCAGGCTTGCCGATTTCCTATACAACTTCTTTTTTACGTGACAATGTA  1721854
Query  1021     GTTGCGACCTTTCAAAACAGTACAGACTATGTTGAGACTAAGGTTACAGCTTACAGAAAC  1080
Sbjct  1721853  GTTGCGACCTTTCAAAACAGTACAGACTATGTTGAGACTAAGGTTACAGCTTACAGAAAC  1721794
Query  1081     GGAGATTTACTGCTGGATCATAGTGGTGCCTATGTTGCCCAATATTATATTACTTGGGAT  1140
Sbjct  1721793  GGAGATTTACTGCTGGATCATAGTGGTGCCTATGTTGCCCAATATTATATTACTTGGGAT  1721734
Query  1141     GAATTATCCTATGATCATCAAGGTAAGGAAGTCTTGACTCCTAAGGCTTGGGACAGAAAT  1200
Sbjct  1721733  GAATTATCCTATGATCATCAAGGTAAGGAAGTCTTGACTCCTAAGGCTTGGGACAGAAAT  1721674
Query  1201     GGGCAGGATTTGACGGCTCACTTTACCACTAGTATTCCTTTAAAAGGGAATGTTCGTAAT  1260
Sbjct  1721673  GGGCAGGATTTGACGGCTCACTTTACCACTAGTATTCCTTTAAAAGGGAATGTTCGTAAT  1721614
Query  1261     CTCTCTGTCAAAATTAGAGAGTGTACCGGGCTTGCCTGGGAATGGTGGCGTACGGTTTAT  1320
Sbjct  1721613  CTCTCTGTCAAAATTAGAGAGTGTACCGGGCTTGCCTGGGAATGGTGGCGTACGGTTTAT  1721554
Query  1321     GAAAAAACCGATTTGCCACTAGTGCGTAAGCGGACGATTTCTATTTGGGGAACAACTCTC  1380
Sbjct  1721553  GAAAAAACCGATTTGCCACTAGTGCGTAAGCGGACGATTTCTATTTGGGGAACAACTCTC  1721494
Query  1381     TATCCTCAGGTAGAGGATAAGGTAGAAAATGACTAG  1416
Sbjct  1721493  TATCCTCAGGTAGAGGATAAGGTAGAAAATGACTAG  1721458
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Ply allele 9

Alignments

Streptococcus pneumoniae D39, complete genome
Sequence ID: **CP000410.2** Length: 2046116 Number of Matches: 1
Range 1: 1721458 to 1722873

Score	Expect	Identities	Gaps	Strand	Frame
2610 bits(1413)	0.0()	1415/1416(99%)	0/1416(0%)	Plus/Minus	

Features:

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Query  1        ATGGCAAATAAAGCAGTAAATGACTTTATACTAGCTATGAATTACGATAAAAAGAAACTC  60
Sbjct  1722873  ATGGCAAATAAAGCAGTAAATGACTTTATACTAGCTATGAATTACGATAAAAAGAAACTC  1722814
Query  61       TTGACCCATCAGGGAGAAAGTATTGAAAATCGTTTCATCAAAGAGGGTAATCAGCTACCC  120
Sbjct  1722813  TTGACCCATCAGGGAGAAAGTATTGAAAATCGTTTCATCAAAGAGGGTAATCAGCTACCC  1722754
Query  121      GATGAGTTTGTTGTTATCGAAAGAAAGAAGCGGAGCTTGTCGACAAATACAAGTGATATT  180
Sbjct  1722753  GATGAGTTTGTTGTTATCGAAAGAAAGAAGCGGAGCTTGTCGACAAATACAAGTGATATT  1722694
Query  181      TCTGTAACAGCTACCAACGACAGTCGCCTCTATCCTGGAGCACTTCTCGTAGTGGATGAG  240
Sbjct  1722693  TCTGTAACAGCTACCAACGACAGTCGCCTCTATCCTGGAGCACTTCTCGTAGTGGATGAG  1722634
Query  241      ACCTTGTTAGAGAATAATCCCACTCTTCTTGCGGTTGATCGTGCTCCGATGACTTATAGT  300
Sbjct  1722633  ACCTTGTTAGAGAATAATCCCACTCTTCTTGCGGTTGATCGTGCTCCGATGACTTATAGT  1722574
Query  301      ATTGATTTGCCTGGTTTGGCAAGTAGCGATAGCTTTCTCCAAGTGGAAGACCCCAGCAAT  360
Sbjct  1722573  ATTGATTTGCCTGGTTTGGCAAGTAGCGATAGCTTTCTCCAAGTGGAAGACCCCAGCAAT  1722514
Query  361      TCAAGTGTTCGCGGAGCGGTAAACGATTTGTTGGCTAAGTGGCATCAAGATTATGGTCAG  420
Sbjct  1722513  TCAAGTGTTCGCGGAGCGGTAAACGATTTGTTGGCTAAGTGGCATCAAGATTATGGTCAG  1722454
Query  421      GTCAATAATGTCCCAGCTAGAATGCAGTATGAAAAAATAACGGCTCACAGCATGGAACAA  480
Sbjct  1722453  GTCAATAATGTCCCAGCTAGAATGCAGTATGAAAAAATAACGGCTCACAGCATGGAACAA  1722394
Query  481      CTCAAGGTCAAGTTTGGTTCTGACTTTGAAAAGACAGGGAATTCTCTTGATATTGATTTT  540
Sbjct  1722393  CTCAAGGTCAAGTTTGGTTCTGACTTTGAAAAGACAGGGAATTCTCTTGATATTGATTTT  1722334
Query  541      AACTCTGTCCATTCAGGTGAAAAGCAGATTCAGATTGTTAATTTTAAGCAGATTTATTAT  600
Sbjct  1722333  AACTCTGTCCATTCAGGTGAAAAGCAGATTCAGATTGTTAATTTTAAGCAGATTTATTAT  1722274
Query  601      ACAGTCAGCGTAGACGCTGTTAAAAATCCAGGAGATGTGTTTCAAGATACTGTAACGGTA  660
Sbjct  1722273  ACAGTCAGCGTAGACGCTGTTAAAAATCCAGGAGATGTGTTTCAAGATACTGTAACGGTA  1722214
Query  661      GAGGATTTAAAACAGAGAGGAATTTCTGCAGAGCGTCCTTTGGTCTATATTTCGAGTGTT  720
Sbjct  1722213  GAGGATTTAAAACAGAGAGGAATTTCTGCAGAGCGTCCTTTGGTCTATATTTCGAGTGTT  1722154
Query  721      GCTTATGGGCGCCAAGTCTATCTCAAGTTGGAAACCACGAGTAAGAGTGATGAAGTAGAG  780
Sbjct  1722153  GCTTATGGGCGCCAAGTCTATCTCAAGTTGGAAACCACGAGTAAGAGTGATGAAGTAGAG  1722094
Query  781      GCTGCTTTTGAAGCTTTGATGAAAGGAGTCAAGGTAGCTCCTCAGACAGAGTGGAAGCAG  840
Sbjct  1722093  GCTGCTTTTGAAGCTTTGATAAAAGGAGTCAAGGTAGCTCCTCAGACAGAGTGGAAGCAG  1722034
Query  841      ATTTTGGACAATACAGAAGTGAAGGCGGTTATTTTAGGGGGCGACCCAAGTTCGGGTGCC  900
Sbjct  1722033  ATTTTGGACAATACAGAAGTGAAGGCGGTTATTTTAGGGGGCGACCCAAGTTCGGGTGCC  1721974
Query  901      CGAGTTGTAACAGGCAAGGTGGATATGGTAGAGGACTTGATTCAAGAAGGCAGTCGCTTT  960
Sbjct  1721973  CGAGTTGTAACAGGCAAGGTGGATATGGTAGAGGACTTGATTCAAGAAGGCAGTCGCTTT  1721914
Query  961      ACAGCAGATCATCCAGGCTTGCCGATTTCCTATACAACTTCTTTTTTACGTGACAATGTA  1020
Sbjct  1721913  ACAGCAGATCATCCAGGCTTGCCGATTTCCTATACAACTTCTTTTTTACGTGACAATGTA  1721854
Query  1021     GTTGCGACCTTTCAAAACAGTACAGACTATGTTGAGACTAAGGTTACAGCTTACAGAAAC  1080
Sbjct  1721853  GTTGCGACCTTTCAAAACAGTACAGACTATGTTGAGACTAAGGTTACAGCTTACAGAAAC  1721794
Query  1081     GGAGATTTACTGCTGGATCATAGTGGTGCCTATGTTGCCCAATATTATATTACTTGGGAT  1140
Sbjct  1721793  GGAGATTTACTGCTGGATCATAGTGGTGCCTATGTTGCCCAATATTATATTACTTGGGAT  1721734
Query  1141     GAATTATCCTATGATCATCAAGGTAAGGAAGTCTTGACTCCTAAGGCTTGGGACAGAAAT  1200
Sbjct  1721733  GAATTATCCTATGATCATCAAGGTAAGGAAGTCTTGACTCCTAAGGCTTGGGACAGAAAT  1721674
Query  1201     GGGCAGGATTTGACGGCTCACTTTACCACTAGTATTCCTTTAAAAGGGAATGTTCGTAAT  1260
Sbjct  1721673  GGGCAGGATTTGACGGCTCACTTTACCACTAGTATTCCTTTAAAAGGGAATGTTCGTAAT  1721614
Query  1261     CTCTCTGTCAAAATTAGAGAGTGTACCGGGCTTGCCTGGGAATGGTGGCGTACGGTTTAT  1320
Sbjct  1721613  CTCTCTGTCAAAATTAGAGAGTGTACCGGGCTTGCCTGGGAATGGTGGCGTACGGTTTAT  1721554
Query  1321     GAAAAAACCGATTTGCCACTAGTGCGTAAGCGGACGATTTCTATTTGGGGAACAACTCTC  1380
Sbjct  1721553  GAAAAAACCGATTTGCCACTAGTGCGTAAGCGGACGATTTCTATTTGGGGAACAACTCTC  1721494
Query  1381     TATCCTCAGGTAGAGGATAAGGTAGAAAATGACTAG  1416
Sbjct  1721493  TATCCTCAGGTAGAGGATAAGGTAGAAAATGACTAG  1721458
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Ply allele 11

Alignments

Streptococcus pneumoniae D39, complete genome
Sequence ID: **CP000410.2** Length: 2046116 Number of Matches: 1
Range 1: 1721458 to 1722873

Score	Expect	Identities	Gaps	Strand	Frame
2604 bits(1410)	0.0()	1414/1416(99%)	0/1416(0%)	Plus/Minus	

Features:

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Query  1        ATGGCAAATAAAGCAGTAAATGACTTTATACTAGCTATGAATTACGATAAAAAGAAACTC  60
Sbjct  1722873  ATGGCAAATAAAGCAGTAAATGACTTTATACTAGCTATGAATTACGATAAAAAGAAACTC  1722814

Query  61       TTGACCCATCAGGGAGAAAGTATTGAAAATCGTTTCATCAAAGAGGGTAATCAGCTACCC  120
Sbjct  1722813  TTGACCCATCAGGGAGAAAGTATTGAAAATCGTTTCATCAAAGAGGGTAATCAGCTACCC  1722754

Query  121      GATGAGTTTGTTGTTATCGAAAGAAAGAAGCGGAGCTTGTCGACAAATACAAGTGATATT  180
Sbjct  1722753  GATGAGTTTGTTGTTATCGAAAGAAAGAAGCGGAGCTTGTCGACAAATACAAGTGATATT  1722694

Query  181      TCTGTAACAGCTACCAACGACAGTCGCCTCTATCCTGGAGCACTTCTCGTAGTGGATGAG  240
Sbjct  1722693  TCTGTAACAGCTACCAACGACAGTCGCCTCTATCCTGGAGCACTTCTCGTAGTGGATGAG  1722634

Query  241      ACCTTGTTAGAGAATAATCCCACTCTTCTTGCGGTTGATCGTGCTCCGATGACTTATAGT  300
Sbjct  1722633  ACCTTGTTAGAGAATAATCCCACTCTTCTTGCGGTTGATCGTGCTCCGATGACTTATAGT  1722574

Query  301      ATTGATTTGCCTGGTTTGGCAAGTAGCGATAGCTTTCTCCAAGTGGAAGACCCCAGCAAT  360
Sbjct  1722573  ATTGATTTGCCTGGTTTGGCAAGTAGCGATAGCTTTCTCCAAGTGGAAGACCCCAGCAAT  1722514

Query  361      TCAAGTGTTCGCGGAGCGGTAAACGATTTGTTGGCTAAGTGGCATAAAGATTATGGTCAG  420
Sbjct  1722513  TCAAGTGTTCGCGGAGCGGTAAACGATTTGTTGGCTAAGTGGCATCAAGATTATGGTCAG  1722454

Query  421      GTCAATAATGTCCCAGCTAGAATGCAGTATGAAAAAATAACGGCTCACAGCATGGAACAA  480
Sbjct  1722453  GTCAATAATGTCCCAGCTAGAATGCAGTATGAAAAAATAACGGCTCACAGCATGGAACAA  1722394

Query  481      CTCAAGGTCAAGTTTGGTTCTGACTTTGAAAAGACAGGGAATTCTCTTGATATTGATTTT  540
Sbjct  1722393  CTCAAGGTCAAGTTTGGTTCTGACTTTGAAAAGACAGGGAATTCTCTTGATATTGATTTT  1722334

Query  541      AACTCTGTCCATTCAGGTGAAAAGCAGATTCAGATTGTTAATTTTAAGCAGATTTATTAT  600
Sbjct  1722333  AACTCTGTCCATTCAGGTGAAAAGCAGATTCAGATTGTTAATTTTAAGCAGATTTATTAT  1722274

Query  601      ACAGTCAGCGTAGACGCTGTTAAAAATCCAGGAGATGTGTTTCAAGATACTGTAACGGTA  660
Sbjct  1722273  ACAGTCAGCGTAGACGCTGTTAAAAATCCAGGAGATGTGTTTCAAGATACTGTAACGGTA  1722214

Query  661      GAGGATTTAAAACAGAGAGGAATTTCTGCAGAGCGTCCTTTGGTCTATATTTCGAGTGTT  720
Sbjct  1722213  GAGGATTTAAAACAGAGAGGAATTTCTGCAGAGCGTCCTTTGGTCTATATTTCGAGTGTT  1722154

Query  721      GCTTATGGGCGCCAAGTCTATCTCAAGTTGGAAACCACGAGTAAGAGTGATGAAGTAGAG  780
Sbjct  1722153  GCTTATGGGCGCCAAGTCTATCTCAAGTTGGAAACCACGAGTAAGAGTGATGAAGTAGAG  1722094

Query  781      GCTGCTTTTGAAGCTTTGATAAAAGGAGTCAAGGTAGCTCCTCAGACAGAGTGGAAGCAG  840
Sbjct  1722093  GCTGCTTTTGAAGCTTTGATAAAAGGAGTCAAGGTAGCTCCTCAGACAGAGTGGAAGCAG  1722034

Query  841      ATTTTGGACAATACAGAAGTGAAGGCGGTTATTTTAGGGGGCGACCCAAGTTCGGGTGCC  900
Sbjct  1722033  ATTTTGGACAATACAGAAGTGAAGGCGGTTATTTTAGGGGGCGACCCAAGTTCGGGTGCC  1721974

Query  901      CGAGTTGTAACAGGCAAGGTGGATATGGTAGAGGACTTGATTCAAGAAGGCAGTCGCTTT  960
Sbjct  1721973  CGAGTTGTAACAGGCAAGGTGGATATGGTAGAGGACTTGATTCAAGAAGGCAGTCGCTTT  1721914

Query  961      ACAGCAGATCATCCAGGCTTGCCGATTTCCTATACAACTTCTTTTTTACGTGACAATGTA  1020
Sbjct  1721913  ACAGCAGATCATCCAGGCTTGCCGATTTCCTATACAACTTCTTTTTTACGTGACAATGTA  1721854

Query  1021     GTTGCGACCTTTCAAAACAGTACAGACTATGTTGAGACTAAGGTTACAGCTTACAGAAAC  1080
Sbjct  1721853  GTTGCGACCTTTCAAAACAGTACAGACTATGTTGAGACTAAGGTTACAGCTTACAGAAAC  1721794

Query  1081     GGAGATTTACTGCTGGATCATAGTGGTGCCTATGTTGCCCAATATTATATTACTTGGAAT  1140
Sbjct  1721793  GGAGATTTACTGCTGGATCATAGTGGTGCCTATGTTGCCCAATATTATATTACTTGGGAT  1721734

Query  1141     GAATTATCCTATGATCATCAAGGTAAGGAAGTCTTGACTCCTAAGGCTTGGGACAGAAAT  1200
Sbjct  1721733  GAATTATCCTATGATCATCAAGGTAAGGAAGTCTTGACTCCTAAGGCTTGGGACAGAAAT  1721674

Query  1201     GGGCAGGATTTGACGGCTCACTTTACCACTAGTATTCCTTTAAAAGGGAATGTTCGTAAT  1260
Sbjct  1721673  GGGCAGGATTTGACGGCTCACTTTACCACTAGTATTCCTTTAAAAGGGAATGTTCGTAAT  1721614

Query  1261     CTCTCTGTCAAAATTAGAGAGTGTACCGGGCTTGCCTGGGAATGGTGGCGTACGGTTTAT  1320
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Query  1321     GAAAAAACCGATTTGCCACTAGTGCGTAAGCGGACGATTTCTATTTGGGGAACAACTCTC  1380
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Query  1381     TATCCTCAGGTAGAGGATAAGGTAGAAAATGACTAG  1416
Sbjct  1721493  TATCCTCAGGTAGAGGATAAGGTAGAAAATGACTAG  1721458
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Ply allele 12

Alignments

Streptococcus pneumoniae D39, complete genome
Sequence ID: **CP000410.2** Length: 2046116 Number of Matches: 1
Range 1: 1721458 to 1722873

Score	Expect	Identities	Gaps	Strand	Frame
2604 bits(1410)	0.0()	1414/1416(99%)	0/1416(0%)	Plus/Minus	

Features:

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Sbjct  1722873  ATGGCAAATAAAGCAGTAAATGACTTTATACTAGCTATGAATTACGATAAAAAGAAACTC  1722814
Query  61       TTGACCCATCAGGGAGAAAGTATTGAAAATCGTTTCATCAAAGAGGGTAATCAGCTACCC  120
Sbjct  1722813  TTGACCCATCAGGGAGAAAGTATTGAAAATCGTTTCATCAAAGAGGGTAATCAGCTACCC  1722754
Query  121      GATGAGTTTGTTGTTATCGAAAGAAAGAAGCGGAGCTTGTCGACAAATACAAGTGATATT  180
Sbjct  1722753  GATGAGTTTGTTGTTATCGAAAGAAAGAAGCGGAGCTTGTCGACAAATACAAGTGATATT  1722694
Query  181      TCTGTAACAGCTACCAACGACAGTCGCCTCTATCCTGGAGCACTTCTCGTAGTGGATGAG  240
Sbjct  1722693  TCTGTAACAGCTACCAACGACAGTCGCCTCTATCCTGGAGCACTTCTCGTAGTGGATGAG  1722634
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Sbjct  1722633  ACCTTGTTAGAGAATAATCCCACTCTTCTTGCGGTTGATCGTGCTCCGATGACTTATAGT  1722574
Query  301      ATTGATTTGCCTGGTTTGGCAAGTAGCGATAGCTTTCTCCAAGTGGAAGACCCCAGCAAT  360
Sbjct  1722573  ATTGATTTGCCTGGTTTGGCAAGTAGCGATAGCTTTCTCCAAGTGGAAGACCCCAGCAAT  1722514
Query  361      TCAAGTGTTCGCGGAGCGGTAAACGATTTGTTGGCTAAGTGGCATCAAGATTATGGTCAG  420
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Query  421      GTCAATAATGTCCCAGCTAGAATGCAGTATGAAAAAATAACGGCTCACAGCATGGAACAA  480
Sbjct  1722453  GTCAATAATGTCCCAGCTAGAATGCAGTATGAAAAAATAACGGCTCACAGCATGGAACAA  1722394
Query  481      CTCAAGGTCAAGTTTGGTTCTGACTTTGAAAAGACAGGGAATTCTCTTGATATTGATTTT  540
Sbjct  1722393  CTCAAGGTCAAGTTTGGTTCTGACTTTGAAAAGACAGGGAATTCTCTTGATATTGATTTT  1722334
Query  541      AACTCTGTCCATTCAGGTGAAAAGCAGATTCAGATTGTTAATTTTAAGCAGATTTATTAT  600
Sbjct  1722333  AACTCTGTCCATTCAGGTGAAAAGCAGATTCAGATTGTTAATTTTAAGCAGATTTATTAT  1722274
Query  601      ACAGTCAGCGTAGACGCTGTTAAAAATCCAGGAGATGTGTTTCAAGATACTGTAACGGTA  660
Sbjct  1722273  ACAGTCAGCGTAGACGCTGTTAAAAATCCAGGAGATGTGTTTCAAGATACTGTAACGGTA  1722214
Query  661      GAGGATTTAAAACAGAGAGGAATTTCTGCAGAGCGTCCTTTGGTCTATATTTCGAGTGTT  720
Sbjct  1722213  GAGGATTTAAAACAGAGAGGAATTTCTGCAGAGCGTCCTTTGGTCTATATTTCGAGTGTT  1722154
Query  721      GCTTATGGGCGCCAAGTCTATCTCAAGTTGGAAACCACGAGTAAGAGTGATGAAGTAGAG  780
Sbjct  1722153  GCTTATGGGCGCCAAGTCTATCTCAAGTTGGAAACCACGAGTAAGAGTGATGAAGTAGAG  1722094
Query  781      GCTGCTTTTGAAGCTTTGATAAAAGGAGTCAAGGTAGCTCCTCAGACAGAGTGGAAGCAG  840
Sbjct  1722093  GCTGCTTTTGAAGCTTTGATAAAAGGAGTCAAGGTAGCTCCTCAGACAGAGTGGAAGCAG  1722034
Query  841      ATTTTGGACAATACAGAAGTGAAGGCGGTTATTTTAGGGGGCGACCCAAGTTCGGGTGCC  900
Sbjct  1722033  ATTTTGGACAATACAGAAGTGAAGGCGGTTATTTTAGGGGGCGACCCAAGTTCGGGTGCC  1721974
Query  901      CGAGTTGTAACAGGCAAGGTGGATATGGTAGAGGACTTGATTCAAGAAGGCAGTCGCTTT  960
Sbjct  1721973  CGAGTTGTAACAGGCAAGGTGGATATGGTAGAGGACTTGATTCAAGAAGGCAGTCGCTTT  1721914
Query  961      ACAGCAGATCATCCAGGCTTGCCGATTTCCTATACAACTTCTTTTTTTACGTGACAATGTA  1020
Sbjct  1721913  ACAGCAGATCATCCAGGCTTGCCGATTTCCTATACAACTTCTTTTTTTACGTGACAATGTA  1721854
Query  1021     GTTGCGACCTTTCAAAACAGTACAGACTATGTTGAGACTAAGGTTACAGCTTACAGAAAC  1080
Sbjct  1721853  GTTGCGACCTTTCAAAACAGTACAGACTATGTTGAGACTAAGGTTACAGCTTACAGAAAC  1721794
Query  1081     GGAGATTTACTGCTGGATCATAGTGGTGCCTATGTTGCCCAATATTATATTACTTGGAAT  1140
Sbjct  1721793  GGAGATTTACTGCTGGATCATAGTGGTGCCTATGTTGCCCAATATTATATTACTTGGGAT  1721734
Query  1141     GAATTATCCTATGATCATCAAGGTAAGGAAGTCTTGACTCCTAAGGCTTGGGACAGAAAT  1200
Sbjct  1721733  GAATTATCCTATGATCATCAAGGTAAGGAAGTCTTGACTCCTAAGGCTTGGGACAGAAAT  1721674
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Sbjct  1721673  GGGCAGGATTTGACGGCTCACTTTACCACTAGTATTCCTTTAAAAGGGAATGTTCGTAAT  1721614
Query  1261     CTCTCTGTCAAAATTAGAGAGTGTACCGGGCTTGCCTGGGAATGGTGGCGTACGGTTTAT  1320
Sbjct  1721613  CTCTCTGTCAAAATTAGAGAGTGTACCGGGCTTGCCTGGGAATGGTGGCGTACGGTTTAT  1721554
Query  1321     GAAAAAACCGATTTGCCACTAGTGCGTAAGCGGACGATTTCTATTTGGGGAACAACTCTC  1380
Sbjct  1721553  GAAAAAACCGATTTGCCACTAGTGCGTAAGCGGACGATTTCTATTTGGGGAACAACTCTC  1721494
Query  1381     TATCCTCAGGTAGAGGATAAGGTAGAAAATGACTAG  1416
Sbjct  1721493  TATCCTCAGGTAGAGGATAAGGTAGAAAATGACTAG  1721458
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Ply allele 15

Alignments

Streptococcus pneumoniae D39, complete genome
Sequence ID: **CP000410.2** Length: 2046116 Number of Matches: 1
Range 1: 1721458 to 1722873

Score	Expect	Identities	Gaps	Strand	Frame
2610 bits(1413)	0.0()	1415/1416(99%)	0/1416(0%)	Plus/Minus	

Features:

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Query  1        ATGGCAAATAAAGCAGTAAATGACTTTATACTAGCTATGAATTACGATAAAAAGAAACTC  60
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Query  61       TTGACCCATCAGGGAGAAAGTATTGAAAATCGTTTCATCAAAGAGGGTAATCAGCTACCC  120
Sbjct  1722813  TTGACCCATCAGGGAGAAAGTATTGAAAATCGTTTCATCAAAGAGGGTAATCAGCTACCC  1722754
Query  121      GATGAGTTTGTTGTTATCGAAAGAAAGAAGCGGAGCTTGTCGACAAATACAAGTGATATT  180
Sbjct  1722753  GATGAGTTTGTTGTTATCGAAAGAAAGAAGCGGAGCTTGTCGACAAATACAAGTGATATT  1722694
Query  181      TCTGTAACAGCTACCAACGACAGTCGCCTCTATCCTGGAGCACTTCTCGTAGTGGATGAG  240
Sbjct  1722693  TCTGTAACAGCTACCAACGACAGTCGCCTCTATCCTGGAGCACTTCTCGTAGTGGATGAG  1722634
Query  241      ACCTTGTTAGAGAATAATCCCACTCTTCTTGCGGTTGATCGTGCTCCGATGACTTATAGT  300
Sbjct  1722633  ACCTTGTTAGAGAATAATCCCACTCTTCTTGCGGTTGATCGTGCTCCGATGACTTATAGT  1722574
Query  301      ATTGATTTGCCTGGTTTGGCAAGTAGCGATAGCTTTCTCCAAGTGGAAGACCCCAGCAAT  360
Sbjct  1722573  ATTGATTTGCCTGGTTTGGCAAGTAGCGATAGCTTTCTCCAAGTGGAAGACCCCAGCAAT  1722514
Query  361      TCAAGTGTTCGCGGAGCGGTAAACGATTTGTTGGCTAAGTGGCATCAAGATTATGGTCAG  420
Sbjct  1722513  TCAAGTGTTCGCGGAGCGGTAAACGATTTGTTGGCTAAGTGGCATCAAGATTATGGTCAG  1722454
Query  421      GTCAATAATGTCCCAGCTAGAATGCAGTATGAAAAAATAACGGCTCACAGCATGGAACAA  480
Sbjct  1722453  GTCAATAATGTCCCAGCTAGAATGCAGTATGAAAAAATAACGGCTCACAGCATGGAACAA  1722394
Query  481      CTCAAGGTCAAGTTTGGTTCTGACTTTGAAAAGACAGGGAATTCTCTTGATATTGATTTT  540
Sbjct  1722393  CTCAAGGTCAAGTTTGGTTCTGACTTTGAAAAGACAGGGAATTCTCTTGATATTGATTTT  1722334
Query  541      AACTCTGTCCATTCAGGTGAAAAGCAGATTCAGATTGTTAATTTTAAGCAGATTTATTAT  600
Sbjct  1722333  AACTCTGTCCATTCAGGTGAAAAGCAGATTCAGATTGTTAATTTTAAGCAGATTTATTAT  1722274
Query  601      ACAGTCAGCGTAGACGCTGTTAAAAATCCAGGAGATGTGTTTCAAGATACTGTAACGGTA  660
Sbjct  1722273  ACAGTCAGCGTAGACGCTGTTAAAAATCCAGGAGATGTGTTTCAAGATACTGTAACGGTA  1722214
Query  661      GAGGATTTAAAACAGAGAGGAATTTCTGCAGAGCGTCCTTTGGTCTATATTTCGAGTGTT  720
Sbjct  1722213  GAGGATTTAAAACAGAGAGGAATTTCTGCAGAGCGTCCTTTGGTCTATATTTCGAGTGTT  1722154
Query  721      GCTTATGGGCGCCAAGTCTATCTCAAGTTGGAAACCACGAGTAAGAGTGATGAAGTAGAT  780
Sbjct  1722153  GCTTATGGGCGCCAAGTCTATCTCAAGTTGGAAACCACGAGTAAGAGTGATGAAGTAGAG  1722094
Query  781      GCTGCTTTTGAAGCTTTGATAAAAGGAGTCAAGGTAGCTCCTCAGACAGAGTGGAAGCAG  840
Sbjct  1722093  GCTGCTTTTGAAGCTTTGATAAAAGGAGTCAAGGTAGCTCCTCAGACAGAGTGGAAGCAG  1722034
Query  841      ATTTTGGACAATACAGAAGTGAAGGCGGTTATTTTAGGGGGCGACCCAAGTTCGGGTGCC  900
Sbjct  1722033  ATTTTGGACAATACAGAAGTGAAGGCGGTTATTTTAGGGGGCGACCCAAGTTCGGGTGCC  1721974
Query  901      CGAGTTGTAACAGGCAAGGTGGATATGGTAGAGGACTTGATTCAAGAAGGCAGTCGCTTT  960
Sbjct  1721973  CGAGTTGTAACAGGCAAGGTGGATATGGTAGAGGACTTGATTCAAGAAGGCAGTCGCTTT  1721914
Query  961      ACAGCAGATCATCCAGGCTTGCCGATTTCCTATACAACTTCTTTTTTACGTGACAATGTA  1020
Sbjct  1721913  ACAGCAGATCATCCAGGCTTGCCGATTTCCTATACAACTTCTTTTTTACGTGACAATGTA  1721854
Query  1021     GTTGCGACCTTTCAAAACAGTACAGACTATGTTGAGACTAAGGTTACAGCTTACAGAAAC  1080
Sbjct  1721853  GTTGCGACCTTTCAAAACAGTACAGACTATGTTGAGACTAAGGTTACAGCTTACAGAAAC  1721794
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Ply allele 18

Alignments

Streptococcus pneumoniae D39, complete genome
Sequence ID: **CP000410.2** Length: 2046116 Number of Matches: 1
Range 1: 1721458 to 1722873

Score	Expect	Identities	Gaps	Strand	Frame
2610 bits(1413)	0.0()	1415/1416(99%)	0/1416(0%)	Plus/Minus	

Features:

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DNA sequence of pORI280 plasmid

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       190        200        210        220        230        240
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       910        920        930        940        950        960
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GGAAAAAATA TGGGCATTTT TAGTATTTTT GTAATCAGCA CAGTTCATTA TCAACCAAAC

      1450       1460       1470       1480       1490       1500
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      1570       1580       1590       1600       1610       1620
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      1930       1940       1950       1960       1970       1980
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      1990       2000       2010       2020       2030       2040
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      2050       2060       2070       2080       2090       2100
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      2230       2240       2250       2260       2270       2280
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      3250       3260       3270       3280       3290       3300
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CCTTTCGGCG GTGAAATTAT CGATGAGCGT GGTGGTTATG CCGATCGCGT CACACTACGT

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       373         374         375         376         377         378
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       397         398         399         400         401         402
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       403         404         405         406         407         408
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       409         410         411         412         413         414
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       415         416         417         418         419         420
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       439         440         441         442         443         444
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       457         458         459         460         461         462
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       463         464         465         466         467         468
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