Group A Streptococcal Peptides Expressed in HBsAg-S VLPs as a Vaccine Candidate

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Declaration

I certify that the work reported in this thesis is entirely my own effort, except where otherwise acknowledged. I also certify that the work is original and has not been previously submitted for assessment in any other course of study at this or any other institution.

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Signature of Candidate

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Date

Endorsement

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	Date		Date

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Abstract

Streptococcus pyogenes, or Group A Streptococcus (GAS) is responsible for significant patient morbidity and mortality in the developing world and within the Australian Indigenous population. GAS is responsible for a variety of diseases such as invasive necrotizing fasciitis and toxic shock syndrome, as well as non-invasive diseases, such as pharyngitis, impetigo, scarlet fever and otitis media. However, GAS sequelae such as rheumatic fever and rheumatic heart disease are responsible for the highest morbidity. The 30-valent vaccine candidate currently in trials is inappropriately specialised to serotypes present in areas with low GAS incidence, such as the United States.

The difficulty in creation of a suitable vaccine lies in part with the variety of GAS virulence factors. The M protein is a highly abundant, multifunctional immunogenic surface protein which confers resistance to phagocytes and complement mediated protection. As sections of the M protein is highly conserved, it has been the focus of vaccination research. Furthermore, protein fragments J8 and J14 within the M protein have given encouraging results within a mouse model.

Virus-like particle (VLP) technology offers a promising alternative to existing vaccination delivery systems. VLPs are able to induce both cell mediated and humoral immune responses. In this study, the use of a chimeric hepatitis B surface antigen VLP expressing M protein epitopes p145, J8 and J14 for use as a dual vaccine against Hepatitis B virus (HBV) and GAS is investigated. Specifically, PCR generated DNA sequences of J8, J14 and p145 from the M protein of GAS have been cloned into the highly immunogenic ‘a’ determinant region of the HBsAg-S VLP and transformed into human embryonic kidney (HEK293T) cells. Expressed recombinant HBsAg-S-GAS-m protein constructs were assayed by ELISA to confirm presentation of GAS epitopes. ELISA results showing high titres were obtained for VLP:p145 but low titres were obtained for VLP:J8 and VLP:J14. Further sequencing of

plasmid constructs, protein expression and antigenic screening of proteins is required before the study can progress to proof-of-concept murine challenge models.

Abbreviations

GAS	Group A Streptococcus
GBS	Group B Streptococcus
RF	Rheumatic Fever
RHD	Rheumatic Heart Disease
APSGN	Acute post-streptococcal glomerulonephritis
WHO	World Health Organisation
GBM	Glomerular Basement Membrane
SpeB	Streptococcal exotoxin B
VLP	Virus-Like Particles
URTIs	Upper Respiratory Tract Infections
APCs	Antigen Presenting Cells
ELISA	Enzyme-linked Immunosorbent Assay
TCSs	Two Component Signal Transduction Systems
GRAB	G-Related α_2-macroglobulin-binding Protein
SK	Streptokinase
HI	Humoral Immunity
CMI	Cell Mediated Immunity
MEM	Minimal Essential Media
PBS	Phosphate Buffered Saline
HEK293T	Human Embryonic Kidney Cells 293T
HEPEs	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HBS	Hepes Buffered Saline
CFA	Complete Freund's Adjuvant
RT	Room Temperature

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Introduction

1.0 Overview of group A streptococcus (GAS) pathogenesis

The bacterium Group A Streptococcus (GAS) (also known as *Streptococcus pyogenes*) is a Gram positive coccus which is responsible for a variety of infections and associated syndromes (Goering *et al.* 2012). GAS is prevalent in under-privileged communities such as the Australian Indigenous population and much of the developing world (Carapetis *et al.* 2005). GAS can cause a number of non-invasive diseases such as pharyngitis, impetigo, pyoderma, scarlet fever and otitis media as well as invasive diseases including necrotising fasciitis and toxic shock syndrome. Post-infection sequelae, however account for the highest global burden of GAS disease and includes rheumatic heart disease (RHD), rheumatic fever (RF), related endocarditis and stroke, as well as acute post-streptococcal glomerulonephritis (APSGN) (Henningham *et al.* 2013). Whilst research activity in GAS vaccinology has been ongoing for decades, no approved vaccine has yet become available. A broad 30-valent vaccine is under development, however the vaccine focusses on North American and European specific GAS serotypes which may not address the main serotypes present in the developing world. One of the main challenges in vaccine design is the incorporation of the 150 known M protein serotypes to provide cross-serotype protection. Studies of pharyngeal infections in South African school children indicate the vaccine coverage will be between 40-59% of isolates recorded (Dale *et al.* 2011). This indicates that the 30-valent vaccine is an ineffective option for a significant proportion of affected individuals in the developing world (Dale *et al.* 2011). Among the many conserved virulence factors which have been explored for usage in a GAS vaccine, the M protein is a promising target for vaccine research as it is a highly conserved, abundant surface GAS protein (O'Brien *et al.* 2002).

1.1 Distribution and incidence of disease

Currently 18.1 million people worldwide suffer GAS associated infections. GAS associated sequelae makes up the majority of cases with 15.6 million individuals currently affected.

Furthermore, RHD can be fatal and is estimated to cause 233,000 deaths per year (Carapetis *et al.* 2005). APSGN affects 472,000 individuals and causes a further 5,000 deaths annually. Superficial infections occur more frequently, where 6.1 million new cases of pharyngitis arise every year and 1.1 million individuals currently suffer of pyoderma (Carapetis *et al.* 2005). Uncertainty levels and assumptions used in the compilation of GAS data tends towards underestimation, making the true prevalence likely to be greater than stated. Carapetis *et al.* (2005) acknowledges that issues in data collection in developing countries affects quality of the data. RHD specific data, however, has a higher level of quality through rigorous data collection. RHD has a wide geographical distribution, peaking in the following countries as a calculated regional prevalence per thousand: Sub-Saharan Africa at 5.7 cases, South-Central Asia at 2.2 cases, other Asian areas at 0.8 cases, Latin America at 1.3 cases, Middle East and North Africa at 1.8 cases, Eastern Europe at 1 case, Pacific and Indigenous Australia/New Zealand at 3.5 cases and China at 0.8 cases (Carapetis *et al.* 2005).

1.2 Populations at risk

Populations at risk for GAS disease include children and young adults within the developed and developing countries. Less developed countries account for 79% of RHD cases, 95% of RF cases, 97% of APSGN cases and 97% of invasive GAS cases. Indigenous Australians also experience high disease burden (Carapetis *et al.* 2005).

1.2.1 Indigenous Australians

As mentioned above, Indigenous Australians are a demographic susceptible to GAS infection, most commonly suffering superficial skin infections. Furthermore, APSGN, RF and RHD are heavily prevalent in the Indigenous population (Carapetis *et al.* 2005). Indigenous Australians have the highest annual mortality rate of 30.2 per 100,000 individuals. This is more than three times as high as the second highest risk population, Maori New Zealanders at 9.6 per 100,000 individuals (Carapetis *et al.* 2005). Rural communities within the Northern Territory and the Kimberley region in Western Australia

exhibit an annual incidence of RF of between 2-7 cases for every 1000 children between 5-14 years old. Furthermore, up to 3% of Indigenous people in rural communities have established RHD in Australia (Carapetis & Currie 1998).

GAS related skin infections such as scabies and streptococcal pyoderma are endemic in Australian Indigenous communities. Scabies is a parasitic infection caused by the human itch mite or *Sarcopes scabiei,* and is known to spread easily in environments with poor sanitation and overcrowding (FitzGerald *et al.* 2014). There is a distinct link between streptococcal pyoderma and scabies, as demonstrated by Andrews *et al.* (2009) (See Figure 1). For example, both scabies and streptococcal pyoderma have been found in Indigenous Australian populations and Alaskan native populations. Scabies is prevalent in 50% of children and 25% of adults in many remote communities (Carapetis *et al.* 1997). Unusually, there is a distinctly lower rate of GAS throat carriage of between 0-14% in comparison to RHD and skin infections (Van Buynder *et al.* 1992). Household crowding, access to adequate water, hot weather, humidity and lack of personal hygiene are likely to contribute to a high prevalence of GAS disease in the Indigenous Australian population (Figure 1) (Munoz *et al.* 1992).

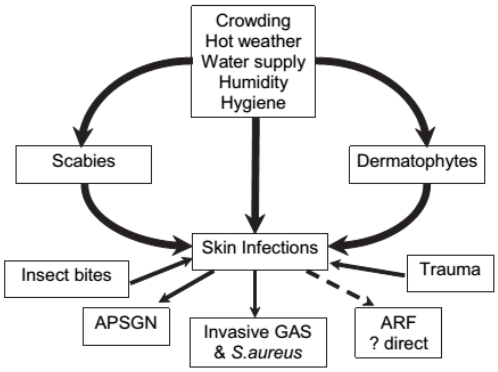

Figure 1 Outcomes and contributing factors to skin infections in Australian Indigenous peoples

A number of contributing factors can culminate to form a skin infection, which may progress to secondary disease states such as acute post-streptococcal glomerulonephritis (APSGN) and acute rheumatic fever (ARF) after GAS infection (Andrews *et al.* 2009).

1.2.2 Paediatric

GAS pharyngitis accounts for 6% of paediatric visits to a medical practitioner, with GAS cultured from 15-36% of children suffering a sore throat in the US (Linder *et al.* 2005). APSGN is also most commonly seen in paediatric patients, equating to 90% of the total population suffering APSGN. The skewed distribution towards paediatric patients is hypothesised to be attributed to the size difference in the glomerular basement membrane (GBM). Children and adults have 2-3 nm and 4-4.5 nm GBM sizes, respectively, making it easier for the immune complex molecule to infect the glomerulus in children rather than adults (Wiwanitkit 2006).

2.0 The biology of group A streptococcus

Infection with GAS begins with the binding of bacterial surface ligands to extracellular matrix components or to specific receptors on dermal epithelial cells. Adhesion is a two-step process, where an initial weak interaction is engaged with the mucosa to overcome electrostatic repulsion. The exchange is finalised through an event conferring tissue specificity and high-avidity adherence. To complete the interchange, a number of host components are known to be involved, such as fibrinogen, fibrin, elastin, vitronectin, laminin, decorin and heparin sulphate-containing proteoglycans (Moschioni *et al.* 2010). In particular, fibrinogen is known to interact and bind with the highly conserved M protein in the β–repeat region near the N-terminus of the protein (Carlsson *et al.* 2005).

As well as cellular adherence, GAS also has the capacity to enter epithelial cells to avoid early host defences and antibiotics. This invasive virulence process is possible through proteins on the cell surface, or invasins such as fibronectin-binding protein and the M protein (LaPenta *et al.* 1994). Further research by Dombek and colleagues (1999) indicates that invasion initiates through a zipper-like mechanism where host microvilli also play a role.

2.1 Virulence factors

2.1.1 M protein

M protein is a highly abundant, multifunctional, immunogenic surface protein. Structurally, the M protein is an alpha-helical coiled coil dimer anchored to the outer streptococcal cell wall via the C terminus. The M protein can be divided up into the hypervariable (A-repeat region), semi-variable (B-repeat region) and conserved regions (C, D, Pro/Gly and Hydrophobic regions) (See Figure 2) (McArthur & Walker 2006). Lancefield GAS classification is measured through N-terminal nucleotide residues of the *emm* gene, found in the aforementioned hyper-variable region. Specifically, Lancefield typing groups beta haemolytic bacteria through cell wall carbohydrate composition. Using the N-terminal nucleotides as a determinant, more than 150 genotypes of GAS have been found thus far (Facklam 1997; McGregor *et al.* 2004). M proteins are divided into Class I or Class II dependent upon their reaction with antibodies against the C repeat region of the M protein; Class I proteins react through the presence of a surface-exposed epitope and Class II does not react (Bessen *et al.* 1989).

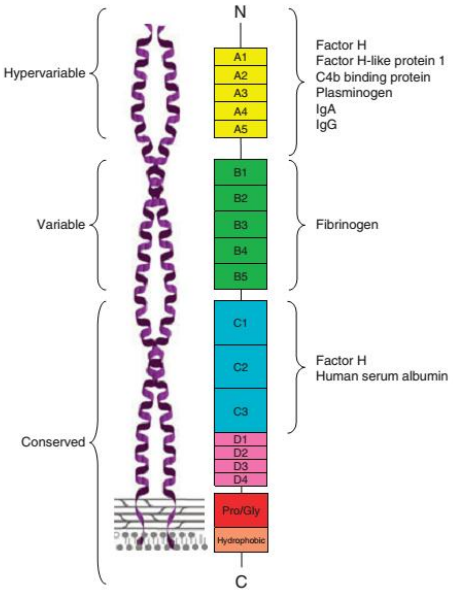

Figure 2 M protein hypervariable, variable and conserved structures
The M protein alpha-helical coiled coil structure with anchored domain. A to C regions of the M protein are multi-functional, and the protein can be divided further into conserved, variable and hypervariable regions. Each colour-coded section can interact with the human plasma proteins indicated (McArthur & Walker 2006).

M Protein antigenic variation contributes to the range of GAS virulence strategies. For example, the ability of M protein to bind to fibrinogen in the B-repeat region interferes with the complement system and contributes to phagocytic resistance (McArthur & Walker 2006; Ringdahl *et al.* 2000). The M protein provides protection against complement-mediated opsonisation and phagocytic resistance. Specifically, the M protein binds C4b-binding protein which inhibits complement activation (Berggård *et al.* 2001). The A-repeat region can bind to IgA, IgG, Factor H, Factor H-like protein 1, C4b binding protein and plasminogen. The C-repeat region can bind Factor H and human serum albumin, further aiding GAS in phagocytic evasion (Berggård *et al.* 2001).

2.1.2 Capsule

The capsule structure contributes considerably to the success of the bacteria. Acapsular strains have been shown to have markedly decreased phagocytic resistance, and a 100-fold decrease in virulence when tested in mice (Wessels *et al.* 1991). The GAS capsule consists of a hyaluronic acid with the degree of encapsulation varying greatly across the serotypes (See Figure 3). GAS has been found to up-regulate hyaluronic acid production to structurally minimise antibody access to bacterial surface protein G-related α_2-macroglobulin-binding protein (GRAB). This mechanism of evasion contributes to the difficulty in creating a functional vaccine as it enables GAS to escape recognition by antibodies (Dinkla *et al.* 2007).

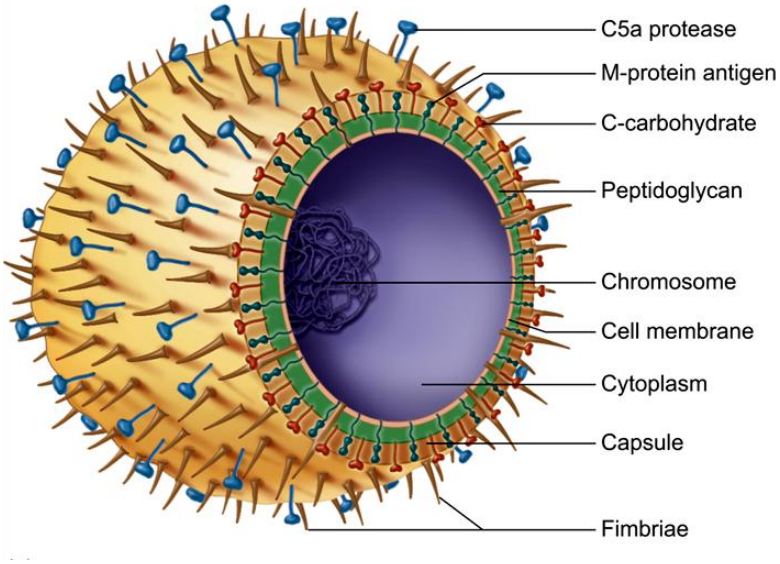

Figure 3 GAS Cross Section
GAS virulence factors include the M Protein, C5a peptidase and capsule as outer structures. Other virulence factors include streptokinase, streptolysin O, cysteine protease SpeB and many others. Taken from Talaro & Talaro (2002).

2.1.3 Streptokinase

Streptokinase (SK) is a plasminogen activator and is a secreted GAS virulence factor with four compact domains. Secretion of SK is associated with APSGN (Simon *et al.* 2014). Specifically, SK binds to plasminogen to induce the structural development of an active site as well as an enzymatic SK-plasminogen complex. SK-plasminogen can sequester substrate plasminogen and convert into the serine protease plasmin. Control over host plasminogen is advantageous to overcome host defences by generating unregulated soluble cell-bound plasmin, which can degrade blood plasma proteins (Simon *et al.* 2014). SK is semi-conserved within GAS and can be classified within one of nine polymorphic genotypes where the main structures such as hydrophobicity are maintained. SK's structural variability arises from a region designated as V1, where it is speculated the genes for nephritis are located (Malke 1993).

2.1.4 Streptolysin O

Streptolysin O is a bacterial toxin of the cholesterol-dependent cytolysins family (Timmer *et al.* 2009). It is known for its ability to form large pores in cell membranes with membrane cholesterol facilitation, but it is also thought to have pore-independent functions (Timmer *et al.* 2009). This ability is a crucial defence mechanism as it acts to prevent phagocytosis (Feil

et al. 2014). A membrane pore is created in a number of steps. Firstly, cholesterol-dependent membrane binding is undertaken in a monomeric form. The pore is completed through oligomerisation, where ring-like structures are formed on the erythrocyte membranes (Hugo *et al.* 1986). Apoptosis of the phagocyte occurs through caspase-dependent pathways, promoted by release of cytochrome c and permeabilisation of mitochondrial outer membranes, leading to decreased cytokine response and greater chance of GAS survival (Timmer *et al.* 2009).

2.1.5 C5a Peptidase

C5a peptidase is a ubiquitously expressed surface proteolytic enzyme which acts as an adhesin and invasin (Cleary *et al.* 2004). It is capable of disrupting the complement pathway via cleavage of chemotaxin C5a at its polymorphonuclear binding site. This proteolysis halts recruitment of C5a-induced granulocytes, further protecting GAS from being overwhelmed by phagocytes and assists in pharyngeal colonisation (Cleary *et al.* 2004).

2.1.6 Cysteine protease SpeB

Cysteine protease SpeB is a highly conserved and multi-functional pyrogenic exotoxin hypothesised to have a role in severe invasive infection and streptococcal toxic shock syndrome (Collin & Olsén 2001). SpeB is able to cleave human immunoglobulins, including IgA, IgM, IgD and IgE (Collin & Olsén 2001). Furthermore, it can cleave vitronectin, fibronectin and host proteins to compromise host tissue integrity (Kagawa *et al.* 2009). It can also spawn biologically active peptides such as interleukin-1, kinins and histamine (Kagawa *et al.* 2000). Through the degradation of host proteins, research conducted by Barnett *et al.* (2013) suggests that a proteolytic SpeB mechanism is utilised by GAS to evade autophagy and enable replication in the cytosol of host cells.

2.2 Genetics including virulence gene control

GAS has been genotyped via M protein typing in an effort to genetically categorise the species. The famous Lancefield method of M protein typing of *emm* GAS species determines the type of opacity factor present through an opacity factor inhibition test. *emm* genes are split up into distinct subfamilies, named from A to E and defined by the sequence differences at the 3' end (McGregor *et al.* 2004). Classically, A to C *emm* pattern strains are recognised as pharyngitis specific, D strains are often isolated from impetigo lesions and E strains are commonly found at all sites (McGregor *et al.* 2004).

The size of the GAS genome ranges between 1.85-1.9 Mb, where 1.7 Mb of the GAS genome is conserved between strains (Wagner & Waldor 2002). Phage transduction is a process through which DNA can be transferred between a bacterium to a phage. It known to be an integral part of GAS survival and development, and phage genomes have the ability to alter the host bacterial properties in any infection stage. Specifically, phages can influence bacterial adhesion, colonisation and invasion, encode bacterial toxins and alter bacterial susceptibility to antibiotics (Wagner & Waldor 2002). Beres *et al.* (2002) found that an average of 56.2% of unique genes between GAS strains is provided by prophage mediated gene transfer.

GAS has a formidable arsenal of virulence factors which enables it to persist and cause infection in a myriad of ways. The control of virulence factors begins with transcription regulators which relay information from environmental signals, usually from host-pathogen interactions. In short, Mga and RofA-like proteins are the two global regulators which pilot the cell as per the signals received by two-component signal transduction systems (TCSs) (Kreikemeyer *et al.* 2003). Mga is a conserved response transcriptional activator which plays a leading role in regulating expression of surface-associated and secreted molecules. Mga specifically regulates the M protein, streptococcal collagen-like protein, serum opacity factor,

C5a peptidase and many other virulence factors essential in host colonisation (Kreikemeyer *et al.* 2003).

If environmental conditions become hostile though lack of nutrient supply or host defence mechanisms, GAS can switch to a stationary growth phase through downregulation of Mga and upregulation of RofA-like protein (Beckert *et al.* 2001).

TCSs are not unique to GAS and function in detection and communication of environmental signals though the transmembrane protein sensor histidine kinase. Currently, 13 TCSs have been identified (Kreikemeyer *et al.* 2003). Notably, these include ihK/Irr, which plays a role in host-cell lysis, GAS neutrophil resistance *in vitro* and in mouse virulence models *in vivo* (Voyich *et al.* 2003). FasBCA is speculated to regulate expression of extracellular matrix adhesins to promote high adherence and internalisation rates (Klenk *et al.* 2005). SilAB regulates IL-8 expression of PrtS/ScpC protease, which specialises in degradation of the murine and human CXC chemokines IL-8, KC and MIP-2 (Hidalgo-Grass *et al.* 2006).

The GAS capsule is essential for pathogenesis. The production of hyaluronic acid, the main ingredient of the capsule, is controlled by an operon made up of three genes: *hasA*, *hasB* and *hasC*. Respectively, these control the production of capsular components hyaluronate synthase, UDP-glucose dehydrogenase and UDP-glucose pyrophosphorylase, a mechanism that is likely to be conserved (Albertí *et al.* 1998). CsrRS is a regulator of the hyaluronic acid capsule biosynthetic operon *hasABC*, and is known to regulate approximately 15% of the GAS genome (Dalton *et al.* 2006).

2.3 Nature and spectrum of GAS infections

GAS infection and sequelae encompasses a vast array of disease, including superficial and invasive infection and associated sequelae (See Table 1).

2.3.1 Common streptococcal throat infection

Strep throat, or acute GAS pharyngitis, makes up approximately one third of all respiratory tract infections in primary care (Little *et al.* 2014). This accounts for approximately 18

million GP visits and over 7 million visits to paediatricians annually in the US (Carapetis *et al.* 2005). Although major complications are rare, usage of antibiotics has been shown to prevent suppurative complications such as quinsy, otitis media, sinusitis and cellulitis by at least 50% (Little *et al.* 2014; Petersen *et al.* 2007).

2.3.2 Rheumatic fever and rheumatic heart disease

RF is a delayed sequelae of acute GAS pharyngitis and is characterised by inflammation of the joints, heart, central nervous system, skin and or subcutaneous nodules (McNamara *et al.* 2008) (Table 1). The Jones criteria (Burke & Chang 2014) states RF can be diagnosed through the presence of a preceding GAS infection, plus the presence of two major manifestations or one major and two minor manifestations of the Jones criteria. Symptoms of the Jones criteria mainly include carditis, polyarthritis and Sydenham's chorea. Further research is needed into pathophysiology and potential biomarkers of RF, as the above symptoms are known to cross over with diseases such as Lyme disease, serum sickness, drug reactions and post-streptococcal reactive arthritis (Burke & Chang 2014).

The pathogenesis of RF and RHD is poorly understood and two hypotheses currently exist which attempt to explain the nature of these disease manifestations. The first hypothesis proposes molecular mimicry and cross reactivity between sarcomeric heart myosin and streptococcal antigen M protein. The second proposes collagen-mediated disease in the valve. Tandon *et al.* (2013) proposes the following inflammatory mechanism: The GAS-m protein's N-terminus has been shown to bind to the CB3 region in collagen type IV, which in turn initiates an antibody response against collagen, resulting in ground substance inflammation. However, Cunningham (2014) proposes that both hypotheses function in tandem. The majority of autoimmune diseases involve more than one auto-antigen, thus cardiac myosin and collagen may act as auto-antigens where one precedes the other. This is supported by data demonstrating an increase in anti-cardiac-myosin and anti-collagen antibody in rheumatic carditis (Martins *et al.* 2008).

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Table 1. Signs/symptoms of GAS superficial and invasive infections, related diseases and sequelae

Disease	***SIGNS AND/OR SYMPTOMS***
Superficial	
Pharyngitis	Sore throat, malaise, fever
Scarlet fever	Deep red rash, "strawberry tongue", exudative pharyngitis
Impetigo	Skin pustules that mature into honey-coloured scabs
Sequelae	
Acute rheumatic fever	Polyarthritis, carditis, rapid and jerky movements, rash, subcutaneous nodules
Rheumatic heart disease	Mitral and/or aortic regurgitation with possible stenosis over time
Acute poststreptococcal glomerulonephritis	Oedema, hypertension, urinary sediment abnormalities, complement deficiency
Invasive	
Bacteremia	High fever, nausea, vomiting
Puerperal sepsis	Fever, chills, abdominal pain in a pregnant or early postpartum woman.
Cellulitis	Acute, tender, erythematous, and swollen area of skin
Necrotising fasciitis	Fever, tender skin lesions, vomiting, diarrhoea, toxaemia, tissue destruction
Streptococcal toxic shock syndrome	High fever, rapid-onset hypotension, accelerated multisystem failure

279 Adapted from Carapetis *et al.* (2005)

2.4 Current treatment options

Antibiotics are the primary treatment for GAS infections. The advantages of penicillin include low cost, efficacy and safety. Other drugs such as cephalosporins, macrolides, erythromycin and clarithromycin have also proven to be effective and are in use in a clinical environment (Bisno *et al.* 2002).

2.4.1 Antibiotic therapy

A meta-analysis of penicillin vs. cephalosporins treatment was undertaken in the context of GAS tonsillopharyngitis, and it was found that the cephalosporin cure rate was twice that of penicillin, making it the superior choice of drug (Casey & Pichichero 2004). However, despite the size and number of trials undertaken by Casey and Pichichero, the study has been criticised for its inconsistencies in control of variables (Bisno 2004). Notably, out of 35 trials, only 9 were investigator-blinded, 6 were double-blinded, 3 reported dropout rates, 9 provided details of patients signs and symptoms at enrolment and 9 based GAS clearance on follow-up throat cultures obtained in the optimal 3 to 14 day period after therapy was completed (Shulman & Gerber 2004). Similar past studies (1953-1993) which retained a high standard of experiment design failed to find evidence of an increase in penicillin failures among pharyngitis patients (Markowitz *et al.* 1993). Furthermore, the advantage of penicillin also lies in its price and spectrum: it is 20 to 30 times cheaper than cephalosporin and has a smaller target range which reduces the selection pressure and the prevalence of other antibiotic resistant bacteria (Shulman & Gerber 2004).

Within GAS strains, macrolide antibiotic resistance is beginning to spread through horizontal transfer of the *mef* gene. This is a concern for individuals who are allergic to β–lactam antibiotics as it equips GAS with a drug efflux pump. Transposon transfer has already spread to *emm* types 1, 2, 3, 4, 9, 12 and 75 (Hadjirin *et al.* 2014). Vaccination to provide long term immunity is essential in worldwide control and elimination of GAS disease.

2.5 GAS vaccine development

Difficulties in the development of a GAS vaccine include serotype diversity and safety concerns. For example, GAS molecules with homology to human proteins could potentially trigger autoimmune sequelae (Kirvan *et al.* 2003). Additionally, understanding of immune protection in humans is incomplete and further epidemiological and combination antigen research is necessary. Ideally, an effective vaccine would prevent pharyngeal colonisation, carriage, invasive infection, asymptomatic GAS infection, RF, RHD, APSGN and toxin mediated complications (Dale *et al.* 2013). Current categories of vaccine exploration include the following: anchored cell wall proteins, cell membrane associated and/or secreted and anchorless vaccine candidates (Table 2).

Vaccine development research is primarily focussed on the highly abundant M protein. Multi-valent vaccinations are designed to target multiple strains, where 6-valent, 26-valent and 30-valent GAS vaccines are in clinical or pre-clinical stages (Steer *et al.* 2013). Despite the number of valencies covered, the 30-valent vaccine would geographically only give good coverage to the US, Canada and Europe, intermediate coverage to Asia and the Middle East and very poor coverage within Africa and the Pacific. However, this concern has been mitigated by research undertaken by Smeesters *et al.* (2008) whose data proposes that M protein based vaccines may evoke cross-protective antibodies. This could broaden the targeted number of serotypes targeted. Furthermore, the new 30-valent M protein based vaccine boasts protection against 98% of known serotypes responsible for pharyngitis and invasive infections (Dale *et al.* 2011).

Table 2. Current vaccination antigens, trials, vaccine types and protein candidates			
Candidates	**Antigen**	**Vaccine type/preclinical data**	**Reference**
Cell Wall Anchored Proteins			
M Protein	N-terminal region: 6-valent, 26-valent and 30-valent antigen	Multivalent recombinant protein/Intramuscular delivery in humans via opsonic antibodies	(Dale *et al.* 2005) (Hu *et al.* 2002) (Dale *et al.* 2011) (Kotloff *et al.* 2004)
	N-terminal peptides linked to tetanus toxoid	Peptide-protein conjugate /Subcutaneous delivery in mice via opsonic antibodies	(Brandt *et al*. 2000)
	N-terminal peptides linked via lipid core peptide	Lipopeptides: IgG and opsonisation/Subcutaneous delivery in mice via peptide specific serum	(Olive *et al.* 2003)
	Heteropolymer (seven N-terminal and one C-terminal peptides)	Peptide polymer/Subcutaneous delivery in mice via systemic IgG and opsonic antibodies	(Brandt *et al*. 2000)
	N-terminal Plasminogen-	Amino acid residues/subcutaneous delivery	(Sanderson-Smith *et al.*

	binding group	in quackenbush mice via peptide specific serum	2008)
	C-terminal region: Whole C-repeat conserved region	Synthetic peptide conjugate/intranasal in mice via peptide specific serum	(Bessen & Fischetti 1988)
	Minimal epitope J8/J14/p145	Self-adjuvanting polyacrylic nanoparticulate delivery/intranasal delivery in mice via peptide specific serum	(Zaman *et al.* 2011)
	C-repeat epitope (StreptInCor vaccine)	C-terminal amino acid residues/subcutaneous delivery in mice via StreptInCor suspension	(Guerino *et al.* 2011)
	Conserved region proteins expressed in *Lactococcus lactis*	C-repeat amino acid residues/nasal and subcutaneous delivery in mice via peptide specific serum	(Mannam *et al.* 2004)
Fibronectin-Binding Protein A	Seven FbaA epitopes co-administered with five M protein fragments	Recombinant multivalent protein/Intraperitoneal delivery in mice via peptide specific serum	(Ma *et al.* 2014)

Protein F1/Streptococcal Fibronectin Binding Protein I	Recombinant H12 co-administered with M protein peptide J14	Recombinant multivalent protein/subcutaneous delivery in mice via peptide specific serum	(Georgousakis *et al.* 2009)
Streptococcal Protective Antigen	N-Spa36 epitopes	Spa antiserum/intraperitoneal delivery in mice	(Ahmed *et al.* 2010) (Dale *et al.* 1999)
***Streptococcus pyogenes* Cell Envelope Proteinase/Spy0416**	Spy0416/SpyCEP epitopes	Recombinant CEP protein/Intramuscular delivery in mice	(Turner *et al.* 2009)
Cell Membrane Associated and/or Secreted GAS Candidates			
C5a Peptidase	C5a Peptidase epitopes (functional in GAS and Group B Streptococcus (GBS))	Recombinant GBS inactive C5a peptidase/Subcutaneous delivery in mice	(Cleary *et al.* 2004)
Streptococcal Hemoprotein Receptor	Shr (CFA/IFA	Purified Shr/intraperitoneal delivery in mice and Shr-expressing *Lactococcus lactis* delivered intranasally in mice.	(Huang *et al.* 2011)
Spe B	SpeB catalytic site co-	Chimeric SpeB and SpeA/Intramuscular	(Ulrich 2008)

	administered with the binding surface of SpeA	delivery in mice	
Anchorless GAS Candidates			
Streptococcal Immunoglobulin-Binding Protein 35	Sib35 epitope	rSib35 protein/subcutaneous delivery in mice	(Okamoto *et al.* 2005)
Arginine Deaminase/Streptococcal Acid Glycoprotein	ADI epitope	ADI adjuvanted with CFA/Intraperitoneal delivery in mice	(Henningham *et al.* 2012)
Trigger Factor	TF epitope	TF adjuvanted with CFA/Subcutaneous delivery in mice	(Henningham *et al.* 2012)

2.6 Virus Like Particle (VLP) technology

Vaccines function as a platform for the presentation of an antigen, so that the body can formulate an immunological memory. Antigen presentation is a crucial part of vaccine success and must accurately replicate the inherent immunostimulation of an infection. VLPs are constructed from viral structural proteins such as the envelope or capsid which can self-assemble. VLPs are produced by many viruses including hepatitis B and human papillomavirus (Zhao *et al.* 2013). VLPs must sufficiently interact with innate immune cells, professional antigen presenting cells (APCs) and adaptive effector/memory cells without causing host damage (Zhao *et al.* 2013) to be utilised as a vaccine. One advantage of VLPs are that they are able to effectively deliver an antigen to professional APC's as well as stimulate both cell-mediated (CMI) and humoral immune (HI) responses. VLP success has been demonstrated through protection against of hand-foot-and-mouth disease, influenza, hepatitis B and human papilloma virus (Bright *et al.* 2008; Bryan 2007; Chung *et al.* 2008).

2.6.1 Overview of the use of VLP technology for human diseases

There are many advantages of VLP technology. VLPs are particulate and have been shown to illicit immune responses without adjuvant usage making them advantageous for vaccination as not many adjuvants are available for human use (Fifis *et al.* 2004). VLP Adjuvant properties stem from the small size of the VLP, which allows easy uptake by dendritic cells for major histocompatibility complex class II (MHC II) cells and subsequent stimulation of the innate immune response (Grgacic & Anderson 2006).

Traditional aluminium based adjuvants are not well paired with all vaccines as they generate a Th2 bias. Th2 humoral responses typically result from infections caused by bacteria and multicellular pathogens, whilst Th1 cell-mediated responses result from intracellular infections (Rosenthal *et al.* 2014). Th1 responses are directed at inducing CMI and have a distinct inflammatory bias. In comparison, Th2 responses reduce inflammation, promote antibody production and are associated with HI (Goering *et al.* 2012). Versatile VLP

technology can be engineered to induce Th1 or Th2 through particle size control. Larger particles encourage increased production of IL-4 for a Th2 response, and smaller particles result in amplified production of IFN-γ for a Th1 response (Rosenthal *et al.* 2014).

Licenced VLP vaccines include Recombivax HB® against hepatitis B and Hecolin®, plus Gardasil® and Cervarix® against HPV (Henningham *et al.* 2013). VLPs are most commonly used to prevent viral pathogens, but there has been evidence of successful delivery of chimeric bacterial antigens using VLPs (Rosenthal *et al.* 2014).

2.6.1.1 Generating recombinant chimeras

As mentioned above, GAS-m protein is constituently expressed, highly immunogenic and promising choice for vaccine development. The highly conserved C-repeat region within the M protein contains the p145 peptide, recognised by antibodies of adults living in an environment with high GAS exposure (see Figure 4). However, challenges for the use of this peptide in a vaccine include potential molecular mimicry as the p145 peptide shares an epitope with the human heart protein myosin (Hayman *et al.* 1997). A study by Hayman *et al.* (1997) utilising mouse anti-p145 sera determined that the M protein peptides containing minimal cross-reactivity were J8 and J14. A follow up study cited by Good and Olive (2003) showed protection following challenge with GAS and production of opsonic antibodies when mice were immunised by J8 or J14 in Complete Freund's Adjuvant (CFA). Notably, a vaccine incorporating the conserved J8 M protein is currently in stage 1 of clinical trials in Australia (Dale *et al.* 2013)

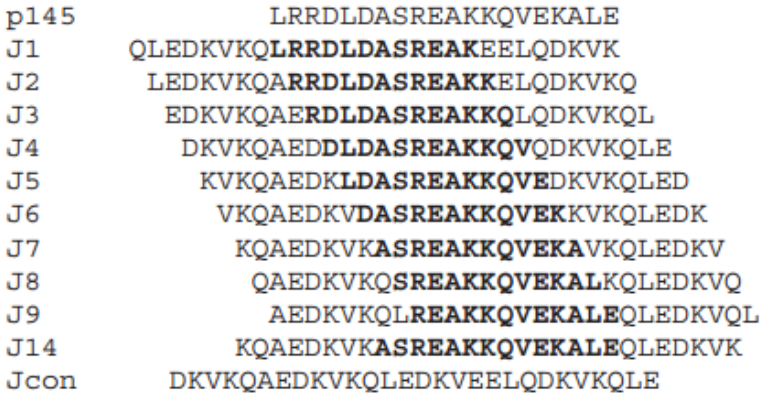


Figure 4 GAS-m Protein C-region Peptides

Research undertaken by Hayman *et al.* (1997) explored a series of overlapping peptides (p145-J14) within the C-region of the GAS-m protein, where Jcon was a non-specific sequence as a control. These peptides were recognised by antibodies within adults living in areas of high GAS exposure, where the bold peptides are the p145 sequence. The p145 sequence is flanked by GCN4 sequences, which is designed to mimic the α-helical folding of M proteins.

Past recombinant chimeric platforms include the hepatitis B virus core, woodchuck hepatitis B virus core, hepatitis B virus S antigen, human papillomavirus, bovine papillomavirus, human immunodeficiency virus (HIV), simian immunodeficiency virus HIV chimera, duck hepatitis B virus and hepatitis E virus (Grgacic & Anderson 2006). In the commercial setting, VLPs are synthesised through insect and yeast cell-based systems for their ease of production, cost efficiency, post-translational modifications and adjustable production size. Bacteria, mammalian and plant cells and cell-free synthesis have been utilised in the laboratory setting to produce VLPs (Rosenthal *et al.* 2014). VLP technology has proven to be safe, effective, long lasting and cost efficient. In particular, the HBsAg-S surface protein is the most common molecule used in VLP technology due to its non-infectious nature and worldwide licence for use (Netter *et al.* 2003).

2.6.1.2 Overview of use of HBsAg-S VLPs

HBsAg-S VLPs stimulate both CMI responses and HI responses and hold potential for usage in dual-vaccination regimens. Desired genes are often cloned into the HBsAg-S a-determinant region, a highly immunogenic tertiary structure (Netter *et al.* 2003). The most

recent successful chimeric HBsAg-S vaccine is GlaxoSmithKline's malaria vaccine Mosquirix®, currently in phase 3 trials (Wilby *et al.* 2012). Similarly, the original hepatitis B vaccine, first licenced in 1981, continues to markedly reduce incidence of hepatitis B worldwide. For example, 18% of Vietnamese children in 1998 were infected with hepatitis B, compared to a drastic reduction to 2.7% in 2013 (Nguyen *et al.* 2014). New HBsAg-S VLP research has also explored the possibility of incorporating bacterial vaccine epitopes. Notably, Kotiw *et al.* (2012) has utilised *Helicobacter pylori* KatA epitopes in conjunction with HBsAg-S surface protein. Results were promising with immunised mice showing increased bacterial clearance, warranting further exploration in bacterial VLP combination research.

2.7 The current study proposal

J8, J14 and p145 from the M protein of *Streptococcus pyogenes* are highly conserved. Furthermore, these peptides provide an adequate antibody response without resulting in cross reactivity to human proteins (Dale *et al.* 2013). In this study J8, J14 and p145 DNA sequences will be generated by PCR and cloned into the 'a' determinant region of HBsAg-S. Recombinant HBsAg-S-GASm VLPs will be generated using a mammalian expression system and assayed for immunogenicity by enzyme-linked immunosorbent assay (ELISA).

2.7.1 Proposed strategy

Indigenous Australians have the highest global RHD and/or RF mortality rate of 30.2 individuals per 100,000 annually (Carapetis *et al.* 2005). The purpose of the study is to target the Australian Indigenous population and developing countries through the development and evaluation of a HBsAg-S-M protein dual vaccine. The HBsAg-S surface protein VLP has proven immunogenic efficacy and safety as numerous licenced vaccines. Furthermore, the M protein epitopes J8, J14 and p145 have demonstrated antigenic and safety properties.

PCR generated DNA sequences of J8, J14 and p145 from the M protein of GAS will be cloned into the 'a' determinant region of the HBsAg-SS and transformed into a mammalian

cell recombinant protein expression system. Expressed recombinant HBsAg-S-GASm constructs will be isolated, purified and assayed by WB and ELISA. Following successful isolation and purification recombinant molecules will be used to vaccinate BALB/C mice in GAS challenge studies. Use of the constructs in challenge models will evaluate their ability to generate an antibody response though VLP antigen delivery (Figure 5).

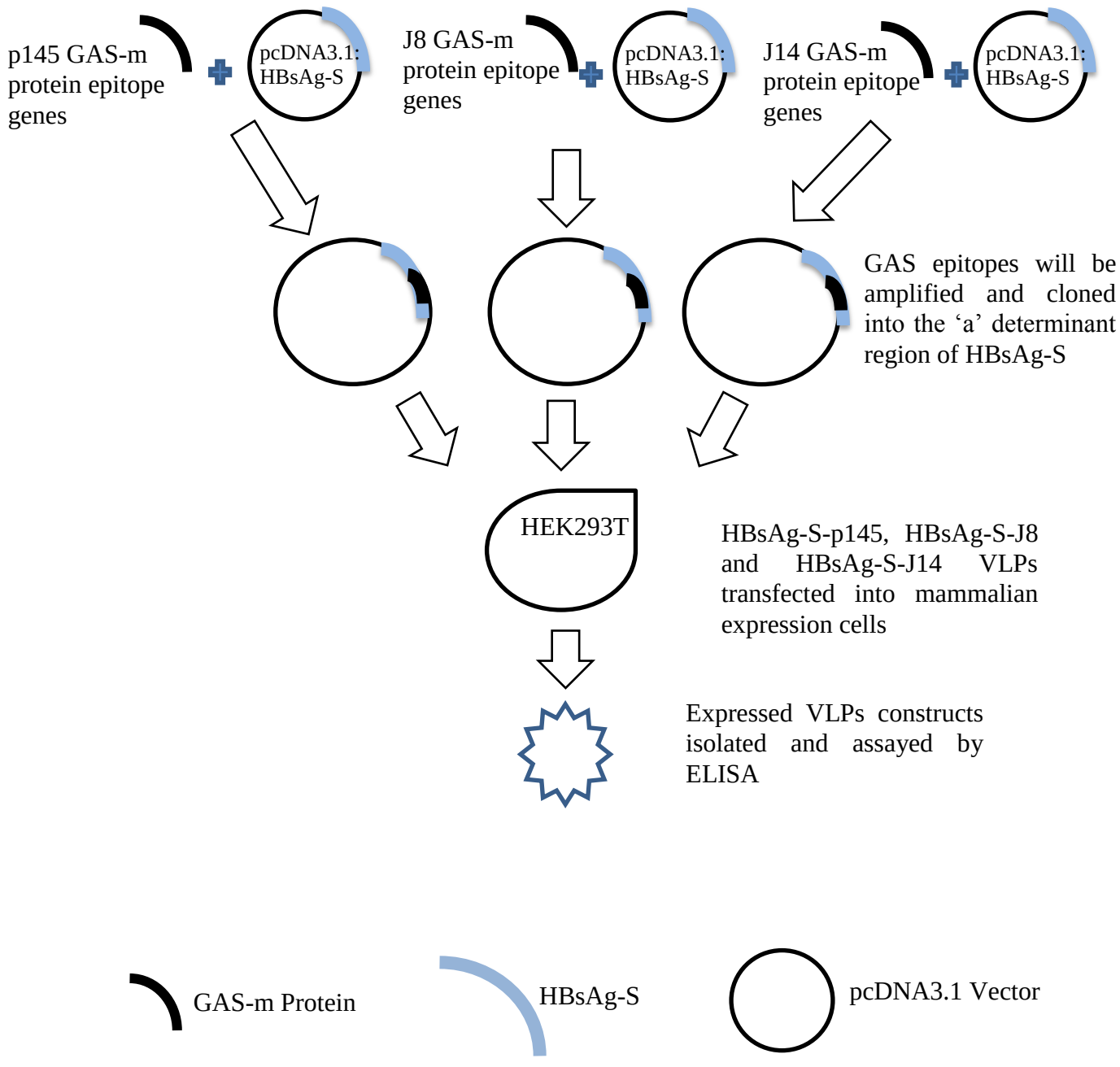


Figure 5 Proposed Strategy Details

Hypotheses

1. GAS antigenic peptides can be expressed in HBsAg-S VLPs to utilise VLPs as a carrier molecule for a dual vaccine.
2. GAS VLPs which are recognised by GAS HBsAg-S sera will be good vaccine candidates to provide protection against GAS in an animal model.

Materials and Methods

3.0 Bacteria and Plasmids

Escherichia coli JM109 (Promega) and TOP10 (Life Technologies) was grown in Luria Bertani (LB) broth or on LB agar containing 100 µg/ml ampicillin. Incubation of plated and liquid bacterial cultures was performed at 37°C in a Bioline Thermocube Incubator (Bioline Alexandria, NSW, Australia), where required shaking was performed at 5 x g. The mammalian protein expression vector was pcDNA3.1:HBsAg-S which was kindly provided by Hans Netter (Monash University, Melbourne, Australia).

3.1 Molecular Analyses

Primers with accompanying forward and reverse sequences are detailed below in Table 3. Primers were purchased from Invitrogen, and all PCR reactions were carried out in a PTC-100™ Programmable Thermal Controller (MJ Research Inc, Quebec, Canada.).

Table 3 Sequence of oligonucleotide primers used

Application	Template DNA		Primer Sequence (5'→ 3')
GAS-m epitope generation	p145	Full sequence	GGA**ACCGGT**CTTCGTCGTGACTTGGACGCATCACG TGAAGCTAAGAAACAAGTTGAAAAAGCTTTAGAA **ACCGGT**TGG
		Forward Primer	GGA**ACCGGT**CTTCGTCGTGACTTGGACGCATCACG TGAAGCTAAGAAAC
		Reverse Primer	CCA**ACCGGT**TTCTAAAGCTTTTTCAACTTGTTTCTT AGCTTCACGTGATG
GAS-m epitope generation	J8	Full sequence	GGC**ACCGGT**CAGGCGGAAGATAAAGTGAAACAGT CACGTGAAGCTAAGAAACAAGTTGAAAAAGCTTTA AAACAGCTGGAAGATAAAGTGCAG**ACCGGT**GGC
		Forward Primer	GGC**ACCGGT**CAGGCGGAAGATAAAGTGAAACAGT CACGTGAAGCTAAGAAACAAGTTG
		Reverse Primer	GCC**ACCGGT**CTGCACTTTATCTTCCAGCTGTTTTAA AGCTTTTTCAACTTGTTTCTTAGCTTC
GAS-m epitope generation	J14	Full sequence	GGC**ACCGGT**AAGCAGGCGGAAGATAAAGTGAAAG CATCACGTGAAGCTAAGAAACAAGTTGAAAAAGCT TTAAAACAGCTGGAAGATAAAGTGAAG**ACCGGT**G GC
		Forward Primer	GGC**ACCGGT**AAGCAGGCGGAAGATAAAGTGAAAG CATCACGTGAAGCTAAGAAACAAGTTG
		Reverse Primer	GTGAAGCTAAGAAACAAGTTGAAAAAGCTTTAGTA CAGCTGGAAGATAAAGTGAAG**ACCGGT**GGC
Amplification	HBsAg-SFwd		GTAGAATTCGCCACCATGGAGAACATCACATCAG
Amplification	HBsAg-SRev		CTGCGGCCGCTTAAATGTATACCCAAAGAC
Sequencing	AOX 1	Reverse Forward	GCAAATGGCATTCTGACATCC GACTGGTTCCAATTGACAAGC

3.2 Amplification of GAS-m Epitopes

Synthesis of GAS-m DNA fragments utilised p145, J8 and J14 forward and reverse primers as shown in Table 3. The sequence 'ACCGGT' was included as a site for the *Age*I restriction enzyme for ease of ligation of fragments into the pcDNA3.1:HBsAg-S mammalian expression vector. Sequences of p145, J8 and J14 were obtained from previous studies by Hayman *et al.* (1997). Expected PCR product sizes for p145, J8 and J14 are 78, 102 and 105 bp respectively. p145, J8 and J14 forward and reverse primers were used to synthesise the

p145, J8 and J14 GAS-m DNA sequences through PCR. Three primer pairs were required to create the fragments as given in Table 3. Each forward primer 5' end contained ACCGGT with three additional N terminal nucleotides to enable restriction digestion with enzyme *AgeI-HF* (New England Biolabs, Arundel, Queensland, Australia), as seen in Table 3 in bold lettering. PCR reactions to generate each fragment contained 5 µl of x10 AccuBuffer (Bioline), 1 µl of 10 mM dNTPs (Promega, Alexandria, NSW, Australia), 1.5 µl of both appropriate forward and reverse primers at 20 pmol/µl (Table 3), 1 µl of Accuzyme™ DNA polymerase (Bioline) and Milli Q water to make reaction volume up to 50 µl. The PCR was performed using the following parameters: 95°C for 3 min, followed by 30 cycles of 95°C for 15 s, 56°C for 15 s and 72°C for 15 s. The resulting PCR products were viewed on a 1% agarose gel as described in section 3.2.1, followed by purification using the Wizard® SV Gel and PCR Clean-Up (Promega).

3.3 Agarose Gel Electrophoresis

Gels were prepared as 1% or 2% agarose in 1X TAE containing 0.75% GelRedDNA Stain (Biotec, Wembley, Australia) for visualisation. The gel was electrophoresed using a Liberty (Biokey, California, USA) or Biorad minisub cell GT electrophoresis tank, depending upon the size of the gel required. The electrophoresis tank was filled with 1X TAE Buffer. Samples were loaded for electrophoresis after mixing, in a ratio of 5:1 with 6X blue loading dye. 5 µl HyperLadder I or V molecular size marker was loaded in a separate well to enable estimation of the size and concentration of DNA in each sample. A Power Pac 200 (Bio-Rad, Gladesville NSW) was used to apply a voltage of 100 V for 60 min per gel. A Fusion FX5 system (Vilber Lourmat, Eberhardzell, Germany) was used to visualise DNA bands under UV light in conjunction with Fusion FX7 software (Peqlab, Erlangen, Germany). When DNA bands required extraction and purification from agarose gel, the Wizard® SV Gel and PCR Clean-Up (Promega) system was utilised.

3.3.1 Wizard® SV Gel and PCR Clean-Up

The Promega (Alexandria, NSW) Wizard® SV Gel and PCR clean-up kit was utilised to purify PCR products. The PCR product or the desired DNA band was removed from the agarose gel and dissolved within 1 µl of membrane binding solution per mg of agarose gel. The solution was vortexed and incubated at 60°C until fully dissolved and transferred to a spin column and collection tube assembly for incubation at RT for 1 min. The assembly was centrifuged at 30 600 x g in Sigma 1-15 Laboratory Centrifuge for 1 min at RT, the flowthrough was discarded. DNA bound to the column membrane was washed with 700 µl of membrane wash solution and centrifuged further at 30 600 x g for 1 min at RT. The flowthrough was discarded and the step was repeated using 500 µl of membrane wash solution (Promega) and centrifuging at 30 600 x g for 5 min at RT. After discarding flowthrough, the spin column was inserted into a sterile 1.5 ml microcentrifuge tube and 50 µl of nuclease free water was pipetted into the spin column. Following incubation at RT for 1 min, the assembly was centrifuged at 30 600 x g for 1 min at RT. The flowthrough containing the DNA of interest was collected in a microcentrifuge tube. The sample was electrophoresed on an agarose gel for visualisation. DNA was stored at -20°C.

3.4 Digestion and Insertion of GAS-m Epitopes into HBsAg-S DNA sequence

As mentioned in section 3.3.1, the primers used to create the GAS-m fragments contained an *Age*I restriction site to enable insertion into pcDNA3.1:HBsAg-S. Digestion was achieved using 5 µl of Cutsmart 1x Buffer (New England Biolabs), 5 units of *AgeI* HF restriction enzyme (New England Biolabs) and 50 ng of the appropriate GAS-m fragment made up to 50 µl. The mixture was then digested for 37°C for 15 min and heated to 65°C for 20 min. The pcDNA3.1 vector underwent a similar digestion, where 100 ng of pcDNA3.1:HBsAg-S, 5 µl of Cutsmart 1x Buffer (New England Biolabs), 5 units of *AgeI* HF restriction enzyme (New England Biolabs) and 40 µl of Milli Q Water was combined. The mixture was digested for

37°C for 15 min. Following digestion 5 units of Antarctic Phosphatase (New England Biolabs) and 5 µl of Antarctic Phosphatase buffer (New England Biolabs) was added. The mixture was further digested at 37°C for 60 min and heat inactivated at 65°C for 20 min. Expected PCR product sizes for HBsAg-S:p145, HBsAg-S:J8 and HBsAg-S:J14 were 766, 784 and 787 bps respectively.

3.5 Plasmid Ligation

Ligation was performed at a ratio of 1:3 vector to insert, where pcDNA3.1:HBsAg-S was the vector and p145, J8 and J14 were the inserts respectively. The Invitrogen (Mulgrave, Victoria) rapid ligation protocol was utilised, where 4 µl of 5x ligase reaction buffer (Invitrogen), 30 fmol of vector DNA, 90 fmol of insert DNA, 1 µl of T4 DNA ligase (Invitrogen) and Milli Q water up to 20 µl were added to a 1.5 ml microcentrifuge tube. Contents were centrifuged briefly and incubated at RT for 5 min.

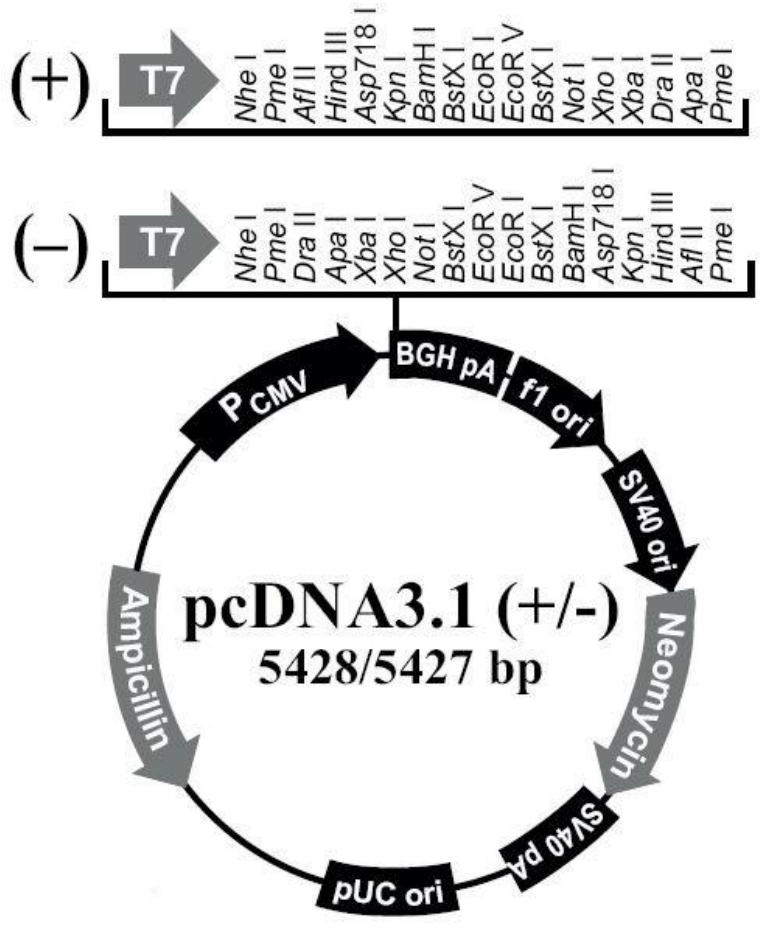


Figure 6 pcDNA3.1 Vector Map

pcDNA plasmid map, detailing locations of the *AgeI* site, Ampicillin resistance gene and mammalian expression promoter. Taken from (Xenbase 2014).

E.coli transformation methods (Invitrogen, Mulgrave, Victoria, Australia). The appropriate amount of One Shot® JM109 or TOP10 *E. coli* was thawed on ice. 5µl of each ligation reaction was pipetted directly into the vial of competent cells and mixed by tapping gently. Cells were incubated on ice for 30 min followed by 30 s in a 42°C water bath. The reaction was placed back into ice and 250 µl of pre-warmed S.O.C medium (Super Optimal Broth, details?) was added using sterile technique. Cells were shaken at 37°C for 1 h and 150 µl of each transformation was pipetted directly onto labelled LB agar plates containing 1 µg/mL of Ampicillin and incubated at 37°C overnight. The ampicillin gene contained in the plasmid vector was crucial to select potential transformants (Figure 6A). Colonies were then repatched onto fresh plates for colony identification. Possible clones were selected through PCR performed using the following parameters: 95°C for 1 min 30 s, followed by 30 cycles of 95°C for 15 s, 55°C for 15 s and 72°C for 1 min 30 s. AOX1 primers were utilised for sequencing verification of plasmid preparations believed to contain GAS-m protein fragments within the HBsAg-S sequence (Table 3).

3.7 Wizard® Plus SV Minipreps DNA Purification

The Promega (Alexandria, NSW) Wizard® Plus SV Miniprep kit was utilised to purify plasmid DNA from *E. coli*. Preparations of 10 ml of LB containing ampicillin were inoculated with single colonies of bacteria. The culture was grown overnight at 37°C with shaking and centrifuged at 2 400 x g in Sigma 3-15 Laboratory Centrifuge (Shropshire, UK) for 5 min at RT. The supernatant was decanted and the pellet resuspended in 250 µl of cell resuspension solution (Promega). 250 µl of cell lysis solution (Promega) was added to the suspension and mixed by inversion, 10 µl of alkaline protease solution was then added and

596 the suspension was inverted four times. The suspension was incubated at RT for 5 min and
597 350 µl of the neutralisation solution (Promega) was added and the suspension was inverted
598 four times before centrifugation at 30,000 x g for 10 min at RT. The supernatant was
599 transferred to a spin column and collection tube assembly and centrifuged at 30 000 x g for 1
600 min at RT, where the flowthrough was discarded. The membrane-bound DNA was washed
601 with 750 µl of wash solution, added to the spin column and centrifuged at 30 000 x g for 1
602 minute at RT. The flowthrough was discarded and the wash step repeated using 250 µl of
603 wash solution followed by centrifugation at 30 000 x g for 2 min at RT. The spin column
604 was re-inserted into a 1.5 ml microcentrifuge tube and 100 µl of nuclease free water was
605 pipetted directly onto the membrane. The assembly was centrifuged at 30 000 x g for 1 min at
606 RT. The flowthrough containing plasmid DNA was harvested and a sample was
607 electrophoresed on a 1% agarose gel for analysis. DNA was stored at -20°C.

608 **3.8 DNA Sequencing**

609 The AOX primer (Table 3) was utilised to enable sequencing of the GAS-m constructs within
610 the HBsAg-S-S sequence of pcDNA3.1. The reaction mixture for each sequencing PCR
611 contained 2 µl of Applied Biosystems (Mulgrave, Australia) 5X sequencing buffer , 1 µl of
612 Big-Dye Terminator sequencing reaction (Applied Biosystems), 3 µl of the appropriate
613 primer at 1 pmol/µl, 250 ng of plasmid DNA and Milli Q water to make the total reaction
614 volume 12 µl. Each reaction was initially heated to 95°C for 2 min, followed by cycling at
615 95°C for 10 s, 52°C for 5 s and 60°C for 3 min, repeated 25 times.

616 Following PCR, 72 µl of 70% isopropanol was added and the reaction mixture vortexed and
617 incubated for 15 min at RT. It was then centrifuged within a Sigma 1-15 Laboratory
618 Centrifuge at maximum speed for 30 min at RT. The supernatant was removed and pellet
619 was briefly centrifuged to remove the remaining droplet. The pellet was rinsed with 300 µl
620 of 70% isopropanol and centrifuged at maximum speed for 5 min. All liquid was removed
621 and samples dried in a fume hood for approximately 1 h. Samples were sent to Queensland

Institute of Medical Research (QIMR, Brisbane, Australia) for sequencing analysis. Results were analysed using Bioedit©, a biological sequence alignment editor (Carlsbad, California).

3.9 Wizard® Plus SV Midipreps DNA Purification

The Wizard® Plus Midiprep kit (Promega, Australia) was utilised to purify *E. coli* plasmid DNA to the quality and amount required for transfection of mammalian cells. All solutions and materials for Wizard® Plus Midiprep kit were obtained from Promega. 100 ml of LB containing 100 µg/ml ampicillin was inoculated with a colony of freshly grown *E. coli* containing the plasmid of interest. The culture was grown overnight at 37°C with shaking. Cells were pelleted at 2 400 x g in a Sigma 3-15 Laboratory Centrifuge and resuspended in 3 ml of cell resuspension solution (Promega). 250 µl of Cell lysis solution (Promega) was added and the suspension inverted 5 times, followed by incubation at RT for 3 min. Neutralisation solution (Promega) was added, and the suspension inverted a further 10 times. Lysate was centrifuged at 2 400 x g for 15 min. A KNF Neuberger (Rowville, Victoria) vacuum manifold was utilised in conjunction with blue PureYield™ Clearing Columns (Promega) and white PureYield™ Binding Columns (Promega). Lysate was pipetted into the column assembly and a vacuum was applied until lysate passed through the membrane, the blue column was then discarded. 5 ml of Endotoxin Removal Wash (Promega) was added and a vacuum applied. 20 ml of Column Wash (Promega) was added and a vacuum applied once more. The membrane was dried by applying a vacuum for 30 s and the binding column was removed from the vacuum manifold. An Eluator vacuum elution device (Alexandria, NSW) was fitted to the vacuum manifold assembly to allow collection of DNA into a 1.5 ml microcentrifuge tube. Finally, DNA was eluted in 600 µl of nuclease free water under a vacuum to obtain purified plasmid DNA.

3.9.1 HEK293T Cell Culture

Human Embryonic Kidney cells 293T (HEK293T ATCC #CRL-1573) were maintained in Minimum Essential Media (MEM) culture media (MEM containing 25 mM 4-(2-

hydroxyethyl)-1-piperazineethanesulfonic acid (HEPEs), Glutamax (Gibco®) and 10% fetal bovine serum (FBS) and incubated in a SANYO Humidified CO_2 Incubator (North Sydney, NSW) at 37°C with 5% CO_2. Cells were passaged regularly to avoid senescence and were grown in conical flasks. To passage, cells were viewed under a Leica Leitz DM IL microscope (North Ryde, NSW) to visually evaluate confluence. Spent media was discarded, 5 ml of phosphate buffered saline (PBS) was added to the flask and incubated for 2 min before being discarded; this wash was repeated. 1 ml of Life Technologies 0.05% trypsin (Mulgrave, Victoria) was added and cells were incubated for <5 min at 37°C with 5% CO_2. After incubation, detached cells were added to 5 ml of MEM culture media to inhibit trypsin activity. Cells were centrifuged at 500 x *g* for 5 min with a 3-15 Laboratory Centrifuge (Sigma, Osterode am Harz, Germany). Supernatant was discarded and cells were re-suspended in the MEM culture media and seeded into new culture flasks at a ratio between 1:3 and 1:10.

3.9.2 HEK293T Transfection and Protein Isolation

For transfection cells were seeded at 1.5 x 10^6 cells/10cm dish in 9 ml of pre-warmed MEM culture media. On the following day the media was replaced approximately 2 h prior to transfection. A mixture of 16 µg of plasmid DNA, 36 µl of 2M $CaCl_2$ and Milli Q water at a final volume of 300 µl was combined and quickly added to 300 µl of 2X HEPES buffered saline (HBS) (containing 10 g/l HEPES; 16 g/l NaCl; 0.74 g/l KCl; 0.27 g/l $Na_2HPO_4.2H_2O$; 2.0 g/l dextrose). The mixture was vortexed and incubated at 37°C for 3 min before being added drop wise to the cells with gentle swirling to facilitate mixing. Cells were incubated at 37°C with 5% CO_2. At 6 days post-transfection, media was taken from the transfection plates and centrifuged at 690 x g for 10 min in a 3-15 Laboratory Centrifuge (Sigma, Osterode am Harz, Germany) to remove cellular debris. Supernatant was gently overlayed onto 2 ml sucrose in STE (100 mM NaCl, 10 mM Tris at pH 8, 1 mM of EDTA) in Beckman ultracentrifuge tubes. Supernatant was centrifuged in a pre-cooled SW41Ti swinging bucket

rotor at 10°C and 172 700 x g for 4 hours. The resulting supernatant was discarded and the pellet resuspended in 200 µl of 1X HBS over two nights, followed by sonication using an Ultrasonic Cleaning bath (Unisonics, Brookvale, NSW) for 5 x 30 s intervals. The supernatant used in subsequent testing by ELISA.

3.9.3 ELISA

Two ELISA tests were undertaken to detect both the presence of my GAS M proteins and HBsAg-s VLPs though usage of J8 and HBsAg-s sera. Proteins were coated as a serial dilution in triplicate wells and tested with polyvalent antibody. Negative and positive control samples were included within these tests. The 'mock' sample referred to a protein harvest where no DNA was transfected into the HEK293T cells, and a 'no protein' sample was also coated to control discrepancies in the ELISA test. Positive controls included Heat-killed GAS bacteria and Engerix B (the current Hepatitis B VLP vaccine).

High-binding 96 well Greiner Bio one plates were coated with 5 µl of protein in carbonate coating buffer (0.1 M $NaHCO_3$ at pH 9.6), in 100µl of coating buffer per well. The plate was sealed and incubated over night at 4°C. The following day, plates were washed twice with PBST (0.05% Phosphate Buffered Saline with Tween20) which contains 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl and 0.05% Tween® 20. 100µl of blocking buffer (2% skim milk in PBS) was added to each well and incubated for 1.5 h. The plate was washed twice with PBST and 100µl of serially diluted primary rabbit sera (anti-HBsAg-S or J8 sera). 1% skim in PBS was added per well and incubated at for 1 h. The plate was washed four times with PBST, and 100µl of anti-rabbit conjugated antibody (Sigma) diluted at 1:3,000 in 1% skim in PBS was added per well and incubated at RT for 1 h. The plate was washed six times with PBST and 100µl of TMB was added to each well. After development in a dimly-lit environment, the reaction was stopped with the addition of 50µl of 2M H_2SO_4. Absorbance was read at 450 nm with a 200rt Biochrom Zenyth Anthos ELISA machine (Cambridge, UK).

Results

4.0 GAS-m epitope Amplification

The p145, J8 and J14 DNA sequences were generated by PCR amplification. Primers were designed with an overlap of approximately 20 base pairs between forward and reverse primers. *Age*I restriction enzyme sites were also included on each end for insertion into the HBsAg sequence, an overhang of 3 nucleotides was included to enable better efficiency of restriction digestion downstream (Figure 7A). Generated p145, J8 and J14 GAS-m fragments had expected sizes of 78 bp, 102 bp and 105 bp respectively and these sizes were confirmed through agarose gel visualisation (Figure 7B).

A

B

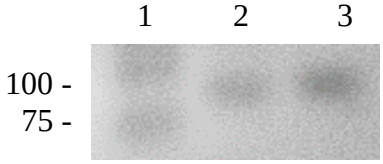


Figure 7 Primer design and matching agarose gel fragments

A. Primer design for generation of p145, J8 and J14, designed to include resitriction enzyme sites for *AgeI* restriction enzyme and synthesised with forward and reverse long primers. B. 2% agarose gel depicting the amplified p145, J8 and J14 DNA respectively in lanes 1, 2 and 3. Sizes depicted approximately mirror the expected sizes of 78 bp, 102 bp and 105 bp. Smear above bands is present as DNA had not been purified as detailed in section 3.2.

4.1 GAS-m Fragment Insertion into HBsAg

HBsAg-s and pcDNA3.1 were digested and ligated first in readiness for GAS-m epitope insertion into the 'a' determinant region of HBsAg-s. Firstly, pcDNA3.1:HBsAg-s post

ligation vector was transformed into JM1091 TOP10 *Escherichia coli* (Promega) for selection of positive uptake through PCR screen as described in section 3.3. Plasmid was visualised on agarose gel (Figure 7) and sequence integrity was confirmed through sequencing. GAS-m constructs p145, J8 and J14 fragments were inserted into the 'a' determinant of the HBsAg-s within pcDNA3.1 through *AgeI* digestion followed by ligation. Expected product output size was 766 bp, 784 bp and 787 bp respectively for p145, J8 and J14 fragments selectively amplified from within pcDNA3.1:HBsAg (Figure 7).

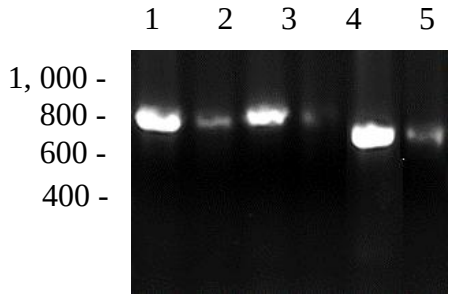


Figure 8 1% Agarose gel of amplified DNA from a colony PCR screen demonstrating insertion of GAS-m fragments into the 'a' determinant region of HBsAg

Wells contain 1 µl and 0.4 µl of each HBsAg:p145 (lanes 1-2), HBsAg:J8 (lanes 3-4) and HBsAg:J14 (lanes 5-6) PCR products respectively. These were amplified through PCR utilising HBsAg forward primers and HBsAg reverse primers.

4.2 PVLP55 Vector Insertion

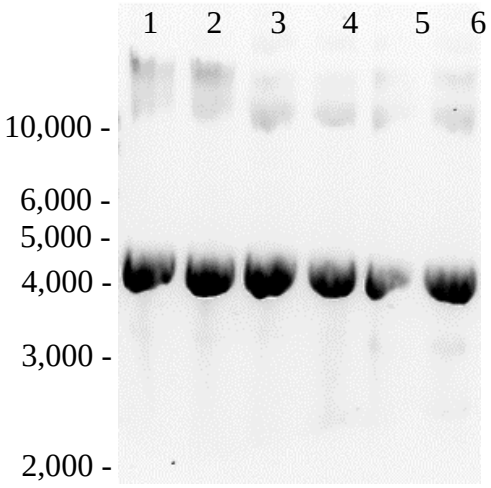


Figure 9 Agarose gel of pcDNA3.1 containing HBsAg:GAS-m fragments
Plasmids were purified from E. *coli* utilising the midiprep technique as described in section 3.2. Wells 1 and 2 contain pcDNA3.1:HBsAg-s:p145, 3 and 4 contain pcDNA3.1:HBsAg-s:J8 and 5 and 6 contain pcDNA3.1:HBsAg-s:J14, where each sample was run on the gel in 0.4 µl (lanes 1, 3 and 5) and 1 µl (lanes 2, 4 and 6).

4.3 DNA Sequence Analysis

Sequencing analysis was carried out to verify correct insertion of the GAS-m epitopes into the HBsAg sequence. Sequencing results from the pcDNA3.1:HBsAg:p145 plasmid confirmed that the inserted p145 sequence is 60 base pairs in length, in the correct orientation and sequence integrity was maintained (Figure 9). Sequencing results for J8 and J14 inserts were not completed.

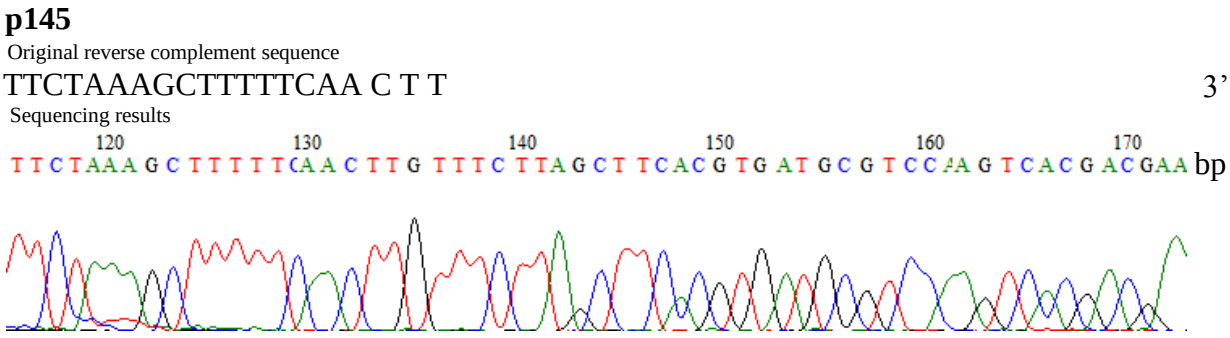


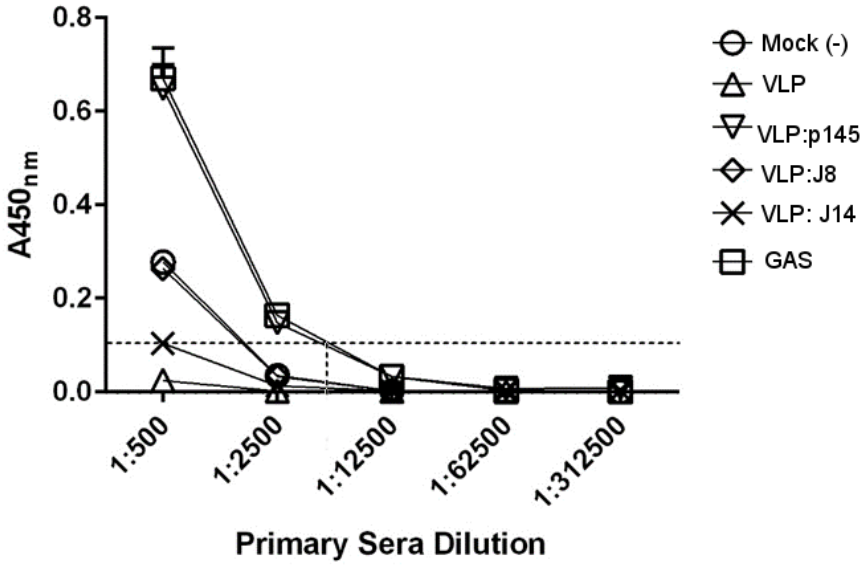
Figure 10 Chromatogram of pcDNA3.1:HBsAg-s:p145 sequencing

Sequencing results for the region covering the p145 insert are displayed alongside the matching original reverse compliment sequences for comparison.

4.4 Detection of HBsAg:GAS:m VLPs by ELISA

ELISA was undertaken to confirm expression of HBsAg VLP and HBsAg:GAS:m VLP constructs. These proteins have been referred to as VLP, VLP:p145, VLP:J8 and VLP:J14 throughout this study. The ELISA plates were pre coated with protein in carbonate coating buffer, and serial dilutions of HBsAg and J8 sera polyvalent rabbit sera were performed in triplicate. Following incubation with the secondary HRP antibody and the addition of TMB substrate absorbance was measured at 450 nm (Figure 10). Titres of VLP:p145 from both HBsAg and J8 sera tests were similar to the heat-killed GAS positive control and higher than the VLP expression control and Engerix B. This indicates that >20 µg/mL of VLP:p145 resulted from protein expression. Engerix B is the current Hepatitis B vaccine and is a formulation containing HBsAg VLPs at 20 µg/mL. However, analysis of the VLP:J8 and VLP:J14 protein samples resulted in a reading lower than the mock (no transfection) negative control.

A



B

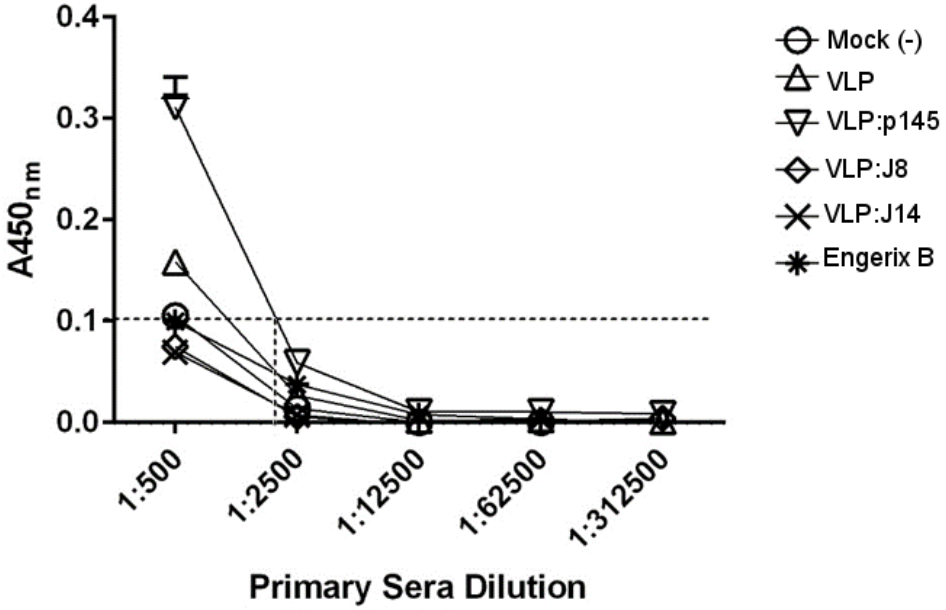


Figure 11 Detection of VLP:p145, VLP:J8 and VLP:J14 by ELISA

Serial dilutions of primary J8 serum (A) or HBsAg-s serum (B) were utilised where the graph displays mean ± standard error for triplicate wells at 450 nm absorbance. Primary sera dilution refers to the ratio at which the protein was diluted. High titres of VLP:p145 were obtained for both J8 and HBsAg-s sera in comparison to the positive controls. Low titres of VLP:J8 and VLP:J14 were obtained in comparison to the negative controls.

Discussion

GAS is responsible for a substantial global disease burden with an estimated 18.1 million individuals currently suffering disease due to GAS infection and sequelae (Carapetis *et al.* 2005). Despite GAS susceptibility to penicillin, the disease burden has not been shown to decrease and effective treatment of sequelae such as RHD can require monthly penicillin injections over many years (Gerber *et al.* 2009). Preventative measures such as improved living conditions and vaccination are the superior solutions in terms of reducing mortality, morbidity and economic costs, including within the developed world.

In this study, GAS M protein epitopes p145, J8 and J14 were amplified by PCR through custom primers and inserted into the 'a' determinant region of HBsAg within a mammalian expression vector.

Sequencing results displayed successful insertion of the p145 gene (Figure 9). Sequencing results for HBsAg:J8 and HBsAg:J14 fragments are incomplete, however no errors have been observed in preliminary sequence data to date. Correct sequencing data is of paramount importance as errors such as double or backwards inserts can occur in recombinant DNA manipulation. The 'a' determinant region of HBsAg-s is located within a double-looped structure where one 22 nm particle contains about 100 HBsAg-s molecules. Incorrect insertion in this area could result in unfavourable assembly of proteins for antibody recognition (Netter *et al.* 2001). As well as sequencing results, DNA purification techniques can also be employed to reach a quality DNA output and remove short primers, unincorporated dNTPs, enzymes, short failed PCR products and salts from PCR reactions. Techniques described in sections 3.2.2, 3.2.4 and 3.2.5 of DNA purification and clean up assisted in this process. Netter *et al.* (2001) undertook a similar study utilising HBsAg-s VLPs where proteins were purified through a 20% sucrose cushion followed by a CsCl density gradient, which was further measured by the Prism HBsAg assay. Examination by

electron microscopy was also performed and compared with wild type HBsAg. Further development on this study could be conducted by mirroring Netter's purification methods. Further techniques such as hydroxyapatite chromatography for the purification of plasmid DNA and affinity tagging for protein purification could be considered (Hilbrig & Freitag 2012; Young *et al.* 2012).

Protein expression was achieved using HEK293 cells. A similar study, undertaken by Kotiw *et al.* (2012), utilised epitopes from the *H. pylori* KatA gene inserted into HBsAg-s. In this study, VLPs were expressed using the HuH7 hepatocellular carcinoma cell line for use in animal vaccination models. Sufficient yield was obtained for animal model testing and HBsAg-s conformation was confirmed through electron micrographs. Furthermore, this method of VLP expression has also shown success in studies by Netter *et al.* (2003) with Hepatitis C VLPs and Schumacher *et al.* (2007) in tumor therapy VLPs. This study, however, utilised HEK293 cells, a predominant cell line used for transient expression of recombinant proteins, where a foreign gene is expressed for a period of time but not integrated into the genome. The HEK293 cell line has the clear advantage of rapid production for usage within a time-restricted study (Geisse & Fux 2009).

ELISA testing was undertaken to confirm expression and measure antigenic recognition of VLP constructs through serial dilution of HBsAg and J8 sera. Results indicated a high VLP:p145 yield for both J8 and HBsAg sera tests. Similar results of the positive control heat-killed GAS and standard VLP controls in comparison to VLP:p145 were obtained with an estimated yield of 20 µg/mL (Figure 10A and 10B), indicating immunogenicity. Studies by Wurm *et al.* (2004) indicate that baseline values of 20-40 µg/mL yields can be obtained at a specific productivity of 1-4pg/cell/day using HEK293 cells, suggesting that VLP:p145 yields within this study are on par with research standards. Within both primary sera tests, VLP:J8 and VLP:J14 proteins were detected at similar or lower titres in comparison to the

negative controls. Low titres of VLP:J8 and VLP:J14 indicate that either the original DNA sequence was incorrect or the correct VLP was unfavourable for antibody detection. The presence of an incorrect DNA sequence is possible as it is still unverified by sequencing results. However, if the latter conclusion is correct then this would suggest that placement of these GAS:m genes for use within VLP may be unsuitable due to incompatibility. Similar findings by Kotiw *et al.* (2012) and Netter *et al.* (2003) support this conclusion, where data has suggested that interference may occur when the HBsAg-s 'a' determinant is disrupted by foreign sequences. This could be due to minor epitopes remaining within the 'a' determinant or elsewhere in the HBsAg molecule, misfolding or unstable expression of HBsAg-s proteins. ELISA testing within this study is a potential limitation as actual expression level may vary as peptides have been inserted into the 'a' determinant region which is highly antigenic.

Further protein verification work and higher yields are required before the project could be continued. This study is further limited by the lack of protein purification and SDS-PAGE results, which could further indicate protein quality.

To increase protein expression yields for animal studies a yeast expression system could be considered. Yeast systems utilising *Saccharomyces cerevisiae* in the development and production of the Hepatitis B vaccine were successful obtaining high yields and successfully demonstrated protection in grivet monkeys (McAleer *et al.* 1984). Yeast expression systems are favourable as they can obtain high protein yields of 1000 µg/mL (Young & Robinson 2014). Additionally, yeast systems hold the advantage of being single cells with fast growth capabilities as well as possessing eukaryotic abilities such as secretory pathways leading to correct protein processing (Porro *et al.* 2005).

Significance

VLPs have a number of advantages as a vaccine as they are particulate in nature, safe, stable and able to carry foreign epitopes. Particulate vaccines are advantageous as they can be efficiently taken up by APCs, this enables the VLP to act as an adjuvant and may mean that

other adjuvants are not required within the vaccine preparation. VLPs are non-infectious, non-replicating and have higher stability than soluble antigens in extreme environmental conditions, this makes VLPs favourable for use in developing countries (Zhao *et al.* 2013). VLP and foreign antigen compatibility make recombinant proteins potentially useful for usage within dual vaccine regimens. Within this study, peptides of GAS M protein were inserted into the 'a' determinant region of HBsAg-s sequence which is highly immunogenic (Netter *et al.* 2003; Vietheer *et al.* 2007).

Approximately 20 GAS vaccine prototypes have been created across the spectrum of GAS cell wall and secreted proteins, yet few have progressed to clinical trials. Evidence has shown that there is a biological feasibility for such a vaccine to exist. For example, GAS pharyngeal studies undertaken in the 1970's successfully demonstrated protection against challenge with a homologous strain of GAS after immunisation with purified M proteins (Polly *et al.* 1975). Preclinical murine studies have demonstrated protection against challenge infections when vaccinated with purified M proteins (Dale *et al.* 2011; Guerino *et al.* 2011). Furthermore, patterns of GAS infection in school aged children who are repetitively exposed to GAS indicate that a threshold level of protective immunity can be achieved (Martin *et al.* 2004).

Despite a long record of research in GAS vaccine development, a protective/acceptable vaccine is not yet in the foreseeable future. The World Health Organisation's roadmap for GAS vaccine development outlines past and present research for future developments is a step forward in collaboration of multi-disciplinary consensus in vaccine licensure, but many challenges are still present (Dale *et al.* 2013). Remaining challenges include safety concerns about the theoretical risk of autoimmune reactions and a necessity for further understanding of the basis for immunological protection in humans. For example, a greater understanding of the contributions of non-M type-specific antigens in inducing protective immunity,

948 immune protection against GAS skin infection and the role of T-cell immunity are necessary
949 (WHO 2014).
950 Suitable GAS peptides intended for vaccination must be conserved throughout GAS
951 serotypes and free of molecular mimicry to human host antigens. To date, the most
952 successful peptides to overcome these challenges have arisen from the M protein. Early
953 studies utilising whole M protein resulted in the development of RF-like symptoms, however,
954 refined attempts such as StreptInCor, J8 and J14 minimal epitope vaccines and the 30-valent
955 vaccine have shown promising results in animal and clinical trials (Dale *et al.* 2011; Guerino
956 *et al.* 2011; Massell *et al.* 1968). However, serotype-specific prototypes such as these have
957 been criticised due to low coverage of strains prevalent in developing countries (Steer *et al.*
958 2009). Thus, the previous 26-valent vaccine which had progressed to Phase II clinical trials
959 in adult human volunteers was re-worked into a 30-valent vaccine to allow greater coverage
960 in the Asia-Pacific region. Criticism regarding coverage has been met with a counter-
961 argument in the light of evidence suggesting cross-protection between *emm*-types may be
962 inferred by the re-developed 30-valent vaccine (Sanderson-Smith *et al.* 2014). However,
963 high valency vaccines such as these may incur a higher production costs making the vaccine
964 prohibitive for widespread use. Both the 30-valent and the minimal epitope J8 vaccine are
965 anticipated to begin phase I trials within adult volunteers as of 2014 (WHO 2014). The main
966 advantage of the 30-valent vaccine lies in the wide coverage through utilisation of fused
967 recombinant peptides from the N-terminal section of M protein. However, conserved M
968 protein vaccines such as StreptInCor and the minimal epitope J8 and J14 vaccines have the
969 greater advantage of consisting of single antigens, lessening the chance of a potential
970 autoimmune reaction (Batzloff *et al.* 2003; Guerino *et al.* 2011).
971 Use of virus-like particles in conjunction with minimal epitope J8 and J14 vaccines are likely
972 to broaden the immune response, making combination vaccines are a more viable approach in

the long term. Despite the multitude of functional GAS vaccine candidates, no commercial vaccine is yet available. It is likely that there is reluctance by large pharmaceutical companies to invest in clinical development of GAS. Questionable markets for a GAS vaccine in affluent countries and the challenges mentioned previously may amount to an adverse commercial risk (WHO 2014). A combination GAS/Hepatitis B vaccine may present a more effective and reliable investment for usage within developed and developing countries alike. Furthermore, worldwide Hepatitis B vaccination is part of the WHO's primary prevention and control framework for global action (WHO 2012). A successful vaccine such as this could allow smarter investing, improve quality of life and reduce mortality across developing and developed countries alike.

Future Directions

A combination vaccine for both GAS and Hepatitis B could be an important piece of the puzzle within global health, and this study had success in the synthesis of VLP:p145.

To progress this study it is essential to verify the sequencing of VLP:J8 and VLP:J14, to confirm correct insertion orientation of GAS:m fragments within the HBsAg 'a' determinant region. Further protein analysis such as SDS-PAGE and western blot and other testing to verify protein purity and configuration could be conducted.

Proof-of-concept animal studies are essential to evaluating the success of this project. Isolated and purified vaccine candidates would be evaluated in GAS challenge studies. Mice would be vaccinated with recombinant VLPs followed by intranasal challenge with GAS mice would then be euthanized and examined for an immunological response. Vaccinated mice serum would be assayed for a specific antibody. GAS load in pharyngeal tissue would be determined by culture and histology 4 days post bacterial challenge. Generation of this data using would evaluate the ability of GAS:m proteins to generate an antibody response and protection from GAS infection when delivered as a dual vaccine within the HBsAg VLP.

998

Conclusion

A successful GAS vaccine has the potential to save over 500,000 premature deaths annually, greatly improve quality of life and reduce the economic burden of common childhood diseases caused by GAS (Carapetis *et al.* 2005).

ELISA assays showed that GAS antigenic peptides can be expressed in HBsAg VLPs for use as a dual vaccine. Specifically, from preliminary results VLP:p145 obtained high protein titres. Further testing of these vaccine candidates still needs to occur and use of proof-of-concept murine challenge models will be essential in assessing their efficacy. GAS vaccines are possible, but not in the foreseeable future despite a long history of developments. Incorporation of GAS:m peptides into a dual or combination vaccine may offer a more appealing solution for widespread GAS protection across developed and developing countries alike.

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