

# **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

.....

Date

## Endorsement

Supervisor: Professor Mike Kotiw

Co-Supervisor: Dr Lisa Seymour

Signature

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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 $\alpha_2$ -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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# 1 Introduction

## 2 1.0 Overview of group A streptococcus (GAS) pathogenesis

3 The bacterium Group A Streptococcus (GAS) (also known as *Streptococcus pyogenes*) is a  
4 Gram positive coccus which is responsible for a variety of infections and associated  
5 syndromes (Goering *et al.* 2012). GAS is prevalent in under-privileged communities such as  
6 the Australian Indigenous population and much of the developing world (Carapetis *et al.*  
7 2005). GAS can cause a number of non-invasive diseases such as pharyngitis, impetigo,  
8 pyoderma, scarlet fever and otitis media as well as invasive diseases including necrotising  
9 fasciitis and toxic shock syndrome. Post-infection sequelae, however account for the highest  
10 global burden of GAS disease and includes rheumatic heart disease (RHD), rheumatic fever  
11 (RF), related endocarditis and stroke, as well as acute post-streptococcal glomerulonephritis  
12 (APSGN) (Henningham *et al.* 2013). Whilst research activity in GAS vaccinology has been  
13 ongoing for decades, no approved vaccine has yet become available. A broad 30-valent  
14 vaccine is under development, however the vaccine focusses on North American and  
15 European specific GAS serotypes which may not address the main serotypes present in the  
16 developing world. One of the main challenges in vaccine design is the incorporation of the  
17 150 known M protein serotypes to provide cross-serotype protection. Studies of pharyngeal  
18 infections in South African school children indicate the vaccine coverage will be between 40-  
19 59% of isolates recorded (Dale *et al.* 2011). This indicates that the 30-valent vaccine is an  
20 ineffective option for a significant proportion of affected individuals in the developing world  
21 (Dale *et al.* 2011). Among the many conserved virulence factors which have been explored  
22 for usage in a GAS vaccine, the M protein is a promising target for vaccine research as it is a  
23 highly conserved, abundant surface GAS protein (O'Brien *et al.* 2002).

## 24 1.1 Distribution and incidence of disease

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

27 Furthermore, RHD can be fatal and is estimated to cause 233,000 deaths per year (Carapetis  
28 *et al.* 2005). APSGN affects 472,000 individuals and causes a further 5,000 deaths annually.  
29 Superficial infections occur more frequently, where 6.1 million new cases of pharyngitis arise  
30 every year and 1.1 million individuals currently suffer of pyoderma (Carapetis *et al.* 2005).  
31 Uncertainty levels and assumptions used in the compilation of GAS data tends towards  
32 underestimation, making the true prevalence likely to be greater than stated. Carapetis *et al.*  
33 (2005) acknowledges that issues in data collection in developing countries affects quality of  
34 the data. RHD specific data, however, has a higher level of quality through rigorous data  
35 collection. RHD has a wide geographical distribution, peaking in the following countries as a  
36 calculated regional prevalence per thousand: Sub-Saharan Africa at 5.7 cases, South-Central  
37 Asia at 2.2 cases, other Asian areas at 0.8 cases, Latin America at 1.3 cases, Middle East and  
38 North Africa at 1.8 cases, Eastern Europe at 1 case, Pacific and Indigenous Australia/New  
39 Zealand at 3.5 cases and China at 0.8 cases (Carapetis *et al.* 2005).

## 40 **1.2 Populations at risk**

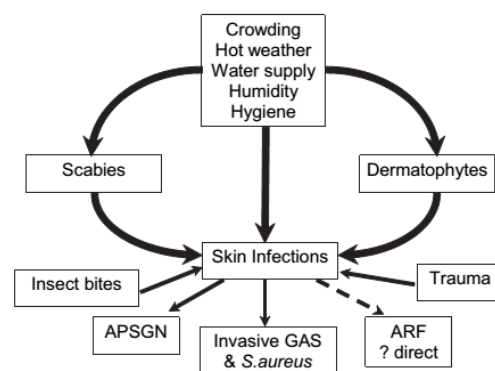
41 Populations at risk for GAS disease include children and young adults within the developed  
42 and developing countries. Less developed countries account for 79% of RHD cases, 95% of  
43 RF cases, 97% of APSGN cases and 97% of invasive GAS cases. Indigenous Australians  
44 also experience high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

### 45 **1.2.1 Indigenous Australians**

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

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

56 GAS related skin infections such as scabies and streptococcal pyoderma are endemic in  
 57 Australian Indigenous communities. Scabies is a parasitic infection caused by the human itch  
 58 mite or *Sarcoptes scabiei*, and is known to spread easily in environments with poor sanitation  
 59 and overcrowding (FitzGerald *et al.* 2014). There is a distinct link between streptococcal  
 60 pyoderma and scabies, as demonstrated by Andrews *et al.* (2009) (See Figure 1). For  
 61 example, both scabies and streptococcal pyoderma have been found in Indigenous Australian  
 62 populations and Alaskan native populations. Scabies is prevalent in 50% of children and  
 63 25% of adults in many remote communities (Carapetis *et al.* 1997). Unusually, there is a  
 64 distinctly lower rate of GAS throat carriage of between 0-14% in comparison to RHD and  
 65 skin infections (Van Buynder *et al.* 1992). Household crowding, access to adequate water,  
 66 hot weather, humidity and lack of personal hygiene are likely to contribute to a high  
 67 prevalence of GAS disease in the Indigenous Australian population (Figure 1) (Munoz *et al.*  
 68 1992).



77 **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).

78

### 79 **1.2.2 Paediatric**

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

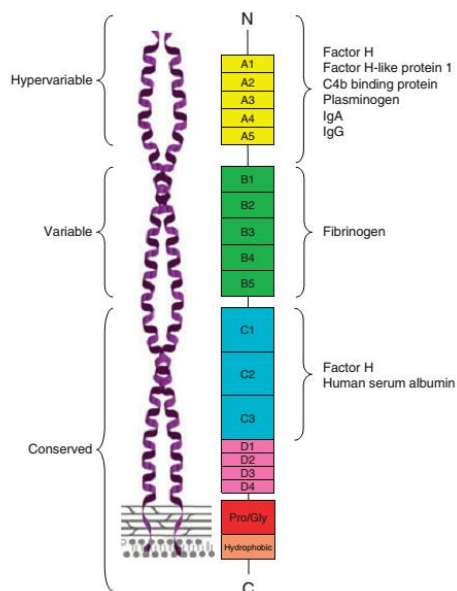
## 88 **2.0 The biology of group A streptococcus**

89 Infection with GAS begins with the binding of bacterial surface ligands to extracellular  
90 matrix components or to specific receptors on dermal epithelial cells. Adhesion is a two-step  
91 process, where an initial weak interaction is engaged with the mucosa to overcome  
92 electrostatic repulsion. The exchange is finalised through an event conferring tissue  
93 specificity and high-avidity adherence. To complete the interchange, a number of host  
94 components are known to be involved, such as fibrinogen, fibrin, elastin, vitronectin, laminin,  
95 decorin and heparin sulphate-containing proteoglycans (Moschioni *et al.* 2010). In  
96 particular, fibrinogen is known to interact and bind with the highly conserved M protein in  
97 the  $\beta$ -repeat region near the N-terminus of the protein (Carlsson *et al.* 2005).  
98 As well as cellular adherence, GAS also has the capacity to enter epithelial cells to avoid  
99 early host defences and antibiotics. This invasive virulence process is possible through  
100 proteins on the cell surface, or invasins such as fibronectin-binding protein and the M protein  
101 (LaPenta *et al.* 1994). Further research by Dombek and colleagues (1999) indicates that  
102 invasion initiates through a zipper-like mechanism where host microvilli also play a role.

## 103 2.1 Virulence factors

### 104 2.1.1 M protein

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



118

### 119 Figure 2 M protein hypervariable, variable and conserved structures

120 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).

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

### 130 **2.1.2 Capsule**

131 The capsule structure contributes considerably to the success of the bacteria. Acapsular  
132 strains have been shown to have markedly decreased phagocytic resistance, and a 100-fold  
133 decrease in virulence when tested in mice (Wessels *et al.* 1991). The GAS capsule consists  
134 of a hyaluronic acid with the degree of encapsulation varying greatly across the serotypes  
135 (See Figure 3). GAS has been found to up-regulate hyaluronic acid production to structurally  
136 minimise antibody access to bacterial surface protein G-related  $\alpha_2$ -macroglobulin-binding  
137 protein (GRAB). This mechanism of evasion contributes to the difficulty in creating a  
138 functional vaccine as it enables GAS to escape recognition by antibodies (Dinkla *et al.* 2007).

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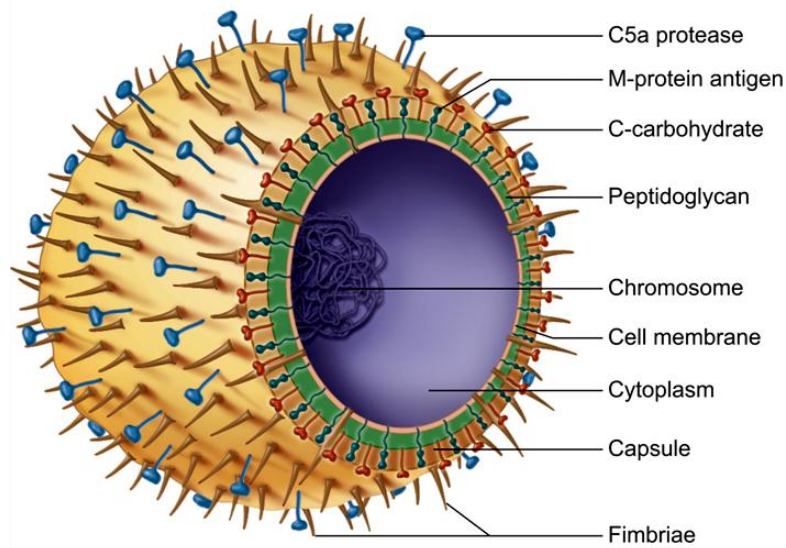
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### Figure 3 GAS Cross Section

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



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

#### 184 **2.1.5 C5a Peptidase**

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

#### 190 **2.1.6 Cysteine protease SpeB**

191 Cysteine protease SpeB is a highly conserved and multi-functional pyrogenic exotoxin  
192 hypothesised to have a role in severe invasive infection and streptococcal toxic shock  
193 syndrome (Collin & Olsén 2001). SpeB is able to cleave human immunoglobulins, including  
194 IgA, IgM, IgD and IgE (Collin & Olsén 2001). Furthermore, it can cleave vitronectin,  
195 fibronectin and host proteins to compromise host tissue integrity (Kagawa *et al.* 2009). It can  
196 also spawn biologically active peptides such as interleukin-1, kinins and histamine (Kagawa  
197 *et al.* 2000). Through the degradation of host proteins, research conducted by Barnett *et al.*  
198 (2013) suggests that a proteolytic SpeB mechanism is utilised by GAS to evade autophagy  
199 and enable replication in the cytosol of host cells.

200

## 201 **2.2 Genetics including virulence gene control**

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

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

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

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

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

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

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

## 246 **2.3 Nature and spectrum of GAS infections**

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

### 249 **2.3.1 Common streptococcal throat infection**

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

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

### 256 **2.3.2 Rheumatic fever and rheumatic heart disease**

257 RF is a delayed sequelae of acute GAS pharyngitis and is characterised by inflammation of  
258 the joints, heart, central nervous system, skin and or subcutaneous nodules (McNamara *et al.*  
259 2008) (Table 1). The Jones criteria (Burke & Chang 2014) states RF can be diagnosed  
260 through the presence of a preceding GAS infection, plus the presence of two major  
261 manifestations or one major and two minor manifestations of the Jones criteria. Symptoms of  
262 the Jones criteria mainly include carditis, polyarthritis and Sydenham's chorea. Further  
263 research is needed into pathophysiology and potential biomarkers of RF, as the above  
264 symptoms are known to cross over with diseases such as Lyme disease, serum sickness, drug  
265 reactions and post-streptococcal reactive arthritis (Burke & Chang 2014).

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

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

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

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

## 280 **2.4 Current treatment options**

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

285

### 286 **2.4.1 Antibiotic therapy**

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

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

## 306 **2.5 GAS vaccine development**

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

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

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



	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 <i>et al.</i> 2011)
	C-repeat epitope (StreptInCor vaccine)	C-terminal amino acid residues/subcutaneous delivery in mice via StreptInCor suspension	(Guerino <i>et al.</i> 2011)
	Conserved region proteins expressed in <i>Lactococcus lactis</i>	C-repeat amino acid residues/nasal and subcutaneous delivery in mice via peptide specific serum	(Mannam <i>et al.</i> 2004)
<b>Fibronectin-Binding Protein A</b>	Seven FbaA epitopes co-administered with five M protein fragments	Recombinant multivalent protein/Intraperitoneal delivery in mice via peptide specific serum	(Ma <i>et al.</i> 2014)

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

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

## 327 **2.6 Virus Like Particle (VLP) technology**

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

### 340 **2.6.1 Overview of the use of VLP technology for human diseases**

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

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

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

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

### 360 **2.6.1.1 Generating recombinant chimeras**

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

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379 p145 LRRDLASREAKKQVEKALE  
 380 J1 QLEDKVKQLRRDLASREAKEELQDKVK  
 J2 LEDKVKQARRDLASREAKKELQDKVKQ  
 J3 EDKVKQAERDLASREAKKQLQDKVKQL  
 381 J4 DKVKQAEDDLASREAKKQVQDKVKQLE  
 J5 KVKQAEDKLDASREAKKQVEDKVKQLED  
 382 J6 VKQAEDKVDASREAKKQVEKKVKQLEDK  
 J7 KQAEDKVKASREAKKQVEKAVKQLEDKV  
 383 J8 QAEDKVKQSREAKKQVEKALKQLEDKVQ  
 J9 AEDKVKQLREAKKQVEKALEQLEDKVQL  
 J14 KQAEDKVKASREAKKQVEKALEQLEDKVK  
 384 Jcon DKVKQAEDKVKQLEDKVEELQDKVKQLE

385  
**Figure 4 GAS-m Protein C-region Peptides**  
 386 Research undertaken by Hayman *et al.* (1997) explored a series of overlapping peptides (p145-J14)  
 387 within the C-region of the GAS-m protein, where Jcon was a non-specific sequence as a control.  
 388 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  $\alpha$ -helical folding of M proteins.

389  
 390 Past recombinant chimeric platforms include the hepatitis B virus core, woodchuck hepatitis  
 391 B virus core, hepatitis B virus S antigen, human papillomavirus, bovine papillomavirus,  
 392 human immunodeficiency virus (HIV), simian immunodeficiency virus HIV chimera, duck  
 393 hepatitis B virus and hepatitis E virus (Grgacic & Anderson 2006). In the commercial  
 394 setting, VLPs are synthesised through insect and yeast cell-based systems for their ease of  
 395 production, cost efficiency, post-translational modifications and adjustable production size.  
 396 Bacteria, mammalian and plant cells and cell-free synthesis have been utilised in the  
 397 laboratory setting to produce VLPs (Rosenthal *et al.* 2014). VLP technology has proven to  
 398 be safe, effective, long lasting and cost efficient. In particular, the HBsAg-S surface protein  
 399 is the most common molecule used in VLP technology due to its non-infectious nature and  
 400 worldwide licence for use (Netter *et al.* 2003).

401 **2.6.1.2 Overview of use of HBsAg-S VLPs**

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

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

## 415 **2.7 The current study proposal**

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

### 422 **2.7.1 Proposed strategy**

423 Indigenous Australians have the highest global RHD and/or RF mortality rate of 30.2  
424 individuals per 100,000 annually (Carapetis *et al.* 2005). The purpose of the study is to target  
425 the Australian Indigenous population and developing countries through the development and  
426 evaluation of a HBsAg-S-M protein dual vaccine. The HBsAg-S surface protein VLP has  
427 proven immunogenic efficacy and safety as numerous licenced vaccines. Furthermore, the M  
428 protein epitopes J8, J14 and p145 have demonstrated antigenic and safety properties.  
429 PCR generated DNA sequences of J8, J14 and p145 from the M protein of GAS will be  
430 cloned into the 'a' determinant region of the HBsAg-SS and transformed into a mammalian

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

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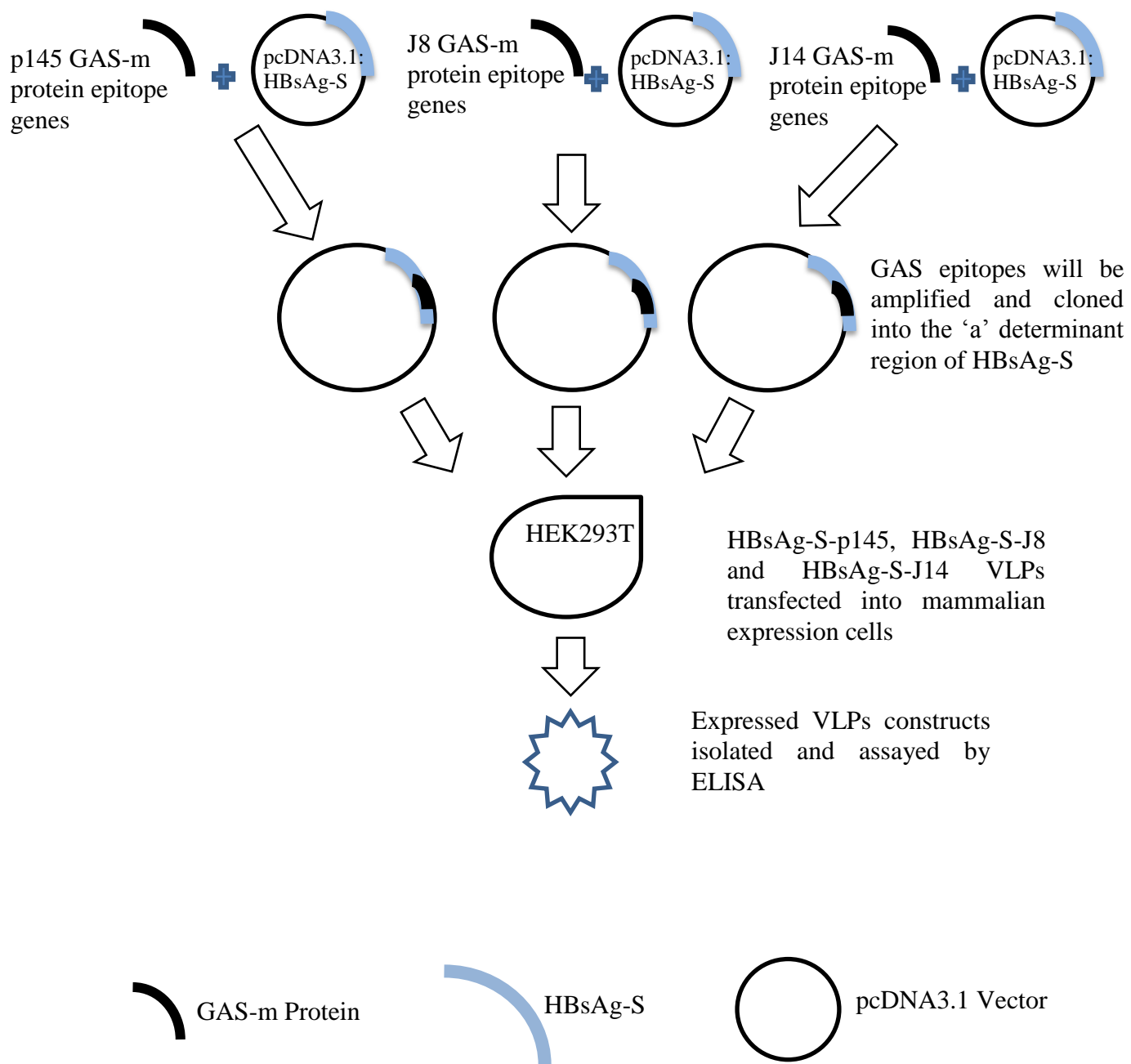
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**Figure 5 Proposed Strategy Details**



448 **Hypotheses**

449 1. GAS antigenic peptides can be expressed in HBsAg-S VLPs to utilise VLPs as a carrier  
450 molecule for a dual vaccine.

451 2. GAS VLPs which are recognised by GAS HBsAg-S sera will be good vaccine candidates  
452 to provide protection against GAS in an animal model.

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## 457 **Materials and Methods**

### 458 **3.0 Bacteria and Plasmids**

459 *Escherichia coli* JM109 (Promega) and TOP10 (Life Technologies) was grown in Luria  
460 Bertani (LB) broth or on LB agar containing 100 µg/ml ampicillin. Incubation of plated and  
461 liquid bacterial cultures was performed at 37°C in a Bioline Thermocube Incubator (Bioline  
462 Alexandria, NSW, Australia), where required shaking was performed at 5 x g. The  
463 mammalian protein expression vector was pcDNA3.1:HBsAg-S which was kindly provided  
464 by Hans Netter (Monash University, Melbourne, Australia).  
465

### 466 **3.1 Molecular Analyses**

467 Primers with accompanying forward and reverse sequences are detailed below in Table 3.  
468 Primers were purchased from Invitrogen, and all PCR reactions were carried out in a PTC-  
469 100™ Programmable Thermal Controller (MJ Research Inc, Quebec, Canada.).  
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484 **Table 3 Sequence of oligonucleotide primers used**

Application	Template DNA		Primer Sequence (5'→ 3')
<b>GAS-m epitope generation</b>	p145	Full sequence	GGAACCGGTCTTCGTCGTGACTTGGACGCATCACG TGAAGCTAAGAAACAAGTTGAAAAAGCTTTAGAA ACCGGTTGG
		Forward Primer	GGAACCGGTCTTCGTCGTGACTTGGACGCATCACG TGAAGCTAAGAAAC
		Reverse Primer	CCAACCGGTTTCTAAAGCTTTTTCAACTTGTTTCTT AGCTTCACGTGATG
<b>GAS-m epitope generation</b>	J8	Full sequence	GGCACCGGTCAGGCGGAAGATAAAGTGAAACAGT CACGTGAAGCTAAGAAACAAGTTGAAAAAGCTTTA AAACAGCTGGAAGATAAAGTGCAGACCGGTGGC
		Forward Primer	GGCACCGGTCAGGCGGAAGATAAAGTGAAACAGT CACGTGAAGCTAAGAAACAAGTTG
		Reverse Primer	GCCACCGGTCTGCACTTTATCTTCCAGCTGTTTTAA AGCTTTTTCAACTTGTTTCTTAGCTTC
<b>GAS-m epitope generation</b>	J14	Full sequence	GGCACCGGTAAGCAGGCGGAAGATAAAGTGAAAG CATCACGTGAAGCTAAGAAACAAGTTGAAAAAGCT TTAAAACAGCTGGAAGATAAAGTGAAGACCGGTG GC
		Forward Primer	GGCACCGGTAAGCAGGCGGAAGATAAAGTGAAAG CATCACGTGAAGCTAAGAAACAAGTTG
		Reverse Primer	GTGAAGCTAAGAAACAAGTTGAAAAAGCTTTAGTA CAGCTGGAAGATAAAGTGAAGACCGGTGGC
<b>Amplification</b>	HBsAg-SFwd		GTAGAATTCGCCACCATGGAGAACATCACATCAG
<b>Amplification</b>	HBsAg-SRev		CTGCGGCCGCTTAAATGTATACCCAAAGAC
<b>Sequencing</b>	AOX 1	Reverse Forward	GCAAATGGCATTCTGACATCC GACTGGTTCCAATTGACAAGC

485

### 486 **3.2 Amplification of GAS-m Epitopes**

487 Synthesis of GAS-m DNA fragments utilised p145, J8 and J14 forward and reverse primers

488 as shown in Table 3. The sequence ‘ACCGGT’ was included as a site for the *AgeI* restriction

489 enzyme for ease of ligation of fragments into the pcDNA3.1:HBsAg-S mammalian

490 expression vector. Sequences of p145, J8 and J14 were obtained from previous studies by

491 Hayman *et al.* (1997). Expected PCR product sizes for p145, J8 and J14 are 78, 102 and 105

492 bp respectively. p145, J8 and J14 forward and reverse primers were used to synthesise the

493 p145, J8 and J14 GAS-m DNA sequences through PCR. Three primer pairs were required to  
494 create the fragments as given in Table 3. Each forward primer 5' end contained ACCGGT  
495 with three additional N terminal nucleotides to enable restriction digestion with enzyme *AgeI*-  
496 *HF* (New England Biolabs, Arundel, Queensland, Australia), as seen in Table 3 in bold  
497 lettering. PCR reactions to generate each fragment contained 5 µl of x10 AccuBuffer  
498 (Bioline), 1 µl of 10 mM dNTPs (Promega, Alexandria, NSW, Australia), 1.5 µl of both  
499 appropriate forward and reverse primers at 20 pmol/µl (Table 3), 1 µl of Accuzyme™ DNA  
500 polymerase (Bioline) and Milli Q water to make reaction volume up to 50 µl. The PCR was  
501 performed using the following parameters: 95°C for 3 min, followed by 30 cycles of 95°C for  
502 15 s, 56°C for 15 s and 72°C for 15 s. The resulting PCR products were viewed on a 1%  
503 agarose gel as described in section 3.2.1, followed by purification using the Wizard® SV Gel  
504 and PCR Clean-Up (Promega).

### 505 **3.3 Agarose Gel Electrophoresis**

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

### 518 **3.3.1 Wizard® SV Gel and PCR Clean-Up**

519 The Promega (Alexandria, NSW) Wizard® SV Gel and PCR clean-up kit was utilised to  
520 purify PCR products. The PCR product or the desired DNA band was removed from the  
521 agarose gel and dissolved within 1 µl of membrane binding solution per mg of agarose gel.  
522 The solution was vortexed and incubated at 60°C until fully dissolved and transferred to a  
523 spin column and collection tube assembly for incubation at RT for 1 min. The assembly was  
524 centrifuged at 30 600 x g in Sigma 1-15 Laboratory Centrifuge for 1 min at RT, the  
525 flowthrough was discarded. DNA bound to the column membrane was washed with 700 µl  
526 of membrane wash solution and centrifuged further at 30 600 x g for 1 min at RT. The  
527 flowthrough was discarded and the step was repeated using 500 µl of membrane wash  
528 solution (Promega) and centrifuging at 30 600 x g for 5 min at RT. After discarding  
529 flowthrough, the spin column was inserted into a sterile 1.5 ml microcentrifuge tube and 50  
530 µl of nuclease free water was pipetted into the spin column. Following incubation at RT for  
531 1 min, the assembly was centrifuged at 30 600 x g for 1 min at RT. The flowthrough  
532 containing the DNA of interest was collected in a microcentrifuge tube. The sample was  
533 electrophoresed on an agarose gel for visualisation. DNA was stored at -20°C.

### 534 **3.4 Digestion and Insertion of GAS-m Epitopes into HBsAg-S DNA** 535 **sequence**

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

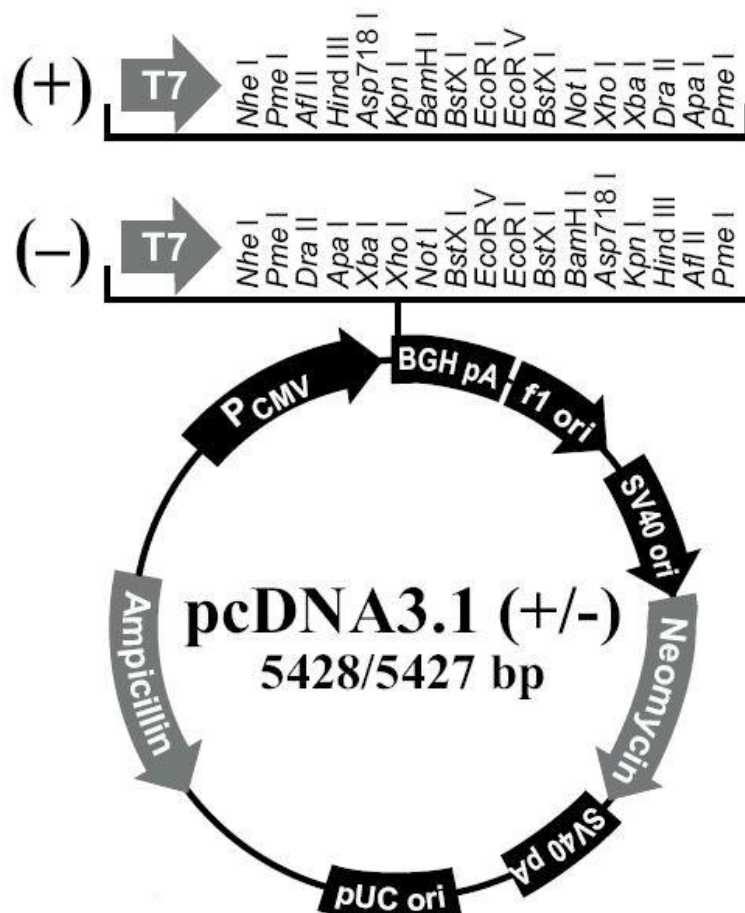
544 37°C for 15 min. Following digestion 5 units of Antarctic Phosphatase (New England  
 545 Biolabs) and 5 µl of Antarctic Phosphatase buffer (New England Biolabs) was added. The  
 546 mixture was further digested at 37°C for 60 min and heat inactivated at 65°C for 20 min.  
 547 Expected PCR product sizes for HBsAg-S:p145, HBsAg-S:J8 and HBsAg-S:J14 were 766,  
 548 784 and 787 bps respectively.

### 549 3.5 Plasmid Ligation

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

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570

### Figure 6 pcDNA3.1 Vector Map

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

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

587

### 588 3.7 Wizard® Plus SV Minipreps DNA Purification

589 The Promega (Alexandria, NSW) Wizard® Plus SV Miniprep kit was utilised to purify  
590 plasmid DNA from *E. coli*. Preparations of 10 ml of LB containing ampicillin were  
591 inoculated with single colonies of bacteria. The culture was grown overnight at 37°C with  
592 shaking and centrifuged at 2 400 x g in Sigma 3-15 Laboratory Centrifuge (Shropshire, UK)  
593 for 5 min at RT. The supernatant was decanted and the pellet resuspended in 250 µl of cell  
594 resuspension solution (Promega). 250 µl of cell lysis solution (Promega) was added to the  
595 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



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

### 624 **3.9 Wizard® Plus SV Midipreps DNA Purification**

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

#### 645 **3.9.1 HEK293T Cell Culture**

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

648 hydroxyethyl)-1-piperazineethanesulfonic acid (HEPEs), Glutamax (Gibco®) and 10% fetal  
649 bovine serum (FBS) and incubated in a SANYO Humidified CO<sub>2</sub> Incubator (North Sydney,  
650 NSW) at 37°C with 5% CO<sub>2</sub>. Cells were passaged regularly to avoid senescence and were  
651 grown in conical flasks. To passage, cells were viewed under a Leica Leitz DM IL  
652 microscope (North Ryde, NSW) to visually evaluate confluence. Spent media was discarded,  
653 5 ml of phosphate buffered saline (PBS) was added to the flask and incubated for 2 min  
654 before being discarded; this wash was repeated. 1 ml of Life Technologies 0.05% trypsin  
655 (Mulgrave, Victoria) was added and cells were incubated for <5 min at 37°C with 5% CO<sub>2</sub>.  
656 After incubation, detached cells were added to 5 ml of MEM culture media to inhibit trypsin  
657 activity. Cells were centrifuged at 500 x g for 5 min with a 3-15 Laboratory Centrifuge  
658 (Sigma, Osterode am Harz, Germany). Supernatant was discarded and cells were re-  
659 suspended in the MEM culture media and seeded into new culture flasks at a ratio between  
660 1:3 and 1:10.

### 661 **3.9.2 HEK293T Transfection and Protein Isolation**

662 For transfection cells were seeded at  $1.5 \times 10^6$  cells/10cm dish in 9 ml of pre-warmed MEM  
663 culture media. On the following day the media was replaced approximately 2 h prior to  
664 transfection. A mixture of 16 µg of plasmid DNA, 36 µl of 2M CaCl<sub>2</sub> and Milli Q water at a  
665 final volume of 300 µl was combined and quickly added to 300 µl of 2X HEPES buffered  
666 saline (HBS) (containing 10 g/l HEPES; 16 g/l NaCl; 0.74 g/l KCl; 0.27 g/l Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O;  
667 2.0 g/l dextrose). The mixture was vortexed and incubated at 37°C for 3 min before being  
668 added drop wise to the cells with gentle swirling to facilitate mixing. Cells were incubated at  
669 37°C with 5% CO<sub>2</sub>. At 6 days post-transfection, media was taken from the transfection plates  
670 and centrifuged at 690 x g for 10 min in a 3-15 Laboratory Centrifuge (Sigma, Osterode am  
671 Harz, Germany) to remove cellular debris. Supernatant was gently overlaid onto 2 ml  
672 sucrose in STE (100 mM NaCl, 10 mM Tris at pH 8, 1 mM of EDTA) in Beckman  
673 ultracentrifuge tubes. Supernatant was centrifuged in a pre-cooled SW41Ti swinging bucket

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

### 678 **3.9.3 ELISA**

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

686 High-binding 96 well Greiner Bio one plates were coated with 5 µl of protein in carbonate  
687 coating buffer (0.1 M NaHCO<sub>3</sub> at pH 9.6), in 100µl of coating buffer per well. The plate was  
688 sealed and incubated overnight at 4°C. The following day, plates were washed twice with  
689 PBST (0.05% Phosphate Buffered Saline with Tween20) which contains 3.2 mM Na<sub>2</sub>HPO<sub>4</sub>,  
690 0.5 mM KH<sub>2</sub>PO<sub>4</sub>, 1.3 mM KCl, 135 mM NaCl and 0.05% Tween<sup>®</sup> 20. 100µl of blocking  
691 buffer (2% skim milk in PBS) was added to each well and incubated for 1.5 h. The plate was  
692 washed twice with PBST and 100µl of serially diluted primary rabbit sera (anti-HBsAg-S or  
693 J8 sera). 1% skim in PBS was added per well and incubated for 1 h. The plate was washed  
694 four times with PBST, and 100µl of anti-rabbit conjugated antibody (Sigma) diluted at  
695 1:3,000 in 1% skim in PBS was added per well and incubated at RT for 1 h. The plate was  
696 washed six times with PBST and 100µl of TMB was added to each well. After development  
697 in a dimly-lit environment, the reaction was stopped with the addition of 50µl of 2M H<sub>2</sub>SO<sub>4</sub>.  
698 Absorbance was read at 450 nm with a 200rt Biochrom Zenyth Anthos ELISA machine  
699 (Cambridge, UK).

## 700 Results

### 701 4.0 GAS-m epitope Amplification

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

709

710 **A**

```
p145 5'...GGA|AgeIACCGGT|CTTCGTCGTGACTTGGACG|CATCACGTGAAGCTAAGAAAC
      GTAGTGCAC TTCGATTCTTTGT|TCAACTTTTTCGAAATCTT |AgeITGGCCA|ACC...3'
J8    5'...GGC|AgeIACCGGT|CAGGCGGAAGATAAAGTGAAACAGTCACGT|GAAGCTAAGAAACAAGTTG
      CTTC GATTCTTTGTT CAAC|TTTTTCGAAATTTTGTGACCTTCTATTTTCACGTC |AgeITGGCCA|CCG...3'
J14  5'...GGC|AgeIACCGGT|AAGCAGGCGGAAGATAAAGTGAAAGCATCAC|GTGAAGCTAAGAAACAAGTT
      CACTTC GATTCTTTGTTCAA|CTTTTTCGAAATCATGTGACCTTCTATTTCACTTC |AgeITGGCCA|CCG...3'
```

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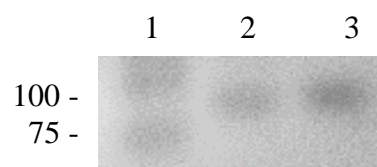
712 **B**

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**Figure 7 Primer design and matching agarose gel fragments**

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

### 721 4.1 GAS-m Fragment Insertion into HBsAg

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

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

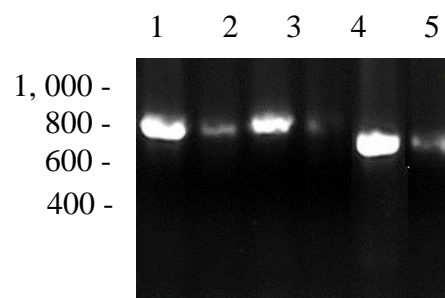
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**Figure 8 1% Agarose gel of amplified DNA from a colony PCR screen demonstrating insertion of GAS-m fragments into the ‘a’ determinant region of HBsAg**

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Wells contain 1  $\mu$ l and 0.4  $\mu$ l 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.

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750 **4.2 PVLP55 Vector Insertion**

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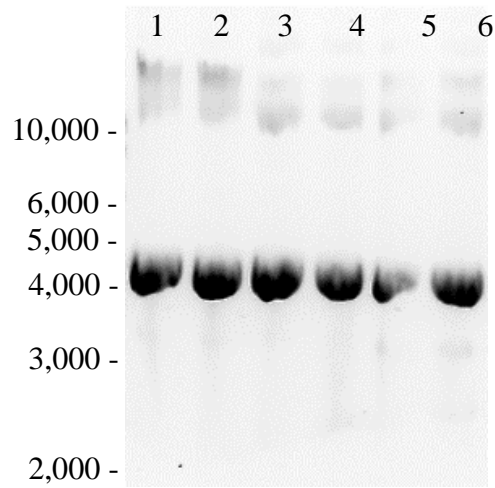
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**Figure 9 Agarose gel of pcDNA3.1 containing HBsAg:GAS-m fragments**

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Plasmids were purified from *E. coli* utilising the midiprep technique as described in section

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3.2. Wells 1 and 2 contain pcDNA3.1:HBsAg-s:p145, 3 and 4 contain pcDNA3.1:HBsAg-

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s:J8 and 5 and 6 contain pcDNA3.1:HBsAg-s:J14, where each sample was run on the gel in

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0.4  $\mu$ l (lanes 1, 3 and 5) and 1  $\mu$ l (lanes 2, 4 and 6).

765

766

767 **4.3 DNA Sequence Analysis**

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

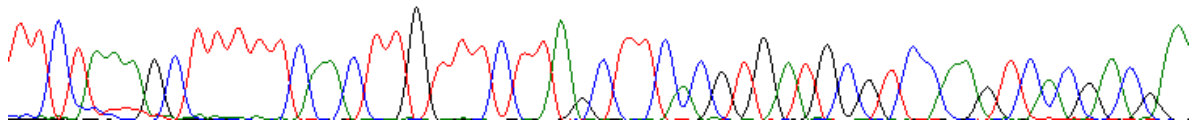
773 **p145**

Original reverse complement sequence

774 5' TTCTAAAGCTTTTTCAA C T T 3'

775 Sequencing results

120 130 140 150 160 170  
T T C T A A A G C T T T T T C A A C T T G T T T C T T A G C T T C A C G T G A T G C G T C C A A G T C A C G A C G A A bp



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

780 Sequencing results for the region covering the p145 insert are displayed alongside the  
781 matching original reverse compliment sequences for comparison.  
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795 **4.4 Detection of HBsAg:GAS:m VLPs by ELISA**

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

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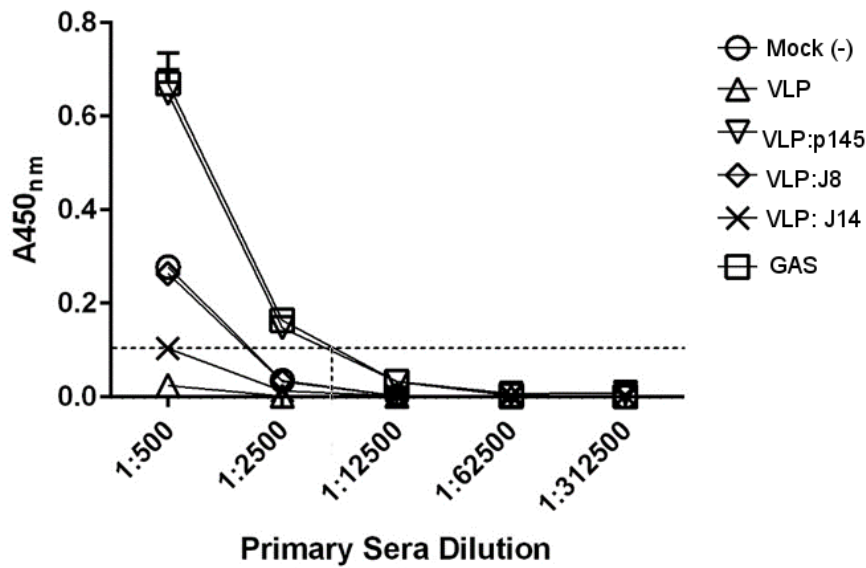
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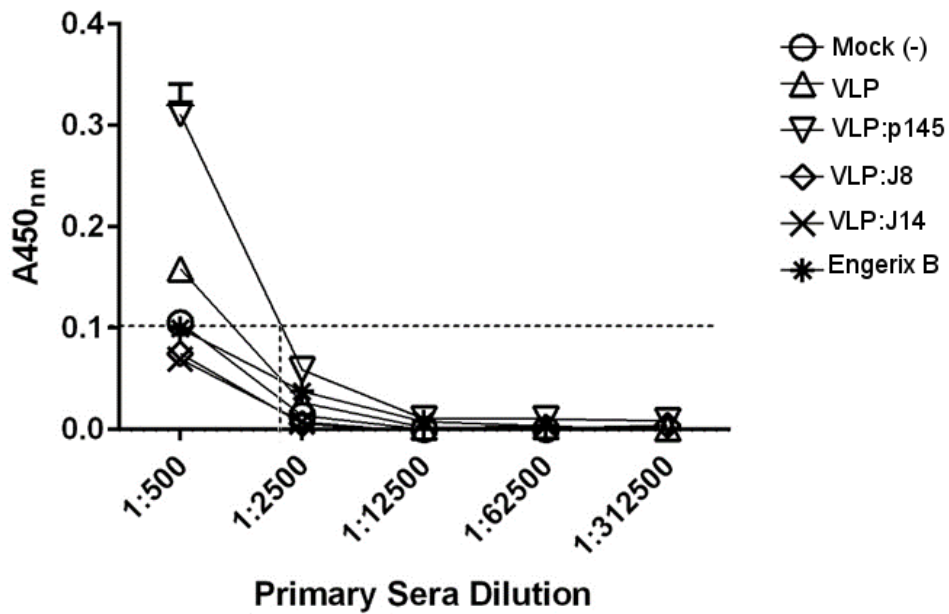
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### Figure 11 Detection of VLP:p145, VLP:J8 and VLP:J14 by ELISA

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Serial dilutions of primary J8 serum (A) or HBsAg-s serum (B) were utilised where the graph displays mean  $\pm$  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.

## 847 **Discussion**

848 GAS is responsible for a substantial global disease burden with an estimated 18.1 million  
849 individuals currently suffering disease due to GAS infection and sequelae (Carapetis *et al.*  
850 2005). Despite GAS susceptibility to penicillin, the disease burden has not been shown to  
851 decrease and effective treatment of sequelae such as RHD can require monthly penicillin  
852 injections over many years (Gerber *et al.* 2009). Preventative measures such as improved  
853 living conditions and vaccination are the superior solutions in terms of reducing mortality,  
854 morbidity and economic costs, including within the developed world.

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

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

872 electron microscopy was also performed and compared with wild type HBsAg. Further  
873 development on this study could be conducted by mirroring Netter's purification methods.  
874 Further techniques such as hydroxyapatite chromatography for the purification of plasmid  
875 DNA and affinity tagging for protein purification could be considered (Hilbrig & Freitag  
876 2012; Young *et al.* 2012).

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

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

897 negative controls. Low titres of VLP:J8 and VLP:J14 indicate that either the original DNA  
898 sequence was incorrect or the correct VLP was unfavourable for antibody detection. The  
899 presence of an incorrect DNA sequence is possible as it is still unverified by sequencing  
900 results. However, if the latter conclusion is correct then this would suggest that placement of  
901 these GAS:m genes for use within VLP may be unsuitable due to incompatibility. Similar  
902 findings by Kotiw *et al.* (2012) and Netter *et al.* (2003) support this conclusion, where data  
903 has suggested that interference may occur when the HBsAg-s 'a' determinant is disrupted by  
904 foreign sequences. This could be due to minor epitopes remaining within the 'a' determinant  
905 or elsewhere in the HBsAg molecule, misfolding or unstable expression of HBsAg-s proteins.  
906 ELISA testing within this study is a potential limitation as actual expression level may vary  
907 as peptides have been inserted into the 'a' determinant region which is highly antigenic.  
908 Further protein verification work and higher yields are required before the project could be  
909 continued. This study is further limited by the lack of protein purification and SDS-PAGE  
910 results, which could further indicate protein quality.

911 To increase protein expression yields for animal studies a yeast expression system could be  
912 considered. Yeast systems utilising *Saccharomyces cerevisiae* in the development and  
913 production of the Hepatitis B vaccine were successful obtaining high yields and successfully  
914 demonstrated protection in grivet monkeys (McAleer *et al.* 1984). Yeast expression systems  
915 are favourable as they can obtain high protein yields of 1000 µg/mL (Young & Robinson  
916 2014). Additionally, yeast systems hold the advantage of being single cells with fast growth  
917 capabilities as well as possessing eukaryotic abilities such as secretory pathways leading to  
918 correct protein processing (Porro *et al.* 2005).

### 919 **Significance**

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

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

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

940 Despite a long record of research in GAS vaccine development, a protective/acceptable  
941 vaccine is not yet in the foreseeable future. The World Health Organisation's roadmap for  
942 GAS vaccine development outlines past and present research for future developments is a  
943 step forward in collaboration of multi-disciplinary consensus in vaccine licensure, but many  
944 challenges are still present (Dale *et al.* 2013). Remaining challenges include safety concerns  
945 about the theoretical risk of autoimmune reactions and a necessity for further understanding  
946 of the basis for immunological protection in humans. For example, a greater understanding  
947 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

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

### 983 **Future Directions**

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

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

990 Proof-of-concept animal studies are essential to evaluating the success of this project.

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





999 **Conclusion**

1000 A successful GAS vaccine has the potential to save over 500,000 premature deaths annually,  
1001 greatly improve quality of life and reduce the economic burden of common childhood  
1002 diseases caused by GAS (Carapetis *et al.* 2005).  
1003 ELISA assays showed that GAS antigenic peptides can be expressed in HBsAg VLPs for use  
1004 as a dual vaccine. Specifically, from preliminary results VLP:p145 obtained high protein  
1005 titres. Further testing of these vaccine candidates still needs to occur and use of proof-of-  
1006 concept murine challenge models will be essential in assessing their efficacy. GAS vaccines  
1007 are possible, but not in the foreseeable future despite a long history of developments.  
1008 Incorporation of GAS:m peptides into a dual or combination vaccine may offer a more  
1009 appealing solution for widespread GAS protection across developed and developing countries  
1010 alike.

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