THE IMMUNOLOGY OF A PUTATIVE RECOMBINANT VACCINE AGAINST *STREPTOCOCCUS PYOGENES* UTILISING HEPATITIS B VIRUS-LIKE PARTICLES AS AN ANTIGEN DELIVERY PLATFORM

A Thesis submitted by

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ABSTRACT

Rheumatic heart disease (RHD) is entirely preventable, yet it claims an estimated 300 000 lives annually. Rates of RHD in children living in remote Australian Aboriginal and Torres Strait Islander communities are among the highest in the world and continue to rise, despite numerous and varied efforts to reduce them. It is predicted that by 2031, 14 500 Australians will be living with the debilitating effects of RHD. The cost associated with the care of Australians with RHD, between 2016 and 2031, is estimated at AUD $ 317 million. RHD is a an autoimmune sequala of a superficial infection caused by the bacterium, *Streptococcus pyogenes* (Strep A) and the most effective way to prevent RHD is to prevent the antecedent infection. The consensus among researchers and medical professionals is that vaccination would be the most effective way to do this. Yet, despite decades of research, there is currently no vaccine available that would protect children from the more than 80 different strains of Strep A endemic in Australia. This thesis describes the design, development, characterisation and evaluation of a putative vaccine against Strep A utilising hepatitis B virus-like particles (VLP) as a platform to deliver the highly immunogenic Strep A epitope, p*17, to the host's immune system. The recombinant chimeric VLPs, VLP-p*17, were expressed in a mammalian protein expression system and spontaneously formed nanoparticles with repetitive surface expression of p*17. BALB/c mice immunised with VLP-p*17, formulated with the commonly used adjuvant Alum, produced high titres (mean 3.7×10^4) of p*17-specific IgG after three 0.5 μg doses. When formulated with the more recently developed saponin-based adjuvant, Vet-SAP®, VLP-p*17 induced significantly higher titres (mean 7.7×10^4) of p*17-specific IgG after three 0.5 μg doses. HBsAg-S is the basis of several human vaccines with proven safety and efficacy, and large-scale manufacturing systems producing HBsAg-S VLPs are already well established. VLP-p*17, formulated with a saponin-based adjuvant has significant potential as a safe, efficacious and cost-effective vaccine against Strep A infections and associated non-infectious complications such as rheumatic heart disease.

CERTIFICATION OF THESIS

I Leanne Mary Dooley declare that the PhD Thesis entitled the immunology of a putative recombinant vaccine against *Streptococcus pyogenes* utilising hepatitis B virus-like particles as an antigen delivery platform is not more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references, and footnotes.

This Thesis is the work of Leanne Mary Dooley except where otherwise acknowledged, with the majority of the contribution to the papers presented as a Thesis by Publication undertaken by the student. The work is original and has not previously been submitted for any other award, except where acknowledged.

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STATEMENT OF CONTRIBUTION

Paper 1:

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Student contributed 80% to this paper. Collectively Tarek Ahmad, Manisha Pandey, Michael Good and Michael Kotiw contributed the remainder.

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Student contributed 70% to this paper. Collectively Tarek Ahmad, Victoria Ozberk, Manisha Pandey, Michael Good and Michael Kotiw contributed the remainder.

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Most of all I thank my husband Paul for his unwavering support and enthusiasm and for reminding me often that what I was doing was really very exciting.

DEDICATION

I dedicate this Thesis to my mother, Joan Mary McPherson,

(30/4/1931 – 23/7/2005)

and my father, Ronald John McPherson.

(19/2/1931 – 1/6/2023)

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ABBREVIATIONS

aa	Amino acid(s)
αHCC	Alpha-helical coiled-coil
AASH	Amino acid sequence homology
AGE	Agarose gel electrophoresis
AGRF	Australian Genome Research Facility
AHA	American Heart Association
AIHW	Australian Institute of Health and Welfare
AMP	Ampicillin
APSGN	Acute post streptococcal glomerulonephritis
ARF	Acute rheumatic fever
BP	Backbone pilin
BPG	Benzathine penicillin G
CAR	Coxsackie and adenovirus receptor
CHIM	Controlled human infection model
CRP	C-reactive protein
CSP	Circumsporozoite protein
DALY	Disability-adjusted life years
DC	Dendritic cell
DIC	Disseminated intravascular coagulation
DMEM	Dulbeco's Modified Eagle's Medium

DMSO	Dimethyl Sulfoxide
DT	Diphtheria toxoid
ESR	Erythrocyte sedimentation rate
FBS	Fetal bovine serum
GAS	Group A Streptococcus
GCS	Group C Streptococcus
GGS	Group G streptococcus
GlcNAc	N-acetyl-βD-glucosamine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome-wide association study
HEK293t	Human Embryonic Kidney cell line 293t
HbsAg-S	Hepatitis B surface antigen / small envelope protein
IFNγ	Interferon gamma
IL	Interleukin
ISD	Invasive Streptococcal diseases
LB	Luria 460 Bertani
MASPs	MBL-associated serine proteases
MBL	Mannose-binding lectin
MHC	Major histocompatibility complex
MM	Molecular mimicry
MR	Mitral regurgitation

MW	Molecular weight
NETs	Neutrophil extracellular traps
NLRP3	Nucleotide binding domain-like receptor protein 3
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PRR	Pattern-recognition receptor
P145	Peptide 145 of *S. pyogenes* M protein
P*17	Peptide 17 (variant 17 of peptide 145)
pVLP	DNA sequence for virus like particle
pVLP55	Plasmid containing Age1 restriction site in the "a" determinant of the DNA sequence for HBsAg-S
RHD	Rheumatic heart disease
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
SpyCEP	*S. pyogenes* cell envelope proteinase (SpyCEP)
SSDE	*Streptococcus dysgalactiae* subspecies *equisimilis*
STSS	Streptococcal toxic shock syndrome
Strep A	*Streptococcus pyogenes* / Group A Streptococcus
TCR	T cell receptor
TGF-β1	Transforming growth factor beta 1
Th	T helper cell

TNFα	Tumor necrosis factor alpha
TLR	Toll-like receptor
UTR	Untranslated region
VLP	Virus-like particle
WHO	World Health Organisation

CHAPTER 1: INTRODUCTION

1.1 Background and rationale behind the research project

Streptococcus pyogenes (Strep A) is typically known for its association with the common and relatively benign childhood infection referred to as "strep throat". However, Strep A is also one of the top three causes of life-threatening maternal sepsis, and the top two causes of toxic shock syndrome and necrotising fasciitis. Strep A is also the cause of serious non-infectious sequalae, the most significant of which are acute rheumatic fever (ARF), and its associated long-term complication, rheumatic heart disease (RHD) (Good, 2020; Ryan, McGrath, & Euler, 2022). Despite universal susceptibility to penicillin, Strep A is associated with an estimated 517 000 deaths globally every year. An estimated 288 000 to 349 000 of these are due to RHD. (Ralph & Carapetis, 2013; WHO, 2020). RHD is entirely preventable and is considered a disease of social disadvantage with the major burden of morbidity and mortality carried by developing countries and Indigenous populations within developed countries including Australia. Aboriginal Australians living in remote communities in the Northern Territory have the highest reported rates of RHD in the world (Rentta et al., 2022; RHDAustralia & McDonald, 2022; Roberts et al., 2015). In 2022, over 9 000 Australians were registered as having RHD and 92% of these were of Aboriginal or Torres Strait islander heritage. The incidence of ARF in these First Nations peoples is approximately 65 per 100 000 compared with 3 per 100 000 for non-Indigenous Australians. Despite well-established screening programs aimed at early diagnosis of RHD in Aboriginal and Torres Strait Islander children, 14% of children with RHD have severe disease, requiring cardiac valve repair surgery, at the time of diagnosis (Katzenellenbogen et al., 2020; RHDAustralia & McDonald, 2022). Children with RHD require monthly injections of penicillin to prevent recurrent ARF and further heart valve damage and may require repeated surgeries to prevent heart failure and death (Reviewed in chapter 3 (Dooley, Ahmad, Pandey, Good, & Kotiw, 2021)). The lifetime treatment cost per child with RHD in remote Indigenous communities in Northern and Central Australia

has been estimated at between one and two million Australian dollars (Hill & Collins, 2019).

Prompt and appropriate treatment of superficial Strep A infections can prevent development of ARF and progression to RHD, however, ARF occurs in the absence of a symptomatic antecedent Strep A infection in up to 60% of cases (Carapetis, Brown, Maguire, & Walsh, 2012). It would seem obvious then that the best way to prevent ARF and RHD would be to prevent the antecedent Strep A infection and that the most efficient way to do this would be to develop a safe and effective vaccine against Strep A. The pathway towards development of a safe and effective vaccine against Strep A has been long and fraught with significant challenges, which include : the vast serotypical and epidemiological diversity of the organism; concerns over the safety of putative vaccines; and the relatively low immunogenicity of minimal bacterial epitopes (reviewed in Chapter 3 (Dooley et al., 2021).

This project addressed the challenges to Strep A vaccine development by: targeting a conserved epitope of Strep A M protein: an epitope that does not differ significantly between different strains; targeting a minimal Strep A B cell epitope that does not induce cross-reactive T cell responses that could induce autoimmune sequalae in vaccinated individuals; and using a hepatitis B virus like particle (VLP) as a platform to significantly enhance the immunogenicity of the Strep A epitope and deliver it to the host in a form that stimulates robust antigen-specific immune responses.

1.2 Research aims, objectives and hypotheses

The aims of this research project were to use recombinant DNA technology to construct a recombinant vaccine against the bacterium *Streptococcus pyogenes* (Strep A) and to evaluate the immunogenicity of the putative vaccine in mice. To this end, the following three studies were undertaken.

Study 1 (Chapter 3): *Rheumatic heart disease: A review of the current status of global research activity*. The main aim of this literature review was to investigate the current knowledge and understanding of Strep A pathogenesis and epidemiology, with a focus on rheumatic heart disease (RHD), and recent advances in Strep A vaccine development. The objectives of this review were to confirm the relevance and significance of this research project, in an Australian and global context, and inform the choice of Strep A epitope and antigen delivery system to be utilised in the putative vaccine.

Study 2 (Chapter 5): *Chimeric hepatitis B surface antigen virus-like particles expressing the Strep A epitope p*17 elicit a humoral response in mice*. The aim of this study was to utilise recombinant DNA technology to express the conserved Strep A epitope, p*17, within the immunodominant "a" determinant region of the hepatitis B small envelope protein (HBsAg-S). The first hypothesis tested in this study was that expression of p*17 within the "a" determinant of HBsAg-S would not compromise spontaneous VLP formation. A second hypothesis was that VLP-p*17 would induce production of p*17-specific IgG in immunised mice.

Study 3 (Chapter 6): *Immunogenicity of a chimeric recombinant virus-like particle as a putative vaccine against Streptococcus pyogenes*. The main aim of this study was to further investigate the immunological value of utilising HBsAg-S VLP as a means of optimising the immunogenicity of the highly conserved Strep A epitope, p*17. In this study, p*17-specific IgG tires (total IgG, IgG1 and IgG2a) were measured in the sera of BALB/c mice immunised with three low doses of VLP-p*17 (0.5 μg/dose) or three standard doses (25 μg/dose) of p*17-DT. A secondary aim of this study was to evaluate and compare the humoral responses induced in mice immunised with VLP-p*17 formulated with three different types of adjuvants.

CHAPTER 2: LITERATURE REVIEW

2.1 Strep A associated disease burden

Streptococcus pyogenes (Strep A), commonly known as Group A Streptococcus (GAS), is a strict human pathogen with no other known reservoir. Strep A is responsible for a wider spectrum of disease than any other bacterial species (Cunningham, 2000). Strep A infections range from mild superficial infections of the throat and skin, commonly known as "strep throat" and "school sores" respectively, to severe invasive Streptococcal diseases (ISD) such as neonatal and maternal sepsis and necrotising fasciitis, and toxigenic diseases such as scarlet fever and catastrophic streptococcal toxic shock syndrome (STSS) (Cunningham, 2000; Stevens & Bryant, 2016).

Severe invasive streptococcal infections are caused by strains of Strep A that are capable of upregulating expression of key virulence factors, which allows invasion of intact and otherwise healthy tissue in sterile anatomical sites (Langshaw, Pandey, & Good, 2018; Stevens & Bryant, 2016). Up to one third of all cases of ISD are rapidly fatal, despite aggressive treatment, which often involves amputation of an infected limb (Stevens & Bryant, 2016).

The pathogenesis of STSS involves production of Strep A exotoxins that act as superantigens binding to class II major histocompatibility complex (MHC) molecules on B cells, macrophages and dendritic cells while simultaneously binding to the T cell receptor (TCR) on T cells (Proft & Fraser, 2016). This simultaneous binding bypasses the usual antigen processing and MHC restriction that is necessary for conventional antigen presentation, and triggers activation events in both cells leading to an acute systemic proinflammatory response and subsequent cytokine storm (Petersson, Forsberg, & Walse, 2004). This in turn results in multiorgan failure, shock, disseminated intravascular coagulation (DIC) and ultimately death within seven days of presentation in 30-50% of cases (Lamagni et al., 2008; Mehta et al., 2006; Thompson, Sterkel, McBride, & Corliss, 2018). The rates of ISD, including STSS, have been rising globally in both

frequency and severity since the 1980s. This rise has been attributed to the emergence and global dissemination of highly virulent covR/S mutant strains of Strep A (Aziz & Kotb, 2008; Ching et al., 2017; Dunne et al., 2022; Langshaw et al., 2018; Nasser et al., 2014; Pandey et al., 2016).

While superficial Strep A infections of the throat and skin, are usually self-limiting, or resolve quickly with appropriate antibiotic therapy, they are associated with the serious post-streptococcal autoimmune diseases, acute rheumatic fever (ARF), rheumatic heart disease (RHD) and acute post streptococcal glomerulonephritis (APSGN) (Cunningham, 2000; Jackson, Steer, & Campbell, 2011). ARF develops in around 0.3-3% of individuals with an untreated or ineffectively treated Strep A infection (usually of the throat, and typically in children between the ages of 5 and 14) two to three weeks after resolution of the infection (Parnaby & Carapetis, 2010). The clinical presentation of ARF includes fever, carditis, migratory polyarthritis, subcutaneous nodules and a characteristic rash referred to as erythema marginatum. These clinical manifestations, which are not all present in all cases of ARF, arise from an aberrant non-suppurative systemic inflammatory response to a typically suppurative, localised Strep A infection (Dooley et al., 2021; Guilherme & Kalil, 2010). Neurological manifestations of ARF, known as Sydenham chorea, can appear several months after the apparent resolution of ARF (Jones, 1944).

The underlying pathology of ARF involves the production of antibodies and activated $CD4^+$ T cells against Strep A antigens, in particular the Group A streptococcal surface carbohydrate, N-acetyl glucosamine (GlcNAc), and M protein (Figures 1 and 2), that cross-react with host tissue causing inflammation in the joints, heart, brain and skin. This cross-reactivity is associated with a phenomenon known as molecular mimicry: structural similarities between cellular components of the pathogen and tissue components in the host that lead to a breakdown in self-tolerance in the host (reviewed in (Dooley et al., 2021)). The inflammation in the skin, brain and joints is usually self-limiting and generally resolves without causing permanent tissue damage. However, in around one third of children with ARF, the post-streptococcal immune-mediated

inflammation of the heart leads to the serious chronic condition known as rheumatic heart disease (RHD) (Guilherme & Kalil, 2002). RHD is characterised by inflammation of the heart valves, referred to as autoimmune valvulitis. Recurrent episodes of ARF typically cause progressive fibrosis of the valves leading to valvular calcification and stenosis and subsequent decompensatory heart failure (Guilherme & Kalil, 2002). While RHD is more commonly associated with recurrent streptococcal infections and repeated episodes of ARF, it can develop after a single episode of ARF. RHD manifests as valvular regurgitation and presents clinically as heart failure with associated extreme fatigue and shortness of breath. Long-term complications of RHD include heart failure, arrhythmia, thromboembolism and stroke (Cunningham, 2000).

Acute post-streptococcal glomerulonephritis (APSGN) is an acute kidney disease caused by the deposition of antigen-antibody complexes in the renal glomeruli and typically follows a streptococcal skin infection known as streptococcal pyoderma, impetigo or "school sores". Most cases of APSGN follow a benign clinical course but acute renal failure can ensue. The long-term implications of APSGN include an increased risk of chronic kidney disease (Hoy, White, Dowling, & Sharma, 2012).

Despite its universal susceptibility to penicillin, the global burden of disease attributed to Strep A remains significant and is probably underestimated due to global inconsistencies in reporting, particularly in resource poor parts of the world where Strep A infections are endemic (Ralph & Carapetis, 2013). The only global epidemiological study of Strep A diseases, by Carapetis et al. (2005), relied on mathematical models to infer global estimates using data from the United States of America collected in the 1950s and more recent data from Australia, New Zealand, India, and Ethiopia. In this study, Carapetis et al. (2005) estimated that globally, Strep A causes 111 million cases of pyoderma, 616 million cases of pharyngitis, 470 000 cases each of ARF and APSGN and 500 000 non-fatal cases of ISD annually. Furthermore, they estimated that Strep A is responsible for 517 000 deaths worldwide each year, which places it among the world's top ten causes of human infection-associated mortality (Ralph & Carapetis, 2013).

Carapetis et al., (2005) attributed the majority of Strep A-related deaths to ISD (including STSS) and post streptococcal sequelae. They estimated that: 663 000 cases of ISD lead to 163 000 deaths annually worldwide; RHD causes an estimated 349 000 deaths annually worldwide; and APSGN causes an estimated 5 000 deaths annually worldwide (Carapetis et al., 2005). Although this study was conducted over sixteen years ago, it is still widely cited as 'a recent global epidemiological study of Strep A-related morbidity and mortality' due to the paucity of more recent comprehensive global data, which tends to focus only on RHD (Ralph & Carapetis, 2013; Steer et al., 2016).

As part of the 2015 Global Burden of Disease study, a systematic review by Watkins et al., (2017) estimated the number of deaths worldwide in 2015 due to RHD alone to be 319 400. More recent data published by WHO estimates that RHD claims 288 348 lives worldwide every year (WHO, 2020). While these figures suggest a decline in the global mortality rates associated with RHD they could reflect a decline that is limited mainly to more resource rich populations. While the true burden of Strep A disease has proven difficult to ascertain, numerous studies confirm that this burden is disproportionately shouldered by people in developing countries and by Indigenous populations in developed countries including Australia (Colquhoun et al., 2015; de Loizaga et al., 2021; Ralph & Carapetis, 2013; Watkins et al., 2017).

2.2 Strep A disease burden in Australia

In Australia, a disproportionate Strep A disease burden is carried by the Indigenous population. In 2018, RHD Australia and McDonald estimated the overall prevalence of RHD in Australia at 1 per 100 000 while the rate in Aboriginal and Torres Strait Islander Australians was 59 per 100 000 (RHDAustralia & McDonald, 2022). The reported rates of ARF and RHD in Australian Aboriginal and Torres Strait Islander people living in remote areas of Northern and Central Australia are consistently among the highest in the world (Carapetis et al., 2005; Ralph & Carapetis, 2013; Watkins et al., 2017; Wyber et al., 2021). The Australian Institute of Health and Welfare reported that, of the 5

385 people with RHD listed on State RHD registers in December 2019, 81% were Indigenous Australians (AIHW, 2020). In 2015–2019, 399 deaths were reported for people with RHD listed on state registers and 72% of these were Indigenous Australians. The median age of RHD-associated death was 52 years for Indigenous Australians compared with 72 years for non-Indigenous Australians. The AIHW also reported that median age for diagnosis of RHD was 22 years for Indigenous Australians compared with 50 years for non-Indigenous Australians. The greatest number and highest rate of new diagnoses of RHD among Indigenous Australians in 2015-2019 was in the Northern Territory (AIHW, 2020). These findings of geographical differences in rates of RHD align with those reported by Colquhoun et al., (2015) who reported that while the overall RHD-related death rate for Indigenous Australians was 18.9 times higher than that for non-indigenous Australians, the rate in the Northern Territory was 54.8 times higher for Indigenous Australians than for non-Indigenous Australians (Colquhoun et al., 2015). Environmental factors, particularly overcrowding, increase the risk of RHD in these populations (reviewed in Chapter 3 (Dooley et al., 2021)).

In addition to the high rates of RHD, children living in remote Aboriginal and Torres Strait Islander communities in the Northern Territory and the Northern regions of Queensland and Western Australia also suffer disproportionately high rates of Strep A pyoderma. A study by Andrews et al. (2009), conducted over a three-year period in five remote indigenous communities in the Northern Territory, showed that almost 70% of children studied had an episode of pyoderma before their first birthday, with most having their first episode at just two months of age. Scabies is also endemic in many Indigenous communities. Andrews et al. (2009) reported that 63% of children in their study had been infested with scabies at some time during their first year of life. They also reported that children with scabies were also likely to have pyoderma and that most cases of pyoderma were due to Strep A infection (Andrews et al., 2009).

Indigenous Australians also suffer higher rates of ISD than non-Indigenous Australians. The overall reported rate of ISD in Australia from 2017 to 2018 was

8.3 per 100 000 (Wright, Langworthy, & Manning, 2021). A retrospective study, conducted by Boyd et al. (2016), of 128 cases of ISD reported in the Northern Territory between 2011 and 2013, found the incidence in the Indigenous population to be 69.7 per 100 000, compared to 8.8 per 100 000 in the non-Indigenous population. In this study, 10 of the 128 cases were fatal (Boyd et al., 2016). A similar disproportionate burden of ISD was reported in Queensland children aged 0-18 years with an annualised incidence of 13.2 per 100 000 for Indigenous children compared with 3.0 per 100 000 for non-Indigenous children (Whitehead, Smith, & Nourse, 2011).

Although now uncommon in the Australian population overall, APSGN is disproportionately high in children living in remote Aboriginal communities where Strep A pyoderma is endemic (Rodriguez-Iturbe & Musser, 2008). From 1991 to 2008, there were 415 confirmed cases and 23 probable cases of APSGN notified in the Northern Territory. Of these, 94.7% were Indigenous Australians (Marshall et al., 2011). Data collected in one remote Aboriginal community in the Northern Territory, indicated that approximately 200 of the 1 519 residents had suffered at least one episode of APSGN and 27 of these had a history of multiple episodes of APSGN, usually during childhood. This study also found increased evidence of diminished kidney function in females with a history of previous APSGN (Hoy et al., 2012).

Strep A disease is on the cusp of recognition as a neglected tropical disease, highlighting its global importance and the need for increased investment in strategies to reduce its disproportionate impact on socioeconomically disadvantaged populations (Good, 2020; Hotez, Aksoy, Brindley, & Kamhawi, 2020). While numerous programs aimed at reducing the rates of Strep A infections have been trialled in at-risk communities, including Australian Aboriginal and Torres Strait islander communities, none have significantly reduced the burden of Strep A disease in these communities (reviewed in Chapter 3 (Dooley et al., 2021)). Effective vaccination is regarded as the most reliable and cost-effective means of reducing the Strep A disease burden in at-risk populations. However, despite decades of research in numerous laboratories

around the world, no Strep A vaccine offering sufficiently broad protection from the diverse strains of Strep A endemic in these populations is currently available (Reviewed in Chapter 3 (Dooley et al., 2021)) (Wright et al., 2021). In May 2018, Member States of the World Health Organization (WHO) unanimously adopted a Global Resolution on ARF and RHD at the World Health Assembly. Part of this resolution focused on development of a safe and effective vaccine against Strep A (WHO, 2018).

2.3 Strep A vaccine research in Australia

Due to the vast diversity and the dynamic nature of Strep A epidemiology in Australia (Marshall et al., 2011), local research has focused on the development of a vaccine targeting the conserved C-terminal region of the Strep A M protein: a helical protein that covers the surface of the bacterium and protects it from complement-mediated phagocytosis (Figure 1) (Dooley et al., 2021; V. A. Fischetti, 1989; V. A. Fischetti, 2016). More specifically, Australian research has focused on peptide 145 (p145), a 20-amino acid peptide from the highly conserved C3 repeat region of the Strep A M protein (Figure 2) (Reviewed in Chapter 3 (Dooley et al., 2021)).

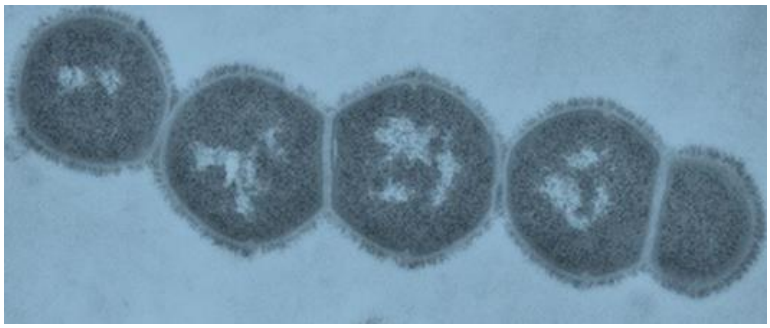

Figure 1. The Streptococcal M protein covers the entire surface of the bacterium and appears "like fuzz on a tennis ball" when viewed by transmission electron microscopy (V. A. Fischetti, 2016).

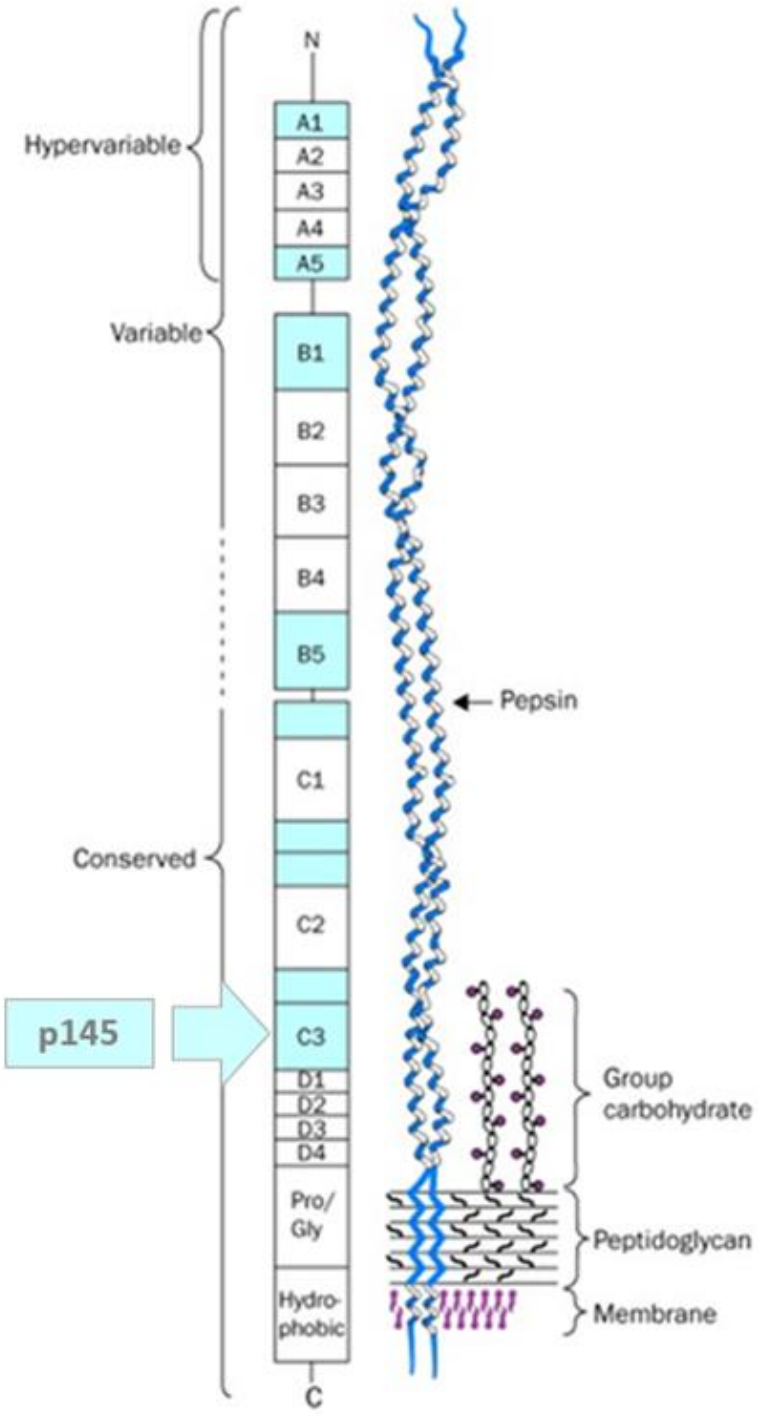

Figure 2. M protein is a coiled-coil protein expressed as a dimer attached to the peptidoglycan cell wall of Strep A. The N-terminus is hypervariable. The C-terminal region is anchored in the cell wall, extends through the cell membrane with the charged tail projecting into the cytoplasm, and is highly conserved. Between these conserved and hypervariable regions is a central domain of intermediate variability consisting of a number of repeat sequences (V. A. Fischetti, 2000).

In addition to the highly conserved nature of p145, which makes it an ideal target for a broad-spectrum vaccine, it was observed that Anti-p145 antibodies develop naturally in response to Strep A infection. Anti-p145 antibodies, capable of opsonising multiple strains of Strep A, were found in the sera of a high proportion of adults in communities where Strep A is endemic (Pruksakorn et al., 1994). However, anti-p145 antibodies were not detected in the sera of children in these communities, which suggested that they develop only after years of exposure (Hayman et al., 1997). Vaccination with p145 or a derivative of p145, could therefore be expected to produce higher titers of these protective antibodies in the sera of vaccinated individuals such that a sufficient level of immunity could be reached at a younger age (Brandt, Hayman, Currie, Pruksakorn, & Good, 1997).

Small bacterial epitopes, like p145 (LRRDLDASREAKKQVEKALE) and its derivatives and variants, are not sufficiently immunogenic to be used alone in putative vaccines. Strep A vaccines currently in development in Australia utilise various different platforms to deliver these minimal epitopes to the immune system including, conjugation to diphtheria toxoid (DT), an alkane backbone, a polyacrylic ester-based dendritic polymer and incorporation into a liposome (reviewed in Chapter 3 (Dooley et al., 2021)).

2.4 Summary of key findings from the literature review

1. Strep A is a significant cause of morbidity and mortality globally, with a disproportionate burden of disease carried by socially and economically disadvantaged populations, including Australian Aboriginal and Torres Strait Islander peoples.

2. Effective vaccination is widely regarded as the most effective means of reducing the burden of Strep A disease in at-risk populations yet no vaccine offering sufficiently broad protection is currently available.

CHAPTER 3: PAPER 1 – Rheumatic heart disease: A review of the current status of global research activity

3.1 Introductory paragraph

While numerous reviews on various aspects of RHD have been published, no single manuscript provides a broad review of the current understandings of the epidemiology, pathogenesis and host-pathogen interactions, genetic and environmental contributors to host susceptibility, and diagnosis, treatment, and prevention of RHD. Chapter 3 addresses this gap in the literature.

3.2 Paper 1

Dooley, L, Ahmad, T, Pandey, M, Good, M & Kotiw M 2021, 'Rheumatic heart disease: A review of the current status of global research activity', *Autoimmunity Reviews,* vol. 20, pp. 102740 https://doi.org/10.1016/j.autrev.2020.102740

3.3 Links and implications for development of a putative recombinant vaccine against Strep A

Table 1 in Dooley et al., (2021) presents the details of 22 different constructs for putative vaccines against Strep A in various stages of development and evaluation globally. Of these, 10 are under development in Australia and all but one of the Australian constructs utilise derivatives or variants of p-145, a conserved peptide of the Streptococcal M protein described in section 2.3 and illustrated in Figure 2. The antigen delivery systems utilised in these vaccines include conjugation to diphtheria toxoid (DT), conjugation to an alkane backbone, incorporation into a polyacrylic ester-based dendritic polymer and incorporation into a liposome.

The approach to antigen delivery in this project was to use recombinant DNA technology to produce a single complete protein with a built-in antigen delivery system. This was achieved by cloning the DNA sequence for p*17 into the DNA sequence for hepatitis B virus small envelope protein (HBsAg-S) to produce virus-like particles (VLPs) expressing the Strep A epitope, p*17.

3.3.1 Rationale behind the use of VLPs as antigen delivery systems

VLPs are viral capsid proteins that self-assemble to form compact and highly ordered nanoparticles, 20-150 nm in diameter. VLPs mimic the live virus in size and form but contain no viral genetic material and are therefore non-infective (Ludwig & Wagner, 2007). VLPs tend to be within the optimal size range for uptake by dendritic cells (DC) and subsequent antigen processing and MHC I and MHC II presentation (Grgacic & Anderson, 2006). By promoting DC maturation and migration, VLPs activate both innate and adaptive immune responses. Due to their particulate nature and their expression of high density and repetitive displays of epitopes, VLPs are also able to stimulate both the humoral and cell-mediated arms of the adaptive immune response (Grgacic & Anderson, 2006). In the laboratory, VLPs are formed by the self-assembly of viral capsid or envelope proteins following recombinant DNA expression in a variety of cell culture systems. Recombinant viral envelope proteins bud from the endoplasmic reticulum, plasma membrane, or associated lipid rafts of the cultured cell, such that the resultant VLPs contain the proteins and the cellular lipids that make up

the viral lipoprotein envelope (Grgacic & Anderson, 2006). Because they are immunogenic but non-infectious, VLPs are a safer alternative to attenuated viruses, such as those used in vaccines against yellow fever, polio, measles, mumps and rubella (Ludwig & Wagner, 2007).

The first VLPs used in human vaccines were derived from envelope proteins of the hepatitis B virus (HBV) expressed in the bakers' yeast *Saccharomyces cerrevisiae* (Blumberg, Millman, & London, 1985). Energix-B (GlaxoSmithKline) and Recombivax HB® (Merck & Co. Inc.), the currently used second-generation hepatitis B vaccines, are derived from the small HBV envelope protein, surface antigen S (HBsAg-S), produced in *S. cerevisiae.* A Third-generation Hepatitis B vaccine, Sci-B-Vac™, comprising VLPs expressing additional HBV surface antigens and produced in mammalian cells, is currently in clinical trials (Elhanan et al., 2018). The recombinant Human Papillomavirus (HPV)-like particle vaccines, Gardasil® (Merck and Co. Inc.) and Cervarix® (GlaxoSmithKline) have demonstrated high protection against oncogenic strains of HPV associated with cancers of the cervix and anus (Rodríguez-Limas, Sekar, & Tyo, 2013). Gardisil® is expressed in *S cerrevisiae* ,and Cervarix® is expressed in the insect cells-baculovirus system (Lowy & Schiller, 2006). In 2012, the hepatitis E VLP vaccine, HEV239 (Hecolin®), produced in *E.coli,* was launched in China (Park, 2012). A phase I clinical trial of the vaccine was completed in the United States in August 2020 (NCT03827395) (Wen et al., 2020; Wu, Chen, Lin, Hao, & Liang, 2016).

In addition to their value as antiviral vaccines, VLPs are highly stable molecules that have demonstrated tolerance to a range of bioconjugation, labelling, and polymerisation techniques making them suitable delivery platforms for a wide spectrum of small molecules including bacterial epitopes (Schwarz & Douglas, 2015).

HBsAg, originally described as the 'Australia antigen' in sera of patients infected with hepatitis B virus (Blumberg & Alter, 1965), forms 22 nm spherical particles each composed of approximately 100 HBsAg monomers. HBsAg monomers consist of three viral envelope proteins: the S, pre-S1 and pre-S2 proteins. The

major structural protein of the Hepatitis B viral envelope, and the most immunologically important, is the S or small HBV envelope protein (HBsAg-S), which is composed of 226 amino acids (aa). HBsAg-S VLPs access both MHC I and MHC II antigen processing pathways in DCs for presentation to CD8+ and CD4+ T cells thereby eliciting adaptive immune responses to associated antigens (Moffat, Cheong, Villadangos, Mintern, & Netter, 2013). Recombinant-derived HBsAg-S molecules spontaneously form VLPs when expressed in a number of different cell types, including mammalian, yeast, and insect cells (Lanford et al., 1989).

Recombinant HBsAg-S is the main component of the safe and effective Energix-B and Recombivax vaccines currently used against HBV infection. Registration as a human vaccine and the presence of well-established high-quality manufacturing processes, along with highly immunogenic epitope presentation, makes HBsAg-S VLPs ideal delivery platforms for foreign epitopes (Lanford et al., 1989; McAleer et al., 1984; Mulder et al., 2012; Netter, Woo, Tindle, Macfarlan, & Gowans, 2003).

The antimalarial vaccine, RTS,S (Mosquirix®), approved for use by European regulators in July 2015, and the more recent candidate vaccine, R21, utilise HBsAg-S as an antigen delivery system (Collins, Snaith, Cottingham, Gilbert, & Hill, 2017; Moorthy & Okwo-Bele, 2015; Wilby, Lau, Gilchrist, & Ensom, 2012). Both vaccines comprise the central repeat and the C-terminus of the circumsporozoite protein (CSP) of *Plasmodium falciparum* fused to the N-terminus of HBsAg-S, a structural alteration that ensures surface expression of CSP but interferes with particle formation. In the RTS,S vaccine, the interference was minimised by incorporation of wildtype HBsAg-S such that each VLP contains a somewhat random mix of both wildtype HBsAg-S and CSP-HBsAg. This dilutes the expression of CSP and triggers a significant immune response against HBsAg (Collins et al., 2017). Rather than fusing or conjugating a Strep A epitope to the N-terminus of HBsAg-S, this project involved the use of recombinant DNA technology to clone the bacterial epitope into a more central, yet still surface-expressed, region of the HBsAg-S called the “a” determinant.

The “a” determinant of HBsAg-S is a highly conformational, hydrophilic domain between positions 122 to 150 (Figure 3) (Gavilanes, Gonzalez-Ros, & Peterson, 1982; Rezaee et al., 2016). The “a” determinant is expressed on the surface of the VLP and represents the immunodominant region of HBsAg-S containing epitopes for induction of a protective humoral immune response (Bhatnagar et al., 1982). Netter et al., (2001) cloned segments of the hepatitis C virus (HCV) E2 protein into the DNA sequence of the “a” determinant region of HBsAg-S successfully producing recombinant chimeric HBsAg-S particles with exposed hepatitis C virus epitopes (Netter, Macnaughton, Woo, Tindle, & Gowans, 2001). Likewise, Kotiw et al. (2012) cloned epitopes from the bacterium *Helicobacter pylori* KAT A gene into the DNA sequence of the “a” determinant region of HBsAg-S successfully producing recombinant HBsAg-S particles with exposed *H. pylori* epitopes (Kotiw et al., 2012).

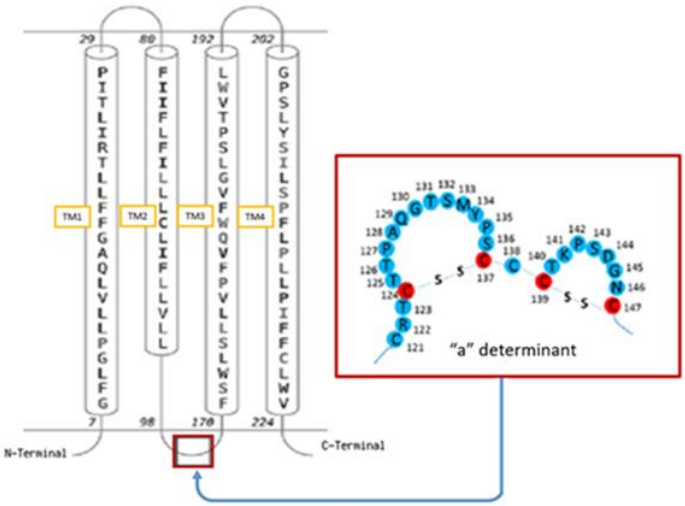

Figure 3. Schematic Representation of the HBsAg-S showing four transmembrane (TM) peptides and the surface expressed “a” Determinant Region. Modified from *(Rezaee et al., 2016)*

Strep A infections are initiated by bacterial colonisation of pharyngeal mucosa or the skin. Protection afforded via vaccination should therefore involve induction of mucosal immune responses. Several VLP-based vaccines targeting mucosal pathogens have progressed to human trials, further supporting the choice of a VLP delivery system for Strep A antigens. These vaccines include ResVax™

(Respiratory Syncytial Virus), NVX-CoV2373 (SARS-CoV-2 Virus), and NanoFlu™ (Influenza Virus) (Nooraei et al., 2021).

Preliminary investigations into the design and production of the plasmid DNA and the recombinant HBsAg-S VLP expressing an epitope from Strep A M protein are described in detail in Chapter 4.

CHAPTER 4: PRELIMINARY INVESTIGATIONS: Design, production, and purification of a recombinant vaccine against Strep A utilising hepatitis B virus-like particles as an antigen delivery platform

4.1 Introduction

The original design for a recombinant VLP-based vaccine against Strep A utilised J8 as the Strep A epitope. As described in Chapter 3, J8 is a chimeric protein consisting of a 12 aa sequence from p145, called J8i, flanked on either side by an eight aa sequence derived from a yeast DNA binding protein: QAEDKVKQSREAKKQVEKALKQLEDKVQ (Hayman et al., 1997; Relf et al., 1996). As shown in Table 1 Chapter 3, J8 was the target antigen of several Strep A vaccines under development in Australia at the time of publication. Studies by our collaborators at the Institute for Glycomics, Griffith University, found that antibodies generated in mice immunised with J8 conjugated to Diphtheria toxoid (MJ8VAX) recognised the parent peptide, p145, and opsonised live Strep A (Good & Yanow, 2016). The safety of MJ8VAX was demonstrated in a Lewis rat model of autoimmune valvulitis in parallel with a rabbit toxicology study (Batzloff et al., 2016). Table 1 Chapter 3 also shows that J8-based vaccines utilised a variety of platforms to deliver the antigen to the immune system. The plan was, therefore, to design and construct a recombinant VLP with surface expression of J8: a Strep A target antigen with already proven efficacy and safety. In contrast to the J8-based vaccine constructs listed in Table 1 Chapter 3, VLP-J8 was designed and produced as a single recombinant protein, rather than a conjugate or fusion of separate molecules, to facilitate enhanced consistency and stability of the putative vaccine. Given the demonstrated ability of recombinant VLPs to induce target-specific B and T-cell responses, VLP-J8 was expected to demonstrate enhanced immunogenicity (Schirmbeck, Melber, Mertens, & Reimann, 1994). Figure 4 illustrates the intended aa sequence of VLP-J8, which included the FLAG® octapeptide (DYKDDDDK), to facilitate protein purification using Anti-FLAG® affinity chromatography.

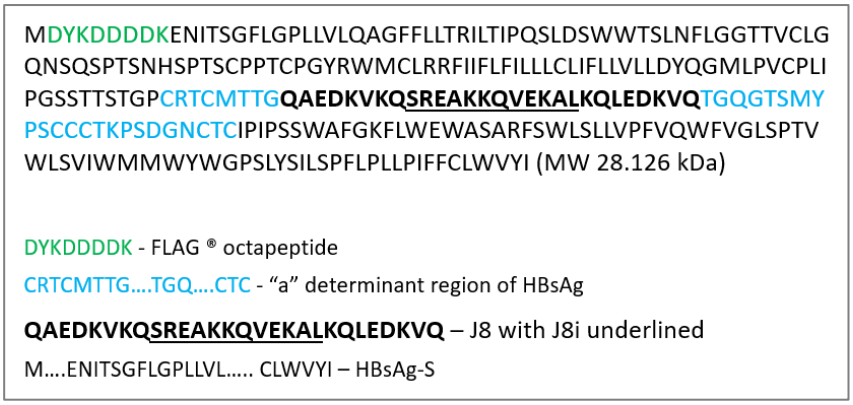

Figure 4. Intended amino acid sequence of VLP-J8: J8 expressed within the "a" determinant region of HBsAg-S. The sequence includes the FLAG® octapeptide sequence at the N-terminus. The calculated MW of the sequence is 28.126 kDa (Bioinformatics.org).

In a subsequent vaccine construct, J8 was replaced by p*17 as the Strep A component. As described in Chapter 3, p*17 is a p145 variant with two amino acid substitutions: p145-LRRDLDASREAK**K**QVE**K**ALE to p*17-LRRDLDASREAK**N**QVE**R**ALE. p*17 demonstrated enhanced, stability, helicity, immunogenicity, and efficacy in a mouse-model of Strep A skin infection (Nordstrom et al., 2017). Figure 5 illustrates the intended aa sequence of the new construct, VLP-p*17, which included a glycine-serine linker to separate the FLAG® octapeptide from the HBsAg-S sequence to enhance VLP purification by Anti-FLAG® affinity chromatography.

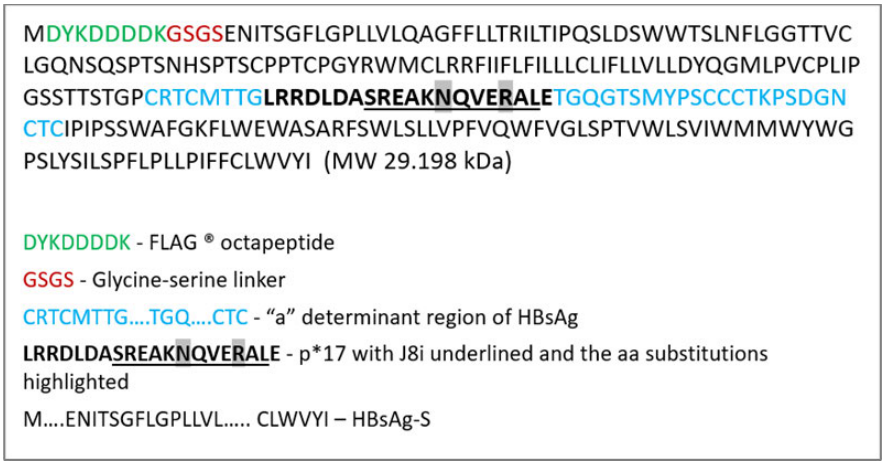

Figure 5. Intended amino acid sequence of VLP-p*17: p*17 expressed within the "a" determinant region of HBsAg-S. The sequence includes the FLAG® octapeptide sequence at the N-terminus followed by a glycine-serine linker. The calculated MW of VLP-p*17 is 29.198 kDa (Bioinformatics.org).

4.2 Materials and Methods

4.2.1 pcDNA3.1 expression vector, containing an engineered *Age*I restriction site in the "a" determinant region of the HBsAg-S insert (pVLP55), was developed and kindly provided by Professor Hans Netter, Victorian Infectious Diseases Reference Laboratory (VIDRL), Melbourne Health, Victoria, Australia (Netter et al., 2001). The DNA sequence for pVLP55 also includes the Kozak sequence to facilitate expression in mammalian cells.

4.2.2 HEK293t cells (ATCC® CRL-3216™), kindly provided by the University of Queensland, St Lucia, Queensland, and approved for use in this project by the University of Southern Queensland Human Research Ethics Committee (approval number: H18REA234). The cells were cultured in 10% FBS DMEM (described below) and passaged three times prior to storage in 10% Dimethyl Sulfoxide (DMSO) at -80°C.

4.2.3 Construction and amplification of plasmid pVLP-J8

The DNA sequence for the J8 insert was constructed by annealing the following oligonucleotides purchased from the Australian Genome Research Facility (AGRF, St Lucia, Queensland): J8 forward primer: 5'CCGGTCAGGCGGAAGATAAAGTGAAACAGAGCCGCGAAGCGAAAAAACAGGTGGAAAAA GCGCTGAAACAGCTGGAAGATAAAGTGCAGA-3' and J8 reverse primer: 5'CCGGTCTGCACTTTATCTTCCAGCTGTTTCAGCGCTTT TTCCACCTGTTTTTTCGCTTCGCGGCTCTGTTTCACTTTATCTTCCGCCTGA-3'. *Age*I restriction sites (A^CCGGT) were included at each end of the sequence, to facilitate ligation of the J8 DNA sequence into the pcDNA3.1:HBsAg-S (pVLP55) expression vector.

To construct the J8 DNA insert, 10 µl of each of the two 100 µM primers were annealed in 25 µl nuclease free water with 5 µl CutSmart® Buffer (New England BioLabs® Inc.) at 95°C for four minutes. The pcDNA3.1 expression vector, pVLP55, was digested using *Age*I-HF® restriction enzyme (New England BioLabs® Inc.) and CutSmart® Buffer. The J8 insert was then ligated into the pVLP55 to form pVLP-J8 (pVLP55+ J8) using T4 DNA ligase (Promega Corporation) and a 1:3 molar ratio of vector:insert in CutSmart® Buffer. The

ligation reaction mix was incubated overnight at 4°C and then transformed in *E. coli* competent cells as follows. 3 µl of ligation reaction mix was added to 25 µl of Invitrogen One Shot™ TOP 10 Chemically Competent *E. coli* (Life Technologies, ThermoFisher Australia) and incubated on ice for 30 minutes, at 42°C for 30 seconds, and then on ice again for five minutes. After addition of 275 µl of pre-warmed (37°C) Invitrogen™ Super Optimal broth with Catabolite repression (SOC) medium (ThermoFisher Scientific), the vial was placed horizontally in a shaking incubator at 37°C and shaken for one hour at 225 rpm. The transformed *E.coli* were then cultured overnight at 37°C on Luria 460 Bertani (LB) agar containing 100 ug/ml ampicillin (Amp) and colonies were screened for the presence of plasmid, pVLP-J8, by polymerase chain reaction (PCR) using the following primers: T7 forward primer: 5'TAATACGACTCACT ATAGGG-3' and HBsAg-S reverse primer: 5'CTGAGCCAGGAGAAAC-3' followed by agarose gel electrophoresis (AGE) of the PCR products on 1% agarose at 100 v for 60 minutes. Colonies that generated a PCR product of the expected size (~1225 bp with the insert compared with 1135 bp without the insert) were subsequently selected for plasmid extraction and cultured overnight at 37°C on LB agar with Amp followed by a second overnight incubation, with shaking at 225 rpm, in LB broth with Amp.

4.2.4 Confirmation of sequence integrity and correct orientation

Because an *Age*1 restriction site was included at each end of the J8 insert, there was a 1:2 chance that the insert would be inserted into pVLP55 in the incorrect (flipped) orientation. To confirm sequence integrity and correct orientation of the J8 insert, plasmids were extracted using the Wizard® Plus SV Miniprep kit (Promega) and sent to AGRF for DNA sequencing. The sequence integrity and correct orientation of the J8 insert was verified in three of five samples sent for DNA sequencing. Subsequently, one of the colonies that produced the pVLP55-J8 in the correct orientation was grown up from frozen stocks as described above with an additional subculture of 600 µl of broth culture in 600 ml LB broth with Amp followed by a third overnight incubation at 37°C, with shaking at 225 rpm. pVLP55-J8 was extracted using NucleoBond® Xtra Maxi Plus EF Endotoxin-free plasmid DNA purification kit (Macherey-Nagel). The concentration of the

extracted and purified plasmid DNA, determined using an Implen NanoPhotometer® (Implen GmbH), was 803 ng/µl. The purified, pVLP55-J8 was then stored at -20°C for subsequent transfection in HEK293t cells.

4.2.5 Secondary structure modelling of VLP-J8

Alpha-helicity is essential to the immunogenicity of derivatives of p145, including J8 (reviewed in Chapter 3 (Dooley et al., 2021)). More specifically, the helicity of the minimal B cell epitope J8i must be preserved in p145-based vaccine candidates. To confirm that J8i is likely to be expressed in its helical form in this construct, the Phyre^2 web portal for protein modelling, prediction and analysis was utilised to analyse VLP-J8. This involved entering the aa sequence for VLP-J8, as shown in Figure 4, into the free Phyre^2 web portal (Kelley, Mezulis, Yates, Wass, & Sternberg, 2015).

4.2.6 Expression of pVLP-J8 in HEK293t cells

Human embryonic kidney (HEK293t) cells were resuscitated from storage at -80°C, in 10% DMSO, by gentle thawing (in the hand) followed by suspension in cell culture medium described below. The DMSO was removed by discarding the supernatant after centrifugation for eight minutes at 125 g. The cells were then resuspended in Dulbeco's Modified Eagle's Medium-High glucose (DMEM Sigma® Life Science) with 10% Fetal bovine serum (FBS, Australia origin Sigma-Aldrich), 1% L-Glutamine 200 mM (GlutaMAX™ 100X Gibco), 2% and Penicillin 5000 U/ml-Streptomycin 5 mg/ml (Sigma-Aldrich). Prior to use, the media was filtered using 0.22 µm Millex®GP syringe filters (Merck Millipore). The cells were transferred to 250 ml CELLSTAR® cell culture flasks with filter caps (Greiner Bio-One, Kremsmünster, Austria) and incubated at 37°C with 5% CO_2 and passaged on alternate days for 10 days by which time the cells were estimated to be 70% confluent.

HEK293t cells were then transfected using FuGENE®HD Transfection Reagent (Promega), following the manufacturer's instructions with a 3:1 ratio of FuGENE®HD to DNA. The transfection mix for each flask consisted of 50 µg pVLP-J8 and 150 µl FuGENE®HD Transfection Reagent (Promega) made up to 3 ml

with culture media as described above, but without the FBS. The transfection mix was vortexed briefly and incubated at room temperature for 15 minutes and then added to flasks containing the cultured HEK293t cells. The transfected HE293t cells, were incubated at 37°C with 5% CO_2 for five days with an additional 10 ml of pre-warmed serum free DMEM added daily. The supernatant from each cell culture flask was harvested on day six post-transfection and analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE).

The supernatant was concentrated using Amicon® Ultra-15 Centrifugal 10K Filter Units (Merck Millipore, Bayswater, Victoria) and then diluted in PBS in 1ml aliquots. The aliquots were analysed using an ARCHITECT chemiluminescent microparticle HBsAg assay (Abbott) at the Pathology Queensland protein laboratory at Royal Brisbane and Women's Hospital, Herston.

4.2.7 Anti-FLAG affinity purification of VLP-J8

FLAG® M2 Purification Kit for mammalian expression systems (Sigma-Aldrich®) was utilised to separate the FLAG-tagged VLP-J8 from other proteins in the cell culture supernatant. Following the manufacturer's instructions for batch absorption of FLAG® fusion proteins using Anti-FLAG® M2 Affinity Gel, 50 ml of cell culture supernatant was added to equilibrated Anti-FLAG® M2 Affinity Gel and incubated at room temperature with gentle mixing for 60 minutes. Following incubation, the resin was collected by centrifugation for five minutes at 1 000 g and then washed in Wash Buffer (0.5M Tris HCl, pH 7.4, 1.5M NaCl) to remove all non-specific (and unbound) proteins. The wash step was repeated until the absorbance reading, at 280 nm, of the wash solution coming off the column was not higher than 0.05 against a wash solution blank. Aliquots of the flowthrough from each wash were subsequently analysed by SDS-PAGE as were aliquots from two final washes of the column prior to elution. Bound proteins were eluted from the column by competitive elution with five one-column volumes of 3X FLAG peptide. Aliquots of eluate were subsequently analysed by SDS-PAGE. The process above was subsequently repeated for a second sample of 500 ml of cell culture supernatant but with incubation at 4°C.

4.2.8 Plasmid DNA for VLP-p*17

The plasmid DNA for VLP-p*17, codon optimised for protein expression in mammalian cells, was purchased as 300 μg of transfection grade plasmid from GenScript Biotech Corporation (GenScript USA Inc.).

4.2.9 Secondary structure modelling of VLP-p*17

The Phyre[2] web portal for protein modelling, prediction and analysis was utilised to predict the secondary structure of VLP-p*17 and confirm the helicity of the J8i variant sequence within p*17.

4.2.10 Expression of VLP-p*17 in HEK293t cells and anti-FLAG purification

VLP-p*17 was expressed in HEK293t cells as described in section 4.2.6 and purified using anti-FLAG affinity purification as described in section 4.2.7.

4.3 Results

4.3.1 Construction and amplification of plasmid pVLP-J8

The DNA sequence for the p145 derivative, J8, was designed to include *Age1* restriction sites at each end and was constructed by annealing two long primers. The resultant DNA segments were amplified by PCR and then ligated into pVLP55: pcDNA3.1 expression vector carrying the HBsAg-S DNA sequence with an *Age1* restriction site in the "a" determinant. The plasmids were cloned in *E.coli,* and 17 single colonies were screened for uptake of pVLP55-J8 by PCR and AGE. As shown in Figure 6, plasmids from colonies 6,7,9,14 and 16 yielded slightly larger PCR products and were therefore most likely to contain the pVLP55-J8. Plasmids were extracted from these five colonies and sent to AGRF for DNA sequencing.

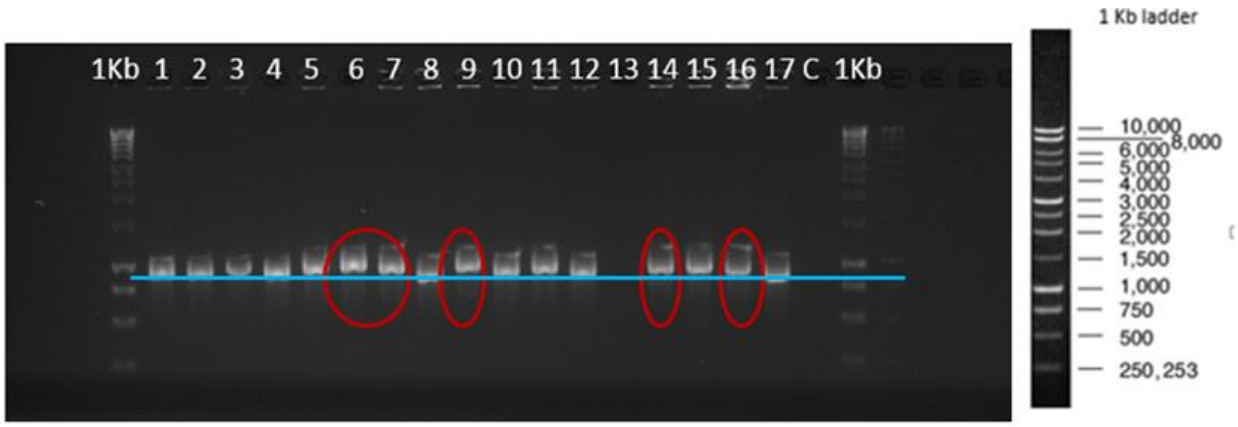

Figure 6. Screening for J8 insert in pVLP55. Colonies 6, 7, 9, 14 and 16 were selected for plasmid extraction based on the slightly larger size of the corresponding PCR product as indicated by AGE.

4.3.2 Confirmation of sequence integrity and correct orientation

The presence of pVLP55-J8 was confirmed in all five samples sent to AGRF for DNA sequencing but the J8 insert was "flipped" in samples 14 and 16. Figure 7 shows the sequencing results for colony six illustrating the correct orientation of the J8 insert within the HBsAg-S sequence and confirming the presence of the Kozac sequence and the FLAG® sequence. These results confirmed the successful cloning of J8 DNA insert into the HbsAg-S DNA at the "a" determinant and marked the first milestone of this project. Colony six was used as a source of pVLP55-J8 from this point on.

Figure 7. Sequencing report for T7FJ8A6_A01 (VLP-J8) plasmid from colony 6 confirms the sequence integrity and correct orientation of the J8 insert in the DNA sequence for the "a" determinant of the HbsAg-S. The sequence upstream of the Kozac sequence and downstream of stop codon for HbsAg-S are part of the DNA sequence for the pcDNA3.1 expression vector.

4.3.3 Secondary structure modelling of VLP-J8

After successful construction of the recombinant plasmid, pVLP55-J8, the predicted secondary structure of VLP-J8, the protein encoded by pVLP55-J8, was analysed using the free Phyre[2] web portal. As described in Chapter 3, J8 comprises a sequence of 12 aa from p-145, flanked on either side by sequences of eight amino acids from the yeast GCN4 DNA binding protein. This 12 aa sequence, referred to as J8i, is a minimal B cell epitope and essentially represents the Strep A, M protein component of J8 (QAEDKVKQSREAKKQVEKALKQLEDKVQ).

The Phyre[2] report (Figure 8) shows predicted alpha helical secondary structure for a 10 aa sequence within the 12 aa J8i sequence. Essentially this result predicts, with a level of confidence from five to seven on a scale of zero to nine, that 83.33% of the J8i epitope will be expressed in alpha-helical configuration in VLP-J8. Furthermore, the transmembrane helix prediction shown in figure 9

forecast that J8i (aa144-156) is likely to be expressed on the surface of the VLP (Kelley et al., 2015) .

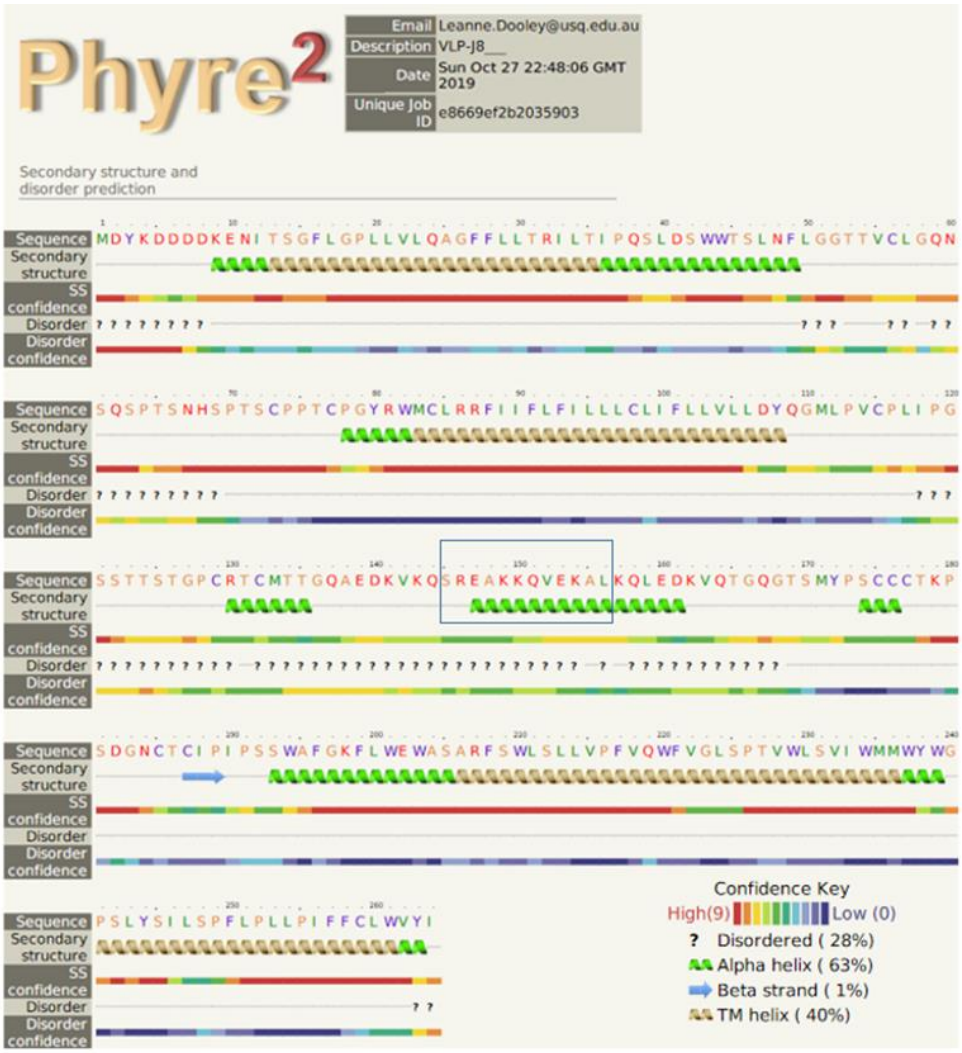

Figure 8. Secondary structure prediction of VLP-J8. The structure of the Strep A p145 component of J8 (J8i: SREAKKQVEKAL) was predicted with medium level confidence to be 83.33% alpha helix (Kelley et al., 2015).

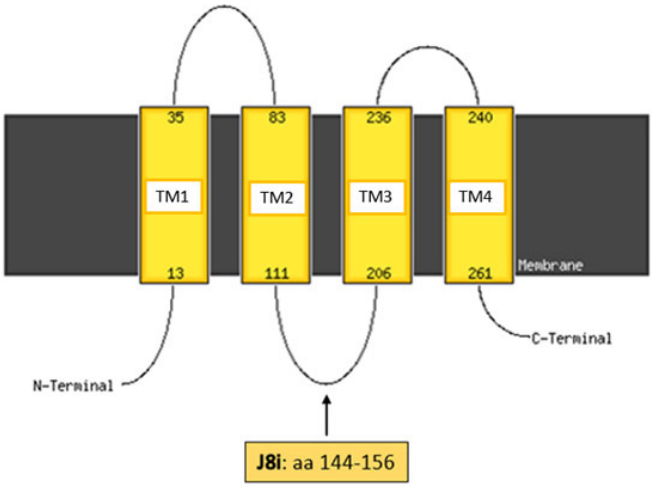

Figure 9. Transmembrane helix prediction for VLP-J8 predicts surface expression of J8i (Kelley et al., 2015).

4.3.4 Expression of pVLP-J8 in HEK293t cells

SDS-PAGE of concentrated HEK293t post-transfection culture supernatant, shown in Figure 10, confirmed the presence of a distinct band at around 28 kDa: the calculated MW of VLP-J8 is 28.126 kDa (Bioinformatics.org). Results of HbsAg quantitation of aliquots of various dilutions of the concentrated post-transfection supernatant are illustrated in Table 1. The HbsAg concentration of the undiluted sample was 225 IU/ml, which was within the analytical range of linearity and, according to the conversion outlined by Deguchi et al. (2004), equates to 900 ng/ml and approximately 1.8 x 10^{11} VLPs/ml (Deguchi et al., 2004). These results showed that VLP-J8 recombinant proteins were recognised by an anti-HbsAg IgM monoclonal antibody used in the Abbott Architect chemiluminescence assay. Successful expression of VLP-J8 marked the second milestone of this project.

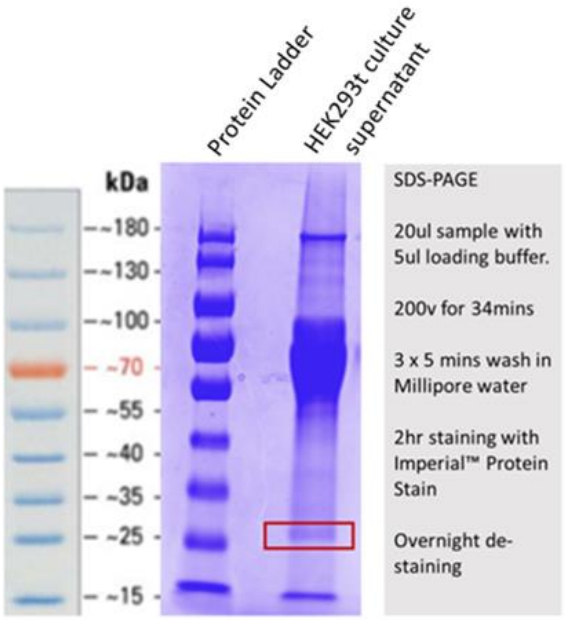

Figure 10. SDS-PAGE of supernatant from transfected HEK293t cells showing the expected band at approximately 28 kDa.

Table 1. HbsAg quantitation of post-transfection cell culture supernatant

HbsAg Quantitation (Abbott ARCHITECT)			
Sample	HbsAg Concentration*		
neat	225 IU/ml	900 ng/ml	1.8 x 10^{11} VLP/ml
1:3	68 x 3 = 204 IU/ml	816 ng/ml	1.6 x 10^{11} VLP/ml
1:6	35 x 6 = 210 IU/ml	840 ng/ml	1.7 x 10^{11} VLP/ml
1:12	17 x 12 = 204 IU/ml	816 ng/ml	1.6 x 10^{11} VLP/ml
* 0.05 IU/ml HbsAg ~ 0.2 ng/ml HbsAg. 0.1 ng/ml HbsAg ~ 2 x 10^{7} VLP /ml (Deguchi et al., 2004).			

4.3.5 Anti-FLAG affinity purification of VLP-J8

The FLAG® peptide sequence DYKDDDDK was included at the N-terminus of VLP-J8 (Figure 4) to facilitate protein purification by Anti-FLAG affinity chromatography. However, SDS-PAGE of the flowthrough and eluate off the Anti-FLAG resin revealed a band at approximately 28 kDa in the flowthrough samples but not in the eluate (Figure 11). These results suggest that the FLAG-tagged

VLP-J8 did not bind to the anti-FLAG resin at 4°C or at room temperature and were confirmed by HbsAg quantitation, with 212 IU/ml measured in pooled flow-through samples and less than 0.05 IU/ml detected in wash and eluate samples.

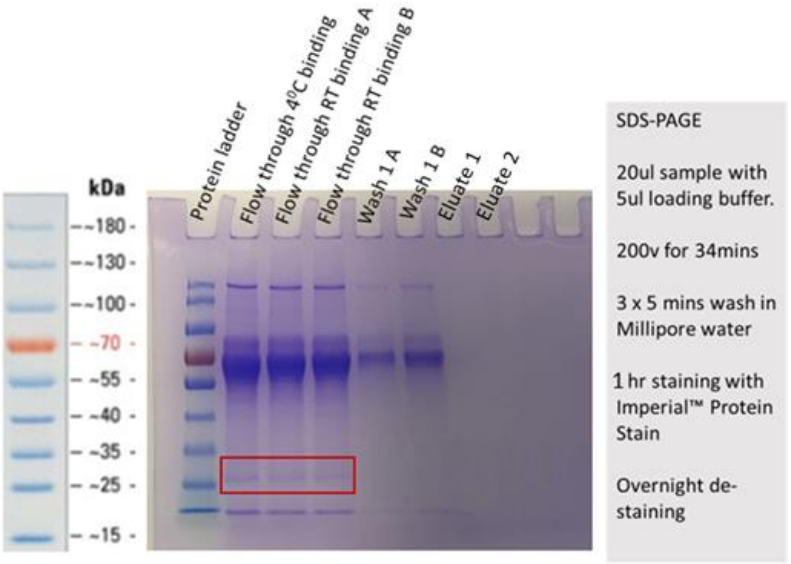

Figure 11. SDS-PAGE of flow through, wash and eluate after Anti-FLAG affinity chromatography for VLP-J8 shows a band at approximately 28 kDa in the flow through but not in the eluate. Imperial™ Protein Stain (ThermoScientific).

4.3.6 The plasmid DNA for VLP-p*17

The DNA sequence for VLP-p*17, codon optimised for protein expression in mammalian cells is shown in Figure 13. The p*17 sequence was inserted into the "a" determinant of HBsAg-S to facilitate surface expression of the Strep A epitope.

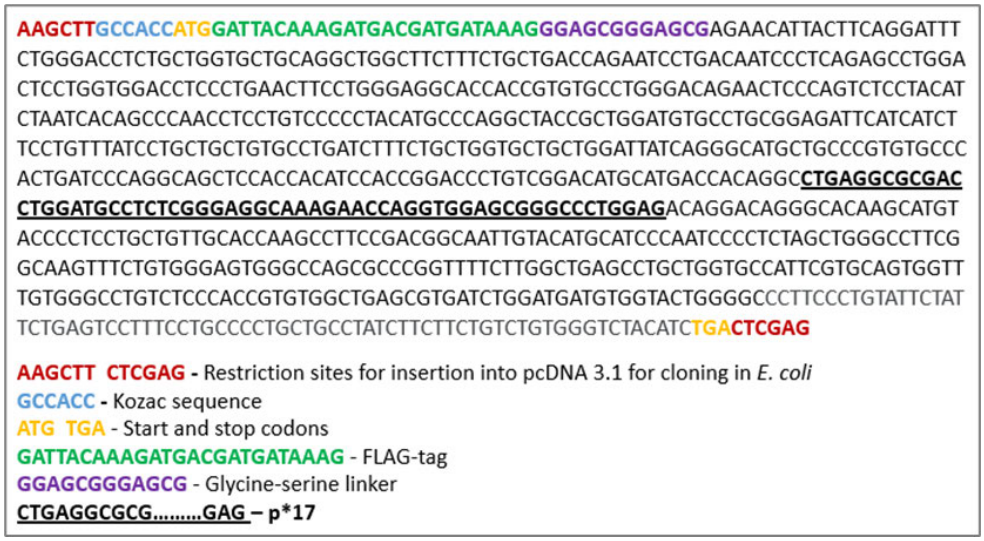

Figure 12. Codon optimised DNA sequence for VLP-p*17 with Glycine-Serine linker, FLAG®-tag and restriction sites for insertion into pcDNA 3.1.

4.3.7 Secondary structure modelling of VLP-p*17

The Phyre[2] report (Figure 13) predicted alpha helical secondary structure for the entire 12aa variant J8i sequence, with medium to high confidence (five to eight on a scale of zero to nine) (Kelley et al., 2015). As was the case for VLP-J8, the transmembrane helix prediction shown in figure 15 confirmed predicted surface expression of p*17 (aa141-160) in the proposed new construct (Kelley et al., 2015).

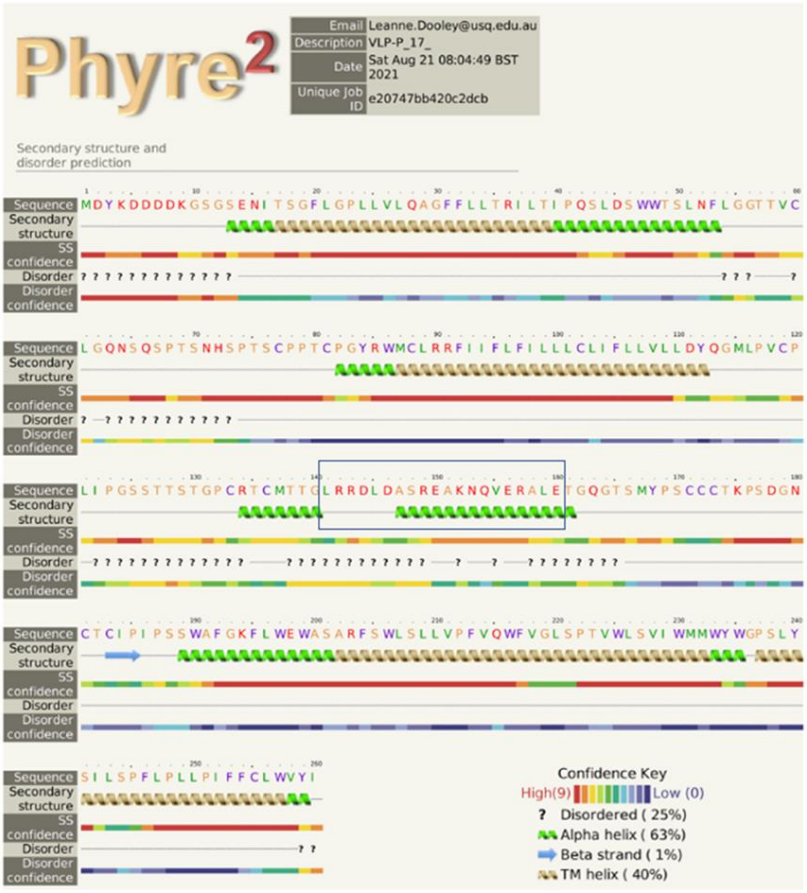

Figure 13. Secondary structure prediction of VLP-p*17. The entire sequence of the J8i variant within p*17 (SREAK**K**QVE**K**AL) is predicted, with medium to high confidence, to be in the alpha helical conformation (Kelley et al., 2015).

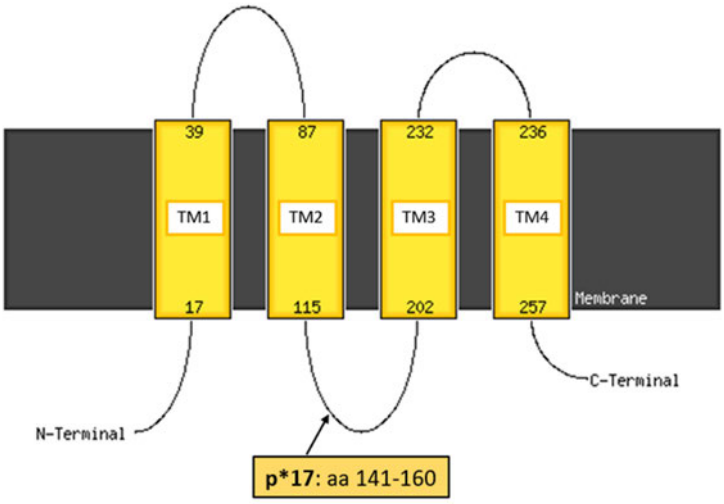

Figure 14. Transmembrane helix prediction for VLP-p*17 predicts surface expression of p*17 (Kelley et al., 2015).

4.3.8 Expression of VLP-p*17 in HEK293t cells and anti-FLAG purification

Transfected HEK293t cells secreted VLP-p*17 but once again, the VLP failed to bind to the anti-FLAG resin as shown in Figure 15.

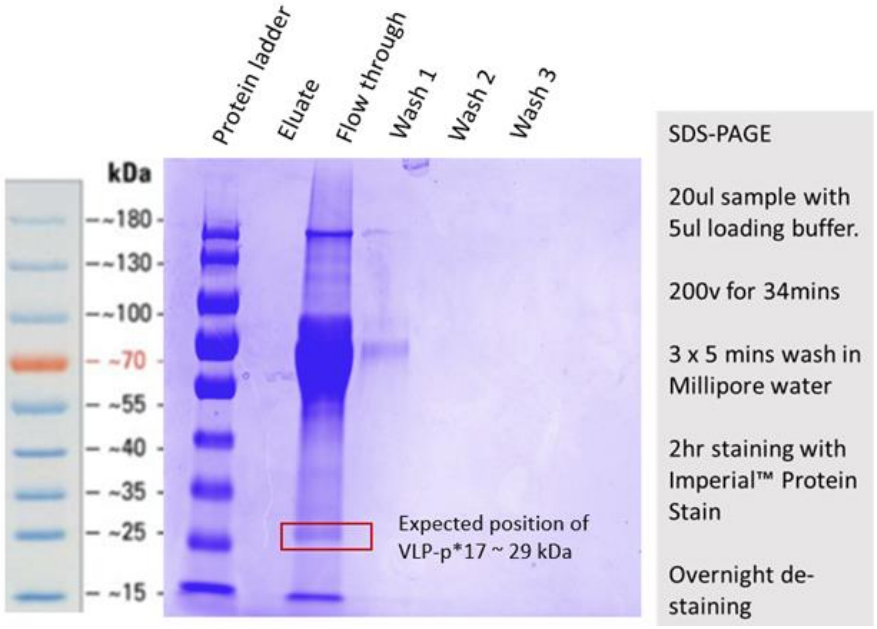

Figure 15. SDS-PAGE of flow through, wash and eluate after Anti-FLAG affinity chromatography shows a band at approximately 29 kDa in the flow through but not in the eluate. Imperial™ Protein Stain (ThermoScientific).

A possible explanation for the failure of binding of FLAG-tagged VLP-p*17 to the anti-FLAG resin could be the tendency of VLPs to spontaneously form particles (as demonstrated by transmission electron microscopy in Chapter 5) that may be too large to bind to the column.

4.4 Discussion and Conclusion

The preliminary investigations described above resulted in successful production of recombinant HBsAg-S VLPs confirmed by the results of HBsAg quantitation shown in Table 1. SDS-PAGE analysis of the cell-culture derived VLPs shown in Figures 10, 11, and 15, indicate the presence of proteins at approximately 28 - 29 kDa, matching the calculated MW of VLP-J8 and VLP-P*17 respectively. These results suggest that the secreted VLPs contain the additional

amino acid sequences inserted into the HBsAg-S. Figures 13 and 14 predict that the alpha-helical secondary structure of the Strep A epitopes will be maintained and that the epitopes will be expressed on the surface of the VLPs. Both of these features are critical for Strep A antigen-specific immunogenicity. The secondary structure of culture derived proteins, including VLPs, can be confirmed by methods such as Raman spectroscopy, circular dichroism spectroscopy, and infrared spectroscopy, Fourier Transform Infrared Spectroscopy and Thermogravimetric Analysis (Pelton & McLean, 2000). However, these analyses require specialised equipment and expertise, were beyond the scope of this study, and are not routinely applied in the early stages of vaccine development (Kotiw et al., 2012; Netter et al., 2001). Failure to purify VLP-J8 using Anti-FLAG affinity chromatography led to the decision to construct a new VLP with inclusion of a glycine-serine linker to separate the FLAG® peptide from the rest of the molecule in the hope that this would help facilitate binding of the VLP to the anti-FLAG resin. In light of new research by our collaborators at the Institute for Glycomics, the decision to substitute J8 with p*17 as the Strep A component of the new vaccine was also made at this stage.

As described in Chapter 3, Nordström et al., (2017), designed and tested 86 variant peptides with single and double amino acid substitutions within the J8i sequence of P145. One of the variants was p*17, (LRRDLDASREAKKQVEKAL with two amino acid alterations LRRDLDASREAK**N**QVE**R**AL). In a mouse-model of Strep A skin infection, p*17 induced a degree of protection that was 10 000-fold greater than that induced by p145 after a single immunisation. Our collaborators at the Institute for Glycomics agreed that p*17 should be the Strep A component of the VLP-based Strep A vaccine and offered to fund the subsequent animal trial described in Chapters 5 and 6.

Failure to purify the recombinant VLPs using Anti-FLAG affinity chromatography, despite inclusion of a glycine-serine linker in the vaccine construct, prompted a decision to attempt partial purification of VLP-p*17 utilising a 20% sucrose gradient and ultracentrifugation.

The production, characterisation, quantitation and semi-purification of VLP-p*17, and evaluation of the immunogenicity of semi-purified VLP-p*17 formulated with adjuvant in BALB/c mice, is described in detail in Chapter 5 (Paper 2).

CHAPTER 5: PAPER 2 – Chimeric hepatitis B surface antigen virus-like particles expressing the Strep A epitope p*17 elicit a humoral response in mice.

5.1 Introductory paragraph

This paper describes the expression, semi-purification and preliminary immunological evaluation of VLP-p*17: a putative recombinant virus-like particle vaccine against Strep A. The study tested the hypotheses that expression of p*17 within the "a" determinant of HBsAg-S will not compromise spontaneous VLP formation, a feature critical for immunogenicity of the putative vaccine. This study also tested the hypothesis that mice immunised with VLP-p*17 formulated with adjuvant will produce measurable titres of p*17-specific IgG antibodies.

5.2 Paper 2

Dooley, L, Ahmad, T, Ozberk, V, Pandey, M, Good, M & Kotiw, M 2022, 'Progress in the development of a novel recombinant virus-like particle vaccine against Strep A infections and rheumatic heart disease', *Heliyon* (submitted for publication November 4, 2022, minor revisions submitted March 14 and May 30, 2023).

Heliyon

Progress in the development of a novel recombinant virus-like particle vaccine against Strep A infections and rheumatic heart disease

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	Tarek Ahmad
	Victoria Ozberk
	Manisha Pandey
	Michael Good
	Michael Kotiw
Abstract:	To optimize immunogenicity, bacterial epitopes in putative vaccine constructs can be presented to immune cells as multiple repeated structures on a defined nanoparticle. Virus-like-particles (VLPs) are viral capsid proteins that self-assemble to form compact and highly ordered nanoparticles that are within the optimal size range for uptake by dendritic cells. VLPs mimic the live virus in size and form but contain no viral genetic material, are therefore non-infective, and are the basis of safe and effective vaccines against Hepatitis B virus (HBV) and Human Papilloma virus (HPV). Due to their particulate nature, molecular stability, and expression of high density and repetitive antigen displays, recombinant cell-culture derived VLPs are ideal platforms for delivery of small molecules including bacterial epitopes. We have developed a putative vaccine by expressing a minimal epitope from the bacterium Streptococcus pyogenes (Strep A) on the surface of a recombinant VLP comprising multiple copies of HBV small envelope protein (HBsAg-S). Strep A is responsible for a wide spectrum of human infections and post-infectious diseases that disproportionately affect children and young adults living in resource-poor communities. No vaccine is currently available to offer sufficiently broad protection from the numerous and diverse strains of Strep A endemic in these at-risk populations. The Strep A antigen targeted by our vaccine construct, is p*17, a cryptic epitope from a highly conserved region of the Strep A M-protein with demonstrated enhanced immunogenicity and broad protective potential against Strep A. To ensure surface expression and optimal immunogenicity, we expressed p*17 within the immunodominant "a" determinant of HBsAg-S. The recombinant VLPs, (VLP-p*17) expressed in HEK293t cells, spontaneously formed 22 nm particles and induced production of high titres of p*17-specific IgG in BALB/c mice immunised with three 0.5 µg doses of VLP-p*17 formulated with adjuvant.
Suggested Reviewers:	
Opposed Reviewers:	

Research Article

Chimeric Hepatitis B surface antigen virus like particles expressing the Strep A epitope p*17 elicit a humoral immune response in mice

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Abstract

To optimize immunogenicity, bacterial epitopes in putative vaccine constructs can be presented to immune cells as multiple repeated structures on a defined nanoparticle. Virus-like-particles (VLPs) are viral capsid proteins that self-assemble to form compact and highly ordered nanoparticles that are within the optimal size range for uptake by dendritic cells. VLPs mimic the live virus in size and form but contain no viral genetic material, are therefore non-infective, and are the basis of safe and effective vaccines against Hepatitis B virus (HBV) and Human Papilloma virus (HPV). Due to their particulate nature, molecular stability, and expression of high density and repetitive antigen displays, recombinant cell-culture derived VLPs are ideal platforms for delivery of small molecules including bacterial epitopes. We have developed a putative vaccine by expressing a minimal epitope from the bacterium *Streptococcus pyogenes* (Strep A) on the surface of a recombinant VLP comprising multiple copies of HBV small envelope protein (HBsAg-S). Strep A is responsible for a wide spectrum of human infections and post-infectious diseases that disproportionately affect children and young adults living in resource-poor communities. No vaccine is currently available to offer sufficiently broad protection from the numerous and diverse strains of Strep A endemic in these at-risk populations. The Strep A antigen targeted by our vaccine construct is p*17, a cryptic epitope from a highly conserved region of the Strep A M-protein with demonstrated enhanced immunogenicity and broad protective potential against Strep A. To ensure surface expression and optimal immunogenicity, we expressed p*17 within the immunodominant "a" determinant of HBsAg-S. The recombinant VLPs, (VLP-p*17) expressed in HEK293t cells, spontaneously formed 22 nm particles and induced production of high titres of p*17-specific IgG in BALB/c mice immunised with three 0.5 μg doses of VLP-p*17 formulated with adjuvant.

1. Introduction

Despite universal susceptibility to penicillin, *Streptococcus pyogenes* (Strep A) claims an estimated 500 000 lives globally every year [1, 2]. The most common causes of Strep A-related deaths are severe invasive infections and rheumatic heart disease (RHD), an autoimmune sequela of superficial Strep A infections [3, 4]. A disproportionate share of the Strep A associated disease burden is carried by children and young adults in developing countries and in Indigenous communities within developed countries, including Australia, Canada, New Zealand, and the United States of America [2, 5]. Vaccination is widely considered the best means of prophylaxis against Strep A yet, despite decades of research, no vaccine offering adequate protection to at-risk populations is currently available [6]. A major challenge to Strep A vaccine development has been the vast serotypical and epidemiological diversity of the organism. Hence most Strep A vaccine candidates currently under development, target conserved epitopes

expressed by all pathogenic strains of the bacterium. Many contemporary Strep A vaccine candidates target epitopes derived from p145, a 20 amino acid peptide within a highly conserved C-terminal region of the Strep A M protein: a dimeric alpha-helical protein that covers the surface of the bacterium and protects it from complement-mediated phagocytosis [7] (Figure 1). The highly conserved nature of p145, and observations that anti-p145 antibodies develop naturally in response to Strep A infection, have made p145 an attractive target for a broad-spectrum Strep A vaccine [8]. Several vaccine constructs utilising a range of different platforms to deliver various derivatives of p145 have been developed by Australian researchers. While these constructs demonstrated immunogenicity, multiple immunisations were required for adequate protection in mouse models of Strep A infection and only one construct, a p145 derivative conjugated to Diphtheria toxoid (MJ8VAX), has progressed to human trials (Reviewed in [6]).

To increase the immunogenicty of p145, Nordstrom et al., (2017), designed 18 peptides through a process of substituting one amino acid at a time in the aa sequence of p145 and assessed the immunogenicity of the new peptides in outbred mice. One of these peptides, p*17 (p145 with two amino acid substitutions as shown in Figure 1), induced more than 10 000-fold greater protection following Strep A challenge when conjugated to diphtheria toxoid (DT) and formulated with Alum, than a p145-DT conjugate formulated with Alum. Furthermore, three immunizations with p145-DT/Alum were required to induce complete protection from Strep A challenge, while only a single immunization with p*17-DT/Alum was required [9].

To optimise immunogenicity, bacterial epitopes in putative vaccine constructs can be presented as multiple repeated structures on a defined nanoparticle such as a virus-like particle (VLP) [10]. VLPs comprising multiple monomers of Hepatitis B virus small envelope protein (HBsAg-S) form the basis of the currently used second generation vaccines against Hepatitis B, Energix-B (GlaxoSmithKline) and Recombivax HB® (Merck & Co. Inc) and the anti-malarial vaccine RTS,S (Mosquirix®) [11-13]. Recombinant-derived HBsAg-S molecules spontaneously polymerise to form 22 nm octahedral particles, each comprising approximately 100 HBsAg-S monomers [14]. Due to their particulate nature, molecular stability, and expression of high density and repetitive displays of epitopes, HBsAg-S VLPs are ideal platforms for delivery of small molecules including bacterial epitopes [10, 11, 15, 16]. The HBsAg-S amino acid sequence contains a highly conformational, hydrophilic domain between positions 122 to 150 referred to as the "a" determinant [17, 18]. The "a" determinant is expressed on the surface of the VLP and contains epitopes for induction of a protective humoral immune response [19]. We hypothesised that insertion of the minimal Strep A epitope p*17 into the "a" determinant of HBsAg-S would preserve the immunogenicity of p*17 and would not inhibit spontaneous polymerisation of the HBsAg-S monomers. We also hypothesised that combining the enhanced immunogencity of p*17 with the natural immunogenicity of the HBsAg-S VLP for epitope delivery, would produce a putative vaccine capable of triggering production of high titre p*17-specific IgG in mice immunised with VLP-p*17 formulated with adjuvant. The aim of this study therefore was to utilise recombinant

DNA technology to express p*17 on the surface of HBsAg-S VLP, to semi-purify the cell-culture-derived VLP-p*17, and to evaluate its immunogenicity in mice.

2. Materials and Methods

Utilizing recombinant DNA technology we designed and produced a chimeric HBsAg VLP with surface expressed p*17, (Figure 1) and evaluated the p*17-specific humoral repsonse in mice vaccinated with three 0.5 μg doses of the putative Strep A vaccine, VLP-p*17.

2.1. DNA expression vector

The DNA sequence for the recombinant HBsAg-S-p*17 construct (VLP-p*17) was designed with the p*17 sequence inserted at an engineered *AGE1* restriction site in the cDNA of HBsAg-S (between amino acids 127 and 128) to direct expression of p*17 in the "a" determinant region of HBsAg-S [15, 20]. The VLP-p*17 gene was codon optimized for expression in mammalian cells, synthesized and cloned into the HindIII/XhoI cloning site of pcDNA3.1(+) by GenScript (Hong Kong) Limited, and purchased as 300 μg of bespoke transfection grade plasmid in 300 μl buffer.

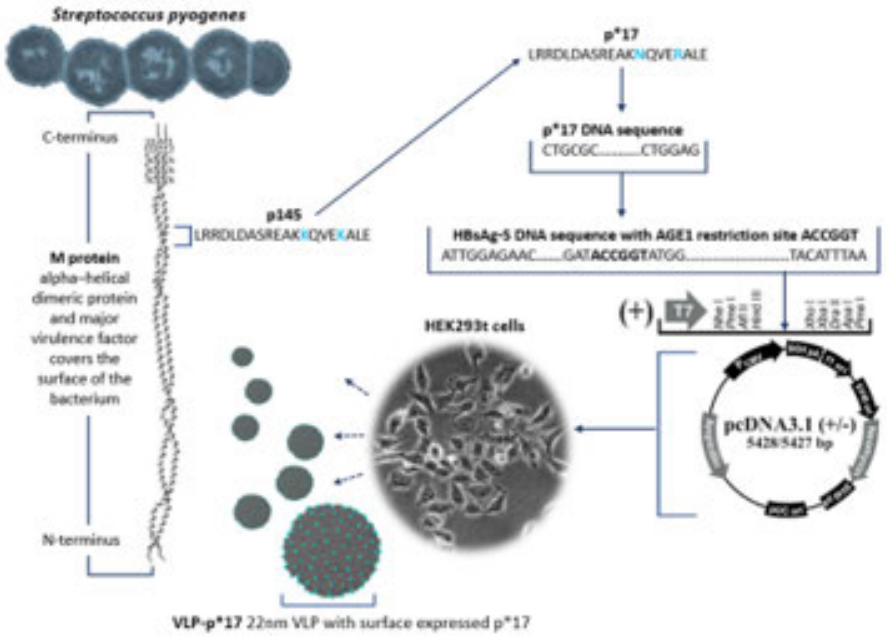

Figure 1. Design and synthesis of VLP-p*17 (amino acid substitutions in p145 to create p*17 are shown in blue). The p*17 DNA sequence was incorporated into the HBsAg-S DNA sequence at an engineered *AGE1* restriction site. The HBsAg-S-p*17 sequence was then inserted into the pcDNA3.1 expression vector and expressed in HEK293t cells, which subsequently secreted recombinant VLP-p*17.

*2.2. Anti-p*17 antibodies*

Sera was raised in BALB/c mice immunized with three doses of p*17-DT/Alum as part of a study by Nordstrom et al., (2017). The sera were tested in ELISA and were shown to contain p*17 specific IgG antibodies (titers 10^5) [9]. The sera were used in this study at a 1:32 000 dilution as a source of anti-p*17 antibodies for Western blotting.

*2.3. Synthesis of cell culture-derived VLP-p*17*

Three 2 ml vials of HEK293t (ATCC® CRL-3216™)cells were resuscitated from storgae, in 10% Dimethyl Sulfoxide at -80°C, by gentle thawing (in a closed hand). The DMSO was removed by discarding the supernatant after centrifugation for eight minutes at 125 g. Cells were immediately resuspended in 2 ml Dulbeco's Modified Eagle's Medium-High glucose (DMEM Sigma® Life Science) with 10% Fetal bovine serum (FBS, Australia origin Sigma-Aldrich), 1% L-Glutamine 200 mM (GlutaMAX™ 100X Gibco), and 2% Penicillin 5000 U/ml-Streptomycin 5 mg/ml (Sigma-Aldrich). The three vials of resuspended cells were then immediately transferred to three 250 ml CELLSTAR® cell culture flasks with filter caps (Greiner Bio-One, Kremsmünster, Austria) each containing 20 ml of cell culture media as described above. Flasks were incubated at 37˚C with 5% CO_2 and cells were passaged on alternate days for 10 days after which time they were estimated to be 70-80% confluent. HEK293t cells were transfected using FuGENE® HD Transfection Reagent (Promega), following the manufacturer's instructions with a 3:1 ratio of FuGENE®HD (900 µl) to DNA (300 µl pcDNA3.1(+)-HBsAg-S-p*17) in 1.8 ml of FBS free DMEM. The transfection mixture was vortexed briefly and incubated at room temperature for 15 minutes after which 1 ml was added to the HEK293t cells in each of the three flasks. The flasked were then gently mixed in a shaking incubator at 37˚C for 10 minutes. The transfected HE293t cells were incubated at 37˚C with 5% CO_2 for five days with an additional 10 ml of pre-warmed FBS free DMEM added to each flask daily. The supernatant from each cell culture flask was harvested on day six post-transfection and analysed by SDS-PAGE using Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad) and following the manufacturer's instructions. A 300 µl sample of crude supernatant from each flask was also analysed for HBsAg concentration using an automated chemiluminescent microparticle assay (Abbott ARCHITECT) [21].

*2.4. Partial purification and quantitation of culture-derived VLP-p*17*

50 ml of crude cell culture supernatant containing the recombinant VLPs was removed from each flask and centrifuged at 2 400 g (Heraeus Multifuge Thermo Scientific) for 10 min, to remove cellular debris, and then transferred to six 38.5 ml Open-Top Thinwall Ultra-Clear™ (Beckman Coulter) centrifuge tubes. The supernatant in each centrifuge tube was then underlaid with 5 ml 20% sucrose in STE buffer (100 mM NaCl, 10 mM Tris, pH8, 1 mM EDTA) and the VLPs were pelleted by ultracentrifugation at 25 000 rpm (Beckman Optima XPN-100) for 16 hours, at 4˚C. Supernatant was discarded and the pelleted VLPs in each of the the six tubes were resuspended in 200 µl STE buffer with three 30 second bursts of low-speed sonication in a sonicator-water bath (GT Sonic) at 4˚C. Partially purified and resuspended VLP samples were further diluted 1:2 in STE

buffer and aliquots were again analysed for HBsAg concentration, as described in section 2.3.The remainder of each sample was then stored in 200 µl aliquots at -80 ˚C until required.

*2.5. Transmission electron microscopy of partially purified VLP-p*17*

Three 200 µl samples of partially purified VLP-p*17 were analysed by transmission electron microscopy (TEM) following a standard negative stain protocol at the Centre for Microscopy and Microanalysis (CMM), University of Queensland, Australia. 4 µl of each sample was placed onto formvar-coated copper grids for 1 minute. Excess sample was removed with damp filter paper after which 1% uranyl acetate was added to each grid for 1 minute and then completely removed with filter paper. The grids were allowed to dry before being viewed in a Hitachi HT7700 transmission electron microscope at 100 kV.

*2.6. Detection of p*17 expression by Western Blot*

Three 200 µl aliquots of partially purified VLP-p*17 in STE buffer were retrieved from frozen storage, thawed at room temperature and analysed for p*17 expression by Western Blot utilizing polyclonal murine anti-p*17-DT sera diluted 1:32 000 in Pierce® Fast Western Antibody Diluent (Thermo Scientific). Proteins were separated by SDS-PAGE (200 v for 30 minutes), transferred to a nitrocellulose membrane, and blotted using a Pierce® Fast Western Blot Kit, with enhanced chemiluminescence (ECL) Substrate (Thermo Scientific) following the manufacturer's instructions.

2.7. *Formulation of VLP-p*17 with adjuvant*

Aliquots of VLP-p*17 in STE buffer were retrieved from frozen storage, thawed at room temperature, and pooled into two lots of equal volume, each of which was then diluted in 30 ml sterile PBS, and stored overnight at 4˚C. The VLPs in PBS where pelleted again by overnight ultracentrifugation at 25 000 rpm and 4˚C. The supernatant was discarded and the VLPs were resuspended in 400 µl sterile PBS to replace the STE buffer with a physiological buffer in preparation for immunization of mice. 50 µl was removed and added to 250 µl PBS for final HBsAg quantitation. The result was 8200 IU/ml, which according to the conversion described by Deguchi et al. (2004), where 0.05 IU/ml HBsAg approximates to 0.2 ng/ml HBsAg, equates to approximately 33 µg/ml HBsAg [22]. Immediately prior to formulation with adjuvant, the VLP-p*17 in PBS was filtered using a 0.22 µm Millex-GP Syringe Filter Unit (Merck Millipore, Ltd., Ireland). For each single vaccine dose, 5 µl of Alum (Vaxine Pty. Ltd., Adelaide, Australia) was added to 15 µl VLP-p*17 and 15 µl sterile PBS for a total volume of 35 µl per dose per mouse.

2.8. Immunization of mice

All protocols were approved by Griffith University Animal Ethics Committee and ratified by the University of Southern Queensland Animal Ethics Committee, in compliance with Australian National Health and Medical Research guidelines. The study involved four groups of 4–6-week-old BALB/c mice (Animal Resource Centre Australia) (n = 5 per

group). Mice were acclimatized for 7 days prior to immunization. On day 0, one group of mice was inoculated i.m. in the hind thigh with 0.5 μg VLP-p*17 formulated with Alum in sterile PBS as described above. The dosage of 0.5 μg for VLP-p*17 was chosen in keeping with the 0.25- 0.5 μg doses used in animal trials of other VLP-based vaccines [11, 15, 20]. A second group was inoculated with 5 μl of Alum in 30 μl sterile PBS as an adjuvant control, and the third group was inoculated with 35 μl sterile PBS alone as a negative control. As a positive control to validate ELISA results, a fourth group was inoculated with 25 μg p*17-DT formulated with 25 μl of Alum. The dosage of 25 μg for p*17-DT was chosen in keeping with that used by Nordstrom et al., (2017). Each mouse received a booster dose, of the same formulation as for the original inoculation, on days 21 and 28. Blood samples (maximum 200 μl) were collected from the submandibular vein of each mouse one day prior to boosting and one week after the final boost. The blood was allowed to clot at 4˚C for at least 4 hr and serum was separated after centrifugation at 1 000 g for 10 min. Sera were stored at -80 ˚C.

2.9. ELISA

Standard ELISA was used to determine murine p*17-specific IgG antibody titres. 96-well microtiter plates (Nunc, Denmark) were coated with 5 μl (10 μg/ml) p*17 peptide (ChinaPeptides) in 10 ml carbonate buffer (pH 9.6) (100 μl/well) for 90 min at 37˚C. Plates were blocked overnight at 4˚C with 150 μl/well 5% skim milk in PBS with 0.05% Tween 20, and then washed twice with PBS/0.05% Tween 20. Murine serum samples were diluted 1/100 in 0.5% skim milk then added in duplicate across the plate continued with doubling dilutions down the plate (100 μl/well). Plates were incubated for 90 min at 37˚C and then washed four times with PBS/0.05% Tween 20. Goat Anti-Mouse IgG (H+L)-HRP conjugate (BIO-RAD, Australia), diluted 1/3000 in 0.5% skim milk was added (100 μl/well) and the plates were once again incubated for 90 min at 37˚C and then washed four times with PBS/0.05% Tween 20. TMB substrate solution (Thermo Scientific) was prepared according to the manufacturer's instructions and 100 μl was added to each well. Plates were incubated at room temperature in the dark for 10 mins, after which time the reactions were stopped with 100 μl of 2M sulfuric acid and the absorbance for each plate was read at 450 nm using a Varioskan LUX Multimode Microplate Reader (ThermoFisher) with SkanIt Software 6.0.2.3 for Microplate Readers. Titers were determined using the mean absorbance of the negative control group (PBS alone) plus three standard deviations as the cut-off.

3. Results

*3.1. Secretion competence of cell culture-derived VLP-p*17*

The molecular weight (MW) of VLP-p*17 (HBsAg-S + p*17) was calculated at 29.198 kDa (Bioinformatics.org). The presence of bands at approximately 29 kDa after SDS-PAGE of the post-transfection cell culture supernatant suggested that the recombinant VLPs (VLP-p*17) were secretion competent (Figure 2). This was confirmed by the results of the HBsAg assay shown in Table 1. The mean HBsAg concentration of the

crude post-transfection supernatant samples was 24.2 IU/ml. According to the conversion described by Deguchi et al. (2004), this result approximates 96.8 ng/ml HBsAg [22].

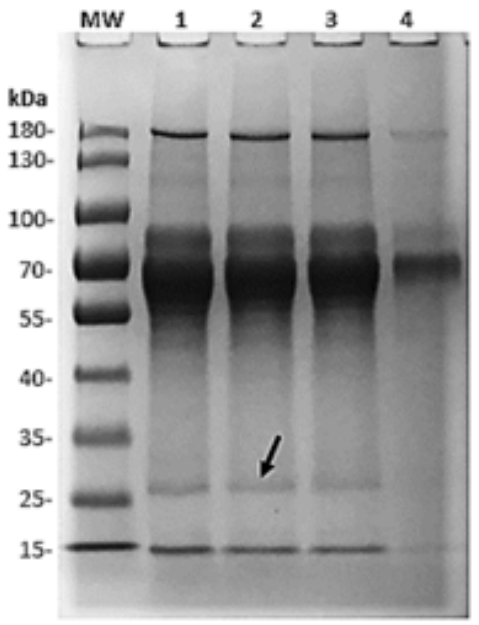

Figure 2. SDS-PAGE of crude supernatant from three cell culture flasks of HEK293t cells (lanes 1,2 and 3) six days post transfection showing a band at approximately 29 kDa (arrow). MW shows a pre-stained 10-180 kDa protein marker (Servicebio®) and lane 4 contained crude pre-transfection cell culture supernatant as a negative control. Imperial™ Protein Stain (Thermo Scientific).

Table 1. HBsAg quantitation in three HEK293t cell culture supernatant samples post transfection with VLP-p*17 pcDNA3.1(+) and post 20% sucrose gradient ultracentrifugation

Sample		HBsAg IU/ml	HBsAg ng/ml[1]	VLP/ml[1]
Crude HEK293t cell culture supernatant post transfection	1	20.26	81.04	1.6×10^{10}
	2	29.93	119.7	2.4×10^{10}
	3	22.41	89.6	1.7×10^{10}
Resuspended pelleted VLPs post ultracentrifugation through 20% sucrose	1	553	2 212	4.4×10^{11}
	2	1 425	5 701	1.1×10^{12}
	3	628	2 515	5.0×10^{11}

[1] 0.05 IU/ml HBsAg ~ 0.2 ng/ml HBsAg and 0.1 ng/ml HBsAg ~ 2×10^7 VLPs [22].

*3.2. Characterization of cell culture-derived VLP-p*17*

To demonstrate particle formation, crude supernatant was concentrated and partially purified by overnight ultra-centrifugation through a 20% sucrose cushion as described above. The pelleted contents were resuspended in 200 µl STE buffer and analysed for HBsAg (Table 1) and visualized using transmission electron microscopy. The TEM

images (Figure 3) confirmed the presence of homologous particles around 22 nm in diameter with visible viral capsomeres. These images are consistent with our previously published TEM images of recombinant HBsAg VLPs [15]. The mean HBsAg concentration of the concentrated and partially purified samples was 868.7 IU/ml, which converts to approximately 3 475 ng/ml. According to Deguchi et al., (2004), 0.1ng/ml HBsAg approximately equals 2 x 10^7 VLPs. Using this calculation, the mean concentration of recombinant HBsAg VLPs in the concentrated and partially purified samples was approximately 6.9 x 10^{11}/ml [22].

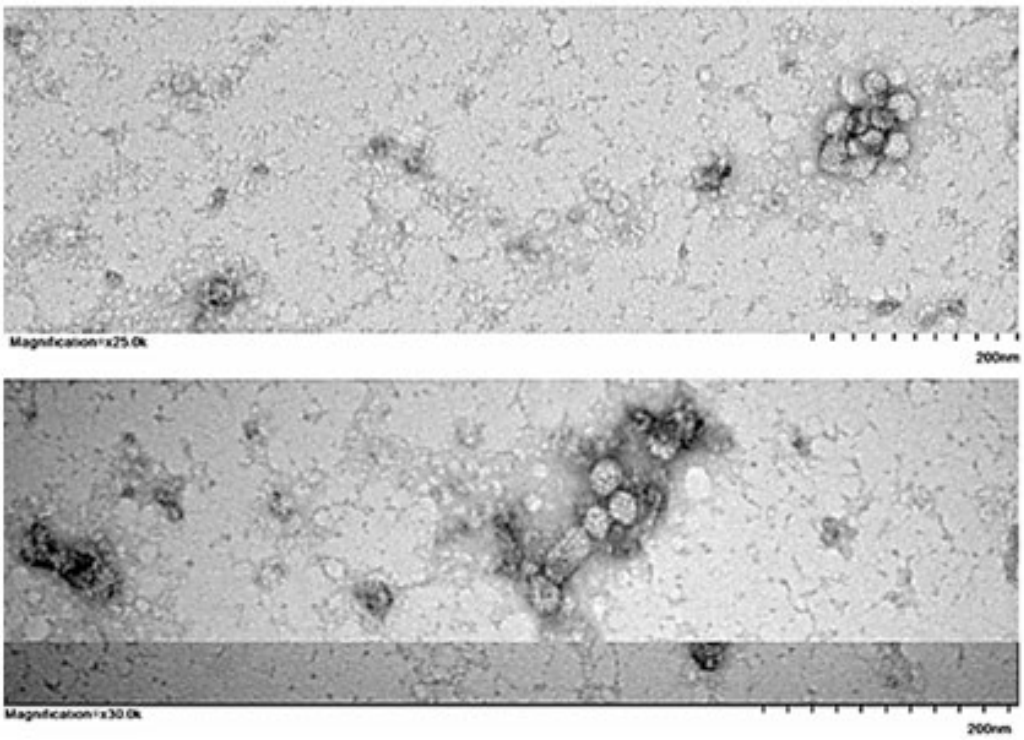

Figure 3. Transmission electron micrograph showing clusters of virus-like particles with visible capsomeres. Using the scale provided, each particle appears to be approximately 22 nm in diameter. Image by Kathryn Green, CMM.

*3.3 Detection of p*17 expression by Western Blot*

Expression of p*17 in the recombinant VLPs was confirmed by Western blot analysis of concentrated and partially purified post-transfection cell culture supernatant using sera from mice previously immunised with p*17-DT/Alum. The anti-p*17-DT antibodies recognized VLP-p*17, confirming the expression of p*17 in the recombinant VLPs (Figure 4). Wild type HBsAg-S VLPs comprise a mixture of non-glycosylated (P24) and glycosylated (GP27) HBsAg monomers [23]. This mix of glycosylated and non-glycosylated forms is reflected in the presence of bands at 31 kDa and 29 kDa. The band at 17 kDa most likely indicates the presence of truncated or degraded monomers that still express p*17.

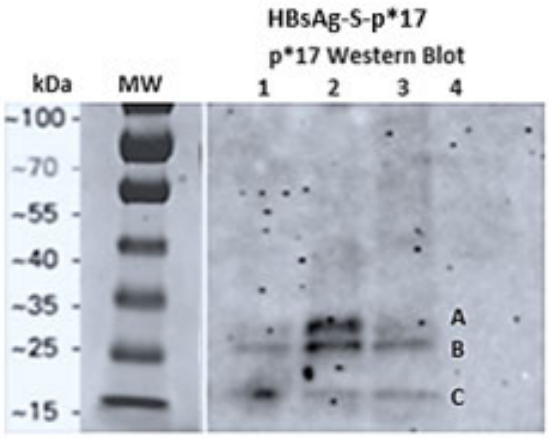

Figure 4. Western blot utilising anti-p*17-DT sera. The panel on the left illustrates the migration of the MW marker (Servicebio®) during SDS-PAGE for comparison with the Western Blot on the right, which illustrates the presence of glycosylated (A) and non-glycosylated (B) HBsAg-S-P*17 monomers, and truncated or degraded monomers (C), in resuspended pelleted VLPs post ultracentrifugation through 20% sucrose cushion (diluted 1:5) in lanes 1, 2 and 3. The darker bands in lane 2, are consistent with the significantly higher concentration of VLP measured in sample 2 as shown in Table 1. Lane 4 contained HBsAg (MyBioSource) as a control for non-specific binding of anti-p*17-DT to the HBsAg-S component of VLP-p*17.

3.4. ELISA

p*17-specific IgG was demonstrated in the sera of all mice immunised with VLP-p*17 or p*17-DT formulated with Alum in sterile PBS and in no mice inoculated with Alum in PBS or with PBS only. While the p*17-specific IgG titers were low (range 2×10^2 - 8×10^2) after the initial inoculation with 0.5 μg VLP-p*17, they increased at least 32-fold after the first booster dose, to greater than 1.28×10^4 (range 1.28×10^4 – 8×10^4) after the second booster dose (Figure 5).

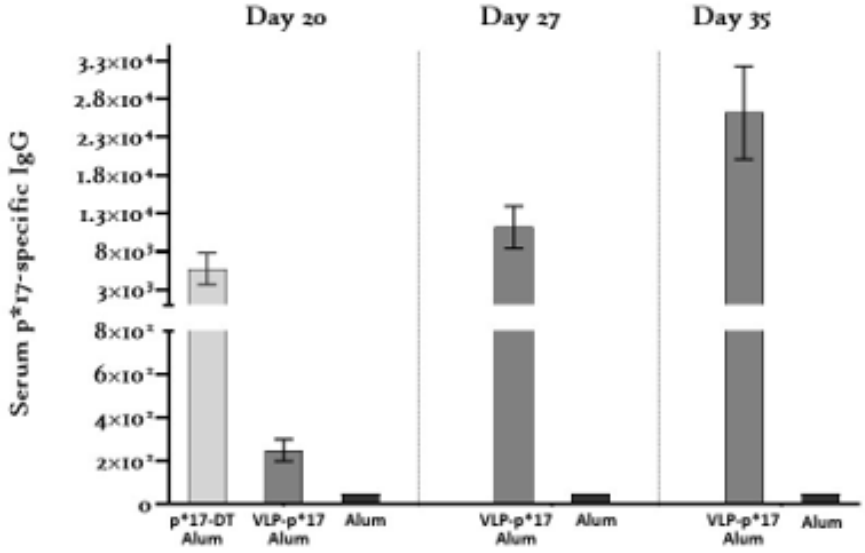

Figure 5. p*17-specific IgG titres in sera collected on days 20, 27 and 35 from mice immunised with VLP-p*17 formulated with Alum in sterile PBS or with Alum only in sterile PBS or with p*17-DT (positive control).

4. Discussion

In this study, we designed and produced a novel Strep A vaccine candidate utilising a recombinant Hepatitis B small envelope protein (HBsAg-S) virus like particle (VLP) as the delivery platform for the cryptic highly conserved and highly immunogenic Strep A M protein epitope, p*17. This putative vaccine, VLP-p*17, induced production of measurable p*17-specific IgG in the sera of mice after a single 0.5 μg dose formulated with the adjuvant Alum (mean titre 3.6 x 10^2). The antibody titres rose approximately 36-fold (mean titre 1.3 x 10^4) after a second 0.5 μg dose and then doubled again (mean titre 3.3 x 10^4) after a third 0.5 μg dose(Figure 5). The data presented in Figure 5 for the positive control group, immunised with 25 μg p*17-DT formulated with 25 μl of Alum, correlates with the high p*17-specific titres demonstrated by Nordström et al., (2017). This group, with the higher inoculum dose, was included in the study only as a control to validate ELISA results in the event that mice immunised with VLP-p*17 failed to produce measurable titres of p*17-specific IgG, which was not the case. However, it was interesting to note that while there was a 50-fold difference in the antigen dose, the difference in the serum p*17-specifi IgG tires between the test group (mean 2.5 x 10^2) and the positive control group (mean 6.4 x 10^3) was only 25-fold. This could suggest enhanced immunogenicity of VLP-p*17 over p*17-DT although a dose-matched trial would be required to demonstrate this conclusively.

Culture-derived VLPs are naturally immunogenic due to their morphological similarity to the parent virus, which extends to both the lipid and protein content of the highly

repetitive viral envelope structure. *In vivo*, because of their size and particulate nature, culture-derived HBsAg VLPs are readily taken up by DCs and are processed via the same immune pathways as is the parent virus [24]. In addition to their value as antiviral vaccines, VLPs are highly stable molecules that have demonstrated tolerance to a range of bioconjugation, labelling, and polymerisation techniques making them suitable delivery platforms for a wide spectrum of small molecules including bacterial epitopes [14]. Furthermore, VLPs can be expressed in, and secreted by, a range of protein expression systems, making them relatively easy and inexpensive to produce [25]. Secretion competence of the recombinant VLP-p*17 expressed in HEK293t cells was confirmed by HBsAg quantitation of the crude and partially purified cell-culture supernatant (Table 1). To facilitate surface expression and ensure optimal immunogenicity of the Strep A epitope, p*17 was expressed within the immunodominant "a" determinant region of HBsAg-S. The inherent immunogenicity of HBsAg-S stems from the spontaneous assembly of individual monomers into highly organised 22nm particles [23]. It was therefore important to determine whether insertion of the foreign peptide sequence, LRRDLDASREAKNQVERALE, into the "a" determinant of the HBsAg-S, enabled particle formation. The TEM images (Figure 3) show homologous particles approximately 22nm in diameter with visible capsomeres confirming that insertion of the 20 mer foreign peptide into the "a" determinant of HBsAg-S did not prevent particle formation. This approach differs from that used in the development of the HBsAg-S VLP-based anti-malarial vaccines, which comprise a peptide from the circumsporozoite protein (CSP) of *Plasmodium falciparum* fused to the N-terminus of HBsAg-S. While this configuration also ensures surface expression of the target antigen, it interferes with particle formation. In the RTS,S anti-malaria vaccine, the interference was minimised by incorporation of wildtype HBsAg-S such that each VLP contained a somewhat random mix of both wildtype HBsAg-S and CSP-HBsAg. This diluted the expression of CSP and thereby reduced the CSP-specific antibody response in vaccinated mice [11]. Rather than fusing or conjugating a Strep A epitope to the N-terminus of HBsAg-S, this project involved the use of recombinant DNA technology to clone the bacterial epitope into a more central, yet still surface-expressed region of the HBsAg-S without compromising particle formation or immunogenicity.

Immunization of mice with 0.5 μg VLP-p*17 formulated with Alum induced measurable titers of p*17-specific IgG after a single dose (mean 3.6×10^2) that increased to greater than 1.28×10^4 (mean 3.3×10^4) after two 0.5 μg booster doses. Higher initial doses of VLP-p*17, such as those used for the p*17-DT positive control group, could potentially induce sufficiently high IgG titres without the need for boosting.

This putative vaccine, VLP-p* 17, has features that make it an attractive construct for a commercially produced vaccine against Strep A: it is a single recombinant protein that spontaneously assembles into stable particles that are of optimal size for recognition and uptake by human immune cells; it presents repetitive displays of the highly immunogenic Strep A epitope, p*17, on the surface of homologous nanoparticles; it can be easily and consistently produced in yeast or mammalian cells; and it is based on the

HBsAg-S VLP, which is already licensed as a human vaccine with associated large-scale quality-controlled manufacturing processes already in place.
While several VLP-based anti-viral and anti-malarial vaccines have been approved for human use, to our knowledge, no VLP-based anti-bacterial vaccine has progressed to clinical trial, further highlighting the novelty of VLP-p*17 [12].

5. Conclusion

In conclusion, we have developed a recombinant HBsAg-S VLP expressing the minimal Strep A M protein epitope, p*17. This construct, VLP-p*17, combines the natural immunogenicity of VLPs with a cryptic highly conserved Strep A epitope that has demonstrated enhanced immunogenicity and efficacy when conjugated with DT. VLP-p*17 induced production of consistently high titers of p*17-specific IgG after three 0.5 μg doses. Furthermore, the parent VLP utilised in this construct, HBsAg-S, is already approved for use in human vaccines, which may remove some obstacles to future human trials of VLP-p*17. A limitation of this study is the partial purifification only of VLP-p*17. Further purification could have resulted in significant loss of VLPs and was not essential for this proof of principle study. However, we acknowledge that impurites in the vaccine formulation could have had an adjuvant effect, or a supressive effect, on the humoral response to immunisation and further studies with more highly purified VLPs should follow. Other limitations of the study are the absence of functional assays of the murine serum p*17-specific IgG and a dose-matched study comparing the antibody responses in mice immunised with VLP-p*17 and mice immunised with p*17-DT, these will be conducted as part of a future study.

Author Contributions

Conceptualization, L.D., M.P., M.G. and M.K.; methodology, L.D., T.A., V.O., M.P. and M.K.; validation, L.D., T.A., M.P. and M.K.; investigation, L.D., T.A. and V.O.; resources, M.K., M.P. and M.G.; writing—original draft preparation, L.D.; writing—review and editing, M.K., M.P. and M.G.; visualization, L.D. T.A and M.K.; supervision, M.K.; project administration, M.K., M.P. and M.G.; All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and was approved by the Human Research Ethics Committee of the UNIVERSITY OF SOUTHERN QUEENSLAND (GLY/07/21/AEC) and the Animal Research Ethics Committee of GRIFFITH UNIVERSITY.

Informed Consent Statement: Not applicable. This study involved the use of commercially available human cell lines only.

Data Availability Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Carapetis, J.R., et al., *The global burden of group A streptococcal diseases.* Lancet Infectious Diseases, 2005. **5**(11): p. 685-694.
2. Ralph, A.P. and J.R. Carapetis, *Group a streptococcal diseases and their global burden.* Current topics in microbiology and immunology, 2013. **368**: p. 1-27.
3. WHO. *Rheumatic Heart Disease*. 2020 August 24 2021]; Available from: https://www.who.int/news-room/fact-sheets/detail/rheumatic-heart-disease.
4. Watkins, D.A., et al., *Global, Regional, and National Burden of Rheumatic Heart Disease, 1990–2015.* New England Journal of Medicine, 2017. **377**(8): p. 713-722.
5. Norton, R., et al., *Invasive group A streptococcal disease in North Queensland (1996 - 2001).* The Indian journal of medical research, 2004. **119 Suppl**: p. 148.
6. Dooley, L.M., et al., *Rheumatic heart disease: A review of the current status of global research activity.* Autoimmunity Reviews, 2021. **20**(2): p. 102740.
7. Fischetti, V.A., *Surface Proteins on Gram-Positive Bacteria.* Microbiol Spectr, 2019. **7**(4).
8. Pruksakorn, S. and B. Currie, *Towards a vaccine for rheumatic fever: Identification of a conserved target epitope on M protein.* Lancet, 1994. **344**(8923): p. 639.
9. Nordstrom, T., et al., *Enhancing vaccine efficacy by engineering a complex synthetic peptide to become a super immunogen.* J Immunol, 2017. **199**(8): p. 2794-2802.
10. Ludwig, C. and R. Wagner, *Virus-like particles-universal molecular toolboxes.* Curr Opin Biotechnol, 2007. **18**(6): p. 537-45.
11. Collins, K.A., et al., *Enhancing protective immunity to malaria with a highly immunogenic virus-like particle vaccine.* Scientific Reports, 2017. **7**(1): p. 46621.
12. Nooraei, S., et al., *Virus-like particles: preparation, immunogenicity and their roles as nanovaccines and drug nanocarriers.* Journal of Nanobiotechnology, 2021. **19**(1): p. 59.
13. Elhanan, E., et al., *A randomized, controlled clinical trial to evaluate the immunogenicity of a PreS/S hepatitis B vaccine Sci-B-Vac™, as compared to Engerix B(®), among vaccine naïve and vaccine non-responder dialysis patients.* Clin Exp Nephrol, 2018. **22**(1): p. 151-158.
14. Mulder, A.M., et al., *Toolbox for Non-Intrusive Structural and Functional Analysis of Recombinant VLP Based Vaccines: A Case Study with Hepatitis B Vaccine.* PLOS ONE, 2012. **7**(4): p. e33235.
15. Kotiw, M., et al., *Immunological response to parenteral vaccination with recombinant hepatitis B virus surface antigen virus-like particles expressing Helicobacter pylori KatA epitopes in a murine H. pylori challenge model.* Clin Vaccine Immunol, 2012. **19**(2): p. 268-76.
16. Moffat, J.M., et al., *Hepatitis B virus-like particles access major histocompatibility class I and II antigen presentation pathways in primary dendritic cells.* Vaccine, 2013. **31**(18): p. 2310-6.
17. Gavilanes, F., J.M. Gonzalez-Ros, and D.L. Peterson, *Structure of hepatitis B surface antigen. Characterization of the lipid components and their association with the viral proteins.* J Biol Chem, 1982. **257**(13): p. 7770-7.

18. Rezaee, R., et al., *Impacts of the G145R Mutation on the Structure and Immunogenic Activity of the Hepatitis B Surface Antigen: A Computational Analysis*. Hepat Mon, 2016. **16**(7): p. e39097.
19. Bhatnagar, P.K., et al., *Immune response to synthetic peptide analogues of hepatitis B surface antigen specific for the a determinant*. Proc Natl Acad Sci U S A, 1982. **79**(14): p. 4400-4.
20. Netter, H.J., et al., *Antigenicity and immunogenicity of novel chimeric hepatitis B surface antigen particles with exposed hepatitis C virus epitopes*. Journal of virology, 2001. **75**(5): p. 2130-2141.
21. Alves Vianna, C.O., et al., *Evaluation of murine monoclonal antibodies targeting different epitopes of the hepatitis B virus surface antigen by using immunological as well as molecular biology and biochemical approaches*. J Immunol Methods, 2006. **313**(1-2): p. 38-47.
22. Deguchi, M., et al., *Quantitation of hepatitis B surface antigen by an automated chemiluminescent microparticle immunoassay*. Journal of Virological Methods, 2004. **115**(2): p. 217-222.
23. Huovila, A.P., A.M. Eder, and S.D. Fuller, *Hepatitis B surface antigen assembles in a post-ER, pre-Golgi compartment*. J Cell Biol, 1992. **118**(6): p. 1305-20.
24. Al-Barwani, F., et al., *Antigen delivery by virus-like particles for immunotherapeutic vaccination*. Ther Deliv, 2014. **5**(11): p. 1223-40.
25. Schwarz, B. and T. Douglas, *Development of virus-like particles for diagnostic and prophylactic biomedical applications*. Wiley interdisciplinary reviews. Nanomedicine and nanobiotechnology, 2015. **7**(5): p. 722-735.

5.3 Links and implications for further studies

The results of this study confirmed that the molecular design, cell culture, transfection, and semi-purification techniques performed resulted in the production of a recombinant VLP with surface-expressed p*17 that spontaneously formed particles within the range for optimal uptake by immune cells. Furthermore, the results of this study demonstrated that mice immunised with VLP-p*17 produced measurable titres of p*-17 specific IgG after a single 0.5 µg dose. These titres rose to greater than 1.28×10^4 in all immunised mice after a further two 0.5 µg booster doses confirming the antigen-specific immunogenicity of VLP-p*17.

While VLPs are inherently immunogenic, most vaccine formulations include an adjuvant to further enhance the immune response to the target epitope presented by the vaccine. Adjuvants not only boost initial response to the target antigen and minimise the dose of antigen required to stimulate a protective response, they can also help sustain the response and reduce the number of booster doses required (Zhang, Li, Liu, Nandakumar, & Jiang, 2022). In this study,

VLP-p*17 was formulated with the adjuvant Alum (Alhydrogel), a 2% aluminium hydroxide wet gel suspension. While Alum is the most widely used adjuvant in human and animal vaccines, other types of adjuvants have recently been approved for use in human vaccines. These newer adjuvants vary in their modes of action, with subsequent differences in the nature of the immune responses they induce (Zhang et al., 2022).

Chapter 6 (Paper 3) describes a comparative immunogenicity trial of VLP-p*17 formulated with Alum and two more recently developed adjuvants, Vet-SAP®, a saponin-based adjuvant, and Advax™, a delta inulin-based adjuvant.

CHAPTER 6: PAPER 3 – Immunogenicity of a chimeric recombinant virus-like particle as a putative vaccine against S*treptococcus pyogenes*

6.1 Introductory paragraph

This study followed directly from the previous study and further investigated the immunogenicity of p*17 expressed on a chimeric recombinant virus-like particle (VLP-p*17). This study also investigated the effects of two novel adjuvants on the immunogenicity of VLP-p*17 by comparing the serum p*17-specifc IgG titres induced by immunisation of mice with VLP-p*17 formulated with a traditional aluminium salt-based adjuvant, with titres induced in mice immunised with VLP-p*17 formulated with the more recently developed adjuvants, Advax™ and Vet-SAP®. The nature of the immune response triggered by the different formulations was further investigated through measurement of serum p*17-specific IgG1 and IgG2a in immunised mice.

6.2 Paper 3

Dooley, L, Ahmad, T, Ozberk, V, Pandey, M, Good, M & Kotiw, M 2022, 'Immunogenicity of a chimeric recombinant virus-like particle as a putative vaccine against S*treptococcus pyogenes',* (to be submitted for publication to an appropriate journal).

Research Article

Immunogenicity of a chimeric recombinant virus-like particle as a putative vaccine against *Streptococcus pyogenes*

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Abstract

Streptococcus pyogenes (Strep A) causes a wider spectrum of human disease than any other bacterium with a disproportionate burden of disease carried by socioeconomically disadvantaged populations. A vaccine that is effective against the multiple strains of Strep A endemic in these populations is considered the most efficient means of delivering prophylaxis against the bacterium. A candidate combinatorial synthetic peptide vaccine, based on the Strep A M protein-derived epitope, p*17, has previously demonstrated high levels of immunogenicity and prophylactic efficacy in a mouse Strep A challenge model. Following these promising results, this study was undertaken to investigate the potential benefits of delivering the p*17 epitope in a chimeric virus-like particle (VLP). The VLP used in this study consists of around 100 monomers of recombinant Hepatitis B small envelope protein (HBsAg-S) with each expressing the p*17 peptide within the surface-expressed, immunodominant "a" determinant region of HBsAg-S. HBsAg-S monomers spontaneously self-polymerise to form icosahedral nanoparticles approximately 22 nm in diameter, which is within the optimal size rage for uptake by dendritic and B cells. Because the recombinant HBsAg-S VLP resembles the infectious hepatitis B virion in size and structure but contains no genetic material, it has an intrinsic advantage of being readily recognised and responded to by the human immune system yet is non-infectious. In this study, we have

demonstrated induction of high titres of p*17-specific IgG antibodies in mice, following two low doses (0.5 µg/dose) of the VLP-p*17 vaccine. These titres were comparable with reported high titres generated by a single dose (25 µg/dose) of a p*17-Diphtheria toxoid conjugate vaccine (p*17-DT). Furthermore, we have also shown that inclusion of the relatively novel saponin-based adjuvant Vet-SAP® in the vaccine formulation induced a significant rise in antibody titre compared to the commonly used standard adjuvant, alum (aluminium hydroxide). The VLP-p*17/Vet-SAP® vaccine formulation was also shown to induce production of p*17-specific IgG1 and IgG2a, with a significant IgG1 bias. This study has highlighted significant advantages in using the HBsAg-S VLP as a delivery vehicle for p*17 in a putative Strep A vaccine. These include generation of high target antigen-specific antibody titres at low dose, molecular stability and reproducibility due to the recombinant nature of the VLP, and its proven track record as a safe and effective vaccine against hepatitis B virus infection: the latter being an attribute that may be beneficial in progressing the putative vaccine to future clinical trials.

1. Introduction

Streptococcus pyogenes (Strep A) is responsible for a wider spectrum of human disease than any other bacterium (Cunningham, 2000). Strep A infections range from mild superficial infections of the throat and skin to severe invasive Streptococcal diseases (ISD) including neonatal and maternal sepsis and necrotising fasciitis, and toxigenic diseases such as scarlet fever and streptococcal toxic shock syndrome (STSS). Superficial Strep A infections are also associated with autoimmune sequelae, the most serious of which are acute rheumatic fever (ARF) and rheumatic heart disease (RHD) (Cunningham, 2000; Stevens & Bryant, 2016). While Strep A is universally susceptible to penicillin, it is responsible for an estimated 517 000 deaths worldwide each year, with a disproportionate share of the disease burden carried by socioeconomically disadvantaged populations (Good, 2020; Ralph & Carapetis, 2013). Effective vaccination is widely regarded as the most reliable and cost-effective means of reducing the Strep A disease burden in at-risk populations (WHO, 2018). However, despite many decades of research globally, no Strep A vaccine offering sufficiently broad protection from the diverse strains of Strep A endemic in these populations is currently available (Dooley,

Ahmad, Pandey, Good, & Kotiw, 2021). Australian researchers have developed several putative Strep A vaccine candidates that aim to address the key challenges of the low inherent immunogenicity of minimal bacterial epitopes, the strain heterogeneity of Strep A, and assessing vaccine efficacy and safety in murine models of Strep A disease (reviewed in Dooley, et al., 2021).

The most promising Strep A vaccine candidate to date, targets the cryptic and highly conserved epitope, p*17, and utilises conjugation to Diphtheria toxoid (DT) as an antigen delivery system. Outcomes of a single dose immunisation of the p*17-DT conjugate formulated with alum hydroxide adjuvant revealed that p*17-DT induced high levels of antibodies to both p*17 and its parent peptide, p145, in vaccinated mice. Furthermore, a single dose immunisation of p*17-DT resulted in a 100-fold reduction in skin levels of the Strep A bacterium and >10 000-fold reduction in blood levels of the bacterium in mice challenged with a live and virulent Strep A inoculum (Nordstrom et al., 2017). Following these promising results, this study was undertaken to investigate the potential benefits of delivering the epitope, p*17, as a chimeric virus-like particle (VLP).

The hepatitis B small envelope protein (HBsAg-S) VLP utilised in this study is a non-infectious cell culture-derived protein that spontaneously polymerises to form 22 nm icosahedral particles. When expressed in mammalian cells, recombinant HBsAg-S VLPs are secreted from the cultured cell and comprise HBsAg-S proteins and cellular lipids (Grgacic & Anderson, 2006). As such, the VLPs mimic the live virus in size and form and are readily recognised and processed by antigen presenting cells such as dendritic cells and B cells. As illustrated in Figure 1, VLPs trigger MHC I and MHC II antigen processing pathways thereby inducing both humoral and cell-mediated responses to target antigens (Moffat, Cheong, Villadangos, Mintern, & Netter, 2013).

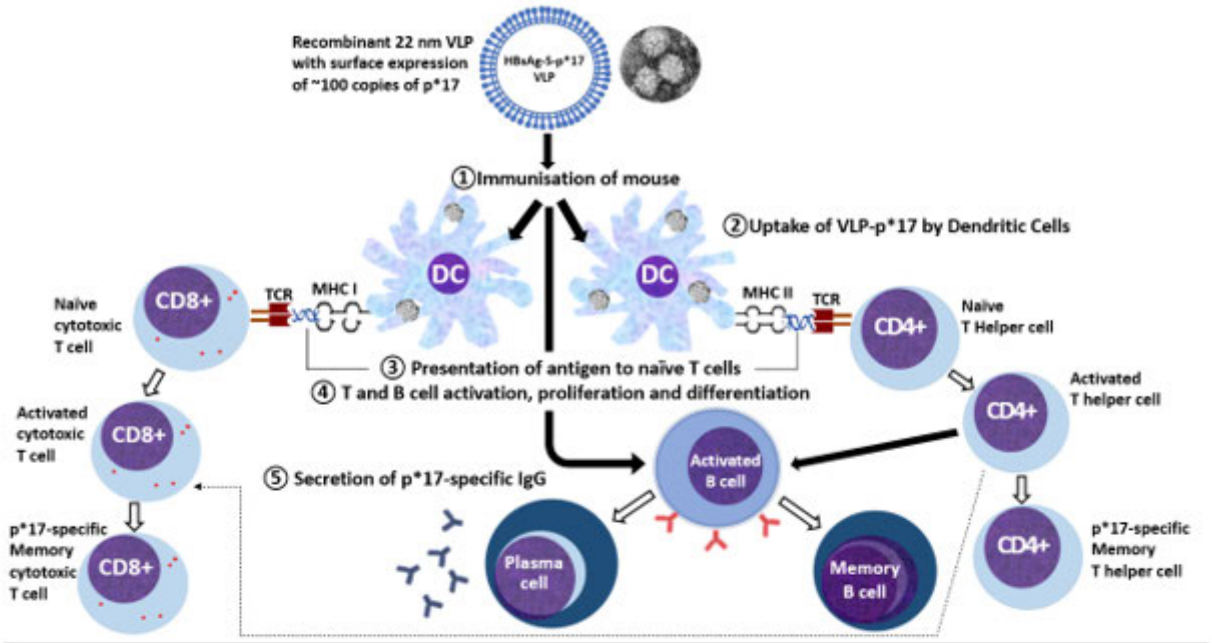

*Figure 1. Immune response to recombinant HBsAg-S-p*17 VLP. VLPs are readily taken up by dendritic cells and presented to CD8+ T cells and CD4+ T cells via MHC I and MHC II antigen presentation pathways respectively. Subsequent activation, proliferation and differentiation of T cells and B cells ultimately leads to generation of p*17-specific activated and memory cytotoxic T cells, memory B cells, and plasma cells secreting p*17-specific IgG.*

Due to their particulate nature, molecular stability, and expression of high density and repetitive displays of epitopes, VLPs are ideal antigen delivery platforms and are the basis of licensed anti-viral and anti-malarial vaccines (Nooraei et al., 2021). Using recombinant DNA procedures, we expressed p*17 within the surface expressed immunodominant "a" determinant region of HBsAg-S such that the resultant chimeric VLPs have the potential to surface-express around 100 copies of p*17 per nanoparticle (Mangold & Streeck, 1993). HBsAg-S VLPs have been used in humans for decades as a safe and effective vaccine against hepatitis B virus, an attribute that may be beneficial in progressing the putative vaccine, VLP-p*17, to future clinical trials.

The principal aim of this study was to determine whether the immunogenicity of the HBsAg-S-p*17 VLP was comparable to that of the p*17-DT candidate vaccine described above. Whilst chimeric VLPs have been shown to be highly immunogenic, and to some extent self-adjuvanting, an adjuvant is typically included in the vaccine formulations to augment its efficacy and promote a desired immune response (Pulendran, Arunachalam, & O'Hagan, 2021; Rivera-Hernandez et al., 2013) . Traditionally,

aluminium salts (Alum) have been the adjuvant used with VLPs. However, novel adjuvant formulations aimed at inducing faster, stronger and more prolonged protective immunity, particularly in weak responders, have emerged (Cimica & Galarza, 2017). Hence, a second aim of this study, was to trial two novel adjuvants, Vet-SAP® and Advax™, in comparison with Alum, for their efficacy in optimising antibody responses to p*17 expressed on HBsAg-S VLPs. Finally, p*17-specific IgG1 and I gG2a titres were determined to define which of the three formulations optimised the Th1 response and were therefore likely to induce antigen-specific cell-mediated responses in addition to the humoral responses measured.

2. Materials and methods

2.1. DNA expression vector

The DNA sequence for the recombinant HBsAg-S-p*17 construct (VLP-p*17) was designed to direct expression of p*17 in the "a" determinant region of HBsAg-S. The VLP-p*17 gene was codon optimised for expression in mammalian cells and synthesised and cloned into pcDNA3.1(+) by GenScript (Hong Kong) Limited.

2.2 Adjuvants

The adjuvants, Alum, Vet-SAP® and Advax™, were kindly provided, ready for use, by Vaxine Pty. Ltd., Adelaide, Australia. The adjuvants were stored at 4^{O}C until required and formulated with antigen, (HBsAg-p*17) according to manufacturer's guidelines.

a. Advax™ comprises of delta inulin particles manufactured under current Good Manufacturing Processes (GMP) and optimised for consistency and maximal adjuvant activity (Petrovsky, 2017). Inulin is a storage polysaccharide found in many food plants including chicory, artichoke, onions and garlic (reviewed in (Barclay, Ginic-Markovic, Cooper, & Petrovsky, 2012)). Delta particles are obtained by precipitation of inulin from water at progressively higher temperatures and are insoluble at body temperature (Cooper & Petrovsky, 2011). Delta inulin enhances both Th1 and Th2 responses and Advax has been proven safe and effective in human trials of influenza vaccines, hepatitis B vaccines, and insect sting allergy vaccines (Petrovsky, 2017; Quan et al., 2021).

b. Vet-SAP® is a veterinary vaccine adjuvant comprising a semi-purified saponin fraction derived from the bark of the *Quillaja Saponaria* (QS) Molina tree. Human QS saponin-based vaccine adjuvants, such as QS-21, are affinity purified and formulated with cholesterol and phospholipids to form 40 nm particles (Reimer et al., 2012). QS saponins have been shown to stimulate long lasting humoral and Th1, Th2 and cytotoxic T cell responses to target antigens (Marty-Roix et al., 2016; Reimer et al., 2012; Takahashi et al., 1990). QS-21 is an adjuvant component of the recombinant Herpes zoster vaccine Shingrix® and RTS,S (Mosquirix), the only vaccine approved against the malarial parasite, *Plasmodium falciparum* (Collins, Snaith, Cottingham, Gilbert, & Hill, 2017; Singh, Song, Choi, Lee, & Nahm, 2020).

*2.3 Production of VLP-p*17*

HEK293t cells were grown in Dulbeco's Modified Eagle's Medium-High glucose (DMEM Sigma® Life Science) with 10% Fetal bovine serum (FBS, Australia origin Sigma-Aldrich), 1% L-Glutamine 200 mM (GlutaMAX ™ 100X Gibco), and 2% Penicillin 5000 U/ml-Streptomycin 5 mg/ml (Sigma-Aldrich) in 250 ml CELLSTAR® cell culture flasks with filter caps (Greiner Bio-One, Kremsmünster, Austria). Flasks were incubated at 37°C with 5% CO_2 and cells were passaged on alternate days for 10 days after which time they were estimated to be 70-80% confluent. HEK293t cells were transfected using FuGENE® HD Transfection Reagent (Promega), following the manufacturer's instructions with a 3:1 ratio of FuGENE®HD to DNA. The transfected HE293t cells, were incubated at 37°C with 5% CO_2 for five days with an additional 10 ml of pre-warmed FBS free DMEM added daily for six days. The cell culture supernatant was harvested and analysed for HBsAg concentration using an automated chemiluminescent microparticle assay (Abbott ARCHITECT) by Pathology Queensland, Herston, Brisbane.

The cell culture derived VLP-p*17 was semi-purified and concentrated by ultracentrifugation at 25 000 rpm at 4°C for 16 h through 20% sucrose in STE buffer. The pelleted VLPs were resuspended in 200 µl aliquots of STE buffer and stored at -80°C until required. Spontaneous formation of 22 nm particles was confirmed by transmission electron microscopy of semi-purified VLP-p*17 (Hitachi HT7700 transmission electron microscope at 100 kV) following a standard negative stain procedure at the Centre for Microscopy and Microanalysis (CMM), University of Queensland, St Lucia (Figure 3).

*2.4 Formulation of VLP-p*17 with adjuvant*

Aliquots of VLP-p*17 were retrieved from frozen storage, thawed at room temperature, pooled, and diluted in 60 ml sterile PBS. The VLPs in PBS were pelleted by overnight ultracentrifugation at 25 000 rpm and 4°C. The supernatant was discarded and the VLPs were resuspended in 400 µl sterile PBS. Immediately prior to formulation with adjuvant, the VLP-p*17 in PBS was filtered using a 0.22 µm Millex-GP Syringe Filter Unit (Merck Millipore, Ltd., Ireland).

For each single vaccine dose, 15 µl VLP-p*17 in PBS (0.5 µg VLP-p*17) was added to the volume of adjuvant recommended by the manufacturer (Alum: 5 µl, Advax™: 20 µl, Vet-SAP®: 1 µl). Sterile PBS was added to make a final volume of 35 µl per dose per mouse.

2.5 Immunisation of mice

All protocols were approved by Griffith University Animal Ethics Committee and ratified by the University of Southern Queensland Animal Ethics Committee, in compliance with Australian National Health and Medical Research guidelines.
The study involved eight groups of 4–6-week-old BALB/c mice (Animal Resource Centre Australia) (n = 5 per group). Mice were acclimatised for one week prior to immunisation. The immunisation scheduled is outlined in Figure 2 and proceeded as follows. On day 0, three groups of mice were inoculated i.m. in the hind thigh with 0.5 µg VLP-p*17 formulated with either Alum, Advax™, or Vet-SAP® at the dose recommended by the manufacturer in a total volume of 35 µl as previously described. Three groups were inoculated with either Advax™, Alum or Vet-SAP®, at the dose recommended by the manufacturer, without VLP-p*17, in a total volume of 35 µl. A positive control group was inoculated with 25 µg p*17-DT formulated with 25 µl Alum, and a negative control group was inoculated with 35 µl sterile PBS. Each mouse received a booster dose, of the same formulation as for the original inoculation, on days 21 and 28. Blood (200 µl) was collected from the submandibular vein of each mouse on days 20, 27 and 35 and left to clot at 4°C for at least 4 hr. After centrifugation at 1000 g for 10 min, serum was separated and stored at -80°C.

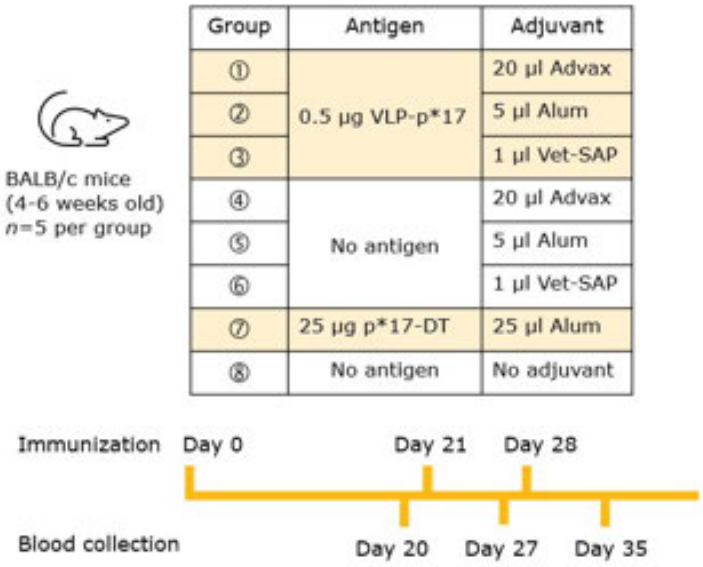

Figure 2. Vaccine formulations and immunization and sample collection schedule

2.6 ELISA

Standard ELISA was used to determine murine p*17-specific IgG antibody titres. 96-well microtiter plates (Nunc, Denmark) were coated with 5 μl p*17 peptide (10 mg/ml) in 10 ml carbonate buffer (pH 9.6) for 90 min at 37°C. Plates were blocked overnight at 4°C with 150 μl/well 5% skim milk containing 0.05% Tween 20, and then washed twice with PBS/0.05% Tween 20. Murine serum samples were diluted 1/100 in 0.5% skim milk then added across the plate continued with doubling dilutions down the plate (100 μl/well). Plates were incubated for 90 min at 37°C and then washed four times with PBS/0.05% Tween 20. Goat Anti-Mouse IgG (H+L)-HRP conjugate (BIO-RAD, Australia), diluted 1/3000 in 0.5% skim milk, was added (100 μl/well) and the plates were once again incubated for 90 min at 37°C and then washed four times with PBS/0.05% Tween 20. TMB substrate solution (Thermo Scientific) was prepared according to the manufacturer's instructions and 100 μl was added to each well. Plates were incubated at room temperature in the dark for 15 mins, after which time the reactions were stopped with 100 μl of 2M sulfuric acid and the absorbance for each plate was read at 450 nm using a Varioskan LUX Multimode Microplate Reader (ThermoFisher) with SkanIt Soft-

ware 6.0.2.3 for Microplate Readers. Titres were determined using the mean absorbance of the negative control group (PBS alone) plus three standard deviations as the cut-off.

ELISA were repeated on the sera from Day 27 and Day 35 bleeds from groups 1, 2 and 3, using initial dilutions of 1/1000 and 1/10000 respectively with doubling dilutions down the plates to determine the final IgG titres.

ELISA for IgG1 and IgG2a were performed only on sera from Day 35 bleeds from groups 1, 2, 3 and 7 with sera from group 8 as a negative control. ELISA for IgG1 were performed as above, substituting Goat Anti-Mouse IgG1-HRP (Invitrogen) as the secondary antibody and 1/10000 as the initial dilution. ELISA for IgG2a were performed as above substituting Goat Anti-Mouse IgG2a-HRP (Invitrogen) as the secondary antibody and 1/200 as the initial dilution.

2.7 Statistical analysis

Statistical analyses of ELISA results for total IgG, IgG1 and IgG2a comparisons following immunisation with VLP-p*17 formulated with Alum, Advax™ or Vet-SAP® were performed using two-way ANOVA with Tukey's Multiple Comparison test. Differences between groups were considered statistically significant at P values of < 0.05. All statistical analyses were performed using GraphPad Prism 9.2.0.332 (GraphPad Software).

3. Results

*3.1 Production of VLP-p*17*

The TEM images (Figure 3) confirmed the presence of numerous homologous particles of around 22 nm in diameter with visible viral capsomeres in the semi-purified VLP-p*17 samples. The size and appearance of the particles is consistent with published images of recombinant and wildtype HBsAg-S VLPs (Kotiw et al., 2012; Netter, Macnaughton, Woo, Tindle, & Gowans, 2001).

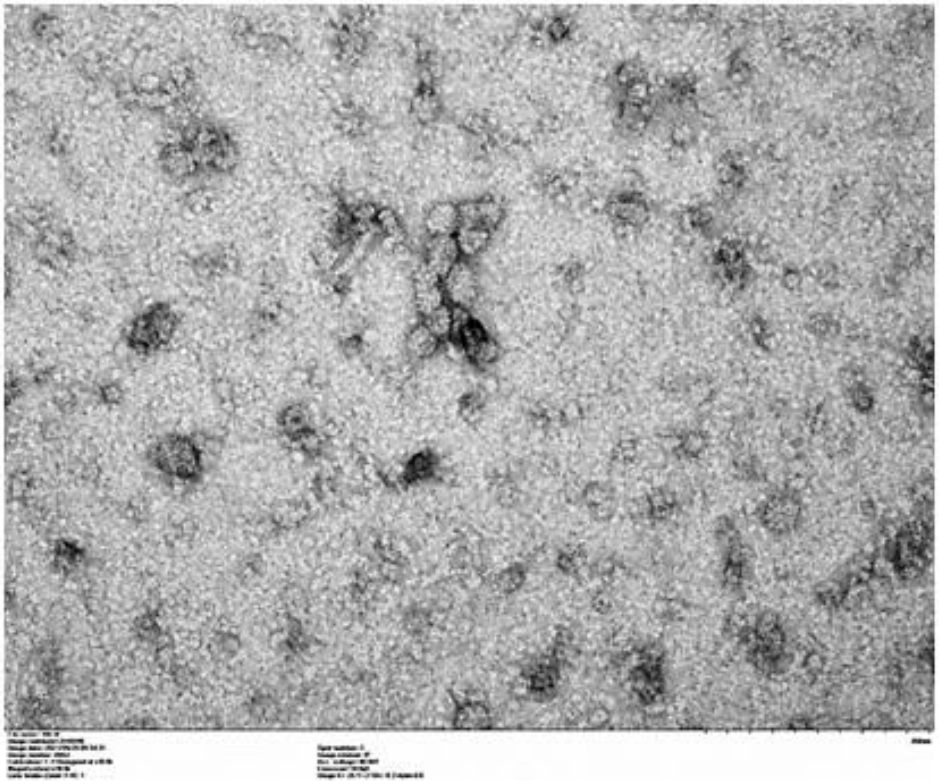

Figure 3. Transmission electron micrograph showing clusters of virus-like particles with visible capsomeres. Using the scale provided, each particle appears to be approximately 22 nm in diameter. Image by Kathryn Green, CMM.

3.2 ELISA

p*17-specific IgG was demonstrated in the sera of all mice immunised with a single dose of VLP-p*17 formulated with adjuvant in PBS, or p*17-DT formulated with Alum in PBS (positive control) and in no mice immunised with adjuvant alone or PBS alone. The serum p*17-specific IgG titres induced 20 days post initial dose were considerably higher in mice inoculated with p*17-DT/Alum (mean 5 760) than in those inoculated with VLP-p*17/Alum (mean 360). Given the fifty-fold difference in inoculum dose (0.5 μg VLP-p*17/0.5 μl Alum, versus 25 μg p*17-DT/25 μl Alum) this sixteen-fold difference in IgG titres was less than might be unexpected.

When comparing IgG titres in the sera of mice immunised with VLP-p*17 formulated with different adjuvants, titres were consistently highest in mice immunised with VLP-p*17 formulated with Vet-SAP® as shown in Figure 4. Although the differences in titres observed in the sera collected on day 20 and day 27 were not statistically significant, in the sera collected on day 35, the differences observed between the VLP-p*17/Advax™

and VLP-p*17/ Vet-SAP® groups (P < 0.005) and VLP-p*17/Alum and VLP-p*17/Vet-SAP® groups (P < 0.005) were statistically significant.

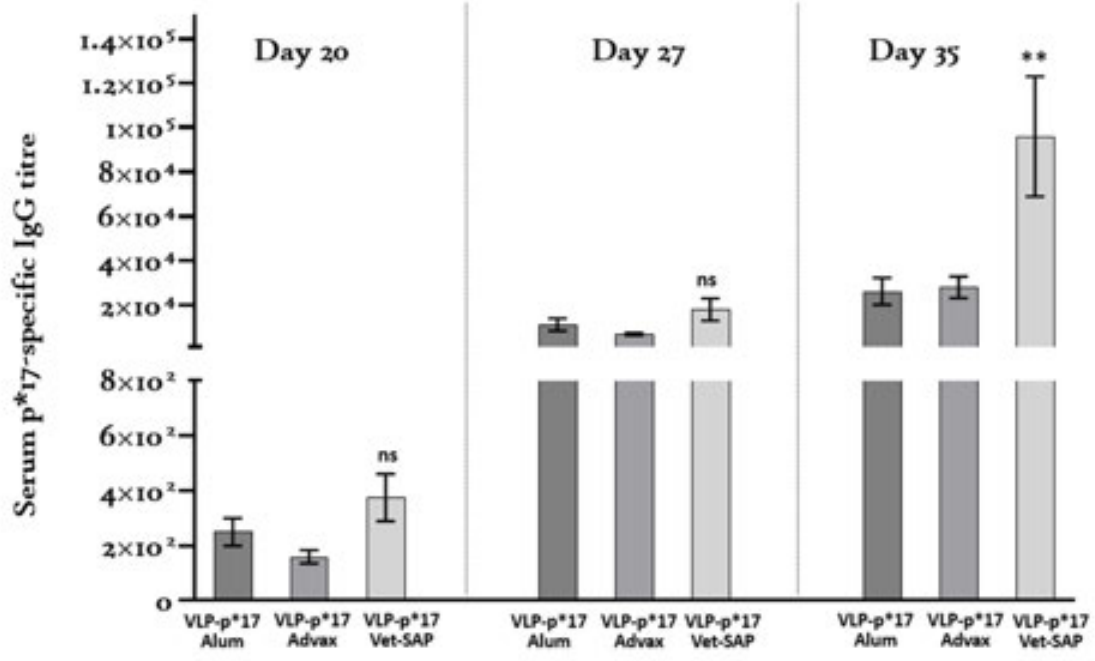

*Figure 4. Serum p*17-specific IgG titres in mice immunised with VLP-p*17/Alum, VLP-p*17/ Advax™ or VLP-p*17/Vet-SAP®. IgG titres were determined by ELISA on sera collected on day 20 (Bleed 1), day 27 (Bleed 2) and day 35 (Bleed 3) post immunisation. Error bars show SEM. Titres for the p*17-DT (positive control) group for bleeds 1, 2 and 3 are not shown but were 5760, > 640 000 and >1 280 000 respectively.*

Figure 5 displays the increases in p*17-specific IgG titres after the first (Day 20 - Day 27) and second (Day 27 – Day 35) booster doses of VLP-p*17 formulated with the three different adjuvants, and the overall increases in p*17-specific IgG titres (Day 20-day 35). The mean increase in p*17-specific IgG titres after the first boost was highest in the VLP-p*17/Alum group (52.8-fold increase) compared with the VLP-P*17/Advax (49.6-fold increase) and VLP-p*17/Vet-SAP® groups (51.6-fold increase), although the differences were not statistically significant. The mean increases after the second boost was greatest in the VLP-p*17/Vet-SAP® group (4.6-fold increase) compared with the VLP-P*17/Advax (4-fold increase) and VLP-p*17/Alum groups (2.175-fold increase), and once again the differences were not statistically significant.

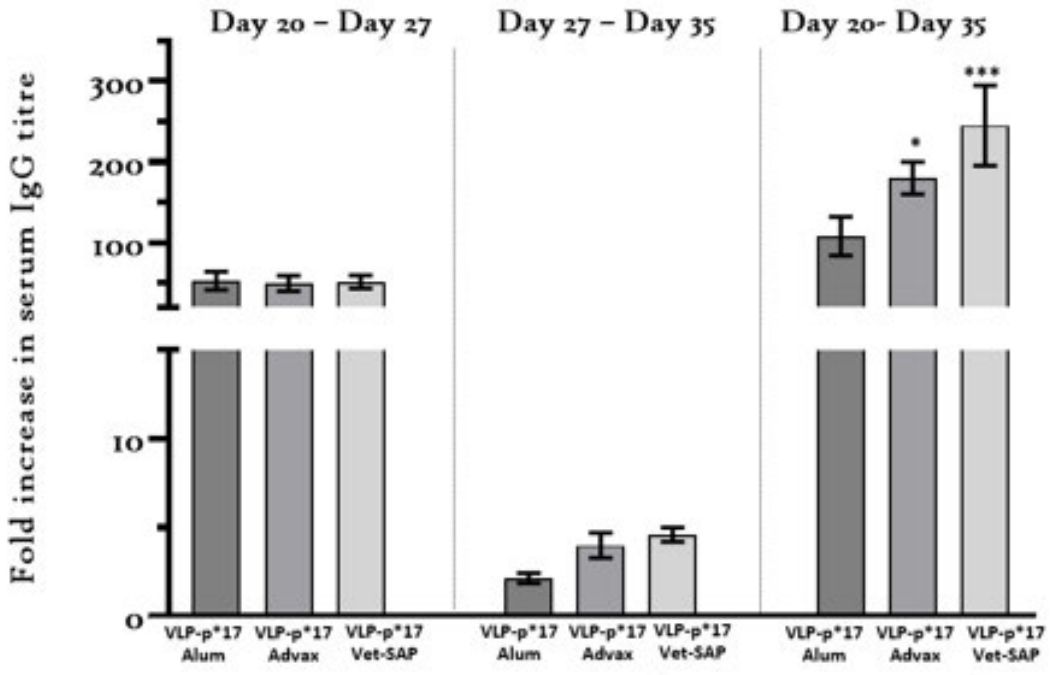

*Figure 5. Fold increase in p*17-specific IgG titres between sera collected on days 20 and 27, between sera collected on days 27 and 35, and between sera collected on days 20 and 35. Error bars show SEM.*

As shown in Figure 6, p*17-specific IgG1 titres were highest in the sera collected on Day 35 from the VLP-p*17/Vet-SAP® group. However, only the difference between the VLP-p*17/Vet-SAP® group and the VLP-p*17/Advax™ group was statistically significant ($P<0.05$). Figure 5 also shows that p*17-specific IgG2a titres were <100 in all mice inoculated with either VLP-p*17/Alum or p*17-DT/Alum. Although the IgG2a titres were higher in the VLP-p*17/Vet-SAP® group than in the VLP-p*17 Advax™ group, the difference was not statistically significant ($P = 0.99$).

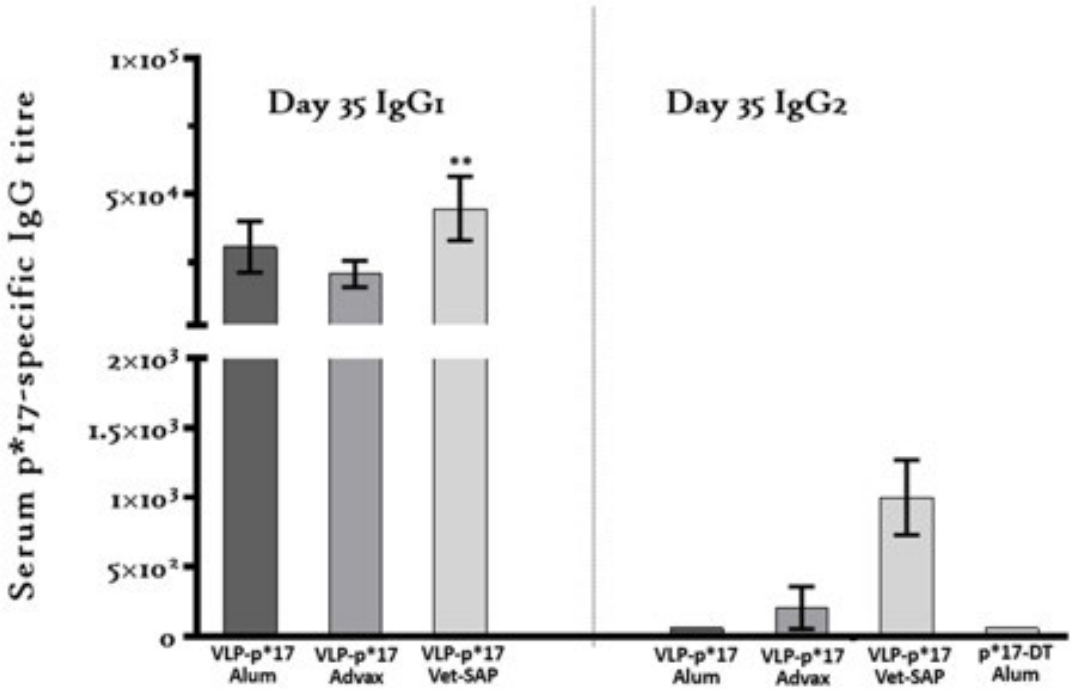

*Figure 6. Serum p*17-specific IgG1 titres in mice immunised with VLP-p*17/Alum, VLP-p*17/Advax™, or VLP-p*17/Vet-SAP®, and serum p*17-specific IgG2a titres in mice immunised with VLP-p*17/Alum, VLP-p*17/Advax™, VLP-p*17/Vet-SAP® or p*17-DT. Titres were determined by ELISA on sera collected on day 35 (Bleed 3) only. Error bars show SEM.*

4. Discussion

In this study we examined the immunogenicity of the conserved Strep A M protein-based epitope, p*17, when delivered by a recombinant chimeric HBsAg-S VLP (VLP-p*17). The induction of high antibody titres and enhanced protective efficacy against dermal and invasive Strep A bacterial challenge in mice immunised with p*17 has been demonstrated previously (Reynolds et al., 2021). The use of chimeric VLPs as antigen delivery platforms offers significant benefits compared to other more traditional vaccine strategies such as: resemblance to the live virion but without replicative potential and associated risk of mutation or reversion to virulence; predictable repetitive antigen display and potential to optimise efficacy via addition of multiple epitopes; and potential utilisation as drug nanocarriers via concurrent use of the internal spaces of the VLP to present additional complimentary immunogenic components to the immunised host. Furthermore, VLPs provide reproducible molecular

stability for large scale vaccine production purposes (Nooraei et al., 2021; Qian et al., 2020; Tariq, Batool, Asif, Ali, & Abbasi, 2022).

In this study we have demonstrated a specific and high titre antibody response in BALB/c mice to the Strep A epitope, p*17, that appears related to the inherent immunogenicity of the HBsAg-S VLP and the surface presentation of large numbers of the highly immunogenic p*17 expressed on the nanoparticle surface. Significant findings include the induction of high p*17-specifc IgG titres with a low dose inoculum (0.5 μg/dose). This study confirmed the very high p*-17 specific IgG titres induced in mice immunised with the p*17-DT conjugate vaccine reported in previous studies, however, these studies used a significantly higher dose inoculum (25-30 ug/dose) (Nordstrom et al., 2017; Reynolds et al., 2021). The relatively high antibody titres (only 16-fold less) induced by the 50-fold lower dose inoculum of VLP-p*17 may be attributed to four intrinsic features of the chimeric nanoparticle including: the optimal size of the nanoparticle; its morphological similarity to the virulent parent virus; its efficient host cell absorption; and the high number of target epitopes presented on the particle surface. As previously mentioned, this inherent immunogenicity of VLPs is further enhanced by inclusion of an adjuvant in the vaccine formulation and the development of novel adjuvants with different modes of action has increased the options available (Cimica & Galarza, 2017; Nooraei et al., 2021; Qian et al., 2020).

To investigate the effects of two novel adjuvants, we compared the p*17 IgG titres produced in mice immunised with VLP-p*17 formulated with either the traditional adjuvant, Alum, or one of the more recently developed adjuvants, Advax™ or Vet-SAP®. Antibody titres were consistently highest in mice immunised with VLP-p*17 formulated with Vet-SAP® as shown in Figure 4. However, only the differences measured in the sera collected on day 35, between the VLP-p*17/Advax™ and VLP-p*17/ Vet-SAP® groups ($P < 0.005$), and VLP-p*17/Alum and VLP-p*17/Vet-SAP® groups ($P < 0.005$) were statistically significant. Regardless of which adjuvant was used, initial titres increased around 50-fold after the first booster dose with the greatest increases seen in the VLP-p*17/Vet-SAP® group. The greatest increase in titres after the second booster dose was an average 3.6-fold increase in the VLP-p*17/Vet-SAP® group. These results demonstrate enhanced total IgG responses, after three 0.5 μg doses, when VLP-p*17

was formulated with Vet-SAP®. Furthermore, only 1 µl of Vet-SAP® per dose was required to elicit these responses compared with 5 µl of Alum and 20 µl of Advax™, providing further evidence of the enhanced adjuvant effects of Vet-SAP®.

IgG1 titres were also highest in the VLP-p*17/Vet-SAP® group although only the difference between this group and the VLP-p*17/Advax™ group was statistically significant ($P < 0.05$). Similarly, p*17-specific IgG2a titres were highest when VLP-p*17 was formulated with Vet-SAP® but the difference was not statistically significant ($p = 0.99$). No IgG2a was detected in the sera of mice immunised with antigen plus Alum (titres < 100). These finding are consistent with those of other studies reporting that Alum induces a predominantly Th2-driven response, while Advax™ and QS-based adjuvants such as Vet-SAP® induce both Th2 and Th1 responses (Rivera-Hernandez et al., 2020).

Th2 responses are predominantly humoral and are generally considered sufficient to resolve extracellular bacterial infections. Th1 responses involve a combination of humoral and cell-mediated immune responses required to resolve intracellular and fungal infections (Magombedze, Eda, & Ganusov, 2014). While Strep A is generally considered an extracellular pathogen, certain strains have the ability for intracellular invasion and persistence within epithelial and endothelial cells and immune cells such as macrophages, neutrophils, and dendritic cells (Bennett-Wood, Carapetis, & Robins-Browne, 1998). This capacity for intracellular survival is thought to contribute to antibiotic treatment failure in recurrent Strep A pharyngitis and recurrent erysipelas (Jendoubi, Rohde, & Prinz, 2019; Pichichero & Casey, 2007). Group C and Group G beta-haemolytic Streptococci are also recognised for their ability to invade epithelial or endothelial cells and these strains have been implicated as causes of ARF and RHD in Australian Aboriginal populations (Rohde, Talay, & Rasmussen, 2012; Sikder et al., 2019; Sikder et al., 2018). This would suggest that adjuvant used in an effective broad-spectrum Strep A vaccine offering protection against the full spectrum of Strep A disease, should be one that elicits both Th1 and Th2 responses but with a Th1 bias to minimise inflammation.

A major challenge in the development of vaccines against mucosal pathogens, such as Strep A, is induction of a robust mucosal response, particularly when the vaccine is administered parenterally. A significant disadvantage of Alum is its inability to induce

mucosal responses to target antigens (Rhee, Lee, & Kim, 2012). Saponins, however, have been shown to elicit strong mucosal responses in animals when used as adjuvants in nasal vaccines. When administered parenterally, saponins induce a cytokine response that favours production of secretory IgA (Chen et al., 2023). Advax has been used in vaccines against seasonal influenza viruses, which have demonstrated efficacy in human trials, suggesting induction of mucosal responses (Petrovsky, 2017). This lends further support for inclusion of saponin or inulin-based adjuvants in preference to the traditional aluminium-based adjuvants in vaccines targeting mucosal pathogens, such as Strep A.

Conclusion

This study suggests that the high immunogenicity of the conserved Strep A epitope p*17 is further enhanced when the epitope is expressed on the surface of a chimeric HBsAg-S VLP. Furthermore, the immunogenicity of the chimeric VLP is enhanced when formulated with the QS saponin-based adjuvant Vet-SAP®.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and was approved by the Human Research Ethics Committee of the UNIVERSITY OF SOUTHERN QUEENSLAND and the Animal Research Ethics Committee of GRIFFITH UNIVERSITY.

Informed Consent Statement: Not applicable. This study involved the use of commercially available human cell lines only.

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Conflicts of Interest: The authors declare no conflict of interest.

References

Barclay, T., Ginic-Markovic, M., Cooper, P., & Petrovsky, N. (2012). The Chemistry and Sources of Fructose and Their Effect on its Utility and Health Implications. *Journal of Excipients and Food Chemicals*(2), 67-82%V 63. Retrieved from https://ojs.abo.fi/ojs/index.php/jefc/article/view/145

Bennett-Wood, V. R., Carapetis, J. R., & Robins-Browne, R. M. (1998). Ability of clinical isolates of group A streptococci to adhere to and invade HEp-2 epithelial cells. *Journal of Medical Microbiology, 47*(10), 899-906. doi:https://doi.org/10.1099/00222615-47-10-899

Chen, K., Wang, N., Zhang, X., Wang, M., Liu, Y., & Shi, Y. (2023). Potentials of saponins-based adjuvants for nasal vaccines. *Front Immunol, 14*, 1153042. doi:10.3389/fimmu.2023.1153042

Cimica, V., & Galarza, J. M. (2017). Adjuvant formulations for virus-like particle (VLP) based vaccines. *Clin Immunol, 183*, 99-108. doi:10.1016/j.clim.2017.08.004

Collins, K. A., Snaith, R., Cottingham, M. G., Gilbert, S. C., & Hill, A. V. S. (2017). Enhancing protective immunity to malaria with a highly immunogenic virus-like particle vaccine. *Sci Rep, 7*(1), 46621. doi:10.1038/srep46621

Cooper, P. D., & Petrovsky, N. (2011). Delta inulin: a novel, immunologically active, stable packing structure comprising β-D-[2 -> 1] poly(fructo-furanosyl) α-D-glucose polymers. *Glycobiology, 21*(5), 595-606. doi:10.1093/glycob/cwq201

Cunningham, M. W. (2000). Pathogenesis of Group A Streptococcal Infections. *Clinical Microbiology Reviews, 13*(3), 470-511. doi:10.1128/cmr.13.3.470-511.2000

Dooley, L. M., Ahmad, T. B., Pandey, M., Good, M. F., & Kotiw, M. (2021). Rheumatic heart disease: A review of the current status of global research activity. *Autoimmun Rev, 20*(2), 102740. doi:https://doi.org/10.1016/j.autrev.2020.102740

Good, M. F. (2020). Streptococcus: An organism causing diseases beyond neglect. *PLoS Negl Trop Dis, 14*(5), e0008095. doi:10.1371/journal.pntd.0008095

Grgacic, E. V., & Anderson, D. A. (2006). Virus-like particles: passport to immune recognition. *Methods, 40*(1), 60-65. doi:10.1016/j.ymeth.2006.07.018

Jendoubi, F., Rohde, M., & Prinz, J. C. (2019). Intracellular Streptococcal Uptake and Persistence: A Potential Cause of Erysipelas Recurrence. *Front Med (Lausanne), 6*, 6. doi:10.3389/fmed.2019.00006

Kotiw, M., Johnson, M., Pandey, M., Fry, S., Hazell, S. L., Netter, H. J., . . . Olive, C. (2012). Immunological response to parenteral vaccination with recombinant hepatitis B virus surface antigen virus-like particles expressing Helicobacter pylori KatA epitopes in a murine H. pylori challenge model. *Clin Vaccine Immunol, 19*(2), 268-276. doi:10.1128/cvi.05295-11

Magombedze, G., Eda, S., & Ganusov, V. V. (2014). Competition for Antigen between Th1 and Th2 Responses Determines the Timing of the Immune Response Switch during Mycobaterium avium Subspecies paratuberulosis Infection in Ruminants. *PLOS Computational Biology, 10*(1), e1003414. doi:10.1371/journal.pcbi.1003414

Mangold, C. M., & Streeck, R. E. (1993). Mutational analysis of the cysteine residues in the hepatitis B virus small envelope protein. *J Virol, 67*(8), 4588-4597. doi:10.1128/jvi.67.8.4588-4597.1993

Marty-Roix, R., Vladimer, G. I., Pouliot, K., Weng, D., Buglione-Corbett, R., West, K., . . . Lien, E. (2016). Identification of QS-21 as an Inflammasome-activating Molecular Component of Saponin Adjuvants. *J Biol Chem, 291*(3), 1123-1136. doi:10.1074/jbc.M115.683011

Moffat, J. M., Cheong, W. S., Villadangos, J. A., Mintern, J. D., & Netter, H. J. (2013). Hepatitis B virus-like particles access major histocompatibility class I and II antigen presentation pathways in primary dendritic cells. *Vaccine, 31*(18), 2310-2316. doi:10.1016/j.vaccine.2013.02.042

Netter, H. J., Macnaughton, T. B., Woo, W. P., Tindle, R., & Gowans, E. J. (2001). Antigenicity and immunogenicity of novel chimeric hepatitis B surface antigen particles with exposed hepatitis C virus epitopes. *Journal of Virology, 75*(5), 2130-2141. doi:10.1128/JVI.75.5.2130-2141.2001

Nooraei, S., Bahrulolum, H., Hoseini, Z. S., Katalani, C., Hajizade, A., Easton, A. J., & Ahmadian, G. (2021). Virus-like particles: preparation, immunogenicity and their roles as nanovaccines and drug nanocarriers. *Journal of Nanobiotechnology, 19*(1), 59. doi:10.1186/s12951-021-00806-7

Nordstrom, T., Pandey, M., Calcutt, A., Powell, J., Phillips, Z. N., Yeung, G., . . . Good, M. F. (2017). Enhancing vaccine efficacy by engineering a complex synthetic peptide to become a super immunogen. *J Immunol, 199*(8), 2794-2802. doi:10.4049/jimmunol.1700836

Petrovsky, N. (2017). *Advax Adjuvant: A Potent and Safe Immunopotentiator Composed of Delta Inulin*: Immunopotentiators in Modern Vaccines. 2017:199-210. doi: 10.1016/B978-0-12-804019-5.00010-4. Epub 2017 Jan 13.

Pichichero, M. E., & Casey, J. R. (2007). Systematic review of factors contributing to penicillin treatment failure in Streptococcus pyogenes pharyngitis. *Otolaryngology–Head and Neck Surgery, 137*(6), 851-857. doi:10.1016/j.otohns.2007.07.033

Pulendran, B., Arunachalam, S. P., & O'Hagan, D. T. (2021). Emerging concepts in the science of vaccine adjuvants. *Nature Reviews Drug Discovery, 20*(6), 454-475. doi:10.1038/s41573-021-00163-y

Qian, C., Liu, X., Xu, Q., Wang, Z., Chen, J. X., Li, T., . . . Xia, N. S. (2020). Recent Progress on the Versatility of Virus-Like Particles. *Vaccines (Basel), 8*(1). doi:10.3390/vaccines8010139

Quan, D. H., Counoupas, C., Nagalingam, G., Pinto, R., Petrovsky, N., Britton, W. J., & Triccas, J. A. (2021). Advax adjuvant formulations promote protective immunity against aerosol Mycobacterium tuberculosis in the absence of deleterious inflammation and reactogenicity. *Vaccine, 39*(14), 1990-1996. doi:https://doi.org/10.1016/j.vaccine.2021.02.041

Ralph, A. P., & Carapetis, J. R. (2013). Group a streptococcal diseases and their global burden. *Current topics in microbiology and immunology, 368*, 1-27. doi:10.1007/82_2012_280

Reimer, J. M., Karlsson, K. H., Lövgren-Bengtsson, K., Magnusson, S. E., Fuentes, A., & Stertman, L. (2012). Matrix-M™ adjuvant induces local recruitment, activation and maturation of central immune cells in absence of antigen. *PloS one, 7*(7), e41451-e41451. doi:10.1371/journal.pone.0041451

Reynolds, S., Pandey, M., Dooley, J., Calcutt, A., Batzloff, M. R., Ozberk, V., . . . Good, M. F. (2021). Preclinical safety and immunogenicity of Streptococcus pyogenes (Strep A) peptide vaccines. *Sci Rep, 11*(1), 127. doi:10.1038/s41598-020-80508-6

Rhee, J. H., Lee, S. E., & Kim, S. Y. (2012). Mucosal vaccine adjuvants update. *Clin Exp Vaccine Res, 1*(1), 50-63. doi:10.7774/cevr.2012.1.1.50

Rivera-Hernandez, T., Hartas, J., Wu, Y., Chuan, Y. P., Lua, L. H., Good, M., . . . Middelberg, A. P. (2013). Self-adjuvanting modular virus-like particles for mucosal vaccination against group A streptococcus (GAS). *Vaccine, 31*(15), 1950-1955. doi:10.1016/j.vaccine.2013.02.013

Rivera-Hernandez, T., Rhyme, M. S., Cork, A. J., Jones, S., Segui-Perez, C., Brunner, L., . . . Walker, M. J. (2020). Vaccine-Induced Th1-Type Response Protects against Invasive Group A Streptococcus Infection in the Absence of Opsonizing Antibodies. *mBio, 11*(2). doi:10.1128/mBio.00122-20

Rohde, M., Talay, S. R., & Rasmussen, M. (2012). Molecular mechanisms of Streptococcus dysgalactiae subsp equisimilis enabling intravascular persistence. *Microbes and Infection, 14*(4), 329-334. doi:https://doi.org/10.1016/j.micinf.2011.10.008

Sikder, S., Rush, C. M., Govan, B. L., Norton, R. E., Cunningham, M. W., McMillan, D. J., . . . Ketheesan, N. (2019). Reply to Dale and Shulman. *J Infect Dis, 219*(4), 675-676. doi:10.1093/infdis/jiy561

Sikder, S., Williams, N. L., Sorenson, A. E., Alim, M. A., Vidgen, M. E., Moreland, N. J., . . . Ketheesan, N. (2018). Group G Streptococcus Induces an Autoimmune Carditis Mediated by Interleukin 17A and Interferon gamma in the Lewis Rat Model of Rheumatic Heart Disease. *J Infect Dis, 218*(2), 324-335. doi:10.1093/infdis/jix637

Singh, G., Song, S., Choi, E., Lee, P. B., & Nahm, F. S. (2020). Recombinant zoster vaccine (Shingrix(®)): a new option for the prevention of herpes zoster and postherpetic neuralgia. *Korean J Pain, 33*(3), 201-207. doi:10.3344/kjp.2020.33.3.201

Stevens, D. L., & Bryant, A. E. (2016). Severe Group A Streptococcal Infections. In J. J. Ferretti, D. L. Stevens, & V. A. Fischetti (Eds.), *Streptococcus pyogenes: Basic Biology to Clinical Manifestations*. Oklahoma City: University of Oklahoma Health Sciences Cente.

Takahashi, H., Takeshita, T., Morein, B., Putney, S., Germain, R. N., & Berzofsky, J. A. (1990). Induction of CD8+ cytotoxic T cells by immunization with purified HIV-1 envelope protein in ISCOMs. *Nature, 344*(6269), 873-875. doi:10.1038/344873a0

Tariq, H., Batool, S., Asif, S., Ali, M., & Abbasi, B. H. (2022). Virus-Like Particles: Revolutionary Platforms for Developing Vaccines Against Emerging Infectious Diseases. *Frontiers in Microbiology, 12*. doi:10.3389/fmicb.2021.790121

WHO. (2018). 71st World Health Assembly adopts resolution calling for greater action on rheumatic heart disease. Retrieved from https://www.who.int/ncds/management/rheumatic-heart-disease-resolution/en/

CHAPTER 7: DISCUSSION AND CONCLUSION

7.1 Discussion

The primary aim of the research described in this thesis was to construct a recombinant vaccine against the bacterium *Streptococcus pyogenes* (Strep A) and to evaluate the immunogenicity of the putative vaccine in mice. This chapter will summarise the main findings from this research.

The initial review of the literature, presented in Chapter 2, highlighted the significance of Strep A as a major human pathogen responsible for disproportionate morbidity and mortality in socially disadvantaged populations. Those most at risk are children living in developing countries or in Indigenous communities in developed counties including Australia (May, Bowen, & Carapetis, 2016).

Strep A infections are a cause of significant suffering and premature death among children and young adults living in remote Indigenous communities in central and northern Australia where overcrowding and inadequate access to health care services make infection control extremely difficult (Ralph et al., 2018; White et al., 2010). While children in these communities suffer alarmingly high rates of Strep A pyoderma and disproportionately high rates of APSGN and ISD, the major mortality and morbidity attributed to Strep A in these children is due to RHD. Children with symptomatic RHD can suffer extreme fatigue and severe shortness of breath. As a result, they are often unable to go to school or play, or in some cases even walk. Repeated Strep A infections and recurrent bouts of ARF cause progressive damage to the heart valves which eventually need to be surgically repaired or replaced, at a cost of between $100,000 and $200,000 per surgery. Some children require repeated surgeries to repair and replace the damaged valves with tissue grafts from animals (Keenan, Newland, Baker, Rice, & Bennetts, 2019). These children suffer significant emotional trauma when taken from their families and communities to spend months in the alien environment of a large hospital in a major city, typically Melbourne, Sydney or Brisbane. They

also miss considerable amounts of schooling (Hill & Collins, 2019). After surgery, children with RHD require penicillin injections every 28 days for a minimum of 10 years (RHDAustralia, 2020). Missing a single injection can mean that the child becomes reinfected by Strep A and suffers further heart valve damage, which may require further surgical repair or replacement. Even without reinfection, tissue graft valves breakdown after around five years and eventually require replacement with a mechanical valve. The human heart has four valves and children with RHD often have more than one valve that requires repair. This presents additional challenges to cardiac surgeons and increases the risk of surgical complications (Keenan et al., 2019). Children with mechanical heart valves require life-long oral anticoagulant therapy (Warfarin) and regular blood tests to monitor therapy, in addition to regular monthly penicillin injections. The injections need to be administered slowly (up to two minutes) and are painful (RHDAustralia, 2020). These services are difficult to access for people living in remote Aboriginal communities: a factor that may lead to lapses in their treatment or necessitate their relocation to a community that has appropriate medical services. In some Aboriginal communities, children as young as nine require surgery to repair damaged heart valves and children as young as 15 require the insertion of mechanical heart valves after repeated surgical valve repairs (Hill & Collins, 2019). Life expectancy in children, after insertion of a mechanical heart valve is only around 10 years, mainly due to the risks associated with anticoagulant therapy: over warfarinisation can lead to bleeding and under warfarinisation can lead to thrombosis. The risk of premature death from thrombosis in females with mechanical heart valves is particularly high because warfarin is teratogenic and therefore cannot be used during pregnancy (Sullivan et al., 2020; Wyber et al., 2020).

The review of the literature also found that while numerous programs aimed at reducing the rates of Strep A infections have been trialled in Australian Aboriginal and Torres Strait islander communities, none have significantly reduced the burden of Strep A disease in these communities. Effective vaccination is widely regarded as the most reliable and cost-effective means of reducing the Strep A disease burden in at-risk populations (Cannon et al., 2018).

This view was formalised in 2018 when part of the Global Resolution on ARF and RHD unanimously adopted by Member States of the World Health Organisation (WHO) at the World Health Assembly, focused on development of a safe and effective vaccine against Strep A (WHO, 2018).

The aim of the review paper, published in *Autoimmunity Reviews* and presented in Chapter 3 (Paper 1), was to construct a broad review of the current understandings of the epidemiology, pathogenesis and host-pathogen interactions, genetic and environmental contributors to host susceptibility, and diagnosis, treatment, and prevention of RHD. While numerous previously published reviews addressed one or two of these topics, the manuscript presented in Chapter 3 provides a detailed update on the status of research and understanding across all of them. While the current understandings of the pathogenesis and host-pathogen interactions, and genetic and environmental contributors to host susceptibility, were investigated from a global perspective, epidemiology, diagnosis, treatment and prevention were reviewed primarily from an Australian perspective. The following key points were highlighted: In Australia, streptococcal pyoderma (in addition to streptococcal pharyngitis) and Group C and Group G Streptococcus (in addition to Strep A) have been implicated as antecedents to ARF; the rates of RHD in remote Indigenous communities are persistently among the highest in the world; government register-based programs coordinate disease screening and delivery of prophylaxis with variable success; and Australian researchers, particularly our collaborators at the Institute for Glycomics, Griffith University, have made significant progress in the development of a broad-spectrum vaccine against Strep A.

The review of Strep A vaccine research, summarised in Chapter 3, Table 1, was aimed at informing a novel approach to the design of a putative Strep A vaccine, in particular the choice of target antigen. Due to the heterogeneity of the Strep A strains endemic in Australia, local research targets conserved antigens, particularly peptide sequences in the C repeat regions of the Step A M protein. As described in detail in Chapter 4, our initial vaccine construct targeted J8, a derivative of p145, a 20 aa peptide in the C3 repeat region of M protein (LRRDLDASREAKKQVEKALE). J8 comprises a minimal B cell epitope (J8i:

SREAKKQVEKAL) within p145, flanked on either side by sequences of eight amino acids from the yeast GCN4 DNA binding protein to maintain the natural alpha-helicity of J8i (Relf et al., 1996). As shown in Chapter 3 Table 1, J8 was the Strep A target antigen of the leading Strep A candidate vaccines in development in Australia at the time of publication. Chapter 3 Table 1 also highlights the different ways in which various vaccine constructs delivered J8 to the immune system suggesting that, while J8 was considered an optimal Strep A target antigen at that time, research into the ideal antigen delivery system was ongoing. A secondary aim of the general literature review, presented in Chapter 2, was therefore to investigate the use of VLPs, in particular HBsAg-S VLPs, as antigen delivery platforms in putative vaccines against bacterial pathogens. While no VLP-based antibacterial vaccine is currently licensed for use in humans, the literature confirmed the safety and efficacy of VLP-based antiviral and antimalarial vaccines. This in turn confirmed the novelty of a recombinant HBsAg-S VLP-based vaccine against the bacterium *Streptococcus pyogenes*. The literature review also validated the rationale behind expressing foreign epitopes within the immunodominant "a" determinant region of HBsAg-S.

Chapter 4 provided a detailed description of the design and production of VLP-J8, the initial vaccine construct, and VLP-p*17, the putative vaccine that was progressed to the animal trials described in Chapters 5 and 6. Two critical considerations in the design of the VLP-J8 and VLP-p*17 were, surface expression of the Strep A antigen, and preservation of the natural alpha-helical secondary structure of the Strep A antigen, particularly the J8i sequence. The Phyre^2 report for VLP-p*17, presented in Figure 14, shows predicted alpha helical secondary structure for the entire 12aa sequence of the variant J8i epitope compared with the Phyre^2 report for VLP-J8, presented in Figure 9, which shows predicted alpha helical secondary structure for only 83.3% of the J8i epitope. This concurs with enhanced helicity of p*17 reported by Nordstrom et al., (2017) and further supported the decision to progress VLP-p*17 as a putative Strep A vaccine in place of VLP-J8.

Production of VLP-J8, started with two long DNA primers, which were annealed to produce the double stranded DNA to be inserted into the HBsAg-S DNA sequence. The insert was digested and then ligated into the pcDNA3.1 expression vector containing the DNA sequence for HBsAg-S with an *Age*I restriction site in the "a" determinant region. The plasmid DNA was transformed in *E. coli,* then extracted and sequenced to confirm the insertion of J8, in the correct orientation, within the "a" determinant region of the HBsAg-S as shown in Figure 8. The plasmid with correct orientation of the J8 sequence was amplified in *E. coli*, extracted and transfected into HEK239t cells for protein expression. While these procedures resulted in successful production of recombinant VLP as demonstrated by the results shown in Chapter 4 Table 1, they were extremely laborious and resulted in a relatively low yield. However, the results were proof of principle prompting investigation into means of increasing yield and reducing time and labour.

For the production of VLP-p*17, the recombinant DNA was designed with assistance from Professor Hans Netter (Victorian Infectious Diseases Reference Laboratory, Melbourne Health), and the DNA sequence was codon optimised, synthesised and cloned into the pcDNA3.1(+) by GenScript (Hong Kong) Limited. This was cost-effective, significantly reduced labour, and improved yield as described in Chapter 5 (Paper 2).

The paper submitted to the journal *Heliyon* and presented in Chapter 5 (Paper 2) describes the design, production, characterisation and semi-purification of VLP-p*17, a recombinant HBsAg-S VLP-based vaccine against Strep A infections and rheumatic heart disease. The results of the study described in this paper support the hypothesis that expression of p*17 within the "a" determinant of HBsAg-S will not compromise spontaneous VLP formation. Cell culture-derived VLP-p*17 self-assembled into 22 nm particles visible by electron microscopy and recognised by p*17 specific antibodies, as demonstrated in Chapter 5 Figures 3 and 4 respectively. This marked a major milestone in the project and led to the generous offer from Professor Michael Good to facilitate and fund an

immunogenicity trial of VLP-p*17 in mice at the Animal Facility at Griffith University.

The results of the immunogenicity trial supported the hypothesis that mice immunised with VLP-p*17 formulated with adjuvant will produce measurable titres of p*17-specific IgG antibodies. BALB/c mice immunised with three doses of 0.5 µg VLP-p*17, formulated with the adjuvant Alum, produced titres of p*-17 specific IgG in excess of 1.28×10^4 and no adverse effects were reported in any immunised mice. This marked a second major milestone and a significant achievement.

In 2013 a joint initiative, between the Australian and New Zealand Governments, called the Coalition to Accelerate New Vaccines for group A Streptococcus (CANVAS), was established to "tackle rheumatic fever, rheumatic heart disease (RHD) and a range of serious infections caused by the bacterium Group A Streptococcus " (Telethon Kids Institute, 2016). The project is funded by the National Health and Medical Research Council (NHMRC) and Health Research Council (New Zealand) and incorporates an objective pre-clinical and clinical evaluation of leading Spy vaccine candidates currently in development.

To be eligible for consideration by CANVAS, vaccine candidates must not cause autoimmune cross reactivity with human tissue and the lead vaccine candidate will be selected on the basis of efficacy against the Strep A strains circulating in the target population: Australian Aboriginal and Torres Strait Islander populations in Australia or Māori and Pacific populations in New Zealand. VLP-p*17 will satisfy both of these conditions. The CANVAS project will also investigate the economic viability of Strep A vaccine candidates against other prevention and treatment strategies.

While our approach utilised a mammalian cell protein expression system, VLPs can be produced more cost effectively in yeast cells. Transformed yeast cells can grow to extremely high densities facilitating high volume production of VLPs in commercial-scale fermenters (Tariq, Batool, Asif, Ali, & Abbasi, 2022). The cost of production of a recombinant VLP-based Strep A vaccine would be comparable to the cost of production of the current HBV vaccines. The implementation of the National Newborn Hepatitis B Vaccine program in 2000 has led to a significant

decline in the rates of HBV infection in Aboriginal women and children in the Northern Territory (Liu et al., 2012). A safe, efficacious VLP-based Strep A vaccine could therefore be a cost-effective means of lowering the rates of Strep A infections and autoimmune sequalae in these and other socially and economically challenged communities.
The success of this project has implications not only for development of a safe and effective vaccine against Strep A, it paves the way for future development of HBsAg-S VLP-based vaccines against other bacterial pathogens for use in humans and animals.

The study reported in Chapter 6 (Paper 3), which will be submitted for publication in an appropriate journal such as *MDPI Vaccines* or *Journal of Immunology*, is a direct progression of the study described in Chapter 5 (Paper 2). This paper includes measurement of IgG titres in sera from groups of BALB/c mice immunised with different formulations of VLP-p*17 plus adjuvant, groups immunised with adjuvant alone, and one group immunised with p*17-DT, the current lead Strep A vaccine candidate (Table 1 Chapter 6). The latter group was included as a positive control for ELISA should immunisation with VLP-p*17 fail to induce production of measurable p*17-specific IgG. The primary aims of this study were to investigate the immunological value of delivering p*17 expressed on a chimeric HBsAg-S VLP and to investigate the effects of two novel adjuvants on the immune response to VLP- p*17. The results of this investigation (Figure 4, Chapter 6) show that significantly higher p*17-specific IgG titres were generated in triple immunised mice when VLP-p*17 was formulated with Vet-SAP® than when VLP-p*17 was formulated with Alum or Advax™ ($P < 0.005$). Triple immunised mice also produced higher titres of p*17-specific IgG1 when VLP-p*17 was formulated with Vet-SAP® rather than Alum or Advax™ although only the difference between the VLP-p*17/Vet-SAP® and VLP/ Advax™ groups was statistically significant ($P < 0.05$). P*17-specific IgG2a was not detected in mice when the antigen was formulated with Alum, regardless of whether the antigen delivery platform utilised was DT or HBsAg-S VLP. Although not statistically significant ($P = 0.99$), IgG2a titers were higher in the VLP-p*17/Vet-SAP® group than in the VLP-p*17 Advax™ group. These results suggest that Advax™ and Vet-

SAP® are both likely to trigger cell-mediated responses against the target antigen in addition to robust antibody-mediated responses but that responses are likely to be greater when the antigen is formulated with Vet-SAP®. Based on these results, future immunogenicity or efficacy studies of VLP-p*17, and those of any future vaccines we develop, will most likely include Vet-SAP® (for veterinary vaccines) or another QS saponin-based adjuvant (for human vaccines).

7.2 Limitations

A possible limitation of this study is the omission of a ninth group of mice immunised with p*17-DT at doses of 0.5 µg with 5 µl of Alum. This would have allowed a direct dose-matched comparison of the immunogenicity of VLP-p*17 with that of p*17-DT.

A second potential limitation of this research is the omission of a bacterial challenge study to evaluate the efficacy of VLP-p*17 in an animal model of Strep A pharyngitis or pyoderma. The antigen dose for a bacterial challenge study is typically 25-30 µg per dose per mouse. This equates to a total of 375-450 µg for a triple-dose study involving a test group of five mice. The total amount of VLP-p*17 produced after six rounds of transfection and cell culture, and six overnight ultra-centrifugations at the University of Queensland, was only 45 µg. However, p*17, conjugated with DT and formulated with Alum, has demonstrated enhanced protective efficacy in mouse models of Strep A infections (Nordstrom et al., 2017). The results of this project demonstrated that p*17 induced high titres of p*17 specific IgG when expressed on HBsAg-S VLP and formulated with Alum, Advax™ or Vet-SAP®. It is therefore likely that VLP-p*17, administered at standard doses of 25-30 µg, will provide an equal or even greater level of protection than p*17-DT.

7.3 Future directions

The results of this project demonstrated that VLP-p*17 is highly immunogenic, inducing production of high titres of serum p*17-specific IgG with three low

doses: only 0.5 µg each. Assessing the efficacy of VLP-p*17 in mice challenged with a mix of virulent strains of Step A, following procedures developed by our collaborators at the Institute for Glycomics (Pandey et al., 2016), is the next step in assessing the value of VLP-p*17 as an effective vaccine against Strep A infections and post-infectious sequelae. To this end, the cost of having VLP-p*17 produced by GenScript, in sufficient quantity for a bacterial challenge study, is being explored.

The immunogenicity of VLP-p*17 could be further enhanced by cloning additional Strep A epitopes into the VLP to prime the immune system to recognise additional components of the bacterium. These additional epitopes could include cell wall-associated and shed virulence factors such as *S. pyogenes* cell envelope proteinase (SpyCEP). Targeting additional virulence factors has been shown to enhance protection against hypervirulent strains of Strep A capable of upregulating expression of these virulence factors (Pandey et al., 2016). Similarly, VLPs containing a mix of HBsAg-S monomers expressing different Strep A antigens could be produced to induce production of multiple clones of immune cells primed to target different Strep A virulence factors.

The procedures used to design, produce and semi-purify VLP-p*17 can be readily applied to other manipulations of HBsAg-S for production of other recombinant antibacterial vaccines for use in animals and humans. Furthermore, because recombinant VLPs have an empty core surrounded by a permeable membrane, they can also be utilised as carriers of tumour antigens and small anti-tumour molecules for treatment of malignant diseases. In the same way that p*17 was expressed on the VLP surface, receptor molecules can be expressed on the VLP surface to facilitate uptake by specific cells, ensuring targeted delivery of the antigen or agent (Nooraei et al., 2021). This work could therefore add value to projects investigating the anti-tumour properties of naturally occurring and synthesised molecules (Khan et al., 2022).

While HBsAg-S, the small (S) envelope protein, is the immunodominant HBV surface protein, the natural HBV envelope comprises two additional proteins: the

middle (M) and large (L) envelope proteins, also referred to as Pre-S1 and Pre-S2. All three envelope proteins are coded by the same gene and have identical C-terminals. Clinical trials of new generation HBV vaccines, incorporating the S and M envelope proteins or all three envelope proteins, have shown that these constructs trigger faster and stronger immune responses than those induced by VLPs comprising only HBsAg-S (Shouval, Roggendorf, & Roggendorf, 2015). The processes described in Chapters 5 and 6 could be used to incorporate additional HBV envelope proteins into VLP-p*17 to further enhance its immunogenicity.

Finally, the success of this research project was made possible by support from scientists from other institutions, who contributed time and resources often without remuneration. Without the animal trial in particular, there was no way of knowing whether or not VLP-p*17 had any potential as a vaccine against Strep A. This highlights the critical importance of the collaborations established during the course of this research that will hopefully carry over to future projects.

7.4 Conclusion

This project involved the design and production of a recombinant chimeric VLP that induced production of high titres of peptide-specific anti-Strep A antibodies in vaccinated mice after three minimal doses of just 0.5 μg per dose. This putative vaccine, VLP-p* 17, has features that make it an attractive construct for a commercially produced vaccine against Strep A: it is a single recombinant protein that spontaneously assembles into stable particles that are of optimal size for recognition and uptake by human immune cells; it presents repetitive displays of the highly immunogenic Strep A epitope, p*17, on the surface of homologous nanoparticles; it can be easily and consistently produced in yeast or mammalian cells; and it is based on the HBsAg-S VLP, which is already licensed as a human vaccine with associated large-scale quality-controlled manufacturing processes already in place. VLP-p*17 has substantial potential as a safe and effective vaccine against *Streptococcus pyogenes*, a bacterium responsible for significant morbidity and mortality, particularly among children and young adults living in resource poor communities. As such, it could contribute

significantly to the improvement of health outcomes for Australian Aboriginal and Torres Strait Islander children.

REFERENCES

AIHW 2020, 'Acute rheumatic fever and rheumatic heart disease in Australia, 2015–2019', retrieved from https://www.aihw.gov.au/reports/heart-stroke-vascular-diseases/acute-rheumatic-fever-and-rheumatic-heart-disease/contents/summary

Andrews, RM, Kearns, T, Connors, C, Parker, C, Carville, K, Currie, BJ & Carapetis, JR 2009, 'A regional initiative to reduce skin infections amongst aboriginal children living in remote communities of the Northern Territory, Australia', *PLoS Neglected Tropical Diseases, vol. 3, no.* 11, e554.

Aziz, RK, & Kotb, M 2008, 'Rise and persistence of global M1T1 clone of Streptococcus pyogenes', *Emerging Infectious Diseases,* vol. 14, no. 10, pp. 1511-1517.

Batzloff, MR, Fane, A, Gorton, D, Pandey, M, Rivera-Hernandez, T, Calcutt ,A, Yeung, G, Hartas, J, Johnson, L, Rush, CM, McCarthy, JS, Ketheesan, N, Good, MF 2016, 'Preclinical immunogenicity and safety of a Group A streptococcal M protein-based vaccine candidate' *Human Vaccines & Immunotherapeutics, vol.* 12, no. 12, pp. 3089-3096.

Bhatnagar, PK, Papas, E, Blum, HE, Milich, DR, Nitecki, D, Karels, MJ, & Vyas, GN, 1982 'Immune response to synthetic peptide analogues of hepatitis B surface antigen specific for the a determinant', *Proceedings of the National Academy of Sciences of the United States of America,* vol. 79, no. 14, pp. 4400-4404.

Blumberg, BS, & Alter, HJ 1965, 'A "new" antigen in leukemia sera', *Journal of the American Medical Association*, vol. 191, no. 7, pp. 541-546.

Blumberg, BS, Millman, I, & London, WT 1985, 'Ted Slavin's blood and the development of HBV vaccine' *New England Journal of Medicine, vol. 312, no.* 3, pp. 189.

Boyd, R, Patel, M, Currie, BJ, Holt, DC, Harris, T & Krause, V 2016, 'High burden of invasive group A streptococcal disease in the Northern Territory of Australia', *Epidemiology and infection,* vol. 144, no. 5, pp. 1018.

Brandt, ER, Hayman, WA, Currie, B, Pruksakorn, S & Good, MF 1997, 'Human antibodies to the conserved region of the M protein: opsonization of heterologous strains of group A streptococci' *Vaccine,* no. 15, no. 16, pp. 1805-1812.

Cannon, JW, Jack, S, Wu, Y, Zhang, J, Baker, MG, Geelhoed, E, Fraser, JD & Carapetis, JR 2018, 'An economic case for a vaccine to prevent group A streptococcus skin infections', *Vaccine,* vol. 36, no. 46, pp. 6968-6978.

Carapetis, JR, Brown, A, Maguire, GP & Walsh, W 2012, 'The Australian guideline for prevention, diagnosis and management of acute rheumatic fever and

rheumatic heart disease', *National Heart Foundation of Australia and the Cardiac Society of Australia and New Zealand*.

Carapetis, JR, Steer, AC, Mulholland, EK, & Weber, M 2005, 'The global burden of group A streptococcal diseases' *Lancet Infectious Diseases, vol. 5, no.* 11, pp. 685-694.

Ching, NS, Crawford, N, McMinn, A, Baker, C, Azzopardi, K, Brownlee, K, Lee, D, Gibson, M, Smeesters, P, Gonis, G, Ojaimi, S, Buttery, J & Steer, AC 2017, 'Prospective Surveillance of Pediatric Invasive Group A Streptococcus Infection', *Journal of the Pediatric Infectious Diseases Society*, vol. 28, no. 1, pp. 46-52.

Collins, KA, Snaith, R, Cottingham, MG, Gilbert, SC, & Hill, AVS 2017, 'Enhancing protective immunity to malaria with a highly immunogenic virus-like particle vaccine', *Scientific Reports,* vol. 7, no. 1, pp. 46621.

Colquhoun, SM, Condon, JR, Steer, AC, Li, SQ, Guthridge, S & Carapetis, JR 2015, 'Disparity in Mortality From Rheumatic Heart Disease in Indigenous Australians', *Journal of the American Heart Association, vol. 4, no.* 7, e001282.

Cunningham, MW 2000, 'Pathogenesis of Group A Streptococcal Infections', *Clinical Microbiology Reviews,* vol. 13, no. 3, pp. 470-511.

Deguchi, M, Yamashita, N, Kagita, M, Asari, S, Iwatani, Y, Tsuchida, T, Iinuma, K & Mushahwar, IK 2004, 'Quantitation of hepatitis B surface antigen by an automated chemiluminescent microparticle immunoassay, *Journal of Virological Methods,* vol. 115, no. 2, pp. 217-222.

de Loizaga, SR, Beaton, AZ, Nascimento, BR, Macedo, FVB, Spolaor, BCM, de Pádua, LB, Ribeiro, TFS, Oliveira, GCF, Oliveira, LR, de Almeida, LFR, Moura, TD, de Barros, TT, Sable, C & Nunes, MCP 2021, 'Diagnosing rheumatic heart disease: where are we now and what are the challenges?' *Expert Review of Cardiovascular Therapy*, vol. 19, no. 9, pp. 777-786

Dooley, LM, Ahmad, TB, Pandey, M, Good, MF & Kotiw, M 2021, 'Rheumatic heart disease: A review of the current status of global research activity' *Autoimmunity Reviews,* vol. 20, no. 2, pp. 102740.

Dunne, EM, Hutton, S, Peterson, E, Blackstock, AJ, Hahn, CG, Turner, K, & Carter, KK 2022, 'Increasing Incidence of Invasive Group A Streptococcus Disease, Idaho, USA, 2008-2019', *Emerging Infectious Diseases,* vol. 28, no. 9, pp. 1785-1795.

Elhanan, E, Boaz, M, Schwartz, I, Schwartz, D, Chernin, G, Soetendorp, H, Gal Oz, A, Agbaria, A & Weinstein, T 2018, 'A randomized, controlled clinical trial to evaluate the immunogenicity of a PreS/S hepatitis B vaccine Sci-B-Vac™, as compared to Engerix B(®), among vaccine naïve and vaccine non-responder dialysis patients', *Clinical and Experimental Nephrology,* vol. 22, no. 1, pp. 151-158.

Fischetti, VA 1989, 'Streptococcal M protein: molecular design and biological behavior', *Clinical Microbiology Reviews,* vol. 2 no. 3, pp. 285-314.
Fischetti, VA 2000, 'Surface proteins on Gram-Positive bacteria', in Fischetti, VA, Novick, RP, Ferretti, JJ, Stevens, DL, Portnoy, DA & Rood, JI (Eds.), *Gram-Positive Pathogens,* pp. 11-24, Washington DC: ASM Press.

Fischetti, VA 2016, 'M Protein and Other Surface Proteins on Streptococci', in Ferretti, JJ, Stevens, DL & Fischetti, VA (Eds.), *Streptococcus pyogenes : Basic Biology to Clinical Manifestations,* University of Oklahoma Health Sciences Center.

Gavilanes, F, Gonzalez-Ros, JM & Peterson, DL 1982,' Structure of hepatitis B surface antigen. Characterization of the lipid components and their association with the viral proteins', *Journal of Biological Chemistry,* vol. 257, no. 13, pp. 7770-7777.

Good, MF 2020, 'Streptococcus: An organism causing diseases beyond neglect', *PLoS Neglected Tropical Diseases,* vol. 14, no. 5, e0008095.

Good, MF & Yanow, SK 2016, 'Cryptic epitope for antibodies should not be forgotten in vaccine design', *Expert Review of Vaccines,* vol. 15, no. 6, pp. 675-676.

Grgacic, EV & Anderson, DA 2006, 'Virus-like particles: passport to immune recognition', *Methods,* vol. 40, no. 1, pp. 60-65.

Guilherme, L & Kalil, J 2002, 'Rheumatic fever: the T cell response leading to autoimmune aggression in the heart', *Autoimmunity Reviews,* vol. 1, no. 5, pp. 261-266.

Guilherme, L & Kalil, J 2010, 'Rheumatic fever and rheumatic heart disease: cellular mechanisms leading autoimmune reactivity and disease', *Journal of Clinical Immunologly,* vol. 30, no. 1, pp. 17-23.

Hayman, WA, Brandt, ER, Relf, WA, Cooper, J, Saul, A & Good, MF 1997, 'Mapping the minimal murine T cell and B cell epitopes within a peptide vaccine candidate from the conserved region of the M protein of group A streptococcus', *International Immunology,* vol. 9, No. 11, pp. 1723-1733.

Hill, M & Collins, S 2019, 'Take Heart: The quest to rid Australia of rheumatic heart disease', *Moonshine Agency*, Retrieved from http://toolkit.takeheart.tv

Hotez, PJ, Aksoy, S, Brindley, PJ & Kamhawi, S 2020, 'What constitutes a neglected tropical disease?', *PLoS Neglected Tropical Diseases,* vol. 14, no. 1, e0008001.

Hoy, WE, White, AW, Dowling, A & Sharma, SK 2012, 'Post-streptococcal glomerulonephritis is a strong risk factor for chronic kidney disease in later life', *Kidney international,* vol. 81, no. 10, pp. 1026-1032.

Jackson, SJ, Steer, AC & Campbell, H 2011, 'Systematic review: Estimation of global burden of non-suppurative sequelae of upper respiratory tract infection:

rheumatic fever and post-streptococcal glomerulonephritis' *Tropical Medicine and International Health,* vol. 16, no. 1, pp. 2-11.

Jones, TD 1944, 'The diagnosis of rheumatic fever', *Journal of the American Medical Association,* vol. 126, no. 8, pp. 481-484.

Katzenellenbogen, JM, Bond-Smith, D, Seth, RJ, Dempsey, K, Cannon, J, Stacey, I, Wade, V, de Klerk, N, Greenland, M, Sanfilippo, FM, Brown, A, Carapetis, JR, Wyber, R, Nedkoff, L, Hung, J, Bessarab, D & Ralph, AP 2020, 'Contemporary incidence and prevalence of rheumatic fever and rheumatic heart disease in Australia using linked data: The Case for policy change', *Journal of the American Heart Association,* vol. 9, no. 19, e016851.

Keenan, NM, Newland, RF, Baker, RA, Rice, GD & Bennetts, JS 2019, 'Outcomes of redo valve surgery in Indigenous Australians', *Heart, Lung and Circulation,* vol. 28, no. 7, pp. 1102-1111.

Kelley, LA, Mezulis, S, Yates, CM, Wass, MN & Sternberg, MJE 2015, 'The Phyre2 web portal for protein modeling, prediction and analysis', *Nature Protocols,* vol. 10, no. 6, pp. 845-858.

Khan MI, Bouyahya A, Hachlafi NEL, Menyiy NE, Akram M, Sultana S, Zengin G, Ponomareva L, Shariati MA, Ojo OA, Dall'Acqua S & Elebiyo TC 2022, 'Anticancer properties of medicinal plants and their bioactive compounds against breast cancer: a review on recent investigations', *Environmental Science and Pollution Research,* vol. 29, no. 17, pp. 24411-24444.

Kotiw, M, Johnson, M, Pandey, M, Fry, S, Hazell, SL, Netter, HJ, Good, MF & Olive, C 2012, 'Immunological response to parenteral vaccination with recombinant hepatitis B virus surface antigen virus-like particles expressing *Helicobacter pylori* KatA epitopes in a murine *H. pylori* challenge model', *Clinical and Vaccine Immunology,* vol. 19, no. 2, pp. 268-276.

Lamagni, TL, Neal, S, Keshishian, C, Alhaddad, N, George, R, Duckworth, G, Vuopio-Varkila, J & Efstratiou, A 2008, 'Severe *Streptococcus pyogenes* infections, United Kingdom, 2003-2004', *Emerging Infectious Diseases,* vol. 14, no. 2, pp. 202-209.

Lanford, RE, Luckow, V, Kennedy, RC, Dreesman, GR, Notvall, L & Summers, MD 1989, 'Expression and characterization of hepatitis B virus surface antigen polypeptides in insect cells with a baculovirus expression system', *Journal of Virology,* vol. 63, no. 4, pp. 1549-1557.

Langshaw, EL, Pandey, M & Good, MF 2018, 'Cellular interactions of covR/S mutant group A Streptococci', *Microbes and Infection,* vol. 20, no. 9, pp. 531-535.

Liu, B, Guthridge, S, Li, SQ, Markey, P, Krause, V, McIntyre, P & Kaldor, JM et al. 2012, 'The end of the Australia antigen? An ecological study of the impact of universal newborn hepatitis B vaccination two decades on', *Vaccine,* vol. 30, no. 50, pp. 7309-7314.

Lowy, DR & Schiller, JT 2006, 'Prophylactic human papillomavirus vaccines', *The Journal of clinical investigation,* vol. 116*,* no. 5, pp. 1167-1173.

Ludwig, C & Wagner, R 2007, 'Virus-like particles-universal molecular toolboxes', *Current Opinions in Biotechnology,* vol. 18, no*.* 6, pp. 537-545.

Marshall, CS, Cheng, AC, Markey, PG, Towers, RJ, Richardson, LJ, Fagan, PK, Scott, L, Krause, VL & Currie, BJ 2011, 'Acute post-streptococcal glomerulonephritis in the Northern Territory of Australia: A review of 16 years data and comparison with the literature', *American Journal of Tropical Medicine and Hygiene,* vol. 85, no. 4, pp. 703-710.

May, PJJ, Bowen, AC & Carapetis, JR 2016, 'The inequitable burden of group a streptococcal diseases in indigenous Australians: We need to fill evidence gaps and make clinical advances to reduce these diseases of disadvantage, *Medical Journal of Australia,* vol. 205, no. 5, pp. 201-203.

McAleer, WJ, Buynak, EB, Maigetter, RZ, Wampler, DE, Miller, WJ & Hilleman, MR 1984, 'Human hepatitis B vaccine from recombinant yeast', *Nature,* vol. 307, no. 5947, pp. 178-180.

Mehta, S, McGeer, A, Low, DE, Hallett, D, Bowman, DJ, Grossman, SL & Stewart, TE 2006, 'Morbidity and mortality of patients with invasive group A streptococcal infections admitted to the ICU', *Chest,* vol. 130 no. 6, pp. 1679-1686.

Moffat, JM, Cheong, WS, Villadangos, JA, Mintern, JD & Netter, HJ 2013, 'Hepatitis B virus-like particles access major histocompatibility class I and II antigen presentation pathways in primary dendritic cells', *Vaccine,* vol. 31, no. 18, pp. 2310-2316.

Moorthy, VS & Okwo-Bele, J 2015, 'Final results from a pivotal phase 3 malaria vaccine trial', *The Lancet,* vol. 386, no*.* 9988, pp. 5-7.

Mulder, AM, Carragher, B, Towne, V, Meng, Y, Wang, Y, Dieter, L, Potter, CS, Washabaugh, MW, Sitrin, RD & Zhao, Q 2012, 'Toolbox for non-intrusive structural and functional analysis of recombinant VLP based vaccines: A case study with hepatitis B vaccine', *PloS one,* vol. 7, no. 4, e33235.

Nasser, W, Beres, SB, Olsen, RJ, Dean, MA, Rice, KA, Long, SW, Kristinsson, KG, Gottfredsson, M, Vuopio, J, Raisanen, K, Caugant, DA, Steinbakk, M, Low, DE, McGeer, A, Darenberg, J, Henriques-Normark, B, Van Beneden, CA, Hoffmann, S and Musser, JM 2014, 'Evolutionary pathway to increased virulence and epidemic group A Streptococcus disease derived from 3,615 genome sequences' *Proceedings of the National Acadademy of Sciences U S A, vol. 111, no.* 17, e1768-1776.

Netter, HJ, Macnaughton, TB, Woo, WP, Tindle, R & Gowans, EJ 2001, 'Antigenicity and immunogenicity of novel chimeric hepatitis B surface antigen particles with exposed hepatitis C virus epitopes', *Journal of Virology,* vol. 75, no. 5, pp. 2130-2141.

Netter, HJ, Woo, WP, Tindle, R, Macfarlan, RI & Gowans, EJ 2003, 'Immunogenicity of recombinant HBsAg/HCV particles in mice pre-immunised with hepatitis B virus-specific vaccine', *Vaccine,* vol. 21, no. 21-22, pp. 2692-2697.

Nooraei, S, Bahrulolum, H, Hoseini, ZS, Katalani, C, Hajizade, A, Easton, AJ & Ahmadian, G 2021, 'Virus-like particles: preparation, immunogenicity and their roles as nanovaccines and drug nanocarriers', *Journal of Nanobiotechnology,* vol. 19, no. 1, pp. 59.

Nordstrom, T, Pandey, M, Calcutt, A, Powell, J, Phillips, ZN, Yeung, G, Giddam, AK, Shi, Y, Haselhorst, T, von Itzstein, M, Batzloff, MR & Good, MF 2017, 'Enhancing vaccine efficacy by engineering a complex synthetic peptide to become a super immunogen', *Journal of Immunology,* vol. 199, no. 8, pp. 2794-2802.

Pandey, M, Mortensen, R, Calcutt, A, Powell, J, Batzloff, MR, Dietrich, J & Good, MF 2016, 'Combinatorial synthetic peptide vaccine strategy protects against hypervirulent CovR/S mutant Streptococci', *Journal of Immunology, vol. 196, no.* 8, pp. 3364-3374.

Park, SB 2012, 'Hepatitis E vaccine debuts', *Nature,* vol. 491, no. 7422, pp. 21-22.

Parnaby, MG & Carapetis, JR 2010, 'Rheumatic fever in Indigenous Australian children', *Journal of Paediatrics & Child Health,* vol. 46, no. 9, pp. 527-533.
Pelton, JT & McLean, LR 2000 'Spectroscopic Methods for Analysis of Protein Secondary Structure', *Analytical Biochemistry,* vol. 277, *no.* 2, pp. 167-176.

Petersson, K, Forsberg, G & Walse, B 2004, 'Interplay between superantigens and immunoreceptors', *Scandinavian Journal of Immunology,* vol. 59, no. 4, pp. 345-355.

Proft, T & Fraser, JD 2016, 'Streptococcal superantigens: Biological properties and potential role in disease', in Ferretti, JJ, Stevens, DL & Fischetti, VA (Eds.), *Streptococcus pyogenes : Basic Biology to Clinical Manifestations,* University of Oklahoma Health Sciences Center.

Pruksakorn, S, Brandt, E, Good, MF, Currie, B, Martin, D, Galbraith, A, Phornphutkul, C, Hunsakunachai, S & Manmontri, A 1994, 'Towards a vaccine for rheumatic fever: identification of a conserved target epitope on M protein of group A streptococci', *The Lancet,* vol. 344, no. 8923, pp. 639-642.

Ralph, AP & Carapetis, JR 2013, 'Group A streptococcal diseases and their global burden', *Current topics in Microbiology and Immunology,* vol. 368, pp. 1-27.

Ralph, AP, de Dassel, JL, Kirby, A, Read, C, Mitchell, A, Maguire, GP & Carapetis, JR 2018, 'Improving Delivery of Secondary Prophylaxis for Rheumatic Heart Disease in a High Burden Setting: Outcome of a stepped Wedge, Community, Randomized Trial', *Journal of the American Heart Association,* vol. 7, no. 14, e009308.

Relf, WA, Cooper, J, Brandt, ER, Hayman, WA, Anders, RF, Pruksakorn, S, Currie, B, Saul, A & Good, MF 1996, 'Mapping a conserved conformational epitope from the M protein of group A streptococci', *Peptide Research,* vol. 9, no. 1, pp. 12-20.

Rentta, NN, Bennett, J, Leung, W, Webb, RH, Jack, S, Harwood, M, Baker, MG, Lund, M & Wilson, N 2022, 'Medical treatment for rheumatic heart disease: A narrative review', *Heart, Lung and Circulation*.

Rezaee, R, Poorebrahim, M, Najafi, S, Sadeghi, S, Pourdast, A, Alavian, SM, Alavian, SE & Poortahmasebi, V 2016, 'Impacts of the G145R mutation on the structure and immunogenic activity of the hepatitis B surface antigen: A computational analysis', *Hepatitis Monthly,* vol. 16, no. 7, e39097.

RHDAustralia 2020, 'The 2020 Australian guideline for prevention, diagnosis and management of acute rheumatic fever and rheumatic heart disease*', RHD Australia, r*etrieved from https://www.rhdaustralia.org.au/arf-rhd-guideline

RHDAustralia & McDonald, M 2022, 'Fast facts infographic 2022', *RHDAustralia*. retrieved from https://www.rhdaustralia.org.au/resources/fast-facts-infographic-2022

Roberts, KV, Maguire, GP, Brown, A, Atkinson, DN, Remenyi, B, Wheaton, G, Ilton, M & Carapetis, JR 2015, 'Rheumatic heart disease in Indigenous children in northern Australia: differences in prevalence and the challenges of screening', *Medical Journal of Australia,* vol. 203, no. 5, e221-227.

Rodriguez-Iturbe, B & Musser, JM 2008, 'The current state of poststreptococcal glomerulonephritis', *Journal of the American Society of Nephrology,* vol. 19, no. 10, pp. 1855-1864.

Rodríguez-Limas, WA, Sekar, K & Tyo, KEJ 2013, 'Virus-like particles: the future of microbial factories and cell-free systems as platforms for vaccine development', *Current Opinion in Biotechnology,* vol. 24, no. 6, pp. 1089-1093.

Ryan, PA, McGrath, D & Euler, CW 2022, 'Watch your Strep: *Streptococcus pyogenes* is a preventable cause of maternal death', *Future Microbiology,* vol. 17, no. 5, pp. 319-323.

Schirmbeck, R, Melber, K, Mertens, T & Reimann, J 1994, 'Antibody and cytotoxic T-cell responses to soluble hepatitis B virus (HBV) S antigen in mice: implication for the pathogenesis of HBV-induced hepatitis', *Journal of Virology,* vol. 68, no*.* 3, pp. 1418-1425.

Schwarz, B & Douglas, T 2015, 'Development of virus-like particles for diagnostic and prophylactic biomedical applications', *Wiley interdisciplinary reviews. Nanomedicine and nanobiotechnology,* vol. 7, no. 5, pp. 722-735.

Shouval, D, Roggendorf, H & Roggendorf, M 2015, 'Enhanced immune response to hepatitis B vaccination through immunization with a Pre-S1/Pre-S2/S Vaccine', *Medical Microbiology and Immunology,* vol. 204, no. 1, pp. 57-68.

Steer, AC, Carapetis, JR, Dale, JB, Fraser, JD, Good, MF, Guilherme, L, Moreland, NJ, Mulholland, EK, Schodel, F & Smeesters, PR 2016, 'Status of research and development of vaccines for *Streptococcus pyogenes*', *Vaccine,* vol. 34, no. 26, pp. 2953-2958.

Stevens, DL & Bryant, AE 2016, 'Severe Group A Streptococcal infections', in Ferretti, JJ, Stevens, DL & Fischetti, VA (Eds.), *Streptococcus pyogenes: Basic biology to clinical manifestations*, Oklahoma City: University of Oklahoma Health Sciences Cente.

Sullivan, EA, Vaughan, G, Li, Z, Peek, MJ, Carapetis, JR, Walsh, W, Frawley, J, Rémond, M, Remenyi, B, Jackson Pulver, L, Kruske, S, Belton, S & McLintock, C 2020, 'The high prevalence and impact of rheumatic heart disease in pregnancy in First Nations populations in a high-income setting: a prospective cohort study', *British Journal of Obstetrics and Gynaecology,* Vol. 127, no. 1, pp. 47-56.

Tariq, H, Batool, S, Asif, S, Ali, M & Abbasi, BH 2022 'Virus-Like Particles: Revolutionary Platforms for Developing Vaccines Against Emerging Infectious Diseases', *Frontiers in Microbiology,* vol. 12, e790121

Telethon Kids Institute. (2016). Group A Streptococcal Diseases: Coalition to advance new vaccines against group A streptococcus (CANVAS). Retrieved from http://www.telethonkids.org.au/our-research/projects-index/g/group-a-streptococcal-diseases-coalition-to-advance-new-vaccines-against-group-a-streptococcus-(canvas

Thompson, KM, Sterkel, AK, McBride, JA & Corliss, RF 2018, 'The shock of Strep: Rapid deaths due to Group A Streptococcus', *Academic Forensic Pathology,* vol. 8, no. 1, pp. 136-149.

Wannamaker, LW 1973 'The chain that links the heart to the throat', *Circulation,* vol. 48, no. 1, pp. 9-18.

Watkins, DA, Johnson, CO, Colquhoun, SM, Karthikeyan, G, Beaton, A, Bukhman, G, Forouzanfar, MH, Longenecker, CT, Mayosi, BM, Mensah, GA, Nascimento, BR, Ribeiro, ALP, Sable, CA, Steer, AC, Naghavi, M, Mokdad, AH, Murray, CJL, Vos, T, Carapetis, JR & Roth, GA 2017, 'Global, regional, and national burden of rheumatic heart disease, 1990–2015, *New England Journal of Medicine,* vol. 377, no. 8, pp. 713-722.

Wen, GP, He, L, Tang, ZM, Wang, SL, Zhang, X, Chen, YZ, Lin, X, Liu, C, Chen, JX, Ying, D, Chen, ZH, Wang, YB, Luo, WX, Huang, SJ, Li, SW, Zhang, J, Zheng, ZZ, Zhu, J & Xia, NS 2020, 'Quantitative evaluation of protective antibody response induced by hepatitis E vaccine in humans', *Nature Communications,* vol. 11, no. 1, pp. 3971.

White, H, Walsh, W, Brown, A, Riddell, T, Tonkin, A, Jeremy, R & Kritharides, L et al. 2010 'Rheumatic heart disease in indigenous populations', *Heart Lung Circulation,* vol. 19, no.5-6, pp. 273-281.

Whitehead, BD, Smith, HV & Nourse, C 2011, 'Invasive group A streptococcal disease in children in Queensland', *Epidemiology & Infection,* vol. 139, no. 4, pp. 623-628.

WHO 2018, '71st World Health Assembly adopts resolution calling for greater action on rheumatic heart disease', retrieved from https://www.who.int/news/item/25-05-2018-seventy-first-world-health-assembly-update-25-may

WHO 2020, 'Rheumatic Heart Disease', retrieved from https://www.who.int/news-room/fact-sheets/detail/rheumatic-heart-disease

Wilby, KJ, Lau, TT, Gilchrist, SE & Ensom, MH 2012, 'Mosquirix (RTS,S): a novel vaccine for the prevention of *Plasmodium falciparum* malaria', *Annals of Pharmacotherapy,* vol. 46, no. 3, pp. 384-393.

Wright, CM, Langworthy, K & Manning, L 2021, 'The Australian burden of invasive group A streptococcal disease: a narrative review', *Internal Medicine Journal,* vol. 51, no. 6, pp. 835-844.

Wu, X, Chen, P, Lin, H, Hao, X & Liang, Z 2016, 'Hepatitis E virus: Current epidemiology and vaccine', *Human Vaccines & Immunotherapeutics,* Vol. 12, no. 10, pp. 2603-2610.

Wyber, R, Noonan, K, Halkon, C, Enkel, S, Cannon, J, Haynes, E, Mitchell, AG, Bessarab, DC, Katzenellenbogen, JM, Bond-Smith, D, Seth, R, D'Antoine, H, Ralph, AP, Bowen, AC, Brown, A & Carapetis, JR 2020, 'Ending rheumatic heart disease in Australia: the evidence for a new approach', *Medical Journal of Australia,* vol. 213, Supp. 10, pp. 3-31.

Wyber, R, Wade, V, Anderson, A, Schreiber, Y, Saginur, R, Brown, A & Carapetis, JR 2021, 'Rheumatic heart disease in Indigenous young peoples', *The Lancet Child & Adolescent Health,* vol. 5, no. 6, pp. 437-446.

Zhang, N, Li, K, Liu, Z, Nandakumar, KS & Jiang, S 2022, 'A Perspective on the roles of adjuvants in developing highly potent COVID-19 vaccines', *Viruses,* vol. 14, no. 2, pp. 387