doi: 10.1016/j.mimet.2017.01.010.

Development of a high-throughput opsonophagocytic assay for the determination of functional antibody activity against *Streptococcus pyogenes* using bioluminescence

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Working title: GAS bactericidal assay using bioluminescence

Abstract: (50 words): The lack of standardised protocols for the assessment of functional antibodies has hindered *Streptococcus pyogenes* research and the development of vaccines. A robust, high throughput opsonophagocytic bactericidal assay to determine protective antibodies in human and rabbit serum has been developed that utilises bioluminescence as a rapid read out.

Keywords: Group A streptococcus (GAS); bactericidal assay; bioluminescence; Lancefield assay; correlates of protection; protective antibodies.

Group A streptococcus (GAS) or *Streptococcus pyogenes* is a major human pathogen that causes a range of diseases, from minor localised skin infections and pharyngitis to severe conditions such as streptococcal toxic shock syndrome and necrotising fasciitis (Carapetis et al., 2005, Cunningham, 2008, Walker et al., 2014). Recurring pharyngitis and skin infections have been linked to the development of acute rheumatic fever (ARF) and rheumatic heart disease (RHD), autoimmune conditions with high mortality rates (Carapetis et al., 2016, Cunningham, 2016, Martin et al., 2015). Much of the GAS disease burden exists in resource poor settings, which can make efficient treatment and control difficult. This has fuelled efforts to develop a GAS vaccine with several candidates in various stages of development (Moreland et al., 2014, Steer et al., 2009, Steer et al., 2016). However, progress is slowed by the lack of a robust assay to assess the protective capacity of vaccine antisera. The two main types of immunoassay currently used are enzyme-linked immunosorbent assays (ELISAs) and functional bactericidal assays. While ELISAs are highly standardised, reproducible and allow for a high-throughput, they are limited by the fact that the measured immune response does not distinguish between functional and non-functional antibodies. Existing functional bactericidal assays to determine protective antibodies are based on the classical Lancefield assay (Lancefield, 1959). These indirect bactericidal assays are low throughput, labour-intensive and require fresh human whole blood as a source of phagocytes, resulting in high variability between experiments (Tsoi et al., 2015).

Here we show the development of a robust, high-throughput opsonophagocytic assay that reflects protective capacity by determining functional antibodies. The assay overcomes the challenges of previous assays by using rabbit peripheral blood leukocytes (PBLs) as the source of phagocytes and an exogenous source of complement, thereby mimicking *in vitro* the *in vivo* process of host effector cell-mediated killing following bacterial opsonisation with specific antibodies. Furthermore, our assay introduces bioluminescence as a rapid, high throughput detection method of bacterial survival.

To obtain specific antiserum for assay development, female New Zealand White rabbits were immunised subcutaneously with 50μg recombinant M1 protein in Incomplete Freund's adjuvant (Sigma-Aldrich) at 0, 2, 4, and 6 weeks. Recombinant M1 protein was cloned from genomic DNA of the GAS strain SF370 using the primers ctagGGATCCaacggtgatggtaatcctagg and ctagGAATTCctgtctcttagtttccttcattgg. Protein was expressed in *E. coli* BL21(λDE3)pLysS using the expression vector pET32a3c, and purified by nickel-affinity chromatography as previously described (Young et al., 2014). M1 antiserum was obtained two weeks after the final injection and reactivity against recombinant M1 confirmed by ELISA (Figure 1A). Affinity sepharose was made by coupling recombinant M1 protein to CNBr-Activated Sepharose 4B (GE Healthcare) according to manufacturer's instructions. M1 antiserum was depleted by overnight end-over-end rotation at 4°C of antiserum mixed with affinity sepharose in a 1:1 ratio. Reactivity of the anti-M1 serum by ELISA was abolished after depletion over an M1-affinity column, but was retained when antiserum was passed over a control sepharose column demonstrating the presence of M1-specific antibodies in the serum (Figure 1A). To standardise complement activity in the experimental set up, all sera used were heat-inactivated for 30 min at 56°C and exogenous complement activity added with commercial rabbit complement (Sigma-Aldrich).

In the indirect bactericidal assay complement is a source of variability, with complement activity influenced by the health status of the donor as well the anticoagulant, timing and storage conditions used during the blood draw. Besides non-standardised complement activity, human whole blood frequently contains pre-existing anti-GAS antibodies that need to be screened for prior to use (Moreland et al., 2014). Isolating the source of phagocytes, together with the use of exogenous complement, overcomes these problems. To isolate fresh rabbit PBLs, rabbit blood was taken by venepuncture of the ear marginal vein in heparinised vacutainers (BD) and mixed with a 5-fold volume of 3% dextran (molecular weight, 500,000,

Sigma-Aldrich), 0.9% NaCl and incubated for 45min at room temperature (RT). Following dextran erythrocyte sedimentation PBLs contained in the supernatant were pelleted (10min, 500xg at RT) and any remaining erythrocytes lysed by resuspension in sterile MilliQ H_2O for 30s followed by immediate restoration of tonicity with an equal volume of 1.8% (w/v) NaCl. PBLs were pelleted (10min, 500xg at RT) and resuspended in pre-warmed (37°C) Hanks' Balanced Salt solution (HBSS) containing 0.5mM $MgCl_2$, 1.3mM $CaCl_2$, $HBSS^{++}$ Gibco) at a final concentration of $5x10^6$ cells/ml.

To prepare bacteria for the assays, the GAS strains M1 (strain SF370, ATCC reference 700294) and a M1-protein deletion mutant (ΔM1) (Loh et al., 2013) were cultured in Brain Heart Infusion (BHI) medium (Difco, BD). Overnight cultures were diluted 1:100 in fresh broth and grown to mid-log phase (OD_{600} of 0.4 to 0.6), before addition of 10% sterile glycerol (v/v) and freezing at -80°C. To enumerate bacteria, several aliquots were defrosted by incubation at 37°C for 5min, serially diluted and plated for colony count after incubation over night at 37°C. To prepare the bacterial inoculum for the experimental set up bacterial aliquots were rapidly defrosted, pelleted and resuspended in $HBSS^{++}$.

Initial opsonophagocytic assays were performed by incubation of $2x10^3$ bacteria with 20% serum and 2% rabbit complement for 30 min at RT prior to addition of $4x10^5$ isolated lymphocytes made up to a total assay volume of 500µl with $HBSS^{++}$. Bacterial survival was determined after 3 h incubation at 37°C with end over end rotation by plating on BHI agar (Difco, BD) and CFU count the next day. A minimum 5-fold increase in bacterial growth was observed with naïve serum over the 3 h incubation period (Figure 1B). Inclusion of anti-M1 antiserum in the experimental set-up significantly reduced the growth of WT bacteria, but did not affect the growth of GAS ΔM1 (Figure 1B). When individual components such as the PBLs or complement were left out of the experimental set up, comparable bacterial growth to the naïve serum control was observed (Figure 1B). These results confirmed that the use of isolated sources of complement (commercial rabbit serum) and phagocytic cells (rabbit

leukocytes) provides a solid basis towards the establishment of a more standardised opsonophagocytic assay. Equally important, the removal of whole blood as a source of phagocytes and the associated light absorption by haemoglobin, enabled us to investigate the use of bioluminescence as an approach to simply and rapidly assess bacterial survival without the need of time-consuming plate enumeration.

Bioluminescence was introduced into GAS strains by transfection with the stabilised, bioluminescence reporter plasmid pLZ12Km2-P23R:TA_Ffluc and showed bioluminescence signals that were proportional to the number of viable cells (Loh and Proft, 2013). All plasmid-containing strains were cultured in BHI medium supplemented with 200µg/ml kanamycin and aliquots were generated as outlined above. Opsonophagocytic assays were performed as before and samples were enumerated by plating in parallel to measuring light emission in an EnSpire multilabel plate reader (PerkinElmer) after addition of 100µg/ml luciferin substrate at 1:1 (v/v). The percentage of bacterial killing was determined as [(CFU or BLU with naive $serum_{t=3}$ − CFU or BLU with immune $serum_{t=3}$) ÷ CFU or BLU with naive $serum_{t=3}$] × 100.

Both detection methods showed comparative killing (Figure 1C), confirming the suitability of bioluminescence as fast read out of bacterial survival to replace time consuming plating and CFU counts. After the establishment of bioluminescence as a read out, the total assay volume was downscaled to 100µl and the complete experimental set up moved to a white, flat-bottom, 96-well plate (Lab supply ltd.) format. From there on bioluminescence opsonophagocytic assays were performed by incubation of $2x10^3$ bacteria with 20% serum and 2% rabbit complement for 30 min at RT prior to addition of $8x10^4$ isolated PBLs and incubation in a ThermoMixer (Eppendorf) for 3 h at 37°C, 300rpm. Downscaling reduces use of reagents, while at the same time increasing the number of possible samples to be tested in a single assay. This might be of importance in future applications, when sera to be tested are of limited availability, such as in clinical trials or for the analysis of patient samples.

The killing observed in our assay set up was anti-M1 antibody specific, as depletion of the antiserum over a M1-affinity column significantly reduced killing of the wt strain, but had no significant effect on the ΔM1 strain (Figure 1D). Passing of antiserum over a control sepharose column had no effect on the ability of the antiserum to induce bacterial killing.

In order to broaden the application of the assay to human antibodies, we tested the use of pooled human intravenous immunoglobulin (IVIG, Intagram® P, CSL Behring). Human intravenous immunoglobulin (IVIG) is a mixture of polyclonal IgG antibodies isolated and pooled from thousands of healthy human donors. IVIG has been suggested as adjunct therapy for streptococcal toxic shock syndrome due to the high levels of antibodies against GAS (Linner et al., 2014, Schrage et al., 2006, Sriskandan et al., 2006). ELISA analysis of IVIG revealed reactivity against strain-specific hypervariable region peptides of the M proteins of GAS serotypes M1, M6 and M89 (aa1-50 of the corresponding mature M protein [M1 SF370, M6 MGAS10394, M89 GAS131361], chemically synthesised by GenScript USA Inc) (Figure 2A). This reactivity was confirmed by flow cytometry analysis of whole bacteria (data not shown). GAS strains MGAS10394 (M6, ATCC BAA-946) and 89/54 (M89, clinical isolate (Steemson, 2014)) were labelled with the bioluminescent plasmid as described (Loh and Proft, 2013) and used in the bioluminescence opsonophagocytic assay adapted for human IVIG as follows. Human IVIG in PBS, pH 7.4, 20% (v/v) fetal bovine serum or control (pH 7.4, 20% (v/v) fetal bovine serum) was incubated with $2x10^3$ bacteria and 2% commercial rabbit complement serum for 30 min at RT prior to addition of $8x10^4$ isolated PBLs and incubation in a ThermoMixer C (Eppendorf) for 3 h at 37°C with agitation (300rpm). Bacterial killing was detected with bioluminescence as outlined above, and percentage killing calculated as [(BLU with control $buffer_{t=3}$ − BLU with $IVIG_{t=3}$) ÷ BLU with control $buffer_{t=3}$] × 100. While antibody responses against all three strains were detected by ELISA and flow cytometry, bacterial killing was only observed for the M1 and the M89 strain (Figure 2B). The lack of killing for M6 suggests the anti-M6 antibodies in IVIG are non-functional and highlights the importance of

evaluating functional antibodies in opsonophagocytic assays rather than total antibodies as in traditional immunoassays such as ELISAs. These results show that our bioluminescence opsonophagocytic assay can successfully be used to determine functional antibodies in both rabbit and human polyclonal preparations. Additional optimisation of the assay could include testing of murine antiserum, further broadening the application possibilities of this assay.

Conclusion

A good antibody response does not always correlate with protective capacity and traditional methods such as ELISAs fail to distinguish between functional and non-functional antibodies. However, for the development of vaccine candidates, evaluation of functional antibodies and bactericidal activity are crucial. We have developed a robust assay that allows easy assessment of protective antibodies from human and rabbit serum. This high throughput, opsonophagocytic assay for GAS could greatly advance vaccine development as it enables rapid assessment of vaccine candidates in rabbits, as well as samples from future clinical studies in man. The versatility of the plasmid used to introduce bioluminescence into the target GAS strains (with over 50 strains labelled in our laboratory to date), together with the linear correlation between light and CFU observed, will make testing for functional, cross protective antibodies against large panels of GAS strains possible in the future.

This work was supported by research grants from the Health Research Council of New Zealand (HRC 15/172) and the School of Medicine Foundation, The University of Auckland (3710302). JMSL is a New Zealand Heart Foundation Research Fellow.

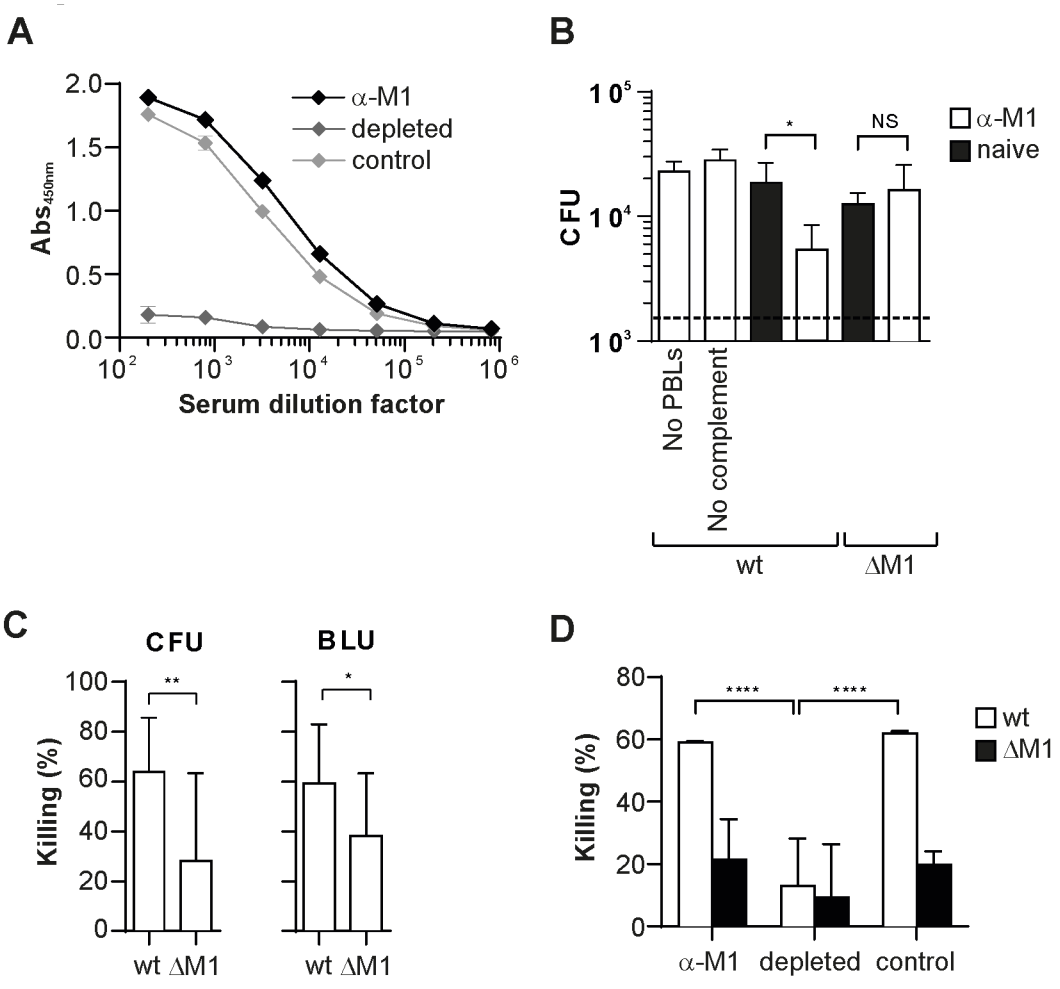

Figure 1: Establishment of a GAS opsonophagocytic assay using bioluminescence

A) ELISA showing reactivity levels of M1 antiserum (α-M1), M1-affinity depleted antiserum (depleted) and antiserum passed over a sepharose control column (control) against recombinant M1 protein. Data shown as mean ± SD. **B)** Tube-based opsonophagocytic assay of wild-type GAS M1 (wt) and GAS M1 deletion mutant (ΔM1) after incubation with or without isolated PBLs/complement as determined by CFU counts. Dashed line indicates CFU of inoculum. Data combines four independent experiments and is shown as mean ± SD, analysed by Wilcoxon matched-pairs signed rank test, * $P < 0.05$, NS=not significant. **C)** Comparison of bacterial killing as determined by CFU counts and bioluminescence units (BLU) in a tube-based assay format. Bacterial killing (%) was determined as [($CFU_{t=3}$ naive serum − $CFU_{t=3}$ immune serum) ÷ $CFU_{t=3}$ naive serum] × 100. For bioluminescence read-out, killing (%) was

191 determined as [($BLU_{t=3}$ naive serum − $BLU_{t=3}$ immune serum) ÷ $BLU_{t=3}$ naive serum] × 100.
192 Data combines nine independent experiments and is shown as mean ± SD, analysed by paired
193 t test, * $P < 0.05$ and ** $P < 0.01$. **D)** Plate-based opsonophagocytic assay of GAS M1 wt (wt)
194 and GAS deletion mutant (ΔM1) with M1 specific antiserum (α-M1), M1-affinity depleted
195 antiserum (depleted) and antiserum passed over a sepharose control column (control). Data
196 combines three independent experiments and is shown as mean ± SD, statistically analysed by
197 one-way ANOVA with Tukey's multiple comparisons test, *** $P < 0.001$.
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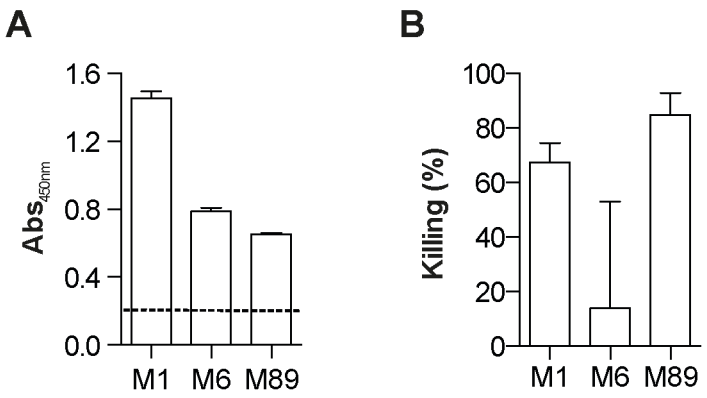

Figure 2: Establishment of the GAS opsonophagocytic assay using human antibodies

A) Reactivity of human IVIG against the hypervariable region peptides of GAS serotypes M1, M6 and M89 as determined by ELISA. Dashed line indicates reactivity two SD above the background control. **B)** Bacterial killing of GAS serotypes M1, M6 and M89 after opsonisation with human IVIG. Data combines two independent experiments and is shown as mean ± SD.

References

Carapetis, J.R., Beaton, A., Cunningham, M.W., Guilherme, L., Karthikeyan, G., Mayosi, B.M., Sable, C., Steer, A., Wilson, N., Wyber, R., Zuhlke, L., 2016. Acute rheumatic fever and rheumatic heart disease. Nature Rev. 2, 15084.

Carapetis, J.R., Steer, A.C., Mulholland, E.K., Weber, M., 2005. The global burden of group A streptococcal diseases. Lancet Infect. Dis. 5, 685-694.

Cunningham, M.W., 2008. Pathogenesis of group A streptococcal infections and their sequelae. Adv. Exp. Med. Biol. 609, 29-42.

Cunningham, M.W., 2016. Post-Streptococcal Autoimmune Sequelae: Rheumatic Fever and Beyond. In: J. J. Ferretti, D. L. Stevens, V. A. Fischetti (Eds.), *Streptococcus pyogenes*: Basic Biology to Clinical Manifestations, University of Oklahoma Health Sciences Center (c) The University of Oklahoma Health Sciences Center., Oklahoma City (OK).

Lancefield, R.C., 1959. Persistence of type-specific antibodies in man following infection with group A streptococci. J. Exp. Med. 110, 271-292.

Linner, A., Darenberg, J., Sjolin, J., Henriques-Normark, B., Norrby-Teglund, A., 2014. Clinical efficacy of polyspecific intravenous immunoglobulin therapy in patients with streptococcal toxic shock syndrome: a comparative observational study. Clin. Infect. Dis. 59, 851-857.

Loh, J.M., Proft, T., 2013. Toxin-antitoxin-stabilized reporter plasmids for biophotonic imaging of Group A streptococcus. Appl. Microbiol. Biotechnol. 97, 9737-9745.

Loh, J.M.S., Adenwalla, N., Wiles, S., Proft, T., 2013. *Galleria mellonella* larvae as an infection model for group A streptococcus. Virulence. 4, 419-428.

Martin, W.J., Steer, A.C., Smeesters, P.R., Keeble, J., Inouye, M., Carapetis, J., Wicks, I.P., 2015. Post-infectious group A streptococcal autoimmune syndromes and the heart. Autoimmun. Rev. 14, 710-725.

Moreland, N.J., Waddington, C.S., Williamson, D.A., Sriskandan, S., Smeesters, P.R., Proft, T., Steer, A.C., Walker, M.J., Baker, E.N., Baker, M.G., Lennon, D., Dunbar, R., Carapetis, J., Fraser, J.D., 2014. Working towards a group A streptococcal vaccine: report of a collaborative Trans-Tasman workshop. Vaccine. 32, 3713-3720.

Schrage, B., Duan, G., Yang, L.P., Fraser, J.D., Proft, T., 2006. Different preparations of intravenous immunoglobulin vary in their efficacy to neutralize streptococcal superantigens: implications for treatment of streptococcal toxic shock syndrome. Clin. Infect. Dis. 43, 743-746.

Sriskandan, S., Ferguson, M., Elliot, V., Faulkner, L., Cohen, J., 2006. Human intravenous immunoglobulin for experimental streptococcal toxic shock: bacterial clearance and modulation of inflammation. J. Antimicrob. Chemoth. 58, 117-124.

Steer, A.C., Batzloff, M.R., Mulholland, K., Carapetis, J.R., 2009. Group A streptococcal vaccines: facts versus fantasy. Curr. Opin. Infect. Dis. 22, 544-552.

Steer, A.C., Carapetis, J.R., Dale, J.B., Fraser, J.D., Good, M.F., Guilherme, L., Moreland, N.J., Mulholland, E.K., Schodel, F., Smeesters, P.R., 2016. Status of research and development of vaccines for *Streptococcus pyogenes.* Vaccine. 34, 2953-2958.

Tsoi, S.K., Smeesters, P.R., Frost, H.R., Licciardi, P., Steer, A.C., 2015. Correlates of Protection for M Protein-Based Vaccines against Group A Streptococcus. J. Immunol. Res. 2015, 167089.

Walker, M.J., Barnett, T.C., McArthur, J.D., Cole, J.N., Gillen, C.M., Henningham, A., Sriprakash, K.S., Sanderson-Smith, M.L., Nizet, V., 2014. Disease manifestations and pathogenic mechanisms of Group A Streptococcus. Clin. Microbiol. Rev. 27, 264-301.