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Abstract: Group A Streptococcus (GAS) or Streptococcus pyogenes is
responsible for an estimated 500,000 deaths worldwide each year.
Protection against GAS infection is thought to be mediated by
phagocytosis, enhanced by bacteria-specific antibody. There are no
licenced GAS vaccines, despite many promising candidates in preclinical
and early stage clinical development, the most advanced of which are
based on the GAS M-protein. Vaccine progress has been hindered, in part,
by the lack of a standardised functional assay suitable for vaccine
evaluation. Current assays, developed over 50 years ago, rely on non-
immune human whole blood as a source of neutrophils and complement.
Variations in complement and neutrophil activity between donors result in
variable data that is difficult to interpret. We have developed an
opsonophagocytic killing assay (OPKA) for GAS that utilises
dimethylformamide (DMF)-differentiated human promyelocytic leukemia cells
(HL-60) as a source of neutrophils and baby rabbit complement, thus
removing the major sources of variation in current assays. We have
standardised the OPKA for several clinically relevant GAS strain types
(emm1, emm6 and emm12) and have shown antibody-specific killing for each
emm-type using M-protein specific rabbit antisera. Specificity was
demonstrated by pre-incubation of the antisera with homologous M-protein
antigens that blocked antibody-specific killing. Additional
qualifications of the GAS OPKA, including the assessment of the accuracy,
precision, linearity and the lower limit of quantification, were also
performed. This GAS OPKA assay has the potential to provide a robust and
reproducible platform to accelerate GAS vaccine development.

Highlights (for review)

1. Opsonophagocytic killing assay (OPKA) developed for a number of clinically relevant Group A *Streptococcus* (GAS).

2. Homologous rabbit full-length M-protein antisera and human IVIg shown to effectively kill GAS strains expressing *emm*-types 1, 6 and 12.

3. Killing of bacteria is effectively inhibited by homologous antigen but not heterologous antigens proving assay specificity.

4. GAS OPKA shown to be highly precise with intra-assay and inter-assay coefficients of variation less than 30%.

*Abstract

1   Group A *Streptococcus* (GAS) or *Streptococcus pyogenes* is responsible for an estimated

2   500,000 deaths worldwide each year. Protection against GAS infection is thought to be mediated by

3   phagocytosis, enhanced by bacteria-specific antibody. There are no licenced GAS vaccines, despite

4   many promising candidates in preclinical and early stage clinical development, the most advanced of

5   which are based on the GAS M-protein. Vaccine progress has been hindered, in part, by the lack of a

6   standardised functional assay suitable for vaccine evaluation. Current assays, developed over 50

7   years ago, rely on non-immune human whole blood as a source of neutrophils and complement.

8   Variations in complement and neutrophil activity between donors result in variable data that is

9   difficult to interpret. We have developed an opsonophagocytic killing assay (OPKA) for GAS that

10   utilises dimethylformamide (DMF)-differentiated human promyelocytic leukemia cells (HL-60) as a

11   source of neutrophils and baby rabbit complement, thus removing the major sources of variation in

12   current assays. We have standardised the OPKA for several clinically relevant GAS strain types

13   (*emm1, emm6* and *emm12*) and have shown antibody-specific killing for each *emm*-type using M-

14   protein specific rabbit antisera. Specificity was demonstrated by pre-incubation of the antisera with

15   homologous M-protein antigens that blocked antibody-specific killing. Additional qualifications of

16   the GAS OPKA, including the assessment of the accuracy, precision, linearity and the lower limit of

17   quantification, were also performed. This GAS OPKA assay has the potential to provide a robust and

18   reproducible platform to accelerate GAS vaccine development.

DEVELOPMENT OF AN OPSONOPHAGOCYTIC KILLING ASSAY FOR GROUP A STREPTOCOCCUS

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1. INTRODUCTION

Group A *Streptococcus* (GAS) or *Streptococcus pyogenes* is a species of Gram-positive bacteria responsible for a significant worldwide burden of disease [1-4]. Clinical syndromes resulting from GAS infection include superficial infections, such as pyoderma and pharyngitis, severe invasive GAS disease (iGAS), acute rheumatic fever (ARF) and associated rheumatic heart disease (RHD), and post-streptococcal glomerulonephritis. Conservative estimates suggest that more than 33 million people are afflicted with severe GAS disease that results in roughly 500,000 deaths each year, with RHD responsible for two thirds of this mortality [5].

Despite the substantial morbidity and mortality GAS infection poses, there are currently no licenced vaccines against GAS [6, 7]. Epidemiological data reveals that the majority of infections occur within the ages of 1-15, suggesting that immunity to GAS develops with age [8, 9]. First identified by Rebecca Lancefield, the M-protein is a major GAS virulence factor that comprises a hypervariable N-terminus, which forms the basis for GAS strain typing (known as *emm*-typing), and a conserved C-terminus [10, 11]. The M-protein has multiple functions, including an ability to subvert phagocytosis by binding multiple host proteins [11, 12]. Antibodies specific to the M protein have long been known produce a protective immune response and as such the M protein is being extensively investigated as a vaccine antigen [10, 13]. M-protein based vaccines are generally composed of either the hypervariable N-terminal region of multiple *emm*-types or the conserved C-terminal region and have been shown in early stage clinical trials to be safe and well tolerated [10, 14-18]. Non-M-protein based vaccines are also in development, though none have yet been tested in human trials. These tend to comprise a combination of conserved GAS antigens that have been identified through reverse vaccinology or studies on GAS virulence [19-21]. Such antigens include C5a peptidase, serine esterase, GAS carbohydrate, fibronectin-binding protein, serum opacity factor, SpyCep and T-antigens [6, 7, 14, 22-25].

25   The progress of GAS vaccine development has been hindered, in part, by the lack of a

26   reliable and fully standardised functional assay that assesses immunity to GAS. The bactericidal assay

27   used most widely to assess the immunogenicity of GAS vaccine antigens is known as the Lancefield

28   assay [13, 26-29]. The Lancefield assay measures the growth and survival of GAS in fresh human

29   blood from an immune individual (direct) or non-immune whole blood supplemented with serum

30   from an immune individual or animal immunised with an antigen of interest (indirect) [28, 29].

31   Natural variations in complement and neutrophil activity that exist between individuals often result

32   in variable data that is difficult to interpret, and does not allow for comparison of results between

33   laboratories [21, 30]. In addition, widespread natural exposure to GAS renders identifying a non-

34   immune donor (i.e. an individual with minimal reactivity to the bacterium) laborious [24, 29].

35   The need for improved assays to measure GAS vaccine efficiency has been highlighted as a

36   priority by the international vaccine community and was recently recognized by the Coalition to

37   Accelerate New Vaccines Against Streptococcus (CANVAS) [6, 14, 18, 24]. CANVAS is an Australian

38   and New Zealand government-sponsored GAS vaccine development programme that aims to

39   overcome key developmental hurdles for GAS vaccines. The coalition was formed in response to the

40   unacceptably high rates of ARF and RHD in Indigenous populations in Australia and New Zealand [31,

41   32]. To overcome the issues of variability associated with the traditional Lancefield methods, we

42   have developed a GAS opsonophagocytic killing assay (OPKA) that uses an exogenous source of both

43   complement and phagocytic cells. This assay is adapted from a validated pneumococcal OPKA which

44   makes use of baby rabbit complement (BRC) as the exogenous source of complement and

45   dimethylformamide (DMF) -differentiated human promyelocytic leukemia cells (HL-60) as the

46   exogenous source of phagocytic cells [33-36]. Using high titre anti-M protein rabbit sera and human

47   intravenous immunoglobulin, we have developed and optimised an OPKA for a number of clinically

48   relevant GAS strains.

2. MATERIALS AND METHODS

2.1. Bacterial Strains

The following GAS strains were used in this study: emm1 (strains 43, 02-12 and GAS05134), emm12 (strains 611020, 611025 and GAS09437) and emm6 (strain GASOPA6_02). These emm-types were selected because they belong to distinct *emm*-protein clusters in the recently described *emm* cluster typing system [37]. Additional strain details are listed in **Table 1**. Working stocks of bacterial strains were generated by streaking a fleck of frozen master stocks onto a horse blood agar plate (Fisher Scientific, Leicestershire, UK), which was incubated overnight at 37°C, 5% CO_2. Todd-Hewitt Broth (THB; Sigma-Aldrich Company Ltd, Dorset, UK) was inoculated with a single colony and grown at 37°C, 5% CO_2 to an OD_{600} (optical density at 600nm) of 0.6-0.7; considered to be the late-log phase of growth. Once the required OD_{600} was reached, cultures were mixed 1:1 with STGG medium (3% w/v tryptone soya broth (Thermo Fisher Scientific Oxoid Ltd, Basingstoke, UK), 0.5% w/v D-glucose (Sigma-Aldrich Company Ltd), 10% v/v glycerol (Sigma-Aldrich Company Ltd) in sterile, pyrogen-free water (Baxter Healthcare UK, Newbury, UK) and stored in 0.5ml aliquots at -80°C until required.

2.2. Antisera

Two sets of sera were used to optimise the GAS OPKA: human intravenous immunoglobulin (IVIg; Euglobulin®; Baxter, Berkshire, UK) and rabbit anti-full-length M-protein sera. To generate M-proteins for rabbit vaccinations *emm*1 (SF370) and *emm*6 (MGAS10394) were amplified from genomic DNA. The primer pairs ctagGGATCCaacggtgatggtaatcctagg and ctagGAATTCctgtctcttagtttccttcattgg were used for *emm1*, and cgcGGATCCagagtgtttcctagggggacg and ctagGAATTCctgtctcttagtttccttcattgg for *emm6*. The genes were cloned into pET32a3c sequences and the sequences were confirmed by Sanger sequencing. For *emm*12 (HKU16) the gene was synthesised (Life technologies) and inserted into pET151/D-TOPO. Each of the three proteins were expressed with the signal sequence and transmembrane regions removed (M1, aaN42-Q449; M6, aaR43-T358; M12, aaD42-Q540) in BL21(DE3) pLysS *E.coli* and purified by immobilised-metal affinity

74 chromatography (IMAC) as previously described [37, 38]. The His_6 tag was cleaved from the

75 recombinant M proteins with 5 μg/ml 3C-His_6 protease (M1 and M6) or a 1:100 ratio of rTEV-His_6

76 protease to M protein (M12) for 16 hours at 4°C. The M-proteins were separated from the cleaved

77 His_6 tag and the rTEV-His_6 protease by IMAC and further purified by gel filtration. Female New

78 Zealand White rabbits were immunised subcutaneously with 200μg of the purified, recombinant M1,

79 M6 or M12 proteins in Incomplete Freund's adjuvant (Sigma-Aldrich) at 0, 2 and 4 weeks prior to

80 exsanguination. The reactivity of immune sera to the recombinant M proteins was confirmed by

81 ELISA (endpoint titre >100,000).

82 *2.3. HL-60 Cells*

83 Master stocks of HL-60 cells were prepared from frozen commercial stock (ATCC Standards

84 UK, Middlesex, UK). Working stocks of HL-60 cells were differentiated into neutrophil-like cells by

85 culture in 0.8% DMF (Sigma-Aldrich Company Ltd) in M2 medium (10% v/v FetalClone1 (Hyclone, GE

86 Healthcare Life Sciences, Buckinghamshire, UK), 1% L-Glutamine (Invitrogen, Life Technologies Ltd,

87 Paisley, UK), in RPMI 1640 (Invitrogen)) at a concentration of $4x10^5$ cells per ml. Cells were

88 differentiated at 37°C, 5% CO_2 for five to six days. On the day of the assay, cells were washed with

89 HBSS without Ca/Mg (Invitrogen), followed by HBSS with Ca/Mg (Invitrogen) and re-suspended at

90 $1x10^7$ cells per ml in fresh opsonisation buffer (10% v/v defined FBS (Hyclone), 0.1% w/v gelatin

91 (Sigma-Aldrich Company Ltd) in HBSS with Ca/Mg). The phenotype of differentiated HL-60 cells was

92 assessed by flow cytometry using mouse anti-human CD35 FITC conjugated antibody (Serotec, Bio-

93 Rad, Kidlington, UK) and/or mouse anti-human CD71 PE-conjugated antibody (Serotec).

94 Differentiated cells were accepted for use in the assay if the up-regulation of CD35 was ≥55% of the

95 cell population and CD71 expressing was down-regulated by ≥15% when compared to the working

96 stock preparation.

2.4. Complement

In order to reduce levels of non-specific killing of bacteria in the assay, new batches of baby

99   rabbit complement were pre-absorbed as previously described with minor changes [39]. Briefly,

100 200ml of three to four-week baby rabbit complement (Pel-Freez, AR, USA) was defrosted in ice-cold

101 water with constant agitation. Whilst still in ice-cold water, the defrosted complement was

102 inoculated with 10 colonies per 50ml of an overnight horse-blood agar culture of *Staphylococcus*

103 *aureus* (strain 6538) and incubated on a roller for 30 minutes at 4°C. The complement was then

104 vacuum filtered with a 0.22μM, aliquoted on ice and stored at -80°C until required.

105 *2.5. Opsonophagocytic Killing Assay*

106   Working stocks of bacteria were defrosted and washed and diluted in OPS buffer to 120,000

107 CFU per ml. Bacteria were added to samples serially diluted (in duplicate) with OPS buffer in a

108 round-bottomed 96-well plate and incubated for 30 minutes at room temperature on a mini-orbital

109 shaker at 700rpm. Diluted active or heat-inactivated BRC was added to each well followed by DMF-

110 differentiated HL-60 cells and the plate was incubated for an additional 90 minutes at 37°C, 5% CO_2

111 on a mini-orbital shaker at 700rpm. The ratio between HL-60 cells and bacteria in each assay was

112 approximately between 500-2000 HL-60 cells to one bacterial cell. Two complement only controls

113 (referred to as control A and control B hereafter) were included on each plate to calculate the level

114 of non-specific killing in an assay. Control A contains bacteria, differentiated HL-60 cells and heat-

115 inactivated complement only and control B contains bacteria, differentiated HL-60 cells and activate

116 complement only.

117   After the second incubation, plates were placed on ice for at least thirty minutes to stop the

118 reaction. After mixing well, 10μl of each well was 'spotted' onto Todd-Hewitt-yeast agar plates (THB

119 supplement with 0.5% w/v yeast extract (Sigma-Aldrich Company Ltd) and 1.5% w/v bacteriological

120 agar (Sigma-Aldrich Company Ltd), which was tilted to allow the spots to spread across one quarter

121 of the plate and left to dry at room temperature. Once dry, 20ml autoclaved overlay agar (THB

122 supplement with w/v yeast extract, 0.75% w/v bacteriological agar and 0.0025% 2, 3, 5-

123 tetraphenyltetrazolium chloride (Sigma-Aldrich Company Ltd) was poured over each plate and

124 allowed to set. Plates were then incubated overnight at 37°C, 5% CO_2.

125 The number of CFU per spot was enumerated using an automated colony counter

126 (ProtoCOL3; Synbiosis, Cambridge, UK). The percentage killing at each dilution of a sample was

127 calculated as: (CFU[control B]-CFU[antisera])/CFU[control B])*100 and the dilution of the sample

128 resulting in 50% killing was calculated as the opsonic index (OI) or opsonic titre. The percentage of

129 non-specific killing in each assay was calculated as: 1-(CFU[control B]/CFU[control A])*100. Assays

130 were accepted if levels of non-specific killing were ≤35% and the CFU of both complement controls

131 were between 50-200. Furthermore, serum samples were only reported as positive if the maximum

132 level of killing achieved was ≥70%. These acceptance criteria are comparable to those used for the

133 validated pneumococcal OPKA and are included to ensure the reproducibility and quality of assay

134 results [36].

135 *2.6 Determination of Assay Specificity*

136 Rabbit anti-full-length M-protein sera were pre-incubated with a titration of full-length M-

137 protein or OPS buffer only (0% inhibition control) for thirty minutes at room temperature. Washed

138 and diluted bacteria were then added to the pre-incubated samples and the assay was continued as

139 previously described. The percentage inhibition of killing at each concentration of antigen was

140 calculated as: (CFU[antisera:antigen]-CFU[0% inhibition control])/(CFU[control B]-CFU[0% inhibition

141 control])*100.

142 # 3. RESULTS

143 The GAS OPKA was adapted from a validated pneumococcal OPKA with several modifications

144 with respect to incubation times and the preparation and amount of exogenous BRC added [33, 34].

145 *3.1 GAS OPKA Optimisation*

146   Prior to performing the GAS OPKA, the dilution of bacterial working stocks resulting in spots

147 containing between 150 and 200 CFU was determined for each strain. The concentration of BRC is

148 critical to maintain non-specific killing below 35%, the acceptance criteria for the assay. To optimise

149 the assay with respect to non-specific killing, bacteria were incubated with a serial dilution of pre-

150 absorbed BRC in the presence of differentiated HL-60 cells. Representative complement titration

151 data of CFU at each concentration of heat-inactivated or active BRC and the level of non-specific

152 killing for GAS strain M1(43) is shown in **Figure 1**. In this example, the concentration of complement

153 resulting in <35% non-specific killing was 2.1%. Across all seven strains used in this study the optimal

154 concentration of baby rabbit complement was 2.1% for GAS strains M1(43), M1(02-12),

155 M1(GAS05134), M12(611020), M12(GAS09437) and 3.1% for GAS strains M12(611025) and

156 M6(GASOPA6_02) (Table 2).

157 *3.2 GAS OPKA*

158   Once assay conditions were optimised for each GAS strain, the ability of human IVIg and

159 rabbit anti-full-length M-protein sera to kill GAS was assessed. Representative data for two GAS

160 strains M1(43) and M12(611020) tested against human IVIg, rabbit antisera from the homologous

161 M-type (M1 or M12), matched pre-immunisation sera and rabbit anti-sera for the heterologous M-

162 types are shown in **Figure 2**. There was a marked reduction in CFU of M1(43) in the presence of the

163 M1 anti-sera and IVIg, but not for the pre-immune sera or the M6 and M12 antisera. Similarly, there

164 was a marked reduction in CFU for the M12(611020) strain in the presence of M12 anti-sera and

165 IVIg, but not for pre-immune sera, or the M1 and M6 antisera. This marked M type-specific killing is

166 further demonstrated by the OIs determined for each sera against the seven GAS strain as

167 summarized in **Table 2**. Killing of all strains by homologous full-length M-protein antisera and human

168 IVIg had an average OI of 4089 and 6497, respectively. In contrast no killing by heterologous antisera

169 or pre-immunisation sera was observed for any of the strains tested.

3.3 GAS OPKA Specificity

171   The specificity of the GAS OPKA was determined by assessing the ability of homologous and

172 heterologous purified M-protein to inhibit the killing of bacteria by rabbit anti-full-length M-protein

173 sera. Representative data of percentage inhibition of killing by rabbit anti-full-length M1 sera, pre-

174 incubated with full-length M1, M6 or M12, run against the GAS strain M1(43) is shown in *Figure 3a*.

175 In this example, maximum inhibition of killing achieved by the homologous antigen (M1) was 86.5%

176 and the maximum inhibition of killing by the heterologous antigens (M6 and M12) was 9.7% and

177 10.7%, respectively. By fitting a sigmoidal standard curve to log[x] transformed data, the

178 concentration of homologous M1 antigen resulting in 50% inhibition of killing of strain M1(43) in this

179 example was interpolated to be 72.4ng/ml. Across all the seven strains investigated the average

180 concentration of homologous antigen resulting in 50% inhibition of killing was 131ng/ml ±76.25. The

181 maximum inhibition of killing by homologous and heterologous antigens for each GAS strain is

182 detailed in *Table 3*.

183 *3.4 GAS OPKA Precision*

184   GAS OPKA precision was assessed by running rabbit anti-full-length M-protein antisera three

185 times either on the same plate with one control (intra-assay coefficient of variation) or on three

186 different plates with individual controls (inter-assay coefficient of variation) with each GAS strain.

187 Representative data from each of the three runs used to calculate the intra-assay coefficient of

188 variation and inter-assay coefficient of variation for GAS strain M1(43) are shown in *Figure 3a* and

189 *Figure 3b*, respectively. The intra-assay and inter-assay coefficients of variation for each optimised

190 strain are detailed in *Table 3*. The intra-assay coefficient of variation of the GAS OPKA was assessed

191 to be 15.4% and the inter-assay coefficient of variation of the GAS OPKA was assessed to be 17.9%.

192 *3.5 Relative Accuracy*

193   The relative accuracy of the GAS OPKA was assessed by running rabbit full-length M-protein

194 antisera through the GAS OPKA at four different starting dilutions. The percent agreement (whether

195   OIs are within a factor of three of each other) and coefficient of variation between the OIs for each

196   of these four samples was calculated for each strain. Representative data for the calculation of the

197   relative accuracy of GAS strain M1(43), is shown in *Figure 3d*. In this example, rabbit anti-full-length

198   M1 was pre-diluted to a factor of 4, 16, 32 and 64 and had OIs between 2060-2967 against M1(43)

199   resulting in a coefficient of variation of 15.21%. The relative accuracy of each optimised GAS strain is

200   detailed in *Table 3*. The average coefficient of variation across all seven GAS strain studied was

201   calculated as 15.59% and OI agreement was at 100% for all strains.

202   *3.6 Linearity*

203   The linearity of the GAS OPKA was assessed by running non-immune rabbit serum, spiked

204   with four different concentrations of full-length M-protein antisera, through the GAS OPKA. The

205   correlation (R-squared) between initial sample dilution (log transformed) and resultant OI (log

206   transformed) of the four samples was calculated for each strain. Representative data for the

207   calculation of OPKA linearity for GAS strain M1(43) is shown in *Figure 3e*. In this example, rabbit anti-

208   full-length M1 was pre-diluted to a factor of 4, 16, 32 and 64 and had OIs of 2700, 740, 604 and 128

209   against M1(43), respectively. The correlation (R-squared) was calculated as 0.993 with a slope of -

210   1.096±0.065. The linearity for each optimised GAS strain is detailed in *Table 3*. Across all seven

211   strains, the average correlation (R-squared) between initial sample dilution (log transformed) and

212   resultant OI (log transformed) was 0.987 with an average gradient (slope) of -1.07.

213   *3.7 Lower Limit of Quantification*

214   The lower limit of quantification (LLOQ) of the GAS OPKA was assessed by spiking low

215   concentrations of full-length M-protein-rabbit serum into heat-inactivated non-immune rabbit

216   serum for each corresponding GAS strain to produce a sample with an opsonic index of 4-12. Each

217   sample was generated individually four times and run through the assay in duplicate. Representative

218   data for the calculation of the LLOQ of GAS strain M1(43), is shown in *Figure 3f*. In this example, the

219   average OI of these spiked samples was calculated as 8.75±0.5. The LLOQ for each of the seven GAS

220   strains is detailed in **Table 3**. The average LLOQ across the seven strains was calculated as 7.9.

4. DISCUSSION

222   We have optimised an OPKA for a number of clinically relevant GAS strain types (*emm1,*

223   *emm6* and *emm12*). Additionally, we have taken the first steps towards qualifying and standardising

224   the GAS OPKA; assay specificity, precision, relative accuracy, linearity and lower limit of

225   quantification [40]. Both IVIg and anti-M protein specific sera generated in rabbits were able to kill

226   each of the GAS strains used in study. The specificity of the GAS OPKA was established by measuring

227   the ability of homologous or heterologous full-length M-protein to block the ability to kill GAS. For

228   each of the strains tested, homologous antigen was able to block >70% of killing whereas

229   heterologous antigen was only able to block 10% thus proving assay specificity. The strains used in

230   this study strains belong to three distinct *emm*-cluster types: this cluster system classifies M-proteins

231   based on their functional and structural features and the lack of heterologous killing observed in our

232   study is in line with the distinct cluster phylogeny of emm1 (cluster A-C3), emm6 (single protein

233   cluster M6) and emm12 (cluster A-C4) [37]. The ability of certain M-proteins, including M1 used in

234   this study, to non-specifically bind human immunoglobulin via the Fc region is well described [37, 41,

235   42]. At the highest concentration tested (1.25μg/ml), soluble M1 protein did not significantly inhibit

236   killing by either the rabbit anti-M6 or rabbit anti-M12 serum. This suggests that the affinity of the

237   non-specific interaction between soluble M1-IgG Fc is lower than the affinity of the specific

238   interaction between antisera and surface bound M-protein such that opsonophagocytosis occurs.

239   While *emm*1 is the most common GAS *emm* type globally, each of the *emm*-types used in

240   this study is typically considered to be associated with disease in the developed world [43]. As such,

241   cumulatively *emm1*, *emm6* and *emm12* contribute to approximately 34% of GAS related disease in

242   Established Market Countries but only 10% and 3% of GAS-related disease in Africa and Pacific Island

243   nations/Indigenous Australians, respectively [43]. Furthermore, there is a large degree of *emm*-type

244 diversity associated with ARF in Maori and Pacific children in New Zealand with *emm1*, *emm6* and

245 *emm12* rarely observed [44]. Optimising GAS OPKA conditions for additional strains that are clinically

246 relevant to low and middle-income countries, and Indigenous populations at high risk of GAS disease

247 in New Zealand and Australia, will form a significant part of future work.

248 The GAS strains in this study were cultured to late exponential phase (an OD_{600} of 0.6-0.7)

249 following the current multiplexed opsonisation assay for *Streptococcus pneumoniae* [45]. Licenced

250 pneumococcal vaccines are composed of the polysaccharides that make up the bacterial capsule and

251 culturing bacteria to an OD_{600} of 0.7 ensures sufficient capsule growth required for the assessment

252 of pneumococcal polysaccharides antibody function [46]. As with *Streptococcus pneumoniae*, GAS

253 capsule expression peaks during this exponential growth-phase [47]. For non-M protein GAS

254 antigens that may be obscured by excessive capsule expression, culturing of GAS strains to late

255 exponential phase may prove ineffective and optimisation of assays with early log-phase GAS maybe

256 be required.

257 The inter-assay and intra-assay coefficients of variation of the GAS OPKA were assessed and

258 found to be <30% for all strains which is acceptable for a dynamic biological assay such as the assay

259 described here. The average LLOQ was assessed to be 7.9, the relative accuracy coefficient of

260 variation was 15.6% and the assay linearity correlation was 0.987; all well within the acceptable

261 levels of variation for an assay of this complexity [39, 48, 49]. Full GAS OPKA standardisation will

262 require additional qualification as recommended by the ICH Harmonised Tripartite Guidelines for the

263 validation of analytical procedures [40]. These additional qualifications will need to include an

264 assessment of assay robustness and reproducibility between laboratories.

265 Correlates of protection do not currently exist for immunity to a GAS infection [9]. The

266 standardised, reproducible and specific opsonophagocytic killing assay described here should enable

267 a correlate to be developed when used in conjunction with convalescent GAS sera from individuals

268 who have recovered from a GAS infection or vaccine trials with a clinical endpoint. While our study

269   has demonstrated that the assay should enable correlates to be determined for M-protein based

270   vaccines, it also has the potential to become the gold-standard assay for vaccines comprised of

271   conserved, cell wall-associated GAS antigens. In conclusion, this standardised GAS OPKA should

272   provide a reliable method to assess GAS immunity and support the licensure of GAS vaccine

273   candidates.

AUTHORS' CONTRIBUTION

DG, NJM, JRC and JDF conceived of the presented idea and consulted throughout. SJ wrote the manuscript in consultation with NJM, SS, JRC, JDF and DG. SJ, MZ, NJM, JR and MSJL performed the experiments. GAS strains were provided by the CANVAS strain selection group (including PRS and NJM) and SS. SS also provided advice and expertise on GAS biology and human IVIg. DG supervised the findings of this work.

CONFLICTS OF INTEREST

Authors declare no conflicts of interest.

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Tables

Table 1 GAS Strains

Summary table of the GAS strains used in this study with additional details of M-clusters, clinical syndrome association, country of origin and year of isolation.

M- type	M-cluster	Strain ID	Clinical syndrome	Country of origin	Year of isolation
1	A-C3	*43*	Pharyngitis	UK	2009
		02-12	Pharyngitis	Brazil	2012
		GAS05134	Acute rheumatic fever	New Zealand	2005
12	A-C4	*611020*	Pharyngitis	Australia	2011
		611025	Pharyngitis	Australia	2011
		GAS09437	Acute rheumatic fever	New Zealand	2009
6	Single protein M-cluster	*GASOPA6_02*	Pharyngitis	UK	2009

Table 2 Opsonophagocytic Killing Assay Summary

Summary table of GAS OPKA assay conditions (optimal concentration of baby rabbit complement and second incubation times), mean levels of non-specific killing and mean opsonic indexes achieved with human IVIg, pre and post rabbit anti-full-length M-protein antisera. The mean levels of non-specific killing were calculated from seven sequential assays. Mean opsonic indexes were calculated from three sequential assays.

M-Protein		1			12			6
Strain ID		*43*	*02-12*	*GAS05134*	*611020*	*611025*	*GAS09437*	*GASOPA6_02*
Optimal Concentration of Complement		*2.1%*	*2.1%*	*2.1%*	*2.1%*	*3.1%*	*2.1%*	*3.1%*
Non-specific killing *(n=7)*		*20.3*	*13.4*	*6.87*	*13.5*	*20.2*	*13.0*	*30.6*
Incubation Time *(mins)*		*60*	*60*	*60*	*60*	*90*	*60*	*60*
GMT Opsonic Index *(n=3)*	**Pre-immunisation**	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*
	Homologous	*2307*	*2753*	*2571*	*7610*	*4685*	*2902*	*5794*
	Heterologous (1)	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*
	Heterologous (2)	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*	*<10*
	IVIg	*4840*	*3443*	*4303*	*3360*	*12599*	*15663*	*1271*

Table 3 GAS OPKA Qualifications Summary

Summary table of assay qualifications. The maximum percentage inhibition of killing by rabbit anti-full-length M-protein sera (pre-incubated with purified homologous or heterologous purified M-protein) is shown for each strain (n=1). Intra-assay and inter-assay coefficients of variation were calculated by running rabbit anti-full-length M-protein sera three times on the same assay or three individual assays, respectively. The coefficient of variation of opsonic indexes is shown for each strain (n=3). The relative accuracy was assessed by running rabbit full-length M-protein antisera, diluted to four different factors with OPS buffer. The percent agreement (whether OIs are within a factor of 3 of each other) and coefficient of variation (%) between the OIs for each of these four samples is shown (n=4). The linearity of the GAS OPKA was assessed by running non-immune rabbit serum, spiked with four different concentrations of full-length M-protein antisera. The correlation (R-squared) between initial sample dilution (log transformed) and resultant OI (log transformed) of the four samples is shown (n=4). The lower limit of quantification (LLOQ) was assessed by running full-length M-protein-spiked rabbit serum against each GAS strain. These samples were generated by spiking heat-inactivated non-immune rabbit serum with sufficient homologous rabbit anti-full-length M-protein antisera to produce a sample with an opsonic index of 4-12. The average OI is shown for each run (n=4).

| M-Protein		1			12			6
Strain ID		*43*	*02-12*	*GAS05134*	*611020*	*611025*	*GAS09437*	*GASOPA6_2*
Specificity (n=1)	Inhibition by homologous antigen (%)	*87*	*73*	*85*	*83*	*100*	*85*	*71*
	Inhibition by heterologous antigen (%)	*11*	*12*	*10*	*9*	*11*	*11*	*6*
Precision (n=3)	Intra-assay coefficient of variation (%)	*24.7*	*10.8*	*21.3*	*19.5*	*16.2*	*6.7*	*8.9*
	Inter-assay coefficient of variation (%)	*26.4*	*20.0*	*18.0*	*21.5*	*20.9*	*9.4*	*9.0*
Relative Accuracy (n=4)	Agreement (%)	*100*	*100*	*100*	*100*	*100*	*100*	*100*
	Coefficient of variation (%)	*15.21*	*14.5*	*8.69*	*17.0*	*20.8*	*16.1*	*22.3*
Linearity (n=4)	R-squared	*0.993*	*0.996*	*0.997*	*0.966*	*0.976*	*0.988*	*0.993*
	Slope	*-1.10*	*-1.14*	*-1.04*	*-1.18*	*-1.01*	*-1.11*	*-0.92*
LLOQ (n=4)		*8.75*	*14.75*	*7.50*	*6.50*	*5.00*	*6.75*	*6.25*

Figures
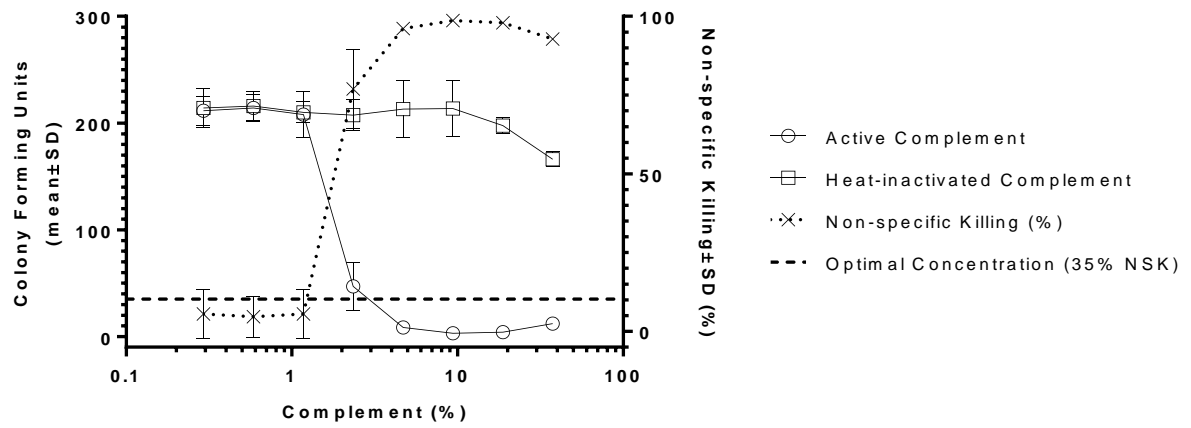
Fig 1 GAS OPKA Optimisation
The optimal concentration of BRC for GAS OPKA was calculated by incubating bacteria with a serial dilution of BRC in the presence of differentiated HL-60 cells. **b**, Representative data showing the number of CFU (left-y-axis) and percentage non-specific killing (right-y-axis, dotted line) at each dilution of heat-inactivated (open squares) or active (open circles) BRC with GAS strain M1(43) and differentiated HL-60 cells. Dilutions of BRC resulting in <35% non-specific killing were used as the optimal dilution (right-y-axis, dashed line).
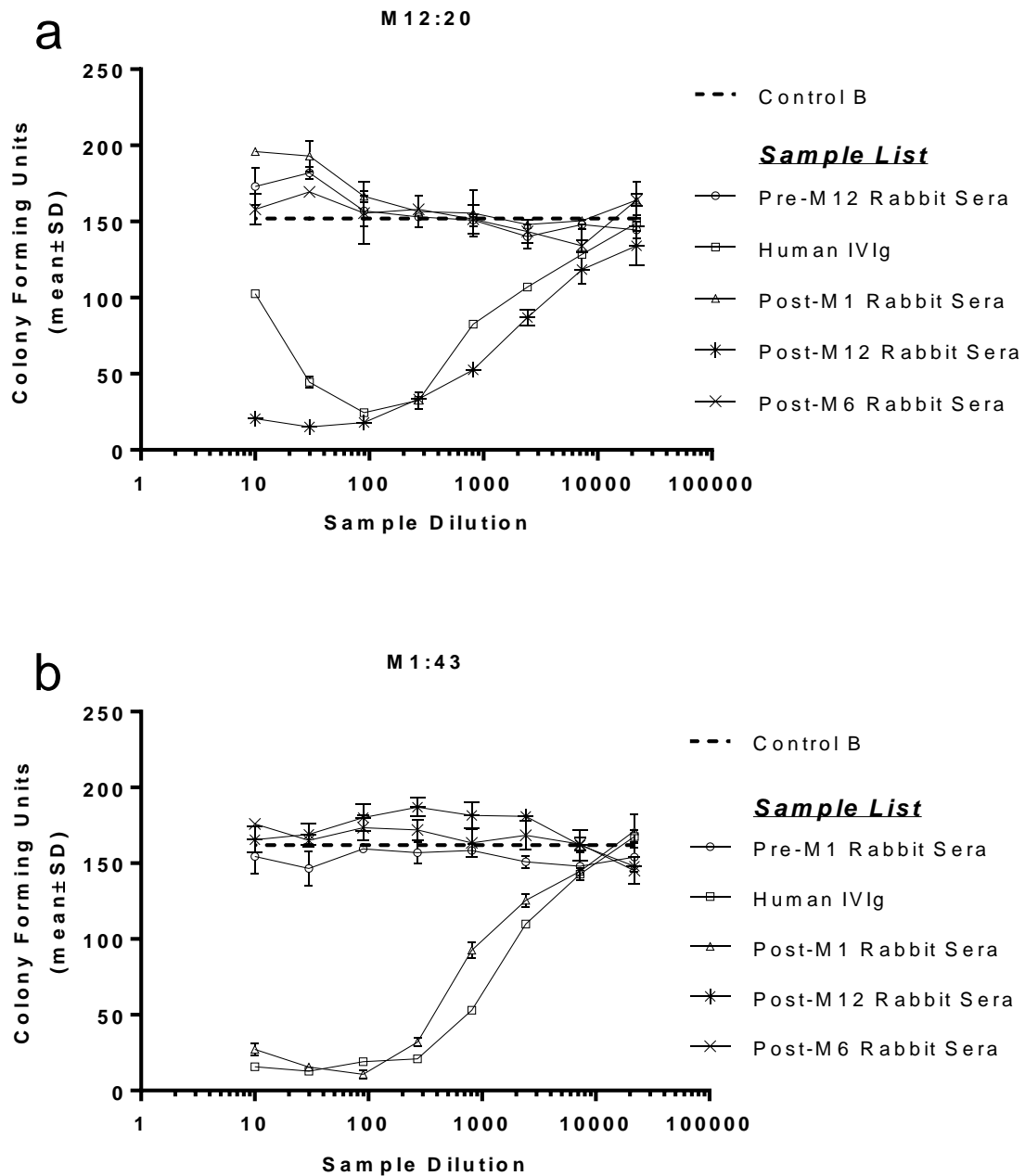
Fig 2 – Opsonophagocytic Killing Assay
Representative GAS OPKA data for GAS strains M1(43) (**a**) and M12(611020) (**b**). Both strains were run with human IVIg (open squares), rabbit anti-M1 sera (open triangles), rabbit anti-M6 sera (crosses), rabbit anti-M12 sera (stars) and either pre-M1 immunisation sera or pre-M12 immunisation sera (open circles). The average number of colony forming units (CFU) at each sample dilution is shown with the active complement control (control B – dashed line).
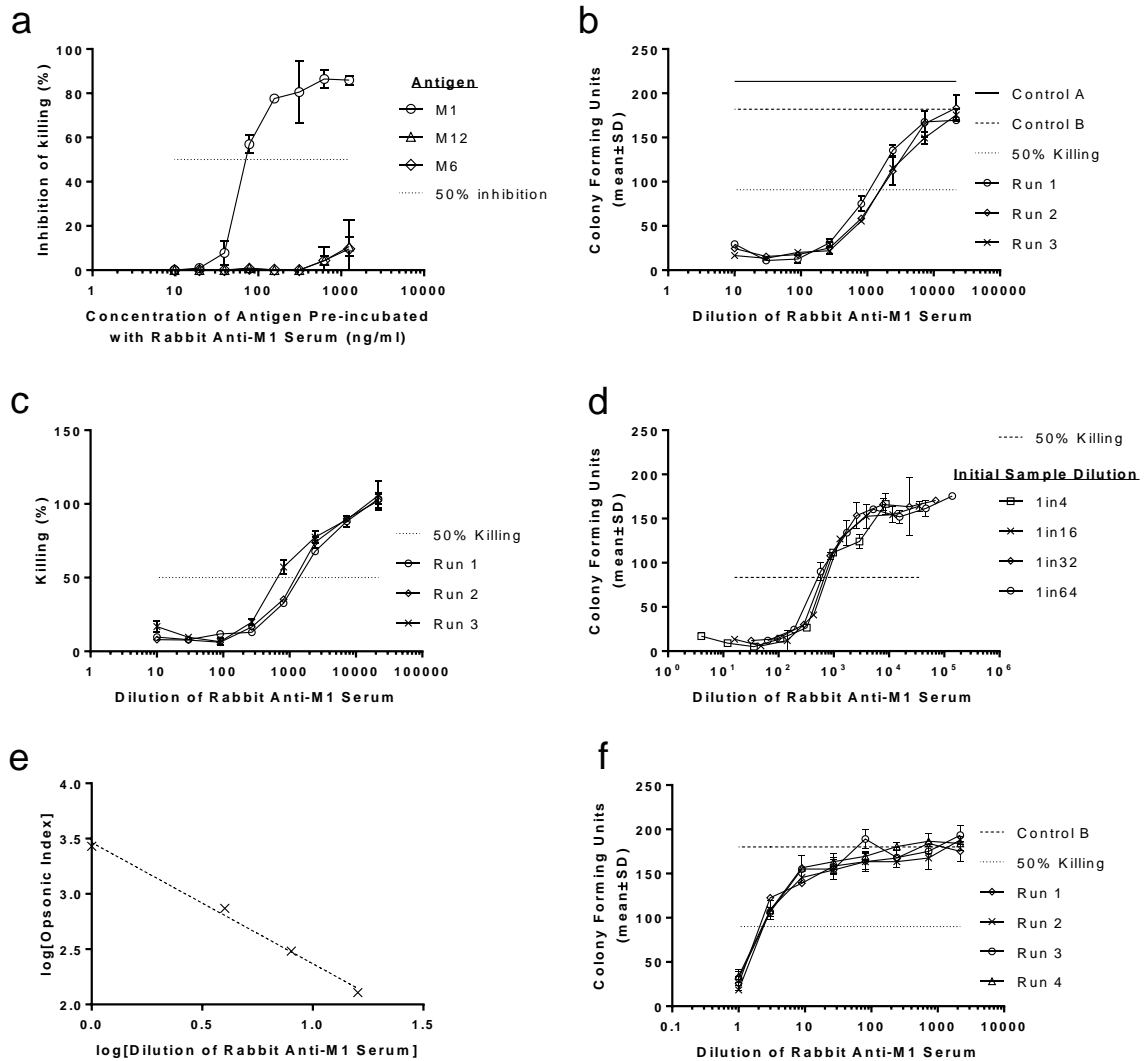
Fig 3 GAS OPKA Qualifications

a, Percentage inhibition of killing of the GAS strain M1(43) by rabbit anti-M1 serum pre-incubated with purified M1 (open circles), M6 (open diamond) or M12 (open triangle) protein. The maximum inhibition of killing achieved by homologous antigen (M1) was 86.5% whereas maximum inhibition by heterologous antigen was 9.7% (M6) and 10.7% (M12). **b**, Rabbit anti-full-length M-protein antiserum was run against GAS strain M1(43) three times on the same plate to calculate the intra-assay coefficient of variation of the opsonic index (50% killing – dotted line). The average number of colony forming units (CFU) at each sample dilution for the three runs is shown with heat-inactivated (A – solid line) and active (B – dashed line) complement controls. **c**, Rabbit anti-full-length M-protein antisera was run against GAS strain M1(43) three times on three different plates with individual controls to calculate the inter-assay coefficient of variation of the opsonic index (50% killing – dotted line). The average percentage killing of M1(43) at each sample dilution for the three runs is shown. **d**, Rabbit anti-full-length M-protein antiserum was run against GAS strain M1(43) at four different dilution on the same plate to calculate the relative accuracy. The average number of CFU at each sample dilution for the four initial dilution is shown with the 50% killing line (dashed line). **e**, Non-immune rabbit serum was spiked with four different concentrations of rabbit anti-full-length M-protein antiserum and run against GAS strain M1(43) to assess assay linearity. The opsonic index at each sample dilution (both log transformed) is shown with the line of linear regression (dashed line). **f**, Non-immune rabbit serum was spiked with sufficient rabbit anti-full-length M-protein antiserum to produce an opsonic index of 4 and run against GAS strain M1(43) to assess the lower limit of quantification. The average number of CFU at each sample dilution of four runs is shown with the 50% killing line (dotted line) and CFU with active complement (control B – dashed line).