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Development and evaluation of a new triplex immunoassay that detects Group A *Streptococcus* antibodies for the diagnosis of rheumatic fever

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ABSTRACT

Objectives: Streptococcal serology is a cornerstone in the diagnosis of acute rheumatic fever (ARF), a post-infectious sequalae associated with Group A *Streptococcus* infection. Current tests that measure anti-streptolysin-O (ASO) and anti-DNaseB (ADB) titres require parallel processing, with predictive value limited by the slow rate of decay in antibody response. Accordingly, our objective was to develop and assess the diagnostic potential of a triplex bead-based assay, which simultaneously quantifies ASO and ADB together with titres for a third antigen, SpnA.

Methods: Our previous cytometric bead assay was transferred to the clinically appropriate Luminex platform by coupling streptolysin-O, DNaseB and SpnA to spectrally unique magnetic beads. Sera from over 350 subjects, including 97 ARF patients, were used to validate the assay and explore immunokinetics.

Results: Operating parameters demonstrate the triplex assay produces accurate and reproducible antibody titres which, for ASO and ADB, are highly correlative with existing assay methodology. When ARF patients were stratified by time (days following hospital admission) there was no difference in ASO and ADB between <28 and 28+ days groups. However, for anti-SpnA there was a significant decrease (P<0.05) in the 28+ day group, indicative of faster anti-SpnA antibody decay.

Conclusions: Anti-SpnA immunokinetics support very recent Group A *Streptococcus* infection, and may assist in diagnostic classification of ARF. Further, bead-based assays enable streptococcal serology to be performed efficiently in a high-throughput manner.

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Key words: group A streptococcus; SpnA; streptolysin-O; DNaseB; serology; acute rheumatic fever; immunoassay

INTRODUCTION

Group A Streptococcus (GAS; *Streptococcus pyogenes*) causes a broad range of diseases in humans, including skin and throat infections that may lead to more serious autoimmune sequelae such as acute rheumatic fever (ARF) and post-streptococcal glomerulonephritis (PSGN) (1). ARF precedes rheumatic heart disease (RHD), which is responsible for major morbidity and mortality in resource-poor countries and in certain Indigenous populations in high-income countries (2). In particular, Māori and Pacific children in New Zealand, and Aboriginal children in Australia, have some of the highest incidences of ARF and RHD in the world (3, 4).

Accurate diagnosis of ARF is essential to ensure timely hospitalisation, clinical management and initiation of secondary prevention (monthly benzathine penicillin injections) to reduce the risk of RHD. The Jones criteria are a set of clinical guidelines that forms the basis of ARF diagnosis; these criteria specifically include laboratory evidence of a previous GAS infection (5). Since ARF occurs 2-4 weeks after an initial GAS infection, the causative bacteria are often undetectable by culture at the time of clinical presentation. Streptococcal serology, the measurement of antibodies produced in response to a GAS infection, is therefore commonly used as definitive laboratory evidence of infection (6). Current ARF guidelines require a patient to have elevated antibody titres to one of two GAS antigens – Streptolysin O (SLO) and Deoxyribonuclease B (DNaseB) (7, 8).

Demonstrating a rise in anti-SLO (ASO) and anti-DNaseB (ADB) antibody titres for the diagnosis of post-streptococcal diseases requires paired serum measurements at both the acute and convalescent stage of infection. This can be impractical to obtain in patients with suspected ARF (9), such that an upper limit of normal (ULN) cut-off is often applied in lieu of the gold standard two-fold rise in titre (8, 10). ULN cut-offs are defined as the 80th

71 percentile of ASO and ADB titres in a matched healthy population, and can be challenging to
72 establish in GAS endemic settings, resulting in a broad range of ULN recommendations for
73 ASO and ADB globally (9).

74 ASO is generally measured by an automated turbidimetric or nephelometric assay that utilises
75 an international standard of pooled sera (8, 11). While there is a nephelometric assay for
76 ADB, it is commonly measured with an enzyme inhibition assay. This inhibition assay is only
77 semi-quantitative and there are no international reference sera available for ADB, making
78 these assays less standardised (8). With currently available methods, the titres for ASO and
79 ADB need to be determined in two separate assays in parallel, which impacts on laboratory
80 efficiency. In addition to the limitation associated with parallel processing, the antigens
81 themselves have inherent limitations. The gene encoding DNaseB shows allelic variation and
82 is not universally present across all GAS strains. This can lead to false negatives, where a
83 lack of ADB response may be the result of a true GAS infection with a strain not carrying a
84 DNaseB encoding gene (12). Furthermore, the immunokinetics of ASO and ADB complicate
85 interpretation. The titres to both antigens can stay elevated for many weeks after infection
86 and in some individuals elevated titres have been observed 12 months after a GAS infection
87 (13). This can lead to false-positives, where elevated antibody titres do not necessarily
88 represent a recent infection, but are the consequence of slow antibody decay following a GAS
89 infection that occurred months or even years prior.

90 To circumvent some of the limitations with current streptococcal serology, our laboratory
91 recently developed a proof-of-concept multiplex immunoassay using cytometric bead array
92 (CBA) technology (14). The assay simultaneously measured ASO and ADB titres as well as
93 antibody titres to a third, novel GAS antigen, *Streptococcus pyogenes* nuclease A (SpnA,
94 Spy0747), which is a highly conserved and immunogenic cell-wall anchored nuclease (15,
95 16). However, the CBA platform is not currently suitable for routine use in clinical

laboratories due to a lack of automation and reliance on a flow cytometer for read-out. Accordingly, the aims of the current study were to: (i) design and optimise the multiplex immunoassay technology for the clinically available Luminex bead-based platform and; (ii) assess the efficiency and accuracy of the resulting triplex assay in diagnosing ARF in a large clinical cohort. The Luminex system is based on spectrally unique magnetic beads that enable simultaneous measurement of antibodies against multiple analytes in a single reaction well. Compared with CBA, Luminex assays tend to be more reproducible, can be automated, and the machines are widely used in clinical diagnostic laboratories (17-19).

METHODS

Study subjects

Human sera were obtained from several studies conducted in the North Island of New Zealand. Each had appropriate ethical approval and all participants (or their parents or legal guardians) provided written informed consent. All ARF cases (n=97) were diagnosed according to the New Zealand modification of the Jones criteria (7, 20). Sixteen cases were recruited via two small studies conducted at Waikato Hospital (2012–2015; ethics CEN/12/06/017) and Starship Hospital (2004-2006; ethics AKX/2002/08). The remaining 79 cases were recruited as part of the Rheumatic Fever Risk Factors (RF RISK) study (21). This nationwide study was conducted between 2014-2017 (ethics 14/NTA/53) and aimed to identify modifiable risk factors for ARF in New Zealand. Sera was also obtained from 120 healthy controls recruited into the RF RISK study and highly matched with the ARF cases for age, ethnicity and social deprivation. Forty-five of these participants were excluded from analysis as they reported a definite or probable sore throat or skin infection in the 4 weeks preceding enrolment, such that the final healthy control group comprised 75 participants. Demographics for all ARF cases and healthy controls are shown in Table 1. Finally, to enable

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comparison of the triplex assay with existing commercial serology assays, residual sera (n=180) were stored following routine streptococcal serological testing in 2018 at Labtests, a community laboratory that serves the greater Auckland region (ethics 17/CEN/230). Demographic information was not collected for these samples.

Recombinant antigens

Recombinant DNaseB **(**rDNaseB) and rSpnA were expressed and purified as previously described (14). The *slo* gene was cloned using GeneArt Gene Synthesis technology (ThermoFisher). DNA encoding a SLO double mutant (aa32-571) with substitutions at amino acid sites P427L and W535F was synthesised and cloned into pET151/D-TOPO for expression of a detoxified SLO construct with an N-terminal His6-tag. The protein was expressed in *Escherichia coli* BL21AI cells after induction with 1 mM IPTG and 0.2% L-arabinose (Alfa Aeser) at 37 °C for 2.5 hours. rSLO was purified from *E. coli* cell lysate using Ni2+ NTA immobilised metal affinity chromatography and the His6–tag was cleaved using recombinant tobacco etch virus protease at a 1:20 ratio of protease to protein. The protein was subject to a final purification step using a Superdex200 10/300 column (GE Healthcare) in phosphate buffered saline (PBS) and fractions containing rSLO were pooled following verification by SDS-PAGE.

Bead coupling

The three antigens were covalently coupled to MagPlex® microspheres (beads) by carbodiimide chemistry using the xMAP® Antibody Coupling Kit (Luminex Corporation), according to the manufacturer's instructions. Briefly, magnetic beads were washed with activation buffer followed by a 20 min incubation with EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) and Sulfo-NHS (N-hydroxysulfosuccinimide). Each protein antigen was coupled to a spectrally distinct bead region; rSLO to region 72, rDNaseB to region 30 and rSpnA to region 78. Small-scale

coupling reactions with protein concentrations ranging from 10–30 µg per 2.5 x 10^6 beads were performed by incubating the proteins with beads for 2 hours at room temperature to determine optimal coupling concentrations for each protein. For large-scale reactions 12.5 x 10^6 beads were incubated with proteins for 2 hours at room temperature. Protein-coupled beads were washed and enumerated with a haemocytometer, and stored at 4ºC protected from light until further use.

Triplex immunoassay overview

Serum samples were diluted 1:8000 in assay buffer (AB) containing PBS supplemented with 0.5% IgG-free bovine serum albumin (BSA). The diluted sera (30 µl) were added into duplicate wells of a 96-well U-bottom plate (Greiner) and 30 µl of bead solution consisting of equal parts SLO, DNaseB and SpnA-coupled beads was added to samples for a final concentration of 50 beads/µl/antigen. Plates were incubated for 1 hour at room temperature and then washed twice with AB using a handheld magnet (Luminex Corporation). Phycoerythrin (PE)-labelled Donkey Anti-human IgG (Fcy-specific) detection antibody (Jackson Immunoresearch) was added to wells and incubated for 1.5 hours at room temperature. Beads were washed twice, re-suspended in 100 µl Drive Fluid (Luminex Corporation), and analysed on a MagPix® instrument (Luminex Corporation). All incubations were performed on a plate shaker at 800rpm, protected from light.

Standard material was obtained by purifying IgG antibodies specific for rDNaseB, rSLO and rSpnA from pooled human immunoglobulin (intravenous immunoglobulin, IVIG, Intragam® P) as previously described (14). The specificity of the purified IgG antibodies was confirmed by ELISA. A nine-point standard curve was created for each antigen by mixing known starting concentrations of specific IgG in AB (600 ng/ml for SLO, 400ng/ml for DNaseB and 250ng/ml for SpnA) and performing a three-fold dilution series. Standard curves were fitted

using a five-parameter regression formula generated by the xPonent software version 4.2 (Luminex Corporation). Net median fluorescence intensity (MFI) values obtained for test sera were converted to concentration (μg/ml) based on the IgG standard curves.

Assay sensitivity and precision

The lower limit of detection (LLOD) for each antigen was defined as the lowest concentration of analyte whose MFI response was greater than the blank plus three standard deviations (22) across 12 independent assays. Intra-assay variability was determined by calculating the coefficient of variation (CV; standard deviation divided by the mean) from 20 replicates of one sample in a single plate. Inter-assay variability was determined by calculating the CV from eight serum samples that exhibited varying degrees of reactivity against the three antigens, in eight independent assay runs (23).

Comparison with commercial assays

ASO and ADB titres were determined at Labtests, Auckland, New Zealand and LabPLUS, Auckland City Hospital, New Zealand. ASO titres were measured by the turbidimetric technique using the human anti–streptolysin-O kit on a SPAplus analyzer (The Binding Site, CA, USA) and reported in international units (IU/ml). ADB titres were measured by an enzyme inhibition assay following manufacturer instructions and titres reported in U/ml (bioMerieux, Marcy l'Etoile, France). Both assays provide an inexact, titre range for samples with a low concentration of antibodies; <25 IU/ml for ASO and <100 U/ml for ADB. Mid-titre values of 12.5 IU/ml for ASO and 50 U/ml for ADB were therefore used to define samples that fell within these ranges.

Upper Limit of Normal

192 The sensitivity of each antigen as a serological marker in ARF diagnosis was assessed by
193 applying experimentally determined upper limit of normal (ULN) cut-offs that were
194 calculated using a nonparametric method (24). ULN values were determined as the 80th
195 percentile of titres in the 75 healthy controls recruited in the RF RISK study (21), with those
196 that reported a sore throat or skin infection in the 4 weeks preceding enrolment having
197 already been excluded. This exclusion is in line with previous studies that have removed
198 individuals with recent streptococcal infections from ULN estimates (24, 25). ULN were
199 determined from titres measured with the triplex assay as well as titres measured with
200 commercial serological methods.

201 ***Statistical Analyses***

202 Differences in antibody responses between ARF cases and healthy controls for the three
203 antigens were analysed using Mann-Whitney U unpaired *t*-test. For the comparison of the
204 triplex measurements with commercial methods, the strength of correlation was calculated
205 using Spearman's *r*. Linear regression analysis was also performed on these data to obtain
206 equations that enabled conversion of triplex concentrations (μg/ml) into internationally
207 recognized units (IU/mL for ASO and U/mL for ADB). The diagnostic sensitivity of the
208 three antigens (SLO, DNaseB, SpnA) was compared using a chi-squared test. All statistical
209 analyses were performed in GraphPad Prism (version 8.0) and a *P*-value of ≤ 0.05 was
210 considered significant.

211

212 **RESULTS**

213 ***Optimisation and validation of the triplex Luminex assay***

In order to expand the utility of a bead-based assay for streptococcal serology, the assay was transferred from our previously described CBA beads (14), to the clinically relevant Luminex platform. The optimal coupling concentration for each of the highly purified (>95%) recombinant SLO, DNaseB and SpnA antigens to spectrally unique magnetic beads was determined. This was defined as the concentration that gave the highest MFI across a panel of reference sera with known titres (previously determined by CBA) in single-plex assays. The optimal coupling concentrations were 12.5 μg of SLO and SpnA, and 10 μg of DNaseB per 2.5 x 10^6 beads.

Next, beads coupled with each of the three antigens were mixed in equal parts and the concentration of antibodies for each antigen was determined in a triplex format for the panel of reference sera. The antibody titres measured in the triplex format correlated strongly with the single-plex assays, indicating no cross-reactivity between the different beads (R^2 values >0.999 for all antigens in a linear regression analysis, data not shown). After trialling various test serum concentrations, a 1:8,000 dilution was found to give MFI's within the log-linear range for all three antigens. Comparison of the MFI at this dilution with the previous CBA beads for 33 samples showed significant correlations ($p < 0.0001$) for all three antigens (Figure S1), such that 1:8,000 was used in all subsequent triplex assays.

Following optimisation, the assay operating characteristics were determined and are summarised in Table 2. The lower limit of detection (LLOD) was <0.1 ng/ml for all three antigens. The intra-assay coefficient of variation (CV)'s were <5% and inter-assay CVs were <13% for each of the three antigens. These characteristics confirm that the Luminex-based triplex assay is a reliable and repeatable method for quantifying serum antibody titres to GAS antigens.

Comparison with existing streptococcal serological methodologies

To compare the triplex assay with existing commercial methodologies, experimentally determined titres were compared with titres determined by the turbidimetric technique (ASO) and the semi-quantitative enzyme inhibition assay (ADB). The 318 serum samples analysed included residual sera from a community laboratory (n=180) as well as sera from ARF cases and controls (n=138) for which clinical titres were available. As seen in Figure 1a, there is a highly significant correlation between the concentration of ASO IgG determined in the triplex assay and the commercial methodology (Spearman's r =0.950, P-value <0.0001). A significant correlation also exists between the concentration of ADB IgG determined by the triplex assay and the commercial methodology (Spearman's r=0.924, P-value <0.0001). However, as previously described (14), there is a lack of precision of the traditional ADB enzyme inhibition assay, which produces titre ranges rather than continuous values. This can be seen visually as a 'stacking' of points in Figure 1b.

The lack of precise titres for ADB in the enzyme inhibition assay is further illustrated by plots of antibody titre distribution (Figure S2). The continuous distribution of ASO is largely the same between the turbidimetric and triplex assay. In contrast, the discontinuous distribution of ADB titres in the enzyme inhibition assay is improved by the Luminex methodology. The triplex assay therefore offers increased precision for ADB, compared to traditional methods.

Streptococcal antibody titres in ARF

The ability of the triplex assay to detect streptococcal antibodies in ARF was assessed in sera obtained from 97 ARF cases and the 75 healthy controls (Table 1). As was expected, IgG titres against all three antigens were significantly elevated (P < 0.001) in children with ARF compared to healthy control children (Figure 2A). Serum samples for each ARF case were obtained at varying time points after hospital admission, with recruitment of some patients

being delayed for over 4 weeks. This provided an opportunity to assess the impact of time on serum antibody levels. ARF cases were stratified into two groups; those whose serum was collected < 28 days after hospital admission (n=72) and those whose serum was collected 28+ days after hospital admission (n=25). As ARF tends to develop 2-4 weeks after an initial GAS infection, the 28 day stratification essentially grouped cases as those with a GAS infection in the preceding 6-8 weeks, or greater. As shown in Figure 2B, there was no significant difference in the median ASO and ADB titres between the <28 and 28+ days groups (P = 0.513 and 0.963, respectively). However, for anti-SpnA, there was a significant decrease (P < 0.05) in the median antibody titre in the 28+ day group compared with the <28 day group, suggesting a faster decay in anti-SpnA IgG over time.

Utility of the triplex assay and SpnA in ARF diagnosis

The ability of the triplex assay to detect a previous GAS exposure for ARF diagnosis was assessed using an upper limit of normal (ULN) approach. ULN was experimentally determined from the 75 healthy controls recruited in the RF RISK study, who had no recent history of sore throat or skin infection (Figure 2A). The resulting ULN values of 354 μg/ml for SLO, 117 μg/ml for DNaseB and 64 μg/ml for SpnA were applied to the ARF cases to determine the diagnostic sensitivity of each antigen (Table 3). There was no significant difference (P = 0.853) in sensitivity of each of the three antigens in detecting true positives in the <28-day ARF group. The sensitivity for each of the three antigens in this group was high, ranging from 85-88%. In contrast, in the 28+ day ARF group there was a significant difference in sensitivity between the antigens (P = 0.001) , with ASO and ADB very high at 92 and 96%, respectively, compared with anti-SpnA at 60%. This mirrors the significant decrease in anti-SpnA IgG titres seen between the <28 day and 28+ ARF group (Figure 2B) and is further evidence that anti-SpnA IgG decays faster than ASO and ADB.

286 ***Impact of ULN values on diagnostic sensitivity***

287 The relationship between the experimentally determined ULN in this study and the ULN that
288 are included in current ARF diagnostic guidelines was explored by conversion of triplex
289 assay units (μg/ml) to the commonly reported clinical units for streptococcal serology (IU/ml
290 for ASO and U/ml for ADB). The rationale for this conversion was based on the significant
291 correlations between the ASO and ADB titres determined by the triplex assay and the
292 commercial methodologies (Figure 1). By solving the linear equation of the regression lines,
293 the experimentally determined ULN for the 75 healthy controls equated to 412 IU/ml for
294 ASO and 367 U/ml for ADB, respectively (Table S1). This is very similar to the ULN
295 determined in the same 75 healthy controls when titres were measured using the
296 commercially available assays in clinical laboratories (422 IU/ml for ASO, 400 U/ml for
297 ADB), demonstrating the accuracy of interpolating the triplex assay units to clinical units.

298 Next, the linear regression approach was utilised to convert the ULN for ASO and ADB
299 reported in ARF diagnostic guidelines in New Zealand (7) and Australia (25) to triplex assay
300 units (Table S1). There are substantial differences in ULN applied in ARF diagnostic
301 guidelines globally, which is exemplified by the differences between New Zealand and
302 Australia. New Zealand guidelines stipulate a higher, all ages ULN, compared to the lower,
303 age-specific ULN applied in Australia (9). These differences are reflected in sensitivity
304 calculations for ASO and ADB in the triplex assay when the interpolated, clinical ULN are
305 applied (Table S2). The sensitivity of ADB in the triplex assay is 51% when the current
306 New Zealand cut-off is applied compared with 75% using Australian age specific cut-offs,
307 and 88% with the experimentally determined ULN. For ASO, sensitivity is 82% in the triplex
308 assay using the current New Zealand cut-off compared with 97% using Australian age
309 specific cut-offs, and 88% with the experimentally determined ULN.

310

DISCUSSION

In this study a triplex Luminex assay that measures serum IgG concentration to three GAS antigens (SLO, DNaseB, and SpnA) with high efficiency and accuracy was developed and evaluated. The multiplex nature of the assay ensures that titres are measured simultaneously, in a single tube, from a very small volume of sera (2 µl). This provides efficiency advantages over current commercial methodology for ASO and ADB that include turbidimetric, nephelometric or enzyme inhibition assays and require separate reactions with each antigen run in parallel assays (8, 9, 14). The accuracy of the triplex assay described here is highlighted by the significant correlation with ASO and ADB titres determined from the commercial assays currently used in the New Zealand clinical setting. Of note, the enzyme inhibition assay for ADB produces titre ranges, which results in a discontinuous distribution of values as observed in this study and previously (14, 25). In contrast, the newly developed triplex assay provides continuous values for all three antigens, resulting in enhanced precision for ADB titres in particular.

The predictive value of elevated ASO and ADB for the diagnosis of GAS immune sequelae is influenced by immunokinetics, and the slow rate of antibody decay following GAS infection in some individuals (13). While induction of a long-lived antibody response is a desired feature of a vaccine antigen, it is not ideal for serological diagnostics where the goal is to identify individuals who have experienced a recent GAS infection. Indeed, the knowledge that SLO antibodies stay elevated for extended periods after infection (13), combined with the high sequence conservation across GAS strains (26), has provided rational for including SLO in combination vaccines (15, 27). This highlights a need to characterise additional GAS antigens, which are not being developed as vaccines, and have favourable immunokinetics for serological diagnostics. SpnA is not only highly conserved and immunogenic (15), but is

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unlikely to be used in a vaccine as it failed to provide protection in a mouse infection model (28).

This study has built on our previous findings regarding the potential utility of SpnA in streptococcal serology (14), by comparing the sensitivity of anti-SpnA with ASO and ADB in a larger cohort of ARF patients. While the sensitivity of all three antigens was comparable in cases whose serology was performed within 4 weeks of hospital admission, the significant reduction in anti-SpnA tires in cases for whom serology was delayed suggests anti-SpnA IgG decay more rapidly following a GAS infection. The biological basis for the differences in IgG immunokinetics between GAS antigens is yet to be elucidated but may be driven by differences in CD4+ T-helper cells and long-term memory responses. Mechanism aside, the short-lived nature of the anti-SpnA IgG response following GAS infection is a highly desirable trait for diagnostic streptococcal serology as it decreases the likelihood of false-positives.

The predictive value of streptococcal serology in the diagnosis of ARF is influenced by the ULN cut-off that is applied. This was clearly demonstrated in a recent audit of ARF patients in New Zealand and the Northern Territories of Australia, in which the high ULN cut-offs for ASO and ADB used in New Zealand resulted in undercounting of definite and probable cases (9). In the present study, the experimentally determined ULN was derived from 75 highly matched healthy children who did not have a recent sore throat or skin infection. When expressed in units commonly reported in the literature these values are 412 IU/ml for ASO and 367 U/ml for ADB, respectively, which are markedly lower than those currently specified in New Zealand guidelines. A greater proportion of ARF cases in our study thus met the experimentally determined ULN for ASO and ADB, compared with when the ULN from current New Zealand guidelines were applied to our cohort. This highlights how crucial it is to have accurate ULNs for the diagnosis of ARF when paired sera are unable to be

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obtained (12). Moreover, it provides further evidence for New Zealand to consider updating ULN in future ARF guidelines, particularly given the current ULN were determined over 35 years ago (7), and are higher than those recommended elsewhere in the world (9).

Previous studies of ASO and ADB in ARF have been inconsistent with respect to trends in the antigen specific responses. A recent audit in New Zealand and the Northern Territory of Australia found ARF cases in both countries more likely to have elevated ASO than ADB, while an older Northern Territory study found that ADB was more likely to be elevated (29). Our previous study in a small ARF cohort (n=16) found anti-SpnA and ASO were more likely to be elevated compared with ADB (14), yet in this study there was no significant difference between the three antigens in patients for which sera were obtained within 4 weeks of hospital admission. This highlights the complexity of antigen specific serological response following GAS infection and is consistent with a recent study in GAS pharyngitis, which found no clear pattern of immune responses against conserved GAS antigens, and where the need for a larger antigen panel to increase the sensitivity of assays to detect a recent GAS infection was described (30).

Limitations of this study include the relatively small sample size used in determining experimental ULN such that age-specific values were not practical to obtain, and an all ages ULN was applied. In addition, paired sera from ARF cases were not available meaning temporal responses in individuals could not be investigated. However, the significant reduction in anti-SpnA titres in ARF patients with delayed serology is strong evidence that the immunokinetics of anti-SpnA IgG differ from that of ASO and ADB, and further studies in participants with confirmed GAS pharyngitis and skin infections, which incorporate temporal samples, are planned.

Conclusion

In summary, this study describes the development and evaluation of a Luminex-based triplex assay that simultaneously measures ASO, ADB and anti-SpnA in very small volumes of patient sera. The diagnostic utility of anti-SpnA was confirmed in a large cohort of ARF patients, with an immunokinetic profile that suggests a recent GAS exposure. As such this triplex assay has the potential to improve both the efficiency and accuracy of streptococcal serology for the diagnosis of post-streptococcal sequelae.

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507

FIGURE LEGENDS

Figure 1. Scatter plots showing the correlation between the triplex Luminex assay and clinically available methodologies for ASO (n=317) (A) and ADB (n=318) (B). Spearman's *r* and *P*-values as shown. The dashed lines represents the linear regression equation, see also Table S1.

Figure 2. A – Violin plots showing serum titres for ASO (i), ADB (ii) and anti-SpnA (iii) determined by the triplex Luminex assay in ARF cases (blue) and controls (yellow). Dashed lines represent experimentally determined ULN values. B - Tukey box and whisker plots showing serum titres for ASO (i), ADB (ii) and anti-SpnA (iii) determined by the triplex assay for ARF patients stratified into serum collected <28 days after hospital admission (dark green) and 28+ days after hospitalisation (light green). *P*-values were determined by Mann-Whitney U unpaired two-tailed analysis.

523 **TABLES**

524

525 **Table 1.** Demographics of the study participants included in the calculation of experimental

526 ULN values and the diagnostic sensitivity of each antigen

	ARF (n=97)	Healthy children (n=75)
Age (years)		
Median	11	11.7
Range	4-20	6-18
Male sex, N (%)	67 (69)	46 (61)
Ethnicity, N (%)		
Maori	42 (43)	30 (40)
Pacific	54 (56)	43 (57)
Other	1 (1)	2 (1)

527

528 **Table 2.** Summary of assay sensitivity and precision

529

Assay characteristic	ASO	ADB	Anti-SpnA
Lower limit of detection	0.04 ng/ml	0.02 ng/ml	0.06 ng/ml
Intra-assay CV[a]	4.8 %	4.6 %	4.5 %
Inter-assay CV[a]	12.4 %	12.5 %	11.6 %

530 [a]CV, coefficient of variation

531

532 **Table 3.** Diagnostic sensitivity of ASO, ADB and anti-SpnA in the triplex assay, with
533 sensitivity defined as the number of ARF patients with antibody titres above the
534 experimentally determined ULN cut-offs. ARF patients were stratified into those with sera
535 obtained <28 days after hospital admission and 28+ after hospital admission. *P*-values were
536 determined by 3 x 2 χ^2 test.

537

	ASO	**ADB**	**Anti-SpnA**	***P*-value**
ARF <28 days, n=72 (%)	63 (88)	63 (88)	61 (85)	*0.853*
ARF 28 + days, n=25 (%)	23 (92)	24 (96)	15 (60)	*0.001*

538