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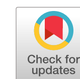
The novel Group A Streptococcus antigen SpnA combined with bead-based immunoassay technology improves streptococcal serology for the diagnosis of acute rheumatic fever



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Abstract *Objectives:* Streptococcal serology provides evidence of prior Group A Streptococcus (GAS) exposure, crucial to the diagnosis of acute rheumatic fever (ARF) and post-streptococcal glomerulonephritis. However, current tests, which measure anti-streptolysin-O and anti-DNaseB antibodies, are limited by false positives in GAS endemic settings, and incompatible methodology requiring the two tests to be run in parallel. The objective was to improve streptococcal serology by combining the novel GAS antigen, SpnA, with streptolysin-O and DNaseB in a contemporary, bead-based immunoassay.

Methods: Recombinant streptolysin-O, DNAseB and SpnA were conjugated to polystyrene beads with unique fluorescence positions so antibody binding to all three antigens could be detected simultaneously by cytometric bead array. Multiplex assays were run on sera collected in three groups: ARF; ethnically matched healthy children; and healthy adults.

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Results: The ability of the antigens to detect a previous GAS exposure in ARF was assessed using the 80th centile of the healthy children group as cut-off (upper limit of normal). SpnA had the highest sensitivity at 88%, compared with 75% for streptolysin-O and 56% for DNaseB.
Conclusions: SpnA has favorable immunokinetics for streptococcal serology, and can be combined with anti-streptolysin-O and anti-DNaseB in a multiplex format to improve efficiency and accuracy.
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Introduction

Group A Streptococcus (GAS; *Streptococcus pyogenes*) causes a range of diseases from mild pharyngitis and skin infections through to serious invasive disease.[1] The bacterium is also associated with the post-infectious immune sequelae of acute rheumatic fever (ARF) and post-streptococcal glomerulonephritis (PSGN). ARF, and associated rheumatic heart disease, continue to be the major cause of acquired heart disease in the developing world, and are estimated to cause at least 350,000 premature deaths per year.[2] While the disease is now largely restricted to resource-poor countries, ARF also persists in indigenous populations in selected developed countries. Aboriginal children in Australia and Maori and Pacific children in New Zealand experience some of the highest disease incidence rates in the world.[3,4] The disease burden of PSGN is also significant, with over 95% of the 470,000 cases occurring in resource-poor settings.[5] Outbreaks of PSGN are observed in settings where impetigo is endemic, and recent data suggest a link between PSGN and the high rates of end-stage renal failure in the Aboriginal population of Australia.[6]

Streptococcal serology is crucial to the diagnosis of these post-infectious immune sequelae because they occur several weeks after the GAS infection that initiates them. This delay of 2–4 weeks means culture of the causative bacterial strain is often no longer possible. The measurement and interpretation of streptococcal serologic tests is complex and many clinical and technical aspects should be considered.[7,8] During a GAS infection the immune system will generate antibodies to a large array of GAS antigens, but the titres to just two antigens are currently measured in clinical practice.[8] These antigens are streptolysin-O (SLO) and deoxyribonuclease-B (DNaseB), with the serological tests referred to as anti-streptolysin-A (ASO) and anti-deoxyribonuclease-B (ADB), respectively. SLO is a secreted, pore forming cytolysin, while DNase-B is a secreted enzyme that degrades DNA. ASO and ADB titers are interpreted by comparison of acute with convalescent samples and a demonstration of a two-fold rise in titre between these two-time points. However, obtaining paired sera is not always practical in a clinical setting. When single serum samples are available, titres are compared to a reference upper limit of normal (ULN). The ULN for streptococcal serology is defined as the 80th percentile in a matched, normal, healthy population.[8]

ASO titres are commonly measured using nephelometric or turbidimetric assays in an automated manner. An international standard for ASO was established by the World Health Organization (WHO) over 50 years ago[9] and ASO values are normally reported as international units per milliliter (IU/mL). The SLO antigen is chromosomally encoded and highly conserved across GAS strains. Of note, SLO is also produced by Group G and Group C Streptococcus (*Streptococcus dysgalactiae* subspecies *equisimilis*) that are antigenically similar and have an overlapping spectrum with GAS.[10] It follows that an elevated ASO titre can arise from a prior infection with either GAS or GGS/GCS.[8]

In contrast with SLO, there are no reference sera available for DNaseB, and as such ADB tests are less standardized and are used to provide additional information to the ASO test.[11] ADB titres are commonly measured using an enzyme inhibition assay in which the ability of serum to neutralize the activity of DNaseB is detected by a colored dye. These tests are time consuming and only semi-quantitative. There are also latex agglutination and nephelometric tests for ADB, but these are less commonly installed in the analyzers used in many contemporary clinical laboratories, hence the continued use of a semi-quantitative, enzyme inhibition assay. Furthermore, the DNaseB antigen is not uniformly expressed across GAS strains. Many variants have been described and these are often encoded by mobile genetic elements (bacteriophage).[7] If the infecting strain does not contain the *dnaseB* gene, an elevated ADB titre will not be observed.

The accuracy of immunodiagnostics tests such as ASO and ADB is reliant on the immunokinetics of the antibody response, that is, the rate at which antibody concentrations rise and decay in response to an antigen stimulus. The most comprehensive data on the kinetics of ASO and ADB comes from a study of 160 children with GAS pharyngitis by Johnson et al.[12] In contrast to previous classical descriptions that the ASO and ADB titres peak at 3–5 weeks and 6–8 weeks, respectively, followed by a steady return to pre-infection levels, the Johnson study showed marked variability. ASO titers were frequently elevated for extended periods following infection with 67% of children having titres above pre-infection levels for more than 1 year. Similarly, 56% of the children maintained ADB titres above pre-infection levels for more than 1 year following infection.[12] Furthermore, 22% of *bona fide* GAS pharyngitis cases were associated with a rise in titre in only one antigen (e.g. either ASO or ADB, but not both), emphasizing the importance of measuring titres to more than one GAS antigen to minimize false negatives.

The prolonged elevation of titres observed for ASO and ADB, and associated risk of false-positives, together with the semi-quantitative nature of the current ADB assays highlights the pressing need to improve streptococcal serology. Consequently, the aims of this study were to i) assess the utility of a novel GAS antigen *Streptococcus pyogenes* nuclease A (SpnA, spy0747) for streptococcal serology, and ii) to assess the feasibility of using a bead-based immunoassay platform to measure titres to ASO, ADB and SpnA simultaneously. SpnA is a cell wall-anchored DNase and an important

GAS virulence factor.[13,14] It is highly conserved and has previously been shown to be immunogenic in assays with sera from patients with GAS disease.[15,16] Furthermore, our laboratory observed low levels of antibodies to SpnA in a limited number of healthy individuals.[13,16] Low background titres in a healthy, control population are a desired feature for diagnostic streptococcal serology. In this study, we show that anti-SpnA is more sensitive than ASO and ADB in the diagnosis of ARF, and describe methodology that enables streptococcal titres for the three antigens to be determined rapidly in a single assay with multiplex bead-based technology.

Methods

Study subjects

In this study, human sera were obtained from four different sources. Patients with ARF were diagnosed according to the New Zealand modification of the Jones criteria[17] and recruited while hospitalized at Starship Children's Hospital in Auckland between 2004–2006 (n = 8) and Waikato Hospital in Hamilton between 2012–2015 (n = 8). Sera from healthy children, aged six, matched to the cases by ethnic identification, were obtained from the children of women recruited into the Auckland arm of the SCOPE study.[18] Finally, sera were obtained from unmatched, healthy volunteers aged 20 years or older recruited at the University of Auckland as an additional control group. Demographics are shown in Table S1. All participants had provided written informed consent and appropriate ethical board approval was obtained for each of the four sites.

Antigen preparation

The recombinant antigens utilized in this study were all prepared in their mature form without N–terminal signal sequences. SLO, with a molecular weight of 64.4 kDa (aa 34–571) and an N–terminal His_6-tag was purchased from Fitzgerald Industries International. The gene encoding DNaseB was amplified from *S. pyogenes* SF370 (ATCC 700294) genomic DNA using the following primers: forward, 5′–CACCATGCGACAAACACAGGTCTCAAATGATGTTG-3′ and reverse, 5′–TTTCTGAGTAGGTGTACCGTTATGGTAGTTAATGG-3′ for cloning into pET101/D–TOPO (Life Technologies) using Topo cloning methodology. The resulting vector encodes DNaseB (aa 43–271) followed by a C-terminal His_6-tag for a total molecular weight of 29 kDa. The protein was expressed in *Escherichia coli* BL21λDE3 cells with 1 mM IPTG induction at 37 °C for 3 hours in Lennox broth (LB) media supplemented with ampicillin and 0.1% glucose. SpnA was amplified from *S. pyogenes* SF370 genomic DNA with primers that contained *Kas*I and *Xho*I restriction enzyme sites (underlined): forward, 5′–AAAGGCGCCCGCCAAAATTTGACTTATGCCAA-3′ and reverse, 5′–AAACTCGAGCTATTTGGAAAATGATAATTGAAGTAACA-3′. The resulting *spnA* amplicon (aa 28–854) was cloned into a pProExHta vector that encodes an N–terminal His_6–tag and transformed into *E. coli* BL21 λDE3 cells. Protein expression was induced with 0.3 mM IPTG at 18 °C for 16 hours in LB containing ampicillin. Both rDNaseB and rSpnA were purified from *E. coli* cell lysate using standard Ni^{2+}-NTA affinity chromatography. The His_6–tag was cleaved off rSpnA using recombinant tobacco etch virus protease (1:100 ratio of rTEV to recombinant protein) to yield an 85 kDa protein. The purity of all antigens was verified by SDS-PAGE.

Purification of antigen specific IgG

IgGs specific for SLO, DNaseB and SpnA were purified from pooled human immunoglobulin (intravenous immunoglobulin, IVIG (Intragam® P)). Affinity columns for each of the GAS antigens were generated by covalently coupling the antigens to agarose resin via their primary amines using an AminoLink® Coupling kit (Thermo Scientific). A 5 ml solution of IVIG was diluted four-fold with phosphate buffered saline (PBS) (pH 7.4) and passed over the resin to allow antibody binding. The resin was washed four times with PBS to remove unbound antibody. Bound antibody was eluted using 0.2 M glycine-HCl buffer (pH 2.5-3.0) and immediately neutralized with 1M Tris buffer (pH 9). Trace IgA was removed using Melon spin columns (Thermo Scientific) and the resulting antigen-specific IgG was concentrated using a centrifugal filter (Merck Millipore). To confirm that the eluted IgG was specific for the antigen it was isolated against, Enzyme-Linked Immunosorbent Assays (ELISA) were performed. Plates were coated with antigen at 5 μg/ml and blocked with PBS supplemented with 0.1% Tween-20 and 5% skim milk powder (PBST-5% milk) for 1 hour at room temperature (RT). Purified IgG was added for 1 hour at RT and binding was detected using an anti-human horseradish peroxidase (HRP) secondary antibody (1:3000; Santa Cruz Biotechnology) as previously published.[19]

Cytometric bead array assay

Each of the antigens was coupled to polystyrene beads using an amine-to-sulfhydryl crosslinker, sulfo-SMCC, according to the manufacturer's instructions (Functional beads, Becton-Dickinson). Briefly, color-coded 7.5 μm polystyrene beads were prepared for conjugation by adding 25 mM dithiothreitol (DTT). The target antigen (90 μg) was modified by adding 44 μg/ml sulfo-SMCC solution and unreacted protein was removed using a Bio-Rad spin column (Biorad). The modified protein and functional beads were then mixed and incubated at room temperature for 1 hour before adding N–Ethylmaleimide (44 μg/ml) and incubating for a further 15 min. The washed, conjugated beads were stored at 4 °C protected from light. Each antigen was conjugated to beads containing differing ratios of fluorophores (APCy7 and APC) to allow detection at unique positions on a scatter plot of fluorescence using two detectors (FL3 and FL4) on a flow cytometer. Beads were selected to ensure maximum separation between the antigen coated beads as follows: rSLO, position E4; rDNaseB, position A4; and rSpnA, position A9.

Incubations for Cytometric Bead Array (CBA) experiments were conducted in duplicate in 96-well U-bottom plates. Singleplex assays were performed by incubating rSLO-, rDNaseB- and rSpnA-coated beads separately for 1 hour at RT with serum samples diluted 1:10,000 in assay diluent. To detect levels of serum antibody binding, R-Phycoerythrin (PE) conjugated donkey anti-human IgG Fcγ–specific antibody (Jackson ImmunoResearch) was added at a concentration of 1:100 for

2 hours at RT. Multiplex bead assays were performed by following the same protocol except the rSLO-, rDNaseB- and rSpnA-coupled beads were mixed at equal ratios prior to adding sera samples diluted 1:10,000. A 1:30 dilution of PE-conjugated donkey anti-human IgG Fcγ–specific antibody was used for detection in the multiplex assays. Bead position and PE-signal were detected using a flow cytometer (Accuri C6, Becton–Dickinson) equipped with 488 nm (blue) and 640 nm (red) lasers. Quantification of serum antibody was based on median fluorescence intensity (MFI) of PE.

A seven-point standard curve was created for each antigen by mixing known starting concentrations of SLO-, DNaseB- and SpnA-specific antibody in one tube and performing a two-fold dilution series. A starting concentration of 500 ng/ml was used for SLO and DNaseB, whereas 1500 ng/ml was used for SpnA. The beads were incubated with each of the diluted standards for 1 hour, followed by detection with a PE secondary antibody as described above. MFI values were converted into concentration (μg/ml) and a five-parameter logistic regression equation was used to generate a standard curve for each antigen using Flow Cytometric Analysis Program (FCAP) Array software, version 3 (Becton–Dickinson). When study subject sera were run in a multiplex assay alongside the seven-point standard curves for each antigen, MFI was converted into concentration (μg/ml) using the FCAP Array software. The lower limit of detection for each antigen was defined as the lowest concentration on the standard curve whose MFI was greater than 3 standard deviations above the blank (where the blank is beads plus secondary antibody only) as previously published.[20]

Data analysis and statistics

Upper limit of normal (ULN) values were calculated for each antigen by ranking the antibody concentrations for each of the healthy children sera samples and determining the 80th centile in Microsoft Excel (version 15.24). Statistical analysis and graphs were prepared using GraphPad Prism (version 7a). All correlations were analyzed using linear regression.

ASO and ADB titres using commercial assays

Both ASO and ADB titres were determined at Labtests Pathology, Auckland, New Zealand. ASO titers (IU/ml) were measured by a turbidimetric technique using the human anti–streptolysin-O kit on a SPAplus analyzer (The Binding Site, CA, USA). ADB titres (U/mL) were measured by an enzyme inhibition assay (bioMerieux, Marcy l'Etoile, France). This assay provides an inexact figure for low titres of <100 U/ml; a midtiter value of 50 U/ml was estimated for samples that fell in this range.

Results

Bead coupling and proof of concept for a multiplex assay

To generate GAS-antigen coupled beads for analysis in this study, highly purified preparations of each of the three antigens were produced recombinantly in *E. coli*. The secreted proteins (SLO and DNaseB) were expressed without their N-terminal signal sequence and SpnA was expressed without the N-terminal signal sequence and truncated at K854 upstream of the sortase motif for improved protein stability. Each of the three proteins was coupled to fluorescent polystyrene beads that were distinguishable on a two-color fluorescence plot by flow cytometry. Various serum dilutions and concentrations of the anti-IgG detection reagent were tested to determine the log-linear range and saturation point of the assay. A 1:10,000 serum dilution was found to generate fluorescence in the log-linear range for all three antigens.

In order to assess whether the titres of the three antigens could be measured simultaneously, the results of singleplex assays were compared with multiplex assays. Singleplex assays were performed in which each bead was incubated with sera from 10 participants diluted 1:10,000. These participants were chosen as previous ELISA had shown that reactivity against the three antigens ranged from low to high, ensuring a good spread of MFI for optimizing the CBA assays (Fig. 1). These same 10 sera were then tested in multiplex format where the three antigen beads were mixed in equal parts and incubated with the test sera in a single assay well. The MFI in these multiplex assays showed a strong correlation with the singleplex MFI for each antigen as shown in Fig. 1 (R^2 values: SLO = 0.999; DNaseB B = 0.998; and SpnA = 0.998). This indicates there was no interference or IgG cross-reactivity between the beads and demonstrates the feasibility of a multiplex assay comprising the three streptococcal antigens.

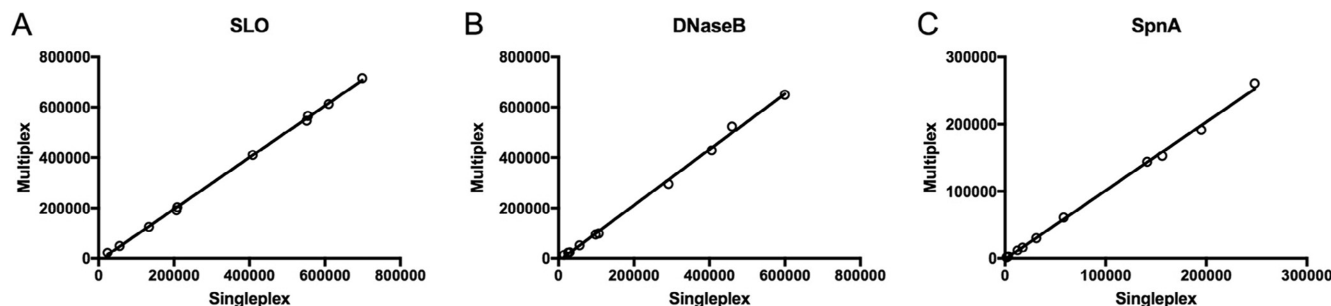

Fig. 1. Scatter plots showing the correlation between singleplex and multiplex Median Fluorescence Values (MFI) determined by the Cytometric Bead Array for SLO (A), DNaseB (B) and SpnA (C) in 10 serum samples. Linear regression analysis was performed and R^2 were determined as follows: SLO = 0.999; DNaseB B = 0.998; and SpnA = 0.998.

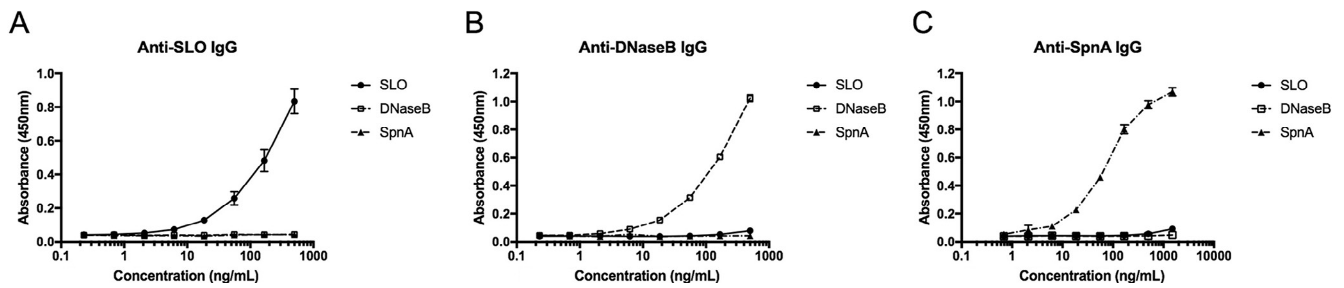

Fig. 2. ELISA for the purified IgG against the three Group A Streptococcus antigens. The antibodies were purified from IVIG using affinity chromatography resulting in IgG with specificity for SLO (A), DNaseB (B) and SpnA (C). The error bars represent standard deviations.

Standardization and precision of the multiplex CBA assay

Standard curves for each of the three antigens were generated to determine the concentration of antibodies binding the antigen-coupled beads and enable comparison between assays. IgG specific for SLO, DNaseB and SpnA were purified from IVIG by affinity chromatography. The specificity of the purified antibodies was verified by ELISA. As shown in Fig. 2, the SLO-, DNaseB- and SpnA-specific antibodies only showed reactivity to their corresponding antigen and no detectible reactivity with the other two antigens. The purified IgG was used to generate seven-point standard curves for each antigen (Fig. S1). Known concentrations of purified IgG were diluted twofold with starting concentrations of 500 ng/ml for anti-SLO and anti-DNaseB and 1500 ng/ml for anti-SpnA. These diluted standards were incubated with the antigen-coupled beads in a multiplex format and standard curves were fitted using a five-parameter logistic formula. Standard curves were highly reproducible, with a fitting accuracy of at least 98%, demonstrating the utility of affinity purified, polyclonal antibodies as reference standards for serological responses to these antigens.

The purified antibody standards were used to determine lower limits of detection for the three antigens in the CBA assay: anti-SLO, 1 ng/ml; anti-DNaseB, 0.1 ng/ml; and anti-SpnA 0.1 ng/m. The Coefficient of Variability (CV) for the multiplex assay was assessed using the same 10 sera utilized in the singleplex/multiplex comparison. The concentrations of IgG for these 10 sera were measured in assays incorporating the IgG standard curves and the average intra- and inter-assay CVs were <4% and <15%, respectively, for each of the antigens (Table S2). These CVs demonstrate the multiplex bead-based assay has good precision and is repeatable. The reproducibility of both the standard curve and the assay test results means these reagents can be utilized to check the coupling efficacy and integrity of future batches of antigen-coupled beads.

Measuring antibody titres in ARF patient sera

To assess the utility of the SpnA antigen and bead-based technology in clinical streptococcal serology, the multiplex assay was performed on all study subjects (Table S1). The concentration of IgG specific for SLO, DNaseB and SpnA could be determined for all 47 participants in one experiment, performed by a single operator, in 1-day. As shown in Fig. 3, the mean antibody titres in ARF samples were significantly higher than the mean titres in both the healthy children and healthy adult control groups for each of the three antigens. In keeping with observations in previous studies,[21] the titres for ASO and ADB were higher and showed more spread in healthy children than in healthy adults as illustrated by the larger confidence intervals in Table 1. Notably, the titres for

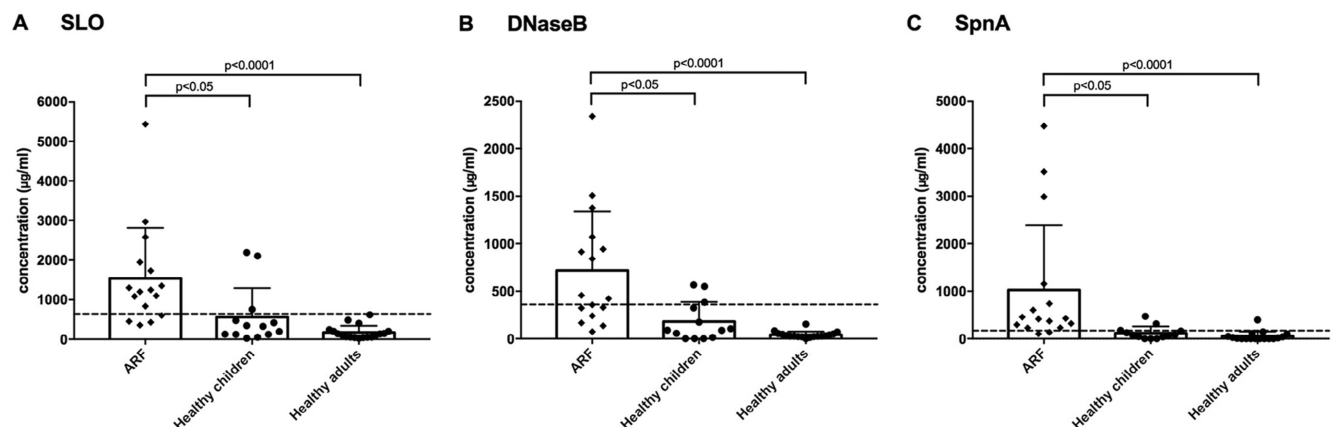

Fig. 3. Scatter plots showing serum antibody concentrations determined by Cytometric Bead Array for SLO (A), DNaseB (B) and SpnA (C). The ULN values for each antigen is shown (dotted line). Kruskal–Wallis one-way analysis of variance was performed to determine p-values.

Table 1 Summary statistics for the concentration of antibodies (μg/mL) specific for SLO, DNaseB and SpnA determined by the Cytometric Bead Array assay.

Antigen	Acute rheumatic fever (n = 16)	Healthy children (n = 13)	Healthy adults (n = 18)
Streptolysin-O			
Mean (95% CI)	1539 (859.9–2218.0)	559 (115.2–1003.0)	173 (89.6–256.2)
Median (95% CI)	1225 (601.4–1948.0)	326 (123.7–757.9)	123 (53.6–201.4)
DNaseB			
Mean (95% CI)	718.5 (387.4–1050)	180.9 (56.2–305.5)	41.7 (24.7–58.7)
Median (95% CI)	439.5 (240.9–1071)	89.01 (0–383.7)	29.38 (22.8–49.6)
SpnA			
Mean (95% CI)	1029 (304.9–1752)	119.8 (36.7–202.9)	52.2 (1.3–103.1)
Median (95% CI)	422.2 (229.1–1156)	76.01 (0–177.3)	7.4 (0–49.7)

SpnA in healthy children were similar to those in healthy adults, and had narrower confidence intervals compared to those for ASO and ADB in the healthy, ethnically matched children group (Table 1). This supports previous observations that background titres for SpnA are low in healthy individuals.[13]

The ability of the antigens to detect a previous GAS exposure for ARF diagnosis was assessed using ULN values. The ULN, or 80th centiles, were calculated from the healthy children group as 644, 360 and 170 μg/ml for SLO, DNaseB and SpnA, respectively. The lower ULN for SpnA reflects the reduced titres seen in the healthy children compared with SLO and DNaseB. These experimentally determined cut-offs, shown as a dotted line in Fig. 3, were then applied to the ARF samples to determine the sensitivity of each antigen. That is the number of true positives that were detected based on whether an observed titre was above the ULN. DNaseB was the least sensitive detecting just 9 out of 16 ARF samples (56%). SLO showed intermediate sensitivity by detecting 12 out of 16 ARF samples (75%). SpnA showed the highest sensitivity detecting 14 out of 16 ARF samples (88%).

Comparison with existing serological tests

To compare our multiplex CBA assay with existing, commercially available methodology, sera from 20 participants for which sufficient volumes were available, were subject to ASO and ADB testing at a commercial laboratory. ASO was measured using the widely employed turbidimetric technique and exact values in international units (IU/ml) were obtained. In contrast the ADB titres were measured using an enzyme inhibition assay that provides titre ranges (100, 200, 300, 400, 600, 800, 1200 and ≥1600). As shown in Fig. 4A, there is an excellent correlation between the concentration of ASO IgG determined in our CBA assay and the commercially available turbidimetric technique ($R^2 = 0.968$). As shown in Fig. 4B, there is also a good correlation between the concentration of ADB IgG determined in our CBA assay and the commercially available enzyme inhibition assay ($R^2 = 0.934$). However, the lack of precision of the ADB enzyme inhibition assay is also illustrated in Fig. 4B. Three samples were classified as "1200" in the enzyme inhibition assay, yet the concentration of anti-DNaseB IgG measured in our CBA assay was 1508, 1070 and 914 μg/ml, respectively (boxed data points).

The linear equation of the regression lines in Fig. 4 enabled conversion of our experimentally determined ULN expressed as μg/ml to ULN in IU/ml commonly reported in the literature. The ASO ULN of 644 μg/ml equates to 324 IU/ml and the ADB ULN of 360 μg/ml equates to 410 U/ml.

Discussion

This study describes the characterization of SpnA as a new antigen with utility in streptococcal serology. SpnA showed improved sensitivity for ARF diagnosis compared with the antigens currently used in clinical streptococcal serology, SLO and DNaseB. The improved sensitivity is likely a result of the immunokinetics of the antibody response to SpnA. A major short-coming of both the ASO and ADB tests is that the

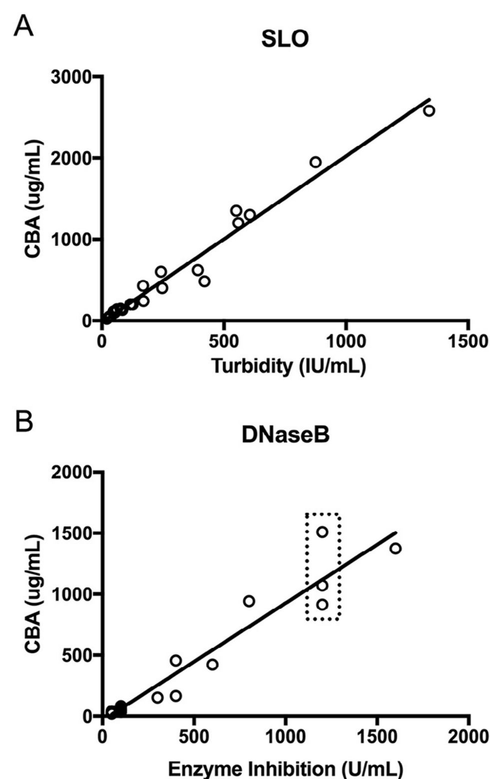

Fig. 4. Scatter plots showing the correlation between commercially available tests and the Cytometric Bead Array for SLO (A) and DNaseB (B). Linear regression analysis was performed and R^2 were determined as SLO = 0.968 and DNaseB B = 0.934.

antibody titres to SLO and DNaseB can stay elevated for many months after a GAS infection resulting in false positive results.[12] Previous studies suggested that SpnA was highly immunogenic,[15] but that the antibody response maybe short-lived.[13] Our results support these findings – the mean concentration of SpnA antibodies in healthy children was lower and had narrower confidence intervals compared with SLO and DNaseB antibodies. This reduced spread, combined with the lack of healthy children with elevated SpnA titres, resulted in a lower ULN for SpnA. The ULN cut-off is crucial for diagnosis of ARF and PSGN as these sequelae develop several weeks after a GAS infection, making use of paired serology to observe an increase in titres challenging.[8] ULN for ASO and ADB are known to vary with age and geography and titres in healthy adults tend to be lower than in children.[21,22] Here we determined ULN for ASO, ADB and anti-SpnA in a group of healthy six–year old Maori and Pacific children, with these ethnic groups associated with higher risk for developing ARF in New Zealand.[3] When the ULN were applied to ARF sera, SpnA had the highest sensitivity of the three antigens at 88%. The sensitivity of ASO in our assay format was 75%, which is comparable to the 72.5% observed in an Australian ARF study using existing clinical assays.[22] The sensitivity of DNaseB in our assay format was lower at 56%. Reduced sensitivity of ADB compared with ASO in diagnosing post-infectious sequelae is well documented[7,8,22] and due in part to the variable expression of DNaseB across GAS strains.

The bead-based methodology used in this study enabled the determination of streptococcal titres to be multiplexed. Excellent correlations were observed for all three antigens in multiplex assays compared with singleplex assays indicating there is no interference or cross–reactivity between the antigen-coated beads and serum antibodies in this assay format. The ability to multiplex, combined with the wide-dynamic range of fluorescent bead systems, enables the titres for the three antigens to be measured in parallel with a single serum dilution (1:10,000). The ability to multiplex offers an efficiency advantage over the nephelometric, turbidimetric and enzyme inhibition assay methods currently used in clinical laboratories to measure ASO and ADB, which need to be run separately thus taking extra time and effort for laboratory staff. Importantly, the titres for ASO and ADB determined using our bead-based assay format correlated very well with existing methodology. The slightly higher correlation for ASO compared with ADB was expected since both ASO turbidimetry assays and our bead-based system are quantitative and provide exact titre values, while the commercially enzyme-inhibition assay for ADB only provides titre ranges.[23]

There are several limitations with this study. The healthy, ethnically matched sera samples used to determine ULN were from a group of children that were all aged six years. Titres for ASO and ADB have previously been shown to peak in children aged 6–15 years old[11,21,24] and a ULN cut-off should ideally be determined in a healthy cohort matched by age, as well as ethnicity. Having control sera from healthy children aged 6–15 years, the same age range as the ARF cases in this study, may have increased the accuracy of the ULN values determined. However, when our experimentally determined ULN are expressed in units commonly reported in literature they are 324 IU/ml for ASO and 410 U/ml for ADB, respectively. These values are comparable to those determined in larger studies in Fiji (ASO 276 IU/ml and ADB 499 IU/ml in 186 children aged 5–14 years)[21] and Melbourne, Australia (ASO 320 IU/ml and ADB 300 IU/ml in 66 children aged 4–14 years)[24] and suggests the ULN calculated in this study maybe representative of a larger age matched cohort.

A further limitation is the sample size, with only 16 sera samples from patients with ARF tested. Future studies planned in our laboratory include testing the utility of the multiplex test on a much larger cohort of patients with post-streptococcal immune sequelae and comparing these to healthy control groups matched by age, ethnicity and area of residence. This will enable more accurate determination of sensitivity and specificity of anti-SpnA and accurate calculations of the minimum sample size needed for clinical evaluation studies.[25] Studies in cellulitis and invasive disease, where determining whether GAS is the causative organism can be problematic, are also planned. The transfer of the assay to the Luminex® platform, routinely utilized in clinical laboratories in resource rich settings for measuring a broad range of immunological analytes, and a lateral flow test more appropriate for resource poor settings will be explored.

Conclusions

In summary, this study describes a new GAS antigen, SpnA, which has favorable immunokinetics for streptococcal serology. Anti-SpnA alone had higher sensitivity when compared to the existing antigens, SLO and DNaseB, currently used in clinical streptococcal serology. A multiplex bead-based immunoassay has been developed that enables ASO, ADB and anti-SpnA to be determined in a single test. This assay should markedly increase both the efficiency and accuracy of streptococcal serology for the diagnosis of ARF and PSGN, diseases associated with significant global morbidity and mortality.

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Appendix. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jinf.2017.12.008.

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