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RESEARCH PAPER

Development of a multiplexed opsonophagocytic killing assay (MOPA) for group B *Streptococcus*

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ABSTRACT

Group B *Streptococcus* (GBS) is a leading cause of sepsis in infants as well as chorioamnionitis in pregnant women. Opsonophagocytic killing assays (OPAs) are an essential technique in vaccine studies of encapsulated bacteria for estimating serotype-specific functional antibody levels *in vitro*. Here, we developed a three-fold multiplexed OPA (MOPA) to enable practical, large-scale assessment of GBS vaccine immunogenicity, including against serotypes Ia, III, and V. First, three target bacteria strains resistant to streptomycin, spectinomycin, or kanamycin were generated by natural selection through exposure to increasing antibiotic concentrations. Since a high level of nonspecific killing (NSK) of serotype V was observed in a 12.5% baby rabbit complement (BRC) solution, the BRC concentration was optimized. The final GBS-MOPA BRC concentration was 9%, which resulted in less than 20% NSK. The specificity was measured by preabsorbing serum with inactivated GBS. The opsonic index (OI) of preabsorbed serum with the homologous serotype GBS was significantly reduced in all three serotypes tested. The accuracy of the MOPA was compared with that of a single OPA (SOPA) with 35 serum samples. The OIs of the MOPA correlated well with those of the SOPA, and the r^2 values were higher than 0.950 for all three serotypes. The precision of the MOPA assay was assessed in five independent experiments with five serum samples. The inter-assay precision of the GBS-MOPA was 12.5% of the average coefficient of variation. This is the first report to develop and standardize a GBS-MOPA, which will be useful for GBS vaccine development and evaluation.

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capsular polysaccharide; immunoassay; opsonophagocytic killing assay; *Streptococcus agalactiae*

Introduction

Streptococcus agalactiae (group B streptococcus, GBS) remains a leading cause of sepsis and meningitis in infants.[1-8] Across diverse ethnic groups, 5 to 43.6% of all pregnant women are asymptomatically colonized with GBS in the vagina, leading to vertical transmission to the newborn during vaginal delivery.[9-11] A meta-analysis reported an overall estimated incidence of invasive GBS disease as 0.53 occurrences per 1000 live births, with a 9.6% case fatality rate.[12] Recent reports indicated that GBS has also emerged as a frequent cause of invasive infections in pregnant women and non-pregnant adults with underlying medical conditions.[13-24] Similar to pneumococcal infections, the incidence of GBS infection increases with age and has been reported to be as high as 26 infections per 100,000 adults in the elderly population aged ≥65 years.[24-26] Notably, the case fatality rate for the elderly is estimated at 15%, much higher than the 4 to 6% for young infants.[25] Prenatal GBS culture screening and intrapartum antibiotic prophylaxis (IAP) for high risk pregnant women have been effective in reducing early-onset disease (EOD), but not in eliminating it.[24,27] Moreover, this approach is not able to prevent late-onset disease (LOD) or infection in adults.[28] Considering the pitfalls of the current prenatal strategy and the high burden of GBS infections in the elderly, the development of an efficacious GBS vaccine has been eagerly requested.[29-31]

Several polysaccharide conjugate vaccines (PCVs) have completed phase I/II clinical trials. These vaccines have been evaluated by estimating the levels of maternal transferred polysaccharide (PS)-specific IgG in infant serum using an enzyme-linked immunosorbent assay (ELISA).[31] However, ELISA results might not always reflect functional antibody levels for encapsulated bacteria, as is the case for pneumococcal vaccines.[32-34] The first WHO technical consultation on GBS vaccines was held in April 2016. Among other topics, this consultation considered how GBS vaccine efficacy will be evaluated in a phase III trial because of the low baseline incidence of GBS invasive disease (primary endpoint).[31]

The results of *in vitro* opsonophagocytic killing assays (OPAs) are proven to correlate well with the functional efficacy of pneumococcal vaccines, because host protection against pneumococcus is mainly mediated by opsonin-dependent phagocytosis.[35,36] For development of a GBS vaccine and

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clinical trials of vaccine candidates, a GBS-specific OPA is required. Pregnant women are the main target of GBS vaccines, and the maternal transfer of specific anti-PS IgG must be assessed using only an extremely small amount of serum from the newborn. Nahm *et al.* modified the classical OPA into a multiplexed opsonophagocytic killing assay (MOPA) for pneumococcus, thereby reducing the amounts of serum sample, reagents, and time required by two-fold or four-fold.[37,38] In this study, we developed, standardized, and validated a three-fold multiplexed GBS-OPA (GBS-MOPA) to enable practical, large-scale assessment of GBS vaccine immunogenicity against serotypes Ia, III, and V.

Results

GBS-MOPA optimization

The GBS-MOPA was adopted from the previously described pneumococcal MOPA protocol[37] by adding several minor modifications specific for GBS. The first issue to overcome was that the GBS colonies spotted on THY agar (THA) plates floated onto the overlay agar during incubation. This problem was solved by using TSA (1.5% agar) plates with a THA (0.75% agar) overlay.

Second, significant nonspecific killing (NSK) of GBS was observed, particularly for serotype V, whose NSK was estimated to be as high as 80%. Several lots of complement collected from 3- to 4-week-old baby rabbits were tested and yielded consistent results (data not shown). Previously, NSK was observed in multiple pneumococcal serotypes. This phenomenon is likely caused by an unknown factor(s) in baby rabbit complement capable of opsonizing or killing GBS.[37] Pneumococcal NSK was shown to be dramatically reduced by performing the phagocytosis phase in a 5% CO_2 incubator.[37] We also found that NSK of serotype V was markedly reduced in a 5% CO_2 incubator, but remained around 50%. Thus, the complement concentration was further adjusted. A human serum pool (pool 1) and a convalescent serum (FGBS5) from the patient with GBS infection were used to evaluate the complement concentration-dependent NSK. As shown in Fig. 1, 12.5% complement (the concentration recommended in the pneumococcal MOPA) yielded around 84% NSK against GBS serotype V, whereas a less than 20% NSK was observed at 9.0% and 7.5% complement conditions. Of note, no significant effects on NSK or OPA activities at these concentrations were observed for the other two serotypes (Ia and III). Thus, the optimal complement concentration was set at 9% for the GBS-MOPA.

Finally, the effector-to-target (E:T) ratio was optimized. To this end, the effect of altering the number of HL-60 cells at a constant number of GBS cells (1,000 CFUs/well of each strain) was investigated. The OIs for serotypes Ia, III, and V decreased more than 30% at E:T ratios ranging from 100:1 to 50:1. However, the reduction was modest (less than 20%) with E:T ratios ranging from 400:1 to 150:1 (data not shown). Thus, the optimal E:T ratio was set at 200:1 for the GBS-MOPA protocol.

Validation of the MOPA

Specificity

No available reference serum from a GBS vaccinee is currently available for measuring the specificity of the GBS-MOPA. Thus, the assay specificity for neutralizing serum samples was determined by adsorbing serotype-specific antibodies using inactivated serotype-specific GBS. As shown in Fig. 2, the OIs of native serum were 199, 17,496, and 1,275 against serotypes Ia, III, and V, respectively. In contrast, the OIs of pre-adsorbed serum were undetectable against Ia and V and 1,498 against serotype III, which was 11.7 times lower than that of the native serum. Pre-adsorption with the heterologous GBS serotype resulted in an OI reduction of 0–30%, indicating the possible presence of cross-reactive antibodies in the serum (Fig. 2). The OI reductions of the various serum samples were diverse.

Complement concentration	Opsonic index		Non-specific killing
	Pooled sera	FGBS 5	
12.5%	17,496	17,496	84%
9.0%	1,368	3,479	20%
7.0%	1,944	2,770	18%

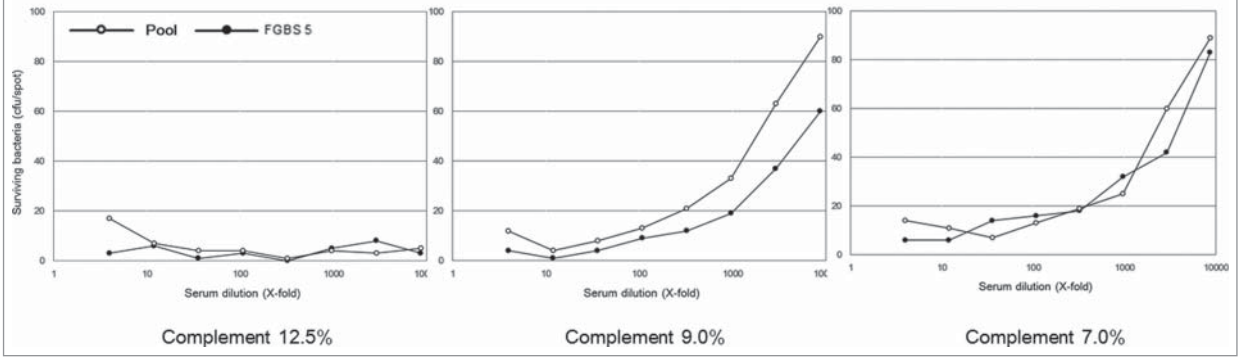

Figure 1. Effect of concentration of baby rabbit complement (BRC) on non-specific killing (NSK) of group B streptococcus (GBS) serotype V. (A) Opsonic indexes (OIs) and NSK ratios at different BRC concentrations in the GBS multiplexed opsonophagocytic killing assay (MOPA). (B) GBS-MOPA results obtained using different BRC concentrations ranging from 7.0% to 12.5%.

Serotype-specific adsorption	OI for serotype Ia		OI for serotype III		OI for serotype V	
	PBS	Inactivated bacteria	PBS	Inactivated bacteria	PBS	Inactivated bacteria
Serotype Ia	199	4	17,496	17,496	2,552	1,984
Serotype III	235	199	17,496	1,498	2,831	2,552
Serotype V	294	246	17,496	17,496	1,275	2

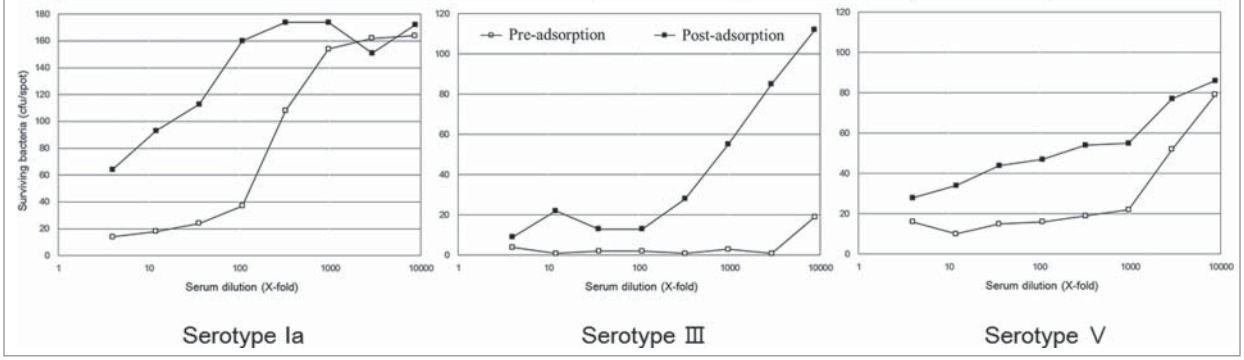

Figure 2. Specificity analysis of the group B streptococcus (GBS) multiplexed opsonophagocytic killing assay (MOPA). Specificity of the GBS-MOPA was analyzed by comparing pre-adsorption (open circle) and post-adsorption (closed circle) opsonic indexes (OIs) of the serum samples with homologous/heterologous GBS. Results with homologous adsorption are presented in the graphs. The y-axis and x-axis indicate the number of bacteria and the three-fold serum dilution number, respectively.

Accuracy

The assay accuracy was also examined by comparing the values of the MOPA to those of the previously described SOPA.[37] To this end, 35 serum samples (20 subjects with previous GBS infection and 15 healthy subjects) were tested in both assays, and the obtained MOPA (*y*-axis) and SOPA (*x*-axis) OIs were compared (Fig. 3). The dashed and solid lines in the graph indicate identity and two-fold deviation from identity, respectively. Although 15 of the 105 data points deviated more than two-fold from identity, the OIs of both assays were well correlated, with *R*-squared correlation coefficient (r^2) values exceeding 0.950.

Precision (reproducibility)

The reproducibility of the GBS-MOPA under normal operating conditions (inter-assay variation) was evaluated in five independent experiments performed by two independent operators using five serum samples on four non-consecutive days. The mean OI, standard deviation, and coefficient of variation of the five independent experiments were calculated. The coefficients of variation (CV) of the three serotypes ranged from 0% to 31.2% (average 12.5%) (Table 1). For serotype Ia, 4 of the 5 serum samples showed consistently undetectable opsonization, with an OI <4.

Discussion

While GBS is considered an important pathogen with a large disease burden among neonates and infants, no GBS vaccine is yet available.[29-31,39] Currently, both GlaxoSmithKline (GSK) and Pfizer are in early phase development of an investigational multivalent polysaccharide conjugate vaccine, while MinervaX

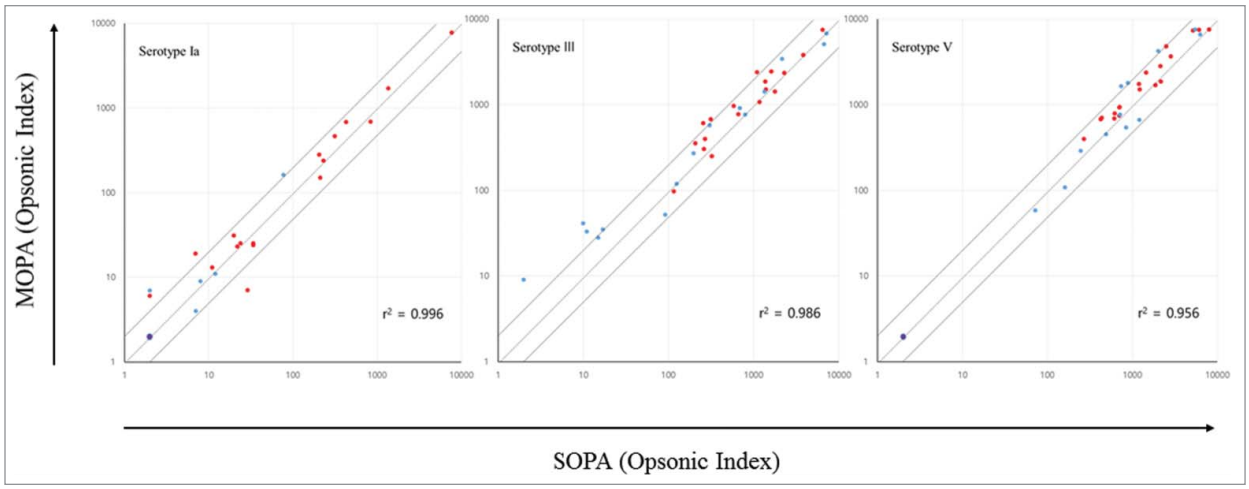

Figure 3. Comparison of opsonic indexes (OIs) obtained from the group B streptococcus (GBS) multiplexed opsonophagocytic killing assay (MOPA) versus those from the GBS single opsonophagocytic killing assay (SOPA). Twenty serum samples from subjects who had recovered from previous GBS infection (red circles) and 15 serum samples from healthy adults without any prior episode of GBS infection (blue circles) were tested in the MOPA (y-axis) and SOPA (x-axis). The dashed line and solid line indicate the line of identity and two-fold deviation from identity, respectively. OIs were estimated to be below the detection limit (assigned as 2, purple circle) in 13 samples (3 from subjects with previous GBS infection and 10 from healthy subjects) for serotype Ia and in 3 samples (3 from healthy subjects) for serotype V.

Table 1. Inter-assay precision of the group B streptococcus multiplexed opsonophagocytic killing assay (GBS-MOPA).

	STREP-Ia		SPEC-III		KAN-V	
Sample	Mean ± SD	CV (%)	Mean ± SD	CV (%)	Mean ± SD	CV (%)
Sample 1	2 ± 0	0	599.8 ± 174.3	29.1	753.8 ± 122.4	16.2
Sample 2	87.1 ± 23.4	26.8	6467.4 ± 1328.9	20.5	30.7 ± 27.9	9.1
Sample 3	2 ± 0	0	10.1 ± 1.8	17.9	7.8 ± 3.4	4.3
Sample 4	2 ± 0	0	14.3 ± 3.1	21.8	724.3 ± 230.9	31.9
Sample 5	2 ± 0	0	2.5 ± 0.4	4.3	2.7 ± 1.6	6.1

recently completed a phase I trial of a protein-based GBS vaccine containing a fusion protein of the N-terminal domains of two alpha-like proteins (Rib and AlpC).[40-43] Since the efficacy of candidate GBS vaccines in clinical trials has only been measured by ELISA, which might not accurately distinguish the functional activities of low and high avidity antibodies, an OPA is needed to assess the efficacy of candidate GBS vaccines.[41] To develop an OPA against GBS, we reasoned that the assay should be (1) multiplexed, because the amount of infant serum available for testing is extremely small; and (2) compatible with the pneumococcal OPA (e.g. reagents and protocol), because the primary users of the newly developed GBS-OPA protocol are predicted to be predominantly users of the pneumococcal OPA. Therefore, these users can easily adapt to the proposed OPA for evaluating GBS vaccines.

In this study, we developed a three-fold multiplexed GBS-OPA targeting serotypes Ia, III, and V whose coverage in EOD and LOD is around 81% on a systematic meta-analysis.[12] Similar to certain pneumococci serotypes, GBS serotype V showed a particularly high level of NSK. This high level was observed even after incubation in 5% CO_2 during the phagocytosis phase, which has been shown to have significantly reduced pneumococcal NSK.[37] As reported previously, the level of bacterial capsule expression and complement concentration are critical factors influencing NSK; therefore, these factors should be optimized to reliably measure the opsonophagocytic killing (OPK) of encapsulated bacteria by functional antibodies.[35,44] For example, pneumococci with small capsules are considered to have higher sensitivity to complement-mediated NSK, while pneumococci with large capsules are likely to be relatively resistant to OPK. We found highly variable levels of NSK and OPK when the OPA was performed with several GBS serotype isolates (data not shown). However, NSK of GBS was significantly and consistently reduced (<30%) in the assay by using 9% BRC instead of 12.5% BRC, as recommended by the pneumococcal MOPA protocol. A previous study showed that the optimal complement concentration in the pneumococcal OPA ranged from 8 and 16%, depending on the serotype.[34] Thus, we chose 9% BRC as the optimal concentration for the GBS-MOPA. At this concentration, NSK was consistently lower than the 40% observed against Ia and III. However, it is not possible at this point to determine whether the high level of NSK of serotype V is due to differences in the level of capsule expression in different GBS serotypes.

The GBS-MOPA was validated with respect to specificity, accuracy, and precision. Analysis of assay specificity showed that the GBS-MOPA was sufficiently specific, since adsorption of the sera with homologous inactivated GBS resulted in significant reductions of OIs. However, we also found modest reductions (0–30%) of OIs with heterologous serotype adsorption (Fig. 2). These cross-reactive antibodies might bind to conserved antigen(s) expressed on the surface of GBS. When we examined reactive surface antigen protein(s) in the sera by Western blotting, at least three conserved cell wall-anchoring proteins were found in the sera with high OIs (data not shown). To better assess the specific activity of serotype-specific functional anti-CPS antibodies, pre-adsorption of serum with either non-capsulated GBS strains or surface protein extracts should be considered. In addition, the accuracy and precision of our GBS-MOPA were acceptable when compared with the pneumococcal MOPA. Although the OIs of the MOPA were slightly higher than those of the SOPA, correction of the OIs with standard serum values should theoretically reduce these assay variations.[45] As Burton et al. suggested, reference standard sera would be required for calibration of the OPA in the initial evaluation of new assays or periodic assessment of established assays.[45] In addition, these will be quite useful for the normalization of OPA results from different laboratories.[45]

In April 2016, the WHO convened the first consultation on GBS vaccine development. This meeting was focused on the GBS maternal immunization program, which is aimed at reducing newborn and young infant infections worldwide. One of the issues considered in the meeting was the need for an efficient functional efficacy test to accelerate the pipeline of GBS vaccine development. Thus, a standardized functional efficacy assay is required to evaluate clinical efficacy in the process of vaccine approval and licensure. In this study, we developed and validated three new target strains for the GBS-MOPA. We are currently developing three additional target serotypes (Ib, IV, VI), which will cover around 92% of all GBS infections. These strains will be deposited at the Korean Collection for Type Cultures (KCTC) and the American Type Culture Collection (ATCC) for distribution worldwide.

Materials and methods

The study was conducted in two phases for the development and optimization of the MOPA for GBS. First, target bacterial strains were generated, and OPA conditions including type of agar plate, complement concentration, and effector-to-target (E:T) ratio were optimized. Second, the assay specificity, accuracy, and precision were investigated as described in the pneumococcal MOPA protocol (www.vaccine.uab.edu). The study was approved by the ethics committee of Korea University Guro Hospital (IRB No. KUGH16119).

Serum samples

This study was approved by the ethics committee of Korea University Guro Hospital (IRB No. KUGH14106) and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice. The institutional review board of KUGH waived the requirement for written informed consent for the use of deidentified remnant serum samples. A human serum pool (pool 1) was prepared by mixing sera from 15 healthy adults aged ≥19 years. This pool was used for quality control of the OPA. Twenty serum samples were from subjects

who had recovered from previous GBS infection, and 15 serum samples were collected from healthy adults without any prior episode of GBS infection. All study samples were obtained from subjects who had not received any antibiotics in the three days prior to blood sampling. These samples were used to optimize and validate the GBS MOPA.

Generation of antibiotic-resistant GBS strains and GBS working stocks

The GBS strains used in this study are described in Table 2. Spectinomycin-resistant, streptomycin-resistant, and kanamycin-resistant variants of GBS serotypes Ia, III, and V were generated by natural selection. Specifically, cells were grown in Todd-Hewitt broth (Becton Dickinson; Sparks, MD) with 0.5% yeast extract (Becton Dickinson) (THY broth) in the presence of increasing antibiotic concentrations. Antibiotic-resistant GBS strains were cultured in THY broth at 37 °C until reaching an optical density at 600 nm (OD_{600}) of 0.8–1.0. Bacterial working assay stocks were cryopreserved at −80 °C in THY containing 15% glycerol until use.

GBS opsonophagocytic killing assay

The GBS multiplexed opsonophagocytic killing assay (GBS-MOPA) consisted of a modified version of the pneumococcal MOPA.[38,46,47] In brief, HL-60 cells were differentiated into granulocytic cells by culturing in RPMI 1640 medium supplemented with 10% fetal calf serum (FBS; HyClone, Logan, UT) and 0.8% dimethylformamide (Fisher Scientific, Pittsburgh, PA) for 5 days. Differentiated HL-60 cells were diluted to 10^7 cells/ml with opsonization assay buffer B [OBB; Hanks' buffer supplemented with 0.1% gelatin (Sigma-Aldrich) and 10% FBS]. Thirty microliters of each test serum sample was serially diluted 3-fold with OBB in 96-well plates. The assay used 10 μL of stock and diluted sera. Ten microliters of heat-inactivated complement was used as a negative control. Frozen working stocks of each of the three target GBS strains were thawed immediately before use and washed twice with OBB, after which the cells were resuspended and adjusted to 1×10^5 CFU/mL in OBB. The three GBS strains were mixed identically, and 10 μL of the GBS mixture was then added to each well. After 30 min of incubation at room temperature, 42.8 μl of differentiated HL-60 cell suspension (4.28×10^5 cells per well) and 7.2 μl of baby rabbit complement (BRC; Pel-Freez Biological; Rogers, AR) were added to each well, after which the mixture was incubated for 45 min at 37°C with shaking. Afterward, 10 μL of the final reaction mixture from each well was spotted onto tryptic soy agar containing 1.5% bacto-agar (TSA; Becton Dickinson). Next, 25 mL of THY with 0.75% bacto-agar containing one of three antibiotics and 2,3,4-triphenyltetrazolium chloride (TTC; Sigma-Aldrich) was overlaid onto the spotted plates, after which the plates were incubated overnight at 37°C. Surviving bacterial colonies on the plates were enumerated, and the opsonic indexes (OIs) of the serum samples were determined. The OI was defined as the last dilution of the serum sample yielding a CFU reduction of 50% compared to the negative control, to which 10 μL of heat-inactivated complement was added instead of diluted serum. An undiluted serum sample killed 50% of the GBS, yielding an OI of 4. A control serum sample (pool 1) was included in each assay to monitor assay reproducibility.

For the GBS single opsonophagocytic killing assay (GBS-SOPA), the same protocol was performed as for the GBS-MOPA, except that the GBS strains were prepared as single GBS serotypes rather than a mixture of 3 serotypes. After following the same protocol used in the GBS-MOPA, 5 μL of the final reaction mixture from each well was spotted onto tryptic soy agar containing 1.5% bacto-agar (TSA; Becton Dickinson). Next, 25 mL of THY with 0.75% bacto-agar containing only 2,3,4-triphenyltetrazolium chloride (TTC; Sigma-Aldrich) was overlaid onto the spotted plates, after which the plates were incubated overnight at 37°C. After overnight incubation, the bacterial colonies on the agar plates were counted using National Institute of Standards and Technology, US's Integrated Colony Enumerator.

Table 2. Bacterial strains used in the group B streptococcus multiplexed opsonophagocytic killing assay (GBS-MOPA).

MOPA strain	Serotype	ATCC No.	Resistant antibiotic and concentration
STREP-Ia (A909)	Ia	12401	Streptomycin (300 mg/L)
SPEC-III (NEM316)	III	12402	Spectinomycin (300 mg/L)
KAN-V (NCTC10/84)	V	49447	Kanamycin (300 mg/L)

Specificity determination by serum sample adsorption

Assay specificity was determined by pre-adsorption of serum samples with formalin-inactivated hetero-serotype or homo-serotype GBS. The indicated GBS serotypes (Ia, III, and V) were grown in THY broth to an OD_{600} of 0.5. Fifty milliliters of each GBS culture broth was mixed with 0.2% formalin (100 μl, v/v) and incubated at 37°C for 2 hr. Inactivated GBS cells were then harvested by centrifugation and resuspended in phosphate-buffered saline (PBS) with 15% glycerol. One milliliter aliquots were stored at −80 °C. For adsorption, the GBS aliquots were washed twice with OBB by centrifugation at 13,000 × g for 2 min, followed by resuspension in 1 mL of OBB. Fifty microliters of inactivated GBS was mixed with 450 μl of serum sample, followed by incubation at 4°C for 2 hr with shaking. The GBS cells were then removed by centrifugation (13,000 × g, 5 min), and 400 μl of absorbed serum was collected and used immediately for the GBS-MOPA.

Statistical analysis

All statistical analyses were performed using SPSS 18.0 (SPSS Korea, Seoul, Republic of Korea). As for the precision, test conditions were considered equivalent and reproducible with CV of ≤20%. With respect to accuracy, r^2 values ≥0.95 on linear correlation analysis was considered acceptable.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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