Immunochromatographic Detection of the Group B Streptococcus Antigen from Enrichment Cultures

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Group B Streptococcus (GBS; Streptococcus agalactiae) is a comensal bacterium found in the genital and lower intestinal tracts, and it has been isolated from 10 to 30% of pregnant women (1–3). Transmission of this organism from GBS carrier women to neonates was reported to occur in about 50% of deliveries, and 1 to 3% of the affected neonates were assumed to develop severe GBS infections, such as neonatal pneumonia, sepsis, and meningitis. The mortality of early-onset GBS infections within the first week has decreased from 50% in the 1970s to 4 to 6% in recent years due to the advancement of intrapartum and neonatal care. There is still a high mortality rate among preterm infants compared with full-term infants (4–7). Moreover, a serious, as-yet-unresolved problem is that up to 50% of survivors suffer from neurological sequelae, including mental retardation, spastic quadriplegia, blindness, and deafness (8–10).

The Centers for Disease Control and Prevention (CDC) published guidelines for prevention of GBS infections in 1996 (11), and its 2002 version recommended that culture-based GBS screening be conducted during the 35th to 37th weeks of gestation (12). For GBS carrier pregnant women, intrapartum antibiotic prophylaxis (IAP) with penicillin or ampicillin has been performed since the early 1990s to 0.34 to 0.37 cases in recent years in the United States (13). Prophylaxis (IAP) with penicillin or ampicillin has been performed. The incidence of early-onset GBS disease has been remarkably decreased, from 1.7 cases per 1,000 live births in the early 1990s to 0.34 to 0.37 cases in recent years in the United States (13).

To improve the sensitivity of GBS detection, the guidelines recommend an enrichment culture using Lim broth or modified Granada medium (13). The cells producing red to orange pigment in Granada medium are classified as beta-hemolytic GBS. Microorganisms grown in the enrichment culture are streaked onto the sheep blood agar plate, and suspected colonies are subjected to further GBS identification tests, such as the latex aggregation test, CAMP test, or conventional identification tests. The whole procedure to identify GBS by the culture method requires several days. Since culture methods may fail to detect nonhemolytic GBS, alternative GBS detection methods, such as the DNA probe test and PCR, have been developed recently (14–16).

This article reports the development of an immunochromatographic test (ICT) that can detect the GBS antigen from an overnight enrichment broth culture within 15 min. Newly developed monoclonal antibodies that recognize surface immunogenic protein (Sip) of GBS were used (17). The reliability of this newly developed ICT was assessed using vaginal swabs, and the results were compared with those from culture methods.

(A part of this work was presented at the 57th meeting of the Eastern Branch of the Japanese Society of Chemotherapy and received a Research Promotion Award.)

MATERIALS AND METHODS

Expression and purification of His-tagged rSip. DNA was purified from the nonhemolytic Streptococcus agalactiae strain KUB791 using the DNeasy Blood & Tissue Kit (Qiagen K.K., Tokyo, Japan). The sip gene was amplified by PCR using a pair of primers designed in this laboratory, 5' - CATCAGTTGTATACAAAAAGGTATCTTG-3' and 5'- CATCAGTTGTATACAAAAAGGTATCTTG-3', containing the NcoI and XhoI restriction sites, respectively. PCR was carried out with PrimeSTAR

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HS DNA polymerase (TaKaRa Bio Inc., Shiga, Japan) under the following conditions. After initial denaturation of DNA at 98°C for 30 s with a Gene Amp PCR System 9700 thermal cycler (Applied Biosystems Inc., Tokyo, Japan), the program was set at 98°C for 10 s, 55°C for 5 s, and 72°C for 2 min for a total of 26 cycles and then at 72°C for 5 min for the final extension. Amplified sip DNA was ligated to the pETBlue2 vector containing a sequence encoding hexahistidine (Novagen, Madison, WI). Escherichia coli Novablu(DE3) cells were transformed with pETBlue2-sip by a previously described method (18). The transformants were cultured in LB broth (Becton, Dickinson and Co., Tokyo, Japan) containing 50 μg/ml of carbenicillin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 34 μg/ml of chloramphenicol (Wako Pure Chemical Industries, Ltd.) at 37°C until the absorbance at 578 nm reached 0.5. Hexahistidine-tagged recombinant Sip (rSip) was expressed by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG); Wako Pure Chemical Industries, Ltd.), and the culture was incubated at 37°C for an additional 2 h. The soluble fraction containing rSip was extracted by ultrasonic oscillation and then applied to and eluted from a nickel-ion immobilized-metal affinity chromatography (IMAC) resin column (Bio-Rad Laboratories, K.K., Tokyo, Japan) by a previously described method (18). The protein concentration was quantified by the Lowry method with bovine serum albumin (BSA) as the standard (Thermo Fisher Scientific K.K., Kanagawa, Japan).

Electrophoresis and Western blotting. Crude extracts of the transformant cells and purified rSip were subjected to sodium dodecyl sulfate-polyacrylamide gel (12%) electrophoresis (SDS-PAGE). Protein bands were visualized by staining with Coomassie brilliant blue (CBB) or the Western blotting method using the anti-His tag antibody (Qiagen K.K., Tokyo, Japan). For Western blotting, proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (ATTO Corporation, Tokyo, Japan). The membrane was treated as follows: blocked with phosphate-buffered saline (PBS) containing 4.0% Block Ace (DS Pharma Biomedical Co., Ltd., Osaka, Japan) and 0.1% Tween 20 (Wako Pure Chemical Industries, Ltd.) for 1 h at 24°C, washed 4 times with PBS containing 0.1% Tween 20, incubated with 100 ng/ml of monoclonal antibodies at 24°C for 1 h, washed 4 times with PBS containing 0.1% Tween 20, incubated with 2,000-fold-diluted horseradish peroxidase (HRP)-conjugated anti-mouse Ig (Dako Japan, Tokyo, Japan) at 24°C for 1 h, washed 4 times with PBS containing 0.1% Tween 20, and then soaked in the TMB membrane peroxidase substrate system (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) for color development.

Preparation of anti-Sip monoclonal antibodies. Anti-Sip monoclonal antibodies were raised against the purified rSip protein and two synthetic peptides. The synthetic peptide antigens were designed using antigenicity prediction programs (19, 20), and two expected peripheral peptides were chosen. One peptide corresponded to the 313th to 336th amino acid residues of the Sip protein, and an additional cysteine residue was added at the N terminus (peptide 313–336, NH2-CNAVAAPHENA GLOPHVAAKKEVKA-OH) (Biologica, Nagoya, Japan). Another peptide was used corresponded to the 200th to 217th amino acid residues of the Sip protein, with an additional cysteine residue (peptide 200–217, NH2-CEV PAAKEEVKPTQTSVSO-OH). These peptides were coupled to keyhole limpet hemocyanin with N-(6-maleimidocaproyloxy) succinimide (Dojindo Laboratories, Kumamoto, Japan). Mice were immunized with these antigens, and monoclonal antibodies were obtained by a previously described method (18).

Specificity test of monoclonal antibody. Whole-cell extracts were prepared from nine GBS strains representing different serotypes (Table 1). Other Streptococcus strains tested were Streptococcus pneumoniae ATCC 49619 (American Type Culture Collection), Streptococcus pyogenes ATCC 12344 (group A streptococcus), Streptococcus dysgalactiae subsp. equisimilis KUB794 (group C streptococcus), and Streptococcus dysgalactiae subsp. equisimilis ATCC 12394 (group G streptococcus). Cells were grown on sheep blood agar overnight, suspended in 0.125 M Tris-HCl (pH 6.8) containing 5% 2-mercaptoethanol, 2% SDS, 5% glycerol, and 0.02% bromophenol blue, and then boiled for 5 min. The centrifuged supernatant was subjected to SDS-PAGE (12%), and then the protein bands were blotted onto a PVDF membrane. The PVDF membrane was treated as follows: blocked with PBS containing 2% BSA and 0.1% Tween 20 overnight at 4°C, washed 4 times with PBS containing 0.1% Tween 20, incubated with 100 ng/ml of monoclonal antibodies at 24°C for 1 h, washed 4 times with PBS containing 0.1% Tween 20, and incubated with 10,000-fold-diluted HRP-conjugated anti-mouse Ig (Dako Japan, Tokyo, Japan) at 24°C for 1 h. Subsequent washing and color development steps were similar to the above.

Preparation of the immunochromatographic test strip. The membrane with immobilized antibodies was prepared by a previously described method using the anti-rSip monoclonal antibody (clone R68) and anti-mouse IgG antibody, to serve as test and control lines, respectively (18). These monoclonal antibodies were chosen for high sensitivity on the ICT. Colloidal gold-conjugated IgG was prepared by a previously described method using anti-synthetic peptide (peptide 313–338) monoclonal antibody (clone 6H8), absorbed into a fiberglass pad (Millipore, Billerica, MA), and air dried. The membrane, the fiberglass pad, an absorbent pad (Millipore), and a sample pad (Whatman Japan K. K., Tokyo, Japan) were laminated on an adhesive sheet and cover by a clear film.

Performance evaluation of ICT. The detection limit of ICT was evaluated using nine GBS strains representing serotypes (Table 1). Bacterial cells were grown on sheep blood agar at 35°C for 18 h and suspended in sterile saline. The viable cells were counted on sheep blood agar. The Sip antigen was extracted by mixing 25 μl of a serially diluted cell suspension with 250 μl of 0.5 M NaOH containing 0.02% Triton X-100 (Wako Pure Chemical Industries, Ltd.). It was then left to stand for 3 min, and the solution was neutralized with 250 μl of 0.6 M tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES) containing 0.15 M HCl and 0.02% Triton X-100. The test strip assembly was placed in a test tube containing extract, and the chromatography was developed for 10 min at room temperature. The reaction products at the test line and the control line were observed macroscopically.

The cross-reactivity of ICT was evaluated using the following microorganisms grown on sheep blood agar at 35°C overnight: Staphylococcus aureus FDA209P, Staphylococcus epidermidis KUB795 (laboratory stock), Staphylococcus haemolyticus ATCC 29970, Staphylococcus lugdunensis ATCC 43809, Streptococcus pyogenes ATCC 12344, group C Streptococcus KUB794 (laboratory stock), Streptococcus dysgalactiae subsp. equisimilis

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**Table 1 GBs strains used in this study**

| GBS strain | Serotype | Source |
|------------|----------|--------|
| KUB 791    | II       | Clinical isolate, laboratory stock |
| KUB 159a   | Ia       | Clinical isolate, laboratory stock |
| KUB 174a   | Ib       | Clinical isolate, laboratory stock |
| KUB 161a   | II       | Clinical isolate, laboratory stock |
| ATCC 12403a| III      | ATCC   |
| ATCC 49446a | IV      | ATCC   |
| ATCC BAA-611a,b | V | ATCC   |
| KUB 171a   | VI       | Clinical isolate, laboratory stock |
| KUB 166a,b | VII      | Clinical isolate, laboratory stock |
| KUB 178a   | VIII     | Clinical isolate, laboratory stock |
| IID 1621b  | Ia       | Institute of Medical Science, the University of Tokyo |
| IID 1622b  | Ib       | Institute of Medical Science, the University of Tokyo |
| IID 1624b  | II       | Institute of Medical Science, the University of Tokyo |
| IID 1625b  | III      | Institute of Medical Science, the University of Tokyo |
| KUB 953b   | VI       | Clinical isolate, laboratory stock |
| KUB 987b   | VIII     | Clinical isolate, laboratory stock |

*Used for the specificity test of monoclonal antibodies by Western blotting.
*Used for the detection limit test of ICT.
RESULTS

Preparation of His<sub>6</sub>-tagged rSip and anti-Sip monoclonal antibodies. The amplified sip gene of 1,316 bp was cloned into pETBlue2, which carries a lac operator and a sequencing code for hexahistidine. E. coli strain Tuner(DE3) pLacI cells harboring the pETBlue2-sip plasmid grown in the presence and absence of IPTG. The rSip fraction was purified from the cells in the absence of inducer; lane 2, crude soluble fraction from the cells in the presence of IPTG; lane 3, affinity-purified rSip.

FIG 1 SDS-PAGE profiles of the crude extracts and purified rSip. The soluble fraction was prepared from E. coli strain Tuner(DE3) pLacI cells harboring the pETBlue2-sip plasmid grown in the presence and absence of IPTG. The rSip fraction was purified by Ni resin affinity chromatography. The rSip fraction was subjected to SDS-PAGE. (a) The soluble fraction (7.5 μg) and purified rSip (2 μg) were applied. (b) The soluble fraction (50 ng) and purified rSip (10 ng) were applied, run through SDS-PAGE, and electroblotted onto a PVDF membrane. The membrane was stained with the Western blotting method using the anti-His tag antibody. Lane M, molecular mass markers; lane 1, crude soluble fraction from the cells in the absence of inducer; lane 2, crude soluble fraction from the cells in the presence of IPTG; lane 3, affinity-purified rSip.

Performance of ICT. The ICT was prepared using R6E8 and S6H8 monoclonal antibodies. This combination of antibodies yielded the optimum sensitivity and specificity on the ICT. The S6H8 antibody, raised against peptide 313–336, was conjugated with a colloidal gold particle that served as the Sip detector, and the other antibody, R6E8, raised against rSip, was immobilized on a nitrocellulose membrane that captured the S6H8-gold-colloid-Sip complex, thereby forming an antigen sandwich with two monoclonal antibodies.

Purified rSip was used to first evaluate the detection limit of the ICT. The ICT was able to detect levels as low as 0.5 ng/ml of rSip (Fig. 3). The intensity of the test lines increased as the concentration of rSip was increased up to 50 ng/ml. In the next experiment, whole-cell extracts from the nine GBS strains with different serotypes were subjected to the ICT (Table 2). The results showed that the detection limit of the ICT varied from one strain to another, ranging from 9.5 × 10<sup>5</sup> to 3.7 × 10<sup>6</sup> CFU/ml of GBS (Table 2). To test cross-reactivity of the ICT, 26 strains from 24 bacterial species and 2 strains of Candida species were examined at cell densities of 10<sup>4</sup> CFU/ml and 10<sup>5</sup> CFU/ml, respectively. The results clearly showed that none of these microorganisms yielded false-positive reactions (data not shown).

Reactivity of the ICT was evaluated using 229 strains of clinically isolated and identified GBS. Among these, 228 strains showed positive results. However, only 1 strain yielded a negative result even though the culture contained GBS cells at the level of approximately 10<sup>5</sup> CFU/ml (see Discussion).

Evaluation of the ICT using vaginal swabs. Practical application of the ICT with clinical specimens was tested using 260 vagi-
nal swabs. The swab specimens were first subjected to enrichment culture in GBS medium F, a modified version of Granada medium; 236 were turbid by 24 or 48 h of incubation, and 24 were clear even after 48 h of incubation (Fig. 4). Among the 236 turbid cultures, 17 produced red to orange pigments, a typical property of hemolytic GBS, which was equivalent to 6.5% of the tested vaginal swabs. The remaining 219 were pigment negative. All the turbid cultures were streaked on ChromID StreptoB agar plates, and suspected GBS colonies were subjected to GBS identification tests by latex aggregation and PCR. All the pigment-positive cultures contained GBS without exception, whereas only 12 out of 219 pigment-negative cultures appeared to be GBS positive. The following numbers and species of microorganisms were identified from 236 turbid cultures: 149 strains of *E. faecalis*, 5 of *C. albicans*, 11 of coagulase-negative staphylococcus, 7 of *E. faecium*, 5 of *C. glabrata*, 5 of *L. acidophilus*, 4 of *E. coli*, 2 of *P. aeruginosa*, 2 of *Enterococcus gallinarum*, and 1 strain each of group A streptococcus, group G streptococcus, *S. aureus*, *Streptococcus bovis*, and *Candida parapsilosis*.

All turbid cultures, regardless of pigment production, were subjected to the ICT (Fig. 4). The test revealed that 17 pigment-positive cultures were Sip antigen positive, without exception. The remaining 219 were pigment-negative cultures, equivalent to 84.2% of the total. Among 12 GBS-positive nonpigmented cultures, 10 were Sip antigen positive and the remaining 2 were Sip antigen negative. Among 29 GBS-positive specimens detected by the culture method, 27 were Sip antigen positive. Therefore, the sensitivity of the ICT was 93.1%. Among 207 GBS-negative, nonpigmented cultures, only 1 was Sip antigen positive by the ICT (see Discussion), and the remaining 206 were Sip antigen negative (Fig. 4). Therefore, the specificity of the ICT was 99.5%.

**DISCUSSION**

Transmissions of GBS from carrier women to neonates during delivery cause pneumonia, meningitis, sepsis, and other diseases. To prevent such infections, GBS screening for pregnant women at the 35th to 37th week of gestation has been recommended (13). The GBS detection methods used to date include culture-based conventional method, the PCR amplification of the GBS gene (15, 16), the DNA probe hybridization test (14), and immunoassay (24–26). The culture method requires an enrichment culture using Lim broth or modified Granada medium to improve the GBS detection frequency compared with the classical method, which uses smearing of a vaginal or rectal swab directly onto an agar plate.
This method, however, requires two culture steps and further GBS identification tests and therefore is time-consuming. In addition, this method may overlook nonhemolytic GBS, which appears in about 1 to 5% of the total number of GBS isolates (13, 29, 30). The PCR method and the DNA probe hybridization test require equipment and extensive labor. Therefore, development of a rapid GBS detection method that is capable of detecting even nonhemolytic GBS has long been awaited.

In the present study, we investigated a rapid GBS detection immunochromatography method targeted at the GBS-specific Sip antigen, which is commonly expressed in GBS strains regardless of serotype. Although several other surface antigens, such as α-protein, β-protein, Rib, C5a peptidase, and the FbsA protein, are identified in GBS strains, they are not common to all serotypes of GBS strains (31). The conventional immunological GBS identification methods, including the latex aggregation test (25, 26) and enzyme immunoassay (24), detect group-specific C-carbohydrate antigens. However, it was reported that this method showed cross-reactivity with \textit{E. faecalis}, \textit{C. albicans}, and others, which were often isolated from vaginal and rectal specimens (32–34). Our newly developed ICT was able to detect GBS in the range of $9.5 \times 10^5$ to $3.7 \times 10^6$ CFU/ml and showed similar detection limits among GBS cells expressing different serotypes.

Cross-reactivity of the ICT was tested using bacterial and fungal strains frequently isolated from the vaginal and rectal specimens and, in addition, 8 streptococcal species. The results showed absolutely no false-positive reaction. It was reported that commercially available group B streptococcus identification reagents cross-react with \textit{Streptococcus porcinus} and \textit{Streptococcus pseudoporcinus} (35, 36). The former is often associated with cervical lymph node infection in swine, and the latter is isolated from female genital tracts at a frequency of about 5.4% (37–39). Our ICT showed no detectable cross-reactivity with these microorganisms. Thus, our ICT is suitable for use as a GBS detection tool.

Among 229 clinical isolates identified to be GBS, 1 strain was found to be Sip negative by the present ICT. To search for the cause of this discrepancy, we analyzed the DNA sequence of the \textit{sip} gene from this strain and found that the gene had a 4-bp deletion from the 651th to the 654th bases compared with the \textit{sip} gene sequence of \textit{S. agalactiae} ATCC BAA-611 deposited in the GenBank database (accession number NC_004116). The \textit{sip} gene with a 4-bp deletion was also deposited in the GenBank database (accession number DQ914266) as the pseudo \textit{sip} gene. This deletion caused a frameshift mutation and a termination codon from the 697th to 699th nucleotides, producing a truncated Sip protein having 232 amino acid residues. The calculated molecular mass of the truncated Sip appears to be 25 kDa instead of the 53 kDa of the full-length Sip. Reactivity of two monoclonal antibodies with the truncated Sip was tested by the Western blotting method, and the results confirmed that they were not reactive with the cells producing the truncated Sip (\textit{Fig. 5}). The other antipeptide antibody raised against the 200th to 217th residues of Sip (S4H5) was found to be reactive with the protein band from cells producing the truncated Sip, and its molecular mass appeared to be 25 kDa, as expected (\textit{Fig. 5}).

Since the anti-synthetic-peptide monoclonal antibody used for ICT was prepared against the sequence from the 313th to 336th amino acid residues of Sip, this antibody was unreactive with cells producing the truncated Sip. An antigenic determinant in another monoclonal antibody (R6E8) used for ICT is not known, but it is likely that the antibody recognizes the amino acid residues located within the truncated region.

Only one strain showed a false-positive result with the ICT among 260 clinical vaginal swabs. To determine the cause of the
false-positive reaction, DNA was extracted from the remaining contents of the specimen transport tube and the dltS gene was subjected to PCR amplification. The PCR products showed the presence of the dltS gene, suggesting that the specimen contained dead GBS cells or their debris. This case suggested that living GBS cells might have been present in the patient’s vagina even though the culture method failed to detect GBS. Alternatively, it is likely that the patient was treated with a chemotherapeutic agent(s).

Among 219 pigment-negative enrichment swab cultures, 12 and 10 cultures were shown to be GBS positive by the culture methods and the ICT, respectively. Two Sip-negative swab cultures that were GBS positive by the culture-based method contained a few GBS colonies and abundant E. faecalis organisms. To search for the cause of these false-negative results, the GBS strains isolated from these cultures were subjected to the ICT again. Both strains showed clear GBS-positive reactions. It was reported that moderate or heavy contamination with E. faecalis suspension of 10^8 CFU/ml and then subjected to the ICT. The detection limit of GBS from vaginal swabs is an excellent technique that is readily applicable for clinical use.

FIG 5 Reactivity of monoclonal antibodies with false-negative GBS cells. Whole-cell extracts of the false-negative GBS strain were subjected to SDS-PAGE and electroblotted onto PVDF membranes. The reactivities of three monoclonal antibodies were tested by the Western blotting method: S6H8 and R6E8 were the antibodies used for the ICT; S4H5 is another monoclonal antibody that was raised against a synthetic peptide corresponding to the 200th to 217th amino acid residues of Sip (peptide200-217). Lane M, molecular mass markers; lane 1, ICT-negative GBS; lane 2, GBS ATCC BAA-611; lane 3, recombinant Sip.

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