Reverse Line Blot Assay for Direct Identification of Seven *Streptococcus agalactiae* Major Surface Protein Antigen Genes

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We developed a multiplex PCR-based reverse line blot hybridization assay (mPCR/RLB) to detect the genes encoding members of the family of variable surface-localized proteins of *Streptococcus agalactiae* (group B streptococcus [GBS]), namely, Bca (C), Rib, Epsilon (Epsilon/Alp1/Alp5), Alp2, Alp3, and Alp4, and the immunoglobulin A binding protein, Bac (Cβ). We used the assay to identify these genes in a collection of well-characterized GBS isolates and reference strains. The results showed that mPCR/RLB avoids the common problems of cross-reaction and nontypability associated with protein typing using antisera. It is as sensitive as, but more practical than, separate gene-specific PCRs and would be suitable for large molecular epidemiological studies of GBS.

Group B streptococcus (GBS) (*Streptococcus agalactiae*) is an important cause of sepsis, especially in neonates (19). In addition to capsular polysaccharide antigens, some *S. agalactiae* surface proteins are potential components of a protein-based GBS vaccine (16), and the use of some as carriers in polysaccharide conjugate vaccines is under investigation (5, 17). Identification of surface-localized protein antigens can facilitate studies of the epidemiology and pathogenesis of GBS infection (14).

The genes encoding the proteins Co (bca), Alp2 and Alp3 (Co-like 2 and 3) (alp2 and alp3), Epsilon (epsilon [alp1/ alp5]), and Rib (rib) have been well studied, and their gene sequences are published in GenBank (2, 16, 18, 23). They belong to a family of surface-localized proteins containing internal tandem repeats, variation in the numbers of which causes variation in protein size and antigenicity (16, 23). The gene encoding the immunoglobulin A (IgA) binding protein, Cβ (bac), also has been well described (6, 7). In a previous study, we developed individual gene-specific PCRs for *bca*, *rib*, *alp2*, *alp3*, and *bac* (12), and Creti et al. have recently described a multiplex PCR method for *bca*, *rib*, *alp2/alp3*, *alp4*, and *epsilon* (2). In this study, we developed a multiplex PCR (mPCR)-based reverse line blot (RLB) hybridization assay and used it to identify seven protein antigen genes in several sets of well-characterized GBS isolates.

**MATERIALS AND METHODS**

**GBS isolates and serotyping.** The GBS isolates used in this study were the following: 27 reference strains used in our previous studies (Table 1); 83 isolates provided by Nicola Jones, Oxford, United Kingdom, which had been characterized by multilocus sequence typing (MLST) (8); 58 isolates provided by Dele Davies and Shannon Manning, Michigan State University, East Lansing, MI, which also had been characterized by MLST (3); and 50 isolates provided by Catherine Lachenaier, Channing Laboratory, that previously had been used in a serological study of protein surface antigens (15).

Results of capsular polysaccharide serotyping were provided by the donor laboratories (1, 13).

**Oligonucleotide design.** We designed two specific PCR primers and two specific probes for each of the seven GBS protein genes, using published GenBank sequences. We also designed one GBS-specific primer pair and two GBS-specific probes (9) (Table 2).

**Primer design.** The primers were designed to have similar physical characteristics in order to allow simultaneous amplification in a multiplex reaction. Their lengths were between 20 and 32 bp, melting temperatures between 57.2°C and 65.8°C, and amplicon sizes in the range of 193 to 624 bp (Table 2). The specificities of primer sequences were determined by comparison with GenBank, using FASTA, to ensure that there would be minimal risk of cross-hybridization with other regions of the GBS genome or unrelated genes. All multiplex PCR primers were 5′ biotinylated to allow detection of hybridization with a streptavidin peroxidase substrate.

**Probe design.** To avoid optimal hybridization under the common conditions, probes were designed to have similar physical characteristics: lengths between 20 and 24 bp and melting temperatures between 57.1°C and 63.7°C (Table 2). FASTA searches were performed to compare their sequences with those in GenBank. All probes have a 5′ amine group to facilitate covalent linkage to the nylon membrane and allow membranes to be stripped and reused repeatedly.

**Multiplex PCR system.** The 25-μl eight-primer-pair multiplex mixture was prepared as follows: 5 μl template DNA, 0.25 μl of each of all forward (50 pmol/μl) and reverse (50 pmol/μl) primers, 1.25 μl deoxynucleoside triphosphates (2.5 mM of each deoxynucleoside triphosphate), 2.5 μl 10× PCR buffer, 3 μl 25 mM (4.5 mM final) MgCl2, 0.1 μl QIAGEN hotstart Taq polymerase (5 units/μl); also, water was added to 25 μl. The PCR program was performed according to the QIAGEN Hotstart Taq polymerase kit instructions: 95°C for 15 min, 1 cycle, 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, 35 cycles, 72°C for 10 min, 1 cycle, and 22°C hold. A total of 8 μl of each PCR product was separated by electrophoresis on a 1.5% agarose gel to confirm successful amplification (13). The remaining PCR products were used for RLB hybridization (5 μl of PCR product for each).

**RLB hybridization.** The RLB hybridization assay was based on the method described previously (21, 22), except that the hybridization temperature was 60°C, the conjugate used was streptavidin-peroxidase (Roche Diagnostics Co.) diluted 1:4,000 in 2× SSPE (SSPE is 0.18 M NaCl, 10 mM NaH2PO4, and 1 mM EDTA [pH 7.7])–0.5% sodium dodecyl sulfate, and the time of exposure to X-ray...
The sensitivity and specificity of mPCR/RLB were compared with those of single-gene-specific PCR. For all 27 reference strains (Table 1) and 191 isolates studied (Table 3), the results of mPCR/RLB were the same as those of single-gene-specific PCR.

Comparison of mPCR/RLB with conventional protein serotyping. Of the 50 isolates for which protein subtyping had been done previously, using antisera, 48 had reacted with two or more antisera (15), indicating a high degree of cross-reactivity. By contrast, mPCR/RLB showed complete specificity, with no additional antisera (15), indicating a high degree of cross-reactivity. By contrast, mPCR/RLB showed complete specificity, with no cross-reactions between different gene probes (Table 3). How- ever, one isolate (no. 24) contained two different protein genes that hybridized with Co gene (bca) probes also hybridized with Cib gene (bac) probes.

Comparison of mPCR/RLB and single-gene-specific PCR. To confirm the sensitivity and specificity, we compared the results of mPCR/RLB with those of single-gene-specific PCR. For all 27 reference strains (Table 1) and 191 isolates studied (Table 3), the results of mPCR/RLB were the same as those of single-gene-specific PCR.

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DISCUSSION

In this study, we developed an mPCR/RLB method to identify 6 GBS surface protein genes and the IgA binding protein,
TABLE 2. Oligonucleotide primers used in this study

| Primer*  | Target | Tm (°C)* | GenBank accession no. | Sequence† |
|----------|--------|----------|-----------------------|-----------|
| cbfSb    | cbf    | 59.93    | X72754                | 326 ATG ATG TAT CTA TCT GCT GAA GCT CTA TG 352 |
| Sag59PS  | cbf    | 58.55    | X72754                | 368 TTT TCA CCA GCT GTA TTA GAA GTA GTA 391 |
| Sag190Pa  | cbf    | 59.74    | X72754                | 522 GCT CCC TGA ACA TTA TCT TGT AT 300 |
| cbfAb    | cbf    | 59.74    | X72754                | 585 CCG AAT GAA GTC TTT AAT TTT TC 353 |
| bcaSb    | bca    | 60.45    | M97256                | 313 GCT TAC ATA GAT TTA TAT GAT GTA AAA TTA GG 344 |
| bcaSp    | bca    | 57.62    | M97256                | 370 GTT TTA CAA CAA GGT TTT ACC ACA GC 392 |
| bcaAp    | bca    | 58.60    | M97256                | 579 CT TAT CCC TCA AGG TGT TGT GGA 560 |
| bcaAb    | bca    | 58.91    | M97256                | 637 CAG TAC GAC TTT CTT CCG TC 618 |
| alp23Sb  | alp2/3  | 59.89    | AF208158              | 1254 CAT GGA AGT GAC AAT TAT GAA AG 1276 |
| alp2Sp   | alp2   | 59.24    | AF208158              | 1366 CTI CCC CCA CAA ATT AAG 1386 |
| alp2Ap   | alp2   | 58.25    | AF208158              | 1576 CTG TTG ACT TCT CTA GAT AGC GC 1554 |
| alp2Ab   | alp2   | 60.81    | AF208158              | 1603 CTA CTG TAA CTT CTA CAG GAA CTT C 1579 |
| Alp23Sb  | alp2/3  | 59.89    | AF291065              | 1534 CAT GGA AGT GAC AAT TAT GAA AG 1556 |
| alp3Sp   | alp3   | 57.11    | AF291065              | 1643 GTT CTT CCG CTG AAG TAG AG 1662 |
| alp3Ap   | alp3   | 60.17    | AF291065              | 1711 CGG TGT TTT TCC TAC TTT GAC 1691 |
| ribSb    | rib    | 63.88    | U58333                | 219 AGA TAC TGT GTT TGC AGC TGA AGT AA 244 |
| ribSp    | rib    | 59.07    | U58333                | 256 GTG TTT CTA GCT TAA ACA AAA ATC ATC 279 |
| ribAp    | rib    | 57.92    | U58333                | 448 C AAC AGT AGA TAA TCC AAC AGG 427 |
| ribAb    | rib    | 57.16    | U58333                | 577 CTA TTT TCT CTC TAA AAG GGA CAG 554 |
| alp4Sp   | alp4   | 61.47    | AJ488912              | 158 TGT TAG CAG CTG AAG TAG TAC AAG 181 |
| alp4Ap   | alp4   | 63.70    | AJ488912              | 187 GCT GCA ACA TTA AAT ACT GCC AT 209 |
| alp4Ab   | alp4   | 60.62    | AJ488912              | 325 TGT AAT AAA TAG CAG TGT ATC CCG 302 |
| epsilonSb | epsilon | 64.75    | U33554                | 350 GCA TAA CCT TTT ACC CTG TGT O 329 |
| epsilonSp | epsilon | 58.30    | U33554                | 304 CTG TTT CAG CTG ACG TG 323 |
| epsilonAp | epsilon | 60.91    | U33554                | 452 CCC TTC TAA TTT TCA TCC AAC AAA C 473 |
| epsilonAb | epsilon | 62.52    | U33554                | 685 GGA TCA TTT GCA TTT TCA ATT AC 663 |
| GBS1360Sb | bac    | 65.82    | X59771                | 719 CA GTA CAT CCT TCT GAC TAT CAT CAT CG 695 |
| GBS1716Sp | bac    | 58.32    | X59771                | 1337 AAG GCT ATG AGT GAG AGC TG 1360 |
| GBS1716Ap | bac    | 59.28    | X59771                | 1697 AAA AGT GAT TCG AAG AGC AC 1716 |
| GBS1937Ab | bac    | 64.80    | X59771                | 1735 AGA TAC GAA GTC CAA CG 1716 |
| GBS1937Sb | bac    | 64.80    | X59771                | 1960 CTG CTC TGG TTT CTA GAG AAC TG 1937 |

* Suffixes: “b” indicates primers 5' labeled with biotin; “p” indicates probes 5' labeled with amine.

† The primer Tm values are those provided by the primer synthesizer (Sigma-Aldrich).

§ Numbers represent the numbered base positions at which primer sequences start and finish (numbering start points refer to the start point 1 of the corresponding gene GenBank accession number).

The species-specific primers have been previously published (9). All other primers were designed specifically for this study.

Cj, and used it to further characterize 27 reference strains and 191 isolates. With the exception of a single reference strain (no. 24 [70339] [NCTC 12907]), which contained both bca and rib, all isolates contained only one of the six members—rib, bca, epsilon/alp1/alp5, alp2, alp3, or alp4—of the variable surface protein gene family. The Cj gene (bac) was present with one or other of these genes (usually bca) in 8 of 27 reference strains and 16 of 191 isolates (most of which belonged to molecular serotype [MS] Ib).

Our study confirmed previously reported relationships between cps serotypes and surface protein gene profiles (10, 12, 16). For example, most serotype III isolates (our molecular serosubtypes [mss] III-1 and III-2) were associated with rib (20), and mss III-3 with alp2 (16). Serotype Ib was associated with bca and bac (14), and serotypes V and VIII with alp3 (16). However, since the relationship was not absolute, different combinations of cps serotype and protein gene profile identified many serovariants, which will be useful in epidemiological studies and in formulation of conjugate vaccines (14, 16).

Most of the 50 isolates which had been subtyped using antisera against surface proteins express “laddering” patterns in Western blots with two or more antisera (15). This serological cross-reactivity probably arises from conserved epitopes present within more than one surface protein. However, in our study, all strains tested possessed one and, with a single exception, only one of the six members of the surface protein antigen gene family—rib, bca, epsilon, alp2, alp3, and alp4 (14, 16). These results indicate that immunologic typing of GBS surface proteins with currently available antisera is potentially misleading and should be used with caution, especially for epidemiological studies.

Unlike the use of antisera, molecular methods of protein “typing” do not demonstrate protein expression, which is clearly important for virulence. However, since the results of existing immunological typing methods are so difficult to interpret, further investigation is needed to identify reliable markers of gene expression. Our mPCR/RLB assay is an appropriate method for epidemiological typing, especially when combined with other molecular markers, as in our three-component system (10). If combined with an immunological assay, such as a Western blot, the mPCR/RLB would clarify the interpretation of results.

The use of a polyvalent GBS conjugate vaccine, containing all of the prevalent capsular polysaccharides, has been proposed as a strategy for the prevention of GBS infections. One or more of the GBS surface proteins could be conjugated with GBS polysaccharides and independently contribute to protective immunity against the corresponding protein-containing

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serotypes in an effective vaccine. In this context, a rapid, reliable molecular test capable of identifying the GBS surface protein genes, such as mPCR/RLB, would facilitate cost-effective epidemiological studies of circulating GBS strains to identify protein genes profiles.

This mPCR/RLB method has a number of advantages over our previous protein gene profiling method (12). It is easy to perform and does not require the use of gels; amplification products of 43 isolates can be tested simultaneously, and therefore, the method would be suitable for high-throughput GBS typing (4). Furthermore, it can detect all seven protein genes in a single PCR and on one membrane, which can be reused more than 20 times. The assay (for 43 isolates) can be completed within one working day. Although we have not tested its sensitivity compared with culture, it has the potential to be used directly for clinical specimens (4).

Our method also has practical advantages over the multiplex

**FIG. 1.** Results of mPCR/RLB hybridization for 27 reference strains. Lanes 1 to 27 represent the 27 reference strains, in the same order as in Table 1; the 20 labeled probes are in the same order as in Table 2. Duplicate rows for the same probe represent different probe concentrations (0.2 or 0.4 pM, respectively). Probe targets in rows from top to bottom are as follows: rows 1 and 2, GBS species specific; 3 and 4, blank; 5 to 8, Co or Bca protein gene (protein gene profile code A [see Table 1, footnote a]); 9 to 12, blank; 13 to 16, alp2; 17 to 20, alp3; 21 to 24, blank; 25 to 28, alp4; 29 to 31, rib (protein gene profile code R); 32 to 35, epsilon/alp1/alp5; 36 and 37, Cb or Bac protein gene (protein gene profile code B); 38 and 39, blank; 40 and 41, GBS species-specific gene. PCR product of strain no. 18 hybridized with GBS-specific probes but with none of the protein gene probes. However, on retesting, its PCR product hybridized with alp3 probes.

**TABLE 3.** The relationship between protein gene profiles and capsular polysaccharide synthesis gene MS for 191 GBS isolates

| MS (n) | No. of isolates with protein antigen gene profilea |
|-------|-----------------------------------------------|
|       | A | AB | R   | Epsilon | Alp2 | Alp3 | Alp4 |
| Ia (18)| 1 |    | 17  |          |      |      |      |
| Ib (16)| 1 | 15 |     |          |      |      |      |
| II (7) | 2 | 1  | 3   | 1        |      |      |      |
| III (96)| 6 | 85 | 4   | 1        |      |      |      |
| IV (1) | 1 |    |     |          | 10   |      |      |
| V (13) | 3 | 10 |     |          |      |      |      |
| VI (20)| 19|    | 1   |          |      |      |      |
| VII (2)|   |    |     |          | 2    |      |      |
| VIII (18)| |   | 18  |          |      |      |      |
| Total (191)| 29 | 16 | 88  | 23       | 4    | 31   |      |

a n, number of isolates.
b Protein antigen gene profile codes are the following: A, bac positive; B, bac positive; R, rib positive; Alp2, alp2 positive; Alp3, alp3 positive; Alp4, alp4 positive; Epsilon, epsilon (epsilon/alp1/alp5) positive.
PCR described by Creti et al. (2). First, it does not involve the use of gels, and the use of two probes to identify PCR products increases the sensitivity (by amplifying the signal) and specificity (the result is considered positive only if both probes are hybridized). Second, unlike Creti et al., we can distinguish alp2 from the closely related alp3 gene by RLB. This is important, because these proteins classically cross-react with typing antisera but are clearly associated with different capsular polysaccharide serotypes (alp2 with msst III-3 [3, 10] and alp3 with serotypes V and VIII [16]). Third, our mPCR/RLB method also includes the Cβ (IgA binding) protein gene, which is an important virulence factor.

Finally, because this method uses only 20 of 43 lanes in the MiniBlotter, additional targets can be added to both mPCR and RLB, such as serotype-specific cps sequences (11) or mobile genetic elements, which are included in our three-component genotyping system (10), or antibiotic resistance genes.

In conclusion, our mPCR/RLB assay is a more sensitive and specific method for the identification of GBS surface protein gene profiles than the use of antisera and more efficient and convenient than separate PCRs or multiplex PCR with gel electrophoresis. Application of PCR/RLB in future epidemiological studies and for surveillance will facilitate the appropriate formulation of candidate GBS vaccines.

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