Evaluation of a Multiplex PCR-Based Reverse Line Blot-Hybridization Assay for Identification of Serotype and Surface Protein Antigens of *Streptococcus agalactiae*

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A 33-primer multiplex PCR-based reverse line blot (mPCR/RLB) assay was developed to identify *Streptococcus agalactiae* serotypes and surface protein antigen genes simultaneously. It was evaluated by using 551 clinical isolates. The mPCR/RLB assay was more sensitive than conventional serotyping, especially for protein antigen typing, but otherwise the results correlated well.

Recently, we developed separate multiplex PCR-based reverse line blot-hybridization (mPCR/RLB) assays to identify molecular serotypes (MS) (2) and protein antigen gene profiles (PGPs) (9) of *Streptococcus agalactiae* (group B streptococcus [GBS]). The aim of this study was to develop an assay that would identify MS and PGPs simultaneously and could potentially replace the use of antisera for typing. To evaluate the new combined assay, we compared the results with those of conventional serotyping for 27 well-characterized reference strains and 551 consecutive GBS isolates referred for serotyping to the Streptococcus Reference Laboratory, Institute of Environmental Science and Research (ESR), Porirua, New Zealand, some of which have been studied previously (3, 4).

Conventional serotyping (CS) was performed as described before (8). Rabbit antisera were raised at ESR, using standard methodology (WHO International Reference Centre for Streptococcus Typing, Prague, Czech Republic) and GBS serotype strains obtained from the New Zealand Reference Culture Collection Medical Section. Antisera were not raised against serotypes VI to VIII because they were very rare in New Zealand at the time. Antisera were checked for specificity against type-specific antigens for all strains.

mPCR/RLB primers and probes from our previous studies (2, 9) were combined together in the same mPCR/RLB assay for this study. The 17 targets were one GBS species-specific target (*cfb*), nine MS-specific targets (serotypes Ia to VIII) (2, 9), and seven PGP-specific targets (genes encoding Bca/Cα, Rib, Alp1 [Alp1/Alp5/Epsilon], Alp2, Alp3, Alp4, and the immunoglobulin A binding protein Bac/Cβ) (9). In all, there were 33 primers (*alp2* and *alp3* share the same forward primer) and 34 probes (two for each target). mPCR/RLB was performed as previously described (2, 9), except that for mPCR, the 33 primers were combined in the same tube with 0.2 μl (1 unit) rather than 0.1 μl (0.5 unit) QIAGEN Hotstar *Taq* polymerase

(5 units/μl); for RLB, 17 probe pairs (see above) were used on the same membrane.

The MS and PGPs of all 27 reference strains (Table 1), as identified by the 33-primer mPCR/RLB assay, were identical to those obtained previously by PCR and sequencing (3, 4), separate MS 20-primer mPCR/RLB (2) and PGP 13-primer mPCR/RLB (9) assays, and CS for those that were serotypeable (25 reference strains) and for which protein type results were available (9 reference strains). The CS results and mPCR/RLB results for 551 clinical isolates are shown in Table 2. mPCR/RLB results for 40 isolates from 1997 to 1998 are shown in Fig. 1.

mPCR/RLB identified the MS of all 551 isolates, including 65 (12%) that were nonserotypeable by CS. MS III, Ia, V, Ib, and II accounted for 85% of isolates. In New Zealand, MS VI and VIII were first isolated in 1998 and MS VII was first isolated in 2001; 11 of 551 (1.9%) isolates (16% of those that were nonserotypeable by CS) belonged to these MS. There were 5 of 484 isolates for which the results of both CS and MS were available but discordant. These isolates were sent to Central Public Health Laboratory, Health Protection Authority, United Kingdom, for serotyping, with the following results: two (previously identified by CS as serotypes III and Ib) were nonserotypeable (mPCR/RLB identified them as MS Ia and V, respectively); one identified as serotype Ib by CS was retyped as serotype VI, which was concordant with the MS result; one previously identified by CS as serotype III was retyped as serotype V, which agreed with the MS result; and one previously identified by CS as serotype Ib and by mPCR/RLB as MS V was confirmed to be serotype Ib. The mPCR/RLB assay was repeated for this isolate, and the result also confirmed that it was MS Ib, suggesting that the wrong isolate was tested or the result was wrongly transcribed the first time. Thus, in all, there were 67 (12%) isolates that were nonserotypeable by CS. The final results are shown in Table 2.

The majority of isolates (401 [73%]) were nontypeable using antisera against the R and C antigens. R protein antigens were identified in 96 (17%) isolates, and C antigens were identified in 54 (10%) of 551 isolates. However, at least one protein antigen gene was identified by mPCR/RLB in 547 (99%) iso-

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TABLE 1. Results of CS, MS identification based on PCR and sequencing, and mPCR/RLB for 27 reference strains

Strain	CS ^a	MS	PGP ^a
O90 (Rockefeller University) ^b	Ia	Ia	Alp1
H36B (ATCC 12401; NCTC 8187) ^b	Ib	Ib	Bca and Bac
18 RS 21 (NCTC 11079) ^b	II	II	Rib
M 781 (ATCC BAA-22) ^b	III	III	Rib
3139 ^b	IV	IV	Alp1
CJB 111 (ATCC BAA-23) ^b	V	V	Alp3
SS 1214 ^b	VI	VI	Alp1
7271 ^b	VII	VII	Alp3
JM9 130013 ^b	VIII	VIII	Alp3
A909 (NCTC 11078) ^b	Ia	Ia	Bca and Bac
BM110 ^b	III/R4/Rib	III	Rib
NZRM 908 (NCDC SS 615) ^c	Ia	Ia	Alp2
NZRM 909 (NCDC SS 618) ^c	Ib	Ib	Bca and Bac
NZRM 910 (NCDC SS 700) ^c	Ia/c	Ia	Bca and Bac
NZRM 911 (NCDC SS 619) ^c	II	II	Rib
NZRM 912 (NCDC SS 620) ^c	III	III	Alp2
NZRM 2832 (Prague 1/82; ATCC 49446) ^c	IV	IV	Alp1
NZRM 2833 (Prague 10/84; ATCC 49447) ^c	V	V	Alp3
NZRM 2834 (Prague 118754) ^c	VI	VI	Bca and Bac
NZRM 2217 (Prague 25/60; ATCC 49448) ^{c,d}	NT-R	II	Alp4
O90 (ATCC 12400) ^e	Ia	Ia	Alp2
A909 (NCTC 11078) ^e	Ia-AB	Ia	Bca and Bac
335 (NCTC 12906) ^e	Ia-A	Ia	Alp1
70339 (NCTC 12907) ^e	Ia-BR4	Ia	Bca, Bac, and Rib
65604 (3) ^e	III-R4	III	Rib
15626/81 (3) ^e	IV-B	IV	Bca and Bac
Pattison (NCTC 9828) ^e	NT-R3R4	II	Alp4

^a NT, nonserotypeable; Bca, Cα; Bac, Cβ; Alp1, Alp1/Alp5/Epsilon.

^b Eleven GBS reference strains from Lawrence Paoletti and Catherine Lachenauer, Channing Laboratory, Boston, Mass.

^c Nine GBS reference strains from Diana Martin, Streptococcus Reference Laboratory, ESR, Wellington, New Zealand.

^d This isolate is the atypical reference strain Prague 25/60, which was nonserotypeable using capsular polysaccharide antisera but was assigned to molecular serotype II. It expresses a protein that is antigenically similar to R (and is used to generate R antiserum), encoded by the gene *alp4*.

^e Seven GBS reference strains from Johan Maeland, Department of Microbiology, School of Medicine, Norwegian University of Science and Technology, Trondheim, Norway.

lates (Table 3); *bca* was the most common and usually was present with another protein gene (most commonly *bac*), as previously reported (9). Apart from *bac*, protein genes were almost always mutually exclusive, except for the case of a single atypical isolate which had both *bca* and *rib*. Of the 96 isolates with R proteins, 1 had no detectable protein genes, 71 (73%) contained *rib*, 23 (24%) contained *alp3*, and 1 contained *alp1*. Apart from the first and last isolates, these results were consistent between CS and mPCR/RLB. Of the 54 isolates that were positive for the C protein, 34 (62%) contained *alp1*, 19 (35%) contained *bac* (of which 10 also had *bca*), and 1 con-

tained *alp2*. Only the last result was inconsistent between CS and mPCR/RLB. As previously demonstrated, the identification of protein antigens using antisera is difficult, and cross-reactions are common (6). However, in general, isolates that express the R protein, as demonstrated using antisera, contain *rib* or *alp3*, and those that express the C protein contain *alp1* or *bac* (Cα), with or without *bca* (Cβ) (5, 7, 9).

In this study, the relationships between MS and PGPs were generally the same as previously demonstrated, namely, MS Ia and IV strains carried *alp1*, MS Ib strains carried *bca* and *bac*, MS II strains carried various genes, MS III strains carried *rib*, and MS V and VIII strains carried *alp3* (Table 3) (3). A minority of isolates had an atypical combination, suggesting the occurrence of gene recombination. The identification of these atypical isolates will be important for disease surveillance in relation to potential vaccine use.

Some probes reacted (usually very weakly) with amplicons from more than one serotype. For example, amplicons from most MS Ia and V isolates cross-hybridized weakly with *VcpsHSp* and *IacpsHSp*, as previously described (2). However, the combined results for *VcpsHAp/VcpsHSp* and *IacpsHAp/IacpsHSp* clearly distinguished between serotypes Ia and V (Fig. 1). Otherwise, the sensitivity and specificity of mPCR/RLB were considerably better than those of CS and similar to those of individual serotype-specific PCRs for confirmed GBS isolates.

The development of multivalent conjugate GBS vaccines containing the most important serotypes and, potentially, spe-

TABLE 2. Comparison of CS for capsular type with mPCR/RLB results for 551 GBS clinical isolates

CS	No. of isolates	No. of isolates with MS by mPCR/RLB							
		Ia	Ib	II	III	IV	V	VI	VII
Ia	122	122							
Ib	58		58						
II	43			43					
III	149				149				
IV	16					16			
V	94						94		
NT ^a	69	6	18	7	8	2	17	6	2
Total	551	128	76	50	157	18	111	6	2

^a NT, nontypeable (includes one isolate which was retyped as MS VII by CS).

FIG. 1. Results of mPCR/RLB assay for 40 consecutive isolates. Sample 35 was buffer, which was used as a negative control. Probes sag59Sp and sag190Ap are group B streptococcus specific. Molecular serotype probes are named according to the corresponding serotype, the *cps* gene target (e.g., *cpsH*, *cpsK*, etc.), and whether the probe is sense (S) or antisense (A). Protein gene probes are named for the corresponding protein, except for GBS1716Ap and GBS1716Sp, which are the sense (S) and antisense (A) probes targeting the C β gene (*bac*) (3, 4).

sific GBS protein antigens is under way. Epidemiological studies and surveillance of GBS serotype and PGP distributions for different diseases, age groups, and countries and over time are needed to support vaccine development. The relatively recent emergence of serotypes VI, VII, and VIII demonstrates the ongoing evolution of GBS. Our mPCR/RLB assay for detection of MS and PGPs will provide a convenient and inexpen-

sive tool for ongoing surveillance. The mPCR/RLB assay is easy to perform and does not require the use of expensive commercial antisera (which are available only for some serotypes) or in-house antisera, which have variable specificity and quality and frequently cross-react. The amplification products of 43 isolates can be tested simultaneously in the mPCR/RLB assay, making the assay suitable for high-throughput epidemiological studies of GBS (2). Because PCR detects the presence of capsular genes, it can characterize isolates with low or absent capsular expression, which are nonserotypeable by CS. The mPCR/RLB assay has enabled, for the first time, the objective, simultaneous identification of GBS capsular types and protein antigens without the need for interpretation requiring an experienced operator. It also has the potential to be used directly for clinical specimens (1).

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TABLE 3. Relationships between MS and PGPs for 551 GBS clinical isolates

MS	No. of isolates	No. of isolates with PGP ^a							None ^b
		<i>bca</i> only	<i>bca</i> and <i>bac</i>	<i>alp1</i>	<i>alp2</i>	<i>alp3</i>	<i>rib</i>	<i>rib</i> and <i>bac</i>	
Ia	127	7	3	112	4	1			
Ib	77	3	70			4			
II	50	13	9	6	1	12	8		1
III	158		1		14	5	137		1
IV	18			17			1		
V	110	1	3	3		98	2	1	2
VI	6	4	1	1					
VII	2			1		1			
VIII	3					3			
Total	551	28	87	140	19	124	148	1	4

^a No isolates contained *alp4*.
^b None of the seven PGPs were detected.

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