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Multiplex PCR Assay for Rapid and Accurate Capsular Typing of Group B Streptococci[▽]

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We developed a simple, specific, and sensitive two-multiplex-PCR assay that enabled the detection of all known group B streptococcal (GBS) capsular polysaccharides. This test is well adapted for GBS capsular polysaccharide typing in large-scale epidemiological studies.

Group B streptococci (GBS; *Streptococcus agalactiae*) are a leading cause of invasive infections in neonates and a serious cause of mortality or morbidity in adults with underlying diseases (13). Nine distinct capsular polysaccharide (CPS) serotypes have been described (6, 7). The CPS is commonly used for strain typing. The commercial kits most widely used are based on latex agglutination (LA), but these tests are only moderately reliable, resulting in nontypeability (NT) or erroneous serotyping of the isolates. Therefore, molecular capsular typing techniques are attractive because they are reproducible, specific, and easy to perform. Different genotypic methods have been described for the molecular capsular typing of GBS (1, 2, 8, 9, 14, 17). However, while these techniques are relatively easy to perform in the routine laboratory, they all involve the conjunction of two different techniques, e.g., PCR plus sequencing, PCR plus hybridization, or PCR plus enzymatic restriction. We report in this work on a simple multiplex PCR assay which enables the detection of all known GBS CPSs.

The DNA sequences of the *cps* operons of all GBS CPSs that have been described have recently been made available (3). The nine *cps* DNA sequences were analyzed by using Beacon Designer 5.1 software to generate CPS-specific primer pairs,

TABLE 1. CPS type-specific primers and prediction of PCR products by computer simulation

Primer name	Sequence (5′ to 3′)	Gene target(s)	Amplicon size(s) (bp)	GenBank accession no. of targeted operon
Ia-F	GGTCAGACTGGATTAATGGTATGC	*cps1aH*		AB028896
Ia-R	GTAGAAATAGCCTATATACGTTGAATGC	*cps1aH*	521 and 1,826	
Ib-F	TAAACGAGAATGGAATATCACAAACC	*cps1bJ*		AB050723
Ib-R	GAATTAACTTCAATCCCTAAACAATATCG	*cps1bK*	770	
II-F	GCTTCAGTAAGTATTGTAAGACGATAG	*cps2K*		AY375362
II-R	TTCTCTAGGAAATCAAATAATTCTATAGGG	*cps2K*	397	
III-F[a]	TCCGTACTACAACAGACTCATCC	*cps1a/2/3I*		AF163833
III-R[a]	AGTAACCGTCCATACATTCTATAAGC	*cps1a/2/3J*	1,826	
IV-F	GGTGGTAATCCTAAGAGTGAACTGT	*cps4N*		AF355776
IV-R	CCTCCCCAATTTCGTCCATAATGGT	*cps4N*	578	
V-F	GAGGCCAATCAGTTGCACGTAA	*cps5O*		AF349539
V-R	AACCTTCTCCTTCACACTAATCCT	*cps5O*	701	
VI-F	GGACTTGAGATGGCAGAAGGTGAA	*cps6I*		AF337958
VI-R	CTGTCGGACTATCCTGATGAATCTC	*cps6I*	487	
VII-F	CCTGGAGAGAACAATGTCCAGAT	*cps7M*		AY376403
VII-R	GCTGGTCGTGATTTCTACACA	*cps7M*	371	
VIII-F	AGGTCAACCACTATATAGCGA	*cps8J*		AY375363
VIII-R	TCTTCAAATTCCGCTGACTT	*cps8J*	282	
dltS-F	AGGAATACCAGGCGATGAACCGAT	*dltS*		AL766853
dltS-R	TGCTCTAATTCTCCCCTTATGGC	*dltS*	952	

[a] Primers III-F and III-R never yielded the expected amplicon with DNA templates extracted from all GBS serotype II strains tested in this study.

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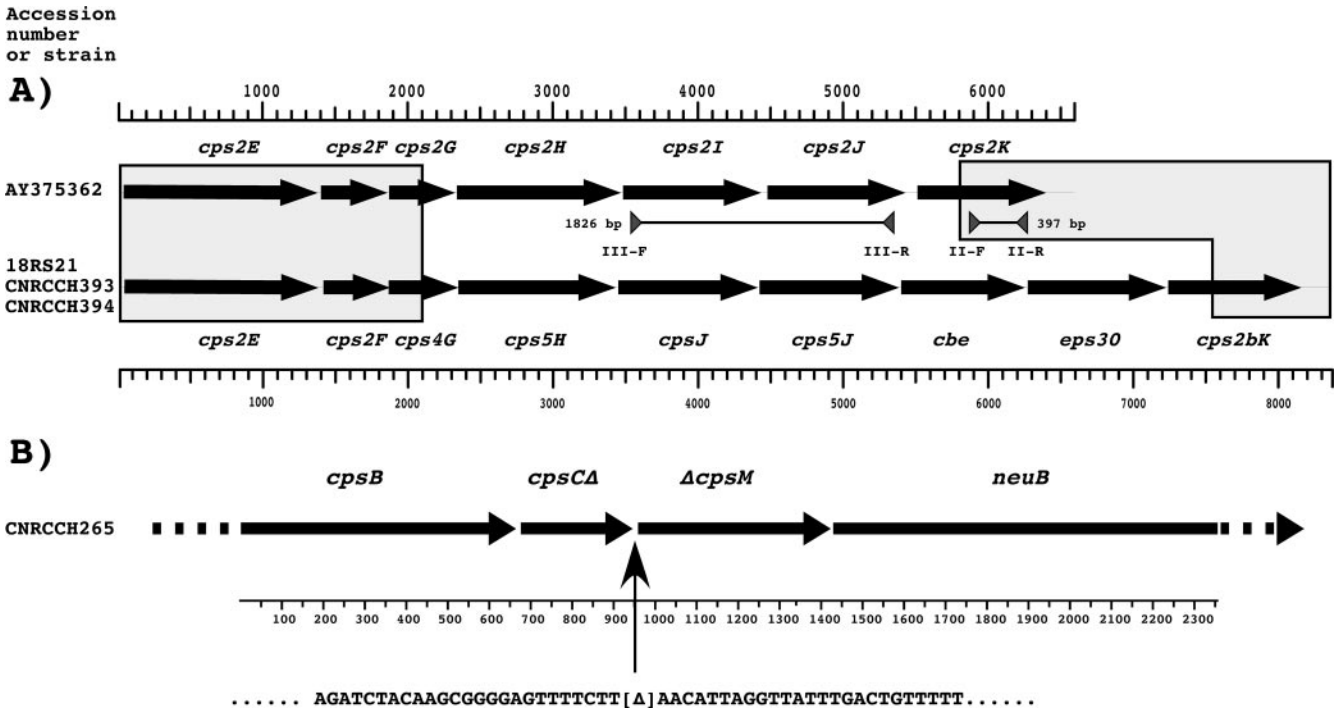

FIG. 1. Schematic representation of GBS *cps* loci. (A) Sequence analysis of two unrelated *cps* loci encoding the GBS serotype II capsular polysaccharide in strains CNRCCH393 and CNRCCH394. The gene nomenclatures are those used in the published sequence with GenBank accession number AY375362, thought to constitute the prototype sequence for type II *cps* loci (3), and the published sequence with GenBank accession number AAJO01000077 (strain 18RS21). The gray boxes indicate regions of sequence identity. Note that primers II-F and II-R match the 3′ moiety of *cps2K* present in all sequences, whereas primers III-F (specific for the 5′ extremity of *cps2I*) and III-R (specific for the 3′ extremity of *cps2J*) appear to be specific for the sequence with GenBank accession number AY375362. Sequencing of the strain CNRCCH393 and CNRCCH394 *cps2* loci was done by chromosome walking between the 5′ and 3′ ends of *cps2E* and *cps2K*, respectively. Scales are indicated in base pairs. (B) Sequence analysis of the *cps* locus of NT strain CNRCCH265. Sequencing of this locus was done by chromosome walking between the 5′ and 3′ ends of *cpsB* and *neuB*, respectively. This strain should synthesize a COOH-truncated form of CpsC (CpsCΔ) but should not express any CpsM derivatives (ΔCpsM) because the appropriate translational signals have been deleted. The scale is indicated in base pairs.

which enabled the amplification of fragments of different sizes that could be easily discriminated by agarose gel electrophoresis. Primer specificity was tested against the sequences in the GenBank database by using BLAST searches to verify the absence of serendipitous similarities. PCR simulations were carried out by using AmplifX 1.37 software. Primers that met these criteria and that were specific for sequences corresponding to CPS types Ia, Ib, II, IV,V, VI, VII, and VIII were identified; and the most appropriate pairs, which were selected on the basis of similar melting temperatures and the ability to generate distinguishable amplicon sizes, were retained (Table 1). Due to the high degree of sequence similarity of these loci, we failed to define primers specific for CPS type III. We therefore selected the pairs with the lowest potential for cross-hybridization with other *cps* operons. As shown in Table 1, all but one of the primer pairs were predicted to be CPS type specific, whereas the primer pair used to detect type III strains was expected to cross-react with type Ia and II strains. Moreover, the size differences between the amplicons allowed us to readily identify each CPS type, based on the electrophoretic mobility of the corresponding PCR product (Table 1).

The specificity and efficiency of each primer pair used separately were determined by PCR with DNA extracted from 33 GBS strains representative of all nine serotypes ($n = 5$ strains each for types Ia, Ib, II, III, IV, and V and $n = 3$ strains each for types VI, VII, and VIII). This analysis included the sequenced strains A909 (type Ia), NEM316 (type III), and 2603 V/R (type V) (5, 15, 16). The expected PCR patterns were obtained with all primer pairs and strains except primers III-F and III-R, which did not yield the expected 1,826-bp fragment with the five serotype II strains tested (data not shown). The specificities of the PCRs were assessed by sequencing the PCR products derived from the 33 strains. As expected, all sequenced amplicons displayed >98% identity with the corresponding CPS reference sequence.

Our finding that primers III-F and III-R did not hybridize with any serotype II clinical isolates suggested that the sequence with GenBank accession number AY375362 (3) might not be representative of *cps* operons encoding the serotype II CPS. Sequencing of the central region of the *cps* operons containing the genes presumably targeted by primers III-F and III-R of two unrelated CPS type II GBS isolates (isolates CNRCCH393 and CNRCCH394) revealed that these loci were identical to that of type II strain 18RS21 (Fig. 1) (4, 15). However, comparison of the strain 18RS21 sequence and the sequence with GenBank accession number AY375362 revealed that only their 5′ and 3′ extremities were identical, whereas the internal segments were different in size and gene content (Fig. 1A). Based on our results and those presented in previous reports (10, 17), we propose that the strain 18RS21 sequence

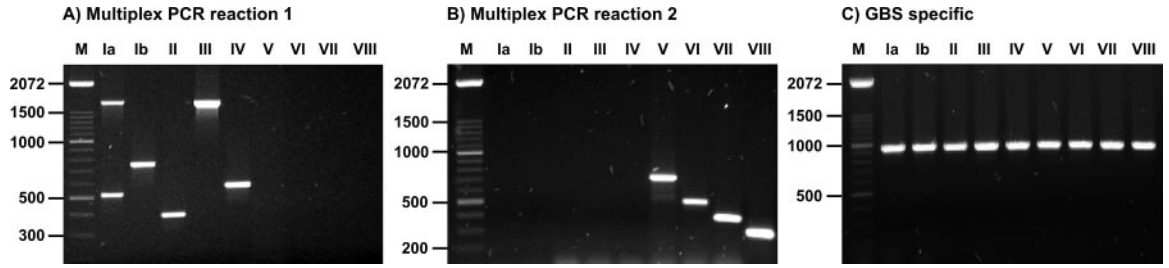

FIG. 2. Representative PCR multiplex reactions. The CPS types of the strains analyzed are indicated above their respective lanes. (A) Multiplex PCR for detection of CPS types Ia, Ib, II, III, and IV; (B) multiplex PCR for detection of CPS types V, VI, VII, and VIII; (C) GBS-specific PCR. Lanes M, molecular size standard (100-bp ladder; Invitrogen). The numbers on the right of each panel are in base pairs.

should be considered the *cps2* sequence prototype. The 18RS21 and AY375362 sequences could be designated *cps2a* and *cps2b*, respectively. Thus, at least two different *cps2* loci, *cps2a* and *cps2b*, could encode serotype II CPS.

Our aim was to develop a simple multiplex PCR assay that enables accurate GBS CPS typing. Two primer mixes (with mix I containing primer pairs specific for CPS types Ia, Ib, II, III, and IV and mix II containing primer pairs specific for CPS types V, VI, VII, and VIII) were used in separate PCRs. In every reaction, the CPS type could be unambiguously determined, with each type possessing a characteristic electrophoretic pattern (Fig. 2A and B). In this assay, the systematic use of two PCR mixes per strain provided an internal negative control, as only one mix should produce a PCR fragment. A third PCR (with primer pair dltS-F and dltS-R) targeting the GBS-specific *dltS* gene (11) was also included as an internal positive control (Fig. 2C).

Four hundred twenty-six human nonredundant GBS isolates collected in different French geographical areas between 2004 and 2006 were studied. Fifty-three (12.4%) strains were isolated from patients with GBS invasive diseases: 47 (11%; 16 from neonates, 2 from children, and 29 from adults) were from blood cultures and 6 (1.4%; all from neonates) were from cerebrospinal fluid. Two hundred thirty-two strains (54%) were isolated from vaginal samples from pregnant women, and 70 strains (16%) were from colonized but noninfected neonates. Seventy-one additional strains (16.6%) were obtained from urine samples ($n = 56$) or pus from various sites ($n = 15$). All GBS isolates were serotyped by agglutination with a commercial standard kit from Essum AB (Umea, Sweden) containing immunoglobulin G binding particles coated with rabbit antibodies specific to capsular serotypes Ia, Ib, II, III, IV, and V. LA serotyping (LAS) allowed us to identify the capsular serotypes of 93% (397/426) of the strains studied (Table 2). All GBS strains were also tested by using the multiplex PCR system illustrated in Fig. 2. Following PCR with the dltS primer pair, all strains yielded the expected PCR product, which confirmed that the DNA preparations were devoid of PCR inhibitors and that the corresponding strains were GBS. A capsular genotype was assigned to 99.7% (425/426) of the isolates (Table 2). Among the 397 strains typeable by both methods, the results of PCR CPS typing and LAS were in agreement for 394 isolates (99%). The three discordant strains (LAS and PCR CPS typing results for the three strains, III and IV, respectively; V and Ib, respectively; and V and III, respectively; Table 2) were each retested in three independent LAS experiments, and the PCR products were checked by sequencing. The same discordances between the phenotypic and the genotypic assays were obtained. The CPS types of these three strains determined by PCR were considered to be correct, based on the assumption that genotypic methods are more reliable than phenotypic methods.

Among the 29 (7%) NT strains that did not react or that gave weak polyagglutination by LAS, 22 (5.1%) were assigned by PCR to type Ia ($n = 16$), Ib ($n = 1$), II ($n = 2$), IV ($n = 1$), and V ($n = 2$). Six strains (1.4%) were assigned by PCR to type VI ($n = 1$) and type VII ($n = 5$) and were classified as NT by LAS due to the absence of the corresponding antisera in our kit. A single strain, CNRCCH265, could not be typed by either LAS or the PCR assay and was further studied. We searched by PCR for the presence of genes *cpsA* to *-E*; *cpsL*; and *neuB*, *-A*, *-C*, and *-D*, which are conserved in all nine GBS *cps* operons (3). This analysis indicated that the genes *cpsC* to *-E* and *cpsL* were apparently missing from this strain (data not shown). Sequence analysis revealed that a single large deletion that resulted in an out-of-frame fusion between the first half of *cpsC* and the second half of *cpsM* occurred in strain CNRCCH265 (Fig. 1B). The loss of the genes *cpsC* to *-M* is consistent with its NT phenotype. GBS isolates that are NT due to the presence of mutations or insertion sequences in the *cps* biosynthetic genes have been described (2, 3, 8, 9, 12, 14, 17). However, we describe here the first molecular character-

TABLE 2. Concordance matrix comparing PCR typing and LAS for 426 GBS clinical isolates from various sources

Serotype obtained by LAS	No. of strains typed as follows by PCR[a]:										
	Ia	Ib	II	III	IV	V	VI	VII	VIII	NT	Total
Ia	32										32
Ib		13									13
II			28								28
III				189	1						190
IV					22						22
V		1		1		110					112
VI							0				0
VII								0			0
VIII									0		0
NT	16	1	2		1	2	(1)	(5)		1	29
Total	48	15	30	190	24	112	1	5	0	1	426

[a] The six strains in parentheses were typed as CpsVI and CpsVII by PCR but were considered NT by LAS because the kit used did not contain the corresponding specific antisera.

ization of a GBS clinical isolate bearing a large deletion within the *cps* operon.

In conclusion, the multiplex PCR assay described in this work provides a simple tool for GBS CPS typing. This sensitive and specific method enables the characterization of all known GBS CPSs, thereby reducing the rate of detection of NT isolates. This assay is therefore particularly well adapted for GBS CPS typing in large-scale epidemiological studies.

Nucleotide sequence accession numbers. The GenBank/EMBL accession numbers of the sequences derived from strains 18RS21, CNRCCH393, and CNRCCH265 are AAJO01000077, AM498296, and AM498295, respectively.

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