AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Clinical Microbiology®

BACTERIOLOGY

Identification of Group B Streptococcus Capsule Type by Use of a Dual Phenotypic/Genotypic Assay

Areej Alhhazmi,[a,c] Armaan Pandey,[a] Gregory J. Tyrrell[a,b]

The Division of Diagnostic and Applied Microbiology, Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, Alberta, Canada[a]; The Provincial Laboratory for Public Health (Microbiology), Edmonton, Alberta, Canada[b]; Medical Laboratory Technology, College of Applied Medical Sciences, Taibah University, Madinah, Saudi Arabia[c]

ABSTRACT The group B streptococcus (GBS) capsular polysaccharide (CPS) is an important virulence factor which is also used for GBS typing. There are 10 CPS types (Ia, Ib, and II to IX). GBS that do not phenotypically type are considered nontypeable. All genes required for CPS synthesis are found on the GBS *cps* operon, which contains a highly variable CPS-determining region (*cpsG-cpsK*). The objective of this study was development of an assay to detect sialic acid on the GBS cell surface, followed by a genotypic PCR CPS typing assay. Sialic acid is located at the terminal end of the side chain of all known GBS CPS types. Sialic acid can be bound to commercially available lectins such as slug *Limax flavus* lectin. Biotinylated *L. flavus*-streptavidin-peroxidase complex was used in an enzyme immunoassay and dot blot assay to detect sialic acid. This was followed by a PCR typing scheme that was developed to target the serotype-determining region of the *cps* locus for Ia, Ib, and II to IX. Sialic acid from the CPS types Ia, Ib, and II to IX was detectable on the GBS cell surfaces of all previously identified CPS-typed GBS strains assayed. This was followed by the real-time PCR typing assay which successfully identified CPS Ia, Ib, and II to IX types. The combination of phenotypic and genotypic assays provides an accurate tool for detection of CPS expression and assignment of CPS typing. These assays have the potential to be used for CPS typing in large-scale epidemiological studies.

KEYWORDS RT-PCR, streptococcus, *Streptococcus agalactiae*, group B streptococcus, lectin, serotyping, sialic acid

Group B streptococci (GBS) are recognized as a leading cause of neonatal invasive disease, as well as invasive disease, in immunocompromised patients and in elderly individuals (1–7). An important virulence factor of GBS is a capsular polysaccharide (CPS); there are ten antigenic CPS variants designated Ia, Ib, and II to IX (8–11). In North America and a number of European countries, five CPS types (Ia, Ib, II, III, and V) cause the bulk of invasive disease cases, with CPS III causing a higher rate of disease among neonates and CPS types Ia and V causing higher rates among adult patients (12–23). Interestingly, recent studies have noted the emergence and circulation of CPS IV, a previously uncommon serotype, as an important cause of both neonatal and adult infections (14, 19, 24–28).

As attention is focused on invasive GBS (iGBS) disease with the emergence of new strains and increased antibiotic resistance, the potential to prevent iGBS disease via vaccination becomes more attractive (21, 29). If a GBS vaccine is developed for use that is based on selected capsule types, it will become important to monitor distribution patterns of CPS types in circulation in the target population using sensitive and specific methods for determining CPS types.

Received 18 February 2017 **Returned for modification** 10 April 2017 **Accepted** 8 June 2017

Accepted manuscript posted online 14 June 2017

Citation Alhhazmi A, Pandey A, Tyrrell GJ. 2017. Identification of group B streptococcus capsule type by use of a dual phenotypic/genotypic assay. J Clin Microbiol 55:2637–2650. https://doi.org/10.1128/JCM.00300-17.

Editor Robin Patel, Mayo Clinic

Address correspondence to Gregory J. Tyrrell, greg.tyrrell@ahs.ca.

TABLE 1 Strains used in study

Strain type	Designation	Source or reference
GBS		
Ia	A909	70
Ib	NCS4	71
II	NCS6	71
III	COH1	72
IV	NCS11	71
V	NCS13	71
VI	NCS14	71
VII	7271	73
VIII	JM9	74
IX	ATCC 27412	
S. pneumoniae serotype 14	10SR3072	This study
E. coli	ATCC 25922	

Common phenotypic methods of GBS CPS type identification are based on serological assays such as capillary precipitin (30), immunodiffusion (30, 31), latex agglutination (32, 33), coagglutination (34), or enzyme immunoassay (35), which have proven invaluable for identifying CPS types. However, these assays may have complicated interpretations resulting in a number of incorrect typing assignments due to poor capsule expression, capsule operon mutations, rearrangements, or limited accuracy. Molecular typing based on PCR assays has been already developed; however, these assays target capsule gene detection rather than capsule expression. Moreover, they tend to involve a combination of two different techniques, e.g., PCR plus sequencing (36), PCR plus blot hybridization (37, 38), PCR plus enzymatic restriction (39), or multiplex PCR plus agarose gel electrophoresis (40, 41). A real-time PCR assay is a more attractive molecular method for assigning CPS type than conventional PCR since it is usually more rapid.

The CPS type-specific epitopes of each GBS polysaccharide are created by different arrangements of four component sugars (glucose, galactose, *N*-acetylglucosamine, and sialic acid) into a unique repeating unit. Interestingly, all of these structures contain a terminal sialic acid (Neu5Ac) bound to galactose in an α2-3 linkage (40, 42, 43). The conservation of Neu5Ac among all known GBS capsular types suggests that this structural feature is essential to GBS capsular polysaccharide pathogenicity. We propose that sialic acid on the surfaces of GBS capsule positive cells can be used as a universal phenotypic method to ascertain capsule expression, followed by a CPS-specific real-time PCR assay.

RESULTS

Detection of sialic acid from 10 GBS CPS types using a lectin enzyme immunoassay (EIA). Understanding that sialylation is essential for full GBS capsule biosynthesis and loss of sialylated capsule reduces the amount of CPS expressed on the bacterial cell surface by 80% (43), we hypothesized that sialic acid could be used as a recognition moiety for capsule expression. The commercially available biotinylated lectin from the slug *Limax flavus* was selected to be used in our sialo-lectin binding assay. *L. flavus* lectin was selected because it reacts with sialic acid in any linkage (44, 45), whereas other sialic acid-specific lectins recognize only specific glycosidic linkages of sialic acid or other carbohydrate moieties (45–48). Sialic acid is conserved at the terminus of the side chain of all GBS capsule types.

To examine the use of sialo-lectin binding to detect GBS CPS expression, 10 GBS strains were selected representing all recognized GBS CPS types (Ia, Ib, and II to IX) for CPS extraction (Table 1). Recognition of the immobilized sialic acid from the 10 assayed CPS type bacterial isolates by biotinylated *L. flavus* lectin validated the presence of CPS. It also provided a dose-dependent signal that exhibited a saturating signal at a lectin concentration of 10 μg/ml (Fig. 1). Therefore, the working concentration of biotinylated *L. flavus* lectin with all the CPS types in the study was restricted to 10 μg/ml. The

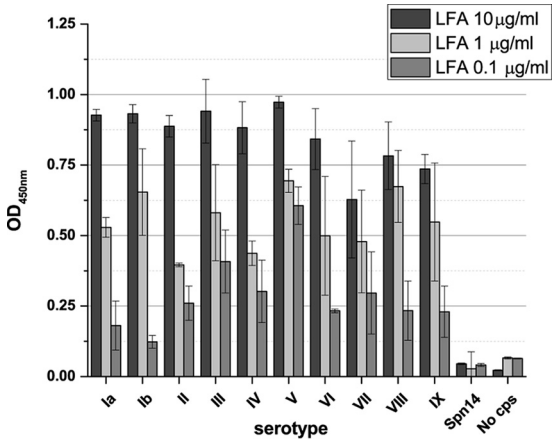

FIG 1 Recognition of GBS CPSs by the lectin EIA. Crude GBS CPS preparations (Ia, Ib, and II to IX) were incubated with *L. flavus* lectin specific for Neu5Ac. Three concentrations of biotin-*L. flavus* lectin (LFA) were used (10, 1, and 0.1 μg/ml), while 1 μg of HRP-labeled streptavidin/ml was used. Data are expressed as mean OD_{450} values with the standard deviations (SD) for at least three independent experiments.

average absorbance at 450 nm for all CPS types was 0.85 $\pm$ 0.079 with a 95% confidence interval (CI) of 0.77 to 0.93 (Fig. 1). For the asialo-CPS (spn14) isolate with no sialic acid in its capsule and the well with no capsule material, average absorbances of 0.45 nm (95% CI = 0.44 to 0.45) and 0.022 nm (95% CI = 0.02 to 0.03) were detected, respectively. The upper absorbance limit of the 95% CI of the negative control (spn14) preparation was 0.45. This value was used as a cutoff for detecting the presence of sialic acid in further experiments.

Detection of sialic acid from the 10 GBS CPS types using a lectin dot blot assay. To verify that the sialo-lectin binding phenotypes from the 10 assayed CPS type isolates by the lectin EIA was not due to endogenous sialic acids coextracted from the bacteria during the capsule extraction process, whole bacteria were assayed in a lectin dot blot assay. As shown in Fig. 2, sialic acid was uniformly expressed in all the assayed strains, whereas no signal was detected on the spot corresponding to the asialo-CPS (spn14) isolate. The results suggested that both the lectin EIA and the lectin dot blot assays could be used as simple phenotypic assays to determine CPS expression for GBS.

GBS CPS typing assay. (i) Assignment of CPS types for types Ia, Ib, and II to IX using a singleplex real-time PCR CPS typing assay. Representative CPS DNA operon sequences for CPS Ia, Ib, and II to VIII and a partial CPS DNA operon sequence for IX were obtained from the NCBI website (http://www.ncbi.nlm.nih.gov/). The sequences of these strains were analyzed to generate *cps*-specific primer pairs and dually labeled probes, which enabled the amplification of DNA amplicons to be easily discriminated by specific probes. A CPS-specific gene scheme was developed based on comparison analyses of *cps* genes. A unique region(s) of the *cps* gene(s) was identified for each CPS type based on the nucleotide sequence comparison with other CPS types. From this information, a set of primers for each CPS type, Ia, Ib, and II to IX, was designed to uniquely target each of the 10 CPS types (Table 2). The identification of CPS types Ia, Ib, and II to IX depends on the ability of the primer pairs to amplify and probe

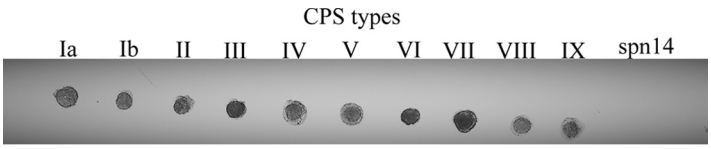

FIG 2 Recognition of GBS CPSs by dot blot enzyme-linked immunosorbent assay. GBS type Ia, Ib, and II to IX whole bacteria (10^7 CFU) were incubated with *L. flavus*. An spn14 isolate was used as a negative control.

TABLE 2 Primer pairs and probes used in the singleplex and multiplex RT-PCR assays

Identifier	CPS type	Sequence (5′–3′)	Duplex reaction
Primer			
cpsO-F	V	AACAGAGGCCAATCAGTTGCA	1
cpsO-R		CGGCATTGGTAGCTTTCTGTATG	
cpsI-F	VI	TTCACCTTCTGCCATCTCAA	1
cpsI-R		AAGGGATAGTCGCGTAAAAGTC	
cpsG-F	III	AAACGGGTTACTCAGACTTCG	2
cpsG-R		TCACCAAACTGCTTTCTCCTAG	
cpsK-F	II	GCTATTCCCTACATGGAAGATGG	2
cpsK-R		TTACTGAAGCCATGATATCGGG	
cpsM-F	VII	CCTTTGAGAGTTCATAACTGTT	3
cpsM-R		GTCCTCTAATTGCACCAATAAT	
cpsR-F	VIII	CCAGATGGGCATGAGTGGTTAC	3
cpsR-R		CAGTCCCATAGGCGATGTAGG	
cpsN-F	IV	GTATGCTTTCGTGTCTGATTATGC	4
cpsN-R		ATTGATCCAAAACCCAAACCTG	
cpsH-F	Ia	TTAATTTGCGATCCGGGAGTAG	4
cpsH-R		GCAGGCCACTTTTGTAGAAATAG	
cpsJ-F	Ib	TGGGATATAGAGATTTAGTACCTGTTG	5
cpsJ-R		ATTGGTTTGTGATATTCCATTCTCG	
cpsO-F	IX	CTGATGATCTTTGTTCGCCATTT	5
cpsO-R		ACAAGGGTGATCCTCAATTCC	
Probe			
cpsO	V	Cal Fluor 560-CAACGGAGTACTTAGGTGTACAGGAGA-BHQ1	1
cpsI	VI	FAM-ACAATGGAGGTGCATCATCAGCA-BHQ1	1
cpsK	II	Cal Fluor 560-TCTTGTCACAAAGACCATCTGGAGCG-BHQ1	2
cpsG	III	FAM-ATTGTTATCACACATGGCGGCCC-BHQ1	2
cpsM	VII	Cal Fluor 540-CTAGGGAGTTAAGTTATGATGTGA-BHQ1	3
cpsR	VIII	FAM-TGGAGTATTCCTGTACGTCGCTATTGGA-BHQ1	3
cpsN	IV	Cal Fluor 560-CCTCTCCAGGTAGCTCACAAGCAAA-BHQ1	4
cpsH	Ia	FAM-TTGAATGCGACCCCAAAGGGAGA-BHQ1	4
cpsJ	Ib	FAM-CCGATTTTGAAATCAGCCAGAGCTCCT-BHQ1	5
cpsO	IX	Cal Fluor CAACATGAAACTGGTGCTGACCTTGT-BHQ1	

to identify a specific fragment for the following *cps* genes: *cpsH*, *cpsJ*, *cpsK*, *cpsG*, *cpsN*, *cpsO-V*, *cpsI*, *cpsM*, *cpsR*, and *cpsO*-IX, respectively.

To verify the specificity of the designed primer pairs and probes, they were BLAST searched against the NCBI nonredundant sequence database to confirm the absence of serendipitous similarities. Based on this analysis, the designed primer pairs and probes were determined to have 100% specificity for the identification of GBS CPS types, except for CPS type III. The primers and probe targeting *cpsG* were similar to CPS types V and VI. However, the primers for CPS V and VI are specific for the identification of these CPS types. An algorithm was constructed to allow identification of the 10 CPS types, as shown in Fig. 3. The specificity and efficiency of each primer pair used separately were determined by singleplex reverse transcription-PCR (RT-PCR) with DNA extracted from 10 GBS strains representing all GBS recognized CPS types (Table 3) that were used as reference strains. Specific and characteristic PCR patterns were obtained with all primer pairs and probes for each CPS type (Table 4). No signal was obtained for the negative controls, isolates spn14 and *Escherichia coli* ATCC 2592 (data not shown).

The standard curve and efficiency of each primer pair was determined by using a series of diluted genomic DNA extracts from the recognized CPS types to determine the smallest amount of DNA detected while maintaining a desirable efficiency. The minimum concentration of purified genomic DNA required for the cpsH, cpsK, cpsJ, cpsN, and cpsI primer pairs was 30 ng, whereas for the cpsG, cpsM, cpsO-V, and cpsO-IX primers 20 ng was required (Fig. 4). For the cpsR primer set, 40 ng was required for the detection. The designed primers have high amplification efficiency ranging between 90.9 and 99.9%, as shown in Table 3, indicating that the assay is robust and reproducible.

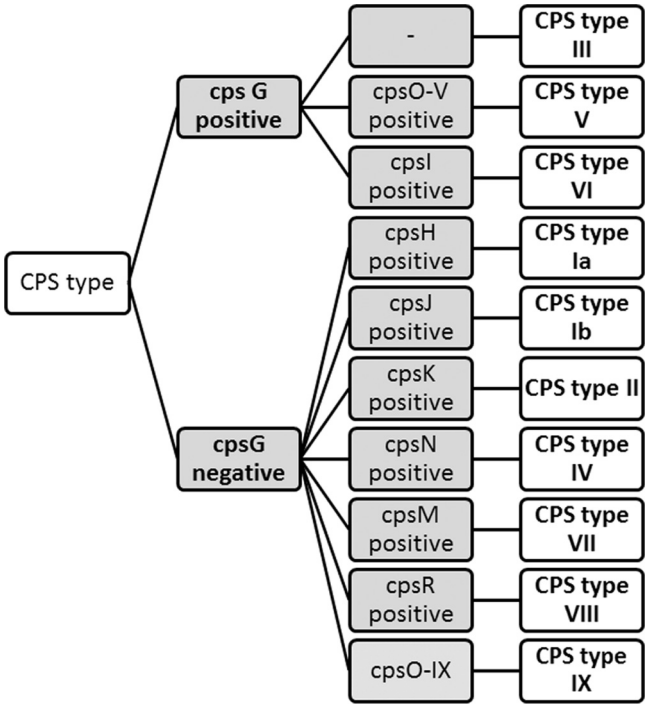

FIG 3 Algorithm to identify GBS CPS types Ia, Ib, and II to IX. Gray boxes are *cps* target genes, and white boxes are GBS CPS types.

(ii) Assignment of CPS types using a duplex RT-PCR GBS typing assay. Five duplex reactions (1 to 5) (Table 2) containing primer pairs and probes specific for CPS types V/VI, III/II, VII/VIII, Ia/IV, and Ib/IX were used in a real-time PCR platform. The 10 GBS CPS reference strains previously assayed in the singleplex PCR typing assay were analyzed using the duplex real-time PCR assay to assign CPS type. The duplex real-time PCR assay allowed identification of the CPS type for the GBS strains shown in Table 5.

A collection of 70 previously serotyped GBS clinical isolates representing all recognized CPS types were analyzed to determine the reliability of the duplex real-time PCR typing with the exception of CPS VII. No CPS type VII except for the reference strain was present in our collection; therefore, no clinical CPS VII isolates were assayed (15). We compared the CPS typing by the duplex RT-PCR assay against serotyping by the immunodiffusion assay for CPS types Ia, Ib, and II to VIII or a previously described PCR assay (40) for CPS type IX. A concordance of 97.2% for the duplex RT-PCR GBS typing and the phenotypic serotyping assay (immunodiffusion) was observed. No discordant results were obtained between the molecular assay and the serological assay for all CPS types, with the exception of CPS type Ib. Two isolates were typed by the serological assay as CPS type Ib, and yet the molecular assay assigned CPS type II for these isolates

TABLE 3 Efficiency calculated for primers for real-time PCR GBS CPS typing

cps gene target	Efficiency (%)
cpsH	92.60
cpsK	99.87
cpsJ	90.94
cpsG	92.31
cpsN	99.79
cpsO-V	98.56
cpsI	96.14
cpsM	91.84
cpsR	98.60
cpsO-IX	96.68

TABLE 4 Singleplex real-time PCR for GBS typing[a]

Gene target	Mean C_T ± SD for CPS type:									
	Ia	Ib	II	III	IV	V	VI	VII	VIII	IX
cpsH	23.9 ± 0.2									
cpsJ		24.1 ± 0.5								
cpsK			26.8 ± 1.5							
cpsG				18.4 ± 1.6		23.2 ± 2.2	26.4 ± 2.7			
cpsN					18.5 ± 0.0					
cpsO-V						20.0 ± 1.4				
cpsI							21.5 ± 0.6			
cpsM								26.2 ± 2.3		
cpsR									23.5 ± 1.1	
cpsO-IX										17.6 ± 0.1

[a]Data are expressed as mean C_T values for at least three independent experiments.

(Table 6). To confirm CPS genotyping of these two isolates as CPS type II, a previously described conventional PCR assay (41) was performed to distinguish between CPS types II and Ib. The PCR assay verified these isolates as CPS type II (Fig. 5).

Assignment of CPS type to a collection of clinical NT isolates using lectin EIA/lectin dot assays and a duplex RT-PCR GBS typing assay. To determine the robustness of our assay algorithm, we assayed 159 GBS clinical isolates from Alberta, Canada, collected from 2003 to 2013 that were determined to be nontypeable (NT) in a double immunodiffusion assay. These GBS isolates were assayed for the presence of sialo-CPS using the lectin EIA after CPS extraction. A total of 47.2% (75/159) of the capsule preparations from the NT isolates reacted positively with *L. flavus* lectin, suggesting the presence of capsule (data are not shown). The lectin dot blot assay supported the results obtained from the lectin EIA since all 75 isolates spotted in the lectin dot blot assay displayed spots darker than the spots from isolates with an optical density (OD) below 0.45 in the lectin EIA (Fig. 6), suggesting the presence of sialic acid.

To prove the stability and veracity of the duplex RT-PCR typing assay, the capsule types of the sialo-CPS-positive NT strains were assayed. DNA was extracted, and PCR amplification with the *cfb* primer pair was performed. 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 (data not shown). A duplex RT-PCR CPS typing assay was then performed to identify CPS genotypes. A capsular genotype was assigned for 71 of 75 isolates (94.7%). The majority of the strains were typed as CPS type

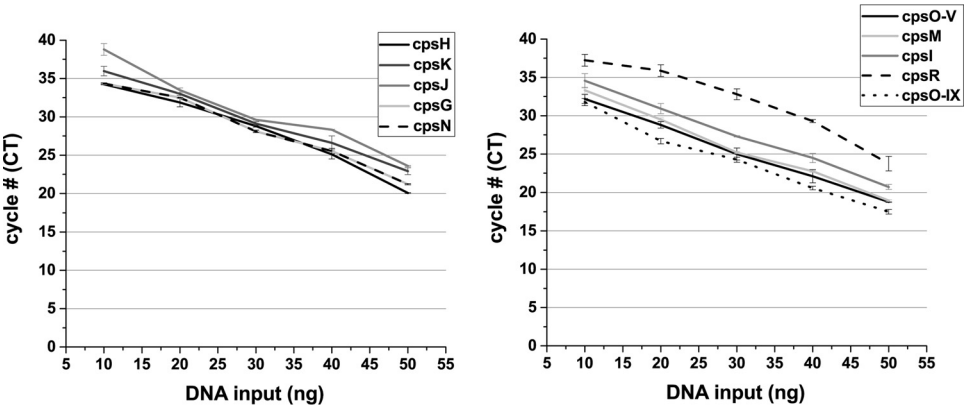

FIG 4 Standard curves and efficiencies calculated for primers for real-time PCR GBS CPS typing. Tenfold dilutions of DNA template (10, 20, 30, 40, and 50 ng) were plotted against the C_T value for each dilution to generate a standard curve. From this standard curve, the smallest amount of DNA detected and the C_T slope were determined for the primers cpsH, cpsK, cpsJ, cpsG, cpsN, cpsO-V, cpsI, cpsM, cpsR, and cpsO-IX to identify CPS types Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX, respectively. The efficiency values were measured for each primer pair using the C_T slope method. The amplification efficiency was calculated according to the following equation: Ex = 10(−1/slope) − 1, where Ex is the efficiency. Data are expressed as mean C_T values with the SD for at least three independent experiments.

TABLE 5 Duplex real-time PCR for GBS CPS typing[a]

Reaction	*cps* gene	CPS type	Mean C_T ± SD
1	*cpsO*	V	18.04 ± 1.33
	cpsI	VI	18.07 ± 0.07
2	*cpsG*	III	16.77 ± 0.70
	cpsK	II	24.88 ± 0.50
3	*cpsM*	VII	19.70 ± 1.34
	cpsR	VIII	22.44 ± 0.22
4	*cpsH*	Ia	24.55 ± 0.04
	cpsN	IV	18.12 ± 0.02
5	*cpsJ*	Ib	19.97 ± 1.41
	cpsO	IX	17.84 ± 0.06

[a]Data are expressed as mean C_T values for at least three independent experiments.

V (53.3%, 40/75). A total of 14.7% (11/75) of the isolates were typed as CPS type III, 8% (6/75) as type II, 5% (4/75) as type Ia, 5% (4/75) as Ib, 4% (3/75) as type VI, 2.7% (2/75) as type IV, and 1.3% (1/75) as type IX. Four isolates failed to be assigned a CPS type.

To test the ability of the duplex PCR assay to assign CPS types to GBS isolates, we randomly selected 32 of the remaining 84 GBS isolates that did not react with *L. flavus* lectin in EIAs and dot blot assays and assayed them in the duplex real-time PCR assay. The capsule types identified were CPS types Ia (3.1%, 1/32), Ib (31.3%, 10/32), II (3.1%, 1/32), III (6.3%, 2/32), V (18.3%, 6/32), VI (3.1%, 1/32), and IX (28.1%, 9/32). Four isolates failed to be assigned a capsule type. This demonstrated the ability of the duplex RT-PCR assay to genotypically identify CPS types which failed to express sialic acid on their surfaces, suggesting the absence of capsule.

DISCUSSION

GBS CPS is a well-known protective antigen against GBS (29, 49). Past efforts to develop GBS vaccines have focused primarily on the use of capsular polysaccharides from more common types associated with GBS disease (29, 49). For this avenue of vaccine development to be effective requires inclusion of the most relevant CPS types in the target population (29, 49). Here, we present a dual GBS typing system that provides information regarding capsule expression and identification of capsule type.

The first step of the proposed GBS typing system involves the detection of sialic acid and confirmation of capsule expression. GBS CPS is terminally linked to sialic acid. Sialylation is an essential process for full GBS CPS biosynthesis and expression. A past study reported that sialylation of GBS capsular polysaccharide is required for full synthesis of CPS by GBS (43). These investigators found an 80% reduction in surface associated CPS produced by asialo mutant strains that had a deletion in the *cpsK* gene encoding sialyltransferase compared to the parental strain (43). This group also re-

TABLE 6 Comparison of typing results obtained with the immunodiffusion assay (the serological method) and the duplex real-time PCR assay using a collection of GBS clinical strains

CPS type determined by PCR	Result for CPS type determined by immunodiffusion assay										Concordance (%)
	Ia	Ib	II	III	IV	V	VI	VIII	IX[a]	Total	
Ia	8/8									8	100
Ib		6/8								6	75
II		2	6/6							8	100
III				10/10						10	100
IV					8/8					8	100
V						11/11				11	100
VI							5/5			5	100
VIII								4/4		4	100
IX									10/10	10	100
Total	8	8	6	10	8	11	5	4	10	70	97.2

[a]CPS type IX isolates were typed by a PCR assay as previously described (40).

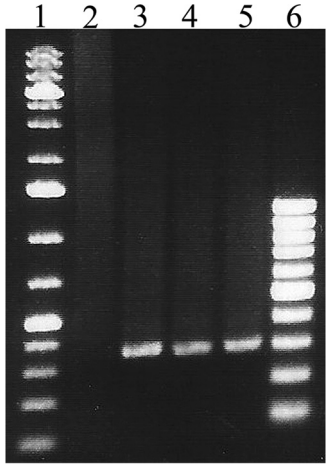

FIG 5 GBS PCR typing of the two isolates that displayed a discrepancy between the immunodiffusion assay (CPS type Ib) and duplex real-time PCR typing (CPS type II). To identify CPS type II, a previously described PCR assay (41) was used. Lanes: 1, 1-kb DNA ladder; 2, CPS type Ib genomic DNA (gDNA); 3, CPS type II gDNA; 4, 04SR421 gDNA; 5, 13SR567 gDNA; 6, 100-bp DNA ladder.

ported the same scenario with an asialo mutant with a deletion in *neuA* encoding CMP-sialic acid synthase (50). The lectin from *L. flavus* agglutinin was chosen in our study to detect sialic acid from GBS CPS types for two reasons. First, *L. flavus* lectin binds to Neu5Ac regardless of its linkage. This was optimal for our assay since sialic acid in the capsule among the known GBS CPS types has different linkages. Second, to date, nonspecific binding has not been identified for *L. flavus* lectin (51). For these reasons, biotinylated *L. flavus* lectin was selected.

Serological CPS typing is the most common method for GBS CPS assignment, but the proportion of NT isolates is significant and has increased over time (11, 37, 52). This leads to misrepresentation of some of the CPS types. The sensitivity of the serological assays used to detect GBS capsule polysaccharide can vary. These methods depend on the quality of the antibodies, the technical experience of the operator, and the expression of a detectable amount of capsule (53). A study by Kilian and coworkers (54) identified limitations associated with GBS latex agglutination serotyping. The agglutination assay was found to be less sensitive than the flow cytometric assay. Approximately one-half of the strains assigned as NT in the agglutination test were found to express type-specific polysaccharides by the flow cytometric method (54). To overcome these limitations, we developed a sialo-lectin assay for which sialic acid could be used as a universal approach to detect capsule expression without the need for

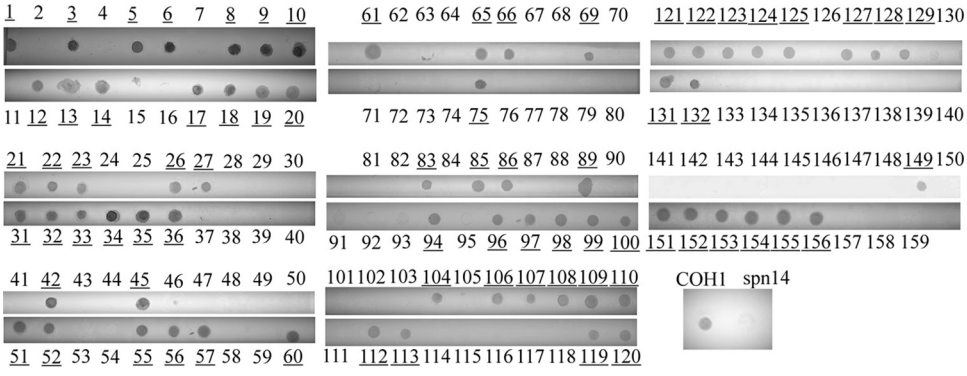

FIG 6 A lectin dot blot assay was performed for GBS isolates that failed to be assigned a CPS type by the immunodiffusion assay (NT isolates). Underlined dots are positive for sialic acid. There are 75 positive spots. GBS whole bacteria (10^7 CFU) were incubated with *L. flavus*; the positive control was CPS type III GBS strain COH1. The negative control was the spn14 isolate.

antibodies. Moreover, the use of biotinylated lectin in a streptavidin-horseradish peroxidase (HRP) detection and amplification system has the advantage of detecting a small amount of sialylated capsule (43, 46, 55, 56). Two assays, lectin EIAs and lectin dot blot assays, were described in our work to identify isolates with capsule. We also used these assays to characterize isolates in our collection that were identified as NT by immunodiffusion assay. Based on our results, there were 52.8% (84/159) NT isolates that lacked sialic acid expression on the bacterial surface. However, 47.2% (75/159) of our serologically NT isolates expressed sialic acid on the surface, suggesting the presence of the capsule. The immunodiffusion assay is less sensitive than the sialo-lectin methods, as confirmed by our observation that 47.2% of the strains assigned as NT in this assay were found to express sialylated capsule polysaccharide when examined by the sialo-lectin assays. The 84 sialic acid negative isolates in the lectin EIAs and lectin dot blot assays presumably do not express capsular polysaccharides. Mechanisms of capsule loss could potentially be caused by genetic alterations such as the insertion or deletion of DNA fragments in the *cps* operon or mutations in specific conserved genes, such as *cpsE*, *cpsF*, *cpsA*, and *cpsG*, located in the *cps* locus (37, 57, 58).

The second step of our proposed GBS typing system is identification of the CPS type by PCR. Several molecular typing-based PCR assays have been developed (36, 38, 40, 41, 59). These assays target *cps*-determining genes in the *cps* operon. PCR assays are an attractive approach to GBS CPS typing because of their high discriminatory power for identifying CPS types. Such PCR assays were able to assign a CPS type to isolates that failed to be identified by serological assay methods (36, 40, 41, 59). Although PCR-based assays for typing GBS work well, a number of limitations have been identified. Generally, these assays target capsule genes rather than expression; hence, the *cps* locus identified by the PCR-based assays does not confirm whether the CPS is expressed or not. Moreover, to identify CPS types by the available PCR-based assays, two different techniques are required, such as PCR plus sequencing (36), PCR plus blot hybridization (37, 38), PCR plus enzymatic restriction (39), or multiplex PCR plus agarose gel electrophoresis (40, 41). The involvement of two molecular techniques to identify CPS types only at the genotypic level can consume time and may involve increased expense when techniques such as sequencing and blot hybridization are used. Also, these assays may not always be accurate. For example, the multiplex PCR assay described by Imperi et al. (40) that was designed to distinguish among types Ia, Ib, and II to IX was found to misidentify some CPS type Ib and IV strains as CPS type Ia (54, 60). In addition, a two-set multiplex PCR assay (set 1, types Ia, Ib, II, III, and IV; set 2, types V, VI, VII, and VIII) developed by Poyart et al. (41) failed to distinguish between CPS types VII and IX (54). Also, the CPS-specific PCR assay described by Kong et al. (36, 38) did not include a PCR specific for CPS type II, VII, or VIII (54). To address these issues, we developed a duplex real-time PCR assay that identifies CPS type based on detecting the presence of capsular genes. The real-time PCR technique used in our typing system is a more attractive approach to assigning CPS types than conventional PCR. The main advantages of the TaqMan real-time PCR assay are its high sensitivity and reliability (one step and gel free) with a specific set of primers and probes to identify each CPS type. The assay is easy to perform and does not require the use of antisera. The method also has time advantages over previously described molecular methods (36, 40, 41, 59) because it requires only a single PCR for amplification and detection and can be completed within 1 h. To overcome GBS typing inaccuracies using PCR, previous investigators have developed a GBS CPS typing algorithm that includes three previously described PCR assays (36, 38, 40, 41). This approach resulted in a GBS CPS typing accuracy of 99% (60). Our duplex real-time PCR assay had a level of accuracy of 100%. We believe that our assay would be ideally suited for high-throughput GBS epidemiological studies in which a large collection of GBS isolates requires CPS typing. A limitation of our assay was the inability to type a small subset of isolates. Four isolates of the 75 isolates that were sialic acid positive and 2 isolates of the 32 randomly selected sialic acid-negative isolates failed to be typed by our PCR assay. Possible reasons for this may be the presence of an insertion(s) or mutation(s) impairing primer and/or probe binding.

Alternatively, these isolates may represent new CPS types resulting from capsular switching events, in which GBS has acquired new capsule genes by horizontal gene transfer, followed by genetic recombination. Recently, a short report by Breeding et al. (61) described a real time-PCR CPS typing assay for GBS. These authors examined a collection of 21 clinical GBS isolates using their real-time PCR assay compared to latex agglutination and found 100% concordance for their assay, suggesting that the real-time PCR assay performs well. Our algorithm differs in that the presence of capsule is also determined phenotypically and assignment of CPS type is done by real-time PCR. Nonetheless, the assay described by Breeding et al. and our assay algorithm demonstrate the strength of the real-time PCR for GBS CPS typing.

In summary, we propose a new GBS typing system for which the first step is to confirm capsule expression using sialo-lectin assays, followed by duplex real-time PCR typing that identifies the *cps* genotype. The combination of sialo-CPS lectin binding and duplex real time-PCR typing assays provides a simple and reliable tool for CPS expression confirmation and GBS CPS typing, respectively. This sensitive and specific method enables the characterization of GBS CPS types Ia, Ib, and II to IX, thereby reducing the rate of detection of NT isolates. It is therefore particularly well adapted for GBS CPS typing in large-scale epidemiological studies.

MATERIALS AND METHODS

Bacterial strains used. Ten GBS strains with known CPS types were used as reference strains for CPS typing (Table 1). Seventy GBS clinical isolates previously typed by the phenotypic immunodiffusion assay and 159 GBS clinical isolates that previously failed to be typed (nontypeable [NT]) by the phenotypic immunodiffusion assay were included in this study (15). A *Streptococcus pneumoniae* serotype 14 isolate (spn14) and *Escherichia coli* ATCC 25922 were used as control strains (Table 1) (62, 63). GBS isolates were cultured on Columbia blood agar plates (Dalynn Biologicals, Canada) containing 5% sheep blood overnight at 37°C. They subsequently were inoculated into Todd-Hewitt broth (Becton Dickinson, USA) and incubated overnight at 37°C for use in the experiments described here.

Identification of GBS. Isolates were identified on the basis of colony morphology, beta-hemolysis, Gram stain, and Lancefield grouping with type B antisera (Oxoid, Canada) (64). GBS isolates were additionally confirmed as GBS using a conventional PCR-based assay targeting the *cfb* gene that encodes the Christie-Atkins-Munch-Petersen factor (65).

GBS CPS typing. (i) Double immunodiffusion assay. Phenotypic CPS typing was performed using a Lancefield heat-acid extraction assay, followed by a double immunodiffusion method described previously (30, 31). The immunodiffusion assay of GBS CPS typing used for this study was based on reactions with antisera raised against CPS types Ia, Ib, and II to VIII. A type-specific antiserum panel was prepared in rabbits as previously described (30, 31).

(ii) CPS type IX identification. PCR CPS typing was used for the identification of CPS IX using the primers cpsI-7-9-F (5′-CTGTAATTGGAGGAATGTGGATCG) and cpsI-9-R (5′-AATCATCTTCATAATTTATCTCC CATT), which amplify target regions specific to CPS IX as previously described (40).

(iii) CPS type II identification. To identify CPS type II, a previously described PCR assay (41) was performed using the primers II-F (5′-TCCGTACTACAACAGACTCATCC) and II-R (5′-TTCTCTAGGAAATCAA ATAATTCTATAGGG), which amplify target region specific for CPS type II (397 bp).

Genomic DNA extraction. Genomic DNA extraction was performed as follows. Overnight broth cultures (1.5 ml) were centrifuged for 10 min at 3,000 × *g*. The pellet was resuspended in 500 μl of 1× phosphate-buffered saline (PBS; 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4, and 1,000 ml of H_2O [pH 7.2]) and washed two times with PBS. Genomic DNA was extracted by using a DNA Mericon kit (Qiagen, Germany). Extracted genomic DNA was concentrated and dissolved in 30 μl of Qiagen elution buffer or water and then stored at −20°C. RNase pretreatment was done prior to quantification of genomic DNA.

Isolation of CPS. CPS from GBS strains was isolated as previously described (43, 66–68), with modifications. Bacteria were grown overnight in 200 ml of Todd-Hewitt broth (THB) with 3% neopeptone for 24 h at 35°C, diluted to 2 liters in fresh THB, and grown to an optical density at 600 nm (OD_{600}) of 0.7. The cultures were chilled on ice, and the cells were pelleted and washed twice with ice-cold PBS. The cells were then resuspended in 200 ml of lysis buffer (25 mM sodium phosphate buffer, 10 mM $MgCl_2$, 40% [wt/vol] sucrose, 13.3 U/ml mutanolysin [Sigma-Aldrich, Canada]; pH 7.0), followed by incubation for 19 h at 37°C with end-over-end mixing. Protoplasts were removed by centrifugation, and the mutanolysin extract was treated with DNase buffer (3.5 ml, 400 mM Tris, 60 mM $MgCl_2$, 20 mM $CaCl_2$, pH 7.5), along with sodium azide to a 0.05% final concentration. DNase (300 U) and RNase (200 μg) were added, and the sample was incubated for 24 h at 37°C with rocking. After centrifugation at 3,200 × *g* for 30 min at 4°C to pellet precipitated material, pronase (0.5 mg predigested for 2 h at 50°C to destroy glycosidases in the preparation) was added, along with 0.1 ml $CaCl_2$ (10 mM final concentration), and the sample was incubated for 17 h at 37°C with rocking. The remaining insoluble cell wall fragments and cell bodies were removed by centrifugation. The supernatant was collected and stored at 4°C.

Lectin enzyme immunoassay (lectin EIA). Capsule extract (200 μl), along with 0.15 M sodium carbonate, was used to coat the wells of 96-well EIA plates (MP Biomedicals, USA) at 4°C overnight. The detection method was based on biotinylated lectin extracted from *L. flavus* slug agglutinin (EY Laboratories, USA). The amount of bound biotinylated *L. flavus* was quantified by using HRP-labeled streptavidin (Vector Laboratories, USA). Streptavidin was selected as a detection method because it had no carbohydrate groups to which lectins may incidentally bind, and it provided an amplification step to detect small amounts of sialylated CPS (43, 46, 55, 56). The plates were washed with PBS for 15 min and blocked with 1% bovine serum albumin (BSA) in PBS for 4 h at 37°C, and then 50-μl portions of three dilutions (10, 1, and 0.1 μg/ml) of biotinylated *L. flavus* in PBS with 1% BSA and 0.05% Tween 20 (PBSAT) were added to the wells. After incubation for 1 h at room temperature, the plates were washed with PBS for 15 min, and 50 μl of HRP conjugated to streptavidin (1 μg/ml) in PBSAT was added to each well. After incubation for 1 h at room temperature, the plates were washed with PBS for 15 min. Then, 50 μl of HRP substrate (1-Step Ultra TMB-ELISA; Thermo Scientific, USA) was added to each well, followed by incubation at 37°C for 15 min. The absorbance was read on a microplate reader (Dynex Technologies, USA) at 450 nm. To identify isolates that were sialic acid positive, the average absorbance (95% CI) value was calculated for the negative-control spn14 (no sialic acid in its capsule), and this value was subtracted from the absorbance values of the assayed GBS isolates.

Lectin dot blot assay. Late exponentially growing bacteria were washed with PBS and resuspended in PBS to give an OD_{600} of approximately 2. The bacterial suspension was spotted onto nitrocellulose membrane (20 μl/spot) using a Biodot apparatus (Bio-Rad, USA) and dried for 30 min at room temperature. The membranes were washed for 15 min with TBS (6.05 g of Tris and 8.76 g of NaCl in 1,000 ml of H_2O [pH 7.5]) and then incubated with blocking buffer (5% skim milk and 0.1% Tween 20 in TBS) for 1 h at 37°C. Membranes were subsequently washed with TBS for 15 min, incubated with biotinylated *L. flavus* lectin (10 μg/ml in blocking buffer) for 1 h, washed three times (15 min each time) with TBS, and then incubated with HRP-conjugated streptavidin (1 μg/ml in blocking buffer). After incubation for 1 h at room temperature, the membranes were washed with TBST three times (15 min each time) and then twice with TBS (15 min each time). Detection was performed using 4-chloro-1-naphthol solution (Sigma-Aldrich, Canada). A positive spot was identified as a clearly defined spot at the site where the bacteria were applied, and a negative result was identified as a trace reaction or the absence of any reaction.

Primer design for real-time PCR GBS typing assay. Representative isolates of GBS CPS types with complete *cps* operon sequences available in GenBank were included in this study (Ia, CP000114; Ib, AB050723; II, AY375362.1; III, HG939456.1; IV, AF355776.1; V, AE009948.1; VI, AF337958.1; VII, AY376403; and VIII, AY375363.1). The deduced nucleotide sequences of GBS capsular genes *cpsG*, *-H*, *-I*, *-J*, *-K*, *-M*, *-N*, and *-O* available for serotypes Ia, Ib, and II to VIII in the data bank were aligned by the Muscles program (http://www.ebi.ac.uk/Tools/msa/muscle/help/) (69), and then distinctive genes or regions were chosen to infer the primer and probe sequences listed in Table 2. The partial CPS IX sequence was also included in the multiple-sequence alignment (GenBank accession no. GQ499301). CPS types Ia, Ib, and II to IX were identified based on the ability of the primer pairs to amplify an amplicon and the probe to bind the *cps* genes *cpsH*, *cpsJ*, *cpsK*, *cpsG*, *cpsN*, *cpsO*-V, *cpsI*, *cpsM*, *cpsR*, and *cpsO*-IX. Probes used in the singleplex and duplex real-time PCR assays are labeled with a fluorescent reporter dye and a quencher dye. The sequences of the 10 primer pairs and 10 TaqMan probes used are given in Table 2.

Singleplex real-time PCR assay. A real-time PCR mixture was prepared in a final volume of 20 μl. TaqMan Fast Universal PCR master mix (2×) (Applied Biosystems, USA) was used. Reaction mixtures included 0.18 μM reverse primer, 0.18 μM forward primer, and 0.5 mM probe. Two microliters of extracted DNA was used for each reaction. An RT-PCR assay was performed in a TaqMan RT-PCR Applied Biosystems 7500. The cycling conditions were denaturation at 95°C for 1 min, followed by 40 cycles of amplification at 95°C for 3 s and 60°C for 30 s. The rate of temperature increase was 1°C/s (or 0.5°C/s), and the fluorescence was acquired once.

Duplex RT-PCR assay. Two fluorogenic probes were utilized in each duplex reaction as in Table 2. The first probe was covalently labeled at the 5′-terminal nucleotide with the FAM (6-carboxyfluorescein) reporter dye and at the 3′-terminal nucleotide with the BHQ1 (Black Hole Quencher 1) quencher. The second probe was labeled with Cal Fluor 540 or 560 (Integrated DNA Technologies [IDT], USA) reporter dye at the 5′-terminal nucleotide and again with the BHQ1 quencher dye at the 3′-terminal nucleotide. The duplex RT-PCR mixture was prepared in a final volume of 20 μl. TaqMan Fast Universal PCR master mix (2×; Applied Biosystems) was used, and a PCR was performed in the TaqMan RT-PCR Applied Biosystems 7500 as in the singleplex reaction. However, the duplex mixtures include two reverse primers (0.18 μM), two forward primers (0.18 μM), and two probes (0.5 mM) that target two *cps*-specific regions. Four microliters of extracted DNA was used for each reaction. The cycling conditions were denaturation at 95°C for 1 min, followed by 40 cycles of amplification at 95°C for 3 s and 60°C for 30 s. The rate of temperature increase was 1°C/s (or 0.5°C/s), and the fluorescence was acquired once.

Standard curve and efficiency measurements. A standard curve was established for each primer pair and probe targeting *cps* genes (*cpsH*, *cpsJ*, *cpsK*, *cpsG*, *cpsN*, *cpsO*-V, *cpsI*, *cpsM*, *cpsR*, and *cpsO*-IX) in the PCR assay. Tenfold dilutions of the template were generated and a plot of the threshold cycle (C_T) versus the DNA concentration was constructed. From this standard curve, information about the smallest amount of DNA detected and the C_T slope were determined. The efficiency values were measured for each primer pair using the C_T slope method. The amplification efficiency was calculated according to the following equation: $Ex = 10(-1/\text{slope}) - 1$, where Ex is the efficiency.

ACKNOWLEDGMENTS

We gratefully acknowledge the diagnostic laboratories in Alberta that identified and submitted for CPS typing group B streptococcal isolates from cases of invasive disease.

This study was supported by Alberta Health.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts relevant to the content of the manuscript have been disclosed.

REFERENCES

1. Schuchat A. 1999. Group B streptococcus. Lancet 353:51–56. https://doi.org/10.1016/S0140-6736(98)07128-1.
2. Baker CJ, Barrett FF. 1973. Transmission of group B streptococci among parturient women and their neonates. J Pediatr 83:919–925. https://doi.org/10.1016/S0022-3476(73)80524-4.
3. Baker CJ, Barrett FF. 1974. Group B streptococcal infections in infants: the importance of the various serotypes. JAMA 230:1158–1160. https://doi.org/10.1001/jama.1974.03240080040025.
4. Berardi A, Rossi C, Lugli L, Creti R, Bacchi Reggiani ML, Lanari M, Memo L, Pedna MF, Venturelli C, Perrone E, Ciccia M, Tridapalli E, Piepoli M, Contiero R, Ferrari F, GBS Prevention Working Group. 2013. Group B streptococcus late-onset disease: 2003-2010. Pediatrics 131:e361–e368. https://doi.org/10.1542/peds.2012-1231.
5. Campbell JR, Hillier SL, Krohn MA, Ferrieri P, Zaleznik DF, Baker CJ. 2000. Group B streptococcal colonization and serotype-specific immunity in pregnant women at delivery. Obstet Gynecol 96:498–503. https://doi.org/10.1016/S0029-7844(00)00977-7.
6. Edmond KM, Kortsalioudaki C, Scott S, Schrag SJ, Zaidi AK, Cousens S, Heath PT. 2012. Group B streptococcal disease in infants aged younger than 3 months: systematic review and meta-analysis. Lancet 379:547–556. https://doi.org/10.1016/S0140-6736(11)61651-6.
7. Franciosi RA, Knostman JD, Zimmerman RA. 1973. Group B streptococcal neonatal and infant infections. J Pediatr 82:707–718. https://doi.org/10.1016/S0022-3476(73)80604-3.
8. Doran KS, Nizet V. 2004. Molecular pathogenesis of neonatal group B streptococcal infection: no longer in its infancy. Mol Microbiol 54:23–31. https://doi.org/10.1111/j.1365-2958.2004.04266.x.
9. Herbert MA, Beveridge CJ, Saunders NJ. 2004. Bacterial virulence factors in neonatal sepsis: group B streptococcus. Curr Opin Infect Dis 17:225–229. https://doi.org/10.1097/00001432-200406000-00009.
10. Rubens CE, Wessels MR, Kuypers JM, Kasper DL, Weiser JN. 1990. Molecular analysis of two group B streptococcal virulence factors. Semin Perinatol 14:22–29.
11. Slotved HC, Kong F, Lambertsen L, Sauer S, Gilbert GL. 2007. Serotype IX, a proposed new *Streptococcus agalactiae* serotype. J Clin Microbiol 45:2929–2936. https://doi.org/10.1128/JCM.00117-07.
12. Davies HD, Raj S, Adair C, Robinson J, McGeer A, Alberta GBS Study Group. 2001. Population-based active surveillance for neonatal group B streptococcal infections in Alberta, Canada: implications for vaccine formulation. Pediatr Infect Dis J 20:879–884. https://doi.org/10.1097/00006454-200109000-00011.
13. Phares CR, Lynfield R, Farley MM, Mohle-Boetani J, Harrison LH, Petit S, Craig AS, Schaffner W, Zansky SM, Gershman K, Stefonek KR, Albanese BA, Zell ER, Schuchat A, Schrag SJ. 2008. Epidemiology of invasive group B streptococcal disease in the United States, 1999-2005. JAMA 299:2056–2065. https://doi.org/10.1001/jama.299.17.2056.
14. Teatero S, McGeer A, Low DE, Li A, Demczuk W, Martin I, Fittipaldi N. 2014. Characterization of invasive group B streptococcus from the greater Toronto area, Canada. J Clin Microbiol 52:1441–1447. https://doi.org/10.1128/JCM.03554-13.
15. Alhhazmi A, Hurteau D, Tyrrell GJ. 2016. Epidemiology of invasive group B streptococcal disease in Alberta, Canada 2003-2013. J Clin Microbiol 54:1774–1781. https://doi.org/10.1128/JCM.00355-16.
16. Barcaite E, Bartusevicius A, Tameliene R, Kliucinskas M, Maleckiene L, Nadisauskiene R. 2008. Prevalence of maternal group B streptococcal colonisation in European countries. Acta Obstet Gynecol Scand 87:260–271. https://doi.org/10.1080/00016340801908759.
17. Blumberg HM, Stephens DS, Modansky M, Erwin M, Elliot J, Facklam RR, Schuchat A, Baughman W, Farley MM. 1996. Invasive group B streptococcal disease: the emergence of serotype V. J Infect Dis 173:365–373. https://doi.org/10.1093/infdis/173.2.365.
18. Figueira-Coelho J, Ramirez M, Salgado MJ, Melo-Cristino J. 2004. *Streptococcus agalactiae* in a large Portuguese teaching hospital: antimicrobial susceptibility, serotype distribution, and clonal analysis of macrolide-resistant isolates. Microb Drug Resist 10:31–36. https://doi.org/10.1089/107662904323047772.
19. Florindo C, Damiao V, Silvestre I, Farinha C, Rodrigues F, Nogueira F, Martins-Pereira F, Castro R, Borrego MJ, Santos-Sanches I, Group for the Prevention of Neonatal GBS Infection. 2014. Epidemiological surveillance of colonising group B Streptococcus epidemiology in the Lisbon and Tagus Valley regions, Portugal (2005 to 2012): emergence of a new epidemic type IV/clonal complex 17 clone. Euro Surveill 19:pii20825.
20. Gherardi G, Imperi M, Baldassarri L, Pataracchia M, Alfarone G, Recchia S, Orefici G, Dicuonzo G, Creti R. 2007. Molecular epidemiology and distribution of serotypes, surface proteins, and antibiotic resistance among group B streptococci in Italy. J Clin Microbiol 45:2909–2916. https://doi.org/10.1128/JCM.00999-07.
21. Harrison LH, Elliott JA, Dwyer DM, Libonati JP, Ferrieri P, Billmann L, Schuchat A. 1998. Serotype distribution of invasive group B streptococcal isolates in Maryland: implications for vaccine formulation. J Infect Dis 177:998–1002.
22. Lamagni TL, Keshishian C, Efstratiou A, Guy R, Henderson KL, Broughton K, Sheridan E. 2013. Emerging trends in the epidemiology of invasive group B streptococcal disease in England and Wales, 1991-2010. Clin Infect Dis 57:682–688. https://doi.org/10.1093/cid/cit337.
23. Persson E, Berg S, Trollfors B, Larsson P, Ek E, Backhaus E, Claesson BE, Jonsson L, Radberg G, Ripa T, Johansson S. 2004. Serotypes and clinical manifestations of invasive group B streptococcal infections in western Sweden 1998-2001. Clin Microbiol Infect 10:791–796. https://doi.org/10.1111/j.1469-0691.2004.00931.x.
24. Ferrieri P, Lynfield R, Creti R, Flores AE. 2013. Serotype IV and invasive group B streptococcus disease in neonates, Minnesota, USA, 2000-2010. Emerg Infect Dis 19:551–558. https://doi.org/10.3201/eid1904.121572.
25. Kiely RA, Cotter L, Mollaghan AM, Cryan B, Coffey A, Lucey B. 2011. Emergence of group B streptococcus serotype IV in women of childbearing age in Ireland. Epidemiol Infect 139:236–238. https://doi.org/10.1017/S0950268810001275.
26. Diedrick MJ, Flores AE, Hillier SL, Creti R, Ferrieri P. 2010. Clonal analysis of colonizing group B streptococcus, serotype IV, an emerging pathogen in the United States. J Clin Microbiol 48:3100–3104. https://doi.org/10.1128/JCM.00277-10.
27. Amin A, Abdulrazzaq YM, Uduman S. 2002. Group B streptococcal serotype distribution of isolates from colonized pregnant women at the time of delivery in United Arab Emirates. J Infect 45:42–46. https://doi.org/10.1053/jinf.2001.0990.
28. Teatero S, Athey TB, Van Caeseele P, Horsman G, Alexander DC, Melano RG, Li A, Flores AR, Shelburne SA, McGeer A, Demczuk W, Martin I, Fittipaldi N. 2015. Emergence of serotype IV group B streptococcus adult invasive disease in Manitoba and Saskatchewan, Canada, is driven by clonal sequence type 459 strains. J Clin Microbiol 53:2919–2926. https://doi.org/10.1128/JCM.01128-15.
29. Johri AK, Paoletti LC, Glaser P, Dua M, Sharma PK, Grandi G, Rappuoli R. 2006. Group B streptococcus: global incidence and vaccine development. Nat Rev Microbiol 4:932–942. https://doi.org/10.1038/nrmicro1552.
30. Johnson DR, Ferrieri P. 1984. Group B streptococcal Ibc protein antigen: distribution of two determinants in wild-type strains of common serotypes. J Clin Microbiol 19:506–510.
31. Lancefield RC, McCarty M, Everly WN. 1975. Multiple mouse-protective antibodies directed against group B streptococci: special reference to antibodies effective against protein antigens. J Exp Med 142:165–179. https://doi.org/10.1084/jem.142.1.165.
32. Slotved HC, Elliott J, Thompson T, Konradsen HB. 2003. Latex assay for serotyping of group B streptococcus isolates. J Clin Microbiol 41:4445–4447. https://doi.org/10.1128/JCM.41.9.4445-4447.2003.

33. Park CJ, Vandel NM, Ruprai DK, Martin EA, Gates KM, Coker D. 2001. Detection of group B streptococcal colonization in pregnant women using direct latex agglutination testing of selective broth. J Clin Microbiol 39:408–409. https://doi.org/10.1128/JCM.39.1.408-409.2001.
34. Håkansson S, Burman LG, Henrichsen J, Holm SE. 1992. Novel coagglutination method for serotyping group B streptococci. J Clin Microbiol 30:3268–3269.
35. Arakere G, Flores AE, Ferrieri P, Frasch CE. 1999. Inhibition enzyme-linked immunosorbent assay for serotyping of group B streptococcal isolates. J Clin Microbiol 37:2564–2567.
36. Kong F, Gowan S, Martin D, James G, Gilbert GL. 2002. Serotype identification of group B streptococci by PCR and sequencing. J Clin Microbiol 40:216–226. https://doi.org/10.1128/JCM.40.1.216-226.2002.
37. Kong F, Lambertsen LM, Slotved HC, Ko D, Wang H, Gilbert GL. 2008. Use of phenotypic and molecular serotype identification methods to characterize previously nonserotypeable group B streptococci. J Clin Microbiol 46:2745–2750. https://doi.org/10.1128/JCM.00189-08.
38. Kong F, Ma L, Gilbert GL. 2005. Simultaneous detection and serotype identification of *Streptococcus agalactiae* using multiplex PCR and reverse line blot hybridization. J Med Microbiol 54:1133–1138. https://doi.org/10.1099/jmm.0.46244-0.
39. Sellin M, Olofsson C, Håkansson S, Norgren M. 2000. Genotyping of the capsule gene cluster (cps) in nontypeable group B streptococci reveals two major cps allelic variants of serotypes III and VII. J Clin Microbiol 38:3420–3428.
40. Imperi M, Pataracchia M, Alfarone G, Baldassarri L, Orefici G, Creti R. 2010. A multiplex PCR assay for the direct identification of the capsular type (Ia to IX) of *Streptococcus agalactiae*. J Microbiol Methods 80:212–214. https://doi.org/10.1016/j.mimet.2009.11.010.
41. Poyart C, Tazi A, Réglier-Poupet H, Billoët A, Tavares N, Raymond J, Trieu-Cuot P. 2007. Multiplex PCR assay for rapid and accurate capsular typing of group B streptococci. J Clin Microbiol 45:1985–1988. https://doi.org/10.1128/JCM.00159-07.
42. Cieslewicz MJ, Chaffin D, Glusman G, Kasper D, Madan A, Rodrigues S, Fahey J, Wessels MR, Rubens CE. 2005. Structural and genetic diversity of group B streptococcus capsular polysaccharides. Infect Immun 73:3096–3103. https://doi.org/10.1128/IAI.73.5.3096-3103.2005.
43. Chaffin DO, Mentele LM, Rubens CE. 2005. Sialylation of group B streptococcal capsular polysaccharide is mediated by *cpsK* and is required for optimal capsule polymerization and expression. J Bacteriol 187:4615–4626. https://doi.org/10.1128/JB.187.13.4615-4626.2005.
44. Knibbs RN, Osborne SE, Glick GD, Goldstein IJ. 1993. Binding determinants of the sialic acid-specific lectin from the slug *Limax flavus*. J Biol Chem 268:18524–18531.
45. Wasylnka JA, Simmer MI, Moore MM. 2001. Differences in sialic acid density in pathogenic and nonpathogenic *Aspergillus* species. Microbiology 147:869–877. https://doi.org/10.1099/00221287-147-4-869.
46. Rogerieux F, Belaise M, Terzidis-Trabelsi H, Greffard A, Pilatte Y, Lambré CR. 1993. Determination of the sialic acid linkage specificity of sialidases using lectins in a solid-phase assay. Anal Biochem 211:200–204. https://doi.org/10.1006/abio.1993.1257.
47. Imberty A, Gautier C, Lescar J, Pérez S, Wyns L, Loris R. 2000. An unusual carbohydrate binding site revealed by the structures of two *Maackia amurensis* lectins complexed with sialic acid-containing oligosaccharides. J Biol Chem 275:17541–17548. https://doi.org/10.1074/jbc.M000560200.
48. Shibuya N, Goldstein IJ, Broekaert WF, Nsimba-Lubaki M, Peeters B, Peumans WJ. 1987. The elderberry (*Sambucus nigra* L.) bark lectin recognizes the Neu5Ac(α2-6)Gal/GalNAc sequence. J Biol Chem 262:1596–1601.
49. Rodriguez-Granger J, Alvargonzalez JC, Berardi A, Berner R, Kunze M, Hufnagel M, Melin P, Decheva A, Orefici G, Poyart C, Telford J, Efstratiou A, Killian M, Krizova P, Baldassarri L, Spellerberg B, Puertas A, Rosa-Fraile M. 2012. Prevention of group B streptococcal neonatal disease revisited. The DEVANI European Project. Eur J Clin Microbiol Infect Dis 31:2097–2104. https://doi.org/10.1007/s10096-012-1559-0.
50. Wessels MR, Haft RF, Heggen LM, Rubens CE. 1992. Identification of a genetic locus essential for capsule sialylation in type III group B streptococci. Infect Immun 60:392–400.
51. Geisler C, Jarvis DL. 2011. Effective glycoanalysis with *Maackia amurensis* lectins requires a clear understanding of their binding specificities. Glycobiology 21:988–993. https://doi.org/10.1093/glycob/cwr080.
52. Jones N, Bohnsack JF, Takahashi S, Oliver KA, Chan MS, Kunst F, Glaser P, Rusniok C, Crook DW, Harding RM, Bisharat N, Spratt BG. 2003. Multilocus sequence typing system for group B streptococcus. J Clin Microbiol 41:2530–2536. https://doi.org/10.1128/JCM.41.6.2530-2536.2003.
53. Afshar B, Broughton K, Creti R, Decheva A, Hufnagel M, Kriz P, Lambertsen L, Lovgren M, Melin P, Orefici G, Poyart C, Radtke A, Rodriguez-Granger J, Sørensen UB, Telford J, Valinsky L, Zachariadou L, Efstratiou A, The DEVANI Study Group. 2011. International external quality assurance for laboratory identification and typing of *Streptococcus agalactiae* (group B streptococci). J Clin Microbiol 49:1475–1482. https://doi.org/10.1128/JCM.02365-10.
54. Yao K, Poulsen K, Maione D, Rinaudo CD, Baldassarri L, Telford JL, Sorensen UB, Kilian M. 2013. Capsular gene typing of *Streptococcus agalactiae* compared to serotyping by latex agglutination. J Clin Microbiol 51:503–507. https://doi.org/10.1128/JCM.02417-12.
55. Duk M, Lisowska E, Wu JH, Wu AM. 1994. The biotin/avidin-mediated microtiter plate lectin assay with the use of chemically modified glycoprotein ligand. Anal Biochem 221:266–272. https://doi.org/10.1006/abio.1994.1410.
56. Diamandis EP, Christopoulos TK. 1991. The biotin-(strept)avidin system: principles and applications in biotechnology. Clin Chem 37:625–636.
57. Creti R, Imperi M, Pataracchia M, Alfarone G, Recchia S, Baldassarri L. 2012. Identification and molecular characterization of a *Streptococcus agalactiae* strain lacking the capsular locus. Eur J Clin Microbiol Infect Dis 31:233–235. https://doi.org/10.1007/s10096-011-1298-7.
58. Ramaswamy SV, Ferrieri P, Madoff LC, Flores AE, Kumar N, Tettelin H, Paoletti LC. 2006. Identification of novel *cps* locus polymorphisms in nontypeable group B streptococcus. J Med Microbiol 55:775–783. https://doi.org/10.1099/jmm.0.46253-0.
59. Wen L, Wang Q, Li Y, Kong F, Gilbert GL, Cao B, Wang L, Feng L. 2006. Use of a serotype-specific DNA microarray for identification of group B streptococcus (*Streptococcus agalactiae*). J Clin Microbiol 44:1447–1452. https://doi.org/10.1128/JCM.44.4.1447-1452.2006.
60. Brigtsen AK, Dedi L, Melby KK, Holberg-Petersen M, Radtke A, Lyng RV, Andresen LL, Jacobsen AF, Fugelseth D, Whitelaw A. 2015. Comparison of PCR and serotyping of group B streptococcus in pregnant women: the Oslo GBS Study. J Microbiol Methods 108:31–35. https://doi.org/10.1016/j.mimet.2014.11.001.
61. Breeding KM, Ragipani B, Lee KD, Malik M, Randis TM, Ratner AJ. 2016. Real-time PCR-based serotyping of *Streptococcus agalactiae*. Sci Rep 6:38523. https://doi.org/10.1038/srep38523.
62. Kolkman MA, Morrison DA, Van Der Zeijst BA, Nuijten PJ. 1996. The capsule polysaccharide synthesis locus of *Streptococcus pneumoniae* serotype 14: identification of the glycosyl transferase gene cps14E. J Bacteriol 178:3736–3741. https://doi.org/10.1128/jb.178.13.3736-3741.1996.
63. Kolkman MA, Wakarchuk W, Nuijten PJ, van der Zeijst BA. 1997. Capsular polysaccharide synthesis in *Streptococcus pneumoniae* serotype 14: molecular analysis of the complete cps locus and identification of genes encoding glycosyltransferases required for the biosynthesis of the tetrasaccharide subunit. Mol Microbiol 26:197–208. https://doi.org/10.1046/j.1365-2958.1997.5791940.x.
64. Facklam RR, Padula JF, Wortham EC, Cooksey RC, Rountree HA. 1979. Presumptive identification of group A, B, and D streptococci on agar plate media. J Clin Microbiol 9:665–672.
65. Ke D, Ménard C, Picard FJ, Boissinot M, Ouellette M, Roy PH, Bergeron MG. 2000. Development of conventional and real-time PCR assays for the rapid detection of group B streptococci. Clin Chem 46:324–331.
66. Calandra GB, Cole RM. 1980. Lysis and protoplast formation of group B streptococci by mutanolysin. Infect Immun 28:1033–1037.
67. Cieslewicz MJ, Kasper DL, Wang Y, Wessels MR. 2001. Functional analysis in type Ia group B streptococcus of a cluster of genes involved in extracellular polysaccharide production by diverse species of streptococci. J Biol Chem 276:139–146. https://doi.org/10.1074/jbc.M005702200.
68. Wessels MR, Benedí WJ, Jennings HJ, Michon F, DiFabio JL, Kasper DL. 1989. Isolation and characterization of type IV group B streptococcus capsular polysaccharide. Infect Immun 57:1089–1094.
69. Edgar RC. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 5:113. https://doi.org/10.1186/1471-2105-5-113.
70. Madoff LC, Michel JL, Kasper DL. 1991. A monoclonal antibody identifies a protective C-protein alpha-antigen epitope in group B streptococci. Infect Immun 59:204–210.
71. Tyrrell GJ, Kennedy A, Shokoples SE, Sherburne RK. 2002. Binding and

invasion of HeLa and MRC-5 cells by *Streptococcus agalactiae*. Microbiology 148:3921–3931. https://doi.org/10.1099/00221287-148-12-3921.
72. Rubens CE, Heggen LM, Haft RF, Wessels MR. 1993. Identification of *cpsD*, a gene essential for type III capsule expression in group B streptococci. Mol Microbiol 8:843–855. https://doi.org/10.1111/j.1365-2958.1993.tb01631.x.
73. Perch B, Kjems E, Henrichsen. 1979. New serotypes of group B streptococci isolated from human sources. J Clin Microbiol 10:109–110.
74. Wagner M, Murai T, Wagner B, Günther E, Jelinkova J. 1994. JM9 strains, a new type of group B streptococci from Japan. Zentralbl Bakteriol 280:488–496. https://doi.org/10.1016/S0934-8840(11)80508-6.