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Multiplex PCR Assay That Identifies the Major Lipooligosaccharide Serotype Expressed by *Moraxella catarrhalis* Clinical Isolates

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A heterologous cluster of glycosyltransferase genes was identified in the three *Moraxella catarrhalis* LOS serotype strains. Multiple PCR primers designed to this region amplified products that differentiate between the serotypes more rapidly and efficiently than previously described serological analyses. This assay will be valuable for clinical and research-based studies.

Moraxella catarrhalis, a gram-negative diplococcus, is considered a significant cause of acute otitis media in children and lower respiratory infections in adults with chronic obstructive pulmonary disease (COPD) (12, 21, 29). A number of putative virulence factors have been described for *M. catarrhalis* (8, 11, 12, 17, 20), including a surface-exposed lipooligosaccharide (LOS) (6, 18, 23, 32). Structural and serological studies with *M. catarrhalis* have described only three different LOS serotypes (termed A, B, and C), which vary in length and content of the oligosaccharide branches (3–5, 13, 15, 28). One serological study by Vaneechoutte et al. grouped clinical isolates into serotypes A (60%), B (30%), and C (5%), with 5% of the strains unidentified (28). That has been the only study to investigate the prevalence of specific *M. catarrhalis* LOS serotypes in the population. The difficulties with serological determinations of *M. catarrhalis* LOS expression are the limited quantities of antibodies, the absolute requirement for purified sample, and the potential for cross-reactivity between serotypes A and C (13, 24, 25).

Recently, a cluster of three glycosyltransferase (*lgt*) genes were identified and characterized in a strain of *M. catarrhalis* 7169 expressing serotype B LOS (6). Primers 406 and 408 (Table 1) designed to this region were subsequently used in PCRs with chromosomal DNA from *M. catarrhalis* 25238, the previously defined LOS serotype A strain, and *M. catarrhalis* RS10, the previously defined LOS serotype C strain (Table 2) (3, 4). PCR was performed in 50-μl reaction mixtures containing PCR SuperMix (Invitrogen, Carlsbad, CA), 20 pmol/μl of each primer, and 1 μl chromosomal DNA prepared as previously described (26). Amplifications were performed in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol for 25 cycles with an annealing temperature of 53.1°C and extension time of 4 min. Primers 406 and 408 (Table 1) produced an amplicon of 4.3 kb in serotype A and C strains, which was 1 kb larger than the product amplified in the serotype B strain, 7169 (data not shown).

Sequence analyses (MacVector 7.2 software; Accelrys, San Diego, CA) of the entire region amplified by primer 406 and flanking primer 704 (Table 1) identified an additional open reading frame upstream of the original *lgt* cluster described in 7169 and in the same orientation as *lgt1*, as illustrated in Fig. 1A and C. A ClustalW alignment with the translated sequence of this open reading frame (Lgt4) in both strains revealed 46% identity and 60% similarity to Lgt1, an α(1-2) glucosyltransferase in *M. catarrhalis* 7169 (6). The 5′ end of this cluster in serotype A and C strains contained DNA sequence that is homologous to the 5′ region of the *lgt* cluster in 7169, as depicted in all three clusters in Fig. 1A to C. Additional sequence analysis of the cluster in serotype A-expressing strains revealed a region of sequence divergence located at the 3′ end of $lgt2_A$, depicted in Fig. 1A. A ClustalW alignment with the translated sequence of $Lgt2_A$ revealed 61% identity and 75% similarity to $Lgt2_{B/C}$ (formally Lgt2), a β(1-4) galactosyltransferase in *M. catarrhalis* 7169 (6). A ClustalW alignment with the translated sequences of Lgt1 and Lgt3 from both serotypes A and C revealed ≥98% identity to the respective homologous Lgt enzymes identified in *M. catarrhalis* 7169 (6). These observations were confirmed by sequencing the entire *lgt* cluster in a second serotype C strain, CCUG 26391, and another serotype A strain, 27335 (Table 2) (data not shown).

Due to the homologous DNA sequence 5′ of the *lgt* cluster in all three serotype strains, we implemented a strategy of multiplex PCR in which primers 406 and 649 were the forward primers for serotype B/C strains and serotype A strains, respectively, and primer 408 was the reverse primer for all three serotypes, as depicted in Fig. 1. This single PCR produces amplicons of 1.9 kb, 3.3 kb, and 4.3 kb for serotypes A, B, and C, respectively. Figure 2 (lanes 1 to 3) is a representative agarose gel depicting the amplicons resulting from this multiplex PCR using chromosomal DNA from *M. catarrhalis* LOS serotypes A, B, and C, the control strains ATCC 25238, 7169, and RS10, respectively (Table 2) (3, 4, 6). Other *M. catarrhalis* clinical isolates (Table 2) were also tested, some representing a geographically diverse panel (Fig. 2, lanes 14 to 18), as well as isolates from a local population of adults with COPD (Fig. 2, lanes 4 to 8) and children with otitis media with effusion (OME) (Fig. 2, lanes 9 to 13). Overall, 152 *M. catarrhalis*

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TABLE 1. Nucleotide sequence of oligonucleotide primers used for PCR-based LOS typing and sequencing in this study

Primer	Sequence[a]	Brief description
Pr 406	CAAAAGAAGACAAACAAGCAGC	Primer (sense) designed for sequencing the entire *lgt* cluster and flanking DNA in all LOS serotype strains; multiplex PCR primer used to distinguish between different LOS serotypes (6)
Pr 408	CATCAAAAACCCCCCTACC	Multiplex PCR primer (antisense) used to distinguish between different LOS serotypes
Pr 649	ATCCTGCTCCAACTGACTTTC	Multiplex PCR primer (sense) used to distinguish between different LOS serotypes (primer only binds in LOS serotype A strains)
Pr 704	GCCACCAAACTATTCACGC	Primer (antisense) designed for sequencing the entire *lgt* cluster and flanking DNA (downstream of *lgt3*) in all LOS serotype strains

[a] All primers are listed in the 5′ to 3′ direction.

TABLE 2. *M. catarrhalis* strains used in this study

Strain	Description	Source and/or reference
M. catarrhalis strains		
3P3B1	Adult COPD sputum isolate	Timothy Murphy (VA Medical Center, Buffalo, N.Y.) (1)
5P26B1	Adult COPD sputum isolate	Timothy Murphy (VA Medical Center, Buffalo, N.Y.)
7P94B1	Adult COPD sputum isolate	Timothy Murphy (VA Medical Center, Buffalo, N.Y.)
18P6B1	Adult COPD sputum isolate	Timothy Murphy (VA Medical Center, Buffalo, N.Y.)
12P80B1	Adult COPD sputum isolate	Timothy Murphy (VA Medical Center, Buffalo, N.Y.)
51P9B1	Adult COPD sputum isolate	Timothy Murphy (VA Medical Center, Buffalo, N.Y.) (1, 22)
HF-006	Pediatric middle-ear isolate	Howard Faden (Children's Hospital, Buffalo, N.Y.) (7)
HF-039	Pediatric middle-ear isolate	Howard Faden (Children's Hospital, Buffalo, N.Y.)
HF-165	Pediatric middle-ear isolate	Howard Faden (Children's Hospital, Buffalo, N.Y.)
HF-218	Pediatric middle-ear isolate	Howard Faden (Children's Hospital, Buffalo, N.Y.)
HF-2246	Pediatric middle-ear isolate	Howard Faden (Children's Hospital, Buffalo, N.Y.)
7169	Pediatric middle-ear isolate; LOS type B strain	Howard Faden (Children's Hospital, Buffalo, N.Y.) (6, 9, 18, 19)
2951	Nasopharyngeal isolate; LOS type A strain	Mike Apicella (Iowa City, Iowa) (32)
27335	Unknown origin isolate from Paris, France	Mark Achtman (Max-Planck Institute, Germany)
035E	Middle-ear fluid isolate	Eric Hansen (Dallas, Tex.) (10)
ATCC 25238	LOS serotype A control strain	American Type Culture Collection (ATCC) (4)
ATCC 43617	Transtracheal aspirate	ATCC (30)
CCUG 3292	LOS serotype B control strain	Culture Collection, University of Göteborg, Göteborg, Sweden (CCUG) (5)
CCUG 26391	LOS serotype C strain	CCUG (14)
RS10	LOS serotype C control strain	Motiur Rahman (3)
Non-*M. catarrhalis* strains		
Acinetobacter baumanni strain 19606		ATCC
Moraxella bovis strain 10900		ATCC
Moraxella caviae strain 14659		ATCC
Moraxella nonliquefaciens strain 17593		ATCC
Moraxella osloensis strain 15276		ATCC
Neisseria cinerea strain 658		Dave Dyer (Oklahoma City, Okla.)
Neisseria gonorrhoeae (two strains) strain GC6 and GC10		Dave Dyer (Oklahoma City, Okla.)
Neisseria meningitidis strain 121		Dave Dyer (Oklahoma City, Okla.)
Haemophilus ducreyi (two strains) strains 35000 and CIP542		2
Haemophilus influenzae (two strains) strains 7502 and 2019		Timothy Murphy (VA Medical Center, Buffalo, N.Y.)
Haemophilus parainfluenzae strain 9P20		Timothy Murphy (VA Medical Center, Buffalo, N.Y.)
Klebsiella pneumoniae strain 94-339-0352		Thomas Russo (Buffalo, N.Y.)
Enterobacter aerogenes strain 94-347-0274		Thomas Russo (Buffalo, N.Y.)
Escherichia coli (two strains) strain CP9 and XL-1 Blue		Thomas Russo (Buffalo, N.Y.) (27) and Stratagene (La Jolla, Calif.)
Pseudomonas aeruginosa strain 94-343-0448		Thomas Russo (Buffalo, N.Y.)
Proteus mirabilis strain 94-341-0610		Thomas Russo (Buffalo, N.Y.)

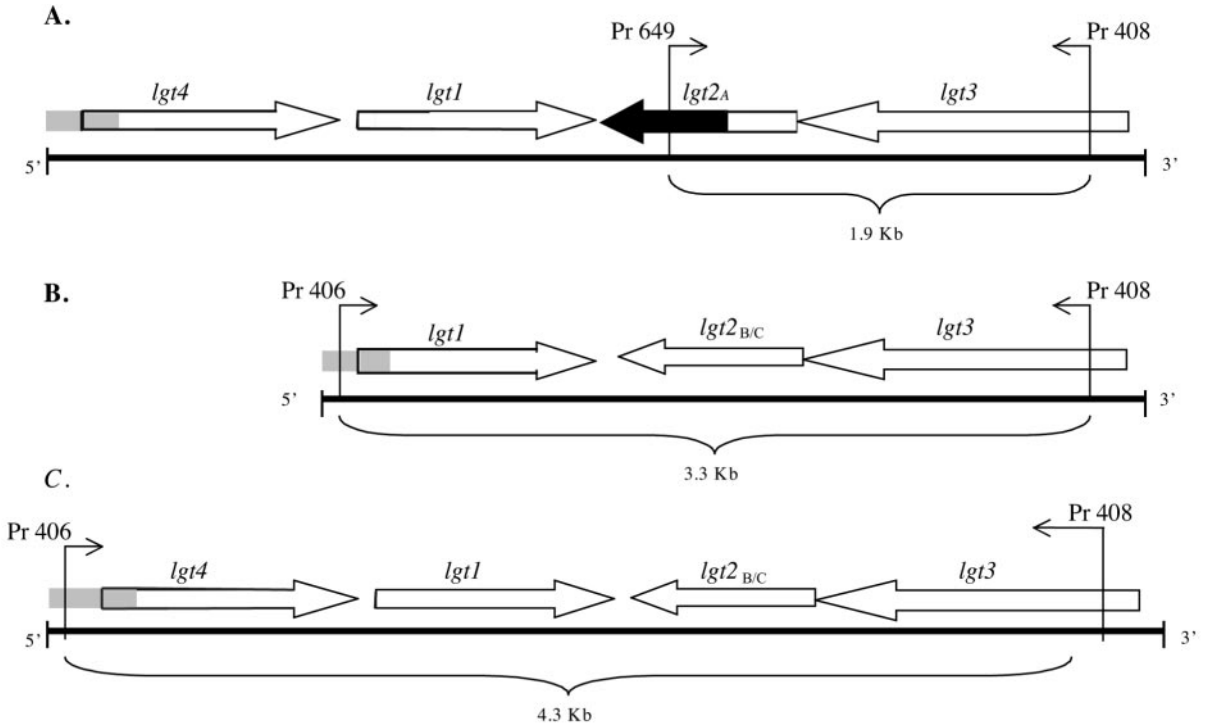

FIG. 1. Organization of *lgt* clusters in strains of *M. catarrhalis* expressing different LOS serotypes and binding sites of the primers used in the multiplex PCRs. (A) Representative (putative) *lgt* cluster in a LOS serotype A genome from *M. catarrhalis* strain ATCC 25238 (NCBI accession number DQ137417). (B) Representative *lgt* cluster in a LOS serotype B genome from *M. catarrhalis* strain 7169 (Edwards, 2005). (C) Representative (putative) *lgt* cluster in a LOS serotype C genome from *M. catarrhalis* strain RS10 (NCBI accession number DQ137418). The 5′ end of the cluster in serotype A and C strains contained DNA sequence that is homologous to the 5′ region of the *lgt* cluster in 7169, as depicted by gray shading in all three clusters (A to C). Black sequence portion in panel A represents a region of sequence divergence at the 3′ end of $lgt2_A$.

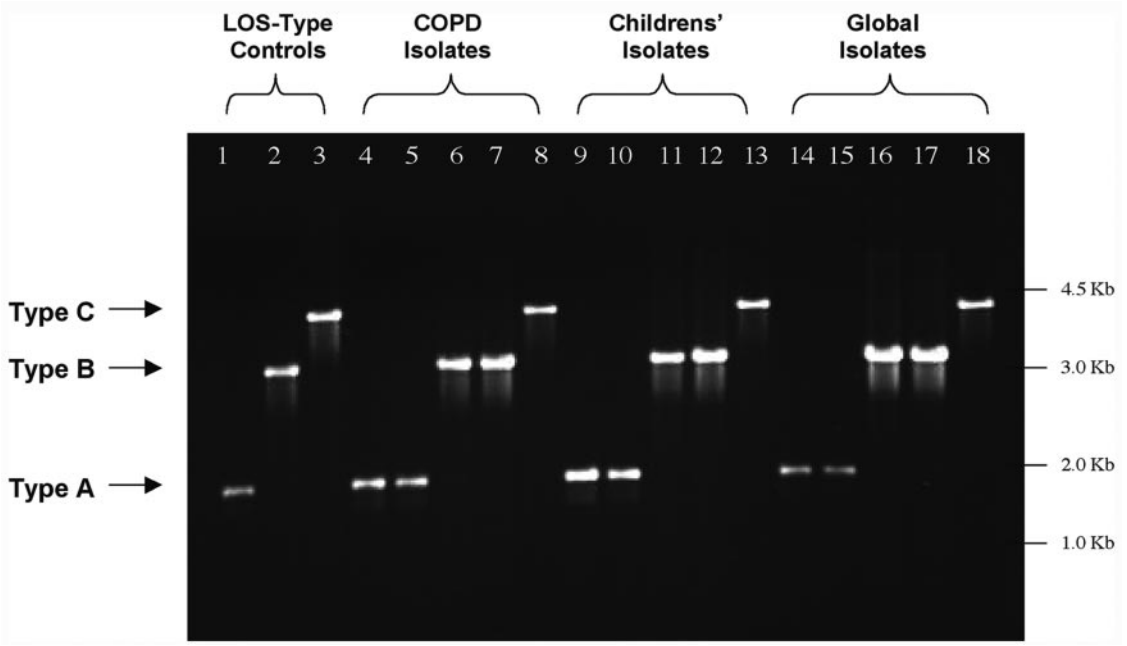

FIG. 2. Representative multiplex PCR test using primers 406, 408, and 649 (Table 1) with chromosomal DNA isolated from various *M. catarrhalis* isolates. Lanes 1 to 3 show amplicons from the three LOS serotype control strains: LOS serotype A, ATCC 25238 (lane 1); LOS serotype B, 7169 (lane 2); and LOS serotype C, RS10 (lane 3). Lanes 4 to 8 contain amplicons from five COPD patients' isolates: 3P3B1 (lane 4), 51P9B1 (lane 5), 5P26B1 (lane 6), 18P6B1 (lane 7), and 12P80B1 (lane 8). Lanes 9 to 13 contain amplicons from five children's OME isolates: HF-039 (lane 9), HF-2246 (lane 10), HF-165 (lane 11), HF-218 (lane 12), and HF-006 (lane 13). Lanes 14 to 18 contain amplicons from five geographically diverse (global) isolates: 2951 (lane 14), 035E (lane 15), CCUG 3292 (lane 16), ATCC 43617 (lane 17), and CCUG 26391 (lane 18).

TABLE 3. LOS serotypes expressed by *M. catarrhalis* strains isolated from different populations of patients as determined by multiplex PCR

Origin	No. isolates expressing serotype/ total isolates (%)		
	A	B	C
COPD patient isolates	42/52 (81)	8/52 (15)	2/52 (4)
Children (OME isolates)	32/50 (64)	17/50 (34)	1/50 (2)
Global (geographically diverse) strains	23/50 (46)	20/50 (40)	7/50 (14)
Total	97/152 (64)	45/152 (30)	10/152 (6)

strains were typed using this system, and the results of these reactions are summarized in Table 3. The percentage of strains expressing serotype A, B, or C LOS was 64%, 30%, and 6%, respectively, which is consistent with the previous data (13, 28). However, the percentage of *M. catarrhalis* isolates expressing serotype A LOS was relatively high for the COPD isolates (81%) and relatively low for the diverse global isolates (46%). At this time it is difficult to determine the significance of this observation, and further analyses are needed to determine whether LOS serotype is a factor for tissue tropism (i.e., inner ear or lung), colonization, or subsequent infection.

In order to confirm that the multiplex PCR method specifically correlated with the LOS serotype expressed by clinical isolates, compositional analysis and mass spectroscopy were performed as described previously (16). LOS was purified from a selected group of *M. catarrhalis* clinical isolates representing the three major serotypes, and oligosaccharides were isolated as described previously (6, 31). A portion (100 μg) of each OS was methanolyzed, trimethylsilylated, and analyzed by gas chromatography-mass spectrometry (MS). The glycosyl composition analyses for the OSs from *M. catarrhalis* strains 3P3B1 and 12P80B1 detected galactose, glucose, 3-deoxy-D-*manno*-octulosonic acid (KDO), and 2-acetamido-2-deoxy glucose as the major monosaccharide constituents, whereas the *M. catarrhalis* strain 43617 OS contained galactose, glucose, and KDO as the major monosaccharide constituents, with the absence of 2-acetamido-2-deoxy glucose residues (Table 4).

Methylation analysis of the strain 3P3B1 OS revealed the presence of terminal galactopyranose, terminal glucopyranose, 2-linked glucopyranose, 4-linked glucopyranose, 4-linked galactopyranose, 2,3,6-linked glucopyranose, and terminal 2-acetamido-2-deoxy glucopyranoside, indicating that 3P3B1 expresses serotype A LOS. Analysis of the strain 12P80B1 OS revealed the same profile as 3P3B1, except this sample contained 4-linked 2-acetamido-2-deoxy glucopyranoside rather than terminal 2-acetamido-2-deoxy glucopyranoside, indicating that 12P80B1 expresses serotype C LOS. Analysis of the strain 43617 OS revealed the presence of terminal galactopyranose, terminal glucopyranose, 2-linked glucopyranose, 4-linked glucopyranose, 4-linked galactopyranose, and 2,3,6-linked glucopyranose, with the absence of the 2-acetamido-2-deoxy glucopyranoside residues, indicating that 43617 expresses serotype B LOS. Analyses of the molecular masses of the OSs by negative-ion matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) MS, using dihydroxybenzoic acid as the matrix, as previously described (16), were heterogeneous and consistent with the composition and linkage analyses (data not shown). The results of the mass spectrometric analysis of the OSs are summarized in Table 4. These structural analyses were also performed with *M. catarrhalis* clinical isolates 035E (serotype A), 5P26B1 (serotype B), and CCUG 26391 (serotype C) (strains referenced in Table 1) and were consistent with the above analyses (data not shown).

TABLE 4. Ions observed from MALDI-TOF MS analyses of OSs from LOS isolated from different strains of *M. catarrhalis* and their proposed composition

Oligosaccharide	Ion $[M-H]^-$	Proposed composition
3P3B1 (serotype A)	1736	$Gal_3Glc_5GlcNAc_1KDO$
	1574	$Gal_2Glc_5GlcNAc_1KDO$
	1412	$Gal_1Glc_5GlcNAc_1KDO$
43617 (serotype B)	1858	Gal_4Glc_6KDO
	1695	Gal_3Glc_6KDO
	1533	Gal_2Glc_6KDO
	1371	Gal_1Glc_6KDO
12P80B1 (serotype C)	1898	$Gal_4Glc_5GlcNAc_1KDO$
	1736	$Gal_3Glc_5GlcNAc_1KDO$
	1574	$Gal_2Glc_5GlcNAc_1KDO$

The species specificity of the multiplex PCR assay was also evaluated with chromosomal DNA isolated from 20 strains representing 16 different species, within the genus *Moraxella*, as well as other mucosal pathogens and commensal organisms (Table 2). The LOS serotype-specific primers did not amplify products from any of the 20 heterologous strains tested in this study (data not shown).

In conclusion, the results in this study indicate that this multiplex PCR test is a specific and highly effective assay for the identification of LOS serotypes expressed by *M. catarrhalis* strains. In addition, this system may have the added advantage of identifying a clinical isolate as an *M. catarrhalis* strain, although more data are needed. A single-step PCR method may prove to be a useful tool for basic research studies designed to determine the role of LOS in relation to colonization and pathogenesis, as well as provide a more specific means for clinical investigation of the type of *M. catarrhalis* strains infecting specific patients.

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