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Rapid and Accurate Determination of Lipopolysaccharide O-Antigen Types in *Klebsiella pneumoniae* with a Novel PCR-Based O-Genotyping Method

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***Klebsiella pneumoniae*, a Gram-negative bacillus that causes life-threatening infections in both hospitalized patients and ambulatory persons, can be classified into nine lipopolysaccharide (LPS) O-antigen serotypes. The O-antigen type has important clinical and epidemiological significance. However, *K. pneumoniae* O serotyping is cumbersome, and the reagents are not commercially available. To overcome the limitations of conventional serotyping methods, we aimed to create a rapid and accurate PCR method for *K. pneumoniae* O genotyping. We sequenced the genetic determinants of LPS O antigen from serotypes O1, O2a, O2ac, O3, O4, O5, O8, O9, and O12. We established a two-step genotyping scheme, based on the two genomic regions associated with O-antigen biosynthesis. The first set of PCR primers, which detects alleles at the *wzm-wzt* loci of the *wb* gene cluster, distinguishes between O1/O2, O3, O4, O5, O8, O9, and O12. The second set of PCR primers, which detects alleles at the *wbbY* region, further differentiates between O1, O2a, and O2ac. We verified the specificity of O genotyping against the O-serotype reference strains. We then tested the sensitivity and specificity of O genotyping in *K. pneumoniae*, using the 56 K-serotype reference strains with known O serotypes determined by an inhibition enzyme-linked immunosorbent assay (iELISA). There is a very good correlation between the O genotypes and classical O serotypes. Three discrepancies were observed and resolved by nucleotide sequencing—all in favor of O genotyping. The PCR-based O genotyping, which can be easily performed in clinical and research microbiology laboratories, is a rapid and accurate method for determining the LPS O-antigen types of *K. pneumoniae* isolates.**

Klebsiella pneumoniae is an encapsulated Gram-negative bacillus that frequently causes outbreaks of nosocomial infections in hospitalized patients (1–3). It can also affect ambulatory persons and cause community-acquired invasive diseases, including pyogenic liver abscess, endophthalmitis, meningitis, empyema, lung abscess, and necrotizing fasciitis (4–9). The worldwide emergence of multidrug-resistant strains and hypervirulent strains of *K. pneumoniae* has become an increasing clinical challenge and public health concern (10, 11).

K. pneumoniae strains can be distinguished by their capsular polysaccharide (CPS) K-antigen types (77 serotypes) and lipopolysaccharide (LPS) O-antigen types (9 serotypes) (2). The K type and O type of *K. pneumoniae* both have important clinical and epidemiological significance (8, 12, 13). K1 clonal complex 23 is a newly emerged hypervirulent clade, which causes pyogenic liver abscesses with septic ocular or central nervous system complications (8, 14, 15). Likewise, O1 is associated with hypervirulent strains that cause pyogenic liver abscess (12). The O:K typing also helps establish the clonality of *K. pneumoniae* in nosocomial outbreak investigations (13). K-genotyping methods are now available for quickly and correctly determining the K types in *K. pneumoniae* (8, 16, 17). On the other hand, there is still a lack of genotyping techniques for identifying the O types of *K. pneumoniae* strains.

A rapid and accurate O-genotyping method for *K. pneumoniae* is desirable, because O-serotyping methods have several notable limitations. Traditional O serotyping such as tube agglutination is interfered with by the masking effect of heat-stable CPS (18), which necessitates the use of capsule-free mutants (19). The introduction of an inhibition enzyme-linked immunosorbent assay (iELISA) has obviated the need for mutants (18, 20, 21). Nevertheless, the procedure is still cumbersome and the reagents are not commercially available. Furthermore, serological cross-reactivity exists between biochemically distinctive O types due to similarities in their O-antigen structures (19, 21, 22). For example, serological cross-reactions occur between O1, O2(2a), and O2(2a,2c) because the O-antigen polysaccharides in O1 (D-galactan I and D-galactan II), O2(2a) (D-galactan I alone), and O2(2a,2c) (D-galactan I and 2c polysaccharide) have a common element: D-galactan I (23, 24). The technical difficulties in O serotyping have hindered the clinical and epidemiological applications of *K. pneumoniae* O typing.

To overcome the limitations of conventional O-serotyping methods, we designed a novel PCR-based O-genotyping approach. The main genetic determinant of *K. pneumoniae* O antigen is the *wb* gene cluster (25–28), which contains the *wzm* and

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wzt genes, which encode an ATP-binding cassette transporter for O-polysaccharide biosynthesis (29). The *wbbY* region, which contains the *wbbY* gene, which encodes a glycosyltransferase, is also involved in the O-antigen synthesis (30). In *K. pneumoniae* O1, D-galactan I synthesis requires the *wb* gene cluster only (25), while D-galactan II synthesis further requires the *wbbY* region (30). Therefore, we hypothesized that O1, O2(2a), and O2(2a,2c) have identical *wb* gene clusters for D-galactan I synthesis, yet they can be distinguished by the existence or absence of specific alleles at the *wbbY* region for synthesis of a second O-polysaccharide (D-galactan II or 2c polysaccharide); meanwhile, each of the other O types (O3, O4, O5, O8, O9, and O12) has a unique allele at the *wb* gene cluster for generating type-specific O-polysaccharides. In other words, one assay (*wb*) would allow us to separate the O1/O2a/O2ac strains from the other O types, and the second assay (*wbbY*) would further differentiate between O1, O2a, and O2ac.

This study aimed to sequence and analyze the genetic determinants of the nine O serotypes (O1, O2a, O2ac, O3, O4, O5, O8, O9, and O12) that have been found in clinical *K. pneumoniae* isolates (13, 18, 20, 21, 31) and to create and validate a novel genotyping scheme for determining the O-antigen types in *K. pneumoniae*.

MATERIALS AND METHODS

Bacterial strains. Nine *Klebsiella* O-serotype reference strains and 77 *Klebsiella* K-serotype reference strains (of which 56 are *K. pneumoniae*) were purchased from Statens Serum Institut (Copenhagen, Denmark) (see Tables S1 and S2 in the supplemental material). The O serotypes of the 77 K-serotype reference strains were previously determined using an iELISA by Hansen et al. (18). The O2ac reference strain 5053 and six K-serotype reference strains (K4, K27, K28, K35, K43, and K59) are the prototype strains of the serotype O2 subgroups established by Ørskov (32). The taxonomic designation of the 77 K reference strains was reevaluated and updated using *rpoB* gene sequencing by Brisse et al. (16, 33). An epidemiological sample of 87 nonduplicate *K. pneumoniae* strains, which were consecutively collected from routine cultures in patients with community-acquired *K. pneumoniae* infections at National Taiwan University Hospital (Taipei, Taiwan), were used to evaluate the performance of our O-genotyping method in clinical settings. The study procedure was reviewed and approved by National Taiwan University Hospital's Institutional Review Board (no. 201506057RINA). All strains were stored at −80°C before use.

Sequencing the *wb* gene cluster. We sequenced the *wb* gene clusters of O1, O2ac, O3, O4, O5, O8, and O12 reference strains, using PCR primers designed from published nucleotide sequences of the following strains: *K. pneumoniae* NTUH-K2044 (serotype O1; complete genome: GenBank accession number NC012731) (34), *K. variicola* 342 (O serotype uncertain; complete genome: accession no. NC011283) (33, 35), *K. pneumoniae* KPNIH31 (O serotype uncertain; complete genome: accession no. CP009876) (36), *K. pneumoniae* KT769 (serotype O5; partial *wb* gene cluster: accession no. AF189151) (27), *K. terrigena* 889 (serotype O8; partial *wb* gene cluster: accession no. L41518) (16, 37), and *K. pneumoniae* KT776 (serotype O12; partial *wb* gene cluster: accession no. AF097519) (28). The PCR products were generally 1 to 3 kb in size. When the standard PCR was not successful, we used the long and accurate PCR (LAPCR) method (38), which yielded products of up to 8 kb. Nucleotide sequencing was performed using an ABI 3730 DNA sequencer at the Sequencing Laboratory of the National Taiwan University Hospital.

Sequencing the *wbbY* region. The *wbbY* region was originally characterized and sequenced by Hsieh et al. (30) in an O1 serotype strain: *K. pneumoniae* NTUH-K2044 (GenBank accession no. KJ451390). We sequenced the *wbbY* region in an O2ac serotype strain: *K. pneumoniae* subsp. *ozaenae* D5050 (the K4 reference strain) (see Table S2 in the supplemental material), using PCR primers designed from the whole-genome shotgun sequence of *K. pneumoniae* subsp. *ozaenae* ATCC 11296 (accession no. NZ_CDJH00000000.1).

Extraction of genomic DNA. The pellet from the overnight culture preparation was lysed by cell lysis solution (1% sodium dodecyl sulfate, 200 mM Tris-HCl buffer [pH 8.5], and 100 mM EDTA [pH 8.0]). The protein components were then precipitated by 10 M ammonium acetate. The genomic DNA in the supernatant was extracted by phenol-chloroform.

Genotyping procedure. Genomic DNA (100 ng) extracted from the strains tested was used as the template. Standard *Taq* polymerase (DreamTaq; Thermo Fisher Scientific, Waltham, MA) was used for PCR, with 0.4 μM primers and 2.5 mM Mg^{2+} in a final reaction volume of 50 μl. The cycle was set as 96°C for 3 min and then 30 cycles of the following steps: 96°C for 30 s, 56°C for 15 s, and 72°C for 1 min. Afterwards, the samples were incubated at 72°C for 10 min.

Sensitivity and specificity of O genotyping. We examined the specificity of O genotyping against the nine O-serotype reference strains. We then assessed the sensitivity and specificity of O genotyping in *K. pneumoniae* strains, using the 56 *K. pneumoniae* K-serotype reference strains with known O serotypes (see Table S2 in the supplemental material) (18). The sensitivity and specificity of O genotyping (against the iELISA) are assessed by the true-positive rate (i.e., the proportion of strains with a given O serotype that did have the corresponding O genotype) and the true-negative rate (i.e., the proportion of strains without a given O serotype that did not have the corresponding O genotype), respectively (39). For strains with inconsistent results between the iELISA and O genotyping, we sequenced the PCR products to resolve the discrepancy.

Nucleotide sequence accession numbers. We deposited complete nucleotide sequence of the *wb* gene clusters from the O1, O2ac, O3, O4, O5, O8, and O12 reference strains in GenBank under accession no. AB819964, AB795943, AB795941, KU310493, AB819962, AB819963, and AB795942, respectively. We deposited the nucleotide sequences of the *wzm* and *wzt* genes from the O9 reference strain in GenBank under accession no. LC107950. We also deposited the complete nucleotide sequence of the *wbbY* region from the O2ac serotype strain D5050 in GenBank under accession no. LC074715. We performed sequence analysis and annotation using Sequencher (Gene Codes, Ann Arbor, MI) and the National Center for Biotechnology Information (NCBI) BLAST. The phylogenetic trees were constructed using Molecular Evolutionary Genetics Analysis (MEGA) (http://megasoftware.net/) (40), based on the maximum likelihood method with 2,000 bootstrap replicates.

RESULTS

Nucleotide sequence diversity in the *wb* gene clusters of O1, O3, O4, O5, O8, O9, and O12. Figure 1 shows the organization of the *wb* gene clusters in O1, O2ac, O3, O4, O5, O8, and O12 reference strains, all of which contain the *wzm* and *wzt* genes (shown in gray). The nucleotide sequences at the *wzm-wzt* loci are <70% similar between the O1, O3, O4, O5, O8, and O12 reference strains. In contrast, the sequences are nearly identical between the O1 and O2ac reference strains at *wzm* (99.6% similar) and *wzt* (97.2% similar).

To confirm the identity in the nucleotide sequences at the *wzm-wzt* loci between O1, O2a, and O2ac, we sequenced 10 K reference strains of a known O1 serotype (K1, K2, K7, K8, K10), O2 serotype (K3, K27, K28) (for which the principal antigen inhibited in the O2 iELISA system is O2a) (18), or O2ac serotype (K4, K5) (see Table S2 in the supplemental material). The *wzm* and *wzt* genes are nearly identical among the 10 O1, O2, and O2ac strains (see File S1 in the supplemental material). The phylogenetic analysis of *wzm* and *wzt* shows that all of the sequenced O1, O2, and O2ac strains ($n = 12$) form a single clade that is separate from the O3, O4, O5, O8, and O12 strains (Fig. 2 and 3).

Since the previous iELISA studies showed that O9 is closely

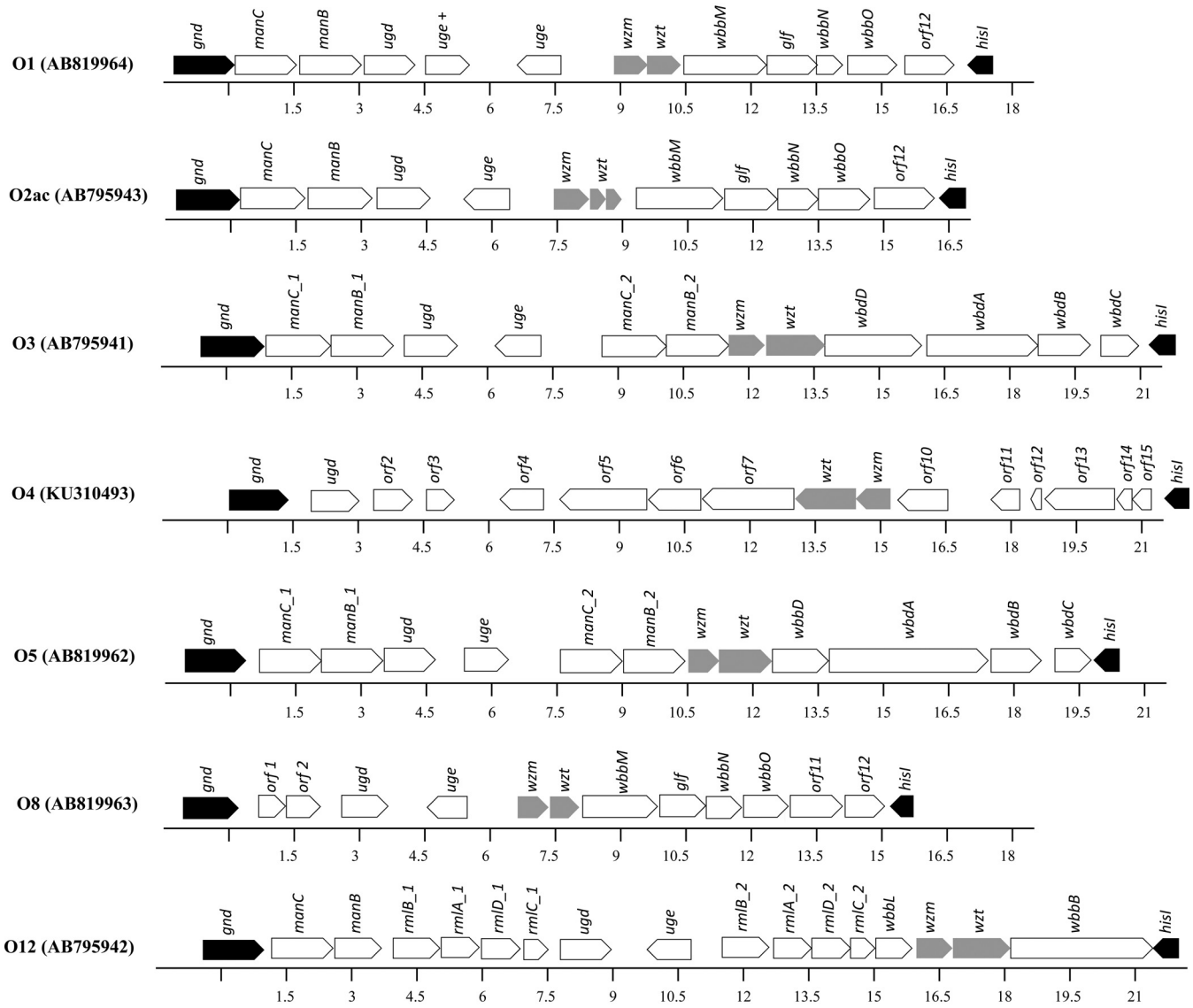

FIG 1 Organization of the *wb* gene clusters in the O1, O2ac, O3, O4, O5, O8, and O12 reference strains. The *wb* gene clusters are located between the highly conserved *gnd* and *hisI* genes (black color). The nucleotide position (in kilobases) is relative to the first nucleotide of the complete sequence submitted to GenBank. The *wzm* and *wzt* loci are shown in gray. The O1 reference strain has an additional *uge* gene, which is not present in the other O1 strains. The O2ac reference strain has a truncated *wzt* gene because of a frameshift mutation (secondary to the addition of two DNA bases, adenine and cytosine, at nucleotides 324 and 325, respectively), which is not present in the other O2ac strains.

related to O2 (18), we sequenced the *wzm-wzt* region of the O9 reference strain (see Table S3 and File S2 in the supplemental material) and compared it with that of the O2ac reference strain. We found that the nucleotide sequences are only 78 to 79% similar between O9 and O2ac.

Based on the diversity in nucleotide sequences, we designed the first set of PCR primers to detect the O1/O2, O3, O4, O5, O8, O9, and O12 alleles at the *wzm-wzt* loci of the *wb* gene clusters (Tables 1 and 2).

Nucleotide sequence diversity at *wbbY* loci of O1 and O2ac. Figure 4 shows the organization of the *wbbY* region in O1 and O2ac serotype strains, both of which contain the *wbbY* gene (shown in gray). Nucleotide sequences of the O1 allele (2,223 nucleotides) and the O2ac allele (1,926 nucleotides) are nearly identical up to nucleotide 1896, after which the sequence similarity is <60%. Based on this difference, we designed a second set of PCR primers to detect the O1 and O2ac alleles at the *wbbY* locus (Tables 1 and 2).

Two-step O-genotyping scheme. We initially tested all strains using the first set of PCR primers, which detect the O1/O2, O3, O4, O5, O8, O9, and O12 alleles at the *wzm-wzt* loci of the *wb* gene clusters. To enhance the specificity, we designed two primer pairs for each allele at the *wb* gene cluster. Only the strains that tested positive for both primer pairs are classified as having a specific genotype. Strains that did not belong to the above genotypes are considered nontypeable. The strains with the O1/O2 allele were further tested using the second set of PCR primers, which detect the O1 and O2ac alleles at the *wbbY* locus. The strains with the

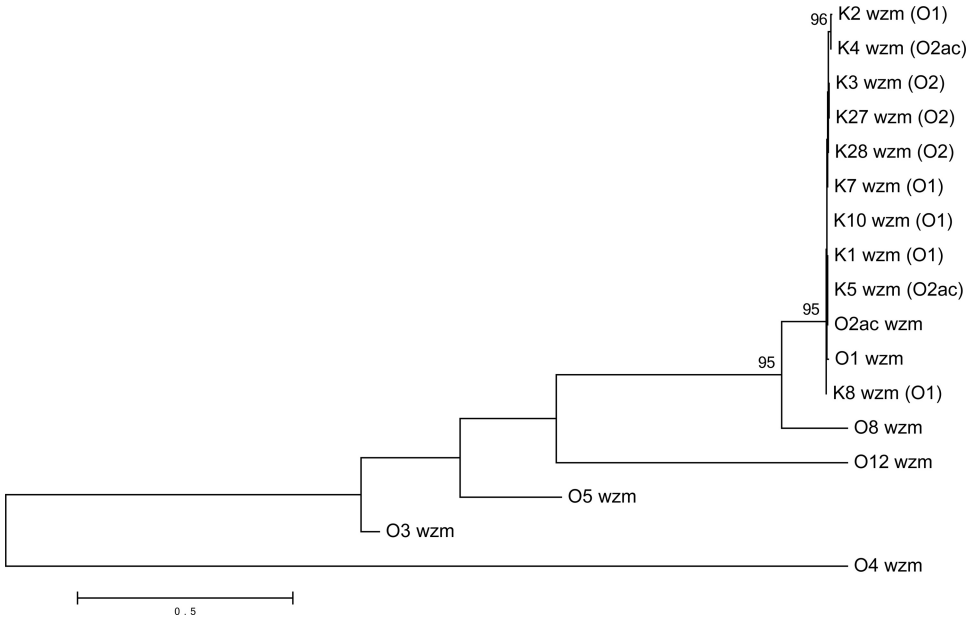

FIG 2 Phylogenetic relationships among the *wzm* genes from O1, O2ac, O3, O4, O5, O8, and O12 reference strains, as well as from the 10 K reference strains of an O1, O2, or O2ac serotype by iELISA (for which the principal antigen inhibited in the O2 iELISA system is O2a) (18). Bootstrap percentages of remarkable nodes are shown (distance scale, 0.1 represents 10% difference between sequences).

O1/O2 allele that did not react with the O1- or O2ac-specific primers are considered the O2a genotype.

Specificity against O reference strains. We verified that O genotyping is specific against the nine O-serotype reference strains. In the *wb* assay, each of the PCR primer pairs targeting the O3, O4, O5, O8, O9, and O12 alleles reacts with its corresponding reference strain, without cross-reacting with the other reference strains; those targeting the O1/O2 allele react only with the O1 and O2ac reference strains. In the *wbbY* assay of the O1 and O2ac reference strains, the PCR primers are also specific, without cross-reactions.

Sensitivity and specificity in 56 *K. pneumoniae* K reference strains. There is a very good correlation between the iELISA O

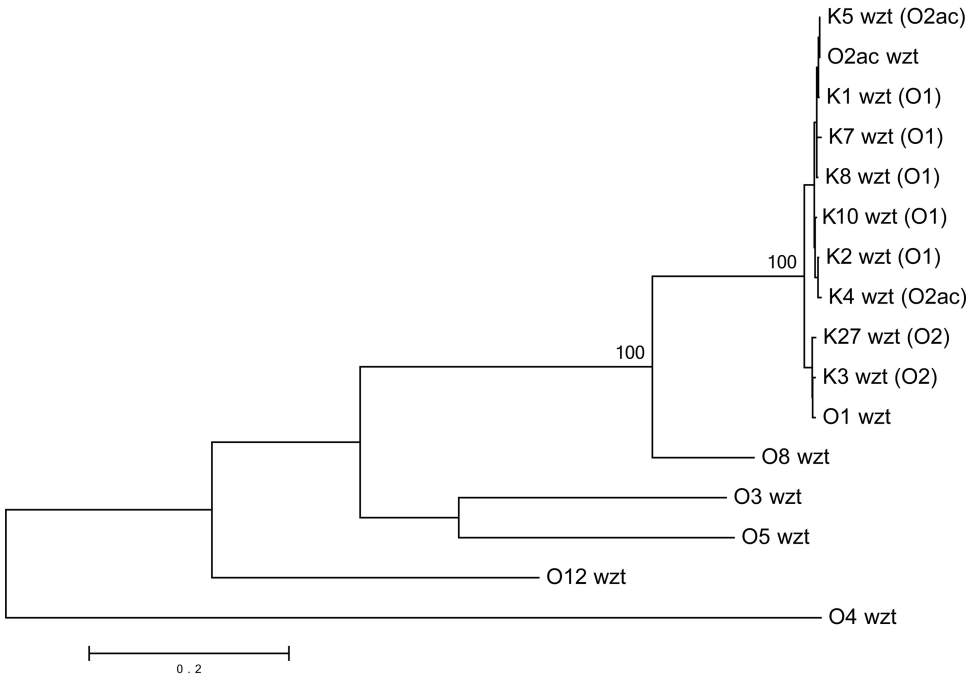

FIG 3 Phylogenetic relationships among the *wzt* genes from the O1, O2ac, O3, O4, O5, O8, and O12 reference strains and from the 10 K reference strains of an O1, O2, or O2ac serotype by iELISA (for which the principal antigen inhibited in the O2 iELISA system is O2a) (18). Bootstrap percentages of remarkable nodes are shown.

TABLE 1 *Klebsiella pneumoniae* O-genotyping scheme

Genotype			Serotype			
Designation	*wb* allele	*wbbY* allele	Designation[a]	Biochemical component(s)[b]	Antigen factor(s)[a]	Reference strain(s)
O1	O1/O2	O1	O1	D-Galactan I, D-galactan II	O2a, O1	Friedländer 204
O2						
O2a	O1/O2	None	O2(2a)	D-Galactan I	O2a	5758, 2482
			O2(2a,2f,2g)	D-Galactan I (with D-Gal*p* side group)	O2a, O2f, O2g	6613[c]
O2ac	O1/O2	O2ac	O2(2a,2c)	D-Galactan I, 2c polysaccharide	O2a, O2c	5053[d]
			O2(2a,2c,2d)		O2a, O2c, O2d	D5050[e]
O3	O3	NA[f]	O3	O3 polysaccharide	O3	636/52
O4	O4	NA	O4	O4 polysaccharide	O4	Mich.61
O5	O5	NA	O5	O5 polysaccharide	O5	5710/52
O7[g]	NS[g]	NA	O7	O7 polysaccharide	O7	264(1)[h]
O8	O8	O1	O8	O-acetylated D-galactan I, D-galactan II	O2a, O1	889
O9	O9	NA	O9	O-acetylated D-galactan I with D-Gal*p* side group	O2a, O9	1205
			O2(2a,2e)		O2a, O2e	7444[i]
O12	O12	NA	O12	O12 polysaccharide	O12	708

[a] From Ørskov and Ørskov (19), Ørskov (32), Trautmann et al. (21), and Hansen et al. (18). O2b is not a true O-antigenic factor (24). O2(2a,2e,2h) is biochemically closely related to O9 and O2(2a,2e), with minor differences in the stoichiometry of the D-Gal*p* side chain addition and O-acetylation of the D-Gal residue (43). We found that the prototype O2(2a,2e,2h) strain (*K. michiganensis* 2212/52) does not react with the O9, O1/O2, or other O-type *wb* primers. O6 is identical to O1 (19). O10 is not *Klebsiella* (21). O11 (for which the biochemical components have not yet been elucidated) is serologically closely related to O4 (21).

[b] From Ørskov and Ørskov(19), Whitfield et al. (23, 24), Kelly et al. (41, 43), and Vinogradov et al. (22).

[c] The D-galactan I in strain 6613 has a D-Gal*p* side group (43). The genetic determinant for addition of the D-Gal*p* side group is located outside the *wb* gene cluster (43). Therefore, O2(2a,2f,2g) has the same O1/O2 *wb* allele as O2(2a). The genes responsible for the addition of the D-Gal*p* side group have not yet been identified.

[d] Strain 5053 has a truncated *wzt* gene, which retains a certain degree of function that allows the expression of O antigen (24, 47). Compared with strains D5050 (K4 reference strain) and E5051 (K5 reference strain), both of which have a normal *wzt* gene, strain 5053 produces significantly more low-molecular-weight D-galactan I in its SDS-PAGE LPS profile (24).

[e] Strain D5050 has a normal *wzt* gene. Compared with strain 5053, which has a truncated *wzt* gene, strain D5050 produces predominantly high-molecular-weight 2c polysaccharide in its SDS-PAGE LPS profile (24).

[f] NA, not applicable.

[g] Currently nontypeable. NS, not sequenced yet.

[h] Taxonomic reevaluation using *rpo* sequencing in 2013 found that strain 264(1) is of the species *K.* (*Raoultella*) *terrigena* (16).

[i] The structure of O-polysaccharide in strain 7444, O2(2a,2e), is identical to that of O9 (43). Strain 7444 reacts with the O9-specific *wb* primers, but not with the O1/O2 *wb* primers.

serotypes and the PCR-based O genotypes (Table 3). The sensitivities and specificities, respectively, of O genotyping (against iELISA) are 100% (24/24) and 94% (30/32) for O1, 80% (4/5) and 96% (49/51) for O2, 100% (2/2) and 100% (54/54) for O2ac, 86% (6/7) and 100% (49/49) for O3, 100% (3/3) and 98% (52/53) for O4, 100% (3/3) and 100% (53/53) for O5, 100% (1/1) and 98% (54/55) for O8, and 100% (1/1) and 100% (55/55) for O12. The four strains (K9, K13, K17, and K38) that did not express O antigen and were thus nontypeable by iELISA (18) were found to be genotypes O2a, O1, O4, and O1, respectively. There were another three strains that expressed O antigen and had discordant results between iELISA and O genotyping, including K6 (iELISA nontypeable versus genotype O2a), K50 (serotype O3 versus O-genotyping nontypeable), and K68 (serotype O2 versus genotype O8). These discrepancies were resolved by nucleotide sequencing, all in favor of O genotyping. With nucleotide sequencing as the gold standard, O genotyping has 100% sensitivity and 100% specificity.

Nucleotide diversity between *Klebsiella* species. To explore whether the O-genotyping method primarily developed for *K. pneumoniae* may also be applied to other *Klebsiella* species, we sequenced *wzm* and *wzt* in the nine K reference strains with an O3 serotype; these included four *K. pneumoniae* strains (K31, K33, K51, K55), four *K. variicola* strains (K48, K49, K53, K54), and one *K. oxytoca* strain (K74) (see File S3 in the supplemental material). At *wzm*, the sequences are 98 to 99% similar among the different strains of a given species but are only 80 to 81% similar between *K. pneumoniae* and *K. oxytoca*. At *wzt*, substantial diversity (nucleotide sequence similarity of 77 to 81%) between the two species is also observed. The significant sequence variations across species indicate a need to design the O-genotyping PCR primers separately for different *Klebsiella* species.

O-genotype distribution in 87 clinical *K. pneumoniae* strains. The 87 strains are isolates from blood ($n = 82$), liver abscesses ($n = 4$), or urine ($n = 1$). The O1 genotype is the most common ($n = 56$; 64%), followed by the O2a ($n = 17$; 20%), O3 ($n = 5$; 6%), and O5 ($n = 4$; 5%) genotypes (Table 4). Overall, 83 (95%) of the 87 strains are typable.

DISCUSSION

We have successfully developed and validated a PCR-based O-genotyping method for the rapid and accurate determination of O-antigen types in *K. pneumoniae*. This new technique overcomes several limitations of conventional serotyping methods. First, O genotyping directly detects the genetic determinants of O antigen and thus avoids the problems of the capsular masking effect and serological cross-reaction. Second, unlike O antisera, PCR primers can be commercially synthesized with high quality at a low cost, which allows the O-genotyping procedure to be easily performed in clinical and research microbiology laboratories.

In addition to the technical convenience, our O-genotyping method accurately identifies the O-antigen types of *K. pneumoniae* isolates. There is a very good correlation between the O genotypes and classical O serotypes, with a complete match in

TABLE 2 Primers used for *Klebsiella pneumoniae* lipopolysaccharide O genotyping by allele-specific PCR

O type detected	Primer	Nucleotide sequence	Primer position[a]	PCR product size (kb)
Allele at the *wzm-wzt* loci of the *wb* gene cluster[b]				
O1/O2	wb O1/O2-A-F	5′-CGCTATAAGAGCAGCATGCTAG-3′	7470–7491	1.3
	wb O1/O2-A-R	5′-CGATATCACCTACTGCCAGA-3′	8703–8722	
	wb O1/O2-B-F	5′-TTGTTGAGCCTGACAGGATC-3′	7317–7336	1.6
	wb O1/O2-B-R	5′-GCCATTGCTTGCTTGTACAG-3′	8890–8909	
O3	wb O3-A-F	5′-CTATCGCTACCGTGGCTTTA-3′	11093–11112	0.8
	wb O3-A-R	5′-TCTCGTCCACAATATCAGCG-3′	11840–11859	
	wb O3-B-F	5′-GCCTACAGTATCTACCTCTG-3′	11271–11290	0.9
	wb O3-B-R	5′-CGGTAAAGTCAGGATGGAAG-3′	12154–12173	
O4	wb O4-A-F	5′-CAGAAGCGCGAGTTAATCTG-3′	15212–15231	0.7
	wb O4-A-R	5′-GGTCCAGTTAGGCTCAATTC-3′	14554–14573	
	wb O4-B-F	5′-GTCAGCGGGAATTATTGGAC-3′	14258–14277	1.2
	wb O4-B-R	5′-CTTGAGATCCAGAATGCCAC-3′	13077–13096	
O5	wb O5-A-F	5′-GCTACCAAACCAGTATGCTG-3′	10564–10583	1.8
	wb O5-A-R	5′-AGGTGCGTACTGGAAGTATG-3′	12365–12384	
	wb O5-B-F	5′-GGTGATGAAAGCCAGAATGC-3′	10649–10668	1.4
	wb O5-B-R	5′-CAGTGCCTGAAACAGTTTGC-3′	12052–12071	
O8	wb O8-A-F	5′-CGTGGCAATGGTTTGCTAGT-3′	7784–7803	1.2
	wb O8-A-R	5′-TCAATCCACACAACTCGGTC-3′	8994–9013	
	wb O8-B-F	5′-GCTAGTTCGGCAACTAACTCAC-3′	7798–7819	0.8
	wb O8-B-R	5′-AGTTCCAGCATCGAAGCAACTC-3′	8617–8638	
O9	wb O9-A-F	5′-CGCGCTCAGTTATTCCATTG-3′	305–324	1.0
	wb O9-A-R	5′-CTGGCTGATGACAGAGAATC-3′	1258–1277	
	wb O9-B-F	5′-GCATTCCTGTTCGTGTATGG-3′	379–398	0.9
	wb O9-B-R	5′-ATGTCACCGACAGCAAGTAC-3′	1308–1327	
O12	wb O12-A-F	5′-CTGCAGATGGCTAAACGTGA-3′	16269–16288	0.6
	wb O12-A-R	5′-CCGTTCGGGCTTGTTCAATA-3′	16877–16896	
	wb O12-B-F	5′-GAAGTCGACTTTGCTGCAGA-3′	17238–17257	1.0
	wb O12-B-R	5′-ACGTTGATCAAGCTCCTCTC-3′	18227–18246	
Allele at the *wbbY* loci of the *wbbY* region[c]				
O1	wbb O1-F	5′-GATTTCACTTTCCGGGCAAC-3′	2191–2210	1.1
	wbb O1-R	5′-GGCTTGCTGAATCACAAGAC-3′	1136–1155	
O2ac	wbb O2ac-F	5′-AAACATCGCTGACTCGAGTC-3′	1905–1924	1.0
	wbb O2ac-R	5′-CGACTATGATCGTACCAACG-3′	879–898	

[a] For the *wb* gene cluster, the primer position is relative to the first nucleotide of the corresponding complete *wb* gene cluster sequence submitted to GenBank. For the primers targeting O1/O2, O3, O4, O5, O8, and O12 alleles, the reference sequences are the following: O2ac, AB795943; O3, AB795941; O4, KU310493; O5, AB819962; O8, AB819963; and O12, AB795942, respectively (Fig. 1; the positions of the corresponding genes, *wzm* and *wzt*, are shown in gray). For O9, the primer position is relative to the first nucleotide of the *wzm-wzt* region sequence submitted to GenBank (accession no. LC107950). The primer position is relative to the first nucleotide of the *wbbY* alleles. For the primers targeting the O1 and O2ac *wbbY* allele, the reference sequences are the following: O1, KJ451390; and O2ac, LC074715, respectively (see Fig. 4, the positions of the corresponding gene, *wbbY*, are shown in gray color).

[b] To enhance specificity, we designed two PCR primer pairs for each allele at the wb gene cluster. Only the strains that tested positive for both primer pairs are classified as having a specific genotype.

[c] The pair of primers wbb O1-F and wbb O1-R, which detects the allele for D-galactan II, also reacts with O8 (which contains D-galactan II and *O*-acetylated D-galactan I).

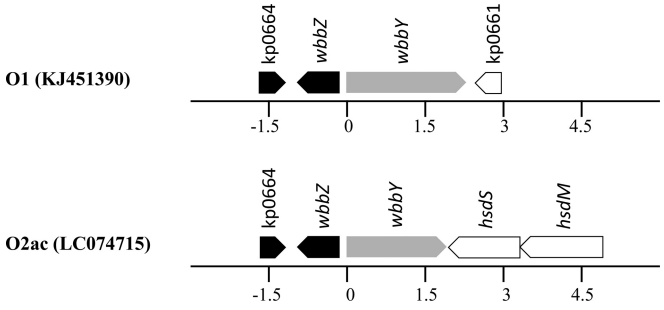

FIG 4 Organization of the *wbbY* regions in *Klebsiella pneumoniae* NTUH-K2044 (serotype O1) and *K. pneumoniae* subsp. *ozaenae* D5050 (serotype O2ac). *wbbZ* is highly conserved (black). The *wbbY* locus is shown in gray. The nucleotide position (in kilobases) is relative to the first nucleotide of the *wbbY* gene.

100% (9/9) of the O reference strains and 88% (49/56) of the K reference strains. For the three strains that express O antigen and had discordant results between iELISA and O genotyping, nucleotide sequencing evidently supports the accuracy of O genotyping in all of the cases. With nucleotide sequencing as the gold standard, the O-genotyping method has 100% sensitivity and 100% specificity in *K. pneumoniae*.

The nine O types (O1, O2, O2ac, O3, O4, O5, O8, O9, and O12) that are typable by our method together represent the great majority (83/87, 95%) of the *K. pneumoniae* clinical isolates in an epidemiologically representative sample in the current study. This validates the applicability and good performance of our O-genotyping method in the clinical setting. Similarly, based on a large

TABLE 3 Comparison of O-typing results between iELISA and genotyping, with discrepancies resolved by nucleotide sequencing, in 56 *Klebsiella pneumoniae* K-serotype reference strains

K type	Species[a]	Strain designation	iELISA[b]	Genotyping	Sequencing
1	*K. pneumoniae*	A 5054	O1	O1	
2	*K. pneumoniae*	B 5055	O1	O1	
3	*K. pneumoniae*	C 5046	O2	O2a	
4	*K. pneumoniae* subsp. *ozaenae*	D 5050	O2ac	O2ac	
5	*K. pneumoniae* subsp. *ozaenae*	E 5051	O2ac	O2ac	
6	*K. pneumoniae* subsp. *ozaenae*	F 5052	NT[c] O^+	O2a	O2a
7	*K. pneumoniae*	Aerogenes 4140	O1	O1	
8	*K. pneumoniae*	Klebs. 1015	O1	O1	
9	*K. pneumoniae*	Klebs. 56	NT O^-	O2a	O2a
10	*K. pneumoniae*	Klebs. 919	O1	O1	
11	*K. pneumoniae*	Klebs. 390	O3	O3	
12	*K. pneumoniae*	Klebs. 313	O1	O1	
13	*K. pneumoniae*	Klebs. 1470	NT O^-	O1	O1
14	*K. pneumoniae*	Klebs. 1193	O5	O5	
15	*K. pneumoniae*	Mich. 61	O4	O4	
16	*K. pneumoniae*	2069/49	O1	O1	
17	*K. pneumoniae*	2005/49	NT O^-	O4	O4
18	*K. pneumoniae*	1754/49	O1	O1	
19	*K. pneumoniae*	293/50	O1	O1	
20	*K. pneumoniae*	889/50	O1	O1	
21	*K. pneumoniae*	1702/49	O1	O1	
22	*K. pneumoniae*	1996/49	O1	O1	
23	*K. pneumoniae*	2812/50	O1	O1	
24	*K. pneumoniae*	1680/49	O1	O1	
25	*K. pneumoniae*	2002/49	O3	O3	
27	*K. pneumoniae*	6613	O2	O2a	
28	*K. pneumoniae*	5758	O2	O2a	
30	*K. pneumoniae*	7824	O1	O1	
31	*K. pneumoniae*	6258	O3	O3	
33	*K. pneumoniae*	6168	O3	O3	
34	*K. pneumoniae*	7522	O1	O1	
36	*K. pneumoniae*	8306	O4	O4	
37	*K. pneumoniae*	8238	O1	O1	
38	*K. pneumoniae*	8414	NT O^-	O1	O1
39	*K. pneumoniae*	7749	O1	O1	
40	*K. pneumoniae*	8588	NT O^-	NT	
42	*K. pneumoniae*	1702	O4	O4	
43	*K. pneumoniae*	2482	O2	O2a	
45	*K. pneumoniae*	8464	O1	O1	
46	*K. pneumoniae*	5281	O1	O1	
47	*K. pneumoniae*	9682	O1	O1	
50	*K. quasipneumoniae* subsp. *similipneumoniae*	1303/50	O3	NT	NT[d]
51	*K. pneumoniae*	4715/50	O3	O3	
52	*K. pneumoniae*	5759/50	NT O^-	NT	
55	*K. pneumoniae*	3985/51	O3	O3	
60	*K. quasipneumoniae* subsp. *similipneumoniae*	4463/52	O5	O5	
61	*K. pneumoniae*	5710/52	O5	O5	
62	*K. pneumoniae*	5711/52	O1	O1	
63	*K. pneumoniae*	5845/52	O1	O1	
64	*K. pneumoniae*	NCTC 8172	O1	O1	
67	*K. terrigena* (previously identified as *K. pneumoniae*)	264 (1)	O7	NT	
68	*K. terrigena* (previously identified as *K. pneumoniae*)	265 (1)	O2	O8	O8
69	*K. terrigena* (previously identified as *K. pneumoniae*)	889	O8	O8	
80	*K. quasipneumoniae* subsp. *similipneumoniae*	708	O12	O12	
81	*K. pneumoniae*	370	NT O^+	NT	
82	*K. pneumoniae*	3454-70	NT O^+	NT	

[a] Taxonomic designation updated in 2013 (16). *K. quasipneumoniae* subsp. *similipneumoniae*, previously *K. pneumoniae* phylogroup II-B (33).
[b] iELISA results reported by Hansen et al. (18). The principal antigen inhibited in the O2 iELISA system is O2a (18).
[c] NT, nontypeable.
[d] Nucleotide sequencing was unsuccessful (i.e., all of the PCR primer pairs designed from the *wb* sequence of the O3 reference strain yielded negative results) (see Table S4 in the supplemental material), which indicates that this strain was incorrectly assigned to the O3 serotype.

TABLE 4 O-genotype distribution of 87 clinical *Klebsiella pneumoniae* strains

O genotype	No. (%) of strains[a]
O1	56 (64)
O2a	17 (20)
O2ac	0 (0)
O3	5 (6)
O4	0 (0)
O5	4 (5)
O8	0 (0)
O9	0 (0)
O12	1 (1)
NT[b]	4 (5)
Total	87 (100)

[a] Consecutively collected from the patients with community-acquired *K. pneumoniae* infections diagnosed at the National Taiwan University Hospital from 1 July 2006 to 31 July 2007. For each patient, only one isolate was included. The 87 strains are isolates from blood ($n = 82$), liver abscesses ($n = 4$), or urine ($n = 1$).
[b] NT, nontypeable.

survey in Denmark, Spain, and the United States (18), these nine O types account for up to 99.6% (528/530) of all O-typable *K. pneumoniae* clinical isolates. In another classical seroepidemiological study in Germany, the nine O types also accounted for up to 93.8% (315/336) of all O-typable *Klebsiella* clinical isolates (21). Both studies show that O7, the type not included in our current O-genotyping scheme, is extremely rare among *K. pneumoniae* clinical isolates. In fact, a reevaluation using phylogenetic analysis of the *rpoB* gene sequence in 2013 found that *K. pneumoniae* strain 264(1), the strain in which the O7 serotype was originally described, is actually *K.* (*Raoultella*) *terrigena*, rather than *K. pneumoniae* (16).

It should be emphasized that O-genotyping complements, rather than replaces, iELISA. Since mutations within the *wb* and *wbbY* gene cluster(s) might abolish O-antigen expression on the cell surface, strains of a given O genotype may not exhibit the corresponding phenotype. This is shown by the four *K. pneumoniae* strains that have a detectable O genotype yet are nontypeable by iELISA because they did not produce O antigen. As bacterial virulence depends not only on the genotype but also on the phenotype actually expressed, iELISA remains an essential phenotyping tool for *K. pneumoniae*.

With the novel O-genotyping technology, our study provides new and important evidence for previously unresolved questions. There have been controversies about the status of O8 and O9 in the *Klebsiella* O-type classification system (18, 21). Although O8 is biochemically discernible from O1 by the O-acetylation of D-galactan I (41), serological methods are unable to separate O8 from O1 (18, 21). It has been proposed that genetic methods might be able to distinguish between these two types (18), since the *wb* gene cluster of O8 is substantially different from that of O1 (37, 41). In the present study, using allele-specific PCR primers targeting the *wzm-wzt* loci of the *wb* gene cluster, we demonstrate that O8 can be explicitly discriminated from O1. This finding confirms that O8 is a distinct O type. Likewise, O9 is biochemically discernible from O2(2a) (by the O-acetylation of D-galactan I and the α-D-Gal*p* residues attached to alternate D-galactan I-repeating units) (22, 42), yet there is serological cross-reactivity between O9 and O2(2a) (18, 21). Hansen et al. considered O9 as part of the O2 group (18, 43). Conversely, Trautmann et al. have described O9 as a distinct serotype, based on its possession of an additional, non-O2 antigenic factor (21). In the current study, we found substantial diversity in the nucleotide sequences of the *wzm-wzt* loci between the O9 and O2ac reference strains. Moreover, the successful development and validation of the O9-specific *wb* primers also support the classification of O9 as a discrete O type.

Our two-step genotyping scheme precisely corresponds to the structural composition of the O antigen. The O1 and O2(2a,2c) antigens contain both D-galactan I and another O polysaccharide (23, 24), while the O2(2a) antigen contains only D-galactan I. Accordingly, we defined the O1 and O2ac genotypes as having not only the genes (at the *wb* gene cluster) for D-galactan I but also that (at the *wbbY* region) for the second O polysaccharide and defined the O2a genotype as having the genes for D-galactan I only. Both the *wb* and *wbbY* assays are required to distinguish between O1, O2a, and O2ac. In contrast, the first assay (*wb*) alone is sufficient to specifically identify genotypes O3, O4, O5, O9, and O12, each of which has a unique O polysaccharide (22) and thus is defined as having a specific allele at the *wzm-wzt* loci. Interestingly, the O8 antigen also has two polysaccharides: O-acetylated D-galactan I and D-galactan II (41). We did find that the O8 reference strain reacts with the second set of PCR primers targeting the allele for D-galactan II (Tables 1 and 2). However, since the *wb* gene cluster of O8 is markedly different from that of O1, the first assay (*wb*) alone is adequate to unequivocally identify O8. Another example of a good correlation between biochemical structure and O genotyping is our result for O9 and O2(2a,2e). Studies revealed that O9 and O2(2a,2e) polysaccharides have identical structures that are different from that of the O2(2a) antigen (43). In keeping with previous biochemical findings, we show that both the O9 and O2(2a,2e) reference strains react with the O9 *wb* primers but not with the O1/O2 *wb* primers (Table 1).

O genotyping can provide important information regarding the pathogenic ability of *K. pneumoniae* strains to cause invasive infections. Studies have suggested that O1 is a major virulence factor in *K. pneumoniae* (12, 30, 44). Compared with non-O1 strains, the O1 strains are generally more resistant to the bactericidal effect of human serum (12), which has been attributed to the D-galactan II component in O1 antigen (30, 44). One study further shows that the association of O1 with invasive *K. pneumoniae* strains causing pyogenic liver abscesses (90.5% [38/42]) is greater than that with noninvasive strains (28.1% [9/32]) (12). Nevertheless, two classical O-seroepidemiological studies found a lack of significant differences in the distribution of O1 between invasive and noninvasive strains (18, 21). This seeming inconsistency between studies may be due to the fact that *K. pneumoniae* virulence also depends on the K-antigen type; K1 is the most virulent, followed by K2, K5, K20, and K54 (8). To characterize the significance of the O-antigen types in *K. pneumoniae* pathogenicity, analysis of both the O type and K type would be required in future studies. Our O-genotyping technique facilitates studies on the role of the O type in the pathogenesis of invasive *K. pneumoniae* diseases.

When combined with K genotyping (8, 16), O genotyping also helps determine the clonality of *K. pneumoniae* isolates. The O:K-typing system (with nine O types and 77 K types) has a discriminative power comparable to that of pulsed-field gel electrophoresis (PFGE) (13). Unlike PFGE, which still lacks an international database for real-time pattern recognition of *K. pneumoniae* strains, the O:K genotyping method yields definitive O:K types

and thus facilitates the direct discrimination of *K. pneumoniae* strains isolated from different geographical locations or time periods (13). Furthermore, by targeting the two major virulent factors (LPS and CPS) in *K. pneumoniae*, O:K typing can also complement multilocus sequence typing (MLST) (which targets housekeeping genes in the genomic background) (45) for the purposes of tracing the source and transmission of *K. pneumoniae* in large-scale epidemiological investigations (46). Hence, the O:K genotyping method helps to investigate outbreaks, evaluate trends, and detect emerging strains of *K. pneumoniae*.

Given the greater convenience and high accuracy, our PCR-based O-genotyping technique represents an important breakthrough in the methodology of *K. pneumoniae* LPS O-antigen typing. The O:K genotyping system provides a useful tool for the clinical and epidemiological investigations of *K. pneumoniae* and its associated diseases.

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