JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1999, p. 56–62 Vol. 37, No. 1
0095-1137/99/$04.00+0

Klebsiella pneumoniae Lipopolysaccharide O Typing: Revision of Prototype Strains and O-Group Distribution among Clinical Isolates from Different Sources and Countries

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Received 15 June 1998/Returned for modification 24 August 1998/Accepted 19 October 1998

We have previously described an inhibition enzyme-linked immunosorbent assay method for the O typing of O1 lipopolysaccharide from *Klebsiella pneumoniae* which overcomes the technical problems and limitations of the classical O-typing method. In this study, we have extended the method to all of the currently recognized O types. The method was validated by studying the prototype strains that have defined the O groups by the classical tube agglutinatination O-typing method. Based on these results, we confirmed the O types of 60 of 64 typeable strains, and we propose a revised O-antigenic scheme, with minor but necessary changes, consisting of serogroups or serotypes O1, O2, O2ac, O3, O4, O5, O7, O8, and O12. Application of this typing method to 638 *K. pneumoniae* clinical isolates from Denmark, Spain, and the United States from different sources (blood, urine, and others) showed that up to 80% of these isolates belong to serotypes or serogroups O1, O2, O3, and O5, independently of the source of isolation, and that a major group of nontypeable isolates, representing about 17% of the total, consists of half O^+ and half O^- strains. Differences were observed, however, in the prevalence of the lipopolysaccharide O types or groups, depending on the country and isolation source.

Klebsiella spp., particularly *Klebsiella pneumoniae*, are important causes of nosocomial infections (9, 25). *K. pneumoniae* infections may occur at almost all body sites, but the highest incidence is found in the urinary and respiratory tracts. The main populations at risk are neonates, immunocompromised hosts, and patients predisposed by prior surgery, diabetes, malignancy, etc. (9, 14, 16). The existence of multiply antibiotic-resistant *K. pneumoniae* strains is notorious and has complicated therapy. Mortality rates of up to 50% have been found in respiratory tract infections. As an alternative to antibiotic treatment, prevention and/or treatment of *Klebsiella* infections by immunotherapy have received more attention in recent years.

K. pneumoniae typically expresses both lipopolysaccharide (LPS, O antigen) and capsule polysaccharide (K antigen) on its surface, and both LPS and capsule contribute to the pathogenicity of this species. Prevention of *K. pneumoniae* infections has been attempted by active and passive immunizations with a capsular polysaccharide vaccine including 24 of the 77 currently recognized K serotypes (5, 7). In contrast to the large number of capsular serotypes, only nine LPS O groups have been recognized, although some of these are still under debate. O1, the most common serogroup among clinical isolates (1), has been found to be exposed on the surface of most strains (30), and antibodies against it are protective in animal models (27, 30). The remaining eight O groups are O2, O2ac, O3, O4, O5, O7, O8, and O12.

In contrast to *Escherichia coli* and *Salmonella* spp., where O typing is a very useful epidemiological tool and a predictive factor for pathogenicity or virulence, O typing of *K. pneumoniae* has not been routinely performed. The main reasons for this are that (i) the capsular polysaccharide is heat stable, thus making it necessary to isolate unencapsulated mutants for test tube agglutination, and (ii) O typing has less discriminatory power than K typing (nine O groups versus 77 K types) (26). The introduction of an inhibition enzyme-linked immunosorbent assay (iELISA) method (1) has made O typing of *K. pneumoniae* easier. At the same time, several research groups have investigated the structures and genetics of *K. pneumoniae* O antigens (10, 19, 28, 36). However, very little is known about the distribution of the different O groups in clinical specimens. Since Kauffmann and Ørskov described the O groups in the 1940s and 1950s, only four publications, two from Japan in the beginning of the 1980s (11, 23) and two recent papers from Germany (32, 33), have studied the O-group distribution in *Klebsiella*.

Here we present the results obtained by an extension of the iELISA O1 typing method to all nine currently known *Klebsiella* O groups. We propose a revised O-antigenic scheme for the 77 K prototype strains and show the O-group distribution of 638 clinical isolates from Denmark, Spain, and the United States.

MATERIALS AND METHODS

Strains and growth conditions. Strains used to validate the iELISA typing methods were obtained from The International *Escherichia* and *Klebsiella* Reference Centre (WHO), Statens Serum Institut, Copenhagen, Denmark. Clinical isolates were obtained from the clinical microbiology laboratories of the following institutions: Hvidovre Hospital (Copenhagen, Denmark), 226 blood and 49 urine isolates; Hospital Ramón y Cajal (Madrid, Spain), 103 blood and 16 urine isolates; Hospital Son Dureta (Palma de Mallorca, Spain), 102 urine isolates and

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TABLE 1. Strains used for LPS purification and coating of iELISA plates, strains used for immunization, the antigen or epitope recognized by the iELISA systems, and working antiserum dilutions

iELISA O type determined by	LPS source strain (serotypes)	Antiserum raised against strain (serotypes)	Antigen or epitope recognized[a]	Antiserum dilution
O1	Friedländer 204 (O1:K$^-$)	C3 (O1:K66)	D-gal II	1/5,000
O2	1205 (O2 or O9:K72)	1205	O2a (D-gal I)	1/1,000
O2ac	5053 (O2a,2c:K$^-$)	5053	O2c	1/600
O3	636/52 (O3;K58)	390 (O3:K11)	O3	1/2,000
O4	Mich. 61 (O4:K15)	1702 (O4:K42)	O4	1/400
O5	5710/52 (O5:K61)	4425/51 (O5:K579)	O5	1/2,000
O7	264(1) (O7:K67)	264(1)	O7	1/2,000
O8	889 (O2 or O8:K69)	889[b]	*O*-Acetyl D-gal I	1/200
O12	702 (O12:K80)	702	O12	1/4,000

[a] Antisera were previously absorbed with an O$^-$ mutant (strain KT793) to remove core-specific antibodies.
[b] Antiserum was absorbed with mutant strain 52145-4 (D-gal I$^-$ D-gal II$^+$).

32 isolates from other sources; Hospital de la Macarena (Seville, Spain), 28 urine isolates. Isolates from the United States were obtained from the Nosocomial Pathogens Laboratory Branch-Hospital Infections Program of the Centers for Disease Control and Prevention (Atlanta, Ga.). Isolates were identified in the laboratories of origin in accordance with standard procedures (12). Strains were routinely grown at 37°C in Luria-Bertani broth (LB) or on LB–1.5% agar and kept at −80°C in glycerol broth. For result analyses, urine isolates from Spanish hospitals were pooled, as no differences between them were seen.

Purified LPS and LPS-containing extracts. The LPSs used to coat iELISA plates were purified by the phenol-water method of Westphal and Jann (35). LPS-containing extracts from 4-ml overnight cultures (37°C, 200-rpm shaking, LB medium) of clinical isolates were obtained by a phenol-water minipreparation method (2). Phenol was eliminated from the LPS-containing extracts by chloroform extraction and ethanol precipitation. Precipitates containing LPS were suspended in 1 ml of distilled water and directly used at the inhibition step of iELISA typing experiments.

Antisera. Immune sera containing anti-LPS antibodies were obtained by repeated intravenous immunization of New Zealand White rabbits with formalin-killed bacterial cells of the strains detailed in Table 1 and by following the protocol described by Edwards and Ewing (8). Antisera (1 ml, 1:10 dilution) were absorbed by incubations (2 × 1 h at 37°C and then overnight at 4°C) with 10^{11} cells of *K. pneumoniae* KT793, an O$^-$:K$^-$ mutant derived from strain C3 (3), to remove core-specific antibodies that may cross-react in the iELISA. For the O8 iELISA system, further absorptions with whole cells from strain 52145-4, a mutant derived from strain 52145 which expresses D-galactan II (D-gal II) but does not express D-gal I, were performed under the conditions detailed above.

iELISA O typing. Each iELISA plate was coated with purified LPS from one of the nine strains in Table 1 at 1 μg/well and incubated overnight at 4°C in 50 mM bicarbonate buffer (pH 9.6). After washing, the plates were blocked by incubation with 5% skim milk and washed. Next, the plates were incubated with 50 μl of LPS-containing extracts from the strains under investigation (typing) and 50 μl of the appropriate dilution of the homologue antiserum against the LPS used to coat the plate (Table 1). After washing, the plates were incubated with alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (Sigma; 1/3,000 dilution), washed, and developed for 1 h with 1-mg/ml *p*-nitrophenyl phosphate in 25 mM bicarbonate buffer (pH 9.6)–1 mM $MgCl_2$, and the A_{405} was recorded. Incubations were carried out at 37°C for 1 h, washes were done with phosphate-buffered saline (PBS)–0.05% Tween 20, and antisera and skim milk were diluted in PBS–0.05% Tween 20. Each LPS-containing extract from every strain or isolate studied was tested in all of the iELISA systems described in Table 1. Control wells (50 μg of LPS purified from all of the strains shown in Table 1) were included in each iELISA plate.

O typing by tube agglutination. Classical O typing was performed by tube agglutination or agglutination in microtiter systems in plastic trays as described elsewhere (26) by using O antisera raised against *K. pneumoniae* nonencapsulated forms or the corresponding *E. coli* O antisera (25).

Electrophoretic separation and Western blot analysis of LPS. An LPS extract from proteinase K-treated whole cells was used (15). Extracts were analyzed by electrophoresis in 15% polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE) and detected by silver staining (34). LPSs separated by SDS-PAGE were transferred to Immobilon P membranes (Millipore, Madrid, Spain) by using the buffers and conditions described by Towbin et al. (31), except that 1 mA and 1 h were used for electrophoretic transfer. Membranes were blocked for 1 h with 5% skim milk in PBS, washed, and incubated with the appropriate dilution of anti-LPS rabbit serum. After washing, membranes were incubated with the appropriate dilution of alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G, washed, and developed with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium (4). Incubations were carried out for 1 h at 37°C.

Statistical analyses. Analyses of serotype distribution among clinical isolates were performed by using the chi square and Fisher *t* tests.

RESULTS

Validation of the iELISA typing methods and revision of the typing scheme. In order to validate the iELISA methods for *Klebsiella* O typing and to revise the O-group distribution among the K-type strains, we studied all of the 77 prototype K-type strains reviewed by Ørskov and Ørskov (26). Results from these studies are shown in Table 2, which contains the iELISA values for strains selected from among the 77 prototype strains, as well as the O2 group strains defined by Ørskov (25) and the reference strains Friedländer 204 (O1:K$^-$) and 378 (O11:K78) (25, 26). Some strains have been included in Table 2 to show the typical or expected reactions for a given O type or group, while other strains are shown because their reactions were in conflict with the previous typing scheme. Strains not shown in Table 2 produced results in total agreement with those reported by Ørskov. Strains shown in Table 2 could be grouped according to their reactions in the following iELISA systems: O1, O2, O2ac, O3, O4, O5, O7, O8, and O12.

The O1 group of strains was not homogeneous. Some strains, including the prototype strain Friedländer 204 (O1:K$^-$), were inhibiting only in the O1 iELISA, i.e., strains A5054 (O1:K1), B5055 (O1:K2), and 889/50 (O1:K20); a second group of strains, represented by strain NCTC 8172 (cited in the literature as either O1:K64 or O6:K64), gave good inhibition values in both the O1 and O2 iELISA systems; and a third group of strains did not inhibit in the O1 system and gave no or variable inhibition in the O2 system: strains Br22 (O1:K32), SW4 (O1:K65), 265 (O1:K68), and 325 (O1:K79). Since only the first two groups, expressing the major O1 antigen (D-gal II polysaccharide; see Discussion), can be considered O1 strains, the four strains from the third group do not belong to serogroup O1.

Antigenic heterogeneity of the O2 serogroup has previously been reported. By using an extended antigenic formula (O2a; O2a, 2b; . . . ; O2a, 2h), the O2 group was divided into eight serotypes (25). We used strain 1205 (O2 or O9:K72) to raise antibodies for the typing of O9 strains, but the results in Table 2 clearly show that the antigen recognized by our iELISA system has to be the O2a antigen (D-gal I polysaccharide; see Discussion), which is the principal antigen of the O2 group. This assumption is supported by the fact that strains harboring the O2a antigen, or a major part of it, even with the additional antigens 2b, 2d, 2e, 2f, 2g, or 2h, inhibited in the O2 iELISA system. These strains did not inhibit in the O1 or O2ac systems. However, strains expressing both the O2a and O2c antigens inhibited in the O2ac iELISA system, but they did not inhibit in the O2 system, as could be expected. Strain 5052 (O2a,2c) could not be typed by any of the iELISA systems (Table 2) and was negative by Western blotting with anti-O2a,2c serum (data

TABLE 2. iELISA values for representative *Klebsiella* O- and K-antigen reference strains[a]

Serotypes (strain[s])	ELISA value (A_{405}, 10^3)								
	O1	O2	O2ac	O3	O4	O5	O7	O8	O12
O1:K$^-$ (Friedländer 204)	297	947	—[b]	—	—	—	—	827	—
O1:K1 (A5054)	92	1,092	—	—	—	—	—	896	—
O1:K2 (B5055)	37	933	—	—	—	—	—	1,088	—
O1:K20 (889/50)	119	746	—	—	—	—	—	1,112	—
O1:K32 (Br22)	1,164	163	—	—	—	—	—	1,164	—
O1 or O6:K64 (NCTC 8172)	102	247	—	—	—	—	—	1,062	—
O1:K65 (SW4)[c]	1,096	915	1,015	1,214	923	1,040	905	1,050	1,010
O1:K68 (265)	1,141	107	—	—	—	—	—	975	—
O1:K79 (325)	1,010	255	—	—	—	—	—	1,070	—
O2a:K3 (C5046)	1,000	90	1,000	—	—	—	—	898	—
O2a:K28 (5758)	882	108	979	—	—	—	—	876	—
O2a:K43 (2482)	1,103	83	1,011	—	—	—	—	948	—
O2a,2b:K2 (7380)	950	73	1,080	—	—	—	—	1,210	—
O2a,2e:K35 (7444)	1,085	170	1,141	—	—	—	—	612	—
O2a,2e,O2h:K59 (2212/52)	1,235	324	908	—	—	—	—	745	—
O2a,2f,2g:K27 (6613)	863	86	1,012	—	—	—	—	1,005	—
O2 or O9:K72 (1205)	1,205	138	1,128	—	—	—	—	579	—
O2a,2c:K− (5053)[d]	1,013	1,254	74	—	—	—	—	1,128	—
O2a,2c:K5 (E5051)	990	92	150	—	—	—	—	1,022	—
O2a,2c:K6 (F5052)[c]	1,021	1,289	971	—	—	—	—	1,077	—
O2(a),2c,2d:K4 (D5050)	946	1,172	106	—	—	—	—	1,008	—
O2(a),2f:K23 (Br37m)	1,057	705	1,000	1,055	906	974	1,051	989	976
O3:K11 (390)	—	—	—	385	—	—	—	—	—
O3:K58 (636/52)	—	—	—	195	—	—	—	—	—
O3:K25 (2002/49)	—	—	—	156	—	—	—	—	—
O3:K31 (6258)	—	—	—	161	—	—	—	—	—
O4:K42 (1702)	—	—	—	—	144	—	—	—	—
O4:K15 (Mich. 61)	—	—	—	—	91	—	—	—	—
O11:K78 (378)	—	—	—	—	101	—	—	—	—
O5:K57 (4425/51)	—	—	—	—	—	72	—	—	—
O5:K61 (5710/52)	—	—	—	—	—	83	—	—	—
O7:K67 (264 (1))	—	—	—	—	—	—	272	—	—
O8 or O2:K69 (889)	139	1,098	—	—	—	—	—	250	—
O12:K80 (702)	—	—	—	—	—	—	—	—	126
R:K18 (1754/49)[c,f]	177	—	—	—	—	—	—	—	—
R:K36 (8306)[c,f]	—	—	—	—	178	—	—	—	—
R:K14 (Klebs. 1193)[c,f]	—	—	—	—	—	80	—	—	—
R:K60 (4463/52)[c,f]	—	—	—	—	—	80	—	—	—
R:K56 (3534/51), R:K81 (370), R:K82 (3454-70)[c,f]	—	—	—	—	—	—	—	—	—
R:K9 (56), R:K13 (1470), R:K17 (2005/49), R:K38 (8414), R:K40 (8588), R:K52 (5759/50)[e,f]	—	—	—	—	—	—	—	—	—

[a] Reviewed by Ørskov and Ørskov (25, 26). The extended formula (25) has been used for serogroup O2. Horizontal gaps separate the O groups determined by Ørskov and Ørskov (25, 26).
[b] —, A_{405}, 1,000 ± 200 (no inhibition).
[c] Smooth LPS by SDS-PAGE.
[d] K$^-$ mutant from strain E5051 (25).
[e] Rough LPS by SDS-PAGE.
[f] R, rough LPS or not determined according to Ørskov and Ørskov (26).

not shown). Finally, we found that strain Br37m [O2(a),2f:K23] gave no inhibition in any of the iELISA systems, except for minor inhibition in the O2 iELISA system.

For the O3 group, we first tried the homologous iELISA system, i.e., antiserum raised against strain 390 (O3:K11) and plates coated with LPS extracted from it. With this typing system, not all of the O3 strains reported by Ørskov (25) were detected, and several of the clinical isolates that were nontypeable by iELISA were clearly O3 by the classical tube agglutination method (data not shown). By using a heterologous typing system, iELISA plate wells coated with LPS from strain 636/52 (O3:K58), and antiserum against strain 390 (O3:K11), all O3 strains reported by Ørskov (25) were detected. With this iELISA system, *E. coli* Bi316/42 serogroup O9, immunologically identical to *Klebsiella* O3 (25), was also recognized, and the number of clinical isolates typed as O3 increased.

As for serogroup O3, the homologous typing systems for serogroups O4 and O5 were unable to detect all of the known

O4 and O5 strains reported by Ørskov and Ørskov (26). By using the heterologous systems described in Table 1, we could correctly type these strains, as well as *E. coli* O20, corresponding to *K. pneumoniae* O4, and *E. coli* O8, which is identical to *K. pneumoniae* O5. Finally, it is interesting to review the results for strain 378, which in 1963 was proposed as the prototype for serotype O11 (6). Our results clearly demonstrate that strain 378 (O11:K78) is typeable by the O4 iELISA.

For both the O7 and O12 serotypes, there were no additional control reference strains, but Table 2 shows that they produced good inhibition values in their homologous iELISA systems and that these typing systems did not recognize other known serotypes. Also, for serogroup O8, which is defined by strain 889 (O8 or O2:K69), no other reference strain has been published. Strain 889 was the only strain in Table 2 giving good inhibition in the O8 iELISA system, but it also produced, as expected (see Discussion), good inhibition in the O1 iELISA system.

We have finally studied one group of reference strains described by Ørskov and Ørskov as either rough or not determined (26). By SDS-PAGE and silver staining, and by iELISA, we could divide this group into three: (i) strains with rough LPS (Table 2); (ii) strains with smooth LPS that could be typed by the iELISA systems, i.e., strain 1754/49 (R:K18), typed as O1, strain 8306 (R:K36), typed as O4, and strains Klebs. 1193 (R:K14) and 4463/52 (R:K60), typed as O5; and (iii) strains with smooth LPS but nontypeable by any of the iELISA systems, i.e., strains 3534 (R:K56), 370 (R:K81), and 3454-70 (R:K82). Since all of the above strains are K prototype strains, (O):K antisera were available from Statens Serum Institut. We isolated spontaneous nonencapsulated mutants from the three strains above and tested them against their (O):K antisera by the classical tube agglutination method, confirming the presence of anti-O antibodies. LPS was purified from these three strains and used to coat iELISA plates. High titers (1/1,600 to 1/25,600) of specific anti-O antibodies were confirmed. iELISA experiments were then performed in these three systems to test all of the nontypeable strains, both reference and clinical strains. No K-type reference strain could be typed in any of these three iELISA systems. However, two clinical isolates were recognized by an iELISA with LPS from strain 370 (R:K81) and its antiserum, suggesting the existence of a previously unrecognized O serogroup.

In summary, considering the 77 K-type reference strains (serotypes K1 through K72, K74, and K79 through K82) reviewed in reference 26, the classical tube agglutination method for O typing assigned 64 of these strains to nine different O groups while 13 strains were either rough or nontypeable (26). We could confirm the O serotypes of 60 of the 64 typeable strains by iELISA. For three strains described as O1, we found a different serotype (O2), and one strain described as O1 was nontypeable in our experiments. Additionally, among the 13 strains rough or nontypeable by iELISA, we assigned 4 to previously defined serogroups, and by SDS-PAGE and silver staining, the rest of these strains were further subdivided into O^+ (3 strains) and O^- (6 strains). These results show that the iELISA method described here can reliably be used for O typing instead of the more laborious tube agglutination method. Also, based on these results, we propose a revised O-antigenic scheme for the 77 K-type strains, with minor but necessary changes (see Table 3).

O-group distribution of *K. pneumoniae* clinical isolates. By using the validated iELISA and the O-antigenic scheme in Table 3, we O grouped 638 clinical isolates from different countries and sources of isolation. Isolates were divided into three groups according to their sources of isolation: blood (n = 348 isolates), urine (n = 210), and other (n = 80). The isolates were from Spain (n = 281), Denmark (n = 275), and the United States (n = 82), and their O-group distribution is shown in Table 4.

TABLE 3. Proposed O-group distribution of the 77 K-antigen reference strains described in reference 26

O group	K type(s)
O1	1, 2, 7, 8, 10, 12, 16, 18, 19, 20, 21, 22, 23, 24, 26, 29, 30, 34, 37, 39, 41, 44, 45, 46, 47, 62, 63, 64, 66, 70
O2	3, 27, 28, 32, 35, 43, 59, 68, 72, 79
O2ac	4, 5
O3	11, 25, 31, 33, 48, 49, 50, 51, 53, 54, 55, 58, 74
O4	15, 36, 42
O5	14, 57, 60, 61, 71
O7	67
O8	69
O12	80
NT[a] O^-	9, 13, 17, 38, 40, 52
NT O^+	6, 56, 65, 81, 82

[a] NT, nontypeable.

The total distribution, independently of the country and source, was as follows: O1 was the most frequent serogroup (39.3%), followed by O3 (15.5%) and O2a (15.0%). Strains belonging to serogroups O4, O5, and O12 were less frequently found (3.3, 9.1, and 0.3%, respectively). No strains belonging to serogroup O2ac or O7 were found. However, the second largest group of strains consisted of nontypeable isolates (17.4%); of these isolates, nearly half were O^- by SDS-PAGE (8.3% of the total). Thus, overall, 78.9% of the clinical isolates belong to only four O groups (O1, O2, O3, and O5).

For each isolation source, we have found major differences in serotype distribution, depending of the country of origin. Among the blood isolates, the most prevalent serogroup was O1 in both Spain (44%) and Denmark (39%), while O1 (26%) ranked second to O2 (37%) in the United States. In Spain, the next most prevalent serogroups where O2, O3 (14% each), and O5 (8%). Serogroup O3 was the second most prevalent in Denmark (24%), followed by O5 and O2 (11% each).

Among urine isolates, O1 was the most frequent serogroup in all three countries, at 35, 57, and 40% for Spain, Denmark, and the United States, respectively. In Spain, as for the blood isolates, serogroups O2 and O3 ranked next in prevalence among urine isolates (17 and 13%, respectively). In Denmark, the ranking of serogroups O1, O3, and O5 was as found among the blood isolates, but the prevalence of O3 among urine isolates was only one-third of that found in blood (8 versus 24%). Urine isolates from the United States that did not belong to serogroup O1 were basically nontypeable: by SDS-PAGE, 20% were O^+ and 27% were O^-.

A miscellaneous group of strains from Spain and the United States designated "other" in Table 4, was also studied. In Spain, O1 and O5 (28 and 22%) were the major serogroups, whereas O1 and O2 (41 and 31%) were the major ones in the United States. This rank of serogroups for the U.S. isolates was the reverse of that observed for blood isolates in the United States, where O2 predominated over O1 (37 versus 26%).

DISCUSSION

We have validated an iELISA method for O typing of the nine known *Klebsiella* O groups and used it to study the O-group distribution in clinical samples from different origins and sources. Below, we will discuss the results and problems found with each of the iELISA systems.

TABLE 4. Distribution of 638 *K. pneumoniae* clinical strains into O groups according to their sources of isolation and geographic origins

Source (no.) and origin	No. of isolates	% (subtotal) of strains in serogroup:					% Nontypeable[a]	
		O1	O2	O3	O4	O5	O^+	O^-
Blood (348)								
Spain	103	43.7	13.6	13.6	1.9	7.7	8.7	10.7
Denmark[b]	226	38.1	11.0	24.3	1.7	11.5	6.6	5.7
United States	19	26.3 (39.3)	36.8 (13.2)	5.3 (20.1)	5.3 (2.0)	5.3 (10.0)	10.5 (7.5)	10.5 (7.5)
Urine (210)								
Spain[b]	146	34.9	17.1	13.0	5.5	8.2	12.3	8.2
Denmark	49	57.1	6.1	8.2	2.0	8.2	6.1	12.2
United States	15	40.0 (40.4)	6.7 (13.8)	0.0 (10.9)	6.7 (4.7)	0.0 (7.6)	20.0 (11.4)	26.6 (10.4)
Other[c] (80)								
Spain	32	28.1	18.7	6.2	6.2	21.9	3.1	15.6
United States	48	41.7 (36.2)	11.2 (26.2)	8.3 (7.5)	4.2 (5.0)	0.0 (8.7)	14.6 (10.0)	0.0 (6.2)
Total % (no.)	99.5 (638)	39.3 (251)	15.0 (96)	15.5 (99)	3.3 (21)	9.1 (58)	9.1 (58)	8.3 (53)

[a] Nontypeable strains gave no inhibition in any of the iELISA typing systems. O^+ and O^- strains were determined by SDS-PAGE and silver staining of their LPSs.
[b] Two strains belonging to serotype O12 (a urine isolate from Spain and a blood isolate from Denmark) and one belonging to O8 (a blood isolate from Denmark) have been removed from the table for clarity, thus causing a total percentage of only 99.5%.
[c] Other, human isolates of a different or unknown source.

The O1 antigen is composed of two polysaccharides called D-gal I and D-gal II (37). D-gal II is a high-molecular-mass polysaccharide, it is the principal LPS antigen expressed by all O1 strains, and it is responsible for resistance to the complement bactericidal activity of nonimmune serum (22, 29). D-gal I is low in molecular mass and links D-gal II to the core LPS (21) in O1 strains. D-gal I is also expressed by O2 group strains and has been shown to be identical to the O2a antigen (36). The fact that D-gal I is expressed in variable amounts in different O1 strains explains the different degrees of inhibition we detected for some O1 group strains in the O2 iELISA. This variable degree of cross-reaction between O1 and O2 has also been reported by others (32, 33). Four strains previously reported as O1 by tube agglutination (25) could not be confirmed as such by Mizuta et al. (23). By using the iELISA methods, three of these strains were O2 and one was nontypeable.

It has been proposed that serogroup O2 could be divided into serogroups O8 and O9. We thus developed iELISA systems based on prototype O8 and O9 strains. Serogroup O8 will be discussed below. Serogroup O9 was proposed with strain 1205 (O2 or O9:K72) as the prototype (6). However, both serological (17) and structural (18) studies have demonstrated that the LPS from this strain and that from O2 group strain 7444 (O2a,2e:K35) are identical. These data clearly indicate that the LPS of the prototype O9 strain belongs to the O2 group. Our iELISA based on strain 1205 recognized all of the O2 group strains described by Ørskov (25), having in common the O2a antigen independently of the presence of additional antigen 2b, 2d, 2e, 2f, 2g, or 2h. Very recently, Trautmann et al. have described O9 as a separate serogroup (33). For the serological and structural reasons mentioned above, and given that no O9 strains were found among 378 clinical isolates in the study referred to, we have considered O9 not as a separate group but as part of the O2 group.

We obtained no inhibition in the O2 iELISA system with strains expressing the O2a,2c antigens. These results coincide with those obtained by Whitfield et al. by Western blotting with a monoclonal antibody specific for D-gal I (37) and with the typing results reported by Trautmann et al. (32). In both of the studies referred to, the explanation given for the nonrecognition of the O2a antigen expressed by O2a,2c strains was that the 2c antigen masked the 2a antigen. Although we do not have an alternative explanation, we find it hard to believe in a masking effect in phenol-extracted samples analyzed by Western blotting or iELISA. The number of O2a,2c clinical isolates found in our study and in that of Trautmann et al. (33) is either none or very low. However, as a high percentage (9 of the 11 strain studied) of O2a,2c *K. ozenae* strains has been reported (32) and since the O2a,2c strains are recognized by neither the O2 iELISA (this work and reference 33) nor Western blotting (37), we believe that O2a,2c has to be kept as a separate serotype.

Strain 5052 (O2a,2c) was nontypeable in any of the iELISA systems and was negative by Western blotting. These results agree with those obtained by Whitfield et al. (37) and may be explained by greatly reduced production of O-substituted LPS molecules. Strain Br37m produced no inhibition in the iELISA systems, except for minor inhibition in the O2 system. These results are consistent with the report of Ørskov demonstrating that this particular strain contains only a minor part of the O2a antigen (25).

For O3 typing, we had to use a heterologous iELISA system to detect all of the O3 strains among the 77 K-type strains. This coincides with previous descriptions (32) and may be due to the heterogeneity in the O3 group reported by Ørskov (25).

For O4 and O5 typing, we also had to use heterologous iELISA systems, and we detected two additional O5 strains among strains previously described as rough LPS or not determined. Strain 8306 was identified as O4, in agreement with a previous report (24). Strain 378 was initially described as the prototype for serotype O11. It was later found, both serologically (24) and structurally (unpublished studies by W. Nimmich referred by Kenne and Lindberg [20]), that the O4 and O11 LPSs are identical. Ørskov's data (25) suggest that the O4 group may be heterogeneous, with strain 1702 expressing an additional epitope or antigen compared to strain Mich. 61, which expresses the O4 type antigen. According to a recent paper (33), 21 of 23 O4 strains have an additional "partial or decorative" antigen defining serogroup O11, besides the major O4 antigen. These strains represent only 5.6% of the clinical isolates studied, and there are no clinical data suggesting that this additional antigen plays a role in virulence or pathogenicity. For all of the above reasons, we are more inclined, for the moment, to keep O4 as a single serogroup. The superiority of heterologous (Table 1), rather than homologous, iELISA systems for detection of serogroups O3, O4, and O5 might be due

to the existence of multiple epitopes in their LPSs and the use of polyclonal antisera. Under these conditions, if the epitope defining the serogroup is underrepresented, the use of heterologous iELISA systems is advantageous.

The O8 serogroup was proposed by Durlakowa (6), based on strain 889 (O8 or O2:K69), but to our knowledge, no other O8 reference strain has been published. We found that several clinical isolates that inhibited the O1 iELISA also produced variable degrees of inhibition in the O8 iELISA. As shown by structural analysis (19), the basis for the discrimination between O1 and O8 is the presence in the O8 LPS of a partially O-acetylated D-gal I polysaccharide. Both O1 and O8 LPSs have a D-gal II polysaccharide in common, besides the nonacetylated part of D-gal I. Thus, it would be expected that O8 strains, besides inhibiting the O8 iELISA, could also inhibit the O1 system (D-gal II) and even, to some degree, the O2 iELISA (D-gal I). We have only found one O8 strain among the 638 clinical isolates studied; thus, it seems questionable whether all O8 strains can be serologically recognized. Our results agree with those of Trautmann et al. (33), who were unable to separate O1 and O8 by serological methods. Since a different gene organization has been demonstrated between O1 strains and the O8 strain (19), genetic methods might allow distinction of O1 and O8. Because the O1-O8 group accounts for about 40% of the clinical isolates, further subdivision of the group might be clinically informative.

Distribution of O types among clinical isolates. We found that O1 was the major group (38%), followed by serogroups O3, O2 (15% each), and O5 (9%). This general trend coincides with those reported from Japan (11) and Germany (33). In all three studies, about 80% of the strains belonged to these four O groups. The O1 predominance among clinical isolates has also been reported before (1, 23). Only two previous studies (1, 33) have included blood isolates: in one study, only serotype O1 was determined (38%, $n = 60$), while the second study showed that 85.9% of the blood isolates ($n = 79$) belonged to the four serotypes mentioned above. We found that 74 to 85% of our 348 blood isolates, depending on the country of origin, belonged to these four serotypes. Blood isolates from the United States ($n = 19$) are an exception, as O2 (37%) predominated over O1 (26%). Also, among the urine isolates, O1 is the leading serogroup. The high percentage of nontypeable urine (and blood) isolates in the United States is noticeable, but since the data for this country are based on few isolates (urine, $n = 15$; blood, $n = 19$), further studies are needed to verify these differences.

Comparison between urine ($n = 49$) and blood ($n = 226$) isolates from Denmark shows that serogroup O3 is more prevalent in blood than in urine (24 versus 8%, $P < 0.05$), while the opposite is true for serogroup O1 (38 versus 57%, $P < 0.05$). A recent work reported no differences in serotype distribution between invasive and noninvasive isolates in Germany (33). However, the same trends that we just described for Denmark can be seen in Table 2 of the above reference: in Germany, O3 was significantly more prevalent in blood than in urine (30 versus 19%, $P < 0.05$), and O1 predominated in urine versus blood (45 versus 37%, $P < 0.05$). The most frequent portal of entry for these *Klebsiella* bacteremic isolates in Denmark was urine (14), thus suggesting enhanced virulence or an additional pathogenic factor(s) for these O3 strains. We found no differences in O-type distribution between blood and urine isolates from Spain.

The second largest group of strains in our study consisted of nontypeable isolates (17.4%), with approximately half O^- and half O^+ by SDS-PAGE. As the iELISA systems used could correctly type the majority of the prototype strains, and we could even type four of these previously given as nontypeable, and since an iELISA prepared with LPS from a previously nontypeable strain could identify two strains among the clinical isolates, these data suggest that the number of nontypeable O^+ isolates found in this study is not due to failure of the method. Rather, these data suggest that the nontypeable group might consist of several undefined and infrequent O groups. Regarding the nontypeable O^- isolates, they may produce LPS amounts below the detection limits of the silver staining method, although we repeated SDS-PAGE with overloaded samples. Many of these O^- strains were isolated from blood, and even when we analyzed their LPSs by SDS-PAGE and silver staining after growing them in serum, we were unable to detect high-molecular-mass LPS molecules (data not shown). Most strains were also serum resistant (data not shown). It would be appealing, but it is beyond the scope of this work, to study the mechanisms that these O^- isolates use to resist complement-mediated killing, since the LPS O side chain is the major factor of complement resistance in *K. pneumoniae* (29).

Results from this work point to three directions of future research. Firstly, since more than 75% of the clinical isolates belong to just four serogroups, regardless of the country of origin and the type of isolate, an obvious conclusion is that a tetravalent LPS-based vaccine could be formulated and tested for protection in animal models. Secondly, although most clinical isolates belong to just a few serotypes, the combination of O and K types produces higher discriminatory power (13); thus, the benefits of O:K serotyping as an improved epidemiological tool requires evaluation. Thirdly, many years of serotyping of related organisms, such as *E. coli* and *Salmonella* spp., has established the virulence of different serotypes and their association with certain syndromes. In these species, knowledge of the serotypes helps in the treatment of the patients and may predict the outcome of the infectious process. We are just beginning to collect such data for O groups in *Klebsiella*, and future studies using O (and K) serotyping in controlled clinical settings are needed to confirm these differences and to provide further epidemiological and predictive information associated with the O serogroups.

ACKNOWLEDGMENTS

This work was supported by grants from the Comisión Interministerial de Ciencia y Tecnología (CICYT) of the Spanish Government. S.H.A., S.A., and D.A. were supported by predoctoral fellowships/postdoctoral contracts from CICYT.

We thank R. Cantón, J. Martínez-Beltrán, L. Martínez-Martínez, and C. O'Hara, from the institutions listed in Materials and Methods, for providing us with clinical isolates and A. Igelmo (Universidad de las Islas Baleares) for helpful discussions on the statistic analyses. We are indebted to I. Ørskov and D. Kasper for critical reading of the manuscript.

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