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Development of an Enzyme-Linked Immunosorbent Assay Method for Typing and Quantitation of *Klebsiella pneumoniae* Lipopolysaccharide: Application to Serotype O1

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We describe a method for the typing and quantitation of *Klebsiella pneumoniae* serotype O1 lipopolysaccharide (LPS) based on inhibition in an enzyme-linked immunosorbent assay of a reaction of known O1 LPS antigen and anti-O1 antibody by unknown LPS extracts. Serotype O1 was found in 32% of the 124 *K. pneumoniae* clinical isolates tested, showing that this serotype is frequent among the eight O serotypes which have been described previously.

Klebsiella pneumoniae is one of the leading causes of bacteremia caused by gram-negative-rods (6, 11) and is an important opportunistic pathogen, especially for the immunocompromised patient (3, 4, 9). Unlike with other enterobacteria, both the capsule and lipopolysaccharide (LPS) are constitutively present and have been established as essential virulence determinants (8, 14, 15, 18). In *K. pneumoniae*, unlike in other enterobacteria, K-antigen (capsular polysaccharide) determination is the basis of serological-type determination, while O-antigen determination is not performed. Two reasons explain the paucity of O serotyping with *K. pneumoniae*. First, although up to 12 O serotypes have been described for this species (12, 13), only 8 distinct serotypes are presently considered (10, 12, 13): O1, O3, O4, O5, O7, O8, O9, and O12. Second, the copious capsule, which may effectively mask the O antigen (16), is immunogenic, and the preparation of O-antigen-specific sera requires the use of capsule-deficient mutants (12, 13). Therefore, there is no information on the distribution of different O-antigen types, and the existence of additional O types may have gone unrecognized. We report here the development of an enzyme-linked immunosorbent assay (ELISA) method which overcomes the technical limitations cited above. By this ELISA method, the LPS is released into a fluid phase by phenol treatment and is assayed by an ELISA inhibition approach. Therefore, nonagglutination (i.e., noninhibition by this method) does not have to be taken into consideration when one is dealing with strains whose LPS is masked by the capsule. Also, since purified LPS is used to coat the ELISA plates, only O-antigen-specific antibodies are assayed, and an antiserum containing both O and K antibodies can be used. Finally, for the same reason, although the LPS-containing extracts obtained by the phenol treatment do in fact contain other bacterial antigens (for example, K antigen), this ELISA method only quantitates LPS.

K. pneumoniae clinical isolates were obtained and identified according to standard methods by the following tests (7): adonitol, arginine, DNase, H_2S, motility, ornithine, phenylalanine, arabinose, mannitol, melibiose, raffinose, salicin, sorbitol, trehalose, and xylose to place the isolates in the genus *Klebsiella* and indole, malonate, *o*-nitrophenyl-β-D-galactopyranoside, and Voges-Proskauer to identify to species level. Strains of known LPS types were also used as controls, and they are listed in Fig. 1. LPS was released from 5×10^9 bacterial cells by the phenol-water method of Westphal and Jann (17) with silica gel-containing tubes as described previously (1). Aqueous phases from the phenol extraction (LPS-containing extracts) were precipitated with ethanol, dissolved in 0.5 ml of water, and used as inhibitors for typing and quantitation. Immune sera containing antibodies against O1 LPS (anti-LPS) were obtained by repeated immunizations of rabbits with formalin-killed bacterial cells of *K. pneumoniae* C3 (serotype O1:K66) (5). Type O1 LPS from strain C3 was isolated and purified away from the capsular polysaccharide by repeated ultracentrifugation (2).

ELISA plates were coated with serotype O1 LPS from strain C3 (1 μg per well) by overnight incubation at 4°C in 50 mM bicarbonate (pH 9.6). After being washed with phosphate-buffered saline (PBS)–0.05% Tween 20 (PBST), plates were incubated at 37°C for 1 h with PBST–5% skim milk. Two ELISA approaches were then followed. For LPS quantitation, plates were incubated with 50 μl of anti-LPS serum (usually diluted 1:1,000) together with 50 μl of serial dilutions of the inhibitor (either LPS-containing extracts from the different strains or known amounts of LPS purified from strain C3). After being incubated and washed, the plates were incubated with alkaline phosphatase-labeled goat anti-rabbit immunoglobulin G (usually diluted 1:3,000) (Sigma). Finally, *p*-nitrophenyl phosphate at 1 mg/ml in 25 mM bicarbonate buffer (pH 9.6)–1 mM $MgCl_2$ was added, and the A_{405} was read after incubation at 37°C for 30 min. Incubations with the antisera were made at 37°C for 1 h, and washing steps with PBST were included after each incubation. Percent inhibitions were calculated by comparing wells without inhibitor with wells incubated in the presence of inhibitors. For typing, the ELISA procedure was as described above, except that only a duplicate aliquot of 50 μl of

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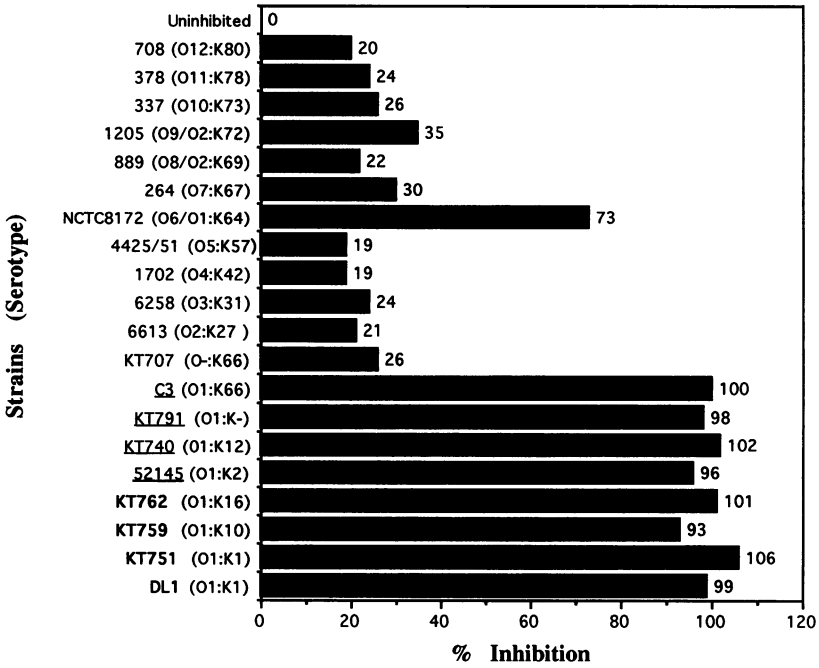

FIG. 1. ELISA inhibition values of the O1–anti-O1 reaction obtained with extracts containing LPS from different *Klebsiella* serotypes. Underlined strains have LPS exposed on the cell surface, while strains in boldface type do not (16). All other, non-O1 strains are reference strains for the typing of *Klebsiella* LPS (13). Non-O1 strains include the following type strains for *Klebsiella* O serotypes that have been deleted from the current typing scheme: O2 (strains are either O8 or O9), O6 (immunologically and structurally identical to O1), O10 (the type strain was later identified as *Enterobacter aerogenes*), and O11 (immunologically identical to O4) (10).

LPS-containing extract (undiluted) from each strain studied was used as inhibitor.

The specificity of the typing procedure was assessed by studying bacterial strains of known LPS and capsular serotypes. For this purpose, the final ELISA values (A_{405}) were normalized by considering as 0% the inhibition values of the uninhibited plate wells (usually A_{405} of 1.0) and as 100% those inhibition values obtained in the homologous situation, i.e., inhibition values of wells inhibited with a serotype O1 strain (usually A_{405} of 0.2). A representative example of the results obtained is shown in Fig. 1. It can be seen that strains of serotype O1 inhibited the binding of specific antibodies to LPS-coated wells, whereas strains of other LPS serotypes gave low inhibition values. To test the usefulness of the method, we also included O1 strains whose LPS is not surface exposed (strains DL1, KT751, KT759, and KT762). As shown in Fig. 1, these strains as well as other strains whose LPS is surface exposed (strains C3, KT791, KT740, and 52145) gave high inhibition values and could unequivocally be assigned to serotype O1. This shows that the method is suitable for O typing, independent of whether LPS is present on the cell surface.

The prevalence of LPS serotype O1 among 124 *K. pneumoniae* clinical isolates was studied by this method. Of the 124 isolates studied, 40 strains (32.2%) were serotype O1. Serotype O1 was found for 14 of the 44 strains isolated from urine (31.8%), 23 of 60 strains isolated from blood (38.3%), and 3 of 20 strains isolated from other sites (15%). This is, to our knowledge, the first report on the distribution of *K. pneumoniae* O1 LPS. Thus, although we cannot conclude that O1 is the most frequent serotype, the >30% value found for O1 in our study shows that it is one of the most frequent among the eight O serotypes previously described. The typing method described here should theoretically be suitable for the determination of other *K. pneumoniae* O serotypes, and this type of study would necessarily precede the development of LPS-based immunotherapy for *K. pneumoniae* infections. Such a strategy should be considered in view of the present work and since it has been reported previously that O1 LPS is exposed on the cell surface of many *K. pneumoniae* capsular serotypes (16) and that antibodies against serotype O1 are opsonic (18) and protective (16).

The amount of LPS produced by different clinical isolates was quantitated by ELISAs measuring inhibition. By using known amounts of O1 LPS purified from strain C3 as inhibitors, we obtained an inhibition curve that showed a direct correlation between the amount of inhibitor and the inhibition of the LPS–anti-LPS reaction at inhibition values of between 20 and 80% (data not shown). Therefore, this linear part of the inhibition curve was used as a standard curve to quantitate the amount of LPS present in a given cellular extract. The inhibition curves obtained with serial dilutions of LPS-containing extracts from two different strains (5×10^9 cells) are shown in Fig. 2. The slope of each curve is the same, thereby indicating quantitative rather than qualitative differences in the extracts, i.e., both strains produce O1 LPS in different amounts. The inhibition curves are otherwise linear at inhibition values of between 20 and 80%. Therefore, points between these two inhibition values are used to interpolate in the standard curve, allowing quantitation of the amount of LPS contained in each extract. By following this procedure, amounts of 0.35 and 4.18 μg of LPS were found in the LPS-containing extracts of strains M39 and M35, respectively (Fig. 2, closed and open circles, respectively).

The amount produced by each of the 124 strains tested (5

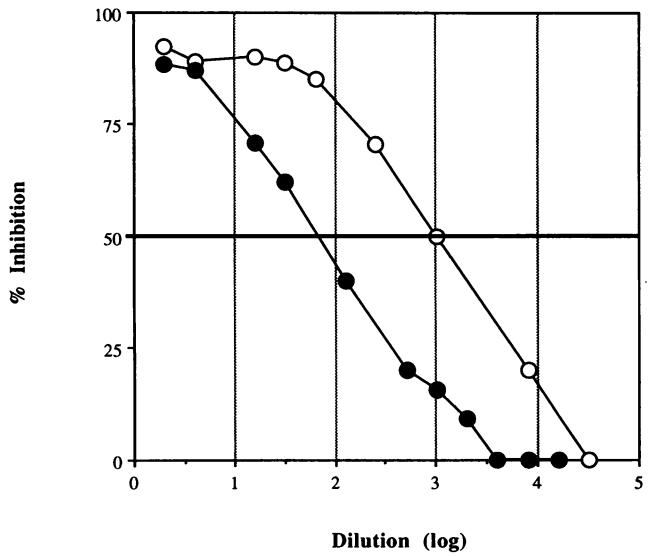

FIG. 2. ELISA inhibition values of the O1–anti-O1 reaction obtained with dilutions of LPS-containing extracts from two serotype O1 strains. Strains M35 (open circles) and M39 (closed circles) produce large and small amounts of LPS, respectively.

$\times 10^9$ cells) ranged between 0.3 and 4.2 μg of LPS, with mean amounts of 2.3, 2.2, and 2.5 μg of LPS produced by bacteremic, urinary, and other isolates, respectively. Therefore, we have not been able to detect significant differences in LPS production by clinical *K. pneumoniae* strains from different infection sites. The method, however, should be suitable for the quantitation of LPS and assessment of its importance in other biological phenomena.

By the agglutination method, the best way to identify the O antigen is to isolate unencapsulated mutants and to test boiled cultures for agglutination in O antiserum raised against unencapsulated mutants (12, 13). If such a mono-O-specific antiserum is not available, Ørskov and Ørskov (12, 13) suggest an antiserum containing O and K antibodies as an alternative, but then it is essential that the strain to be typed is unencapsulated. Clearly, this method requires the extra work of isolating unencapsulated mutants, and it also raises questions about the techniques necessary to screen for such a mutant and ensure that it is completely devoid of capsule. The ELISA method described here requires the extra work of LPS extraction and its assay, but it allows typing and quantitation of LPS in nonagglutinable (nontypeable) strains.

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