CHARACTERISATION OF BACTERIA

Evaluation of a competitive ELISA method for the determination of *Klebsiella* O antigens

M. TRAUTMANN, A.S. CROSS*, G. REICH†, H. HELD*†, R. PODSCHUN‡ and R. MARRE

Department of Medical Microbiology and Hygiene, University of Ulm, D-89075 Ulm, Germany, *University of Maryland Cancer Center, Baltimore, MD 21201-1595, USA, †Department of Internal Medicine, Klinikum Rudolf Virchow, Free University, D-13353 Berlin and ‡Department of Medical Microbiology, University of Kiel, D-24105 Kiel, Germany

Strains of *Klebsiella* spp. are often inagglutinable by O-specific antisera because of the copious capsule produced by most isolates. A competitive ELISA method based on the observation that bacterial supernates containing homologous O antigen specifically inhibited the reaction of type-specific antisera with purified LPS coated on ELISA plates was used to examine the O antigen of 82 isolates of different *Klebsiella* species and subspecies. The O antigens O1/2ab (19 isolates), O2ab (13 isolates), O2ac (11 isolates) and O3 (16 isolates) were found to account for > 70% of the O antigenic types. Overall, 65 (79%) of the strains could be assigned to a specific O serogroup. The method is suitable for examining the role of individual O antigens in systemic *klebsiella* infections such as nosocomial septicaemia and pneumonia.

Introduction

Organisms of the genus *Klebsiella* are a frequent cause of nosocomial infections including pneumonia [1], septicaemia [1–3], and urinary tract infections [1, 4]. In bacteraemic gram-negative infections, *klebsiellae* usually rank second only to *Escherichia coli* and are isolated more frequently than *Pseudomonas aeruginosa* [3, 5]. However, in contrast to the latter two pathogens, whose somatic antigens have been studied extensively both with respect to pathogenicity and their possible role as a target for immunotherapy [6–9], relatively little attention has been paid to such issues in *Klebsiella* spp. Because the presence of a large capsule is the most prominent feature of *Klebsiella* isolates, most investigators have focused on the role of the capsule as a virulence factor and on the distribution of capsular types in clinical material [10–14]. More recently, it has been recognised that the somatic lipopolysaccharide (LPS, O antigen) produced by *Klebsiella* spp. may also play an important role in the pathogenesis of blood stream [15, 16] and pulmonary infection [17] by this organism. While the latter observations have been made for strains of serotype O1 which occur most frequently in clinical material [18–21], little is known about the occurrence and pathogenic potential of strains with other O antigens.

During the last five decades, 12 *Klebsiella* O antigen serogroups have been described. However, subsequent examinations revealed that four of these, namely O6, O8, O9 and O11, were identical with, or closely related to, others described previously [22–26]. Serogroup O10 was deleted after it was found that the respective prototype strain was motile and, therefore, had to be relocated to the genus *Enterobacter* [22]. At present, serogroups O1–O5, O7 and O12 are recognised as being clearly distinct [27]. Within serogroup O2, Ørskov identified eight subgroups all of which shared a common “2a” antigen [18]. Recently, it has been shown that the O1 antigen comprises two different specificities one of which, termed D-galactan I, appears to be serologically identical to the O2a antigen [28]. Thus, the correct designation for this O antigen may be O1/2a.

In a previous report describing a monoclonal antibody against LPS of *Klebsiella*, we also studied the relative importance of O serogroups of *Klebsiella* other than O1 in clinical material [29]. In this study, a competitive ELISA method which enables the reliable identification of the O antigen in clinical isolates of *Klebsiella* is described in more detail. This method may serve as a tool for further, more extensive studies, which may characterise the importance of individual O antigens in defined clinical settings such as septicaemia and pneumonia. If relatively few O antigens are found in a significant portion of clinical isolates from cases

of invasive disease, then an O antigen-based polyvalent vaccine may be feasible for the immunotherapy or prophylaxis of klebsiella infection.

Materials and methods

Bacteria

The *Klebsiella* O antigen reference strains *Klebsiella* Friedländer 201 (O1:K-), 7380 (O2ab:K-), 5053 (O2ac:K-), 390 (O3:K11), 1702 (O4:K42), 4425:51 (O5:K57), 264(1) (O7:K67) and 708 (O12:K80), and additional strains harbouring homologous O antigens namely *Klebsiella* 2002/49 (O3:K25), Mich. 61 (O4:K15), 5710/52 (O5:K61) and *E. coli* 8188/41 (O19a,19b) whose O antigen has been shown to be serologically identical to *Klebsiella* O1 [30] were obtained from Drs I. and F. Ørskov, International *Klebsiella* and *E. coli* Reference Centre, Statens Seruminstitut, Copenhagen, Denmark. The O antigen of *E. coli* Bort (O18:H7:K1) which has been used previously in our laboratories [31] was found not to be related to any known *Klebsiella* O antigen. Therefore, this strain was included as a negative control in all serological studies. Clinical isolates of *K. pneumoniae* and *K. oxytoca* were collected in the Department of Medical Microbiology, University of Ulm, and identified by the API 20E system. Additional biochemical tests were performed as described previously [32]. *K. terrigena* [33], *K. ozaenae* and *K. rhinoscleromatis* strains were from the collection of the Department of Medical Microbiology, University of Kiel, Germany. Additional strains included were *K. ozaenae* NCTC 9657, NCTC 5050, NCTC 5051; *K. rhinoscleromatis* NCTC 1936, NCTC 5046 and ATCC 9436.

Lipopolysaccharides

The hot phenol-water method [34] was used to prepare lipopolysaccharides from all O antigen reference strains, *E. coli* 8188/41 and *E. coli* Bort. Capsular material was removed by precipitation with cetylmethylammonium-bromide (Cetavlon, Merck, Darmstadt, Germany) 1.25% as described previously [35]. Final LPS preparations were examined for protein by determination of the absorbance (A) at 280 nm and for the presence of residual capsular polysaccharide (CPS) by the determination of glucuronic acid [36].

Production of antisera

Outbred rabbits were immunised with the *Klebsiella* O antigen reference strains, additional *Klebsiella* strains possessing known O antigens, and with *E. coli* 8188/41. Bacteria used for immunisation were grown in trypticase soy broth at 37°C for 4–5 h, washed twice in physiological saline and adjusted in saline to give suspensions containing 1×10^8 cfu/ml. Non-capsulate organisms were inactivated at 60°C for 1 h. Capsulate strains were boiled for 2 h to destroy the K antigens.

Suspensions were stabilised by the addition of formalin 0.5% and kept at 4°C. Rabbits (mean weight 3.5 kg) were immunised intravenously into the ear vein with the following volumes of bacterial suspension: 0.2 ml (day 0), 0.5 ml (day 5), 1.0 ml (day 10), 1.5 ml (day 15), 2.0 ml (day 20). Seven days later, 30–50 ml of venous blood were taken from each rabbit and, after centrifugation, the serum was removed and stored.

Because klebsiellae are known to possess common O antigen epitopes [29] and, possibly, common protein antigens, sera were absorbed with the heterologous O antigen reference strains. For this purpose, 40 ml of serum were mixed thoroughly with a bacterial pellet containing $c. 10^9$ cells and incubated on crushed ice for 1 h with repeated vortex mixing. The suspension was then centrifuged and the supernatant serum was absorbed three more times in the same manner. Final serum supernates were filter-sterilised (0.45 µm-pore Millex HA filter unit; Millipore, Molsheim, France) and frozen in 1-ml volumes at -25°C or lyophilised.

Direct ELISA

To determine the titre of O-specific antisera, 96-well flat-bottomed microtitration plates (Greiner, Nürtingen, Germany) were coated (100 µl/well) with purified LPS 25 µg/ml in PBS, pH 7.4. After incubation at 4°C for 18 h and removal of antigen, 200 µl of "filling-buffer" (PBS, pH 7.4, containing bovine serum albumin 0.5%, casein 0.5% and sodium azide 0.1%) were added to each well to block non-specific binding sites. After incubation for 1 h at 37°C, plates were washed three times with PBS. Two-fold dilutions of O-specific antisera in "filling buffer" were added to duplicate wells (100 µl/well) and incubated overnight at 4°C. The plates were then washed three times with PBS, and 100 µl of appropriately diluted alkaline phosphatase-conjugated anti rabbit-IgG(γ) monoclonal antibody (Sigma) were added to each well. After incubation at 4°C for 4 h, the plates were washed and reactions were developed by adding to each well 200 µl of substrate solution (*p*-nitrophenyl phosphate 1 mg/ml in 1 M diethanolamine buffer, pH 9.8). The OD₄₀₅ of the wells was read in a Titertek Multiscan Plus reader (Flow Laboratories, Helsinki, Finland) after incubation for 30 min. Titres were defined as the highest dilution of antiserum yielding a mean OD₄₀₅ ≥ 0.300.

Competitive ELISA

Optimal serum dilutions and coating conditions for the competitive ELISA were selected on the basis of chessboard titrations with LPS concentrations from 0.5 to 32 µg/ml and serum dilutions from 100 to 51 200. In preliminary experiments, it was found that an OD₄₀₅ of 0.3–0.4 obtained without inhibition provided optimum sensitivity for competition, resulting in OD₄₀₅ values near to 0 after inhibition with homologous supernates.

Klebsiella strains to be tested were grown overnight on trypticase soy agar, suspended at a density of $c. 10^9$ cells/ml in PBS, pH 7.4, and boiled for 2 h to destroy most of the capsular material and proteins and to release membrane-bound LPS. After centrifugation (4000 g, 30 min) the clear supernates containing soluble LPS were harvested and stored at 4°C. For ELISA inhibition, 150 µl of appropriately diluted O-specific antiserum were mixed with 150 µl of boiled bacterial supernate and vortex mixed every 15 min during incubation for 2 h on crushed ice. Inhibition mixtures were pipetted (100 µl/well) into duplicate wells of LPS-coated microtitration plates and incubated at 4°C for 18 h. After removal of the mixture and washing, the plates were processed further as described for the direct ELISA. Each plate contained a positive control (supernate of the O reference strain harbouring the same O antigen coated on the plate) and a negative control (supernate of *E. coli* Bort). Developing times for each plate were adjusted to yield a mean OD₄₀₅ of 0.3–0.4 for the negative control.

Results

Purity of LPS preparations

The protein content of all LPS preparations was < 1% of dry weight. The glucuronic acid content, as a marker for CPS, was 0.3% (*E. coli* O19), 4.5% (O1), 3.9% (O2ab), 1.8% (O2ac), 1.7% (O3), 9.5% (O4), 2.7% (O5), 4.4% (O7) and 10.7% (O12) of the LPS dry weight. The glucuronic acid content of LPS preparations from non-capsulate organisms (O1, O2ab and O2ac) was, at first sight, surprising, but may possibly be explained by the presence of CPS precursor molecules in the outer membrane of these organisms.

Specificity and antibody titres of antisera

Before absorption, ELISA titres of individual rabbit antisera against the homologous antigens ranged from 6400 (O2ab) to > 102400 (O1). However, there were various cross reactions, in particular between antigens O1–O5. After cross-absorption, ELISA titres against

homologous O antigens were ≥ 1600 , whereas titres against heterologous O antigens were < 100. Cross-absorptions were not performed for sera 7380 (O2ab) and 5053 (O2ac) to avoid removal of specific antibodies against the "2a" epitope. Furthermore, although the antiserum to strain Friedländer 201 (O1) could be fully absorbed with strain 7380 (O2ab) without loss of specific activity, absorption of serum 7380 (O2ab) with strain Friedländer 201 resulted in complete loss of activity against LPS O2ab. Therefore, it was concluded that the correct antigenic formula of strain Friedländer 201 was O1/2ab, and serum O2ab was used without previous absorption with strains 5053 and Friedländer 201.

Establishment of competitive ELISA

To reach optimal sensitivity for the detection of each individual antigen, both the amount of antibody and the concentration of LPS used for coating the microtitration plates were minimised as much as possible on the basis of chess board titrations. The final reaction conditions are summarised in Table 1.

To examine the sensitivity and specificity of the inhibition reaction, antisera were mixed with increasing concentrations of homologous and heterologous LPS. Examples of the resulting inhibition curves are given in Fig. 1. While no inhibition was seen with heterologous LPSs, homologous LPS at 0.06–1 µg/ml resulted in > 50% inhibition (Table 1).

Concentration of LPS in bacterial supernates

The concentrations of LPS present in boiled supernates of *Klebsiella* strains to be tested were examined by a slightly modified version of the inhibition reaction. Antiserum against *E. coli* 8188/41 was diluted 200-fold and allowed to react with LPS O1 at a coating concentration of 2 µg/ml. With purified LPS O1 as an inhibitor, a log-linear standard curve was obtained (Fig. 2a). On the same microtitration plate, 10-fold dilutions of bacterial supernates from 19 strains previously identified as O1 containing an unknown

Table 1. ELISA systems for identification of *Klebsiella* O antigens

Test for O antigen	LPS prepared from		Coating concentration (µg/ml)	Organism used to prepare O antiserum (working dilution)	Sensitivity (µg LPS/ml)*	Organism used to prepare antiserum for confirmation
	strain	serotype				
O1	Friedländer 201	O1:K-	2	Friedl. 201 (102400)	0.06	<i>E. coli</i> 8188/41
O2ab	7380	O2ab:K-	8	7380 (1600)	0.06	...
O2ac	5053	O2ac:K-	4	5053 (12800)	0.06	...
O3	390	O3:K11	4	390 (12800)	1.0	2002/49 (O3:K25)
O4	1702	O4:K42	8	1702 (400)	1.0	Mich. 61 (O4:K15)
O5	4425/51	O5:K57	4	4425/51 (25600)	0.25	5710/52 (O5:K61)
O7	264(1)	O7:K67	2	264(1) (12800)	0.125	...
O12	708	O12:K80	4	708 (25600)	0.25	...

*Defined as > 50% inhibition compared to control.

Fig. 1. Examples of the sensitivity and specificity of ELISA inhibition reactions: **a**, the solid phase antigen was LPS O1 from strain Friedländer 201 (2 µg/ml) in reaction with serum to *E. coli* 8188/41 at a final dilution of 800; **b**, the solid phase antigen was LPS O5 from strain 4425/51 in reaction with antiserum to the same strain at a dilution of 25 600. Inhibitors were LPS O1 (△), LPS O3 (●), LPS O5 (□).

amount of LPS were also tested. From the mean OD₄₀₅ produced by these supernates at an appropriate dilution, the concentration of LPS was calculated from the standard curve. LPS concentrations ranged between 135 and 790 µg/ml and were thus far above the maximum concentration of 1 µg/ml needed for > 50% inhibition in each of the reaction systems (Fig. 2b).

Confirmatory tests with additional O-specific antisera

Because LPS preparations were not completely free of contaminating capsular material, it could not be excluded that capsule-mediated inhibition might occur in some instances. Therefore, confirmatory reactions

Fig. 2. Quantitation of LPS in boiled supernates of *Klebsiella* O1 strains: **a**, antiserum against *E. coli* 8188/41 was used at a final dilution of 200 to achieve higher OD values for construction of a standard curve; **b**, concentrations of LPS O1 in 19 supernates as calculated from the standard curve (horizontal bars indicate mean and 95% confidence interval).

were performed in those O serogroups for which reference strains other than the O antigen prototype strain were available. Thus, sera were produced against *E. coli* 8188/41 (O19, identical with *Klebsiella* O1) [30], and *Klebsiella* strains 2002/49 (O3), Mich. 61 (O4) and 5710/52 (O5). Because these strains belonged to K types other than those from which the LPS preparations were made, inhibition produced with the respective sera could be assumed to be solely due to O antigen homology.

Typing of *Klebsiella* isolates

The validity of the typing method was evaluated in a pilot study of 82 clinical and laboratory *Klebsiella* isolates in which individual bacterial supernates were allowed to react with each of the LPS preparations and antisera available. As can be seen from the mean OD values summarised in Table 2, reactions were usually clear-cut with OD values > 0.3 for heterologous and < 0.1 for homologous antigens. In every instance, strains identified as belonging to serogroup O1 on the basis of a positive inhibition reaction with serum to strain Friedländer 201 also reacted with serum to *E. coli* 8188/41, confirming the identity of these two O antigens. Fourteen of these strains also completely inhibited the reaction of serum 7380 (O2ab) with LPS O2ab, thus justifying the designation of these strains as O1/2ab. A partial inhibition of the O2ab reaction was seen with five other strains belonging to serogroup O1. This partial inhibition could not be explained by a reduced concentration of LPS in the samples because all of these specimens contained > 100 µg of O1 LPS/ml as determined by quantitative inhibition ELISA. Rather, this phenomenon appeared to be due to a less intense expression, or even absence, of the O2ab epitope in these strains.

Twenty-four isolates were identified as belonging to serogroup O2; 13 of them showed complete inhibition with antiserum 7380 (O2ab) but no inhibition was seen with antiserum 5053 (O2ac). It should be stressed that these two sera were not cross-absorbed with the corresponding strains in order not to remove antibodies against the 'O2a' component. However, the reaction pattern of the sera showed that antibodies against 'O2a' were lacking in serum 5053 (O2ac), possibly

because the 2a component was masked by the immunodominant 'c' antigen. Consequently, strains identified as O2ac on the basis of a complete inhibition reaction with serum 5053 showed only insignificant inhibition with serum 7380 (O2ab).

Sixteen isolates reacted strongly with serum 2002/49 (O3) and were, therefore, assigned to serogroup O3. Interestingly, none of these strains reacted with the homologous serum produced with strain 390 (O3:K11) while strain 390 itself produced complete inhibition (data not shown). At present, we have no explanation for this phenomenon although it appears possible that the O3 antigen reference strain (strain 390) contains more than one O antigen epitope, and that clinical isolates, as well as strain 2002/49, lack one of these components.

In the four strains identified as serotype O5 by reaction with serum 4425/51, reactions with the confirmatory serum 5710/52 were also positive. Two strains belonging to serogroup O7 were identified by their reaction with serum 264(1). Since no confirmatory serum was available in this case, and since LPS O7 contained 4.4% CPS (K67), the capsule type of these strains was examined. The two isolates belonged to capsule serotypes K32 and K47, which excludes a CPS-mediated false-positive reaction. In the material examined, no strains of serogroups O4 and O12 were identified, and a total of 17 strains could not be assigned to a known O serogroup.

The relative frequency of individual O groups in different *Klebsiella* spp. is summarised in Table 3. The fact that all *K. rhinoscleromatis* strains belonged to the O2ab group is in complete accordance with the findings of Kauffmann [30]. He also examined three isolates of *K. ozaenae* which were found to belong to the O2ac group [30]. In this study, two *K. ozaenae* isolates contained the O2ab antigen and nine isolates the additional 'c' antigen (Table 3).

Discussion

Sero-epidemiological investigations of *Klebsiella* O antigens have been hampered by technical problems

Table 2. Reaction pattern obtained with supernates of 82 *Klebsiella* isolates

O group assigned	No. of isolates	Mean OD _{405 nm} (SD) produced with antiserum								
		Frdl. 201 (O1)	<i>E. coli</i> 8188/41 (O19)	7380 (O2ab)	5053 (O2ac)	2002/49 (O3)	1702 (O4)	4425/51 (O5)	264(1) (O7)	708 (O12)
O1/2ab	14	0.00 (0.00)	0.01 (0.00)	0.01 (0.01)	0.48 (0.13)	0.38 (0.06)	0.43 (0.15)	0.59 (0.13)	0.47 (0.09)	0.45 (0.08)
O1(2ab)	5	0.01 (0.00)	0.01 (0.02)	0.18 (0.09)	0.44 (0.13)	0.30 (0.06)	0.42 (0.06)	0.46 (0.02)	0.53 (0.14)	0.42 (0.02)
O2ab	13	0.59 (0.09)	0.45 (0.08)	0.02 (0.02)	0.41 (0.19)	0.32 (0.07)	0.38 (0.15)	0.48 (0.07)	0.49 (0.07)	0.44 (0.06)
O2ac	11	0.56 (0.05)	0.42 (0.03)	0.27 (0.08)	0.01 (0.02)	0.33 (0.09)	0.48 (0.11)	0.47 (0.07)	0.41 (0.04)	0.46 (0.04)
O3	16	0.47 (0.11)	0.39 (0.08)	0.37 (0.10)	0.53 (0.14)	0.02 (0.02)	0.39 (0.07)	0.45 (0.04)	0.39 (0.06)	0.47 (0.07)
O5	4	0.49 (0.07)	0.37 (0.03)	0.32 (0.08)	0.47 (0.07)	0.36 (0.08)	0.46 (0.12)	0.00 (0.00)	0.35 (0.08)	0.44 (0.05)
O7	2	0.60 (0.13)	0.47 (0.04)	0.46 (0.08)	0.41 (0.02)	0.29 (0.04)	0.37 (0.04)	0.48 (0.02)	0.03 (0.00)	0.35 (0.03)
None	17	0.54 (0.11)	0.38 (0.07)	0.31 (0.14)	0.47 (0.13)	0.35 (0.08)	0.37 (0.10)	0.56 (0.09)	0.48 (0.13)	0.43 (0.08)

Table 3. Distribution of O serogroups in individual *Klebsiella* spp.

Species	No. of isolates	Number of strains belonging to O group							
		O1/2ab	O1(2ab)	O2ab	O2ac	O3	O5	O7	NT
<i>K. pneumoniae</i>	22	3	1	...	2	7	...	...	9
<i>K. oxytoca</i>	24	8	1	1	...	9	4	...	1
<i>K. terrigena</i>	21	3	3	6	...	...	...	2	7
<i>K. ozaenae</i>	11	...	...	2	9	...	...	...	...
<i>K. rhinoscleromatis</i>	4	...	...	4	...	...	...	...	...
Total	82	14	5	13	11	16	4	2	17
Percentage	100	17	6	16	13	20	5	2	21

NT, non-typable.

associated with O antigen typing. While the O antigens of other enterobacteria are readily identified by simple slide agglutination tests [37, 38], *Klebsiella* strains are often O inagglutinable because of the copious capsule produced by most isolates [27, 30]. Furthermore, although O-specific antisera are usually produced by immunisation with boiled organisms, the capsular material may retain some of its immunogenicity. Thus, so-called O-specific sera in reality are OK-sera, and agglutination reactions may be due to K antigen homology. Therefore, Kauffmann [30] and Ørskov [18], who identified the first *Klebsiella* O antigens, produced their antisera with acapsular mutants. Furthermore, such acapsular mutants had also to be raised from the strains to be tested to allow agglutination to take place [18, 30].

Because isolation of acapsular mutants is both tedious and time-consuming, several investigators took advantage of the fact that *Klebsiella* O antigens are released into the medium during growth in broth. Thus, Fujita and Matsubara, with latex particles coated with O-specific antibodies and bacterial culture supernates for agglutination, were able to identify the O antigen in 328 of 361 clinical isolates of *Klebsiella* [19]. Others have used bacterial supernates to inhibit type-specific prototype reactions measured by passive haemagglutination [20] or ELISA [21, 29]. However, with the exception of our own preliminary study [29], these investigations were limited to serogroup O1.

In the present study, several ameliorations were made compared to previously described methods. Thus, while Alberti *et al.* precipitated the O antigen from bacterial supernates by means of the phenol-water method [21], we found that large amounts of O antigen could be released simply by boiling freshly harvested bacteria. By quantitative inhibition, 135–790 µg/ml of LPS were detectable in boiled supernates of O1 strains. Preliminary chessboard titrations of the antisera were used to dilute specific antibodies as much as possible and to minimise the amount of antigen coated on microtitration plates. By this means, a sensitivity of at least 1 µg of homologous LPS/ml was reached in each of the systems, making the method sensitive enough to detect LPS even in cases when other supernates would

contain somewhat lower amounts of LPS than the O1 strains tested.

In spite of the removal of CPS by cetavlon precipitation, final LPS preparations usually contained 2–5% (in two cases > 9%) CPS as judged by the glucuronic acid method. Therefore, the possibility of K antigen-based false-positive reactions was not completely excluded, necessitating cross-checking of each inhibition reaction with confirmatory sera raised against strains of heterologous K types. Because discrepancies between primary and confirmatory reactions were not found, it must be assumed that boiling of the strains used for immunisation and, additionally, of strains to be tested, sufficiently prevented K antigen-mediated reactions. In several instances, in particular O2ab and O2ac, reference strains containing homologous O antigens were not available. However, since antisera against serogroups O2ab and O2ac were raised with non-capsulate strains, positive reactions in these systems may be assumed to be due to O antigen homology.

Several novel observations were made when the typing system was applied to clinical isolates. Firstly, although 19 isolates were identified as belonging to serogroup O1, 14 of these also reacted strongly with the O2ab serum (Table 2). This contrasts with earlier studies by Kauffmann [30] and Fujita and Matsubara [19] who found a positive reaction with serum O2ab (produced with strain 7380 as in the present study) in only two of 121 strains of serogroup O1 [19]. However, it has been shown recently that the O1 antigen produced by a reference strain (O1:K20) contained two O antigen epitopes, identified as D-galactan I and II [28]. Immunologically, D-galactan I was found to correspond to the O2a antigen [28, 39]. The present study confirms that clinical isolates of serogroup O1 may in fact harbour an LPS epitope indistinguishable by conventional serology from that of O2a strains, and the designation O1/2a may be appropriate for these isolates. However, although only a limited number of strains was included in this study, five O1 strains were identified in which the O2a epitope was only poorly expressed or absent. Our previous study has demonstrated O1 strains that did

not contain a genus-specific common LPS epitope defined by a monoclonal antibody and otherwise expressed by most *Klebsiella* isolates [29]. Thus, the O1 serogroup appears to be antigenically heterogeneous, a phenomenon that has to be examined further by detailed immunochemical and molecular analyses.

Thirteen and 11 strains harboured the O2ab and O2ac antigens, respectively. Because the O2a antigen is the only true LPS antigen in strain 7380 while the 'b' antigen appears to arise from a different cellular component [39], the O serogroup of the first group of strains might also be expressed as O2a. Interestingly, although the two sera were not cross-absorbed in order not to remove 2a-specific antibodies, serum 5053 did not recognise O2a strains. Conversely, serum 7380 was inhibited only slightly by strains harbouring the O2c epitope. These findings are in complete accordance with Whitfield *et al.* who reported that the O2c antigen appeared to be associated with a longer polysaccharide chain structure than that in O2a and therefore it masks the O2a antigen in strains possessing both O antigen epitopes [39].

A peculiar observation was made in the system established to detect the O3 antigen. Antiserum raised against the reference strain 390 (O3:K11) reacted strongly with LPS extracted from this strain, and this reaction was inhibited both by purified LPS and the boiled supernate of strain 390. However, no clinical isolate reactive in this system was detected in a first series of experiments. Later, the confirmatory serum to strain 2002/49 (O3:K25) was found to react as well with LPS 390. Sixteen isolates exhibiting the O3 antigen were clearly identified in this system. At present, we have no ready explanation for this finding. However, since heterogeneous LPS antigens have been found in serogroups O1 and O2, it is possible that the serogroup O3 strains comprise more than one O antigen. The subgroup represented by the clinical isolates and strain 2002/49 might lack a partial antigen present in strain 390. This would explain the lack of inhibition of serum 390 by supernates of these isolates. Further studies involving a larger number of O3 strains from clinical material are clearly warranted to clarify this issue.

Although a few isolates could be assigned to serogroups O5 and O7, no strains reactive with O4 and O12 antisera were detected. In addition, 17 (21%) of the strains could not be O serogrouped, a percentage considerably larger than that reported for isolates from Japan [19]. It was interesting to note that non-typable isolates occurred rather frequently in the newly described species *K. terrigena* [33]. Further studies involving a larger number of isolates and including additional sera raised against non-typable strains will have to clarify whether other, hitherto unrecognised, O antigens may play a role. Also, such studies that are

currently under way in our laboratory will have to relate the expression of certain O antigens to the clinical disease state, thus allowing an assessment of the pathogenic potential of individual O antigens for the manifestation of invasive *klebsiella* infection.

We thank Dr E. Furer, Berne, Switzerland, for biochemical examinations of LPS preparations and J. Steiger for technical assistance.

References

- Horan T, Culver D, Jarvis W *et al.* Pathogens causing nosocomial infections. Preliminary data from the National Nosocomial Infections Surveillance System. *Antimicrob Newsletter* 1988; **5**: 65–67.
- Bodey GP, Elting LS, Rodriguez S, Hernandez M. *Klebsiella* bacteremia. A 10-year review in a cancer institution. *Cancer* 1989; **64**: 2368–2376.
- Feldman C, Smith C, Levy H, Ginsburg P, Miller SD, Koornhof HJ. *Klebsiella pneumoniae* bacteraemia at an urban general hospital. *J Infect* 1990; **20**: 21–31.
- Gouby A, Neuwirth C, Bourg G *et al.* Epidemiological study by pulsed-field gel electrophoresis of an outbreak of extended-spectrum β -lactamase-producing *Klebsiella pneumoniae* in a geriatric hospital. *J Clin Microbiol* 1994; **32**: 301–305.
- Geerdes HF, Ziegler D, Lode H *et al.* Septicemia in 980 patients at a University hospital in Berlin: prospective studies during 4 selected years between 1979 and 1989. *Clin Infect Dis* 1992; **15**: 991–1002.
- Cryz SJ, Pitt TL, Furer E, Germanier R. Role of lipopolysaccharide in virulence of *Pseudomonas aeruginosa*. *Infect Immun* 1984; **44**: 508–513.
- Pennington JE, Pier GB, Sadoff JC, Small GJ. Active and passive immunization strategies against *Pseudomonas aeruginosa* pneumonia. *Rev Infect Dis* 1986; **8** Suppl 4: S426–S433.
- Cryz SJ, Furer E, Cross AS, Wegmann A, Germanier R, Sadoff JC. Safety and immunogenicity of a *Pseudomonas aeruginosa* O-polysaccharide toxin A conjugate vaccine in humans. *J Clin Invest* 1987; **80**: 51–56.
- Cross A, Artenstein A, Que J *et al.* Safety and immunogenicity of a polyvalent *Escherichia coli* vaccine in human volunteers. *J Infect Dis* 1994; **170**: 834–840.
- Casewell M, Talsania HG. Predominance of certain *Klebsiella* capsular types in hospitals in the United Kingdom. *J Infect* 1979; **1**: 77–79.
- Ullmann U. The distribution of *Klebsiella pneumoniae* serotypes from different sources and their sensitivity to cephalosporins. *Infection* 1983; **11** Suppl 1: S28–S31.
- Podschun R, Heineken P, Ullmann U, Sonntag HG. Comparative investigations of *Klebsiella* species of clinical origin: plasmid patterns, biochemical reactions, antibiotic resistance and serotypes. *Zentralbl Bakteriol Mikrobiol Hyg A* 1986; **262**: 335–345.
- Simoons-Smit AM, Verweij-Van Vught AMJJ, MacLaren DM. The role of K antigens as virulence factors in *Klebsiella*. *J Med Microbiol* 1986; **21**: 133–137.
- Held TK, Trautmann M, Mielke MEA, Neudeck H, Cryz SJ, Cross AS. Monoclonal antibody against *Klebsiella* capsular polysaccharide reduces severity and hematogenic spread of experimental *Klebsiella pneumoniae* pneumonia. *Infect Immun* 1992; **60**: 1771–1778.
- McCallum KL, Schoenhals G, Laakso D, Clarke B, Whitfield C. A high-molecular-weight fraction of smooth lipopolysaccharide in *Klebsiella* serotype O1:K20 contains a unique O-antigen epitope and determines resistance to nonspecific serum killing. *Infect Immun* 1989; **57**: 3816–3822.
- Tomas JM, Benedi VJ, Ciurana B, Jofre J. Role of capsule and O antigen in resistance of *Klebsiella pneumoniae* to serum bactericidal activity. *Infect Immun* 1986; **54**: 85–89.
- Straus DC, Atkisson DL, Garner CW. Importance of a lipopolysaccharide-containing extracellular toxic complex in infections produced by *Klebsiella pneumoniae*. *Infect Immun* 1985; **50**: 787–795.

18. Ørskov I. O antigens in the *Klebsiella* group. *Acta Pathol Microbiol Scand* 1954; **34**: 145–156.
19. Fujita S, Matsubara F. Latex agglutination test for O serotyping of *Klebsiella* species. *Microbiol Immunol* 1984; **28**: 731–734.
20. Mizuta K, Ohta M, Mori M, Hasegawa T, Nakashima I, Kato N. Virulence for mice of *Klebsiella* strains belonging to the O1 group: relationship to their capsular (K) types. *Infect Immun* 1983; **40**: 56–61.
21. Alberti S, Hernández-Allés S, Gil J *et al.* Development of an enzyme-linked immunosorbent assay method for typing and quantitation of *Klebsiella pneumoniae* lipopolysaccharide: application to serotype O1. *J Clin Microbiol* 1993; **31**: 1379–1381.
22. Ørskov I, Fife-Asbury MA. New *Klebsiella* capsular antigen, K82, and the deletion of five of those previously assigned. *Int J Syst Bacteriol* 1977; **27**: 386–387.
23. Kaluzewski S. Somatic antigens of *Klebsiella* strains K63-K72. *Med Dosw Mikrobiol* 1965; **17**: 283–290 (in Polish).
24. Nimmich W, Korten G. Die chemische Zusammensetzung der *Klebsiella*-Lipopolysaccharide (O-Antigene). *Pathologia et Microbiologia* 1970; **36**: 179–190.
25. Kenne L, Lindberg B. Bacterial polysaccharides. In: Aspinall GO (ed) *The polysaccharides*, vol 2. Orlando, Academic Press, 1983: 287–363.
26. Kelly RF, Severn WB, Richards JC *et al.* Structural variation in the O-specific polysaccharides of *Klebsiella pneumoniae* serotype O1 and O8 lipopolysaccharide: evidence for clonal diversity in *rfb* genes. *Mol Microbiol* 1993; **10**: 615–625.
27. Ørskov I, Ørskov F. Serotyping of *Klebsiella*. *Methods Microbiol* 1984; **14**: 143–164.
28. Whitfield C, Richards JC, Perry MB, Clarke BR, MacLean LL. Expression of two structurally distinct D-galactan O antigens in the lipopolysaccharide of *Klebsiella pneumoniae* serotype O1. *J Bacteriol* 1991; **173**: 1420–1431.
29. Trautmann M, Vogt K, Hammack C, Cross AS. A murine monoclonal antibody defines a unique epitope shared by *Klebsiella* lipopolysaccharides. *Infect Immun* 1994; **62**: 1282–1288.
30. Kauffmann F. On the serology of the *Klebsiella* group. *Acta Pathol Microbiol Scand* 1949; **26**: 381–406.
31. Cross AS, Gemski P, Sadoff JC, Ørskov F, Ørskov I. The importance of the K1 capsule in invasive infections caused by *Escherichia coli*. *J Infect Dis* 1984; **149**: 184–193.
32. Podschun R. Phenotypic properties of *Klebsiella pneumoniae* and *K. oxytoca* isolated from different sources. *Zentralbl Hyg* 1990; **189**: 527–535.
33. Podschun R, Ullmann U. Isolation of *Klebsiella terrigena* from clinical specimens. *Eur J Clin Microbiol Infect Dis* 1992; **11**: 349–352.
34. Westphal O, Jann K. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure. *Methods Carbohydr Chem* 1965; **5**: 83–91.
35. Cryz SJ, Fürer E, Germanier R. Purification and vaccine potential of *Klebsiella* capsular polysaccharides. *Infect Immun* 1985; **50**: 225–230.
36. Blumenkrantz N, Asboe-Hansen G. New method for quantitative determination of uronic acids. *Anal Biochem* 1973; **54**: 484–489.
37. Gransden WR, Eykyn SJ, Phillips I, Rowe B. Bacteremia due to *Escherichia coli*: a study of 861 episodes. *Rev Infect Dis* 1990; **12**: 1008–1018.
38. Kasama H. Serological classification of *Pseudomonas aeruginosa* by a slide agglutination test. *J Clin Microbiol* 1978; **8**: 181–188.
39. Whitfield C, Perry MB, MacLean LL, Yu SH. Structural analysis of the O-antigen side chain polysaccharides in the lipopolysaccharides of *Klebsiella* serotypes O2(2a), O2(2a, 2b), and O2(2a, 2c). *J Bacteriol* 1992; **174**: 4913–4919.