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Immunochemical characterisation of *Vibrio cholerae* O139 O antigens and production of a diagnostic antiserum without absorption

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Rabbits and mice immunised with chemically extracted O-antigens (O-Ags) of *Vibrio cholerae* O139 (O-AgB and O-AgD) developed antibodies (Abs) which appeared to be highly specific in ELISA for the relevant antigens and *V. cholerae* O139 strains without absorption, in contrast to the Abs against the heated O-Ag (O-AgH). An ELISA test based on the use of these Abs was shown to detect *V. cholerae* O139 strains down to concentrations of (9.4×10^4)–(7.5×10^5) vibrios/ml and demonstrated no cross-reaction with other vibrios including representatives of serogroup O22. Native and proteinase K-treated O-AgB, O-AgD, O-AgH, as well as whole-cell lysates of *V. cholerae* O139 strains of different origin were used in immunoblotting with these Abs. Clear differences in the patterns of zones of specific reaction between chemically extracted and heated O-Ags and between lipopolysaccharide profiles of the *V. cholerae* O139 strains of different origin were observed. Serogroup-specific protein bands in the native O-AgB and O-AgD preparations were defined. The approach described for obtaining serogroup-specific Abs against vibrios and other bacteria seems to be promising for the development of specific diagnostic tests and further investigation of bacterial antigenic structure.

Introduction

One of the methods used for accurate identification of *Vibrio cholerae* is serotyping, which allows the differentiation of the vibrios on the basis of their heat-stable antigens [1–5]. It is supposed that this O antigen (O-Ag) corresponds to the polysaccharide side chains of lipopolysaccharide (LPS) [3, 5] and represents the main chemotaxonomic marker for most of the bacteria [6, 7]. Being simple, rapid and inexpensive, serotyping is used successfully for differentiation of vibrios within the Vibrionaceae and for epidemiological typing in outbreaks caused by *V. cholerae* O1 or O139, the second known aetiological agent of cholera [1]. Recently, diagnostic antiserum against *V. cholerae* O139 strain was obtained by immunisation of rabbits with heat-stable O139 antigen [2]. However, when tested for specificity in the agglutination test it reacted with *V. cholerae* O22 reference strain 169-68 [2, 4] and so for practical use needed special absorption.

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The current study describes a new mild method of O-Ag extraction from *V. cholerae* O139. When used for immunisation, the prepared O-Ag induced highly specific antiserum which did not cross-react with other *V. cholerae* strains, including O22. The antibodies purified from this antiserum were used for development of a rapid, cheap, strictly specific ELISA test for detection of *V. cholerae* O139.

Materials and methods

Bacterial strains

V. cholerae strains used in this study are listed in Table 1. Another 29 strains of other bacteria (*Escherichia coli*, 10; *Shigella* spp., 7; *Salmonella* spp., 6; *Proteus* spp., 2; *Staphylococcus* spp., 4) were also employed in this study. The growth medium for *V. cholerae* strains was Luria-Bertani broth (LB) or LB agar. The vibrios were grown at 37°C for 18 h. *V. cholerae* O139 strain P16064 was also grown in an M-19 apparatus (Brunswick) at 37°C, pH 7.8 ± 0.2, in a broth containing tryptic hydrolysate of casein (amino-nitrogen 0.35%, NaCl 0.5%, disubstituted sodium phosphate 0.05%) with aeration and glucose supplementation. The

Table 1. *V. cholerae* strains used in this study

Strain	Description	Reference or source
V. cholerae O139		
MDO25	Toxigenic clinical isolate from India	34
MDO12	Toxigenic clinical isolate from India	23, 35
SG25	Toxigenic clinical isolate from India	23, 36
VO7	Toxigenic clinical isolate from India	23, 36
PO2	Toxigenic clinical isolate from India	23, 36
SG24	Toxigenic clinical isolate from India	23, 36
VO15	Toxigenic clinical isolate from India	23, 36
AP1	Toxigenic clinical isolate from India	23, 36
AP2	Toxigenic clinical isolate from India	34
VO2	Toxigenic clinical isolate from India	23, 36
MO28	Toxigenic clinical isolate from India	34
PO7	Toxigenic clinical isolate from India	34
MO45	O139 epidemic reference strain	2, 25, 37
62	Toxigenic clinical isolate from India	34
65	Toxigenic clinical isolate from India	34
71	Toxigenic clinical isolate from India	34
88	Toxigenic clinical isolate from India	34
P16064	Toxigenic 1993 clinical isolate from a patient (Azov Hospital, Rostov, Russia), imported case from India	4, 38
P16065	Toxigenic 1993 clinical isolate from a patient (Azov Hospital, Rostov, Russia), imported case from India	4
P16131	Toxigenic 1993 clinical isolate from a patient (Azov Hospital, Rostov, Russia), imported case from India	34, Institute 'Microbe' Collection
1311	Toxigenic clinical isolate from Kirghizstan, imported case from Pakistan	34, Institute 'Microbe' Collection
1306	Toxigenic clinical isolate from Kirghizstan, imported case from Pakistan	34, Institute 'Microbe' Collection
V. cholerae non O1, non-O139		
NCTC4711	O2 reference strain	R. Sakazaki
B4202-64	O5 reference strain	R. Sakazaki
10317	O8 reference strain	R. Sakazaki
218-68	O10 reference strain	R. Sakazaki
B8645-64	O14 reference strain	R. Sakazaki
223-68	O16 reference strain	R. Sakazaki
10332-62	O20 reference strain	R. Sakazaki
109-68	O21 reference strain	R. Sakazaki
169-68	O22 reference strain	2
14438-62	O24 reference strain	R. Sakazaki
10438-62	O27 reference strain	R. Sakazaki
5473-62	O31 reference strain	R. Sakazaki
5473-62	O38 reference strain	R. Sakazaki
P912	Clinical O45 isolate from Russia, 1968	Institute 'Microbe' Collection
P1114	Clinical isolate O47 from Russia, 1968	Institute 'Microbe' Collection
V. cholerae O1		
569B	Clinical isolate from India, 1960, serotype Inaba, biotype classical	4, 23, 38
B53-1	Isolated in India in 1962, serotype Inaba, biotype classical	Institute 'Microbe' Collection
35-A-3	Clinical isolate from India, 1960, serotype Inaba, biotype classical	Institute 'Microbe' Collection
1488A	Isolated from India, 1937, serotype Inaba, biotype classical	Institute 'Microbe' Collection
71	Isolated in Korea, 1946, serotype Inaba, biotype classical	Institute 'Microbe' Collection
325	Isolated in India, 1946, serotype Inaba, biotype classical	Institute 'Microbe' Collection
M59	Clinical isolate from the USSR, 1948, serotype Inaba, biotype classical	Institute 'Microbe' Collection
266	Isolated in India, 1949, serotype Inaba, biotype classical	Institute 'Microbe' Collection
P-1	Isolated in India, 1958, serotype Inaba, biotype classical	Institute 'Microbe' Collection
M818	Clinical isolate from the USSR, 1970, serotype Inaba, biotype El Tor	Institute 'Microbe' Collection
M1042	Clinical isolate from the USSR, 1970, serotype Inaba, biotype El Tor	Institute 'Microbe' Collection

Table 1. (*Continued*).

Strain	Description	Reference or source
15/66	Isolated in Pakistan, 1966, serotype Inaba, biotype El Tor	Institute 'Microbe' Collection
M311	Clinical isolate from the USSR, 1966, serotype Inaba, biotype El Tor	Institute 'Microbe' Collection
M415	Clinical isolate from the USSR, 1967, serotype Inaba, biotype El Tor	Institute 'Microbe' Collection
9352	Isolated in Pakistan, 1969, serotype Inaba, biotype El Tor	Institute 'Microbe' Collection
M704	Clinical isolate from the USSR, 1970, serotype Inaba, biotype El Tor	Institute 'Microbe' Collection
M871	Clinical isolate from the USSR, 1971, serotype Inaba, biotype El Tor	Institute 'Microbe' Collection
M874	Clinical isolate from the USSR, 1971, serotype Inaba, biotype El Tor	Institute 'Microbe' Collection
M47	Isolated in 1946, serotype Ogawa, biotype classical, source unknown	Institute 'Microbe' Collection
M35	Isolated in the USSR in 1943, serotype Ogawa, biotype classical	Institute 'Microbe' Collection
267	Isolated in India, 1946, serotype Ogawa, biotype classical	Institute 'Microbe' Collection
1001	Isolated in China, 1949, serotype Ogawa, biotype classical	Institute 'Microbe' Collection
16017	Isolated in India, 1954, serotype Ogawa, biotype classical	Institute 'Microbe' Collection
C79	Isolated in Cambodia, 1958, serotype Ogawa, biotype classical	Institute 'Microbe' Collection
3 Dhaka	Clinical isolate from Pakistan, 1958, serotype Ogawa, biotype classical	Institute 'Microbe' Collection
41	Clinical isolate from India, 1960, serotype Ogawa, biotype classical	Institute 'Microbe' Collection
6	Clinical isolate from Afghanistan, 1960, serotype Ogawa, biotype classical	Institute 'Microbe' Collection
M1284	Clinical isolate from the USSR, 1983, serotype Ogawa, biotype El Tor	Institute 'Microbe' Collection
4564/4	Clinical isolate from the USSR, 1970, serotype Ogawa, biotype El Tor	Institute 'Microbe' Collection
Malaka 1	Clinical isolate from India, 1963, serotype Ogawa, biotype El Tor	Institute 'Microbe' Collection
4/64	Isolated in Pakistan, 1964, serotype Ogawa, biotype El Tor	Institute 'Microbe' Collection
M309	Clinical isolate from Afghanistan, 1965, serotype Ogawa, biotype El Tor	Institute 'Microbe' Collection
M230	Clinical isolate from the USSR, 1965, serotype Ogawa, biotype El Tor	Institute 'Microbe' Collection
6/67	Isolated in India, 1967, serotype Ogawa, biotype El Tor	Institute 'Microbe' Collection
M885	Clinical isolate from the USSR, 1970, serotype Ogawa, biotype El Tor	Institute 'Microbe' Collection
9341	Isolated in Pakistan, 1969, serotype Ogawa, biotype El Tor	Institute 'Microbe' Collection

broth culture was then centrifuged and the supernate and cells were disinfected separately by the addition of formaldehyde 0.6% and 2%, respectively, and used for O-Ag extraction.

O-Ag preparations

Three modifications of O-Ag extraction from *V. cholerae* O139 strain P16064 were used. The Boivin procedure [8] was employed for extraction of O-AgB. Briefly, 0.5 N trichloroacetic acid was added 1:1 v:v to the vibrio cell suspension in phosphate-buffered saline (PBS). Then, O-AgB was precipitated by the addition of cold (−30°C) ethanol 96% v/v with subsequent lyophilisation.

The method of Dzhaparidze *et al*. [9] was used for extraction of O-AgD. Briefly, a cell-free formaldehyde-treated culture supernate was treated with ammonium sulphate 25–50% w/v saturated solution, dialysed, purified by gel filtration through TSK-gel HW-60 and HW-75 and lyophilised.

The Shimada procedure [2] was used for preparation of O-AgH. Briefly, the broth culture was heated at 100°C for 2 h and centrifuged at 2000 *g* for 15 min. The pellet was washed twice with saline and resuspended in saline to twice the original volume.

The Boivin method [8] was also used for extraction of O-Ags from *V. cholerae* O1 reference strains – *V.*

cholerae 569B and *V. cholerae* El Tor M-818. The method of Shimada *et al*. [2] was employed for preparing O-Ags from *V. cholerae* O139 strains MO45, P16065, MDO12, 1306 and O22 strain 169-68.

Specific immunoglobulins and antisera

Different groups of 6–8-week-old female BALB/mice, 50 animals in each, treated intraperitoneally with 0.5 ml of 'Pristane' (Sigma) were given five intraperitoneal doses, at 2-week intervals, of 25–50 μg of O-AgB, O-AgD or 2×10^8 cells of *V. cholerae* O139 P16064 or MO45 strains killed with sodium merthiolate at a concentration of 1 in 10 000. On the third day after the last immunisation the mice were inoculated intraperitoneally with 1.5×10^6 cells of the myeloma cell line Sp2/0-Ag 14 and ascitic fluids containing specific antibodies were obtained. Rabbits (six animals) were given five intravenous injections of 0.2, 0.3, 0.5 and 1.0 mg of O-AgB separated by 3-day intervals and were exsanguinated 7 days after the last dose. The same schedule was used for immunisation of a group of rabbits (five animals) with O-AgD. Immunoglobulins were isolated from the ascitic fluids and sera by precipitation with ammonium sulphate 45% w/v saturated solution followed by purification through Sephadex G-50.

The commercial reference horse absorbed polyvalent O1, RO and monospecific Inaba and Ogawa antisera (Institute 'Microbe', Russia) were employed for studying O-Ag specificity.

SDS-PAGE and immunoblotting

LPS profiles of proteinase K-treated (PK-treated) whole-cell lysates of *V. cholerae* strains (5.0×10^8 microbial cells/lane) [10] and O-Ags extracted from *V. cholerae* O139 (10 μg/lane) were determined by SDS-PAGE in acrylamide 12.5% gels [11]. The protein mol. wt markers included cytochrome C (12.5 kDa), trypsin inhibitor (soybean; 21.5 kDa), carbonic anhydrase (30 kDa), ovalbumin (46 kDa), bovine serum albumin (69 kDa) and lysozyme (14.3 kDa) (Amersham). The gels were either silver stained according to Tsai and Frasch [12] or electrotransferred to nitrocellulose for Western blot analysis [13]. Specific bands were visualised with peroxidase-labelled mouse or rabbit IgG to *V. cholerae* O139 O-AgB [14].

ELISA

Indirect ELISA. This method was used for studying the specificity of the O-AgB and O-AgD preparations and the relevant immunoglobulins. Briefly, ELISA plates (96-well) were sensitised (/well) with 100 μl of each preparation at a concentration of 10 μg/ml of PBS or of suspension containing 1×10^9 cells of different strains of *V. cholerae* in 1 ml of PBS and incubated overnight at 4°C. The wells were washed three times with washing buffer (PBS containing Tween-20 0.05%). The wells were then saturated with bovine serum albumin (BSA) 1% in PBS for 1 h at 37°C and washed as above. Then, dilutions of mouse or rabbit IgG against O-AgB or O-AgD of 1.0 mg–1 ng/ml of PBS were added and the plates were re-incubated for 1 h at 37°C. After washing, peroxidase-labelled rabbit anti-mouse or sheep anti-rabbit IgG (Gamaleya Institute, Russia), respectively, diluted in diluting buffer (BSA 1% in PBS) were added and the plates were incubated for 1 h at 37°C. The wells were washed six times. As a substrate, 100 μl of 2,2′-azinobis(3-ethylbenzthiazoline sulphonic acid) (Sigma) 22.3 mg/ml in 0.05 mM citrate buffer (pH 4.0) with H_2O_2 0.0003% was used. The OD_{405} was measured in a Titertec Multiscan spectrophotometer (Flow Laboratories). An OD_{405} of 0.2 above background was considered a positive result. Normal horse serum (Serva) was used as a negative control.

Direct double-antibody sandwich ELISA. Doubling dilutions of *V. cholerae* O1 and O139 O-AgB and O-AgD 1.0 mg–1 ng/ml and suspensions of *V. cholerae* strains, (2×10^9)–(2×10^3) microbial cells, were added to wells previously coated with 100 μl of antibodies (Abs) to O-AgD at 10 μg/ml in PBS. Then, 100 μl of peroxidase-labelled mouse or rabbit Abs to O-AgD were added to each well. The wells were washed, blocked and screened as described above.

Direct double-antibody sandwich ELISA. This approach was also used for studying antibody response to protein or carbohydrate parts of O-AgB. Briefly, suspensions of *V. cholerae* strains P16064, P16131, 169-68, 569B, M818 and M47 at a concentration of 1×10^9 microbial cells/ml of PBS, native and PK-digested, and O-AgB at a concentration of 0.1 mg/ml of PBS, native or PK-digested, were added to wells of 96-well ELISA plates previously coated with 100 μl of Abs to O-AgB at 10 μl/ml in PBS in triplicate. Then, 100 μl of peroxidase-labelled mouse or rabbit Abs to O-AgD were added to each well. The wells were washed, blocked and screened as described above.

Results

Chemical characteristics of O-AgB and O-AgD

O-AgB and O-AgD were found to possess similar chemical composition, but O-AgB contained more protein and lipid and less carbohydrate (Table 2).

Serological reactivity of O-Ags and Abs

In indirect ELISA, O-AgB and O-AgD demonstrated no positive reaction with commercial antisera O, RO, Inaba and Ogawa. At the same time, Abs to O-AgB and O-AgD were absolutely specific for *V. cholerae* O139 strains (Table 3), as well as for the O-Ags extracted from the relevant strains. No reaction of Abs

Table 2. Chemical analysis of O-AgB and O-AgD extracted from *V. cholerae* O139 strain P16064, percentages of dried mass

Antigen	Protein	Carbohydrate	Nucleic acids	KDO	Glucosamine	Aldoheptose	Lipids
O-AgB	48.0 ± 0.6	30.0 ± 0.1	5.0 ± 0.8	4.0 ± 0.2	2.3 ± 0.3	5.5 ± 0.4	17.0 ± 2.0
O-AgD	35.0 ± 0.3	38.0 ± 0.5	4.0 ± 0.2	3.5 ± 0.2	2.0 ± 0.2	5.0 ± 0.6	12.0 ± 1.4

Table 3. Specificity of murine Abs to *V. cholerae* O139 O-AgB and O-AgD and murine Abs to the whole cells of *V. cholerae* O139 strains in indirect ELISA

Bacteria tested	Number of strains	Percentage of positive reactions with murine Abs to *V. cholerae* O139			
				Whole cells	
		O-AgB	O-AgD	P16064	MO45
V. cholerae O1	36	0	0	100	100
V. cholerae non-O1	14	0	0	23	23
V. cholerae O139	22	100	100	100	100
V. cholerae O22	1	0	0	0	0
E. coli	10	0	0	40	40
Shigella spp.	7	0	0	0	0
Salmonella spp.	6	0	0	0	0
Proteus spp.	2	0	0	0	0
Staphylococcus spp.	4	0	0	0	0

to O-AgB and O-AgD with O-Ags of *V. cholerae* O1 (Inaba and Ogawa of both biotypes, classical and El Tor) was registered.

Development of ELISA

In the sandwich ELISA, as well as in the indirect ELISA (Table 3), only the 22 *V. cholerae* strains belonging to O139 serogroup were detected; the other bacteria were not. Table 4 shows the minimal concentration of O139 vibrios/ml detected by this method.

Table 4. Minimum concentration of vibrios/ml detected in ELISA

V. cholerae O139 strains	Concentration of vibrios, microbial cells/ml, detected
MDO25	7.5×10^5
62	7.5×10^5
MDO12	7.5×10^5
71	3.8×10^5
P16031	7.5×10^5
SG25	3.8×10^5
VO7	1.5×10^6
55	1.8×10^5
P16065	7.5×10^5
PO2	7.5×10^5
SG24	7.5×10^5
VO15	9.4×10^4
AP2	3.8×10^5
1311	3.8×10^5
88	7.5×10^5
VO2	3.8×10^5
AP1	3.8×10^5
1306	3.8×10^5
P16064	7.5×10^5
MO45	1.5×10^6
MO28	1.5×10^6
PO7	1.5×10^5

Antibody response to protein or carbohydrate parts of O-AgB

In the sandwich ELISA, positive reactions were obtained only with *V. cholerae* O139 strains and native and PK-digested O-AgB. The OD_{405} means for native whole cells were three or four times higher than those of PK-digested cells. However, in both cases the OD_{405} means were significantly (7.7–9.1-fold) higher than the negative control. The OD_{405} means for both native and PK-digested O-AgB were also markedly (4.9-fold) higher than the negative control. There was no reaction with other vibrios (Table 5).

SDS-PAGE and immunoblotting

LPS profiles of the PK-digested O139 strains tested were very similar to each other and possessed two predominant higher and lower mol.-wt components differing by their length. None of the strains produced a ladder pattern typical of *V. cholerae* O1, but a few (two or three) bands of intermediate mol. wt appeared

Table 5. Comparison of specific interactions of native or PK-digested O-AgB and *V. cholerae* O1 and non-O1 strains in direct sandwich ELISA

Strain	OD_{405} mean (SD)	
	Native	PK-digested
V. cholerae P16064	1560.0 (214.3)	383.0 (41.3)
V. cholerae P16131	997.3 (77.3)	322.3 (88.1)
V. cholerae 169-68	41.6 (0.4)	42.3 (0.4)
V. cholerae 569B	41.3 (0.4)	41.3 (0.4)
V. cholerae M818	42.0 (0.7)	43.3 (0.4)
V. cholerae M47	42.0 (0)	42.6 (0.4)
O-AgB	271.3 (15.3)	205.0 (15.1)
PBS (negative control)	42.0 (0)	42.0 (0)

consistently for each strain (Fig. 1). There were no whole-cell lysates in which only lower mol.-wt components were visualised. In immunoblotting with murine Abs, a specific reaction was readily observed only with low mol.-wt bands similar to those of LPS molecules without O-chains, although some differences between the strains were noted (Fig. 2). The zones of specific reaction in *V. cholerae* strains P16064, P16065, MDO12 and SG24 were larger than those in the other strains, icluding the reference strain MO45. The zones of specific reaction of Abs with O-AgH isolated from the same strains were significantly smaller. The profiles of O-AgB and O-AgD were similar in size and intensity with the zones of specific reaction of the relevant strains tested (Fig. 3). No reaction with *V. cholerae* O22 O-AgH was observed. Abs gave strongly positive immunoblotting with rapidly migrating carbohydrate zones and two peptide bands in native (not PK-digested) O-AgB, O-AgD (53.7 and 36.3 kDa) and the whole-cell lysates of *V. cholerae* O139 strains (69.2 and 20.0 kDa).

Rabbit Abs gave the same patterns in immunoblotting with both native or PK-digested whole-cell lysates and O-Ags.

Discussion

V. cholerae is classified on the basis of its somatic antigens (O-Ags) into serovars or serogroups [3, 5, 15]. At present at least 155 serogroups of *V. cholerae* are known [1]; of these 154 are of non-O1 vibrios that are non-agglutinable with polyvalent O1 antiserum. Within each serogroup the vibrios are agglutinated with a homologous O antiserum. To prepare O antiserum, rabbits are immunised with the cultures heated at 100°C for 2 h. For practical use, the diagnostic antiserum must be absorbed with some *V. cholerae* strains to remove cross-reacting agglutinins [3, 5]. Reference strain 169-68, representing serogroup O22, is used for absorption of O139-specific antiserum [2, 4], because the O-Ag of this strain is closely related to that of strain O139 [2]. However, they are not completely identical [2], as the homology of *rfb* genes of *V. cholerae* O22 and O139 is 91% [16]. In this study, O-Ags extracted by the classical Boivin method [8] and by the original Dzhaparidze method [9] were found to bear the epitopes specific for *V. cholerae* O139, because immunisation of both rabbits and mice with these preparations induced production of specific Abs which gave no cross-reaction with other vibrios, including

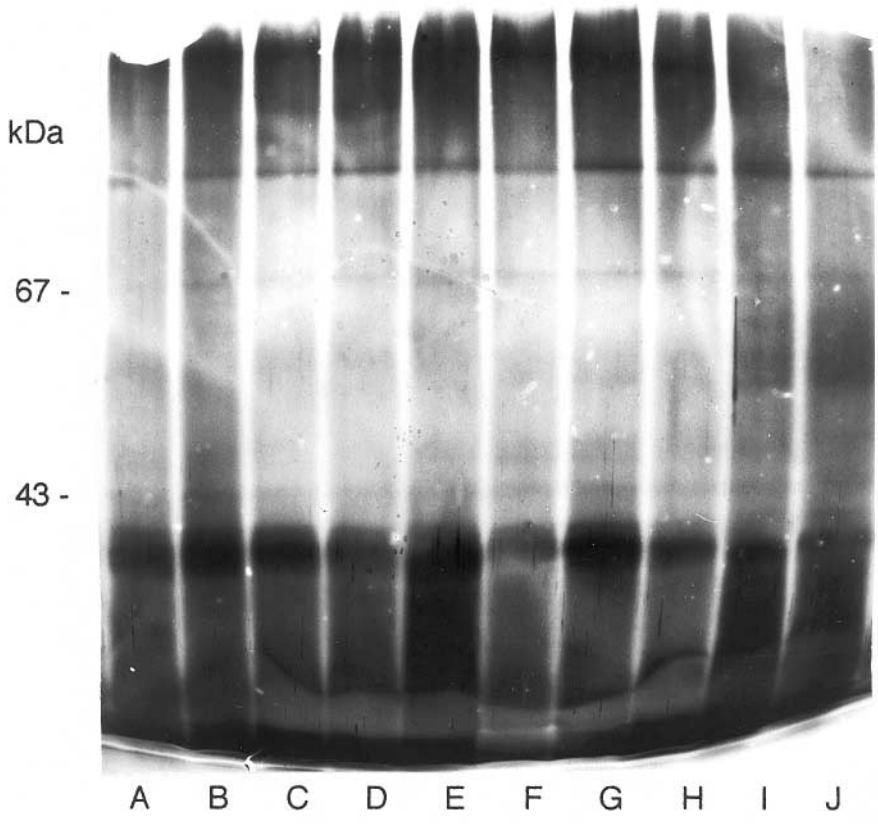

Fig. 1. SDS-PAGE of PK-digested whole-cell lysates of *V. cholerae* O139 strains. Lane **A**, 1306; **B**, MO28; **C**, AP1; **D**, AP2; **E**, VO20; **F**, 1311; **G**, PO7; **H**, 88; **I**, MO45; **J**, P16064. Number on the left indicates mol. wt of the standard proteins employed.

Fig. 2. Immunoblotting of PK-digested O-Ags and whole-cell lysates of *V. cholerae* O139 with mouse Abs to O-AgB. Lane **A**, P16163; **B**, 70; **C**, SG24; **D**, 1306; **E**, MDO12; **F**, P16065; **G**, 55; **H**, MO45, **I**, P16064, **J**, O-AgD; **K**, O-AgB.

Fig. 3. Immunoblotting of O-Ags and whole-cell lysates of *V. cholerae* O139 (lanes **G–K**) and O22 (lane **A**) with Abs. Lane **A**, O-AgH (169-68); **B**, O-AgH (1306); **C**, O-AgH (MDO12); **D**, O-AgH (P16065); **E**, O-AgH (MO45); **F**, O-AgH (P16064); **G**, 1306; **H**, MDO12; **I**, P16065; **J**, MO45; **K**, P16064; **L**, O-AgD; **M**, O-AgB.

strain O22, without any absorption (Table 3). This allowed the development of a highly specific and sensitive ELISA for detection of *V. cholerae* O139 with 100% efficacy. These data were confirmed by the absence of reaction of Abs with O22 antigen in immunoblotting (Fig. 3, lane A).

It is known that immunisation with LPS or wild types of enteropathogenic bacteria induces an antibody response against long 'O' polysaccharide side-chains [17] determining the serological specificity of the bacteria but not against core saccharide or lipid A which bear some common group-specific epitopes [18, 19]. In the native LPS molecule, these epitopes are covered or masked by the 'O' polysaccharide chain and are not available for interaction with the effectors of the immune system [17]. Unmasking of these epitopes occurs either after LPS hydrolysis by heating at 100°C for 4–6 h in dilute acid, or in acid-free conditions after incubation for 10–20 min at different temperatures (20–100°C) (mild hydrolysis) [20–22]. Thus, preparing O-Ag by heating *V. cholerae* cultures at 100°C for 2 h possibly leads to partial degradation of poly- or oligo-saccharides and unmasking of group-

specific epitopes. These are the epitopes that can induce antibodies cross-reacting with the antigens of closely related micro-organisms following immunisation. This is supported by the observation that the antiserum against O-AgH reacted with two strains, *V. cholerae* O139 and O22, whereas the antiserum against chemically extracted O-Ag reacted only with *V. cholerae* O139 strains. Moreover, the antiserum against *V. cholerae* O139 whole cells reacted with the relevant strain and a number of strains of enteropathogenic bacteria, but not with *V. cholerae* O22 (Table 3). This may mean that induction of the antibody response against the common epitopes of the strains in question becomes possible only after unmasking of these epitopes in the heated antigens, which are partially degraded carbohydrate components of *V. cholerae* O139. This degradation may be a result of heating for 2 h at 100°C (mild hydrolysis). Indeed, the hot phenol-water technique has been shown to provide partial degradation of LPS during the isolation procedure [20, 21].

Additional evidence for this supposition is the difference in the pattern of the zones of specific interaction of the O-AgsH and the LPS profiles of the relevant strains in immunoblotting. In the first case, the zones of specific interaction had significantly less length and intensity than those of the LPS profiles, especially in the strains isolated in Russia (Fig. 3, lanes D and F or I and K, respectively). At the same time, the pattern of the zones of specific interaction of the chemically isolated O-Ags (O-AgB and O-AgD) was similar to that of the LPS profiles (Fig. 3, lanes M, L and K; Fig. 2, lanes K, J and I, respectively). The data obtained led to the conclusion that partial degradation of O-AgH does take place.

Interestingly, the antibodies obtained in the current study reacted in immunoblotting with O-AgH, chemically extracted O-Ag and the carbohydrate component of *V. cholerae* O139 located in the lower part of the blot and associated with core and lipid A. O-Specific polysaccharide chains responsible for serological specificity of micro-organisms are located in the upper and medium part of the gels [23, 24]. This was previously demonstrated with the help of mono- and poly-clonal antibodies specific for *V. cholerae* O139 [23–25] and other bacteria [19]. This leads to the conclusion that the O139 serogroup-specific determinants of the O-Ag are localised in the region previously associated only with core or lipid A [23, 26, 27]. On the other hand, they could be the epitopes located in the inner core region which are recognised by species-specific monoclonal antibodies [19]. However, core oligosaccharide determines the serological specificity only in gram-negative bacteria lacking the ability to synthesise an O antigen, i.e., producing the typical R-LPS [22]. On the contrary, O-antigenic side-chain consisting of a variable number of repeating saccharide units provides serological specificity in bacteria possessing S-type LPS [6, 7, 26]. However, all *V. cholerae* O139 strains used in this study possessed the higher and intermediate mol. wt carbohydrate components which usually correspond to O-chain of S-LPS [19, 26, 27]. There were no strains with only low mol.-wt bands, although they did not produce the typical long O-side chain polysaccharides (Fig. 1) and probably produced the short chains [28]. Thus, the patterns visualised in *V. cholerae* O139 strains were not typical for either smooth or rough LPS observed in *V. cholerae* O1 or a number of mutants of *S. minnesota* and *S. typhimurium* [23, 26]. Moreover, the Boivin method was used, which is more effective for isolation of O-chains [8]. The second method of O-Ag extraction from the culture fluid is based on the fact that the O polysaccharide (side) chains are located at a distance of 10–12 nm from the microbial cell wall [29] and can be extracted easily from the culture fluid of vibrios [9]. Both preparations were eluted as three fractions when separated by gel chromatography through TSK-gel HW-75. O-Antigenic activity was registered in the second fraction. The subsequent high-performance liquid chromatography revealed a single peak without additional signals in each of the samples which was determined as O-AgB and O-AgD, respectively (data not shown). The relevant preparations were used as immunogens for mice and rabbits. Thus, isolated O-Ags possibly contained mainly O-side chains of *V. cholerae* O139 LPS. Further evidence of this supposition was the positive reaction in sandwich ELISA with the fraction of hydrolysed O-AgD corresponding to O-side chains which was 100 times stronger than with the fraction corresponding to core polysaccharide. This can be explained by the reaction of Abs with a specific epitope located nearest to the core O-side chain region (data not shown). Finally, the data obtained suggest that either core region epitopes are responsible for serogroup specificity of *V. cholerae* O139 or O-chain epitopes are visualised on the blots as the core–lipid A fraction. This component possesses serogroup specificity of *V. cholerae* O139 and is a conserved element inherent in all strains used in the current study.

It should be emphasised that in immunoblotting of native whole-cell lysates of *V. cholerae* O139 strains and O-Ags with Abs, their reactivity with carbohydrate and protein bands of both O-Ags (O-AgB and O-AgD) was demonstrated. This means that both portions of O-Ags induced the antibody response, and this could be explained by the presence of marked quantities of protein in these preparations (Table 2).

The same proteins were visualised in whole-cell lysates and O-Ags, although they had different electrophoretic mobility. Moreover, carbohydrate bands in the whole-cell lysates were smaller and occupied less circumscribed space compared with the O-Ags in the PK-digested lysates. These data present evidence that strong ‘carbohydrate component–protein’ interaction occurs in *V. cholerae* O139. This conclusion agrees

with a number of reports about protein–LPS interactions and the functional association of LPSs with outer-membrane proteins (OMPs) [6–8, 30]. An important role for LPS, especially core region, in the correct folding and assembly of bacterial OMPs has been demonstrated [31]. Furthermore, as the Abs obtained in the current study were highly specific, both portions of the preparations are supposed to be serogroup-specific. The comparative electrophoresis of whole-cell lysates of *V. cholerae* O1, non-O1 and O139 strains showed the presence of proteins with mol. wts of 69.2 and 20.0 kDa in only *V. cholerae* O139 strains (data not shown). These proteins are probably located in an outer membrane, but not in a capsule of *V. cholerae* O139, because Abs reacted in ELISA with whole merthiolate-killed bacterial cells of both capsulate and non-capsulate vibrios [32]. Moreover, specific capsule-associated proteins were found to have different mol. wts – 38 and 61 kDa [33]. This strongly indicates that *V. cholerae* produces some proteins with serogroup-specific activity, although the serological relationship and cross-reactivity of the protein components of O-Ags of gram-negative bacteria were postulated earlier [6]. As the reaction of Abs obtained was observed with protein as well as with carbohydrate portions of O-Ags, the serogroup-specific proteins can be supposed to shield cross-reacting core-associated epitopes providing specific interaction with homologous Abs. However, Abs reacted not only with native vibrios but also with PK-digested bacteria in immunoblotting (Figs. 2 and 3) and ELISA (Table 5). No cross-reactions with other bacteria which could be provided by highly conserved core region epitopes of Enterobacteriaceae were registered in the ELISA. Furthermore, no reaction with *V. cholerae* O22 strain was visualised in any of the analyses (Tables 3 and 5; Fig. 3, lane A). Moreover, protein-free (PK-digested) O-AgB induced antibodies highly specific for *V. cholerae* O139 strains in BALB/c mice (data not shown). This provides strong evidence that the carbohydrate component of chemically isolated O-Ags of *V. cholerae* O139 bears serogroup-specific epitopes.

The ELISA developed in this study has significant advantages over slide agglutination tests for detection of *V. cholerae* O139 described previously [2, 4]. Firstly, the ELISA is based on the use of strictly specific Abs without absorption, whereas in an agglutination test antiserum absorbed with several cross-reacting *V. cholerae* strains is used. As a rule, absorption leads to significant decrease of diagnostic titre [5]. Also, spontaneous auto-agglutination and, consequently, false-positive reactions can often be registered in the slide agglutination technique [5]. It should be mentioned that this method is suitable only for laboratory identification of cultures of *V. cholerae* O139 isolates, and not for its detection in native specimens contaminated with different biological agents. In contrast, the ELISA is effective for detection of *V. cholerae* O139 in various specimens [25, 32]. The direct dot-ELISA for rapid diagnosis of cholera O139 is based on the use of monoclonal antibodies [25] obtained by hybridoma technology, which requires special expensive equipment and reagents and trained personnel. The method of obtaining specific Abs used for the development of this new test system is cheap, simple and can be performed in laboratories with the minimum of equipment. At the same time this ELISA has the same sensitivity and specificity as the direct dot-ELISA.

Furthermore, merthiolate-killed micro-organisms were used for the ELISA. With this method of killing bacteria the serogroup-specific epitopes may remain in their native form and a highly specific interaction of these epitopes and the relevant Abs can occur. This can be confirmed by the parallel results of slide agglutination and ELISA the same panel of live bacteria (data not shown).

In summary, rabbit and mouse Abs against chemically extracted O-Ags of *V. cholerae* O139 were obtained and shown to be specific for the relevant strains in ELISA without any absorption. This is a promising approach for the development of serogroup-specific Ab-based diagnostic tests that may be important when identifying vibrios and other bacteria. Further studies defining species- and serogroup-specific proteins of the vibrios will extend our knowledge of *V. cholerae* O-Ags and their contribution to biological activity and cholera pathogenesis. The ELISA developed here can be recommended as a specific diagnostic test for cholera cases caused by *V. cholerae* O139.

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