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Modified Enzyme-Linked Immunosorbent Assay for Detecting Enteroinvasive *Escherichia coli* and Virulent *Shigella* Strains

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Immune sera were produced in rabbits with living cells of an enteroinvasive O143 strain of *Escherichia coli*. To remove O and K antibodies, sera were absorbed with an avirulent derivative of the same strain. In the enzyme-linked immunosorbent assay, absorbed sera reacted specifically with only virulent *Shigella* strains and enteroinvasive *E. coli* strains of different geographical origin, regardless of species or serogroups. The investigation of 83 strains indicated complete agreement between enzyme-linked immunosorbent assay results and those of the keratoconjunctivitis test. It is assumed that the absorbed immune sera reacted with a possible virulence marker antigen. This inexpensive and simple method provides an alternative to other virulence tests. It has a definite advantage for screening large number of isolates within 24 h.

In humans, dysentery is frequently caused by virulent *Shigella* and enteroinvasive *Escherichia coli* (EIEC) strains. In disease production bacterial penetration of intestinal epithelial cells is the most important step (6). This attribute may be associated with a 140-megadalton (Md) plasmid (4, 8, 10, 12) or, in case of *S. sonnei*, with a 120-Md plasmid (9). These plasmids code for outer membrane proteins required in the penetration process (3).

The most frequent test for virulence of these strains consists of the inoculation of guinea pig eyes, the keratoconjunctivitis test (11). In addition, oral infection of starved guinea pigs (2) or monkeys (6) have been used. Bacterial invasiveness can be evaluated in vitro by the bacterial penetrating capacity of cultured mammalian cells (6).

These tests allow the study of only a limited number of strains; they are time consuming, cumbersome, costly, and require special technology. Thus, the application of these procedures to epidemiological studies involving large numbers of isolates is of very limited value.

Previously, we have reported that an enzyme-linked immunosorbent assay (ELISA) procedure can distinguish virulent and avirulent *Shigella* spp., EIEC and non-EIEC strains (7). This differentiation was achieved with an absorbed immune serum that contained ELISA-reactive antibodies specific for a possibly unique antigen site (virulence marker antigen [VMA]) of the virulent strains.

In this paper we describe in detail how this modified ELISA procedure has been adjusted to identify virulent *Shigella* and EIEC strains. Our goal was to develop a test, suitable for screening large number of isolates.

MATERIALS AND METHODS

Bacterial strains. A total of 62 *E. coli* and 21 *Shigella* strains were included in the study. Of these, 50 *E. coli* and 13 *Shigella* strains were isolated in Hungary (Public Health Station, County Baranya, and Institute of Microbiology, University Medical School, Pécs). Those strains kindly provided by Betty R. Davis, L. R. Trabulsi, and J. P. Sansonetti are listed in Table 1. Strains were serogrouped by slide agglutination with diagnostic immune sera prepared according to the method of Edwards and Ewing (1). Samples of strains were stored in deep agar at room temperature and in nutrient broth containing 15% glycerol at −20°C.

Keratoconjunctivitis test. The guinea pig keratoconjunctivitis test was performed according to the method of Serény (11).

Plasmid detection. Plasmids were isolated and treated with electrophoresis according to the method of Kado and Liu (5). The molecular weight standards used were pCW3 (110 Md), pCW1 (70 Md), and pCW2 (41 Md), kindly provided by C. Waalwijk, Department of Medical Microbiology, Free University, Amsterdam, The Netherlands. Additionally, *Shigella dysenteriae* 1 47-80 obtained from J. P. Sansonetti, Institut Pasteur, Paris, France, which had a known 140-Md plasmid was included as a control in each isolation procedure and each gel.

Production of VMA specific antibodies. For production of VMA-specific antibodies, a virulent isolate of *E. coli* O143 (strain no. 2) was used (7). Three rabbits were immunized intravenously at 4-day intervals with 0.2, 0.5, 1.0, and 2.0 ml, respectively, of a suspension containing 10^9 living bacteria per ml. Thereafter, 2.0-ml booster doses were given at week 2 and 8. Blood was obtained on day 4 after the last injection. Tube agglutination titers of the three immune sera varied from 1:640 to 1:1,280 with living and from 1:5,120 to 1:10,240 with boiled cells of the homologous strain.

These sera were absorbed with a Serény test negative derivative (no. 2/33) of strain no. 2 which was obtained by serial passages on nutrient agar plates. This derivative was identical in serotype, biotype, antibiotic susceptibility, and plasmid pattern to parent strain no. 2.

One sample of each sera was diluted in phosphate-buffered saline (PBS), pH 7.2, to 1:5; 1 g (wet weight) of living avirulent bacteria (no. 2/33) was added per ml of each serum. Mixtures were incubated for 2 h at 37°C and overnight at 4°C and centrifuged, and the entire procedure was repeated with boiled cells.

Absorbed sera did not agglutinate either live or boiled cells of strain no. 2. Sera were membrane filtered and stored at −20°C without preservative. The sera used in the ELISA were designated as VMA-specific antibodies (VMA-Ab-1, -2, -3).

ELISA. Wells of Dynatech MicroELISA plates (type M 129 A) were sensitized overnight at 4°C with 100 μl of a

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TABLE 1. Geographical and serological distribution of strains isolated outside Hungary

Strains	Supplier code	Serény test reaction	Source
E. coli			
O124	931-78	+	B. R. Davis
O124	1457-75	+	
O124	3144-67	−	
E. coli			
O28ac	14185-83 HU	+	L. R. Trabulsi
O29	127-82 FAV	+	
O124	280-83 FAV	+	
O136	282-83 FAV	+	
O143	267-83 FAV	+	
O144	88-82 FAV	+	
O152	2855-83 HU	+	
O164	Saigon	+	
O167	226-83 FAV	+	
S. dysenteriae			
1	47-80	+	J. P. Sansonetti
1	D989	−	
1	1007-74	−	
Shigella boydii			
2	2854-67	+	
4	874-74	+	
4	1-81	−	
5	Prigent	−	
7	19-79	−	

bacterial suspension diluted in coating buffer (Na_2CO_3, 1.59 g, and $NaHCO_3$, 2.93 g, in 1,000 ml of distilled water [pH 9.6]) containing 10^9 living bacteria per ml. Microbial counts were adjusted photometrically at 690 nm. The sensitized plates were washed three times with PBS containing 0.05% Tween 20. The washing step was repeated after each incubation. Next, 100 μl of the VMA-Ab, diluted appropriately in PBS with 0.05% Tween 20, was added. Plates were incubated again for 1 h at 30°C, and then 100 μl of the conjugate (anti-rabbit immunoglobulin G labeled with horseradish peroxidase [DAKO] diluted 1:2,000 in PBS with 0.05% Tween 20 containing 0.5% bovine serum albumin [Serva]) was added. After further incubation for 1 h at 30°C, the reaction was developed by adding 150 μl of the substrate (10 mg of *o*-phenylenediamine-dihydrochloride [Fluka] in 30 ml of citric acid buffer [pH 6.0]). The reaction was stopped after 10 min at 30°C by adding 50 μl of 4 N sulfuric acid.

In each experiment, strains were tested in three parallels. Optical density (OD) was measured at 492 nm with a Dynatech ELISA reader. Wells without bacteria served as blanks. Additional controls were wells with normal rabbit serum and wells without VMA-Ab. The blanks were used to set the photometer at 0.

RESULTS

Optimalization of the test. ELISA reactivity of one of the three VMA-Abs (VMA-Ab-1) was tested against three pairs of Serény-test-positive and -negative *E. coli* strains of serogroups O124, O136, and O143, respectively. A pair of virulent and avirulent *Shigella flexneri* 2a isolates were also tested. Different dilutions of VMA-Ab-1 were used in the ELISA. OD values are shown in Table 2.

VMA-Ab-1 reacted with the Serény test-positive strains at a remarkably higher dilution than it did with the negative strains. Even dilutions of 1:2,000 and 1:1,000 gave OD values substantially higher with the keratoconjunctivitis-positive than with the negative isolates. This difference did not depend on the serogroups.

To exclude the possibility that VMA reactivity is restricted to the serum of a single rabbit, reactions of VMA-Ab-1, -2, and -3 were compared in a 1:1,000 dilution against EIEC and non-EIEC strains (Table 3). It can be seen that antibodies produced in the other two rabbits differentiate between EIEC and non-EIEC strains similarly.

To rule out the possibility that difference in OD values between Serény test-positive and -negative isolates was due to their adhering capability to ELISA plates, we carried out the following experiment. Plates were sensitized with virulent strain no. 2 and with its avirulent counterpart no. 2/33. The plates were reacted with serial twofold dilutions of up to 1:32,000 of an immune serum raised against the avirulent strain no. 2/33, supposedly containing O- and K- but not VMA-specific antibodies.

Both clones reacted with this immune serum up to a dilution of 1:16,000. At each dilution applied, OD values did not differ according to virulent and avirulent counterparts.

In these experiments neither controls nor blanks gave OD values above 0.10.

In further studies VMA-Ab-1 was used in a dilution of 1:1,000. We examined a number of strains of different geographical origin.

Reactivity of VMA-Ab-1 with strains of different geographical origin. In this part of the study we tested 62 *E. coli* and 21 *Shigella* strains of different geographical origin. *E. coli*

TABLE 2. Reactivity of VMA-Ab-1 with virulent and avirulent strains in different dilutions

Strains	Code	Virulence[a]	OD values[b] in dilution:			
			1:200	1:500	1:1,000	1:2,000
E. coli						
O143	no. 2	+	1.33	0.99	0.95	0.56
	no. 2/33	−	0.04	0.02	0.00	0.00
O124	no. 34	+	1.23	0.98	0.90	0.50
	no. 27	−	0.14	0.08	0.02	0.00
O136	no. 16	+	1.06	0.85	0.65	0.56
	no. 70	−	0.02	0.00	0.00	0.00
S. flexneri 2a	K478	+	0.66	0.55	0.52	0.36
	20014	−	0.02	0.00	0.00	0.00

[a] Virulence was estimated according to the method of Serény (11).
[b] ODs represent mean values of three parallels.

TABLE 3. Reactivity of the three VMA-Abs with EIEC and non-EIEC strains

Strains	Code	Virulence[a]	OD values[b]		
			VMA-Ab-1[c]	VMA-Ab-2	VMA-Ab-3
E. coli					
O143	no. 2	+	1.03	0.89	0.79
	no. 2/33	−	0.00	0.00	0.00
O124	no. 34	+	0.92	0.87	0.52
	no. 27	−	0.02	0.00	0.00
O136	no. 78	+	0.93	0.90	0.67
	no. 70	−	0.02	0.00	0.00
O164	Saigon	+	0.88	0.90	0.72
	no. 110	−	0.03	0.02	0.00

[a] Virulence was estimated according to Serény / 11 /.
[b] ODs represent mean values of three parallels.
[c] Sera were used at a dilution of 1:1,000.

strains belonged to nine serogroups, and the members of the *Shigella* genus represented nine serotypes of four species.

All strains were inoculated into guinea pig eyes. By the Serény test, 56 isolates proved to be positive. All of them harbored the 140-Md plasmid, or in the case of *Shigella sonnei*, the 120-Md plasmid.

Although the 140-Md plasmid could be detected in avirulent derivative no. 2/33 by gel electrophoresis, none of the wild-type, avirulent isolates harbored it. The *S. sonnei* phase II strains did not contain the 120-Md plasmid.

Then strains were tested in ELISA with VMA-Ab-1 in a dilution of 1:1,000 (Table 4). The strains that were positive by the keratoconjunctivitis test reacted strongly with the immune serum (OD range, 0.40 to 1.60). For these strains, the mean OD value was 0.83 ± 0.25 standard deviations. Contrary to this, Serény test-negative isolates had a low OD value (range, 0.00 to 0.15), with a mean of 0.037 ± 0.026 standard deviations. According to these OD values we considered strains as VMA positive when the OD value exceeded 0.33, i.e., the mean − 2 standard deviations of the Serény test-positive isolates. The difference between the OD values of the two groups was found to be significant at $P < 0.001$ by the two-tailed Student's t test. At this dilution VMA-Ab gave a clear-cut differentiation between virulent and avirulent isolates. This difference was so pronounced that positive strains could be spotted with the naked eye.

TABLE 4. Comparison between virulence and VMA reactivity

Strains	Virulence[a]	No. of strains tested	VMA		OD range[b]
			Positive	Negative	
E. coli					
O28ac	+	1	1	0	0.48
	−	6	0	6	0.02–0.05
O29	+	3	3	0	0.43–0.93
	−				
O124	+	30	30	0	0.50–1.60
	−	2	0	2	0.02–0.05
O136	+	3	3	0	0.40–0.93
	−	1	0	1	0.03
O143	+	4	4	0	0.42–1.15
	−	3	0	3	0.00–0.05
O144	+	1	1	0	0.65
	−				
O152	+	1	1	0	0.85
	−	3	0	3	0.02–0.05
O164	+	1	1	0	0.87
	−	2	0	2	0.02–0.05
O167	+	1	1	0	0.70
	−				
S. dysenteriae 1	+	1	1	0	0.57
	−	2	0	2	0.00–0.15
S. flexneri					
2a	+	3	3	0	0.52–1.31
	−	1	0	1	0.00
3	+	2	2	0	0.96–1.60
	−	1	0	1	0.02
S. boydii					
2	+	1	1	0	0.89
	−				
4	+	1	1	0	0.66
	−	1	0	1	0.02
5	+				
	−	1	0	1	0.02
7	+				
	−	1	0	1	0.06
S. sonnei					
I	+	3	3	0	0.51–0.77
II	−	3	0	3	0.00–0.02
Total	+	56	56	0	0.40–1.60
	−	27	0	27	0.00–0.15

[a] Virulence was estimated by the method of Serény (11).
[b] Range of OD mean values as estimated by three parallel wells at 492 nm.

DISCUSSION

The determination of virulence of *E. coli* and *Shigella* strains has both clinical and epidemiological importance. The presently available methods for the study of invasive capabilities are applicable to individual isolates. However, for screening large numbers of bacterial isolates for epidemiological purposes, these methods are unsuitable. A rapid and economical test is needed for the identification of virulent isolates.

The examination of 83 *E. coli* and *Shigella* strains indicates that the modified ELISA meets these requirements. On the basis of results for 56 Serény test-positive and 27 Serény test-negative strains of different geographical origin and different serogroups, it can be concluded that in the ELISA VMA-Ab reacts with a unique antigenic moiety expressed by the enteroinvasive strains.

Although VMA-Ab differentiates between virulent and avirulent strains, OK antibodies reacted equally with homologous virulent and avirulent counterparts, suggesting that there is no difference in the rate of bacterial attachment to the ELISA plates.

Whether this VMA is a real virulence factor or only a marker antigen closely related to it, requires further investigations. It was proven that in the case of *Shigella* and *E. coli* strains the presence of a large plasmid is essential for enteroinvasiveness (4, 8–10, 12). The genetic background of VMA is not yet known. It is remarkable that except for strain no. 2/33 no VMA-negative isolate contained the 140-Md plasmid. The fact that the above clone harbored a plasmid of this size does not exclude the possibility that this plasmid may be responsible for the expression of VMA. It could be present with a mutation undetectable by the gel electrophoretic method used.

Regardless of the nature of the association between VMA and enteroinvasiveness, this ELISA technique can be used to identify and select EIEC and virulent *Shigella* strains within 24 h. It is suitable to test large numbers of strains or colonies efficiently and economically. We recommend this ELISA, especially for laboratories working in the developing areas of the world.

Further improvement of the test, to detect VMA directly from fecal samples, is in progress.

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