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A simple enzyme-linked immunosorbent assay for the detection of *Aeromonas* spp.

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ABSTRACT

An indirect enzyme-linked immunosorbent assay employing antisera against somatic antigen and outer membrane protein of a rough *Aeromonas* strain was developed for the detection of *Aeromonas* spp. The test detected *Aeromonas* irrespective of its species or serotype. The optimum detection level was found to be 10^6/ml. The cross-reactions were observed only with *Vibrio cholerae* (O39) among the 15 different organisms belonging to *Enterobacteriaceae, Vibrionaceae* and a few Gram-positive pathogens tested. Preliminary trials on food samples revealed the test to be efficient in detecting *Aeromonas* spp.

Key words: *Aeromonas* spp., ELISA, outer membrane protein, food

Introduction

Mesophilic aeromonads, the Gram-negative bacteria of ubiquitous nature, are implicated as a causative agent of enteric and systemic infection (JANDA et al., 1983; KHARDORI and FAINSTEIN, 1988; KROVACEK et al., 1993). Water and food are considered to be the major source of infection in human beings (FRICKER and TOMPSETT, 1989; WADSTROM and LJUNGH, 1991).

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Mesophilic aeromonads are phenotypically classified into three species, namely *A. hydrophila*, *A. sobria* and *A. caviae*. However, the genus *Aeromonas* is diverse and now, 14 hybridization and several serotypes are recognized (CARNAHAN and JOSEPH, 1993; SAKAZAKI and SHIMADA, 1984). The most widely used system of serotyping based on somatic (O) antigen recognizes 44 serogroups and an additional 52 provisional serogroups within the genus *Aeromonas* (JANDA et al., 1996). Some of these serogroups (O:11, O:34 and O:16) are considered to be more pathogenic (THOMAS et al., 1990; JANDA et al., 1996). However, SINGH and SANYAL (1992) have shown that all *Aeromonas* have pathogenic potentials.

Conventionally, *Aeromonas* spp. are isolated and identified by cultural methods which are time consuming and labour intensive. Recent attempts to develop rapid immunological and biotechnological methods for the identification of *Aeromonas* spp. are serotype or hybridization group specific (MERINO et al., 1993; LUDWIG et al., 1994; KHAN and CERNIGLIA, 1997). There is no immuno-assay available which can identify all *Aeromonas* types. This paper reports a simple indirect-ELISA which can identify *Aeromonas* spp. irrespective of its serotype or species.

Materials and methods

Bacterial strains. The bacterial strains used in this study are listed in Table 1. All the strains were maintained on nutrient agar slants. Serotyping of the *Aeromonas* strains was kindly carried out by courtesy of Dr. T. Shimada, Chief Laboratory of Enteric Infection 1, National Institute of Health, Tokyo 162, Japan.

Outer membrane protein (OMP) extraction. The OMP from a rough strain (VPH 5) of *A. hydrophila* was prepared following the method of CROSA and HODGES (1981). The bacteria were recovered by centrifugation from overnight grown culture in 100 ml of trypticase soya broth. They were suspended in 3 ml of 10 mmol/L tris buffer containing 0.3% (w/v) NaCl (pH 8.0) and sonicated three times for 30 s with an MSE sonicator. The preparation was centrifuged at 10000 g for 2 min and supernatant subjected to further centrifugation for 1 h at 17000 g at 4 ^{o}C. The cell envelope material was incubated with 3% sodium lauroyl sarcosinate

(sarkosyl) (w/v) in 10 mmol/L tris buffer at room temperature for 20 min. OMP was collected by centrifugation at 17000 g for 1h. The OMP was analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to LAEMMLI (1970) using 12.5% (w/v) acrylamide in the separating gel and 3% (w/v) acrylamide in the stacking gel. The gel was stained with coomassie brilliant blue.

Antiserum against whole cell antigen of Aeromonas. Whole cell antigen against a rough strain of *A. hydrophila* (VPH5) was prepared as per the method of SHIMADA and KOSAKO (1991). Briefly, 10 ml of infusion broth (Difco) was inoculated with *A. hydrophila* (VPH5) and incubated overnight at 35 °C with continuous shaking. The culture was heated at 100 °C for 2 hrs and then washed twice in sterile normal saline (NSS). Finally, the packed cells were re-suspended in 20 ml of NSS and used as antigen.

For immunization, adult rabbits were given six intravenous injections with increasing doses of 0.5, 1.0, 2.0 and 4.0 ml of antigen at 4-day intervals. The rabbits were bled 7 days after the final immunization.

Antiserum against OMP. Antiserum against OMP of *A. hydrophila* (VPH5) was raised in rabbits as per the method described by SANTOS et al. (1996), with some modifications. OMP (500 µg protein) was emulsified with an equal volume of Freunds complete adjuvant (FCA, Difco). Rabbits were injected subcutaneously in a divided dose at six dorsal sites. Two booster doses were given with OMP (250 µg protein) emulsified with an equal volume of Freunds incomplete adjuvant (Difco) at two-week intervals. Rabbits were bled 15 days after final immunization.

Indirect ELISA. The indirect ELISA was performed as per the method of ENGVALL and PERLMANN (1971), with certain modifications. The test was standardized by checker board analysis. Various cell concentrations (antigen) ranging from 10 to 10^9 cells/ml were reacted with 1:10 to 1:1280 dilutions of anti-whole cell serum and 1:100 to 1:1280 for anti-OMP serum. Coating was accomplished by incubating plates overnight at 4 °C with 100 µl of antigen diluted in carbonate-bicarbonate buffer (0.01 M, pH 9.6). Anti-rabbit HRPO conjugate was used in 1:4000 dilution. The substrate used was o-phenylene diamine dihydrochloride (OPD) at 1 mg/ml concentration in citrate buffer (0.05 M, pH 4.6) and the reaction was stopped

Table 1. *Aeromonas* strains and other organisms used in the present study

Organism	Strain N^o	Serotype	Source	Donor
A. hydrophila	*VPH5*	*Rough*	*Sewage*	-[1]
A. hydrophila	69	Rough	Quail egg	-
A. hydrophila	G3	O38	Goat milk	-
A. caviae	10	Rough	Fish	-
A. caviae	F7	O38	Fish	-
A. caviae	F17	O38	Fish	-
A. sobria	C5	O16	Chicken meat	-
A. sobria	C11	O16	Chicken meat	-
A. sobria	C23	O16	Chicken meat	-
A. sobria	Buff1	unknown	Buffalo milk	-
A. sobria	R5	O37	Mastitis case	-
A. sobria	F14	O16	Fish	-
A. sobria	Floor2	O16	Floor (soil)	-
A. sobria	F6	O6	Fish	-
A. sobria	F2	O73	Fish	-
A. sobria	AC5	O60	Fish	-
V. cholerae	-	*O19*	-	Dr. G.B. Nair[2]
V. cholerae	-	O39	-	Dr. G.B. Nair
V. cholerae	-	O59	-	Dr. G.B. Nair
V. parahaemolyticus	VP202	-	-	Dr. G.B. Nair
V. fulvialis	AS236	-	-	Dr. G.B. Nair
Plesiomonas shigelloides	SW4	-	-	-
Klebsiella spp.	Oxy1	-	-	-
Proteus spp.	Proteus	-	-	-
Morganella spp.	M49M	-	-	-
Bacillus cereus	VPHE15	-	-	-
Bacillus cereus	NCTC45	-	-	-
Salmonella Derby	E17	-	-	Dr. J.C. Verma[3]
Enterobacter cloacae	E222	-	-	Dr. J.C. Verma
Pseudomonas spp.	E241	-	-	Dr. J.C. Verma
Salmonella Paratyphi B	E120	-	-	Dr. J.C. Verma
Salmonella Typhimurium	E270	-	-	Dr. J.C. Verma
Shigella flexneri	MTCC1457	-	-	Dr. J.C. Verma
Staphylococcus spp.	E2	-	-	Dr. J.C. Verma
Citrobacter freundii	E217	-	-	Dr. J.C. Verma
Escherichia coli	F2	-	-	-

[1]Strains were maintained in our own laboratory.
[2]Dr. G. B. Nair, Deputy Director, National Institute of Cholera and Enteric Diseases, Calcutta
[3]Dr. J. C. Verma, Senior Scientist, National Salmonella Centre, IVRI, Izatnagar.

with 2 N H_2SO_4. The plates were read at 492 nm in ELISA reader (ECIL, India). A positive to negative ratio of >2 was taken as positive.

For 15 other *Aeromonas* strains a similar procedure was followed except that the plates were coated with cell concentrations of 10^6 to 10^9 cells/ml and reacted with anti-whole cell (1:160 and 1:320) and anti-OMP (1:3200) sera.

Cross-reaction studies were carried out by coating the plates with 10^9 cells/ml of different organisms (Table 1).

Assessment of indirect ELISA for the detection of Aeromonas in foods. The chicken meat samples were homogenized with 0.1% peptone water and divided into two parts. One part was also inoculated with one ml culture (10^9 cells/ml) of *Aeromonas* spp., after two-step enrichment in alkaline peptone water (APW) for 18 and 6 hrs at 37 °C. The cells were harvested by centrifugation (5000 rpm, 30 min), washed once with carbonate buffer (0.01 M, pH 9.6) and re-suspended in the same buffer to give a final concentration of 10^9 cells/ml. Indirect ELISA was performed as described above by using harvested cells or antigen and anti-OMP serum (1:3200) as antibodies. The same samples were also processed conventionally for isolation of *Aeromonas* spp. by the microbiological method (LEE and DONOVAN, 1985).

Results and discussion

This paper describes an indirect ELISA for the detection of *Aeromonas* irrespective of species or serotype. The standardization of cell concentration and antisera dilution by checker board analysis revealed that approximately a minimum of 10^7 cells/ml of *Aeromonas* (VPH5) resulted in optimal reaction at 1:640 dilution of anti-whole cell serum. Positive reactions were also observed with lower concentration of cells (10^6/ml) employing lower antiserum dilutions (1:10 to 1:320) but plateau was reached at 10^7 cells/ml. The plateau was maintained until 10^9 cells/ml up to 1:1280 antiserum dilution.

On standardization of the test using anti-OMP serum a minimum of 10^6 cells/ml of homologous antigen (VPH5) could be optimally detected with antiserum dilutions up to 1:6400. These findings are in agreement with other ELISA systems, where optimum reactions at a cell concentration of

10^6/ml or thereabouts has been achieved (CHAND et al., 1989; LEE et al., 1990). MERINO et al. (1993) were able to detect as low as 10 cells per 100 ml of peptone water, which might have been due to the specific detector antibody against S layer of *A. hydrophila* (O:11) used by them.

Testing of 15 other *Aeromonas* strain belonging to *A. hydrophila* (69, G3), *A. sobria* (C5, C11, C23, Buff 1, R5, F14, Floor 2, F6, F2, AC5) and *A. caviae* (10, F7 and F17) and representing different serogroups at cell concentrations between 10^9 to 10^6 cells/ml yielded a positive reaction with both anti-whole cell (1:160 and 1:320) and anti-OMP sera (1:3200). Use of antiserum against a rough strain of *Aeromonas* spp. had potential advantages as all strains of *Aeromonas*, irrespective of their serotype, possess R-agglutinins (SAKAZAKI and SHIMADA, 1984), which helps in the detection of all types of *Aeromonas* strain.

The results of studies on cross-reactions with other organisms (Table 1) using anti-whole cell serum (1:160) revealed negative reactions at 10^9 cells/ml concentration, except for *V. cholerae* (O:19, O:39, O:59), *V. parahaemolyticus* and *V. fulvialis*. On further study it was observed that these cross-reacting organisms gave positive reactions up to 10^6 cells/ml concentration with anti-whole cell serum (1:160). However, by using 1:3200 dilution of anti-OMP serum reactions most organisms were eliminated, except for *V. cholerae* (O:39) which reacted positively only at a concentration of 10^8 cells/ml. The cross-reactions with *Vibrio* spp. might have been due to the presence of R-agglutinins (SHIMADA et al., 1984).

Application of the test for the detection of *Aeromonas* from natural and experimentally inoculated chicken meat samples resulted in the positivity of all 10 experimentally inoculated samples and one of the 10 natural samples. The results were in total agreement with those of the conventional microbiological method indicating the efficacy of test.

Thus, indirect ELISA developed in this study will prove useful in detecting *Aeromonas* spp. from foods where vibrio group of organisms are not expected. Further work is in progress to eliminate cross-reactions with *Vibrio* spp.

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SAŽETAK

Neizravni imunoenzimni test za dokazivanje bakterije roda *Aeromonas* razvijen je uz upotrebu antiseruma za somatski antigen i protein vanjske membrane. Testom su dokazane bakterije roda *Aeromonas* neovisno o vrsti i serotipu. Pokazao se najpovoljnijim na razini 10^6 bakterijskih stanica u mililitru materijala. Križne reakcije ustanovljene su samo za bakteriju *Vibrio cholerae* (O39) unutar skupine od 15 različitih mikroorganizama iz porodice *Enterobacteriaceae*, *Vibrionaceae* te nekoliko drugih gramnegativnih bakterija. Preliminarni pokusi pokazali su da je test pogodan za dokazivanje bakterija roda *Aeromonas* u uzorcima hrane.

Ključne riječi: *Aeromonas* spp., imunoenzimni test, membranski protein, hrana