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Enzyme immunoassay for shrimp vibriosis

Y. L. Song[1,*], S. P. Lee[2], Y. T. Lin[2], C. C. Chen[1]

[1] Department of Zoology, National Taiwan University, Taipei, Taiwan, Republic of China
[2] Institute of Fisheries Science, National Taiwan University, Taipei, Taiwan, Republic of China

ABSTRACT: In an effort to detect vibriosis caused by *Vibrio vulnificus* and *V. harveyi* in cultured shrimp in Taiwan, several enzyme immunoassays (EIA) were compared for sensitivity, reproducibility, time consumption, technical complexity and instrumentation requirements. For field applications, the indirect immunodot blot assay using avidin-biotin complex (ABC) proved to be the best method for detection of subclinical vibriosis. This method can detect 10^3 organisms per 100 µl of hepatopancreas homogenates, and color results are clear-cut and available within 5.5 h. These test kits are made specific by using monoclonal antibody reagents. Field surveys including shrimp from larval to adult stages are described in order to determine the feasibility of the ABC immunodot blot assay. This technology may help management to change their emphasis from disease control to health monitoring and thereby provide greater benefit to shrimp farmers.

INTRODUCTION

Since 1987, mass mortalities have occurred in shrimp intensively reared at different locations in Taiwan. Affected shrimp displayed nonspecific signs, including poor growth rate, anorexia, rough shell and milky musculature. The hepatopancreas showed an albinic and atrophic appearance. Bacterial infection was found in the hepatopancreas. Strains isolated from infected shrimp were characterized, and the dominant group was identified as *Vibrios* (Cheng 1989, Huang 1989). In order to detect vibriosis in cultured shrimp, several immunoassays were compared for their efficacy in field conditions. Ideally, the assay should be specific, sensitive, rapid, easy to perform, inexpensive and should not require special instrumentation. In this study, we have described an indirect immunodot blot assay using an avidin-biotin complex for the detection of shrimp pathogens in hepatopancreas homogenates.

Vibrio harveyi has been shown to be the dominant species among *Vibrio* spp. strains isolated from moribund tiger shrimp (Huang 1989). The same species has also been reported to cause mortalities in *Penaeus monodon* hatcheries in the Philippines (Lavilla-Pitogo 1988). Pathogenicity of *V. harveyi* for *Penaeus monodon* and *Penaeus orientalis* has been proven by artificial infection using both injection and water-borne routes (Huang 1989, Liu et al. 1989, Lavilla-Pitogo et al. 1990). *Vibrio vulnificus* is an opportunistic pathogen indigenous to estuarine and marine environments year-round. Studies have shown that it can be isolated from seawater, sediment, plankton, shellfish, crabs (Tamplin et al. 1982) and eels (Tison et al. 1982). This organism was isolated from cultured shrimp and eels in Taiwan and was shown to be virulent not only for the original hosts but also for mice (Song et al. 1990). The human health risks of *V. vulnificus* are high, as a mortality rate of about 50 % was seen in persons who were infected by this organism through food-borne exposure (Blake et al. 1979, Tacket et al. 1984, Morris & Black 1985, Johnston et al. 1986). Based on their prevalence, pathogenicity and broad host range, *V. harveyi* and *V. vulnificus* were selected as the target organisms for screening in this study.

MATERIALS AND METHODS

Monoclonal antibodies. Because *Vibrio vulnificus* and *V. harveyi* are serologically cross-reactive, specific monoclonal antibodies (Mabs) were developed against each species. Monclonal antibodies D3A6, D3B3, C3D5 and C1C2, raised against *V. vulnificus*, reacted with 10 *V. vulnificus* strains comprising 2 different biogroups (Tison et al. 1982). No cross-reactions were observed among 14 non-*V. vulnificus* strains comprising 12

* Addressee for correspondence

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species and 5 genera (Table 1). The Mabs L4F4 and J3C4, raised against *V. harveyi*, reacted with the type strain ATCC14126 and 23 local strains of the same species. No cross-reactions were observed among 16 non-*V. harveyi* strains comprising 13 species and 5 genera (Table 1). The production and characterization of these Mabs have been detailed by Lin (1990) and Chen (1991). Isotypes of the immunoglobulins produced by each hybridoma were determined: D3A6, D3B3 and C1C2 were IgG1; C3D5 and L4F4 were IgG3; and J3C4 was IgG2a. The affinity of these Mabs for surface epitopes of *V. vulnificus* and *V. harveyi* was determined using the indirect fluorescent antibody technique (Lin et al. 1987). Because none of the individual Mabs raised against *V. vulnificus* could react with a commonly expressed surface epitope in all *V. vulnificus* strains tested, Mabs D3A6, D3B3, C3D5 and C1C2 were mixed and used in the 6 assays to avoid discrimination among strains. Using the additivity test (Friguet et al. 1983), Mabs L4F4 and J3C4 were determined to recognize different surface epitopes of *V. harveyi*. These 2 Mabs were therefore mixed and used in the 6 assays for detection of *V. harveyi*.

Rabbit anti-*Vibrio* sera. Immunogen of *Vibrio vulnificus* (strain TG617) was prepared by inactivation of whole cells at 65 °C for 1 h. The concentration of immunogen suspension was adjusted to 10^9 ml^{-1}. Then it was mixed 1 : 1 with Freund's complete adjuvant and 1 ml was injected into a New Zealand white rabbit subcutaneously between the scapulae. At 2 wk intervals, boosters were administered by injection of the immunogen mixed 1 : 1 in Freund's incomplete adjuvant. Final bleeding was done when agglutination titers of antiserum reached 1 : 1024. The antisera were lyophilized and stored at −20 °C for future use. Rabbit anti-*V. harveyi* (strain ATCC14126) serum was also prepared using the procedures described above. These polyclonal antisera were used only in the indirect sandwich enzyme immunoassay (EIA).

Preparation of antigens. Antigens were made using strains of *V. vulnificus* TG617 and *V. harveyi* ATCC14126 which were grown on tryptic soy broth (TSB, Difco) supplemented with 2.5 % NaCl and shaken for 18 h at 28 °C. Cells were inactivated overnight using 0.3 % formalin. Bacterial cells were harvested by centrifugation at 11 000 × *g* for 25 min at 4 °C, washed

Table 1. Specificity of monoclonal antibodies (Mabs) tested by indirect enzyme immunoassay (EIA). ND: not determined

Bacterial species and strain	Indirect EIA reaction					
	D3A6	D3B3	C3D5	C1C2	L4F4	J3C4
Vibrio vulnificus						
Biogroup I. ATCC27562	+	−	−	−	−	−
TG617	+	+	+	+	ND	ND
A2	−	+	+	+	ND	ND
79-08-09	−	−	+	−	ND	ND
LSU11-3M-30	+	+	+	+	ND	ND
Biogroup II. ET-519	+	−	+	−	ND	ND
ET-7618	+	+	+	+	ND	ND
ES-7602	−	−	−	+	ND	ND
KV-3	+	−	+	+	ND	ND
HM1-1	+	−	+	−	ND	ND
Vibrio harveyi ATCC14126	−	−	−	−	+	+
23 local strains	ND	ND	ND	ND	+	+
Vibrio alginolyticus ATCC17749	−	−	−	−	−	−
Vibrio anguillarum serovar A	−	−	−	−	−	−
serovar B	−	−	−	−	−	−
serovar C	−	−	−	−	−	−
Vibrio cholerae Inaba strain	ND	ND	ND	ND	−	−
Ogawa strain	ND	ND	ND	ND	−	−
Vibrio damsela ATCC33539	−	−	−	−	−	−
Vibrio fluvialis ATCC33809	−	−	−	−	−	−
Vibrio ordalii ATCC1669	−	−	−	−	−	−
Vibrio parahaemolyticus ATCC17802	−	−	−	−	−	−
Vibrio tubiashii ATCC19105	−	−	−	−	−	−
Aeromonas hydrophila ATCC7966	ND	ND	ND	ND	−	−
Aeromonas salmonicida	−	−	−	−	ND	ND
Edwardsiella tarda 2K	−	−	−	−	−	−
Escherichia coli WP2	−	−	−	−	−	−
Pseudomonas fluorescence ATCC13525	−	−	−	−	−	−

twice with PBS (pH 7.0) and resuspended to 10^8 ml^{-1}. They were then frozen at −20 °C for future comparison of immunoassays. In order to compare the sensitivity of EIAs, 10-fold serial dilutions of antigens were prepared in carbonate coating buffer (0.05 M, pH 9.6) or phosphate buffer (0.01 M, pH 7.0).

Comparison of immunoassays. The immunodot blot assay was compared with the indirect EIA and the indirect sandwich EIA (Fig. 1). Indirect EIA used in this study was carried out according to Austin et al. (1986). The indirect sandwich EIA technique was a modification of the procedures reported by Smith (1981). The immunodot blot assay used in this study was a partial modification of the methods described by Hawkes et al. (1982) and Hsu & Leong (1985). The procedure for the immunodot blot is summarized as follows:

(1) Prepare the polyvinylidine difluoride (PVDF) membrane (Millipore) by rinsing with 100% methanol to decrease its hydrophobicity.
(2) Rinse with nanopure water.

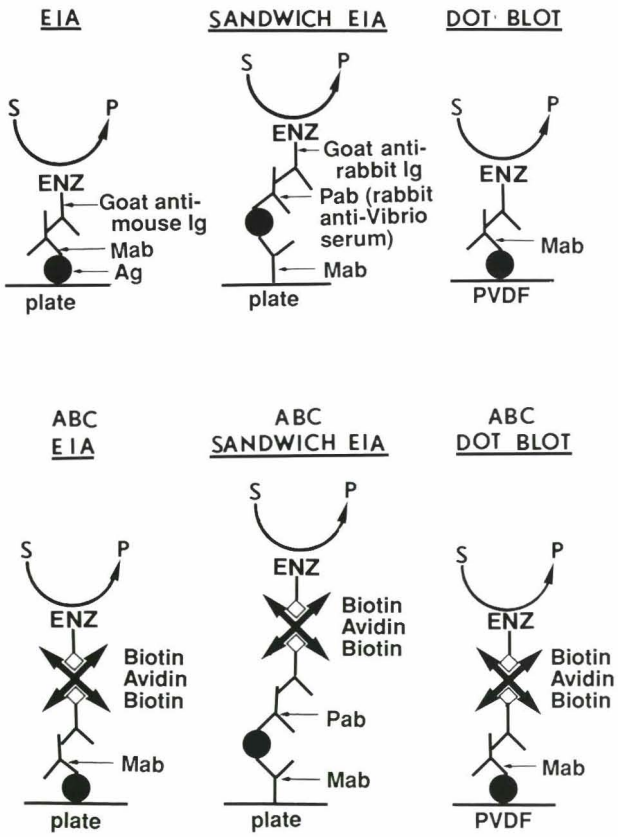

Fig. 1. Diagram of enzyme immunoassay (EIA) methods compared in this study: EIA, sandwich EIA, and immunodot blot assay. The 3 methods were also tested using avidin-biotin complex (ABC) to increase sensitivity. S: substrate; P: product; ENZ: enzyme; Ig: immunoglobulin; Mab: monoclonal antibody; Ag: *Vibrio vulnificus* or *V. harveyi* cell; Pab: polyclonal antibody; PVDF: polyvinylidine difluoride membrane

(3) Immerse in a solution of phosphate buffered saline (PBS, 2 M, pH 7.0).
(4) Shake gently on a reciprocal shaker for 15 min at 25 °C.
(5) Rinse with nanopure water.
(6) Immediately place the membrane into a Hybrid Dot 96-well Filtration Manifold (BRL).
(7) Add 100 µl of each of the serially diluted antigen preparations to the membrane surface of each well by the vacuum process.
(8) Remove the membrane from the Filtration Manifold.
(9) Immerse in a blocking solution of Gelatin-NET (Gelatin 0.25%, NaCl 0.15M, EDTA 5 mM, Tween-20 0.05%, Tris base 50 mM) at room temperature and gently shake for 30 min.
(10) Wash with a solution of PBS 0.02 M, pH 7.0, with 0.05% Tween-20 (PBST) at room temperature for 15 min.
(11) Dilute mouse ascitic fluids containing anti-*V. vulnificus* Mabs (D3A6, D3B3, C3D5 and C1C2) or anti-*V. harveyi* Mabs (L4F4 and J3C4) 20× and 200×, respectively.
(12) Mount the Mabs onto the membrane and incubate 1 h at 37 °C.
(13) Wash the membrane twice with PBST.
(14) Transfer the membrane into a solution containing horseradish peroxidase conjugated goat anti-mouse Ig (2000× dilution with Gelatin-NET, Pierce).
(15) Incubate at 37 °C for 40 min and then at 4 °C for 20 min.
(16) Rinse the membrane with PBST.
(17) Immerse the membrane in a freshly prepared H_2O_2 solution containing the insoluble 3-Amino-9-Ethylcarbazole (AEC; Pierce) for color development.
(18) Shake for 15 to 30 min until red dots appear.
(19) Rinse the membrane with nanopure water.
(20) Store the membrane in the dark.

Application of avidin-biotin complex (ABC). In order to increase the sensitivity of immunoassays, avidin was used as a bridge to cross-link biotins which were conjugated to secondary antibodies and to the enzyme. Biotinylated goat-anti-mouse Ig (220× dilution; Pierce) was used in both the indirect ELISA and the immunodot blot assay, while biotinylated goat-anti-rabbit Ig (220× dilution; Pierce) was used in the indirect sandwich ELISA. The procedure for the application of the ABC is summarized as follows:

(1) Add the biotinylated secondary antibodies to the EIA plate or PVDF membrane processed as described above (steps 1 to 13).
(2) Incubate at 37 °C for 1 h.
(3) Wash the plate or membrane with PBST.

(4) Add a freshly prepared solution containing avidin and biotinylated peroxidase to the plate or membrane.
(5) React at 37 °C for 1 h.
(6) Wash the plate or membrane with PBST.
(7) Add a H_2O_2 solution containing the color developer 2,2-azino-di-[3-ethyl-benzathiazoline sulfonate] diammonium salt (ABTS, used in EIA plate) or AEC (used in PVDF membrane).
(8) Observe with the naked eye or an ELISA reader (Emax, Molecular Devices) at a wavelength of 405 nm.

The sensitivity (number of bacteria detected per 100 µl of sample) was determined as the highest dilution of antigens in the well or dot in which visible color occurs. The reproducibility (trials of same sensitivity/number of trials), time requirement, technical complexity and need for special instrumentations in each assay were recorded.

Elimination of non-specific reaction in the EIAs using sodium dodecyl sulfate (SDS). It was understood that hepatopancreas peroxidase would contribute to the enzyme reaction in the EIAs when using peroxidase-conjugated antibody. Seven initial concentrations of SDS, 0.25, 0.5, 1.0, 2.0, 2.5, 5 and 10 % in 0.85 % NaCl solution, were tested for peroxidase inhibitory activity. The hepatopancreas from healthy shrimp was removed aseptically and homogenized in an equal volume of a sterile 0.85 % NaCl solution. Tissue debris was removed by sedimentation at 4 °C for 1 h. Equal volumes of 100 µl filter-sterilized supernatant (0.22 µm, Millipore) and SDS solution were mixed. After reacting at room temperature for 15 min, each mixture was bound to the membrane by the vacuum process. The membrane was then color-developed using the ABC immunodot blot assay.

To determine the influence of the added 10 % SDS or other potentially inhibitory substances naturally present in the hepatopancreas on the reaction between bacteria harbored in hepatopancreas and antibody in the ABC immunodot blot assay, hepatopancreas of healthy shrimp was artificially inoculated with a known number of live *Vibrio vulnificus*. The hepatopancreas was then removed, homogenized and sedimented. The final number of bacteria in each 100 µl supernatant fluid of hepatopancreas homogenate were from 10^8 to 10^2 cells (a logarithmic decrease). This supernatant fluid was mixed with an equal volume of 10 % SDS solution. The mixture was bound to the PVDF membrane and the color developed with the ABC immunodot blot assay.

Feasibility of the ABC immunodot blot assay in a field survey. A total of 602 larval to adult shrimp, ranging from 0.6 to 23.5 cm in body length, were sampled from Tainan (Taiwan) hatcheries in July and August 1990, and at Tungkang, Chiatung and Linyuan in April 1991. No mortalities or symptomatic diseases were present at the time of sampling. Bacterial isolations were made from the hepatopancreas on thiosulfate-citrate-bile salt-sucrose agar supplemented with 2 % NaCl (TCBS). Both green and yellow colonies on TCBS were subcultured on tryptic soy agar supplemented with 2.5 % NaCl (TSA). Each isolate on TSA was tested for susceptibility to both the vibriostatic agent, 2,4-diamino-6,7-diisopropyl pteridine phosphate (0/129, 150 µg; CPM, Taipei), and the antibiotic novobiocin (30 µg; BBL, Hong Kong). Sensitive colonies were characterized according to Baumann et al. (1984). Meanwhile, the hepatopancreas of each individual was further processed to detect the presence of *Vibrio vulnificus* and *V. harveyi*. Equal volumes of 100 µl supernatant from each hepatopancreas homogenate and 10 % SDS solution were mixed and then tested using the ABC immunodot blot assay.

RESULTS

Comparison of immunoassays

The comparative ability of the 6 different immunoassays to detect the potential shrimp pathogens *Vibrio vulnificus* and *V. harveyi* was examined (Table 2). It was found that the sensitivity of the indirect EIA was limited to 10^5 bacteria per 100 µl of hepatopancreas homogenate. Enhancement of sensitivity by 1 order of magnitude was achieved in 3 of 5 trials through the use of ABC. Sandwich EIA, with or without the application of ABC, had a sensitivity of 10^3 bacteria per 100 µl of hepatopancreas homogenate in at least 50 % of the trials. Instead of a 96-well polystyrene microtiter plate, the PVDF membrane was used as the solid phase in the indirect immunodot blot assay. Owing to the high affinity of proteins for the PVDF membrane, a 10-fold increase in sensitivity was obtained in the indirect immunodot blot assay compared with the indirect EIA. The indirect immunodot blot assay using ABC, however, was shown to be the most sensitive, detecting 100 organisms per 100 µl of hepatopancreas homogenate (Fig. 2). This result was reproducible, color development was clear and distinguishable with the naked eye with no special instrument required.

Influence of SDS and other potential inhibitory substances in the hepatopancreas on the ABC immunodot blot assay

A 10 % initial concentration SDS (5 % in the final hepatopancreas extract) was shown to be high enough

Table 2. Comparison of immunoassays for shrimp vibriosis. EIA: enzyme immunoassay; ABC: avidin-biotin complex; R: ELISA reader; E: naked eye

Indirect assay	ABC	Sensitivity[a]	Reproducibility[b]	Technical steps	Background	Reading	Time (h)
EIA	–	10^5	6/7	5	low	R/E	4.5
	+	10^4	3/5	6	high	R/E	5.5
Sandwich EIA	–	10^3	5/8	6	moderate	R/E	5.5
	+	10^3	2/4	7	high	R/E	6.5
Immunodot BLOT	–	10^4	4/5	5	low	E	4.0
	+	10^2	6/6	6	low	E	5.5

[a] Bacterial cells per 100 µl of hepatopancreas homogenate
[b] Trials of same sensitivity/number of total trials

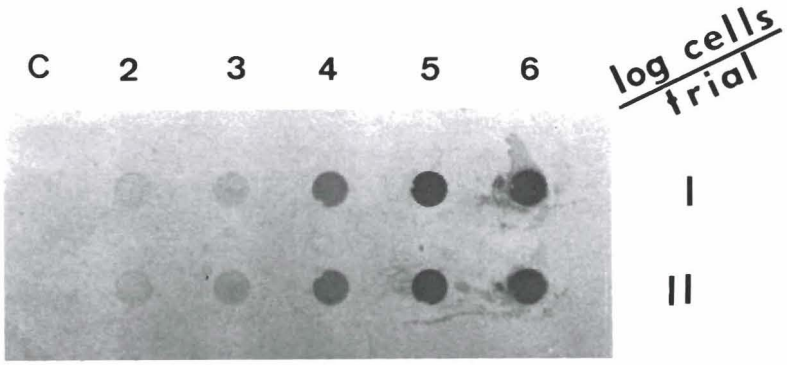

Fig. 2. Sensitivity of ABC immunodot blot assay for *Vibrio vulnificus*

to completely eliminate the interference of tissue peroxidase in the ABC immunodot blot assay (Fig. 3). When hepatopancreas was artificially inoculated with a known number of live *Vibrio vulnificus* in the presence of 5% (final) SDS, it was found that the sensitivity of the ABC immunodot blot assay was 10^3 bacteria per 100 µl of hepatopancreas homogenate SDS (Fig. 4). The sensitivity of the ABC immunodot blot assay for these bacteria which were inoculated in the hepatopancreas decreased 10-fold in comparison to the sensitivity for pure bacterial cultures (Figs. 2 & 4).

Feasibility of ABC immunodot blot assay in the field survey

Vibrio strains isolated from hepatopancreas of shrimp collected in the field survey were characterized (Table 3). Both *Vibrio vulnificus* and *V. harveyi* strains were Gram-negative rods, could grow at 40°C and utilize chitin, cellobiose, casein and tyrosine, and were lysine decarboxylase positive. Of the 447 *V. harveyi* strains isolated 78 were luminous while 39 *V. vulnificus* strains and the remaining *V. harveyi* strains were non-

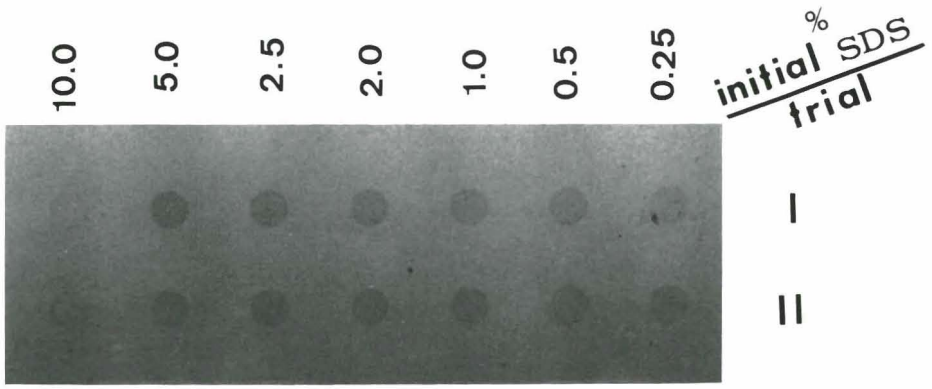

Fig. 3. Effect of sodium dodecyl sulfate on hepatopancreas peroxidase in the ABC immunodot blot assay

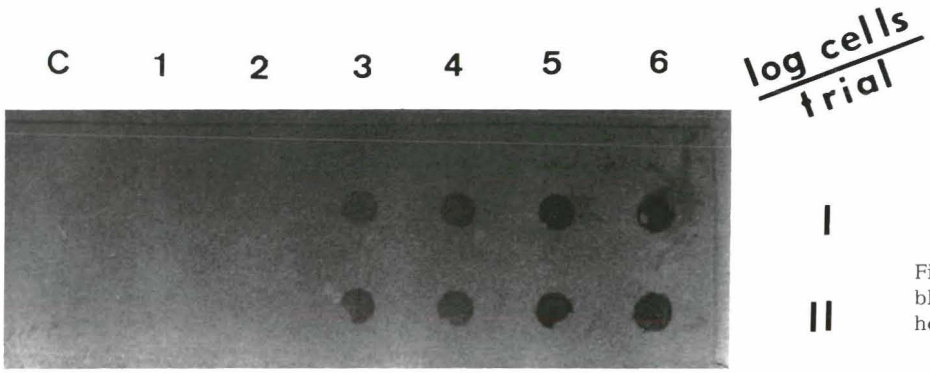

Fig. 4. Sensitivity of ABC immunodot blot assay for *Vibrio vulnificus* in the hepatopancreas with the presence of sodium dodecyl sulfate

Table 3. Differential characteristics of *Vibrio vulnificus* and *V. harveyi* isolated in the field survey. TCBS: thiosulfate-citrate-bile salt-sucrose; ONPG: *o*-nitrophenyl-B-D-galactopyranoside

Test	*V. vulnificus* (n = 39)	*V. harveyi* (n = 447)
Colony on TCBS agar	G/Y	G/Y
Susceptibility to:		
0/129 (150 µg)	+	+
Novobiocin (30 µg)	+	+
Gram stain	–	–
Morphology	rod	rod
Luminescence	–	+ (78)[a]
Growth at 40C	+	+
Lysine decarboxylase production	+	+
ONPG	+	–
Utilization of:		
Chitin	+	+
Cellobiose	+	+
Casein	+	+
Tyrosine	+	+
Serine	–	+

[a] Number of luminous strains

luminous. *V. harveyi* strains utilized serine, but *V. vulnificus* strains did not. *V. vulnificus* strains were able to decompose ONPG, but *V. harveyi* strains could not.

Among the 602 shrimp collected, 379 shrimp were shown to harbor *Vibrio* spp. Hepatopancreas homogenates were checked for the presence of *Vibrio vulnificus* and *V. harveyi* using both classical bacterial identification and the ABC immunodot blot assay. The proportion of shrimp harboring *V. vulnificus* or *V. harveyi* in each collection remained identical or increased when the ABC immunodot blot assay was used (Table 4).

DISCUSSION

The immunodot blot assay without application of ABC proved to be superior to other assays tested in relation to reproducibility, technical complexity, time consumption and special instrumentation requirements. However, the detection limit may be too low to diagnose, for example, the *Vibrio vulnificus* in oysters, where concentrations ranged from 10^3 to 10^5 bacteria g^{-1} of tissue homogenate (Bryant et al. 1987, Kaysner et al. 1989). Development of a diagnostic kit to be used not only for symptomatic infections but also for early-infected or subclinical carriers would be very useful. In the field survey, the application of the PVDF membrane with the aid of the Hybrid Dot 96-well Filtration Manifold allowed diagnosis of a large number of samples. Visual interpretation of the results will make it practical for future use in hatcheries. Basic laboratory facilities, however, including a vacuum line for filtration, are needed and there are problems of technical complexity and high cost to be solved.

Bacterial isolation and characterization confirmed the accuracy of the ABC immunodot blot assay, demon-

Table 4. Diagnosis of shrimp harboring *Vibrio* spp., *V. vulnificus* and *V. harveyi* using bacterial isolation (BI) and the indirect ABC immunodot blot assay (IDB)

Location	No. of shrimp collected	Body length (cm)	No. of shrimp harboring:				
			Vibrio spp.	*V. vulnificus*		*V. harveyi*[a]	
			BI	BI	IDB	BI	IDB
Tainan	212	0.6– 2.1	131 (62)[b]	4 (2)	4 (2)	40 (19)	46 (22)
Tainan	135	0.8– 3.5	118 (81)	6 (4)	10 (7)	47 (35)	53 (39)
Tungkang	78	6.5–10.5	48 (62)	3 (4)	5 (6)	40 (51)	45 (58)
Chiatung	116	12.2–16.3	40 (34)	1 (1)	1 (1)	21 (18)	24 (21)
Linyuan	61	19.1–23.5	42 (69)	0 (0)	2 (3)	28 (46)	35 (57)
Total	602		379 (63)	14 (2)	22 (4)	176 (29)	203 (34)

[a] Luminous and nonluminous strains
[b] Numbers in parenthesis represent percentage

strating that carriers of *Vibrio harveyi* and *V. vulnificus*, detected by bacterial isolation, were also positive in the ABC immunodot blot assay. The opposite, however, was not true, suggesting that bacterial isolation from the hepatopancreas on TCBS agar may not be as sensitive as the ABC immunodot blot assay from organ homogenates. This may be because only viable bacteria can be cultured whereas both viable and dead cells are detected in the ABC immunodot blot assay. The presence of SDS may not only eliminate the interference of tissue peroxidase but also disrupt the bacterial cells in the hepatopancreas. Attachment of bacterial fragments of the PVDF membrane should be greater than that of intact cells; thus a higher dose of antigens would be bound on the solid phase and better sensitivity obtained.

Because the immunodot blot assay has the advantage of detecting both culturable and non-culturable cells, handling between collection and processing to ensure organisms' viability (Oliver 1981) is less critical. Besides, rapid identification tests will probably improve ecological and clinical investigations of shrimp vibrios. These advances will reduce the need for many biochemical tests. With improvements in direct identification techniques using Mabs, the need for culture techniques should be substantially reduced.

Shrimp from larvae to adult were screened for the presence of *Vibrio vulnificus* and *V. harveyi*. Results showed that shrimp of all stages carried these vibrios. This discovery ruled out the previous conjecture that shrimp in the larval stage were more easily susceptible to *Vibrio* infection, and the results are consistent with observations from Malaysian shrimp ponds, where mortalities occurred throughout the year and were equally likely to occur in any pond (Anderson & Shariff 1987).

Only 2 % (assayed with the method of bacterial isolation) to 4 % (assayed with the method of ABC immunodot blot) of the shrimp harbored *Vibrio vulnificus*. Mortalities or symptomatic disease were not present at the time of sampling, suggesting that *V. vulnificus* is an opportunistic pathogen. However, the positive chitinoclastic activity of our isolates suggests a mechanism for bacterial penetration of the shrimp exoskeleton. Baumann et al. (1980) showed that all pathogenic *Vibrio* species elaborate an extracellular chitinase. Furthermore, even if *V. vulnificus* is only present opportunistically, its virulence to many hosts including humans should be noted. This study showed that 29 % (bacterial isolation) to 34 % (ABC immunodot blot) of the shrimp harbored *V. harveyi*, suggesting that *V. harveyi* is not pathogenic but rather endemic and possibly becomes pathogenic when shrimp are stressed by other factors. However, these results should not be taken to mean that no other virulent organisms were involved in the shrimp epidemics. While 63 % of the shrimp were found to be carriers of genus *Vibrio*, only 31 % (2 + 29 %) of the shrimp were carriers of *V. vulnificus* and *V. harveyi*. Therefore, other species could be involved in mortalities, including *V. damsela* (Song et al. 1992), *V. nereis*, *V. tubiashi*, and *V. anguillarum*, etc. (Huang 1989). Mixed infection might also account for the shrimp epidemics. In any case, the ABC immunodot blot assay can be an effective tool to monitor for the presence of *Vibrio* spp. and to detect increases prior to symptomatology and mass mortalities which result in serious economic losses to shrimp farmers.

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