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Source: Journal of Wildlife Diseases, 21(3) : 211-218

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-21.3.211

Journal of Wildlife Diseases, 21(3), 1985, pp. 211–218

DETECTION OF *VIBRIO ANGUILLARUM* ANTIGEN BY THE DOT BLOT ASSAY

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ABSTRACT: The dot blot assay, modified and adapted for detection of antigens from *Vibrio anguillarum* in fish tissues, was specific for *V. anguillarum* and did not react with antigens of *V. ordalii*, *Pseudomonas* sp., or *Yersinia ruckeri*. The blot assay enabled detection of as little as 2.3 ng of a mixture of protein antigens obtained from cell-free extracts of *V. anguillarum*; it was about 100 times more sensitive than either the indirect fluorescent antibody technique or bacterial isolation for detecting *V. anguillarum* in fish tissues.

INTRODUCTION

Certain vibrios are pathogens for marine and estuarine animals. As an example, vibriosis is a serious disease of anadromous salmonids. In North America, the effects of vibriosis have been most severe in salmonid aquaculture operations in the Pacific Northwest (Cisar and Fryer, 1969; Evelyn, 1971; Novotny et al., 1975). However, vibriosis is a pandemic disease that lacks host specificity. It has also been reported in many nonsalmonid species of marine fishes, including ayu (*Plecoglossus altivelis*) (Aoki and Kitao, 1978), eel (*Anguilla japonicum*) (Muroga et al., 1976), herring (*Clupea harengus pallasi*) (Pacha and Kiehn, 1969), sole (*Solea solea*) (Fluchter, 1979), striped bass (*Morone saxatilis*) (Toranzo et al., 1983), and yellowtail (*Seriola quinqueradiata*) (Jo et al., 1979). Although vibriosis is primarily a disease of marine fishes, epizootics have also occurred among rainbow trout (*Salmo gairdneri*) cultured in freshwater (Ross et al., 1968; Giorgetti and Ceschia, 1982), and even in fishes in tropical aquaria (Hacking and Budd, 1971).

Although considerable heterogeneity exists among nonpathogenic marine vibrios (Staley and Colwell, 1973), *Vibrio anguillarum* is most frequently implicated as the etiologic agent of vibriosis in diseased fish from the Pacific Northwest (Rucker, 1959). Harrel et al. (1976) isolated a second pathogenic vibrio that had fastidious growth characteristics and differed serologically from *V. anguillarum*; this vibrio was later classified as a distinct species, *V. ordalii* (Schiewe et al., 1981).

Detection and diagnosis of *V. anguillarum* and *V. ordalii* have been achieved by isolation, biochemical identification, and serological confirmation. Two serodiagnostic tests used for other pathogenic bacteria of fish also have been adapted for the detection of vibriosis: macroscopic slide agglutination (Conroy and Withnell, 1974) and indirect fluorescent antibody (Bullock and Stuckey, 1975). Immunoenzyme assays have also been developed to detect fish pathogens. The most common procedure thus far developed is the enzyme-linked immunosorbent assay (ELISA) that has been adapted to detect certain bacterial (Rodgers, 1981; Smith, 1981) and viral (Dixon and Hill, 1981) pathogens of fish. An alternative enzyme immunoassay that detects protein antigens bound to nitrocellulose (NC) paper was decribed by Hawkes et al. (1982) and is available as a commercially prepared kit. Our study was conducted to adapt the Bio-Rad Immun-Blot (GAR-HRP) Assay (Bio-Rad Laboratories, 1983) for serodiagnostic detection of *V. anguillarum*.

MATERIALS AND METHODS

Pure cultures of *V. anguillarum* and *V. ordalii* (Table 1) were examined for biochemical

Received for publication 26 October 1984.

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TABLE 1. Source of bacteria used in this study.

Bacterium and identification number	Source	Year isolated
Vibrio anguillarum		
8.3	Willow Beach, Arizona	1963
8.5	ATCC #14033	1963
8.9	Willow Beach, Arizona	1964
8.11	Eastern Fish Disease Laboratory, West Virginia	1967
8.13	Lint Slough, Oregon	1972
8.14	Little Port Walter, Arkansas	1972
8.15	Lumi Indian Aquaculture Project, Washington	1972
8.17	Eastern Fish Disease Laboratory, West Virginia	1973
Vibrio ordalii		
V1669	Manchester, Washington	1975
MSC-275	Oregon State University, Oregon	1975
MSC-876	Oregon State University, Oregon	1976
Pseudomonas sp.		
8.18	Eastern Fish Disease Laboratory, West Virginia	1975
Yersinia ruckeri		
11.40	State Hatchery, North Carolina	1978

reactions consistent for each species. We identified each isolate as the genus *Vibrio* by using the following criteria: gram-negative motile bacilli, cytochrome oxidase positive, ferment glucose anaerogenically, and sensitive to novobiocin and the vibriostatic agent 0/129 (Evelyn, 1971). Additional biochemical reactions were performed as described by Schiewe et al. (1981) to distinguish *V. anguillarum* from *V. ordalii*. Reference strains of *Yersinia ruckeri* and *Pseudomonas* sp. were used as controls.

After biochemical reactions were established, each strain of *Vibrio* was cultured in brain–heart infusion (BHI) broth (Difco) supplemented with 1% NaCl. Cultures were incubated for 24 hr at 25 C and then centrifuged at 5,000 RPM for 5 min in a Fisher Model 59 microfuge. Spent media were pipetted into sterile vials and frozen at −20 C as soluble antigen. Pelleted bacteria were resuspended in 1.0 ml of sterile phosphate buffered saline (PBS) and also frozen at −20 C as whole cell antigens. Similar antigens were also prepared for *Y. ruckeri* and *Pseudomonas* sp.

We attempted to detect these antigens by the dot blot assay, following the manufacturer's directions (Bio-Rad Laboratories, 1983). Nitrocellulose sheets were placed in 20 mM Tris, 500 mM NaCl, pH 7.5 (TBS) for 5 min. These sheets were removed from the TBS and allowed to air dry; then 1 μl of the whole cell and soluble antigens from each strain of bacteria were blotted onto the NC sheets. Additionally, 1 μl of PBS was blotted on the NC sheets as a negative control.

Antigen-blotted NC sheets were placed in 3% gelatin–TBS for 30 min and reacted either with a specific rabbit antiserum against *V. anguillarum* or with normal rabbit serum. The rabbit sera were obtained from the Biologics Section of the National Fish Health Research Laboratory. Both specific and control sera were diluted 1:50 in 1% gelatin–TBS and incubated at room temperature with the antigen-blotted NC sheets for 2 hr with gentle agitation. Nitrocellulose sheets were then removed from these primary antibody solutions, washed briefly with deionized water, and rinsed for a total of 20 min in two changes of TBS. Nitrocellulose sheets were reacted for 2 hr with a 1:3,000 secondary antibody solution of goat anti-rabbit IgG–horseradish peroxidase conjugate diluted in 1% gelatin–TBS. Nitrocellulose sheets were washed again with water and rinsed in TBS, as previously described. After the final TBS rinse, NC sheets were developed with 0.015% H_2O_2 and 4-chloro-1-naphthol for 5 to 15 min. When antigen was detected, it appeared as blue dots against a white background (Fig. 1). Color development was halted by placing NC sheets in deionized water for 10 min.

To determine the accuracy of the dot blot assay, the whole cell bacterial antigens were examined by the indirect fluorescent antibody test (IFAT) as described by Bullock and Stuckey (1975). Both primary and secondary antibody dilutions were similar to those used in the dot blot assay.

Because the dot blot assay was designed to bind protein antigens to NC sheets, a cell-free antigen extract from *V. anguillarum* (8.13) was prepared as described by Cipriano et al. (1981). *Vibrio anguillarum* (8.13) was incubated in 1 liter of BHI broth supplemented with 1% NaCl on a rotary shaker at 150 RPM for 72 hr at 25 C. This culture was centrifuged at 3,896 *g* for 30 min at 4 C in a Sorval RC-5 centrifuge. Spent medium was decanted and brought to 80% saturation with crystalline $(NH_4)_2SO_4$ by constant stirring. The mixture was centrifuged

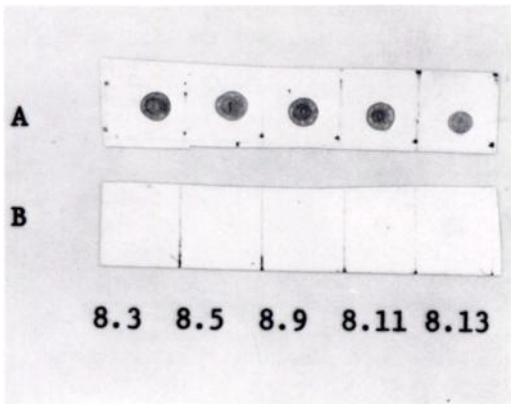

FIGURE 1. Results of dot blot reaction for specific detection of *Vibrio anguillarum* antigen. Soluble antigens from *V. anguillarum* strains 8.3, 8.5, 8.9, 8.11, and 8.13 were blotted onto nitrocellulose (NC) sheets. Nitrocellulose sheet A was reacted with a rabbit antiserum to *V. anguillarum* and NC sheet B with normal rabbit serum. Goat anti-rabbit serum conjugated with horseradish peroxidase was used as a secondary antibody, and color reactions were developed with 0.015% H_2O_2 and 4-chloro-1-naphthol.

again for 30 min at 3,896 *g* to precipitate proteins contained in the spent medium. Precipitated proteins were dissolved in 10 ml of deionized water and dialyzed against PBS for 48 hr at 4 C. The dialyzed material was designated as a cell-free antigen extract and contained 1.17 mg protein per milliliter (Bradford, 1976). Serial twofold dilutions of the cell-free extract were prepared in PBS and the sensitivity of the dot blot assay was tested.

Western blot immunoassays were conducted to further examine the antigenicity of the *V. anguillarum* cell-free antigen extract. Polyacrylamide slab gels (1.5 mm thick) were prepared using a 12% resolving gel and a 4% stacking gel as described by Laemmli (1970). Sonicated cell antigen and cell-free antigen extract of *V. anguillarum*, with appropriate molecular weight markers, were boiled for 5 min in sample buffer; 20 µl samples were then loaded into the gel slot. Electrophoresis was begun at 15 mA until the bromphenol blue tracking dye reached the gel interface. Current was then increased to 25 mA with a 200-V limit. Electrophoresis was conducted at room temperature; upon completion, gels were stained with 0.01% Coomassie blue in 25% methanol–10% acetic acid, and then destained in the same solution without dye.

Polyacrylamide gels were transferred immediately to 200 ml of electrode buffer for the electrotransfer of proteins from SDS gels as described by Towbin et al. (1979). Gels were soaked for 10 min in electrode buffer and placed in contact with a sheet of NC (BA-85, 0.45 µm; Schleicher and Schuell, Inc., Keene, New Hampshire 03431, USA). The gel and NC sheets were soaked in buffer and sandwiched between layers of soaked GB002 filter paper (Schleicher and Schuell) in a TE-52 transfer apparatus (Hoefer Scientific Instruments, San Francisco, California 94107, USA). Proteins were electrotransferred from acrylamide gels to NC sheets in 45 min at 12 C, according to the manufacturer's instructions. Proteins transferred to NC sheets were visualized either by Coomassie blue or by the dot blot assay.

Infectivity studies were then conducted with disease-free brook trout (*Salvelinus fontinalis*) to determine the ability of the dot blot assay to detect antigen from *V. anguillarum* (8.13) in diseased tissues. Each of 20 brook trout (4.2 g) was injected intraperitoneally with 0.1 ml of a *V. anguillarum* viable cell suspension containing 2.0×10^8 cells/ml of PBS. Ten other brook trout were similarly injected with a comparable suspension of viable bacteria from *Y. ruckeri* (11.40) and an additional 10 fish were injected only with 0.1 ml of PBS. Each group of brook trout was maintained for 72 hr in pathogen-free spring water at 12.5 C. After 72 hr, dead fish were removed and the remainder were killed in tricaine methanosulfonate. Kidney extracts were prepared from diseased and control trout as described by Kimura and Yoshimizu (1981). The entire kidney from each fish was excised and homogenized in 0.5 ml of PBS. After homogenized kidneys were boiled for 30 min at 100 C and centrifuged at 5,000 RPM for 5 min in a Fisher Model 59 microfuge, the supernatant was used as an antigen source. Each supernatant suspension (1 µl) was blotted onto NC sheets as previously described. Rabbit antiserum to *V. anguillarum* was used as a specific test serum and normal rabbit serum was used as a control.

Additional infectivity studies were then conducted to compare the sensitivities of the blot assay with the IFAT. Brook trout (47.4 g) were divided into eight groups of 10 fish each, and each fish was injected with 0.2 ml of one of the dilutions of viable *V. anguillarum* cells containing from 2.4×10^9 to 2.4×10^3 cells/ml. Fish in a final group were similarly injected with PBS. Mortality began 48 hr after injection, and the remaining fish were killed and necropsied, and their kidneys were cultured on BHI

TABLE 2. Biochemical reactions performed to differentiate *Vibrio anguillarum* from *V. ordalii*.*

Test	Bacterium and strain number										
	Vibrio anguillarum								*Vibrio ordalii*		
	8.3	8.5	8.9	8.11	8.13	8.14	8.15	8.17	V1669	MSC-275	MSC-876
Growth at 37 C	+	W	–	W	+	W	W	W	–	–	–
Gelatin hydrolysis	+	+	+	+	+	+	+	+	W	W	W
Arginine decarboxylase	+	+	+	+	+	+	+	+	–	–	–
Simmon's citrate	W	+	+	+	+	+	+	+	–	–	–
Christensen's citrate	+	+	+	+	+	+	+	+	–	–	–
Voges Proskauer	+	+	+	+	+	+	+	+	–	–	–
ONPG	+	+	W	+	+	+	+	+	–	–	–
Fermentation of:											
Sorbitol	+	+	+	+	+	+	+	+	–	–	–
Glycerol	+	+	+	+	+	+	+	+	–	–	–
Trehalose	+	+	+	+	+	+	+	+	–	–	–

* W = weak positive reaction.

agar supplemented with 1% NaCl. Cultures were incubated for 48 hr at 25 C. Growth was tentatively identified as *V. anguillarum* if the organisms were gram-negative, motile bacilli that were cytochrome oxidase positive, anaerogenic fermentors of glucose, and sensitive to both novobiocin and the vibriostat 0/129. The identifications were further confirmed by macroscopic slide agglutination with specific antiserum to *V. anguillarum* (Conroy and Withnell, 1974).

After necropsy, kidney preparations for immunodetection were prepared as previously described. We performed IFAT's on kidney homogenates and blot assays on the kidney extract antigen. In addition to specific antiserum, normal rabbit serum was also reacted in both assays with duplicate samples as a control.

RESULTS

All *Vibrio* isolates used in this study were gram-negative, motile bacilli that were cytochrome oxidase positive, anaerogenically fermented glucose without gas formation, and were sensitive to novobiocin and the vibriostatic agent 0/129. Additional biochemical criteria that differentiated *V. anguillarum* from isolates of *V. ordalii* are listed in Table 2. On the basis of these results, isolates V1669, MSC-275, and MSC-876 were identified as *V. ordalii*, and isolates 8.3, 8.5, 8.9, 8.11, 8.13, 8.14, 8.15, and 8.17 as *V. anguillarum*. Isolate 8.18 was a gram-negative, motile rod that was cytochrome oxidase positive and sensitive to novobiocin; however, this bacterium oxidized glucose and was therefore tentatively identified as a *Pseudomonas* sp. *Yersinia ruckeri* (11.40) showed biochemical reactions consistent for this species (O'Leary et al., 1979).

Initial dot blot results in which whole-cell antigens and soluble antigen preparations of test bacteria were used indicated that both whole cells and soluble antigens of *V. anguillarum* could be detected by the dot blot assay (Table 3). Additionally, the specificity of the dot blot assay was comparable to that of IFAT. When rabbit antiserum prepared against *V. anguillarum* was used, both serodiagnostic tests gave positive test results for antigen preparations prepared from test strains of *V. anguillarum*. However, neither assay gave positive reactions for the three strains of *V. ordalii*, nor the two control bacterial antigens—*Pseudomonas* sp. and *Y. ruckeri*.

Sensitivity studies indicated that the dot blot assay gave positive reactions to a 1:512 dilution of cell-free extract antigen of *V. anguillarum* (8.13). Although stock concentrations of the cell-free extract con-

TABLE 3. Results of dot blot and indirect fluorescent antibody tests (IFAT) to detect specific antigen preparations of bacteria used in this study.

Antigen source	IFAT	Dot blot	
	Cell antigens	Cell antigens	Soluble antigens
Vibrio anguillarum			
8.3	+	+	+
8.5	+	+	+
8.9	+	+	+
8.11	+	+	+
8.13	+	+	+
8.14	+	+	+
8.15	+	+	+
8.17	+	+	+
Vibrio ordalii			
V1669	−	−	−
MSC-275	−	−	−
MSC-876	−	−	−
Pseudomonas sp.			
8.18	−	−	−
Yersinia ruckeri			
11.40	−	−	−

tained 1.17 mg of protein per milliliter, only 1 μl from each serial twofold dilution of antigen was blotted onto NC. Therefore, the actual sensitivity of the dot blot assay detected 2.3 ng of *V. anguillarum* antigen per microliter of suspension.

The SDS–polyacrylamide gel electrophoresis studies further indicated that both the cell lysates and cell-free extracts of *V. anguillarum* were complex mixtures of proteins (Fig. 2). Fewer protein bands were visualized in the cell-free extract than in the cell lysate preparation. However, proteins in cell-free extracts appeared to have analogous counterpart proteins in the cell lysate.

Western blot immunoassays confirmed the antigenicity of the cell lysate and cell-free extracts. When these preparations were probed with rabbit antiserum to *V. anguillarum* cells, similarities were found in proteins with molecular weights of about 22.5 kilodaltons within both cell lysate and cell-free extracts (Fig. 2).

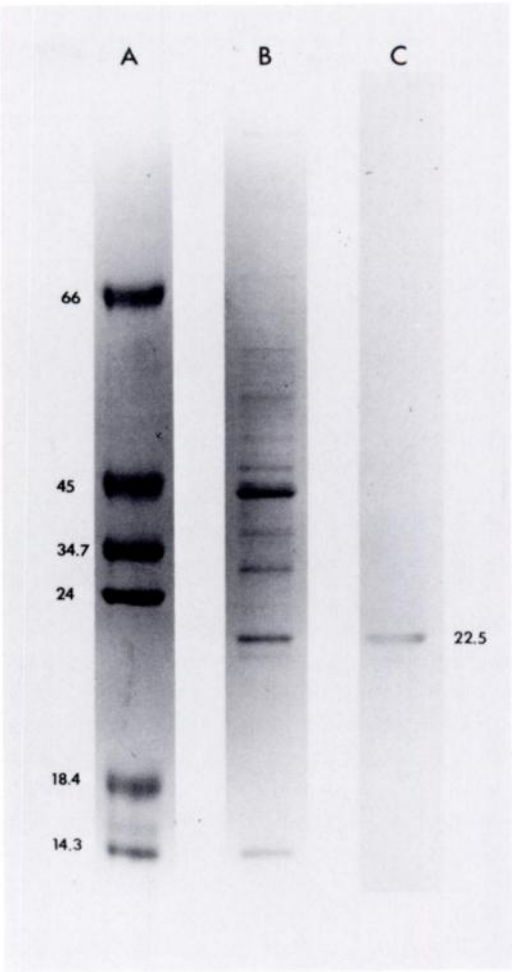

FIGURE 2. Protein and antigenic analysis of *Vibrio anguillarum* cell-free extract by SDS polyacrylamide gel electrophoresis and immunoblotting. (A) Coomassie blue-stained gel of molecular weight markers. Bands are identified in kilodaltons at left. (B) Coomassie blue-stained gel of *V. anguillarum* cell-free extract. (C) Replica of (B) electroblotted onto nitrocellulose and probed with *V. anguillarum* antiserum. Major immunoreactive protein labeled at right in kilodaltons.

Because the western blot immunoassays indicated that cell-free extracts of *V. anguillarum* were antigenic, the dot blot was further developed to detect *V. anguillarum* antigen in kidney tissues of infected trout. In preliminary infectivity studies, all brook trout injected with 2.0×10^7 cells

TABLE 4. Comparative sensitivities of the dot blot, indirect fluorescent antibody test (IFAT), and bacterial culture for detection of *Vibrio anguillarum* in kidneys of infected brook trout.[a]

Infective dose[b]	Dot blot	IFAT	Culture
4.8×10^8	10	7	10
4.8×10^7	10	6	10
4.8×10^6	7	3	10
4.8×10^5	7	3	4
4.8×10^4	6	1	1
4.8×10^3	6	0	0
4.8×10^2	4	0	0
0	0	0	0

[a] Number of fish positive for *V. anguillarum* within each group of 10 brook trout.

[b] Number of bacteria injected intraperitoneally in 0.2 ml of saline per fish.

of *V. anguillarum* and killed 72 hr postinjection gave positive results for *V. anguillarum* by the dot blot method. None of the fish injected with either *Y. ruckeri* or PBS gave positive results for *V. anguillarum*. Furthermore, all of the infected and control brook trout that were reacted with normal rabbit serum as the primary antibody in the dot blot assay gave negative results.

The sensitivity of the dot blot was compared with both the IFAT test and culture; our results indicated that the dot blot was more sensitive than either (Table 4). Among fish injected with graded dilutions of *V. anguillarum* and killed 48 hr postinjection, dot blot assay detected antigen in four of 10 fish injected with 4.8×10^2 cells. By contrast, endpoint detection limit for both culture and IFAT was demonstrated by one positive fish at the 4.8×10^4 dilution. The dot blot was at least 100 times more sensitive than bacterial isolation of IFAT for practical detection of *V. anguillarum* antigen. No antigen was detected in fish injected with PBS or in the duplicate set of samples in which normal rabbit serum was used as the primary antibody.

DISCUSSION

Our results indicated that the dot blot assay can be adapted for in vitro and in vivo detection of *V. anguillarum* antigens. The assay showed specificity comparable to that observed with IFAT. When available antiserum was used, neither serodiagnostic assay gave positive reactions when reacted with antigens from *V. ordalii*, *Pseudomonas* sp., or *Y. ruckeri*. However, both IFAT and the dot blot tests were able to detect antigens of *V. anguillarum*. In vitro, the dot blot assay was able to detect as little as 2.3 ng of antigen from *V. anguillarum*. Moreover, the assay was about 100 times more sensitive than IFAT for identification of antigen in tissues.

Primary (1:50) and secondary (1:3,000) antisera concentrations used in these assays were adapted from manufacturer's instructions and double checker-board titration with test antigens. The effectiveness of any serodiagnostic assay is highly dependent on antisera used for detection. Antibody titrations should therefore always be performed on each lot of antiserum to determine optimal working concentrations.

Both ELISA and dot blot assays are similar enzyme immunoassays performed on different support substrates. In ELISA tests, antigen is usually bound to polystyrene plates, whereas in the blot assay protein antigens are bound to nitrocellulose sheets. Equipment involved with the dot blot assay is less expensive than that used in ELISA. Therefore, the dot blot can be more economically advantageous than ELISA for the small diagnostic laboratory. However, ELISA may still provide a more versatile and accurate estimation of antigen than is derived from the blot assay.

In this study we adapted the dot blot assay for detection of *V. anguillarum* antigen in diseased fish tissues. However, by changing the primary antiserum used, one can adapt the assay for detection of other fish pathogens. Currently, we have mod-

ified procedures and have used the dot blot test to detect *Aeromonas salmonicida*, the cause of furunculosis; *Yersinia ruckeri*, the cause of enteric redmouth disease; and *Renibacerium salmoninarum*, the cause of bacterial kidney disease.

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