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FLUORESCENT ANTIBODY ENUMERATION OF *VIBRIO CHOLERAE* IN THE MARINE ENVIRONMENT

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ABSTRACT - From a series of microcosm studies performed in our laboratory, it has been observed that over time *V. cholerae* and related potential human pathogens enter a viable but nonculturable state. Direct viable counts by epifluorescent microscopy consistently remain higher than corresponding plate counts. Thus, the assumption that pathogens "die-off" or "decay" in the marine environment must be re-evaluated, since stressed or nutrient starved cells are unable to grow and be enumerated by standard plate count methods. Indirect immunofluorescent microscopy offers a more sensitive detection system for environment sampling for human pathogens. Field studies indicate that cultures positive for *V. cholerae* O1 are also positive by fluorescent antibody staining; however, the reverse was not necessarily true. By adapting epifluorescent techniques for environmental studies, a more realistic, i.e., valid estimate of *V. cholerae* O1 population size can be obtained directly from environmental samples.

Key words : *V. cholerae*, microcosm, viable, non-cultivable, immunofluorescent-epifluorescent

RÉSUMÉ - D'après une série d'études de microcosmes réalisées dans notre laboratoire, il a été observé que *V. cholerae* et d'autres pathogènes potentiels pour l'homme sont toujours à l'état viable mais non cultivable en milieu de culture. Les comptages directs de bactéries viables par microscope à épifluorescence restent nettement plus forts que les numérations sur milieu de culture correspondantes. Aussi, l'hypothèse selon laquelle les bactéries pathogènes "meurent" ou "disparaissent" dans l'environnement marin doit être revue, puisque les cellules stressées ou à jeun sont incapables de se développer et d'être dénombrées par les méthodes de culture classiques. La microscopie indirecte par immunofluorescence permet une détection plus sensible pour les pathogènes de l'homme dans les échantillons naturels. Les études indiquent que les cultures positives pour *V. cholerae* O1 sont aussi positives par la coloration fluorescente des anticorps ; cependant, l'inverse n'est pas forcément vérifié. En adaptant les techniques d'épifluorescence pour les études dans le milieu naturel, une estimation plus proche de la réalité de la taille des populations de *V. cholerae* O1 peut être obtenue directement.

Mots clés : *V. cholerae*, microcosme, viable, non cultivable sur milieu de culture, immunofluorescence, épifluorescence.

Determining the number of pathogens and indicator organisms in the marine environment is imperative for assessing the public health safety of coastal and estuarine waters. Bacteria entering coastal waters confront a variety of temperature, salinity, and nutrient concentration changes, with all parameters not necessarily optimal, or even permissive, for growth and replication at any given time. Is there then, a rapid "die-off" during unfavorable conditions as some investigators suggest (Mitchell, 1968), or rather, do the organisms employ a survival strategy until more satisfactory conditions for growth occur? Extensive studies employing microcosms in the laboratory, using *Escherichia coli*, *Vibrio cholerae*, *Salmonella* and *Shigella* spp., have shown that these organisms persist in

a dormant state, and are not enumerated in the dormant state because they are incapable of growth in conventional culture media. The cells are viable, but non-culturable. Therefore, waters, the quality of which are judged acceptable, may indeed be hazardous, placing the public health safety of those waters in question.

By adapting indirect immunofluorescent-epifluorescent techniques developed in our laboratory for environmental studies, a more realistic estimate of population size has been obtained (Xu, *et al.*, 1983 and 1984). An evaluation of this new approach, compared with conventional enumeration methods was conducted for waters of Chesapeake Bay, Louisiana, Puerto Rico, Zimbabwe (Africa) and Bangladesh for the presence of *V. cholerae* serovar O1 (Colwell, *et al.*, 1984, Xu, *et al.*, 1984). Determinations were performed in sewage and water samples using the three-tube, three-dilution, most probable number (MPN) index. Loopfuls from each tube of alkaline peptone enrichment broth (peptone, 1% w/v; NaCl; 1% w/v ox-bile; 0.5% w/v, pH 8.6, were transferred to thiosulfate-citrate-bile salts agar (TCBS, Oxoid, Columbia, MD USA) and suspect colonies were examined by slide agglutination, employing commercial antiserum (Difco Laboratory, Detroit, MI) for the presence of the O1 antigen. A loopful was also spread on a clean glass slide for fluorescent antibody staining (FAB) and an MPN enumeration by microscopic detection was made (Xu, *et al.*, 1984).

Slides were air-dried, fixed with alcohol, and air dried once more. A drop of RITC for background staining (Rhodamine isothiocyanate-conjugated bovine serum albumin, BBL Microbiology Systems, Cockeysville, MD) was placed on each slide and incubated in a moist chamber for 30 minutes at 35°C. Phosphate buffered saline (PBS) was then used to wash each slide. Next, a drop of specific antiserum was placed on the slides, followed by incubation. After another washing with PBS, one drop of FITC (fluorescein-isothiocyanate conjugate of goat anti-rabbit globulin antiserum, Difco) was delivered to the slides. A final incubation period with subsequent washing followed. After air drying, a cover-glass was mounted on each slide using FA mounting fluid (Difco, pH 9.0.). Slides were viewed using a Zeiss standard 18 microscope equipped with a 12.5 x eyepiece, 100 x neofluor objective, IVFL epifluorescence condenser, 100 W halogen lamp, BP 450-490 band pass filter, FT 510 beam splitter, and LP 520 barrier filter (Carl Zeiss Inc., New-York, NY) or equivalent.

Upon examination stained preparations of *V. cholerae* revealed rod-shaped cells, with distinct curvature, in some cases, and a peripheral green band just below the cell wall was visible.

Results of MPN enumerations of sample collections in Louisiana are presented in Table I. Only one sewage sample in June, 1981 proved positive for *V. cholerae* O1, using standard culture methods, i.e. media and slide agglutinations. In contrast, all water, sewage and sediment samples from June and September were positive by fluorescent staining, yielding a significantly higher bacterial count per 100 ml volume of water.

Canals and village water sources in Bangladesh were the sites of sampling in April and May, 1982. To determine the presence or absence of *V. cholerae* O1, 100 ml volumes of water were filtered through 0.45 µm filters (Gelman, GN-6) which were placed in enrichment broths. Broths were incubated at 30°C for 12 h, after which 1.0 ml aliquots were removed and dispensed to fresh enrichment broth. Incubation at 37°C followed for an additional 22 h. Loopfuls of inoculum were transferred, after each incubation stage, to taurocholate-tellurite-gelatin agar and slides. Slide agglutinations were performed on portions of bacterial colonies and fluorescent antibody staining was done to determine the presence of the *V. cholerae* O1 antigen. From a total of 52 water samples collected,

seven were positive for *V. cholerae* O1 by conventional culture methods. Over 3,000 colonies were picked for slide agglutination for this determination. Using fluorescent antibody techniques, however, 51 out of the 52 samples were confirmed as positive for *V. cholerae*. All samples positive by culture, were positive by staining. Also, recovery of *V. cholerae* on media was higher at the earlier stages of enrichment incubation, and not recoverable at later periods. Fluorescent antibody results remained positive throughout.

Date	Source (1)	<i>Vibrio cholerae</i> MPN (2)		
		Culture		Epifluorescent
		Total (3)	O1	O1
6/15/81	W	24	<3.0	460
6/15/81	SD	1.1	<0.3	>110
6/23/81	Sw	11,000	16	>1100
9/14/81	W	29/4,600	<3.0	150
9/14/81	Sd	1.2/21	<0.3	4.2
9/15/81	Sw	11,000	<3.0	20
12/7/81	W	<3.0	<3.0	<3.0
12/7/81	Sd	<0.3	<0.3	<0.3
12/15/81	Sw	<3.0	<3.0	<3.0

Table 1 : *Vibrio Cholerae* culture and epifluorescent MPN results of Louisiana samples.

(1). Water : W, Sediment : Sd, Sewage : Sw. (2). *V. cholerae* MPN per liter of water or per gram sediment. (3). Total *Vibrio cholerae* population independent of Serovar.

It is apparent from these results that the fluorescent antibody technique is a more sensitive mechanism for detecting the presence of *V. cholerae* O1, compared to traditional culture methods. This also holds true for other bacteria when appropriate specific antisera are used.

Additional fluorescent techniques have been applied in the laboratory to study this non-culturability phenomenon. Direct viable counts (DVC) (Kogure *et al.*, 1979), offer viewing and enumeration of viable cells by direct microscopy. Yeast extract is added to a water sample to provide nutrient for growth and replication. With the addition of nalidixic acid, cell wall formation is inhibited. Therefore, substrate responsive cells, i.e. viable cells, appear elongated when stained with acridine orange. Acridine orange direct counts (AODC) (Hobbie, *et al.*, 1977) is a fluorescent procedure used to enumerate total bacteria in a sample. These enumeration techniques, along with the fluorescent antibody procedure described above (modified for enumeration by using filtration procedures), along with the traditional plate count and MPN methods (Russek *et al.*, 1982), have been incorporated in studies examining the survival patterns of selected pathogens with laboratory microcosms.

Aged estuarine water, for which the pH and salinity were adjusted was used to simulate conditions in the environment over time. No additional nutrients were introduced, in order to induce a dormant state for the analyses. A strain of *Salmonella enteritidis*, serogroup C, isolated from the Potomac River, (Roszak *et al.*, 1983), was studied in microcosms prepared with water collected from the Potomac River. Approximately 1×10^5 cells per ml were introduced on Day 1. *Salmonella* Poly A-1 and Vi antisera (Difco) was used for FAB staining; Veal Infusion and Xylose Lysine Decarboxylase agar (XLD,

Difco) for plate counts; and Dulcitol Broth, (Difco), Selenite Cysteine Broth (Difco) and XLD agar for MPN determinations. Results (Fig. 1) showed that the AODC remained constant, at 10^5 cells per ml, during the study. Counts for FAB and DVC were 0.5 log lower than the AODC. All plate counts and MPN estimates, however, exhibited a rapid decline and became non-culturable on the media employed for enumeration by days 2 and 3.

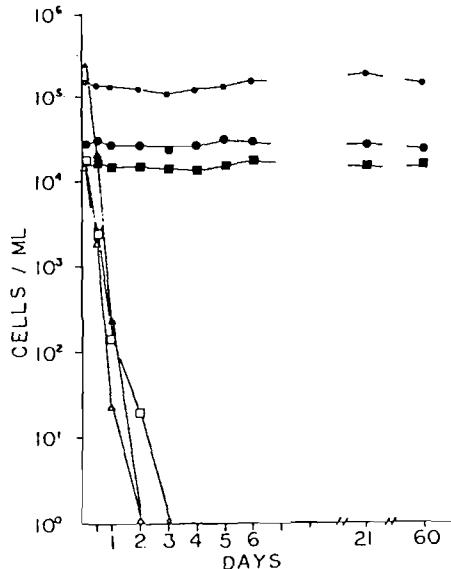


Figure 1 : *Salmonella enteritidis* cultures exposed to river water in microcosms. O : Acridine orange Direct Count, ● Fluorescent antibody Count, ■ Direct viable Count, □ Plate Count, Veal Infusion, △ Plate Count, XLD, Δ MPN-Salmonella Enrichment.

E. coli strain H10407 was introduced at a concentration of 1×10^7 cells/ml into a microcosm containing Chesapeake Bay water (salinity 15‰). AODC remained at the initial concentration (Fig. 2). Over a period of 19 days, viable counts declined only by 0.5 log. Significantly, plate counts on TSA and Eosin Methylene Blue agar (EMB, Difco) dropped from 10^6 on Day 1 to 10^4 on Day 7, and then to 10^3 cells/ml on Day 14.

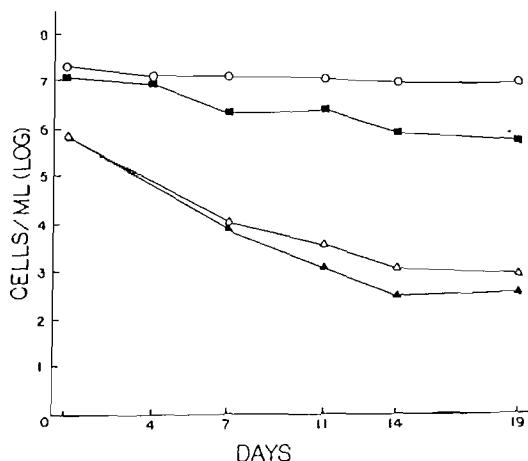


Figure 2 : *Escherichia coli* cultures exposed to Chesapeake Bay water in microcosms. O Acridine Orange Direct Count, ■ Direct Viable Count, △ Plate Count, TSA, △ Plate Count, EMB.

Microcosms of *Shigella sonnei* strain 53 G, and *Shigella flexneri*, strain M42-43, were prepared using Chesapeake Bay water (15°/oo, 18°C). TSA and MacConkey agar were used to recover culturable cells. *Shigella* Poly B (Difco) was employed for the FAB staining. When 1×10^6 cells/ml were inoculated into the system, the AODC and FAB counts remained at the same concentration (Fig. 3, 4). DVC declined by 1.0 log. Both organisms became non-culturable, with *S. flexneri* plate counts reaching 0 cfu significantly earlier than *S. sonnei*.

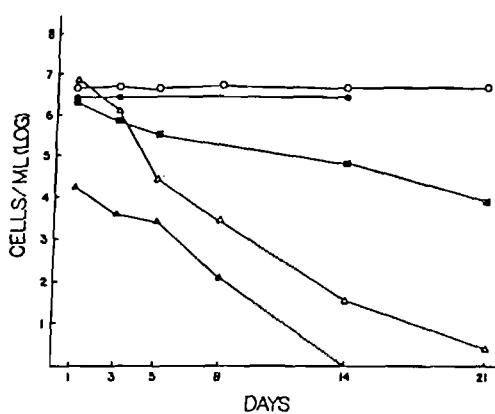


Figure 3 : *Shigella sonnei* cultures exposed to Chesapeake Bay water in microcosms. ○ Acridine Orange Direct Count, ● Fluorescent Antibody Count, ■ Direct Viable Count, △ Plate Count, TSA, □ Plate Count, MacConkey.

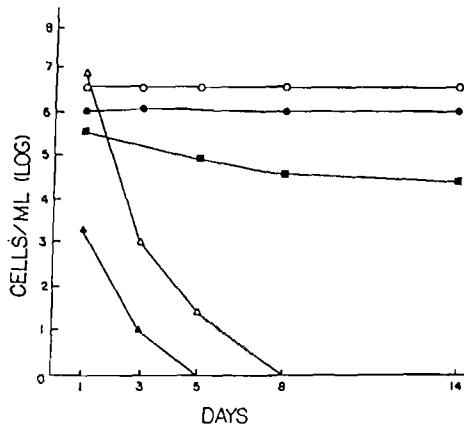


Figure 4 : *Shigella flexneri* cultures exposed to Chesapeake Bay water in microcosms. ○ Acridine Orange Direct Count, ● Fluorescent Antibody Count, ■ Direct Viable Count, △ Plate Count, TSA, □ Plate Count, MacConkey.

Microcosm experiments were designed to explore the survival patterns of *V. cholerae* CA 401, serotype O1, a toxin positive strain (Fig. 5). Two flasks of Patuxent River water (salinity 15°/oo, pH 7.4) were inoculated with 10^6 cells/ml. In both systems, AODC and FAB counts remained constant at 10^6 cells/ml for the week following inoculation, and DVC counts remained constant at 10^4 cells/ml. Upon withdrawing samples 1 h post-inoculation, plate counts on TCBS and TSA plates for Microcosm 1 were 10^4 cfu/ml on both media types. For Microcosm 2, counts were 10^4 and 10^5 cfu/ml respectively. After 24 h, all plate counts dropped to 0, and remained non-culturable for the duration of the experiment.

In situ experiments were performed using membrane chambers submerged in semi-tropical waters at Bimini, Bahamas (Fig. 6) (Grimes *et al.*, 1984). *E. coli* strain H10407 was added at a concentration of 2.4×10^6 cells/ml into 3 chambers. An additional chamber received no bacteria, and served as a control. Fluorescent enumeration procedures and plate counts on 1/8 brain heart infusion agar (BHI, Difco) were conducted on samples removed at 0, 13, 25, 38, and 112 h. The FAB and DVC held constant at 10^5 cells/ml. *E. coli* was not present in the control chamber, which proved impervious to environmental *E. coli* species. Plate counts on BHI dropped from 10^4 cfu/ml at 0 h, to 1 cfu/ml at 13 h and remained as such for the duration of the experiment.

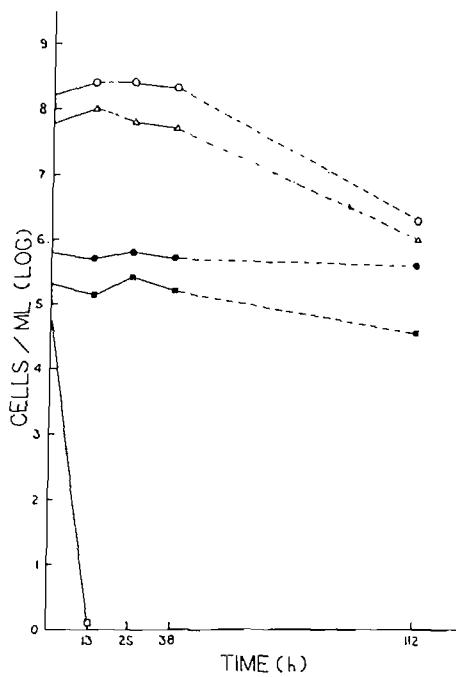


Figure 5 : *V. cholerae* CA 401 cultures exposed to Patuxent River water in microcosms. ○ Acridine Orange Direct Count, ● Fluorescent Antibody Count, ■ Direct Viable Count, △ Plate Count, TSA.

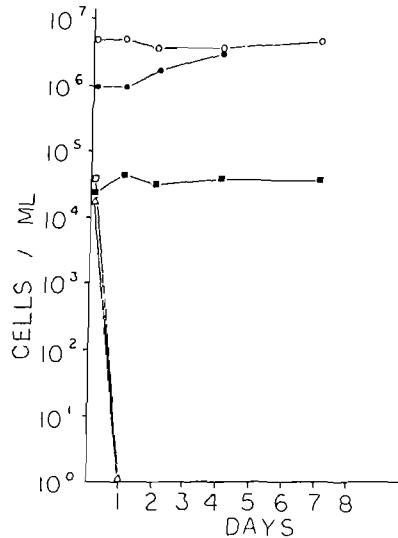


Figure 6 : *E. coli* H10407 cultures exposed to semi-tropical waters in membrane chambers. ○ Acridine Orange Direct Count, ● Fluorescent Antibody Count, ■ Direct Viable Count, △ Plate Count, TSA, ▲ Acridine Orange Direct Count of control chamber.

Clearly, a non-culturable, but viable and dormant stage exists for those bacteria examined to date. Viable counts by fluorescent microscopy consistently remained higher than corresponding plate counts. The use of immunofluorescent-epifluorescent methods in the laboratory has provided us with clues to the status of bacteria in the environment. Since non-culturable cells are incapable of growing on standard enumeration media, it can then be concluded that the traditional culture methods to assess microbial populations are inadequate. Of prime concern is the question of whether viable but non-culturable bacteria remain virulent.

A ligated ileal loop assay (Spira *et al.*, 1981) was performed to answer this important question. The non-culturable cells from both microcosms of *V. cholerae* CA 401 were harvested by centrifugation and resuspended in river water to a concentration of 10^8 cells/ml, comprising a DVC count of ca. 10^6 cells/ml. Rabbits were prepared for surgery; seven loops, separated by interloops, were ligated with catgut. Ca. 1.0 ml volumes of the following solutions were injected into individual loops: 1) T_1N_1 broth (1% tryptone, 1% NaCl; the original growth medium for strain CA 401), 2) CA 401 (fully viable, pure culture, 10^6 cells/ml); 3) sterile Patuxent river water; 4) Microcosm 1 cell 5; 7) Microcosm 2 cells. Ileal loop assay was done ca. 30 h post surgery. When the intestine was excised, positive virulence response was noted in all microcosm loops, and all were similar in appearance to the CA 401 control loop, i.e. distended, hemorrhagic, and filled with fluid. The river water loop returned to its original size. The T_1N_1 loop was distended, but not hemorrhagic. An average of 4 ml of fluid was aspirated from each microcosm loop.

The fluid was transferred to a tube of BHI broth, streaked to plates of TCBS and TSA and prepared for AODC, FAB and DVC counts. Results showed that CA 401 cells from all microcosms injected into the rabbit ileum, were recoverable in BHI broth and on TSA and TCBS plates. Surprisingly, all plate cultures grew as pure cultures. Slide agglutination tests were 4⁺ positive with *V. cholerae* O1 antisera. DVC counts increased to 10⁸ to 10⁹ cells/ml. AODC counts increased from 10⁸ to 10¹⁰ cells/ml. Transfers from the T₁N₁ loop revealed no growth on TSA, TCBS, and EMB plates and in BHI broth. Microscopic examination of the T₁N₁ loop revealed large rods in chains, consistent with the morphology of gut anaerobes.

A similar ileal loop assay was performed using the viable, but non-culturable cells recovered from membrane chambers. Aspirated fluid from the loops were streaked to EMB and BHI-Instant Ocean plates. *E. coli* grew on all the media tested.

In conclusion, immunofluorescent-epifluorescent techniques have provided information concerning the survival patterns of selected bacteria in the aquatic environment. Although "die-off" is indicated by conventional plating techniques, fluorescent microscopy reveals that viable pathogens continue to be viable and to remain present in far greater numbers than reported. Thus, bacteria possess a "starvation-survival" mechanism (Baker *et al.*, 1983; Hoppe, 1976; Jones *et al.*, 1981; Mitchell, 1968; Morita, 1983; Novitsky *et al.*, 1977 and 1978; Stevenson, 1978; and Tabor *et al.*, 1981.) during the absence of suitable energy-yielding substrates, and prevail in a dormant phase until suitable environmental conditions return. The latter may occur in the water column or in animal hosts. The results of the animal studies dramatically demonstrate that non-recoverable cells continue to harbor the potential for virulence, the public health implications of which cannot be ignored. Reevaluation of the traditional enumeration procedures clearly are needed.

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