PD-AAW-695
52028

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: 10/8/87

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 4.178

2 cp FINAL - Aug 87 rec'd

Attachment

2 cp July 86

2 cp Feb 86 (rec'd 10/8/87)

2 cp Path. Potential, Oct 85 rec'd 10/8/87

2 cp Vibrio cholerae abstract rec'd 10/8/87

2 cp A Rapid Test for Cholerae ... July 87 rec'd 10/8/87

2 cp Fluorescent Antibody/1987 rec'd 10/8/87

2 cp Modif'd T aurocholate/Dec 85 rec'd 10/8/87

2 cp The Role of Enteric Path/1988 rec'd 10/8/87

2 cp Vibrio hist/Non-Cholerae/1985 rec'd 10/8/87

4.178
PDAAW-695

FINAL REPORT

submitted to

AGENCY FOR INTERNATIONAL DEVELOPMENT
Grant No. DPE-5542-G-SS-4060-00

Period Covered
March 1984 - August 1987

Submitted by

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August 1987

Rec'd in A.I.D. OCT 8 1987

Introduction

The principal objective of this research was to determine factors influencing the ecology of *Vibrio cholerae*, including physical, chemical, and biological parameters of the aquatic environment. Epidemiological data concerning transmission of *V. cholerae* in the environment to human populations was also collected and analyzed.

Specific objectives were to determine the distribution, growth, and survival of *V. cholerae* and related bacteria in the aquatic environment; to expand the application of direct detection of microorganisms by immunofluorescent techniques; to measure physico-chemical parameters and biological associations, and to determine genetic factors influencing the ecology and pathogenicity of these organisms. Also, methods suitable for isolation and identification of pathogenic bacteria from environmental samples were developed from the data gathered during this study. A summary of specific aims follows.

Section I

Refining the immunofluorescent direct microscopic method for detecting specific microorganisms in the aquatic environment

Enumeration of indicator organisms or, preferably, direct detection of pathogens in the aquatic environment, is essential for estimation of water quality. Public health safety is determined from the results of such tests and the choice of method for assessment depends on specificity, reproducibility, and statistical validity, in conjunction with time and cost efficiency, and ease of operation. Immunofluorescent detection methods fulfill these criteria and are being used to detect a variety of organisms in environmental samples (6, 10, 14, 15, 17).

The viable, but non-culturable stage of bacteria has been established by our laboratory (3, 10-12). In addition, results of studies have shown that nutrient-starved and "injured" cells are incapable of growing on standard culture media and, therefore, "starved" or "injured" indicator organisms will not respond when standard cultivation methods for assessing microbial populations are employed. The virulence of viable, but non-culturable cells may be retained, and this phenomenon has been demonstrated by animal studies (3, 16).

Fluorescent antibody (FA) procedures can overcome the problem of organisms remaining viable but yielding "no growth" in standard tests, since the presence, absence, or number of specific cells comprising such populations can be detected microscopically.

Critical to the success of the FA staining procedure is the specificity of the antibody utilized. For that reason, it was our objective to prepare a monoclonal antibody specific to the 01 antigen of *Vibrio cholerae*.

Production of monoclonal antibodies to the Vibrio cholera 01 antigens

It was our objective to produce monoclonal antibodies (mAb) that would react only with *V. cholerae* 01 antigen, for use in the fluorescent antibody-direct viable count assay, immunoblotting assay, and agglutination tests.

It has long been known that antibodies employed in agglutination assays are often not useful for tests of greater sensitivity. For example, serum that agglutinates with *V. cholerae* 01 will cross-react with many non-*V. cholerae* 01 bacteria when used in fluorescent antibody or enzyme-linked immunosorbent assays. Such limitations of polyvalent serum antibodies led to production of monoclonal antibodies with greater specificity.

Monoclonal antibodies were prepared by standard methods. Briefly, Balb C mice were immunized with 1 x 10^7 CFU of a mixture of whole, formaldehyde-killed cells of *V. cholerae*, serotypes Inaba and Ogawa. Immunizations were performed on days 1, 21, 35, and 38. Tail vein blood was tested at selected timed intervals by whole-cell enzyme-linked immunosorbent assay (ELISA). At day 35, antibody titers were observed to be greater than 50,000. On day 38, mouse splenocytes were fused with SP2/O myeloma cells by the method of Lane (9). Hybridoma supernatants were screened by ELISA, using whole cell and

Ogawa lipopolysaccharide-coated microtiter plates. Hybridoma supernatants testing positive were cloned by limiting dilution and, thereafter, stored in liquid nitrogen.

Specificity of the mAb was tested by whole cell ELISA assays, including the bacterial species listed in Table 1. MAb 4AG6 was found to react only with V. cholerae 01 strains and lipopolysaccharide (LPS) prepared from V. cholerae 01 strains.

Other hydridomas produced mAbs (LPS and non-LPS specific) that cross-reacted with a variety of bacterial species. 4AG6 reacted with both Ogawa and Inaba serotypes. This finding was in agreement with results for antibody reacting with the A antigen of 01 LPS. This antigen, unlike B and C, has recently been reported to be specific to 01 V. cholerae (13).

The value of the 01 monoclonal in fluorescent antibody assays was confirmed by testing a variety of bacterial strains frequently found in environmental samples (Table 2).

All cultures were grown overnight in T_1N_1 broths (1% w/v tryptone; 1% w/v NaCl) at 25° C or 35° C, depending on the bacterial strain. Loopfuls of culture were transferred to a tube of phosphate buffered saline (PBS), until a concentration of 10^4 - 10^7 cells/ml, determined by acridine orange direct count, was achieved. At this point, 2.0 ml of the PBS suspension was filtered through an 0.2μ x 25mm polycarbonate membrane filter (Nuclepore Co., Pleasanton, CA), and stained by the fluorescent antibody procedure (18). One hundred fields of each filter were examined by fluorescent microscopy to assess cross-reactivity between the mAb and test strains. Bacterial strains tested are listed in Table 2. Results showed that all Vibrio cholerae 01 strains appeared as small distinct rods surrounded by a bright peripheral fluorescent green band. Fluorescent cells were not observed in preparations of the other 75 bacterial species tested.

Field trials in Bangladesh using the V. cholerae 01 monoclonal antibody

In the conventional FA procedure, all organisms possessing a species-specific antigen can be detected. However, it is not possible to determine which cells are viable and which are dead. By combining the indirect fluorescent antibody procedure of Xu et al. (18) for direct detection of bacteria in natural water samples with the direct microscopic viable count of Kogure et al. (8), enumeration of viable, specific populations of bacteria can be achieved. This membrane filter procedure is described in detail by Brayton et al. (1).

Briefly, environmental samples are incubated with yeast extract and nalidixic acid. Substrate responsive cells, i.e. viable cells, elongate and, after staining with specific antiserum and fluorescein conjugate, viable V. cholerae 01 cells appear as long, peripherally fluorescent, green-banded bacilli, when viewed by microscopic examination. Using an ocular reticule, the number of viable cells per ml can be calculated.

The mechanism of nalidixic acid centers on its ability to inhibit DNA gyrase. At the concentration employed in this study, protein and RNA synthesis continue, but cell division is interrupted. As a consequence,

those cells exposed to the nalidixic acid and yeast extract mixture elongate (5, 8).

The method permits filtration of increasing volumes of water when the bacterial count is low. The maximum volume filtered will be dependent only on the degree of turbidity of the water. If the turbidity of the water sample is high, the sample can be prefiltered through a 1.2μ filter (Gelman Science Inc., Ann Arbor, MI) to remove large particulate matter.

The protocol for cultivation and enumeration of V. cholerae 01 involves sample filtration, transfer of the filter to thiosulfate-citrate-bile salts-sucrose agar (TCBS Oxoid, Columbia, MD) incubation for 24 h, followed by biochemical testing of all sucrose positive colonies with subsequent serological confirmation. For most probable number (MPN) determinations, samples are enriched, employing alkaline peptone broth and incubation for 6 h. Then a loopful from each tube is streaked onto TCBS agar. The same biochemical and serological protocols as above are followed. Inaccurate enumeration can result if V. cholerae is overgrown by other organisms during the enrichment procedure and, therefore, fails to appear on the plating medium. Confirmation of identification and enumeration in both procedures may require several weeks. In comparison, the immunofluorescent technique is not time consuming and is cost-effective. A total FA count of V. cholerae 01, using the membrane filtration method (yeast extract-nalidixic acid step is eliminated to count live and dead cells), can be accomplished in 3 h. The fluorescent antibody-direct viable count (FA-DVC) can be completed the same day, or by the next day, depending on the length of the incubation period required.

A field trial using the FA-DVC and employing 01 antigen-specific mAb was conducted, with water samples collected in Bangladesh (2). This procedure was compared with standard cultural methods to determine the relative sensitivity and rapidity of the two methods.

Sampling for the experiments was undertaken in January 1986, at villages surrounding Matlab, where cholera is endemic. For MPN estimates of V. cholerae 01 (4), 100 ml, 10 ml, and 1.0 ml volumes of water were inoculated into alkaline peptone broth tubes, using three replicates of each volume. Tubes were incubated at 35° C for 6h, after which a loopful from each tube was streaked onto TCBS agar. Plates were incubated at 35° C for 18-24 h, at which time yellow colonies appearing on the agar were picked and subcultured to tryptic soy agar (TSA, Difco, Detroit, MI) amended with 0.5% w/v NaCl. After incubation at 35° C, slide agglutination tests were performed on all isolates using V. cholerae poly 01 antiserum (Difco). Any isolate yielding a positive test was confirmed by fluorescent antibody staining, using mAb.

Fluorescent microscopy enumeration included acridine orange direct counts (AODC), following the protocol of Hobbie et al. (7), and FA for total V. cholerae 01, following the protocol of Xu et al. (1).

Enumeration data for water samples from village sites are shown in Table 3. AODC, employed for the purpose of enumerating the total bacteria, revealed tube well counts of 10^4 - 10^6 cells/ml. River water samples contained 10^6 cells/ml, while pond water counts ranged from 10^6 - 10^7

cells/ml.

The FA results for total V. cholerae 01 indicated all tube well samples to be negative. The river water sample collected adjacent to the Kadamtali village, contained 10^2 V. cholerae 01 per ml, while 10^3 per ml were present in all other river and pond water samples.

Results of viable V. cholerae counts, enumerated by the FA-DVC procedure, yielded similar findings. As might have been predicted, V. cholerae 01 could not be detected by FA-DVC in tube well water samples examined in this study. Both the pond and river water samples from Kadamtali yielded 10^2 viable V. cholerae 01 per ml. All other FA-DVC enumerations for pond and river water samples were in the range of 10^3 cells/ml.

As shown in Table 3, all MPN estimates for water samples were < 0.3 V. cholerae 01 cells per 100 ml, except for the river water sample collected at the village of Gajipur, which contained 2.0 cells per 100 mls. This sample yielded the only culturable V. cholerae 01 in the sample set. No other isolates were recovered after screening all yellow colonies growing on TCBS culture plates by slide agglutination.

In all river and pond water samples examined in this study, total V. cholerae 01 cells comprised 0.01 to 0.1% of the total microbial population. Of the V. cholerae 01 present, 16 to 85% were viable when examined by FA-DVC.

Although the tube well water samples yielded high AODC counts, results of FA and FA-DVC counts indicated V. cholerae 01 were not present. Tube wells have been installed in many villages to provide safe drinking water.

The International Center for Diarrhoeal Disease Research - Bangladesh (ICDDR-B) conducts epidemiological examinations by conventional culture methods at those villages reporting cholera cases. Attempts to isolate V. cholerae 01 from food and water sources in the villages is not always successful (unpublished data). Our attempts to culture V. cholerae 01 were unsuccessful in all but one water sample, which had been collected at the same time samples for FA analysis were collected. In contrast using the FA-DVC method, viable V. cholerae 01 could be detected and enumerated in all river and pond water samples examined. Viable V. cholerae 01 was detected in all samples that were culture positive, as well as those that were culture negative.

The advantage of the FA-DVC far exceeds conventional plating methods for viable bacteria. Plate counts considerably underestimate viable populations in the aquatic environment. No culture medium is capable of supporting growth of all microorganisms. It is possible that all viable bacteria are not capable of enlarging in the DVC procedure, with yeast extract as a substrate. Therefore, this procedure may also underestimate the true viable count. However, the FA-DVC more closely portrays the viable environmental population than traditional cultural methods. Also, the difference in results between the cultural and FA procedures provides strong evidence of the existence of viable but non-culturable V. cholerae in natural environments of cholera-endemic areas (3).

Direct membrane filtration and replica-plating method for detection of 01 serogroup Vibrio cholerae by monoclonal antibody enzyme-linked immunoassay

Detection and enumeration of culturable V. cholerae 01 is complicated by many factors. These include the sensitivity of the methodology, overgrowth of V. cholerae in culture by other endogenous bacteria, and the selection of V. cholerae-like colonies on the surface of agar media. Standard methodologies typically include broth culture of water samples, followed by plating onto selective, differential, and non-selective media. A common problem is that bacteria occurring with V. cholerae 01 will overgrow it. This interferes with subsequent isolation and characterization of the organism. These problems could be overcome if all colonies on agar media could be screened for a specific V. cholerae 01 characteristic, such as the 01 antigen.

As stated above, one of the goals of this research was to develop and implement an immunoassay for rapid and sensitive screening of V. cholerae 01 colonies on agar media. This screening technique was facilitated by the use of anti-01 antigen-specific monoclonal antibody.

Two membrane immunoassays were developed to detect V. cholerae 01 in aquatic systems. The first assay was developed to screen all colonies on agar following standard methods of culture. Bacterial colonies were replica-plated from standard selective and non-selective agar media to nylon membranes, transferred to selective and non-selective agar media, then assayed by enzyme-immunoassay.

Previous experiments established that anti-01 monoclonal antibody did not crossreact with other non-01 bacterial isolates (see above experiments). Results of these experiments showed that the replica-plating method allowed detection of all V. cholerae 01 colonies. After replica-plating, 01 colonies were detected in less than 4 hours. Because of the specificity of the monoclonal antibody and the sensitivity of the method, this assay eliminates the time-consuming and labor-intensive activities of selecting numerous colonies for further biochemical and serological testing.

The methodology was evaluated in a field study during a cholera outbreak in Louisiana in October, 1986. We were contacted by Louisiana State Health Officials of the outbreak and offered the opportunity to employ our method in a field trial.

Water samples suspected to be contaminated with V. cholerae 01 were filtered and tested by colony immunoassay. The Louisiana State Health Department personnel tested the same samples using conventional methodologies. We detected and enumerated V. cholerae 01 in a sample of water that also tested positive by the Health Department. No other samples tested positive. Our results were obtained 24 hours earlier than the Health Department.

The field study also allowed us to recognize limitations of the assay. Examination of sewage samples was not possible because of the large number of non-cholera vibrios which also grew on the selective media. Also, the presence of sulfur-reducing bacteria results in deposits of H_2S which react

with enzyme substrate. This is not a serious limitation, however, since the method still allows screening of all colonies, thus increasing the probability of detecting 01 colonies.

We conclude that this method is valuable for rapid detection of culturable *V. cholera* 01 and should be tested in future cholera outbreaks in the United States and abroad.

Literature Cited

1. Brayton, P.R. and R.R. Colwell. 1987. Enumeration of viable Vibrio cholerae cells by the fluorescent antibody staining technique. J. Microbiol. Meth. (in press).

2. Brayton, P.R., M.L. Tamplin, A. Huq, and R.R. Colwell. 1987. Vibrio cholerae 01 cells in Bangladesh waters: enumeration by fluorescent antibody direct viable count. Submitted to Appl. Environ. Microbiol.

3. Colwell, R.R., P.R. Brayton, D.J. Grimes, D.B. Roszak, S.A. Huq, and L.M. Palmer. 1985. Viable but non- culturable Vibrio cholerae and related pathogens in the environment; implications for release of genetically engineered microorganisms. Biotechnology. 3:817-820.

4. Franson, Mary H. (ed.). Standard methods for the examination of water and wastewater. Sixteenth edition. American Public Health Assoc. Washington, D.C.

5. Goss, W.A., W. H. Deitz, and T.M. Cook. 1965. Mechanism of action of nalidixic acid on Escherichia coli. II. Inhibition of deoxyribonucleic acid synthesis. J. Bacteriol. 89:1068-1074.

6. Gray, L.D. and A.S. Kreger. 1985. Identification of Vibrio vulnificus by indirect immunofluorescence. Diagn. Microbiol. Infect. Dis. 3:461-468.

7. Hobbie, J.E., R.J. Daley, and S. Jasper. 1977. Use of nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33:1225-1228.

8. Kogure, K., U. Simidu, and N. Taga. 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. 25:415-420.

9. Lane, R.D. 1985. A short duration polyethylene gylcol fusion technique for increasing production of monoclonal antibody-secreting hybridomas. J. Immunol. Methods. 81:223-228.

10. Pearson, A., R. Colwell, J. Byrd, J. Dennis, J. Grimes, M. Hoods, D. Hussong, D. Hutchinson, J. Lee, M. O'Brien, J. Stott and J. Walker. 1986. Discovery of viable but non-culturable Legionella pneumophila by immune epifluorescence microscopy: evidence of amplification and sources in five case studies. Bio/Technology (submitted).

11. Rollins, D.M., and R.R. Colwell. 1986. Viable but non-culturable stage of Camplobacter jejuni and its role in survival in the natural aquatic environment. Appl. Environ. Microbiol. 52:531-538.

12. Roszak, D.B., D.J. Grimes, and R.R. Colwell. 1984. Viable but non-recoverable stage of Salmonella enteritidis in aquatic systems. Can. J. Microbiol. 30:334-338.

13. Shimaza,T., R. Sakazaki, and M. Oue. 1987. A bioserogroup of marine vibrios possessing somatic antigen factors in common with Vibrio cholerae 01. J. Appl. Bacteriol. 62:453-456.

14. Sauch, J.R. 1985. Use of immunofluorescence and phase contrast microscopy for detection and identification of Giardia cysts in water samples. Appl. Environ. Microbiol. 50:1434-1438.

15. Schmidt, E.L., R.O. Bankole and B.B. Bohlool. 1968. Fluorescent antibody approach to study of Rhizobia in soil. J. Bacteriol. 95:192-198.

16. Singh, A., R. Yeager, and G.A. McFeters. 1986. Assessment of in vivo revival, growth, and pathogenicity of Escherichia coli strains after copper- and chlorine- induced injury. Appl. Environ. Microbiol. 52: 832-837.

17. Strayer, R.F. and J.M. Tiedje. 1978. Application of the fluorescent-antibody technique to the study of a methanogenic bacterium in lake sediments. Appl. Environ. Microbiol. 35:192-198.

18. Xu, H.-S, N.C. Roberts, L.B. Adams, P.A. West, R.J. Siebeling, A. Huq, M.I. Huq, R. Rahman, and R.R. Colwell. 1984. An indirect fluorescent antibody staining procedure for detection of Vibrio cholerae serovar 01 cells in aquatic environmental samples. J. Microbiol. Meth. 2:221-231.

Table 1. Bacteria and LPS Tested in Monoclonal Antibody Cross-Reaction Experiments employing ELISA.

Bacterial species		Strains
Vibrio cholerae	O1	ATCC14033, ATCC14035, HV113, UM4635, 599655, 194, 192, 395, 195, 01, N16961, 193, 102
LPS prepared from *Vibrio cholerae* O1 strains		5875, 569B, ATCC14033, ATCC14035, 411, NIH41
Vibrio cholerae	non-O1	N2030H, SG8268, SG7509, 43, 42, N8246, 905, 13, 163, 19
LPS prepared from *Vibrio cholerae* non-O1 strains serotyped according to the LSU system		G, K, O, Q, X, Y, FF, UU, XX
Vibrio fluvialis		UM4476, UM4122
Vibrio parahaemolyticus		UM4111, UM4324, UM4237, UM4108, ATCC17802
Vibrio logii		ATCC15382
Vibrio splendidus		UM4259
Vibrio mimicus		UM4074, UM4053
Vibrio tubiashi		3358
Vibrio natriegens		UM4384
Vibrio furnissii		UM4158, UM4137
Vibrio campbellii		UM4344
Vibrio alginolyticus		UM4367, UM4109
Vibrio vulnificus		UM4304
Vibrio anguillarum		UM4248, UM4393
Vibrio carchariae		ATCC35804
Proteus vulgaris		UM769, ATCC13315
Pseudomonas aeruginosa		ATCC10145
Staphylococcus aureus		ATCC13598

Bacterial species	Strains
Flavobacterium meningosepticum	ATCC13253
Streptococcus liquefaciens	ATCC10447
Pseudomonas putida	ATCC12633
Shigella sonnei	UM773
Shigella flexneri	UM774, ATCC12022
Escherichia coli	HB101
Aeromonas hydrophila	UM4396
Alcaligenes faecalis	ATCC8750
Plesiomonas shigelloides	ATCC14029
Enterococcus faecalis	ATCC19433
Salmonalla typhimurium	ATCC14028
Klebsiella pneumoniae	ATCC13883
Citrobacter freundii	ATCC8090
Serratia marcescens	ATCC13880

Table 2. Bacterial strains screened for cross-reaction by fluorescent antibody assay, employing monoclonal antibody.

V. cholerae 01	ATCC[a] 14035
V. cholerae 01	ATCC 14033
V. cholerae 01	59965
V. cholerae non-01	SG 7509
V. cholerae non-01	2030H
V. cholerae non-01	SG 8268
V. cholerae non-01	N 8246
V. cholerae non-01	42
V. cholerae non-01	90
V. cholerae non-01	13
V. cholerae non-01	19
V. cholerae non-01	43
V. mimicus	ATCC 33653
V. mimicus	UM[b] 4053
V. mimicus	UM 4074
V. proteolyticus	ATCC 15338
V. vulnificus	ATCC 27562
V. vulnificus	UM 4304
V. diazotrophicus	ATCC 33466
V. alginolyticus	ATCC 17749
V. alginolyticus	UM 4109
V. parahaemolyticus	ATCC 17802
V. parahaemolyticus	ATCC 27969
V. parahaemolyticus	UM 4111
V. parahaemolyticus	UM 4324

V. parahaemolyticus	UM 4108
V. parahaemolyticus	UM 4237
V. damsela	ATCC 35083
V. damsela	ATCC 33539
V. carchariae	ATCC 35804
V. anguillarum	ATCC 19264
V. anguillarum	UM 4393
V. anguillarum	UM 4248
V. aestuarianus	ATCC 35048
V. cincinnatiensis	ATCC 35912
V. natriegens	ATCC 14048
V. natriegens	UM 4384
V. harveyi	ATCC 14126
V. ordallii	ATCC 33509
V. campbellii	ATCC 25920
V. campbellii	UM 4344
V. fluvialis	ATCC 11328
V. fluvialis	UM 4476
V. fluvialis	UM 4122
V. tubiashii	3358
V. tubiashii	3359
V. splendidus	UM 4259
V. splendidus	UM 4424
V. pelagius	UM 4422
V. pelagius	UM 4494
V. furnissii	UM 4137
Pseudomonas aeruginosa	ATCC 10145

Pseudomonas putida	ATCC 12633
Pseudomonas solonacerium	ATCC 11696
Shigella sonnei	UM 773
Shigella flexneri	ATCC 12022
Shigella flexneri	UM 774
Serratia liquefaciens	NCTC[c] 10442
Serratia marcescens	ATCC 13880
Proteus vulgaris	ATCC 13315
Proteuz vularis	UM 769
Flavobacterium meningosepticum	ATCC 13253
Photobacterium angustum	ATC 25915
Plesiumonas shigelloides	ATCC 14030
Escherichia coli	H10407
escherichia coli	ATCC 11303
Escherichia coli	ATCC 11775
Enterococcus faecalis	ATCC 19433
Salmonella typhimurium	ATCC 14208
Klebsiella pneumoniae	ATCC 13883
Citrobacter freundii	ATCC 8090
Enterobacter aerogenes	ATCC 13048
Aeromonas punctata	ATCC 15468
Aeromonas media	ATCC 33907
Aeromonas hydrohila	ATCC 99071
Aeromonas hydrophila	ATCC 15467
Aeromonas hydrophila	UM 4396
Alcaligenes faecalis	ATCC 8750

- ATCC - American Type Culture Collection, Rockville, MD
- UM - culture collection at University of Maryland
- NCTC - National Collection of Type Culture, Central Public Health Laboratory, London, England

Table 3. Enumeration of *Vibrio cholerae* O1 in Bangladesh waters by cultural and fluorescent antibody methods

Sampling Date	Site	Source	AODC[A] cells/ml	FA[B] cells/ml	FA-DVC[C] cells/ml	MPN[D]/ 100 mls
1-8-86	Goalyaor	River	2.2×10^6	9.9×10^3	1.6×10^3	< 0.3
1-8-86	Goalyaor	Tube well	2.5×10^6	0	0	< 0.3
1-8-86	Shahwazkandi	Pond	2.7×10^7	7.7×10^3	5.9×10^3	< 0.3
1-8-86	Shahwazkandi	Tube well	3.2×10^5	0	0	< 0.3
1-8-86	Shahwazkandi	River	5.9×10^6	5.7×10^3	3.4×10^3	< 0.3
1-8-86	Kadamtali	Tube well	8.5×10^4	0	0	< 0.3
1-8-86	Kadamtali	Pond	1.9×10^7	2.9×10^3	9.4×10^2	< 0.3
1-8-86	Kadamtali	River	6.8×10^6	4.1×10^2	1.6×10^2	< 0.3
1-19-86	Sarderandi	Pond	4.4×10^6	2.9×10^3	1.5×10^3	< 0.3
1-19-86	Sarderandi	River	2.9×10^6	5.4×10^3	1.9×10^3	< 0.3
1-19-86	Uddamdi	Pond	2.0×10^7	6.1×10^3	1.4×10^3	< 0.3
1-19-86	Uddamdi	River	4.9×10^6	3.5×10^3	1.8×10^3	< 0.3
1-19-86	Gajipur	Pond	4.8×10^7	4.1×10^3	3.5×10^3	< 0.3
1-19-86	Gajipur	River	1.9×10^6	4.4×10^3	1.6×10^3	2.0
1-19-86	Charmokundi	Pond	4.9×10^6	3.9×10^3	2.0×10^3	< 0.3
1-19-86	Charmokundi	2nd pond	2.5×10^6	4.9×10^3	1.6×10^3	< 0.3

[A] AODC - acridine orange direct count.

[B] FA - fluorescent antibody count.

[C] FA-DVC - fluorescent antibody - direct viable count.

[D] MPN - most probable number estimate.

Section II

Effects of microcosm salinity and organic substrate concentration on production of Vibrio cholerae enterotoxin.

The incidence of cholera, as other enteropathogenic diseases, ranges from sporadic outbreaks to epidemics that characteristically occur in seasonal cycles (1, 3, 7, 8). Indeed, the cyclical nature of the occurrence of disease caused by *Vibrio cholerae* can be influenced by many variables, including the habits of individuals and a variety of environmental factors. The former has been well-studied in classical epidemiology, especially in the examination of the direct transmission of diarrheal disease. The latter, however, is of special interest, from the perspective of the microbiologist, because the physiological state of the organism, *V. cholerae*, can be directly affected by temperature, salinity, nutrient concentration, and other environmental factors. If these also affect potential pathogenicity (for example, production of enterotoxin), then the environment can, indeed, influence the incidence of the disease in a very direct way (2).

In this study, the effect of salinity and organic nutrient concentration on production of enterotoxin by *V. cholerae* was studied in order to gain information about the physiological and potentially pathogenic state of *V. cholerae* in the natural environment and, therefore, how it may be transmitted to humans from aquatic sources.

Toxigenic isolates of *V. cholerae* were used in all experiments, including classical biotype strains 569B, CA401, ATCC 14035, HV113, University of Maryland isolates 4507, 4510, International Center for Disease Research, Bangladesh (ICDDR,B) isolates 599655, 599687, 599716, 599717, and ICDDR,B El Tor biotype VCELOG52. Stock cultures were maintained in liquid nitrogen and bench cultures were maintained on peptone agar, which comprised 1% peptone, 1% NaCl, and 1.5% agar, pH 7.4. Bacteria were transferred from peptone agar to 100 ml of broth consisting of 3% casamino acids, 0.3% yeast extract, 0.2% glucose, 0.05% KH_2PO_4, pH 8.4 (CAYEG; 9), and incubated for approximately 15 h at 37°C. One ml of a 15 h culture was added to 100 ml of freshly prepared CAYEG broth and incubated at 37°C to mid-to-late logarithmic growth phase, after which cells were washed twice in phosphate buffered saline (PBS) at 1500 x g for 10 minutes. Cells were resuspended in PBS at approximately 10^8 CFU per ml.

Microcosms consisted of 50 ml of defined seawater medium, previously described by Singleton et al. (10). Concentration of components in 32 ppt seawater were as follows (g/L): NaCl, 23.926; $MgCl_2$ $6H_2O$, 10.83; Na_2SO_4, 4.004; $CaCl_2$, 1.147; KCL, 0.677; $NaHCO_3$, 0.0.196; KBr, 0.098; H_3BO_3, 0.026; NaF, 0.003; $SrCl_2$, 0.024. Each microcosm contained 48.5 ml seawater, 0.1 ml bacteria in PBS, and 1 ml organic nutrient (CYG) in H_2O. CYG consisted of 30 g casamino acids, 3.0 g yeast extract and 2.0 g glucose per liter. The resulting microcosms contained approximately 10^6 CFU per ml. Organic nutrient concentration in individual microcosms was adjusted to 10.0, 1.0, 0.1, 0.01, or 0.001 g per liter, Control microcosms received only bacteria and PBS. In some experiments, individual salt components were deleted from microcosms. Microcosms were incubated at 37°C for 15 h with

agitation at 100 rpm. Microcosm salinity was measured with a salinometer (American Optical Corporation, Keene, New Hampshire). In some experiments, microcosms were prepared using seawater collected from the open ocean at Hogsty Atoll in the Bahamas. Microcosms of various salinities were prepared using natural and artificial seawater diluted with sterile, distilled, deionized water.

Toxin production was also measured in CAYEG and syncase broth (4), incubated at 37°C for 15 h at 150 rpm.

Cholera toxin (CT) was detected using a modified enzyme-linked immunosorbent assay (ELISA, 5). Polystyrene (Costar, Cambridge, Massachusetts) or polyvinyl chloride (Falcon, 3912, Becton Dickinson, Oxnard, California) 96-well microtiter plates were coated with 0.1 ml of anti-cholera toxin serum (S12 rabbit serum Peterson, National Institute of Health, Bethesda, Maryland) diluted 1:10,000 with 0.1 M carbonate buffer, pH 9.6. Broth and microcosm samples were dialysed three times against 100 volumes of PBS at 6°C. One-tenth milliter of samples was added to coated plates and incubated for one hour at room temperature. Bound toxin was detected using anti-cholera toxin IgG mAb 227-65, and affinity-purified horseradish peroxidase-conjugated goat anti-mouse IgG (Capell, Malvern, Pennsylvania). The developing substrate employed was 2,2'-azinodi-3-ethylbenzthiazoline sulfonic acid (A-1888, Sigma Chemical Company, St. Louis, Missouri) in 0.05 M citric acid, pH 4.0. Negative controls consisted of plate wells receiving uninoculated microcosms or broths. Purified cholera toxin used for positive controls, and in some microcosms, was purchased from Sigma Chemical Company, St. Louis, Missouri. Cholera toxin was stored at 5°C at 1 ml of protein/ml in buffer composed of 0.05M Tris, 0.001M Na_2-EDTA, 0.003M NaN_3 and 0.2M NaCl.

The Y-1 adrenal cell assay was used to measure the biological activity of cholera toxin (6).

Toxin production increased when V. cholerae strains were grown in 10 to 20 ppt seawater and 1g CYG/L (Fig. 1). This effect was not related to CFU concentration at 10, 20, and 30 ppt, although CT levels were associated with CFU concentration of strain 599687 (Table 1). When salinity was increased in 5 ppt increments, 20 to 25 ppt was optimal for strain 569B (Fig. 2). In contrast, when the concentration of CYG was increased, e.g. from 1 to 10 g/L, the enhanced production of CT production were at 30 ppt. Toxin was not detected in microcosms when the organic nutrient concentration was less than 0.1 g/L (Fig. 3).

Toxin was added to 10 ml of 0, 0.5, 10, 15, 20, 25 and 30 ppt sterile seawater (final concentration of CT of 1 ug/ml) to determine if CT was stable at various concentrations of salt. Results of ELISA and Y-1 cell assays confirmed that biological and antigenic properties of cholera CT were stable under conditions of the experiments employed in this study (Fig. 3). Extended measurements of CT over a 6 month period yielded results showing the CT was remarkably stable at salinities ranging from 10 to 30 ppt. Very high concentrations of salt, e.g. 25 to 30 ppt, provided greatest stability.

The effects of individual salt components on CT production were tested by deleting individual salts of 20 ppt (1 g/L CYG) microcosm medium. CT was not detected in microcosms when sodium bicarbonate or potassium chloride were individually deleted (Table 2). Elimination of NaCl, $MgCl_2$ $6H_2O$ Na_2SO_4, $CaCl_2$, or $SrCl_2$ did not significantly reduce CT production ($P<0.05$). However, a small reduction in CT ($P<0.05$) was observed when KBr, H_3BO_3, or NaF were deleted.

Seven of 10 strains of V. cholerae produced more CT in defined seawater medium (20 ppt, 10g CYG/L) than in CAYEG (15 g/L) or syncase 35 g/L) (Fig. 4). The enhanced production of CT in seawater was not related to CFU/ml Table 1), particularly since CFU/ml syncase broth was higher than in seawater or CAYEG.

Many reports have been published describing properties and mechanisms of pathogenicity of bacteria and providing an understanding of disease processes in general. However, little is known about environmental factors affecting the physiological state of enteropathogenic bacteria and control of pathogenicity.

These experiments elucidated the effect of marine and estuarine environments on the physiology of V. cholerae. Since estuarine and brackish waters have been identified as a source of V. cholerae, factors of these waters may affect the viability and physiology of V. cholerae and influence the transmission of cholera (3).

The findings reported here also demonstrate a salinity dependency of V. cholerae, measured at a nutrient concentration of 0.1 to 1.0 g/L, nearly 2-fold greater than that reported by Singleton et al. (10). It is probable that there is a greater sensitivity of direct methods in measuring viability, which allows detection of effects of nutrient at lower concentrations than is possible with ELISA.

The results of this study show that at high concentrations of organic nutrient, e.g. 10 g substrate per liter, toxin production increases with salinity, in the range of 0 ppt to 30 ppt. However, when organic nutrient is less than 1 g/L, toxin production is markedly enhanced at salinities of 10 to 25 ppt. The salinity effect is concluded to be directly related to the rate of toxin production into the extracellular environment and not to the concentration of culturable cells or stability of toxin in various salinity strengths.

Sodium bicarbonate and potassium chloride were essential salts for CT production at 20 ppt. Importantly, seawater medium, in general, supported higher CT production than CAYEG or syncase media, even though the seawater concentration was 1.5 to 3.5 fold less. The fact that seawater medium enhances toxin production and survival of V. cholerae further supports studies which show V. cholerae to be autochthonous to estuarine environments (2, 3).

Both chemical and physical factors of the aquatic environment have a significant effect on the physiology of V. cholerae, reflected in the production of enterotoxin associated with clinical disease. We believe that the altered physiology of cells responding to physico-chemical variations in

the natural environment is reflected in enteropathogenic disease processes.

Literature Cited

1. Black, R.E., K.H. Brown, S. Becker, A.R.M.A. Alim and M.H. Merson. 1982. Contamination of weanling foods and transmission of enterotoxigenic Escherichia coli diarrhoea in children in rural Bangladesh. Trans. R. Soc. Trop. Med. Hyg. 76:259-264.

2. Colwell, R.R., P.R. Brayton, D.J. Grimes, D.B. Roszak and L.M. Palmer. 1985. Viable but non-culturable Vibrio cholerae in the environment: implications for release of genetically engineered microorganisms. Bio/Technology 3:817-820.

3. Colwell, R.R., R.J. Seidler, J. Kaper, S.W. Joseph, S. Garges, H. Lockman, D. Maneval, H.B. Bradford, N. Roberts, E. Remmers, I. Huq, and A. Huq. 1981. Occurrence of Vibrio cholerae serotype 01 in Maryland and Louisiana estuaries. Appl. Environ. Microbiol. 41:555-558.

4. Finkelstein, R.A., P. Atthasampunna, M. Chulasamaya, and P. Charunmethee. 1966. Pathogenesis of experimental cholera: biological activities of purified procholeragen A. J. Immunol. 96:440-449.

5. Ketyi, I. and A.S. Pacsa. 1980. Estimation of Vibrio cholerae and Escherichia coli heat-labile enterotoxin by enzyme-linked immunosorbent assay (ELISA). Acta Microbiol. Acad. Sci. Hung. 27:89-97.

6. Maneval, D. R. , R.R. Colwell, S.W. Joseph, R. Grays and S.T. Donta. 1980. A tissue culture method for the detection of bacterial enterotoxins. J. Tissue Culture Methods 3: 85-90.

7. Martin, A.R., W. H. Mosley, B.S. Binapani, A. Shamsa and I. Huq. 1969. Epidemiological analysis of endemic cholera in urban East Pakistan 1964-1966. Am. J. Epidemiol. 89:572-582.

8. McCormack, W.M., W.H. Mosley, M. Fahimmuddin, and A.S. Benenson. 1969. Endemic Cholera in East Pakistan. Am. J. Epidemiol. 89:393-404.

9. Sack, D.H., S. Huda, P.K.B. Neogi, R.R. Daniel, and W.M. Spira. 1980. Microtiter ganglioside enzyme-linked immunosorbent assay for Vibrio and Escherichia coli heat-labile enterotoxins and antitoxin. J. Clin. Microbiol. 11:35-40.

10. Singleton, F.L., R.A. Attwell, M.S. Jangi, and R.R.Colwell. 1982. Influence of salinity and organic nutrient concentration on survival and growth of Vibrio cholerae in aquatic microcosms. Appl. Environ. Microbiol. 43:1080-1085.

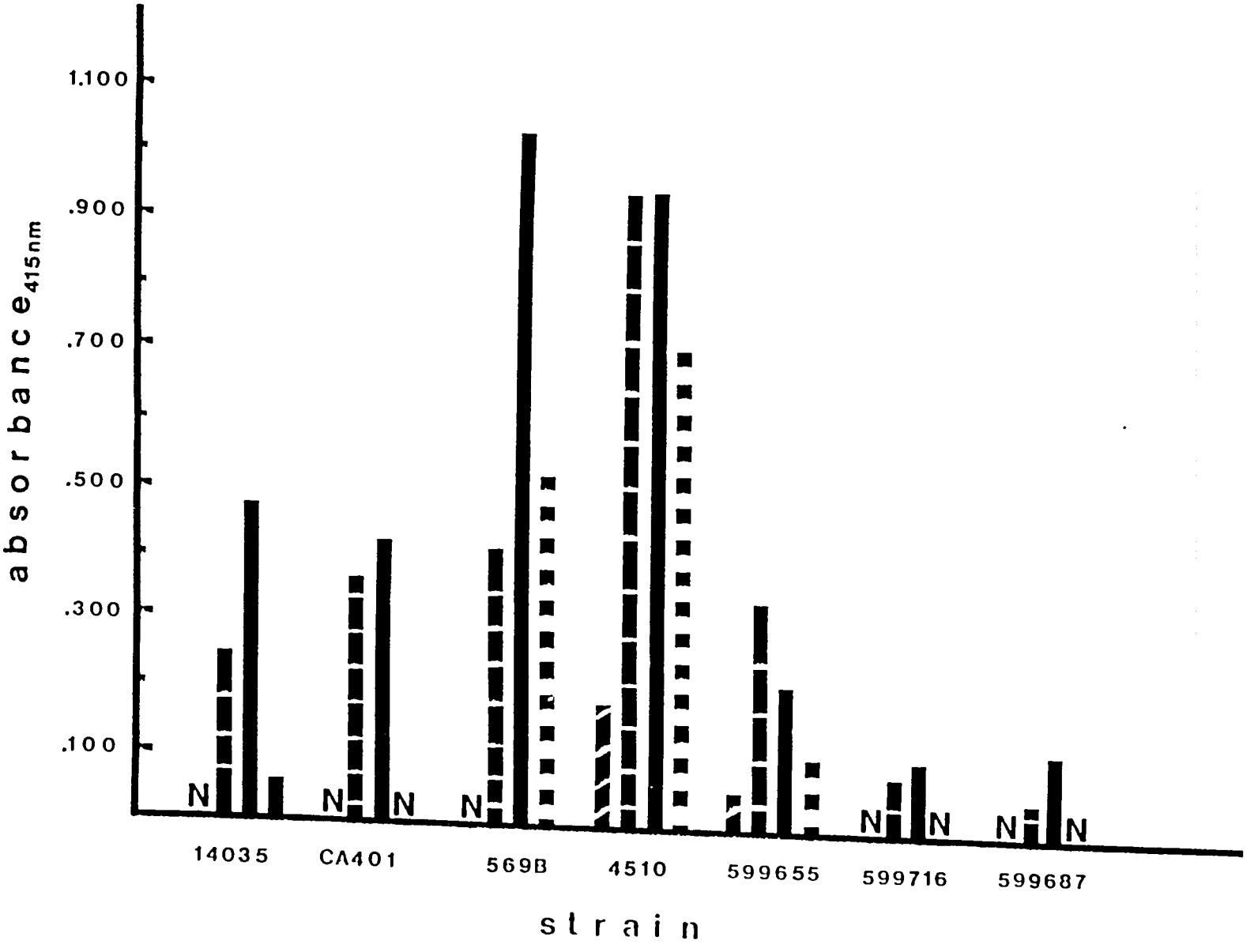

Figure 1. Effect of salinity on toxin production by V. cholerae strains. Conditions for incubation: 37°C, 100 rpm, 15 hr, 1 g CYG/L seawater. Toxin measured by CT ELISA (415nm). Absorbance units were corrected for control microcosms receiving PBS, not bacteria. Salinities tested were 0 ppt (▨), 10 ppt (▬), 20 ppt (■) and 30 ppt (□). N - not detected.

Table 1. Effect of salinity and organic nutrient on cell concentration of *V. cholerae* strains.

	colony-forming units per ml ($\times 10^7$)						
	salinity				media		
Strain	0 [a]	10	20	30	seawater [b]	syncase	CAYEG
14035	1.2×10^{-4}	175	95	192	205	330	42
CA401	3.0×10^{-4}	128	125	160	45	67	70
599655	1.8×10^{-3}	5.3	3.5	7.1	71	325	73
599716	5.3×10^{-4}	8.0	16	8.1	70	325	265
599687	3.5×10^{-4}	7.0	140	7.5	59	275	315
588717	4.5×10^{-4}	15	3.9	4.8	NT [c]	NT	NT
VCELOG52	$<5.0 \times 10^{-5}$	4.0	3.5	7.4	NT	NT	NT
4510	1.5×10^{-2}	53	53	45	60	255	40
569B	9.4×10^{-4}	72	76	100	46	405	6.6

[a] 0, 10, 20, and 30 ppt salinity microcosms were incubated at 37°C, 100 rpm, for 15 hr; seawater (1 g CYG/L), syncase, and CAYEG media were incubated at 37°C, 150 rpm, for 15 hr

[b] seawater - 20 ppt, 10 g CYG/L

[c] NT - not tested

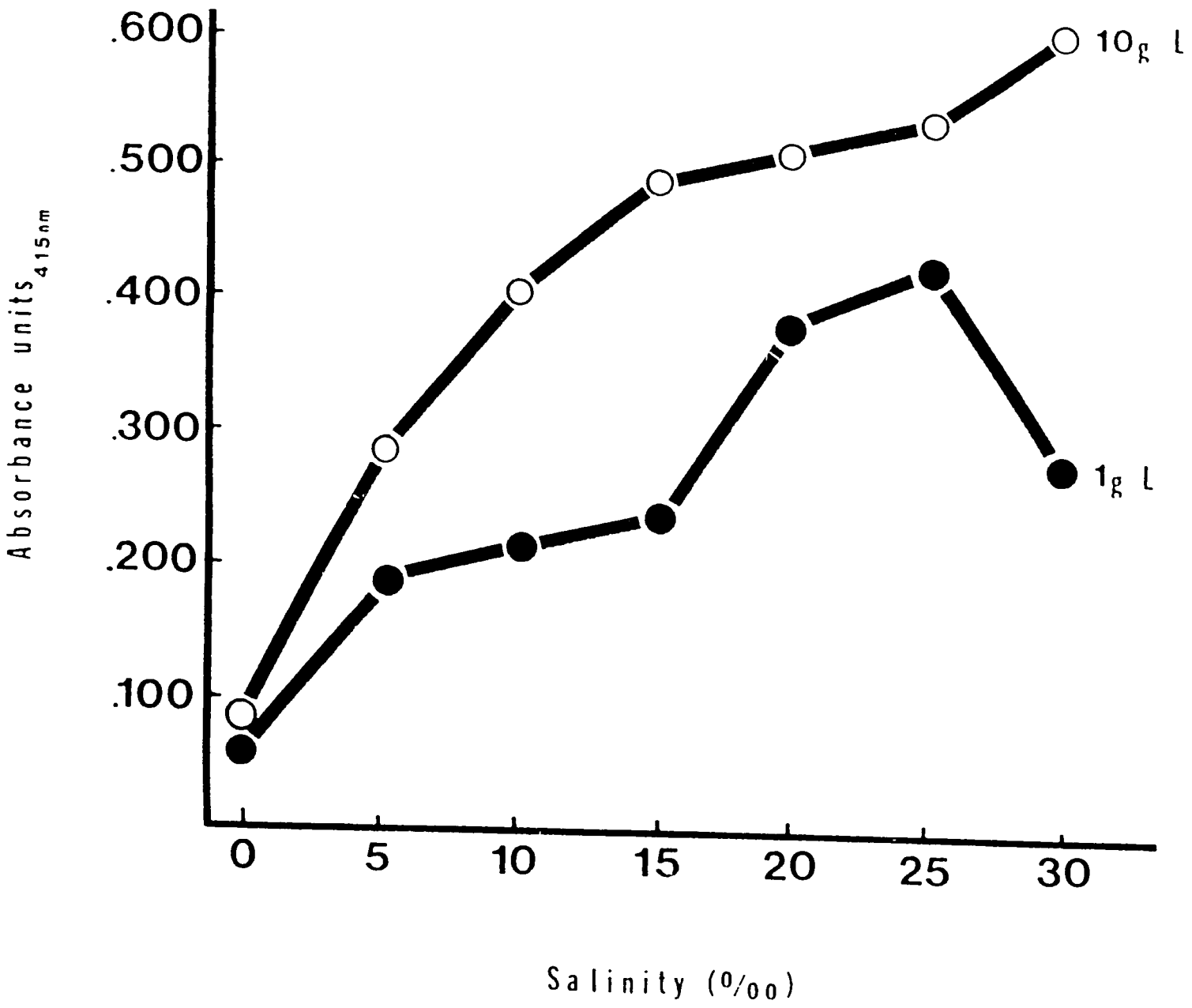

Figure 2. Effect of salinity and organic nutrient contration (○ - 10g CYG/L; ● - 1g CYG/L) on production of cholera toxin by V. cholerae 569B.

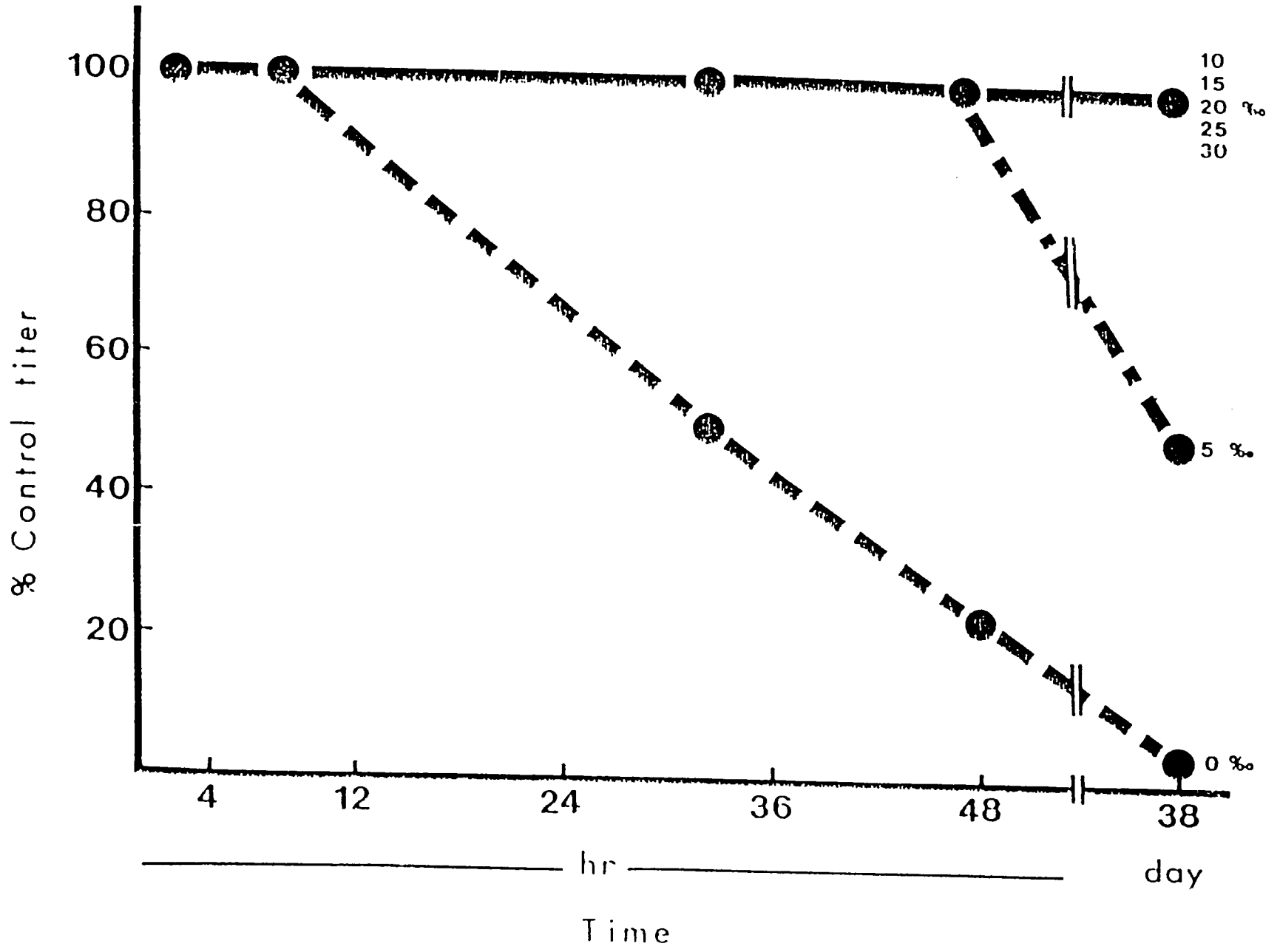

Figure 3. Effects of salinity on antigenic structure and biological activity of cholera toxin. Ten ml of each sterile microcosm contained 1 ug of toxin per ml. Samples were incubated at 25C and tested using ELISA and Y-1 assays. Values are given as percent of PBS control receiving 1 ug of toxin per ml on day of assay.

Table 2. Effects of seawater medium components on production of cholera toxin.

Salt component deleted from seawater medium	CT ELISA [a] (absorbance 415nm)
None	0.118
NaCl	0.118
$MgCl_2$ $6H_2O$	0.126
Na_2SO_4	0.125
$CaCl_2$	0.101
KCl	0.000 [b]
$NaHCO_3$	0.000
KBr	0.083
H_3BO_3	0.079
NaF	0.046
$SrCl_2$	0.096

[a] CT - cholera toxin ELISA; absorbance values were corrected for microtiter plate wells receiving uninoculated microcosms

[b] CT levels in microcosms with KCl, $NaHCO_3$, KBr, H_3BO_3, NaF, or $SrCl_2$ deleted were significantly lower than complete medium

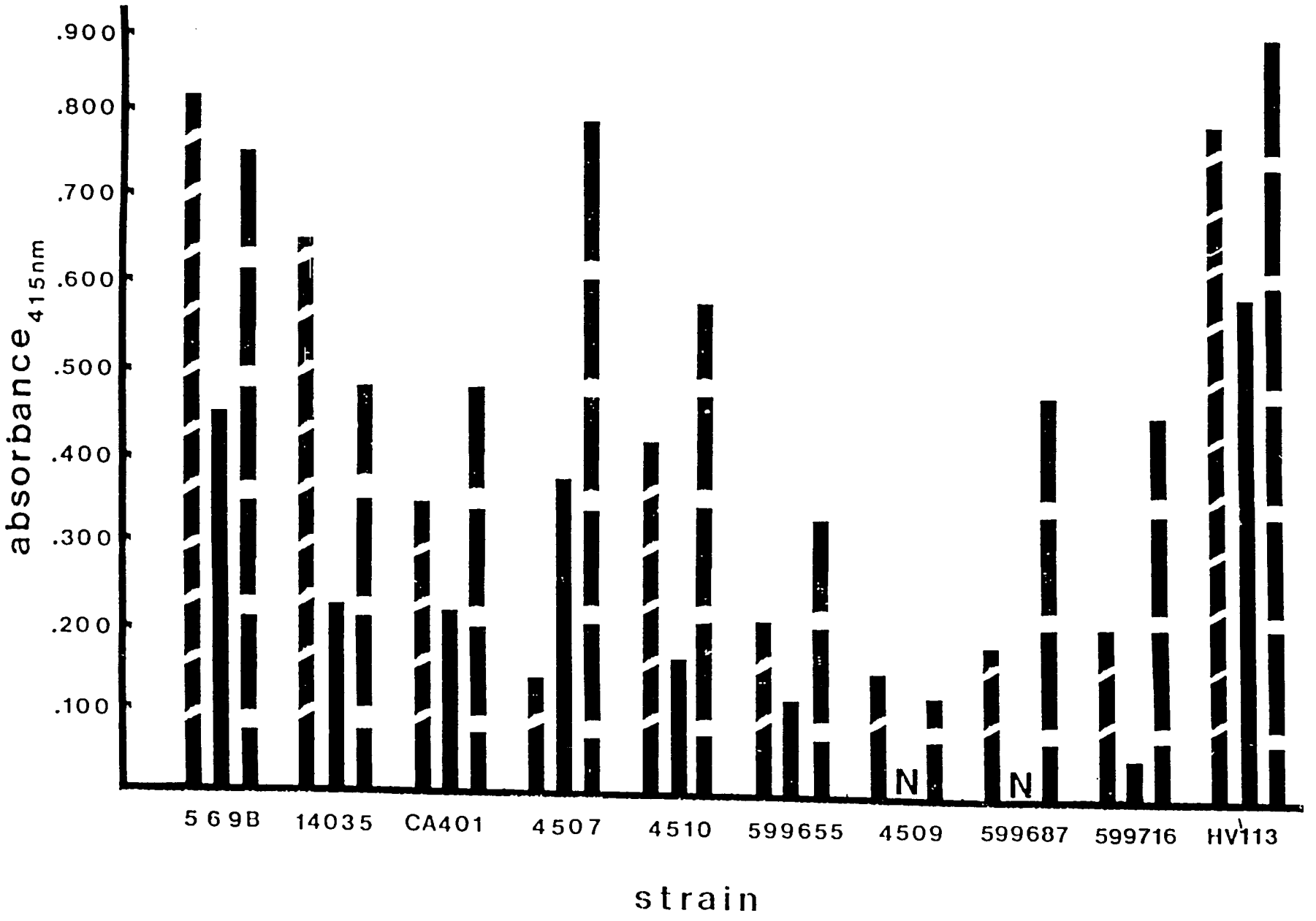

Figure 4. Comparison of toxin production in defined seawater medium (▨), CAYEG (■), and syncase (□) broth. Absorbance values for CT ELISA were corrected for uninoculated control cultures. Seawater - 10 g CYG/L).

N - not detected.

Section III

To study growth and survival of pathogenic bacteria in the aquatic environment; to identify physico-chemical parameters that may influence the ecology and pathogenicity of these organisms.

We hypothesize that the viable, but non-culturable form of aquatic *V. cholerae* 01 is important in the transmission of cholera in cholera-endemic regions. In the present experiments, we investigated survival of *V. cholerae* in Bangladesh waters commonly used by villagers for a variety of domestic activities.

River, pond and tube well waters were collected at several Matlab villages where cases of cholera were reported (Table 1). Approximately one liter of water was collected in sterile polypropylene containers. At the time of collection, salinity, conductivity, and temperature were measured with a YSI mode 33 S-C-T meter (Yellow Springs Instruments Co., Inc., Yellow Springs, Ohio). Measurement of total organic carbon was performed by Dr. Lloyd Wolfinbarger, Jr., Department of Biological Sciences, Old Dominion University, Norfolk, Virginia. The data in Table 1 show that temperature, pH, and salinity were similar for all water samples tested. In contrast, variation was noted in conductivity and total organic concentration (Table 1). Tube well water displayed the highest conductivity. Measurements of total organic carbon showed that the concentrations ranged from 2.9 mg/L to 11.6 mg/L. All samples, with the exception of Shahwazkandi pond water (i.e. 11.6 mg/L), yielded total organic concentrations in the range of approximately 3 to 6 mg/L.

Survival experiments were conducted using clinical *V. cholerae* isolates in pond, river, and tube well waters. *V. cholerae* 01 strains employed in these studies included American Type Culture Collections (ATCC; Rockville, Maryland) 14035, and Bangladesh isolates 599655 (classical biotype), 599717 (classical biotype), 49 (El Tor biotype), 51 (El Tor), and 52 (El Tor). *V. parahaemolyticus* ATCC 17802 and *V. vulnificus* C7184 were studied in separate experiments.

Vibrio isolates were grown in broth composed of Casamino acids (Difco) (30 g/L), yeast extract (Difco) (3 g/L), glucose (2 g/L), and K_2HPO_4 (0.5 g/L), pH 7.4 (11). Cultures were incubated for 15 h at 37°C, with rotary agitation at 100 rpm. Cells were harvested and washed with PBS (NaCl, 7.65 g/L; Na_2HPO_4, 0.21 g/L; pH 7.4) by centrifugation at 3000 x g. The bacterial suspension was adjusted to approximately 2 x 10^3 CFU/ml by spectrophotometric measurement at 420 nm. Microcosms were prepared using 125 ml Erlenmeyer flasks containing 50 ml of autoclaved sample water. Vibrios were added to microcosms to a final concentration of 10^5 CFU/ml, and incubated at 25°C with 100 rpm rotary agitation. Samples of the microcosms were removed at selected time intervals to determine CFU/ml on TSA and TCBS media, in triplicate.

Figure 1 shows survival patterns of *V. cholerae* ATCC 14035 exposed to eight different water samples for 72 hours. CFU's decreased 0.4 to 1.4 $\log_{10}$ units within 2 h, declining an additional 0.1 to 1.1 log units by 24 h. From 24 to 72 h, CFU's increased 0.4 to 1.1 log units in all water samples, with the exception of sample 8, where the CFU count decreased an

additional 0.7 units. Over the entire period of 72 h incubation, CFU/ml changed from an increase of 0.4 to a decrease of 3.0 log units, with a mean value of -1.3. Therefore, after 72 h of incubation, V. cholerae ATCC 14035 showed similar survival patterns in pond, river, and tube well waters and remained detectable at a mean value of 5.0×10^3 CFU/ml.

In similar experiments, 3 classical and 3 El Tor V. cholerae 01 Bangladesh clinical isolates were tested in laboratory microcosms of two river and two pond water samples. Survival patterns were observed for a total of 100 h. The results of these survival studies showed that CFU generally increased between 10 h and 30 h and remained at the same approximate concentration at 100 h. (Figure 2). However, CFU/ml decreased for two V. cholerae El Tor strains at a time interval between 10 h and 30 h in water samples 12 and 15. El Tor and classical biotypes, and Inaba and Ogawa serotypes showed similar survival patterns in pond and river waters. Total cell counts, determined by acridine orange staining, showed that the bacterial numbers increased in all water samples at 100 hours (Table 2). These experiments demonstrated that the six strains of V. cholerae 01 examined in this study, representing El Tor and classical biotypes, survived a minimum of 100 h in natural waters of Matlab.

From these results and those of previous experiments conducted in Matlab, we hypothesize that the waters of Matlab act as reservoirs of V. cholerae disease. Since these waters favored the survival of this pathogenic bacterium, then cholera could be acquired by contact with contaminated water.

To test this hypothesis, we studied the survival of V. parahaemolyticus and V. vulnificus, two halophilic Vibrio species that are not associated with large numbers of cases of water-associated disease in Matlab (personal communication). They, however, do cause disease in areas of Bangladesh where human populations come into contact with marine and estuarine water (personal communication).

V. parahaemolyticus ATCC 17802 and V. vulnificus C7184 were grown in TSA broth and added to microcosms of pond and river water, as described above. After incubation for one h, V. parahaemolyticus was not detected in water samples 3 and 5 (Table 3). Samples 7 and 8 contained only 2.5×10^2 CFU/ml, respectively. Similarly, V. vulnificus was detected only in sample 8 (i.e. 2.5×10^2 CFU/ml). After 24 h of incubation, both V. parahaemolyticus and V. vulnificus CFU were not detected in any microcosm.

The survival patterns of V. parahaemolyticus and V. vulnificus were markedly different from V. cholerae. The former species rapidly died in waters as early as one h after exposure. Results of previous work has shown that estuarine and marine environments favor the survival and isolation of V. parahaemolyticus (5, 10, 14). Therefore, the lack of V. parahaemolyticus and V. vulnificus disease in Matlab is supported by their respective survival patterns in Matlab waters.

Previous laboratory experiments have suggested that estuarine salinities are optimal for V. cholerae survival, however, the present experiments showed that survival of V. cholerae was extended in fresh waters. It is possible that the higher organic content of Matlab water

versus synthetic estuarine water, as well as trace salts and biotic factors (e.g. plankton) may contribute to V. cholerae 01 survival under conditions that are very different from those of laboratory microcosms. Indeed, Huq et al. (3, 4) have shown that copepods enhance V. cholerae survival in fresh water environments, and other investigators have reported that specific forms of chitin, a compound found in abundance in aquatic environments, enhance V. cholerae survival (1, 2, 8, 9).

In conclusion, results of the experiments reported here demonstrate that fresh water environments of Matlab, Bangladesh enhance the survival of V. cholerae 01 strains for extended periods of time. This suggests that if clinical strains of V. cholerae 01 enter the aquatic environments of Matlab (e.g. via fecal contamination), these waters can extend the survival time of V. cholerae in the environment. If the water is used by villagers following fecal contamination, the V. cholerae may be infective.

Attachment of Vibrio Cholerae 01 to plankton surfaces

The aquatic ecology of V. cholerae 01 in cholera-endemic regions of the world can be influenced by a variety of biological and physiochemical factors. Several reports describe the effect of temperature, salinity and nutrient on V. cholerae survival and physiology (6, 7, 12, 13, 15). However, little is known of interactions between V. cholerae and biological factors of aquatic ecosystems, and the effect of transmission of cholera in human populations.

Early reports by Huq et al. (3) demonstrate that live planktonic copepods of Chesapeake Bay and Patuxent River enhance the survival of V. cholerae 01 in laboratory microcosms.

The objective of this research was to study the attachment of clinical V. cholerae 01 isolates to plankton of Bangladesh waters. Fluorescein-labelled mAb, specific for the 01 antigen, was used in a direct qualitative analysis of attached V. cholerae 01. Plankton populations were sampled during the Spring 1987 cholera season in Matlab, Bangladesh.

Plankton were collected from river, canal, and pond water of Matlab, Bangladesh with a nylon Nytex plankton net (64μ pore size). Plankton were resuspended in autologous water and transferred to the laboratory. An approximate 10^7 CFU/ml suspension of clinical V. cholerae 01 Bangladesh isolates were prepared in autologous water. Ca. 0.9 ml of plankton were added to polypropylene cups fitted with nylon Nytex screen fitted to one end. These containers were placed in wells of a 24-well cluster plate. One hundred microliters of bacteria were added to plankton samples and incubated for 60 minutes at room temperature (approximately 24°C). Nylon mesh-fitted containers were rinsed with PBS and fixed with 2% formaldehyde for 15 minutes at room temperature. Samples were rinsed with PBS, then placed in wells containing 1.0 ml diluted anti-01 mAb. After incubation for 60 min., samples were rinsed and transferred to wells containing 1.0 ml of fluorescein-conjugated goat anti-mouse IgG. Following incubation for 60 min., samples were rinsed with PBS and viewed under 10X and 40X with a microscope fitted for fluorescent microscopy.

Results of these experiments showed the V. cholerae 01 attached to

moulted copepod exoskeletons (i.e. exuviae) in high numbers. Fewer vibrio cells attached to live copepods. Four of 5 V. cholerae strains examined attached to copepod moults. One strain, however, attached to both live copepod moults (Table 4).

V. cholerae 01 also attached to a Volvox species, a colonial form of phytoplankton. This attachment to Volvox sp. appeared in a "ring" pattern. When natural, autochthonous bacteria were visualized by acridine orange stain, endogenous bacteria attached to similar surfaces (i.e. to copepod moults) as exogenous V. cholerae 01.

These experiments demonstrate that the surfaces of plankton serve as attachment sites for V. cholerae 01 and suggest that in Bangladesh aquatic systems the ecology of V. cholerae is intertwined with the ecology of the plankton in those waters. The relevance of this finding to the epidemiology of cholera is under investigation.

Literature Cited

1. Amuko, K., S. Shimodor, T. Imoto, S. Miake, and A. Umeda. 1987. Effects of chitin and its soluble derivatives on survival of Vibrio cholerae 01 at low temperature. Appl. Environ. Microbiol. 53:603-605.

2. Boyer, J. 1986. End products of anaerobic chitin degradation by salt marsh bacteria as substrates for dissimilatory sulfate reduction and methanogensis. Appl. Environ. Microbiol. 52:1415-1418.

3. Huq, A., E.G. Small, P.A. West and R.R. Colwell. 1984. The role of planktonic copepods in the survival and multiplication of Vibrio cholerae in the aquatic environment. In R.R. Colwell (ed.), Vibrios in the Environment, John Wiley and Sons, New York.

4. Huq, A., E.B. Small, P.A. West, M.I. Huq, R. Rahman and R.R. Colwell. 1983. Ecological relationships between Vibrio cholerae and planktonic crustacean copepods. Appl. Environ. Microbiol. 45:275-283.

5. Kaneko, T. and R.R. Colwell. 1973. Ecology of Vibrio parahaemolyticus in Chesapeake Bay. J. Bacteriol. 113:24-32.

6. Miller, C.J., B.S. Drasar and R.G. Feachem. 1984. Response of toxigenic Vibrio cholerae 01 to physico-chemical stresses in aquatic environments. J. Hyg. Camb. 93:475-495.

7. Miller, C.J., B.S. Drasar, R.G. Feachem and R.J. Hayes. 1986. The impact of phsico-chemical stress on the toxigenicity of Vibrio cholerae. J. Hyg. Camb. 96:49-57.

8. Nalin, D.R. 1976. Cholera, copepods, and chitinase. Lancet. ii:958.

9. Nalin, D.R., V. Daya. A. Reid, M.M. Levine, and L. Cisneros. 1979. Absorption and growth of Vibrio cholerae on chitin. Infect. Immun. 25:768-770.

10. Oliver, J.D., R.A. Warner and D.R. Cleland. 1982. Distribution and ecology of Vibrio vulnificus and other lactose-fermenting vibrios in coastal waters of the Southeastern United States.

11. Sack, D.H., S. Huda, P.K.B. Neogi, R.R. Daniel, and W.M. Spira. 1980. Microtiter ganglioside enzyme-linked immunosorbent assay for Vibrio and Escherichia coli heat-labile enterotoxins and antitoxin. J. Clin. Microbiol. 11:35-40.

12. Singleton, F.L., R. Atwell, S. Jangi and R.R. Colwell. 1982. Effects of temperature and salinity on Vibrio cholerae growth. 44:1047-1058.

13. Singleton, F.L., R. Atwell, S. Jangi and R.R. Colwell. 1982. Influence of salinity and organic nutrient concentration on survival and growth of Vibrio cholerae in aquatic microcosms. Appl. Environ. Microbiol. 43:1080-1087.

14. Tamplin, M.L. and R.R. Colwell. 1986. Effects of microcosm salinity and organic substrate concentration on production of Vibrio cholerae enterotoxin. Appl. Environ. Microbiol. 52:297-301.

15. Tamplin, M.L., G.E. Rodrick, N.J. Blake and T.R. Cuba. 1982. Isolation and characterization of Vibrio vulnificus in two Florida estuarines. Appl. Environ. Microbiol. 44:1466-1470.

Table 1. Environmental and clinical data for Bangladesh village water supplies.

sample #	village	water source	temperature (°C)	pH	salinity (°/oo)	conductivity (mhos/cm)	total organic carbon (mg/L)	cholera cases [a]
1	Goalvaor	river	21.5	6.0	0.0	170	4.4	5
2	Goalvaor	tube well	16.0	6.0	0.4	650	2.9	
3	Shahazkandi	pond	21.0	6.0	0.0	105	11.6	1
4	Shahazkandi	tube well	25.0	7.0	0.5	850	5.6	
5	Shahazkandi	river	21.0	6.0	0.0	220	4.6	
6	Kadamtali	tube well	26.0	6.0	0.5	1000	6.6	0
7	Kadamtali	pond	21.0	6.0	0.0	50	4.8	
8	Kadamtali	river	21.0	6.0	0.0	100	NT [b]	
9	Sarderandi	pond	22.5	6.0	0.0	180	NT	2
10	Sarderandi	river	21.5	6.0	0.0	185	NT	
11	Uddandi	pond	20.5	6.0	0.0	160	NT	2
12	Uddandi	river	24.0	6.0	0.0	100	NT	
13	Gajipur	pond	24.0	6.0	0.0	190	NT	3
14	Gajipur	river	23.0	6.0	0.0	100	NT	
15	Charmokundi	pond	22.0	6.0	0.0	80	NT	3
16	Charmokundi	pond	20.0	6.0	0.0	60	NT	

[a] total cholera cases reported per village 2 weeks pre- and post-sampling
[b] NT - not tested

Table 2. Changes observed in total cell counts of V. cholerae classical and El Tor biotypes exposed to Bangladesh river and pond water in microcosm flask tests.

Isolate	Water Source			
	River 10 [a]	River 12	Pond 13	Pond 15
1 [b]	0.9 [c]	5.9	10.5	1.2
2	5.8	2.6	5.5	2.2
3	10.6	2.0	1.5	1.8
4	11.3	3.9	2.0	5.5
5	7.6	1.5	3.8	2.3
6	10.5	5.0	3.1	2.1

[a] River 1 - sample No. 10, River 2 - sample No. 12, Pond 1 - sample No. 13, Pond 2 - sample No. 15

[b] V. cholorea strains 1-3 were classical strains of V. cholerae and 4-6 were El Tor strains

[c] Net fold increase in total cells/ml from time of inoculation to 100 hr.

Table 3. Survival of *V. parahaemolyticus* 17802 and *V. vulnificus* C7184 in Bangladesh water microcosms

Water sample	Time (hr) *V. parahaemolyticus* 2	*V. parahaemolyticus* 24	*V. vulnificus* 2	*V. vulnificus* 24
3	ND [a]	ND	ND	ND
5	ND	ND	ND	ND
7	2.4 [b]	ND	ND	ND
8	2.7	ND	2.4	ND

[a] ND - not detected
[b] log CFU/ml microcosm

Table 4. Attachment of *V. cholerae* 01 to copepods.

Species	ATTACHMENT Live	Exuviae	Location
ZOOPLANKTON			
COPOPODS			
Acartia sp.	yes	yes	River
Cyclops spp.	yes	yes	River & Ponds
Diaptomus sp.			Ponds
Senecella sp.	yes	?	River
CLADOCERANS			
Bosmina sp.	yes	yes	River
Daphnia sp.			Ponds
Ceriodaphnia sp.			Ponds
Diaphanosoma sp.	yes	yes	River & Ponds
Bosminopsis sp.			Ponds
ROTIFERS			
Brachionus spp.	yes	yes	River & Ponds
PHYTOPLANKTON			
Volox sp.	yes	yes	River & Ponds
Pediastrum simplex	yes	yes	River & Ponds
Anacystis sp.	yes		River & Ponds
Spirulina sp.	no		River & Ponds

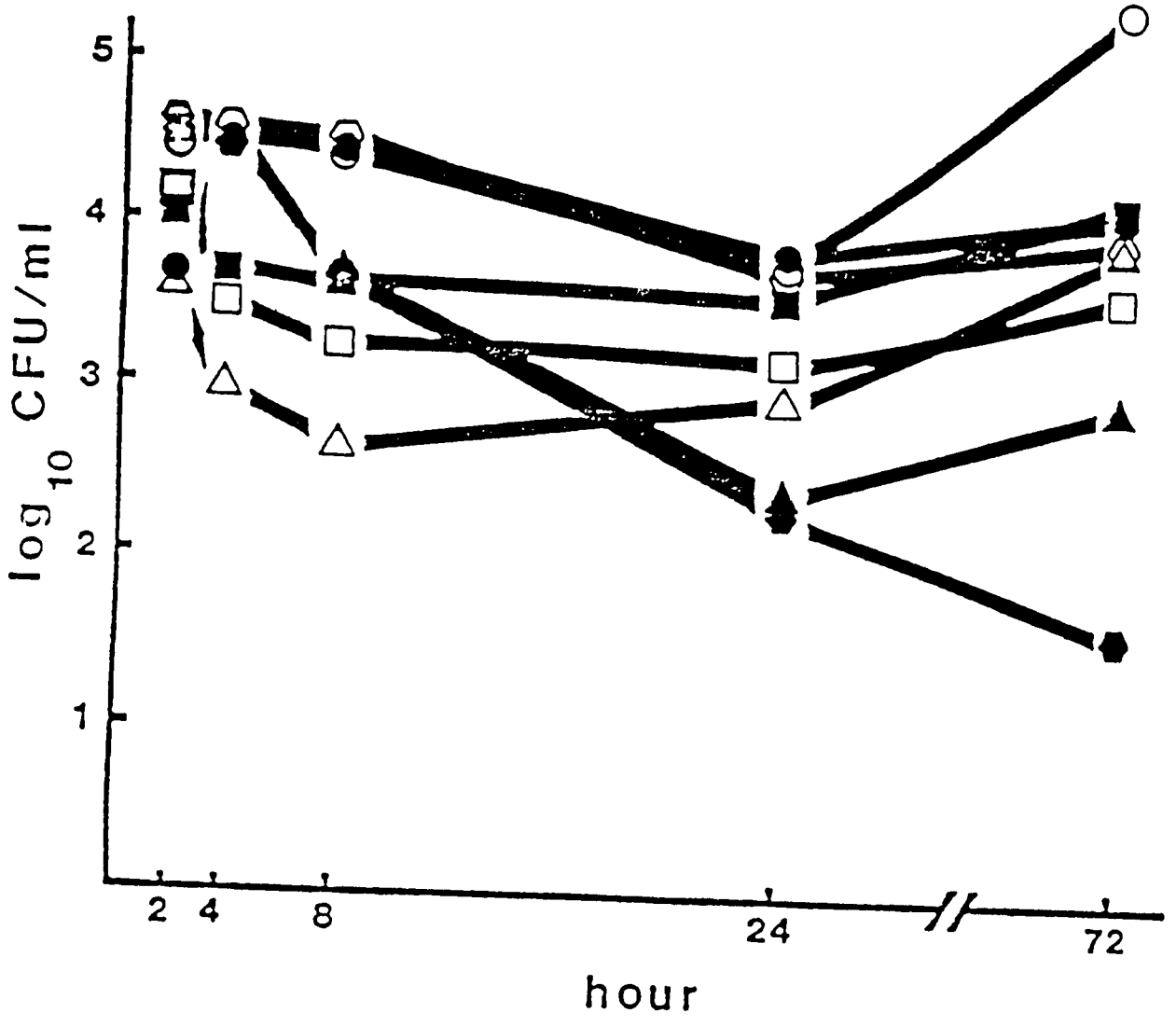

Figure 1. Survival patterns of V. cholerae ATCC 14035 in 8 Bangladesh water microcosms. Microcosms were inoculated at 10^5 CFU/ml, and incubated at 25°C and 100 rpm. Water samples were #1 (○), #2 (□), #3 (⬡), #4 (△), #5 (●), #6 (■), #7 (⬢), and #8 (▲).

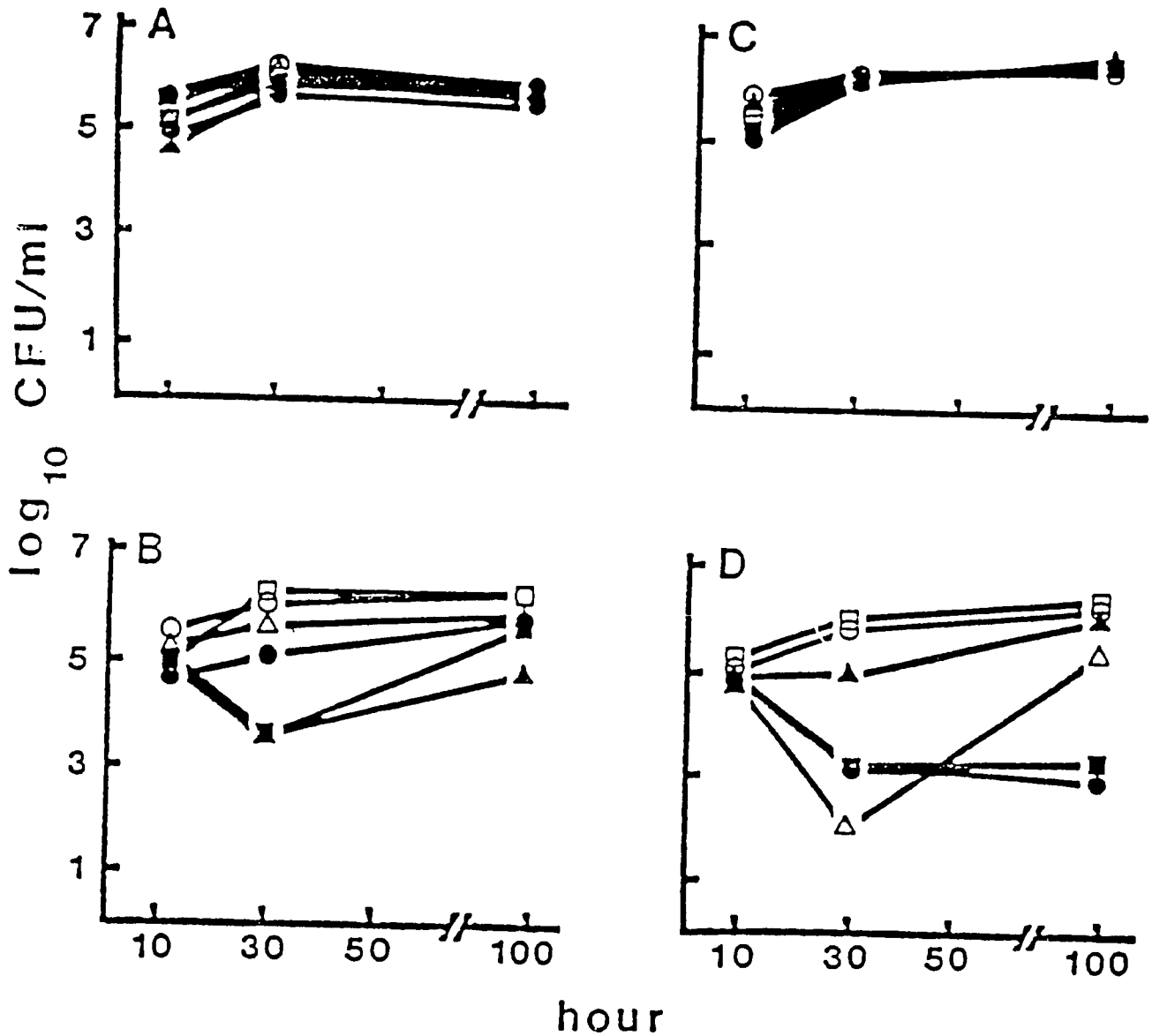

Figure 2. Survival patterns of classical and El Tor V. cholerae biotypes in river and pond water microcosms. Three classical (○,□,△) and 3 El Tor (●,■,▲) strains were inoculated at 10^5 CFU/ml in each of 2 river (A, sample #10; B, sample #12) and 2 pond water micrcosms (C, sample #13; D, sample #15) Conditions of incubation were 25°C and 100 rpm.

Section IV

Development of methods for recovery of cells exposed to the environment for the purpose of increasing understanding of the "non-recoverability" phenomenon of bacteria.

The viable but non-culturable stage of bacteria has been established (1, 3, 4, 5). Experiments were undertaken to devise media and/or methods to recover cells that have entered the dormant, non-culturable state in aquatic environments. If successful, a better understanding of the dormancy phenomenon, as well as a means of more effective assessment of contamination of public waters can be achieved.

A microcosm of sterile Chesapeake Bay water and *V. cholerae* strain ATCC 14035 was assembled according to methods described by Colwell *et al* (1). Cell growth was monitored by microscopy (DVC/FA/AODC) and plate culture until cells were "viable but non-culturable."

PART A: Aliquots of microcosm cells described above were dispensed into sterile test tubes and grown under specified conditions of temperature, gaseous atmosphere and with various growth factors, nutrients, etc. (see below). These "modified" cells served as the source of inocula for routine recovery plate media:

-- Experiment 1: Tube contained yeast extract (0.0313%, 0.00625%, 0.0125%, 0.1%, 0.25%) and NaCl (1%). Incubation temps: 4°C, 25°C and 35°C. Gaseous atmosphere: aerobic, anaerobic, *Campylobacter* cultivation atmosphere (i.e. 5% oxygen, 10% CO_2, 85% N_2).

-- Experiment 2: Experiment 1 above was repeated but with the addition of nalidixic acid to each tube at a final concentration of 0.025%.

-- Experiment 3: Experiment 1 above was repeated but modified as follows:

a) Tubes contained yeast extract, 0.25%; NaCl, 0.5%; $MgSO_4$, 0.2%; and histidine, 1.0%

b) As in (a) above but to each tube was added EDTA (10^{-5}M).

c) As in (a) above but to each tube was added Tween 80 (0.05%)

d) Incubation temperatures (a, b, c): 25°C, 35°C. Gaseous atmosphere (a, b, c): See experiment 1.

-- Experiment 4: Experiment 1 above was repeated but modified as follows:

a) Tubes containing RPMI 1640 tissue culture medium (Flow Laboratories) were prepared. The following were added individually or in combination:
   (1) Gramicidin D (13 mg/ml)
   (2) Gramicidin S (6.5 mg/ml)
   (3) Chitin (0.05%)
   (4) Sodium pyruvate (1%)
   (5) Foetal bovine serum (5%)

b) Incubation temperature: 35°C
Gaseous atmosphere: aerobic

c) Tubes contained RPMI 1640 and the antibiotic triethylene-tetramine dihydrochloride (TRIEN) at final concentrations 0.0025 M and 0.10 M.

-- Experiment 5: Experiment 4 (b) was repeated but modified as follows:
(a) Each tube contained the flowing added individually or in combination:
(1) Ferric ammonium citrate 1.0%
(2) Asparagine (2.5g/L)
(3) Glucose (0.3%)
(4) $MgSO_4$ (0.005%)
Incubation temperatures and gaseous atmosphere as in Experiment 4 (a) above.

-- Experiment 6: As in Experiment 1 but modified as follows:
a) Tubes contained nutrient broth, Na/Cl (0.5%) and TRIEN (See Experiment 4 [c] above).
b) Incubation temperature: 35°C

Part b: Biphasic (liquid/solid) cell culture systems were prepared according to the methods outlined by Krieg and Gerhardt (2). The agar (solid) layer, containing various nutrients, growth factors, metal ions, ionophores etc., was overlaid with aliquots of microcosm cells in the "viable but non-culturable" phase (see above). Cells under these conditions served as inocula for routine recovery plate media:

-- Experiment 1.
a) Agar layer contained yeast extract (0.25%), NaCl (0.5%), and EDTA (10^{-6}M).
b) Incubation temperature: 35°C
Gaseous atmosphere: aerobic

-- Experiment 2. As in Experiment 1 above but the following were added individually and in combination:
(1) iron (as ferrous sulfate) 1.0%
(2) sodium pyruvate (1.0%)
(3) $MgSO_4$ (0.005%)

-- Experiment 3. Experiment 1 was repeated with the following modifications.
a) The agar layer contained yeast extract (0.25%) and NaCl (0.5%)
b) The following were added individually and in combination:
(1) Gramicidin S (6.5% mg/ml)
(2) Gramicidin D (13 mg/ml)
(3) Chitin (0.05%)
(4) Ferric ammonium citrate (1.0%)
(5) Foetal bovine serum (5.0%)
(6) Glucose (0.3%)
(7) $MgSO_4$ (0.05%)
(8) Sodium pyruvate (1.0%)
(9) Histidine (1.0%)
(10) Asparagine (2.5 g/l)

c) Incubation temperature and gaseous atmosphere as in Experiment 1 above.

Results: Recoverability of the cells was not improved by the experiments detailed in parts A and B above. Only by animal passage has good recovery been obtained to date (1).

PART C: Aliquots of V. cholerae microcosm cells in the "viable but non-culturable" phase were inoculated into microtitre tissue culture wells containing Henle intestinal epithelial cells. The V. cholerae cells grown under these conditions served as the source of inocula for routine recovery plate media.

Results: Recoverability of the cells was not improved by the use of tissue culture.

Literature Cited

1. Colwell, R.R., P.R. Brayton, D.J. Grimes, D.B. Roszak, S.A. Huq, and L.M. Palmer. 1985. Viable but non-culturable Vibrio cholerae and related pathogens in the environment: implications for release of genetically engineered microorganisms. Biotechnology. 3:817-820.

2. Krieg, N., and P. Gerhardt. 1981. In: Manual of methods for general bacteriology. Gerhardt et al. (eds). p. 146

3. Pearson, A., R. Colwell, J. Byrd, J. Dennis, J. Grimes, M. Hood, D. Hussong, C. Hutchinson, J. Lee, M. O'Brien, J. Scott and J. Walker. 1986. Discovery of viable but non-culturable Legionella pneumophila by immune epifluorescence microscopy: evidence of amplification and sources in five case studies. Bio/Technology (submitted).

4. Rollins, D.M., and R.R. Colwell. 1986. Viable but non-culturable stage of Campylobacter jejuni and its role in survival in the natural aquatic environment. Appl. Environ. Microbiol. 52:531-538.

5. Roszak, D.B., D.J. Grimes and R.R. Colwell. 1984. Viable but non-recoverable stage of Salmonella enteritidis in aquatic systems. Can J. Microbiol. 30:334-338.

Section V

To develop methods suitable for isolation and identification of pathogenic bacteria from environmental samples.

A modified taurocholate-tellurite-gelatin agar (TTGA) was developed to improve the differentiation of *Vibrio* spp. isolated from the environment (3). 4-methylumbelliferyl-β-D-galactoside (150 g/ml) was incorporated into the TTGA medium to allow for direct detection of β-D-galactosidase (β -gal activity. In addition to other differential features of the TTGA medium, the modified version provided easy differentiation of *Vibrio cholerae* (β-gal +) from *Vibrio parahaemolyticus* (β-gal-). A preliminary study was conducted in Dhaka, Bangladesh, to compare TTGA and modified TTGA media using fresh clinical specimens. Stool and rectal swab specimens, collected from diarrhoeal patients at the ICDDR/B hospital in Dhaka and which were found to be presumptively positive for *V. cholerae* by dark field microscopy (motility immobilization test), were processed as follows: Three plates each of TCBS, TTGA and modified TTGA media inoculated in parallel using the fresh clinical specimens. *V. cholerae* isolates in TCBS were presumptively identified according to colony morphology and sucrose fermentation. Slide agglutination using polyvalent *V. cholerae* antisera was done. The following features were recorded on TTGA and modified TTGA media: Gram reaction, catalase, tellurite reduction, gelatinase, -D-galactosidase and in some cases slide agglutination with *V. cholerae* polyvalent antisera.

V. cholerae was isolated and presumptively identified on TCBS, TTGA, and modified TTGA media. There was excellent agreement in results for *V. cholerae* recovery and presumptive identification for the three media. The features of the *V. cholerae* isolates recovered on TTGA and modified TTGA media are recorded in Table 1, with excellent agreement in results for both media.

Addition of the β-D-galactosidase substrate to modified TTGA medium yielded improved presumptive identification of *V. cholerae*. Addition of the substrate did not affect the performance of TTGA medium in the recovery of *V. cholerae* from fresh clinical specimens. Both TTGA and modified TTGA, unlike TCBS, allowed for ready determination of slide agglutination, supporting the findings of Gangarosa *et al*. (1) and Morris *et al*. (2).

In addition, a rapid, simple-to-perform, and inexpensive technique to detect chitobiase activity has been developed (4). 4-methylumbelliferyl-N-acetyl- -D-glucosaminide was incorporated into a filter paper disc and organisms were spot tested for activity. Results were compared to those obtained by a conventional plate method for chitinase activity using colloidal chitin as substrate. There was excellent agreement in results for both methods. The method can be used in routine taxonomy protocols for the identification of microorganisms from environmental and clinical sources.

Table 1

Features of *V. cholerae* isolate on TTGA[a] and modified TTGA[b]

Feature	No. Isolates Tested	No. (%) Positive	
		TTGA	Modified TTGA
Gram negative	15	15 (100)	15 (100)
Oxidase	25	24 (96)	24 (96)
Catalase	25	25 (100)	25 (100)
Tellurite reduction	25	24 (96)	24 (96)
Gelatinase	25	23 (92)	23 (92)
Slide agglutination	11	11 (100)	11 (100)

[a] TTGA, taurocholate-tellurite-gelatin agar

[b] modified TTGA, contained 4-methylumbelliferyl β-D-galactoside.

Literature Cited

1. Gangarosa, E.J., W.E. DeWitt, I. Huq, and A. Zafiri. 1968. Laboratory methods in cholera: isolation of Vibrio cholerae (El Tor and Classical) on TCBS medium in minimally equipped laboratories. Trans. R. Soc. Trop. Med. Hyg. 62:693-699.

2. Morris, G. K., M.H. Merson, I. Huq, A.K.M.G. Gibrya, and R. Black. 1979. Comparison of four plating media for isolating Vibrio cholerae. J. Clin. Microbiol. 9:79-83.

3. O'Brien, M. and R.R. Colwell. 1985. Modified taurocholate-tellurite-gelatin agar for improved differentiation of Vibrio species. J. Clin. Microbiol. 22:1011-1013.

4. O'Brien, M., and. R.R. Colwell. 1987. A rapid test for chitinase activity that uses 4-methylumbelliferyl-N-acetyl- -D-glucosaminide. Appl. Environ. Microbiol. 53:1713-1720.

Section VI

Additional studies conducted in Bangladesh. Isolation of *Plesiomonas shigelloides* from the environment: evaluation of inositol-bile-salts-brilliant green (IBB) medium.

P. shigelloides has been implicated as a diarrhoeal pathogen. The majority of *P. shigelloides* isolates have been recovered from specimens of human and animal origin. It has been found in fresh water and occasionally in seawater, although the aquatic distribution of *P. shigelloides* is limited by its minimum growth temperature of 8°C and the fact that it does not survive well in seawater (2,4).

Schubert (3) devised a selective/differential (IBB) medium for isolation of *P. shigelloides* from clinical sources. Experience with this medium in isolating *P. shigelloides* from the environment is lacking. Therefore, the objective of this study was to isolate *P. shigelloides* from environmental water sources in Bangladesh and to evaluate the efficacy of IBB medium for recovery of *P. shigelloides* from the environment.

Water samples were collected from river and village water sources in the Matlab environs of Bangladesh. Sample sites were chosen at random. *Ca.* 240 ml. volumes were collected for processing. The following physical parameters were recorded for each sample: temperature, pH, salinity and conductivity. Fifty ml. aliquots of each undiluted sample were filtered through either a 0.22 m and/or 0.45 membrane filter. Each filter was placed on a IBB plate (1 filter per plate) and incubated aerobically for 24-48h at 37°C. Plates were examined for inosital fermenting (IBB+) colonies and screened as shown in Figure 1. Due to time constraints, some water samples were processed as follows: 0.01 ml aliquots of undiluted water samples were plated directly onto IBB medium. IBB- colonies were screened as shown in Figure 1.

P. shigelloides was isolated and presumptively identified from five water samples out of a total of 34 water source samples. Growth of *P. shigelloides* in the environmental samples were impeded by heavy overgrowth of other environmental bacteria. Also, *P. shigelloides* appeared to be present in very low numbers.

Other workers have reported similar findings (1). Furthermore, *P. shigelloides* demonstrates seasonal variation in population numbers in the environment (2). Diluting the raw sample did not improve quantification as *P. shigelloides*, following the biochemical screen. Thus, other environmental bacteria can mimic *P. shigelloides* in growth and inositol-fermenting characteristics on IBB medium.

These results and those of other studies (1) suggest a need for an improved selective/differential medium for recovery of *P. shigelloides* from environmental sources.

Literature Cited

1. Koburger, J.A. and M.L. Miller, M.L. 1984. Plesiomonas shigelloides: A new problem for the oyster industry? Proceedings of the Ninth Annual Tropical and Subtropical Fisheries Conference of the Americas. pp. 337-342.

2. Sakazaki, R. and A. Balows. 1981. pp. 1272-1301. The genera Vibrio, Plesiomonas, and Aeromonas. The Prokaryotes: A Handbook on Habitats, Isolation and Identification. M.P. Starr et al. (eds.). Springer-Verlag: Berlin.

3. Schubert, R.H.W. 1977. Weber den Nachweis von Plesiomonas shigelloides. Habs and Schubert, 1962, und ein Elektivmedium, den Inositol-Brillantgrun-Gallesalz-Agar. Z. Rodenwaldt-Arch. 4:97-103.

4. Von Graevenitz, A. 1985. pp. 278-281. Aeromonas and Plesiomonas. In Manual of Clinical Microbiology. Lennette et al. (eds.). ASM: Washington, D.C.

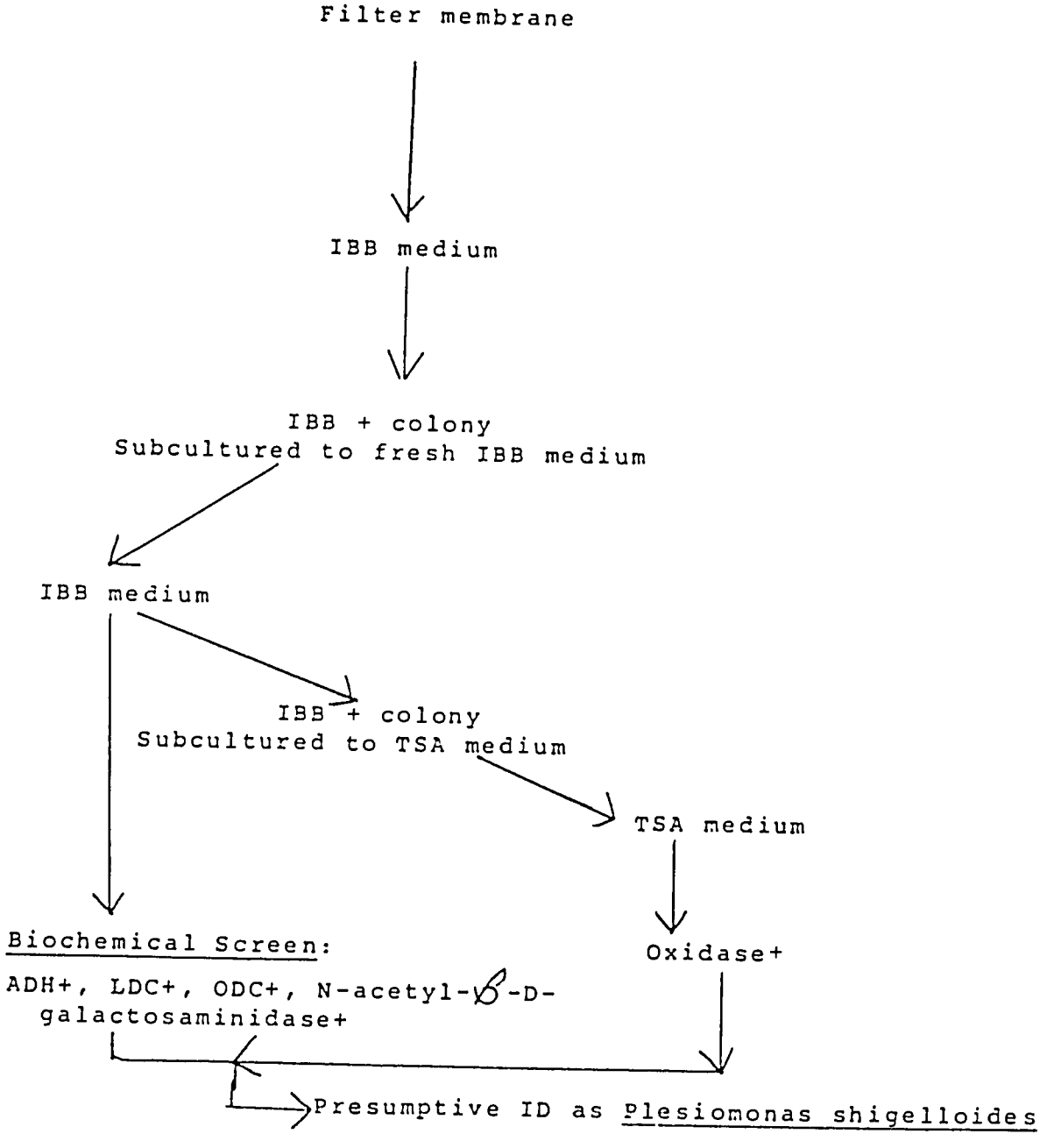

Figure 1. Screening procedure for isolation and presumptive identification of P.shigelloides from environmental water samples in Bangladesh.

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VIABLE BUT NON-CULTURABLE *VIBRIO CHOLERAE* AND RELATED PATHOGENS IN THE ENVIRONMENT: IMPLICATIONS FOR RELEASE OF GENETICALLY ENGINEERED MICROORGANISMS

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In a series of microcosm and field studies, we have observed that over time *V. cholerae* and related human pathogens enter a viable but non-culturable state. Direct viable counts by epifluorescent microscopy are consistently higher than corresponding plate counts. Thus, the assumption that microorganisms, including 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. Furthermore, animal passage reveals pathogenicity persisting for such "non-viable" cells. Indirect immunofluorescent microscopy offers a more sensitive detection system for environmental sampling for human pathogens and therefore, a more valid estimation of population size. One implication of these findings for the release of genetically engineered organisms is that highly specific methods of detection and monitoring are required, with direct detection by fluorescent antibody the most reliable at the present time.

Determining the number of pathogens and indicator organisms in the aquatic environment is imperative for assessing public health safety. Bacteria entering natural waters are confronted with 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. Extensive studies employing laboratory microcosms, using *Escherichia coli*, *Vibrio cholerae*, *Salmonella* and *Shigella* spp., have shown that these organisms persist in a dormant state. The cells are incapable of growth in conventional culture media, and are said to be viable, but non-culturable. Therefore, waters, the quality of which are judged acceptable by conventional culture methodology, may indeed be hazardous, placing the public health safety of those waters in question.

RESULTS

We adapted indirect immunofluorescent-epifluorescent techniques to environmental samples in order to obtain a more realistic estimate of population size[1,2]. The fluorescent antibody (FA) procedure utilizes a species-specific antiserum and a fluorescent dye. Upon examination with an epifluorescence microscope, stained cells present a peripheral green band visible beneath the cell wall. An evaluation of this new enumeration approach, compared with conventional culture methods, was conducted for waters of Chesapeake Bay, Louisiana, Puerto Rico, Zimbabwe (Africa), Brazil, and Bangladesh for the presence of *Vibrio cholerae* serovar O1[3].

Results of Most Probable Number (MPN) enumerations of sample collections in Louisiana are presented in Table 1. 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 antibody staining, yielding a significantly higher bacterial count per 100 ml volume of water[3].

Canals and village water sources in Bangladesh were the sites of samplings in April and May, 1982. From a total of 52 water samples collected, seven were positive for *V. cholerae* O1 by conventional culture methods. 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 fluorescent antibody 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.

Additional fluorescent counting techniques have been applied in the laboratory to elucidate the non-culturability phenomenon. Direct viable counts (DVC) permit viewing and enumeration of viable cells by direct microscopy[4]. Yeast extract added to water samples provides nutrients for growth and replication. With addition of nalidixic acid, septum formation is inhibited. Therefore, substrate responsive cells, i.e., viable cells, appear elongated when stained with acridine orange (AO). Staining with fluorescent antibody, instead of AO, provides a species-specific DVC (FA-DVC). Acridine orange direct count (AODC) is a procedure used to enumerate total bacteria[5]. These procedures, along with standard plate counts were utilized to monitor survival patterns of selected pathogens studied in laboratory microcosms.

Aged estuarine water, adjusted to a suitable pH and salinity was assembled to simulate environmental conditions. No additional nutrients were introduced in order to induce a dormant state.

E. coli strain H10407 was inoculated into a microcosm,

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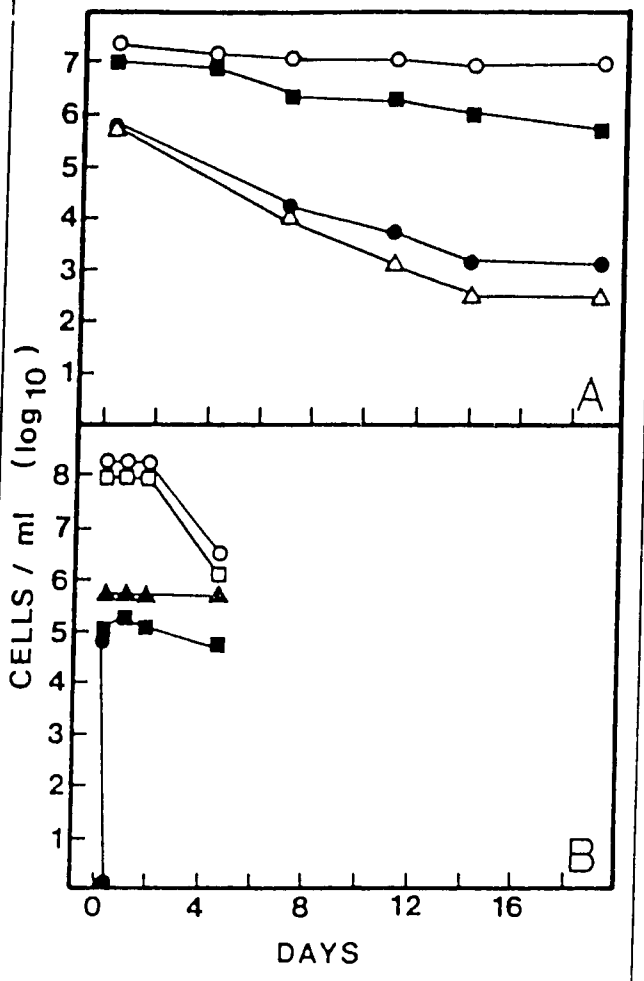

FIGURE 1 *Escherichia coli* H10407 exposed to Chesapeake Bay water in microcosms (A) and Bimini Lagoon water by means of membrane chamber (B). ○ Acridine Orange Direct Count; □ Acridine Orange Direct Count-control; ▲ Fluorescent Antibody Count; ● Standard Plate Count; △ EMB Agar Count; ■ Direct Viable Count

containing Chesapeake Bay water, at a concentration of 10^7 cells/ml (salinity 15‰). AODC remained at the initial concentration (Fig. 1A). Over a period of 19 days, direct viable counts declined only by 0.5 log. Significantly, plate counts on Tryptic Soy Agar (TSA) and Eosin Methylene Blue Agar (EMB) dropped from 10^6 cells/ml on Day 1, to 10^4 on Day 7, and then to 10^3 on Day 14.

Shigella sonnei was introduced at a concentration of 10^6 cells/ml into a microcosm prepared with Chesapeake Bay water (salinity 15‰). The AODC remained at the initial concentration (Fig. 2). The DVC declined by 1.0 log. The organism became non-culturable on MacConkey Agar by Day 14, and on TSA by Day 21. *Shigella flexneri* exhibited a similar pattern; however, this strain reached 0 cfu at a rate three times faster than *S. sonnei*.

Figure 3 illustrates the survival patterns of the toxin positive strain *V. cholerae* CA 401 in microcosms. Two flasks of Patuxent River water were inoculated with 10^6 cells/ml. In both systems, AODC and FA 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 at one hour post-inoculation, plate counts on Thiosulfate-citrate-bile salts-sucrose agar (TCBS) and TSA plates for microcosm 1 were 10^4 cfu/ml on both media types. For microcosm 2, counts were 10^4 and 10^3

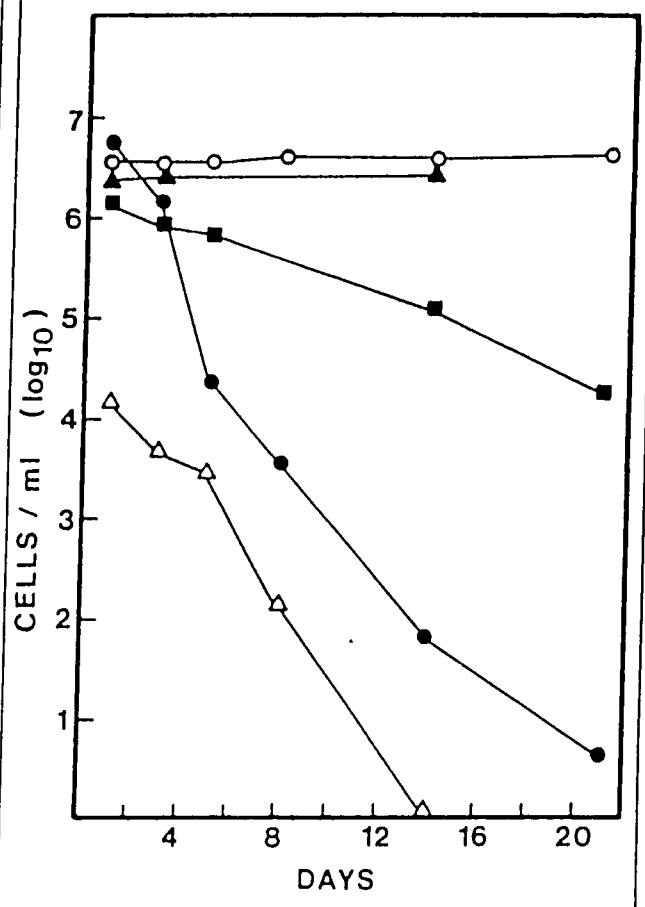

FIGURE 2 *Shigella sonnei* 53 G exposed to Chesapeake Bay water in microcosms. ○ Acridine Orange Direct Count; ▲ Fluorescent Antibody Count; ■ Direct Viable Count; ● Plate Count, TSA; △ Plate Count, MacConkey.

cfu/ml respectively. After 24 hours, all plate counts dropped to 0, and remained non-culturable for the duration of the microcosm experiment.

Results compiled by Roszak et al.[7] for *Salmonella enteritidis* followed a similar trend regarding the fate of nutrient starved cells. FA and DVC dropped 0.5 log below the AODC, while all plate counts and MPN estimates exhibited rapid decline and became non-culturable by the second and third day of the microcosm experiment.

Membrane chambers were used to conduct *in situ* experiments in the semi-tropical waters of Bimini, Bahamas (salinity 38‰, mean temperature 25°C). They were initially inoculated with 10^6 cells/ml of *V. cholerae* CA 401 and *E. coli* H10407. Enumerations were conducted on samples removed at 0, 13, 25, 38, and 112 hours. All FA, DVC and plate counts of *V. cholerae* dropped to zero. Values plotted for *E. coli* are arithmetic means of the results from the 3 inoculated chambers (Fig. 1B). The FA and FA-DVC held constant at 10^5 cells/ml. Plate counts on Brain Heart Infusion Agar (BHI) dropped from 10^4 cfu/ml at 0 hours, to <1 cfu/ml at 13 hours and remained as such for the term of the experiment. *E. coli* was not present in the control chamber, which proved impervious to environmental *E. coli* species, but not to marine bacteria, as reflected in the high AODC and control AODC (this chamber contained filtered [0.2 μm] seawater at the start of the experiment). Of interest is the fact that this *E. coli* strain did not go completely non-culturable in the laboratory experiments described above. However, conditions in the laboratory microcosms were not as harsh (15‰ salini-

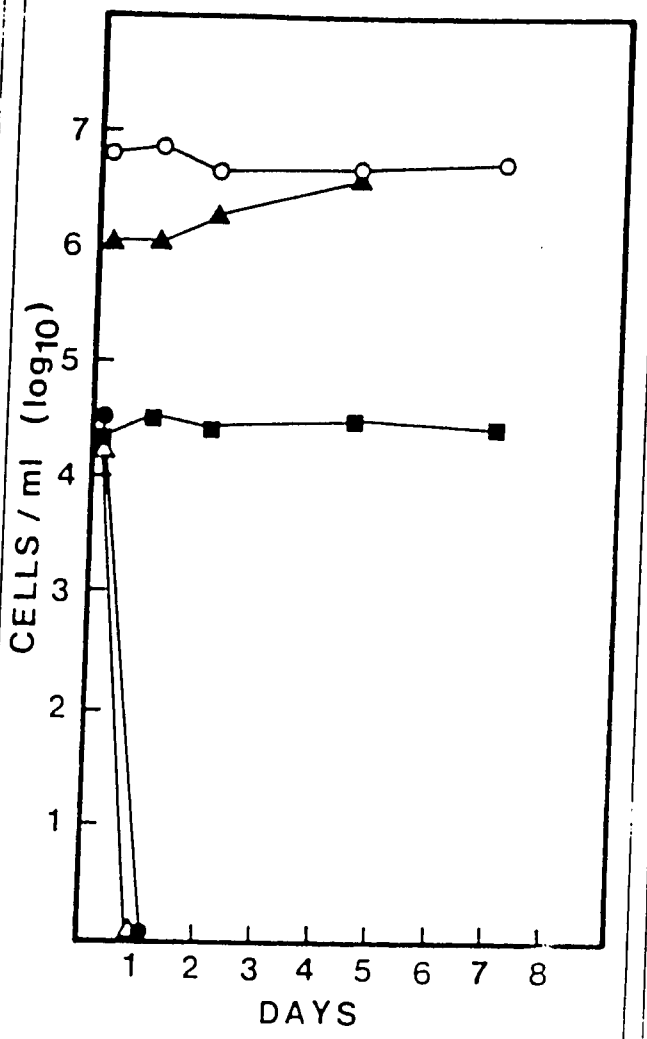

FIGURE 3 *Vibrio cholerae* CA 401 exposed to Patuxent River water in microcosms. ○ Acridine Orange Direct Count. ▲ Fluorescent Antibody Count. ■ Direct Viable Count. △ Plate Count, TCBS. ● Plate Count, TSA

TABLE 1 *Vibrio cholerae* culture and epifluorescent MPN results of Louisiana samples

		MPN Index[2]		
		Culture		Epifluorescent
Date	Source[1]	Total[3]	01	01
6/15/81	W	24	<3.0	460
6/15/81	SD	1.1	<0.3	>110
6/23/81	SW	11,000	16	>1,100
9/14/81	W	29/4,600[4]	<3.0	150
9/14/81	SD	1.2/21[4]	<0.3	4.2
9/15/81	SW	11,000	<3.0	20
12/07/81	W	<3.0	<3.0	<3.0
12/07/81	SD	<0.3	<0.3	<0.3
12/15/81	SW	<3.0	<3.0	<3.0

[1]W = water; SD = sediment; SW = sewage.
[2]*V. cholerae* MPN index per liter of water or gram of sediment.
[3]Total *Vibrio cholerae* concentration, independent of serovar.
[4]Two numbers because a 4-fold dilution was used; the lower 3 tubes give one MPN index, and the upper 3 another index.

ty, 25°C, filtered [0.2 μm] and autoclaved water).

The non-culturable, but viable and dormant stage in bacteria examined to date reveals viable counts by fluorescent microscopy to be consistently higher than corresponding plate counts. Since non-culturable cells are incapable of growing on standard media, it can be concluded that traditional culture methods to assess microbial populations are inadequate. Of prime concern is the question of whether viable but non-culturable bacteria remain virulent.

Rabbit ligated ileal loop assays were performed to answer this important question[7]. Non-culturable cells from microcosms of *V. cholerae* CA 401 were harvested by centrifugation, resuspended to a concentration of 10^8 cells/ml (constituting a 10^6 DVC) and injected into the intestinal loops. Positive virulence responses were observed in all microcosm-injected loops and were similar in appearance to the CA 401 control loop, i.e., distended, hemorrhagic, and filled with fluid. Fluid was aspirated and transferred to tubes of BHI broth, streaked to plates of TCBS and TSA, and prepared for AODC, FA and DVC. *V. cholerae* CA 401 cells from all microcosms injected into rabbit loops were recoverable in BHI broth and on TSA and TCBS plates. Surprisingly, all plate cultures grew as pure cultures on the selective medium. Slide agglutination tests were positive using *V. cholerae* 01 antisera on TSA isolates. DVC counts increased to 10^8 to 10^9 cells/ml. Transfers from the T_1N_1 (1 percent tryptone, 1 percent NaCl) control loop revealed no growth on TSA, TCBS, and EMB plates, or in BHI broth. The river water control loop was negative for CA 401.

The non-culturable *E. coli* withdrawn from the submerged membrane chambers produced fluid accumulation, but no hemorrhaging in a separate ileal loop experiment. Aspirated fluid was streaked to EMB and 1/8 strength BHI-Instant Ocean plates. *E. coli* was recovered on all the media tested and its biochemical profile was confirmed on API 20E identification strips. It was shown to be strain H10407 by virtue of detecting the plasmid bands characteristic of that strain[7].

DISCUSSION

The immunofluorescent-epifluorescent method has elucidated survival patterns of bacteria in the aquatic environment. Although "die-off" has been reported when conventional plating methods were used, results obtained by fluorescent microscopy reveal that pathogens remain viable in far greater numbers than previously reported. The "starvation-survival" mechanism[8-12] elicited during absence of suitable energy yielding substrates is prevalent in the natural environment. Such bacteria persist in the dormant phase until suitable conditions for outgrowth return. The latter may occur directly in the water as, for example, during a plankton bloom or in an animal host. Results of animal studies demonstrate that non-culturable cells continue to harbor the potential for virulence, both virulence plasmids and the clinical manifestation of virulence, i.e., engorged ileal loops.

The public health significance of these findings is obvious. Not so obvious is the relevance of these findings for deliberate release of genetically engineered microorganisms to the environment. It will not be possible to depend on plating methods for recovery and monitoring of such microorganisms but, rather, direct detection methods of high specificity will be required. Such methods include, but are not limited to, immunofluorescence-epifluorescence microscopy coupled with highly specific methods for molecular genetic tagging now being developed in our laboratory (MacDonell and Colwell, unpublished results).

EXPERIMENTAL PROTOCOL

Field testing. In Louisiana, enumerations were performed using a three-tube, three-dilution, MPN index[19]. Loopfuls from each tube of Alkaline Peptone Enrichment Broth (peptone, 1 percent w/v; NaCl, 1 percent w/v; ox-bile, 0.5 percent w/v, pH 8.6) were transferred to TCBS plates and suspect colonies were examined by slide agglutination, employing commercial antiserum (Difco) for the presence of the O1 antigen. A loopful was also spread on a clean glass slide for fluorescent antibody staining and an MPN enumeration by microscopic detection was made.

In Bangladesh, 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 hours, after which 1.0 ml aliquots were removed and dispensed to fresh enrichment broth. Incubation at 37°C followed for an additional 22 hours. Loopfuls of inoculum were transferred, after each incubation stage, to Taurocholate-Tellurite-Gelatin Agar[20] and slides. Slide agglutinations were performed on portions of 3,000 bacterial colonies and fluorescent antibody staining was done to determine the presence of *V. cholerae* O1 antigen. The fluorescent antibody staining procedure is described elsewhere[2].

Laboratory microcosms. Microcosm water was prefiltered, autoclaved and placed in acid washed flasks. Rubber stoppers were fitted with serum stoppered glass tubing to permit aseptic inoculation of the bacteria and withdrawal of the microcosm water with a needle and syringe. Overnight broth cultures of each bacterial strain were centrifuged (5000 rpm) for 20 minutes. The cells were washed and centrifuged three times with sterile microcosm water. Experimental organisms included *E. coli* strain H10407, *Shigella sonnei* strain 53-G, *S. flexneri* strain M42-43, and *Vibrio cholerae* strain CA 401, serotype O1.

Environmental microcosms. Environmental chambers were described by Mach and Grimes[21]. They were fitted with 76 mm dia. 0.4 μm pore size polycarbonate membranes (Nuclepore Corp., Pleasanton, CA). Each chamber was filled with filtered (0.2 μm) seawater that was collected from the test site, observed for leaks, and then allowed to equilibrate in the test site for 3–5 hours. Cells were incubated overnight in 1/8 BHI-Instant Ocean broth (Aquarium Systems, Mentor, OH) centrifuged and washed in filtered seawater. Two chambers each received a suspension of *E. coli* H10407, a third received *E. coli* and *V. cholerae* CA 401 and a fourth served as an uninoculated control. All chambers were secured to a sunken barge located ca. 110 m from shore in Nixon's Harbor, South Bimini, Bahamas.

Ligated ileal loop assay. The non-culturable cells from both *V. cholerae* CA 401 laboratory microcosms were harvested by centrifugation, and resuspended in sterile microcosm water. A rabbit was prepared for surgery. 7 loops separated with interloops, were ligated with catgut. Ca. 1.0 ml volumes of the following solutions were injected into individual loops: (1) T_1N_1 broth, (2) CA 401 (fully viable, pure culture, 10^6 cells/ml); (3) sterile Patuxent River water (used in the microcosms); (4) microcosm 1 cells, (5) microcosm 2 cells, (6) microcosm 1 cells, (7) microcosm 2 cells. The loop assay was done ca. 30 hours post surgery.

For the environmental microcosm, the non-culturable cells were withdrawn and concentrated ca. 112 hours post inoculation into the chambers. Pellets were resuspended in sterile Instant Ocean, and injected into ligated ileal loops. API 20E strips (Analytab Products, Plainview, NY) provided confirmation of *E. coli*. Plasmid extraction and analysis followed the methods of Kado and Liu[22].

Acknowledgments

The authors are grateful to K. M. S. Aziz and I. Huq (Bangladesh), I. Martin (Brazil), and P. Cross (South Africa), all of whom assisted in the collection of water samples for this project. The authors are also grateful for the advice and assistance of A. Huq, N. Roberts, and H. Bradford. This work was supported, in part, by the National Science Foundation (BSR-84-01397), Office of Naval Research (N00014-81-K-0638), Environmental Protection Agency (CR812246-01), World Health Organization (C6/181/70), Agency for International Development (DPE-5542-G-SS-4060-00), and Maryland Sea Grant College through the National Oceanic and Atmospheric Administration (R/P-15).

Received 20 June 1985; accepted 3 July 1985.

References

1. Xu, H. S., N. Roberts, F. L. Singleton, R. W. Atwell, D. J. Grimes, and R. R. Colwell. 1982. Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. Microb. Ecol. 8:313–323.
2. Xu, H. S., N. C. Roberts, L. B. Adams, P. A. West, R. J. Siebeling, A. Huq, M. I. Huq, R. Rahman, and R. R. Colwell. 1984. An indirect fluorescent antibody staining procedure for detection of *Vibrio cholerae* serovar O1 cells in aquatic environmental samples. J. Microbiol. Methods 2:221–231.
3. Colwell, R. R., H. S. Xu, N. Roberts, S. Siebeling, A. Huq, P. West, I. Huq, K. M. S. Aziz, D. Maneval, P. Brayton, D. Roszak, and M. MacDonell. 1984. Ecology and systematics of *Vibrio cholerae* in endemic and non-endemic areas. U.S.-Japan Cholerae Conference, Washington, D.C.
4. Kogure, K., U. Simidu, and N. Taga. 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. 25:415–420.
5. Hobbie, J. E., R. J. Daley, and S. Jasper. 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33:1225–1228.
6. Palmer, L. M., A. M. Bava, D. J. Grimes, and R. R. Colwell. 1984. Molecular genetic and phenotypic alteration of *Escherichia coli* in natural water microcosms containing toxic chemicals. FEMS Microbiol. Lett. 21:169–173.
7. Roszak, D. B., D. J. Grimes, and R. R. Colwell. 1984. Viable but non-recoverable stage of *Salmonella enteritidis* in aquatic systems. Can. J. Microbiol. 30:334–338.
8. Spira, W., R. B. Sack, and Froehlich, J. 1981. Simple adult rabbit model for *Vibrio cholerae* and enterotoxigenic *Escherichia coli* diarrhea. Infect. Immun. 32:739–747.
9. Macrina, F. L., D. J. Kopecko, K. R. Jones, D. J. Ayers, and S. M. McCowen. 1978. A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. Plasmid 1:417–420.
10. Baker, R. M., F. L. Singleton, and M. A. Hood. 1983. The effects of nutrient deprivation on *Vibrio cholerae*. Appl. Environ. Microbiol. 46:930–941.
11. Hoppe, H. G. 1976. Determination and properties of actively metabolizing heterotrophic bacteria in the sea, investigated by means of autoradiography. Mar. Biol. (Berlin) 36:291–302.
12. Jones, J. L. and M. E. Rhodes-Roberts. 1981. The survival of marine bacteria under starvation conditions. J. Appl. Bacteriol. 50:246–258.
13. Mitchell, R. 1968. Factors affecting the decline of non-marine microorganisms in seawater. Water Res. 2:535–543.
14. Morita, R. Y. 1983. Starvation-survival of heterotrophs in the marine environment. Adv. Microb. Ecol. 6:171–198.
15. Novitsky, J. A. and R. Y. Morita. 1977. Survival of a psychrophilic marine *Vibrio* under long-term nutrient starvation. Appl. Environ. Microbiol. 46:930–941.
16. Novitsky, J. A. and R. Y. Morita. 1978. Possible strategy for the survival of marine bacteria under starvation conditions. Mar. Biol. 48:289–294.
17. Stevenson, L. H. 1978. A case for bacterial dormancy in aquatic systems. Microb. Ecol. 4:127–133.
18. Tabor, P. S., K. Ohwada, and R. R. Colwell. 1981. Filterable marine bacteria found in the deep sea: distribution, taxonomy and response to starvation. Microb. Ecol. 7:67–83.
19. Russek, E. and R. R. Colwell. 1983. Computation of most probable numbers. MPNs. Appl. Environ. Microbiol. 45:1646–1650.
20. Monsur, K. A. 1961. A highly selective gelatin taurocholate tellurite medium for the isolation of *V. cholerae*. Trans. R. Soc. Trop. Med. Hyg. 55:110–112.
21. Mach, P. A. and Grimes, D. J. 1982. R Plasmid transfer in a wastewater treatment plant. Appl. Environ. Microbiol. 44:1095–1103.
22. Kado, C. I. and Liu, S. T. 1981. Rapid procedure for detecting isolation of large and small plasmids. J. Bacteriol. 145:1365–1373.

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JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1985, p. 1011–1013
0095-1137/85/121011-03$02.00/0
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Vol. 22, No. 6

Modified Taurocholate-Tellurite-Gelatin Agar for Improved Differentiation of *Vibrio* Species

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Received 12 June 1985/Accepted 26 August 1985

A total of 78 strains, representing 21 *Vibrio* species, were examined by using taurocholate-tellurite-gelatin agar (TTGA) medium and modified TTGA medium containing 4-methylumbelliferyl-β-D-galactoside (150 μg/ml). Modified TTGA medium allowed for simple and direct detection of β-D-galactosidase (β-gal) activity. This feature, in conjunction with other differential characteristics of TTGA medium, gave improved differentiation of the vibrios tested. The modified TTGA medium allowed for easy differentiation of *Vibrio cholerae* (β-gal$^+$) from *Vibrio parahaemolyticus* (β-gal$^-$). The 4-methylumbelliferyl-β-D-galactoside substrate is inexpensive and very stable. Incorporation into the agar did not affect the performance of TTGA as a differential medium. The assay for β-gal activity with this substrate was specific and sensitive.

There are several media, in addition to TCBS (thiosulfate-citrate-bile salts-sucrose) agar, employed for isolation and presumptive identification of *Vibrio* spp. Taurocholate-tellurite-gelatin agar (TTGA) medium, devised by Monsur (9) and recommended by the World Health Organization (15), has several distinct advantages over TCBS agar. It is relatively inexpensive to prepare and there are no problems in follow-up serological tests (4, 10). Furthermore, oxidase activity can be determined directly from the plate (10). A major drawback to the widespread acceptance of TTGA medium as a primary isolation medium for vibrios has been the failure of the medium to provide adequate differentiation between *Vibrio cholerae* and *Vibrio parahaemolyticus* (10). Preliminary studies in our laboratory, using 4-methylumbelliferyl-conjugated substrates, showed that β-D-galactosidase (β-gal) activity can be a useful differential character for vibrios. The substrates can be readily incorporated into selective media (13), and detection of enzyme activity with 4-methylumbelliferyl-conjugated substrates is both highly specific and sensitive (11). The objective of the present study was to evaluate the modified TTGA medium, which contains 4-methylumbelliferyl-β-D-galactoside, for the differentiation of *Vibrio* spp.

MATERIALS AND METHODS

Bacterial strains. A total of 78 strains, representing 21 *Vibrio* spp. and including both clinical and environmental isolates, were examined. Type and reference strains (American Type Culture Collection, Rockville, Md.) were included. The *V. cholerae* strains employed were O1 and non-O1 serotypes. Identification of the strains was confirmed by the criteria of West and Colwell (14); and details of procedures used are provided by Molitoris et al. (submitted for publication) and West et al. (submitted for publication). Strains in nutrient broth to which 12% glycerol was added were maintained under liquid nitrogen at −170°C.

Inocula. A loopful (5 to 15 colonies) of a nutrient agar plate culture incubated at either 35°C (clinical str ins) or 25°C (environmental strains) for 24 to 48 h was used.

Media. TTGA medium was prepared by the method of Furniss et al. (3). For the modified medium, 4-methylumbelliferyl-β-D-galactoside (Sigma Chemical Co., St. Louis, Mo.) was added to molten TTGA medium to a final concentration of 150 μg/ml. Both TTGA and modified TTGA media were autoclaved at 121°C for 15 min. Both TTGA and modified TTGA plates were used fresh. Nutrient agar was prepared by the method of West and Colwell (14).

Procedure. TTGA and modified TTGA media were inoculated in parallel with each of the test strains by using a wire loop (4 mm diameter) and a 16-streak dilution procedure to obtain isolated colonies. Some strains were tested for susceptibility to the vibriostatic agent (2,4-diamino-6,7-diisopropyl-pteridine) by the method of West and Colwell (14) with sterile antimicrobial assay disks (BBL Microbiology Systems, Cockeysville, Md.) impregnated with 10 and 150 μg of the 0/129 compound, respectively. TTGA and modified TTGA plate cultures were incubated at either 35°C (clinical strains) or 25°C (environmental strains). Unless specified otherwise, results were recorded after incubation for 24 to 48 h. β-Gal activity was tested by direct examination of plate cultures under UV light (366 nm) after incubation overnight and at 24 to 48 h. Positive reactions were indicated by bright-blue fluorescence of the individual colonies and confluent growth due to the formation of the reaction by-product 4-methylumbelliferone (11, 13). The standard tube method of Lowe (7), employing the *o*-nitrophenyl-β-D-galactopyranoside compound (Sigma) and with 1.0% NaCl added, instead of 0.5% NaCl, was also done. Tellurite reduction, gelatinase production, and susceptibility to 0/129 were determ ined by direct visual examination of cultures on agar plate The following characteristics were also determined directly from plate cultures: Gram reaction by the KOH method of Gregerson (5), oxidase (6), catalase (1), and catalase reaction speed (1).

A number of strains were tested twice. When discrepancies in results on both types of media occurred, the strains were retested.

RESULTS

Excellent correlation was observed in test results for both TTGA and modified TTGA media. Results for the 78 vibrios examined on modified TTGA medium are presented in Table 1. All but two of the strains grew well on TTGA and modified

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TABLE 1. Results for 78 strains of 21 *Vibrio* species on modified TTGA medium containing 4-methylumbelliferyl-β-D-galactoside

Strain (no. examined)	No. (%) of positive strains[a]							
	Growth	Tellurite reduction	Gelatinase	Catalase reaction speed[b]			β-Gal[c]	
				R	M	D	4-MUCS	ONPG
V. cholerae (26)	26 (100)	26 (100)	26 (100)	2 (8)	6 (23)	18 (69)	26 (100)	26 (100)
V. parahaemolyticus (20)	20 (100)	17 (85)	20 (100)	1 (5)	1 (5)	18 (90)	0 (0)	0 (0)
V. alginolyticus (4)	4 (100)	2 (50)	4 (100)	0 (0)	2 (50)	2 (50)	0 (0)	0 (0)
V. anguillarum (1)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	1 (100)
V. campbelli (1)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
V. carchariae (1)	1 (100)	1 (100)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)
V. cincinnatiensis (1)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	1 (100)
V. damsela (2)	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
V. diazotrophicus (1)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	1 (100)
V. fluvialis (5)	5 (100)	5 (100)	4 (80)	0 (0)	1 (20)	4 (80)	5 (100)	5 (100)
V. harveyii (1)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)
V. metschnikovii[d] (1)	0 (0)							
V. mimicus (3)	3 (100)	3 (100)	3 (100)	0 (0)	2 (66)	1 (33)	3 (100)	3 (100)
V. natriegens (1)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
V. nereis (1)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
V. nigrapulchritudo (1)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	1 (100)	1 (100)
V. ordalii (1)[d]	0 (0)							
V. pelagius (1)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)
V. proteolyticus (1)	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)
V. vulnificus (5)	5 (100)	4 (80)	4 (80)	1 (20)	1 (20)	3 (60)	3 (60)	3 (60)

[a] Includes weakly positive strains
[b] R, Rapid (0 s); M, medium (1 to 2 s); D, delayed (>2 s).
[c] 4-MUCS, 4-Methylumbelliferyl-conjugated substrate; ONPG, *o*-nitrophenyl-β-D-galactopyranoside.
[d] No growth on TTGA medium without added 4-methylumbelliferyl-β-D-galactoside substrate.

TTGA media. Excellent correlation was noted in results for Gram reaction and oxidase and catalase on both media. All of the 76 strains were gram-negative, oxidase and catalase positive, when tested on either medium. Susceptibility to 0/129 was found to be variable and species dependent. However, there was a very good correlation in this feature on both TTGA and modified TTGA media.

Results for tellurite reduction, gelatinase production, and catalase reaction speed on modified TTGA medium are given in Table 1. There was a very good correlation in results for these three features on both TTGA and modified TTGA media. For some strains, in particular *V. parahaemolyticus*, tellurite reduction was more obvious on modified TTGA medium than on TTGA medium. We can offer no explanation for this observation. Many strains gave a delayed (i.e., >48 h) or weak reaction for tellurite reduction and gelatinase on both media. Occasionally, strains of some of the species demonstrated different catalase reaction speeds on both media.

Table 1 also shows results for β-gal activity by the vibrios on modified TTGA medium. The β-gal activity was not detected on TTGA medium for any of the strains when the 4-methylumbelliferyl-β-D-galactoside substrate was omitted. For strains showing β-gal activity, interstrain and interspecies variation was observed. Most of the *V. cholerae* strains gave very strong reactions, i.e., brilliant-blue fluorescence, after incubation for 24 to 48 h. Only weak reactions were observed for strains of *V. anguillarum*, *V. carchariae*, and *V. cincinnatii*. For some species, e.g., *V. cincinnatii*, the β-gal activity was observed only after prolonged incubation. Most of the β-gal-producing strains showed strong activity within 24 h and many after incubation overnight. As evident from Table 1, these strains for species in which at least one or more strains from a given source were examined.

DISCUSSION

The results shown in Table 1 demonstrate that β-gal activity, tested with 4-methylumbelliferyl-β-D-galactoside, is a useful character for differentiation of *Vibrio* spp. on TTGA medium. *V. cholerae* (β-gal$^+$) was easily distinguished from *V. parahaemolyticus* (β-gal$^-$), overcoming an inherent problem with TTGA medium. This finding is particularly valuable since sucrose-fermenting variants of *V. parahaemolyticus*, which would otherwise be discarded without further characterization after isolation on TCBS, can be detected with the modified TTGA medium. Results of the present study also show that β-gal activity on modified TTGA medium, in conjunction with tests for Gram reaction, oxidase activity, tellurite reduction, and gelatinase production, is useful in grouping and differentiating *Vibrio* spp. However, many more strains need to be examined. The results for these characters agree well with those of previously published reports (2, 12, 14). Catalase reaction speed and susceptibility to the agent 0/129 on modified TTGA medium may also prove to be useful differential tests, although more strains need to be examined. Both tests have proven useful in differentiation among *Pseudomonas* spp. (1) and *Vibrio* spp. (2, 12, 14), respectively. Testing for susceptibility to 0/129 with selective media can be criticized, however.

All of the characteristics can be determined directly from the primary isolation medium, which offers an additional advantage since, in many cases, presumptive identification can be achieved within 24 h or less. The 4-methylumbelli

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reaction by-product, 4-methylumbelliferone, is highly fluorescent at this pH, rendering the test more sensitive. Positive and negative reactions of colonies growing on this medium cannot be confused. However, caution should be exercised in delaying observation of plate cultures for β-gal activity, especially for mixed cultures, since diffusion of the fluorescent by-product could lead to erroneous interpretation.

Fortuitously, the results show that the addition of the substrate to TTGA medium did not affect its performance as a differential medium. The cost benefit of incorporating the substrate into TTGA medium is very good (additional cost per plate, less than $0.05). This makes modified TTGA medium particularly attractive since TCBS agar is expensive (10). In light of the disadvantages of TCBS agar (2, 8, 10, 14) and the advantages of the modified TTGA medium detailed above, the modified TTGA medium containing 4-methylumbelliferyl-β-D-galactoside is concluded to be a preferred alternative for differentiation and presumptive identification of *Vibrio* spp.

ACKNOWLEDGMENTS

We thank P. Brayton, E. Molitoris, C. Kaysner, S. Gelbart, and T. Overman for supplying the strains used in this study.

This work was funded by National Science Foundation grant BSR-84-01397.

LITERATURE CITED

1. Chester, B. 1979. Semiquantitative catalase test as an aid in identification of oxidative and nonsaccharolytic gram-negative bacteria. J. Clin. Microbiol. 10:525–528.
2. Farmer, J. J., III, F. W. Hickman-Brenner, and M. T. Kelly. 1985. *Vibrio*, p. 282–301. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
3. Furniss, A. L., J. V. Lee, and T. J. Donovan. 1978. The vibrios. Public Health Laboratory Service monograph no. 11. Her Majesty's Stationery Office, London.
4. Gangarosa, E. J., W. E. DeWitt, I. Huq, and A. Zafiri. 1968. Laboratory methods in cholera: isolation of *Vibrio cholerae* (El Tor and Classical) on TCBS medium in minimally equipped laboratories. Trans. R. Soc. Trop. Med. Hyg. 62:693–699.
5. Gregersen, T. 1978. Rapid method for distinction of gram negative from gram positive bacteria. Eur. J. Appl. Microbiol. Biotechnol. 5:123–127.
6. Kovacs, N. 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature (London) 178:703.
7. Lowe, G. H. 1962. The rapid detection of lactose fermentation in paracolon organisms by the demonstration of β-D-galactosidase. J. Med. Lab. Technol. 19:21.
8. McCormack, W. M., W. E. DeWitt, P. E. Bailey, G. K. Morris, P. Soerharjono, and E. J. Gangarosa. 1974. Evaluation of thiosulfate-citrate-bile salts-sucrose agar, a selective medium for the isolation of *Vibrio cholerae* and other pathogenic vibrios. J. Infect. Dis. 129:497–500.
9. Monsur, K. A. 1961. A highly selective gelatin-taurocholate-tellurite medium for the isolation of *Vibrio cholerae*. Trans. R. Soc. Trop. Med. Hyg. 55:440–442.
10. Morris, G. K., M. H. Merson, I. Huq, A. K. M. G. Kibrya, and R. Black. 1979. Comparison of four plating media for isolating *Vibrio cholerae*. J. Clin. Microbiol. 9:79–83.
11. O'Brien, M., and G. H. G. Davis. 1982. Enzymatic profile of *Pseudomonas maltophilia*. J. Clin. Microbiol. 16:417–421.
12. Sakazaki, R., and A. Balows. 1981. The genera *Vibrio*, *Plesiomonas*, and *Aeromonas*, p. 1272–1301. *In* M. P. Starr, H. Stolz, H. G. Truper, A. Balows, and H. G. Schlegel (ed.), The prokaryotes: a handbook on habitats, isolation and identification of bacteria. Springer Verlag, Berlin.
13. Trepeta, R. W., and S. C. Edberg. 1984. Methylumbelliferyl-β-D-glucuronide-based medium for rapid isolation and identification of *Escherichia coli*. J. Clin. Microbiol. 19:172–174.
14. West, P. A., and R. R. Colwell. 1984. Identification and classification of Vibrionaceae—an overview, p. 285–363. *In* R. R. Colwell (ed.), Vibrios in the environment. John Wiley & Sons, Inc., New York.
15. World Health Organization. 1974. Guidelines for the laboratory diagnosis of cholera. World Health Organization, Geneva.

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Microbiological Sciences Vol. 3, No. 11, 1986

The fate of enteric pathogenic bacteria in estuarine and marine environments

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Sufficient laboratory and field data are now available to hypothesize that enteric pathogens survive for very long periods of time in sea-water. In fact, these Gram-negative bacteria probably enter into dormancy, during which they remain viable and potentially virulent, yet are non-culturable when traditional bacteriological methods are employed. Increasing use of the world's oceans for discharge of domestic wastes may result in public health problems in the future from the allochthonous human pathogens accumulating in the marine environment at disposal sites.

Introduction

Survival of enteric pathogens in estuarine and marine environments has been the subject of extensive study for nearly a century. The conclusion reached by various investigators over the years has been consistent, namely that allochthonous pathogenic bacteria do not survive in salt-water.[1-3] This conclusion has been drawn from results showing inability to culture pathogens from sea-water samples plated on standard bacteriological culture media, after the bacteria had been discharged to an estuary or the sea (or added to sea-water in laboratory simulations, i.e., in salt-water microcosms). Results of such studies indicate a steady decline with time, in cultivable bacteria, until the pathogens can no longer be grown in or on culture media. Variables postulated as responsible for the decline in numbers of culturable bacteria include sedimentation,[4] predation,[5] parasitism,[6] sunlight,[7] temperature,[8,9] osmotic stress,[10] toxic chemicals,[11] and the high salt concentration of sea-water.[9,12] A variety of mathematical formulae have been published which describe observed declines.[13] Some investigators have constructed 'decay models' for specific habitats.[2,14] It is, in general, concluded that the fate of enteric pathogens in salt-water is, eventually, death. This review will examine recent data which suggest that enteric pathogenic bacteria have quite a different fate in sea-water. For the purpose of this review, 'enteric' will be used in the epidemiological sense, i.e. to describe those bacteria that are associated with the intestines of warm-blooded animals and are known to cause gastrointestinal disease, as opposed to the taxonomic use of the term 'enteric', referring to members of the family Enterobacteriaceae.

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Received 22 January, 1986.

Viable but non-culturable bacteria

Studies conducted in our laboratory over the past decade have focussed on the incidence, distribution, and survival of Gram-negative enteric bacteria in estuarine and marine habitats.[15,16] Xu *et al.*[17] showed that a decline in cultivable bacteria in a system does not correlate with cell death in the case of *Escherichia coli* and *Vibrio cholerae* exposed to salt-water (15‰ salinity) microcosms.

Methods used to monitor the fate of *E. coli*, *V. cholerae* and other species in salt-water microcosms are listed in Table 1. In the microscopic methods for

Table 1. *Methods used to detect cells in aquatic systems.*[17,18,20,29]

Direct counts[1]	
AODC	Fluorochrome specific for nucleic acid; used to count all cells (living and dead).
FAC	Antigen-specific fluorochrome; used to count all cells carrying a specific antigen(s) (living and dead).
DVC	Fluorochrome for counting physiologically responsive (living) cells, either nucleic acid- or antigen-specific.
Indirect counts[1]	
MPN	Only living cells capable of growth in the broth medium employed are enumerated (media can be selective and/or differential); when combined with FAC also allows enumeration of non-culturable cells.
HPC	Counts only living cells capable of growth on the medium employed (media can be selective and/or differential).
Animal model and tissue culture	
	Provides growth conditions (not yet defined, in most cases) for non-culturable (dormant) cells; useful also for detecting virulent cells.

[1]AODC, acridine orange direct count; FAC, fluorescent antibody count; DVC, direct viable count; MPN, most probable number; HPC, heterotrophic plate count.

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enumeration, intact cells take up the stain, either acridine or antibody conjugated to fluorescein isothiocyanate (indirect fluorescent antibody method). Counting is done using epifluorescent microscopy. Application of the direct viable count (DVC)[18] was important in documenting that cells do not necessarily die when exposed to sea-water. Briefly, the DVC employs yeast extract as a growth-stimulating nutrient and nalidixic acid as a DNA gyrase inhibitor.[18] Other substrates have also been tested, showing the influence of substrate and substrate concentration on substrate response.[19] Cells respond to nutrient by enlarging, but division is inhibited by nalidixic acid. Recent evidence suggests that nalidixic acid may also facilitate uptake of nutrient.[20] The enlarged cells enumerated by the acridine orange direct count (AODC), or by the fluorescent antibody count (FAC), have been shown to be metabolically active by autoradiographic methods (D.B. Roszak and R.R. Colwell, unpublished results). The DVC, as a measure of viability, has been correlated with results of other methods by other investigators.[21] In addition to the DVC (a direct microscopic method), indirect counts were also used to assess viability. Specifically, most probable number (MPN) indices[22] and heterotrophic plate counts (HPCs) by the spread-plate method[23] were used to detect viable, culturable cells.

Results typical of those obtained in the initial microcosm study by Xu *et al.*[15] and in subsequent experiments are shown in Figure 1. Total counts, either AODCs or FACs, remain relatively constant with time, indicating that cellular integrity is maintained. However, viability cannot be demonstrated by either the AODC or the FAC, unlike conventional cultural methods and the DVC method.[18] Indirect counts made using broth culture (MPN) decline more slowly than those on solid media (HPC), especially when selective agar media are employed. Nevertheless, both exhibit significant negative slope compared to the total direct counts (AODCs and FACs, Figure 1). In general, but depending on the bacterial species employed, the salinity of the water and the temperature of incubation, MPN and HPC indices approach zero within 8 to 20 d, after which time the cells cannot be transferred, i.e. growth no longer occurs even though the DVC indicates continued presence of viable cells. Concentration of large volumes of water containing the viable but non-culturable cells does not provide growth. These viable but non-culturable cells can be considered either starved[24] or dormant.[25]

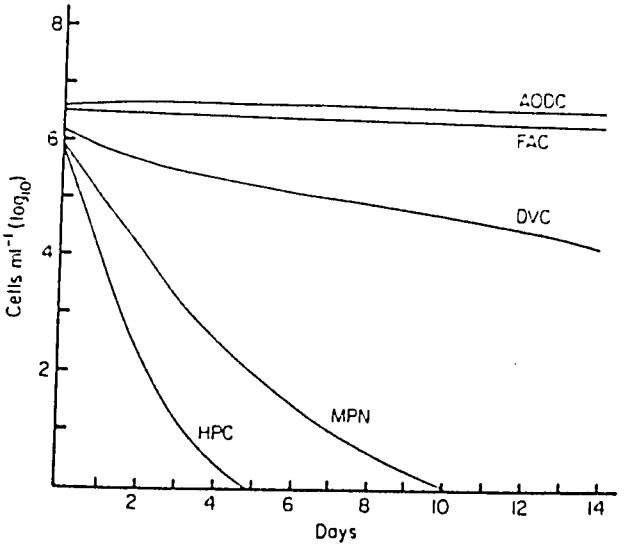

Figure 1. *Enumeration of enteric pathogens by several methods. Results shown provide evidence for viable but non-culturable organisms. (See Table 1 for abbreviations.)*

E. coli H10407, an enterotoxigenic pathogen isolated from a patient with diarrhoea,[26] was exposed to sea-water of 15‰ salinity in a microcosm maintained at 18°C. Results comparable to those shown in Figure 1 were obtained.[27] When the non-culturable cells were analysed for characteristic plasmid bands (Table 2) by direct extraction of cells harvested from the microcosm without regrowth on any medium, there was no evidence of plasmid DNA in the ethidium bromide-exposed gels. However, when a portion of the harvested cells were grown on Brain Heart Infusion agar and then re-examined for plasmids, all three plasmid bands were visible.

Salmonella enteritidis serogroup C_1 was also examined.[28] Unlike either *E. coli* or *V. cholerae*, it was possible to resuscitate *S. enteritidis* 4 d after the non-culturable state was achieved. Recovery was accomplished by harvesting viable but non-culturable cells from the sea-water microcosm, placing the cells in an equal volume of full-strength Veal Infusion broth and, following incubation at 25°C for 25 h, plating aliquots of water from the recovery microcosm on to Veal Infusion agar at selected time intervals. Very small, atypical colonies appeared on the agar plates (colonies which normally would have been rejected as *S. enteritidis*).[28] Resuscitation was repeated after the cells had been in the viable but non-culturable state for 21 d, but was unsuccessful despite persistent elevated DVC counts.

Success in recovering otherwise viable but non-culturable cells from sea-water microcosms has been achieved by introduction into ligated rabbit ileal loops.[29] Viable but non-culturable microcosm cultures of both *E.*

Table 2. *Plasmid-borne virulence markers carried by* Escherichia coli *H10407.*[26]

Plasmid	Size	Marker[1]
pCS1	60	CFA I ST
pJY11	42	LT A LT B ST
pTRA	42	unknown
cryptic	4	unknown

[1] CFA, colonizing factor antigen; ST, heat-stable toxin; LT, heat-labile toxin.

coli H10407 and *V. cholerae* CA 401 have been recovered from loops, providing simultaneous recovery of otherwise non-culturable cells and detection of enterotoxigenicity.[30] *E. coli* H10407 was used in a membrane chamber study conducted in Nixon's Harbour, South Bimini, Bahamas[31] with very similar results.

Allochthonous versus autochthonous pathogens

Bacteria indigenous to a given habitat are *autochthonous* to that habitat, while those foreign to the habitat are *allochthonous*. Most enteric pathogens are allochthonous to estuarine and marine environments, although some of the more notable pathogens are truly autochthonous, e.g., *V. cholerae*,[32] *V. parahaemolyticus*[33] and *Aeromonas hydrophilla*.[34]

It has already been shown that both autochthonous and allochthonous species are capable of entering a viable but non-culturable state.[17,27,28,30,31] Unlike allochthonous bacteria, however, autochthonous enteric pathogens not only survive in sea-water, but grow, if suitable nutrient is present. The nutrient may be supplied by wastewater and/or surface run-off entering estuaries or coastal zones.[35-36] This situation has led many investigators to conclude that wastewater is only a point source of pathogens, rather than a source of nutrients as well which may, in turn, stimulate growth of the autochthonous pathogens. Such erroneous interpretations of data are largely responsible for the belief that autochthonous enteric pathogens such as *V. cholerae* are human gut inhabitants, only transiently waterborne following input of faecal matter from an infected host.[37]

Wastewater and surface run-off to estuaries and sea-water constitues a serious public health hazard. Allochthonous pathogens entering the sea may enter the viable but non-culturable state. Autochthonous human pathogens, as well, are supplied with growth-stimulating nutrients, and increase in number, a phenomenon documented for at least two pathogens.[35,36] Similarly, anthropogenic wastes may stimulate growth of other non-human pathogens, for example, pathogens of migratory waterfowl.[38] In addition to nutrients, wastes may contain chemicals toxic for some species of bacteria, eliminating (selecting against) those bacteria sensitive to the chemicals, resulting either in populations of bacteria resistant to the chemicals or populations capable of chemical degradation. Such a result has been demonstrated repeatedly for antibiotics selecting for resistant strains.[39] Furthermore, *in situ* transmission of plasmid-encoded antibiotic resistance has been demonstrated.[40,41] Thus, an additional public health threat can occur, namely the selection for resistant and potentially virulent strains of allochthonous and autochthonous pathogens.

Movement of pathogens through the water column

It is difficult to quantify, spatially and temporally, the movement of human enteric pathogens, or bacteria in general, through sea-water. Few studies have been conducted, and the rapidity with which allochthonous pathogens enter the viable but non-culturable state complicates interpretation of resultant data. The relative contributions of physical and chemical parameters, such as sedimentation, advection, lysis and accumulation at interfaces, to survival of human pathogens in sea-water are not fully understood at this time.

The contention that large bodies of water, especially the ocean, afford rapid and effective dilution of wastes is no longer without dispute. Results of several studies have demonstrated that particulate wastes discharged to the ocean are not rapidly dispersed but, instead, tend to remain associated and move in response to ocean currents.[42,43] Some particulates sink[44] whereas others meander with gyres and warm core rings.[45-48] Bacterial particulates tend to accumulate at interfaces, where they attach to surfaces and to each other.[49] Still others become airborne, for example coliforms become associated with lipoidal surface particulates (7300 coliforms per mg of hexane-extractable material, on average, have been calculated).[50] Presumably other enteric bacteria (including pathogens) have a similar tendency to partition on to/into lipoidal particulates. In such a micro-environment they would be protected from chlorination at the wastewater treatment plant and from the high salinity of water in estuaries and the ocean. It has been observed that lipoidal particulates are buoyant and can ascend from deep water, through the pycnocline, to the surface. Once surfaced, the particulates are rapidly dispersed toward shore, mainly by wind and wave action. Similar observations have been made in studies of wastewater discharges in Puerto Rico and France.[51,52] It can be concluded that human pathogens can accumulate at surfaces, and in numbers large enough to constitute an infective dose. Unfortunately, by currently accepted enumeration methods such assemblages of cells are counted as a single colony on a solid medium or as a single MPN unit.

Sediment-bound bacteria are subject to vertical fluxes and can be resuspended in the water column following a variety of physical forces. Forces capable of initiating this type of resuspension include storms, dredging,[4,53] navigation, natural upwellings,[43] bioturbation (sediment disturbance by benthic invertebrates, primarily oligochaetes and chironomids), currents and wave action. For example, faecal coliform concentrations increased five-fold as a result of dredging polluted sediment from the Mississippi River.[53] Once resuspended, bacteria can be picked up by (i) a potential host and (ii) non-host animals and transmitted via the food chain to a potential host. The bacteria may also simply resettle into the bottom sediment: for example, it was hypothesized that sediment-bound *Shigella* spp. could have been resuspended by dredging activity in the Mississippi River and thereby involved in a swimming-associated outbreak of shigellosis.[53]

Some cells enter the viable but non-culturable state and others are sedimented to the bottom, so enteric pathogens will cease to be detected in the water column

if conventional methods are employed. Upon entering the sediment, if not already in that state, viable bacteria may become non-culturable.

Animal association

Enteric pathogens frequently become associated with aquatic animals ranging from microscopic invertebrates to marine mammals. These associations can be accidental and transient, as seems to be the case with fish and *Salmonella* spp.,[54] or they can be very specific and long lasting, as demonstrated for copepods and *V. cholerae*[55] and *V. parahaemolyticus*.[56] In the case of copepods and *V. parahaemolyticus*, seasonal growth of copepods has been correlated with growth of *V. parahaemolyticus* and, in turn, increased incidence of gastroenteritis caused by this pathogen.[56] In the case of *V. cholerae*, copepods may act as an overwintering substrate for the bacteria.[55]

Shellfish-borne enteric disease is, of course, a direct result of animal associations. This type of disease is usually of viral aetiology,[57] although bacteria such as *E. coli*, *Campylobacter jejuni*, *Salmonella* spp., *Plesiomonas shigelloides* and *Vibrio* spp. can be involved. There have been almost 12000 cases of shellfish-borne disease reported in the United States and Canada since 1900 (US Government, General Accounting Office, HRD-84-36, June 14, 1984).

In addition to associations with invertebrates, bony fish and marine mammals, enteric pathogenic bacteria have recently been isolated from sharks.[58] These bacteria appear to be part of the autochthonous flora of healthy, free-ranging sharks[58] although, under conditions of stress, they may cause disease in their shark host. It is possible that these pathogens could be passed to humans through shark bites[59] and improperly cooked meat (P.R. Brayton and D.J. Grimes, unpublished results).

Conclusions

It can no longer be argued that inability to culture a given enteric pathogen from estuarine or marine water samples is prima-facie evidence that the pathogen is either dead or absent from the sample. Enteric pathogens can enter a viable but non-culturable state. Table 3 summarizes the salient features of viable but non-culturable bacteria. These dormant bacteria can then follow a variety of pathways in the ocean. Figure 2 illustrates the fate of enteric pathogens in wastes discharged to estuarine and marine environments.

Table 3. *Selected characteristics of viable but non-culturable bacteria.*

Culture
(1) No growth on or in standard culture media.
(2) Resumption of growth under appropriate conditions.

Cytology
(1) Cells appear intact when stained by AODC and/or FAC methods.
(2) Cells are substrate-responsive, demonstrated by the DVC method and autoradiography.
(3) Cells may undergo changes in size (viz: smaller) and/or shape (viz: rounded).

Virulence
(1) Viable but non-culturable cells remain virulent.

Serious thought is now being given to expanding ocean disposal of wastes; concurrently, the population

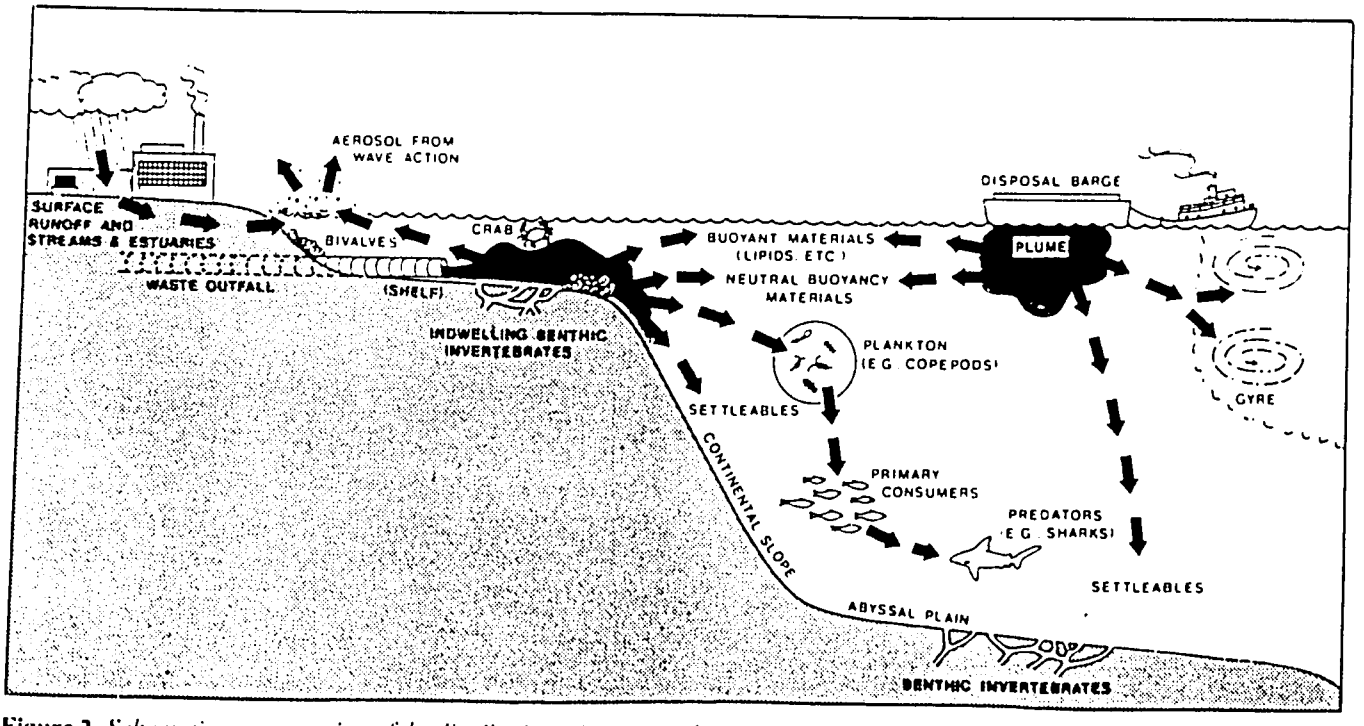

Figure 2. *Schematic representation of the distribution of enteric pathogens in wastes discharged into the Atlantic Ocean.*

of coastal cities is increasing. The public health problem of enteric pathogens in sea-water must be recognized, especially in estuaries, coastal waters and deep ocean disposal sites.

Acknowledgements

The authors gratefully acknowledge Professors R.Y. Morita and G.E. Jones for helpful discussions concerning survival and dormancy, and Professors K. Kogure, U. Simidu and N. Taga with whom we have worked over the years.

The research reported in this review was supported, in part, by grants from the National Oceanic and Atmospheric Administration (NA79AA-D-00062), the National Institutes of Health (R22-AI-14242-07), the National Science Foundation (BSR-84-01397 and INT-82-12853), the World Health Organization (C6/181/70 (A)), the Agency for International Development (DPE-5542-G-55-4060-00), the University of Maryland Sea Grant College (NA84-AA-D-00014), and the US Army Corps of Engineers (DACW376M-2345).

References

1 ZoBell CE. Bactericidal action of sea-water. *Proceedings of the Society of Experimental Biology and Medicine* 1936; 34: 113–16.

2 Chamberlin CE, Mitchell R. A decay model for enteric bacteria in natural waters. In: Mitchell R, ed. *Water Pollution Microbiology*, vol. 2. New York: John Wiley & Sons, Inc., 1978: 325–48.

3 Baross JA, Hanus FJ, Morita RY. Survival of human enteric and other sewage microorganisms under simulated deep-sea conditions. *Applied Microbiology* 1975; 30: 309–18.

4 Grimes DJ. Release of sediment-bound fecal coliforms by dredging. *Applied Microbiology* 1975; 29: 109–11.

5 Sieburth JMcN. Protozoan bacterivory in pelagic marine waters. In: Hobbie JE, Williams, PJleB, eds. *Heterotrophic Activity in the Sea*. New York: Plenum Press, 1984: 405–44.

6 Mitchell R. Factors affecting the decline of non-marine microorganisms in seawater. *Water Research* 1968; 2: 535–43.

7 Kapuscinski RB, Mitchell R. Solar radiation induces sublethal injury in *Escherichia coli* in seawater. *Applied and Environmental Microbiology* 1981; 41: 670–4.

8 Faust MA, Aotaky AE, Hargadon MT. Effect of physical parameters on the *in situ* survival of *Escherichia coli* MC-6 in an estuarine environment. *Applied Microbiology* 1975; 30: 800–6.

9 Singleton FL, Attwell R, Jangi S, Colwell RR. Effects of temperature and salinity on *Vibrio cholerae* growth. *Applied and Environmental Microbiology* 1982; 44: 1047–58.

10 Carlucci AF, Pramer D. An evaluation of factors affecting the survival of *Escherichia coli* in seawater II. Salinity, pH, and nutrients. *Applied Microbiology* 1960; 8: 247–50.

11 Jones GE. Effect of chelating agents on the growth of *Escherichia coli* in seawater. *Journal of Bacteriology* 1964; 87: 483–99.

12 Anderson IC, Rhodes M, Kator H. Sublethal stress in *Escherichia coli*: a function of salinity. *Applied and Environmental Microbiology* 1979; 38: 1147–52.

13 Teltsch B, Shuval HT, Tadmor J. Die-away kinetics of aerosolized bacteria from sprinkler application of wastewater. *Applied and Environmental Microbiology* 1980; 39: 1191–7.

14 Kay D, McDonald A. Predicting coliform concentrations in upland impoundments: design and calibration of a multivariate model. *Applied and Environmental Microbiology* 1983; 46: 611–18.

15 Sayler GS, Nelson, Jr JD, Justice A, Colwell RR. Distribution and significance of fecal indicator organisms in the Upper Chesapeake Bay. *Applied Microbiology* 1975; 30: 625–38.

16 Kaper JB, Sayler GS, Baldini MM, Colwell RR. Ambient-temperature primary nonselective enrichment for isolation of *Salmonella* spp. from an estuarine environment. *Applied and Environmental Microbiology* 1977; 33: 829–35.

17 Xu H-S, Roberts N, Singleton FL, Attwell RW, Grimes DJ, Colwell RR. Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. *Microbial Ecology* 1982; 8: 313–23.

18 Kogure K, Simidu U, Taga N. A tentative direct microscopic method for counting living marine bacteria. *Canadian Journal of Microbiology* 1979; 25: 415–20.

19 Peele ER, Colwell RR. Application of a direct microscopic method for enumeration of substrate-responsive marine bacteria. *Canadian Journal of Microbiology* 1981; 27: 1071–5.

20 Dougherty TJ, Saukkonen JJ. Membrane permeability changes associated with DNA gyrase inhibitors in *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* 1985; 28: 200–6.

21 Tabor PS, Neihof RA. Direct determination of activities for microorganisms of Chesapeake Bay populations. *Applied and Environmental Microbiology* 1984; 48: 1012–19.

22 MacDonell MT, Russek E, Colwell RR. An interactive microcomputer for the computation of most probable number. *Journal of Microbiological Methods* 1984; 2: 1–7.

23 Klein DA, Wu S. Stress: a factor to be considered in heterotrophic microorganism enumeration from aquatic environments. *Applied Microbiology* 1974; 27: 429–31.

24 Morita RY. Starvation and miniaturisation of heterotrophs, with special emphasis on maintenance of the starved viable state. In: Fletcher M, Floodgate GD, eds. *Bacteria in Their Natural Environments*. Orlando, Florida: Academic Press, Inc. 1985: 111–30.

25 Stevenson LH. A case for bacterial dormancy in aquatic systems. *Microbial Ecology* 1978; 4: 127–33.

26 Evans DG, Silver RP, Evans, Jr DJ, Chase DG, Gorbach SL. Plasmid-controlled colonization factor associated with virulence in *Escherichia coli* enterotoxigenic for humans. *Infection and Immunity* 1975; 12: 656–67.

27 Palmer LM, Baya AM, Grimes DJ, Colwell RR. Molecular genetic and phenotypic alteration of *Escherichia coli* in natural water microcosms containing toxic chemicals. *FEMS Microbiology Letters* 1984; 21: 169–73.

28 Roszak DB, Grimes DJ, Colwell RR. Viable but nonrecoverable stage of *Salmonella enteritidis* in aquatic systems. *Canadian Journal of Microbiology* 1984; 30: 334–8.

29 Spira WM, Sack RB, Froehlich JL. Simple adult rabbit model for *Vibrio cholerae* and enterotoxigenic *Escherichia coli* diarrhea. *Infection and Immunity* 1981; 32: 739–47.

30 Colwell RR, Brayton PR, Grimes DJ, Roszak DB, Huq SA, Palmer LM. Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms. *Bio/Technology* 1985; 3: 817–20.

31 Grimes DJ, Colwell RR. Viability and virulence of *Escherichia coli* suspended by membrane chamber in semitropical ocean water. *FEMS Microbiology Letters* 1986; 34: 161–5.

32 Colwell RR, Seidler RJ, Kaper J, Joseph SW, Garges S, Lockman H, Maneval D, Bradford H, Roberts N, Remmers E, Huq I, Huq A. Occurrence of *Vibrio cholerae* serotype 01 in Maryland and Louisiana estuaries. *Applied and Environmental Microbiology* 1981; 41: 555–8.

33 Joseph SW, Colwell RR, Kaper J. *Vibrio parahaemolyticus* and related halophilic vibrios. *CRC Critical Reviews in Microbiology* 1982; 10: 77–124.

34 Kaper JB, Lockman H, Colwell RR, Joseph SW. *Aeromonas hydrophila*: ecology and toxigenicity of isolates from an estuary. *Journal of Applied Bacteriology* 1981; 50: 359–77.

35 Rippey SR, Cabelli VJ. Membrane filter procedure for enumeration of *Aeromonas hydrophila* in fresh waters. *Applied and Environmental Microbiology* 1979; 38: 108–13.

36 Watkins WD, Cabelli VJ. Effect of fecal pollution on *Vibrio parahaemolyticus* densities in an estuarine environment. *Applied and Environmental Microbiology* 1985; 49: 1307–13.

37 Glass RI, Becker S, Huq MI, Stoll BJ, Khan MU, Merson MH, Lee JV, Black RE. Endemic cholera in rural Bangladesh, 1966–1980. *American Journal of Epidemiology* 1982; 116: 959–70.

38 Friend M. Wildlife health implications of sewage disposal in

wetlands. In: Godfrey PJ, Kaynor ER, Pelczarski S, Benforado J, eds. *Ecological Considerations in Wetlands Treatment of Municipal Wastewaters*. New York: Van Nostrand Reinhold Company 1985: 262-7.

39 Goossens H, De Mol P, Cognau H, Levy J, Grados O, Ghysels G, Innocent H, Butzler J-P. Comparative *in vitro* activities of aztreonam, ciprofloxacin, norfloxacin, ofloxacin, HR 810 (a new cephalosporin), RU28965 (a new macrolide), and other agents against enteropathogens. *Antimicrobial Agents and Chemotherapy* 1985; 27: 388-92.

40 Mach PA, Grimes DJ. R-plasmid transfer in a wastewater treatment plant. *Applied and Environment Microbiology* 1982; 44: 1395-403.

41 Morrison WD, Miller RV, Sayler GS. Frequency of F116-mediated transduction of *Pseudomonas aeruginosa* in a freshwater environment. *Applied and Environmental Microbiology* 1978; 36: 724-30.

42 Ketchum BH, Kester DR, Park PK (eds). *Ocean Dumping of Industrial Wastes*. New York: Plenum Press, 1981.

43 Duedall IW, Ketchum BH, Park PK, Kester DR (eds). *Wastes in the Ocean*, vol. 1. *Industrial and Sewage Wastes in the Ocean*. New York: John Wiley & Sons, Inc., 1983.

44 Hargrave BT. Sinking of particulate matter from the surface water of the ocean. In: Hobbie JE, Williams PJleB, eds. *Heterotrophic Activity in the Sea*. New York: Plenum Press, 1984: 155-78.

45 Flierl GR. Simple models of waste disposal in a gyre circulation. In: Deudall IW, Ketchum BH, Park PK, Kester DR, eds. *Wastes in the Ocean*, vol. 1. *Industrial and Sewage Wastes in the Ocean*. New York: John Wiley & Sons, Inc., 1983: 69-85.

46 Duncan CP, Schladow SG, Williams WG. Surface currents near the Greater and Lesser Antilles. *International Hydrographic Review* 1982; 59: 67-78.

47 Grimes DJ, Singleton FL, Colwell RR. Allogenic succession of marine bacterial communities in response to pharmaceutical waste. *Journal of Applied Bacteriology* 1984; 57: 247-61.

48 Ducklow HW. Geographical ecology of marine bacteria: physical and biological variability of the mesoscale. In: Klug MJ, Reddy CA, eds. *Current Perspectives in Microbial Ecology*. Washington, DC: American Society for Microbiology, 1984: 22-31.

49 Marshall KC. Bacterial adhesion in oligotrophic habitats. *Microbiological Sciences* 1985; 2: 321-6.

50 Bracewell LW, Selleck RE, Carter R. Contribution of wastewater discharges to ocean surface particulates. *Journal of the Water Pollution Control Federation* 1980; 52: 2230-45.

51 Grimes DJ, Singleton FL, Stemmler J, Palmer LM, Brayton P, Colwell RR. Microbiological effects of wastewater effluent discharge into coastal waters of Puerto Rico. *Water Research* 1984; 18: 613-19.

52 Beucher M, Plusquellec A, LeGal Y, Cleret JJ. Mise en evidence et evaluation de l'enrichissement bacterien du film de surface des eaux littorales a proximite d'effluents urbains. *Proceedings of the 2nd International Colloquium on Marine Bacteriology* 1985; Brest, France.

53 Grimes DJ. Bacteriological water quality effects of hydraulically dredging contaminated Upper Mississippi River bottom sediment. *Applied and Environmental Microbiology* 1980; 39: 782-9.

54 Lawton RL, Morse EV. *Salmonella* survival in fresh water and experimental infections in goldfish, *Carassius auratus*. *Journal of Environmental Science and Health. Part A. Environmental Science and Engineering* 1980; 15: 339-58.

55 Huq A, Small EB, West PA, H MI, Rahman R, Colwell RR. Ecological relationships between *Vibrio cholerae* and planktonic crustacean copepods. *Applied and Environmental Microbiology* 1983; 45: 275-83.

56 Kaneko T, Colwell RR. The annual cycle of *Vibrio parahaemolyticus* in Chesapeake Bay. *Microbial Ecology* 1978; 4: 135-55.

57 Goyal SM. Viral pollution of the marine environment. *CRC Critical Reviews in Environmental Control* 1984; 14: 1-32.

58 Grimes DJ, Brayton P, Colwell RR, Gruber SH. Vibrios as autochthonous flora of neritic sharks. *Systematic and Applied Microbiology* 1985; 6: 221-6.

59 Buck JD, Spotte S, Gadbaw JJ Jr. Bacteriology of the teeth from a great white shark: potential medical implications for shark bite victims. *Journal of Clinical Microbiology* 1984; 20: 849-51.

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Curing salts in meats; Sulphite preservation of meat products; The use of carbon dioxide and the preservation of milk; Preservation of milk samples; Preserving stored crops with urea; Preservation of on-farm pre-packed beetroot; Preservatives for use in the wool textile industry; Preservation treatment of textile materials and their assessment; Wood preservation and fungal testing; Evaluation of biocides for use on building materials; Test procedures for marine anti-fouling paints; Techniques for the evaluation of biocide activity against sulphate-reducing bacteria; Preservatives available for use; Preservation of mixtures, suspensions and syrups; Preservation of pharmaceutical products; Preservation of cosmetic and toiletry product; Non-plasmidic resistance towards preservatives of pharmaceutical products; The concentration component of disinfectants and preservatives.

Late 1986. 288 pages, 51 illustrations. Cloth, about £32.00 ISBN 0 632 01727 9

Blackwell Scientific Publications Ltd Osney Mead, Oxford OX2 0EL

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Journal of Microbiological Methods 0 (1987) 000–000
Elsevier

JMM 00208

Fluorescent antibody staining method for enumeration of viable environmental *Vibrio cholerae* 01

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(Received 10 June 1987) (Revised version received 8 January 1986) (Accepted 9 January 1987)

Summary

A membrane filtration method has been developed which is useful for enumeration of viable *Vibrio cholerae* 01 in environmental water samples by immunofluorescent staining. The samples are incubated with yeast extract and nalidixic acid. Substrate responsive cells, i.e., viable cells, elongate and after staining with specific antiserum and fluorescein conjugate, viable *V. cholerae* cells appear as long, peripheral fluorescent green banded bacilli when viewed under the microscope. Using an ocular reticule, the number of viable cells per ml can be calculated. The procedure has been adapted for use with other bacterial species if specific antisera is employed.

Key words: Fluorescent antibody; enumeration; viable; *Vibrio cholerae* 01

Introduction

Enumeration of indicator organisms or, preferably, direct detection of pathogens in the aquatic environment is essential if water quality is to be estimated. Public health safety, of course, is determined from the results of such tests. The choice of method for assessment depends on specificity, reproducibility and statistical validity, in conjunction with time and cost efficiency and ease of operation. Immunofluorescent-epifluorescent detection methods fulfill these criteria and are being used to detect a variety of organisms [4, 8, 11, 12, 14].

The viable, but non-culturable stage of bacteria has been established [1, 8–10]. In addition, results of studies have shown that nutrient starved and injured cells are incapable of growing on standard culture media, and, therefore, indicator organisms in that state will not respond to standard methods for assessing microbial

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populations. The virulence of viable but non-recoverable cells may persist, demonstrated by animal studies [1, 13].

Fluorescent antibody (FA) procedures can overcome the problem of organisms remaining viable but yielding 'no growth' in standard tests, since the presence, absence, or size of specific cell populations can be easily detected microscopically. In the conventional FA procedures, all organisms possessing a species-specific antigen can be observed. However, it is not possible to determine which cells are viable and which are dead. The method described here is advantageous because it allows detection of viable cells. By combining the indirect fluorescent antibody procedure of Xu et al. [15] for direct detection of bacteria in natural water samples, with the direct microscopic viable count, by Kogure et al. [7], enumeration of viable, specific populations of bacteria can be achieved. We described here a procedure we have used to detect viable *V. cholerae* serovar 01. The method, however, is adaptable to any organism, provided specific antisera are available.

Materials and Methods

1. Collect water samples in sterile containers following standard procedures for sample collection and employing aseptic techniques. Add filter-sterilized and autoclaved solution of yeast extract to obtain a final concentration of 0.025%. It is recommended that the concentration of yeast extract be prepared so that 1.0 ml of 0.25% yeast extract solution can be added to a 10 ml sample.

2. Incubate for 20 min at a temperature appropriate to the environment from which the sample was collected.

3. Prepare an 0.2% solution of nalidixic acid (Sigma Chemical Co., St. Louis, MO) in 0.05 N NaOH; filter sterilize; add 0.1 to 10 ml of sample.

4. Incubate at the appropriate environmental temperature for 12 h.

5. Filter the water sample through an 0.2 μ × 25 mm polycarbonate membrane filter (Nuclepore Co., Pleasanton, CA), mounted on a 25 mm glass filtration unit (Millipore Corp., Bedford, MA). Filters are prestained overnight in 0.2% irgalan black (Ciba-Geigy Corp., Greensboro, NC), dissolved in 2% acetic acid and rinsed several times in distilled water before use. (Prestained filters are available from the Nuclepore Co.) Place filters in a 30 × 10 mm petri dish.

6. Fix cells to filters by placing petri dishes on the surface of a test tube rack partially submerged in a 55 °C water bath. Incubate for 20 min.

7. Prepare a 1:20 dilution of rhodamine isothiocyanate-bovine serum albumin (RITC, Difco, Detroit, MI). Place one drop on the surface of the membrane, and place a coverslip on top of the membrane to spread the reagent across the filter.

8. Incubate 35–37 °C for 30 min in a dark moist chamber.

9. Rinse filters three times with phosphate-buffered saline (PBS, per liter: NaCl, 8.5 g; Na_2HPO_4, 9.1 g; KH_2PO_4, 1.5 g; pH 7.3) and allow the filters to soak for 10 min in the petri dish. Remove PBS and air dry the filters. Care must be taken to ensure that the top of the filter always remains upright throughout the procedure.

10. Add one drop of *V. cholerae* 01 antiserum and place a coverslip on top of the drop.

11. Repeat steps 8 and 9.

12. Add one drop of fluorescein isothiocyanate anti-rabbit goat serum (FITC, BBL, Cockeysville, MD). Place coverslip on top.

13. Repeat steps 8 and 9.

14. Mount each filter on a glass slide with a coverslip using FA mounting fluid, pH 9 (Difco).

15. Examine with a microscope equipped with a neofluor objective, IVFL epifluorescence condenser, 100 W halogen lamp, BP 450–490 band pass filter, FT 510 beam splitter and LP 520 barrier filter, or equivalent. Slides should be examined as soon after preparation as possible to avoid fading of the intensity of the fluorescence. Slides should be stored in a dark box until used. The *V. cholerae* 01 cells that are counted are those that appear as long and/or fat rods possessing a peripheral green band just below the cell wall (Fig. 1). Calculate total viable cells/ml of sample, according to the size of the ocular reticule employed in the microscope [6].

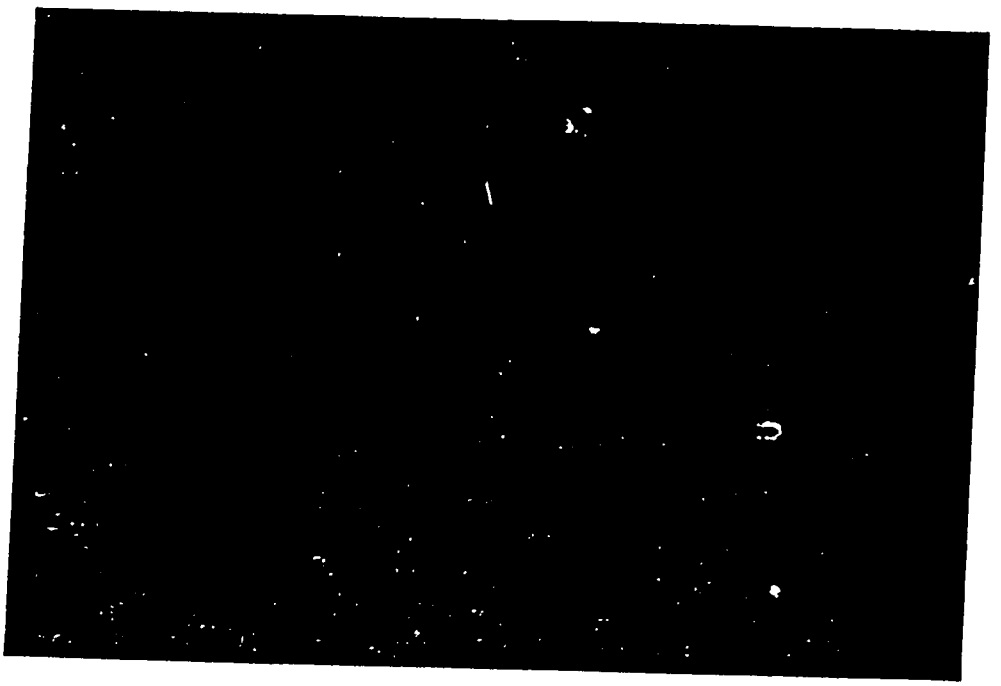
Discussion

The mechanism of nalidixic acid centers on its ability to inhibit DNA gyrase. At the concentration employed in this study, protein and RNA synthesis continue, but cell division is interrupted. As a consequence, the cells exposed to the nalidixic acid and yeast extract mixture elongate [3, 7].

The length of the yeast extract-nalidixic acid incubation is at the discretion of the investigator. Bacterial cells that are starved or injured may need a longer time to respond to added substrate. It is suggested that 6, 12 and 24 h time intervals be used initially, in order to establish which incubation interval produces the largest number of elongated cells per sample. Experience has shown that adjacent sampling sites in the field require the same length of time for incubation, providing a guide for future FA-DVC work in the same geographical area.

For convenience, formaldehyde may be added to samples after incubation, stopping the reaction (0.2 ml of filtered 37% formaldehyde per 10 ml of sample). The samples can then be stored at 4°C. During processing, dried filters may be frozen after the filtration step, or after the RITC and/or antiserum application. For best results, the filters should be viewed the day the FITC is applied.

It must be emphasized that the reliability of the FA-DVC procedure depends on



Water sample containing viable and non-viable *V. cholerae* 01 cells.

the specificity of the antiserum used. The antiserum must be carefully examined for possible cross-reactivity with other bacterial species, and should be absorbed with those strains to eliminate binding activity. Experimental control slides must be prepared to test each new batch of antiserum and FITC to determine which concentration of each reagent will produce the maximum intensity of fluorescent cells.

The filter method permits filtration of increasing volumes of water when the bacterial count is low. The maximum volume filtered will be dependent only on the degree of turbidity of the water. If the turbidity of the water sample is high, the sample can be prefiltered through a 1.2 μm filter (Gelman Sciences Inc., Ann Arbor, MI) to remove large particulate matter. The yeast extract-nalidixic acid incubation can then follow immediately. FITC has a tendency to absorb non-specifically to organic particles and detritus. RITC counterstaining can limit this problem because

the counterstain occupies active sites on the particle surfaces. The red background provides good contrast for fluorescent cells.

Polycarbonate filters are essential for the success of the FA-DVC procedure [5]. The flat surface and uniform pore size of the polycarbonate filter prevents bacteria from being imbedded inside the membrane and, instead, retains the sample on the surface. Irgalan black dye eliminates autofluorescence of the filter.

The membrane filter (MF) method has gained acceptance and recognition as an effective, reproducible and rapid procedure [2]. It is convenient, in that samples can be collected, incubated, fixed and stored for later staining.

The cultural protocol for enumeration of *V. cholerae* 01 involves sample filtration, transfer of the filter to thiosulfate-citrate-bile salts-sucrose agar (TCBS), incubation for 24 h, followed by biochemical testing of all sucrose positive colonies with subsequent serological confirmation. For most probable number (MPN) determinations, samples should be enriched, employing alkaline peptone broth and incubation for 6 h. Then, a loopful from each tube is streaked onto TCBS agar. The same biochemical and serological protocols as above should be followed. Inaccurate counts can result if *V. cholerae* is overgrown by other organisms during the enrichment procedure and, therefore, fails to appear on the plating media. Final identifications and counts for both procedures can extend for several weeks. In comparison, the immunofluorescent technique is very time and cost effective. A total FA count of *V. cholerae* 01 using the MF method (yeast extract-nalidixic acid step is eliminated to count live and dead cells) can be prepared in 3 h. The viable count, or FA-DVC, can be completed the same day, or by the next day, depending on the length of the incubation period. The time of the staining procedure can be further reduced if an antiserum-FA conjugate is available. This direct staining process would then involve two, rather than three, reagent applications.

The advantage of the FA-DVC far exceeds the conventional plating methods for viable bacteria. Plate counts considerably underestimate the viable population in the aquatic environment. No culture media is capable of supporting growth for all organisms. It is probable that all viable bacteria are incapable of enlarging in the DVC procedure with yeast extract as a substrate, and therefore this too, underestimates the true viable count. However, the FA-DVC can more closely portray the viable environmental population than traditional culture methods.

Acknowledgements

This work was supported by grants from the Agency for International Development (Grant No. DPE-5542-G-55-4060-00) and the World Health Organization (Grant No. C6/181/70).

References

1 Colwell, R.R., Brayton, P.R., Grimes, D.J., Roszak, D.B., Huq, S.A. and Palmer, L.M. (1985) Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms. Bio/Technol. 3, 817–820.

2 Franson, H. (ed.) (1985) Standard methods for examination of water and wastewater. Sixteenth Edn. American Public Health Association, Washington, DC.

3 Goss, A., Deitz, W.H. and Cook, T.M. (1965) Mechanism of action of nalidixic acid on *Escherichia coli*. II. Inhibition of deoxyribonucleic acid synthesis. J. Bacteriol. 89, 1068 – 1074.

4 Gray, L.D. and Kreger, A.S. (1985) Identification of *Vibrio vulnificus* by indirec immunofluorescence. Diagnost. Microbiol. Infect. Dis. 3, 461 – 468.

5 Hobbie, J.E., Daley, R.J. and Jasper, S. (1977) Use of nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33, 1225-1228.

6 Jones, J.G. (1979) A guide to methods for estimating microbial numbers and biomass in fresh water. Freshwater Biological Assoc. Scientific Publication No. 39.

7 Kogure, K., Simidu, U. and Taga, N. (1979) A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. 25, 415 – 420.

8 Pearson, A., Colwell, R.R., Byrd, J., Dennis, J., Grimes, J., Hood, M., Hussong, D., Hutchinson, C., Lee, J., O'Brien, M., Stott, J. and Walker, J. (1986) Discovery of viable but non-culturable *Legionella pneumophila* by immune epifluorescence microscopy: evidence of amplification and sources in five case studies. Bio/Technol. (submitted).

9 Rollins, D.M. and Colwell, R.R. (1985) Viable but non-culturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. Appl. Environ. Microbiol. (in press).

10 Roszak, D.B., Grimes, D.J. and Colwell, R.R. (1984) Viable but non-recoverable stage of *Salmonella enteritidis* in aquatic systems. Can. J. Microbiol. 30, 334 – 338.

11 Sauch, J.F. (1985) Use of immunofluorescence and phase contrast microscopy for detection and identification of *Giardia* cysts in water samples. Appl. Environ. Microbiol. 50, 1434 – 1438.

12 Schmidt, E.L., Bankole, R.O. and Bohlool, B.B. (1968) Fluorescent antibody approach to study of *Rhizobia* in soil. J. Bacteriol. 95, 492—498.

13 Singh, A., Yeager, R. and McFeters, G.A. (1986) Revival, growth and pathogenicity of *Escherichia coli* after copper and chlorine-induced injury. Abstract. Annual Meeting of the American Society of Microbiology.

14 Strayer, R.F. and Tiedje, J.M. (1978) Application of the fluorescent-antibody technique to the study of a methanogenic bacterium in lake sediments. Appl. Environ. Microbiol. 35, 192 – 198.

15 Xu, H.S., Roberts, N.C., Adams, L.B., West, P.A., Siebeling, R.J., Huq, A., Huq, M.I., Rahman, R. and Colwell, R.R. (1984) An indirect fluorescent antibody staining procedure for detection of *Vibrio cholerae* serovar 01 cells in aquatic environmental samples. J. Microbiol. Methods 2, 221 – 231.

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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1987, p. 1718–1720
0099-2240/87/071718-03$02.00/0
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Vol. 53, No. 7

A Rapid Test for Chitinase Activity That Uses 4-Methylumbelliferyl-*N*-Acetyl-β-D-Glucosaminide

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Received 14 July 1986/Accepted 14 April 1987

A total of 101 strains of bacteria from environmental and clinical sources, most of which were gram negative, were tested for chitobiase activity by using a filter paper spot test with 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide as the substrate. The results were compared with those obtained by a conventional plate method for chitinase activity by using colloidal chitin as the substrate. There was excellent agreement in the results for both methods. The filter paper spot test with 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide has the advantages of being rapid, simple to perform, and inexpensive. This method should be adaptable to a wider range of microorganisms, particularly those with unusual growth requirements.

Except for cellulose, chitin is probably the most abundant polysaccharide in nature, being a primary constituent of shells in crustaceans and insects (9). Centrate diatoms produce significant amounts of crystalline chitin (3; R. Smucker and R. Dawson, J. Exp. Mar. Biol. Ecol., in press). Chitin also occurs in some protozoa and fungi (16, 23). Chitinase activity has been found in a wide variety of microorganisms (2, 5, 7, 12–15, 19, 22, 23). Chitinase activity plays an important role in the ecology of many marine bacteria (23). For some pathogenic bacteria, such as *Vibrio cholerae*, interactions between the organism and chitinaceous materials have been postulated as playing a role, albeit indirectly, in the epidemiology of disease (6, 10).

Tests for bacterial chitinase activity have relied on conventional plate methods, such as those described by Skerman (17) and West and Colwell (19) in which colloidal chitin is incorporated into a nutrient agar base optimal for the growth of the test organism. Chitinase activity is indicated by zones of clearing around the inoculum growth. While the test itself is straightforward, preparation of the substrate medium is laborious and complicated. Furthermore, the plate test is an insensitive assay for chitinase activity; e.g., some organisms require a long incubation period (up to 7 days or longer). Problems may also be encountered with organisms which have fastidious or unusual growth requirements. Thus, it is understandable why many laboratories are reluctant to include chitinase activity as part of an enzyme profile.

A major end product of chitin degradation by chitinase (or endochitinase) is chitobiose, which is further degraded by the enzyme chitobiase (7, 8, 13). The test described here, which uses 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (4-MUF.GlcNAc), is an assay for chitobiase activity, the inference being that chitobiase activity is produced concomitantly with chitinase activity. The following study was done to find a better method for determining chitinase activity. The test described is an assay for *N*-acetyl-β-D-glucosaminidase activity, the logic being that chitin is essentially a biopolymer of GlcNAc subunits (14, 20, 22).

The strains used in this study are listed in Table 1. No attempt was made to confirm the identity of all of the strains tested. However, whenever possible, American Type Culture Collection type or reference strains or well-characterized isolates received from other laboratories were examined. Many of the strains tested in this study have been used in other studies (11), and they include both environmental and clinical isolates. For the chitinase plate test and culture media, nutrient agar was prepared as described by West and Colwell (19), and brain heart infusion agar and tryptic soy agar were prepared by adding bacteriological agar (Difco Laboratories, Detroit, Mich.) (final concentration, 1.5% [wt/vol]) to brain heart infusion broth (Difco) and tryptic soy broth (Difco), respectively.

The rapid test method was as follows. The 4-MUF.GlcNAc (Sigma Chemical Co., St. Louis, Mo.) stock substrate solution was prepared by dissolving 50 μmol in 2.0 ml of dimethylformamide (Sigma). For the test, 0.6 ml of this solution was diluted in 9.4 ml of phosphate buffer (0.1 M [pH 7.4]) (4). The substrate solutions (stock and buffered) were used immediately, without sterilization, or stored at −20°C. Both solutions were stable for several months at this temperature.

One to five colonies from cultures grown from 24 to 48 h on nutrient agar (*Vibrio* spp.), brain heart infusion agar (*Enterococcus* [*Streptococcus*] *faecalis* and *Enterococcus* [*Streptococcus*] *faecium*), or tryptic soy agar plates were vigorously rubbed onto Whatman no. 1 filter paper, and 20 μl of the 4-MUF.GlcNAc-buffered substrate solution was added. The substrate only and the organism plus the solvent were included as controls.

After incubation at 37°C (or 25°C for *Vibrio* strains tested on nutrient agar) for 10 min, each test spot was covered with 1 drop of a saturated sodium bicarbonate solution and exposed to UV light at a wavelength of 366 nm. The sodium bicarbonate solution enhances the fluorescence intensity of the reaction by-product, methylumbelliferone (18).

Depending on the fluorescence intensity, reactions were graded as positive, weakly positive, or negative. In positive reactions, substrate-organism mixtures produced a strong, light-blue fluorescence.

The conventional plate method for determining chitinase activity was a modification of the method of West and Colwell (19) in that tryptic soy agar and brain heart infusion agar were used as alternative preprepared medium bases in addition to nurient agar and a 20% (wt/vol) concentration of colloidal chitin suspension was used. West and Colwell (19) suggest a 15% (wt/vol) concentration. Briefly, a colloidal chitin suspension (20% [wt/vol]) was prepared and added to

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TABLE 1. Results of chitobiase and chitinase assays with 101 bacterial strains

Gram reaction/strain	No. tested	No. (%) positive[a]	
		Chitobiase	Chitinase
Gram positive			
Bacillus subtilis	1	1 (100)	0 (0)
Micrococcus luteus	1	0 (0)	0 (0)
Staphylococcus aureus	1	0 (0)	0 (0)
Enterococcus (Streptococcus) faecalis	1	1 (100)	1 (100)
Enterococcus (Streptococcus) faecium	2	2 (100)	0 (0)
Gram negative			
Aeromonas hydrophila	6	6 (100)	6 (100)
Aeromonas caviae	6	6 (100)	6 (100)
Aeromonas sobria	5	5 (100)	5 (100)
Agrobacterium tumefaciens	1	1 (100)	0 (0)
Enterobacter aerogenes	1	0 (0)	0 (0)
Escherichia coli	7	1 (14)	0 (0)
Klebsiella pneumoniae	1	0 (0)	0 (0)
Plesiomonas shigelloides	10	10 (100)	10 (100)
Proteus vulgaris	1	0 (0)	0 (0)
Pseudomonas aeruginosa	1	0 (0)	0 (0)
Pseudomonas putida	2	0 (0)	0 (0)
Salmonella typhi	7	0 (0)	0 (0)
Shigella dysenteriae 1	4	0 (0)	0 (0)
Shigella sonnei	1	0 (0)	0 (0)
Shigella boydii	1	0 (0)	0 (0)
Vibrio alginolyticus	2	2 (100)	2 (100)
Vibrio campbelli	1	1 (100)	1 (100)
Vibrio carcharae	1	1 (100)	1 (100)
Vibrio cholerae	10	10 (100)	10 (100)
Vibrio cincinnatiensis	1	1 (100)	1 (100)
Vibrio damsela	2	2 (100)	2 (100)
Vibrio diazotrophicus	1	1 (100)	0 (0)
Vibrio fluvialis	3	3 (100)	3 (100)
Vibrio gazogenes	1	0 (0)	0 (0)
Vibrio metschnikovii	1	1 (100)	1 (100)
Vibrio mimicus	2	2 (100)	2 (100)
Vibrio natriegens	1	1 (100)	0 (0)
Vibrio nereis	1	1 (100)	1 (100)
Vibrio nigrapulchritudo	1	1 (100)	1 (100)
Vibrio ordalii	1	0 (0)	0 (0)
Vibrio parahaemolyticus	6	6 (100)	6 (100)
Vibrio pelagius	1	1 (100)	1 (100)
Vibrio proteolyticus	1	1 (100)	1 (100)
Vibrio tubiashii	1	1 (100)	1 (100)
Vibrio vulnificus	3	3 (100)	3 (100)

[a] Includes weakly positive reactions. For chitobiase, the rapid filter paper spot test with 4MUF.GlcNAc as the substrate was used. For chitinase activity, a conventional plate assay with colloidal chitin as the substrate was used.

molten bacteriological-grade agar to a final concentration of 1% (vol/vol) and poured as an overlay on a prepared medium base. Thus, the *Vibrio* strains were tested on chitin-nutrient agar, *E. faecalis* and *E. faecium* were tested on chitin-brain heart infusion agar, and chitin-tryptic soy agar was used for all other test organisms. Plates were spot inoculated (four spots per plate) with growth (one to five colonies) from a plate culture of the test organism grown on its optimal medium (without chitin) for 24 to 48 h, as indicated above. Except for the *Vibrio* test strains, which were incubated at 25°C, the chitin plate cultures were incubated at 37°C for at least 7 days to observe clearing zones around growth, indicating chitin hydrolysis. A narrow (1 to 2 mm in width) clearing zone extending from the periphery of the spot growth was considered a positive reaction.

Many of the *Vibrio* test strains were able to grow on tryptic soy agar; therefore, in a separate series of experiments, we tested for chitobiase and chitinase activities on this medium and at 37°C by using the methods described above.

The results for chitobiase (rapid test) and chitinase (conventional plate method) activities are given in Table 1. Overall, there was excellent agreement in the results for both methods. Furthermore, the results obtained here for chitinase activity by the conventional plate method are in very good agreement with the results reported by other workers (2, 7, 19). Certain test organisms, e.g., *Agrobacterium tumefaciens*, *Bacillus subtilis*, *E. faecium*, *Vibrio diazotrophicus* and *Vibrio natriegens*, showed chitobiase activity but no chitinase activity by the conventional plate method. This was most likely a result of the latter being a less sensitive method. Enzyme assays that use 4-methylumbelliferyl-conjugated substrates are notably more sensitive than conventional enzyme test methods (M. O'Brien, Ph.D. thesis, University of Queensland, St. Lucia, Australia, 1985). We never observed chitinase activity in the conventional plate method without observing concomitant chitobiase activity in the rapid test.

The degree of activity for chitobiase and chitinase was not necessarily directly correlated. For example, while many of the strains tested positively with the rapid test, yielding strong reactions within 10 min, corresponding tests with the conventional plate method produced varied results. For some strains, there was strong chitobiase activity and weak chitinase activity; for others, although there was strong chitobiase activity, positive plate tests required incubation for 4 days or longer. Some test organisms, e.g., *Aeromonas* spp., *Vibrio damsela*, *Vibrio nigrapulchritudo*, showed very strong *N*-acetyl-β-D-glucosaminidase activity before the addition of the bicarbonate solution.

Results of tests for enzyme activities in *Escherichia coli* proved interesting, since one of the two strains tested was the recipient of plasmid DNA from chitobiase-positive clones but was otherwise identical to the first strain (21). The chitobiase gene was isolated originally from *Vibrio vulnificus* by cloning chromosomal DNA (21). Very strong chitobiase activity was detected in the transformed strain. The plasmidless strain, as well as five other *E. coli* strains examined, was negative for chitobiase and chitinase activity by both test methods.

Comparable results were obtained with *Vibrio* strains tested for chitobiase activity on tryptic soy agar and for chitinase activity on chitin-tryptic soy agar at 37°C. Some of the strains did show stronger reactivities with both assays, probably more a reflection of the higher incubation temperature than of media differences.

The test for chitobiase activity described in this study has many advantages over the conventional plate method for chitinase activity. It is rapid and simple to perform and uses a substrate that is relatively inexpensive and very stable. The test is specific and sensitive.

Different incubation temperatures may be used for the rapid test, and a variety of growth media may be used as the source of the inoculum. Chitinase is produced in chitinase-positive bacteria even when they are grown in a rich organic medium, since only chitin or glucosamine has been shown to induce chitin hydrolysis (1). Furthermore, the rapid test may be more adaptable to microorganisms whose unusual growth requirements do not allow for determination of chitinase activity by the conventional plate method. For example, we are at present carrying out enzyme profiles of barophilic

bacteria by using chitobiase and other 4-methylumbelliferyl-conjugated substrates, since conventional enzyme assay methods are largely inappropriate for this group of bacteria (W. L. Straube and M. O'Brien, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, I-37, p. 171). Thus, many useful applications of the test described here can be seen.

We acknowledge P. Brayton, G. Francis, S. Gelbart, W. L. George, D. J. Grimes, A. Huq, J. M. Janda, B. Kay, C. Kaysner, M. Korshed, J. Lee, M. Miller, S. Muralidhar, T. Overman, C. Pasko-Kolva, R. Rahman, and C. C. Somerville for supplying strains used in this study. Richard Smucker provided a helpful review of the manuscript.

Funding for this work was provided in part by the U.S. Environmental Protection Agency (cooperative agreement CR812246-01), the National Science Foundation (grant BSR-84-01397), and the National Institutes of Health (grant R22-AI-14242).

LITERATURE CITED

1. **Bennett, C. B., and M. A. Hood.** 1980. Effects of cultural conditions on the production of chitinase by *Bacillus megaterium*. Dev. Ind. Microbiol. **21**:357–363.
2. **Clarke, P. H., and M. V. Tracey.** 1956. The occurrence of chitinase in some bacteria. J. Gen. Microbiol. **14**:188–196.
3. **Falk, M., D. G. Smith, J. McLachlan, and A. G. McInnes.** 1966. Studies on chitan (β1-4)-linked 2-acetamido-2-deoxy-D-glucan) fibers of the diatom *Thalassiosira fluviatilis* Bustedt. II. Proton magnetic resonance, infrared, and X-ray studies. Can. J. Chem. **44**:2269–2281.
4. **Gomori, G.** 1955. Preparation of buffers for use in enzyme studies. Methods Enzymol. **1**:138–146.
5. **Hsu, S. C., and J. L. Lockwood.** 1975. Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. Appl. Microbiol. **29**:422–426.
6. **Huq, A., E. B. Small, P. A. West, M. I. Huq, R. Rahman, and R. R. Colwell.** 1983. Ecological relationships between *Vibrio cholerae* and planktonic crustacean copepods. Appl. Environ. Microbiol. **45**:275–283.
7. **Janda, J. M.** 1985. Biochemical and exoenzymatic properties of *Aeromonas* species. Diagn. Microbiol. Infect. Dis. **3**:223–232.
8. **Jeuniaux, C.** 1986. Chitinase. Methods Enzymol. **8**:645–650.
9. **Muzzarelli, R. A. A.** 1977. Chitin. p. 155–181. Pergamon Press, Inc., Elmsford, N.Y.
10. **Nalin, D. R., V. Daya, A. Reid, M. M. Levine, and L. Cisneros.** 1979. Adsorption and growth of *Vibrio cholerae* on chitin. Infect. Immun. **25**:768–770.
11. **O'Brien, M., and R. R. Colwell.** 1985. Modified taurocholate-tellurite-gelatin agar for improved differentiation of *Vibrio* species. J. Clin. Microbiol. **22**:1011–1013.
12. **O'Brien, M., and G. H. G. Davis.** 1982. Enzymatic profile of *Pseudomonas maltophilia*. J. Clin. Microbiol. **16**:417–421.
13. **Ohtakara, A., M. Mitsutomi, and Y. Uchida.** 1979. Purification and some properties of chitinase from *Vibrio* sp. J. Ferment. Technol. **57**:169–177.
14. **Reid, J. D., and D. M. Ogrydziak.** 1981. Chitinase-overproducing mutant of *Serratia marcescens*. Appl. Environ. Microbiol. **41**:664–669.
15. **Reynolds, D. M.** 1954. Exocellular chitinase from a *Streptomyces* sp. J. Gen. Microbiol. **11**:150–159.
16. **Ruiz-Herrera, J.** 1978. The distribution and quantitative importance of chitin in fungi. p. 11–21. *In* R. A. A. Muzzarelli and E. R. Pariser (ed.), Proceedings of the 1st International Conference on Chitin/Chitosan. MIT Sea Grant Report MITSG 78-7, index no. 78-307-Dmb. Massachusetts Institute of Technology, Cambridge, Mass.
17. **Skerman, V. B. D.** 1969. Abstracts of microbiological methods. John Wiley & Sons, Inc., New York.
18. **Slifkin, M., and G. M. Gil.** 1983. Rapid biochemical tests for the identification of groups A, B, C, F, and G streptococci from throat cultures. J. Clin. Microbiol. **18**:29–32.
19. **West, P. A., and R. R. Colwell.** 1984. Identification and classification of *Vibrionaceae*: an overview. p. 285–363. *In* R. R. Colwell (ed.), Vibrios in the environment. John Wiley & Sons, Inc., New York.
20. **White, A., P. Handler, and E. L. Smith (ed.).** 1968. Principles of biochemistry. McGraw-Hill Book Co., New York.
21. **Wortman, A. T., C. C. Somerville, and R. R. Colwell.** 1986. Chitinase determinants of *Vibrio vulnificus*: gene cloning and the applications of a chitinase probe. Appl. Environ. Microbiol. **52**:142–145.
22. **Zikakis, J. P. (ed).** 1984. Chitin, chitosan, and related enzymes. Academic Press, Inc., New York.
23. **Zobell, C. E., and S. C. Rittenberg.** 1938. The occurrence and characteristics of chitinoclastic bacteria in the sea. J. Bacteriol. **35**:275–287.

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PATHOGENIC POTENTIAL, INCLUDING PRODUCTION OF CHOLERA TOXIN, IN VIABLE BUT NOT RECOVERABLE *VIBRIO CHOLERAE* 01 IN ENVIRONMENTAL MICROCOSMS

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Presented to 21st Joint Conference on Cholera,
National Institutes of Health
Bethesda, Maryland
October, 1985

Rec'd in S OCT 8 1987

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ABSTRACT

Vibrio cholerae grown in microcosms can enter into and persist in a dormant state in which the cells, although viable, are non-culturable by routine laboratory culturing methods. Microcosms were constructed, using aged estuarine water adjusted to a suitable pH and salinity, to simulate conditions of the estuarine environment. Bacterial survival was monitored by the following procedures: (1) plate counts on appropriate selective and non-selective media; (2) indirect fluorescent antibody (FA) staining to enumerate cells reacting with 01 antiserum; (3) acridine orange direct counts (AODC) to enumerate total number of bacteria present; and (4) direct viable counts (DVC) to determine substrate-responsive (viable) bacteria.

The toxin-producing, 01 serovar of *V. cholerae*, CA 401, was inoculated into microcosms to a final concentration of 10^6 CFU/ml and monitored for eight days. AODC and FA counts remained constant at 10^6 CFU/ml and the DVC count at 10^6 CFU/ml. Plate counts on Tryptic Soy Agar (TSA, Difco) and Thiosulfate-citrate-bile salts-sucrose agar (TCBS, Oxoid) dropped from 10^4 CFU/ml at one hour post-inoculation to <0 CFU/ml after 24 hours. To determine whether viable, but non-culturable *V. cholerae* CA 401 cells remained virulent, filtered, washed, and resuspended non-culturable cells from microcosms (10^8 total cells, of which 10^6 were "viable," i.e., substrate-responsive cells by the method of Kogure *et al.*, 1979) were injected into rabbit ileal loops. A viable, i.e., platable, culture of *V. cholerae* CA 401 was injected into loops, separated by uninoculated loops, as positive control. When the ileal loops were examined *ca.* 30 hours post surgery, a positive virulence response was noted in all

experimental loops, similar to the *V. cholerae* CA 401 control. The experimental and control loops were distended, hemorrhagic, and filled with fluid. *V. cholerae* CA 401 cells were recoverable from all positive loops using Brain Heart Infusion Broth (BHI, Difco), TSA and TCBS plates. The viable, but non-culturable, i.e., "dormant," cells were recovered by animal passage and retained the potential for virulence. We have further observed that cholera toxin is stable in seawater microcosms for periods of time exceeding 28 days, and that significant quantities of toxin are produced by *V. cholerae* strain 569B in microcosms at a salinity of 25 parts per thousand and at concentrations of organic nutrient ranging from 0.1 to 10 g/liter. Experiments in progress are aimed at elucidating the salinity and organic nutrient concentrations required for cholera toxin production in microcosms by viable, but non-culturable cells and the biological activity of the toxin produced therein.

To date much is known of pathogenic mechanisms of bacteria. However, there is little knowledge of how environmental factors affect survival, growth, and production of pathogenic properties by bacteria in the natural environment. Our laboratory has recently obtained evidence that *Vibrio cholerae*, a major cause of epidemic bacterial diarrhoeae in various geographical areas of the world, undergoes both physiological and morphological changes in marine and estuarine water environments (1, 11). In addition, we have demonstrated that *V. cholerae* can exist as a "viable, but non-culturable" cell, yet can return to the culturable state, inducing positive ileal loops in the rabbit intestine (1). Thus it is important to understand how the pathogenic properties of *V. cholerae* can be affected by environmental factors, such as water temperature, salinity, pH, and/or organic content.

As a result of a series of studies carried out in our laboratory over the past several years, it has been observed that, over time, *V. cholerae* and related human pathogens enter a viable but non-culturable state under conditions simulating that of the natural environment (1). Direct viable counts by epifluorescent microscopy consistently remain higher than corresponding plate counts because 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 environmental sampling for human pathogens.

By adapting the indirect, immunofluorescent-epifluorescent methods developed in our laboratory for use in microbial ecology, a more accurate estimate of total population size can be obtained (11, 12). An application

of this new approach, compared with conventional enumeration methods was made in several countries, besides the United States. Results of these studies are reported here.

In Brazil in September, 1982, alkaline peptone enrichment broths (peptone, 1% w/v; NaCl, 1% w/v; ox-bile, 0.5% w/v; pH: 8.6) were inoculated with water and plankton samples. After incubation, loopfuls from each tube were removed and streaked to Thiosulfate-citrate-bile salts-sucrose agar (TCBS, Oxoid, U.S.A. in Columbia, MD) and suspect colonies were examined by biochemical tests, and 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 (FA) staining (12). A comparison of results between the cultural method and the fluorescent antibody staining method are shown in Table 1. No V. cholerae O1 colonies were isolated by plating; however, five of six field samples proved positive by fluorescent staining.

By modifying the collection procedure, MPN estimates for V. cholerae, O1, were calculated by inoculating a 3-tube, 3-dilution alkaline peptone enrichment series. Both TCBS plates and slides were inoculated from each of the tubes after inoculation with water samples and incubation for 6-8 h. In Zimbabwe, field studies were conducted in April, 1984. Water samples were collected from farm areas, river washing sites near villages, animal habitats on the banks of the Zambesi River above Victoria Falls, and MPN results are shown in Table 2. Population sizes were estimated and were consistently higher by the fluorescent antibody assay, compared with cultural counts.

Field samples collected in South Africa at Soldana Bay, Capetown, and in the Transvaal in the North near the Limpopo River were assayed for *V. cholerae* 01. Fluorescent antibody results ranged from a low of 14 cells/100 ml of seawater along the coast of Langebaan, to a high of >2400 cells/100 ml of algal and sea urchin pool water. Water samples collected near a fish cannery in Soldhana yielded 240 *V. cholerae* non-01 cells/100 ml.

From our results, the fluorescent antibody technique proved more sensitive for detecting the presence of *V. cholerae* 01, especially when it is in the "environmental stage," compared to traditional culture methods. This also held true for other bacteria, when appropriate specific antisera were used.

Other fluorescent antibody-specific methods have been applied in the laboratory to study the non-culturability phenomenon. Direct viable counts (DVC) (4) permit observation and enumeration of viable cells by direct microscopy. The method is relatively simple, i.e. yeast extract is added to a water sample to provide nutrient for growth and replication. With addition of nalidixic acid (0.01% to 0.002%), 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) (2), an epifluorescent microscopy procedure used to enumerate total bacteria in a sample, along with the fluorescent antibody procedure described above, and traditional plate count and MPN methods (7), have been incorporated in studies examining survival patterns of selected pathogens in laboratory microcosms.

Microcosm experiments, designed to explore survival of V. cholerae CA 401, a toxin positive strain (Fig. 1), were carried out using aged estuarine water, for which the pH and salinity were adjusted. These microcosms simulated conditions in the environment over time. No additional nutrients were introduced, in order to induce a dormant state in the culture. Two flasks of Patuxent River water were inoculated with 10^6 cells/ml.

In both systems, AODC and FA counts remained constant at 10^6 cells/ml during the week following inoculation, and DVC counts at 10^4 cells/ml. Upon withdrawing samples 1 h post-inoculation, plate counts on TCBS and Tryptic Soy Agar (TSA, Difco) plates using inocula from microcosm 1 were 10^4 cfu/ml, on both media. For microcosm 2, counts were 10^4 and 10^5 cfu/ml, respectively. Plate counts made 24 h after inoculation dropped to 0, and the microcosm remained non-culturable for the duration of the experiment.

Clearly, a non-culturable, but viable and dormant stage can be induced in the bacterial species examined to date. Of prime concern is the question of whether these viable but non-culturable bacteria remain virulent. Thus, the following experiments were done.

Ligated ileal loop assays (9) were performed. 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. Rabbits were prepared for surgery and, in one experiment, 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 cells; (5) microcosm 2 cells; (6) microcosm 1 cells; (7) microcosm 2 cells. Ileal loop assays were 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.

In this experiment, fluid was transferred to a tube of Brain Heart Infusion broth (BHI, Difco), streaked onto plates of TCBS and TSA, and prepared for AODC, FA and DVC counts. Results showed that CA 401 cells from all microcosms, when injected into the rabbit ileum, could be recovered using BHI and on TSA and TCBS plates. Slide agglutination tests were positive with _V. cholerae_ 01 antisera. DVC counts increased to 10^8 to 10^9 cells/ml. AODC counts increased from 10^8 to 10^{10} cells/ml. Transfers from the T_1N_1 loop revealed no growth on TSA, TCBS, and Eosin Methylene Blue (EMB, Difco) plates and in BHI broth.

Similar results with enteropathogenic _E. coli_ were also obtained. Rabbit ileal loop assays were performed using _E. coli_ H10407 cells that were non-culturable, having been held for 42 d in a microcosm. AODC and DVC counts were 10^3 and 10^2 cells/ml, respectively. Rabbits were sacrificed 48 h after inoculation. In one of the experiments, no swelling or fluid accumulation was observed in the microcosm sample-inoculated loops. Sterile saline was injected into and then aspirated from the loops. Results showed recovery of _E. coli_ from all microcosm loops. Biochemical

testing of the isolates, including plasmid band profile analysis, confirmed the organism to be the E. coli strain H10407 which had been injected. There was no growth from the aspirate of uninoculated loops. One out of the 18 interloops grew E. coli, but the plasmid profiles of it were negative. Also, biochemical test results differed from that of the H10407. These results provide further evidence that cells, other than V. cholerae, which are non-recoverable by standard culture methods can be recovered by ileal loop passage.

Other microcosms examined, included V. cholerae strain CA 401. It showed a DVC of 10^2 cells/ml after being non-culturable for 8 months. The organism was not recovered by ileal loop passage.

Autoradiography was done to answer the question of whether all or a few of the viable, but non-recoverable cells were active. Microcosms from which samples for autoradiographic slides were to be removed were prepared and inoculated as for survival study microcosms. In addition to culturable and microscopic enumeration, microautoradiographic analysis was also performed (10). Salmonella enteritidis and E. coli microcosm samples were incubated with 1 uCi/ml (U ^{14}C) glutamic acid, [methyl ^{3}H] thymidine or [^{3}H] adenine for 20 min to 3 h. Concentrations were determined on the basis of results of preliminary studies showing the lowest concentration yielding readily observed silver grains associated with cells, which could be distinguished from background. At the appropriate time, the reaction was halted by addition of 2% formaldehyde and refrigeration overnight (5°C). Acridine orange direct counts and direct viable counts performed prior to the autoradio- graphic preparation, as well as plate counts made the next morning, provided information as to the appropriate volumes of sample to be filtered for microautoradiography.

There were few (0-2%) examples of elongated cells which did not exhibit any associated silver grains and these were comparable to the number of elongated cells observed in acridine orange controls, but up to 28% of the cells appeared to be active by association with silver grains while not exhibiting elongation (Table 3). In addition to being responsive by the DVC method, viable but non culturable cells were found to be metabolically active, evidenced by uptake of radiolabelled glutamic acid, thymidine and adenine. While cells incubated with radiolabelled substrate alone, i.e., without added nalidixic acid or yeast extract, did not show significant uptake, results of combining radiolabelled substrates with DVC methods were significant. At least 90% of DVC enumerated cells were shown to be metabolically active. Less than 2% of the elongated cells showed no sign of uptake. Interestingly, some small cells, i.e., cells which had not elongated, evidenced by DVC, showed metabolic activity, i.e., took up radiolabelled substrate. These cells represent up to 28% of the total population of cells, lending credence to the postulation that there is a linear response by bacteria to nutrient limited environments. The permeability increase in the cell produced by the nalidixic acid appears to permit uptake of substrate. However, elongation was not observed at this advanced stage of response to nutrient limitation. This phenomenon was observed only in later stage microcosms, when few or no cells were able to be resuscitated, possibly representing further progression into dormancy, the reversal of which remains to be elucidated.

V. cholerae was grown in a defined salt medium (i.e. microcosm) (8) to determine the effect of salinity, organic substrate concentration, type of organic substrate, temperature and pH on production of cholera toxin (CT).

CT antigen was assayed by the ELISA method (3), using monoclonal antibodies (6). Biological activity of toxin was measured by the y-1 adrenal cell assay (5). At 10 g of organic substrate (casamino acids:yeast extract: glucose; 15:1.5:1) per liter of defined medium, the maximum quantity of toxin was produced at salinities between 15 to 30 parts per thousand (ppt) (Figure 2). However, at 1 g of organic substrate per liter, maximum production occurred at 20-25 ppt, decreasing at both higher and lower salinities (Figure 2). Biological activity and antigenic structures of CT were also very stable in microcosms with salinities from 15-30 ppt (Figure 3). Lower salinities resulted in a marked reduction of toxin activity (Figure 3). Addition of concentrations of tryptone, and conditions of lower temperature and reduced pH resulted in less toxin production. These results can be related to the physiological state of V. cholerae and its potential pathogenicity in the aquatic environment, as well as to the epidemiology of the disease. With this information, it will be possible to predict more precisely which water samples may contain potentially enteropathogenic V. cholerae. Experiments in progress are designed to elucidate the mechanism of the above factors as they may affect colonization of intestinal epithelium and the pathogenic disease processes in animal models.

Studies are being done on serum drawn from free-ranging, neritic sharks collected in Bimini, Bahamas. It has been found that the serum is capable of agglutinating V. cholerae, serovar 01, along with other Vibrio spp. found in the aquatic environment and associated with sharks. To date, V. cholerae 01 has not been isolated from sharks, but the bacterium has

been detected in the seawater column by fluorescent antibody procedures (12). Microagglutination titers of V. cholerae with shark serum are shown in Table 4. Interestingly, higher titers were reported from the nurse sharks (family Ginglymostomatidae), correlating with the habitat of the species. These animals are typically slow-moving and bottom-dwelling, feeding on lobster, shrimp, crabs, squid, sea urchins and small fish. The animals comprising food for the shark are also common hosts for Vibrio spp. and may explain why the shark demonstrates antibodies against vibrios.

The ability of members of the genus Vibrio to degrade chitin appears to be important for survival and in seasonal distribution. To initiate studies of regulation and distribution of this function in nature, the chitobiase gene from Vibrio vulnificus, ATCC 27562, has been cloned in E. coli strain DH1 using pBR322 as the cloning vector (Wortman et al., manuscript in preparation). In E. coli host cells containing the chimeric plasmid, chitobiase activity is cell associated and constitutively expressed. Subcloning is in progress to define more precisely the chitinase determinants which can be used as a genetic probe to assay for the presence of related gene sequences in Vibrio and related species and to study the evolution of the chitinase gene complex.

Cloning of genes involved in sucrose fermentation has also been initiated. Mutants of a non-01 V. cholerae strain which are unable to ferment sucrose were obtained by Tn5 mutagenesis. Most mutants had also lost the ability to ferment one or more additional sugars. The mutants were deficient in sucrose transport and sucrose hydrolase activity both of which are inducible activities in the wild type strain. A segment of a Tn5 inactivated gene from a mutant strain deficient only in sucrose fermentation was

isolated by cloning into the plasmid vector, pUC9. This segment from the sucrose specific mutant was nick translated with ^{32}P dCTP and used as a hybridization probe with restriction endonuclease digested genomic DNA from Vibrio strains. No DNA homologous to the probe DNA was detected in the five V. mimicus strains examined or in 4 of 5 sucrose negative V. cholerae strains. Some sucrose positive species--V. anguillarum, V. alginolyticus and V. diazotrophicus--had DNA homologous to the probe. Studies to further characterize sucrose utilization in V. cholerae and to determine distribution of related genes in other Vibrio species are in progress.

Arabinose fermentation is a variable characteristic in Vibrio parahaemolyticus. The arabinose genes of an arabinose positive strain of Vibrio parahaemolyticus have been cloned in E. coli. The cloned segment restores arabinose fermentation ability in an E. coli strain deleted for the arabinose operon as well as complementing ara point mutations. The cloned genes will be used to assess the molecular genetic basis of intraspecific variation in arabinose utilization.

In summary, the ecological aspects of V. cholerae have provided insight into the epidemiology of this organism.

LITERATURE CITED

1. Colwell, R.R., P.R. Brayton, D.J. Grimes, D.B. Roszak and L.M. Palmer. 1985. Viable but non-culturable Vibrio cholerae in the environment: implications for release of genetically engineered microorganisms. Biotechnology 3:817-820.
2. Hobbie, J.E., R.J. Daley and S. Jasper. 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33:1225-1228.
3. Ketyi, I. and A.S. Pacsa. 1980. Estimation of Vibrio cholerae and Escherichia coli heat-labile enterotoxin by enzyme-linked immunoabsorbent assay (ELISA). Acta Microbiol. Acad. Sci. Hung. 27:89-97.
4. Kogure, K., U. Simidu and N. Taga. 1979. A tentative direct microscopic method for converting living bacteria. Can. J. Microbiol. 25:415-420.
5. Maneval, D.R., R.R. Colwell, S.W. Joseph, R. Grays and S. T. Donta. 1980. A tissue culture method for the detection of bacterial enterotoxins. J. Tissue Culture Methods _:85-90.
6. Remmers, E.F., R.R. Colwell and R.A. Goldsby. 1982. Production and characterization of monoclonal antibodies to cholera toxin. Infect. Immun. 37:70-76.
7. Russek, E., and R.R. Colwell. 1982. Computation of most probable numbers (MPN). Appl. Environ. Microbiol. 45:1646-1650.
8. Singleton, F.L., R.A. Attwell, M.S. Jangi and R.R. Colwell. 1982. Influence of salinity and organic nutrient concentration on survival and growth of Vibrio cholerae in aquatic microcosms. Appl. Environ. Microbiol. 43:1080-1085.

9. Spira, W., R.B. Sack and J.L. Froehlich. 1981. Simple adult rabbit model for Vibrio cholerae and enterotoxigenic Escherichia coli diarrhea. Infect Immun. 32:739-747.

10. Tabor, P.S., and R.A. Neihof. 1984. Direct determination of activities for microorganisms of Chesapeake Bay populations. Appl. Environ. Microbiol. 48:1012-1019.

11. Xu, H., N. Roberts, F.L.Singleton, R.W. Atwell, D.J. Grimes and R.R. Colwell. 1982. Survival and viability of nonculturable Escherichia coli in the estuarine and marine environment. Microb. Ecol. 8: 313-323.

12. Xu, H., N.C. Roberts, L.A. Adams, P.A. West, R.J. Siebeling, A. Huq, M.I. Huq, R. Rahman and R.R. Colwell. 1984. An indirect fluorescent antibody staining procedure for detection of Vibrio cholerae samples. J. Microbiological Meth. 2:221-231.

TABLE 1

A COMPARISON BETWEEN EPIFLUORESCENT AND CULTURAL METHODS FOR DETECTION OF *V. CHOLERAE*, SEROVAR 01 IN BRAZIL, 1982

SITE	DETECTION OF *V. CHOLERAE* SEROVAR 01	
	EPIFLUORESCENT	CULTURE
BILLINGS RESERVOIR, RIO GRANDE RIVER WATER	+	-
BILLINGS RESERVOIR, RIO GRANDE RIVER PLANKTON	-	-
JURUBATUBA STREET, SEWAGE WATER	+	-
ORQUI DA' RIO, SEWAGE WATER	+	-
SAN LOURENCO, SEAWATER	+	-
SAN LOURENCO, CONCENTRATED PLANKTON	+	-

TABLE 2

A COMPARISON BETWEEN EPIFLUORESCENT AND CULTURAL METHODS FOR DETECTION OF *V. CHOLERAE*, SEROVAR 01 IN ZIMBABWE, 1984

SITE	MPN ESTIMATE/100 ML SAMPLE EPIFLUORESCENT	CULTURE
VICTORIA FALLS:		
ALLIGATOR SPILLWAY AT ZAMBEZE	11	< 3
HIPPOPOTAMUS WATERING SITE	15	< 3
ZAMBEZI RIVER, NON-FLOWING INLET	14	< 3
MAZOE RIVER:		
FARM WELL WATER	12	< 3
WASHING PLACE AT RIVER	19	< 3
WASHING PLACE	150	< 3
BELOW DAM	15	< 3
SETTLED POOL WATER	7	< 3
ROCK POOL WATER	15	< 3
STAGNANT POOL WATER	33	3

TABLE 3. Results of Microautoradiography

Day	% of Acridine Orange Direct Count								
	Plate Count		Direct Viable		Active			Active Direct Viable	
	E.c.	S.e.	E.c.	S.e.	E.c.	S.e.	N.f.	E.c.	S.e.
0	100	100	100	100	5-30	50-05	0	98-100	100
2	40	74	53	78	11-22	2-44	0	96-100	93-100
5		67		74		8-10	0		95-100
9	29		45		1-11		0	96-100	
10		20		61		16-43	0		92-100
28		0		11		10-17	7		92-100
30	0.02		3		2-7		0-2	91-100	
56	0		4		6-		4-28	100	

E.c. = *E. coli*.
S.e. = *S. enteritidis*.
N.f. = non-filamentous but active.
Plate Count and Direct Viable Count listed as mean.
Active counts listed as ranges.

TABLE 4

MICROAGGLUTINATION TITERS OF SHARK SERUM WITH *V. CHOLERAE*, ATCC 14035

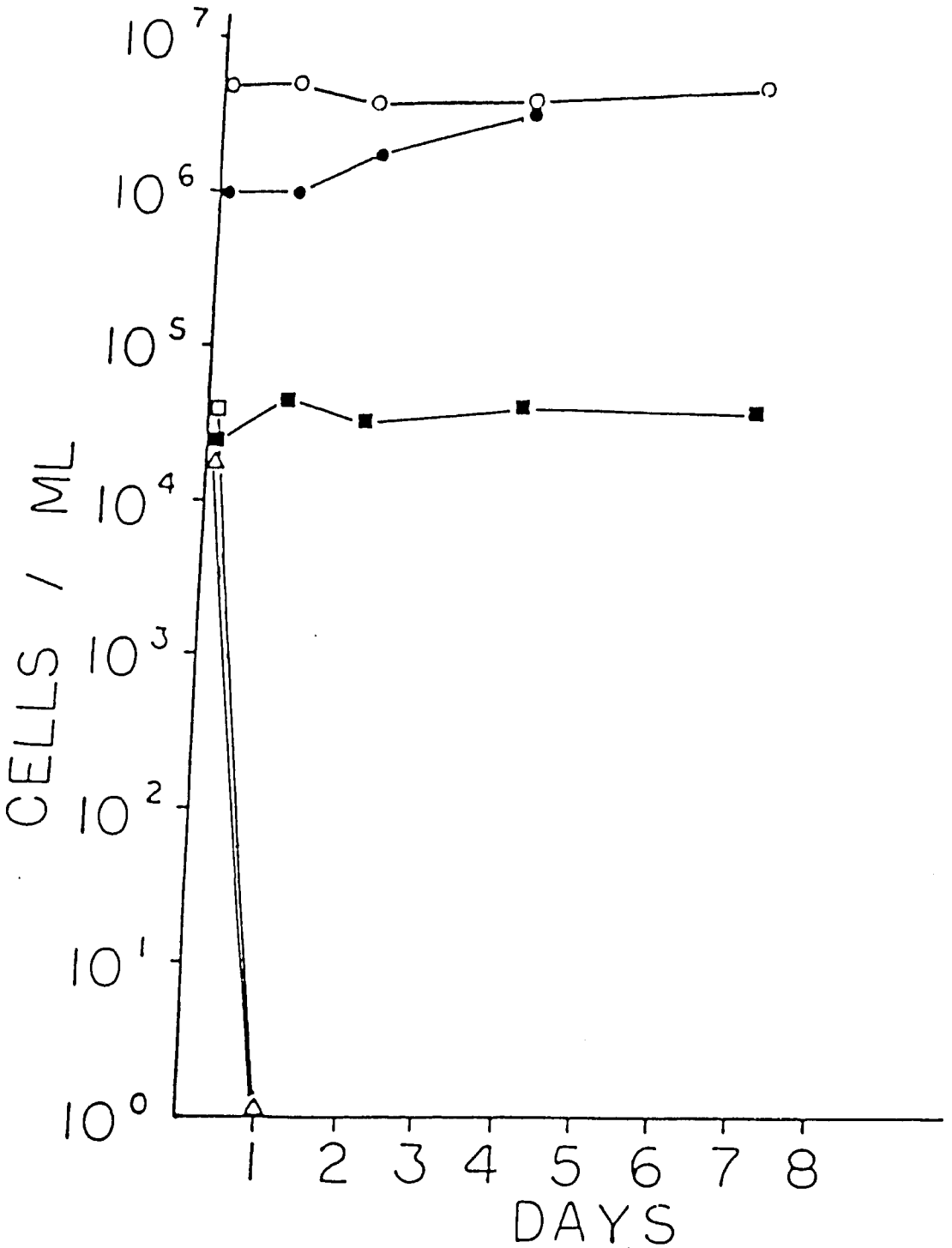
CODE	SPECIES	TITER
184	NURSE	1:640
LL	NURSE	1:1024
95563	NURSE	1:512
698	LEMON	1:2
476	LEMON	1:4
700	LEMON	1:128
	LEMON PUP	1:2
699	LEMON	1:256
678	LEMON	1:2
642	LEMON	1:8
	BLACKTIP	1:10

CAPTIONS TO FIGURES

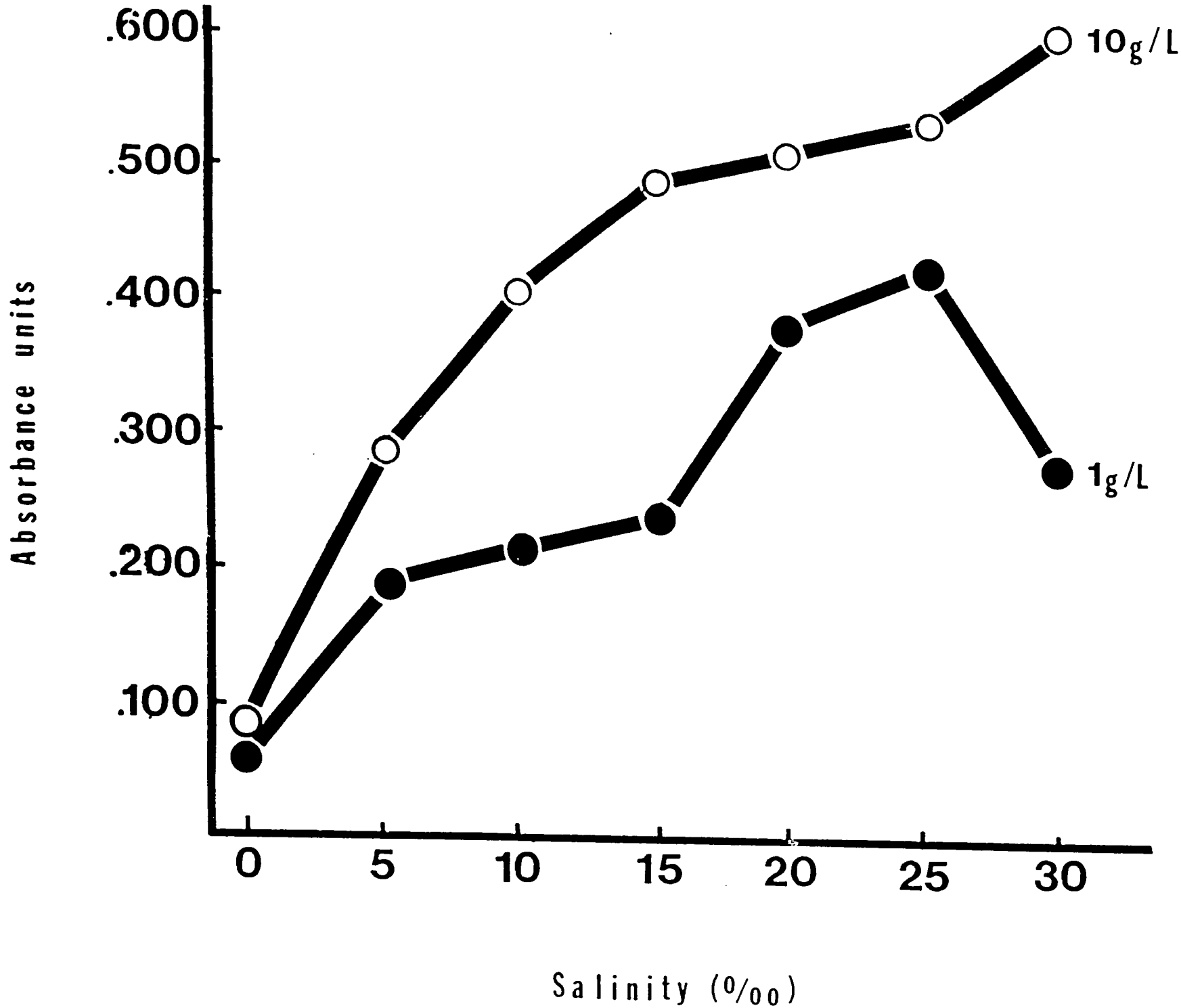
Figure 1. *V. cholerae* CA 401 cultures exposed to Patuxent River water in microcosms. ○ AODC, ● FA, ■ DVC, △ Plate counts, TCBS, □ Plate counts, TSA.

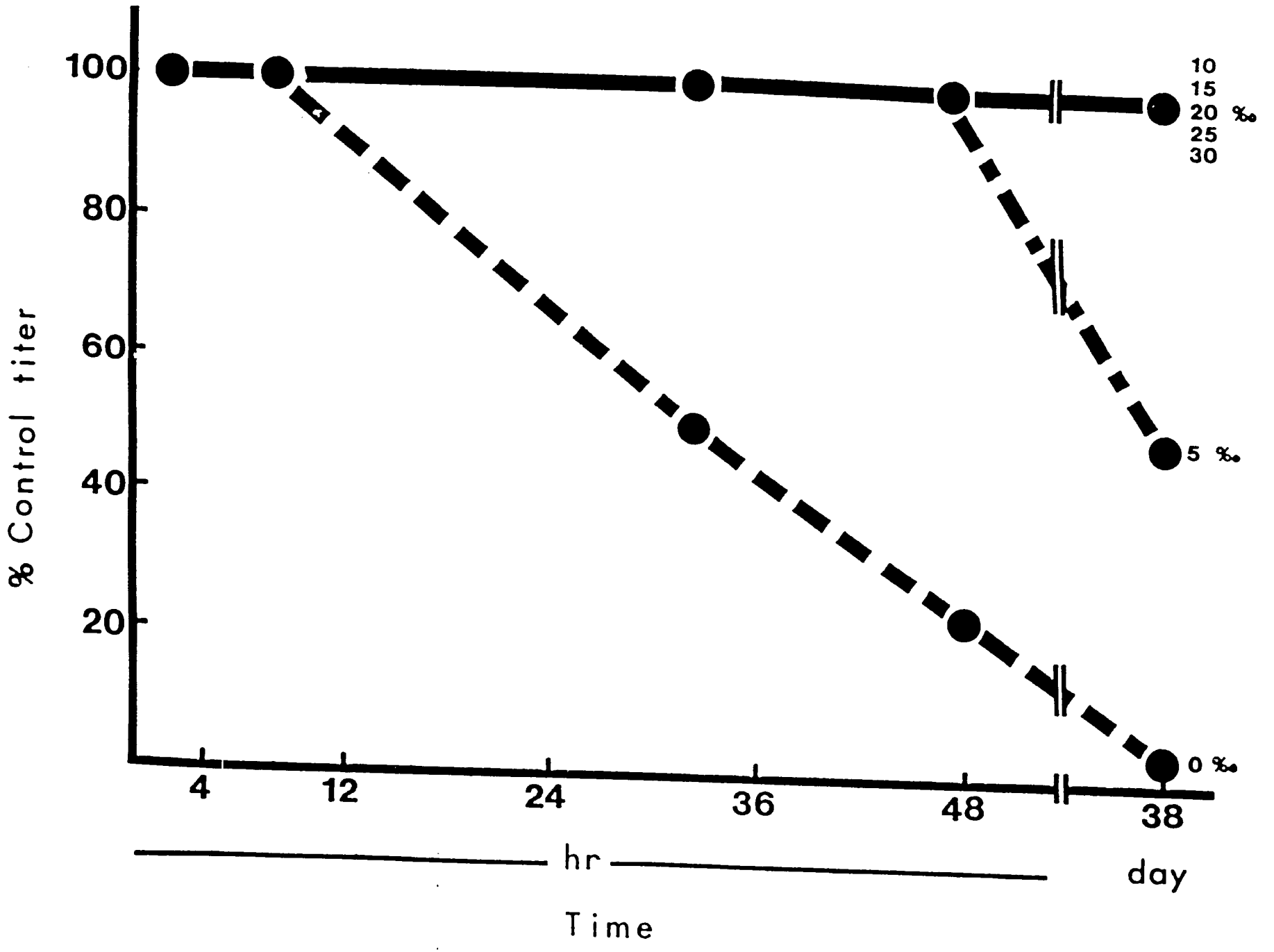
Figure 2. Plot of cholera toxin levels (y-axis) measured in absorbance units at 415 nm versus salinity (x-axis) (0-30 ppt, in increments of 5). Incubation time = 18h.

Figure 3. Plot of y-1 adrenal cell response (% of control titer, y-axis) to toxin incubated at various salinities, under sterile conditions, at 25 C for times ranging from 0-48 hours and at 38 days (x-axis).



9V


.600
.500
.400
.300
.200
.100
Absorbance units
0
5
10
15
20
25
30
Salinity (0/00)
10g/L
1g/L


100
80
60
40
20
% Control titer
10
15
20 ‰
25
30
5 ‰
0 ‰
4
12
24
36
48
38
hr
day
Time

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Vibrio cholerae O1 in Bangladesh waters: enumeration by fluorescent antibody-direct viable count

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Submitted to: Applied and Environmental Microbiology
February, 1987

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Rec'd in S... OCT 8 1987

Abstract

A field trial to enumerate *Vibrio cholerae* O1 in aquatic environments in Bangladesh was conducted, comparing fluorescent antibody-direct viable count (FA-DVC) with cultural detection by the most probable number index (MPN). Specificity of a monoclonal antibody prepared against the O1 antigen was assessed and incorporated into the fluorescent staining method. All pond and water samples yielded higher counts of viable *V. cholerae* O1 by FA-DVC than MPN. Fluorescent microscopy is a more sensitive detection system than cultural methods, allowing the enumeration of both culturable and non-culturable cells, and therefore, providing a more precise monitoring of microbiological water quality.

Introduction

Vibrio cholerae serovar O1 is the causative agent of epidemic cholera in Bangladesh. Its persistence and transmission is fostered by environmental and climatic conditions and sanitary practices. Frequent exposure by villagers to contaminated surface waters makes it mandatory that improved detection methods for *V. cholerae* be developed to evaluate water quality and assess the risk for disease.

Recent laboratory studies have shown that the organism can exist in a viable but non-culturable state. Furthermore, animal models have demonstrated that such cells can regain culturability and remain pathogenic (2). As a result of these findings, alternatives to standard culture methods were sought to detect and enumerate non-culturable *V. cholerae* serovar O1.

In this report, we describe a field trial using fluorescent antibody in a direct viable count (FA-DVC), as described by Brayton et al. (1), for detection and enumeration of *Vibrio cholerae* O1 occurring in the natural environment. Fluorescent microscopy was compared with standard cultural methods to determine the relative sensitivity and rapidity of the two methods.

Materials and Methods

Sample Collection

Sampling for the experiments was conducted in January, 1986, at villages surrounding Matlab, Bangladesh where cholera is endemic. The region is located 30 miles southeast of the capital city of Dhaka and lies in the delta formed by the Meghna and the Ganges Rivers.

All water samples were collected in sterile containers from village tube wells, ponds and the adjacent river. Temperature, salinity, and pH were measured using a YSI meter (Yellow-Springs Instrument Co., Yellowsprings, Ohio). Initial processing of samples was done at the Matlab laboratory branch of the International Center for Diarrhoeal Disease Research-Bangladesh (ICDDR-B), within two hours after collection.

Most Probable Number enumeration

For most probable number (MPN) estimates of *V. cholerae* O1 (4), 100 ml, 10 ml, and 1.0 ml volumes of water were inoculated into alkaline peptone broth tubes (APB; per liter: peptone, 10 g; NaCl, 10 g; sodium taurocholate, 5 g; sodium carbonate, 1 g; ph, 8.6) using three replicates of each volume. Tubes were incubated at 35° C for 6h, and then a loopful of each tube was streaked to thiosulfate citrate bile-salts sucrose agar (TCBS, Oxoid, Columbia, MD). Plates were incubated at 35° C for 18-24 h, at which time yellow colonies were picked and subcultured to tryptic soy agar

(TSA, Difco Labs, Detroit, MI) amended with 0.5% w/v NaCl. After incubation at 35° C, slide agglutination tests were performed on all isolates using V. cholerae poly O1 antiserum (Difco). Any isolate yielding a positive test was confirmed by fluorescent antibody staining, using monoclonal antibody (see below).

Fluorescent microscopy enumeration

Because the pond water samples examined in our study were relatively turbid, prefiltering through a 1.2µm filter (Gelman Sciences Inc., Ann Arbor, MI) was necessary.

Acridine Orange Direct Counts (AODC) were completed on all samples following the protocol of Hobbie et al. (6). Each sample was formalin fixed (2% v/v) and refrigerated until counts were performed.

Fluorescent antibody counts (FA) for total V. cholerae O1 were determined by the method of Xu et al. (23). However, V. cholerae monoclonal antibody specific for the A antigen of O1 lipopolysaccharide (M.L. Tamplin, manuscript in preparation) and goat antimouse fluorescein isothiocyanate (Difco) were employed as reagents.

Fluorescent antibody direct viable counts (FA-DVC) of V. cholerae O1 were done following the protocol of Brayton et al. (1).

Specificity determination of the monoclonal antibody

To confirm the specificity of monoclonal antibody to V. cholerae O1 antigen, fluorescent antibody screening was conducted using a variety of bacterial strains that may be

present in environmental samples. All cultures were grown overnight in T_1N_1 broths (1% w/v tryptone; 1% w/v NaCl) at 25° C or 35° C, depending on the requirements of the bacterial strain. Loopfuls of culture were transferred to a tube of phosphate buffered saline (PBS; per liter: NaCl, 8.5 g; Na_2HPO_4, 9.1 g; KH_2PO_4, 1.5 g; pH = 7.3), until a suspension yielding a concentration of 10^6 - 10^7 cells/ml, determined by AODC, was achieved. At this point, 2.0 ml of the PBS suspension was filtered through an 0.2μm x 25mm polycarbonate membrane filter (Nuclepore Co., Pleasanton, CA), and stained by the fluorescent antibody procedure. One hundred fields of each filter were examined by fluorescent microscopy to assess cross-reactivity between the monoclonal antibody and test strains. Bacterial strains included in the study are listed in Table 1.

Results

Screening of bacterial strains for cross-reactivity with monoclonal antibody

All *Vibrio cholerae* O1 strains appeared as small distinct rods surrounded by a bright peripheral fluorescent green band. Fluorescent cells were not observed in preparations of the other 75 bacterial species tested.

Temperature and pH results

The temperature of the river and pond water samples examined in this study ranged from 21 to 24° C. All pH readings were 6.0, and salinity values 0.0 °/oo. For tube wells, temperature readings ranged from 16 to 26° C, pH 6 to 7, and salinity 0.4 to 0.5 °/oo.

Enumeration of *V. cholerae* O1 in water

All Bangladesh village sites that were sampled were connected by the same river. In some villages, small ponds were sampled. Both the river and pond water sources are used for bathing, washing, occasional drinking and cooking. All villages had tube wells, installed to provide safe drinking water.

Enumeration data for water samples from village sites are shown in Table 2. AODC, done to enumerate total bacteria, revealed tube well counts of 10^4 -10^6 cells/ml. River water samples contained 10^6 cells/ml, while pond water

counts ranged from 10^4 - 10^7 cells/ml.

The FA results for total V. cholerae O1 indicated all tube well samples to be negative. The river water sample, collected adjacent to the Kadamtali village, contained 10^2 V. cholerae O1 per ml, while 10^3 per ml were present in all other river and pond water samples.

Results of viable V. cholerae O1 counts, enumerated by the FA-DVC procedure, yielded similar findings. As might have been predicted, V. cholerae O1 could not be detected by FA-DVC in tube well water samples examined in this study. Both the pond and river water samples from Kadamtali yielded 10^2 viable V. cholerae O1 per ml. All other FA-DVC enumerations for pond and river water samples were in the range of 10^3 cells/ml.

As shown in Table 1, all MPN estimates for water samples were <0.3 V. cholerae O1 cells per 100 mls, except for the river water sample collected at the village of Gajipur, which contained 2.0 cells per 100 mls. This sample yielded the only culturable V. cholerae O1. No other isolates were recovered after screening all yellow colonies growing on TCBS culture plates by slide agglutination.

Discussion

Critical to the success of the fluorescent antibody staining procedure described here is the specificity of the antibody that is utilized. Results of the screening for cross-reactivity demonstrated extraordinarily high specificity of the monoclonal antibody for the *V. cholerae* O1 antigen. Because the reagent was to be employed in a microscopic procedure, a large number of bacterial strains were tested for cross-reactivity. By filtering a 10^6-10^7 cell per ml suspension onto a filter, and staining by FA, a large population of cells could be examined in 100 microscopic fields, and therefore, any individual cells expressing antigens common to the O1 antigen would be detected. No cross-reactivity was observed when representatives of the Vibrionaceae, Aeromonadaceae, Pseudomonadaceae, Enterobacteriaceae, Micrococcaceae, and Streptococcaceae were tested. On the other hand, all *V. cholerae* O1 cells appeared bright and distinct when stained by FA.

The MPN method was used to enumerate culturable *V. cholerae* O1. Sucrose positive colonies were examined by slide agglutination, a tedious and time-consuming task, requiring a minimum of 3 days from the time of sample collection to completion. *V. cholerae* O1 was not cultured from any sample, except river water at Gajipur. Those isolates that were positive by slide agglutination were confirmed microscopically by using FA staining and employing

monoclonal antibody.

By fluorescent microscopic methods, enumeration of environmental samples was accomplished easily and more rapidly, e.g. a complete AODC required ca. 10 min per sample. These results served as a reference for determining what proportion of the total bacterial population in the water sample was comprised of V. cholerae 01.

The FA procedure was used to enumerate the total V. cholerae 01 population. The FA-DVC variation enumerates viable cells only. In this procedure, water samples were incubated with yeast extract and nalidixic acid. Substrate-responsive cells, i.e. viable cells, elongate because of inhibition of DNA gyrase (5,9). After staining with specific antibody and fluorescein conjugate, viable V. cholerae 01 cells appeared as long, fluorescent, green-banded bacilli, when viewed under the microscope (1).

Approximately 3 hours is required to complete the staining process for FA enumeration. An additional 6, 12, or 24 hours is needed for the FA-DVC procedure, taking into account the requirement for supplemental incubation. The filter method utilized in these techniques permitted filtration of increasing volumes of water. The maximum volume filtered was dependent only on the turbidity of the water.

In all river and pond water samples examined in this study, total V. cholerae 01 cells comprised 0.01 to 0.1 % of the total microbial population. Of the V. cholerae 01 population present, 16 to 85% were viable when examined by

FA-DVC.

Although the tube well water samples yielded high AODC, results of FA and FA-DVC indicated V. cholerae O1 were not present. Tube wells have been installed in many villages to provide a source of safe drinking water. However, studies have shown that the rate of cholera infection is the same among those inhabitants drinking water from tube wells as those who do not. This finding was attributed to the multipurpose use of contaminated surface water routinely used for bathing, cooking and washing (10,20,21).

The ICDDR-B conducts epidemiological examinations by conventional culture methods, at those villages reporting cholera cases. Attempts to isolate V. cholerae O1 from food and water sources in the villages is not always successful (unpublished data). Our attempts to culture V. cholerae O1 were unsuccessful in all but one water sample which had been collected at the same time samples for FA analysis were collected. Using the FA-DVC method, viable V. cholerae O1 could be detected and enumerated in all the river and pond water samples examined. Viable V. cholerae O1 was detected in all samples that were culture positive, as well as culture negative.

It is apparent from these results that the FA procedure is a more sensitive detection method than standard cultural procedures. The significant difference in results between the cultural and FA procedures provides additional evidence for the existence of the viable but non-culturable stage of

V. cholerae in the natural environment in a cholera-endemic area (2).

Many environmental and laboratory studies have been done in which growth and survival of *V. cholerae* in water have been examined (3,11,18,19,22). Nutrient content, salinity, temperature, pH and size of inoculum are some of the factors contributing to the persistence and recoverability of the organism. Survival of *V. cholerae* in water may extend from hours to months. Viability in these experiments was determined by colony forming units appearing on plating media.

More recent studies have shown that a variety of bacteria may enter a dormant state when physical and chemical conditions are less than optimal. The organisms remain viable, but non-culturable (2,15,16, D.B. Roszak, Ph.D. thesis, University of Maryland, College Park, 1985). The FA-DVC technique has made it possible to detect, as well as enumerate, specific viable cells that yield "no growth" by standard methods of cultivation.

It is not certain that all viable cells are capable of elongating under the outlined experimental conditions of the DVC method. Yeast extract may be an inadequate substrate for bacteria in advanced stages of dormancy, as suggested by the results of Peele and Colwell (14). Furthermore, some bacteria may be resistant to the action of nalidixic acid. However, the FA-DVC method yields significantly greater success in detecting viable *V. cholerae*, even though it may possibly

underestimate the total viable count. Only viable cells are capable of elongation when exposed to the testing regime and both laboratory and field data indicate that the FA-DVC always exceeds plate counts (2,15,16, D.B. Roszak, Ph.D.thesis, University of Maryland, College Park, 1985).

The role of zooplankton in the ecology of V. cholerae has been studied, i.e., providing a substrate for attachment and nutrient, as well as a vehicle for transmission to humans. Chitin, found on the surface of copepods is capable of protecting microorganisms from the bacteriocidal action of gastric acids (7,8,12,13). That non-culturable bacteria can attach to zooplankton during unfavorable environmental conditions, and, by ingestion, pass into the human gut where optimal conditions for growth, replication and pathogenic mechanisms exist, must be considered. Thus, viable but non-culturable bacteria cannot be ignored, especially since virulence of these bacteria as well as "injured" bacteria have been demonstrated in animal studies (2,17).

In conclusion, the FA-DVC provides a highly specific and sensitive detection system. Culturable, as well as non-culturable bacteria can be observed and enumerated relatively quickly and easily. Village water sources in Bangladesh should be monitored by FA-DVC for viable V. cholerae O1 and correlated with the incidence of disease. With the information needed becoming available, it will be possible to predict the risk of contracting cholera and explain the epidemiology of the disease.

References

1. Brayton, P.R; and R.R. Colwell. 1987. Enumeration of viable Vibrio cholerae cells by the fluorescent antibody staining technique. J. Microbiol. Meth. (in press).

2. Colwell, R.R.; P.R. Brayton; D.J. Grimes; D.B.Roszak; S.A. Huq; and L.M. Palmer. 1985. Viable but non-culturable Vibrio cholerae and related pathogens in the environment: implications for release of genetically engineered microorganisms. Biotechnology. 3:817-820.

3. Feachem, R; C. Miller; and B. Draser. 1981. Environmental aspects of cholera epidemiology. II. Occurrence and survival of Vibrio cholerae in the environment. Trop. Dis. Bull. 78:865-880.

4. Franson, Mary H. (ed). Standard methods for the examination of water and wastewater. Sixteenth edition. American Public Health Assoc. Washington, D.C.

5. Goss, William A.; William H. Deitz; and Thomas M. Cook. 1965. Mechanism of action of nalidixic acid on Escherichia coli. II. Inhibition of deoxyribonucleic acid synthesis. J. Bacteriol. 89:1068-1074.

6. Hobbie, J.E.; R.J. Daley; and S. Jasper. 1977. Use of

12. Nalin, David R. 1976. Cholera, copepods, and chitinase. Lancet. ii:958.

13. Nalin, D.R.; V. Daya; A. Reid; M.M. Levine; and L. Cisneros. 1979. Adsorption and growth of Vibrio cholerae on chitin. Infect. Immun. 25:768-770.

14. Peele, E.R., and R.R. Colwell. 1981. Application of a direct microscopic method for enumeration of substrate-responsive marine bacteria. 27:1071-1075.

15. Rollins, D.M.; and R.R. Colwell. 1986. Viable but non-culturable stage of Campylobacter jejuni and its role in survival in the natural aquatic environment. Appl. Environ. Microbiol. 52:531-538.

16. Roszak, D.B.; D.J. Grimes; and R.R. Colwell. 1984. Viable but non-recoverable stage of Salmonella enteritidis in aquatic systems. Can. J. Microbiol. 30:334-338.

17. Singh, A.; R. Yeager; and G.A. McFeters. 1986. Assessment of in vivo revival, growth, and pathogenicity of Escherichia coli strains after copper- and chlorine-induced injury. Appl. Environ. Microbiol. 52:832-837.

18. Singleton, F.L., R.A. Attwell, M.S. Jangi, and R.R. Colwell. 1982. Influence of salinity and organic nutrient concentration on survival and growth of Vibrio cholerae in aquatic microcosms. Appl. Environ. Microbiol. 43:1080-1085.

19. Singleton, F.L., R. Attwell, S. Jangi, and R.R. Colwell. 1982. Effects of temperature and salinity on Vibrio cholerae growth. Appl. Environ. Microbiol. 44:1047-1058.

20. Sommer, Alfred; and William E. Woodward. 1972. The influence of protected water supplies on the spread of Classical/Inaba and El Tor/Ogawa cholera in rural east Bengal. Lancet. 2:985-987.

21. Spira, W.M.; M.U. Khan; Y.A. Saeed; and M.A. Sattar. 1980. Microbiological surveillance of intra-neighbourhood El Tor cholera transmission in rural Bangladesh. Bulletin of the World Health Organization. 58:731-740.

22. Tamplin, M.L., and R.R. Colwell. 1986. Effects of microcosm salinity and organic substrate concentration on production of Vibrio cholerae enterotoxin. Appl. Environ. Microbiol. 52:297-304.

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23. Xu, H.-S; N.C. Roberts; L.B. Adams; P.A. West, R.J. Siebeling; A. Huq; M.I. Huq; R.Rahman; and R.R. Colwell. 1984. An indirect fluorescent antibody staining procedure for detection of Vibrio cholerae serovar O1 cells in aquatic environmental samples. J. Microbiol Meth. 2:221-231.

Table 1. Bacterial strains screened for cross-reaction wit monoclonal antibody.

V. cholerae 01	ATCC 14035
V. cholerae 01	ATCC 14033
V. cholerae 01	599655
V. cholerae non-01	SG 7509
V. cholerae non-01	2030H
V. cholerae non-01	SG 8268
V. cholerae non-01	N 8246
V. cholerae non-01	42
V. cholerae non-01	90
V. cholerae non-01	13
V. cholerae non-01	19
V. cholerae non-01	43
V. mimicus	ATCC 33653
V. mimicus	UM 4053
V. mimicus	UM 4074
V. proteolyticus	ATCC 15338
V. vulnificus	ATCC 27562
V. vulnificus	UM 4304
V. diazotrophicus	ATCC 33466
V. alginolyticus	ATCC 17749
V. alginolyticus	UM 4109
V. parahaemolyticus	ATCC 17802
V. parahaemolyticus	ATCC 27969

V. parahaemolyticus	UM 4111
V. parahaemolyticus	UM 4324
V. parahaemolyticus	UM 4108
V. parahaemolyticus	UM 4237
V. damsela	ATCC 35083
V. damsela	ATCC 33539
V. carchariae	ATCC 35804
V. anguillarum	ATCC 19264
V. anguillarum	UM 4393
V. anguillarum	UM 4248
V. aestuarianus	ATCC 35048
V. cincinnatiensis	ATCC 35912
V. natriegens	ATCC 14048
V. natriegens	UM 4384
V. harveyi	ATCC 14126
V. ordallii	ATCC 33509
V. campbellii	ATCC 25920
V. campbellii	UM 4344
V. fluvialis	ATCC 11328
V. fluvialis	UM 4476
V. fluvialis	UM 4122
V. tubiashii	3358
V. tubiashii	3359
V. splendidus	UM 4259
V. splendidus	UM 4424
V. pelagius	UM 4422
V. pelagius	UM 4494

V. furnissii	UM 4137
Pseudomonas aeruginosa	ATCC 10145
Pseudomonas putida	ATCC 12633
Pseudomonas solonacerium	ATCC 11696
Shigella sonnei	UM 773
Shigella flexneri	ATCC 12022
Shigella flexneri	UM 774
Serratia liquefaciens	NCTC[c] 10442
Serratia marcescens	ATCC 13880
Proteus vulgaris	ATCC 13315
Proteus vulgaris	UM 769
Flavobacterium meningosepticum	ATCC 13253
Photobacterium angustum	ATCC 25915
Plesiomonas shigelloides	ATCC 14030
Escherichia coli	H10407
Escherichia coli	ATCC 11303
Escherichia coli	ATCC 11775
Enterococcus faecalis	ATCC 19433
Salmonella typhimurium	ATCC 14028
Klebsiella pneumoniae	ATCC 13883
Citrobacter freundii	ATCC 8090
Enterobacter aerogenes	ATCC 13048
Aeromonas punctata	ATCC 15468
Aeromonas media	ATCC 33907
Aeromonas hydrophila	ATCC 99071
Aeromonas hydrophila	ATCC 15467
Aeromonas hydrophila	UM 4396

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Alcaligenes faecalis	ATCC 8750

[a] ATCC - American Type Culture Collection, Rockville, MD

[b] UM - culture collection at University of Maryland

[c] NCTC - National Collection of Type Cultures, Central Public Health Laboratory, London, England

Acknowledgments

This research was funded by National Institutes of Health grant R22-AI-14242, U.S. Aid for International Development grant DOE-542-G-55-4060-00 and World Health Organization grant C6/181/70.

We wish to thank I. Knight and J. Beaver for assistance in screening bacterial strains for cross-reactivity with monoclonal antibody. We are grateful for assistance we received in Bangladesh from Dr. B. Kay and our other colleagues at the International Center for Diarrheoal Disease Research, Bangladesh.

TABLE 2. ENUMERATION OF *VIBRIO CHOLERAE* O1 IN BANGLADESH WATERS BY CULTURAL AND FLUORESCENT ANTIBODY METHODS

SAMPLING DATE	SITE	SOURCE	AODC[A] CELLS/ML	FA[B] CELLS/ML	FA-DVC[C] CELLS/ML	MPN[D]/ 100 MLS
1-8-86	GOALVAOR	RIVER	2.2×10^6	9.9×10^3	1.6×10^3	< 0.3
1-8-86	GOALVAOR	TUBE WELL	2.5×10^6	0	0	< 0.3
1-8-86	SHAHWAZKANDI	POND	2.7×10^7	7.7×10^3	5.9×10^3	< 0.3
1-8-86	SHAHWAZKANDI	TUBE WELL	3.2×10^5	0	0	< 0.3
1-8-86	SHAHWAZKANDI	RIVER	5.9×10^6	5.7×10^3	3.4×10^3	< 0.3
1-8-86	KADAMTALI	TUBE WELL	8.5×10^4	0	0	< 0.3
1-8-86	KADAMTALI	POND	1.9×10^7	2.9×10^3	9.4×10^2	< 0.3
1-8-86	KADAMTALI	RIVER	6.8×10^6	4.1×10^2	1.6×10^2	< 0.3
1-19-86	SARDERANDI	POND	4.4×10^6	2.9×10^3	1.5×10^3	< 0.3
1-19-86	SARDERANDI	RIVER	2.9×10^6	5.4×10^3	1.9×10^3	< 0.3
1-19-86	UDDAMDI	POND	2.0×10^7	6.1×10^3	1.4×10^3	< 0.3
1-19-86	UDDAMDI	RIVER	4.9×10^6	3.5×10^3	1.8×10^3	< 0.3
1-19-86	GAJIPUR	POND	4.8×10^7	4.1×10^3	3.5×10^3	< 0.3
1-19-86	GAJIPUR	RIVER	1.9×10^6	4.4×10^3	1.6×10^3	2.0
1-19-86	CHARMOKUNDI	POND	4.9×10^6	3.9×10^3	2.0×10^3	< 0.3
1-19-86	CHARMOKUNDI	2ND POND	2.5×10^6	4.9×10^3	1.6×10^3	< 0.3

[A] AODC - ACRIDINE ORANGE DIRECT COUNT.

[B] FA - FLUORESCENT ANTIBODY COUNT.

[C] FA-DVC - FLUORESCENT ANTIBODY - DIRECT VIABLE COUNT.

[D] MPN - MOST PROBABLE NUMBER ESTIMATE.