Short Communication

**Enumerations of *Vibrio cholerae* in Aquatic Environments
by MPN-16S rRNA Hybridization Method**

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(Received August 17, 1991-Accepted February 29, 1992)

Key words : 16S rRNA, *Vibrio cholerae*, MPN

Various methods are available for the detection and enumeration of microorganisms in aquatic environments. Advantages and disadvantages of these methods have been discussed⁹⁾. Historically, the methods to determine most probable number (MPN) of bacteria have been employed for the sanitary qualification of drinking water, particularly for the estimation of coliform bacteria. This method has also been widely used for the detection of other microorganisms in environments. Jannasch and Jones⁷⁾ compared various methods for the enumeration of bacterial population in seawater, and found that the extinction dilution method gave values much higher than plate count methods. Furthermore, the combination of the MPN method with a subsequent plating on selective media provides an accurate estimation of particular bacterial groups^{3,8,12)}. More accurate technique for the direct detection in combination with the MPN method would further give more reliable estimation of particular groups of bacteria.

Recently, an application of molecular biology techniques has proved to be quite valuable for ecological studies of microorganisms, especially for the identification and enumeration of specific

bacteria in natural environments¹¹⁾. Therefore, we tried to develop a sensitive hybridization technique using oligonucleotide probes. As a gene probe, 16S rRNA was considered to be suitable at the present time, because its base sequence in many bacteria was completely determined and known as reflecting phylogenetic relationships among prokaryotic microorganisms¹⁵⁾. Furthermore, many of sequencing data of 16S rRNA have been progressively accumulated for bacteria during the past decade, and some particular segments of 16S rRNA sequence are known to be species or genus specific. If we can use specific probes for hybridization tests of some target bacteria, it enable us to detect and differentiate easily individual bacterial groups in natural samples.^{1,4,5,13)}

In this paper, the combined use of 16S rRNA hybridization with a routine MPN method and its availability to detect and enumerate a certain bacterial group in natural environments are discussed. For the development of this method, *Vibrio cholerae* was chosen as a test bacterium, because this particular organism is a typical pathogen in *Vibrionaceae*, and well-known as a causative agent of human cholera. It is also known that this bacterium is widely present in aquatic environments^{8,16)}.

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On the basis of 16S rRNA sequencing data of marine vibrios¹⁴⁾, we have chemically synthesized two cholera-specific cDNA probes (CHO. 1 : CGTCAAATGATTAAGGT, CHO. 2 : CTCCACCTCGCGGTATC; covering the base position of 468–484 and 1260–1276, respectively) and labelled the probes with Texas-red (sulfonyl chloride) via an amine-linker (Milligen/Bioscience). Texas-red is a derivative of rhodamine 101, and has an ability to conjugate with an amino-group at the 5' end of the linker as well as fluorescein isothiocyanate (FITC) conjugates. In a previous paper, Tsukamoto tested several strains of *V. cholerae*, including Eltor Ogawa (IID 936), Eltor Inaba (IID 1655), Classical Inaba (IID 467 and IID 935), and Classical Ogawa (IID 934), and confirmed the presence of the same two sequences at the target portions in all these strains¹⁴⁾. Therefore, it was considered that the cholera probes could react with many strains of *V. cholerae* independently on their serotypes. The hybridization specificity of the cholera probes was checked with 6 species belonging to genus *Vibrio*. As a result, the cross-reaction of the test probes was recognized only with *Vibrio mimicus*¹⁰⁾.

An universal cDNA probe common to all eubacterial 16S rRNA was also synthesized (universal: ACGGGCGGTGTGTAC) and labelled with FITC.

A fresh water sample was collected from Kanda River and seawater samples were collected from Harumi Pier and Aburatsubo Inlet in Kanagawa Prefecture from April to May in 1991 (Fig. 1). Kanda River and Harumi Pier are both located in Tokyo metropolitan area and eutrophicated due to municipal wastes.

The MPN of bacteria was determined by a three-dilution, three-tube replication series. The broth employed was alkaline peptone medium (A.P.) containing polypeptone (10g/liter) and NaCl (10 g/liter) at pH 8.5^{8,12,16)}. Total MPN was estimated by turbidity assay after 24 h of incubation at 25°C and the occurrence of *V. cholerae* in growth-positive tubes was detected by the fluores-

Fig. 1. Sampling locations

A: Kanda River B: Harumi Pier C: Aburatsubo Inlet.

cent oligonucleotide probe technique as follows. The bacteria in the growth-positive tubes were fixed by adding a paraformaldehyde solution at a final concentration of 3%, then precipitated by centrifugation, and cell suspensions were smeared onto gelatin subbed slides. Simultaneous hybridization with both of the universal and cholera probes was performed as described by Amann *et al.*¹⁾. Simultaneously, vibrios in water samples were enumerated by the MPN method with thiosulfate-citrate-bile salt-sucrose (TCBS) broth after incubation for 24 h at 37 °C. The TCBS medium is routinely used for the selective isolation and cultivation of *V. cholerae* and enteropathogenic vibrios. In this investigation, we used 1/2 TCBS medium as well because the growth inhibition of 1×TCBS could be too strong for starved bacteria in natural environments. Heterotrophic bacteria in water samples were obtained by spread plating onto nonselective media, tryptic soy agar (TSA) or ZoBell 2216E.

The slides after hybridization were observed under an epifluorescent microscope (Olympus BHS-RFK) with blue and green filter sets and a high-pressure mercury bulb at a magnification of

Fig. 2. Cells of *Vibrio cholerae* hybridized with cholera probes fluoresced orange and other bacteria hybridized with the universal probe fluoresced green under blue excitation.

$\times 1000$. With a blue excitation filter set, the eubacteria hybridized with the cholera probes gave orange fluorescent colour, while other bacteria hybridized with the universal probe showed green fluorescent colour (Fig. 2). The discrimination between *V. cholerae* and other bacteria was very easy due to the difference in the colours of Texas-red and FITC.

Results of enumerations are shown in Table 1. The occurrence of *V. cholerae* in the samples were confirmed by the MPN-16S rRNA hybridization method. From the data of sample F shown in

Table 1, the number obtained by using cholera-probes (A.P. *V. cholerae*) was estimated to be 1.5×10^0 cells/ml, the total bacterial number obtained by turbidity assay with A.P. medium was estimated to be 1.1×10^2 cells/ml (A.P. total), and the number obtained by using 1/2 TCBS broth was estimated at 3.9×10^{-1} cells/ml. It was demonstrated that one hundredth to one tenth of viable bacteria which grew in A.P. medium were *V. cholerae* as confirmed by the MPN-16S rRNA hybridization method. Furthermore, the numbers of *V. cholerae* obtained with the A.P. - 16S rRNA method were much higher than the numbers of pathogenic vibrios detected by both media of TCBS and 1/2 TCBS.

In this work, the combined use of the 16S rRNA hybridization method with a MPN determination by TCBS medium was also tried, but it was unsuccessful. It was possibly due to that some constituents of the TCBS medium interfered in vitro hybridization reaction.

The synthesized cholera probes showed cross-reaction with *Vibrio mimicus* during hybridization, as the two bacteria share quite similar 16S rRNA sequence¹⁴. It is also possible that the cholera probes could react with *V. cholerae* of

Table 1. Comparison of bacterial number obtained by three different methods.

Date	April 12	May 5 (1991)	May 25
Source ^a	F	E	M
AODC (cells/ml)	2.2×10^6	8.4×10^6	3.5×10^6
Plate counts (CFU/ml)			
: Tryptic soy agar	3.0×10^3	N.T. ^b	N.T.
: ZoBell 2216E	N.T.	3.3×10^4	1.4×10^3
MPN (cells/ml)			
: TCBS ^c	$< 3.0 \times 10^{-2}$	$< 3.0 \times 10^{-2}$	$< 3.0 \times 10^{-2}$
: 1/2TCBS ^c	3.9×10^{-1}	1.2×10^0	3.6×10^{-2}
: A.P. (total) ^d	1.1×10^2	2.4×10^1	2.9×10^0
: A.P. (<i>V. cholerae</i>) ^e	1.5×10^0	2.1×10^0	2.3×10^{-1}

^a F, Fresh water from Kanda River; E, Eutrophic seawater from Harumi Pier; and M, Mesotrophic seawater from Aburatsubo Inlet.

^b Not tested.

^c MPN of pathogenic vibrios.

^d Total MPN determined with A.P.

^e MPN of *V. cholerae* determined by the MPN-16S rRNA hybridization method.

serotypes other than 0-1 (so-called non-agglutinable vibrios). However, the independence of the probes from serotypes would not decrease the value of the cholera probes, since the ecological and ethiological significance of *V. cholerae* of such serotypes has been recognized during the past decade^{2,8)}. Moreover, there may be many unknown bacteria which have never been cultivated, but only detectable by this new technique⁶⁾. After all, those bacteria were, even if detected, closely related phylogenetically to *V. cholerae*, having portions of the same nucleotide sequence in their 16S rRNA where the cholera probes can bind specifically.

For the detection and enumeration of genuine *V. cholerae*, another cholera probe with higher specificity may be available. The sequence analysis study on 23S rRNA would give us other phylogenetically specific information hidden in the RNA structure.

Acknowledgement

We thank Dr. Yoko Katayama, Faculty of Agriculture, Tokyo Univ. of Agriculture and Technology, for her guidance on the oligonucleotide purification.

References

- 1) Amann, R.I., L. Krumholz, and D.A. Stahl, 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J. Bacteriol.* **172**: 762-770.
- 2) Colwell, R.R., J. Kaper, and S.W. Joseph, 1977. *Vibrio cholerae*, *Vibrio parahaemolyticus*, and other Vibrios: occurrence and distribution in Chesapeake Bay. *Science*. **198**: 394-396.
- 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. *Bio/Technology*. **3**: 817-820.
- 4) DeLong, E.F., G.S. Wickham, and N.R. Pace, 1989. Phylogenetic stains: Ribosomal RNA-based probes for the identification of single cells. *Science*. **243**: 1360-1363.
- 5) Giovannoni, S.J., E.F. DeLong, G.J. Olson, and N.R. Pace, 1988. Phylogenetic group-specific oligonucleotide probes for identification of single microbial cells. *J. Bacteriol.* **170**: 720-726.
- 6) Giovannoni, S.J., T.B. Britschgi, C.L. Moyer and K.G. Field, 1990. Genetic diversity in Sargasso Sea bacterioplankton. *Nature*. **345**: 60-63.
- 7) Jannasch, H.W., and G.E. Jones, 1959. Bacterial populations in sea water as determined by different methods of enumeration. *Limnology and Oceanography*. **4**: 128-139.
- 8) Kaper, J., H. Lockman, R.R. Colwell, and S.W. Joseph, 1979. Ecology, serology, and enterotoxin production of *Vibrio cholerae* in Chesapeake Bay. *Appl. Environ. Microbiol.* **37**: 91-103.
- 9) Karl, D.M. 1986. Determination of *in situ* microbial biomass, viability, metabolism, and growth, p. 85-159. In J.S. Poindexter and E.R. Leadbetter (ed.), *Bacteria in nature*. Vol. **2**, Plenum Press, New York.
- 10) Nishimura, M., K.K. Tsukamoto, K. Kogure, K. Ohwada, and U. Simidu, 1991. Submitted for publication.
- 11) Pickup, R.W., 1991. Development of molecular methods for the detection of specific bacteria in the environment. *J. Gen. Microbiol.* **137**: 1009-1019.
- 12) Roberts, N.C., R.J. Siebeling, J.B. Kaper, and H. B. Bradford Jr., 1982. *Vibrio* in the Louisiana Gulf coast environment. *Microb. Ecol.* **8**: 299-312.
- 13) Tsien, H.C., B.J. Bratina, K. Tsuji, and R.S. Hanson, 1990. Use of oligodeoxynucleotide signature probes for identification of physiological groups of methylotrophic bacteria. *Appl. Environ. Microbiol.* **56**: 2855-2865.
- 14) Tsukamoto, K.K., 1991. Study on classification and identification of marine bacteria, mainly family *Vibrionaceae*. Ph. D dissertation. The Univ. of Tokyo.
- 15) Woese, C.R., 1987. Bacterial evolution. *Microbial. Rev.* **51**: 221-271.
- 16) 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. Method.* **2**: 221-231.