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Specific Detection of Toxigenic *Vibrio cholerae* Based on *in situ* PCR in Combination With Flow Cytometry[1]

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Objective To develop an *in situ* PCR in combination with flow cytometry (ISPCR-FCM) for monitoring cholera toxin positive *Vibrio cholerae*. **Methods** In running this method, 4% paraformaldehyde was used to fix the *Vibrio cholerae* cells and 1 mg/mL lysozyme for 20 min to permeabilize the cells. Before the PCR thermal cycling, 2.5% glycerol was added into the PCR reaction mixture in order to protect the integrality of the cells. **Results** A length of 1037bp DNA sequence was amplified, which is specific for the cholera toxin gene (*ctxAB* gene). Cells subjected to ISPCR showed the presences of *ctxAB* gene both in epifluorescence microscopy and in flow cytometric analysis. The specificity and sensitivity of the method were investigated. The sensitivity was relatively low (10^5 cells/mL), while the specificity was high. **Conclusion** We have successfully developed a new technique for detection of toxigenic *Vibrio cholerae* strains. Further study is needed to enhance its sensitivities. ISPCR-FCM shows a great promise in monitoring specific bacteria and their physiological states in environmental samples.

Key words: *Vibrio cholerae*; Detection technique; *in situ* PCR; Flow cytometry

INTRODUCTION

Vibrio cholerae is a well-known human pathogen causing cholera epidemics worldwide. Of at least 206 known serogroups of *V. cholerae*, only two serogroups O1 and O139 strains are responsible for epidemic outbreak of cholera[1]. Most strains in environment are not toxigenic to humans. Contrary to the early idea that *V. cholerae* persists only in humans living in endemic areas and only survives in the aquatic environment for a short time, *V. cholerae*, including the toxigenic strains, are now known to be indigenous to aquatic environment[2-3]. Chakraborty *et al*.[4] discovered that lateral transfer of genes can readily occur in the aquatic environment. Waldor and Mekalanos[5] reported that the *ctxAB* gene that encodes the cholera toxin can be transferred by a filamentous phage CTXΦ. Recently, a study of the genetic relationship and diversity of *V. cholerae* isolated from both clinical and environmental sources indicates that some clinical strains are closely related to their environmental counterparts[6]. These studies are suggestive of the possibility that environmental strains of *V. cholerae* may have developed the ability to adapt themselves to the intestinal environment through acquisition of virulence genes. In addition, under stress conditions in the natural habitat, *V. cholerae* can be converted to another life form, named a viable but non-culturable (VBNC) form, which cannot be recovered by any standard culture technique[7]. The *V. cholerae* strains of VBNC also carry the virulence genes[8]. Therefore, it is important to identify and analyze the environmental *V. cholerae* strains carrying virulence gene. It is necessary to develop new and effective alternates to culture-based techniques.

Early studies relied heavily on culture enrichment and biochemical identification to estimate the most probable number of *V. cholerae* in water. But these methods are labor intensive and only semi-quantitative. Fluorescence antibody direct count, albeit capable of detecting non-culturable *V. cholerae,*

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is only available for special serogroups (O1 and O139) and does not provide information on the overall abundance of *V. cholerae* in the relevant aquatic environment. Though PCR can be used to detect pathogenic vibrios directly in environment, most of these methods are still semi-quantitative, and not easy to be adopted to study the distribution and abundance of a particular microbial species/group in the natural community.

Flow cytometry (FCM) is useful in rapid enumeration of bacteria from aquatic environment[9], but it lacks the necessary resolution power to differentiate various bacterial species/strains. To overcome this shortage, *in situ* PCR (ISPCR) comes to play. ISPCR is a technique that amplifies a specific gene or genes within a cell or tissue. It has the specificity and even sensitivity of the conventional PCR. Hence, coupling FCM with ISPCR would empower a technique to detect a specific gene and enumerate cells containing that gene. Hence, it would be logical to utilize ISPCR-FCM to study the VBNC. Since 1995, several FCM analyses of bacteria subjected to ISPCR or *in situ* RT-PCR prior to the analysis have been reported[10-13]. Despite their current limitations, ISPCR-FCM shows a great promise in monitoring specific bacteria and their physiological states in environmental samples.

In this study, we successfully developed an ISPCR-FCM method specific for the detection and enumeration of toxigenic *V. cholerae* strains. Briefly, we amplified a DNA fragment (1037 bp) of the *ctxAB* gene inside the cells with a pair of fluorescent dye-labeled primers. Then, the cells were analyzed using epifluorescence microscopy and flow cytometry. Our data demonstrate that ISPCR-FCM is a promising tool for specifically detecting and enumerating toxigenic *V. cholerae* strains.

MATERIALS AND METHODS

Bacterial Strains

The toxigenic *V. cholerae* was isolated from clinic. The non-O1/non-O139 *Vibrio. cholerae*, *Vibrio. parahaemolyticus*, *Vibrio. minicus*, and *Vibrio. alginolyticus* were isolated from surface water. The strains were stored at -70℃ in Luria-Bertani (LB) broth containing 20% glycerol. All strains were confirmed by biochemical reactions and serological test, and the presence of *ctxAB* gene was confirmed by DNA probe hybridization.

Experimental Design

Culture was grown in LB broth at 37℃. Bacteria at the exponential phase were harvested by centrifugation at 4000 rpm for 5 min at 4℃, washed twice in phosphate buffer saline (PBS, pH 7.4). Cell suspension was mixed with freshly prepared 4% paraformaldehyde in PBS (pH7.4) at the ratio of 1:3 (v/v), and fixed at 4℃ for 1 h. Cells were then washed twice in PBS and mixed with an equal volume of absolute ethanol sterilized by filtration, then stored at -20℃. Before ISPCR, the cells were washed twice in PBS and permeabilized with 1 mg/mL lysozyme at 30℃ for 20 min.

For the PCR, primers were *ctxAB*-F (5'-agtcaggtggtcttatgcc-3') and *ctxAB*-R (5'-ttgccatactaattgcgg-3'). The primer of *ctxAB*-FAM-R was labeled at the 5' end of *ctxAB*-R with a fluorescent dye FAM. To confirm that the *ctxAB* genes could be successfully amplified using the pairs, we first purified genomic DNAs of relevant bacterial strains and then ran conventional PCR on them. A PCR contained 5 μL 1 mmol/L dNTP, 1 μL each primer, 10 μL 10×buffer with $MgCl_2$ and 0.5 μL *Taq* DNA polymerase (per 100 μL reaction mixture). This was made up to 90 μL with sterilited water, before adding 10 μL target DNA. PCR was run for 30 cycles (denaturation at 94℃ for 20 s, annealing at 50℃ for 20 s, and extension at 72℃ for 40 s). A final extension step was performed at 72℃ for 3 min, after which samples were held at 4℃. Products from these reactions were checked by agarose gel electrophoresis and stained with ethidium bromide. After confirmation of successful amplification, another PCR was run to test the amplification efficiency of fluorescent dye-labelled primers using the same PCR conditions as the conventional one. Once the result showed no variation with that of the conventional PCR, we proceeded to evaluate various conditions for the ISPCR.

ISPCR was run with 10 μL aliquot of permeabilized cells (10^8 cells/mL) rather than purified genomic DNAs. ISPCR conditions were the same as above with some minor modifications, *viz.* adding 2.5% glycerol to the PCR mixture. Cells with ISPCR reaction mixture but excluding *Taq* DNA polymerase were used as one of the negative controls. Cells were washed twice with PBS before analysis.

To confirm the successful *in situ* amplification of our target gene, we extracted DNA from the cells and ran gels as follows: cells in the PCR reaction system were lysed with the addition of 0.05 volume 10% SDS and 0.05 volume proteinase K (2 mg/mL). Samples were shaken at 37℃ until clear before undergoing phenol/chloroform extraction. DNA was ethanol-precipitated and resuspended in 1 × TE. Amplified DNA was electrophoresed with ethidium

bromide. Gel was examined under U.V. Illumination.

The ISPCR-amplified cells were spotted onto a slide and examined using epifluscence microscopy with 1000 magnification (eclipse E400, Nikon, Japan). Both target and control cells were viewed under blue light excitation (492 nm) using the filter B-2A. Images were obtained using a digital imaging system (Cool PIX 4500, Nikon, Japan) mounted onto the epifluscence microscope using the objective, Plan Fluor 100×/1.30 oil.

For the flow cytometer, a Coulter EPICS Elite flow cytometer was used. Samples were excited with an argon ion laser operating at a wavelength of 488 nm. For analysis of the *ctxAB* gene, green fluorescence was captured using a 518-nm band pass filter. To determine the level of background fluorescence from the FAM-labelled primers, unlabeled cells stained with fluorescein-primer under identical conditions of ISPCR, without the addition of *Taq* DNA polymerase, were used. To avoid the interference of fluorescence from bacteria, the cells in the same PCR mixture omitting fluorescent dye-labelled primer were used as the control.

ISPCR-FCM was run on several closely related vibrios. When tested individually, ISPCR-FCM targeting *ctxAB* toxin gene was run on the bacterial strains using the parameters set up above. To test the sensitivity of ISPCR-FCM, we serially diluted the *ctxAB*$^+$ *V. cholerae* from 10^8 down to 10^2 cells/mL, and then ran ISPCR-FCM on them.

RESULTS

Confirmation of ctxAB Primers

Our initial work further confirmed that *ctxAB* gene could be specifically amplified using *ctxAB*-F and *ctxAB*-R prime pairs, and that no cross-reaction was observed when tested on several closely related vibrios as well as other relevant bacteria. To further confirm that the amplicons are the target gene we sequenced some of the PCR products. By BLAST, they displayed significant or nearly identical DNA homology with the previously published sequences of *V. cholerae ctxAB* gene (data not shown).

After the confirmation of specificity and the working of conventional PCR, we then ran PCR with fluorescent dye-labelled primers, *viz.* *ctxAB*-FAM in an attempt to optimize PCR reactions. PCR with conventional primer pairs as well as with the fluorescent dye-labelled ones gave the same results, indicating that fluorescent dye on the PCR primer could not interfere with PCR reaction at all.

Modifications of Fixation and Permeabilization of Cells

To amplify the target DNA sequences within the intact *V. cholerae* cells, cells were fixed and permeabilized for the reagents to access the target DNA. To optimize the fixation efficiencies, two different fixatives and their conditions, *i.e.* 4% paraformaldehyde (4% PFA) and ethanol were used. In the case of ethanol, our results showed that ethanol was not the best choice whatever the different lengths of fixation time and temperatures (room temperature or -20℃), which caused destruction of most of the cells. On the other hand, with PFA as a fixative there was no such problem. With 4% PFA as the fixative, 1 h of fixation was sufficient. *ctxAB* gene within the *V. cholerae* cells was amplified and the amplicons were retained inside the cells. Hence, 1 h fixation with PFA at 4% was optimal in fixing *V. cholerae*.

To test whether a treatment with lysozyme represents a critical step for ISPCR, we tried different digestion time after 4% PFA fixation. Incubation for 5 min was too short and only few cells were stained by the dye. Incubation for 20 min, however, gave the best results while incubation for 30 min yielded a diffuse fluorescence out of cells, suggesting overdigestion. From these results, we chose 20-min incubation for the lysozyme treatment step. We also tested the effect of 4% PFA alone without lysozyme. After ISPCR, there was no detectable fluorescence under epifluorescence microscope, which implied that no ISPCR reaction occurred at all. This further demonstrated the role of lysozyme in the enhancement of cell membrane permeability.

Maintenance of Cell Integrity During ISPCR Thermal Cycling

To test whether *V. cholerae* cells were able to withstand repetitive heating, cells were placed in normal PCR buffer and put through 30 thermal cycles. Cells were enumerated before and after this treatment. Changes were noticeable under the microscope, and the changes prevented cell counts even after two thermal cycles, suggesting that without any protection, repetitive heating in the ISPCR would destroy the cells. Therefore, to improve cell's resistibility to heat, we added glycerol into the PCR reaction mixture. Experiments showed that 2.5% glycerol in reaction mixture would give the best results. Microscopic count revealed that although there were observed changes, they nevertheless did not affect the subsequent operation and the cells remained intact. We therefore included 2.5% glycerol

in the subsequent ISPCR reaction mixture.

Validation of the ISPCR-FCM

To examine the validity of the ISPCR-FCM, cells were first checked by epifluorescence microscopy after ISPCR. When *ctxAB* gene was amplified, cells showed up green (Fig.1). In all ISPCR reactions, a negative control consisting of cells and fluorescent dye-labelled primers without *Taq* DNA polymerase was included. The resulting cells could not be visualized clearly under blue light excitation.

As epifluorescence microscopy is a tedious and labor-intensive process, it is not an ideal method to screen a large amount of samples. Therefore, alternatives should be sought after. Here, we demonstrate the feasibility of employing FCM as an alternative in screening the ISPCR samples.

After ISPCR was confirmed to be successful by the epifluorescent microscopy, cells were subsequently analyzed by flow cytometry, and results are shown in Fig. 2. Our results showed that 100% of the *V. cholerae* cells were amplified by ISPCR. The labelled *V. cholerae* cells were well separated from the unlabeled cells. This result clearly demonstratled that ISPCR-FCM could be used to screen and enumerate the toxigenic *V. cholerae* strains.

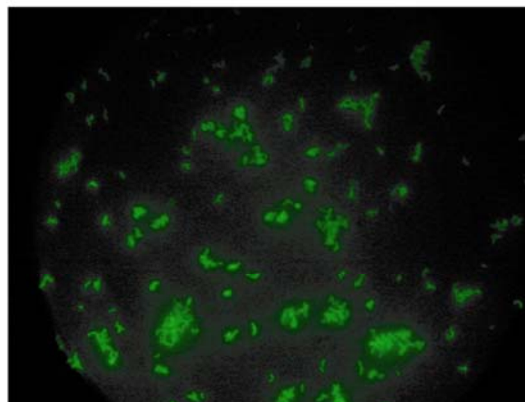

Fig. 1. *in situ* polymerase chain reaction (PCR) analysis of *V. cholerae* for the presence of *ctxAB* gene: Epifluorescence microscopic picture of the cells subjected to ISPCR using fluorescein-labelled primer specific for *ctxAB*. Cells were used in the reaction at the density of 10^8 cells/mL.

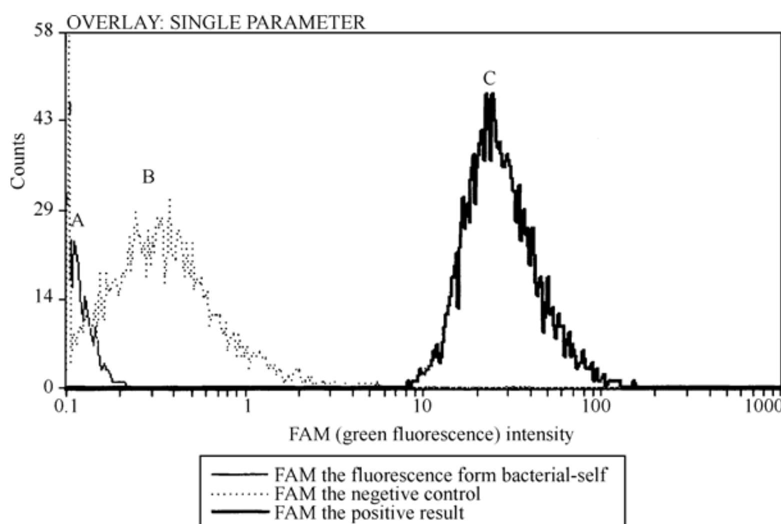

Fig. 2. Flow cytometer histograms demonstrating viability assessment of the $ctxAB^+$ bacteria using ISPCR. (A) Histogram profile of the cells subjected to ISPCR without the FAM-labeled primers; (B) Histogram profile of the cells subjected to ISPCR without the enzyme *Taq* polymerase; (C) Histogram profile of the cells subjected to ISPCR.

Confirmation of ISPCR Amplified Products

To confirm the *in situ* amplification of our target gene, cells were lysed immediately after ISPCR, and the amplified DNA was extracted and electrophoresed in ethidium bromide-stained agarose gel to check if the PCR products had the expected size. As shown in Fig. 3, the *ctxAB* genes (1037 bp length) amplified by ISPCR was identical with the other amplicons by conventional PCR. The amplified products were sequenced and displayed significant identical DNA homology with the previously published sequences of *ctxAB* gene by BLAST (data not shown).

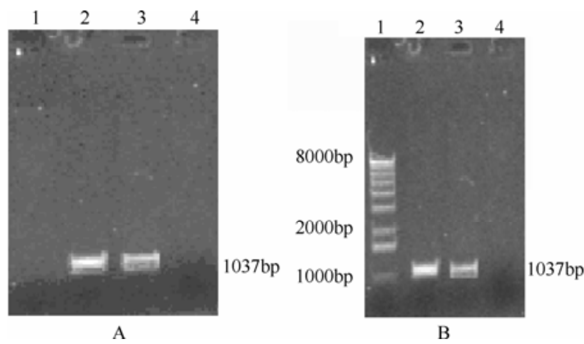

FIG. 3. Agarose gel electrophoresis detecting the *ctxAB* genes in toxigenic *V. cholerae* after ISPCR. (A) Agarose gel electrophoresis without ethidium bromide; (B) Agarose gel electrophoresis with ethidium bromide. Lanes: 1, molecular mass marker (Do16-2); 2, conventional PCR using the FAM-labelled primers; 3, amplified DNA extracted from the cells after ISPCR; 4, conventional PCR using the primer pair *ctxAB*-F and *ctxAB*-R. An identical 1037 bp product was obtained in all PCR.

Specificity and Sensitivity of ISPCR-FCM

Specificity tests clearly showed no cross-reaction of ISPCR primers with other tested bacteria. To test the sensitivity of ISPCR-FCM, we serially diluted the *V. cholerae* from 10^8 down to 10^2 cells/mL, and then ran ISPCR-FCM on them. Results showed that due to the multiple step processing, the sensitivity of ISPCR-FCM was slightly hampered. Up to now, we could only detect/enumerate as low as 10^5 cells/mL in a sample.

DISCUSSION

Porter *et al*.[12] reported that ISPCR could not successfully amplify a 1500-bp target sequence of 16S rrn. Haase *et al*.[14] considered that the product length is of critical importance, as too short, it would leak from cells, and too large, defeated. Keeping them in mind, we successfully *in situ* amplified genes within 1500 bp in this study. By employing our ISPCR approach, we could obtain approximately 100% of the *V. cholerae* cells amplified and no leakage of amplicons was observed.

Ramaiah and Karina[13] reported that ethanol could be used as a good fixative and permeabilizing agent because of its lipid-solubilizing properties and extraction of high molecular proteins from the cells. Nevertheless, close microscopic analysis of cells subjected to ISPCR showed that a significant fraction of the ethanol fixed cells was destroyed as compared with paraformaldehyde fixed cells. In view of this finding, we resorted to paraformaldehyde as the fixative throughout our experiments. Contrary to the previous report that specific amplification of target sequence could not be achieved in paraformaldehyde-fixed cells by ISPCR[11], our results proved the otherwise, even though paraformaldehyde could give birth to nucleic acid damage because of the methylene bridges between functional groups. Based on our ISPCR results, we do not consider that using paraformaldehyde as a fixative is the critical step leading to no results for ISPCR. Instead, we think that the characteristics of the examined cells determine what kinds of fixative should be used.

Porter *et al*.[12] reported that about 50% of fixed cells could withstand repeated heating and be used to detect specific gene sequences inside intact cells. However, in our study, the thermal cycling was very destructible to *V. cholerae* cells, even only with the fixed cells without any PCR reaction mixtures. To circumvent this problem, we tested glycerol for this purpose. As glycerol can protect bacteria under very low temperature (-70℃), it may also be used when bacteria are exposed to high temperature. In addition, glycerol can improve the PCR amplification efficiency[15]. Our results confirm our thinking and show that glycerol is effective in maintaining bacterial morphology throughout the thermal cycling. By cell counts, our results show that the rate of cell loss can be reduced to 10% after the whole analysis process of ISPCR-FCM.

The extent of freshness of the fixed cells is another factor that could lead to the failure of ISPCR. As some pathologists reported that the longer the samples are stored, the lower the probability of success is to amplify the target DNA sequence inside intact cells by ISPCR, the *V. cholerae* cells face the same problem. We tested the cells stored for 3 weeks at -20 ℃ after fixation. ISPCR-amplified cells showed no fluorescence at all as examined by FCM, confirming the importance of freshness of the prepared samples. So, the fixed cells should be discarded if kept for 3 weeks or longer.

Detection of amplified products can be achieved using either direct incorporation of labeled nucleotides[12-13] and fluorescent primers[16], or by subsequent *in situ* hybridization of the amplified DNA[17]. In this study, we used the fluorescence-labelled primers for the detection of ISPCR-amplified products inside intact bacterial cells. For this detection, we have to face two kinds of noise of ISPCR. One is the binding of fluorescence-labelled primers to the cellular material, the other is the amplification of non-specific DAN sequence[11]. Negative ISPCR controls were also analyzed by FCM,

which contained equal amounts of fluorescent-labelled primers, void of Taq DNA polymerase. As a result, the control cells were separated from the positive cells containing amplicons under identical settings. So, this noise could not interfere with the flow cytometric measurements of positive cells virtually. As to the amplification of non-specific DAN sequence, we made sure that the fluorescence-labelled primers had no cross-reaction with foreign bacteria, especially the other relevant bacteria, then to further confirm the *in situ* amplification of our target gene. Cells were lysed immediately after ISPCR, and the amplified DNA was extracted and electrophoresed in ethidium bromide-stained agarose gel to check if the PCR products had the expected size. The results further show that the specificity of the method is high. So, our data clearly demonstrate that as a novel method, ISPCR-FCM can be used to specifically detect/enumerate the toxigenic *V. cholerae* strains, albeit with the shortage of relative lack of sensitivity. This is due to the multiple processing of the method, which causes the loss of some of the target cells. Further study should be carried out to shorten the procedures so as to enhance its sensitivity.

The *ctxAB* gene has been used as a marker for the txoigenic *V. cholerae* strains, whose presence indicates the possible presence of cholera. Either molecular methods or routine culture can be used to detect this bacterial pathogen. But both are unfit for detecting the bacteria from environment directly. The former needs nucleic acid rather than cells, the latter is very laborious and does not detect the VBNC bacteria. The combination of ISPCR and FCM is a relatively simple and promising tool for detecting toxigenic strains from environment. The ISPCR-FCM technique has an advantage for VBNC cells over other methods in that it can be directly applied to screening and enumerating the VBNC cells. Apart from VBNC cells, this technique can also be used to analyze and enumerate normal forms of *ctxAB*-possessing *V. cholerae* cells within a complex microbial community, aiding in the prediction of potential cholera outbreak.

Our further studies are to investigate environmental bacteria in coastal or river waters directly. Although there are a number of challenges ahead, such as the high salinity, natural inhibitors, planktons, the combination of ISPCR and FCM remains a promising tool for the effective detection of a particular group of bacteria in the environment. How to improve the sensitivities of this new method is still a big problem.

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