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SHORT COMMUNICATION

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The detection of picoplankton 16S rDNA in cases of drowning

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Abstract Picoplankton belonging to the *Synechococcus* genus in cyanobacteria (approximately 1 μm in size) are found ubiquitously in Lake Biwa, Japan. However, they could not be morphologically discriminated from other bacteria by microscopy. In this study we attempted to use picoplankton for the diagnosis of drowning by PCR analysis of the 16S ribosomal DNA (rDNA). We designed primers complementary to the variable regions of 16S rDNA of the picoplankton we had sequenced. A comparison was made of the PCR products from the three picoplanktons, five other cyanobacteria, *Melosira* (diatom), *Staurastrum* (green alga), bacteria from Lake Baikal, and humans. The picogram order of template DNA from picoplankton was specifically amplified by the primers. When the template of picoplankton was mixed with human lung tissue, at least 10 ng of template DNA was needed to obtain a PCR product. The isolation of the picoplankton from human lung tissue increased the sensitivity of PCR more than a hundred-fold. The specific PCR products of the picoplankton were obtained from formalin-fixed drowning tissue. Molecular biological diagnosis of drowning was successful using picoplankton 16S rDNA.

Key words Picoplankton · Drowning · 16S rDNA · PCR

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Introduction

A diagnosis of death by drowning is normally based on findings from the external examination of the body and autopsy together with laboratory investigations. Revenstorf [1] proved drowning through the demonstration of algae by direct microscopical examination of water from subpleural lung tissue from all parts of the lungs. The detection of diatoms in organs using the chemical digestion method is routinely performed on cadavers for the diagnosis of drowning [2, 3]. Recently, a safer digestion method with solubilizer instead of strong acids was reported [4]. The enzymatic method which has the advantage of detecting zooplankton and phytoplankton including diatoms was also demonstrated [5]. However, a method to detect minute plankton samples has not yet been developed. Planktons are classified into picoplankton (2 μm or less), nanoplankton (2 μm–20 μm) and microplankton (20 μm–200 μm). Nanoplankton and microplankton detected in organs are utilized in signs of drowning. Picoplankton belonging to the *Synechococcus* genus in cyanobacteria (approximately 1 μm in size) are found ubiquitously in Lake Biwa, Japan. Although the three strains were isolated and designated brown, pink, and green according to their color in the cultivation, they can not be morphologically discriminated from other bacteria by microscopy. Since they have a similar size and shape to other bacteria, a differentiation between picoplanktons and other bacteria was examined by the autofluorescence of picoplankton under epifluorescence microscopy [6]. In this study, we attempted to use the picoplanktons for the diagnosis of drowning by PCR analysis of the 16S rDNA. The secondary structures of 16S rDNA in both prokaryotes and eukaryotes are similar and most of the sequence is relatively conserved, however, prokaryotic 16S rDNA contains nine variable regions [7]. Sequence comparisons of the variable regions of 16S rDNA contained sufficient information to allow the measurement of both close and distant phylogenetic relationships. We searched a DNA data base and designed specific primers that were complementary to the variable

regions of 16S rDNA of the picoplankton we had sequenced [8, 9]. The amplification of three picoplankton, five other cyanobacteria, *Melosira* (diatom), *Staurastrum* (green alga), bacteria from Lake Baikal and human DNA were investigated using the primers. We performed isolation of the picoplankton from human tissue and applied this method to formalin-fixed human tissue from cases of drowning.

Materials and methods

Isolation of plankton

Water samples from Lake Biwa (Shiga prefecture) were aseptically concentrated onto 2 μm pore size filters under moderate vacuum, further diluted with sterile CT medium [10], and incubated at 20° C under low fluorescent light with a 12:12 light and dark cycle. By repeated dilution and subsequent cultivation, the three picoplankton strains (brown, green and pink), *Melosira* (diatom), and *Staurastrum* (green alga) were obtained in a pure form. The five other cyanobacteria (*Synechococcus* S7002, *Synechocystis* PCC6803WT, *Chlorogloeopsis* PCC6912, *Anabaena* PCC7120, *Chroococcidiopsis* PCC7203) and bacteria from Lake Baikal were isolated on CT medium plus 1% agar and cultivated.

DNA extraction of plankton

Each picoplankton strain, five other cyanobacteria, *Melosira*, *Staurastrum* and bacteria from Lake Baikal were harvested aseptically by filtration. They were resuspended in 400 μl TE buffer plus 10% SDS and lysed by freezing and thawing, extracted once in phenol, once in phenol/chloroform then precipitated using absolute ethanol at –85° C. The DNA was then pelleted by centrifugation, washed in 80% ethanol, vacuum-dried briefly and resuspended in 100 μl sterilized TE buffer. The amount of DNA was then quantified fluorometrically.

Sample preparation

Aliquots of DNA from the brown strain ranging from 10 ng to 1 μg were thoroughly mixed with 30 mg human lung tissue in 400 μl BCL buffer (10 mM Tris-HCl pH8.0, 5 mM $MgCl_2$, 32 mM Sucrose, 1% Triton) containing 50 mg of Proteinase K, plus 10% SDS, and incubated at 70° C for 1 h. The mixture was then extracted according to the method described.

Sample pretreatment

After mixing 100 mg human lung tissue and 10^2–10^5 cells of the green strain, the mixture was homogenized in 1 ml TE buffer and centrifuged at 6000 rpm for 5 min. The supernatant was extracted by the organic solvent method and concentrated with DNA ultrafilter tubes (molecular weight cut-off = 30,000, Takara, Japan).

Samples without pretreatment were incubated at 70° C for 1 h and DNA was also extracted by the method described.

PCR conditions

The primers for amplification of the picoplankton in the fifth and sixth variable regions of 16S rDNA are as follows:

forward primer TAAACGATGAATGCCAGACGTCGGC

reverse primer AAAGACCCATCTCTGGATCCCCGAC

PCR conditions were as follows: in a total volume of 50 μl were mixed DNA of each plankton as template, primers each at 1 μM, deoxynucleotide triphosphates (Takara, Japan) each at 250 μM, and 1 × amplification buffer (10 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 50 mM KCl). After denaturing at 98° C for 5 min, 0.5 unit of Taq DNA polymerase (Takara, Japan) was added to the mixture. Amplification reactions were carried out in 0.2 ml microAmp reaction tubes (Perkin Elmer, USA) with a Perkin Elmer Cetus 9600 thermal cycler. Cycling conditions for 38 cycles were as follows: 94° C for 1 min, 65° C for 1 min, and 72° C for 1 min with an additional extension step of 72° C for 10 min. After electrophoresis in 2% NuSieve 3:1 agarose gel (FMC BioProducts, USA), amplification products were identified by staining with ethidium bromide and visualization with UV light.

Results

The templates of cyanobacterial picoplanktons (brown, pink, and green strains) were specifically amplified by the primers. However, the PCR products of the five other cyanobacteria, *Melosira* (diatom), *Staurastrum* (green alga), bacteria from Lake Baikal and human DNA were not detected. (Fig. 1) The segment sizes of PCR products were approximately 210 bp in length. When PCR was performed using 10 ng–1 pg of the green strain in a 50 μl reaction volume, up to 10 pg of the template could be amplified. (Fig. 2) In addition, similar results were obtained

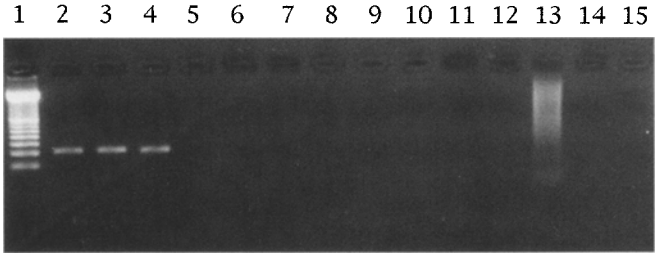

Fig. 1 Agarose gel electrophoresis of PCR products amplified from various templates. The 100 bp ladder (Pharmacia, Sweden) was used as molecular weight standard. Lane 1, 100 bp ladder size standard; lanes 2–4, products from brown, green, pink strain of picoplankton, respectively; lanes 5–9, products from five cyanobacteria (*Synechococcus* S7002, *Synechocystis* PCC6803WT, *Chlorogloeopsis* PCC6912, *Anabaena* PCC7120, *Chroococcidiopsis* PCC7203); lane 10, *Melosira* (diatom); lane 11, *Staurastrum* (green alga); lane 12, bacteria from Lake Baikal; lanes 13 and 14, human DNA from blood and human DNA in a hundred-fold dilution; lane 15, negative control (distilled water blank)

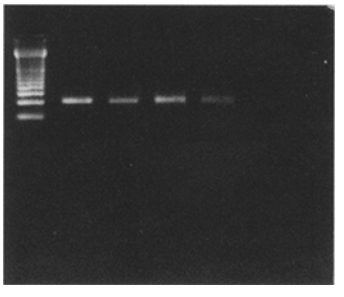

Fig. 2 Agarose gel electrophoresis of PCR products amplified from different concentrations of the green strain. Lane 1, 100 bp ladder size standard; lanes 2–6, products from 10 ng, 1 ng, 100 pg, 10 pg, 1 pg of green strain, respectively; lane 7, negative control (distilled water blank)

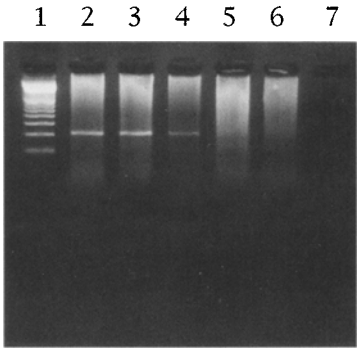

Fig. 3 Agarose gel electrophoresis of PCR products amplified from the mixture of the different concentrations of the brown strain with 30 mg of human lung tissue. Lane 1, 100 bp ladder size standard; lanes 2–6, products from 1 μg, 100 ng, 10 ng, 1 ng, 100 pg of the brown strain in final concentrations, respectively; lane 7, negative control (distilled water blank)

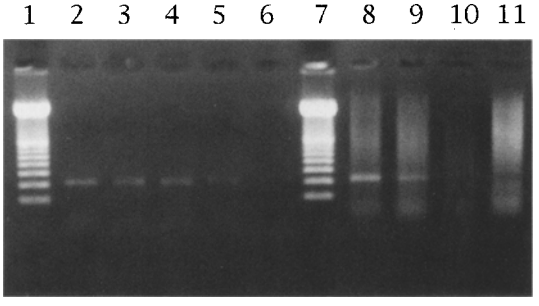

Fig. 4 Agarose gel electrophoresis of PCR products amplified from the mixture of the different cell numbers of the green strain with 100 mg of human lung tissue. Lanes 2–5 pretreated samples (isolation of the picoplankton from human lung tissue) and lanes 8–11, non-treated samples; lanes 1 and 7, 100 bp ladder size standard; lanes 2–5 and 8–11, products from 10^5 cells, 10^4 cells, 10^3 cells, 10^2 cells of the green strain in final cell numbers, respectively; lane 6, negative control (distilled water blank)

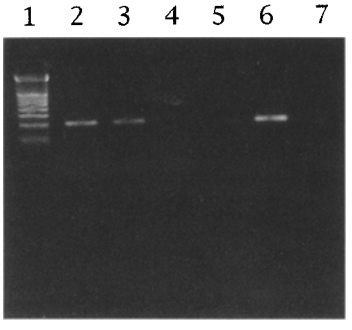

Fig. 5 Agarose gel electrophoresis of PCR products amplified from formalin-fixed human tissues. lane 1, 100 bp ladder size standard; lane 2, formalin-fixed human lung tissue found in river downstream from Lake Biwa; lane 3, formalin-fixed human liver tissue; lane 4, negative control (human lung tissue) lane 5, negative control (human liver tissue); lane 6, picoplankton of green strain; lane 7, negative control (distilled water blank)

from the brown strain, whereas up to 100 pg template of the pink strain was amplified (data not shown). On the other hand, we experimentally mixed a series of ten-fold dilutions of the brown strain template with 30 mg of human lung tissue. In the case of human lung tissue containing at least 10 ng of the brown strain template, the PCR product could be demonstrated (Fig. 3). Therefore, the pretreatment of samples (isolation of the picoplankton from lung tissue by centrifugation at 6000 rpm) was tested. Consequently, the sensitivity of PCR increased 100 times compared with samples without pretreatment. (Fig. 4) When we applied this method to a drowned body that was found in the river downstream from Lake Biwa, the specific PCR products were obtained from formalin-fixed human lung and liver. (Fig. 5) In addition, similar results were obtained from kidney and spleen (data not shown).

Discussion

Small amounts of the template from picoplankton were specifically amplified by the primers we designed. The picoplankton could not be morphologically discriminated from other bacteria by microscopy because of a similar size and shape. But our new molecular biological method solved this problem. It has been considered that the PCR analysis of the 16S rDNA is useful for the diagnosis of drowning by detection of the picoplanktons. After mixing the template from picoplankton with human lung tissue, however, most of the DNA extracted from the mixture was human DNA and the DNA from picoplankton was a minor component. In other words, the template of picoplankton was diluted by the human DNA. Since the picoplankton could be precipitated by centrifugation at more than 9000 rpm, we separated the picoplankton from human tissue by centrifugation at 6000 rpm 5 min. Consequently, the sensitivity of PCR increased 100 times compared with samples without isolation. Therefore, it has been considered that pretreatment of the samples (isolation of the picoplankton from human lung tissue) is essential for the practical examination. The picoplankton in Lake Biwa belongs to the *Synechococcus* genus in cyanobacteria that is the most ubiquitous picoplankton both in marine and fresh water. Comparing the picoplankton 16S rDNA sequence obtained by us with other known sequences, the picoplankton in Lake Biwa was more similar to the marine bacterioplankton (cyanobacterial picoplankton) in the Sargasso Sea of the West Indies [9]. When PCR simulation using Amplify computer software was carried out by primers selected from the third and fourth variable regions of 16S rDNA (V3 and V4 primer), the same length of PCR products were obtained (Fig. 6). A search of the DNA data base from Gene Bank in Internet showed that the sequence of these primers has similar to that of close phylogenetically related species. The sequence of *E. coli* and other bacteria did not agree with that of the V3 and V4 primers. This indicated that the genus-specific sequences in the variable regions of 16S rDNA are useful as primers for marine and fresh water picoplankton. However, the ubiquitous location of picoplanktons in Lake Biwa is a disadvantage for the inference of the site of drowning. The qualitative or quantitative identification of various planktons is essential to the analysis of the site of

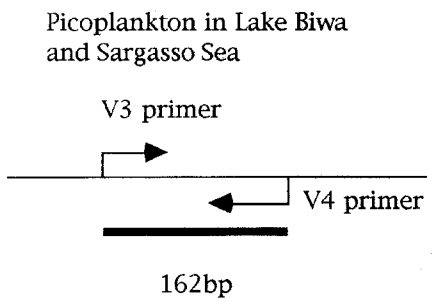

Fig. 6 PCR simulation using Amplify computer software. The sequence of primers in third and fourth variable regions of 16S rDNA as follows; V3 primer: AAGAAGAAGATCTGACGGT. V4 primer: GGAGTTAAGCTCCACGCTTT

drowning. If molecular biological methods are applied to many kinds of plankton, it is possible to detect nanoplankton and microplankton including picoplankton that cannot be morphologically discriminated by microscopy. Since sequence comparisons of the variable regions of 16S rDNA contained sufficient information to allow the measurement of both close and distant phylogenetic relationships, species or genus specific primers may be designed for variable regions of 16S rDNA. When multiple PCR is performed using the species or genus specific primers, various numbers and sizes of the PCR products depending on the different variable regions of 16S rDNA are obtained. In addition, it is considered that the fingerprint-like pattern can be applied to locate the site of drowning. Consequently, the combination of this method with the traditional chemical digestion method [2, 3], a new digestion method with the solubilizer [4] and the enzymatic digestion method [5] are more suitable for the identification of the site of drowning.

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