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Real-time PCR assay for the detection of picoplankton DNA distribution in the tissues of drowned rabbits

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ABSTRACT

The detection of plankton DNA is one of the important methods for the diagnosis of drowning from post-mortem tissues. This study investigated the quantities of picoplankton (Cyanobacteria) DNA in the lung, liver, kidney tissues and blood of drowned and non-drowned rabbits, and the sensitivity of detection of picoplankton DNA by polymerase chain reaction (PCR) detect for the diagnosis of death from drowning. For this purpose, the DNA of the 16S ribosomal RNA gene of picoplankton was quantitatively assayed from the tissues of drowned and non-drowned rabbits immersed in water after death. Each of the liver, kidney and lung tissues and blood were obtained from drowned and non-drowned rabbits. Picoplankton DNA in the tissues was extracted using the DNeasy® Blood & Tissue kit to determine the yield of picoplankton DNA from each tissue. TaqMan real-time PCR was performed for quantitative analysis of picoplankton DNA. Target DNA was detected in the liver, kidney and lung samples obtained from the drowned rabbits, while no picoplankton DNA was detected in the non-drowned rabbit tissues (except in lung samples). The results verified that direct PCR for the detection of picoplankton DNA is useful for the diagnosis of drowning. Although we observed seasonal changes in the quantity of picoplankton in river water, we were able to detect DNA from various organs of drowned bodies during the season when picoplankton were not the most abundant.

1. Introduction

In forensic medicine, the diagnosis of a corpse immersed in water, i.e. differentiation between death from drowning or on entering the water, is primarily made using the diatom test by acid digestion [1,2]. However, this test is technically complicated and requires a larger quantity of sample. Moreover, the procedure is hazardous due to the use of a strong acid, and is time consuming [3]. Alternative methods for the diagnosis of drowning by detecting the genes of picoplankton living in water using molecular biological techniques have been reported [4]. The method of examining a corpse immersed in water using the polymerase chain reaction (PCR) to amplify specific plankton genes has been recently evaluated [5,6]. By performing one PCR amplification using various organs of a corpse immersed in water, this method is anticipated to permit rapid and accurate diagnosis of death from drowning by identifying the genes of phytoplankton that had entered solid organs via blood circulation.

Synechococcus is a unicellular cyanobacterium that is very widespread in the marine environment. Its size varies from 0.8 μm to 1.5 μm. Synechococcus is one of the most important components of the prokaryotic autotrophic picoplankton in the temperate to tropical oceans and fresh water. The genus was first described in 1979 [7,8] and was originally defined to include "small unicellular cyanobacteria with ovoid to cylindrical cells that reproduce by binary traverse fission in a single plane and lack sheaths" [9]. The currently used PCR method for the detection of plankton requires DNA purification, necessitating the treatment of tissues as well as the differentiation between sea water and fresh water plankton. Thus this method has the disadvantages of being labor intensive and having the risk of contamination [10]. To overcome these issues, we first targeted the Synechococcus sp (Bacteria; Cyanobacteria; *Oscillatoria psychidae*; and Chroococcales), so that at least, the PCR primers can be matched to 50% cyanobacteria species, a picoplankton found in both sea and fresh water, and designed primers specific for its 16S ribosomal RNA

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genes (16S rDNA). Then, we used the direct PCR method without DNA extraction to examine autopsy cases diagnosed as death from drowning from autopsy findings and diatom test by acid digestion and succeeded in detecting plankton DNA using minute quantities of tissues from organs of the drowned bodies [11].

In the case of death on entering the water, diatom analysis by acid digestion may detect plankton from the lung, but unlikely from the liver or kidney because blood circulation had already stopped when entering the water [12]. However, because PCR has a high sensitivity of detection, further verification by animal experiments is necessary to establish direct PCR as a method of diagnosing death from drowning. Moreover, the relationship between the time of immersion in water and the quantitative distribution of planktons in solid organs such as the lung, liver and kidney, as well as the effect of this relationship on the detection of plankton DNA by the direct PCR method, needs to be elucidated. In the present study, we produced a model of drowning using rabbis and examined the distribution of plankton DNA in the blood and various organs by real time PCR. Moreover, we used a direct PCR method to detect plankton DNA from the organs of drowned rabbits and rabbits immersed in water after death (non-drowned rabbits).

2. Materials and methods

2.1. Experimental protocol and sample collection

A total of 12 Japanese white rabbits weighing between 2.0–2.5 kg were used in the experiments (the use of animals approved by the Animal Experiment Committee of Nihon University School of Medicine). Water was collected from the middle reach of the Edo River (Saitama, Japan), in summer (July) and transferred immediately into a bath. This water was used to immerse the rabbits. Six rabbits in the drowned group were divided into two subgroups: three rabbits were immersed in water for approximately 2 h and three rabbits were immersed for 3 days. Six rabbits in the control non-drowned group were euthanized by the intravenous injection of pentobarbital. The non-drowned group was also divided into two subgroups: three rabbits were immersed in water for approximately 2 h and three rabbits were immersed for 3 days. The water was approximately 50 cm in depth and the water temperature was 20–23°C. The drowned rabbits floated to the surface of the water, while the non-drowned rabbits were found under the surface of water (but did not sink to the bottom). During organ sampling, approximately 1 ml of heart blood was collected from each rabbit. The blood and organ samples were assayed for plankton DNA.

To investigate the seasonal changes in the quantity of plankton in the river water, we collected 1 ml of water from the middle reach of the Edo River in January, April, July and October. The water of each of the three samples was quantitatively assayed for picoplankton DNA in different seasons.

2.2. Isolation of picoplankton DNA from rabbit tissues

Autopsies were conducted on the rabbits of both groups. All the appliances and the containers were washed with distilled water and sterilized before use; and the appliance was changed according to the organ, taking care to prevent contamination. The lung, liver and kidney of each rabbit were removed and stored in a freezer until use. For DNA extraction, approximately 1 g of tissue from each organ was sampled, minced finely with a scalpel and placed in liquid nitrogen. These tissues were used as homogenates. Then 30 mg of the homogenate was used for DNA extraction using the DNeasy® Blood & Tissue kit (Qiagen, Chatsworth, CA, USA). Blood sample (100 μl) and water sample (1 ml water was centrifuged, and then 800 μl of the supernatant was removed) were also subjected to DNA extraction by the same method. *Euglena gracilis* provided by the National Institute for Environmental Studies (Tsukuba, Japan) was used as the standard organism for amplifying the plankton DNA. All DNA solutions collected from the DNeasy Spin Column were eluted with 100 μL of Buffer AE.

2.3. Quantitative determination of picoplankton DNA in rabbit tissues

Quantitative analysis of picoplankton DNA was conducted using the Step One Plus™ Real Time PCR System (Applied Biosystems, Foster, USA) using 1 μl of DNA solution extracted from each rabbit organ, rabbit blood and river water sample. The sequences of primers and probes used in real time PCR were the same as those reported previously [11]. The real time PCR reaction mixture (total volume 20 μl) contained 10 μl of 2 × TaqMan® Fast Advanced Master Mix (Applied Biosystems), 0.5 μl of 20 × TaqMan Probe & Primer Mix, 1.0 μl of DNA solution, and 8.5 μl of nuclease-free water. The real-time PCR thermal cycling protocol was 95 °C for 20 s followed by 50 cycles of 95 °C for 1 s and 60 °C for 20 s. The standard curve was plotted from 1 ng of *Euglena gracilis* DNA by diluting 10, 100, 1000 and 10,000 times, and its average R^2 is 0.983. Although *Euglena gracilis* is neither a picoplankton nor a cyanobacterium, the PCR primers can detect 16S rDNA as a target for PCR amplification.

2.4. Direct PCR amplification from the tissues of drowned and non-drowned rabbits

Then, we verified whether picoplankton DNA could be detected from various organs in drowned and non-drowned rabbits by direct PCR without DNA extraction. Five milligrams of each organ was treated by the digesting buffer, and direct PCR amplification was performed with the same PCR primers described above. Although the specificity of the PCR primers has not been confirmed using actual genomic DNA from various microbes, PCR can detect sequences matching several picoplankton species according to published databases. The PCR products were analyzed by 5% polyacrylamide electrophoresis [11]. In addition, the PCR products obtained from purified DNA of blood and river water were also analyzed by the same method.

3. Results

Real-time PCR quantification of the 16S rDNA in the lung, liver and kidney tissues of drowned rabbits immersed in water for 2 h showed that the picoplankton DNA could be detected from all the three organs of all six rabbits (Fig. 1). A total of 30 mg of each organ yielded more than 20 ng of 16S rDNA. No remarkable differences in quantity were observed among the lung, liver and kidney tissues. The presence of plankton DNA was also confirmed by analysis of 100 μl of blood. In rabbits that entered water after death (non-drowned group) and immersed for 2 h, while a small quantity of plankton DNA was detected in the lung sample of one rabbit, the DNA was undetectable from the liver, kidney and blood samples.

We also quantified the plankton 16S rDNA in the lung, liver and kidney tissues of drowned and non-drowned rabbits immersed in water for 3 days using the same real time PCR method. The results were similar to those obtained from drowned rabbits immersed for 2 h (data not shown). The plankton DNA was quantified from the lung, liver, kidney, and blood of all six rabbits. In the non-drowned rabbits immersed for 3 days, while a small quantity of plankton DNA was detected from the lung samples of two