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A novel PCR–DGGE-based method for identifying plankton 16S rDNA for the diagnosis of drowning

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Abstract

The diagnosis of drowning is one of the most difficult issues in forensic practice. We have developed a sensitive and specific PCR and DGGE method for identifying the 16S rDNA of plankton, which exists ubiquitously in all types of water. In order to evaluate the usefulness of this method for diagnosis of drowning, we used this method for detection of plankton 16S rDNA in drowned rabbits and non-drowned rabbits submerged after death, as well as two human drowning cases. Plankton DNA was identified from lung, liver, kidney, blood and brain of the drowned victims, and the DGGE patterns were helpful in indicating the site of drowning. Plankton DNA was also identified from two lung samples obtained from non-drowned rabbits. The results show that the new PCR–DGGE-based method is a potentially useful tool for diagnosing drowning.

Keywords: Drowning; Plankton; 16S rDNA; PCR; DGGE

1. Introduction

The diagnosis of drowning for bodies freshly retrieved from water is mainly based on some ''drowning signs'', such as the presence of fine froth at the mouth or nostrils, emphysema aquosum and impress of ribs on lungs, and so on. For the decomposed corpses found in water, however, the diagnosis of drowning is rather difficult because those ''drowning signs'' were destroyed. Although diagnostic value of diatom test for drowning is controversial, most forensic experts still consider it a relatively reliable and useful diagnostic tool in forensic practice [1–6].

There are essentially three methods commonly used for the diatom test: disorganization with strong acids, enzymatic digestion with proteinase K, and solubilization with Soluene-350 [7]. The acid digestion method has been widely used in China. However, diatoms may be absent from the victims of drowning because: (1) they are not present in the drowning medium (medium itself, seasonal variations, pollution, etc.); (2) they do not penetrate the alveolo-capillary barrier; or (3) they are partially or entirely lost or destroyed during the sample preparation process [3,4,8,9]. So it is essential to develop reliable methods for the identification of diatoms and other planktonic organisms which may all penetrate in the organism of drowning victims. A few other methods have been attempted to identify plankton other than diatoms in victims of drowning [10,11].

With the development of molecular biology, the application of DNA methods, especially the detection of genes encoding small-subunit ribosomal RNA (16s rDNA), provides a promising means for plankton detection in tissues and diagnosis of drowning. Kane et al. [12,13] designed two pairs of specific primers according to the sequence information of picoplankton in Lake Biwa, Japan, and successfully used them to amplify the segments of 16S rDNA of picoplankton from the tissues of drowning victims. With this sensitive and specific method, the identification of plankton no longer depends on their morphologic characteristics. Nübel et al. [14] also designed a set of phylum-special primers for selective amplification of the 16S rDNA segments of cyanobacteria and diatoms from a variety of natural settings and succeeded in identifying different strains using denaturing gradient gel electrophoresis (DGGE) methods.

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In the present study, we developed a sensitive and specific PCR and DGGE method for the analysis of 16S rDNA of plankton and tested it using an experimental model and two human drowning cases.

2. Materials and methods

2.1. Experimental animals

Thirty white rabbits of either sex, weighing 2.4–3.4 kg, provided by the Center of Experimental Animals, Tongji Medical College, Huazhong University of Science and Technology, were divided randomly into three groups: drowning ($n = 12$), postmortem submersion($n = 12$) and control ($n = 6$). Animals were treated in accordance with the Guidelines for the Care and Use of Laboratory Animals of Tongji Medical College, Huazhong University of Science and Technology, China.

2.2. Procedures of drowning and tissue preparation

Moshui Lake and Donghu Lake (Wuhan, China) were selected as the drowning sites for the experiment. For the drowning group, 12 white rabbits (six for Moshui Lake and six for Donghu Lake) were placed into the cages and submerged into the water, in a depth of 30 cm, for 1 min, and then taken out of the water; 30 s after the animals were taken out, they were re-submerged into the water in the same depth until the animal dead. For the postmortem submersion group, 12 white rabbits (six for Moshui Lake and six for Donghu Lake) were sacrificed through closed brain injury by hitting their heads using a caoutchouc hammer covered with a soft cotton cushion, and then were submerged into the water in the same depth as the drowning group for 6 h. For the control group, six rabbits were sacrificed by closed brain injury without postmortem disposal.

Washed with tap water, the corpses were transferred to the super-clean bench. After opening of the thoracic cavity and abdominal cavity, the heart blood was taken from left ventricle using a disposable syringe and transferred without addition of any chemical additives to a clean tube, and then the lung, liver and kidney were taken after the organ surface had been rinsed with tri-distilled water, in which no plankton was detected by light microscope without any staining. In addition, the brain tissue was also removed from cranial cavity. Two grams of each kind of the tissue was minced and then homogenized with small quantity of plankton-free water by a homogenizer.

Two millilitres of water samples were also collected at the same sites the rabbits had been submerged.

2.3. Plankton isolation from tissues using Percoll

Percoll®, a well-referenced medium for density gradient centrifugation of cells, was used to isolate plankton species from rabbit or human tissues or blood according to the procedure described by Terazawa and Takatori [15]. Briefly, 8 ml of Percoll® (Amersham Biosciences, Sweden) and 2 ml of the tissue homogenate or heart blood were mixed well with a vortex mixer and placed in a centrifugation tube (10 ml volume, NALGENE, American). After centrifugation at 17,000 rpm for 60 min at 12 °C, the upper layer containing cell debris was removed with a pipette. To the residual Percoll portion, which has a higher density, were added two volumes of plankton-free tri-distilled water; this was stirred with the mixer, centrifuged at 6000 rpm for 15 min and the supernatant was then removed. After had been added plankton-free distilled water and re-suspension, centrifuged at 12,000 rpm for 15 min, as much of the final washing solution was removed as possible. The sediment was used for DNA extraction.

Two millilitres of water samples from the sites the rabbits had been submerged were centrifuged at 12,000 rpm for 15 min, and then washed the sediment with plankton-free distilled water twice. The final sediment was used for DNA extraction.

2.4. DNA extraction of plankton

One hundred and fifty microlitres of 5% Chelex-100 were added to each of sediment in a 0.5 ml volume tube. The solution was incubated at −80 °C for 30 min. It was vortexed at high speed for 5 s when it thawed. After the same freeze–thawing procedure was repeated once, DNA was extracted by Chelex-100 method [16].

Table 1
The primer sequences for amplification of the 16S rDNA of plankton

Primer	Sequence
CYA-F	5′-GGGGAATYTTCCGCAATGGG-3′
CYA-R (a)	5′-GACTACTGGGGTATCTAATCCCATT-3′
CYA-R (b)	5′-GACTACAGGGGTATCTAATCCCTTT-3′

Note: Y denotes a C/T nucleotide degeneracy. The reverse primer CYA-R was an equimolar mixture of CYA-R (a) and CYA-R (b).

2.5. PCR amplification and product detection

The primer sequences for amplifying the 16S rRNA gene of plankton, which were reported by Nübel et al. [14], were synthesized by Sangon Biotech. Co. Ltd. (China) (shown in Table 1). A 40-nucleotide GC-rich sequence (5′-CGCCCGCCGCGCCCCGCGCCGGTCCCGCCGCCCCCGCCCG-3′) (GC-clamp) was attached to the 5′ end of the forward primer.

PCR was performed in a total reaction volume of 20 μl containing 30 ng plankton genomic DNA, 0.5 mM each primer, 10 mM Tris–HCl buffer (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM each dNTP and 1 U Taq DNA polymerase (BioStar, Canada). The cycle conditions were: 95 °C for 2 min soak, 94 °C for 45 s, 60 °C for 50 s, 72 °C for 1 min, 38 cycles, 72 °C for 10 min final extension.

The amplification products were separated in agarose gel and visualized by ethidium bromide or in denaturing gradient gel and visualized by silver staining [17]. For DGGE analysis, 1-mm-thick polyacrylamide gel (T = 6%, Acr:Bis = 37.5:1) with denaturant gradient from 20% to 60% was used, and the electrophoresis was performed in 1× TAE buffer for 6 h at 150 V.

2.6. Human drowning

Case 1: a 47-year-old female had gone missing after disputation and violence with her husband. Her body was found in a river the next day, and an autopsy was performed 2 days after she had gone missing. Diatom shells were found after chemical digestion with strong acid from 20 g of tissues (lung, liver and kidney). The cause of death was estimated to be drowned based on autopsy findings and diatom test.

Case 2: a 39-year-old female was found in a suburb well after she had gone missing 2 days. An autopsy was performed 3 days after she had gone missing. Although the cause of death was estimated to be drowned based on autopsy findings and investigation information, no diatom shell was found from the digestion residues of 20 g tissues (lung, liver and kidney). No diatom was detected from 15 ml water sample from the same well.

Two grams of tissues (lung, liver and kidney) collected from these two cases were investigated for the 16S rDNA with the same procedure used for the animal experiments and the control group.

3. Results

3.1. Qualitative analysis of plankton 16S rDNA

The length of amplification products with the CYA primer set was 487 bp (including GC-clamp). For the drowning group, the amplification product could be identified from most of the rabbit tissues (Table 2, Fig. 1). The PCR products were detected only in two rabbit lung tissues from the postmortem submersion group, and no amplification product was detected from other tissues of the postmortem submersion group and those of the control group (Table 2, Fig. 2).

Table 2
The number and percentage of PCR products detection in different groups

Group	Positive number and percentage				
	Lung	Liver	Kidney	Blood	Brain
Drowning group ($n = 12$)	12 (100%)	10 (83%)	9 (75%)	10 (83%)	5 (42%)
Postmortem submersion group ($n = 12$)	2 (16.7%)	0 (–)	0 (–)	0 (–)	0 (–)
Control group ($n = 6$)	0 (–)	0 (–)	0 (–)	0 (–)	0 (–)

3.2. DGGE analyses

Anabaena flos-aquae (FACHB-245), *Microcystis aeruginosa* (FACHB-942), *Cyclotella meneghiniana* sp. (FACHB-986) and *Gloeocapsa alpicola* (FACHB-905), obtained from Institute of Hydrobiology, Chinese Academy of Sciences, were used as DGGE marker to indicate the differences of PCR products from different water, as they are commonly distributed in water.

To investigate the relation between plankton detected in drowned rabbits and plankton of the drowning site, DGGE analysis was performed using PCR products from lung samples of drowned rabbits and from water samples of Moshui and Donghu Lakes. Moreover water samples from Yuehu Lake were selected as control. DGGE allowed the discrimination of different PCR products (Fig. 3), which have the same length and could not be discriminated by agarose gel electrophoresis (Fig. 1). As shown in Fig. 3, one to four different bands were discriminated in each sample by DGGE analysis, and the DGGE patterns obtained from lung tissues of the drowned rabbits were similar with that obtained from the water samples of their respective drowning sites. However, they were obviously different with that obtained from the water samples collected in the other sites.

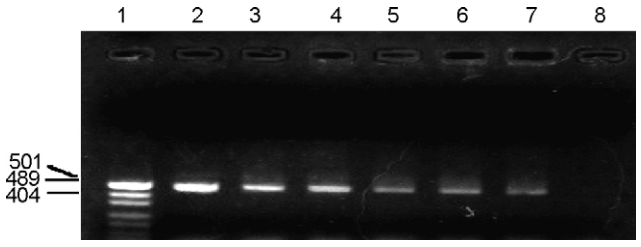

Fig. 1. The agarose gel patterns of amplification products from various tissues of the drowning group. Lane 1: pUC19 DNA/Msplmarker; lane 2: product from water sample; lanes 3–7: products from lung, liver, kidney, heart blood and brain of a drowned rabbit; lane 8: negative control.

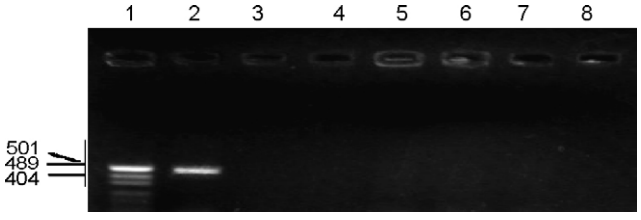

Fig. 2. The agarose gel patterns of amplification products from various tissues of the control group. Lane 1: pUC19 DNA/Msplmarker; lane 2: products from water sample; lanes 3–7: products from lung, liver, kidney, heart blood and brain; lane 8: negative control.

4. Discussion

Plankton, which exists ubiquitously in all types of water, may penetrate into the respiratory system of victims during drowning, and enter internal organs through blood circulation. So, the detection of plankton from various organs or blood of the victims can be used for the diagnosis of drowning. Moreover, the qualitative or quantitative identification of various planktons could be employed for the determination of the drowning sites. Although the most of 16S rDNA sequences are relatively conserved, they contain a few variable regions [18]. Sequence comparison of the variable regions of 16S rDNA can provide sufficient information to allow the discrimination of both close and distant phylogenetic relationships. In the study of Kane et al. [12,13], the genus-specific primer sequences were used to detect the variable regions of 16S rDNA of picoplankton, and the segment sizes of PCR products were approximately 210 bp in length. Although this method was highly sensitive, the information abundance of the amplified products were lower than those in Nübel et al.'s [14] in which 487 bp fragments were amplified. In addition, Abe et al. [19] and Suto et al. [20] also developed a PCR method for identifying plankton in cases drowning by detecting chlorophyll-related genes of *Euglena gracilis* and *Skeletonema costatum*. All these approaches, however, provide only qualitative results because they can only demonstrate the presence of plankton in victim's tissues, and cannot be used to evaluate the site of drowning.

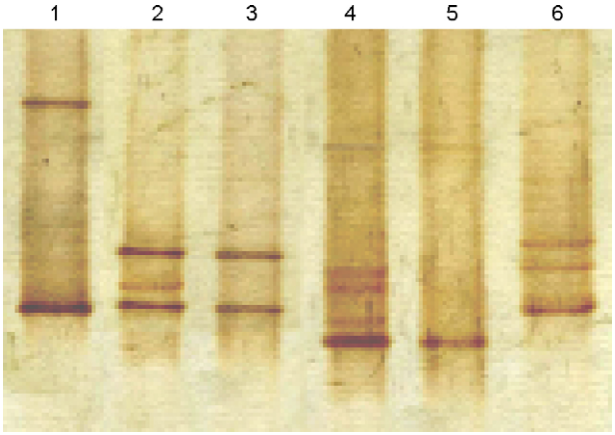

Fig. 3. The DGGE patterns of the amplification products from different samples. Lane 1: products from mixture of *Gloeocapsa alpicola* (upper band), *Microcystis aeruginosa*, *Anabaena flos-aquae* and *Cyclotella meneghiniana* sp. (lower band); lanes 2, 3: products from water sample of Moshui Lake and from lung tissue of a rabbit drowned in it, respectively; lanes 4, 5: products from water sample of Donghu Lake and from lung tissue of a rabbit drowned in it, respectively; lane 6: products from water sample of Yuehu Lake.

DGGE is an extremely sensitive electrophoretic separation technique capable of discriminating among sequence variants that differ by as little as a single nucleotide, and is used extensively to profile complex microbial communities [21,22]. In this study, in order to discriminate the phylogenetic relationships more easily, we selected the same primer set as Nübel et al. [14] did in order to amplify longer DNA segments. The products amplified from all samples were of same size (Fig. 1). After DGGE analysis, however, one to four different bands were identified in each sample. The DGGE profiles obtained from lung tissues of the drowned rabbits were very similar with those from water samples from the drowning sites, and they were obviously different from those from water samples of non-drowning sites (Fig. 3).

In general, 16S rRNA genes are considered to be functionally and structurally more conservative than protein-coding genes, and thus the 16S rRNA sequence identity may not be sufficient to guarantee correct determination of species identity [23]. Indeed, in this study, we found that *Microcystis aeruginosa*, *Anabaena flos-aquae* and *Cyclotella meneghiniana* sp. were not differentiated by DGGE analysis and they had the same DGGE band patterns (Fig. 3). The *rpoC*1 gene, encoding the γ-subunit of RNA polymerase, has been used as an alternative target for analysis of plankton phylogeny and community structure [24,25]. However, the sequence data available for these genes are still limited. It should be noted that the primers used in plankton detection were always designed on the basis of limited sets of data. Further study might reveal better sequences for the identification of drowning.

It is important for the diagnosis of drowning to effectively isolate plankton from the tissues of drowning victims. In a previous study, we found that if planktons were not separated from tissues of drowning victims, the detection sensitivity was obviously lower because of the interference caused by the amount of tissue DNA [26]. In order to solve this problem, in this study, we selected Percoll® as separation medium, and planktons were isolated effectively from blood and tissue samples obtained from the bodies of drowning victims. By using this separation technique, positive results were obtained from 2 g of tissues or 2 ml of heart blood from the experimental animals and two human drowning victims. In addition, case 2 also proved that this method is helpful in the identification of death by drowning in diatom-absent water.

In this study, plankton DNA was also found from two lung samples obtained from non-drowned rabbits possibly because of postmortem plankton penetration into the respiratory system, but no plankton DNA were detected from liver, kidney, blood and brain samples obtained from postmortem submerged rabbits (Table 2). These results indicate that the findings of plankton DNA in lungs alone must be interpreted very cautiously in regard to the diagnosis of death by drowning.

5. Conclusions

We have developed and tested a new PCR–DGGE-based method for identifying plankton. Plankton DNA was identified in lung, liver, kidney, blood and brain in the drowned victims, and the DGGE patterns were helpful to discriminate the site of drowning. Our new PCR–DGGE-based method is a potentially useful alternative method for diagnosis of drowning.

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References

[1] A. Auer, Qualitative diatom analysis as a tool to diagnose of drowning, Am. J. Forensic Med. Pathol. 12 (1991) 213–218.
[2] B. Ludes, M. Coste, A. Tracqui, P. Mangin, Continuous river monitoring of the diatoms in the diagnosis of drowning, J. Forensic Sci. 41 (1996) 425–428.
[3] M.S. Pollanen, C. Cheung, D.A. Chiasson, The diagnostic value of the diatom test for drowning. I. Utility: a retrospective analysis of 771 cases of drowning in Ontario, Canada, J. Forensic Sci. 42 (1997) 281–285.
[4] M.S. Pollanen, The diagnostic value of the diatom test for drowning. II. Validity: analysis of diatoms in bone marrow and drowning medium, J. Forensic Sci. 42 (1997) 286–290.
[5] B. Ludes, M. Coste, N. North, S. Doray, A. Tracqui, P. Kintz, Diatom analysis in victim's tissues as an indicator of the site of drowning, Int. J. Leg. Med. 112 (1999) 163–166.
[6] J. Hürlimann, P. Feer, F. Elber, K. Niederberger, R. Dirnhofer, D. Wyler, Diatom detection in the diagnosis of death by drowning, Int. J. Leg. Med. 114 (2000) 6–14.
[7] M. Ming, X. Meng, E. Wang, Evaluation of four digestive methods for extracting diatoms, Forensic Sci. Int. 170 (2007) 29–34.
[8] J. Timperman, The diagnosis of drowning, A review, Forensic Sci. 1 (1972) 397–409.
[9] L. Sidari, N. Di Nunno, F. Costantinides, M. Melato, Diatom test with Soluene-350 to diagnose drowning in sea water, Forensic Sci. Int. 103 (1999) 61–65.
[10] T. Okada, K. Ide, I. Isahai, M. Okada, Studies on detection method of planktons contained chlorophyll by fluorescence, Nihon Hoigaku Zasshi 37 (1983) 194–197.
[11] J. Qu, E. Wang, A study on the diagnosis of drowning by examination of lung chlorophyll(a) of planktons with a spectrofluorophotometer, Forensic Sci. Int. 53 (1992) 149–155.
[12] M. Kane, T. Fukunaga, H. Maeda, K. Nishi, The detection of picoplankton 16S rDNA in case of drowning, Int. J. Leg. Med. 108 (1996) 323–326.
[13] M. Kane, T. Fukunaga, H. Maeda, K. Nishi, Phylogenetic analysis of picoplankton in Lake Biwa and application to legal medicine, Electrophoresis 21 (2000) 351–354.
[14] U. Nübel, F. Garcia-Pichel, G. Muyzer, PCR primers to amplify 16S rRNA genes from cyanobacteria, Appl. Environ. Microbiol. 63 (1997) 3327–3332.
[15] K. Terazawa, T. Takatori, Isolation of intact plankton from drowning lung tissue by centrifugation in a colloidal silica gradient, Forensic Sci. Int. 16 (1980) 63–66.
[16] P.S. Walsh, D.A. Metzger, R. Higuchi, Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material, Biotechniques 10 (1991) 506–513.
[17] B.J. Bassam, G. Caetano-Anolles, P.M. Gresshoff, Fast and sensitive silver staining of DNA in polyacrylamide gels, Anal. Biochem. 196 (1991) 80–83.
[18] P. De Rijk, J.M. Neefs, Y. Ven de Peer, R. De Wachter, Compilation of small ribosomal subunit RNA sequences, Nucleic Acid Res. 20 (1992) 2075–2089.
[19] S. Abe, M. Suto, H. Nakamura, H. Gunji, K. Hiraiwa, T. Suzuki, T. Itoh, H. Kochi, G. Hoshiai, A novel PCR method for identifying plankton in cases of death by drowning, Med. Sci. Law 43 (2003) 23–30.
[20] M. Suto, S. Abe, H. Nakamura, T. Suzuki, T. Itoh, H. Kochi, G. Hoshiai, K. Hiraiwa, Phytoplankton gene detection in drowned rabbits, Leg. Med. 5 (2003) S142–S144.

[21] G. Muyzer, E.C. De Waal, A.G. Uitterlinden, Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA, Appl. Environ. Microbiol. 59 (1993) 695–700.

[22] M.J. Ferris, G. Muyzer, D.M. Ward, Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community, Appl. Environ. Microbiol. 62 (1996) 340–346.

[23] G.E. Fox, J.D. Wisotzkey, P.Jr. Jurtshuk, How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity, Int. J. Syst. Bacteriol. 42 (1992) 166–170.

[24] K.J. Bergsland, R. Haselkorn, Evolutionary relationships among eubacteria, cyanobacteria, and chloroplasts: evidence from the *rpoC*1 gene of *Anabaena* sp. Strain PCC-7120, J. Bacteriol. 173 (1991) 3446–3455.

[25] B. Palenik, Cyanobacterial community structure as seen from RNA polymerase gene sequence analysis, Appl. Environ. Microbiol. 60 (1994) 3212–3219.

[26] F. He, L. Liu, D. Huang, Q. Yang, X. Zhai, H. Yin, The value of plankton 16S rDNA detection on identification of drowning rat, Chin. J. Forensic Med. 21 (2006) 331–333.