原　　著

A SENSITIVE AND TIME-SAVING METHOD FOR THE DIAGNOSIS OF DROWNING BY MULTIPLEX PCR

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Abstract:For diagnosing death due to drowning, the method of acid digestion of diatoms is widely used to detect plankton in the organs of the corpse. However, the method is limited by its being complex, hazardous, time-consuming, and insufficiently sensitive. We therefore, developed a novel simple method to diagnose death due to drowning, and determined the location of drowning by detecting genes of representative bacteria in the environment. To procure all the information in one step, the multiplex PCR method was designed. For the diagnosis of drowning, the genes of upper respiratory indigenous bacteria, *Streptococcus salivarius* and *Streptococcus sanguinis* were used as indicators. For detection of the location of drowning, *Aeromonas hydrophila* and *Microcystis aeruginosa* were used as indicators of freshwater, and *Vibrio harveyi* as an indicator of seawater. A set of primers was designed for multiplex PCR, to amplify all the bacterial genes simultaneously. Using this method, 47 cases of drowning were examined, and the causes and locations of death were diagnosed.

Key words: Diagnosis of drowning, Multiplex PCR, Aquatic bacteria, Respiratory tract bacteria

Introduction

Diagnosis of death due to drowning is one of the most difficult challenges in forensic medicine. Whether the corpse died by drowning or was placed in water after death needs to be resolved. For this, it is important to detect plankton or other aquatic organisms from the organs of the corpse, besides the other anatomical findings like froth in the air passages and over-inflation of the lungs. Next, if the cause of death has been confirmed to be drowning, the kind of water the person drowned in needs to be identified to find the location of the incident. The method of acid digestion of diatoms has been widely used to detect plankton in the organs[1,2]. However, this method is not sensitive enough, since it can detect only diatoms whose skeletons are retained during strong acid digestion, and cannot be used for water without plankton, like bathtub water. Moreover, the method is complicated, hazardous, time-consuming, and requires a large quantity of sample[3]. Recently, an alternative method for detecting the genes of planktons has been reported[4,5]. Moreover, a method to detect the genes of upper respiratory tract indigenous bacteria, for diagnosis of drowning in water containing no plankton, was reported[6]. However,

these methods are not yet easy to perform, since gene amplification is needed to test several genes to find the organism that is suitable as the marker, depending on the water, and they are not always successful.

In this study, we developed a method to detect several bacterial genes simultaneously, using one multiplex PCR, to diagnose death due to drowning and other associated details. For diagnosis, the genes of upper respiratory tract indigenous bacteria, *Streptococcus salivarius* and *Streptococcus sanguinis*[6] were used as the indicators. For identification of the location of drowning, *Aeromonas hydrophila*[7] and *Microcystis aeruginosa*[8] were used as indicators of freshwater, and *Vibrio harveyi* [9] as an indicator of seawater. Using this method, 47 cases of drowning were examined, and the causes and locations of death diagnosed.

Materials and Methods

Primer design

Primers were designed to amplify DNA from five species of bacteria in one PCR, using Primer-BLAST software (Table 1). Two of the bacteria chosen were freshwater bacteria, *Aeromonas hydrophila* and *Microcystis aeruginosa*, one was a seawater bacterium, *Vibrio harveyi*, and two were upper respiratory tract indigenous bacteria, *Streptococcus salivarius* and *Streptococcus sanguinis*. As shown in Table 2., these bacteria were used as indicators for drowning in freshwater, in seawater, and any unspecified water, respectively. *Aeromonas hydrophila*, *Streptococcus salivarius*, and *Streptococcus sanguinis* were purchased from the Japan Collection of Microorganisms in the RIKEN BioResource Research Center (JCM). *Microcystis aeruginosa* and *Vibrio harveyi* were purchased from Microbial Culture Collection in the National Institute for Environmental Studies (NIES).

Table 1. Amplification primers to detect five species of bacteria for multiplex PCR

Target bacterial species	Target gene	Primer sequence (5'-3')	PCR Product (bp)
Streptococcus salivarius	fructosyltransferase (Ftf)	F : AAATTGCAAGATGCTCACCC	117
		R : ACCAAAGAGACGGTCACCTG	
Microcystis aeruginosa	global nitrogen regulator (NtcA)	F : GGTGGATCTAAAATTGTCTCACCAAG	143
		R : GACGGGATTATGGACGGTAATTTTC	
Aeromonas hydrophila	extracellular serine protease	F : TCTCTGCTCAATCGCGAGAC	227
		R : CCATCCATGGTGACGGACTC	
Streptococcus sanguinis	chaperonin (GroEL)	F : TTGTTGCAGATGATGTGGACG	290
		R : ACGGTTAGCAATGGCTTCAG	
Vibrio harveyi	S-ribosylhomocysteinase (LuxS)	F : ATAAGAACCCGTTGTGTCGC	402
		R : CAGGTATCGCATTGGTTGTG	

Table 2. Bacterial species used for the indicators of drowning

Indicated location	Bacterial species	Strain*
Freshwarter	*Aeromonas hydrophila*	JCM2360
	Microcystis aeruginosa	NIES843
Seawater	*Vibrio harveyi*	NBRC15632
Drowning	*Streptococcus salivarius*	JCM5707
(Respiratory tract indigenous bacteria)	*Streptococcus sanguinis*	JCM5708

* These strains were used for extraction of standard DNAs

Culture of standard bacterial strains

Aeromonas hydrophila was cultured on DHL (Difco Laboratories, Sparks, MA, USA) agar medium at 37 ° C for 24 h; *Streptococcus salivarius* and *Streptococcus sanguinis* were cultured on SCD (Nissui, Tokyo, Japan) agar medium at 37℃ for 24 h; *Microcystis aeruginosa* was cultured at 25℃ for 1 week at MA (NIES, Ibaraki, Japan) medium, and *Vibrio harveyi* was cultured on a Marine Broth medium (Difco Laboratories, Sparks, MA, USA) at 25℃ for 24 to 48 h.

Sample collection

Tissue samples of lungs, liver, kidney, respiratory tract, cardiac blood, and pleural effusion were collected aseptically from 47 bodies autopsied in our laboratory. Forty-one of these bodies were found in freshwater areas, and five were found in a bathtub. All the cases were diagnosed as death by drowning from anatomical observations, with or without detection of diatoms from the tissues by the acid digestion method. Two liters of water, at the location where the drowned bodies were found, were collected in each autopsy case.

For the control, tissue samples from 5 autopsy specimens (3 not decomposed, 2 decomposed), due to factors other than drowning, and peripheral blood and oral mucosa of two healthy volunteers were collected.

This project was approved by the Ethical Committee of Nara Medical University. (accepted number : 1561)

Preparation of DNA templates

Finely cut 5 g of tissues, and 3 ml of cardiac blood and pleural effusion were digested in Proteinase K-containing solution for 6 h in the case of tissues, and 1 h for other cases, followed by DNA extraction using MagExtractor™Genome (TOYOBO, Osaka, Japan). Approximately 2 L of water from the drowning site was filtered with a membrane filter, and used for DNA extraction using MagExtractor™Genome. The extracted DNA was quantified using a spectrophotometer.

PCR amplification

PCR was carried out using 2.5μl (10ng/μl) of the extracted DNA and KOD FX PCR reagent (TOYOBO, Osaka, Japan) with the total volume being 20μl. Primers were added as 0.23μM for *Aeromonas hydrophila*, and 0.3μM for the others. Amplification was carried out by the step-down PCR method to amplify DNA with high specificity. As shown in Fig. 1., the program comprised 94℃ for 2 mins, followed by 5 cycles of 98℃ for 10 s and 66° C for 20 s, 5 cycles of 98℃ for 10 s and 64℃ for 20 s, 5 cycles of 98℃ for 10 s and 62℃ for 20 s, and 20 cycles of 98℃ for 10 s, 58℃ for 20 s, and 68℃ for 7 min. Amplification was done using GeneAmp PCR System 9700 (Thermo Fisher Scientific, Waltham, MA, USA). The amplified products were detected by electrophoresis on 12% gel of acrylamide/bis (29:1).

PCR conditions		
Predenature:	94℃, 2min.	
Denature:	98℃, 10sec.	5 cycles
Extention:	66℃, 20sec.	
Denature:	98℃, 10sec.	5 cycles
Extention:	64℃, 20sec.	
Denature:	98℃, 10sec.	5 cycles
Extention:	62℃, 20sec.	
Denature:	98℃, 10sec.	20 cycles
Extention:	58℃, 20sec.	
Extention:	68℃, 7min.	

Fig. 1. Stepdown PCR cycle

Results

Specificities of the designed primers

To confirm the specificity of the designed primers, DNA extracted from freshwater and seawater sources, along with DNA from oral mucosa and peripheral blood of a healthy volunteer, as controls, were amplified using the multiplex PCR system. Only *Aeromonas hydrophila* and *Microcystis aeruginosa,* used as indicators of freshwater locations, were detected in DNA extracted from freshwater; no other bacteria of the upper respiratory tract and seawater locations were seen. Similarly, only *Vibrio harveyi,* as an indicator of seawater locations, was detected in DNA extracted from seawater, and only *Streptococcus salivarius* and *Streptococcus sanguinis* in the upper respiratory tract were detected in the DNA extracted from oral mucosa. In DNA extracted from blood, no bacterium was detected. From these results, the specificity of each primer was confirmed (Fig. 2).

Detection of bacteria in the water where drowning occurred

The water in 47 cases of drowning, autopsied in our laboratory, were examined using this method, and in 41 cases (87.2%) either *Aeromonas hydrophila* or *Microcystis aeruginosa,* or both, were detected. This water included canals, ponds, rivers, paddy fields, and dams. From these results it was clear that the designed primers can be used for various kinds of water (Fig. 3).

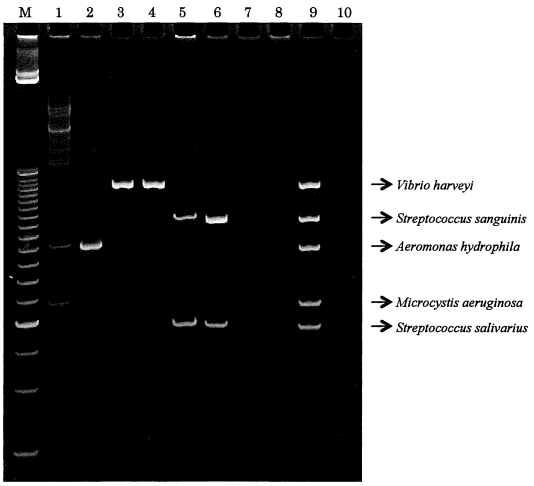

Fig. 2. Specificities of primers
Lane 1 ; freshwater 1, Lane 2 ; freshwater 2,
Lane 3 ; seawater 1, Lane 4 ; seawater 2,
Lane 5 ; oral mucosa 1, Lane 6 ; oral mucosa 2,
Lane 7 ; peripheral blood 1,
Lane 8 ; peripheral blood 2,
Lane 9 ; standard, Lane 10 ; negative control

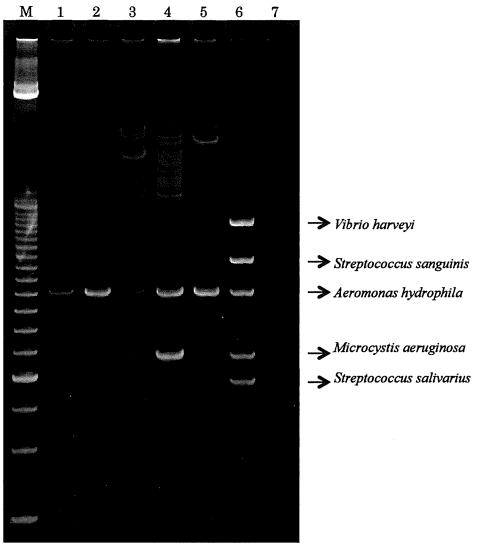

Fig. 3. Detection of the indicator bacteria in drowning waters
Lane 1 ; irrigation canal, Lane 2 ; pond, Lane 3 ; river,
Lane 4 ; paddy field, Lane 5 ; dam, Lane 6 ; standard,
Lane 7 ; negative control

Detection of bacteria in the tissues of a body drowned in water where only *Aeromonas hydrophila* was detected

DNA from lung, liver, kidney, respiratory tract, cardiac blood, and pleural effusion of the

body drowned in water with only *Aeromonas hydrophila* detectable was examined using this multiplex PCR method. As shown in Fig. 4., *Aeromonas hydrophila* (indicator of freshwater area) and *Streptococcus salivarius* (indicator of drowning aspiration) were detected from the lungs and pleural effusion. Only *Aeromonas hydrophila* was detected, weakly, from kidney and cardiac blood, and strongly from pleural effusion. *Aeromonas hydrophila, Streptococcus salivarius,* and *Streptococcus sanguinis* (indicator of drowning aspiration) were detected from the respiratory tract. No bacterium was detected from the liver. *Vibrio harveyi* was not detected from any tissue.

Detection of bacteria in the tissues of a body drowned in water with both *Aeromonas* and *Microcystis*

DNA from the tissues of a body drowned in water with both *Aeromonas hydrophila* and *Microcystis aeruginosa* was examined. As shown in Fig. 5., both *Aeromonas hydrophila* and *Microcystis aeruginosa* were detected from the lungs and pleural effusion, and only *Aeromonas hydrophila* was detected from liver and cardiac blood. *Aeromonas hydrophila, Microcystis aeruginosa, Streptococcus salivarius,* and *Streptococcus sanguinis* were detected from the respiratory tract. Only *Streptococcus salivarius* was detected from lung, cardiac blood and pleural effusion. No bacterium was detected from the kidney. *Vibrio harveyi* was not detected from any tissue.

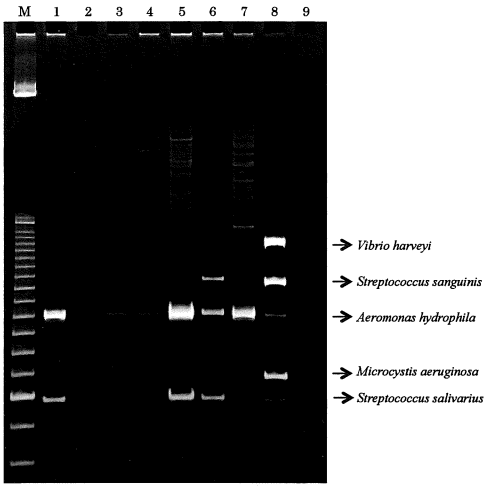

Fig. 4. Detection of bacteria in the tissues from drowned body in freshwater. In case 10, *Aeromonas hydrophila* was detected.
Lane 1 ; lung, Lane 2 ; liver, Lane 3 ; kidney,
Lane 4 ; cardiac blood, Lane 5 ; pleural effusion,
Lane 6 ; respiratory tract, Lane 7 ; water sample,
Lane 8 ; standard, Lane 9 ; negative control

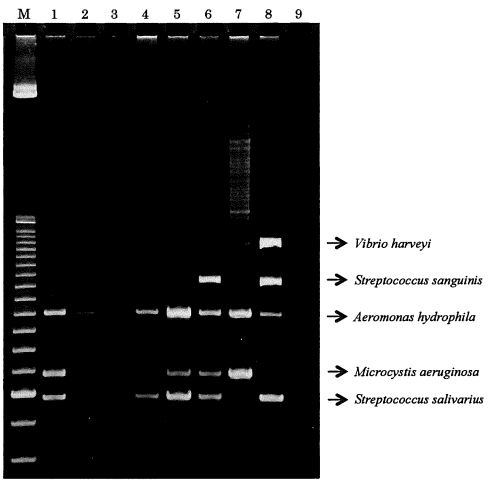

Fig. 5. Detection of bacteria in the tissues from drowned body in freshwater.
In case 8, *Microcystis aeruginosa* and *Aeromonas hydrophila* were detected.
Lane 1 ; lung, Lane 2 ; liver, Lane 3 ; kidney,
Lane 4 ; cardiac blood, Lane 5 ; pleural effusion,
Lane 6 ; respiratory tract, Lane 7 ; water sample,
Lane 8 ; standard, Lane 9 ; negative control

Detection of bacteria in the tissues of a case drowned in bath water

DNA from the tissues of a body drowned in bath water was examined. As shown in Fig. 6., both *Streptococcus salivarius* and *Streptococcus sanguinis* were detected from lung, kidney,

pleural effusion, and respiratory tract. From cardiac blood, only *Streptococcus salivarius* was detected. Neither was detected from the liver. In bath water, no bacteria specific to freshwater or seawater exist. Therefore, the primers for upper respiratory tract bacteria are useful for the diagnosis of drowning.

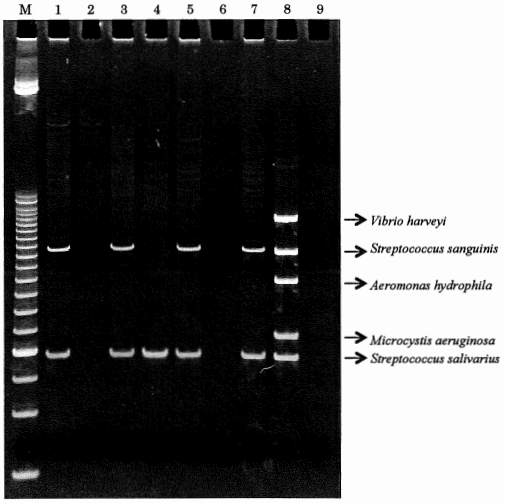

Fig. 6. Detection of bacteria in the tissues from drowned body in bathtub.
In case 3, *Streptococcus salivarius* and *Streptococcus sanguinis* were detected.
Lane 1 ; lung, Lane 2 ; liver, Lane 3 ; kidney,
Lane 4 ; right cardiac blood,
Lane 5 ; left pleural effusion,
Lane 6 ; right pleural effusion,
Lane 7 ; respiratory tract, Lane 8 ; standard,
Lane 9 ; negative control

Evaluation of the multiplex PCR system for application in diagnosis of drowning

In Table 3., the rate of detection of indicator bacteria in the tissues of 41 cases of bodies drowned in water in which freshwater bacteria were detected is presented. The indicator of freshwater could be detected in 80% of cases for plural effusion and 89% of cases for lung, though for other tissues it was around 50%. The indicators of drowning, *Streptococcus*, could be detected at higher rate in 71% of cases for cardiac blood and 91% of cases for plural effusion. The results showed that this method may be useful to diagnose the location of death, even if not exactly. In Table 4., the precise data of 10 representative cases (out of the 41) are listed.

Table 3. Detection rate of bacteria in 41 cases of drowning examined in our laboratory

	A + M	L + N	A + M + L + N	acid digestion method
Lung	89%	90%	100%	71%
Liver	44	40	53	23
Kidney	56	50	61	19
Cardiac blood	52	71	74	-
Pleural effusion	80	91	100	-
Respiratory tract	86	100	100	-

A+M : Detected *Aeromonas hydrophila* or *Microcystis aeruginosa* or both of them
L+N : Detected *Streptococcus salivarius* or *Streptococcus sanguinis* or both of them
A+M+L+N : Detected *Aeromonas hydrophila* or *Microcystis aeruginosa* or *Streptococcus salivarius* or *Streptococcus sanguinis* or some of them

Table 4. Result of detection of bacteria in cases of drowning examined in our laboratory

Case no.		Multiplex PCR			Acid digestion	Overview
		Bacteria in freshwater	Bacteria in trachea	Bacteria in seawater		
		Aeromonas hydrophila *Microcystis aeruginosa*	*Streptococcus salivarius* *Streptococcus sanguinis*	*Vibrio harveyi*		
	Lung	+	+	−	−	
	Liver	+	+	−	−	
	Kidney	+	+	−	−	postmortem 3.5 days
1	Cardiac blood	nt	nt	nt	nt	decomposed
	Pleural effusion	nt	nt	nt	nt	river
	Respiratory tract	+	+	−	nt	
	Water sample	+	−	−	nt	
	Lung	−	+	−	−	
	Liver	−	−	−	−	
	Kidney	−	−	−	−	postmortem 1.5 days
2	Cardiac blood	−	+	−	nt	not decomposed
	Pleural effusion	+	+	−	nt	pond
	Respiratory tract	+	+	−	nt	
	Water sample	+	−	−	nt	
	Lung	−	+	−	nt	
	Liver	−	−	−	nt	
	Kidney	−	+	−	nt	postmortem 3 ～ 3.5 days
3	Cardiac blood	−	+	−	nt	weak decomposed
	Pleural effusion	−	+	−	nt	bathtub
	Respiratory tract	−	+	−	nt	
	Water sample	nt	nt	nt	nt	
	Lung	+	+	−	+	
	Liver	−	−	−	−	
	Kidney	+	−	−	−	postmortem 2 days
4	Cardiac blood	+	+	−	nt	not decomposed
	Pleural effusion	nt	nt	nt	nt	irrigation canal
	Respiratory tract	+	+	−	nt	
	Water sample	+	−	−	nt	
	Lung	+	+	−	+	
	Liver	+	+	−	+	
	Kidney	−	−	−	−	postmortem 2 days
5	Cardiac blood	+	+	−	nt	weak decomposed
	Pleural effusion	nt	nt	nt	nt	river
	Respiratory tract	+	+	−	nt	
	Water sample	+	−	−	nt	
	Lung	+	+	−	+	
	Liver	−	−	−	−	
	Kidney	−	−	−	−	postmortem 2 weeks
6	Cardiac blood	−	−	−	nt	decomposed
	Pleural effusion	nt	nt	nt	nt	pond
	Respiratory tract	+	+	−	nt	
	Water sample	+	−	−	nt	
	Lung	+	−	−	+	
	Liver	+	+	−	+	
	Kidney	+	+	−	+	postmortem 2 days
7	Cardiac blood	−	+	−	nt	not decomposed
	Pleural effusion	nt	nt	nt	nt	river
	Respiratory tract	+	+	−	nt	
	Water sample	+	−	−	nt	
	Lung	+	+	−	+	
	Liver	+	−	−	+	
	Kidney	−	−	−	+	postmortem 6 days
8	Cardiac blood	+	+	nt	nt	not decomposed
	Pleural effusion	+	+	−	nt	pond
	Respiratory tract	+	+	−	nt	
	Water sample	+	−	−	nt	
	Lung	+	+	−	+	
	Liver	+	−	−	−	
	Kidney	−	−	−	−	postmortem 1.5 days
9	Cardiac blood	−	−	−	nt	not decomposed
	Pleural effusion	+	+	−	nt	irrigation canal
	Respiratory tract	−	+	−	nt	
	Water sample	+	−	−	nt	
	Lung	+	+	−	+	
	Liver	−	−	−	−	
	Kidney	+	−	−	−	postmortem 2 days
10	Cardiac blood	+	−	−	nt	not decomposed
	Pleural effusion	+	+	−	nt	irrigation canal
	Respiratory tract	+	+	−	nt	
	Water sample	+	−	−	nt	

nt : not tested

Discussion

In this study, we developed a sensitive and time-saving method for the diagnosis of drowning using multiplex PCR, as an alternative to the method of acid digestion of diatoms, which is complicated, hazardous, time-consuming, and requires a large quantity of sample[3]. Recently, methods of detection of plankton DNA[10] or aquatic bacterial DNA[11] were reported as a drowning diagnosis method using PCR. However, for plankton detection, the sensitivity was low, and for aquatic bacterial detection, it is necessary to perform PCR several times for each bacterial species, which is troublesome and costly. On that point, this Multiplex PCR method detected all indicator bacteria including *Vibrio harveyi* which is an indicator of seawater, *Aeromonas hydrophira* and *Microcystis aeruginosa* which are indicators of freshwater and *Streptococcus salivarius* and *Streptococcus sanguinis* which are indicators of drowning suction, in one PCR. Since it is integrated, it is possible to assess the location of drowning with both seawater and freshwater covered simultaneously. At the same time, drowning can be diagnosed depending on the presence or absence of indicator bacteria for drowning suction. It can be said that it is a more useful method for diagnosis of drowning. Although a PCR method for detecting indigenous bacteria in the upper respiratory tract, an indicator of drowning suction, has been reported[6], it was only used on cardiac blood. However, there are many cases where cardiac blood cannot be collected due to corruption or other related circumstances. In contrast, this method can be used for detection from various tissues, including those of drowning bodies from which it was not possible to collect cardiac blood.

In addition, in the development of this method, the PCR conditions were tweaked. Depending on the dissection case, it may be necessary to extract DNA from a corrupted drowning body or water including a lot of contaminants. However, for DNA extraction, a DNA extraction reagent that removes PCR inhibiting substances such as metals by using the bead method was selected. In addition, since DNA in tissues contains relatively large amounts of human-derived DNA and trace amounts of bacterial DNA to be detected, high specificity is required for the PCR. Therefore, by amplifying at a high annealing temperature in the initial PCR amplification, we adopted a step down PCR method which gradually lowers the temperature and increases the amount of amplification, thereby enhancing the specificity.

For the evaluation of this method, forty-one drowning cases examined in our laboratory were tested using it, and analyzed to determine the rate of detection of indicator bacteria (Table 3). The detection rate with the method of acid digestion of plankton was 71% in lung, 23% in liver, and 19% in kidney, while the detection rate of the total bacteria used as indicators using this method was 100% in lung, 53% in liver, and 61% in kidney. These results demonstrate that this method gives a higher detection rate than the acid digestion test and is a highly sensitive inspection method. In addition, the detection rate in pleural effusion was 100%, while it was not tested by the acid digestion test. It seems that using pleural effusion as a test sample can contribute to improvement of the accuracy of drowning diagnosis. Diagnosis of drowning with pleural effusion is carried out by anatomical examinations, but nothing has been reported about DNA from pleural effusion as a tool in drowning diagnosis. This study contributes the first report on the high sensitivity of drowning diagnosis using pleural effusion DNA.

For drowning in a bathtub, since there are no plankton in tap water, it was not possible to use the acid digestion test, and until now, the diagnosis depended on macroscopic findings. However, we often experience difficulties in diagnosing drowning macroscopically due to corruption. In contrast, using this method it is possible to make a diagnosis even from corrupt tissues (Table 4., case 1, 3, 5 and 6). It can be concluded that this method is useful for diagnosis of drowning.

Improvements in the method may be made by further increasing the detection sensitivity by increasing the number of types of bacteria used as indicators or by preparing primers with higher specificity.

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