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PCR - Capillary Electrophoresis is a New Method for Forensic Diatom Testing

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PCR-Capillary electrophoresis technology was used to identify diatoms in lung, liver and kidney, and to assess the effects of these diatom species. From 20 randomly selected experimental rabbits, which were drowned in the same location, the liver, kidney and lung from each rabbit were removed and diatom DNA was extracted from the tissues of these organs and subsequently amplified by specific primers of the diatom SSU gene. The diatom DNA was then analyzed using PCR-Capillary electrophoresis. When the amount of biomaterial was increased, the number of diatom species detected in the lung, liver and kidney gradually increased, and was statistically significant ($P < 0.01$). Bivariate correlation analysis showed a positive correlation between the quantity and types of diatoms. The proportion of diatom species detected in each group varied. When the quantity was greater than 0.9 g in the lung, samples including two or more types of diatoms predominated. When the quantity was greater than 1.5 g in the liver, samples including one or more types of diatoms predominated. When the quantity was greater than 2 g in the kidney, samples which included one or more types of diatoms predominated. The number of diatom species found in different organs with different tissue mass was significantly different, and provides a reference for the detection of diatoms using PCR-Capillary electrophoresis technology. This technique also has potential in the forensic identification of drowning.

Key words: Forensic medicine; Drowning; Detection of diatom; SSU gene; PCR; Capillary electrophoresis.

There are many water bodies in and around the cities of China. The diatom test is commonly used in the analysis of cause of death. The traditional diatom test is an acid digestion method, however, the diatom detection rate using this method is low, and the method has operational risks and is controversial. In 1996, Kane[1] obtained specific PCR products from picoplankton using formalin-fixed tissue from a drowning incident, and successfully carried out the molecular biological diagnosis of drowning using picoplankton 16S rDNA. In 1994, Ludes[2] used the enzyme digestion method to detect diatoms in organs, and concluded that the enzyme digestion method was superior to the acid digestion method in terms of diatom detection rate. Trypsin had better digestion ability and a higher diatom detection rate than other enzymes. However, enzyme digestion methods only focused on the digestive ability of different enzymes, but not on the number of diatom species detected after digestion. Other diatom detection methods include enzyme digestion using smear microscopy. However, the detection rate using this

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technique is inferior to the molecular biology method. In 2008, Fanggang He [3] used primers designed by N bel[4] to amplify three lake water samples and the lung tissues of animals drowned in two of the three lakes, and successfully confirmed that the amplification products in the drowned animals' lungs were similar to the corresponding drowning site, but significantly different to the non-drowning site. These methods no longer depend on the morphology, physical and chemical characteristics of plankton, but amplify the specific sequences of the plankton at the molecular level. PCR has high sensitivity and specificity, and can effectively eliminate the interference of spoilage organisms. It is a new method for the identification of drowning and the 3130 electrophoresis apparatus is commonly used in the Public Security Bureau. In this research we selected primers designed by LAURA etc.[5] and used PCR-Capillary electrophoresis technology to detect the number of diatom species in organs with different tissue mass. We then compared the PCR-Capillary electrophoresis method with microwave digestion-scanning electron microscopy (SEM) for the detection of diatom species.

MATERIALS AND METHODS

Specimens

Sixty male and female rabbits weighing 2.4-3.4 kg were provided by the Animal Experiment Center of Guangdong province. These rabbits were randomly divided into three groups: the drowned group, the submerged after lethal aeroembolism group, and the lethal aeroembolism group which acted as a control group. In winter, rabbits in the drowned group were placed in a cage which was lowered from the Nansha Humen Bridge into the sea to a depth of 0.5 m for 5 s, raised to the surface for 10 s, and then lowered to the same depth again. These steps were repeated until the rabbits died. The cage was left at a depth of 0.5 m for 24 h. Lung, liver and kidney tissues were obtained after death. In addition, 20 human samples were provided by the Guangzhou Institute of Criminal Science and Technology, including kidney and liver tissues.

Diatom separation

Twenty samples of lung, liver and kidney were obtained from each rabbit. Trypsin (20 mg/ml) was added to lung samples weighing 0.3 g, 0.6 g, 0.9 g, 1.2 g, 1.5 g, 2 g, 2.5 g and incubated at 50°C for 8 h. Liver and kidney samples were also digested and centrifuged at 3000 rpm for 30 min, and the supernatant was discarded.

Diatom DNA extraction

The PowerSoil™ DNA Isolation Kit was used to extract DNA from the sediment which was used in subsequent experiments without further purification.

PCR amplification

The primers reported by LAURA[5] were used to amplify the diatom SSU gene. The sequences of these primers are shown in Table 1. These primers were prepared by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.

Table 1. The diatom SSU primer sequences

Primer	Sequences	Modification
Dia-516R	CTCATTCCAATTGCCAGACC	add fam blue fluorescence to the 5 'end
A145F	CCGTAGTAATTCTAGAGCTAATA	

The volume in the PCR reaction was 50 ul and contained 0.2 mmol/L dNTPs, 3 mmol/L MgCl2, two primers each of 10 mmol/L, 0.1 U/ul Taq DNA polymerase, 2×PCR buffer and template DNA 1 ul (the template concentration was 10 ug/ml). The PCR cycle parameters were as follows: 94°C for 2 min, then 94°C for 30 s, 53°Cfor 45 s, 72°C for 2 min for a total of 35 cycles, and 72°C for 15 min.

Microwave digestion-scanning electron microscopy (SEM)

Microwave Digestion—Vacuum Filtration-Automated Scanning Electron Microscopy (MD-VF-Auto SEM) is a new qualitative and quantitative method of diatom testing for the diagnosis of drowning. This new method is based on a microwave digestion technique, vacuum filtration, and automated SEM,

which achieves maximum recovery of diatoms and identifies diatoms easily by SEM with high resolution[6].

Capillary electrophoresis (CE) technology

Over the past two decades, capillary electrophoresis (CE) has been the subject of extensive development and progress in various DNA-based sieving electrophoresis applications, including Sanger sequencing, forensic short tandem repeat (STR) analysis, clinical genotype screening (SNP), and phylogenetic fingerprinting. CE is currently a highly active area of research in analytical chemistry and is the principle system for nucleic acid analysis due to its high separation efficiency and resolution, fast analysis time, minimum sample requirement, high detection sensitivity and ease of automation[7]. The small surface area of capillaries provides better control of temperature in comparison to slab gels, which minimize Joule heating effects allowing higher electric fields to be used resulting in faster separations[8]. Capillary electrophoresis can distinguish differences in product size of the four most common diatoms in the Guangzhou section of the Pearl River. During PCR, 1 ul PCR product, 9 ul formamide and 0.3 ul internal standard were added. Following CE, different PCR products form different peaks according to the diatom species[9].

RESULTS

Rate of diatom detection

In these experimental rabbits, the rate of diatom detection was 100%, 90% and 85% in lung, liver; kidney tissue samples; the drowned group respectively. However, no specific amplification products were detected in the samples from the control group and the rate of diatom detection was only 15% in the lung sample from the submerged after lethal aeroembolism group. The rate of diatom detection in the drowned group was significantly higher than that in the submerged after lethal aeroembolism group and was statistically significant ($P<0.05$).

The sequences of DNA fragments from specific diatoms

The forward sequence for *Cyclotella meneghiniana* was as follows:

CGTTATACCGACTTCTG GAGGGT AGTATTTATTAGGTA TAGA CCAACACCC CTCGGGG TTGCTTTGGT GATTCAT AATAA CTA ATCGGATCGCATGGC TCCATGCCGG CGATGGAT CATTCAA GTTTCTGCCC TATCA GTTTTGGTTGGGAG TGTATTGGACTCCCAAGA C T T T G A C G G G T A A C G A A T T G T T A GGGCAAGATTTCGGAGAGGGAG CCTGAGAGA CGGCTAC CACATCCAAGG AAGG CAGCAG GCGCGTAAA TTACCC AATACTGAAACAGTG AGGTAGTG ACAATA AATAACA ATGCCGGG CCTTTACA G GTCTG GCATTG GAATGAGA

The reverse sequence for *Cyclotella meneghiniana* was as follows

TGACGGCATGTTATTTATTGTC ACTACCTCACTGTTTCAGTATTGGGTAAT TTACGCGCCTGCTGCCTTCCTTGGATGTGGTA GCCGTCTCTCAGGCTCCCTCTCCGAAATCTTG CCCTAACAATTCGTTACCCGTCAAAGTCTTG GGAGTCCAATA CACTCCCAACCA AAACTG ATAGGGCA GAAACTTGAATGA TCCATCGCC GGCATGGA GCCATGCGATCCGA TTAGTTA TTATGAATCACCAAAGCAACCCCG AGGGG TGTTG GTCTATACCT AATAAATA CTACCC TTCCAG AAGTCGGGTATTGAT GCATGTAT TAG CTCTAGAATTACTACGGA

The forward sequence for *Melosira varians* was as follows:

AGGTGTAGATTGTATTTATTAG GTATCAAACCATCCTCAAT TGAGTATATG GTGATTCATGATATTGGAATGGATCAT ACAGC TTTAAGCTGTTGA CAAGTCATATAA GTTTCTG CCCTATCAG C TTTGGATGGTAGGGT ATTTGCCTACCATGGCA TTCACGGGTAACG GGAGATTA GGGTTTGACTCCGGAGAG GGAGCCTGAGAGACGGCTACCACAT CCAAGGAAGGCAGCAGGCG CGTAAATTA CCCAATCCT AATACA GGGAGG TAGTAAC G A T G A A T A A C A A T G T T G G G C C T T T T CAGGTCTGGCAATTGG AATGAGA

The reverse sequence for *Melosira varians* was as follows:

GGAAAAATGTTATTCATCGTTACT A C C T C C C T G T A T T A G G A T T G G G TAATTTACGCGCCTGCTGCCTTCCTTG GATGT GGTA GCCGTCTCTCAGGCTCCCTC TCCGG AGTCAAACCCTAATCTCCCGTTAC CCGTGAA TGCCATGGTAGGCAAATACCCTACCATC CAAAGCTGATAGGGCAGAAACTTATATGA CTTGTCAACA GCTTAAAGCTG TATGATCCA TTCCAATATCATGAATCACCATATACT CAATTGAGGATGGTTTGATACCTAAT

AAATACAATCTTCCGAAG TCAGATTTTA ATGCATGTA TTAGCTCTAGAATTACTACGGA
The forward sequence for *Nitzschia* sp was as follows:

A G G G T C A T A C C T T C T G G A G TA G T A T T T A T T A G A T T G A A A C C A A C C C C T T C G G G G T G A T G T G G T G A T T C A TAATAAGCTTGCGGATCGCATGGCTTTGCCGGCGATG GATCAT TCAAGTTTCTGCCC TATCAGCT TTGGATGGTAGGGTATTGGCCTAC CATGGC TTTAACGGGTAACGGGAAATTAG GGTTTG ATTCCGGAGAGGGAGCCTGAGAGA CGGCTACCACATCCAAGG AAGGCAGCAGGC G C G T A A A T T A C C C A A T C C T G A C A C A GGGAGGTAGTG ACAATAAATAAC AATGCCGG G CCTTTGTAGGTCTGGCAAT TGGAATGAGA
The reverse sequence for *Nitzschia* sp was as follows:

TAACGGTCATTGTTATTTATTGTCAC TACCTCCCTGTGTCAGGATTGGGTAATTTACG CGCCTGCTGCCTTCCTTGGATGTGGT AGCCGTCTCTCAGGCTCCCTCTCCGGA ATCAAACCCTAATTTCCCGTTACCCG TTAAAGCCATGGTAGGCCAATACCCTA CCATCCAAAGCTGATAG GGCAGAAACTTG AATGATCCATCGCCGGCAAAG CCATGCGA T C C G C A A G C T T A T T A T G A A T C A C C ACATCACCCCGAAG GGGTTGGTTTCAATCTA ATAAATACTACCCCAGAA GGGTATTGA CGCATGTATTAGCTC TAGAATTA CTACGGA
The forward sequence for *Synedra* sp was as follows:

TCTAATCCCGACTTCTGGAGGGGTGT ACTTATTAGATGGAAACCAATGCGGGGCAACCC GGATTCTGGTGATTCATAATAATTAT CGGAT CGATCGTATGATCGATGCATCATTCAAGTTT CTGCCCTATCAGCTTTGGATGGTAGG GTATTGGCCTACCATGGCATTAACG GGTAACGGAGAATTAGGGTTCGATT CCGGAGAGGGAGCCTGAGAAATGGCTACC ACATCCAAGGAAGGCAGCAGGCGCGTAAAT TACCCAATCC TGACACAGGGAGGTAGT GACAATAAATAACAATGT CGGGCCTTCG GGTCTGGCAATTGGAATGAGA
The reverse sequence for *Synedra* sp was as follows:

GGCAAAATGCTTATTTATTGTCACT ACCTCCC TGTGTCAGGATTGGGTAATTTACGC GCCTGCTGCCTTCC TTGGATGTGGTAGCC ATTTCTCAGGCTCCCTCTCCGGAATCGAACCCTAATT CTCCGTTACCCGTTAATGCCATGGTAGGC CAATACCCTACCATCCAAAGCTGATAGGGCAG AAACTT GAATGATGCATCGATCATACGATC G AT C C G A T A A T T A T T A T G A A T C A C C AGAATCCGGGTTGCCCCCGCATTGGTT TCCATCTAATAAGTACACCCCTTCCA GGAAGTCGGGGATT GATGCATGTATTAGC TCTAGAATTACTACGGA

The DNA fragments found in this study were compared with sequences in PubMed, which showed that these fragments were from diatoms.

The amplification product size of the diatom SSU gene

The amplification of the product size of the diatom was approximately 340 bp. The specificity of the primers is shown in Figure 1.

Note: The four most common diatoms[10] in the Guangzhou section of Pearl River (*Cyclotella*

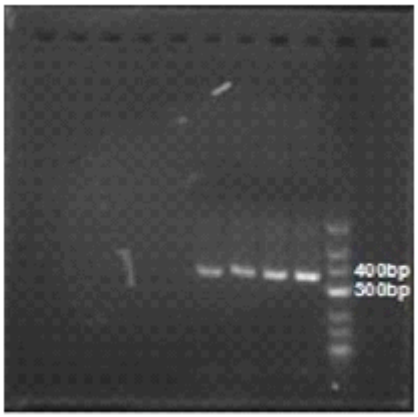

Fig. 1. This figure from left to right shows the human genome, rabbit genome, *Scrippsiella trochoidea*, *Platymonas elliptica*, Prorocentrales, *Nitzschia* sp, *Cyclotella meneghiniana*, *Melosira varians* and *Synedra* sp

meneghiniana, *Melosira varians*, *Nitzschia* sp, *Synedra* sp) were provided by The Institute of Hydrobiology of Chinese Academy of Sciences. The other three types of common algae (*Scrippsiella trochoidea*, *Platymonas elliptica*, Prorocentrales) were provided by The Red Tide and Marine Biology Research Center, Jinan University.

The Capillary electrophoresis results

The Capillary electrophoresis results are shown in Figures 2-7.

Fig. 2. *Cyclotella meneghiniana*

Fig. 3. *Melosira varians*

Fig. 4. *Synedra* sp

Fig. 5. *Nitzschia* sp

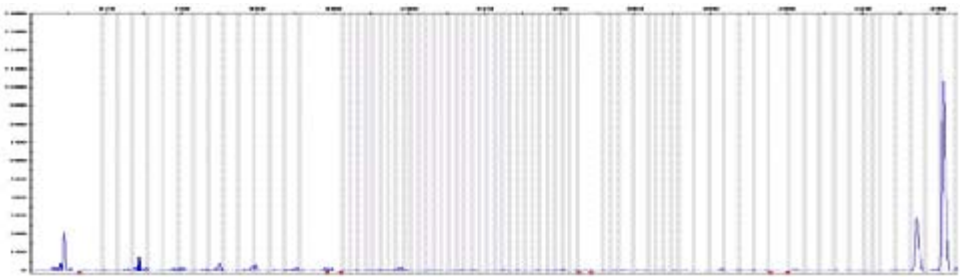

Fig. 6. Capillary electrophoresis results of the diatoms in rabbit liver (*Cyclotella meneghiniana* and *Nitzschia* sp)

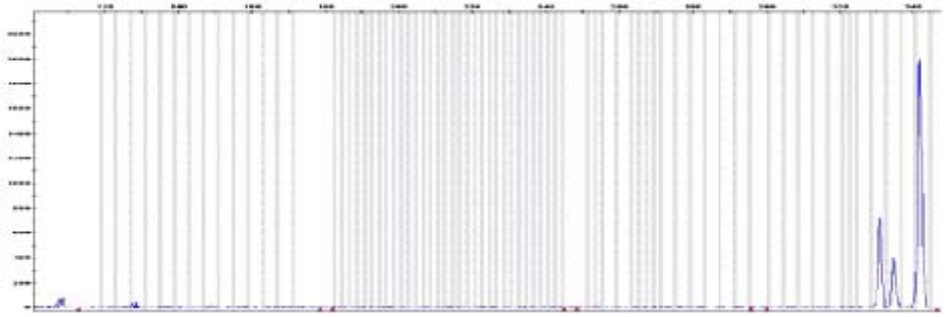

Fig. 7. Capillary electrophoresis results of the diatoms in rabbit lung (*Cyclotella meneghiniana*, *Synedra* sp and *Nitzschia* sp)

The numbers of diatom species detected in the lung, liver and kidney

The numbers of diatom species detected in the lung, liver and kidney following PCR-Capillary electrophoresis are shown in Tables 2-4.

Table 2. The number of diatom species detected in the lung

Types	Types * quantity Cross-tabulation Quantity							
	0.3 g	0.6 g	0.9 g	1.2 g	1.5 g	2 g	2.5 g	Total
0	7(35%)	4(20%)	1(5%)	0(0%)	1(5%)	0(0%)	0(0%)	13(9.3%)
1	10(50%)	8(40%)	3(15%)	2(10%)	3(15%)	3(15%)	3(15%)	32(22.9%)
2	2(10%)	5(25%)	9(45%)	11(55%)	9(45%)	10(50%)	9(45%)	55(39.3%)
3	1(5%)	3(15%)	6(30%)	5(25%)	6(30%)	5(25%)	7(35%)	33(23.6%)
4	0(0%)	0(0%)	1(5%)	2(10%)	1(5%)	2(10%)	1(5%)	7(5%)
Total	20(100%)	20(100%)	20(100%)	20(100%)	20(100%)	20(100%)	20(100%)	140(100%)

Spearman's rho was 0.433 (P<0.01). When the amount of biomaterial was increased, the number of diatom species detected in the lung gradually increased, and was statistically significant (P < 0.01). Bivariate correlation analysis showed a positive correlation between quantity and types of diatoms. The proportion of diatom species detected in each group varied. When the quantity was greater than 0.9 g, samples which included two or more types of diatoms predominated.

Table 3. The number of diatom species detected in the liver

Types	Types * quantity Cross-tabulation Quantity							
	0.3 g	0.6 g	0.9 g	1.2 g	1.5 g	2 g	2.5 g	Total
0	15(75%)	11(55%)	7(35%)	3(15%)	2(10%)	1(5%)	1(5%)	40(28.6%)
1	5(25%)	8(40%)	10(50%)	11(55%)	8(40%)	7(35%)	6(30%)	55(39.3%)
2	0(0%)	1(5%)	3(15%)	5(25%)	7(35%)	8(40%)	9(45%)	33(23.6%)
3	0(0%)	0(0%)	0(0%)	1(5%)	2(10%)	3(15%)	2(10%)	8(5.7%)
4	0(0%)	0(0%)	0(0%)	0(10%)	1(5%)	1(5%)	2(10%)	4(2.9%)
Total	20(100%)	20(100%)	20(100%)	20(100%)	20(100%)	20(100%)	20(100%)	140(100%)

Arman's rho was 0.623 (P<0.01). When the amount of biomaterial was increased, the number of diatom species detected in the liver gradually increased, and was statistically significant (P < 0.01). Bivariate correlation analysis showed a positive correlation between quantity and types of diatoms. The proportion of diatom species detected in each group varied. When the

Table 4. The number of diatom species detected in the kidney

Types	Types * quantity Cross-tabulation Quantity							
	0.3 g	0.6 g	0.9 g	1.2 g	1.5 g	2 g	2.5 g	Total
0	18(90%)	14(70%)	11(55%)	7(35%)	5(25%)	3(15%)	3(15%)	61(43.6%)
1	2(10%)	6(30%)	8(40%)	9(45%)	7(35%)	5(25%)	4(20%)	41(29.3%)
2	0(0%)	0(0%)	1(5%)	3(15%)	6(30%)	8(40%)	9(45%)	27(19.3%)
3	0(0%)	0(0%)	0(0%)	1(5%)	2(10%)	3(15%)	3(15%)	9(6.4%)
4	0(0%)	0(0%)	0(0%)	0(10%)	0(0%)	1(5%)	1(5%)	2(1.4%)
Total	20(100%)	20(100%)	20(100%)	20(100%)	20(100%)	20(100%)	20(100%)	140(100%)

quantity was greater than 1.5 g, samples which included one or more types of diatoms predominated.

Spearman's rho was 0.613 (P<0.01). When the amount of biomaterial was increased, the number of diatom species detected in the kidney gradually increased, and was statistically significant (P < 0.01). Bivariate correlation analysis showed a positive correlation between quantity and types of diatoms. The proportion of diatom species detected in each group varied. When the quantity was greater than 2 g, samples which included one or more types of diatoms predominated.

A comparison of PCR-Capillary electrophoresis with microwave digestion-scanning electron microscopy (SEM) in the detection of diatom types.

Table 5. A comparison of PCR-Capillary electrophoresis and microwave digestion-scanning electron microscopy

	SEM	PCR-Capillary electrophoresis
Sample 1	3	3
Sample 2	2	3
Sample 3	2	3
Sample 4	3	4
Sample 5	2	3
Sample 6	1	3
Sample 7	3	3
Sample 8	2	2
Sample 9	4	3
Sample 10	1	3
Sample 11	2	2
Sample 12	2	2
Sample 13	2	3
Sample 14	3	2
Sample 15	4	3
Sample 16	2	4
Sample 17	3	3
Sample 18	2	2
Sample 19	3	3
Sample 20	2	3

$R_1 = 15$, $R_2 = 63$ ($P < 0.05$). Significantly more diatom types were detected by PCR-Capillary electrophoresis than by microwave digestion-scanning electron microscopy and the difference was statistically significant ($P < 0.05$).

Note: The 20 samples were randomly selected from cases of human drowning and included kidney and liver tissue. The microwave digestion-scanning electron microscopy results of samples 1-20 were provided by Guangzhou Institute of Criminal Science and Technology. The diatom species shown in Table 2 to Table 5 were the four most common diatoms in Pearl River of Guangzhou section.

DISCUSSION

Research on the reliability of the diatom detection method for the diagnosis of drowning has yielded widely divergent results, the most critical of which often rely on studies lacking a rigorous methodology. However, this method represents a useful supportive tool for the diagnosis of death by drowning[11]. At present, although there are a number of reports on diatom detection, the acid digestion method is still the main method used in practice[6]. The diatom detection rate using the acid digestion method is low and is prone to contamination. Thus, it is difficult to eliminate pollution factors and the method is controversial[10]. In the present study, PCR-Capillary electrophoresis technology is used to identify diatoms in lung, liver and kidney of different tissue mass, and to assess the effects of these diatom species. This study was based on molecular biology methods, which were sensitive and specific for the detection of diatoms, and strongly supported the diagnosis of death by drowning. In this study, three other types of common algal (*Scrippsiella trochoidea, Platymonas elliptica,* Prorocentrales) genomic DNA, human genome DNA, rabbit genomic DNA, and diatom genomic DNA were used as templates. Under the same PCR conditions, the products were tested using agarose electrophoresis, and only the diatom genomic DNA showed a positive band, which proved that the primers were specific to the diatoms. The DNA fragment sequences were compared with sequences in PubMed, which proved that the fragments were from diatoms. When the amount of biomaterial was increased, the number of diatom species detected in the lung, liver and kidney gradually increased, which was statistically significant ($P < 0.01$). Bivariate correlation analysis showed a positive correlation between quantity and the types of diatoms. The proportion of diatom species detected in each group varied. Many investigations have focused on the

relation between diatom species and diatom quantity. In general, the sites with more diatom species showed a greater quantity of diatoms[12]. In the present study, due to the number of diatom species, the quantity of diatoms at the site of drowning can be estimated. CE appears to be an economic and rapid option for microsatellite fragment size analysis, and offers good typability, discrimination and ease of use[13]. From the results shown in Fig. 6-7, it can be seen that when the diatom concentrations were similar, the single peaks of the diatom were close, but the height of peaks in the animal samples were different, suggesting that different diatom species in drowned animals showed different diatom quantities. *Melosira varians* appeared to be the dominant diatom species in drowned rabbit organs. Sice Many impurity peaks were observed, thus PCR products should be purified in subsequent studies. When there are many types of diatom DNA in the template, the amplification efficiency of each diatom DNA is different; the peak difference is significant; and parts of the peak are easily suppressed. In future practice, the primers should be improved to ensure the amplification efficiency of each diatom DNA. The next step will be to add more diatom species for the test to build a bigger diatom species library for the detection of more types of diatoms in organs. In addition, large animal experiments should be initiated, and material types and quantity should be increased to reflect human drowning in order to improve the identification of drowning in forensic medicine.

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