Detection of *Aeromonas hydrophila* gyrB and 16S rRNA Genes by PCR Capillary Electrophoresis: Postprint of a Drowning Diagnostic Method

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Abstract

Objective To establish a PCR capillary electrophoresis detection method for Aeromonas hydrophila gyrB and 16S rRNA genes and investigate their application value in the diagnosis of freshwater drowning. Methods DNA was extracted from human, 18 types of planktonic organisms (Candida albicans, Aeromonas hydrophila, and 16 types of algae), and tissue samples (one each of lung, liver, and kidney per case) from 30 cadavers examined by the microwave digestion-vacuum filtration-automated SEM method (28 cases of antemortem freshwater drowning and 2 cases of natural death on land). Amplification was performed using primer AH (gyrB gene) and primer Ah (16S rRNA gene), respectively, and the amplification products were detected by capillary electrophoresis. Results Capillary electrophoresis detection after DNA amplification showed negative results for human, Candida albicans, and the 16 types of algae, while positive results were obtained for Aeromonas hydrophila, with product sizes of 195 bp and 350 bp, respectively. PCR capillary electrophoresis detection of lung, liver, and kidney samples from 28 freshwater drowning cadavers using primers AH and Ah revealed Aeromonas hydrophila detection rates of 96.4%, 71.4%, and 60.7% for primer AH, and 75.0%, 42.9%, and 32.1% for primer Ah, respectively. The calculated overall positive rates for Aeromonas hydrophila detection in the systemic circulation organs of the drowning victims were 82.1% and 53.6%, respectively. Both cadaver samples from the two natural death cases on land showed negative results. There was a significant difference in the detection rates of Aeromonas hydrophila between the two primer pairs AH and Ah ($P<0.05$). Conclusion PCR capillary electrophoresis detection of the Aeromonas hydrophila gyrB gene demonstrates high sensitivity for diagnosing freshwater drowning and can serve as an auxiliary diagnostic method; combined use of the 16S rRNA gene can improve the detection rate of this bacterium and

enhance the evidentiary value of the MD-VF-Auto SEM method in diagnosing freshwater drowning.

Full Text

Preamble

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Abstract

Objective: To establish a PCR-capillary electrophoresis (CE) method for detecting the *gyrB* and 16S rRNA genes of *Aeromonas hydrophila* and evaluate its application in diagnosing freshwater drowning.

Methods: DNA was extracted from human samples, 18 types of plankton (*Candida albicans*, *Aeromonas hydrophila*, and 16 algal species), and tissue samples (lung, liver, and kidney, one sample each per case) from 30 cadavers previously examined by microwave digestion-vacuum filtration-automated scanning electron microscopy (MD-VF-Auto SEM), including 28 freshwater drowning victims and 2 cases of natural death on land. The DNA samples were amplified using primer AH (targeting the *gyrB* gene) and primer Ah (targeting the 16S rRNA gene), and the amplification products were analyzed by capillary electrophoresis.

Results: Capillary electrophoresis following PCR amplification yielded negative results for human, *Candida albicans*, and algal DNA, whereas *Aeromonas hydrophila* DNA showed positive results with product sizes of 195 bp and 350 bp, respectively. In the 28 freshwater drowning cases, the detection rates of *A. hydrophila* in lung, liver, and kidney tissues were 96.4%, 71.4%, and 60.7% using primer AH, and 75.0%, 42.9%, and 32.1% using primer Ah, respectively. The overall positive rates in the systemic organs of drowning victims were 82.1% with primer AH and 53.6% with primer Ah. Both cases of natural death tested negative. The detection rates between the two primer sets were significantly different ($P<0.05$).

Conclusion: PCR-CE detection of the *gyrB* gene of *Aeromonas hydrophila* demonstrates high sensitivity for assisting in the diagnosis of freshwater drowning and can serve as an auxiliary diagnostic method. Combined detection of both the *gyrB* and 16S rRNA genes can improve the detection rate and

strengthen the evidentiary value of MD-VF-Auto SEM for diagnosing freshwater drowning.

Key words: forensic pathology; drowning; *Aeromonas hydrophila*; polymerase chain reaction; capillary electrophoresis

Introduction

Currently, the primary methods for forensic diagnosis of drowning include autopsy, diatom testing, and planktonic bacterial examination [1]. Autopsy-based diagnosis relies on typical drowning features such as froth in the mouth, nose, and airways, pulmonary edema, pleural effusion, and subpleural hemorrhage. However, these features become inconspicuous or absent in advanced decomposition, making cause of death determination difficult [1]. Diatom testing is considered the gold standard for diagnosing drowning in highly decomposed bodies, but conventional methods have drawbacks including hazardous procedures, diatom loss during centrifugation, and low sensitivity [2]. The microwave digestion-vacuum filtration-automated scanning electron microscopy (MD-VF-Auto SEM) method significantly improves the specificity and detection rate of diatom analysis, enabling quantitative and qualitative detection as well as drowning site inference [3]. Nevertheless, this technique requires highly skilled operators, expensive equipment, and lengthy processing times. Therefore, developing alternative detection methods for drowning-associated plankton is crucial to complement existing diagnostic approaches and provide rapid, accurate auxiliary evidence for freshwater drowning determination.

With advances in molecular biology, PCR-based detection of plankton for drowning diagnosis offers advantages of speed, convenience, and high specificity. For instance, Xu et al. [4] utilized magnetic nanoparticles for diatom DNA extraction to enhance PCR detection rates, while Yu et al. [5-6] employed PCR technology to identify microalgae species in tissues of drowned experimental rabbits, obtaining results within one working day. However, no studies have been conducted in China on using *Aeromonas hydrophila* for drowning diagnosis. *Aeromonas hydrophila* is a small (0.2–2.0 m) planktonic bacterium predominantly distributed in freshwater environments. During drowning, this organism enters the victim' s respiratory system with aspirated water and subsequently reaches systemic organs via blood circulation [7]. This study employed two specific primer sets targeting the *gyrB* and 16S rRNA genes of *A. hydrophila*, using PCR-capillary electrophoresis to analyze DNA extracted from 18 standard plankton species. Our objective was to establish a molecular biological method for detecting *A. hydrophila* in tissues of freshwater drowning victims, laying an experimental foundation for using this bacterium as an auxiliary indicator in freshwater drowning identification. This approach also promises to improve the timeliness of cause-of-death determination in water-related fatalities and offers valuable application prospects in criminal investigation.

Methods

1.1 Instruments and Reagents

VeritiTM 96-well Thermal Cycler (Applied Biosystems, USA), 3130 Capillary Electrophoresis Instrument (Applied Biosystems, USA), ThermoMixer (Zhuhai Bomaijie Biotechnology Co., Ltd.), Vortex-Genie (Scientific Industries, USA), and Benchtop Centrifuge (Sigma, Germany) were used.

PowerSoilTM DNA Isolation Kit was purchased from Shenzhen Anbisen Company, 20 mg/mL Proteinase K (Qiagen), Premix Taq polymerase (Takara), formamide (Applied Biosystems), and CC5 ILS500 (Applied Biosystems).

1.2 Materials

1.2.1 Standard Strains: Standard algal strains including *Chlamydomonas*, soil algae, *Tetraselmis tetrathele*, *Pseudanabaena* sp., *Skeletonema*, *Fragilaria* sp., *Anabaena azollae*, *Tetraselmis*, *Navicula* sp., *Cyclotella* sp., *Nitzschia* sp., *Synedra radians*, *Melosira varians*, and *Chlorella* were obtained from the Institute of Hydrobiology, Chinese Academy of Sciences. Standard strains of *Microcystis* and *Distephanus* were purchased from the Research Center for Red Tide and Marine Biology, Jinan University. Standard strains of *Candida albicans* and *Aeromonas hydrophila* were acquired from the Guangdong Institute of Microbiology.

1.2.2 Tissue Samples: Thirty cadaveric tissue samples (lung, liver, kidney) and their MD-VF-Auto SEM examination results were provided by the Guangzhou Institute of Criminal Science and Technology, comprising 28 freshwater drowning cases and 2 natural death cases on land. For PCR analysis, 0.5 g of lung, liver, or kidney tissue was sampled from each case. The overall positive rate of *A. hydrophila* detection in drowning victims was calculated based on positive findings in at least one systemic organ (liver or kidney) in addition to lung tissue.

1.3 DNA Extraction from Algae, Bacteria, and Tissue Samples

Following the method described in reference [8], 0.5 mL of liquid culture containing algal or bacterial standard strains was added to PowerBead tubes. For tissue samples, 0.5 g of each specimen was minced and placed in PowerBead tubes with 10 L of Proteinase K (20 mg/mL), followed by incubation in a ThermoMixer at 56°C for 2 hours and standing for 10 minutes. DNA from tissues and the 18 planktonic species was extracted using the PowerSoilTM DNA Isolation Kit.

1.4 PCR Amplification

Primers AH and Ah, targeting the *gyrB* and 16S rRNA genes of *A. hydrophila* as reported by Suto [9] and Rao et al. [10], were used. The primer sequences

were as follows: - AHF: 5’ -GAACGACGCCTATCAGGAAG-3’ - AHR: 5’ -ACGGAGATAACGGCAATCAG-3’- AhF: 5’-GGGAGTGCCTTCGGGAATCAGA-3’ - AhR: 5’ -TCACCGCAACATTCTGATTTG-3’

All forward primers were labeled with FAM fluorescence at the 5’ end and synthesized by Sangon Biotech (Shanghai). The total PCR reaction volume was 20 L, containing 10 L Premix Taq polymerase, 0.75 L each of forward and reverse primers (10 mol/L), 7.5 L deionized water, and 1 L template DNA. Thermal cycling parameters were: initial denaturation at 94°C for 10 min; 35 cycles of 94°C for 30 s, 61°C for 40 s, and 72°C for 40 s; final extension at 72°C for 10 min for primer AH. For primer Ah: initial denaturation at 94°C for 10 min; 35 cycles of 94°C for 30 s, 63°C for 40 s, and 72°C for 40 s; final extension at 72°C for 10 min.

1.5 Specificity Testing

The two primer sets were used to amplify DNA from 18 standard strains and human genomic DNA, including *Chlamydomonas*, soil algae, *Tetraselmis tetrathele*, *Pseudanabaena* sp., *Skeletonema*, *Fragilaria* sp., *Anabaena azollae*, *Tetraselmis*, *Navicula* sp., *Cyclotella* sp., *Nitzschia* sp., *Synedra radians*, *Melosira varians*, *Chlorella*, *Microcystis*, *Distephanus*, *Candida albicans*, *Aeromonas hydrophila*, human female DNA, and human male DNA.

1.6 Capillary Electrophoresis Detection

PCR products were analyzed by capillary electrophoresis. The loading sample consisted of 9 L formamide, 1 L ILS CC500 internal standard, and 1 L PCR product.

1.7 Product Sequencing

One positive systemic organ sample each from primers AH and Ah was randomly selected and sent to Sangon Biotech (Shanghai) for sequencing. Results were compared against the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

1.8 Statistical Analysis

SPSS 22.0 software was used for statistical analysis. Independent t-tests were employed to compare the two detection methods using primers AH and Ah, with $P<0.05$ considered statistically significant.

Results

2.1 Primer Specificity Verification

Primers AH and Ah were used to amplify DNA from 16 algal species, humans, *Candida albicans*, and *Aeromonas hydrophila*, followed by capillary electrophoresis. Results showed that only *A. hydrophila* genomic DNA yielded amplification

products of 195 bp for the *gyrB* gene and 350 bp for the 16S rRNA gene, consistent with expected sizes. No positive fragments were detected from human, algal, or *Candida* DNA, demonstrating excellent species specificity for both primer sets.

2.2 Detection Results from 30 Cadaveric Tissue Samples by PCR-CE and MD-VF-Auto SEM

DNA was extracted from tissue samples of 30 cases and analyzed by PCR using primers AH and Ah, followed by capillary electrophoresis. The results were compared with MD-VF-Auto SEM findings, with systemic organ results summarized in . The MD-VF-Auto SEM method showed negative diatom detection in lung, liver, and kidney tissues from Cases 1 and 16 (natural deaths). In contrast, all 28 freshwater drowning cases showed positive diatom results in organ samples, yielding an overall positive rate of 100%.

The PCR-CE method demonstrated negative results for Cases 1 and 16, consistent with case histories. For the 28 drowning victims, detection rates of *A. hydrophila* in lung, liver, and kidney tissues were 96.4%, 71.4%, and 60.7% using primer AH, and 75.0%, 42.9%, and 32.1% using primer Ah, respectively. The overall positive rates in systemic organs of drowning victims were 82.1% for primer AH and 53.6% for primer Ah. Primer AH consistently showed higher detection rates than primer Ah across all organ types. The difference in positive rates between the two primers for detecting *A. hydrophila* in drowning victims' organs was statistically significant ($P<0.05$).

2.3 Sequencing and Alignment Results

PCR products amplified from systemic organ samples using primers AH and Ah were sequenced. The sequences could distinguish *Aeromonas* spp. from *Vibrio cholerae*, *Escherichia coli*, and *Plesiomonas shigelloides*, confirming that *gyrB* gene analysis is suitable for bacterial classification and identification of closely related species. For this reason, we selected these two conserved genes as target molecules to establish an accurate and effective method for detecting *A. hydrophila* in cadaveric tissues.

The PCR product sequences were as follows: - (AH): GCAGACTTGGC-
CTTCTTGCTGTAGTCCTCTTTGTCCATGTAGGAGTTGAGGGTACGGGTCAGCGCGGTACGGAAGC
- (Ah): GTCGAGTTGCAGACTCCGATCCGGACTACGACGCGCTTTTTGGGATTCGCT-
CACTATCGCTAGTTTGCAGCCCTCTGTACGCGCCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGG

Sequencing of the AH primer product showed 96–99% homology with *gyrB* gene sequences JX275847, DQ519366, and AY987520 in GenBank, confirming it as a specific band for *A. hydrophila gyrB*. The Ah primer product exhibited >99% homology with 16S rRNA gene sequences EF077527, DQ207728, and DQ990053, confirming its specificity for *A. hydrophila* 16S rRNA.

Discussion

3.1 Utility of 16S rRNA and *gyrB* Genes of *Aeromonas hydrophila* in Drowning Diagnosis

The two target genes selected in this study were the 16S rRNA gene and the DNA gyrase subunit B gene (*gyrB*), both highly conserved across species and widely used for determining phylogenetic relationships [11]. The 16S rRNA gene enables identification of nearly all bacteria at the genus level and serves as a primary marker for bacterial phylogenetic classification [12]. The *gyrB* gene, encoding DNA gyrase subunit B, meets the requirements for bacterial phylogenetic classification [13]. Yanez et al. [14] analyzed *gyrB* sequences from 53 *Aeromonas* strains and found that this gene could discriminate *Aeromonas* species with interspecies similarity rates of 86.7–100%.

Our primers AH and Ah specifically amplified only *A. hydrophila* DNA, producing fragments of 195 bp and 350 bp, consistent with reported product sizes [9,10]. Amplification of human, algal, and *Candida* DNA yielded negative results, demonstrating high primer specificity.

3.2 Application Value of the Two Primer Sets in Drowning Identification

Cases 1 and 16, involving natural deaths on land, showed negative results with both primers, consistent with case details. For the remaining 28 drowning cases, detection rates in lung, liver, and kidney tissues were 96.4%, 71.4%, and 60.7% with primer AH, and 75.0%, 42.9%, and 32.1% with primer Ah, respectively. The overall positive rates in drowning victims' organs were 82.1% for AH and 53.6% for Ah. Primer AH demonstrated superior detection capability compared to Ah, with statistically significant differences in positive rates ($P<0.05$). Analysis using Oligo 7.0 software revealed that primer AH had better overall evaluation scores, conferring greater detection advantage.

In Case 4, primer AH yielded negative results while Ah was positive. The combined positive rate using both primers reached 85.7%, consistent with Uchiyama et al.' s [15] reported 84.0% positive rate for *Aeromonas* in freshwater drowning victims. This demonstrates that combined use of both primers can improve detection rates.

The higher detection rate in lung tissue compared to liver and kidney reflects that planktonic organisms more readily infiltrate the lungs due to water pressure. Therefore, positive lung results alone cannot confirm drowning [16] and must be integrated with findings from other systemic organs. Since *A. hydrophila* primarily inhabits freshwater, this method is applicable to freshwater drowning cases but not seawater or bathtub drowning victims or non-drowning cases [17]. Comprehensive analysis of *A. hydrophila* detection results from multiple systemic organs is essential for determining whether a body recovered from water represents antemortem submersion (i.e., drowning).

3.3 Comparison Between PCR-CE and MD-VF-Auto SEM Methods

The MD-VF-Auto SEM method represents a reliable approach for diatom testing [3]. In this study, it showed negative diatom results in Cases 1 and 16 (natural deaths) and positive results in all 28 freshwater drowning cases, achieving 100% overall positive rate. While this method provides accurate qualitative and quantitative analysis, it requires large tissue samples (2 g lung, 10 g liver/kidney), expensive equipment, 2 days from sampling to results, and can only process one case at a time, demanding highly skilled operators.

In contrast, PCR-CE requires minimal sample amounts (0.5 g per tissue), is simple to operate, rapid (<1 day), and can process multiple samples simultaneously (up to 96). For Cases 5, 10, 13, and 29, MD-VF-Auto SEM detected only 1, 3, 2, and 1 diatoms per 10 g liver tissue, respectively—insufficient for definitive drowning diagnosis [18-19]. Diatom counts exceeding 4 per 10 g are required to confirm antemortem submersion [20]. However, PCR-CE detection of *A. hydrophila gyrB* gene in these cases yielded positive results, providing auxiliary evidence for freshwater drowning when MD-VF-Auto SEM results are inconclusive or diatom counts are low (<3 per 10 g).

Negative results in Cases 11, 19, 22, and 27 may be attributed to low bacterial concentration at the drowning site or excessively low water temperature, resulting in insufficient *A. hydrophila* in the victims' liver and kidney tissues for PCR-CE detection. Although our method' s detection rate is slightly lower than MD-VF-Auto SEM, it is highly suitable for high-throughput screening, requires short processing time, uses standard forensic laboratory equipment, and involves simple technical operations. Therefore, this method provides an important forensic tool offering auxiliary indicators for freshwater drowning diagnosis through planktonic bacterial detection.

Conclusion

The PCR-CE method for detecting *Aeromonas hydrophila gyrB* gene established in this study demonstrates high sensitivity, short processing time, and operational simplicity, making it a valuable auxiliary method for freshwater drowning diagnosis. It shows promising potential for rapid screening of drowning cases and guiding further confirmatory testing. Combined detection of the 16S rRNA gene can improve detection rates and strengthen the evidentiary value of MD-VF-Auto SEM for diagnosing freshwater drowning.

References

[1] Yukawa N, Kakizaki E, Kozawa S. Diatom and laboratory tests to support a conclusion of death by drowning//Essentials of Autopsy Practice. London: Springer, 2013: 1-36.
[2] Xu Q, Liu C, Zhao J, et al. Comparison of digestion methods complied with two nanofiltration membranes on the efficiency of diatoms recovery. J Nanosci

Nanotechnol, 2016, 16(7): 7129-33.
[3] Zhao J, Liu C, Hu SL, et al. Microwave Digestion-Vacuum Filtration-Automated scanning electron microscopy as a sensitive method for forensic diatom test. Int J Legal Med, 2013, 127(2): [page numbers missing in original]
[4] Xu QY, Yu ZL, Mai BS, et al. Magnetic nanoparticles enhanced DNA extraction and detection of forensic algae by multiplex PCR capillary electrophoresis. Nanosci Nanotechnol Letters, 2016, 8(8): 682-7.
[5] Yu Z, Liu C, Hu S, et al. PCR-Capillary electrophoresis is a new method for forensic diatom testing. J Pure Applied Microbiol, 2014, 8(2): 1467-74.
[6] Yu ZL, Liu C, Wang HJ, et al. The effect of enzyme digestion time on the detection of diatom species. Pak J Pharm Sci, 2014, 27(3, S): 691-4.
[7] Kakizaki E, Takahama K, Seo Y, et al. Marine bacteria comprise a possible indicator of drowning in seawater. Forensic Sci Int, 2008, 176(2/3): 236-47.
[8] Li P, Xu QY, Chen L, et al. Detection of algae 16SrDNA specific fragments in the diagnosis of drowning. Journal of Southern Medical University, 2015, 35(8): 1215-8.
[9] Suto M, Kato N, Abe S, et al. PCR detection of bacterial genes provides evidence of death by drowning. Leg Med (Tokyo), 2009, 11(Suppl 1): S354-6.
[10] Rao JJ, Li SS, Huang KH, et al. Establishment of a multiplex PCR detection method for pathogenic Aeromonas hydrophila. Journal of Fishery Sciences of China, 2007, 14(5): 749-55.
[11] Hou XL, Chen Z. A new target for bacterial classification and identification: the gyrB gene. Foreign Medical Sciences: Section of Epidemiology and Infectious Diseases, 2005, 32(1): 38-41.
[12] Liu C, Li JB, Rui JP, et al. Application of the 16S rRNA gene in microbial ecology. Acta Ecologica Sinica, 2015, 35(9): 2769-88.
[13] Izumi S, Yamamoto M, Suzuki K, et al. Identification and detection of *Pseudomonas plecoglossicida* isolates with PCR primers targeting the gyrB region. J Fish Dis, 2007, 30(7): 391-7.
[14] Yanez MA, Catalan V, Apraiz D, et al. Phylogenetic analysis of members of the genus *Aeromonas* based on gyrB gene sequences. Int J Syst Evol Microbiol, 2003, 53(3): 875-83.
[15] Uchiyama T, Kakizaki E, Kozawa S, et al. A new molecular approach to help conclude drowning as a cause of death: Simultaneous detection of eight bacterioplankton species using real-time PCR assays with TaqMan probes. Forensic Sci Int, 2012, 222(1/3): 11-26.
[16] He FG, Huang DX, Liu L, et al. A novel PCR-DGGE-based method for identifying plankton 16S rDNA for the diagnosis of drowning. Forensic Sci Int, 2008, 176(2/3): 152-6.
[17] Rutty GN, Bradley CJ, Biggs MJ, et al. Detection of bacterioplankton using PCR probes as a diagnostic indicator for drowning; the Leicester experience. Leg Med, 2015, 17(5): 401-8.
[18] Ludes B, Quantin S, Coste M, et al. Application of a simple enzymatic digestion method for diatom detection in the diagnosis of drowning in putrified corpses by diatom analysis. Int J Legal Med, 1994, 107(1): 37-41.
[19] Ludes B, Coste M, Tracqui A, et al. Continuous river monitoring of the

diatoms in the diagnosis of drowning. J Forensic Sci, 1996, 41(3): 425-8.
[20] Zhao J, Yuan ZC, Zhang YJ, et al. Comparison of two diatom test methods. Chinese Journal of Forensic Medicine, 2015, 30(1): 62-5.

Note: Figure translations are in progress. See original paper for figures.