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Determination the presence of amplification products of 16s rRNA microcystis aeruginosa as a biomarker of drowning

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Abstract: Forensic medical diagnostics of drowning now is a difficult issue to resolve. Determination of diatom plankton with light microscopy is one of the supplementary methods for diagnostics of drowning. The disadvantage of this method is the use of concentrated acids to destroy the tissues of the organs, which greatly complicates, and sometimes precludes the detection of diatom plankton. In this case, the detection of other phytoplankton species in internal organs is treated as pseudoplankton, but does not have a diagnostic value. We have developed a sensitive and specific method of drowning diagnostics using a pair of specific oligonucleotide primers by the polymerase chain reaction (PCR) method to determine the presence of DNA of Cyanobacteria of the genus Microcystis, namely a fragment of the 16S rRNA gene in the tissues of mice and water samples in order to establish the fact and place of drowning. In order to evaluate the diagnostic value of this method, we conducted an experimental study to detect fragments of the 16S rRNA gene in mice tissues during drowning and post-mortem immersion. The amplification products were found in the tissues of heart, kidneys, liver, spleen, bone tissue, brain tissue, and lungs in case of drowning. During post-mortem immersion products of amplification are detected only in the tissues of lungs. The results indicate that the proposed PCR method is a potentially useful tool for diagnosing of mechanical asphyxia as a result of drowning.
Key Words: Drowning, Forensic pathology, DNA, Polymerase chain reaction, Cyanobacteria.

INTRODUCTION

According to a global report by the World Health Organization, drowning occurs annually with 360,000 people worldwide dying as a result of drowning, accounting for more than 9% of global mortality [1]. More than 90% of deaths due to drowning are recorded in low and middle-income countries. Mechanical asphyxia as a result of drowning in Ivano-Frankivsk region amounted to 354 cases for the period from 2012 to 2016. This is 4,5% of all types of expertise conducted by the Ivano-Frankivsk Regional Bureau of Forensic Medical Examination [2].

Forensic medical diagnostics mechanical asphyxia as a result of drowning is currently a difficult issue for resolution [3-8]. Drowning is a leading cause of unintentional deaths [9]. A number of methods have been developed over the years to determine mechanical asphyxia as a result of drowning [10].

Determination of the presence of diatom algae is one of the supplementary methods of diagnosis, not only drowning but sometimes the place of drowning [11-13]. The scientific community is constantly discussing the authenticity and reliability of the diatom test at drowning [14]. There are even several cases where the expert's findings obtained using diatoms have been challenged and sometimes canceled in court [15].

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In some cases, the presence of diatom algae during drowning is so small that it is not possible to detect them by microscopy [16]. Picoplankton, for example, could not be morphologically discriminated from other bacteria such as Proteobacteria and Bacillus by microscopy [17-19]. In such cases, the presence of phytoplanktonic DNA detected by PCR in tissues during drowning is perhaps the only method for diagnosis of drowning [20-25].

Cyanobacteria is one of the most common phytoplankton groups that can be found not only in standing water of lakes but also in seawater [26]. Microcystis aeruginosa is one of the most common types of cyanobacteria. These are the simplest single-celled algae, in which there is no nucleus and mitochondria. Microscopic colonies of spherical and carnal forms develop in fresh water with standing and slowly flowing water. Cyanobacteria that produce hepatotoxins can be harmful to human and animal health, especially when they have a high concentration of water and cause the formation of blooms [27].

The development of criteria for determination of drowning, using experimental drowning of mice, satisfies the statistical requirements necessary for argumentation. When experimental drowning water with available phytoplankton in it penetrates into closed organs, as in the case of lethal drowning in humans, the differences between experimental and real drowning will be minimal.

MATERIALS AND METHODS

Sample collection of water

For the research, a water sample from the town lake of Tysmenytsia, Ivano-Frankivsk region, Ukraine was conducted. This lake is characterized by lentil conditions, namely the lack of significant movement of water. Water was taken from five different places in a sterile plastic tube with a volume of 10 mL. Coordinates of water collection points: point number 1 - latitude 48°54'4.51"N longitude 24°50'32.93"E; point number 2 - latitude 48°54'5.05"N longitude 24°50'30.49"E; point number 3 - latitude 48°54'8.51"N longitude 24°50'31.06"E; point number 4 - latitude 48°54'10.87"N longitude 24°50'33.80"E; point number 5 - latitude 48°54'7.85"N longitude 24°50'34.20"E.

Before the sampling of water samples with phytoplankton, the temperature of air and water was determined. The temperature was measured using a mercury thermometer with a graduation scale of 0,5° C. The water temperature at the place of drowning was 16°C. All water sampling was carried out between 11 and 13 hours of PM.

Experimental animals

Eighteen white mice of any sex from the clinical and biological base (vivarium) of the Ivano-Frankivsk National Medical University were used during the scientific experiment procedure. All animals were randomly divided into three groups: animals that were drowned (n = 6), animals that were immersed in water after death (n = 6) and control group (n = 6). All mice were derived from the experiment by human methods of killing in accordance with Article 2 of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purpose [28]. The experiment was conducted in September 2017.

Experimental drowning

For experimental drowning, was selected the town lake of Tysmenytsia, Ivano-Frankivsk region, Ukraine, which is characterized by a slow movement of surface waters, standing water (lentil conditions of water movement). Drowning was carried out at the coordinates of point 1: latitude 48°54'4.5 "N longitude 24°50'32.93"E. Height above sea level is 245 m.

The group of animals subjected to drowning (n = 6) was placed in cagesand immersed in water, at a depth of 30 cm, for 1 minute, and then removed from the water. 30 seconds after the animals were taken, out they were again immersed in water at the same depth until the animal died [29]. For post-mortem immersion in water (n = 6) animals were used, which had previously been intraperitoneally injected with a lethal dose of anesthetic. The animals in the cages were immersed in water at the same depth as during drowning. Duration of the immersion was 6 hours. During the autopsy of the mice, a collection of tissues was performed, namely: lung, heart with blood, kidney, liver, spleen, femoral bone, and brain tissue removed from the cranial cavity. Several sets of sterilized instruments and distilled water were used during autopsy to exclude phytoplankton from outside of the samples under investigation. Samples from each type of tissue were crushed and then homogenized with a small amount of non-planktonic water by a homogenizer.

A pure culture of cyanobacteria

Samples of water obtained from the reservoir of the town lake Tysmenytsia were cultivated on a Fitzgerald's liquid medium modified by Zender and Gorham (№ 11) at a temperature of 22-25°C and i artificial llumination of 3000 lux of daylight lamps with alternating light and darkness of 16-8 hours [30-31]. When growing colonies, agar was added to the medium and a series of transplants was made in glass biological test tubes. Thus, we managed to obtain a purely cyanobacterial culture of Microcystis aeruginosa Kütz. emend Elenk. Pure culture served as a marker to denote the differences between products of PCR and reservoirs, as there are different types of cyanobacteria in reservoirs.

Removal of cyanobacteria from the test substances

Plastic tubes of 10 mL with samples of water from the lake were centrifuged at 3000 rpm for 10 minutes. The top layer of water was removed using a pipette. The remainder of the water in the test tubes was transferred to Eppendorf tubes of 2 mL volume, and they were to be frozen.

Removal of cyanobacteria from the test specimens was carried out in accordance with the extraction procedure described by Terazawa and Takatori [32]. The Percoll© solution was added to the tissue homogenate selected for study, the volume was 10 ml. The resulting homogenate was mixed well with the vortex mixer, then centrifuged at 17,000 rpm for 60 minutes at 12°C. The remnants of cells were removed from the top layer of the pipette. To the remainder of Percoll©, a double volume of distilled water was added, stirred and centrifuged at 6000 rpm for 15 minutes, and then the suspension was removed. After adding distilled water, the resulting suspension was centrifuged at 12,000 rpm for 15 min. The last precipitate was used for DNA extraction [21]. The isolated DNA was stored at -20°C in a frozen conditions.

PCR study

The specific synthesized oligonucleotide primers set limiting the amplified region, used to amplify the 16S rDNA of Cyanobacteria Microcystis aeruginosa. The forward primer was 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and the reverse primer was 809R 5'-GCTTCGGCACGGCTCGGGTCGATA-3', which flank the 16S rRNA gene fragment with a length of 782 bp used [33]. The analytical specificity of the primers is confirmed by the results obtained using the computer program " GenBank BLAST" [34]. As a marker of the length of DNA fragments we used pUC19 DNA / Mspl (Hpall) Marker ("Thermo Scientific", EU). PCR was performed in a 25-mL reaction mixture containing 1,0 ml sample of DNA template, each primer at a concentration of 25 mM, 50mM KCl, 10mM Tris-HCl (pH 8.3), 1,5 mM MgCl2, each deoxynucleoside triphosphate at a concentration of 200 mM and 1,25 U of AmpliTaq Gold DNA polymerase [35].

PCR study of the samples was carried out in three stages:

1. Denaturation of nucleic acid, which consisted of 30 cycles of denaturation at 95°C for 30 s;

2. Annealing - 30s at 57°C;

3. Extension (or elongation) at 72°C for 30 s, and PCR was conducted using a thermal cycler.

Electrophoretic separation of PCR products in a 6% polyacrylamide gel for 30 min allows a clear determination of the presence of DNA of the Microcystis aeruginosa species at the appropriate size of the amplified fragment in the biological tissues of the mice that were drowned in the experiment. Gels were stained with ethidium bromide solution. Transilluminator was used for visualization.

RESULTS

Having carried out a polymerase chain reaction with samples of water, obtained from 5 points of the lake, we obtained the following results (Table 1).

The amplification products of 16S rRNA Microcystis aeruginosa were found in all samples of water from five different points of the lake. This indicates that Cyanobacteria Microcystis aeruginosa is presented uniformly throughout the lake surface.

For groups of animals that have been drowned, amplification products can be found in all tissues (Table 2). The lack of amplification products in one case of drowning may be due to the development of asphyctic or dry drowning.

In all 6 mice that were immersed in water after death, amplification products were detected only in lung tissues. Electrophoretic separation of PCR products in a polyacrylamide gel allows a clear determination of the presence of DNA of the Microcystis aeruginosa species at an appropriate size of the amplified fragment in the biological tissues of the mice that were drowned in the experiment. The length of the amplification product is 782 bp.

Comparison of bands on electrophoretic samples obtained with PCR, the study of biological tissues from

Table 1. The availability of 16S rRNA product in water samples

Points	16S rRNA
1	+
2	+
3	+
4	+
5	+

Table 2. The number and percentage of 16S rRNA product in samples of tissues

Samples of tissues	Drowning (n=6)	Post-mortem submersion (n=6)	Control (n=6)
Heart with blood	5 (83.3%)	0(-)	0(-)
Liver	5 (83.3%)	0(-)	0(-)
Kidney	5 (83.3%)	0(-)	0(-)
Spleen	5 (83.3%)	0(-)	0(-)
Bone	5 (83.3%)	0(-)	0(-)
Brain	5 (83.3%)	0(-)	0(-)
Lungs	5 (83.3%)	6(100%)	0(-)

corpses of mice that died during experimental drowning, and electrophoreograms obtained during sampling water from the place of drowning, allows us to determine the fact and the place of drowning by the genetic profile of cyanobacteria of the species Microcystis aeruginosa.

In the case of drowning, we compared the bands of 782 bp amplification products in polyacrylamide gel. It was found the coincidence of the presence of amplification products in the samples of water from the place of drowning and tissues of the lungs, heart with blood, kidneys, liver, spleen, femur bone obtained during experimental drowning (Fig. 1).

In the case of the post-mortem submersion in water, we compared the bands of 782 bp amplification products in the polyacrylamide gel. It was found the coincidence of the presence of amplification products in samples of water from the place of drowning and lung tissues (Fig. 2). In the tissues of heart with blood, kidneys, liver, spleen, bone, brain tissue in case of post-mortem submersion the products of amplification was not found. Also,we did not detect amplification products in tissue samples on the control group of mice.

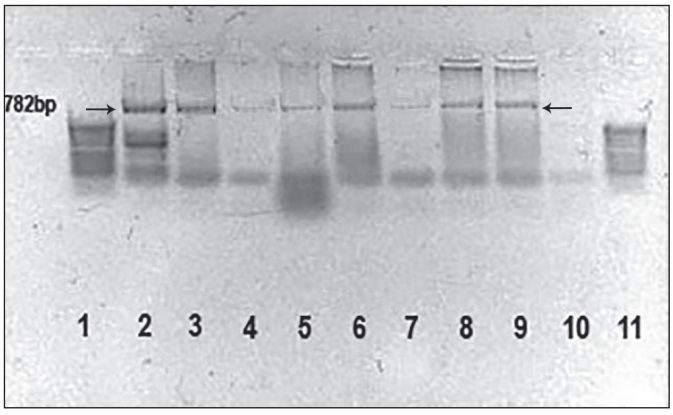

Figure 1. Distribution of amplification products in polyacrylamide gel from samples of tissues of the drowning group. Lane 1: pUC19 DNA / Mspl (Hpall) Marker; lane 2: pure culture of Microcystis aeruginosa sample; lanes 3: water sample collected at the place of drowning; lane 4: spleen; lane 5: heart with blood; line 6: liver; line 7: bone; lane 8: kidney; lanes 9: lungs; line 10: negative control (distilled water); line 11: pUC19 DNA / Mspl (Hpall) Marker.

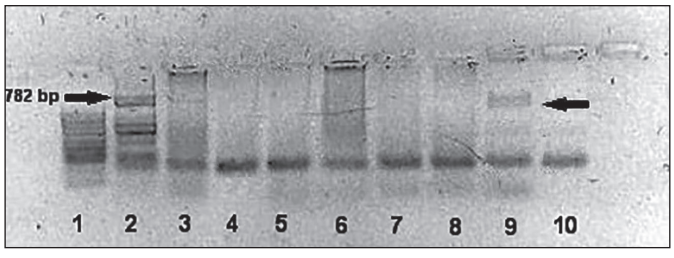

Figure 2. Distribution of amplification products in polyacrylamide gel from samples of tissues of the post-mortem submersion group. Lane 1: pUC19 DNA / Mspl (Hpall) Marker; lane 2: water sample collected at the place of post-mortem submersion; lane 3: heart with blood; lanes 4: liver; line 5: bone; line 6: kidney; lane 7: spleen; lanes 8: brain; line 9: lungs; line 10: negative control (distilled water).

DISCUSSION

The diatom test is currently a "golden standard" for diagnosis of drowning [5]. The analysis of the frequency of detection of diatoms in bodies of people who died of drowning indicates the tendency of decreasing the frequency of their determination in recent years. Such a phenomenon may be due to the method of destruction of the material destined for research, as a result of the use of concentrated acids for the destruction of organs, which sometimes eliminates the possibility of detecting diatomaceous plankton [19, 36-37]. Therefore, the PCR test method proved to be more reliable, sensitive, specific and rapid compared with the diagnosis of the usual diatoms by acid digestion. One of the advantages of using the PCR method is that for a molecular genetic study, usually a very small volume of tissue (1-3 mm^3) [38]. High sensitivity in the detection of phytoplankton allows determining drowning using the minimum amount of material for research and the minimum amount of cyanobacteria in comparison with traditional methods.

Phytoplankton exists in all types of reservoirs. One type of phytoplankton is cyanobacteria. Cyanobacteria are among the most primitive existing plants that contain chlorophyll and are considered to be one of the first photosynthetic of the planet. Microcystis aeruginosa is one of the most common types of cyanobacteria, among all phytoplankton [39]. Microcystis aeruginosa causes the formation of blooms and the death of fish. Since cyanobacteria are small in size from 0,2 to 2,0 μm, they can penetrate the bloodstream of the victims of drowning. [40]. Diatom plankton, which is important for drowning, has a size of about 50 μm. Diatomes of larger sizes do not penetrate the bloodstream, which significantly reduces the plankton kit for diagnosis.

When drowning water with cyanobacteria falls into the distal parts of the respiratory tract, as a result of which the alveoli are filled with water. The mechanism of drowning includes many pathophysiological processes: fear of drowning, diving response, autonomic conflict, upper airway reflexes, water aspiration and swallowing, emesis, and electrolyte disorders [41]. Interalveolar membranes are broken up, blood capillaries and pulmonary surfactants are damaged due to acute respiratory distress and the activation of the early-phase inflammatory mediator caused [42]. Water comes the blood circulation by diffusion, osmosis (haemodilution), and is carried by the blood flow to all internal organs [5]. The discovery of phytoplankton in the closed organs is an important marker for the diagnosis of death in case of drowning [43]. This can be used as a diagnostic criteria for drowning. The presence of phytoplankton in tissues can also be detected even with strongly expressed putrefaction changes [44]. Our study proves that cyanobacteria penetrate into all bodies of drowned animals. An absence of 16S rRNA products of cyanobacterial amplification in

one case of drowning can indicate an asphyctic (spastic) type of drowning. At the same time, there is a stable laryngospasm, as a reaction to irritation of the receptor apparatus of the mucous membrane of the larynx with the liquid, in which there was drowning. This prevents penetration of fluid into the respiratory tract and lungs [45]. This type of drowning is observed from 10-15% up to 35% cases of drowning [45-46]. According to studies by Lunetta P. at all, the deaths of people found in water and having normal lungs or non-detectable fluid in the respiratory tract are about 2% [46]. The sign post-mortem immersion in water is the presence of water in the respiratory tract, which is confirmed by the results of our work. It is also important that the proposed method can be used to determine the place of drowning by comparing the water samples and the presence of Microcystis aeruginosa DNA in the victims of drowning.

In conclusion, since cyanobacteria of the species Microcystis aeruginosa are fairly common, especially in standing water reservoirs, this can be used for establish aspiration of contaminated immersion medium to diagnose mechanical asphyxia as a result of drowning. The presence of specific species of cyanobacteria will allow to establish the place of drowning and to estimate the site of immersion. Since the size of cyanobacteria is 0,2 to 2,0 μm, they penetrate better through an aerogematic barrier than diatom plankton. This suitable combination of biomarkers will help expand the diagnostic range of criteria for diagnosis of drowning.

For the first time in Ukraine we develop a rapid, applicable, and reliable PCR method for detecting of Microcystis aeruginosa that can't be detected by the traditional method of diagnosis using light-optical microscopy.

Successful application of the polymerase chain reaction method allows diagnosis of mechanical asphyxia as a result of drowning, which is especially important in the light of evidence-based medicine.

Key points:

1. Forensic medical diagnostics of drowning with light microscopy now is a difficult issue to resolve.

2. Amplification productsof Microcystis aeruginosa can be used for establishing aspiration of contaminated immersion medium to diagnose mechanical asphyxia as a result of drowning.

3. The presence of specific species of cyanobacteria will allow to establish the place of drowning and to estimate the site of immersion.

4. Cyanobacteria penetrate better through an aerogematic barrier than diatom plankton.

Conflict of interest. The authors declare that there is no conflict of interest.

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