THE DEVELOPMENT OF FORENSIC LABORATORY METHODS FOR DIAGNOSING DROWNING

Ph.D. Thesis

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INTRODUCTION

Difficulties associated with the diagnosis of drowning

According to the World Health Organization (WHO) data (2016), drowning is the 3rd leading cause of death worldwide, accounting for 7% of all injury-related deaths. WHO estimates indicate that 359 000 people died from drowning in 2011. Drowning is defined as "*the process of experiencing respiratory impairment from submersion/immersion in liquid; outcomes are classified as death, morbidity and no morbidity*" (Word Congress of Drowning, 2002). Complete submersion is not necessary, death can occur in a manner when only the face of an individual (nose and mouth) is immersed in liquid.

The diagnosis of drowning has been a troublesome task in forensic medicine. Macroscopic and microscopic autopsy findings of the bodies retrieved from water are difficult to interpret and non-specific to drowning. Moreover, a prolonged post-mortem interval makes the diagnosis extremely challenging and often impossible.

Algae and drowning

Algae are a diverse, ubiquitous group of photosynthetic organisms. Depending on the species, they can be found in freshwater and saltwater and indicate special environmental conditions as well, because they are sensitive to pollutants. There are species, which prefer still water bodies and others can be found more likely in running water bodies. Diatoms (class: *Bacillariophyceae*), which is one of the major group of algae, are small, unicellular eukaryote organisms present in all types of water.

It has been recognised in the 20th century that these uni- and multicellular organisms living in water can form the basis of a laboratory method supporting the diagnosis of drowning due to their size and habitat specificity. In proving drowning, to confirm of the presence of diatom valves in the post-mortem tissues is a key point, therefore it is also appropriate to target broader algal groups instead of concentrating on species. Diatoms with their resistant silica backbone called frustules (hydrated silicon dioxide) are an appropriate group for this purpose. There are conditions, however, when the drowning medium contains only a few or no diatoms. In that case, we need to focus on the detection of other types of algae or bacteria (cyanobacteria, picochlorococcus etc.).

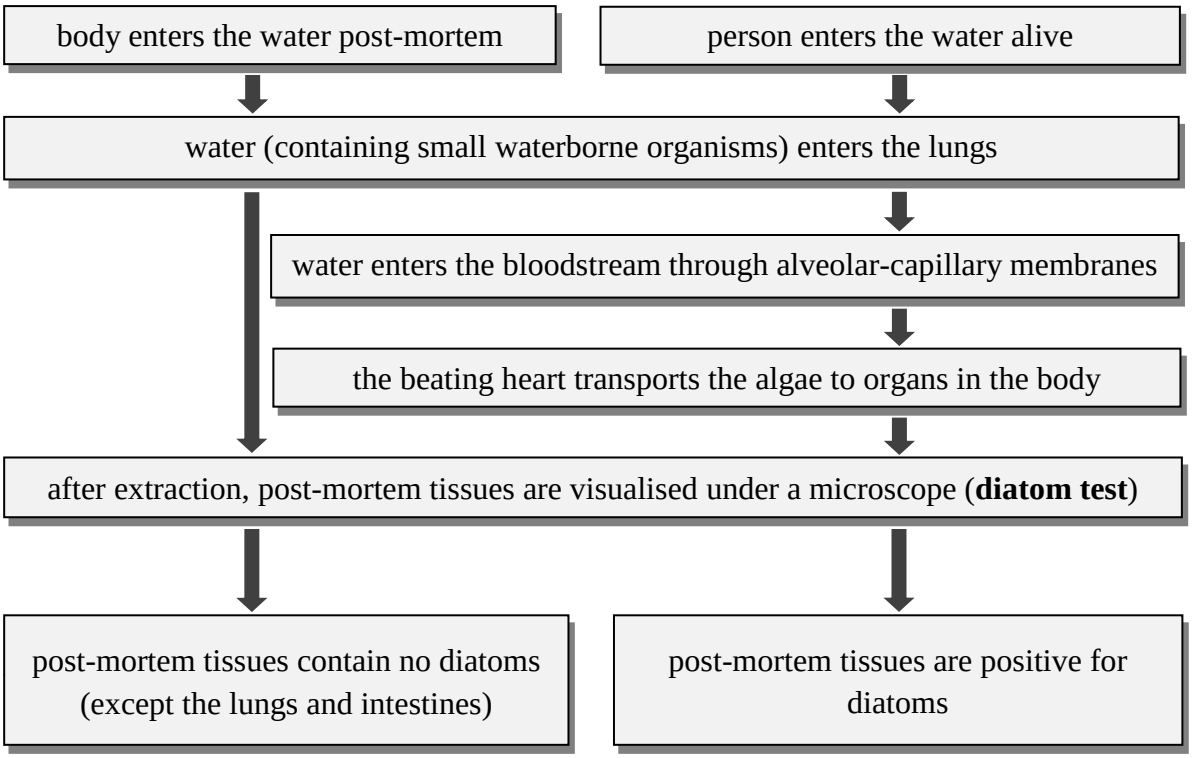
Diagnosing drowning with diatom test

The diatom test, the most widely accepted method for the identification of drowning, has been used since the beginning of the 20th century. *Revenstorf* was the first scientist who published his article in 1904 about using diatoms in the diagnosis of death by drowning. Later on, more scientists observed planktonic species in the blood, brain and bone marrow.

The diatom test is based on the aspiration of water and waterborne diatoms during the process of drowning followed by their distribution in the organs via the circulation. When a person drowns, they inhale water which contains small waterborne organisms. Drowning causes ruptures in the alveolar-capillary membranes of the lungs, therefore planktonic species can enter into the circulation. With the help of the beating heart, algae reach the organs (brain, spleen, liver, kidney, bone marrow etc.) in the body.

When a body enters the water post mortem, algae will not be transported to tissues and organs due to lack of circulation. However, in this case diatoms and other planktonic species still can be observed in the lungs and the stomach. According to the current scientific knowledge, if the body surface is intact, diatoms, cyanobacteria and other types of picoalgae

will not reach the target organs (except the lungs and stomach), when the body is immersed in water.



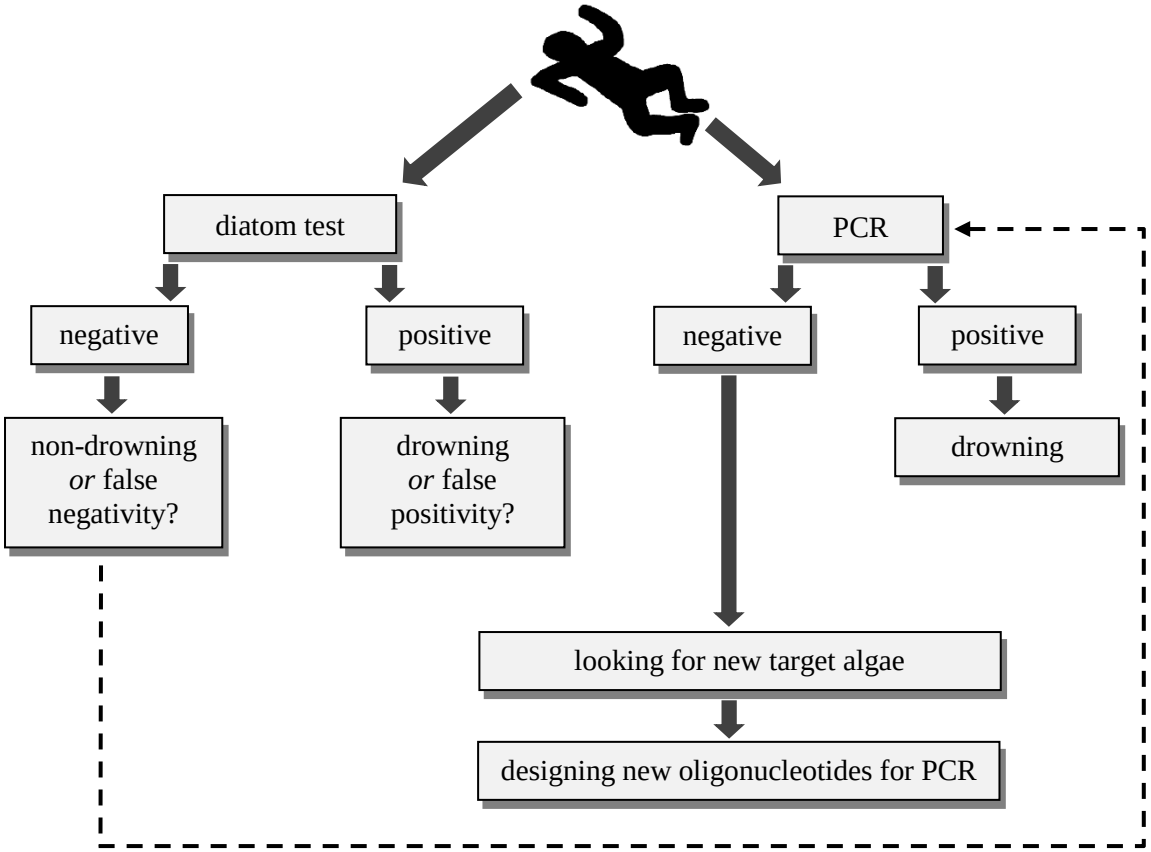
The role of diatom test for diagnosing drowning.

Diatoms originating from the drowned victim's tissues (spleen, bone marrow, etc.) build up a silica wall in the form of orthosilicic acid ($Si(OH)_4$) around their cells which helps in their identification using a microscope. Some studies involve searching for the presence of diatoms and there are reports in which the number of the diatoms are counted.

Drowning has extensively been discussed in medico-legal literature and several experts raises question about the acceptance of diatom test. Though the diatom test is the most widely used test, its reliability is controversial. It has its shortcomings, such as low sensitivity, health-threatening conditions during sample preparation, and loss of some diatom species because of the harsh treatment of the samples.

False negativity may occur, when (**1**) the drowning medium contained only a few or no diatoms; (**2**) drowning occurred within a short time and diatoms could not spread through the body; (**3**) only a few diatom frustules were present in the tissues which lost during the tissue extraction.

The possibility of false positivity is also discussed in the literature. As diatoms are present in the air and soil as well, the presence of the valves in the tissues can be the result of inhalation. *Langer et. al.* (1971) suggested that the inhalation of diatoms present in the surface of tobacco leaves is also possible. *Peabody et. al.* published a study in 1977 about the detectable diatom valves in the coated end of a match (which remain intact after burning), paints and varnishes, which fact raises the possibility of contamination. *Otto* (1961) examined 28 patients' lung with silicosis and observed diatoms in 23 out of 28 individuals' lung. Silicon-containing cosmetics and diatomaceous earth-containing dietary supplements are also present in the market, which can distort the results.



Decision-making possibilities when a body is found in water. If the result of the diatom test is negative, PCR-based test needs to be considered.

In recent years, new molecular biological methods have appeared for the identification of drowning. These methods are based on the fact that in all types of water beside diatoms, several phytoplanktonic strains are found (cyanobacteria, green algae etc.). In the event of drowning, these small waterborne organisms appear in the victim's tissues, which otherwise are not typically found in the human body. Their presence can be demonstrated by the amplification of the specific regions of theor DNA. These sequences are not present in the human genome.

AIMS OF THE STUDY

Proving drowning is still a difficult task. With the invention of polymerase chain reaction (PCR) technique in the end of the 20th century, new opportunities opened up for forensic scientists for designing new laboratory methods.

Based on the above-mentioned information we aimed the followings:

1. to identify and analyse the sites, circumstances and possible risk factors of water-related deaths in South-West Hungary;

2. identification of the representative algal groups in the natural and artificial water of the above-mentioned region of Hungary for the purpose of applying environment-specific oligonucleotides in a novel drowning diagnosis test;

3. to increase the sensitivity and specificity of the microscopic method currently used in medico-legal practice with the extension of the test for detecting other phytoplanktonic species beside diatoms;

4. development of a novel DNA-based method for detecting algae in post-mortem tissues by PCR, which can be used for strengthening the diagnosis of drowning and easily put into forensic laboratory practice.

MATERIAL AND METHODS

In our experiments the following techniques were used:

- drowning-related fatalities in Baranya, Somogy and Tolna counties in the South-West region of Hungary from 1 January 2008 till 31 December 2012 were described in a retrospective analysis (a total of 114 cases) with the examination of the following risk factors: *sex, age, alcohol and drug consumption, when and where drowning death occurred (season and types of waterbodies where the victims were found) and the manner of drowning deaths*
- statistical analysis with GraphPad Prism
- collecting and examining the surrounding natural water bodies for targeting new species which can be used for proving drowning
- classical diatom test (acidic digestion method)
- diatom test based on proteinase K extraction
- evaluating diatom tests with light microscope
- evaluating algae in post-mortem tissues and water derived from the spot with reverse plankton microscope
- identification of cyanobacteria using fluorescence microscope
- isolation of phytoplanktonic DNA from water, algae culture and post-mortem tissues
- PCR with oligonucleotides specific to DNA present in cyanobacteria, green algae and diatoms
- agarose gel electrophoresis

RESULTS AND DISCUSSION

Retrospective analysis of risk factors associated with drowning in South-West Hungary

Many countries including Hungary have no reliable study on major causes and reasons of drowning deaths. The present study was aimed to collect and describe all drowning-related fatalities in Baranya, Somogy and Tolna counties in a 5 years' period.

There was a total number of 114 drowning-related deaths during the five years investigated. We have focused our attention on the gender, age and the possible alcohol consumption of victims, the location of death (type of water), and other factors contributing to the fatalities including drugs, manner of drowning, etc.

Drowning as the cause of death was presumed by autopsy findings and police reports (which also included eyewitnesses' descriptions) and was diagnosed by macroscopic and microscopic findings (histology and diatom test), in some cases it was also confirmed by PCR-based methods (DNA isolation from microalgae). Four out of the 114 cases were unidentified persons, but during autopsy we could classify them by most of their properties (gender, age etc).

We examined the sex distribution the drowned individuals for the purpose to find the most affected group associated with drowning. All studies available in this field report that more males than females get drowned. We also found that males (81 out of 114) are at greater risk of drowning than females (33 out of 114). Death rate of males (71.05%) was more than two times higher than that of females (28.95%). The reason for this may be the consumption of greater amounts of alcohol by men and their relatively high self-esteem. It is also manifested in the circumstances of deaths (e.g. swimming on their own under the influence of alcohol).

The number of drowning fatalities was the highest between ages 51 and 60 (31 of the total 114 cases; 27.19%); moreover, both for men and women the drowning rate was the highest between the ages 51 and 70, in this age group it is 44.73% of all drowning cases during those five years. According to WHO, children under five years of age have the highest drowning mortality rate worldwide. In contrast, we found that children's drowning death rate was surprisingly low in this area of Hungary; there was only one victim in this age group. In the investigated area of Hungary, we found that elderly men are more likely to drown than younger individuals. No data showing a similarly high ratio of elderly victims have been found in other countries, this rate is the highest known in the region of Hungary studied.

From many countries, there is no information about alcohol consumption of victims, although WHO data suggest that it is a preliminary condition for accidental drowning deaths. The blood alcohol concentration (BAC) could be measured in 68 out of 81 (83.95%) male and 29 out of 33 (87.89%) female victims. In these cases with measurable BAC (97 out of 114), 85.29% of males (58 out of 68) and 51.72% of females (15 out of 29) had consumed alcohol. In nearly two third of all the cases (73 out of 114; 64.04%) the blood alcohol level was above 0.021% (0.021 g of alcohol for every dL of individual's blood) and only 24 victims' BAC (21.05%) was proved negative (BAC $\leq$0.020%). Due to the advanced decay stage of decomposition and/or the lack of blood, in 17 cases (14.91%) the BAC level could not be determined. Blood alcohol level in male victims was higher than in females on average, there were 8 males with a blood alcohol concentration close to the toxic level (BAC is above 0.351%), however, the cause of death in those cases was also drowning. Female victims have consumed much smaller amounts of alcohol and it was found typical in the elderly group.

Toxicology analysis identified drugs in 11 out of the 74 cases (5 cases in females, and 6 cases in males; 14.86%): the most common pharmaceutical drug was benzodiazepine (5 cases), followed by citalopram (2 cases), and carbamazepine, noraminophenazon, ibuprofen, venlafaxine in the rest of the cases. In 3 cases the concentration of drugs in blood was found above the toxic level (benzodiazepine, venlafaxine, citalopram). The ages of the above victims were between 54 and 75 years. We also found amphetamine in three cases (17, 20 and 52 years old males). Compared to other countries, this is a very low number of drug use among drowning victims.

In our statistical analysis drowning occurred mostly in warmer months from March till the end of August (74.56%). Nearly half of the fatalities (49.12%) happened in summer. Drowning deaths in autumn and winter are not negligible, either: the causes, similarly to those in other seasons, usually were accidents. However, suicide by drowning happened generally in winter. The seasonal pattern of drowning deaths was similar compared to that of other countries.

Hungary is rich in rivers and lakes, therefore the majority of fatal drowning cases occurred in natural waterbodies. The highest number of drowning fatalities occurred in the River Danube (30 out of 114; 26.32%) and Lake Balaton (22 out of 114; 19.30%). Indoor drowning deaths were rare: there were only four victims who died in either a swimming pool (1 case) or in bathtubs (3 cases). 13 out of 114 drowning deaths occurred near the victims' home (garden pond, ditch, wine cellar) and three cases in the victim's apartment (3 bathtub deaths). Victims were often found in wells in cases of suicide.

Eyewitnesses may contribute to the investigation on the circumstances of fatalities with a lot of data. Accidents are the main cause of drowning deaths in South-West Hungary.

Accidental drowning fatalities happened mainly because of high BAC level, chronic diseases (e.g. diabetes, high blood pressure) or during physical or sports activities. We found that swimming in a body of water is still the recreational sports activity during which the largest number of drowning occurs (22 out of 114 cases). We found 15 suicidal drowning cases (13.16% of all the cases) in the five years investigated and suicidal deaths were more frequent in elderly people (age group of 61-90). In this study the chosen modus operandi of suicidal drowning deaths was jumping in wells or jumping off bridges. In the period investigated there were only two homicidal drowning cases.

The results of this study may draw the attention to the most vulnerable age group, individuals between 51 and 70 years. It became obvious that alcohol consumption and accidental drowning deaths (and to a lesser extent suicidal drowning) show close correlation. As this inquiry shows, it is necessary to consider these risk factors in drowning cases and to avoid alcohol consumption during water activities, because in addition to increasing self-confidence, it reduces the potential of victims' ability to rescuing themselves.

Supporting the diagnosis of drowning: the development of diatom test

The diagnosis of drowning is one of the most difficult task in the field of forensic medicine: autopsy findings are not specific to drowning and the majority of signs are related to asphyxia as well. Moreover, there are signs which can be seen in some cases but do not appear in others. External froth around the mouth and nostrils is one of the major signs of drowning, however, foam can also appear in other types of death associated with pulmonary oedema.

Although diatom test is the most commonly used test for supporting the diagnosis of drowning, it has several disadvantages, therefore increasing the specificity and sensitivity of diatom test was one of the aims of this thesis.

At first, we collected water samples from various water bodies in Hungary and analysed their seasonal variation. The purpose of these tests was to determine those genera that could be the target of a novel molecular biological method as well as to examine the changes in the phytoplankton depending on the environment.

There were a total of 69 water samples collected between 2011 and 2015, which were analysed natively using a light microscope and some of them were subjected to inverted plankton microscopy in Lugol's Solution. Using inverted plankton microscope, the possibility of finding diatoms in a sample is increasing, because with this tool the total amount of diatoms can be visualised by volume.

In the samples investigated the most frequently observed diatom genera were the following: *Cyclotella sp., Cymbella sp., Diatoma sp., Navicula sp., Nitzschia sp.* Besides diatoms, green algae (*Nostoc sp., Pediastrum sp., Scenedesmus sp., Volvox sp.*) and cyanobacteria (*Synechococcus sp.*) were present in a large number. Unicellular picocyanobacteria can be observed and identified by fluorescence microscopy. Independently of seasons, one of the most common picocyanobacteria in Hungary is *Synechococcus sp*., therefore this group was chosen as the target of detection in the molecular biology method. The size of these aquatic organisms is far smaller than that of diatoms, so they can easily enter the blood circulation

The molecular method used in support of the diagnosis of drowning

In all of the cases discussed in this case report, the autopsy findings included the following: dry, voluminous, and distended lungs; petechial bleeding on the lungs (Paltauf's hemorrhages); liquid blood; congestive organs; and anaemic spleen. On histological examinations, drowning signs were confirmed (emphysema aquosum) and other microscopic signs of natural deaths were excluded.

The PCR-based method described in the thesis can be a good candidate for an ancillary assay. Isolating DNA from cyanobacteria and small green algae does not require harmful chemicals (H_2SO_4, HNO_3, and H_2O_2); there are reliable protocols for gaining DNA from even a few organisms present.

We present here four drowning cases which were unusual because the diatom test to diagnose drowning was negative in these cases. The cases described illustrate that in specific water bodies, where low numbers of diatoms or none can be found by microscope, PCR-based techniques relying on the detection of other phytoplanktonic species can facilitate the correct diagnosis.

PCR-based methods are very sensitive for contamination. There are two major routes for possible phytoplankton contaminations: one source is the skin of the drowned individual (because it was in close contact with the drowning medium), and the other is the tap water in the autopsy room. The tap water controls yielded no PCR products in our experiments. We can remarkably reduce the risk of contamination if we are meticulous in sample preparation, and as these cases prove, it is possible to do so. We examined cases where victims were found in water; however, the actual cause of death was natural (not drowning); in these cases, we found no phytoplanktonic DNA during PCR. We used post-mortem tissues from non-drowning (suicide by hanging) cases as negative controls, and the results of these samples were in agreement with the autopsy findings. We examined 33 additional drowning cases, where the results of our PCR-based method and the diatom test were in agreement.

Even though the diatom test is used worldwide to prove or support the diagnosis of drowning, previous studies demonstrate that it may not always provide a positive result, while a negative test result does not exclude drowning. For this reason, relying on the negativity of the tissues exclusively can lead to false conclusions. Though very little is known about the penetration of bacteria and picoplankton in non-drowned victims immersed in water, it seems that they do not passively enter the organs of a body immersed in water. The lack of pulmonary passive diffusion of waterborne bacteria in non-drowning victims was shown by *Lucci et al. (2008)*. We propose to supplement the traditional diatom test with specific PCR-based methods for detecting picoplanktonic and diatom species in the evaluation of drowning cases because they can provide additional evidence of the cause of death.

SUMMARY

In the present doctoral thesis, I determined the main risk groups and summarized the circumstances and main characteristics of all the drowning-related deaths in South-West Hungary between 2008 and 2012. In summary, we can conclude that the most considerable risk factors of drowning are being a male (71.05%), age between 51 and 70 years (44.73%) and alcohol consumption (64.04% of the total number of all deaths).

One of the main aim of the thesis was to increase the specificity and sensitivity of the diatom test, which is currently used worldwide for supporting the drowning diagnosis. The proteinase K method -which is not routinely used in Hungary yet- seemed to be more effective for detecting diatoms in the post-mortem tissues of a drowned individual, than the classical diatom test which is based on acidic digestion. Besides light microscopy, other microscopic techniques (inverted plankton microscope, fluorescence microscope) were applied to analyse the results. These microscopic techniques are routinely used by algologist, but not yet implemented in the field of forensic biology. The potential modification of the classical diatom test in special circumstances of drowning (e.g. drowning medium contains only a few diatoms) was suggested to increase the possibility of detecting algae in the organs post-mortem.

In addition to strengthening the classical diatom test, four drowning cases is presented, in which the diatom tests were negative, however, the autopsy findings suggested drowning. In

those four cases, we supported the diagnosis of drowning by detecting the DNA of several types of algae genera (picocyanobacteria, small green algae, diatoms etc.) in the post mortem tissues.

As a novel drowning diagnosis test, a PCR-based molecular biological method was suggested to put into forensic laboratory practice, which can be easily used routinely after selecting the relevant oligonucleotides for PCR.

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Cumulative impact factor of articles related to thesis: 3,732
Poster presentations related to thesis: 2
Oral presentations related to thesis: 8

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