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Application of a simple enzymatic digestion method for diatom detection in the diagnosis of drowning in putrified corpses by diatom analysis

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Abstract The reliability and applicability of quantitative and qualitative diatom analysis by an enzymatic digestion method in the diagnosis of drowning of putrified bodies has been evaluated. The authors report the analysis of water and organ samples of 12 immersion cases using light microscopy. This study included control organ samples from the bodies of persons who died from causes other than drowning. Organ samples were treated by both chemical and enzymatic methods, the first one using concentrated nitric acid and the second proteinase K. Diatoms were present in most organ samples of the immersed corpses; no diatoms could be found in the control samples. Our experience was that the enzymatic method seemed to be more convenient in terms of rapidity, safety and environmental protection than chemical digestion. The number of diatoms recovered with both methods was similar. Qualitative and quantitative analysis of both water and organ samples of immersion cases supported the diagnosis of death by drowning in 41.6% of the putrified cases studied. The authors suggest that diatom analysis using enzymatic digestion of organs can be used as a criterion for positive diagnosis of drowning in cases involving putrified bodies.

Key words Diatoms · Drowning · Proteinase K digestion · Diagnosis of death

Zusammenfassung Es wird über die Anwendbarkeit der Proteinase K Verdauungsmethode für eine quantitative und qualitative Diatomeen Analyse zur Diagnose des Ertrinkungstodes berichtet. Das Untersuchungsmaterial bestand aus 12 Leichen bei denen die Todesursache Ertrinken durch Autopsie oder Polizeiberichte festgestellt worden war. Zur Kontrolle dienten 5 Leichen von Personen, die an verschiedenen Erkrankungen starben. In den Organen der Kontrollgruppe wurden keine Diatomeen gefunden. Wenn Diatomeen in den Hauptorganen gefunden wurden, waren sie auch in den Lungen nachweisbar. Falls sie in den Lungen nicht vorhanden waren, verlief der Nachweis in den anderen Organen ebenfalls negativ. Die Quantifizierung und die Identifizierung der Kieselalgen und die gleichzeitige Untersuchung der Wasserproben konnten in 41,6% der Fälle den Ertrinkungstod unterstützen. Ein anderer Vorteil der Proteinase K Verdauungsmethode ist, daß sie im Gegensatz zu der Salzsäure-Methode umweltschonend ist.

Schlüsselwörter Diatomeen · Ertrinken · Proteinase K Verdauung · Diagnose der Todesursache

Introduction

Since the diagnosis of drowning is one of the most difficult diagnosis in forensic pathology, a great number of tests have been proposed to confirm the drowning of a victim [4–6, 12, 18, 20, 22, 24–26].

In bodies freshly recovered from water, the best indicators of death by drowning are the presence of fine froth at the mouth and nostrils and the macroscopic and microscopic alterations in the lungs. When bodies remain immersed in water for a long period of time they are often unidentified and there is no information regarding the circumstances of death. In some cases the corpses are badly decomposed and putrefaction induces the disappearance of these macroscopic and microscopic findings and the diagnosis of drowning is practically impossible.

The diatom test has been proposed by several authors to provide supportive evidence of drowning, but the relationship between diatoms and a diagnosis of death by drowning is very controversial. Thus, the reliability of this test is still disputed.

Among the various investigators, Gylseth and Mowé [8], Schellmann and Sperl [23] and Foged [7] are of the opinion

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that since diatoms can be found at autopsy in other subjects, they cannot be used as evidence of drowning. On the other hand, Udermann and Schuhmann [28], Ranner et al. [21], and Auer and Mottonen [1] consider that accurate diatom counts can, in most cases, discriminate between drowning and non-drowning cases, especially when post-mortem mutilation or decomposition has occurred or following resuscitation attempts. Peabody [16, 17] stressed that the diatom method is supportive evidence for the diagnosis of drowning only when performed by an experienced examiner and following rigorous criteria. On the other hand, the finding of diatoms in the organs of immersed corpses is accepted as proof of drowning in many countries, even when the test is performed and interpreted by neophytes.

The diatom investigation must be performed using proper techniques, and ensure the exclusion of contamination of glass containers and reagents and the correct interpretation of the results, which involves a complete taxonomic analysis of the diatoms recovered from water samples and from the organs of the deceased [2, 3].

To look further into this matter, we present our experience in comparing quantitative and qualitative diatom analysis using light microscopy of both water taken at the site of drowning and post-mortem tissues samples recovered during the autopsies of 12 putrefied bodies. The tissues of 5 corpses where drowning was not the cause of death, were also analyzed as control samples.

Materials and methods

The investigations were conducted on material collected during postmortem examination of bodies recovered from water in the Strasbourg area (France), an area covered by the Institut de Médecine Légale.

The 12 corpses chosen for this study were decomposed but not fragmented, dismembered or skeletonized, and drowning was the cause of death. The postmortem period ranged from 20 to 60 days. The majority of the bodies were found in the river Ill (6 bodies) and in canals (3 bodies); the rest were recovered in small lakes (3 bodies).

Two samples of water (100 ml per sample) from the site where the body was found or where the immersion probably occurred, were collected in clean containers as well as stones found in the water, in order to collect attached diatoms by scraping. The samples were centrifuged at 2500 rpm for 15 mins and diatoms were cleaned by incubation in hydrogen peroxide (130 vol.%) at 80° C for 12 h. The fluid obtained was allowed to cool at room temperature and centrifuged at 2500 rpm for 15 mins; the supernatant was decanted and replaced with diatom-free distilled water. The process was repeated 3 times until the fluid was transparent, with a final spin at 3000 rpm to produce a pellet. After removing the supernatant, the sediment was air dried and mounted in Naphrax. Examination of 4 slides was carried out under a light microscope equipped with polarization.

Chemical digestion method

During autopsy, the following samples were taken for diatom analysis: a segment of peripheral lung tissue, kidney, liver and brain. As controls, organ samples from 5 corpses who died from natural causes on dry land were taken and processed in the same manner as the immersion cases.

Blocks of 10 g of each organ (i.e., lung, liver, kidney, and brain) were removed without contamination during autopsies, and minced with scissors. The tissue strips were then dropped into Becher flasks (50 ml) and boiled in twice the volume of concentrated analytic nitric acid (specific gravity 1.42) under a fume hood for 40 h. Further additions of nitric acid were made carefully to the boiling sample until the tissue had dissolved. The fluid obtained was allowed to cool at room temperature and then centrifuged at 2500 rpm for 25 min. The supernatant acid was decanted and replaced with diatom-free distilled water. At this point, the samples were treated in the same manner as the water samples. The precipitate (100 µl/slide) was transferred to a coverglass using a disposable micropipette. Under the light microscope, the diatoms were evaluated both quantitatively and qualitatively and the results were compared with those obtained from the known or suspected site of drowning.

The chemicals and instruments were checked regularly for contamination. Since diatoms are said to dissolve in highly alkaline substances, the equipment was cleaned by immersion in 2 N NaOH for 24 h immediately after each process and rinsed with diatom-free distilled water.

Enzymatic digestion method

Prior to the analysis the SDS and proteinase K buffers were checked for diatoms and filtered.

Blocks of 10 g of tissues (i.e., lung, liver, kidney, and brain) were rinsed and mixed with 500 µl of 10 mg/ml proteinase K and 100 ml of 0.01 *M* Tris-HCl buffer (pH 7.5) containing 2% SDS. The mixture was incubated at 50° C overnight; a further 500 µl of proteinase K (Boehringer Mannheim) was added and the solution was incubated for 8 h. After complete digestion of the tissues, the solution was diluted with 100 ml of distilled water and centrifuged at 3000 rpm for 15 min; the upper layer was then removed. The sediment (100 µl/slide) was transferred onto a coverglass, mounted in Naphrax and examined under the light microscope.

Ashing method

In the cases where enough organ material was available, 10 g of tissue was first dried in a muffle furnace at 80° C for 2 periods of 8 h and at 200° C for a further 8 h; the material was then ashed at 550° C for 8 h. The mineral ash was treated by acid digestion and mounted in Naphrax for light microscope examination.

During analysis of the slides using the 100 × oil immersion objective and the 10 × ocular, we counted the number of diatoms present, whether whole or fragments.

Quantitative analysis was performed by counting the number of diatoms per 100 µl of bottom deposit (after the final centrifugation using acid or enzymatic digestion per slide. The total volume of this deposit represented 10 g of tissue.

The number of genera was estimated by establishing the differences in shape and size of diatoms and comparing them with reference publications on diatoms. Qualitative analysis aimed at identifying and comparing the different species was made following reports of the most common genera of diatoms in the river Ill and with the aid of an expert diatomist, by examining the photographic records.

Results

In all the bodies examined, the chest cavity was closed and there was no direct contact between the immersion water and the lung due to total decomposition of the thorax. Diatoms were found in 11 lung samples, 6 kidney samples, 5 liver samples and 4 brain samples. In all cases where diatoms were recovered from the major circulatory organs, diatoms were also present in the lungs and vice versa. In case 8, where no diatoms were found in the lungs, none were found in the other organs. Cases 1 and 2

Table 1 Results of quantitative analysis of diatoms in the immersion cases.

Case number	Water D per ml	Lung (10 g) D/10 g			Brain D/10 g			Kidney D/10 g			Liver (10 g) D/10 g		
		Acid	PK	Ashing	Acid	PK	Ashing	Acid	PK	Ashing	Acid	PK	Ashing
1	210	73	66	70 + F	0	0	0	6	5	F	0	0	0
2	184	84	88	80 + F	0	0	0	8	9	F	0	0	0
3	265	53	60	57	0	0	0	0	0	0	0	0	0
4	150	108	101	0	0	0	0	0	0	0	0	0	0
5	190	99	105	95	0	0	0	0	0	0	0	0	0
6	340	109	98	ND	0	0	ND	0	0	ND	0	0	ND
7	199	62	70	ND	0	0	0	4	6	F	2	3	F
8	350	4	0	ND	0	0	ND	0	0	ND	0	0	ND
9	410	67	70	ND	7	11	ND	15	10	ND	7	4	ND
10	315	63	69	ND	10	7	ND	2	3	ND	10	6	ND
11	245	50	55	ND	6	8	ND	4	6	ND	8	10	ND
12	290	66	78	ND	7	8	ND	4	5	ND	3	4	ND

ND: Not Done; F: Fragmented frustules; PK; Proteinase K digestion; Acid: Acid digestion; D: Diatoms

concerned suspected neonaticides and the bodies of newborn children found in a river. The goal of the analysis was to determine whether they had breathed. The microscopical examination of the lungs was invalidated by putrefaction which distended the alveoli. Diatoms were found in both lung and kidney samples.

In 41% of the positive lung samples more than 5 different genera and more than 60 diatoms/10 g of tissue were found in 66% of the samples. The 2 digestion methods gave approximately the same yields of diatoms. The maximum diatom content of samples from closed organs involved 3 different genera and was approximately 15 diatoms/10 g of tissue.

Using the ashing method, the same number of genera was found in the lung samples. The number of diatoms in the lung samples was slightly superior to that found with the other method, but in these lung samples we observed significant destruction of the frustules. This was always the case in closed organs where a few fragmented frustules were found. The estimated number of diatoms found in water and in different organ samples (10 g of tissue) in each case is presented in Table 1.

The quantitative analysis of the immersion cases gave the following results: there were 7 cases in which diatoms were found in water, lung and closed organ samples and 4 cases in which diatoms were present in both water and lung samples, but not in other organs. In one case we found diatoms in the water samples and no diatoms in the postmortem tissue.

No control samples showed the presence of diatoms. No diatoms were recovered in samples of distilled water or concentrated nitric acid. After preparation of the buffer solutions with diatom-free water we found 2 diatoms belonging to the genera *Stephanodiscus* in the enzyme buffer solution prior to analysis of case 9. After filtration of the buffer no diatoms were detected. We assumed these diatoms came from the dry stock powder of the reagents.

In the organs of immersed bodies recovered from the river Ill, canals and lakes of the Strasbourg area, the most common genera identified were *Navicula, Frustulia, Cyclotella, Nitzschia, Melosira.* These genera were also identified as dominant genera in the water samples in which more than 10 different genera of diatoms were identified. The most common genera were *Navicula, Frustulia, Cyclotella, Nitzschia, Melosira, Fragilaria, Gomphonema, Synedra, Cymbella, Tabellaria.*

Similar morphological characteristics were found in the diatoms recovered from water, lung and other organ samples in 7 cases (cases 1, 2, 7, 9–12). These were considered as positive cases, indicating death by aspiration of water into the lungs. The results of qualitative analysis in these cases showed good correlation to the conditions of the site of drowning when known (6 cases). Experience suggests that the sizes or shapes of the species identified would not pose any barrier to transport from the lungs into other organs.

Large numbers of diatoms were found in the brain, liver and kidney samples. These tissues could be rapidly dissolved by enzyme digestion but more time and more reagents were necessary using acid digestion.

Discussion

The results of the diatom tests must be interpreted in the light of the possibility of contamination, and of the presence of diatoms in the lungs in non-drowned individuals. The presence of diatoms in the organs of non-drowned persons has been used as one of the arguments against this method [19]. Few diatoms in the organs of non-drowned individuals have already been reported in similar studies [15]. Polson et al. [20] reported that in 10 cases of death due to causes other than drowning, the maximum number of diatoms in 100 g of tissue was 20 in the lungs and 13 in the liver. For Pachar and Cameron [15], control samples had few diatoms (5–25/100 g of tissue) in the lungs and a maximum of 10 in the closed organs. In our study, no diatoms could be recovered from the lungs and organs of non-drowned individuals.

Another source of contamination is represented by reagents and glass containers. In the present study, only once did we recover 2 examples of *Stephanodiscus* in the proteinase K buffer prior to the analysis and we assume that these diatoms came from the stock powder. In fact diatom-free water was used to prepare the reagents.

In our series where drowning was the cause of death, we made a positive diagnosis of drowning using the diatom analysis only when the number of diatoms of similar genera was above a minimal established limit in the lungs and in closed organ samples.

Analysis of the cases showed that the 20 diatoms per microscope slide [1] set for lung samples allowed a sufficient margin for exclusion of sources of contamination. For closed organs, we required more than 5 complete diatoms, with exclusion of fragments, as an indication of aspiration of water. In fact, using the ashing procedure fragments were found in 4 cases which did not allow identification of the diatoms. Therefore in our hands the ashing method was not a suitable method for the analysis of closed organs.

Diatom evidence of drowning implied the presence of diatoms in the immersion fluid. Like Hendey [9, 10], we believe in the need for having water samples for comparative purposes. The samples should be taken from the bed of the river or lake by sampling stones in order to recover diatoms by scraping their surfaces. Auer and Mottonen [1] examined the applicability of quantitative and qualitative analysis of diatoms for the diagnosis of drowning, by using the acid procedure on 107 cases, and proposed the following criteria: when diatoms were found in both lung and other organs (i.e., kidney, liver or brain), evidence of death by drowning was established; when diatoms were found only in the lung, numerous or moderate occurrence of diatoms was regarded as indications drowning, whereas lower numbers were thought to be insufficient to permit a clear conclusion of drowning.

In 7 cases quantitative analysis showed a large number of diatoms in the lungs and in the other organs tested. This number was above the established limit, thus the diagnosis of death by aspiration of water could be sustained. The diagnosis of drowning by diatom analysis was not made unless similar genera were identified in both organ and water samples. This drowning group represented 58.3% of our small series of putrified corpses, which was higher than the 20% reported by Neidhart and Greedyke [14], and similar to the 57.9% reported by Auer and Mottonen [1] in a larger series. In these cases, diatom analysis was the most practical method for diagnosing death by drowning in the decomposed bodies.

In 4 cases (33.33%), numerous diatoms were present in water and in lung samples but not in other organs, which was slightly higher than the 30.8% reported by Auer and Mottonen [1]. It has been suggested that finding diatoms in the lungs alone is strongly indicative of rapid death after a short agonal period [27]. Since the number of diatoms in the lungs was above the established limit, death by drowning could be assumed but no definitive conclusion could be reached, because diatoms could have entered the lungs passively when the body remained immersed in water under increased hydrostatic pressure.

In case 8, where only a few diatoms below the established limit could be found, no reliable conclusions could be reached regarding the mechanism of death.

It could be assumed that the absence of diatoms in the control samples in our series and the few diatoms found by other authors [15], along with the high numbers found in immersion cases, was sufficient proof that quantitative analysis could be used as a supportive method for the diagnosis of drowning. Nevertheless, it should be complemented by qualitative analysis.

Since Pachar and Cameron [15] stated that extraction with concentrated nitric acid could result in a significant loss of diatoms during the process, we concurrently performed an enzymatic digestion procedure. This last method was suitable in terms of rapidity and efficiency and less hazardous and pollutive since the number of diatoms found was similar to that with acid digestion.

Thus, in our experience the proteinase K digestion procedure seemed to be the most suitable for extracting diatoms from putrefied human tissues, and did not require strong acids which are dangerous to deal with and problematic in view of environmental pollution.

We chose to analyze 10 g of tissue, since it was a quantity easier to handle than the 100 g proposed by other authors [15] and more representative of the tissue than the 2 g given by Kobayashi et al. [11] and Matsumoto and Fukui [13].

A difficult interpretation problem was raised by the presence of diatoms in both water and lung samples and their absence in other organs. According to Pachar and Cameron [15], the number of diatoms extracted from organs, lungs excluded, may be related to the rapidity of death (occurring immediately after entering the water) which did not allow penetration of diatoms into the circulation.

For us, a positive diagnosis of drowning could only be made if significant numbers of diatoms of similar genera were found in both water and lung samples and also in other circulatory organs. The proteinase K digestion method was the most suitable procedure for this purpose.

Conclusion

Quantitative and qualitative diatom analysis in putrified bodies can be strong evidence of death due to aspiration of water. The enzymatic digestion method using proteinase K could be the method of choice and provides evidence of drowning, only when a considerable number of diatoms are present in the immersion medium. Diatoms must enter the circulation during the drowning process and reach the circulatory organs. The recovery of diatoms in those organs must be positive in quantity and quality (i.e., same genera), in order to give a positive diagnosis of drowning. A negative result does not exclude death by aspiration of water. Thus, the diatom test is limited and may confirm drowning in only a certain number of cases.

Qualitative diatom interpretation requires assessment by an expert.

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