Int J Legal Med (1995) 108:39–42

TECHNICAL NOTE

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Detection of green algae *(Chlorophyceae)* for the diagnosis of drowning

Received: 26 July 1994 / Received in revised form: 3 April 1995

Abstract The plankton test (generally, diatom test) is one of the methods available to diagnose the cause of death of submerged bodies. The solubilization method using tissue solubilizer Soluene-350 was used in this study to detect not only diatoms but also green algae, based on the fact that the solubilizer does not digest the cell walls of green algae which are made from cellulose. Detection of green algae from organs of submerged cadavers is very informative to determine drowning in fresh water, and also in cases where only few diatoms are detected in the organs.

Key words Drowning · Plankton test · Tissue solubilizer · Green alga · Diatom

Introduction

In forensic autopsies the cause of death should be determined carefully and conclusively. Diagnosis of drowning is one of the most difficult tasks, and is often determined by the detection of plankton in blood, lung, liver, kidney and bone marrow of submerged bodies [1–8]. Several investigators have claimed that plankton cannot be used as evidence of drowning since they can be found in the organs of non-drowned cadavers [9–16]. However, other researchers have recognized the significance of the plankton test because much more plankton can be found in the organs of drowned bodies than in non-drowned corpses [2–6, 8]. For a more exact discrimination between drowning and non-drowning cases, new techniques should be explored to detect plankton more efficiently.

Plankton is usually detected by the disorganization method in which the organs are digested by strong acid at high temperature to microscopically identify the skeleton of diatoms in the digestion residues. However, this method is hazardous and the remaining acid often corrodes the objectives of microscopes [17]. Therefore other microscopical examinations have been reported in which diatoms are extracted from the organs by tissue solubilizer Soluene-350 [18, 19], membrane filter [2, 20], centrifugation in a colloidal silica gradient [21], ashing [8, 17] or proteinase K digestion [8, 22]. Fluorescence of chlorophyll has also been analyzed to demonstrate phytoplankton in the organs by fluorescence microscopy [23] or spectrofluorophotometry [24]. In these methods, the solubilization method using tissue solubilizer is very simple and easy, since it needs neither special equipment nor techniques [18, 19]. In this study, the solubilization method was applied to detect green algae *(Chlorophyceae)* as well as diatoms for the diagnosis of drowning in fresh water.

Materials and methods

Plankton in water from the Yodo River (Moriguchi, Osaka, Japan) were concentrated by centrifugation at 3,000 rpm for 5 min, washed with Milli-Q quality water (free from plankton) and centrifuged again. Living plankton in part of the precipitate were fixed in 60% ethanol and observed microscopically. The remaining precipitate was suspended in an adequate volume of tissue solubilizer Soluene-350 (Packard., Meriden, Conn.) in a glass tube. The solution was incubated at 50° C for 2 h and subsequently at room temperature overnight, for 1 week or 1 month. Following centrifugation at 3,000 rpm for 60 min, one drop of the precipitate was examined microscopically.

Lung, liver and kidney tissues (20 g each) were extracted from 2 drowned corpses. Cases 1: A 26-year-old man found dead in the Yodo River was sent for autopsy to our laboratory to confirm the cause of death. The corpse was putrefied and no injuries were found. The thoracic and abdominal cavities were not exposed to the river water, but black mud was found in the trachea. The lungs were edematous, and wine-red fluid was found in the thoracic cavities. The time since death was estimated to be about 2 months. Lung, liver and kidney samples were extracted for the plankton test. Case 2: An approximately 50-year-old woman was found dead in the Yodo River. The skin of the corpse showed adipocere formation and the thoracic and abdominal cavities had not been exposed to the water. Sand was observed in the trachea, and wine-red fluid was found in the thoracic cavities. The time since death was estimated to be about 3 months. The organs were extracted for the plankton test.

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Organs were also extracted from 2 cadavers where death was due to other causes, and these were regarded as controls in this study. Control 1: A submerged corpse. Cause of death was not drowning but asphyxia. The time since death was estimated to be about 1 month. The thoracic and abdominal cavities were not exposed to river water. Control 2: A traffic accident victim who died of cerebral contusion. This cadaver was without water immersion.

Disposable containers were used for the experiments. The organs and equipment were carefully washed with Milli-Q quality water immediately before the experiments to avoid contamination by airborne plankton [9]. Milli-Q quality water was prepared in a water purifier Milli-XQ (Millipore, Milford, Mass.). Microscopical examination of centrifugation precipitates of Soluene-350 and acetone, was carried out to check that the reagents were not contaminated by plankton.

The organs were cut into small pieces with scissors, and homogenized in 5 vols of Milli-Q quality water. Following centrifugation at 18,000 rpm for 10 min at 4° C, the precipitate was suspended in 8 vols of Soluene-350 in a glass tube. The solution was incubated 50° C for 2 h and subsequently at room temperature overnight. Following centrifugation at 3,000 rpm for 60 min, one drop of the precipitate was examined microscopically.

As an alternative method for digestion of the organs, 5 vols of acetone was mixed with the homogenate and the mixture was centrifuged at 3,000 rpm for 5 min at room temperature. The precipitate was digested by Soluene-350 as described.

Results

Many types of green algae were found in the river water, such as *Hydrodictyaceae, Micractiniaceae, Oöcystaceae, Ueotrichaceae, Desmidiaceae* and *Zygnemataceae,* as well as many types of diatoms, such as *Coscinodiscaceae, Diatomaceae, Achnanthaceae, Naviculaceae, Melosiraceae, Cymbellaceae* and *Nitzschiaceae.* When the green algae were treated with Soluene-350 overnight, the cell walls could be detected in the digestion residue (Fig. 1). Although the digestion for 1 week or more destroyed part of the plankton, several green algae were identified in the residue even after digestion for 1 month. The silica skeleton of diatoms could be easily detected from all these samples.

When the homogenates of the organs were pretreated with acetone, the precipitate formed by centrifugation (3,000 rpm, 5 min) was more condensed than by centrifugation alone (18,000 rpm, 10 min) in the original method. Therefore the amount (8 vols of precipitate) of Soluene-350 was reduced in this modified method. Diatoms and green algae were also detected in the organs, and in particular larger plankton such as *Zygnemataceae* could still be detected in the digestion residue by this method. Since the organs were sufficiently solubilized only by Soluene-350, ultrasonic irradiation was not performed for further digestion [17, 18].

In Case 1, some plankton were detected in the organs; *Melosira* (diatom) and *Staurastrum* (green alga, Fig. 2) from the lung (10 examples of plankton per microscope slide), and a fragment of *Navicula* (diatom) from the liver. In Case 2, many diatom species such as *Navicula* and *Cymbella*, and several green algae such as *Staurastrum* and one species of *Zygnemataceae* (Fig. 3) were detected in the lung (more than 20 examples of plankton), the diatoms and *Zygnemataceae* from the liver (more than 5 ex-

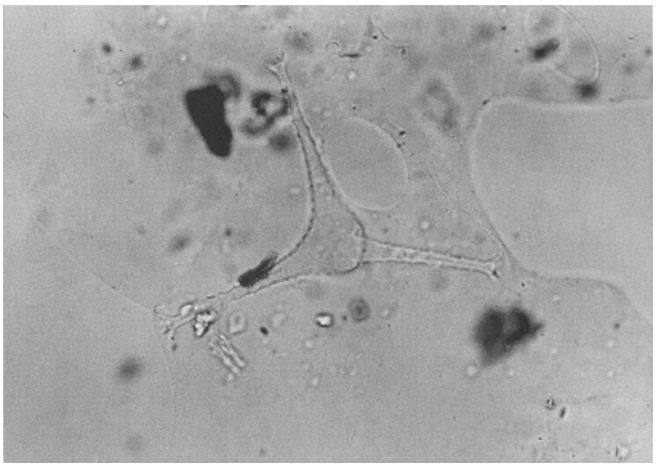

Fig. 1 *Staurastrum* in the precipitate of water from the Yodo River treated with Soluene-350 (× 400)

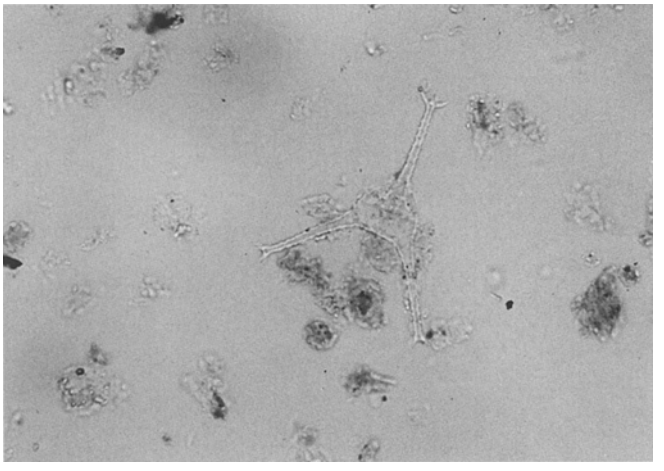

Fig. 2 *Staurastrum* from the lung of the submerged corpse in Case 1 (× 400)

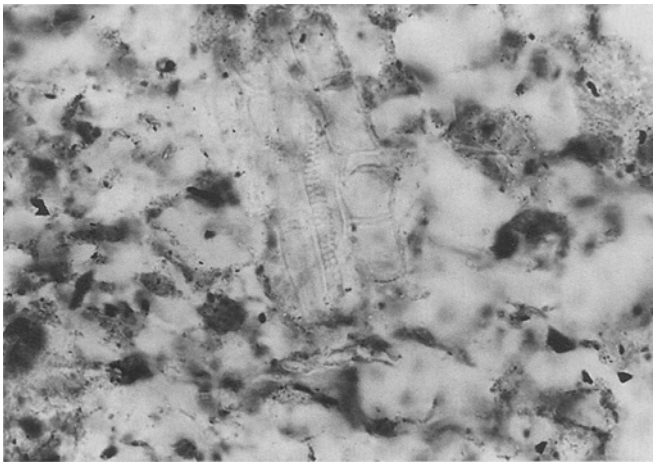

Fig. 3 *Zygnemataceae* from the lung of the submerged corpse in Case 2 (× 400)

amples of plankton), and the diatoms from the kidney (more than 5 examples of plankton). Controls 1 and 2: no plankton was detected in the organs of the cadavers.

Discussion

Soluene-350, which consists of 0.5 *M* quaternary ammonium hydroxide in toluene, is usually used as a solubilizer for liquid scintillation counting [25]. This reagent has been applied to the diatom test for the diagnosis of drowning, and is estimated to be a very simple and less hazardous technique [18, 19].

It is known that more green algae inhabit river and pond water than diatoms, whereas the latter are more commonly found in sea water [26]. Thus, the detection of green algae should be more informative for the diagnosis of drowning in rivers and ponds. Several methods have been reported for detecting green algae, such as a centrifugation method using a colloidal silica gradient [21] and fluorescence analysis of chlorophyll [23, 24]. As this study demonstrates that Soluene-350 does not digest cellulose, it is also applicable to the detection of green algae.

The Yodo River is a large river in the Osaka district, that arises in Biwako Lake and flows into Osaka Bay. Every year several submerged cadavers are found in the river and autopsied in our laboratory. Many species of green algae and diatoms are found in the river water. When living plankton were treated with Soluene-350, some green algae were identifiable by their residual cell walls which consist of cellulose. The cell walls of green algae are thinner and therefore less visible than the shells of diatoms. However, some species of green algae have specific shapes, and could be sufficiently detected even after digestion for 1 month.

In case 2, a submerged cadaver, the green algae *Zygnemataceae* and *Staurastrum* were detected in the organs, strongly supporting that the cause of death was drowning in the river. In Case 1, only a few plankton species (*Merosira* and *Staurastrum*) were found in the lungs. The fact that no complete plankton were found in the liver and kidney might be due to rapid death before penetration of plankton into the circulation [8, 16]. In this case, it was difficult to eliminate the possibility of water invasion into the lungs after death. However, the detection of green algae suggested that the cadaver had been immersed not in sea water but in a river or pond. In general, a larger number of plankton (not only diatoms but green algae) are detectable by this solubilizer method, which will ensure more exact diagnosis of drowning.

Milli-Q quality water throughout the whole procedure was used because distilled water may contain plankton. Use of Milli-Q water, reagents, containers and equipment free from plankton in a clean laboratory may avoid contamination by airborne planktons to some extent, and in this study no plankton were found in the organs of non-drowned cadavers. These were of course not controls in a strict sense, so further examinations on controls from deaths other than drowning but with a comparable post-mortem time of water immersion should be required.

By a modification of the pretreatment using acetone, large algae such as *Zygnemataceae* could be detected in the organs. The reduced centrifugation force was likely to cause less damage to the cell wall of green algae, preventing the destruction and loss of plankton.

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