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Reverse Aqua Regia: A New Method For Extraction Of Diatoms From Human Tissue

Research article

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Abstract

Background: The presence of diatoms in human tissues contributes significantly in determining the mode of death in drowning. Conventional acid digestion of tissues for extraction of diatoms is a time consuming and potentially hazardous technique. The various techniques of extraction and analysis affect the identification of diatoms from human tissues. We have developed and tested a new method which is a simple, safe, time saving, economical extraction technique and extracts diatoms rapidly by removing the extracellular and intracellular organic matter from the siliceous frustules of diatoms. The method was compared with other conventional methods of extraction. This method provides results within 2-3 hours from the tissue samples of suspected drowning cases without interfering with microscopic observation and may be useful in current forensic practices.

Materials and methods: The lung tissue samples and bone marrow from bones (femur and sternum) of 66 human cases suspected for drowning death were taken. The human tissue samples of humans were analyzed by the modified reverse aqua regia digestion method and the conventional acid digestion method simultaneously.

Results: The results showed that the modified reverse aqua regia digestion method was less time consuming having very strong digestive ability with less impurity as the structure of diatoms remained almost intact, diatoms were identified with clear striations and the recovery rate was higher as compared to conventional acid digestion method.

Conclusion: The modified reverse aqua regia acid digestion method is a new scientific approach in the field of forensic diatomology as this is simple and rapid procedure that produces more effective results.

Keywords: Drowning; Diatom Test; Digestion Method; Diatom Extraction; Modified Reverse Aqua Regia; N: Number; WHO: World Health Organization; NCRB: National Crime Record Bureau; DNA: Deoxyribonucleic Acid; PCR: Polymerase Chain Reaction; SEM: Scanning Electron Microscope.

Introduction

Drowning is the most common cause of mortality in India. The establishment of ante-mortem or post-mortem submersion in unnatural deaths plays an important role in investigation of crime cases. Nearly, every hour 42 people and every year, 3.7 lakh people die due to drowning worldwide. Drowning is the third major cause of accidental deaths worldwide [1]. According to National Crime Record Bureau (NCRB) report 2018, drowning was the third major cause of all accidental deaths reported in India [2]. Diatoms are microscopic, unicellular, photosynthetic algal organisms with a diameter of between 2 and 200 microns, although sometimes they can be up to 2 millimeters long. Their uniqueness lies in the hardened silica cell wall (SiO_2) which is resistant to decay and retains diagnostic features enabling species identification and comparison. Diatom is a large group of algae which consists of over 200 genera and 10,000 species, out of which 92 genera and 569 species are reported in India [3]. According to Mann and Droop, there are hundreds of genera and perhaps 20,000 species of diatoms of which only a tenth have been described so far [4]. Diatoms occur in almost every type of habitat, but some species have a narrow range of distribution due to preferable mode of habitat and thus they can be used for biomonitoring [5]. Diatoms have different environmental preferences based on pH, light, temperature, moisture condition, salinity, oxygen, organic and inorganic nutrients, thus the species of diatoms vary from one

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drowning medium to another [6, 7]. It helps to determine the site of drowning by comparing the diatoms species present in tissue sample and water sample of the drowning site [8].

The identification of diatoms in the body tissues to prove death by drowning dates back to the end of the 19th century. In the case of ante-mortem drowning, the water enters into the lungs and then into the bloodstream through ruptures in the peripheral alveoli before being carried to the other organs due to inhalation. The microscopic contents including diatoms present in the drowning medium enter into the blood. The detection of these diatoms is done by using diatom test to ascertain the cause of death. The diatom test is based on the diatom density in the drowning medium and the extreme locations of human body including femur, sternum bone, liver, kidney and brain as several diatoms have passed to these locations from the lungs through blood [9, 10]. The extraction of diatoms from the various tissue samples requires the acid digestion. Therefore, the criteria used for the diatom test is based on the time required for samples digestion, the digestive capability of the method used, reclamation of diatoms after digestion, extraction and destruction of diatom frustules [11].

Due to advancement in the molecular methods in recent years, DNA sequencing has been used in diatom testing of drowning cases [8]. Racz et al. used a polymerase chain reaction (PCR) based method to identify phytoplankton such as cyanobacteria, green algae, etc [12]. Vinayak and Gautam used DNA barcoding to identify diatom species by using short standardized DNA region [13]. Chen et al. classified a supplementary method of classifying the single diatom cells by using the V4 region of 18S rDNA [14]. DNA based techniques are effective in identifying the diatom species than other morphological based methods. However, the use of DNA sequencing in forensic cases is limited as these methods require expensive reagents and instruments which may not be available in forensics laboratories. Zhao et al. developed Microwave-Vacuum Filtration-Automated Scanning Electron Microscopy (MD-VF-Auto SEM) method. In this method, the SEM system automatically scans filter fields from the vacuum and takes pictures of the fields for quantitative and qualitative analysis. The limitation of this method is that the microwave digestion device and electron microscope are not commonly available in conventional forensic laboratories [15]. Some earlier studies conducted on the diatom test directly utilized tissue samples for microscopic examination, but with the intervention of new technologies; the diatom test has also been standardized. However, several methods for digestion of human tissues have been presented in the literature, but they have some limitations. The commonly used method is the acid digestion test using nitric acid which is still widely used for the detection of diatoms. The conventional acid digestion method is a laborious, hazardous and time consuming. The integrity of diatom structure is destroyed due to the harsh nature of the chemicals used in this method. The retrieval of literature showed that there is no study having such a large sample of human tissue digestion by modified reverse aqua regia till date. The new method is advancement in forensic diatom tests for identification and classification of diatoms.

Materials and Methods

This study was conducted on samples of 66 cases of drowning deaths received for examination at Regional Forensic Science Laboratory (RFSL) from January 2016 to December 2018. The diatom extraction of the samples was done by using the conventional acid digestion method and newly developed, tested and validated modified reverse aqua regia digestion method. In case of conventional acid digestion method, the material was extracted from the human lung tissues and bone marrow samples. 50 ml. of nitric acid was added to the beaker containing extracted material and kept for simmering on a hot plate at 60-70°C for 48 hours in a fume hood. The material extracted was centrifuged thrice at 4000 rpm for 10 minutes and washed with double distilled water and finally with ethanol. The acid-resistant extracted material was isolated and control water sample were spread on a glass slide, dried in an oven, and examined microscopically.

The modified reverse aqua regia digestion method was developed at RFSL, Northern Range, Dharamshala, Himachal Pradesh, India. The diatom extraction was done from lung tissue samples and bone marrow. The bones (femur and sternum) were cut using a clean band saw and the bone marrow was scooped out by using a spatula. The lung tissue samples and bone marrow were digested by mixing 15 ml of analytical grade concentrated HNO_3 and 5 ml of HCl in a ratio 3:1 in 5-10 g of tissue samples (lungs and bone marrow), placed into a boiling flask and kept on the hot plate for simmering at 60-70°C without interruption approximately for two hours in an acid digestion block. The fat layer of organic matter was removed and the suspension was taken in to a test tube for centrifugation. The extracted samples were centrifuged at 4000 rpm for 15 minutes. The supernatant was removed and the deposit retained was washed twice with distilled water using centrifugation and finally twice with ethanol. The distilled water removes the extra residual particles and ethanol increases the clarity in the anatomical structure of diatoms. The supernatant was discarded and the residues were transferred on a clean glass slide by using a disposable micropipette, dried in an oven and examined under a microscope at a magnification of 400x as shown in figure 1.

After microscopic observation of both samples of conventional and modified reverse aqua regia method, intra and inter comparison was done between the diatoms present in the digested material extracted from a biological specimen. The qualitative, quantitative, and morphological aspects of every sample of diatoms were recorded to study the utility and validity of both the diatom extraction methods. A comparison was done between both the methods. Validation of new digestive method was done by inter laboratory comparison and similar results were obtained.

Results

In the present study, on comparing both the digestion methods, it was found that the newly developed method was less time consuming having higher digestive capability and higher recovery of diatoms with clear morphological features whereas conventional method took more time, affected the siliceous cell wall of diatoms and gave poor results in identification. The new method for detecting diatoms in human tissues took about 2-3 hours for analysis whereas conventional method took at least 48 hours. The integrity of diatom structure was not disturbed as it remained intact after the digestion process with a clear background with less impurity as shown in figure 2.

Figure 1: Protocol of modified aqua regia method.

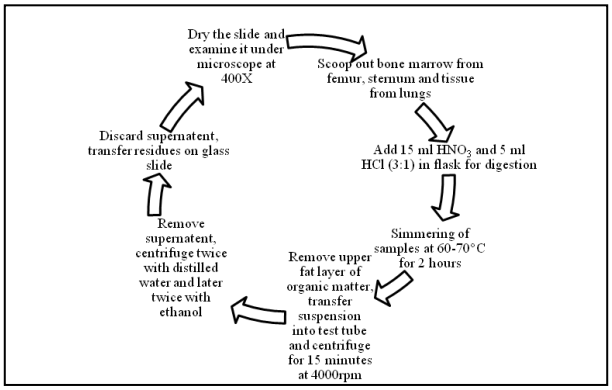
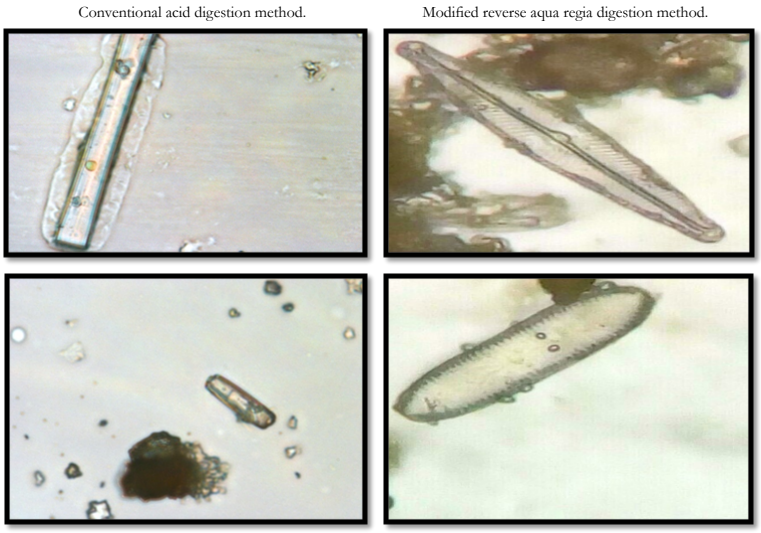


Figure 2: Microscopic images of diatoms under 400x and comparison of residues by using conventional acid digestion method and modified reverse aqua regia digestion method.



There was no or minimal loss of diatoms in this new method. The modified reverse aqua regia is a corrosive mixture of 3 parts of HNO_3 + 1 part of HCl by volumes of concentrated reagents. Nitric acid is an excellent oxidizing agent which allows the organic matter digested faster. When nitric acid and hydrochloric acid are mixed, the chemical reaction that ensued was as:

$$3HNO_3 + HCl \rightarrow [H_2 ((N_3O_9) Cl))]$$

$$\downarrow\uparrow$$

$$2H_2 + N_3O_9Cl$$

Discussion

Some modified strong acid digestion methods are in use in forensic procedures. The modified reverse aqua regia method is a quick, simple and economical technique of diatom extraction and detection from human bone marrow and tissues samples. The digestive ability of the modified reverse aqua regia method was very strong as the structure of diatoms remained almost intact and showed clear diatom identification. In this newly developed method, remarkable collection showing morphological and anatomical features of diatoms of different genera were seen. These results showed that the modified reverse aqua regia could detect sufficient numbers and types of diatoms in small samples even. The conventional method of acid digestion is time-consuming and laborious method. It destroys the integrity of the diatom structure, thus often leading to false negative results. The conventional forensic diatom tests are not a useful tool to provide accurate information about diatoms. The results of our study were in line with the results of Wang et al. who tested lefort aqua regia method on animal tissue samples and showed that the lefort aqua regia digestion method had a superior digestive ability with higher recovery rate and less damage than the conventional acid digestion method. In the study of Wang et al., they used 15ml of concentrated HNO_3 with 5ml of concentrated HCl added in succession, then 2ml of H_2O_2 was dripping in the solution at the rate of 1 drop per 3 sec. The samples were left undisturbed for 15 minutes and then were put into the thermostatic water tank at 85°C for 50 minutes, and during heating, another 3 ml of H_2O_2 was added at the rate of 1 drop per 3 seconds [16]. Fucci developed a method by taking 30% H_2SO_4 and then treated the samples with diluted solution which has an advantage over the classical method. The advantage of this method was that there was a decrease in the presence of destroyed diatoms fragments. The study showed that the classical acid digestion method used for the detection of diatoms was very aggressive and destroyed the diatoms frustules [17].

Modified reverse aqua regia method took very less time in detecting diatoms present in human tissues than the previous techniques as the samples were analyzed within 2-3 hours. Takeichi and Kitamura in their study showed that formalin-fixed samples can also be used for the detection of diatoms by using enzymatic digestion method. The lungs samples were heated in 0.01 M Tris-HCl

buffer (pH-7.5) containing sodium dodecyl sulfate (SDS) with or without glycine, then the lungs samples were subjected to enzymatic digestion with proteinase K, afterwards the samples were heated at 80°C for 6-12 hour and then the samples were analyzed microscopically. They reported that the incubation of digested samples with this method was more appropriate for qualitative and quantitative analysis of diatom. The morphology of diatoms was preserved [18]. Kakizaki et al. used inexpensive reagents such as papain, SDS, and 5 N HCl for extracting diatoms from lung, kidney, and liver tissues and extracted diatoms from tissue samples within 3-5 h. Kakizaki et al. also proposed that the digestion efficiency of papain is better than proteinase K [19].

In modified reverse aqua regia digestion method, the structures of diatoms remained almost intact with a high reclamation rate and were identified with clear striations as compared to the conventional method. Ming et al. in their study compared four digestive methods (nitric acid plus hydrogen peroxide, proteinase K, nitric acid in disorganization can and toluene-350) and showed that the structure of diatoms under the scanning electron microscope (SEM) remained perfect after digestion with proteinase K, but was found to be destructive to some extent in case of nitric acid plus hydrogen peroxide, nitric acid in disorganization can and toluene-350 method. They showed that the diatoms extracted from the proteinase K method had a higher recovery rate and produced more satisfactory results with fewer samples and caused less pollution but the proteinase K method is too expensive to use for diatom testing [20]. DiGiancamillo et al. used a new method with HCl to digest the pig tissues for diatom testing and compared the method to other chemical and enzymatic digestion methods such as H_2O_2+HCl, H_2SO_4+HNO_3, Pronase+Pancreatine and Proteinase K+Pancreatine and reported that HCl method was a more simple, safe, inexpensive, less time consuming with minimal organic residue [21].

Kakizaki and Yukawa in their study proposed a new protocol (rapid enzymatic digestion method) for solubilizing lung tissue sample by using Qiagen proteinase K, Qiagen buffer ATL and 5N HCl within 3 hours. There is limitation in rapid enzymatic digestion method as this method can only be used for small samples of lung tissue, as diatoms are found in ample amount in lungs and not in enclosed organs such as kidney and liver. The numbers and variations in types of diatoms are low in such organs and the method is more expensive and impractical for routine testing in laboratories [22]. Ludes et al. treated organs samples with chemical digestion by using nitric acid, enzymatic digestion by using proteinase K and ashing method. Ludes et al. reported that enzymatic digestion method was more rapid, safe and environment friendly but this method takes eight hours for digestion of liver, lung, kidney and brain samples [23]. The modified reverse aqua regia method was very economical, less time consuming and environment friendly as compared to other methods such as H_2SO_4, nitric acid, chemical and enzymatic digestion using nitric acid and proteinase K, enzymatic digestion with proteinase K, papain, HCl, rapid enzymatic digestion, nitric acid plus hydrogen peroxide, nitric acid in disorganization can and toluene-350 method. All these methods of diatom extraction had different methodology and were comparatively expensive.

In the present study, we detected 15-18 diatoms/100 µl of a pellet in 5-10 gm of lung samples and 30-35 diatoms from the bone marrow of femur and sternum bones. The diatom analysis was considered positive when the number of diatoms was above a minimal established limit i.e., 20 diatoms/100 µl of a pellet obtained from 10 gm of lung samples and 50 diatoms from other organs [24]. The presence of diatoms in lung tissues and bone marrow strengthen the supportive evidence that the person was alive at the time of drowning. Ludes et al. reported that in cases of suspected drowning, 20 diatoms from a 10g of lung sample or five complete diatoms from other organs, including bone marrow are normally required for a positive diagnosis [25].

Conclusion

The modified reverse aqua regia acid digestion method is a simple and considerably fast standardized technique with a higher success rate of diatom extraction within 2-3 hours which may become the gold standard in forensic diatomology. The recovery rate of modified reverse aqua regia acid digestion method is superior to the conventional acid digestion method. The new procedure improves the stability and ensures the complete extraction of diatom from the sample and gives the best in sight into the identification of morphological features of diatoms.

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