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# Marine bacteria comprise a possible indicator of drowning in seawater☆

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**Abstract**

To investigate the effectiveness of marine bacteria as a new marker of drowning in seawater, we determined the optimal conditions of media required to selectively detect marine bacteria and applied the technique to drowned cadavers. We incubated model blood samples ($n = 20$ per group) mixed with seawater, river, tap or muddy water on agar plates (Todd Hewitt, TH; Marine 2216, M2216) and determined the NaCl concentration required to selectively detect marine bacteria. We also used TCBS agar plates without manipulation to isolate *Vibrio* spp. Among the culture media, TH agar was superior. Bioluminescent colonies were detected only in blood mixed with seawater. Blue colonies stained using the cytochrome oxidase test (COT), were detected in blood mixed with both sea and river water. However when the NaCl concentration was above 4%, COT stained colonies were detectable only in blood mixed with seawater. We subsequently used 2, 3 and 4% NaCl in TH and TCBS agar to examine blood from victims who had drowned in seawater ($n = 8$) and in fresh water ($n = 7$), as well as from victims who died near aquatic environments (non drowned; dry-land control, $n = 7$). Bioluminescent colonies were detectable on 2–4% NaCl TH agar only from two victims that drowned in seawater. Bioluminescent colonies did not grow on TCBS agar. Blue colonies from all cadavers that had drowned in seawater (8/8) and in four of those that had drowned in fresh water (4/7) proliferated on TH agar containing 2% and/or 3% NaCl, but at 4% NaCl such colonies were detected only from cadavers that had drowned in seawater (8/8). Colonies from only one cadaver from seawater grew on TCBS agar. Furthermore, neither bioluminescent nor blue colonies were detected on TH agar containing 4% NaCl in samples from two cadavers found in an estuary (brackish water) who were thought to have been carried from areas of fresh water. Homologous analyses of the 16S rRNA gene revealed that the dominant colonies on TH agar containing 4% NaCl were marine bacteria (*Photobacterium*, *Vibrio*, *Shewanella*, *Psychrobacter*). Thus, proliferating bioluminescent and/or blue colonies detected in the blood of immersed cadavers using 4% NaCl TH agar, could help to establish drowning in seawater.


*Keywords:* Bioluminescent bacteria; Diatom; Drowning; Marine bacteria; Plankton; Seawater; Selective medium

## 1. Introduction

A determination of death by drowning is normally based on pathologic findings and laboratory tests, together with environmental aspects and police reports. When signs of typical drowning at autopsy are scarce because little water has been aspirated and/or putrefactive changes have transpired, to establish death by drowning is very difficult and would be mainly dependent on laboratory analyses. However, the most versatile and popular diatom test is often negative, even when drowning in waterways full of diatoms is beyond doubt [1]. In addition, various technical issues as well as specimens from the closed organs of non-drowned bodies can cause false-positive results [2,3]. We searched for a new marker of aquatic microorganisms that are normally absent in the human body but comparatively abundant in sea or river water, to improve the accuracy of laboratory tests.

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Most bacteria that inhabit marine environments are Gram-negative rods, many of which turn blue in the simple cytochrome oxidase test (COT) [4–7]. By contrast, most bacterial groups that proliferate during human decomposition such as enteric and Gram-positive bacteria are not stained by COT [4,5]. Interestingly, bioluminescent bacteria are also widespread in marine environments [8,9].

During the process of drowning, diatoms can penetrate the pulmonary vessels via lacerations of the thinner portion of the alveolo-capillary barrier together with water [10,11], and smaller diatoms can easily enter the blood circulation [12,13]. Because marine bacteria (0.2–2 μm, picoplankton) are smaller than diatoms (2–200<μm; nanoplankton, microplankton and mesoplankton) [14,15], they might penetrate the blood system with aspirated water more easily and some might survive and proliferate in the blood of cadavers.

Marine bacteria can, whereas many species of terrestrial or fresh water bacteria cannot grow in comparatively high salt concentrations such as the 3.4–3.7% NaCl found in seawater [15,16]. In addition, the optimal growth temperature of many species of normal human bacterial flora or human pathogenic bacteria is about 37 °C [17], whereas that of marine bacteria is around 20–25 °C [18].

Here, we investigated a novel method of detecting marine bacteria using selective media combined with COT staining, and showed that the procedure helped to establish drowning in seawater as a cause of death.

## 2. Materials and methods

### 2.1. Detection of aquatic bacteria in seawater and river water

#### 2.1.1. Bacterial culture

Coastal seawater samples ($n = 20$) were collected twice in summer and winter from sandy shores (2 locations) and from rocky areas (2), as well as inside (3) and outside (3) harbors. We also collected fresh water samples from various rivers ($n = 20$).

All water samples were concentrated 1- and 10-fold and then 0.1 ml aliquots were spread in duplicate over culture plates containing Bacto® Todd Hewitt agar (Difco Laboratories, Maryland, USA) [19,20] with 2, 3 and 4% NaCl. The pH of the media was brought to 8.0 to resemble that of seawater and the plates were incubated at 25 °C for 24 h.

#### 2.1.2. Cytochrome oxidase test reagent

The COT reagent was prepared using the modified Gaby-Hadley method [5]. *N,N*-Dimethyl-1,4-phenylenediamine oxalate (1%, Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) was dissolved in 0.5% HCl, and alpha-naphthol (1%, Sigma Chemical Co., Steinheim, Germany) was dissolved in 99.5% ethanol. Both solutions were mixed in equal volumes to generate the COT reagent.

#### 2.1.3. Detection of bacteria

If viable colonies (diameter, about >0.3 mm) appeared after incubation, bioluminescence was examined in the dark, and then the plates were stained with COT and bioluminescent and blue colonies were counted (Fig. 1).

### 2.2. Determination of optimal culture media and conditions for growth of marine bacteria using model blood samples

#### 2.2.1. Preparation of model blood samples

We prepared model blood samples based on the blood of drowned victims found 24 h after death as follows. Normal blood (2.4 ml) was incubated with 0.6 ml of coastal seawater ($n = 20$), river water ($n = 20$), tap water ($n = 20$), or muddy water ($n = 20$) at 25 °C for 24 h. Seawater and river water samples were used immediately after the experiment described in Section 2.1.1. We also collected samples of tap water from various households and muddy water after rainfall from puddles on land located far away from rivers.

The Medical Ethics Committee of Miyazaki Medical College approved the study.

#### 2.2.2. Preparation of culture media

We cultured the blood samples on Bacto® Todd Hewitt broth and Bacto® Marine broth 2216 (Difco Laboratories) to detect heterotrophic marine bacteria [21,22] and on TCBS (Thiosulfate-Citrate-Bile-Sucrose) agar Nissui (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) to detect Vibrios [23]. In addition to marine bacteria, various other types of bacteria can also grow on Todd Hewitt

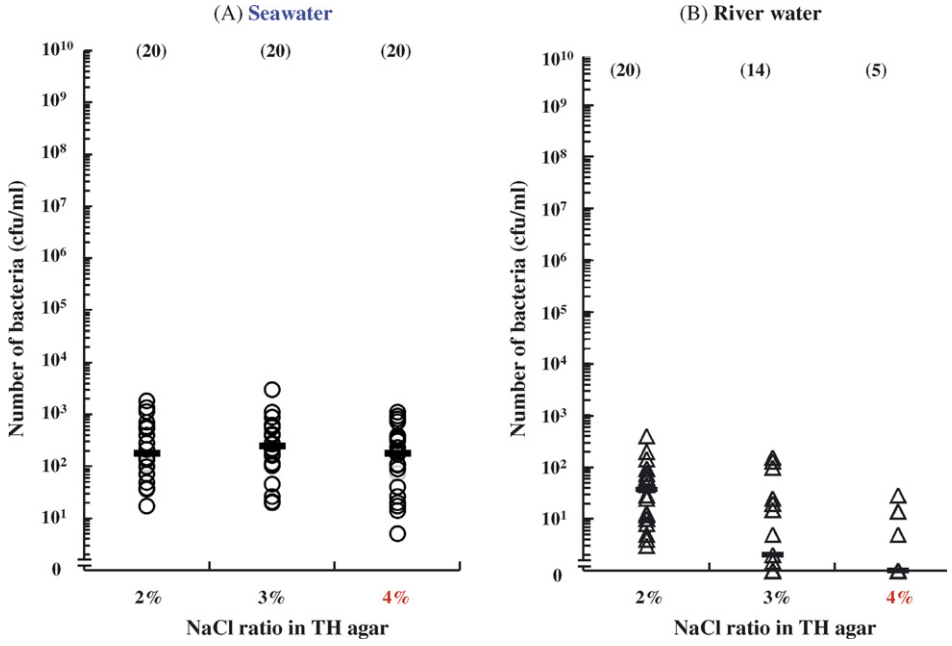


Fig. 1. Numbers of bioluminescent and/or blue (COT-positive) colonies detected in seawater (A) and river water (fresh water) (B) samples using TH agar plates containing 2, 3 and 4% NaCl. Transverse line shows median value. Numbers of water samples containing luminous and/or blue colonies are provided in parentheses.