



Detection of bacterioplankton using PCR probes as a diagnostic indicator for drowning; the Leicester experience



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ARTICLE INFO

Article history:

Received 11 February 2015

Received in revised form 1 May 2015

Accepted 3 June 2015

Available online 23 June 2015

Keywords:

Forensic

Autopsy

Drowning

Bacterioplankton

Real-time PCR

ABSTRACT

Bodies found immersed in water can pose difficulties to the investigating authorities. Pathologists may be assisted with the diagnosis by the use of tests such as the analysis for diatoms or the levels of strontium in the blood, although there is a recognised level of uncertainty associated with these tests. Recent work from Japan has shown that using molecular approaches, most recently real-time polymerase chain reaction (PCR) assays with TaqMan probes for bacterioplankton, it is possible to undertake rapid, less laborious, high throughput tests to differentiate freshwater from marine bacterioplankton and in doing so provide a molecular diagnostic test to assist in the diagnosis of drowning. We report the experiences of a United Kingdom forensic pathology unit in the use of this PCR based system for the diagnosis of drowning. We applied this technique to 20 adult and child cadavers from 4 bath, 12 freshwater, 2 brackish and 2 salt water scenes both from within the United Kingdom and abroad. Drowning was concluded to be the cause of death in 16 of these 20 cases and the PCR method supported this conclusion in 12 of these 16 cases. The PCR did not provide evidence of drowning in the four cases where death was from other causes. We illustrate that this PCR method provides a rapid diagnostic supportive test for the diagnosis of drowning that can be applied to United Kingdom autopsy practice.

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1. Introduction

Bodies found immersed in water can pose difficulties to the investigating authorities. The forensic pathologist must assist in determining whether the deceased was alive or dead prior to entering the water and, if alive, whether the primary cause of death was that of so-called “wet” drowning. All too often, the resulting conclusions may rely on a diagnosis of exclusion [1]. Macroscopic features of drowning such as the classic plume of white froth from the nose or mouth, overinflated, crepitant lungs, pulmonary oedema or water in the stomach may be present or absent depending upon the individual case [2]. Pathologists may be assisted with the diagnosis by the use of tests such as the analysis for diatoms [3] or the levels of strontium in the blood [4] although there is a recognised level of uncertainty associated with these tests.

In 1990 Mishul'skii suggested that the examination of blood for *Pseudomonas putida* and *Pseudomonas fluorescens* could assist in the

diagnosis of freshwater drowning [5]. In 2001 a meeting abstract in the Japanese language proposed the use of water borne bacterial DNA for diagnostic use [6]. This work was expanded upon by Lucci et al., who again studied the presence of faecal bacteria in water environments [7,8]. Although their work appeared promising, doubts still existed in relation to the use of faecal bacteria for the diagnosis of drowning [9]. Following on from this early work, a series of papers has been presented within the peer reviewed literature from Japan concerning the investigation of bacterioplankton, first in blood and latterly in tissue samples for the diagnosis of drowning [10–17]. This work has shown that using molecular approaches, most recently real-time PCR assays with TaqMan probes for bacterioplankton, it is possible to undertake rapid, less laborious, high throughput tests to differentiate freshwater from marine bacterioplankton and in doing so provide a molecular diagnostic test to assist in the diagnosis of drowning [18].

Considering the work from Japan we have applied the PCR technique reported by Uchiyama et al. [16] to 20 cases of bodies retrieved from or adjacent to water and examined by the East Midlands Forensic Pathology Unit (EMFPU), Leicester, United

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Kingdom (UK). Using tissue samples obtained from three body cavities from both adult and child cadavers found in indoors and outdoors scenes, including 4 bath, 12 freshwater, 2 brackish and 2 salt water scenes both from within the UK and abroad we report, for what we believe is the first time from the UK, our experiences in the use of this PCR based system for the diagnosis of drowning. We agree with the conclusions of Uchiyama et al., that the use of this test assists with the rapid diagnosis of drowning to the extent that we have adopted this test as routine in the practice of the EMFPU in the investigation of cases of suspected drowning.

2. Materials and method

Twenty consecutive cases of bodies found in water or adjacent to water where the possibility of drowning was raised, which were referred to the EMFPU and authorised for invasive autopsy examination by HM Coroner, were included in this service evaluation study (Table 1). These included both adults and children and bodies repatriated from overseas. There were no exclusion criteria.

2.1. Samples

In all cases a reference water sample from the point where the body was recovered was requested. Where a sample could not be obtained, for example when a body was repatriated from abroad, a sample of stomach contents was submitted for examination. On one occasion, the body bag was found to contain a quantity of water which had arisen during the body recovery process. A sample was submitted from the water in the bag.

In all cases tissue samples were obtained under as sterile sampling procedures as possible. The first sample was taken from the brain on opening of the skull bones and reflection of the dura (except for cases 1 and 2). The second sample was taken from one of the lungs on opening of the chest cavity (except for cases 13 and 18). The third sample was taken from the spleen on opening of the peritoneal cavity and prior to the removal of any of the other organs (except for case 5). Finally, again prior to removal of the other organs, a sample of kidney was obtained (except for case 18). Liver samples were also taken in some cases (5, 11, and 13–15). For each case a new scalpel blade and a clean, different pair of forceps were used for sampling and tissue handling. All samples were placed into separate sterile universal containers with no preservative. These were placed into a refrigerator at 4 °C before being processed in the Clinical Microbiology laboratory at Leicester Royal Infirmary.

2.2. Autopsy

A full invasive autopsy was undertaken by a forensic pathologist trainee or consultant, working to national forensic pathology guidelines in all cases [19]. The subsequent report was retrospectively interrogated for the age and gender of the deceased, date and time pronounced dead, date of autopsy, date of laboratory investigation and cause of death.

2.3. DNA isolation

Initial treatment of samples for nucleic acid extraction varied depending on type. Up to 50 mg of each tissue sample (<10 mg of spleen) was aseptically transferred to a MagNA Lyser Green Beads tube (Roche Diagnostics Ltd., Mannheim, Germany) containing 500 µL of a 1:1 solution of Bacteria Lysis Buffer (BLB; Roche) and phosphate buffered saline (PBS) and homogenised for 30 s at 6000 rpm in a MagNA Lyser instrument (Roche). Body fluids, including viscous stomach content samples, were prepared by

combining 200 µL of fluid with an equal volume of BLB. Water samples from the source were prepared by centrifuging 10 mL of the neat water at 4500×g and reconstituting any pellet formed in 200 µL of the supernatant plus 200 µL of BLB. All sample types were then digested with the addition of Proteinase K solution (1:20 of final sample volume) at 65 °C for a minimum of 30 min until any solid material had completely lysed. A final digestion step at 99 °C for 10 min completed the tissue lysis. To reduce the risk of carry-over contamination between the samples, each case was processed individually and a fresh set of disposable forceps and scalpel were used to process each tissue. The closed tissues and body fluids were processed first, followed by the lung tissue before handling the water samples last. A negative control sample consisting of molecular grade water spiked with carrier RNA (cRNA) was used through the extraction (treated in the same way as body fluid samples) in order to identify reagent contamination during this process.

Following completion of the lysis step, 400 µL of each of the samples was processed using the MagNA Pure Compact instrument (Roche) with the Nucleic Acid isolation Kit I (Roche) and the DNA_Bacteria_V3_2 protocol, eluting into a final volume of 50 µL. 5 µL of an in-house preparation of T4 bacteriophage was added to each sample during the extraction protocol and used as an internal control (IC) to detect PCR inhibition or extraction failure. The DNA isolated from the samples was stored at 4 °C until the PCR could be performed which was within 24 h of extraction.

2.4. PCR investigations

The purified DNA solution from each sample was tested in duplicate for the presence of eight bacterioplankton DNA targets by TaqMan PCR. Three triplex primer-probe sets to detect species of *Aeromonas*, *Vibrio* and *Photobacterium* [18] (NED-labelled probes were substituted with VIC labelled probes for thermocycler compatibility), and one set for detection of the IC (forward primer 5'-AGCACAATGGTGAGCACAG-3', reverse primer 5'-CACCCGCCCTGTATGATATG-3' and probe ROX-5'-TCGAGGCATGGAATGGTACTGGTGT-3'-BHQ2) were used to test all samples regardless of the water type at the location of the body. Each reaction contained 1× SensiFast™ Probe No-ROX mix (Bioline Reagents Ltd., London, UK), 8 pmol of each primer, 4 pmol of each probe and 5 µL of each sample or appropriate positive or negative PCR control, in a total volume of 20 µL. Genomic DNA of each target bacterioplankton species (a gift from Dr. E. Kakizaki) was used as positive control material and optimised to amplify near to the end of the PCR cycling. Amplification was performed on a Rotor-Gene Q real-time PCR system (QIAGEN, Manchester, UK) using the cycling protocol: 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s and 58 °C for 50 s. Amplification results were accepted only if the positive controls were within the expected range, the negative template control and negative extraction controls were negative and the IC was within the expected range for each extracted sample. If the IC was not within the expected range in any sample the test was repeated with a 1:10 dilution of the DNA extract and the negative extraction control in parallel.

2.5. Assay analytical sensitivity and linearity

Analytical sensitivity and linearity of the PCR assay were assessed by testing in triplicate 10-fold serial dilutions of purified genomic DNA from each target bacterioplankton species. DNA concentrations tested ranged from 1 ng/µL to 0.1 pg/µL. Standard curves, correlation coefficients and reaction efficiency values were calculated for each target using the Rotor-Gene Q software version 2.1 (QIAGEN).

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