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# Distinctive thanatomicrobiome signatures found in the blood and internal organs of humans

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ABSTRACT

According to the Human Microbiome Project, 90% of the cells in a healthy adult body are microorganisms. What happens to these cells after human host death, defined here as the thanatomicrobiome (i.e., thanatos-, Greek *defn.*, death), is not clear. To fill the void, we examined the thanatomicrobiome of the spleen, liver, brain, heart and blood of human cadavers. These organs are thought to be devoid of microorganisms in a healthy adult host. We report that the thanatomicrobiome was highly similar among organ tissues from the same cadaver but very different among the cadavers possibly due to differences in the elapsed time since death and/or environmental factors. Isolation of microbial DNA from cadavers is known to be a challenge. We compared the effectiveness of two methods by amplifying the 16S rRNA genes and sequencing the amplicons from four cadavers. Paired comparisons revealed that the conventional DNA extraction method (bead-beating in phenol/chloroform/bead-beating followed by ethanol precipitation) yielded more 16S rRNA amplicons (28 of 30 amplicons) than a second method (repeated cycles of heating/cooling followed by centrifugation to remove cellular debris) (19 of 30 amplicons). Shannon diversity index of the 16S rRNA genes revealed no significant difference by extraction method. The present report provides a proof of principle that the thanatomicrobiome may be an efficient biomarker to study postmortem transformations of cadavers.



## 1. Introduction

The Human Microbiome Project (HMP) revealed that an adult body contains about ten times more microbial cells than human cells (NIH HMP Working Group et al., 2009; Turnbaugh et al., 2009). What happens to these microbial cells after a person dies is not well known. We *do* know that human cells become hypoxic because blood circulation ceases when the heart stops pumping (Gevers, 1975). Hypoxia triggers the release of intracellular factors that cause the organized degradation of cellular organelles by autolytic enzymes (Proskuryakov et al., 2003). These enzymes cause human cell membranes to lyse, releasing nutrient-rich cellular constituents such as carbohydrates, amino acids, lipids, minerals and water to the surrounding tissues. Then there is a massive increase in microbial abundance because bacteria metabolize these constituents for growth (Paczkowski and Schütz, 2011). A decrease in the availability of oxygen causes a shift from aerobic to anaerobic fermentation resulting in the build-up and release of gases such as $H_2S$, $CO_2$, methane, ammonia, sulfur dioxide and hydrogen (Vass et al., 2002; Vass, 2001). Details on the specific microbial species involved in these processes have not been well studied.

In a healthy adult, most internal organs such as the brain, spleen, liver, and heart are devoid of microorganisms because the immune system keeps them in check. After death, however, the immune system falters and microorganisms proliferate throughout the body beginning in the ileocecal area, spreading to the liver and spleen, and continuing to the heart and brain (Alan and Sarah, 2012). The spread of bacteria to different areas of the body occurs by microbial invasion of the capillaries of the lymphatic and vascular system (Paczkowski and Schütz, 2011) and by invasion of the mucus membranes in the respiratory system (Gill et al., 1976). The identity of the bacteria proliferating in organ tissues is not known — nor is it known if the bacterial composition varies by organ tissue or the blood. The reason for studying microorganisms associated with internal organ tissues is because they are less affected by environmental conditions than those associated with external organ tissues such as the skin or oral mucosa, and they are not directly affected by the proliferation of gut microorganisms that occurs rapidly after human death. To date, no study has examined the microorganisms associated with internal organ tissues (e.g., liver, spleen, heart and brain) after human death using culture-independent methods. In forensic science, it is important to study these microorganisms because the presence/absence and/or abundance of certain bacteria might be

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indicative of the elapsed-time-since-death (i.e., the postmortem interval, PMI) as demonstrated in mouse and swine studies (Metcalf et al., 2013; Pechal et al., 2013). In autopsy microbiology, this information might be important to confirm a suspected antemortem infection — particularly when the cause of death is unknown (Riedel, 2014).

A DNA extraction/sampling method has not been established to study the microorganisms found in organ tissue and blood samples after a host's death, which here we define as the thanatomicrobiome (i.e., thanatos-, Greek *defn.*, death microbiome). We rationalized that a method that provides DNA of sufficient quality and quantity to generate 16S rRNA gene amplicons would be highly desirable. DNA quality and quantity are of particular concern when studying the thanatomicrobiome because the ratio of microbial DNA to human DNA would be anticipated to be low in cadavers with short PMIs (i.e., samples containing few bacteria) and quantity/quality issues could potentially affect the detection of microbial DNA using PCR amplification methods. A method that delivers highly diverse rRNA genes would also be desirable because it would suggest that the method is not biased in terms of extracting DNA from certain types of bacteria over others. Other desirable features of a DNA extraction/sampling method are that it should be simple-to-perform (i.e., requiring low technical expertise) and not involve the use of biohazardous materials.

In this study, we will compare two DNA extraction/sampling methods. One method (the gold standard) involves physically and chemically breaking cells by bead-beating in a phenol/chloroform solution and precipitating the DNA in ethanol, and the other is a simple-to-perform method which involves physically breaking the cells by repeated cycles of heating and cooling, and separating the DNA in solution from cellular debris by centrifugation.

The objectives of this study were two-fold: (i) to determine the best method for extracting/sampling DNA from organ tissues, and (ii) to demonstrate that the devised method is feasible to survey the thanatomicrobiome of different organ tissues and the blood of human cadavers.

## 2. Materials and methods

### 2.1. Cadaver cases

The cadavers were kept in the Alabama Department of Forensic Sciences Medical Laboratory's morgue at 1 °C. The autopsy took place in a work area with temperature of 20 °C. After collection of samples, the samples were transported in a cooler (10 min ride) to Alabama State University and placed in a freezer at −80 °C until the day of analysis. The following metadata was collected on each corpse: age, sex, weight, height, ethnicity, PMI, and rectal temperature upon autopsy (Table S1). Samples were collected from the blood and internal organs (brain, heart, liver, spleen) from 11 corpses. Specifically, portions of the internal organs were dissected using a sterile scalpel and deposited into labeled sterile plastic bags. Blood samples were collected from the heart using a sterile syringe. In some cases, it was not possible to collect blood samples because the blood was coagulated. The samples were transported from the morgue to the laboratory on ice where they were deposited into a −80 °C freezer.

This study was approved by Institutional Review Board under number: 2013CMST004A.

### 2.2. DNA extraction methods

Two DNA extraction methods were used. In the first method (which is a modification of Urakawa et al., 2010), 50 μl of blood or approximately 10 mg of thawed organ tissue was removed using a sterile scalpel and deposited into the Lysing matrix E tube (MP Biomedicals Cat# 116914) containing zirconia/silica beads, 0.5 ml phenol/chloroform/isoamyl alcohol (50:49:1) (TE saturated, pH 8.0) and 0.5 ml of 2× TENS (100 mM Tris–HCl [pH 8.0], 40 mM EDTA, 200 mM NaCl, 2% SDS) buffer (Kuske et al., 1998). The tube was then shaken at speed 6 for 40 s in a bead beater, briefly cooled in ice and centrifuged at 16 K rpm for 5 min. The supernatant was transferred to Phase Lock Gel TM 2.0 ml tube containing 0.3 ml of 7.5 M ammonium acetate and an equal volume of chloroform. The tube was mixed by repeated gentle inversions (10 times) and the supernatant transferred into a new tube containing 0.6 volume of ice cold isopropanol and 3 μl of GlycoBlue. After several inversions, the sample was stored at −80 °C for 10 min. Following centrifugation at 16 K rpm for 5 min, the isopropanol was poured off and the pellet was washed with cold 80% ethanol and allowed to dry for 5 min. The pellet was eluted into 100 to 200 μl of molecular grade water or TE buffer.

In the second method, a sterile cotton applicator tip was dipped into the organ and swabbed on the surface and the tip was deposited into a centrifuge tube containing 1 ml of PBS buffer. The PBS buffer was then heated to 100 °C for 10 min and promptly cooled in ice for 10 min. The heating and cooling steps were repeated once and the tube was centrifuged at 10 K for 5 min to remove the cellular debris (Wan et al., 2011). The upper liquid was then transferred to a clean tube for further processing.

### 2.3. PCR amplification

Prokaryotic 16S rRNA genes were amplified using universal primers (27F and 1492R) using the GemTaq kit from MGQuest (Cat# EP012). The PCR program involved a pre-amplification step of 10 cycles with annealing temperature of 56 °C followed by 20 amplification cycles with annealing temperature 58 °C. In each cycle, the elongation time was 1 min 10 s, at 72 °C. PCR was finalized by extended elongation for 5 min. PCR products were purified with Qiagen columns (California, USA). The purity (A260/A280) and quantity of the DNA for each sample were determined using the NanoDrop (Agilent, USA) after amplification and purification.

### 2.4. 454 pyrosequencing of 16S rRNA genes

Five hundred nanograms of purified PCR product was labeled with a Multiplex Identifier (MID) during the Roche Rapid Library preparation step. Four to twelve MID-tagged sequences, representing each of the samples, were combined in equimolar concentrations and subjected to emPCR and DNA sequencing protocols as specified by the manufacturer's recommendations for the 454 GS Junior Instrument. In cases where there was insufficient number of reads (e.g., <10,000), the emPCR and DNA sequencing were repeated.

### 2.5. Data analyses

The obtained sequences were separated by their respective Multiplex Identifier (MID) and uploaded to the MG-RAST web server (Meyer et al., 2008). The MG-RAST pipeline assessed the quality of sequences, removed short sequences (multiplication of standard deviation of length cutoff of 2.0) and removed sequences with ambiguous bp (non-ACGT; maximum allowed number of ambiguous base pair was set to 5). The pipeline annotated the sequences and allowed the integration of the data with previous metagenomic and genomic samples. The M5RNA database was used as the annotation source, and we set the following cutoffs for annotation: minimum sequence identity of 97%, maximum e-value cutoff at $10^{-5}$, and minimum sequence length of 100 bases. M5RNA includes a non-redundant data of 3.4 million ribosomal genes from SILVA, Greengenes and RDP. Alpha (Shannon) diversity analysis was conducted in MG-RAST.

Orthogonal transformation of the annotated rRNA genes to their principal components (PC) was conducted using normalized abundances. Normalization of the abundance was performed identically to the procedure used by MG-RAST. Specifically, abundances were increased by one, log2 transformed, and centered to produce relative values. In order to standardize relative values they were divided by