Journal of Microbiological Methods 106 (2014) 1–7

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

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

Ismail Can [a], Gulnaz T. Javan [a], Alexander E. Pozhitkov [c], Peter A. Noble [b,c,*]

[a] Forensic Science Program, Alabama State University, Montgomery, AL 36101, United States
[b] PhD Program in Microbiology, Alabama State University, Montgomery, AL 36101, United States
[c] Department of Periodontics, University of Washington, Box 3574444, Seattle, WA 98195, United States

ARTICLE INFO

Article history:
Received 27 June 2014
Received in revised form 23 July 2014
Accepted 23 July 2014
Available online 1 August 2014

Keywords:
DNA extraction methods
Thanatomicrobiome
Postmortem interval
Cadaver

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

* Corresponding author at: PhD Program in Microbiology, Alabama State University, Montgomery, AL 36101, United States. Tel.: +1 334 229 8451.
E-mail addresses: ismail@iyte.net (I. Can), gjavan@alasu.edu (G.T. Javan), pozhit@uw.edu (A.E. Pozhitkov), panoble@washington.edu (P.A. Noble).

http://dx.doi.org/10.1016/j.mimet.2014.07.026
0167-7012/

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

the standard deviation of the log2 values. The data were graphed on a 2 dimensional ordination plot. To determine the relative contribution of the microbial species to each plot, we let X denotes the 19 samples by 456 (cases by species) matrix of the normalized abundance values. The matrix X was used to produce a 19 × 19 matrix D of distances between all pairs of samples. Principal component analysis (PCA) was performed on the matrix of distances, D. The Euclidean distance metric was used to create this matrix. To investigate and visualize differences between the organ tissues/blood, the first two principal components, PC1 and PC2, of the distance matrix D were retained. To establish which species were most prominently responsible for the groupings, the projection of each species onto the (PC1, PC2) plane was calculated; those species with the largest projections are displayed in the right panel of Fig. 2. That is, this figure shows a biplot of the species most highly correlated to PC1 and PC2.

Hierarchical clustering of Matrix X and average linkage was used to construct a dendrogram of the microbial communities by organ tissue and blood sample.

Two-tailed paired t-tests were used to investigate if there were significant differences in the means (alpha = 0.05). These analyses were performed using SAS JMP.

3. Results

3.1. Amplification products by DNA extraction method

We examined 11 cases (19 samples in total) that had PMIs ranging from 20 to 240 h (Table 1). Only samples with known time of death obtained from daily crime reports were used for this research. An amplicon product of ~1500 bp was scored positive for the presence of 16S rRNA genes; while the rest were scored negative for no amplification product (Table 2). We confirmed negatives by re-amplifying the DNA. Regardless of extraction method used, the quantity and quality of the purified amplicons ranged from 10 to 500 ng/μl with an average A260/A280 ratio of 1.9 ± 0.1 a.u.

Our earliest detection of 16S rRNA amplicons occurred in a liver sample from a cadaver with a PMI of 20 h. Our earliest detection of amplicons in all sample organ tissues occurred in a cadaver with a PMI of 58 h. These findings provide a time frame for studying the thanatomicrobiome in organ tissues.

A summary of the presence/absence of PCR amplification products revealed that Method 1 yielded more PCR products (29 out of 30 samples) than Method 2 (19 out of 30 samples) (Table 3). It is possible that these disparities could be due to sample types (i.e., brain, heart, liver, spleen or blood) — however, no pattern was apparent in Table 2.

Eleven samples were scored positive for amplification product using Method 1 but were negative for Method 2 (Table 2). A potential reason for the higher success of Method 1 over Method 2 is the phenol/chloroform step that removes debris that could potentially inhibit PCR amplification of the DNA. Method 2 does not have a phenol/chloroform step — hence the PCR amplification might have been affected by debris.

It should be noted that two samples that scored positive for PCR product using Method 2 did not produce a product using Method 1 (Table 2). Presumably, a low amount of microbial DNA in the heart and blood samples is responsible for these results.

A serious drawback of Method 2 was that four of five samples did not produce an amplification product for organ tissues from a well-decomposed cadaver (i.e., Case 41 with a PMI of 240 h) (Table 2). An amplification product was only produced for DNA extracted from the blood using Method 2. In contrast, Method 1 yielded PCR products for all organ tissues from this cadaver (Table 2).

We sought to sequence additional samples for the paired comparisons of the extraction methods but were not able to do so either because one sample of a pair did not yield a PCR amplicon or because one sample of a pair yielded an insufficient amount of DNA for sequencing (Table 2). It should be noted that a minimum of 500 ng of total DNA is required for 454 DNA sequencing. We were also not able to sequence additional samples due to the limited availability of cadavers.

Taken together, these findings imply a significant weakness in Method 2 since Method 1 yielded more PCR products from different organ tissues and blood samples of cadavers with a range of PMIs.

3.2. Sequencing results by organ tissue/blood sample and case number

Nineteen PCR amplicons were selected for high-throughput DNA sequencing (Table 1). The average (±std) number of reads per sample was 31,540 ± 13,113. The rarefaction curve of most samples approached saturation, indicating sufficient reads for comparison by DNA extraction method and case number (Fig. 1).

Table 1
Total number of sequencing reads by case number, sample type, PMI, and extraction method. Extraction method 1, phenol/chloroform/ethanol; extraction method 2, heating/cooling/centrifugation.

Case number	Organ tissue/blood	Sample_ID	PMI (h)	Extraction	Total reads
8	Blood	Bl_1_8	66.0	1	20,276
8	Blood	Bl_2_8	66.0	2	26,227
8	Heart	H_1_8	66.0	1	24,006
8	Heart	H_2_8	66.0	2	17,777
25	Brain	Br_1_25	58.0	1	6637
25	Heart	H_1_25	58.0	1	32,935
25	Heart	H_2_25	58.0	2	29,598
25	Liver	L_1_25	58.0	1	25,630
25	Liver	L_2_25	58.0	2	38,422
25	Spleen	S_1_25	58.0	1	30,332
25	Spleen	S_2_25	58.0	2	23,073
32	Blood	Bl_1_32	29.5	1	23,036
32	Blood	Bl_2_32	29.5	2	28,764
32	Brain	Br_1_32	29.5	1	67,869
41	Blood	Bl_1_41	240.0	1	37,566
41	Brain	Br_1_41	240.0	1	37,988
41	Heart	H_1_41	240.0	1	42,843
41	Liver	L_1_41	240.0	1	47,492
41	Spleen	S_1_41	240.0	1	38,797

Table 2
Presence/absence of correctly sized PCR product by DNA extraction method and organ tissue/blood sample. Bl, blood; Br, brain; H, heart; L, liver; S, spleen; nd, not determined; +, positive PCR product; −, no PCR product; $+^{w}$, weak band of PCR product. Extraction Method 1, phenol/chloroform/ethanol; extraction Method 2, heating/cooling/centrifugation.

Case number	PMI (h)	Extraction Method 1					Extraction Method 2				
		Bl	Br	H	L	S	Bl	Br	H	L	S
4	36.5	nd	−	−	−	−	nd	−	+	−	−
8	66.0	+	$+^{w}$	$+^{w}$	$+^{w}$	−	$+^{w}$	−	+	−	−
12	20.0	−	−	−	+	−	nd	nd	nd	nd	nd
24	41.5	−	−	−	−	−	−	−	−	−	−
25	58.0	nd	+	+	+	+	nd	−	+	+	+
31	23.0	−	−	+	−	−	−	−	−	−	−
32	29.5	+	+	−	−	$+^{w}$	−	+	−	−	−
35	83.0	nd	nd	+	+	+	nd	nd	+	+	nd
36	32.0	−	−	−	+	−	+	−	−	−	−
39	48.0	nd	+	$+^{w}$	+	nd	nd	+	+	+	nd
41	240.0	+	+	+	+	+	$+^{w}$	−	−	−	−

Table 3
Two-by-two table of samples yielding PCR amplicons by DNA extraction method. Results indicate that extraction Method 1 yielded more ($n = 28$) amplicons than extraction Method 2 ($n = 19$). Extraction Method 1, phenol/chloroform/ethanol; extraction Method 2, heating/cooling/centrifugation.

Extraction method		Method 1	
	PCR amplicon	−	+
Method 2	−	14	11
	+	2	17

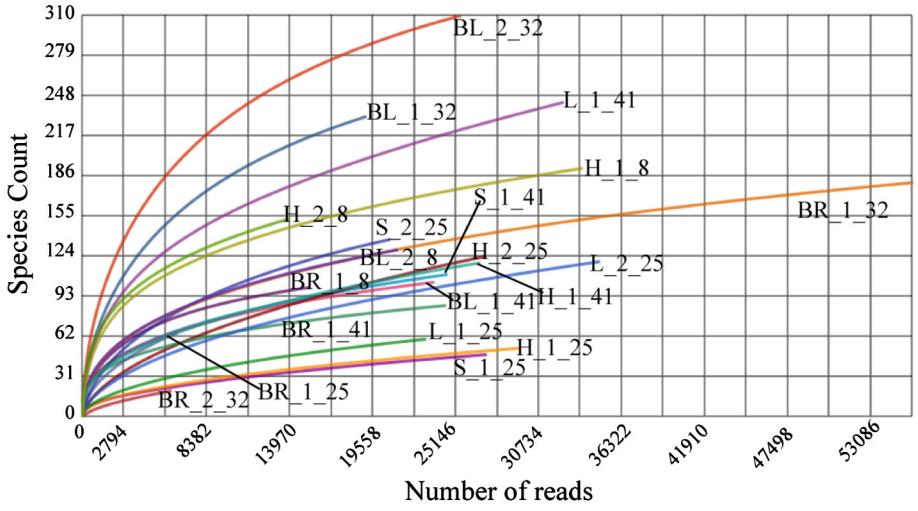

Fig. 1. Rarefaction curves of 16S rRNA genes by sample using M5RNA source data with 97% similarity, 100 bp minimum length and e-value of 10^{-5}. Label coding is shown in Table 1.

The DNA sequencing files can be obtained from the MG-RAST website through the following identifiers: 4552659.3, 4552668.3, 4552669.3, 4552670.3, 4552666.3, 4552665.3, 4552664.3, 4552662.3, 4552663.3, 4552661.3, 4552660.3, 4564139.3, 4564140.3, 4564141.3, 4564142.3, 4564143.3, 4564144.3, 4564145.3, and 4564146.3.

3.3. Microbial diversity and number of microbial species by DNA extraction method

The determinations of the microbial diversity and number of species were based on the taxonomic output from MG-RAST. Although Shannon diversity index was higher for samples processed by Method 2 (6.0 ± 6.0 a.u) than Method 1 (5.2 ± 4.9 a.u.), a paired two-tailed t-test revealed no significant difference by method (Table 4). Similarly, we found a higher number of bacterial species for samples processed by Method 2 (101 ± 53 species) than Method 1 (70 ± 58 species) (Table 4). However, a paired two-tailed t-test revealed no significant difference — although it approached significance ($P = 0.075$; df = 5).

These results indicate that one method was not more advantageous than the other in terms of microbial diversity and number of different species obtained.

3.4. Ordination and clustering results

The ordination plot of the organ tissue and blood samples explained 81.5% of the observed variability. The plot revealed that the thanatomicrobiome varied by case number (and PMI) rather than organ tissue/blood type (Fig. 2, left panel). In other words, specific microorganisms did not appear to be associated with specific organ tissues. The only exception to this rule was the microbial community extracted from Case 8, which will be discussed below. A dendrogram produced by using hierarchical clustering with average linkage of the same data corroborated these findings (Fig. 3).

Table 4
Shannon diversity and number of different microbial species by extraction method. Paired two-tailed t-test (df = 5) found no significant differences in diversity or number of different microbial species by extraction method (alpha = 0.05). Extraction Method 1, phenol/chloroform/ethanol; extraction Method 2, heating/cooling/centrifugation.

Organ tissue/blood/Case	Shannon diversity index		Number of different microbial species	
	Method 1	Method 2	Method 1	Method 2
Blood, Case 32	8.9	12.5	150	203
Blood, Case 8	4.6	3.8	64	65
Heart, Case 8	13.3	14.8	134	120
Heart, Case 25	1.6	1.6	23	71
Liver, Case 25	1.5	1.7	33	71
Spleen, Case 25	1.5	1.9	17	80
Avg ± Stdev	5.2 ± 4.9	6.0 ± 6.0	70 ± 58	101 ± 53

3.5. Projections of microorganisms in the ordination plot

We sought to determine the relative contribution of the bacteria to the ordination plot by projecting the highly contributing bacteria (Fig. 2, right panel). Since the purpose of the projections was predominantly illustrative, the number of displayed species was chosen arbitrarily based on the correlation cutoff of ±0.70 for the species normalized abundances and the *x*- or *y*- ordination.

The projections revealed three groupings. In the lower portion of the ordination plot, bacteria from the genera *Lactobacillus*, *Veillonella*, *Prevotella*, *Streptococcus* and *Gemella* were found to contribute to the microbial composition. Specifically, these bacteria were found in the blood and brain samples in Cases 8 and 32, which had PMIs of 66 and 29.5 h, respectively.

The two other groupings, found in the upper portion of the plot, were mostly composed of members of the genus *Clostridium* (Fig. 2, right panel). The upper right of the ordination were dominated by *Clostridium sordellii*, *Clostridium difficile*, *Clostridium bartlettii*, *Clostridium bifermentans* and *Clostridium limosum* while the upper left was dominated by *Clostridium haemolyticum*, *Clostridium botulinum*, and *Clostridium novyi*, as well as *Escherichia coli* and *Escherichia albertii*. This divergence in *Clostridium* species might be attributed to differences between cadavers and/or PMI since Case 25 had a PMI of 58 h and grouped on the upper right of the plot while Case 41 had a PMI of 240 h and grouped on the upper left. Of note, the heart samples from Case 8 were situated mid-way between Case 41 and Case 32 suggesting that the microbial composition in the heart sample was partially similar to both cases.

In general, these results suggest that microbial species in organ tissues and the blood are similar in samples obtained from the same cadaver and the microbial species varied by cadaver and possibly PMI. Results from the ordination plot, the dendrogram and pie charts suggest that facultative anaerobic bacteria predominate in cadavers with short PMIs and that obligate anaerobic bacteria predominate in cadavers with long PMIs.

4. Discussion

Although the human microbiome has been well studied (~450 articles published in the Web of Science as of June 21, 2014), almost nothing is known about what happens to the microbiome when a

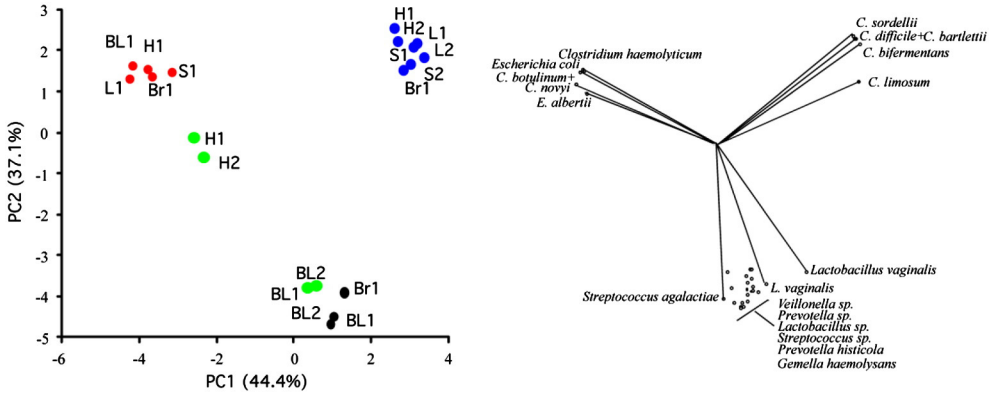

Fig. 2. Left: Ordination plot of microbial communities by organ tissue/blood (BL, blood; L, liver; H, heart; S, spleen; BR, brain), extraction method (1, phenol/chloroform/ethanol; 2, heating/cooling/precipitation), and case number (Case 8, green; Case 25, blue; Case 32, black; Case 41, red). Right: Projections of major microbial species contributing to the groups in the ordination plot. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

human host dies. To fill the void, we sampled organ tissue and blood samples of 11 cadavers having known PMIs with the objective of comparing the microbial communities by extracting the DNA, amplifying the 16S rRNA genes, and sequencing the amplicons using 454 sequencing technology. To our knowledge, this is the first study to compare the thanatomicrobiome of internal organ tissues from cadavers using a culture-independent approach.

4.1. Introduction of a new term

This study introduces the term "thanatomicrobiome", which refers to the microbiome existing in or on an animal host after it dies. We recognize that there ARE other terms used in the forensic literature to refer to organisms involved in decomposing corpses. For example, "necrobiome" refers to "the community of species (e.g., prokaryotic and eukaryotic) associated with decomposing remains of heterotrophic biomass, including animal carrion and human corpses" (Benbow et al., 2013) and "epinecrotic bacterial community" is defined as a subset of the necrobiome that includes "those organisms residing, or moving, on the surface of decomposing remains, whether it is the skin or mucous membrane of a cavity (e.g., mouth)" (Pechal et al., 2013). We assert that neither term is appropriate for this study because the necrobiome encompasses microorganisms, insects, arthropods and other larger organisms that consume/degrade corpses, while the thanatomicrobiome just deals with microorganisms in a decomposing body and not necessarily those on the surface of decomposing remains.

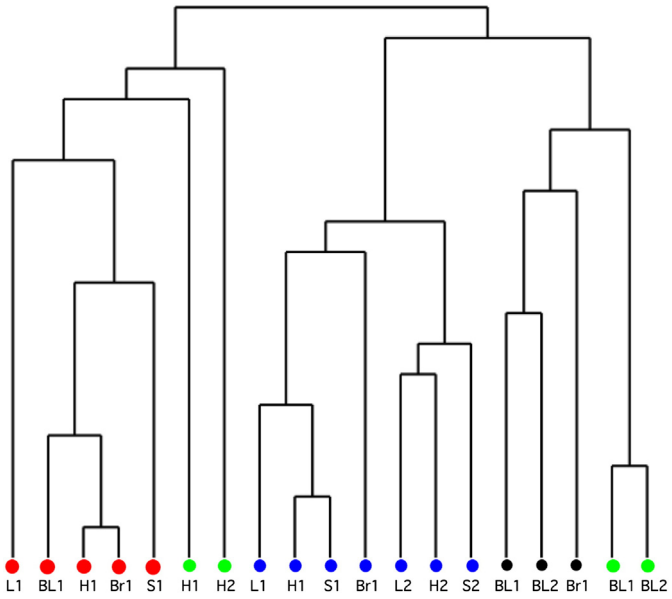

Fig. 3. Dendrogram of microbial communities by organ tissue/blood (BL, blood; L, liver; H, heart; S, spleen; BR, brain), extraction method (1, phenol/chloroform/ethanol; 2, heating/cooling/precipitation), and case number (Case 8, green; Case 25, blue; Case 32, black; Case 41, red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.2. Comparisons with other studies

Our study deviates from those that examined bacterial communities in the skin and abdominal cavity (or other orifices) of decomposing mice, swine, and humans (Metcalf et al., 2013; Pechal et al., 2013; Hyde et al., 2013) because we sampled internal organ tissues that are far less affected by confounding factors that could cloud the understanding of body decomposition. For example, a confounding factor of sampling the skin is that the microorganisms are directly affected by biological and environmental conditions (e.g., insects, clothing, and weather). A confounding factor of sampling microorganisms in the abdominal cavity is that they are mostly composed of those originating from the lower intestine. Furthermore, the thanatomicrobiome of internal organ tissues offers an advantage over other sampling sites because, after a host dies, microorganisms presumably invade organ tissues that were previously devoid of microorganisms. Hence, microorganisms found in these organs are likely key players in body decomposition.

4.3. DNA extraction and microbial survey

When initiating this study, we discovered that an approach for sampling and extracting microbial DNA from organ tissues and the blood has not been established. Several studies have sampled the skin or other decomposing body parts by swabbing the site of interest with a cotton applicator and extracting the DNA using commercial kits (e.g., Qiagen or MoBio PowerSoil) (Metcalf et al., 2013; Hyde et al., 2013). According to Wu et al. (2010), there is considerable variability in the yield and quality of DNA obtained from commercial kits. For example, the amount of DNA yielded from the MoBio PowerSoil kit was approximately 10-fold less than that obtained from the PSP Spin Stool DNA Plus Kit. In addition to this shortcoming, Peng et al. (2013) showed that simple direct boiling methods yielded similar results to those obtained using commercial extraction kits with reduced costs and improved efficiency. Therefore, we chose not to use commercial kits for this study.

It is important to note that other studies have devised their own methods to sample and extract DNA from decomposing bodies. Dickson et al. (2010), for example, collected samples from decomposing swine using swabs that were subjected to chloroform-bead-beating

followed by ethanol precipitation. Pechal et al. (2013) used cotton applicators to sample the oral mucosa and skin of swine. The cells on the applicators were digested with enzymes and the DNA was extracted using phenol/chloroform followed by ethanol precipitation. In our study, we used a phenol/chloroform bead-beating method (Method 1) that typically yields high quality and quantity of DNA (Urakawa et al., 2010) and a heating/cooling and centrifugation method (Method 2) that has been used to extract DNA from fecal samples (Peng et al., 2013) and bacterial colonies on agar plates (Wan et al., 2011). Method 2 offers the advantage over Method 1 in that it requires a low-level of technical expertise to swab, heat, cool, and centrifuge a sample and involves no biohazardous chemicals. A significant finding of this study is that Method 2 yielded comparable results to Method 1 in terms of microbial diversity — provided that an amplification product was produced (Table 4). Moreover, Method 2 apparently yielded more unique species than Method 1 (Table 4) but this difference only approached statistical significance. In retrospect, Method 2 might be improved by incorporating a "clean-up" step that removes proteinaceous debris that presumably effected the PCR amplification of 16S rRNA genes.

Regardless of the DNA extraction/sampling method implemented, the feasibility of surveying the thanatomicrobiome of internal organ tissues (e.g., liver, spleen, heart and brain) was demonstrated in this study. The results showed that most thanatomicrobiome samples grouped by cadaver (or PMI) rather than organ tissue (Fig. 2). An exception to this rule is the microorganisms from the heart and blood from Case 8 that appeared to group apart from one another in the ordination plot. Specifically, in the ordination plot, the microorganisms isolated from the heart from Case 8 appeared to group with all microbial samples from Case 41 (Fig. 2). However, the dendrogram showed this grouping was weak as evidenced by the long similarity length of heart samples from Case 8 (H1 and H2) to all the organ tissue/blood samples from Case 41 (Fig. 3). Similarly, in the ordination plot, microorganisms from the blood of Case 8 grouped with microorganisms from the blood and brain of Case 32. This grouping was also probably not real because the dendrogram showed a long similarity length between the blood samples from Case 8 (BL1 and BL2) and the organ tissues and blood samples from Case 32 (Fig. 3). These findings are corroborated by the microbial community pie charts that show: (i) the heart samples from Case 8 are dissimilar from all the other samples from Case 41 (data not shown) and (ii) the blood samples from Case 8 are dissimilar to the organ tissues and blood samples from Case 32 (Fig. 4). Taken together, the microorganisms obtained from the heart and the blood of Case 8 are different from one another and also different from the other organ tissues and blood samples of other cases for undetermined reasons.

A surprising result was the composition of the microorganisms most responsible for the different groupings in the ordination plot. Specifically, two different groups of the obligate anaerobe *Clostridium* were found in cadavers with different PMIs while facultative anaerobes such as *Lactobacillus* predominate in other cadavers with shorter PMIs. *Clostridium* has enzymes for breaking down lipids and complex carbohydrates associated with human tissue (Janaway et al., 2009), while *Lactobacillus* sp. possess enzymes to convert sugars to acids (Pessione, 2012). It is well documented in decomposing mice and swine studies that bacterial communities undergo shifts in major phyla with time that correspond to well-established visual characteristics of body decomposition (Metcalf et al., 2013; Pechal et al., 2013). It is possible that the differences in the composition of microorganisms observed in our study are due to shifts in major phyla. These findings call for a comprehensive study to validate this assertion. Such a study should involve more cadavers with known PMIs in order to better understand body decomposition and to examine the similarities of the thanatomicrobiome among organ tissues of individual hosts.

To date, only two studies have examined the human thanatomicrobiome using high throughput DNA sequencing technology. One study examined microorganisms in the blood and organs of two drowning victims by amplifying 16S rRNA genes and sequencing

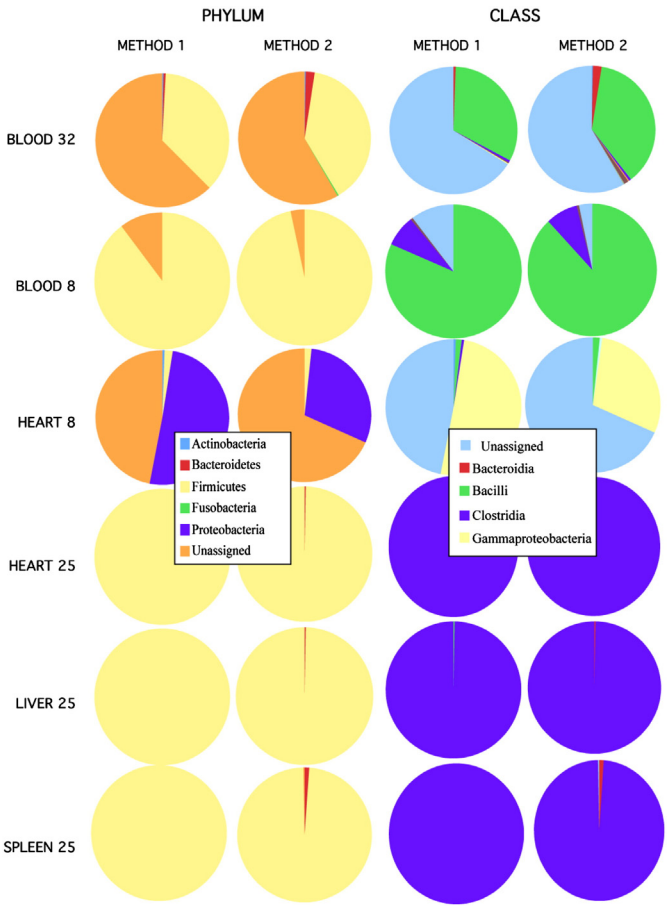

Fig. 4. Comparison of microbial community pie chart by extraction method, organ tissue/blood/case by phylum and class taxonomic resolution.

the amplicons (Kakizaki et al., 2012). We could not compare their results to those of our study because different DNA extraction/sampling methods and different primers were used for amplifying the rRNA genes. In contrast to our study, they observed differences in the microbial community composition in the heart and liver samples from within the same cadaver, but these differences could be attributed to bacteria entering the blood from the lungs together with water and none of our cadavers included drowning victims.

The other study sampled the mouth and rectal orifices of two naturally decomposing cadavers at two sampling times (pre- and post-bloat) (Hyde et al., 2013). They found a shift from aerobic bacteria to anaerobic bacteria in all body sites sampled and demonstrated a variation in community structure by body, sample sites within the same body, and between sampling times. We could not compare their study to ours because they sampled different body sites and used different DNA extraction and amplification protocols.

Interestingly, mice (Metcalf et al., 2013), swine (Pechal et al., 2013), and human (Hyde et al., 2013; Kakizaki et al., 2012) studies and this study have also found similar groups of bacteria (predominately bacteria from Gammaproteobacteria, Lactobacillaceae, and Clostridiaceae) associated with decomposing corpses. Although the composition of these microbial communities might be significantly different, it appears that they are key postmortem taxa involved in decomposing animal remains.

4.4. Relevance to autopsy microbiology

Results from our study have relevance to the field of autopsy microbiology, which is used to confirm the presence of a suspected antemortem infection, identify an infection when the cause of death is unknown, or assess the efficacy of antibiotics in treating an infection (Riedel, 2014;

Morris et al., 2006). Our results show the earliest detection of microorganisms in the liver from a cadaver with a PMI of 20 h and all sampled organ tissues from a cadaver with a PMI of 58 h, which is significant for two reasons. First, autopsy microbiology typically relies on traditional culture-based methods, which have uncertain value since only a fraction (<1%) of microbial cells can be cultured (Amann et al., 1995; Staley and Konopka, 1985). Our study used molecular biology approaches that have no dependence on culturing microorganisms and therefore provide a better picture of who is there. Second, if organ tissues are assumed to be devoid of microorganisms in a live human host, the finding of microorganisms in organ tissues after host death supports the notion that postmortem migration of microorganisms has taken place in the body (Fredetter, 1916; Gradwohl, 1904). Hence, the migration of microorganisms could have a significant effect on the procurement of autopsy microorganisms from cadavers with PMIs of over 20 h. We emphasize this point because autopsies are typically conducted within the first 24 to 48 h after death and therefore the value of results obtained from autopsy microbiology has to be considered within the context of the PMI of the cadaver.

5. Conclusion

Our study demonstrated the feasibility of studying the thanatomicrobiome of organ tissues and blood samples from cadavers by using two DNA extraction/sampling methods. We determined that the conventional phenol/chloroform/bead-beating/ethanol precipitation method was better for extracting DNA from the organ tissues and blood of cadavers than the heating/cooling and centrifugation method because it generated more PCR products. Results suggest that the thanatomicrobiome is similar among the organ tissues and blood sample of the same cadaver. Facultative anaerobic bacteria, such as *Lactobacillus*, predominate in organ tissues and blood samples of cadavers with short PMIs and obligate anaerobic bacteria such as *Clostridium*, predominate in cadavers with longer PMIs.

Competing interests

The authors declare that they have no competing interests.

Author contributions

GJ collected the cadaver samples; GJ, IC, and PAN developed the experimental design; IC and PAN extracted and sequenced the DNA; AP and PAN developed and tested C++ software programs used for analyzing the DNA sequences; IC and PAN carried out the statistical analysis; and all authors participated in writing the manuscript.

Acknowledgments

We would like to thank Medical Examiner, Stephen Boudreau for all of his support and providing us with cadaver samples. We thank Colby Hunter for proof reading the manuscript and the two anonymous reviewers for their insight. This work was supported by institutional funds of the Forensic Science Program at Alabama State University and by National Science Foundation (NSF) grant DBI 10388671.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2014.07.026.

References

Alan, G., Sarah, J.P., 2012. Microbes as forensic indicators. Trop. Biomed. 29, 311–330.
Amann, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59, 143–169.
Benbow, M.E., Lewis, A.J., Tomberlin, J.K., Pechal, J.L., 2013. Seasonal necrophagous insect community assembly during vertebrate carrion decomposition. J. Med. Entomol. 50, 440–450.
Dickson, G.C., Poulter, R.T., Maas, E.W., Probert, P.K., Kieser, J.A., 2010. Marine bacterial succession as a potential indicator of postmortem submersion interval. Forensic Sci. Int. 209, 1–10. http://dx.doi.org/10.1016/j.forsciint.2010.10.016.
Fredetter, J.W., 1916. Bacteremias in the agonal period. J. Lab. Clin. Med. 2, 180–188.
Gevers, W., 1975. Biochemical aspects of cell death. J. Forensic Sci. 6, 25–29.
Gill, C.O., Penney, N., Nottingham, P.M., 1976. Effect of delayed evisceration on the microbial quality of meat. Appl. Environ. Microbiol. 31, 465–468.
Gradwohl, R.B.H., 1904. Importance de l'examen bacteriolique pratique sur les cadavers. Ann. Inst. Pasteur (Paris) 18, 767–773.
Hyde, E.R., Haarmann, D.P., Lynne, A.M., Bucheli, S.R., Petrosino, J.F., 2013. The living dead: bacterial community structure of a cadaver at the onset and end of the bloat stage of decomposition. PLoS ONE 8, e77733. http://dx.doi.org/10.1371/journal.pone.0077733.
Janaway, R., Percival, S., Wilson, A., 2009. Decomposition of human remains. In: Percival, S. (Ed.), Microbiology and Aging. Humana Press, pp. 313–334.
Kakizaki, E., Ogura, Y., Kozawa, S., Nishida, S., Uchiyama, T., Hayashi, T., Yukawa, N., 2012. Detection of diverse aquatic microbes in blood and organs of drowning victims: first metagenomic approach using high-throughput 454-pyrosequencing. Forensic Sci. Int. 220, 135–146. http://dx.doi.org/10.1016/j.forsciint.2012.02.010.
Kuske, C.R., Banton, K.L., Adorada, D.L., Stark, P.C., Hill, K.K., Jackson, P.J., 1998. Small-scale DNA sample preparation method for field PCR detection of microbial sells and spores in soil. Appl. Environ. Microbiol. 64, 2463–2472.
Metcalf, J.L., Wegener Parfrey, L., Gonzalez, A., Lauber, C.L., Knights, D., Ackermann, G., Humphrey, G.C., Gebert, M.J., Van Treuren, W., Berg-Lyons, D., Keepers, K., Guo, Y., Bullard, J., Fierer, N., Carter, D.O., Knight, R., 2013. A microbial clock provides an accurate estimate of the postmortem interval in a mouse model system. Elife 2, e01104. http://dx.doi.org/10.7554/eLife.01104.
Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E.M., Kubal, M., Paczian, T., Rodriguez, A., Stevens, R., Wilke, A., Wilkening, J., Edwards, R.A., 2008. The metagenomics RAST server — a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinforma. 9, 386.
Morris, J.A., Harrison, L.M., Partridge, S.M., 2006. Postmortem bacteriology: a re-evaluation. J. Clin. Pathol. 59, 1–9.
NIH HMP Working Group, Peterson, J., Garges, S., Giovanni, M., McInnes, P., Wang, L., Schloss, J.A., Bonazzi, V., McEwen, J.E., Wetterstrand, K.A., Deal, C., Baker, C.C., Di Francesco, V., Howcroft, T.K., Karp, R.W., Lunsford, R.D., Wellington, C.R., Belachew, T., Wright, M., Giblin, C., David, H., Mills, M., Salomon, R., Mullins, C., Akolkar, B., Begg, L., Davis, C., Grandison, L., Humble, M., Khalsa, J., Little, A.R., Peavy, H., Pontzer, C., Portnoy, M., Sayre, M.H., Starke-Reed, P., Zakhari, S., Read, J., Watson, B., Guyer, M., 2009. The NIH Human Microbiome Project. Genome Res. 19, 2317–2323. http://dx.doi.org/10.1101/gr.096651.109.
Paczkowski, S., Schütz, S., 2011. Post-mortem volatiles of vertebrate tissue. Appl. Microbiol. Biotechnol. 91, 917–935. http://dx.doi.org/10.1007/s00253-011-3417-x.
Pechal, J.L., Crippen, T.L., Benbow, M.E., Tarone, A.M., Dowd, S., Tomberlin, J.K., 2013. The potential use of bacterial community succession in forensics as described by high throughput metagenomic sequencing. Int. J. Legal Med. http://dx.doi.org/10.1007/s00414-013-0872-1 (PMID: 23749255).
Peng, X., Yu, K.Q., Deng, G.H., Jiang, Y.X., Wang, Y., Zhang, G.X., Zhou, H.W., 2013. Comparison of direct boiling method with commercial kits for extracting fecal microbiome DNA by Illumina sequencing of 16S rRNA tags. J. Microbiol. Methods 95, 455–462. http://dx.doi.org/10.1016/j.mimet.2013.07.015.
Pessione, E., 2012. Lactic acid bacteria contribution to gut microbiota complexity: lights and shadows. Front. Cell. Infect. Microbiol. 2, 86. http://dx.doi.org/10.3389/fcimb.2012.00086.
Proskuryakov, S.Y., Konoplyannikov, A.G., Gabai, V.L., 2003. Necrosis: a specific form of programmed cell death? Exp. Cell Res. 283, 1–16.
Riedel, S., 2014. The value of postmortem microbiology cultures. J. Clin. Microbiol. 52, 1028–1033. http://dx.doi.org/10.1128/JCM.03102-13.
Staley, J.T., Konopka, A., 1985. Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annu. Rev. Microbiol. 39, 321–346.
Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., Sogin, M.L., Jones, W.J., Roe, B.A., Affourtit, J.P., Egholm, M., Henrissat, B., Heath, A.C., Knight, R., Gordon, J.I., 2009. A core gut microbiome in obese and lean twins. Nature 457, 480–484. http://dx.doi.org/10.1038/nature07540.
Urakawa, H., Martens-Habbena, W., Stahl, D.A., 2010. High abundance of ammonia-oxidizing Archaea in coastal waters, determined using a modified DNA extraction method. Appl. Environ. Microbiol. 76, 2129–2135.
Vass, A., 2001. Beyond the grave — understanding human decomposition. Microbiol. Today 28, 190–192.
Vass, A.A., Barshick, S.A., Sega, G., Caton, J., Skeen, J.T., Love, J.C., Synstelien, J.A., 2002. Decomposition chemistry of human remains: a new methodology for determining the postmortem interval. J. Forensic Sci. 47, 542–553.
Wan, M., Rosenberg, J.N., Faruq, J., Betenbaugh, M.J., Xia, J., 2011. An improved colony PCR procedure for genetic screening of *Chlorella* and related microalgae. Biotechnol. Lett. 33, 1615–1619. http://dx.doi.org/10.1007/s10529-011-0596-6.
Wu, G.D., Lewis, J.D., Hoffmann, C., Chen, Y.Y., Knight, R., Bittinger, K., Hwang, J., Chen, J., Berkowsky, R., Nessel, L., Li, H., Bushman, F.D., 2010. Sampling and pyrosequencing methods for characterizing bacterial communities in the human gut using 16S sequence tags. BMC Microbiol. 10, 206. http://dx.doi.org/10.1186/1471-2180-10-206.