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Fluorescently labeled bacteria provide insight on post-mortem microbial transmigration

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ABSTRACT

Microbially mediated mechanisms of human decomposition begin immediately after death, and are a driving force for the conversion of a once living organism to a resource of energy and nutrients. Little is known about post-mortem microbiology in cadavers, particularly the community structure of microflora residing within the cadaver and the dynamics of these communities during decomposition. Recent work suggests these bacterial communities undergo taxa turnover and shifts in community composition throughout the post-mortem interval. In this paper we describe how the microbiome of a living host changes and transmigrates within the body after death thus linking the microbiome of a living individual to post-mortem microbiome changes. These differences in the human post-mortem from the ante-mortem microbiome have demonstrated promise as evidence in death investigations. We investigated the post-mortem structure and function dynamics of *Staphylococcus aureus* and *Clostridium perfringens* after intranasal inoculation in the animal model *Mus musculus* L. (mouse) to identify how transmigration of bacterial species can potentially aid in post-mortem interval estimations. *S. aureus* was tracked using *in vivo* and *in vitro* imaging to determine colonization routes associated with different physiological events of host decomposition, while *C. perfringens* was tracked using culture-based techniques. Samples were collected at discrete time intervals associated with various physiological events and host decomposition beginning at 1 h and ending at 60 days post-mortem. Results suggest that *S. aureus* reaches its highest concentration at 5–7 days post-mortem then begins to rapidly decrease and is undetectable by culture on day 30. The ability to track these organisms as they move in to once considered sterile space may be useful for sampling during autopsy to aid in determining post-mortem interval range estimations, cause of death, and origins associated with the geographic location of human remains during death investigations.

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1. Introduction

Identification of a biological indicator that is consistently identified on cadavers and exhibits similar post-mortem patterns regardless of the death circumstances could be crucial in narrowing a post-mortem interval (PMI) range estimate. Only a few studies have focused on commensal microbial communities (*i.e.*, located internally and externally of a living person) for determining dynamics, such as microbial transmigration, under carefully controlled conditions following host death [1–4]. Here we present a controlled study used to determine the bacterial movement of intentionally infected model organisms from a specific anatomical location during the progression of host decomposition. We co-infected mice nasally with *Clostridium perfringens* and a *Staphylococcus aureus* strain with constitutively expressed fluorescent protein. *S. aureus* is a facultative anaerobe of the phylum Firmicutes, and is a natural commensal of the nares [5]. *C. perfringens* is a strict anaerobe and normal commensal of the gastrointestinal tract [6,7]. Both of these bacterial species were previously found associated with the decomposition of human surrogates [3,8,9]. We tracked these bacteria using *in vivo* and *in vitro* imaging and/or culture in order to determine colonization routes associated with bacterial oxygen requirements, anatomical location, and different physiological events of host decomposition. Additionally, a subset of mice was immediately surface sterilized following sacrifice and compared to non-surface sterilized in order to determine the influence of external microbiota on colonization.

2. Materials and methods

2.1. Construction of S. aureus KUB7

To construct a *S. aureus* strain with constitutively expressed fluorescent protein, the gene encoding a codon-optimized DsRed.T3 (DNT) was amplified by PCR from pRFP-F (Table 1) [10] using primers JBKU13 and JBKU14. This construct also contains an optimized ribosome binding site for enhanced expression. The resulting PCR product was cloned downstream of the constitutively expressed P_{sarAP1} promoter in pCM29 [11] using EcoRI and KpnI, replacing the *gfp* gene in this plasmid. The resulting plasmid, pJB1005, was digested with PstI and EcoRI and the fragment containing the P_{sarAP1}::*dsRed* construct was ligated into the same sites of pJC1112 [12], resulting in pJB1008. Next, pJB1008 was transformed into strain DC10B to facilitate electroporation. pJB1008 was isolated from DC10B and used for electroporation of AH1263 containing the integration helper plasmid pRN7023, leading to integration of pJB1008 into the SaPI1 site. Integration was confirmed using primers JCO717 and JBKU13. To ensure loss of pRN7023, the integrated pJB1008 was transferred into AH1263 using Φ11-mediated phage transduction as previously described [13]. Bacterial strains and plasmids used for this study are listed in Table 2.

2.2. Molecular genetic techniques

All manipulations were performed in *Escherichia coli* DH5α. *E. coli* was grown in LB media supplemented with ampicillin (100 μg ml^{-1}) as necessary. *S. aureus* was grown in TSB with erythromycin (5 μg ml^{-1}) or chloramphenicol (10 μg ml^{-1}) as needed for selection. Solid media contained 1.5% agar. Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). High-fidelity PCR for cloning purposes was performed using KOD DNA Polymerase (Novagen, Madison, WI) while chromosome integration confirmation utilized Midas Master Mix (Monserate Biotechnology Group, San Diego, CA). PCR was performed on an Applied Biosystems GeneAmp 9700 (Life Technologies) and products were purified using the DNA Clean and Concentrator-5 kit (Zymo Research, Orange, CA). Plasmids were purified with the Wizard Plus SV Minipreps DNA Purification System (Promega Corporation, Madison, WI). Restriction endonucleases and T4 DNA Ligase were purchased from New England Biolabs (Beverly, MA). All PCR products were sequenced by ACGT, Inc (Wheeling, IL) to ensure there were no unintended changes and sequencing data was analyzed using Vector NTI (ThermoFisher Scientific).

Table 1
Primers used in this study.

Name	Sequence (5′ → 3′)[a]	Reference
JBKU13	*ccggtacc*TGATTAACTTTATAAGGAGGAAAAACATATGG	This study
JBKU14	*ccgaatt*CATCTGTGGTATGGCGCTAG	This study
JCO717	GTGCTTCACCAGCACCACATGCTG	[12]

[a] Lower-case italicized bases are non-homologous nucleotides added for cloning purposes.

Table 2
Bacterial strains and plasmids used in this study and associated references.

	Characteristics[a, b]	Reference
Bacteria		
E. coli DC10B	Δ*hsdRMS* Δ*dcm*	[14]
S. aureus AH1263	CA-MRSA USA300 strain LAC without LAC-p03	[15]
S. aureus KUB7	AH1263 with pJB1008 in SaPI1 site	This study
Plasmid		
pCM29	Amp^r Chl^r Source of P_{sarAP1}	[11]
pJB1005	P_{sarAP1}::*dsRed* in pCM29	This study
pJB1008	P_{sarAP1}::*dsRed* in pJC1112	This study
pJC1112	Ery^r SaPI1 integration plasmid	[12]
pRFP-F	Source of *dsRed*	[10]
pRN7023	Chl^r Integration helper plasmid	[12]

[a] dsRed encodes DsRed.T3(DNT).
[b] Ery^r and Chl^r denote erythromycin and chloramphenicol resistance for *S. aureus*, respectively. Amp^r identifies ampicillin resistance in *E. coli*.

2.2.1. C. perfringens preparation

C. perfringens type A strain WAL 14572 was obtained from ATCC, and maintained anaerobically on reinforced clostridial medium (BD) agar at 37 °C prior to infection. *C. perfringens* was used as a negative control for aerobic nasal inoculation as this species is a strict anaerobe.

2.2.2. Inoculum preparation

S. aureus KUB7 was grown in tryptic soy broth (TSB) to an optical density (OD_{600}) of 0.5–0.55 while *C. perfringens* was grown in 100 mL of reinforced clostridial medium (RCM) broth to an OD_{600} of 0.3–0.35. One milliliter of each culture was aliquoted into a total of 64 tubes containing 1 mL of respective bacterial suspension, then centrifuged at 12,000 × *g* for 10 min to pellet the cells. Each inoculum was individually plated, then *S. aureus* KUB7 was resuspended in 7 μl of TSB and added to *C. perfringens* resuspended in 7 μl of RCM broth containing 60 g/L of sucrose. The final inoculum was 2.8×10^8 CFU/mL and 2.24×10^7 CFU/mL for *S. aureus* KUB7 and *C. perfringens*, respectively.

2.2.3. Animals

Ninety healthy female, SKH-1 Elite female mice, weighing 16–22 g and of 9–12 weeks of age, were obtained from Charles River Laboratories and included in experiments of nares infection studies. SKH-1 mice are hairless, euthymic and immunocompetent. Mice were housed at a constant room temperature (25 °C) with a natural day/night light cycle (12L:12D) in a conventional animal colony at the Mississippi State University Veterinary