HUMAN REMAINS & VIOLENCE

Microbial ecogenomics and methods for investigating clandestine gravesites

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Abstract

In the mid-1990s, the crime scene toolkit was revolutionised by the introduction of DNA-based analyses such as the polymerase chain reaction, low copy number DNA analysis, short-tandem repeat typing, pulse-field gel electrophoresis and variable number tandem repeat. Since then, methodological advances in other disciplines, especially molecular microbial ecology, can now be adapted for cutting-edge applications in forensic contexts. Despite several studies and discussions, there is, however, currently very little evidence of these techniques' adoption at the contemporary crime scene. Consequently, this article discusses some of the popular 'omics' and their current and potential exploitations in the 'forensic ecogenomics' of body decomposition in a crime scene. Thus, together with published supportive findings and discourse, knowledge gaps are identified. These then justify the need for more comprehensive, directed, concerted and global research towards state-of-the-art microecophysiology method application and/or adaptation for subsequent successful exploitations in this additional context of microbial forensics.

Key words: forensic science; crime scene; molecular microbial ecology, ecogenomics

Introduction

The detection of clandestine gravesites and areas of surface body deposition is of vital importance in a range of forensic contexts, but particularly in the investigation of acts of mass violence. Generally this can be achieved through the use of eye witness statements, aerial photography and field walking. However, there are occasions when graves are reopened and the remains moved elsewhere or, indeed, dug and never used. Determining whether or not a grave has been used in these situations can be difficult. Traditional archaeological methods that focus directly on the grave[1] and the presence of small human remains or associated personal effects

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may be helpful.[2] These approaches are, however, dependent on meticulous site excavation, which may not always be achievable. An alternative may be to examine the surrounding soil. Work has shown that decomposition alters indigenous soil microbial community profiles[3] and there is, therefore, potential to use these detectable changes to determine grave locations and use. Although a very technical approach to the investigation of events of mass violence has been cautioned, advanced scientific techniques do have a role to play if deployed sensitively.[4]

The forensic sciences have an ability to co-opt methods and procedures from other disciplines to enhance expertise at a crime scene with forensic evidence. It is well recognised that molecular analyses have revolutionised this discipline as a whole, from collecting evidence and intelligence on site to informing court decisions. Particularly well-publicised applications focussed on body tissues and fluids and human identification and verification.[5] Although only currently offering potential, there is now a developing appreciation that the directed scrutiny of microbial communities can contribute to the study and identification of people and bodies.[6] Within the forensic sciences, there is great debate surrounding the boundaries of the term 'microbial forensics'.[7] However, since our interest focusses on the gravesite and not the individual *per se*, we propose a more encompassing 'forensic ecogenomics' for the application of molecular microbial ecology techniques at the interface of (environmental) forensics, microbiology and archaeology, and thus expand on initial applications of microbiological analyses of soil.[8]

This perspective thus seeks to highlight 'forensic ecogenomics' as another aspect of the 'microbial forensics' category with current applications and the potential exploitation of popular and novel microbial ecology techniques in contemporary crime scene investigations. The ultimate proposal is to make stronger links between soil and aquatic microbial profiles and clandestine burial sites, in particular. Suggestions for additional methodological adoption are also made to ensure that microbial forensics, as an exciting new field of study and investigative discipline, is fit-for-purpose and applicable to wider but relevant contexts.

Specific attention is therefore given to decomposition-related microbioforensic studies published between 2000 and 2014. The application of ecogenomic protocols has then focussed on those with the highest potential for immediate extended applications in real-life scenarios (Table 1). The microecophysiology tools considered include denaturing gradient gel electrophoresis (DGGE), fatty acid methyl ester analysis (FAME), length heterogeneity PCR (LH-PCR), phospholipid fatty acid analysis (PLFA), polymerase chain reaction (PCR), terminal restriction fragment length polymorphisms (t-RFLP), next generation sequencing and microarrays/genechips as used, or for potential application, in measuring ecosystem response to burial and subsequent decomposition.

Burial and decomposition in soil

Soil is recognised in microbial ecology as a complex and heterogeneous environment that supports a vast number of phylogenetically, phenotypically and functionally diverse microbial populations. While the scientific discipline is changing,

until recently soil was often considered in forensic science as essentially a burial medium where human and animal remains decompose. Typically, decomposition processes are mediated by changing indigenous microbial communities, especially bacteria, which are most abundant.[9] According to Carter *et al.*, the burial and subsequent decomposition of cadavers in a grave was often determined to be ecologically localised, releasing organic resources that were spatially and temporally finite. As a result, studies of soil in the burial context tended to focus on the localised influence of temperature or pH on the rate of decomposition.[10] Nevertheless, several researchers reported that chemical, biological and physical changes occur in the burial site in response to the corpse and its resultant decay.[11] These processes are mutual and each impacts on the other. Typically, high temperatures increase the rate of decomposition and, in turn, soil temperatures increase with advanced decay.[12] Fluctuations in pH, sometimes in relation to oxic or anoxic conditions, have been recorded where the production of ammonia and volatile fatty acids led to increases and decreases in soil pH, respectively.

Expectedly, these interacting processes will both lead to changes in, and be partly dependent on, the local microbial communities. Complex scenarios arise, therefore, due to the number of soil types, the way in which the unique and individual properties influence and/or are affected by the added remains, the indigenous microbial communities, local climatic conditions and nutrient availability. As a result, the decompositional effects of these variables and different environmental parameters, such as moisture content, pH, temperature and anion and cation concentrations have been explored in different soils. Thus, Hopkins *et al.* assessed variables in 430-day grave soils of four- to five-month old pigs, which were between 5 and 20 m from the grave of a murder victim. The researchers recorded increases in several parameters including microbial biomass C, microbial respiration, nitrogen mineralisation and pH in comparison to the controls (1 m away from each grave).[13] Haslam and Tibbett made a laboratory-based study of the effects of lamb (*Ovis aries*) skeletal muscle tissue decomposition in three soils with different pH values – a Podzol (pH 4.6), Cambisol Brown Earth (pH 6.4) and Rendzina (pH 7.8) – and found that decomposition and substrate-induced respiration increased in the acidic Podzol in combination with a slightly higher CO2 evolution while the alkaline Rendzina recorded the highest microbial biomass.[14] The effect of moisture on decomposition in Brown Sodosol (loamy sand), Rudosol (sandy) and Grey Vertosol (medium clay) was tested by Carter *et al.* where higher decomposition rates resulted from increased wetness for the two sandy soils.[15]

Overall, the original soil characteristics and the early-phase decomposition by-products select for specific microbial communities and the size of the impacted area. These affect the rate of mid- to late-phase decomposition, subsequent by-products and, in turn, microbial respiration rates and biomass (population size) that show soil-specific responses. For example, Parkinson *et al.* applied PLFA and t-RFLP analyses of bacterial and fungal profiles to human burial soils and reported distinct changes in fungal populations between the early and late phases of cadaver decomposition. These clear delineations were attributed to changes in the amounts and types of substrates that were available as a direct result of decomposition. An

increase in nutrients will thus be accompanied by an abundance of the r-strategist while a decrease in labile substrates will result in higher numbers of the typically slow-growing k-strategists. The authors also suggested that sequential changes in community profiles could be linked, potentially, to post-mortem interval (PMI) estimation as with insect colonisation in forensic entomology.[16]

Length heterogeneity PCR was applied by Moreno *et al.* to study the response of soil microbial communities to cadaver decomposition, particularly in shallow clandestine graves during a sixteen-week period across nine different sites. Together with changes in community compositions of specific functional microbial clades/genes such as nitrogen fixers and the *nifH* gene, the occurrences of different intestinal, oral and skin commensal microbiota were recorded. These were attributed to unique microbiome fingerprints specific to each of the bodies. As a result, the authors proposed that LH-PCR could be another reliable body identification technique in criminal or missing person cases.[17]

Apart from bacteria, other microorganisms such as fungi, and their recognised diversity, can be exploited in a field termed 'forensic mycology'.[18] A comprehensive review by Hawksworth and Wiltshire considered the use of fungi as potential targets for diverse applications including the estimation of time since death, burial location, time of deposition, biological warfare investigations and the enforcement of legislation such as the Drugs Act 2005.[19] Some researchers also recorded the consistent occurrence of fruiting structures in specific fungi, such as *Penicillium* sp., *Aspergillus terreus* and *Eurotium* spp. in response to mammalian/human decomposition.[20] The studies were made *in situ* in clandestine graves and/or with real site materials from different environments including forests and a domestic garden. In particular, Ishii *et al.* reported the first study where taxonomic determinations of the appearance of fungal strains on a decomposing cadaver were made to suggest the PMI.[21] Other strains, such as *Trichophyton mentagrophytes*, were then used *in vitro* on the hair of adult corpses to investigate the perforation test in the gaseous post-mortem period to establish time since death.[22]

Despite these demonstrable achievements, there are several issues to resolve including the dwindling number of specialist mycologists. Also, the numbers (and types) of fungi in a single location are vast with comprehensive inventories impractical and unattainable (particular spores and fruiting bodies of some species are produced infrequently), and appropriate and extensive experimentation on the patterns and rates of growth of species in relation to the post-mortem interval is required.[23] Therefore, robust fungal analysis, dependent particularly on well-established microbial ecology techniques rather than conventional taxonomic methods alone, would enhance the evident and potential value of forensic mycology. Anderson and Cairney present an opportune and succinct review of the molecular techniques used to study fungal diversity and ecology in soils with total/direct 18S rRNA gene or 18S – 23S rRNA gene internal transcribed spacer (ITS) as the principal targets.[24] Furthermore, Damon *et al.* investigated an alternative fungal molecular marker, the mitochondrial cytochrome *c* oxidase 1 (*COX1*) encoding gene, for environmental RNA-based analyses of metabolically active *Agaricomycetes* and Pezizomycotina communities.[25] Although the discourse by

Anderson and Cairney and Damon *et al.* was not intended for the forensic ecogenomic context, we propose that the strengths, limitations and novel approaches highlighted by these researchers are also transferable to, and must be explored for, the advancement and validation of forensic mycology.[26]

Generally, the reports, exemplified above, acknowledge the potential applicability and relevance of the methodologies that are currently adopted in soil-based forensic investigations. They also emphasise the need for additional, robust and sensitive microbial assessments of soils around burial environments and human decomposition to explore local dynamics and functions in more detail and, thus, extend taphonomic research. Also, identification of (core) microbial taxa linked to decomposition 'history' would, potentially, provide stronger or more accurate determinations of PMI.[27] Previous studies have already established that high-throughput sequencing techniques could play a key role in rapidly increasing the necessary comparative libraries.[28] This comprehensive approach would directly address the observation by Tomberlin *et al.* of the need to streamline 'research in decomposition ecology, which promotes quantitative approaches to collecting and applying data to forensic investigations involving decomposing human remains'.[29]

Aquatic environments

Despite the occurrence of human remains in bodies of water, little research has been conducted with a detailed analysis of taphonomic changes due to aquatic submersion.[30] Nevertheless, some studies have explored the potential roles of microbial communities in aquatic ecosystems for their effective and reliable application in human body crime scene investigations. For example, Kakizaki *et al.* used blood from twenty-two cadavers recovered from or near fresh or saline water and normal blood to model blood samples of victims found twenty-four hours after drowning. Culture-dependent and molecular homologous analyses of the 16S rRNA gene were used to determine the occurrence of marine bacteria in blood samples and, potentially, establish a protocol for victims of drowning. The researchers recorded distinct microbial profile differences of cadavers drowned in seawater compared with those drowned in fresh water (river, bathtub) and deposited on dry land. Specifically, homologous 16S rRNA gene analysis of bioluminescent and/or blue colonies on 4% (w/v) NaCl-supplemented TH agar indicated a predominance of marine strains of the *Photobacterium*, *Vibrio*, *Shewanella* and *Psychrobacter* genera in blood of cadavers drowned in seawater. In contrast, non-marine strains, *Aeromonas*, *Vagococcus*, *Staphylococcus* and *Pseudomonas* spp were recovered from cadavers from a river, bathtub and dry land.[31]

Although more traditional diatom analyses can be cumbersome[32], a combination of bacterioplankton cultivation and marine or freshwater diatom counts was used by Kakizaki *et al.* to confirm drowning as the cause of death for a female body that had washed up on a beach after a typhoon. Despite extensive decomposition, the combined analysis of water samples from different organs (such as kidneys, liver and both lungs, including and excluding the pleura) provided conclusive evidence of drowning in fresh or brackish water with low salinity.[33]

In vitro studies have also been used to address knowledge gaps on the potential use of specific aquatic microbiological indicators for drowning and differentiation between incidences in waters of different salinities.[34] These initiatives were complemented further by other research groups such as Dickson *et al.* who applied molecular-based PCR and sequencing of the 16S rRNA gene to determine links between microbial invasion, body decomposition in marine ecosystems and post-mortem submersion interval (PMSI). Analysis of completely submerged pig (*Sus scrofa* L.) carcasses showed a sequential occurrence of specific microbial communities with distinctive diversities and compositions relative to specific PMSIs. Hence, although a predominance of Gammaproteobacteria was generally recorded, novel Bacteroidales genera showed distinct season- and submersion-specific colonisation patterns. Despite these, the authors highlighted deliberations by other workers that microbial composition trends, which then provide PMSI estimates, are dependent on several parameters including temperature, water depth, current, salinity, access to water surface (whether the body remains afloat or sinks), nature of the underlying substrate, water chemistry, presence of scavengers and clothing and trauma. Consequently, the post-mortem submersion interval determinations could probably be site-specific.[35]

These studies highlight that local aquatic environment analysis could be complemented by culture- and molecular-based studies of the microbial communities found in or on victims to provide a substantive link to the crime scene, even if the remains have been moved and/or decomposed. Further detailed, more robust and conclusive research (particularly regarding species distribution) is required, however, before the informed adoption of this approach in the crime investigative toolkit.

Potential impacts of the human microbiome

The human body is host to thousands of different bacterial species (see NIH HMP Working Group *et al.* and Wilson and Kong for comprehensive reviews) and the topmost layer of skin, the epidermis, has local characteristics that result in unique microecosystems.[36] For example, the skin has a low pH, low water activity and secretions from sudoriferous (sweat) and sebacious (oil) glands, with the epidermis contributing dead keratinised epithelial cells. Overall, the inherent properties of each organ lead to differences in bacterial community composition from one area of the body to another. These differences even occur in physiological proximity and/or depths, hence Grice *et al.* observed that hand bacterial profiles are different to those of the forearm.[37]

Furthermore, several symbiotic relationships exist between the human body and a wide range of bacteria, and common examples include the intestinal and skin microbiota.[38] Also, personal lifestyles create distinct microenvironments that select for specific microbial communities or local microbial fingerprints that differ between individuals (Table 2). Thus Fierer *et al.* suggest that 'the collective genomes of our microbial symbionts may be more personally identifying than our own human genomes'.[39] Since these interactions are constant but unique for

each individual, they may afford valuable identification information for forensic investigations ante-mortem. A microbial community must, however, be relatively consistent over time and independent of transitory perturbations to constitute a unique 'microbial fingerprint'.[40] This concept has been tested on different parts of the body including hands, elbows and forearms[41] with the use of culture-based and 16S rRNA gene pyrosequencing analyses where the data recorded some core bacterial taxa together with distinct intra- and interpersonal bacterial associations. The distributions of different phylotypes were also dependent on other parameters including sex. Despite these findings, the occurrence of a microbial fingerprint for forensic applications remains the subject of considerable debate and experimental scrutiny. Its persistence post-mortem, particularly relative to different phases of decomposition, mandates comprehensive studies and is outside the scope of this article.

To date, several investigations of the application of microarrays to explore the human microbiome have been made and/or debated. For example, a review of community composition, dynamics and functional capacity of the human microbiota is presented by Paliy and Agans. The authors highlighted the application of metagenomics, metatranscriptomics, metaproteomics, metabolomics and metabonomics to understand the capacities of microbial communities to function and produce metabolites in response to specific interactions with the environment. Also, recent data comparing child gut microbiomes in response to long-term diets in industrialised (Europe, Amerindian) and emerging (Burkina Faso, Malawi) nations is explored.[42] Similarly, Bergström *et al.* reported on the gut low density array (GULDA), which they developed for rapid, quantitative, cost-effective and high throughput community dynamics analysis of typical and predominant core intestinal microbiome phyla including Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Verrucomicrobia. Despite the small sample size, the study data suggested a potential application of GULDA to assess the response of specific phylogenetic groups as a function of age, diet, functional food, antibiotics and health.[43] Therefore, although experimental analyses are limited or not possible due to ethical guidelines, it seems likely that different human microbiome-based assays that have been used ante-mortem could be applied at real crime scenes to investigate their potential applicability to determine the health, lifestyle, socioeconomic and cultural background of an unidentified victim or suspect.

Although studies on human cadavers are lacking, animal model-based studies have suggested that spatio-temporal shifts in necrobiome community structure and composition can be used, potentially, for PMI estimates.[44] Also, while comprehensive investigations are required, it seems plausible that person-specific microbiota would result in distinct post-mortem decomposition timelines. These would, in turn, lead to divergent effects on the dynamics of the surrounding environmental (burial) microbial communities. Thus post-mortem interval and time-since-burial calculations, as also based on forensic ecogenomic analyses, would be affected directly by the substrates and chemicals released by the decomposing body relative to several parameters such as its size, age, hygiene, diet and health (medication).

Future perspectives

Apart from monitoring the effects of decomposition on microbial community dynamics, other potential applications of forensic ecogenomics may be considered. Some examples include: (i) use of species-specific and hierarchical oligonucleotide primer extension determinations[45] to link environmental degradation to specific pollution sources ('environmental forensics')[46] such as human or animal type faecal matter in surface water; (ii) analysis of samples of the oral microbiome (bite marks and buccal swabs), lip prints and skin microbiota (fingerprints and skin cells) using microarrays or genechips; and (iii) the examination of stable isotope profiles of decomposition products.[47] Although presently theoretical, these innovative and high throughput tools have the potential to extend the forensic toolkit further. The typically rapid generation of large molecular and metabolomic data sets, coupled with robust and proven analysis and conventional intelligence gathering, could facilitate more comprehensive, efficient and expedient interpretation of the complete crime scene data set.

Conclusions

The introduction of DNA-based analysis revolutionised crime scene and forensic investigation protocols. As with any discipline, new challenges necessitate the development of novel approaches and/or the adoption of existing techniques from related fields. For different environmental biotechnologies, this process led to the increasing application of rapid, easy-to-use, high resolution, high throughput, robust and cutting-edge molecular microecophysiology techniques that typically result in data sets of high quantity and quality. Therefore, the aim of this article is to emphasise that the methods now common in exploring the molecular microbial ecology of soils, sediments and water have an exciting role to play in the future investigation of episodes of violence.

Although some key preliminary steps such as sample collection, storage and preparation, especially for representative and uncontaminated DNA/RNA/protein/fatty acid recovery remain critical (and yet could be problematic in remote areas), the advantages and limitations of established and novel ecogenomic tools need to be considered specifically for application in forensic archaeological contexts. Hence, as exemplified in Table 1, rigorous testing and verification of some of these tools prior to their adoption, particularly in the microbioforensics of decomposition, is underway.

Table 1: Current and potential applications of ecogenomic techniques in microbial forensics (adapted from Ralebitso-Senior *et al.*)[48]

Technique	Current and potential applications/observations	
DNA/RNA	Most crime scene science/forensic investigations rely on DNA-based protocols. RNA-based microbial forensics may be required especially for decomposition studies.	
D/TGGE‡	Reliability of DGGE (and other profiling techniques) data as part of a robust intelligence is potentially dependent on the establishment/adoption of specific protocols.[49]	
Q-PCR/RT-PCR‡	Traced sources of environmental degradation. Quantifies the expression of catabolic enzymes for specific substrates in decomposing materials.[50]	
LH-PCR	Identifies composition of specific functional microbial clades/genes for an individual to allow for body identification in criminal or missing person cases. [51]	
Homologous 16S rRNA gene analysis	Establishes the occurrence/predominance of different genera in victims drowned in saline and freshwater.[52]	
t-RFLP	Establishes changes in fugal community structure that could be linked to early and late phases of decomposition. May determine differences in human microbiota.[53]	
FISH-based e.g. MAR-FISH, STAR-FISH	Potential application especially in identifying zones affected by decomposition. Estimation of the size and/or weight of the original buried material.	‡
SIP	GC/MS-based analysis for lipids (FAME). Combination with DNA-/RNA-/protein-/amino acid-based tracking of microbial communities/enzymes in response to decomposition‡. [54]	
HOPE	Afford activity-specific comparisons between pristine and contaminated ecosystems (e.g. *Bacteroidales-Prevotella* distribution/predominance due to farming activities). Quantitative data may allow estimates of contaminant migration relative to source.[55]	
Metagenomic analysis	Applicable: (i) pre-, during and post-decomposition; (ii) above, within, below and away from decomposing material; and (iii) upstream, within and downstream of pollution point.	‡
Microarrays	Potential for bespoke platforms targeting common members of the human microbiota. Use existing arrays, e.g. GeoChip 2.0/3.0 and PhyloChip, in decomposition studies.	‡
Clone libraries	Highly applicable for defined microhabitats and human 'microbial fingerprinting'.[56]	

Sequencing	Identifies phylogenetic members/profiles indicative of and unique to decomposition history, defined 'microbial fingerprints' and tissue-specific human microbiota‡. Provides data to design primers/probes, e.g. for microarrays.[57]	
SDS-PAGE	Applicable for rapid analysis of protein-based response to decomposition.	‡
FAME	Lipid analysis of grave soil confirms disinterred grave location. Can be complemented with microbial community tracking to link profile dynamics as a result of decomposition.[58]	
PLFA	Robust evidence of microbial community structure changes in response to decomposition.[59]	
CLPP	Compares physiological/metabolic profiles between crime- and non-crime-scene samples.	‡

‡ designates techniques that have not yet applied in microbial forensics; FISH: Fluorescent *in situ* hybridization; MAR-FISH: microautoradiography-fluoresence *in situ* hybridization; STAR-FISH: substrate-tracking autoradiography-FISH; SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis; CLPP: Community-level physiological profiling

Table 2: Influences on microbial communities on the person

Lifestyle Variable	Details
Hand sanitation	Increased use of antibacterial hand gels and soaps reduces bacterial adherence and colonisation. They also target specific strains selecting the number and types of skin colonising species.[60]
Antibiotics / medication	These change the chemical composition of skin secretions and, thus, the resident microbial strains. Specifically, antibiotics are bactericidal to species around skin hair follicles.[61]
Smoking	The habit has several effects on the skin including a decrease in moisture making it more difficult for bacteria to find free water in an already dry environment.[62]
Age	Bodily functions change with age potentially affecting amounts/types of secretions hence the commensal microbial profiles. Medical devices (catheters or prostheses) will affect populations.[63]
Sex	Male and female bodies are characterised by different communities, e.g. male skin generally supports higher bacterial density while female skin has a lower density but higher diversity.[64]

Body piercing	Pierced areas are cleansed to reduce risk of infection, which affects the bacterial composition, while silver jewellery may influence bacterial composition due to antimicrobial properties.[65]
Geographical location	Microorganisms are often acquired from the surrounding environment and so it is possible to measure differences in species that are geographically region-specific.[66]
Occupation	The palm, in particular, is in constant contact with other surfaces and/or areas of the body, e.g. manual labourers' hands are mostly subject to harsh treatment and extensive wear and tear depending on the materials handled, hence reducing colonisation by transient bacteria.

Notes

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