



Research paper

Integrating the microbiome as a resource in the forensics toolkit



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ABSTRACT

The introduction of DNA fingerprinting to forensic science rapidly expanded the available evidence that could be garnered from a crime scene and used in court cases. Next generation sequencing technologies increased available genetic data that could be used as evidence by orders of magnitude, and as such, significant additional genetic information is now available for use in forensic science. This includes DNA from the bacteria that live in and on humans, known as the human microbiome. Next generation sequencing of the human microbiome demonstrates that its bacterial DNA can be used to uniquely identify an individual, provide information about their life and behavioral patterns, determine the body site where a sample came from, and estimate postmortem intervals. Bacterial samples from the environment and objects can also be leveraged to address similar questions about the individual(s) who interacted with them. However, the applications of this new field in forensic sciences raises concerns on current methods used in sample processing, including sample collection, storage, and the statistical power of published studies. These areas of human microbiome research need to be fully addressed before microbiome data can become a regularly incorporated evidence type and routine procedure of the forensic toolkit. Here, we summarize information on the current status of microbiome research as applies to the forensic field, the mathematical models used to make predictions, and the possible legal and practical difficulties that can limit the application of microbiomes in forensic science.

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1. Introduction

Advances in DNA analysis in the 1970s, such as Southern blotting [1] and restriction enzyme digests, gave forensic investigators a new tool for criminal investigations, with DNA-based evidence first introduced in a court in 1985 [2]. DNA forensics rapidly matured and the classic techniques, involving the use of minisatellites [3] or a variable number of tandem repeats (VNTRs) [4], became widely used by the 1990s. These inherently polymorphic DNA fragments provide information on an individual's specific DNA 'fingerprint', and can be used to identify suspects and then assist in their conviction through matching the fragment patterns. However, as with all restriction fragment length polymorphism (RFLP)-based methods, the requirement of high molecular weight DNA limited the type of sample that could be processed for forensic purposes [5]. Depending on the forensic question and quality and quantity of DNA available, current standard methods of DNA profiling use one or more of autosomal microsatellites or short tandem repeats (STRs), or lineage markers

including Y chromosome STRs or mitochondrial DNA (mtDNA) [6]. STRs are more precise in determining human identity, and thus were found to be ideally suited for forensic applications when sufficient DNA is available. In contrast, mtDNA is found in much higher copy number than nuclear DNA and can be used in the analysis of heavily degraded DNA when there is insufficient DNA to examine STRs. It is also possible to use mtDNA to determine maternal lineage.

Next generation sequencing (NGS) technologies have exponentially increased the amount of sequencing data that is available for forensic analysis while in parallel significantly reducing the costs associated with the generation of sequencing data [7]. This increase in available sequencing data has expanded the body of useful DNA-based evidence usable for forensic investigation [8]. The human genome, given the enormous amount of discriminatory information contained within, has the potential to be more completely utilized by the forensic community, and integrated approaches analyzing both nuclear and mtDNA can be pursued. Data that is generated from other fields of human genome science such as epigenetics, gene expression data and miRNAs, can now be used to address numerous forensics questions [9].

One area of human biology that holds significant promise for forensic applications is the study of the microbiome (including

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both human and environmental). This exciting new area of research describes the microbial DNA from the microbiome, the collection of microorganisms (predominantly bacteria, with fungi, viruses and microeukarya) that colonize surfaces, including both externally and internally on the human body. This field initially focused on small subunit (SSU) ribosomal (r) sequences, including cloning of the 16S rRNA and fingerprinting methods (DGGE, T-RFLP, ARISA), to uncover the vast richness and diversity of the human and environmental microbiome [10,11]. However, with the reduced costs and increased capabilities of NGS, this field has expanded significantly over the last 10 years enabling researchers to profile hundreds to thousands of DNA samples simultaneously with increased diversity coverage and phylogenetic resolution. NGS has offered new avenues to address critical issues of potential interest to the forensic community. For instance, studies have shown that the human microbiome does not consist of a single species spread over multiple body sites. Instead, distinct bacterial populations exist at different locations in and on the body [12]. We also know that a person's lifestyle can be a major driver of the composition of the microbiome communities across many body sites, including what they eat [13], with whom they live [14], whether they have pets [15], where they are from [16], if they smoke [17,18], their health status [19] and whom they kiss [20].

Humans are not the only potential reservoirs of human-derived microbial species of significance for forensic science. Environmental samples contain human microbial signatures. Research has shown that humans can change the bacterial composition in the environment through direct interaction, including changing the components of the air [15,21,22]. Human microbial signatures can be recovered in several enclosed classrooms [23,24], houses [14], dorm rooms [25], bathrooms [26], and offices [23,27], and even used to predict information about a person interacting with the environment, such as the sex of the inhabitant of dorm rooms [25]. While the signature is strongest immediately following the interactions, it remains detectable for up to several months later. Microbiomes in enclosed built environments are more likely to reflect human microbial signatures compared to natural and open environments [28]. The exchange between environmental and personal microbiomes is also seen on a micro-level, especially for the terms that they are in close contact with their users. Microbiomes harvested from fabrics [29], shoes [30], keyboards [31], and cell phones [30] have all shown that they partially share their user's microbial signatures. The changes are not entirely unidirectional, as the environmental bacteria can also alter the microbiomes of its inhabitants, as was seen between individuals

that live together with cohabitating spouses, children and pets all sharing microbial signatures [14], though bacterial viruses also can lead to increased taxa similarity across families [32].

The ability to capture and leverage these differences in the human and environmental microbiome presents exciting new possibilities for forensic science, including the possibility of linking specific human subjects to a crime scene [8]. However, several questions remain to be addressed before these microbial biosignatures can become routine and highly effective in forensic science. The differences between bacterial communities on the body and in the environment require consideration as to what these communities can reliably address and what statistical power is needed to rely on the microbiome-derived data. Additionally, NGS can generate enough personal information to alter the current legal framework of data collection and application. This review summarizes the current state of human microbiome research in the forensic field, and addresses the possibilities and the limitations, while proposing approaches that can be applied to overcoming some of the potential issues, as visualized in Fig. 1.

2. Human microbiome sequencing techniques

The human microbiome is potentially a very powerful forensics tool given the variety of surfaces that can be used to obtain a sample and the diversity of information available to researchers from these samples. The first large-scale comprehensive survey of the human microbiome, the Human Microbiome Project (HMP), recruited approximately 300 people at two locations in the United States, and sampled 15–18 body sites that represented skin, urogenital tract, oral cavity and gut. The results from this study, which included analysis of both 16S rRNA gene and metagenomic data, indicated that the microbial diversity on an individual body site was more similar to the same body site on a different person than to other body sites on the same person [12]. Later studies also demonstrated that microbiome composition (taxonomic arrangements) vary across time as well [33], with gut and saliva being more consistent in their bacterial compositions than skin. Microbiomes from other body sites, including those of interest to the forensic community like scalp and pubic hair, have begun to be examined for use in forensics [34], but they remain understudied compared to the body sites sampled in the HMP.

Human microbiome diversity can be characterized in several ways. The most common technique is to examine the taxonomic distribution of the species in the sample via targeted sequencing of the variable regions of the 16S rRNA gene. Several bioinformatic

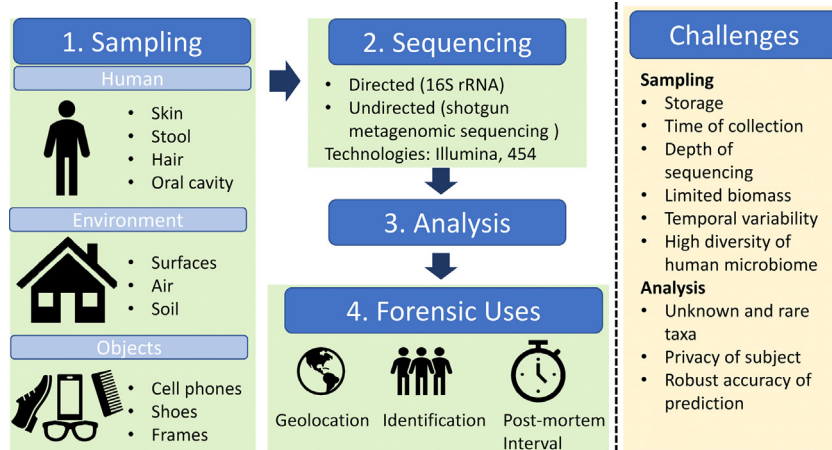


Fig. 1. Current Microbiome and Challenges. A simplified schema summarizing the microbiome analysis pathway including: collecting samples from various reservoirs of microbiomes (Sampling); the different sequencing technologies available to quantify the microbiomes (Sequencing); and possible question that developing analysis can address (Forensic Uses). The current limitations and challenges of these uses are also briefly listed (Challenges). Each topics is addressed in more detail in the text.

suites are available for assembling similar sequences (traditionally at 97% 16S rRNA sequence similarities) into clusters, called Operational Taxonomic Units or OTUs [35–37]. Although it is still an imperfect taxonomic classification scheme, these OTUs correspond, theoretically, to classifying microbial taxa at the species level [38]. Through comparisons to known bacterial species, the programs match OTUs to previously sequenced bacterial taxa and quantify the distribution of different taxa in a given sample. While these suites differ in the exact programs used and assumptions made, they all report similar outcomes [39]. However, the use of targeted 16S rRNA with short amplicon sequences is limited by the impossibility of getting confident resolution at the species level, a limitation not necessarily mitigated by full length 16S rRNA sequences [40].

As sequencing technologies continue to improve and sequencing costs reduce, whole genome shotgun (WGS) methods have begun to be used to evaluate the human microbiome instead of the targeted 16S sequencing. WGS non-specifically targets all the gene content in a given microbial ecosystem and is capable of differentiating microbial species and taxa to a greater extent than 16S rRNA amplicons [41,42]. However, the capacity of WGS of microbiomes to aid in forensic investigations by connecting objects and environments to individuals has been poorly investigated [43], and although suggesting significant potential, it is limited as a forensic tool at this time. The 16S rRNA sequences and their associated metadata are the most commonly published data, and thus more available to be combined into larger datasets, as in meta-studies done by [44,45]. The expanded data size increases the statistical power and therefore the effectiveness of 16S rRNA data as a forensic tool [46]. The Forensics Microbiome Database (FMD) created by our group, which links publicly available 16S rRNA-derived taxa data with their geographic origins down to the city in order to perform geolocation (<http://www.fmd.jcvi.org>), is an example of harnessing meta-samples to perform forensic analyses.

3. Forensic microbiome applications

The potential forensic utility of microbial DNA has been demonstrated for samples collected from highly diverse locations, including the environment (e.g. soils) [47–49], hair [34], skin [31,50] and vagina [51]. Thus, the forensic literature refers to the potential uses of microbial-derived data [52], distinctive signatures [53], and forensic indicators [54]. Examples of the forensic indicators include identification of personal identity, country of origin, and time of death. Since each of these indicators is of interest to forensic scientists, we will take a closer look at these three scenarios to explore how microbiome derived data can be employed in forensic studies.

3.1. Identity

Researches have demonstrated the potential to identify a single individual in large populations, based on microbial fingerprints from metagenomic tags, even over long periods of time [42,55]. It has also been shown that skin bacterial profiles can be confidently used to link specific individuals to objects touched, even after months [56], provided the objects have since remained untouched [31]. Furthermore, it is possible to define specific biogeographical patterns from different objects, based on particular microbiome signals; for instance, toilets, doorknobs and restroom floors faithfully reflect the microbiome characterizing the human gut, skin and soil bacterial profiles respectively [26]. The individualization of a person's microbiome by specific body sites could be useful evidence in forensic investigations. For example, the microbiome left behind by a suspect on a victim could link the specific body site

(s) involved in a sexual assault crime. This information could then be used during sentencing. In the absence of DNA evidence, this would be particularly useful not only for linking the body site to the crime but also the individual. Remarkably, the power of microbial fingerprints for identification purposes extends to ethnicity. Studies combining mitochondrial (mtDNA) haplotypes and mitochondrial single nucleotide polymorphisms (mtSNPs), along with microbiome profiling from the same fecal and vaginal sample show that microbiome patterns can be associated with the ancestral genome background of the host [57]. These observations suggest that microbial and even non-microbial DNA fingerprints from samples of forensic relevance may accurately discriminate single individuals and subpopulations, and link them to sources (objects) of potential physical evidence.

3.2. Geolocation

Microbial communities differ in composition and function across different geographical locations, even at the levels of different cities [23,27,58], due in part to variation in climate, rainfall, altitude and soil as well as the metabolic properties of their host or energy sources available in the environment. Thus, knowledge of specific microbiomes composition in a host or environment can help in specifying its geographic location [58]. For instance, different *Helicobacter pylori* strains can be linked to specific geographic settings [59–61]. It is even possible to determine non-indigenous sources of a disease outbreak by tracing phylogeographic patterns at the strain level in pathogens, as shown in the Cholera outbreak in Haiti [62]. The addition of microbiome data to humanitarian programs that solely use DNA such as DNA-Prokids (<http://www.dna-prokids.org>) [63] could assist authorities in human trafficking cases. Victims, such as small children, could be connected to their families through the microbiome geographical signatures, including possible intermediate locations through which the victim was trafficked. Buildings can also have geographically distinct bacterial signatures, as bacterial 16S rRNA-based taxa abundance vary significantly between offices in Tucson, San Francisco and New York City [58]. Thus, the geographic location of offices across different cities located in different climate zones can be predicted with high accuracy using only the microbiome profile of the office surface [27], suggesting that a suspect could be linked to a crime in a city using the same methods. However, this power of differentiation does not currently extend to buildings located in same city [27].

Some studies have demonstrated microbiome samples harvested from body sites can be related to the hosts' country of origin. Inanimate objects can retain geographic-specific microbiomes, as shown by members of our team, in samples obtained from early 1500 paintings across Europe (Torralba et al; manuscript in preparation). The gut microbiome is the most commonly examined microbiome for geographic information [64,65], and can be collected from toilets [26]. Individuals in diverse geographic locations can be differentiated both by specific microbial sequence signatures [66], and by 16S rRNA-based taxa composition [16]. The relative percentages of two bacterial taxa, Firmicutes and Bacteroides, can be used to map latitude [67]. Although the gut microbiome remains the most referenced for personal geolocation, the microbiomes from other body sites including saliva [68] and skin [69] have also been successfully mined for geolocation information. Machine learning has been the most used for differentiating geographic locations, with random forest analysis used both for human [13] and architectural [58] samples. Ongoing research using the samples in the FMD has demonstrated the power of the random forest classifier to predict geographic location using samples from different body sites based on the microbiome taxa composition (Gomez et al; unpublished data). One potential

limitation of using microbiome methods for geo-sourcing is that geographic location signals can be confounded with the associated lifestyles and societies at specific location, and it is that what is being measured by the bacterial signature [70–72]. Similarly, sudden changes in a person's lifestyle often associated with travel, such as diet or illness, can effect a change in the microbiome over only days [73]. However, the geographic signatures derived from gut microbiomes have been demonstrated to be robust to two possible confounding variables, body mass index and age [74].

3.3. Postmortem interval estimation

Microbial forensics can also use the microbial species associated with a decomposing body (or corpse) to estimate time since death (the postmortem interval or PMI). Studies have shown that there are distinctive bacterial taxonomic signatures that can be defined at different time points [53], and in different body locations, including the gut [75], the bones [52] and skin [76] during decomposition. Studies have developed models for estimating the minimal PMI from the taxa distribution [76–78], with the skin showing the most promise [76]. However, any tax-based calculation of postmortem interval has to account for possible differences deriving from variations in the season [79], temperature (called accumulated degree days or ADD), local soil [80,81] and other variables that can have an effect on taxa distribution. Therefore, equations used to estimate minimal PMI at one location based on microbiome data might not be as accurate at predictions in another location, especially as any machine learning based model is dependent on the starting collection of taxa used as seeds.

4. Forensic microbiome challenges and limitations

The majority of currently available microbiome data is neither comprehensive nor robust, and in order to be applied in forensics, needs to be substantiated. Conventional 16S taxon-based approaches to analyzing microbiome data that are routinely used by the larger scientific community rely heavily on known reference genomes and gene datasets, and result in limited information beyond the relative abundances of microbial species that are present in a sample. In addition to this problem, there are several obvious limitations to this approach exemplified by the fact that almost half of the high-quality sequence reads that are derived from microbiome projects cannot be taxonomically classified [82,83].

The temporal stability, reproducibility, and sensitivity of microbiome-based evidence and analyses are additional critical factors that must be considered for accurately identifying DNA profiles for forensic applications which may increase the variability. The effects of method of sample storage, storage temperature and duration, on microbiome community structure in human microbiome samples have been investigated [84]. There is a consensus that refrigeration or freezing is not associated with significant variation in community composition, so long as sample-stabilizing mechanisms are used. However, there is some disagreement about possible variation in the taxa composition in a sample due to repeated freezing and thawing, and the length of storage at room temperature, all conditions that may occur during the handling of microbiome-based evidence left at a crime scene or on a piece of evidence [85–88]. This variation may be further confounded by the temporal instability of an individual's microbiome sampled at the same location over time, whether a body site [42] object, or environment. The temporal variation is not constant, as some samples, such as gut microbiome, are more robust than others. Similarly, the initial works on the environmental microbiomes have a diverse array of collection techniques: the dust from

the fans were sampled for the dorm rooms [25]; swabs were taken from the subways and parks of New York [83]; specialized collection plates were used in the examination of the different office studies. No meta-study has addressed any possible changes to the results from these different sampling techniques. As such, future studies will need to be conducted in order to provide recommendations for sample collection to enable preservation and to prevent contamination, though the wide variety of surfaces could prevent any generalized rules. Furthermore, a limited amount of available biomass to sample can negatively impact a microbial forensic signature. The variation in microbial biomass from different body sites can diminish the accuracy of correlative analysis across these sites [90–92]. Few, if any, studies have assessed the sensitivity of current technologies to obtain microbiome profiles from samples of trace quantity, though studies have shown that these collection more susceptible to contamination [89].

With the dependence on the comparisons with large datasets while taking into account multiple possible variables, there is a critical need for developing and using novel computational and statistical classification methods that can handle highly dimensional microbial data in a forensic context. This can be a challenging task in light of several possible limitations: the high diversity displayed by human microbiomes; the presence of rare taxa [93]; the propensity of next generation sequencing methods to yield high error rates in taxonomic classification [94]; the fuzzy concept of species in bacteria [95]; and the multifactorial nature of environmental, physiological and genetic microbiome drivers [96]. However, the microbiome field has made significant advances in this research area. Supported by concepts from the machine learning field, it has been shown that supervised classification methods are able to accurately define “microbiome types” and taxa that are highly discriminant of individuals, groups or specific environments, using diverse predictive models. This level of classification resolution is not usually attained based on classic multivariate statistics methods. Machine learning and classification methods as applied in microbial forensic research may be useful in identifying potential contamination sources and labeling errors in samples of forensic relevance [97]. As the use of microbiome data in forensic research is standardized, knowledge of these tools in forensic research is key to significantly advance the field and to fully integrate microbiome-derived data into the forensic community.

Any introduction of novel techniques in forensic science also necessitates changes in the laws and standards governing their collection and use. The human microbiome is no exception, as possible uses in identifying and phenotyping individuals introduce multiple complexities to the laws and regulations governing evidence. Collecting the non-coding DNA used in fingerprinting, storing it, and subsequently maintaining it in a database is relatively accepted, and it has been addressed in case and statutory law, including by the US Supreme Court in *Maryland v. King*. However, as the taxa composition in a microbiome can also reveal details of a person's lifestyle and health, including those not germane to any legal issue, maintaining a similar database for microbiome data would inherently raise privacy issues not shown by DNA fingerprint databases [98]. It is possibly not covered by the ruling in *Maryland v. King* nor would it meet the current legal standard for privacy in some jurisdictions, which is limited to externally visible characteristics [99], concerns that are also raised when discussing storing coding DNA. This however would not inherently preclude collecting microbiome information from an individual or a crime scene as it is still uncertain if courts will treat human DNA differently from bacterial DNA only associated with humans. It remains probable though that the amount of data that can be analyzed and stored will be limited until further regulations are introduced and refined.

The full incorporation of microbiome-derived evidence into the forensics toolkit also requires more robust statistical tests that will give sufficient confidence with the findings. Any scientific evidence is expected to meet the standard introduced in 1993 by the US Supreme Court in their *Daubert v Morrell* decision: validated, peer-reviewed, with a known or potential error rate, and generally acceptable to the relevant community. At this time, studies have used microbiomes to differentiate an individual from a limited group of people, but they do not include a mathematical level of confidence on whether the microbiome could also implicate an individual that is not part of the group sampled [19,27,28,88] [e.g. 19,27,28,88]. Moreover, the use of microbiome-derived identification in court requires demonstrating that the microbiome is from a given individual within an acceptable error rate, especially as the microbiomes are dynamic and can be further influenced by any collection, storage, and analysis. The simplicity of the statistics used to estimate the population probabilities for DNA fingerprinting matching a given individual, which is based on allele proportions in the population, means its use for microbiome data is limited. It is likely instead that the similar microbiome statistics will depend on large publicly available data with the wide variation in body sites, health, and sampling collected into databases, especially as government-based microbiome storage and sharing has a chance of being limited by privacy concerns.

5. Conclusions

As the field of genomics science continues to expand, we have witnessed increasing opportunities to use these NGS technologies in forensic approaches. The promise of using human and environmental microbiome data in the forensic toolbox has even further expanded the possibilities for evaluating a crime scene, the decomposition of a human body, human trafficking etc., and has created significant new potential [e.g. 100]. It is possible can imagine a future where the microbiome samples at a crime scene can be used as evidence for where a suspect had been most recently, the ethnicity of the person, or the familial origin of the victim when the DNA evidence is insufficient. Microbiomes associated with the trafficking of humans, contraband, drugs and more may be used to figure out who has been involved in a crime. Several concerns remain however before these applications can become routine as the majority of data and forensic microbiome information that has been generated to date is neither robust nor has the needed statistical power to allow for widespread applications. To achieve wider application in forensics, reliable microbiome databases are needed that include ranges of metadata associated with humans such as ethnicity, geographic origin, diet, and social information. Many more samples need to be collected and analyzed in a way that is acceptable under the current legal standards for genetic privacy and reliable standards created for the community. By having standardized processes in place we can avoid conflicting results that may be associated with contamination of samples, post-collection alteration of taxa compositions when samples are not collected or stored properly, or the challenges when scientists use different databases and tools to interpret datasets.

It is anticipated that as microbiome science becomes more accepted in forensics, the forensic community will need to be educated on the challenges of using microbiome data, the correct use of databases and informatics tools, the extent of what can be interpreted from these datasets, and need to be open to sharing non-confidential datasets and other information. In addition, multiple additional groups, including legal, regulatory, and health workers as well as immigration officials, will need to be educated on this emerging science to order to fully grasp its potential.

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