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Review Article

Microbial Forensics in Legal Medicine

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Abstract: Application of science in criminal investigation is forensic science. In forensics, findings based on scientific knowledge and technologies are used to serve as witnesses to prove guilt or innocence in criminal and civil matters. It is said that even eye witness may turn hostile, but scientific evidences gathered from the scene of crime after appropriate analysis become solid evidence which does not change with time. Forensic science is an established branch of criminal investigation having several disciplines like forensic chemistry, forensic biology and serology, forensic ballistics, forensic physics, forensic photography and forensic DNA fingerprinting. Microbial forensics is a relatively newly emerging branch which connects microbiology with forensic science, where microbial agents, their origin and their potential effects can be presented as medico legal evidence. It is the interplay of classical microbiology, microbial genomics, phylogenetics and Bioinformatics. The application of microbial forensics is to assist in resolving crimes, with a focus on research and education to facilitate its use in criminal investigations. New technologies, including high throughput DNA sequencing have helped to change many long seeded assumptions about human microbiome. By now the use of microbial forensics is almost negligible but, it has potential and may prove to be concrete if properly investigated. The review presents current status, developments and studies required to make this field vital in forensics.
Keywords: DNA fingerprinting, human, microbial forensics, 16sRNA, molecular characterization.

Introduction

Our body is a house of millions of microbes residing exogenously and endogenously. The exogenous or transient microbes are due to environment, which a person is exposed to, while, the endogenous or the resident microbes are the microbes that reside over the body permanently. Human body is aggregate of microorganisms, that resides on the surface and in deep layers of skin (including in mammary glands), in the saliva and oral mucosa, in the conjunctiva, and in the gastrointestinal tracts. This is also an established fact now that human body has more number of microorganisms than its total body cells. The normal flora of human body consists of a few eukaryotic fungi and protists, but bacteria are the most numerous and obvious microbial components of the normal flora. In 2012, around 200 researchers from some 80 research institutions comprising the Human Microbiome Project (HMP) Consortium have used advanced DNA-sequencing to identify and catalogue the thousands of microorganisms co-existing with humans. Microbial flora has spatial and temporal complexity that differs by individual, body niche, age, geographic location, health status, diet and type of host [1,2] and even within the same individual, the composition of the microbial flora can vary according to changes in diet, stress, sexual behavior, medication, hormonal changes and other host-related factors [2–6]. With the advent of molecular techniques it has become possible now to decipher these variations easily and in comparatively lesser time. The molecular variations between the similar strains can help in determining the origin and transmission routes of a particular sample. So the microbial agents can aid in the geographical identification of human host involved in a crime, which may help in narrowing down the investigation procedures [7]. Microbial forensics is a scientific discipline dedicated to analyzing evidence and it can be defined as the detection of reliably measured molecular variations between related strains and their use to infer the origin, relationships, or transmission route of a particular isolate or forensic sample. So, the major questions which have been addressed after using different methods over the decades are- What microbes are present on the skin surface?, and, How do dermatology practices alter microbial diversity?

Human Microbiome:

The term microbiome was coined by Joshua Lederberg to explain the ecological community of

commensal, symbiotic, and pathogenic microorganisms sharing our body space. Humans are home to complex microbial communities, whose aggregate genomes and their encoded metabolic activities are referred to as the human microbiome [8].

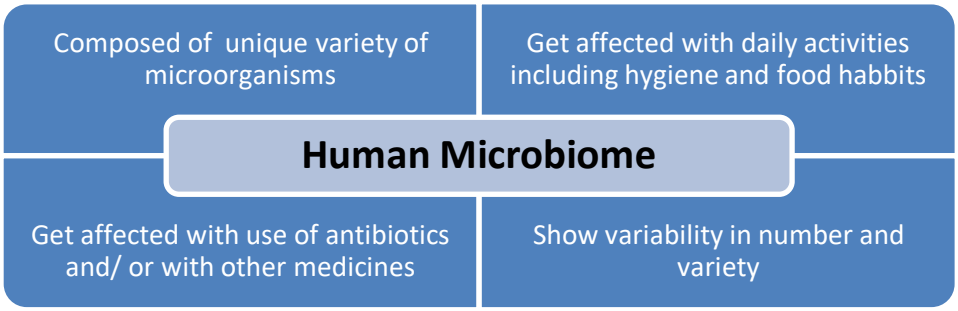


Fig 1: Defining Human microbiome

Just as our human cells contain genetic material that make up our human genome, the much smaller microbial cells in and on our body contain the collection of genes that make up the human microbiome. In current understanding human body is made up of about 10 times more microbial cells (around 10^{14}) than human cells (around 10^{13}).

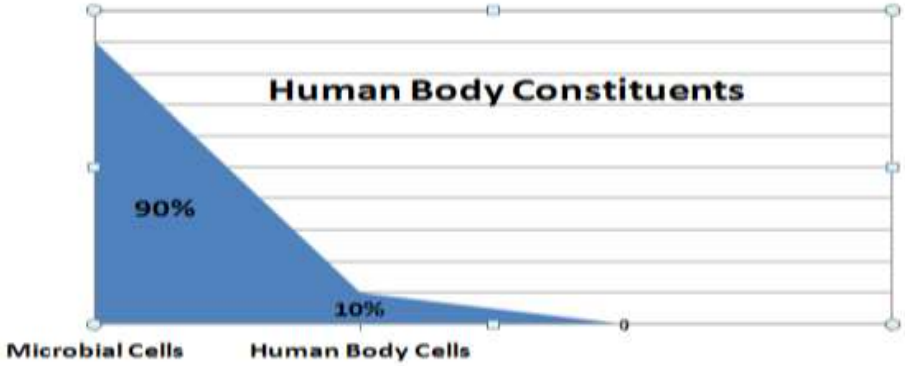


Fig 2: Human body constituents deciphered after Human Genome Project

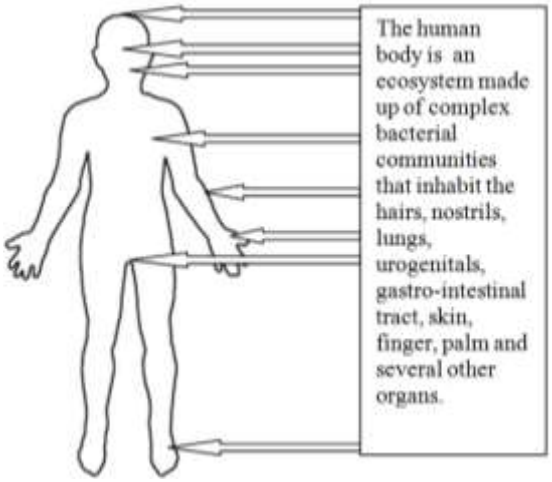


Fig 3: Presence of microorganisms on human body is almost everywhere.

Skin Microbiome:

The skin is the human body's largest organ. Microbial diversity varies across the niches comprising on average 1.8m^2 of adult human skin. Trillions of bacteria, fungi, viruses, archaea and small arthropods colonize the skin surface, collectively comprising the skin microbiome where most of which are harmless or even beneficial to their host. Colonization is driven by the ecology of the skin surface, which is highly variable depending on topographical location, endogenous host factors and exogenous environmental factors [7]. The skin is composed of a variety of niches, including regions with a broad range of pH, temperature, moisture, and sebum content. Furthermore, skin structures such as hair follicles, sebaceous, eccrine, and apocrine glands comprise sub-habitats that may be

associated with their own unique microbiota [9, 10]. For example, hairy, moist underarms lie a short distance from smooth dry forearms, but these two niches are ecologically distinct as are their resident microbial communities [11]. The skin is an ecosystem composed of diverse habitats with an abundance of folds, invaginations and specialized niches that support a wide range of microorganisms. The perception of the skin as an ecosystem composed of living biological and physical components occupying diverse habitats can advance our understanding of the delicate balance between host and microorganism. The diverse population of microbiota on skin as an intricate habitat is generally conceived of as two groups-

- Group I, belongs to the endogenous or resident microbes which are a relatively fixed group of microorganisms that are routinely found in the skin and that reestablish themselves after perturbation.
- Group II, constitutes exogenous or transient microbes which do not establish themselves permanently on the surface, but rather arise from the environment and persist for hours to days.

Many Intrinsic factors, including age, genetic makeup and immune reactivity also influence the composition of skin microbial communities. Environmental factors such as climate and extrinsic factors such as hygiene may also have profound effects on microbial communities. This clearly indicates that microbial flora can be used to individualize for the purpose of scientific investigation. The topography of human skin varies at both microscopic and macroscopic levels. Distinct habitats are characterized by differences in skin thickness, folds and densities of hair follicles and glands. Cutaneous invaginations and appendages, including sweat glands (eccrine and apocrine), sebaceous glands and hair follicles are likely to be associated with their own unique microbiota (Figure 4). For example, sebaceous glands secrete lipid-rich sebum, a hydrophobic coating that protects and lubricates hair and skin. Although sebum generally serves as an antibacterial coating, *Propionibacterium acnes* hydrolyses triglycerides present in sebum, releases free fatty acids that promote bacterial adherence, and then colonizes sebaceous units [12]. Perturbations affecting the host–microorganism relationship can be endogenous (for example, genetic variation that selects for a specific microbial community) or exogenous (for example, hand washing) [8]. Physiological and anatomical differences between male and female cutaneous environments such as sweat, sebum and hormone production, partially account for the microbial differences seen between the genders [9, 10, 13]. A study with larger numbers of subjects will be required to statistically define which bacterial species are unique to certain individuals or body sites. The development of molecular methods to identify microorganisms has led to an emerging view of the resident skin bacteria as highly diverse and variable [8].

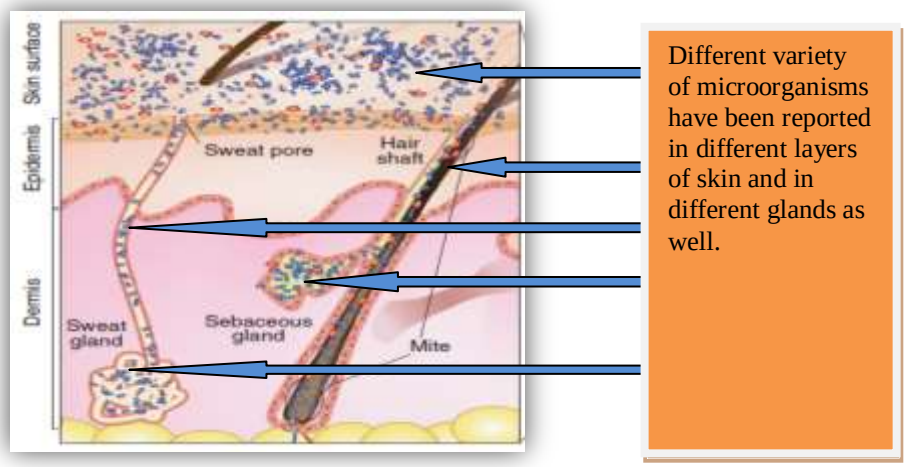


Fig- 4: Schematic of skin histology viewed in cross-section with microorganisms and skin appendages. Microorganisms (viruses, bacteria and fungi, and mites) cover the surface of the skin and reside deep within the hair and glands, After Kong and Segre, 2012 [14].

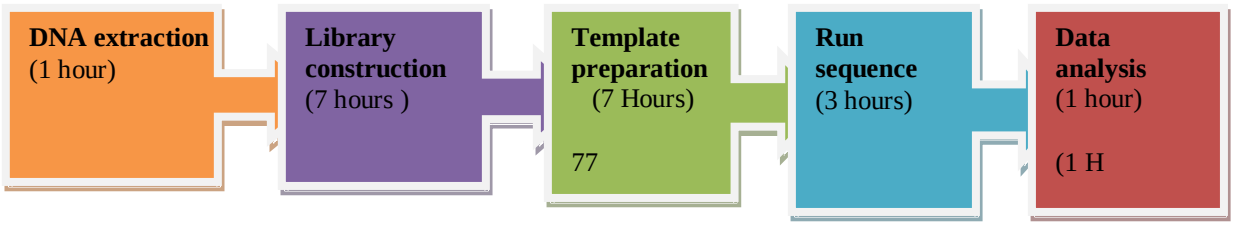
Pre molecular biology era or early culture-based studies to define the microbial skin residents:

Microorganisms colonizing the skin have long been of interest to dermatologists and microbiologists; our knowledge of these microorganisms has until recently, been gleaned through culture-based studies [15]. Historically, culture-based approaches have been the standard for characterizing microbial diversity and *Staphylococcus epidermidis* and other coagulase-negative staphylococci have been regarded as the primary bacterial colonizers of the skin. Other microorganisms that are generally regarded as skin colonizers include coryneforms of the phylum Actinobacteria (the genera *Corynebacterium, Propionibacterium and Brevibacterium*) and the genus *Micrococcus*. Gram-negative bacteria, with the exception of some Acinetobacter spp., are generally not isolated from the skin, but are thought to arise in cultures owing to contamination from the gastrointestinal tract [16]. However, some bacteria

(including, e.g., *Treponema pallidum*) require fastidious growth conditions and are notoriously difficult to isolate. Other bacterial species, e.g., *Staphylococcus aureus*, grow readily under standard culture conditions and subsequently overcrowd more fastidious bacteria. The most commonly isolated fungal species are *Malassezia* spp., which are especially prevalent in sebaceous areas [17]. The role of commensal viruses has not been studied, and investigations are currently limited by the available molecular and microbiological means to identify and characterize viruses [14]. Comprehensive skin microbial surveys performed decades ago were limited by the ability to provide the appropriate growth conditions required to culture and isolate fastidious microbes. Another important aspect to think over is, as we are prejudiced in providing growth media and conditions to isolate and culture unknown numbers of bacteria; therefore the real estimate and the actual number of bacterial species in a sample may not be accessed by using culture based methods. It is well accepted fact that microbes flourish in the context of a large community and only a minority of bacteria thrive in isolation.

Entering the molecular microbiology era to define the microbial residents:

The development of molecular techniques to identify and quantify microbial organisms has revolutionized our view of the microbial world and ushered in another ‘‘gold rush’’ in studying skin microbes. Genomic characterization of bacterial diversity relies on sequence analysis of the 16S ribosomal RNA (rRNA) gene, which is present in all bacteria and archaea but not in eukaryotes. The 16S rRNA gene contains variable regions, enabling taxonomic classification, and conserved regions, serving as binding sites for PCR primers. Importantly, an organism does not need to be cultured to determine its type by 16S rRNA sequencing [18, 19]. Genomic approaches to characterize skin bacteria have revealed a much greater diversity of organisms than that revealed by culture-based methods, predominantly from four different phyla: Actinobacteria, Firmicutes, Bacteroidetes and Proteobacteria. It has been demonstrated by the several groups that the proportion of these bacterial phyla is dependent on the physiology of the skin site, with specific bacteria being associated with moist, dry and sebaceous micro environments [11, 20, 21]. *Propionibacterium* spp. is the dominant organisms in sebaceous areas, which confirms classical microbiological studies. *Staphylococcus* and *Corynebacterium* spp. are the most abundant organisms colonizing moist areas. The most diverse skin sites are the dry areas, with mixed representation from all four phyla. A surprising feature of the microbiota of dry sites, as captured by molecular analysis, is the abundance of Gram-negative organisms, which were previously thought to colonize the skin only rarely, as contaminants from the gastrointestinal tract. The microbiomes of the antecubital fossa, back, nare and plantar heel are more similar to the same site on another individual than to a different site on the same individual [10]. In this sense, the ecological niche, or skin site, is a greater determinant of the microbiota composition than the individual genetic variation among healthy volunteers. Some sites on an individual are similar, such as different sebaceous regions that share common ecological features. Molecular analysis of skin microbiota has also revealed that the temporal variability of the skin microbiome is dependent on the site sampled. In healthy adults, skin niches including the nares, glabella, and external auditory canal demonstrate relative stability as compared with dry regions such as the inner forearm and the heel. In general, contralateral sites on the same individual are more similar to each other than to a corresponding site on another individual. Several studies have assessed skin microbial communities with molecular typing of 16S rRNA genes with interesting results. An early genomics-based skin microbial study demonstrated that the microbial community composition of the palmar surface of the hand was significantly affected by handedness, time since last hand washing, and an individual’s sex [21]. The Knight group later examined whether the residual skin bacteria left on objects could be matched to the individual who touched the object [22]. The authors showed that skin-associated bacteria identified on inanimate objects could be linked to specific individuals. Now next generation sequencing (NGS) is also being utilized for studying microbial flora. Next-generation sequencing refers to non-Sanger-based high-throughput DNA sequencing technologies. Millions or billions of DNA strands can be sequenced in parallel, yielding substantially more throughput and minimizing the need for the fragment-cloning methods that are often used in Sanger sequencing of genomes (http://www.nature.com/subjects/next-generation-sequencing).



NGS workflow: sample to results in less than 24 hours!

Microbial forensics and bioterrorism:

Microbial forensics came into limelight after the Anthrax attack in New York (US) in 2001 and was named bioterrorism [23]. Bioterrorism is the use of biological agents specially the microbial agents to create terror, it has its roots back in 6th Century B.C., when the Assyrians poisoned enemy wells with rye ergot, a fungus that causes convulsions if ingested. In 1754 B.C. during the French and Indian wars, it's suspected that the British forces distribute smallpox-laden blankets to native American Indians who were loyal to the French. Potential agents like *Bacillus anthracis* , *Botulinum toxin, Yersinia pestis* and *variola* viruses are most frequently used as Bio warfare (BW) agents. *B. anthracis* spores can remain stable for almost decades and this long term stability affects the evolution of this pathogen [24]. Ensernik & Ferber have suggested that at least three different strain of each pathogen and upto twenty for the highly pathogenic species should be sequenced since the genomic information can help in pinpointing the source of the pathogen [25]. Genomic sequencing data and detailed information for such strains are not available in many developing countries which make it difficult to trace the source of infection.

Microbial Forensics in medical negligence:

Microbial forensics can be helpful in investigation of the medical negligence cases and attribution of the charges. A forensic investigation in Florida to trace the source and transmission of HIV found out that the deadly virus was originated from a dentist and was passed to the patients [26]. The investigation was done by confirmative sequencing of PCR amplified viral genes from the dentist and the patients. In Sweden, a rape case investigation proved deliberate transmission of HIV-1 from the accused male to the female victim [27]. The comparison of amplified HIV-1 *pol and gag* genes from accused and victim was done and the charges were attributed. In 1998 comparison of amplified *env & gag* genes of HIV-1 from the blood samples of a French orthopedic surgeon and his patient proved transmission of virus from surgeon to his patient during surgical procedures [28].

Microbial forensics and identification of culprits in other crimes:

Microorganisms survive on the biological material which is available on almost all forensic case exhibits and therefore there is every possible chance that they may cause degradation of the biological material present on the article. This aspect of microbial forensics is currently in its developing stage. It will be most effective if there is sufficient basic scientific information concerning microbial genetics, evolution, physiology, and ecology. There have been a few studies on role of microorganisms in solving crimes, but most of these studies are related with the role of microorganisms as pathogens and study of pathogenicity [29-34], stable isotope ratios as a tool the microbial forensics [35-37], potential of bacterial DNA for soil comparison and/or characterization [38,39], a few have dealt with detecting these microorganisms from crime scene material to link suspect with the crime scene [40-43] and a few on isolation of bacterial community from skin or from fingerprint [10, 21,22].

Microbes as a means of identifying individuals:

People or complete bodies are usually identified from their morphological characteristics. In the case of trace evidence recovered during investigation, such as body fluids, human DNA is the best identifier, whilst fingerprints and other contact evidence are analysed using their physical characteristics. However, it is not always possible to get a complete DNA profile and on the other hand, even fingerprints are often smudged and incomplete [44], thus making the identification difficult. Studies on the human microbiome indicate that not only are there major differences in the microbial composition within regions of the body, but that there appear to be consistent differences between individuals [21]. This has led to suggestions that people may have unique microbiota and these could be used as a means of identification [45].

Forensic identification using skin bacterial communities:

The human skin surface harbors large numbers of bacteria that can readily be dislodged and transferred to surfaces upon touching [46, 47]. These skin bacteria may persist on touched surfaces for prolonged periods because many are highly resistant to environmental stresses, including moisture, temperature, and UV radiation [48, 49]. Therefore, we likely leave a persistent “trail” of skin-associated bacteria on the surfaces and objects that we touch during our daily activities. Recent work has demonstrated that our skin-associated bacterial communities are surprisingly diverse, with a high degree of inter individual variability in the composition of bacterial communities at a particular skin location [10, 20,21, 22]. In addition, skin bacterial communities are relatively stable over time: palm surface bacterial communities recover within hours after hand washing [21]; and, on average, interpersonal variation in community composition exceeds temporal variation within people, even when individuals are sampled many months apart [10]. Given that individuals appear to harbor personally unique, temporally stable, and transferable skin-associated bacterial communities, it has been hypothesized that one could use these bacteria as “fingerprints” for forensic identification. To use skin bacteria to link touched surfaces to specific individuals it is suggested that the following criteria must be met: (i) bacterial DNA recovered from touched surfaces allows for adequate characterization and comparison of bacterial communities; (ii) skin bacterial communities persist on surfaces for days to weeks; and (iii) surfaces that are touched can be effectively linked to individuals by assessing the degree of similarity between the bacterial

communities on the object and the skin of the individual who touched the object [22].

Microbial DNA fingerprinting of human fingerprints can be used for forensic purposes:

Human fingertip microflora is transferred to touched objects and may provide forensically relevant information on individual hosts, such as on geographic origins, if endogenous microbial skin species/strains would be retrievable from physical fingerprints and would carry geographically restricted DNA diversity. Kims et al, 2010 tested the suitability of physical fingerprints for revealing human host information, with geographic inference as example, via microbial DNA fingerprinting and showed that the transient exogenous fingertip microflora is frequently different from the resident endogenous bacteria of the same individuals. However, human fingertip microflora left behind on touched objects at crime scenes may potentially contain forensically relevant information that may be useful for human host inferences accessible via microbial DNA fingerprinting of physical fingerprints. For example, if endogenous microbial skin species/strains with a geographically restricted distribution could be retrieved from touched objects via microbial DNA analysis, the geographic origin of the human host individual could be determined indirectly. Information about the geographic region of origin can be relevant in suspect-less forensic cases where the evidence DNA sample does not match either a suspect's DNA profile or any in a criminal DNA database. In such cases, geographic information derived from crime scene samples is expected to reduce the potential pool of suspects by allowing police investigations to concentrate on specific groups of people, i.e., those from a restricted geographic region [50].

Lineage based approach:

Microbial forensic genetic evidence will more likely be analyzed using a lineage-based approach. In other words, sequence similarity and/or genotypic match with microbes may only infer common lineage instead of identity [51].

Problems in forensic DNA profiling:

Human identification based on genomic DNA profiling has wide application in many fields including mass disasters, crime detection and paternity identification [52-54]. Utility of STR markers was accessed long back and these were thought to be important tools for human identity testing for long because of their high degree of variability, ease of use in multiplex amplification formats [52,53]. STR-based multiplex human identification systems have been widely used for many years and became common in forensic studies. Investigation of human derived specimens involves only one species, and forensics experts are able to use a set of only 10 to 16 microsatellite loci on the genome for most identification [52-54]. DNA fingerprinting technology is well defined technology to correlate the crime scene the suspect and the victim conclusively beyond any doubt in the court of law. This is done by generating the DNA profile from the crime scene exhibits received for forensic examination. With the advent of PCR technology, pre-formulated multiplex DNA kits ready to cope up with the amplification inhibitors and even with the very low quantity/degraded DNA, now it is possible to generate DNA profile from most of the samples. But still there remain a considerable number of exhibits which do not provide result or provide a partial DNA profile due to body fluid degradation. There is a need of characterizing microflora over the case exhibits, also this can be helpful in understanding the type of microbial population over the same variety of samples and also about their possible role in degradation of body-fluid [55,56].

Conclusion

Much is to explored in the area of microbial forensics and in the light of above, the following areas seems to be worked upon in the present scenario-

1. Molecular characterization of bacteria from crime scene exhibits,
2. Possible role of bacteria in degradation of biological fluid which may be the probable reason for not getting the results even by using PCR based sensitive techniques like DNA fingerprinting,
3. Molecular characterization of human bacterial microflora,
4. Spatial and temporal variation in human bacterial microflora,
5. To find out link between the personal belongings like cell phone or laptop with the bacterial microflora on fingertip.

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