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Role of microbes in forensic science

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Abstract
Human microbiome refers to all microscopic lifeforms such as bacteria, viruses, algae, and fungi inhabiting the human body. Forensic microbiology involves the identification of microorganisms based on post-mortem interval and their distribution in different parts of the body which helps in individual identification, cause of death determination, geolocation determination of where the corpse was possibly found and body fluid identification. Microbial forensics is used to study the transmission of microbes and diseases caused by microbes in sexual assault cases, bio crimes or any other forms of criminal cases. Advancements in molecular biology and genetics have led to the development of analytical instruments and techniques that help in better analysis of microbial samples and its metabolites. Thanato-microbiology refers to the study of microflora residing on body surfaces which is also an evolving field of study in forensic microbiology which mainly helps in distinguishing one individual from another based on the unique microflora inhabiting their body.

Keywords: Forensic science microbes, thanato-microbiology, pyrosequencing

Introduction
Microorganisms or microbes in short are the smallest, unicellular organisms found. They are both useful and harmful to mankind. They are classified into different types such as bacteria, virus, fungi and protozoa. Bacteria is the most abundantly found group of microorganisms which are usually classified into two types namely archaebacteria and eubacteria. It is believed that there are over ten times more bacteria living within and upon the human body than there are human cells (Turnbaugh *et al*., 2009) [1]. Studies have shown the importance of microbes in forensic post-mortem examination, time since death determination, individual identification through the analysis of microbiota found in body fluids, identification of geographic location where the death might have occurred based on microbial population in the body. Microorganisms such as *Clostridium*, *Lactobacillus*, *Eggerthella*, *and Bacteroides* are found abundantly in the lower gastrointestinal tract whereas *Streptococcus*, *Prevotella*, and *Veillonella* are widely distributed in the upper gastro intestinal tract of the human body. (Zachary *et al*., 2017) [5]. Firmicutes are found in the oral cavity during pre-bloat, while Proteobacteria are found during post bloat (Hyde *et al*., 2013).
Common methods used to identify microorganisms include pyrosequencing and pulsed-field gel electrophoresis. Other methods of detection include16/18S ribosomal RNA (rRNA) gene, Single-nucleotide polymorphisms, internal transcribed spacers, and whole genome shotgun. These genomic methods are useful in forensic science to create genetic profiles and in the identification of entire microbial communities. (Zachary *et al*., 2017) [5]. The 70s and 80s ribosomes that are essential for protein synthesis are made up of 16s and 18s RNA which usually remain highly conserved in a taxonomic phylum but the presence of variable regions with interspecific polymorphisms or mutations help in the identification of individual. Other regions of DNA used for taxonomic analyses are the noncoding regions of DNA between the genes for ribosomal RNA called the internal transcribed spacer (ITS) such as between the 16S and the 23S in bacteria and archaea (Lafontaine and Tollervey, 2001). These regions have a high mutation rate since they are nonessential for survival, and this allows them to be compared across similar species (Baldwin, 1992).

Post Mortem Microbial community and PMI
The microbial community associated with the host after death is referred to as the thanato-microbiome.

Upon death, host tissues and cells decompose and cellular components are released to the surrounding tissues, resulting in significant changes in the host environment over time. Such changes shape, and are influenced by, host microbes as well as environmental microbes, resulting in characteristic microbial community dynamics specific to thanato-microbiome. Post-mortem interval (PMI) can be inferred based on ecological succession patterns of microorganisms on the cadaver. (Wei *et al*., 2018) [14].

After death, the body goes through five main stages of decomposition namely: fresh stage, bloat stage, active decay, advanced decay, and dry remains. The first stage marks the autolysis of cells where the cells undergo damage due to action of body's own enzymes [2]. There is a significant reduction in the body temperature which causes the host's enzymatic activity to be lost but the microbial enzymatic activity continues to take place due to their broader enzymatic activity range. Anaerobic fermentation of bacteria in breaking down the tissues result in the release of gases that are not able to escape from the body giving rise to bloated appearance of the body. At the time of active decay, bacterial community along with scavengers begin breaking down skin and internal tissues leading to a large loss of body mass. Decomposition process takes place until the body is skeletonised [2].

The external and internal body environment plays a significant role in the microbial colonisation of an organism. During life, homeostasis is maintained but after death, homeostasis is lost resulting in changes in pH, temperature leading to bacterial phenotypic and genotypic changes to promote bacterial survival. Bacteria respond by modifying gene expression or movement to a more sustainable environment. This movement, often referred to as bacterial transmigration, is a defining feature during decomposition (Kellerman *et al*., 1976) [7].

Moisture can influence the decomposition of the body by controlling microbial motility, the diffusion of nutrients and waste, and the activity of extracellular enzymes. Microbial mobility in soil is regulated by pore size and the diameter of water filled pores. David *et al*., tested the hypothesis that an increase in moisture content will result in a decrease in cadaver decomposition. They also wanted to determine whether cadaver breakdown significantly differed between soils calibrated to the same matric potential. They collected contrasting soils from various regions of Queensland (Australia). They noted that there was a great loss in cadaver mass in moist soil due to greater enzymatic activity in the presence of moisture in wet soil compared to dry soil [10].

As the environment changes, microbial communities better suited for the new environment will become more dominant and out compete the other inhabitants (Boor, 2006). Studies have shown that aerobic organisms belonging to the genera *Staphylococcus*, *Bacillus*, and *Streptococcus* were detected during early decomposition whereas anaerobic organisms belonging to the genera *Clostridium*, *Proteus*, *and Klebsiella* began to dominate the cadaver as the cadaver progressed through putrefaction. Decomposition studies on non-human models have shown that temperature drove transmigration time and that *Staphylococcal* species were the first to migrate followed by coliforms, and finally anaerobic species, such as *Clostridium* [18].

Post Mortem Interval can be determined by identifying microbial communities present in different parts of the body. Studies say that internal organs such as brain, spleen, liver, and heart are devoid of microorganisms during life but after death microorganisms proliferate throughout the body starting from the ileocecal area spreading to the liver and spleen, and continuing to the heart and brain. The spread of bacteria to different areas of the body occurs by microbial invasion of the capillaries of the lymphatic and vascular system (Paczkowski and Schultz, 2011) and by invasion of the mucus membranes in the respiratory system (Gill *et al*., 1976) [6].

The reason for studying microorganisms associated with internal organ tissues is that they are less affected by environmental conditions than those associated with external organ tissues such as the skin or oral mucosa, and they are not directly affected by the proliferation of gut microorganisms that occurs rapidly after human death (Ismail *et al*., 2014) [21]. In forensic science, it is important to study these microorganisms because the presence/absence and/or abundance of certain bacteria might be indicative of the elapsed time since death (i.e., the post-mortem interval, PMI) as demonstrated in mouse and swine studies (Metcalf *et al*., 2013, Pechal *et al*., 2013). In autopsy microbiology, this information might be important to confirm a suspected antemortem infection — particularly when the cause of death is unknown (Riedel, 2014) [21].

The amount of ethanol formed during the post-mortem interval is considered to depend on the species of microorganisms present, the availability of substrates, the ante-mortem conditions of the deceased and the storage condition of the body prior to collection of specimens for toxicological analysis (Vassiliki *et al*., 2008). Amino acids, lactate, and fatty acids that may be present in the corpse can serve as substrate for microbial growth. Carbon metabolism provides the bacterial cell with energy in the form of reducing equivalents and adenosine triphosphate (ATP), as well as essential biosynthetic precursors. Microbes usually prefer carbohydrates, especially glucose as an energy source. Amino acids generate volatile compounds called higher alcohols which mainly include 1-propanol, 2-methyl-propanol (isobutyl alcohol), 2-methyl-1-butanol (active- or *d*-amyl alcohol) and 3-methyl-1-butanol (isoamyl-alcohol). Hydrolysis of lipids results in the formation of glycerol. *Klebsiella* (*K. pneumoniae*), *Enterobacter* (E. agglomerans) and *Citrobacter* are some of the bacterial species capable of fermenting glycerol. Fatty acids can be degraded by number of anaerobes such as Pseudomonades, Acinetobacter, Bacilli and Coliforms. Among the compounds that have been detected during the analysis of ethanol in post-mortem cases are included various ethyl esters which have been rather considered markers of ante-mortem consumption of ethanol. Esters could be also formed by the microorganisms present during putrefaction. The microbial formation of esters can occur generally by the intracellular reaction between a fatty-acyl-coenzyme A and an alcohol catalysed by an alcohol acyltransferase (or ester synthetase) [8].

Determining cause of death

Cause of death may be natural, accidental, homicidal or suicidal in nature. In case of individuals who have had a history of being diagnosed with a disease prior to death, traces of microbes that have caused the disease may be found while analysing post mortem samples. In drowning cases, microbial communities in the water may be able to provide evidence for the cause of death and the location

where the incident took place. Diatoms are one of the most common microorganisms used to establish drowning as the cause of death [5]. Diatoms are made up of silica wall which makes them resistant to adverse conditions such as degradation from acid, enzymes, and temperature for a long period of time, which allows them to be present in discovered cadavers [5]. Diatoms are typically only present in cases where the individual drowned in a natural environment, such as a lake, river, or sea. Individuals who drown in treated water (e.g., swimming pool) generally present an absence of diatoms, which is most likely due to the water treatment process (Lin *et al*., 2014) [5]. Microbes like Faecal coliforms and faecal streptococci are considered ubiquitous as they can be found in all forms of water ranging from freshwater to sea water but it's less likely to find them in the bloodstream [5]. Migration into the bloodstream along with the drowning medium (e.g., water) may be an indicator of drowning (Lucci *et al*., 2008) [5]. Microorganisms have been used as biological weapons of war to create panic among people and thereby resulting in number of bio crimes. Biological agents like anthrax, botulinum toxin and plague have posed difficulty to public health. The intentional use of microorganisms or their toxins as weapons is almost as old as humanity itself. In the year 1495, the Spanish were known to have mixed wine with the blood of leprosy patients to sell it to their French foes. From this early stage, BW has become more sophisticated, leaning towards the capability of being a weapon of mass destruction when associated with an appropriate delivery system, as specialized munitions on the battlefield and for covert use. Such developments are a direct result of advances in both the fields of microbiology and biotechnology. If an attack is suspected, the microorganism can be isolated and identified using various genetic techniques. The genetic code may be able to provide information that may lead to determining if the strain is related to an environmental isolate or if the pathogen is completely diverse from the communities [22].

Table 1: Classification of potential bioterrorism agents (bacteria, virus, protozoan and toxic) capable of induce diseases in human, according to the unlimited states centre for disease control and prevention (CDC) strategic planning group. (Manuela *et al*., 2020) [22]

Category	Definition	Agent and Disease
A	▪ High-priority agents ▪ Easy to disseminate or transmitted (person to person) ▪ High mortality rates ▪ Potential for major public health impact ▪ Cause public panic and social disruption ▪ Special action for public health preparedness	▪ *Bacillus anthracis* (anthrax) ▪ *Clostridium botulinum* (botulism. toxin) ▪ *Francisella tularensis* (tularemia) ▪ *Yersinia pestis* (plague) ▪ Variola major (smallpox) ▪ Filoviruses (Ebola, Marburg) ▪ Arenaviruses (Lassa, Machupo) ▪ *Bunyaviruses* (Congo-Crimean. Rift Valley) ▪ Flaviviruses (Dengue)
B	▪ Second highest priority agents ▪ Moderately easy to disseminate ▪ Moderate morbidity rates and low mortality ▪ Rates ▪ Specific enhancements of CDCS diagnostic ▪ Capacity and enhanced disease surveillance	▪ *Brucella* spp. (brucellosis) ▪ *Clostridium petfringens* (gangrene and food poisoning, Epsilon toxin) ▪ *Salmonella* spp. (salmonellosis) ▪ *Escherichia coli* 0157:H7 (Hemorrhagic colitis) ▪ *Shigella dysenteriae* (dysentery) ▪ *Burkholderia mallei* (glanders) ▪ *Burftholderia pseudomallei* (melioidosis) ▪ *Chlamydia psittaci* (psittacosis) ▪ *Catlett: bumetii* (Q fever) ▪ *Wbrio cholerae* (cholera) ▪ *Cryptosporidium* parvum (cryptosporidiosis) ▪ *Staphylococcus aunts* (food poisoning, Staphylococcal enterotoxin B) ▪ *Rickettsia prowazekii* (typhus fever) ▪ Alphaviruses (encephalitis) ▪ Caliciviruses (gastroenteritis)
C	▪ Third highest priority agents ▪ Includes emerging pathogens that could be ▪ Engineered for mass dissemination ▪ Availability ▪ Easy to produce and disseminate ▪ High morbidity and mortality rates ▪ Potential for major public health impact	▪ Multidrug-resistant *Mycobacterium tuberculosis* (tuberculosis) ▪ Nipah virus (encephalitis) ▪ Hantavirus (hemorrhagic *fever* with renal syndrome - HERS. cardiopulmonary syndrome - HCPS) ▪ Chikungunya virus (arthritis and rash) ▪ SARS-associated coronavirus (respiratory syndrome) ▪ Highly pathogenic strains Influenza Virus (respiratory syndrome) ▪ Yellow fever (myalgia)

Adapted from hups://emergency.cdc.goviagentjagentlist-categoryasp.

Indicators of body fluids and individual identification

The detection of human body fluids is a crucial step in forensic investigations, helping the determination of the events that took place at the crime scene. For example, identifying saliva on a victim can support the presence of a bite mark, whereas the presence of genital fluids on a particular substrate can support an allegation of a sexual intercourse. In cases of sexual assault, it can be important to determine if stains of *fluor vaginalis* are present on objects, clothes, or furniture. The spatial localization of such

biological samples can suggest a consensual/not consensual sexual relation and can support the testimony of the victim or the suspect. Saliva can be identified by the presence of amylase and by the determination of its specific activity, seminal fluid can be detected by antibodies that are specific for human semenogelin antigen while blood stains are easily recognized by monoclonal antibodies for human glycophorin A or haemoglobin. a multiplex real-time PCR protocol on microflora DNA (mfDNA) has been developed for the discrimination of vaginal fluids from saliva and faecal stains [19].

The human saliva is dominated by five major phyla which include Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes and Fusobacteria. (Sarah *et al*., 2016). Studies have shown that the salivary microbiome exhibits a significant biodiversity. Sarah *et al*., conducted a study by collecting two saliva samples from two different individuals by using a PCR based metagenomic approach to show that the difference in the microbial diversity of saliva sample can distinguish one individual from another [3].

Studies have shown that skin microbiomes can be studied to identify an individual. Sarah *et al*., had conducted a study on skin microbiomes using "hidSkinPlex" sequencing panel which was mainly composed of 286 clade-specific markers from 22 bacterial (and phage) clades selected from the MetaPhlAn2 reference database with > 65% of the markers from the dominant skin flora, *P. acnes.* After collecting skin microbiome samples from eight different individuals the hidSkinPlex was evaluated using skin microbiome samples collected from three skin sites, the toe web/ball of the foot (Fb), the palm of the non-dominant hand (Hp) and the manubrium (Mb) from these individuals. Skin microbiome profiles generated hidSkinPlex from the foot, hand, and manubrium were attributed to their respective individual host with up to 92% (Fb) − 100% (Hp) accuracy. Additionally, body site origin could be predicted with up to 86% accuracy [20].

Hair serves as one of the most common forms of evidence in crime scenes and plays an important role in individual identification. Silvana *et al*., analysed 42 DNA extracts obtained from human scalp and pubic hairs. Using Next Generation Sequencing (NGS) a total of 79,766 reads were generated, yielding 39,814 reads post control and abundance filtering. *Limnohabitans* spp, *Mycoplana* spp and *Neisseriaceae* were abundantly found in scalp hair of human females, scalp hair of males was mainly composed of *Dietziac* and *Corynebacteriaceae* species. Pubic hair of both males and females was mainly composed of *Knoellia subterranea*, *Paracoccus*, *Corynebacterium* and *Lactobacillus* spp, *Anaerococcus, and Prevotella* spp respectively [25].

Faecal material can be found as trace evidence in sexual assault and burglary cases. They can be encountered as visible/invisible smears on items which have been have been inserted anally or in (attempted) burglaries or robberies as intentionally or unintentionally left deposits by a criminal. Frederike *et al*., conducted a study by collecting stool samples from 35 healthy volunteers of different ages. DNA extraction from the sample was done. Samples were homogenised, centrifuged, incubated, and measured. The microarrays were designed such that only the part of the microbial population covered by the selected probes is being analysed. Samples showed abundant presence of *Enterobacteria* [16].

As an alternative to gene expression measurements, microbial markers have been proposed to discriminate between various body fluids. The main idea is to look for the taxonomic composition of bacteria in the various body fluids, and recognize them based on specific patterns in this composition. The standard genetic marker for taxonomic profiling of microbial communities is the small subunit ribosomal RNA gene, also known as the 16S gene. Microbiota-based body fluid recognition is most likely best suited for bacteria-rich body fluids such as saliva, vaginal secretion, faeces, and menstrual blood. (Eirik *et al*., 2017) [19].

Scientists have also proved that there is potential for viral markers to be used for human identification. Targeting viral populations that are part of the core skin virome, such as eukaryotic infecting viral populations not affected by antibacterial agents, not only offers additional biomarkers to those already established but potentially offers increased stability and detection even in the presence of outside environmental contributory factors. Studies have demonstrated human gut viromes tend to be highly individual and temporally stable [17].

Ema *et al*. investigated the temporal human skin virome stability on three body locations (left hand, right hand, and scalp) in 42 study participants using five longitudinal samples taken across a 6-month time period. After the collection and purification of the sample, the resulting viral DNA sample was subjected to whole genome amplification (WGA) using multiple displacement amplification (MDA) implemented with the TruePrime WGA Kit. DNA was sheared using sonication to a mean length distribution of 600 bp. Sonication was performed using a Bioruptor with three cycles of 30s and 90s. Sonicated sample was later subjected to viral identification, taxonomic classification and mapping. Among the double stranded DNA viruses identified, the viral order *Caudovirales* was the most abundant order detected. Papillomaviruses and Polyomaviruses were the most abundant single-stranded DNA viruses [17].

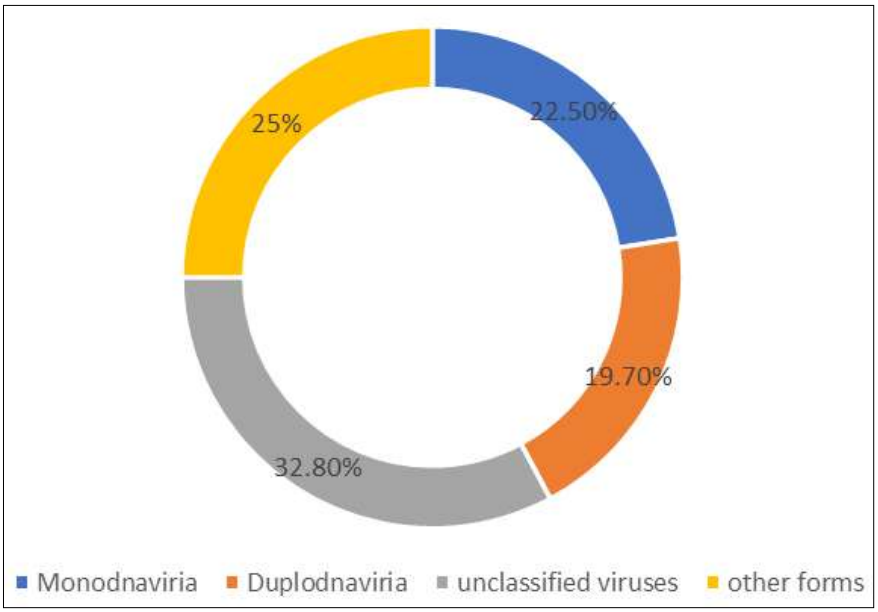

Fig 1: Distribution of viral taxonomy

Considering the most abundant viral families, one could draw clear distinctions between individuals, while still maintaining higher levels of similarity within an individual across multiple skin collection locations. This observation was statistically assessed by comparing the family-level community differences between subjects versus within subjects across locations. It was found that between subjects was significantly more different in viral family level diversity than within subjects across skin site sampling locations. These findings further support the notion that the human skin virome is an individual characteristic and potentially useful for discrimination between different subjects [17].

Determining geographical location

For geolocation determination, microorganisms can be used to distinguish between primary and secondary crime scenes as they differ in composition and function depending on geographical locations, climate (precipitation rates, altitude, temperature, and soil properties) and host properties or energy sources available in the environment (Bruna *et al*., 2023) [24].

Thomas *et al*., analysed oral and stool samples collected from young females grown and raised in four different geographical locations namely Barbados, Chile, South Africa and Thailand. They extracted the DNA from the samples using DNeasy Powersoil DNA Extraction kit. Microbiota profiling was performed targeting the V4 region of the 16S rRNA gene. They studied the participants and observed that half the study population (62%) had normal BMI, with the mean BMI in this range (22.6±5.5). On classifying the subjects based on their diet, 78% of subjects consumed starchy-heavy diet (≥ 4 days a week) of rice, bread and pasta. 66% of them frequently consumed vegetables and fruits and 49% of them frequently consumed dairy products. They also split the study population based on level of tobacco exposure with 51% of the population having never smoked, and 43% being exposed to second-hand smoke through living with a smoker. After computing the microbial diversity of stool samples, *Faecalibacterium* was observed to be most abundant in South African individuals and lower abundance in Thai individuals. *Pseudobutyrivibrio*, *Fusobacterium*, *Christensenellaceae_R-7_group*, *Ruminococcus_1*, *Escherichia-Shigella* are the five taxonomically abundant genera in stool samples obtained from subjects across these four geographical locations. On testing the behavioural factors, Chilean stool microbiota correlated with having never smoked, Pretorians (South African) stool microbiota correlated with BMI categories and the frequency of eating corn/cornmeal, Thai population's stool microbiota was correlated with living with a current smoker and being an ex-smoker and Stool microbiota of the Barbadian population did not significantly correlate with any of the lifestyle behavioural factors tested. The topmost dominant taxa identified among oral microbiota are two *Prevotellaceae* genera, *Pasteurellaceae_unclassified*, *Haemophilus*, *Streptococcus*, *Gemelia*, *Veillonella* and *Neisseria*. Oral microbiota could differentiate geographic locations with 16% variation between countries, where Chilean communities were the most geographically distinct, so that oral microbial community composition may vary according to the lifestyle of populations, as well as stool microbiota. In conclusion, the analysis of oral and stool microbiome can provide important information regarding the geographically location and the influence of populations' diets, behaviours, and lifestyles [23].

Bruna *et al*., reviewed research works involving dust samples, soil samples and other environmental samples to show that the microflora in these samples differed based on geographical location [24].

Fungi are now being used for soil discriminating and fingerprinting to define specific ecosystems. (Ricardo *et al*., 2009) [9]. Isolates of *Aspergillus fumigatus*, a saprophytic mould was studied by Ricardo *et al*., The isolates were genotyped using a previously described microsatellite-based single-multiplex PCR with eight short tandem repeat (STR) markers and an insertion/deletion polymorphism (SNP) of an adenine in marker MC6b. Based on the analysis of samples collected within hospital and outside the hospital, many differences were observed such as presence of additional nucleotides in samples collected outside the hospital [9].

Molecular techniques used to detect microbes of forensic importance

Gas Chromatography-Mass Spectrometry: The odour associated with decomposing remains is mainly caused due to the release of volatile organic compounds (VOC) due to microbial action. Terezie *et al*., studied volatile organic compounds released from three post mortem bacterial isolates namely *Bacillus subtilis*, *Ignatzschineria indica* and *I. ureiclastica*. The three bacteria were isolated from a pig carcass used for decomposition studies. These bacteria were inoculated onto a growth medium and cultured. The analysis was conducted on a Focus GC coupled with a Dual Stage Quadrupole II (DSQ II) Mass Selective Detector (MSD). Detection by GC-MS showed that the most abundant VOC produced by *Bacillus subtilis* was 1-butanol. *Ignatzschineria indica* produced dimethyl disulphide in large amounts and phenol being the most abundantly produced VOC by *I. ureiclastica* [4].

Multi locus variable number tandem repeat analysis (MLVA): This is a PCR based sub typing method which can distinguish different strains of a bacterium according to differences in the number of tandem repeated DNA sequences. Yun *et al*., studied 31 *E. coli* O157:H7 isolates using MLVA combined with automated capillary electrophoresis**.** These 31 DNA strains were isolated from cattle, sheep and pigs in China. Among 31stains, 29 different MLVA profiles were identified. Two isolates with identical MLVA profiles came from cattle and sheep, the other two strains from fowl and sheep had the same profiles [11].

Pyrosequencing

It is a form of DNA extraction method where nucleotides added by DNA polymerase can be detected by using a chemiluminescent enzyme detector. Jennifer *et al*., used pyrosequencing to describe temporal changes during carrion decomposition and identify members of the epi necrotic communities (organisms residing/moving on surface of decomposing remains), which are part of the necrobiome, which has the potential to be useful in PMI estimates. Bacterial community structure was determined by modified bacterial tagged encoded FLX amplicon pyrosequencing). PCR amplification of V1–3 regions of 16S rDNA was performed using the primers for bacterial populations Gray28F (5′ TTTGATCNTGGCTCAG) and Gray519r (5′GTNTTAC NGCGGCKGCTG). Bacterial communities were analysed at both phylum and family taxonomic resolutions [26].

Conclusion

Microbes play an important role in determining post mortem interval, cause of death, individual identification, determination of body fluids and geolocation which helps in linking the crime scene with the victim and suspect. Various molecular techniques such as genome sequencing, pyrosequencing and hyphenated analytical techniques such as gas chromatography-mass spectrometry has resulted in the identification of various types of microorganisms belonging to different phylum on examining body fluids such as saliva, vaginal fluid and blood. Studies have also shown that viruses residing on skin surface can help distinguish one individual from another, fungal strains can be used to identify body's geolocation of origin, algae such as diatoms can help determine cause of death and bacterial population help determine post mortem interval. This way, identification and determination of microorganisms help investigators in solving a criminal case.

Conflict of Interest

Not available

Financial Support

Not available

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