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Investigating the Role of Human Microbiome Profiles in Geographic Identification from Body Fluids

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Abstract

The human microbiome is a complex community of microorganisms which is residing inside As well as outside the human body. This study focuses on the potential of human microbiome Analysis from forensically relevant body fluids for geographic identification. Geographic origin Identification is a crucial aspect in forensic investigations which aids in linking individuals to The scene of crime, a connection between the victim & culprit, in differentiating of primary and Secondary crime scenes. The human microbiome is influenced by diet, lifestyle, climatic Conditions, geographic location so these contribute to the variation of microbial community From individual to individual, location to location as well as the communities will be different And unique in each body fluids. Therefore, the study aims to contribute to the expanding field Of forensic microbiology by providing a tool in investigating geolocations and thereby Enhancing the investigative capabilities in criminal justice system & it is done by using the Microbial communities to identify a geolocation & also by using these communities as Microbial markers in identification of the forensically important body fluids. This study Employed the combination of gram staining & biochemical test which offers a robust and Efficient approach for bacterial identification and classification. The study focused on Identifying the bacterial strains that exhibit significant and reproducible associations with Specific geographic locations. The results showed that the human microbial profile have the Potential to link an individual to a specific location and that the bacterial species can act as Microbial markers in identifying the body fluids. The findings also shed light on the intricate Interplay between human microbial flora, environmental exposures, and microbial ecology Which offers a broader implication for the public health and in understanding human population Migrations. However, application of more sophisticated techniques can enable to delve deeper Into microbial behaviour and for their identification.

Keywords: Human microbiome, Forensic microbiology, Geographical location, Thanatomicrobiome.

1. INTRODUCTION

Forensic science serves as a critical tool in the pursuit of justice, bridging the gap between Science and law enforcement. Whether it is a small town or a developed city, forensic science Has a major role in providing scientific as well as technical assistance to the court, to the Prosecutors and to the scene investigators. From burglaries to murders, forensic science not Only becoming resource used after the crime has been committed but also contributing to the Prevention of future crimes. By applying scientific principles and techniques, forensic science Aids in solving crimes, analysing evidence, and unveiling the

truth behind complex cases. From Examining fingerprints to decoding DNA and analysing digital footprints, this multidisciplinary Field plays an essential role in criminal investigations.The significance of forensic science lies not only in identifying perpetrators but also in Exonerating the innocent, ensuring fairness in the justice system. With advancements in Technology, forensic methods have become more precise and reliable, making them Indispensable in modern crime-solving efforts. Much has happened in the recent years with the Increasing knowledge in technology which is beneficial to the investigators to prove a fact in Question, but the same technology is used by the perpetrators in committing crime which makes It difficult for unveiling the truth. The advancements of technology and its developments have Provided the forensic science with many tools, devices and other applications which allow Forensic a better and optimal acquisition of data, their faster processing and substantially Improving the scientific investigation of crime. Some of the recent advances include DNA Phenotyping for predicting physical traits, AI powered digital forensics, the introduction of Techniques like 3 D imaging and virtual reality for crime scene reconstruction. Along with these Advancements forensic microbiology as well as proteomics also has significantly taken part in The field of criminal investigation. As technology infiltrates in every aspect of our day to day Lives, solving crimes has become advanced. From retinal scanning to trace evidence ananalysi It is vast fast field which is growing day by day. According to the studies conducted in 2024, The major milestones in the field of forensic science includes automated firearm identification, Blood pattern analysis software, sensitive detection methods, omics techniques, carbon dot Powders, nanotechnology, foldscope etc

MICROBE AS A TOOL FOR GEOLOCATION

“Every contact leaves a trace” is the most important and most used axiom in the field of forensic Science, which was first established by Sir Edmond Locard in the early 20 th century. This Statement has been applied to every forensic field by forensic investigators, and it can also be Applied to the forensic microbiology. If a certain location contains a characteristic microbiota That is different from other locations, we can analyse a person’s microbiome and could possibly Establish where they have been, which is precisely the principle behind microbial geolocation Analysis.For geolocation analysis microorganisms can be used to distinguish primary and secondary Crime scenes, to locate clandestine graves and identify the culprit. These associations are made Possible because microbial communities differ in composition and their function depending onThe geographical locations, climate which includes precipitation rates, altitude, temperature and Soil properties and host properties or energy sources which is available in the environment. The Association between the victim or suspects microbiomes can establish the links between them And to the crime scene in cases of human trafficking or source of disease. Microbial geolocation in forensic science leverages the fact that microbial communities vary According to each geographic locations and this fact can be incorporated while investigation Which can provide crucial information’s, and this can be used in various purposes such as:

1. microbial communities as tracers: different environments, including soil, water, and Even human microbiomes harbour unique microbial communities.
2. Geographic variation: the microbial communities are influenced by factors like climate, Soil type, altitude, and human activity which leads to geographic variations.
3. Forensic applications: From the analysis of microbial samples collected from a crime Scene, suspect, or even from the victim’s forensic scientists can potentially determine

The geographic origin of these samples aiding in geolocation. For example, soil Examination, in this analysing the microbial composition of soil found on suspects Shoes and clothing can help determine where they were. From the human microbiome Of victim or suspect can potentially reveal their geographic origin or places they have Visited. Localization through animal microbiome is also possible i.e. to link a person to A crime based on shared microbes with their pets or animals they work with.

METHODS FOR DETECTION OF MICROBES OF FORENSIC IMPORTANCE

The identification and determination of microorganisms provide an aid in a criminal Investigation. So, for the purpose of identification various methods had been employed which Include basic staining methods to advanced molecular techniques.

O 1.6.1. Gram staining

It is a common method which is used to differentiate bacterial species into 2 major groups Based on the physical and chemical properties of the organism's cell wall. Based on the cell Wall properties they are classified into gram negative and gram-positive bacteria. The cell Wall of gram-positive bacteria has a thick peptidoglycan layer, and they retain the primary Stain crystal violet and appear in blue or purple colour under the microscope. In case of Gram-negative bacteria, the cell wall is made up of thin peptidoglycan layer and thick outer Layer, which is formed of lipids, they take up the secondary stain safranin and appear in red To pink colour under microscope.

Biochemical test

The biochemical test is used to identify organisms by analysing their biochemical properties. This test can help identify microbes that have similar size and shape but different biochemical Properties. Some of the examples of biochemical test include catalase test, oxidase test, citrate

Test, coagulase test, MRVP, indole test etc.

1. Catalase test: Determines if a bacterium can produce the enzyme catalase, which breaks Down hydrogen peroxide to oxygen & water.
2. Oxidase test: This test determines if a bacterium can produce cytochrome c oxidase Enzymes.
3. Citrate test: The test determines if a bacterium can use citrate as its main energy source And carbon.
4. Coagulase test: In this test determines if a microbe can produce the coagulase enzyme Which can cause clotting and agglutination.
5. Indole test: Test to determine if a bacteria can produce indole from tryptophan.

O 1.6.3. Molecular techniques

Various sophisticated techniques such as genome sequencing, pyrosequencing, and hyphenated Analytical such as gas chromatography mass spectroscopy has been widely used for the purpose of identification of various types of microbes belonging to different phylum.

1. Gas chromatography mass spectroscopy

It is a powerful analytical technique which combines the action of GC s separation capabilities Along with MS s detection and identification features to analyse volatile and semi volatile Compound enabling the identification of microbial constituents and metabolites.

2. Pyro sequencing

A type of DNA sequencing method which is rapid and accurate used for microbial identification and characterization, particularly for identification of bacterial species, distinguishing strains.

3. Multi locus variable number tandem repeat analysis (MLVA)

This is a PCR based sub typing method which distinguish between different strains of Bacterium according to differences in number of tandem repeats DNA sequences.

SAMPLE COLLECTION

The samples for the study were collected from 2 localities in the Thiruvananthapuram district, Thiruvananthapuram north & Thiruvananthapuram south. The samples for the project include body fluids namely blood, urine & saliva. All the participants were informed about the research, and a signed consent form was collected prior to sample collection. Sample collection forms were filled with details such as name and age of the participants, date of collection and type of sample collected. The blood, urine, and saliva samples were collected from a total of 10 healthy volunteers with no history of antibiotic exposure for past 2 weeks, 5 individuals from TVM north & 5 from TVM south. Laboratory professionals collected 2 ml blood samples from each person in labelled EDTA tubes. The participants were asked to collect their urine and saliva samples in labelled sample bottles. Urine and saliva samples were then kept in the freezer, and blood samples was kept in the refrigerator.

METHODOLOGY

4.3.1. Culture Media preparation

The nutrient agar media about 20 ml for each petri plate was prepared in a conical flask for a total of 15 plates. The prepared nutrient agar was then sterilised in the autoclave at 121 ℃ for 15 – 20 minutes. After sterilisation the prepared media was then poured to 15 sterilised petri plates. The plates are then kept still for 15 minutes in the laminar air flow to allow it to solidify. Once the plates are solidified, the samples were inoculated into the plates. Prior to inoculation labelled the backside of petri plate with credentials such as date, sample name and number, location from which samples collected, and which method is used to inoculate the sample. The entire sampling procedure was done inside laminar air flow.

4.3.2. Culture technique – saliva

Quadrant streak method was used to culture saliva sample.

- Using a Bunsen burner the inoculation loop was heated until red hot. It was allowed to cool before the use.
- Aseptically a loopful of sample was taken from the sample bottle and placed on the agar surface and a series of tight parallel streaks are made in the first quadrant (about ¼ of the plate).
- The loop was again heated in the flame and allowed to cool.

- The plate was rotated to 90 degree and lightly swept across the first quadrant and the loop was sterilized again. The plate was then rotated and repeated the same sweeping procedure.
- The plates are turned upside down and kept in incubation at 37℃ for 24 hours.

4.3.3. Culture technique – Urine & Blood

Spread plate was used to culture both urine and blood samples.

- Using a calibrated pipette, 100 µl of sample was placed in the centre of agar plate.
- Using a sterilised L Rod the sample was evenly spread on to the agar plate by gently rotating the plate.
- After each sample the L rod was shown to flame and rubbed with alcohol to prevent contamination.
- The plates were kept for incubation at 37 ℃ for 24 hours.

After incubation the plates are photographed with the help of white light illuminator. Colony count and colony morphology of each sample in plates were noted down. The colonies with different morphology were noted and labelled with colony numbers for subculturing.

4.3.4. Subculture – quadrant streak method

Based on the morphology of colonies, different colonies were selected for subculturing. After counting the morphologically different colonies, the number of plates to be made was calculated and the required number of media was prepared and plates were made.

- The inoculation loops are sterilised by heating them over a flame until they were red hot and cooled down before picking up the bacterial colony.
- One loopful of colony was transferred on to the agar surface and the mother inoculum was prepared.
- The inoculum was then distributed thinly over the plate by streaking it with loop in a series of parallel lines, in different segments of the plate.
- Culture plates are incubated at 37℃ in incubator for 24 hours.

4.4.SAMPLE ANALYSIS

4.4.1. Gram Staining

- Thin smears are made on clean, grease free slides and air dried.
- Fixation was done by passing the smears over a flame.
- Primary staining was done with pararosaniline dye crystal violet and kept for 1 minute and washed with sterile distilled water.
- Iodine solution (grams iodine) was used as mordant and after 1 minute washed with distilled water.
- Decolourisation was done with ethanol.
- Counterstaining was done with safranine and after 1 minute it was washed, and the slides were kept for drying.

- After the slides are dried, a drop of immersion oil was directly placed on over the smear and placed under 100x oil immersion objective for microscopic examination.

4.4.2. Biochemical Test

Indole test (I)

- 0.05 g of 1 % peptone was dissolved in 5 ml distilled water for each test tubes was prepared and was poured into test tubes using 5 ml pipettes.
- The tubes with media were then then kept for sterilisation in the autoclave at 121℃ for 15 minutes.
- After sterilisation the bacterial colonies were inoculated into the tubes using inoculating loops and incubated at 37℃ for 24 hours.
- After the incubation period a few drops of Kovac's reagent was added through the side walls of test tube and observed the reaction.

Methyl red test (M)

- The MR VP broth prepared was kept for sterilisation in the autoclave at 121℃ for 15 to 20 minutes.
- The bacterial colonies were inoculated into the broth using sterilised inoculation loop and incubated at 37℃ for 24 hours.
- After incubation, 5 drops of methyl red indicator were added into the test tubes and observed the colour.

Vogues – Proskeur test(VP)

- Dissolved in 5 ml distilled water for each test tube and transferred it into test tubes using 5 ml pipettes.
- The MR VP broth prepared was kept for sterilisation in the autoclave at 121℃ for 15 to 20 minutes.
- The bacterial colonies were inoculated into the broth using sterilised inoculation loop and incubated at 37℃ for 24 hours.
- After incubation period 15 drops off Barrits reagent A (α naphthol) followed by 5 drops of Barrits reagent B(KOH).
- The tubes were gently Shaked and kept for 20 minutes, to observe any changes.

Citrate utilization test©

- 0.121 G of citrate agar was dissolved in 5 ml of distilled water and was prepared for all test tubes 5 ml in each tube and was kept for sterilisation at 121℃ for 15 to 20 minutes.
- The agar was poured into test tubes under aseptic conditions and kept in a slanting position till it solidifies.
- The bacterial colonies were inoculated into the citrate agar slants using inoculating loop.
- These slants were then kept for incubation at 37℃ for 24 hours.
- Observation was noted after incubation.

Catalase test

- A clean glass slide was taken and onto it 1 drop of sterile distilled water was added.
- A small amount of bacterial culture was smeared on to the clean glass slide using inoculating loop.
- A drop of 3% hydrogen peroxide was onto the smear.
- Observation was noted.

RESULT

The results obtained from the study showed that the bacterial population differ in each locality & it is possible to use human microbiome in the body fluids to determine a geolocation. The bacterial identification was based on the morphological features, staining properties, and the biochemical properties and the results were compared and interpreted with standard Tripathi et al., (2025), & Ananthanarayanan, R. (2006). The dominant strain of each body fluids was different, and they also differ in each location. The microbial communities in the body fluids were interpreted with respect to the microbial studies conducted in the blood by Martinez., & Wolk, D.M. (2016)., in urine by Imam, T.H. (2024) & in saliva by Nagakubo, D., & Kaibori, Y. (2023)[28].

The results are the outcome of differences in lifestyle, environment, diet, and cultural differences among the individuals and their locality. Different body fluids carry a different type of bacteria that can be identified through the methods used in this study. As far as till now microbes are not yet approved as evidence for individual identification, geolocation interference, and in PMI estimation. This field of study lacks a standardized operating procedure, and specifications for the sample extraction, packaging ,transport and preservation of microbial evidence.

Although only 2 geographical locations were included in this study, we can infer that it is possible to obtain crucial information about the location through body fluids. This study uses basic analysis methods for the identification of bacterial species, furthermore advanced methods such as sequencing can reveal a more conclusive result.

Establishing geolocation is a critical aspect of forensic science, so the aim of the study was to introduce a novel approach to identify geolocations based on the microbiome in body fluids. According to the work conducted by Clarke et al they used stool & oral samples geographically different populations and it was used to distinguish between them. From their study it was found that using the microbial species body fluid identification as well geographical location can be interpreted.in agreement with their study, the results of present study also indicate that the microbial species vary according to each geographical location. In the present study the 2 chosen locations were from the same district then also the microbial population was different in each body fluids. Individuals without preexisting health conditions were selected as participants, but from the analysis of blood sample from TVM N a pathogenic species of bacteria was identified. All the results were interpreted based on their morphological features, gram staining, and the biochemical properties of bacteria.

The total plate count showed that in both TVM S & TVMN more growth was observed in urine samples and least growth was observed in blood samples. The morphological features were determined by

comparing the observed results with standard Tripathi et al., (2025) and the most observed shape of bacteria are rod and cocci (spherical shaped).

From the analysis the bacterial strain in the blood from TVMS was found to be gram negative cocci Veillonella & in subcultures B1C2 & B4C1 gram positive rod Bacillus was found and from TVM N gram negative cocci Acinetobacter was found, both the results were interpreted based on the observed results in gram staining and biochemical test and compared with the study by Martinez & wolk, (2016) to precisely identify the strain .

The analysis of saliva samples from TVM S was found to be gram positive cocci Staphylococcus & in sample S2C1 was Streptococcus & in samples from TVM N Bacillus strain was identified which give both citrate negative and positive result in subcultures & the results was interpreted in comparison with the study by Nagakubo & Kaibori (2023).

Based on the study by Imam, (2024), the microbial strains present in the urine samples from TVM N was found to be gram negative Escherichia coli & in sample U3C2 & TVM S it is found to be gram positive Staphylococcus.

CONCLUSION

Since the year 2001 the interest in the field of forensic microbiology was increased and this led to the emergence of novel methods such as forensic microbiome geolocation, postmortem interval establishment etc. This study was aimed to contributes to the analysis of forensic geolocation using microbiomes. The findings demonstrate that the microbial communities in body fluid exhibit region specific patterns driven by many factors including environmental conditions & dietary. For instance, the individuals from rural region showed a high prevalence of infectious gram-negative bacteria in the blood samples. From the study conducted the observed results showed the presence of both gram negative and gram-positive bacteria was present in the body fluids. And microbiome community varied in each sample and in both locations. The total plate count showed that in both TVM S & TVMN more colonies was present in urine samples. Although bacteria exhibit variety of shapes but in this samples only 2 basic shapes spherical (cocci) and rod-shaped bacteria was observed. Bacterial strains identified were Staphylococcus, Streptococcus, Bacillus, Acinetobacter, Veillonella & Escherichia coli. The selected locations for the study were one urban area and another one was rural area. Although the sample size in this study is limited, it is possible to infer that the chances to obtain information about a location through human body fluids microbiome. This study could allow conclusions to drawn about an individual's geolocation. Also, with the application of advanced methods such as sequencing and electrophoresis the bacteria can be identified, and this could be used as microbial markers to identify the specific body fluid. How ever more research is needed to fulfil the gaps or the questions that remain unanswered in this field of study to understand and provide discriminatory information. Future studies should focus on standardizing methodologies and expanding reference databases to enhance the accuracy and reliability of microbiome-based geolocation in forensic as well as epidemiological contexts.

The implications of the findings are profound for both forensic science and public health. In forensic science microbiome-based geolocation can enhance the precision of identifying an individual's recent

geographic history, particularly in situations where traditional methods like GPS data or isotopic analysis are unavailable. In public health understanding region specific microbial signatures can give information about targeted interventions for diseases influenced by environmental conditions and location specific lifestyles or cultures.

Certain limitations were faced in this study which includes environmental factors such as seasonal changes and individual lifestyle variations (travel history & hygiene practices) were not fully controlled, which potentially introduced noise into the microbial signatures. Expanding the sample size to include underrepresented regions can enhance the robustness of microbial geolocation method. Additionally longitudinal studies tracking individual's microbial profiles over time could give more stability of the microbial signatures in the face of travel or environmental changes. The reliability of the microbiome for forensic applications needs to be further studied and validated and reliable error rates need to be established. Singh et al reported that they introduced the FMD by collecting 16 S rRNA gene sequencing data from publicly available databases to make inferences about geolocation identification. More databases need to be established for this field of study.

In conclusion this study established the microbiome-based geolocation investigation in body fluids as a promising tool in the field of forensic and public health. While challenges remain this research not only advances in understanding the human microbiome environmental interactions but also a new frontier in precision forensics and personalized public health strategies. Beyond the applications listed in this study, inclusion of forensic microbiology could bring breakthroughs in forensic pathology , toxicology and drug abuse testing.

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