Bacterial 16s rRNA and antibiotic susceptibility test - A potential marker for forensic individual identification on the basis of profession

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Abstract

Microorganisms are indistinguishable parts of the environment. They are distributed almost everywhere on the earth's environment and they form the inseparable bond with that particular environment. Similarly, they are distributed inside and outside our body and forms an inextricable conjugation and takes part in the day-to-day's body's internal and external functions. These bacteria also contribute to form a skin microflora of a person staying at the particular region and performing particular type of job. According to Locard's exchange principle when two objects come in contact with each other the exchange of matter takes place from one object to another object of the crime scene which is used in forensic science to connect the suspects with the crime scene. In accordance Pursuant to this fact the skin bacteria can be transferred from one object to another when touched on the scene of crime. Another advent that exactexacts sequences of DNA that encodes for 16s rRNA are not identical between organisms, but stays stable and unchanged throughout the life duration, can be used as an exploratory forensic individualization tool. Literature survey says that the ownership and of the article with the locality of the person, locality of the person, can be identified by Next Generation Sequencing (NGS) on the basis of communities of the skin bacteria. This research is focussed on proving the variations in 16s rRNA sequences clusters on the basis of professions, which can help in identification of the profession and can also give the idea of the region of the person. This attempt will also help in the cases where the degraded DNA is found and cannot be amplified for their short tandem repeats (STRs). In these cases, bBacterial identification can be a useful tool in identifying suspects and workplace or the profession of the suspect/s. In this paper, the results showing clus-

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tering of 16s rRNA sequences is shown clearly on the basis of professions. These clustering results were indicative of possibility of use of sanger sequencing for individualization can be used rather than using expensive analysis like pyrosequencing and NGS with careful sample collection from the scene of crime. The same strains that were used for sanger sequence were also tested for antibiotic susceptibility test, which showed that different professions show difference in antibiotic susceptibility.

Keywords: Forensic microbiology, Bacterial fingerprint, sanger sequencing, Rstudio, cluster analysis Introduction:

1 Introduction

With the advances and multidisciplinary approach has increased in almost all the scientific fields, forensic science is no exception. After 18th Sept 2001 US Anthrax attack in which the letters contained anthrax, mailed in US news agencies. Two suspects (Government scientists) freed as no exact evidence of attack was found. In later years two suspects i.e., Dr.Steven Hatfill and Dr.Bruce Edward Ivins were caught as in the analysis it was found that the spores in the letter were same as their laboratory. Eventually, Dr.Steven Hatfill was exonerated and the second suspect, Dr.Bruce Edward Ivins committed suicide by acetaminophen overdoes, on 29^{th} July 2008 (1).

Various parts of human body harbours bacteria, some of which are helpful and some of them are harmful if increased in the number. These bacteria makes microenvironment of that parts of the body and are responsible for maintaining the skin health (13). One of the studies suggests the presence of 39trillion microbes on and in our body (18). Most of the varieties of bacteria are found in colon part followed by skin (12). These bacteria are highly persistent in harsh weather conditions and are more likely to leave traces on the surfaces on which they come in contact with (20). These microbial communities are very specific to their geolocation and day to day habitat of the host human (15).

The adequate characterization can be made from objects which people have touched (5). Currently, various methods including high throughput sequencing, next generation sequencing, pyrosequencing are the methods of choices for studying skin associated bacteria which can be useful in solving forensic cases. In a recent study published by National Criminal Justice Reference Service in 2020, the characterization of microbial community left behind on the scene of crime was studied by creating the mock crime scene (2). Many previous researches revealed that the microbial communities inside and outside human body are dependent on the biotic and abiotic factors of the environmental, lifestyles, diet, biogeography and health (7). Superficial

skin microbes can be a potential and interesting trace evidence due to its dispersive predisposition, provided the careful collection of these organisms (22). One of the studies revealed the gender of occupants living in the house from the dust collected in AC vents. The results showed differences in the bacterial communities present in the AC vent dust of two genders, but no substantial difference was observed in fungal communities (21). A NIJ study of a mock crime scene revealed that bacterial communities of house occupants and pets, when compared with standards obtained from Hand and nasal swabs were similar before the entry of invaders (2). A study published by Elizabeth, et al in 2009 (17).

For comparison we collected samples from four different profession categories i.e., Garbage collector, College house-keeping, Morgue works and construction workers. 16s rRNA sequencing was carried out for the selected similar colonies between the profession and within the profession in R studio software. These selected sequences were then also subjected for antibiotic susceptibility testing by Kirby-Bauer Method(Hudzicki, 2016).

Even though the Bacterial community doesn't carry the permanent and fixed structure like genomic DNA, but can be used as trace evidence if the collection of the microbial traces is carried out appropriately. All the above findings lay a base to link a crime to the criminal's probable profession by rearing the culturable hand microbes. Apart from 16srRNA sequencing, to check if microbial antibiotic susceptibility of the colonies which are sequenced, can also help in profession wise identification.

2 MATERIALS AND METHODOLOGY

2.1 Collection of the microbes

Direct impressions of the 4 mortuary workers, 4 housekeeping staff of the college, 7 garbage collectors and four construction workers samples of hands were collected on the nutrient Agar plates and mobile phone surface swabs were collected on nutrient agar plates (10). The plates were kept for incubation at 37^{o}C for 24 hrs.

2.2 Selecting the similar species and amplification of 16s rRNA gene

All the incubated Petri plates from four different profession were checked for similar cultures on the basis of colony characteristic. Also, similar colonies were selected from samples belonging to different persons of the same profession as shown below in Figure. 1.

These selected similar colonies were identified, sub-cultured for obtaining pure cultures and subjected to 16s rRNA sequencing using the following forward primer (27F-AGA GTT TGA TCC

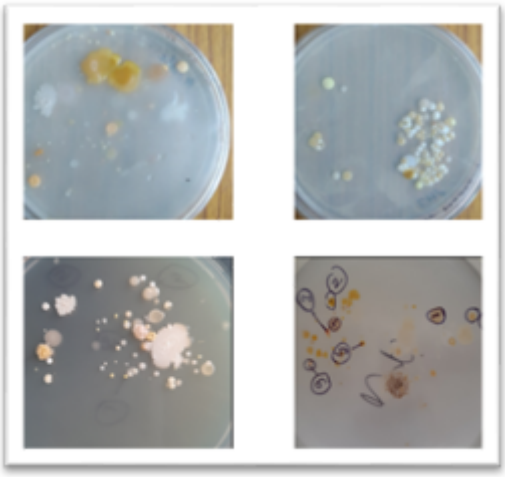

FIGURE 1

A representative image of Construction workers andMorgue workers colony

TGG CTC AG) and reverse primer (1492R-CGG TTA CCT TGT TAC GAC TT). The reaction was carried out in master mix of Taq DNA Polymerase Master Mix RED of 2x concentration. Total reaction volume of 25ul is prepared using 12.5ul of Master mix, 0.5ul of each reverse and forward primers, 2ul of extracted DNA (template DNA) and 9.5ul of denucleated water was used for amplification. The amplification was carried out in thermal cycler with the following conditions. Initial denaturation at 95^0C, followed by 35 cycles at 94^0C for 30 sec, 55^0C for 30 sec, 72^0C for 1min 30 sec and final extension step at 72^0C for 10min. The amplified product of 1.5K was sent for sanger sequencing.

2.3 Sequence processing

The sequences were processed in initially in Bio edit (9) software version number 7.2.5 released on 12^{th} November 2013 was used for making contigs of each reverse and forward sequences obtained from sanger sequencer. These contigs were then aligned using MEGA software using neighbour joining tree. The number of base substitutions per site between sequences are calculated. The analysis was conducted by using Tamura-Nei model (8). The rate variation among sites was modelled with a gamma distribution (shape parameter=1). This analysis was conducted on 24 sequences of 16s rRNA. All the ambiguous positions were removed for each sequence pair. Evolutionary analysis was conducted in MEGA software (19). The pair wise distances obtained from MEGA was plotted in R studio.

2.4 Data Analysis

The data was analysed using RStudio version 2022.02.3+492 "Prairie Trillium" Release (1db809b8323ba0a87c148d16eb84efe39a8e7785, 2022-05-20) for Windows Mozilla/5.0 (Windows NT 10.0; Win64; x64) AppleWebKit/537.36 (KHTML, like Gecko) QtWebEngine/5.12.8 Chrome/69.0.3497.128 Safari/537.36. Packages like Sangerseq, gdata, ggplot2, heatmap was used to generate the cluster dendrogram, PCOA plots for clustering of 16srRNA sequences obtained from four different professions and heat map for antibiotic susceptibility.

TABLE 1

Zone of clearance (in mm)

S.No.	Antibiotic	Code	Dosage used	R	I	S
1.	Clindamycin	CD10	10mcg	<28	–	28-34
2.	Linezolid	LZ	30mcg	20	–	21
3.	Levofloxacin	LE	5mcg	<13	14-16	> 17
4.	Rifampicin	RIF	5mcg	<16	17-19	>20
5.	Gentamicin	Gen10	10mcg	<12	13-14	>15
6.	Vancomycin	VA5	5mcg	<12	–	>12
7.	Ceftazidime	CAZ10	10mcg	–	–	–
8.	Ampicillin/Sulbactam	AS10	10/10mcg	11	12-14	15
9.	Ertapenem	ETP10	10mcg			
10.	Cefazolin	CZ30	30 mcg	14	15-17	18
11.	Trimethoprim	TR	10mcg	–	–	19-26
12.	Penicillin-G(P)	P	1unit	<26	–	>26

2.5 Antibiotic susceptibility

Susceptibility against 12 Antibiotic discs (CD10, LZ, LE5, RIF5, Gen10, VA5, CAZ10, AS10, ETP10, CZ30, TR and P) were checked for all the selected cultures which were given for sanger sequencing. (6). The inoculated broth for 24 hrs at 37^{o}C was spread on the nutrient agar plate with sterile autoclaved earbuds and total of 12 antibiotic discs disc were impregnated on 3 nutrient agar plate with same culture with each disc placed at maximum distance possible from each other(Hudzicki, 2016).

3 Result:

PCR amplification- As per the mentioned conditions the extracted genomic DNA was amplified for 16s rRNA gene. The genomic DNA confirmation was conducted by running 0.8% of electrophoretic agar prepared in 1X TAE buffer with 0.5ul of ethidium bromide in 1X TAE gel running buffer in mini submarine electrophoresis system. The PCR amplification was performed as per the discussed protocol.

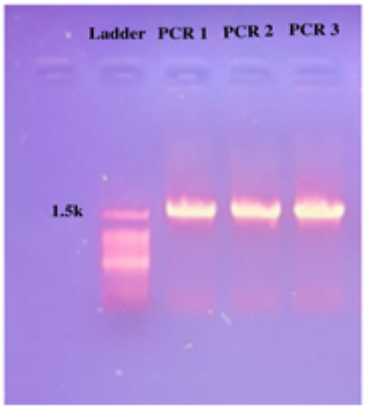

FIGURE 2

A representative image ofthe amplified 16s rRNA gene from three samples

3.1 Clustering of the sequences

The strains collected from four different professions i.e., Garbage collector, College housekeeping, Morgue works and construction workers. When the contigs of the sequences were analysed in MEGA-X software and R studio, it was found that most of the sequences of the bacterial samples collected from house-keeping falls under the same cluster. Construction worker's samples falls under another single cluster, and except one sequence remaining morgue workers sequences are clustered together. Whereas, wide distributed of the sequences throughout the cultures were observed in garbage collectors. This overlapping of the sequences throughout the clusters of housekeeping, morgue workers and construction workers could be due to the reason could be that they collect dry and wet waste from a wider area. These key research findings are showed in the form of PCoA plot and cluster dendrogram.

3.2 Principle coordinate analysis

The pair wise distances were calculated between each sequence discloses that most of the sequences could form 4 different clusters which represents different professions. Fig.2 also

represents that the garbage collects are distributed throughout all four clusters. PCoA1 is first highly variable among whole data and PCoA2 is second most highly variable in whole sequence data.

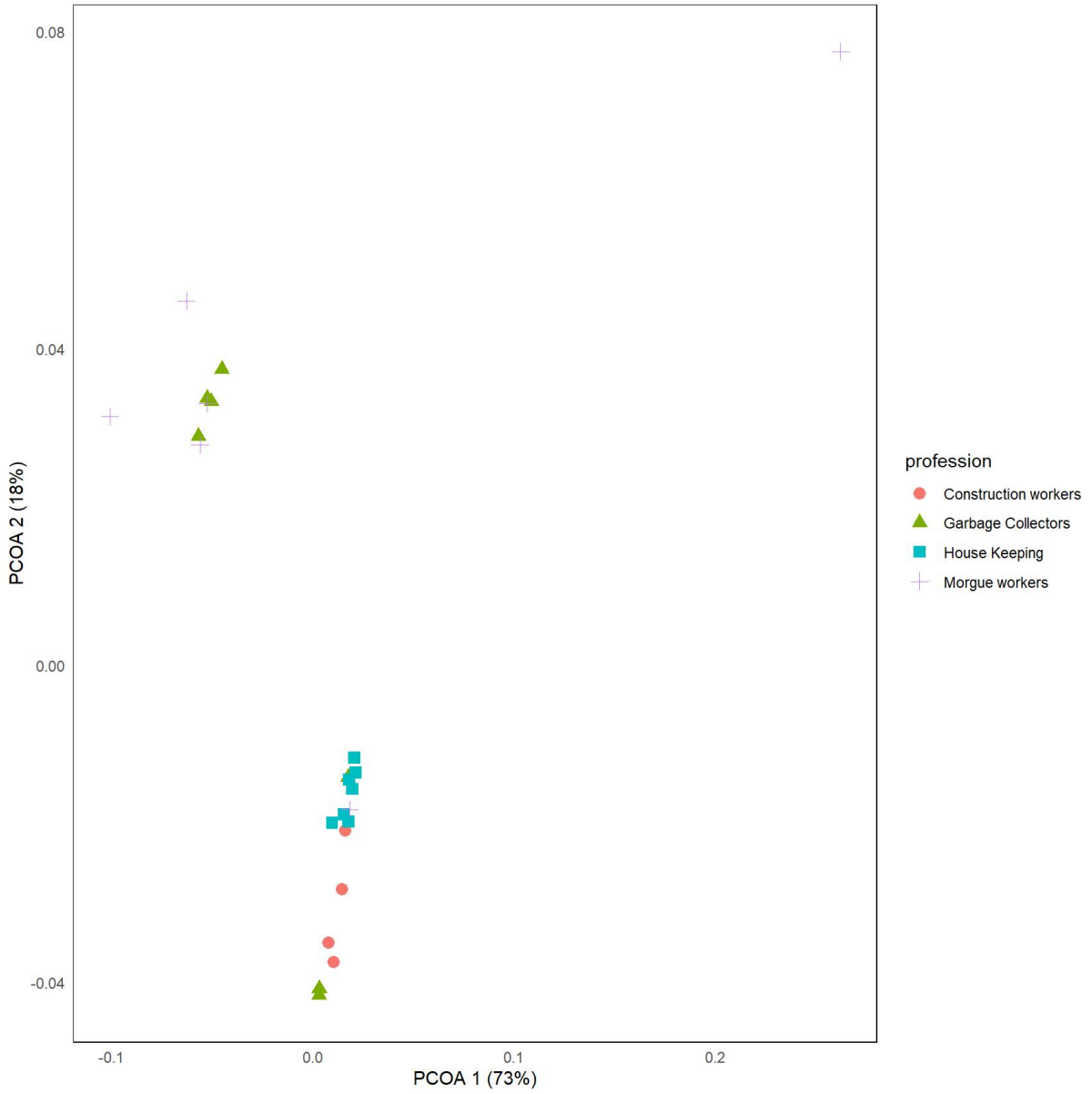

FIGURE 3

Clustering based on pairwise distances between the samples. These samples are represented by colouring clusters based on professions. Red circle represents construction workers, green triangle represents Garbage collectors, blue square represents housekeeping staff and purple plus sign represents morgue workers.

The test for antibiotic susceptibility for 8 colonies of house-keeping and 7 colonies of garbage collectors, 2 mortuary workers and 4 construction workers. Antibiotic susceptibility testing of

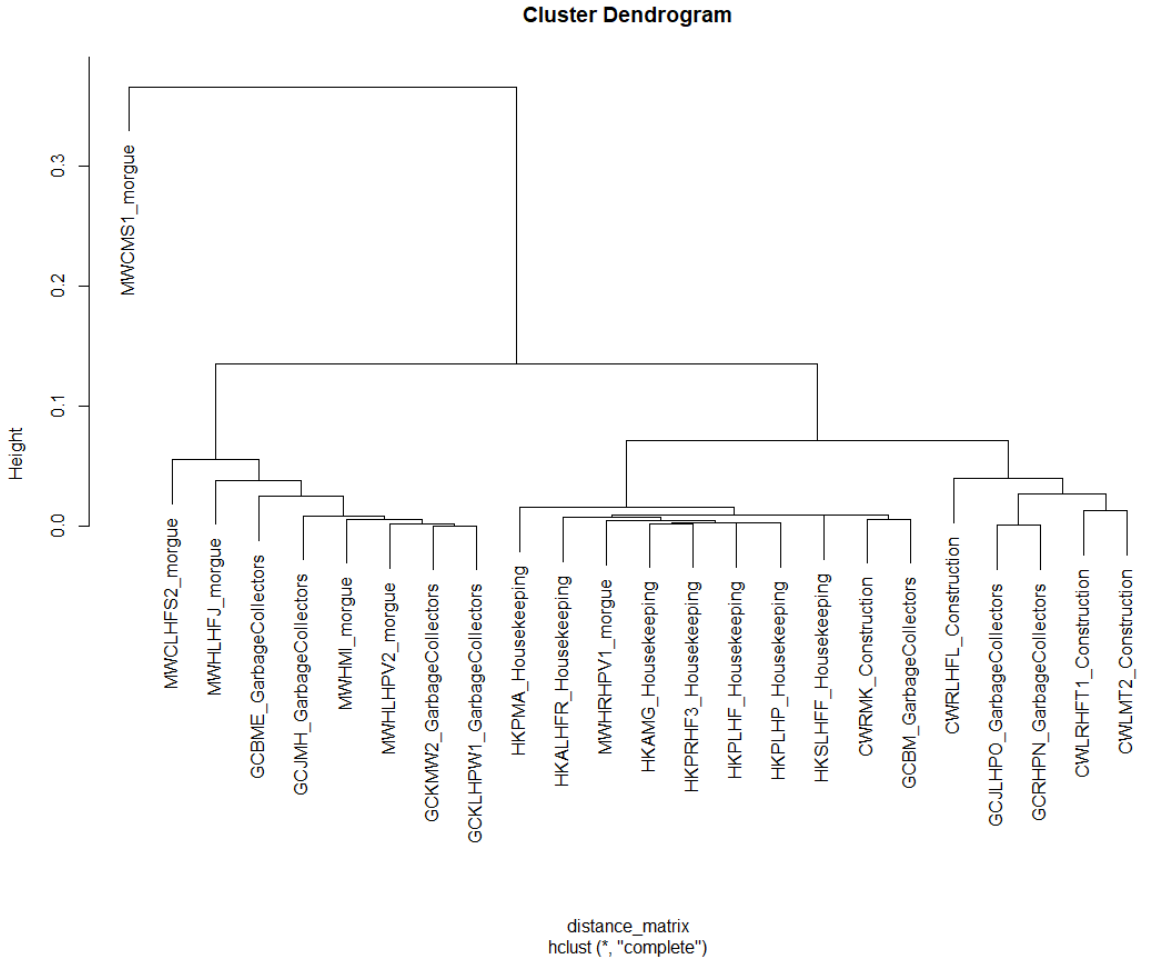

FIGURE 4

Diversity among the 24 sequences of four different professions. The codesstart with MW represents mortuary workers, GC represents garbage collectors, HK represents house keeping staff and CW represents construction workers. From left to right it is observed that the morgue workers, house keeping staff and construction workers are forming three clusters whereas garbage collectors are distributed throughout the group.

21 selected colonies indicates that most of the resistance is showed in the bacterial colonies of housekeeping staff and garbage collector staff followed by construction workers and mortuary workers.

4 Discussion:

Our research findings show even within a same city, 16s rRNA shows profession wise differences. These findings might not be able to identify the exact profession of a person but the position in cluster formation with other collected swabs from the crime scene or the suspects, might be a good indicative of the identification for inclusion or exclusion of the person

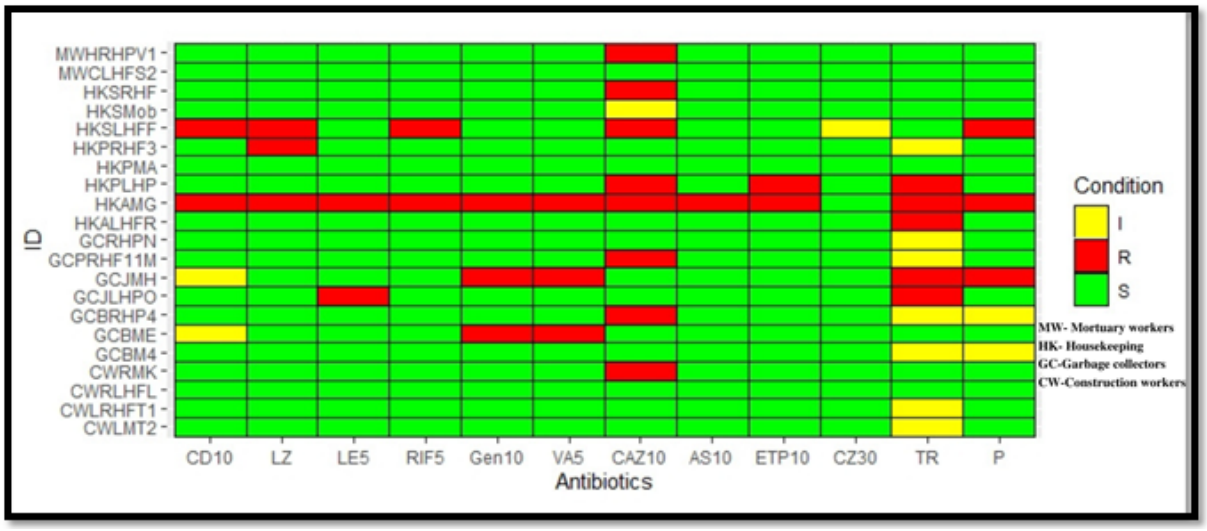

FIGURE 5

Antibiotic susceptibility of various bacterial strains where rows represent antibiotics and columns represents bacterial strains. There are four groups out of which group GC(garbage collectors) and MW(Mortuary workers) shows the highest susceptibility towards the most of the antibiotics followed by housekeeping and garbage collectors.

from the scene of crime. In construction workers and CWLMT2(mobile samples) is near to CWRHFT1(hand samples) and other housekeeping staff (represented by HK) also indicates the transfer of the microbes from hand to mobile. Similar type of experiment was conducted in which bacterial OTUs were of a mobile with its owner were found to be more similar than the mobile belonging to other person (23). Till date many studies were conducted to show that the hand or skin microbes can be transferred to the touched object and these microbial communities changes only on long term exposure into different environment (11; 16). In a research published by J.Craig Venter institute, the accuracy in collecting microbial traces as a evidence from the scene of crime, objects, saliva, stool, etc might lead to the perfect individualization of a person. Provided the samples collection techniques can be improved and practiced under expert's guidance (3). Many microbial researchers have also accepted the facts that the skin and gut microbiomes remains unchanged over a long period (14).

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