International Journal for Multidisciplinary Research (IJFMR)
E-ISSN: 2582-2160 ● Website: www.ijfmr.com ● Email: editor@ijfmr.com

Sequencing And Phylogenetic Analysis of Microbiota Isolated from Different Types of Street Food Samples by Using 16S rRNA Sequencing and Insilico Analysis

Sonali Priyadarsini[1], Pritismita Mahalik[2], Dharitri Priyadarsini[3], Rajat Kumar Nayak[4]

[1]M.Sc. Biotechnology, Reva University, Bangalore, Karnataka
[2]MSc, Biotechnology, Trident Academy of Creative Technology, Bhubaneswar, Odisha
[3,4]Senior Research Associate, SBio Science Pvt Ltd, Bhubaneswar, Odisha.

Abstract
Street food is the one which is sold by vendors with a stall or maybe shop. Here using five different varieties of street food (Panipuri, Bhel, Momo, Dahibara, Chocolate), using different microbial techniques. Different microbes (Gram +ve and Gram -ve) have been observed, and using medicinal plants like (Neem, Tulsi, Parijat) antimicrobial testing has been done which shows the zone of inhibition and calculated result show the high amount of antimicrobial agent is *Nyctanthes arbor-tristis* (Parijat) with a zone of inhibition of 15 mm, 26 mm, 22 mm, 27 mm, 13 mm, etc. for 5 different samples respectively. Studies have come across a previously unidentified microbial variety through the use of molecular methods mainly 16S rRNA sequencing. The identification of novel taxa, a shortage of sequences in nucleotide databases, the existence of species with similar and/or identical 16S rRNA sequences, and nomenclature issues brought on by the assignment of multiple genomovars to a single species or complex are all challenges that must be overcome. Sequence data are analyzed using insilico method. To describe the connections between different bacterial strains, phylogenetic trees can be employed. This can detect strains that are very closely linked to one another and show which bacterial strains have been transmitted to whom.

Keywords: Street food, 16S rRNA, Sequencing, *Nyctanthes arbor-tristis*, insilico analysis, MSA, Phylogenetic tree.

Introduction:

Food and agricultural organization (FAO,1998) define street food as ready-to-eat food and beverages that are sold by vendors and hawkers in streets and other public places for immediate consumption **[1]**. Trading in street food is widespread and diverse. It provides a significant source of income and a cheap source of food for millions of people. Street food comprises 64% of a person's daily intake of calcium, 50% of iron, 60% of vitamin A, 5% of thiamin, and 5% of vitamin C **[2]**. An estimated 40% of the overall energy ingested by people living in Bangkok originates from the approximately 20,000 street

food vendors. An average street supper in Calcutta, India, experienced around thirty grams of protein, 15 grams of fat, and 180 grams of carbohydrates, according to research **[3]**. A varied collection of microorganisms collectively referred to as microbiota can be commensal, pathogenic, or symbiotic. all multicellular organisms, including plants, both inside as well as outside. Microbiota, which additionally goes by the names of bacteria, archaea, protists, fungi, and viruses, have been confirmed to be essential for maintaining the host's immunologic, hormonal, and, metabolic homeostasis **[4]**.

[Fig.1: Different Street foods collected from Bhubaneswar market, Odisha]

Many ranges of microbiota have been isolated from Street food samples like Panipuri, Bhel, Momos, Dahibara, and Chocolates. As microbiota affect health, many antimicrobial agents help to degrade or stop the growth of the microbicide. An antimicrobial agent can be an antibiotic or may be different herbal plants, fruits, vegetables, and peels. Metals can also be used as antimicrobial agents. Many more antimicrobial agents can be used against the microbes and it is called antimicrobial activity. A zone of inhibition is observed on every agent which shows the working ability against the observed microbes. Molecular techniques have enabled researchers to uncover previously unrecognized microbial diversity. These techniques have facilitated the identification and characterization of bacterial species that were difficult to study using traditional culture methods **[5]**.

The computational methods have been tapped to analyze the genetic variability among the closely related species within a bacterial domain based on the comparative analysis of the 16S rRNA sequences. These approaches, comprising marker enzymes that employ in silico DNA restriction breaks down, phylogenetic framework trees, and species-specific conserved motifs, were all employed to recognize the genetic variability among the Helicobacter species in the current study. In order to confirm what was discovered from the 16S rRNA sequences, a phylogenetic analysis using the housekeeping gene was carried out **[6]**.

Phylogenetic trees are indeed useful tools for describing the connections between different bacterial strains and understanding their evolutionary relationships. These trees depict the genetic relatedness among organisms based on their shared ancestry and can provide insights into the transmission and spread of bacterial strains during outbreaks. To construct a phylogenetic tree, scientists analyze the genetic material, typically DNA or RNA sequences, of different bacterial strains. By comparing the sequences, they can identify similarities and differences that reflect the evolutionary history of the

organisms. It's important to note that phylogenetic trees are not the only tool used in outbreak investigations. Other epidemiological and molecular techniques, such as contact tracing and genomic sequencing **[7]**.

Materials and Methods:

Collection of Samples:

Street foods were collected from different vendors of local market, Bhubaneswar, Odisha. A total number of 5 samples were collected including Dahibara, Panipuri, Chocolate, Momo, and Bhel. These samples were then serially diluted to decrease the initial microbial count present.

Isolation of Bacteria:

Different Cultures were made (Primary culture, Sub culture, Pure culture, Broth culture) to isolate bacteria from the 5^{th} dilution (10^{-5}) of food samples. The identification of bacteria was done by colony morphology, Gram's stating, and 16S rRNA sequencing.

Anti-microbial Activity:

The methanol extracts of vegetable and medicinal plants were tested by the agar well diffusion method. The test microorganisms were seeded into respective medium by spread plate method. Impregnated with extracts were placed on test organism seeded on the antibacterial assay plates incubates at 37℃ for 24 hrs. The diameter of the inhibition zones measured in mm.

Sequence Analysis:

Five presumptive positive isolates were selected and further characterized using the 16S rRNA sequence analysis.

DNA Isolation:

Pure cultures of the target bacteria were grown overnight in liquid NB medium for the isolation of genomic DNA. In brief, the bacterial cultures were harvested by centrifuging it at 10,000 rpm for 5 mins at room temperature to collect the pellet. Pellet was washed with 1 ml TE (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA pH 8.0) and resuspended in 0.5 ml SET buffer (75 mM NaCl, 25 mM EDTA pH 8.0, 20 mM Tris-HCl pH 8.0) and incubated at 37°C for 1 hr. To this 1/10 volume of 10% SDS and 100 µg of proteinase K was added and incubated at 55°C for 1 hr. After incubation, 1/3 volume of 5 M NaCl and equal volume of phenol: chloroform: isoamyl-alcohol (25:24:1) were added and incubated at 37°C for further 30 mins. After centrifuging it at 5000 rpm for 15 mins at 4°C, aqueous layer was collected in a fresh eppendorf tube and precipitated with ethanol. The pellet obtained by centrifugation at 10,000 rpm for 5 mins at 4°C was washed with 70% ethanol by centrifugation at 10,000 rpm for 5 mins at 4°C, air dried and dissolved in TE buffer. The concentration and quality of genomic DNA was calculated by the spectrophotometer reading at 260 nm. Integrity of the DNA was checked by running in 1% agarose gel **[8]**.

PCR Amplification of 16S rRNA and Purification:

Extracted DNA templates were subjected to PCR using universal primers (Forward: 5'-AGAGTTTGATCMTGGCTCAG-3' and Reverse: 5'-CGGTTACCTTGTTACGACTT-3') for the

amplification of the 16S rDNA gene fragment. A reaction mixture containing approximately 1 ng of template DNA, 2.5 µl of 10X buffer, 20 pM concentration of each PCR primer, 10 mM dNTP mix and 1 U of Taq polymerase in a total of 25 µl was prepared by adding sterile milli Q water for all the reactions. Thermocycling conditions used was 94°C for 5 minutes for initial denaturation, then denature at 94°C for 30 seconds and annealing at 56°C for 30 seconds, and extension at 72°C for 45 seconds for 30 cycles. Final extension was done at 72°C for 5 minutes; hold temperature was at 4°C. The amplified product of 16S rRNA gene was purified with the PCR purification kit following the standard protocol as supplied by the manufacturer (HiMedia, India). The purification involved adding binding buffer to the PCR mix and centrifuging through filter tubes. The unincorporated nucleotides were removed by adding wash buffer and centrifugation at 10,000 rpm for 2 mins. The PCR products were eluted using elution buffer by centrifugation at 10,000 rpm for 1 min **[9, 10]**.

Computational Analysis:

The 16S rRNA gene sequence of the isolates with the non-redundant collection (GenBank, DDBJ, EMBL & PDB) of sequences was performed using BLAST **[7, 11]**. A pair wise alignment was carried out on both forward and reverse sequence, and the consensus sequence was obtained from the aligned sequence. The consensus sequence was pasted on blast at The National Center for Biotechnology Information (NCBI), to obtain closely related strains. Multiple sequence alignment, Phylogenetic tree analysis was done by using Clustal Omega tool to get the link and ancestors of isolated bacteria **[10,12]**.

Results and Discussion:

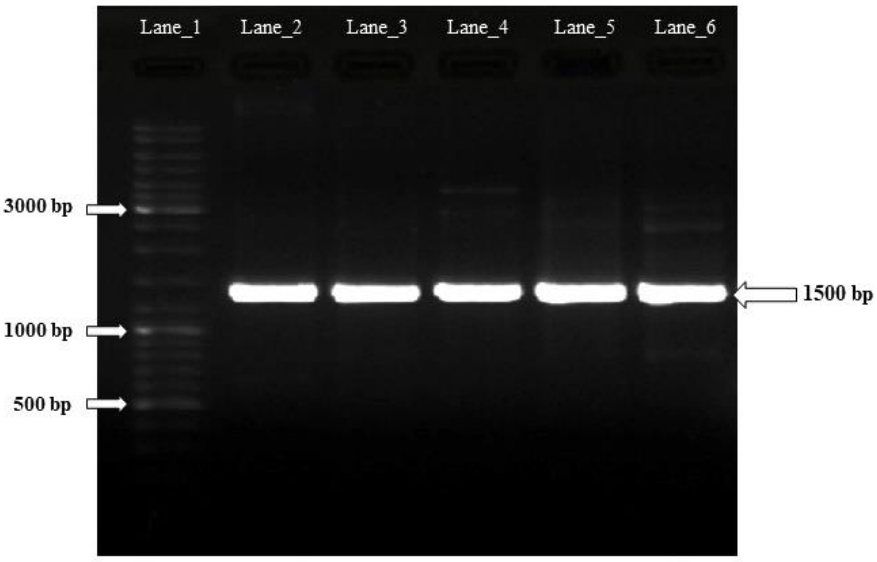
In the present study total 5 samples were collected from different public places of Bhubaneswar. In total 5 isolates were selected from 25 cultures and characterized through colony characters on specific media, and gram staining. Out of 5 isolates, it was found that one isolate is gram negative and other four are gram positive bacteria.

In our result (Table 1) the anti-microbial activity of the methanolic plant extract of Parijat creates maximum zone of inhibition (27mm) against the gram-positive bacteria (*Staphylococcus epidermidis*) and create minimum zone of inhibition (13mm) against the gram-negative bacteria (*Klebsiella pneumoniae*). In case of Tulsi, it creates maximum zone of inhibition (16mm) against the gram-positive bacteria (*Bacillus cereus*) and create minimum zone of inhibition (10mm) against the gram-negative bacteria (*Klebsiella pneumoniae*). Then we see that Neem extract, is most effective (14mm) on the *Staphylococcus epidermidis* and less effective (5.5mm) on the *Klebsiella pneumoniae*.

Isolates	Zone of Inhibition (mm)		
	Neem	Tulsi	Parijat
Staphylococcus aureus	12	15	15
Bacillus cereus	12.5	16	26
Enterococcus faecalis	7.5	11.5	22
Staphylococcus epidermidis	14	14	27
Klebsiella pneumoniae	5.5	10	13

[Table.1: Antimicrobial activity of methanolic extract of medicinal plants (Neem, Tulsi, Parijat) against five different isolates]

Identification of bacteria using Molecular technologies is known to have enabled investigators to examine both human and environmental microbiota more deeply than culture testing has allowed. Figure 2 is the agarose gel electrophoresis showing purified DNA before sequencing **[13]**. Lane 1 is DNA Ladder while lane 2 to 6 are that of the samples. The result of the 16S rRNA sequence analysis in this work shows the presence of *Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae* in the ready to eat street foods. The identification of these very important food borne pathogens up to the specie and sub species level may not have been possible with the traditional phenotypic methods of identification also deployed in this research work. So, the 16S rRNA gene is reported to be universal in bacteria and so relationships can be measured among all bacteria **[14]**.



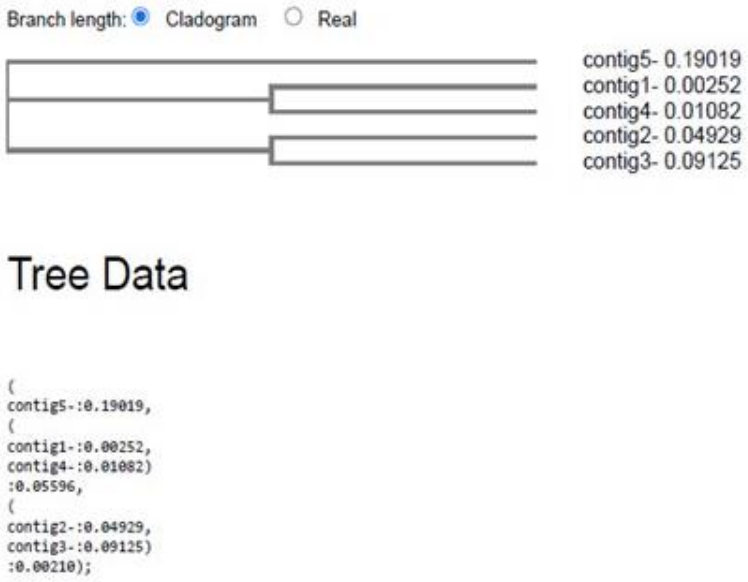
[Fig.2: Agarose gel electrophoresis showing purified DNA before sequencing]

The pair wise comparisons among different species showed a wide variability in Figure 3. The highest percentage similarity was observed between the species *Staphylococcus epidermidis* and *Staphylococcus aureus* isolates (96.67%), *Bacillus cereus* and *Staphylococcus aureus* isolates (89.50%) and *Bacillus cereus* and *Staphylococcus epidermidis* isolates (87.85%), and the least similarity was observed between *Klebsiella pneumoniae* and *Enterococcus faecalis* (71.60%).

PIM	*Klebsiella pneumoniae*	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Bacillus cereus*	*Enterococcus faecalis*
Klebsiella pneumoniae	100.00	74.93	74.37	75.64	71.60
Staphylococcus aureus	74.93	100.00	98.67	89.50	84.40
Staphylococcus epidermidis	74.37	98.67	100.00	87.85	84.26
Bacillus cereus	75.64	89.50	87.85	100.00	85.95
Enterococcus faecalis	71.60	84.40	84.26	85.95	100.00

[Fig.3: Percentage Identity Matrix of five sequences]

The phylogenetic tree (Figure 4) showed that *Klebsiella pneumoniae* was formed a monophyletic clade in the phylogenetic tree with 0.190 branch length. *Staphylococcus aureus* has a relatively deep subline with *Staphylococcus epidermidis*. According to our phylogenetic tree another closely related group consists of *Bacillus cereus* and *Enterococcus faecalis* was found.



[Fig.4: Phylogenetic tree of the bacteria species]

The major occurrence of *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Bacillus cereus*, may be due to poor personal hygiene of the vendors, unhygienic handling of foods, poorly cleaned dishes and use of raw vegetables **[15]**.

Conclusion:

Isolation of microbiota has been done using 5 different street food samples. Using different microbial cultures techniques, different microbes has been isolated. Using medicinal plants and vegetable extracts, the antimicrobial activity has been done. From the antimicrobial activity different zone of inhibition has been observed and by the calculation the highest anti-microbial agent from the medicinal plant is the *Nyctanthes arbor-tristis* (Parijat) with highest of 27 mm, whereas vegetables don't show any antimicrobial activity on the street food samples. Identification of microbiota has been done using a variety of methods one of them is 16S rRNA sequencing. The use of 16S rRNA sequences for studying bacterial phylogeny and taxonomy has very common for several reasons. Genetic similarity of the isolates by sequence analysis were identified. From the sequencing method we have observed species of *Staphylococcus aureus, Bacillus cereus, Enterococcus faecalis, Staphylococcus epidermidis, Klebsiella pneumoniae.* Using Clustal Omega software multiple sequence alignment has been observed with phylogenetic tree showing the ancestors of the sequence isolated.

Acknowledgment:

We are grateful to SBio Science Pvt. Ltd. for supporting us for well-established laboratory facilities and helping us in paper writing.

Conflict of Interest:
Nil

References

1. Das, M., Rath, C. C., & Mohapatra, U. B. (2012). Bacteriology of most popular street food (Panipuri) and inhibitory effect of essential oils on bacterial growth. *Journal of food science and Technology*, *49*, 564-571.
2. Muyanja, C., Nayiga, L., Brenda, N., & Nasinyama, G. (2011). Practices, knowledge and risk factors of street food vendors in Uganda. *Food control*, *22*(10), 1551-1558.
3. Steyn, N. P., Labadarios, D., & Nel, J. H. (2011). Factors which influence the consumption of street foods and fast foods in South Africa-a national survey. *Nutrition Journal*, *10*, 1-10.
4. Peterson, J., & Garges, S. (2009). Mikrobiom und Biofilme. *Genome Res*, *19*(12), 2317-2323.
5. Janda, J. M. (2023). The Molecular Technology Revolution and Bacterial Identification: Unexpected Consequences for Clinical Microbiologists. *Clinical Microbiology Newsletter*, *45*(6), 47-54.
6. Priyadarsini, S., Roy, D., Prusty, P., Sahoo, K. K., Priyadarsini, D., & Nayak, R. K. Docking of Alkaloid as a Source of Potential Anticholinesterase Inhibitors for the Treatment of Alzheimer's Disease. *IJFMR-International Journal For Multidisciplinary Research*, *5*(3).
7. Mahalik, P., Tanya, M., Pradhan, S., Routraya, S., Priyadarsini, D., & Nayak, R. K. Immunoinformatics Approach for the Prediction of Novel Peptide-based Epitope Vaccine Design Against Leishmania Donovani: a Computational Biology Approach. *IJFMR-International Journal For Multidisciplinary Research*, *5*(3).
8. Patel, J. B. (2001). 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. *Molecular diagnosis*, *6*, 313-321.
9. Magray, M. S. U. D., Kumar, A., Rawat, A. K., & Srivastava, S. (2011). Identification of Escherichia coli through analysis of 16S rRNA and 16S-23S rRNA internal transcribed spacer region sequences. *Bioinformation*, *6*(10), 370.
10. Rohinishree Yadahalli, S., & Pradeep Singh, N. (2011). Detection, identification and characterization of staphylococci in street vend foods—characterization of staphylococcus isolates. *Food and Nutrition Sciences*, *2011*.
11. Enabulele, S., & Ehiabhi, G. (2020). Characterisation of Multi-drug Resistant Salmonella spp. Isolated from Ready to Eat Vegetable Salads Using The 16S rRNA Sequence Analysis. *Journal of Basic and Applied Sciences*, *5*(1), 97-112.
12. Budiarso, T. Y., Amarantini, C., & Pakpahan, S. (2021). Biochemical identification and molecular characterization of Klebsiella pneumoniae isolated from street foods and drinks in Yogyakarta, Indonesia using 16S rRNA gene: -. *Biodiversitas Journal of Biological Diversity*, *22*(12).
13. andAkash Kumar, A. K. S. (2022). Medicinal value of the leaves of Nyctanthes arbor-tristis: A review. *Journal of Medicinal Plants*, *10*(1), 205-207.
14. Rai, A., Singh, A., & Sharma, A. (2022). Phytochemical analysis & antimicrobial activity of the leaves of Ocimum sanctum. *Journal of Pharmacognosy and Phytochemistry*, *11*(3), 174-176.
15. Rouhani, R., Heydari, S. B., & SAEIDI, S. (2023). Investigating the Antimicrobial Activity of Different Extracts of AzadirachtaIndica Plant on Antibiotic Resistant Escherichia Coli.