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ISOLATION & MOLECULAR IDENTIFICATION OF STAPHYLOCOCCUS EPIDERMIDIS S13 (PP654467) 16S RRNA GENE FROM POULTRY FECES SAMPLES OF LATUR REGION POULTRY FARM (MS) INDIA

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Abstract

The accumulation of poultry waste, comprising various materials such as broiler and layer carcasses, feathers, bones, blood, hatchery debris, and deceased birds, poses significant environmental and health concerns. These waste materials can lead to microbial contamination, foul odours, and the proliferation of pests like flies and rodents, thereby contributing to environmental pollution. The Samples were collected according to standard microbiological procedures during study period September 2022 to June 2023. 2-5 gm of samples were collected in sterile screw cap tube using sterile spatula and immediately transported with specially prepared ice box to the laboratory for further analysis. Biochemical assays confirmed the bacterial specimens, and molecular characterization was conducted through polymerase chain reaction (PCR) and sequencing of the 16S rRNA gene of *S. epidermidis*. The newly sequenced 16S rRNA gene sequences demonstrated 100% homology to *S. epidermidis*, as analyzed using the NCBI-BLAST tool. Phylogenetic analysis and nucleotide base composition studies were performed using 60 sequences of the 16S rRNA gene from various *Staphylococcus* isolates, including *Staphylococcus epidermidis*. For this purpose, 16S rRNA gene sequences were retrieved from NCBI in FASTA format. The phylogenetic analysis, conducted using the Maximum Likelihood method, revealed the relationships and percent similarity of the *Staphylococcus epidermidis S13 (PP654467)* 16S rRNA gene.

Key Words: Poultry Feces, 16S rRNA gene, BLAST Tool, *Staphylococcus epidermidis.*

Introduction:

The rapid growth of the poultry sector in India has indeed brought about numerous benefits, including increased production, economic growth, and job opportunities. However, alongside these advantages come significant challenges, particularly in managing the resulting poultry waste. The accumulation of poultry waste, comprising various materials such as broiler and layer carcasses, feathers, bones, blood, hatchery debris, and deceased birds, poses significant environmental and health concerns. These waste materials can lead to microbial contamination, foul odours, and the proliferation of pests like flies and rodents, thereby

contributing to environmental pollution. While efforts are being made to find ways to reuse poultry waste, such as converting them into fertilizers or animal feed supplements, there is a pressing need to address the potential health risks associated with these waste materials. One concern is the possibility that these waste deposits could serve as reservoirs for the multiplication of pathogenic microorganisms [Mathan Periasamy et al., 2013]. In intensive poultry production, newly hatched chicks are unable to maintain contact with their mothers, which results in a slower colonization of beneficial microbial flora in their intestinal tracts. Consequently, this makes them more vulnerable to infections from pathogenic microorganisms such as *Salmonella typhimurium, Escherichia coli*, and *Clostridium perfringens* [Mandana Salehizadeh et al., 2020]. This also results in the generation of large quantities of poultry waste, typically consisting of broilers and layers, feathers, bones, blood, hatchery debris, and deceased birds. These wastes pose significant environmental pollution issues due to microbial contamination, offensive odours, and the attraction of flies and rodents. In developing countries like India, proper disposal units for this waste are lacking. However, efforts are underway to repurpose these materials into beneficial products such as fertilizers and animal feed supplements. Given that these wastes consist of tissues and blood, we hypothesize that they may serve as a reservoir for the multiplication of various pathogenic microorganisms capable of causing severe disease outbreaks [Mathan Periasamy et al., 2013 and Adeoye GO et al., 1994]. This study aims to identify potential pathogens that can survive and thrive in poultry waste, which is a crucial step in understanding and mitigating the risks posed by these waste materials. By identifying and studying these pathogens and assess the potential hazards they pose to human and animal health and develop strategies to manage and minimize these risks. Overall, this research addresses an important gap in knowledge regarding the microbial ecology of poultry waste and contributes to efforts aimed at ensuring the sustainable and safe management of poultry production in India. Therefore, the present study was conducted to identify the potential pathogens that can survive in this poultry waste and pose a hazard.

Material and Methods

Study Area

The poultry waste samples were collected from the poultry shed in and around the Udgir, a suburban situated in the east side of Latur, Maharashtra State, India. Samples were collected from two poultry farm of different regions.

Sample Collection

The Samples were collected according to standard microbiological procedures [Microbiologia et al., 2010] and [Steubing, P. M. 1993] during study period September 2022 to June 2023. 2-5 gm of samples were collected in sterile screw cap tube using sterile spatula and immediately transported with specially prepared ice box to the laboratory for further analysis. The media and reagents used for the study such as Nutrient Broth, Nutrient Agar. The collected samples were processed aseptically in the laboratory. 200 mg of each sample were inoculated into the 100 ml of freshly prepared nutrient broth and incubated at 24 hrs at 37^0C. Isolation of chicken feces samples was done on sterile nutrient agar. The plates were incubated for 1 week at 37^0C. The colony characters were observed and gram staining was performed.

Molecular Characterization of *Staphylococcus sp*ecies

Positive samples of *Staphylococcus* species, identified through morphological and biochemical assays, were further analyzed using molecular characterization methods with a PCR-based assay. The details are provided below:

Genomic DNA Extraction from the selected cultures

DNA extraction from bacterial colonies was performed using a Sodium Dodecyl Sulfate (SDS)-based method [Goldenberger D 1995, Natarajan VP, 2016 and Mondeddu Kiran Kumar 2020]. The bacterial cell suspension was treated with a lysis buffer containing SDS, Trisaminomethane Hydrochloric Acid (Tris HCl), and Ethylene-diaminetetra-acetic acid (EDTA). Cell debris and other impurities were removed through several sequential steps involving centrifugation. Genomic DNA was precipitated using chilled ethyl alcohol and collected as a pellet by centrifugation. The pellet was then dissolved in TE buffer and stored at 4°C until further use.

Amplification of 16S rRNA gene using PCR

For the PCR reaction, the total reaction volume was 50 µl, containing 5 µl of DNA template, 1U Ampl Taq DNA polymerase, 10 pmol of each primer (forward and reverse, purchased from Sigma-Aldrich, Hyderabad), 200 µmol of each Deoxyribonucleoside triphosphate, 1.5 mmol of MgCl2, 10 mmol of Tris-HCl (pH 8.8), 50 mmol of KCl, and 0.1% Triton X-100.

Local sequence Alignment

Basic local Alignment Search Tool (BLAST) was performed for the different isolates of *Staphylococcus epidermidis S13 (PP654467)*16S rRNA gene sequence retrieved from NCBI to identify the homology or similarity its relatives in different isolated of *Staphylococcus epidermidis S13 (PP654467)* using the online NCBI-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). This software takes the data in FASTA format and produces the BLAST table.

Phylogenetic Analysis

Phylogenetic analysis of the 16S rRNA gene sequence of *Staphylococcus epidermidis S13 (PP654467)* was conducted using Maximum Likelihood methods with MEGA7. The software generated phylogenetic trees illustrating the ancestral relationships among the sequences. Sequences within the same cluster were found to be closely related.

Result and Discussion

Staphylococcus epidermidis S13 (PP654467) strain 16S rRNA gene, complete sequence were retrieved from the NCBI in FASTA format. The Sequence of the (PP654465) is as the following: PP654465.1 *Staphylococcus epidermidis S13 (PP654467)* strain S11 16S rRNA gene, complete sequence.

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CTAATACCGGATAATATATTGAACCGCATGGTTCAATAGTGAAAGACGGTTTTGCTGTCACTTATAGATG
GATCCGCGCCGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCGTAGCCGACCTGAGA
GGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCC
GCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTT
ATTAGGGAAGAACAAATGTGTAAGTAACTATGCACGTCTTGACGGTACCTAATCAGAAAGCCACGGCTAA
CTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGC
GTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAA
CTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACC
AGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGC
AGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGA
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCC
TCTGACCCCTCTAGAGATAGAGTTTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAA
GTTGGGCACTCTAAGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCC
CCTTATGATTTGGGCTACACACGTGCTACAATGGACAATACAAAGGGTAGCGAAACCGCGAGGTCAAGCA
AATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAACTCGACTATATGAAGCTGGAATCGCTAGT
AATCGTAGATCAGCATGCTACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGA
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Fig. 1: Specific set of primers of *Staphylococcus epidermidis S13 (PP654467)* strain S13 16S rRNA gene Sequence

Specific primer of *Staphylococcus epidermidis S13 (PP654467)* strain 16S rRNA gene sequence was designed using Primer 3 (V040) tool, as illustrated in Figure 1. We obtained a set of primer for 16S rRNA sequence of covering 203 nucleotide sequence length. The length of sense primer is 336 nucleotide (identified with red colour) and antisense primer length is 538 nucleotide (identified with Blue colour). We also check self-complementary alignment of specific primers sequences and PCR Protocols are describing in Table 1.

Amplification of *Staphylococcus epidermidis S13 (PP654467) strain* S11 16S rRNA gene Sequence using PCR

Prepared PCR reaction mixtures were prepared with methods. Amplification of 16S rRNA gene was performed in PCR Thermo cyclers (Applied Biosystems Ver 96) for 30 cycles by using run methods: denaturation at 95ºC for 50 seconds and extension at 72ºC for 1.80 min. The cycles were antedate by a denaturation step at 95ºC for 4 min, afterwards a extensioin step at 72ºC for 3.50 min.

Local sequence alignment

We have sequenced one isolate *Staphylococcus epidermidis S13 (PP654467)*16S rRNA and different isolates of *Staphylococcus epidermidis S13 (PP654467)*16S rRNA gene sequences were retrieved from the NCBI (https://www.ncbi.nlm.nih.gov/nuccore) in FASTA format and performed local sequence alignment by using online NCBI BLAST tool download BLAST table in that description of the gene, accession numbers, percent of similarity, e-value, etc. (Table 2).

```
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any   3' seq
LEFT PRIMER        336   20   59.90   55.00  6.00  2.00
GAAAGCCACGGCTAACTACG
RIGHT PRIMER       538   20   59.99   50.00  4.00  2.00
CATTTCACCGCTACACATGG
SEQUENCE SIZE: 1260
INCLUDED REGION SIZE: 1260

PRODUCT SIZE: 203, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00

    1 CTAATACCGGATAATATATTGAACCGCATGGTTCAATAGTGAAAGACGGTTTTGCTGTCA

   61 CTTATAGATGGATCCGCGCCGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAAC

  121 GATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACT

  181 CCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGC

  241 CGCGTGAGTGATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGGAAGAACAAATGTG

  301 TAAGTAACTATGCACGTCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCA
                                      >>>>>>>>>>>>>>>>>>>

  361 GCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGC

  421 GTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGA

  481 AACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCG
                                           <<<<<<<<<<<<<<<<<<<

  541 CAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGAT

  601 GTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT

  661 GAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTC

  721 CGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAG

  781 CGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCC

  841 TCTGACCCCTCTAGAGATAGAGTTTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCATG

  901 GTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTA

  961 AGCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGTTGACTGCCGGTGACAAACCGGAGG

 1021 AAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACA

 1081 ATGGACAATACAAAGGGTAGCGAAACCGCGAGGTCAAGCAAATCCCATAAAGTTGTTCTC

 1141 AGTTCGGATTGTAGTCTGCAACTCGACTATATGAAGCTGGAATCGCTAGTAATCGTAGAT

 1201 CAGCATGCTACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGA
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Figure 2. ***Staphylococcus epidermidis S13 (PP654467)*****16S rRNA gene Primer3 Output**

Table 1: Detail of Primers sequences used to amplify *Staphylococcus epidermidis S13 (PP654467)*16S rRNA gene

Gene	Primer type	Sequences	Primer Length	PCR Product Size
16S rRNA PCR Primer	Forward Primer	GAAAGCCACGGCTAACTACG	20	203
	Reverse Primer	CATTTCACCGCTACACATGG	20	

Table 2: BLAST table of 16S rRNA of *Staphylococcus epidermidis S13 (PP654467).*

Sr. No	Description	Max Score	Total Score	% identify	Accession No.
1	*Staphylococcus epidermidis S13 (PP654467)* strain S15 16S ribosomal RNA gene, partial sequence	1260	1260	100	PP654467
2	*Staphylococcus epidermidis S13 (PP654467)* strain 3039 16S ribosomal RNA gene, partial sequence	2327	2327	100	MT613456
3	*Staphylococcus epidermidis S13 (PP654467)* strain 607 16S ribosomal RNA gene, partial sequence	2327	2327	100	MT585400
4	*Staphylococcus epidermidis S13 (PP654467)* strain 314 16S ribosomal RNA gene, partial sequence	2327	2327	100	MT573037
5	*Staphylococcus epidermidis S13 (PP654467)* strain 304 16S ribosomal RNA gene, partial sequence	2327	2327	100	MT573035
6	*Staphylococcus epidermidis S13 (PP654467)* strain 468 16S ribosomal RNA gene, partial sequence	2327	2327	100	MT568663
7	*Staphylococcus epidermidis S13 (PP654467)* strain O47 chromosome, complete genome	2327	13911	100	CP040883
8	*Staphylococcus epidermidis S13 (PP654467)* strain 3620 16S ribosomal RNA gene, partial sequence	2327	2327	100	MT538492
9	Staphylococcus sp. strain H34 16S ribosomal RNA gene, partial sequence	2327	2327	100	MT275645

10	*Staphylococcus epidermidis S13 (PP654467)* strain 1910ICU248 16S ribosomal RNA gene, partial sequence	2327	2327	100	MT225635
11	Staphylococcus sp. strain 1910ICU161 16S ribosomal RNA gene, partial sequence	2327	2327	100	MT225634
12	*Staphylococcus epidermidis S13 (PP654467)* strain IGM4-15 16S ribosomal RNA gene, partial sequence	2327	2327	100	MT197256
13	*Staphylococcus epidermidis S13 (PP654467)* strain SESURV_p4_1553 chromosome	2327	13934	100	CP043804
14	*Staphylococcus epidermidis S13 (PP654467)* strain SESURV_p3_1362 chromosome	2327	13934	100	CP043801
15	*Staphylococcus epidermidis S13 (PP654467)* strain SESURV_p1_1200 chromosome	2327	13928	100	CP043796
16	*Staphylococcus epidermidis S13 (PP654467)* strain SESURV_p3_0825 chromosome	2327	16256	100	CP043792
17	*Staphylococcus epidermidis S13 (PP654467)* strain SESURV_p2_0614 chromosome	2327	13939	100	CP043788
18	*Staphylococcus epidermidis S13 (PP654467)* strain SESURV_p1_0557 chromosome	2327	13928	100	CP043777
19	*Staphylococcus epidermidis S13 (PP654467)* strain E73 chromosome, complete genome	2327	11595	100	CP035643
20	Staphylococcus sp. strain TH10 16S ribosomal RNA gene, partial sequence	2327	2327	100	MN049753
21	Staphylococcus sp. strain Fn-1a 16S ribosomal RNA gene, partial sequence	2327	2327	100	MK789749
22	*Staphylococcus epidermidis S13 (PP654467)* strain none genome assembly, chromosome: 1	2327	13906	100	LR735437
23	*Staphylococcus epidermidis S13 (PP654467)* strain none genome assembly, chromosome: 1	2327	13891	100	LR735432
24	*Staphylococcus epidermidis S13 (PP654467)* strain none genome assembly, chromosome: 1	2327	13945	100	LR735429

25	*Staphylococcus epidermidis S13 (PP654467)* strain IRL01 chromosome, complete genome	2327	11589	100	CP045648
26	Staphylococcus sp. strain NAS4 16S ribosomal RNA gene, partial sequence	2327	2327	100	MN519627
27	*Staphylococcus epidermidis S13 (PP654467)* strain IBK-11 16S ribosomal RNA gene, partial sequence	2327	2327	100	MN428237
28	Bacterium strain MTL5-15 16S ribosomal RNA gene, partial sequence	2327	2327	100	MH151226
29	*Staphylococcus epidermidis S13 (PP654467)* strain R2-11-3 16S ribosomal RNA gene, partial sequence	2327	2327	100	MK425675
30	*Staphylococcus epidermidis S13 (PP654467)* strain EnN-5 16S ribosomal RNA gene, partial sequence	2327	2327	100	MN220522
31	Staphylococcus sp. strain AN_A4C 16S ribosomal RNA gene, partial sequence	2327	2327	100	MK426631
32	*Staphylococcus epidermidis S13 (PP654467)* strain PL471 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence	2327	2327	100	MK015803
33	Staphylococcus warneri strain 1XD8_68 16S ribosomal RNA gene, partial sequence	2327	2327	100	MN081698
34	*Staphylococcus epidermidis S13 (PP654467)* strain ACP3 16S ribosomal RNA gene, partial sequence	2327	2327	100	MN068816
35	*Staphylococcus epidermidis S13 (PP654467)* NBRC 100911 DNA, complete genome	2327	13956	100	AP019721
36	*Staphylococcus epidermidis S13 (PP654467)* strain IAE188 16S ribosomal RNA gene, partial sequence	2327	2327	100	MK414887
37	*Staphylococcus epidermidis S13 (PP654467)* strain 4S 16S ribosomal RNA gene, partial sequence	2327	2327	100	MK681421
38	*Staphylococcus epidermidis S13 (PP654467)* strain BMC2N11_1 16S ribosomal RNA (16s ribosomal) gene, partial sequence	2327	2327	100	MH050411

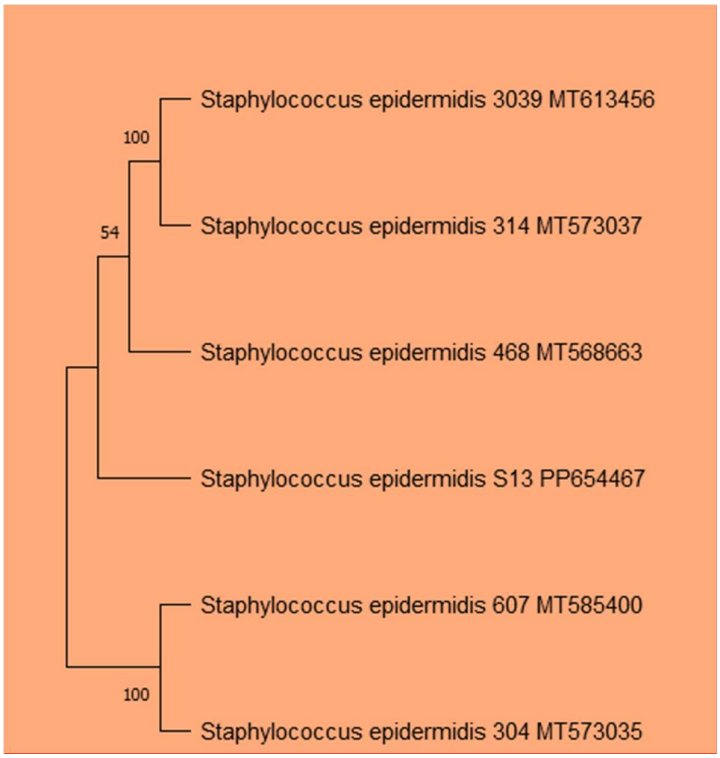
39	*Staphylococcus epidermidis S13 (PP654467)* strain VITAPRRKCU-3 16S ribosomal RNA gene, partial sequence	2327	2327	100	MH118521
40	*Staphylococcus epidermidis S13 (PP654467)* strain CDC120 chromosome, complete genome	2327	13917	100	CP034111
41	Staphylococcus sp. strain InS-282-1 16S ribosomal RNA gene, partial sequence	2327	2327	100	MF070515
42	*Staphylococcus epidermidis S13 (PP654467)* strain 26-63 16S ribosomal RNA gene, partial sequence	2327	2327	100	PP512860
43	*Staphylococcus epidermidis S13 (PP654467)* strain ISP111C 16S ribosomal RNA gene, partial sequence	2324	2324	99.92	MT605362
44	*Staphylococcus epidermidis S13 (PP654467)* strain PL493 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence	2324	2324	99.92	MK015816.
45	*Staphylococcus epidermidis S13 (PP654467)* strain PL452 16S ribosomal RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence	2324	2324	99.92	MK015789
46	*Staphylococcus epidermidis S13 (PP654467)* strain PCU5 16S ribosomal RNA gene, partial sequence	2324	2324	99.92	OR253227
47	Endophytic bacterium strain B14 16S ribosomal RNA gene, partial sequence	2324	2324	99.92	OM938280
48	*Staphylococcus epidermidis S13 (PP654467)* strain Au73 16S ribosomal RNA gene, partial sequence	2324	2324	99.92	MW534868
49	*Staphylococcus epidermidis S13 (PP654467)* strain DFI-22 16S ribosomal RNA gene, partial sequence	2324	2324	99.92	MZ951138
50	*Staphylococcus epidermidis S13 (PP654467)* strain m_5-1 16S ribosomal RNA gene, partial sequence	2302	2302	99.44	MN445604

This BLAST results clear that the newly sequenced (PP654467) *Staphylococcus epidermidis S13 (PP654467)* S13 16S rRNA gene have showing 100% and some are 99% identity with

different strains of *Staphylococcus epidermidis S13 (PP654467)* 16S rRNA genes was submitted at NCBI Genebank

Maximum Parsimony analysis of Taxa

The evolutionary history was inferred using the Maximum Parsimony method. The most parsimonious tree with length = 2319 is shown. The consistency index is 0.952135 (0.942989), the retention index is 0.909388 (0.909388), and the composite index is 0.865859 (0.857543) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (400 replicates) are shown next to the branches [Mathan Periasamy et al., 2013]. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (pg. 126 in ref. [Mandana Salehizadeh et al., 2020] with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). This analysis involved 6 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 1260 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [Adeoye GO et al., 1994]



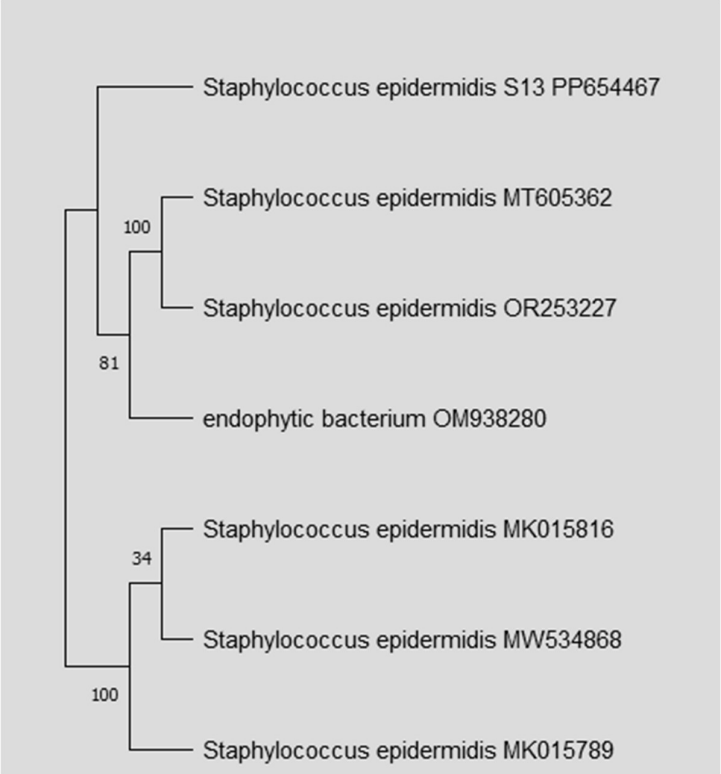


Figure 3: Molecular Phylogenetic Analysis of 16S rRNA gene using Maximum Likelihood Method. The evolutionary history were inferred using maximum pairs

Phylogenetic Analysis

Phylogenetic analysis included the newly sequenced *Staphylococcus epidermidis S13 (PP654467)* 16S rRNA and the NCBI database were searched for 16S rRNA sequences of the different isolates. Alignments of *Staphylococcus epidermidis S13 (PP654467)* 16S rRNA gene sequences were generated using the MEGA11 tool. Individual dendrograms were created using different methods, maximum likelihood methods. Phylogenetic groups and subgroups were defined according to the length and branching order of the integrate gene tree. The resulting groups were supported by high bootstrap values.

In Phylogenetic analysis, aligning nucleotide sequences is a crucial step, especially in studies involving genes from diverse taxa. Although it may seem evident that Phylogenetic analysis must start with proper data alignment, this process remains one of the most challenging and least understood aspects of molecular data analysis. Accurate alignment of genomic sequences is essential for constructing a phylogenetic tree. Additionally, phylogenetic analysis frequently involves examining molecular evolution for signs of directional selection [Mondeddu Kiran Kumar et al., (2020), Hofmann J et al., (2003) and Hsu HW et al., (2005)]. The evolution of the 16S rRNA was examined in various isolates of *Staphylococcus epidermidis S13 (PP654467)*, revealing adaptive changes in their sequences. The phylogenetic analysis of the 16S rRNA gene dataset from *Staphylococcus epidermidis S13 (PP654467)* produced a tree that aligns with the contemporary systematic understanding of the relationships among different species within the Staphylococcus genus, primarily based on DNA sequence homology [Figure 3].

To identify the genus of bacterial isolates collected from poultry feces samples in Latur region; we amplified and sequenced the 16S rRNA gene of the bacterial group. The resulting sequences were then compared against NCBI's 16S rRNA GenBank using BLAST [Mondeddu Kiran Kumar et al., (2020) and Altschul SF et al., (1990)]. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model [Tamura K et al., 1993]. The phylogenetic analysis performed using 20 sequences of 16S rRNA gene from newly and retrieved 16S rRNA sequences, including *Staphylococcus epidermidis S13 (PP654467)*. The consensus tree derived from the 10 most parsimonious trees is presented. Branches corresponding to partitions that appear in fewer than 50% of the trees are collapsed. The consistency index is 1.000000 (1.000000), the retention index is 1.000000 (1.000000), and the composite index is 1.000000 (1.000000) for both all sites and parsimony-informative sites (values in parentheses). The most parsimonious (MP) tree was generated using the Subtree-Pruning-Regrafting (SPR) algorithm [Tamura K et al., 1993]. The MP tree was generated using the Subtree-Pruning-Regrafting (SPR) algorithm with a search level of 0, where the initial trees were constructed through the random addition of sequences (10 replicates). The tree is drawn to scale, with branch lengths calculated using the average pathway method, expressed in units of the number of changes across the entire sequence. The analysis included 39 nucleotide sequences, covering codon positions 1st, 2nd, 3rd, and noncoding regions. All positions with gaps and missing data were excluded. The final dataset comprised 578 positions. Evolutionary analyses were performed using MEGA7 [Kumar S et al., 2016].

Phylogenetic trees were constructed using the Maximum Likelihood method for the sequences of newly isolated bacteria from poultry feces. The Maximum Likelihood method is the most suitable model for understanding the evolutionary history of an organism. Bootstrap consensus trees, inferred from 1000 replicates, were used to represent the evolutionary history of the analyzed taxa. The Maximum Likelihood trees were generated using the Nearest Neighbor-Interchange heuristic algorithm. All positions with gaps and missing data were excluded from the dataset using the Complete Deletion option. Phylogenetic analyses were performed in MEGA11, resulting in three major clusters in Figure 3, it is classified as Clade a (Red Colour) and Clade b (Green Colour). As illustrated in Figure 3, the newly sequenced 16S rRNA gene sequences were clustered with other *Staphylococcus epidermidis S13 (PP654467)* isolates in Clade a, exhibiting 100% homology as determined by local alignment analysis. Clade b Uncultured bacterial culture shown homology with Calde A 99%. Phylogenetic analysis of the 16S rRNA gene of *Staphylococcus epidermidis S13 (PP654467)*, using the Maximum Likelihood method, revealed the relationships and percent similarity of the 16S rRNA gene among different bacterial isolates, including *Staphylococcus epidermidis S13 (PP654467)*. Molecular techniques confirmed the predominant presence of *Staphylococcus epidermidis S13 (PP654467)* in the collected poultry feces samples.

Conclusion

Phylogenetic analysis of Staphylococcus species, including new isolates from Poultry feces sampels , revealed that they belong to the same strain and are affiliated with Staphylococcus equorum . In recent years, Next Generation Sequencing technologies have significantly expanded genome databases, resulting in a remarkable increase in the availability of sequenced genomes, both drafts and complete. However, accurately assigning sequenced strains to their corresponding species using accepted taxonomic tools is essential before conducting

comparative analyses with other genomes. The necessity for whole genome sequences of all type strains, which serve as the only species references publicly available in culture collections, is evident. In the present study, we identified and characterized *Staphylococcus equorum* from poultry feces samples using molecular biology techniques. New 16S rRNA sequences of Staphylococcus equorum isolates were aligned with those of other *Staphylococcus* species, and a phylogenetic tree was constructed to determine the molecular evolution and population structure of Staphylococcus species using bioinformatics tools. The phylogenetic association of the different *Staphylococcus equorum* species were demonstrated through Maximum Likelihood-based phylogenetic analyses of the 16S rRNA sequences. Our study showed evidence of positive selection of the 16S rRNA gene during the divergence of different *Staphylococcus* species isolates throughout evolution. These evolutionary changes have led to necessary modifications in the genetic control of ontogeny, which may have caused adaptive changes in the 16S rRNA gene.

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