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ISOLATION & MOLECULAR IDENTIFICATION OF *STAPHYLOCOCCUS EQUORUM* 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. equorum*. The newly sequenced 16S rRNA gene sequences demonstrated 100% homology to *S. equorum*, 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 equorum*. 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 *equorum* 16S rRNA gene.
Key Words: Poultry Feces, 16S rRNA gene, BLAST Tool,

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, 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 [4, 5] 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 sp.*ecies, 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, et al., 1995., Natarajan VP et al. 2016 and Mondeddu Kiran Kumar et al., 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. Mondeddu Kiran Kumar et al., 2020

Local sequence Alignment

Basic local Alignment Search Tool (BLAST) was performed for the different isolates of *Staphylococcus equorum*16S rRNA gene sequence retrieved from NCBI to identify the homology or similarity its relatives in different isolated of *Staphylococcus equorum* 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. According to Mondeddu Kiran Kumar et al., 2020.

Phylogenetic Analysis

Phylogenetic analysis of the 16S rRNA gene sequence of *Staphylococcus equorum* was conducted using Maximum Likelihood methods with MEGA11. 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 equorum strain S11 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 equorum* strain S11 16S rRNA gene, complete sequence.

```
CGGAGCTAATGCCGGATAACATTTGGAACCGCATGGTTCTAAAGTAAAAGATGGTTTTGCTATCACTTAT
AGATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATACGTAGCCGACC
TGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAAT
CTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACT
CTGTTATTAGGGAAGAACAAATGTGTAAGTAACTGTGCACATCTTGACGGTACCTAATCAGAAAGCCACG
GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAG
CGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTG
GGAAACTTGAGTACAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGA
  ACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAG
GATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGT
GCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGAC
GGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGA
CATCCTTTGAAAACTCTAGAGATAGAGCCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAACTTAGTTGCCAGC
ATTTAGTTGGGCACTCTAGGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATC
ATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGACAATACAAAGGGCAGCTAAACCGCGAGGTC
ATGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCG
CT
```

Specific set of primers of *Staphylococcus equorum* strain S11 16S rRNA gene Sequence

Specific primer of *Staphylococcus equorum* strain S11 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 341 nucleotide (identified with red colour) and antisense primer length is 543 nucleotide (identified with Blue colour). We also check self-complementary alignment of specific primers sequences and PCR Protocols are describing in Table 1.

```
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any    3' seq
LEFT PRIMER        341   20   59.90   55.00  6.00  2.00 GAAAGCCACGGCTAACTACG
RIGHT PRIMER       543   20   59.99   50.00  4.00  2.00 CATTTCACCGCTACACATGG
SEQUENCE SIZE: 1192
INCLUDED REGION SIZE: 1192

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

1 CGGAGCTAATGCCGGATAACATTTGGAACCGCATGGTTCTAAAGTAAAAGATGGTTTTGC

61 TATCACTTATAGATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAG

121 GCAACGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCC

181 AGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGC

241 AACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTATTAGGGAAGAACAA

301 ATGTGTAAGTAACTGTGCACATCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACG
                                          >>>>>>>>>>>>>>>>>>>>
361 TGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAG
    ************************************************************
421 CGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCA
    ************************************************************
481 TTGGAAACTGGGAAACTTGAGTACAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAA
    *****************************************<<<<<<<<<<<<<<<<<<
541 ATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACG
    <<<
601 CTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA

661 ACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAG

721 CACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGC

781 ACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGA

841 CATCCTTTGAAAACTCTAGAGATAGAGCCTTCCCCTTCGGGGGACAAAGTGACAGGTGGT

901 GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC

961 CCTTAAACTTAGTTGCCAGCATTTAGTTGGGCACTCTAGGTTGACTGCCGGTGACAAACC

1021 GGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTG

1081 CTACAATGGACAATACAAAGGGCAGCTAAACCGCGAGGTCATGCAAATCCCATAAAGTTG

1141 TTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCTGGAATCGCT
```

Figure 1. ***Staphylococcus equorum*16S rRNA gene Primer3 Output**

Table 1: Detail of Primers sequences used to amplify *Staphylococcus equorum*16S rRNA gene

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

Amplification of *Staphylococcus equorum 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 equorum*16S rRNA and different isolates of *Staphylococcus equorum*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).

Table 2: BLAST table of 16S rRNA of *Staphylococcus equorum*.

Sr. No	Description	Max Score	Total Score	% identify	Accession No.
1	*Staphylococcus equorum strain* S11 16S rRNA gene, partial sequence	2202	2202	100	PP654465.1
2	*Staphylococcus equorum strain* LS220 16S rRNA gene, partial sequence	2202	2202	100	MT409914.1
3	*Staphylococcus equorum strain* 82b3 chromosome	2202	13136	100	CP041697.1
4	*Staphylococcus equorum strain* JM39 16S rRNA gene, partial sequence	2202	2202	100	MN758799.1
5	*Staphylococcus equorum strain* ATCC 43958 16S rRNA gene, partial sequence	2202	2202	100	MN840040.1
6	*Staphylococcus equorum strain* CR24 16S rRNA gene, partial sequence	2202	2202	100	MN726519.1
7	*Staphylococcus sp.* strain CW523 16S rRNA gene, partial sequence	2202	2202	100	MK034279.1
8	*Staphylococcus sp.* strain XIXJ303 16S rRNA gene, partial sequence	2202	2202	100	MH801105.1
9	*Staphylococcus equorum strain* SP308 16S rRNA gene, partial sequence	2202	2202	100	MN229552.1
10	*Staphylococcus equorum strain* SP185 16S rRNA gene, partial sequence	2202	2202	100	MN229550.1
11	*Staphylococcus equorum strain* PL453 16S rRNA gene, partial sequence; 16S-23S rRNA intergenic spacer, complete sequence; and 23S rRNA gene, partial sequence	2202	2202	100	MK015791.1
12	*Staphylococcus equorum strain* PL455 16S rRNA gene, partial sequence; 16S-23S rRNA intergenic spacer, complete sequence; and 23S rRNA gene, partial sequence	2202	2202	100	MK015790.1
13	*Staphylococcus equorum strain* PL435 16S rRNA gene, partial sequence; 16S-23S rRNA intergenic spacer, complete sequence; and 23S rRNA gene, partial sequence	2202	2202	100	MK015773.1
14	*Staphylococcus equorum strain* PCM 2487 16S	2202	2202	100	MF678878.1

	rRNA gene, partial sequence; 16S-23S rRNA intergenic spacer, complete sequence; and 23S rRNA gene, partial sequence				
15	*Staphylococcus sp.* strain P152-L01a 16S rRNA gene, partial sequence	2202	2202	100	MN043757.1
16	*Staphylococcus equorum*subsp. equorum strain G129 16S rRNA gene, partial sequence	2202	2202	100	MK796070.1
17	*Staphylococcus sp.* strain 1031MS1 16S rRNA gene, partial sequence	2202	2202	100	MK713710.1
18	*Staphylococcus equorum strain* R3 16S rRNA gene, partial sequence	2202	2202	100	MK696401.1
19	*Staphylococcus equorum strain* c7 16S rRNA gene, partial sequence	2202	2202	100	MK696232.1
20	*Staphylococcus sp.* strain CP3V3-05 16S rRNA gene, partial sequence	2202	2202	100	MK534048.1
21	*Staphylococcus sp.* strain CP3V3-04 16S rRNA gene, partial sequence	2202	2202	100	MK534047.1
22	*Staphylococcus sp.* strain CP3V3-03 16S rRNA gene, partial sequence	2202	2202	100	MK534046.1
23	*Staphylococcus sp.* strain CP3V3-02 16S rRNA gene, partial sequence	2202	2202	100	MK534045.1
24	*Staphylococcus equorum*subsp. equorum strain H11M15 16S rRNA gene, partial sequence	2202	2202	100	MK253324.1
25	*Staphylococcus equorum strain* L-5 16S rRNA gene, partial sequence	2202	2202	100	MH727836.1
26	*Staphylococcus sp.* strain Asp_124 16S rRNA gene, partial sequence	2202	2202	100	MK408502.1
27	*Staphylococcus equorum strain* K5 16S rRNA gene, partial sequence	2202	2202	100	MF578770.1
28	Staphylococcus haemolyticus strain K1 16S rRNA gene, partial sequence	2202	2202	100	MF578766.1
29	*Staphylococcus equorum strain* Sp9 16S rRNA gene, partial sequence	2202	2202	100	MH493713.1
30	*Staphylococcus equorum strain* Sp3 16S rRNA gene, partial sequence	2202	2202	100	MH493707.1
31	*Staphylococcus equorum strain* 4-7 16S rRNA gene, partial sequence	2202	2202	100	MH491961.1
32	*Staphylococcus equorum strain* 3-7 16S rRNA gene, partial sequence	2202	2202	100	MH491956.1
33	*Staphylococcus equorum strain* 2-1 16S rRNA gene, partial sequence	2202	2202	100	MH491949.1
34	*Staphylococcus sp.* strain LJ40 16S rRNA gene, partial sequence	2202	2202	100	MG049771.1
35	*Staphylococcus equorum strain* CX-94 16S rRNA gene, partial sequence	2202	2202	100	MH368411.1
36	*Staphylococcus equorum*subsp. equorum strain SML_M139 16S rRNA gene, partial sequence	2202	2202	100	MG937677.1
37	*Staphylococcus equorum*subsp. equorum strain SML_M126 16S rRNA gene, partial sequence	2202	2202	100	MG937672.1
38	Uncultured bacterium clone Wall_B_2 16S rRNA gene, partial sequence	2202	2202	100	MF092670.1
39	Uncultured bacterium clone Wall_B_113 16S rRNA gene, partial sequence	2202	2202	100	MF092653.1
40	Uncultured bacterium clone Shelves_A_87 16S rRNA gene, partial sequence	2202	2202	100	MF092460.1
41	Uncultured bacterium clone Shelves_A_3 16S rRNA gene, partial sequence	2202	2202	100	MF092402.1
42	Uncultured bacterium clone Rinds_B_14 16S rRNA gene, partial sequence	2202	2202	100	MF092292.1
43	Uncultured bacterium clone Rinds_B_1 16S rRNA	2202	2202	100	MF092287.1

	gene, partial sequence				
44	Uncultured bacterium clone Filter_B_85 16S rRNA gene, partial sequence	2202	2202	100	MF091866.1
45	Uncultured bacterium clone Filter_B_35 16S rRNA gene, partial sequence	2202	2202	100	MF091820.1
46	Uncultured bacterium clone Filter_B_114 16S rRNA gene, partial sequence	2202	2202	100	MF091803.1
47	Uncultured bacterium clone Filter_B_110 16S rRNA gene, partial sequence	2202	2202	100	MF091801.1
48	Uncultured bacterium clone Filter_B_105 16S rRNA gene, partial sequence	2202	2202	100	MF091795.1
49	*Staphylococcus sp.* strain F-6 16S rRNA gene, partial sequence	2202	2202	100	MG266295.1
50	*Staphylococcus equorum strain* M8 16S rRNA gene, partial sequence	2202	2202	100	KY008748.1
51	*Staphylococcus equorum strain* JUb173 16S rRNA gene, partial sequence	2202	2202	100	PP723389.1
52	*Staphylococcus equorum strain* JUb153 16S rRNA gene, partial sequence	2202	2202	100	PP723387.1
53	*Staphylococcus equorum strain* JUb140 16S rRNA gene, partial sequence	2202	2202	100	PP723384.1
54	*Staphylococcus sp.* strain S9 16S rRNA gene, partial sequence	2202	2202	100	PP524728.1
55	*Staphylococcus sp.* strain AM6 16S rRNA gene, partial sequence	2202	2202	100	JN975939.1
56	*Staphylococcus equorum*partial 16S rRNA gene, isolate AT2RP19	2202	2202	100	LT797519.1
57	*Staphylococcus equorum*partial 16S rRNA gene, isolate HL2RS21	2202	2202	100	LT797508.1
58	*Staphylococcus equorum*subsp. equorum strain SML_M125 16S rRNA gene, partial sequence	2198	2198	99.92	MG937671.1
59	*Staphylococcus equorum strain* B9_consensus 16S rRNA gene, partial sequence	2198	2198	99.92	PP266359.1
60	*Staphylococcus equorum strain* NR_5-1 16S rRNA gene, partial sequence	2198	2198	99.92	OP862446.1

This BLAST results clear that the newly sequenced (PP654465.1) *Staphylococcus equorum* 16S rRNA gene have showing 100% identity with different *Staphylococcus equorum*16S rRNA genes was submitted at NCBI Genebank and such as *Staphylococcus equorum strain* LS220 MT409914.1 , *Staphylococcus equorum strain* 82b3 chromosome MN758799.1, *Staphylococcus equorum strain* ATCC 43958 16S ribosomal RNA gene, partial sequence MN840040.1, *Staphylococcus equorum* strain CR24 16S ribosomal RNA gene, partial sequence MN726519.1, *Staphylococcus sp.* strain CW523 16S ribosomal RNA gene, partial sequence MK034279.1, *Staphylococcus sp.* strain XIXJ303 16S ribosomal RNA gene, partial sequence MH801105.1, *Staphylococcus equorum* strain SP308 16S ribosomal RNA gene, partial sequence MN229552.1, *Staphylococcus equorum* strain SP185 16S ribosomal RNA gene, partial sequence MN229550.1. However, other uncultured 16S rRNA gene sequence with accession number MF092670.1, MF092653.1, MF092460.1, MF092402.1, MF092292.1, MF092287.1, MF091866.1, MF091820.1, MF091803.1, MF091801.1, MF091795.1 and MG937671.1., PP266359.1., OP862446.1 shown 99.92 homology with newly sequenced of 16S rRNA of *Staphylococcus sp.*

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 equorum* 16S rRNA and the NCBI database were searched for 16S rRNA sequences of the different isolates. Alignments of *Staphylococcus equorum* 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 equorum*, revealing adaptive changes in their sequences. The phylogenetic analysis of the 16S rRNA gene dataset from *Staphylococcus equorum* 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 according to Tamura K, Nei M. 1993. The phylogenetic analysis performed using 20 sequences of 16S rRNA gene from newly and retrieved 16S rRNA sequences, including *Staphylococcus equorum*. 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 according to Tamura K, Nei M. 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. According to Mondeddu Kiran Kumar et al., 2020. 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 (Yellow Colour), Clade b (Red Colour) and Clade c (Purple colour). As illustrated in Figure 3, the newly sequenced 16S rRNA gene sequences were clustered with other Staphylococcus equorum isolates in Clade a, exhibiting 100% homology as determined by local alignment analysis. Clade b Uncultured bacterial culture shown homology with Calde A 100% But Clade c shown 99.92% homology with Clade a. Phylogenetic analysis of the 16S rRNA gene of *Staphylococcus equorum*, using the Maximum Likelihood method, revealed the relationships and percent similarity of the 16S rRNA gene among different bacterial isolates, including *Staphylococcus equorum*. Molecular techniques confirmed the predominant presence of *Staphylococcus equorum* in the collected poultry feces samples.

Conclusion

Phylogenetic analysis of Staphylococcus species, including new isolates from Poultry feces samples, 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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