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Original Article

# PRELIMINARY CHARACTERIZATION OF BLOOD COAGULASE PROTEIN FROM A STAPHYLOCOCCUS STRAIN

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**ABSTRACT**

**Objective:** Coagulase, an extracellular protein produced by *Staphylococcus* species has blood coagulating properties. The objective of the present study was to isolate and characterize the purified coagulase, produced by *Staphylococcus sp.*Cobs2Tis23 (EU246837).

**Methods:** A *Staphylococcus* species was isolated from places around the Brahmaputra river of Assam, India and identified using 16S rDNA primers. The sequenced 1.5 Kb 16S rDNA was clustered hierarchically with reported 16S rDNA sequences available at the NCBI database using the Neighbour Joining Method. Coagulase extracted with ammonium sulphate precipitation was dialyzed. It was further subjected to Column chromatography. The molecular weight of the protein was determined by performing SDS and NON-SDS PAGE. The amino acid compositions of the fractions were analysed through High Pressure Liquid Chromatography. Homology modeling was done to determine the structure of the protein.

**Results:** Best fractions of column chromatography (with coagulase properties) were analyzed and their molecular weight found to be in between 16 kDa to 66 kDa. The amino acid compositions depict a high prominence of Aspartic acid, Glutamic acid and Proline. Theoretical 3D structure of the protein depicts the presence of the one cavity having highest volume and surface volume among five present which is the binding site for prothrombin. The lyophilised samples of the crude protein showed coagulating activity in just 30 seconds.

**Conclusion:** The bacterial strain was identified as: *Staphylococcus sp.*Cobs2Tis23 (EU246837). The protein isolated from the strain showed coagulating property as quick as 30 seconds.

**Keywords**: Coagulase, 16S rDNA, *Staphylococcus*, PCR.

## INTRODUCTION

North-East India is one of the richest biodiversity hotspots in the world. Likewise, Brahmaputra river banks along the southern corridor of Assam consisting of wide geographical and climatic diversity [1] are rich in biodiversity including microbial biodiversity. Earlier investigation involved isolating of the microbial strain from this biodiversity and identifying those [2]. Preliminary study mostly screening tests (microbiological) aptly identified the strain to be Staphylococcus [3]. This species of bacterial strain has been reported to have far reaching implications in clinical microbiological laboratories. The clotting of human plasma by a specific substance (Staphylocoagulase) produced by *Staphylococci Sp.* was investigated [4-5]. However, the ability of certain bacterial species to clot plasma was first reported in 1903 [6].

Also termed as Coagulase, Staphylocoagulase is an extracellular protein which reacts with prothrombin in the blood and enables further conversion of fibrinogen to fibrin, resulting in clotting of blood [7]. Coagulase is a traditional marker for identifying *S. aureas* in the clinic microbiology laboratory; however, there is no overwhelming evidence that it is a virulence factor, although it is reasonable to speculate that the bacteria could protect themselves from phagocytes and immune defenses by causing localized clotting. Characterizing one strain of Staphylococcus from another employs different approaches and techniques like antibiogram analysis, bacteriophage typing, plasmid analysis and protein electrophoresis. We employed the protein electrophoresis approach along with a dendrogram (MEGA 3.1) derived from sequence comparison to study the difference among the bacterial strain and also place our strain in appropriate clad. It has also been found that coagulase enzyme gene approximately contains between 1800bp to 2100bp [8]. Two forms of Coagulase exist [9], a bound form associated with cell (bound coagulase ) and a cell free form that is liberated into the culture medium (free coagulase). Thus the present study emphasize on isolating and characterizing purified coagulase produced by *Staphylococcus sp*.

## MATERIALS AND METHODS

### Isolation and Characterization of Bacterial strain

Isolation of bacterial strain was done by the method of Aneja [10] and it was screened through various biochemical procedures and isolates were maintained in nutrient agar slants (Himedia, India). The bacterial culture was prepared as per the method Sonohara [11]. Briefly; 6g Fluid soybean casein digest broth was prepared and inoculated with bacterial strain. Broth with strain was kept in a shaker at 100 rpm at 28°C for 48 hours. The bacterial strain was characterized by the Gram staining method [12]. A smear was prepared from the overnight culture grown at 37°C in Nutrient agar slants (Himedia, India). Glass slides, pre cleaned overnight in 95% alcohol, were used in the Gram staining process. The smear was prepared on the slide and allowed to air dry. The slide was heat fixed and stained. Gram stained slide was allowed to air dry and was examined under a Phase Contrast Microscope (magnification, 100X).

### Ammonium sulphate precipitation and dialysis

Crude protein was saturated with different concentrations of ammonium sulphate [13]. The precipitated protein obtained was dissolved in phosphate buffer (0.02M, pH 7.2) and dialyzed using dialysis membrane – 50 (Av. Flat width 24.26mm, Av. Diameter 14.3mm and the capacity approximately 1.61ml/cm, Himedia) against phosphate buffer (0.02M, pH- 7.2) to remove excessive salts and stored at 4°C.

### Column chromatography

The chromatographic separation of crude protein was done in column with a height of 0.25m, diameter 0.02m and area 1.5714cm2. The column was packed with Sephadex G-100 and equilibrated with 0.02 M phosphate buffer (pH 7.2). 2 ml of dialyzed protein was allowed to pass through the column. 5ml of fractions were collected until the sample was completely eluted out. Spectrometric readings were taken at 280 nm and the observations recorded and fractions were lyophilised for further tests.

**Blood Coagulating Activity, Bioassay**

Fisher [14] found that clotting took place within the range of pH 6.2 to 7. Blood coagulating property was determined by taking saline water which was taken as control and used concentration of ammonium sulphate dissolved in water as a second control. Crude protein isolated by ammonium sulphate precipitation was taken in a different slide. A bit of lyophilised protein fraction was placed on a fourth glass slide and a drop of fresh blood plasma was added to each. Blood coagulation time was recorded.

**SDS-PAGE and NON-SDS PAGE**

After lyophilisation, protein fractions were dissolved in sample buffer and subjected to SDS-PAGE as described by Laemmle 1970 [15] in gel slabs 0.75mm thick and 0.15m long. Initially, the run was allowed for 15 minutes at 15mA and then at 35mA for 3 hrs until the bromophenol blue marker had reached the bottom of the gel. The gel was stained with coomassie blue overnight and destained until the bands were clearly visible. Similarly Non SDS PAGE was also done to determine the molecular weight and have a comparative study of the whole protein and its peptides.

**HPLC fractionation**

Purifications of different individual amino acids were quantified from the protein fractions collected from column chromatography as per the method followed by Turnel et.al. [16] by using high-pressure liquid chromatography (HPLC) apparatus (Waters 600 controller, Waters inline degasser Symmetry C-18 column 5µm with height and width 4.6 x 250mm, setup with Waters TM 600 Pump and Waters 2489 UV visible detector). The flow rate was set up at 1 ml/min. Absorbance was taken at 280 nm. The 25µl protein sample was injected and the run was allowed for 20 minutes and the graph was recorded.

**Theoretical prediction of protein structure using bioinformatics tools**

The knowledge of a protein's 3D structure is normally required to completely understand its function. Thus the assumption of the structure of the specific protein isolated from the bacterial strain was generated using Homology modeling since no experimental structure is available for this protein. The process through which a tertiary structure was assigned to a given sequence was carried out in three steps, namely: template identification, template alignment, and model building. Finally, the produced model showed was assessed. The prothrombin binding sites were analyzed and the best cavity with highest volume and surface volume was selected [17]. Ramachandran plot analysis was done using Procheck to check bond angle, bond length, and error regions, of the protein structure. Further studies are still on the process with other homology methods to determine the final structure for confirmation.

**DNA extraction**

Prior to extraction of DNA, 5ml of pure bacterial colonies from 24 hours old bacterial culture was centrifuged and the pellet was re-suspended in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). Genomic DNA from bacteria was extracted as per the method of Ausubel [18]. The quality of the extracted DNA was checked by running it on 1% Agarose gel (High EEO). Quantization of genomic DNA was done by spectrophotometer (Model T80+UV/VIS, PG Instruments Ltd., UK) at 260nm.

**PCR amplification and sequencing**

The 16S rDNA sequencing of bacterial strains was done at Bangalore Genei, Bangalore, India. The approximately 1.5 kb 16S rDNA fragment was amplified using consensus primers and Taq DNA polymerase. The PCR product was bi-directionally sequenced using the forward, reverse and an internal primer.

The sequenced data were aligned and analysed for finding the closest homologue for the bacteria. Alignment was developed using a combination of NCBI GenBank and RAPD database. PCR Samples containing final volume of the 25µl reaction mixture containing 2.5 µl of 10x Taq buffer, 10mM of dNTP mix, 10 Picomol of primer, 1 unit of Taq polymerase (MBI Fermentas, USA) and DNA were amplified by a thermal cycler (Thermal Cycler Model 2720, Applied Biosystems, USA). The PCR conditions consisted of 45 cycles at 94°C for 1 min, 37°C for 1 min, 72°C for 2 min and 72°C for 5 min. The PCR amplified product was resolved on 1% agarose gel (High EEO). The gel was visualized and analysed with a Gel documentation system (UVP system, Cambridge, UK).

**Data analysis**

PCR amplified product data obtained were used to estimate the genetic similarity among different isolates on the basis of shared amplification products [19]. A dendogram was constructed by the Unweighted Pair Group Method with Arithmetic mean (UPGMA), [20] to group individuals into discrete clusters. The phylogenetic tree was made in MEGA 3.1 software using the Neighbour Joining method. The gel was visualized by staining with ethidium bromide and photographed under UV light by a Gel Doc-It Digital Imaging System (UVP Ltd. UK).

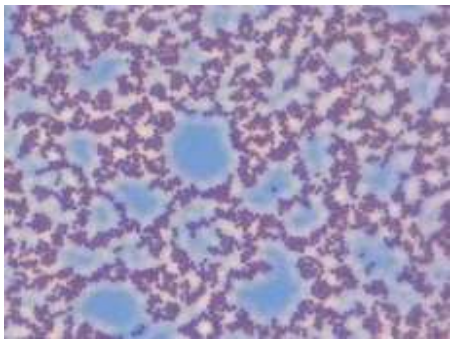


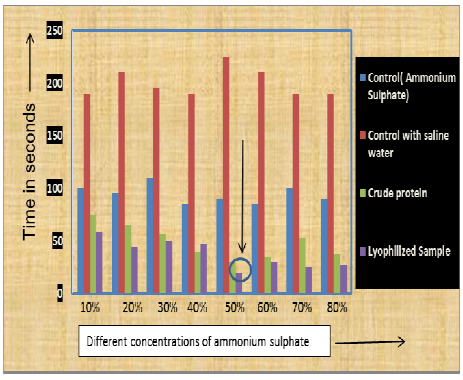
**Fig. 1: It shows Gram staining of Staphylococci sp. Seen under Phase contrast microscope (100X), Grape like clusters forming cocci [21].**

**RESULTS**

As shown in [21], the studied strain appeared to be a gram - positive bacteria using the Gram staining procedure. Its characteristic appearance was grape like clusters forming cocci observed under Phase contrast microscope (Zeiss, Scope.A1, AX10).The dialyzed protein sample obtained was subjected to column chromatography and eluted seven protein fractions from the Sephadex G-100 column in 0.02M phosphate buffer. All the fractions which had a higher absorbance at 280nm were collected and lyophilized.

The extracellular proteins isolated from *Staphylococcus* strain have high coagulation activity. The coagulation time of the human blood plasma was found to be 30±5 seconds when the blood was treated with the third protein fraction of column chromatography. Control blood takes a long time to coagulate. The coagulation time of the crude proteins derived by ammonium sulphate precipitation and their respective lyophilised protein fractions including a control in every test were shown [22].

**Table. 1: It shows bioassay of clotting activity of the protein isolated by Ammonium sulphate precipitation with different concentrations [22].**



In SDS-PAGE, the *Staphylococcus* bacterial proteins got separated based on their molecular weights. In this study the molecular weights of the individual extracellular protein fraction were found to be in between 16kDa and 64kDa [23].

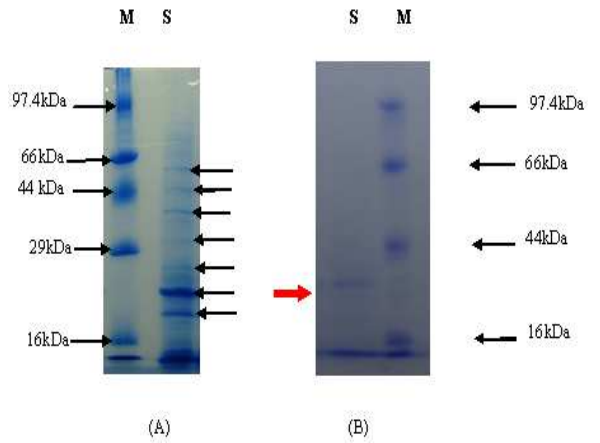
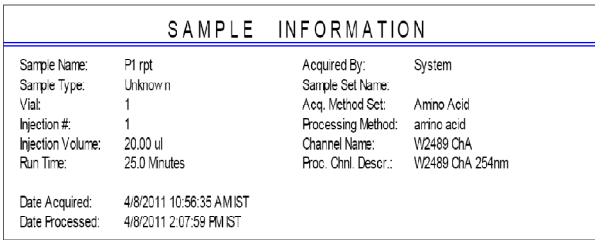



**Fig. 2: It shows (A) SDS-PAGE showing seven protein bands. (B) Non SDS-PAGE showing only one single protein band [23].**

The HPLC was used for analysis and quantification of individual amino acids present in the seven protein fractions purified from the crude sample of *Staphylococcus Sp*. The prominent amino acids found in the fractions were Aspartic acid, Glutamic acid and Proline. The peaks were shown in the form of a graph [24]. Analysis of each amino acid present in the individual protein fractions was done by comparing the peaks of each amino acids with the peaks of the 21 standard amino acids in the standard graph with respect to the retention time. Quantification of the individual amino acids was done by comparing the area of the peak of each amino acid against the area of the standard peak of glycine.


SAMPLE INFORMATION

| | | | |
|---|---|---|---|
| Sample Name: | P1 rpt | Acquired By: | System |
| Sample Type: | Unknown | Sample Set Name: | |
| Vial: | 1 | Acq. Method Set: | Amino Acid |
| Injection #: | 1 | Processing Method: | amino acid |
| Injection Volume: | 20.00 ul | Channel Name: | W2489 ChA |
| Run Time: | 25.0 Minutes | Proc. Chnl. Descr.: | W2489 ChA 254nm |
| Date Acquired: | 4/8/2011 10:56:35 AM IST | | |
| Date Processed: | 4/8/2011 2:07:59 PM IST | | |

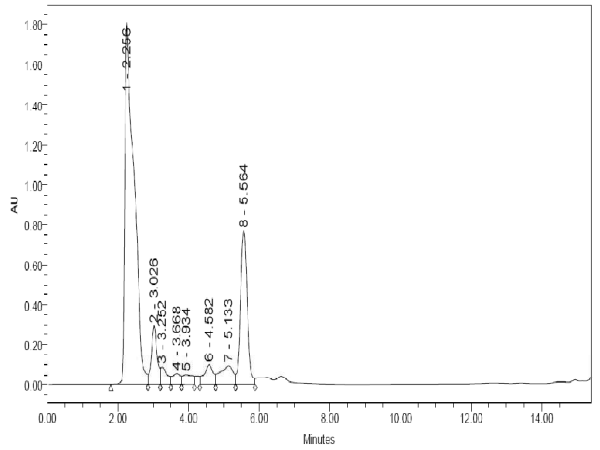



**Fig. 3: It shows the Graph of amino acids (shown in peaks) of the protein fraction 3 isolated from *Staphylococcus* strain showing the retention time (in min) against absorbance [24].**

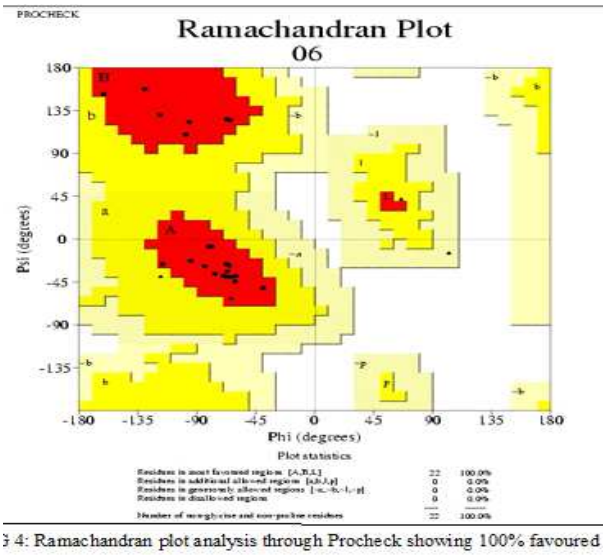



**Fig. 4: It shows Ramachandran Plot analysis through Prochek showing 100% favoured region [25].**

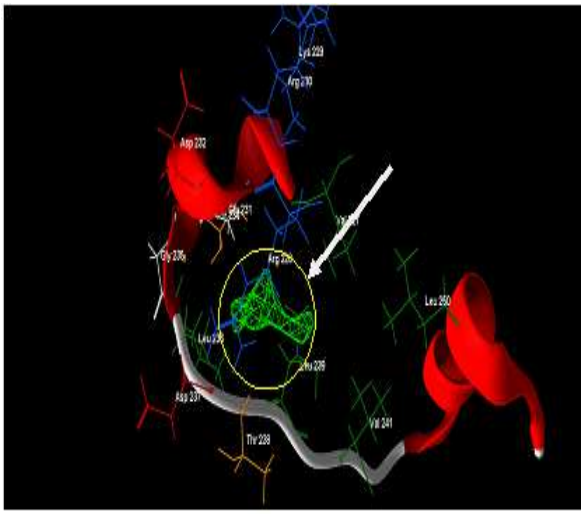


**Fig. 5: It shows probable structure of the protein with predicted site for binding prothrombin [26].**

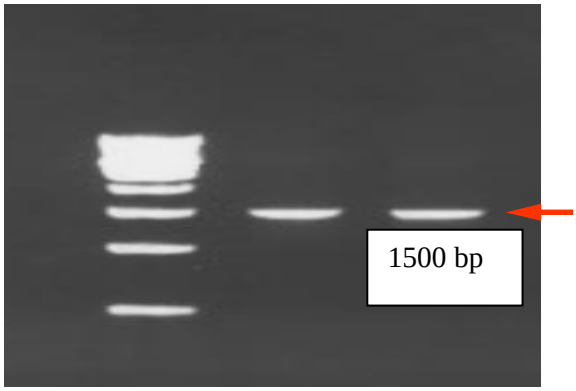



**Fig. 6: It shows Agarose gel (1%) showing the PCR amplified product using bacteria specific primers. Lanes- (M): 500 bp Ladder; and (1, 2): PCR product.(around 1500 bp) [27].**

The 3D assumed structure of the protein showed a loop with two alpha helix and a cavity having volume of 8.704Å and surface volume of 39.68Å. The Ramachandran Plot analysis showed 100% favoured region and 0% in allowed or disallowed region [25]. This gave the probable site for binding of prothrombin [26].

Based on nucleotide homology and phylogenetic analysis, the bacterial strain was identified as: *Staphylococcus sp.*Cobs2Tis23 (EU246837); isolated from *Staphylococcus basab* r- 43 (b) soil sample [27 – 30].

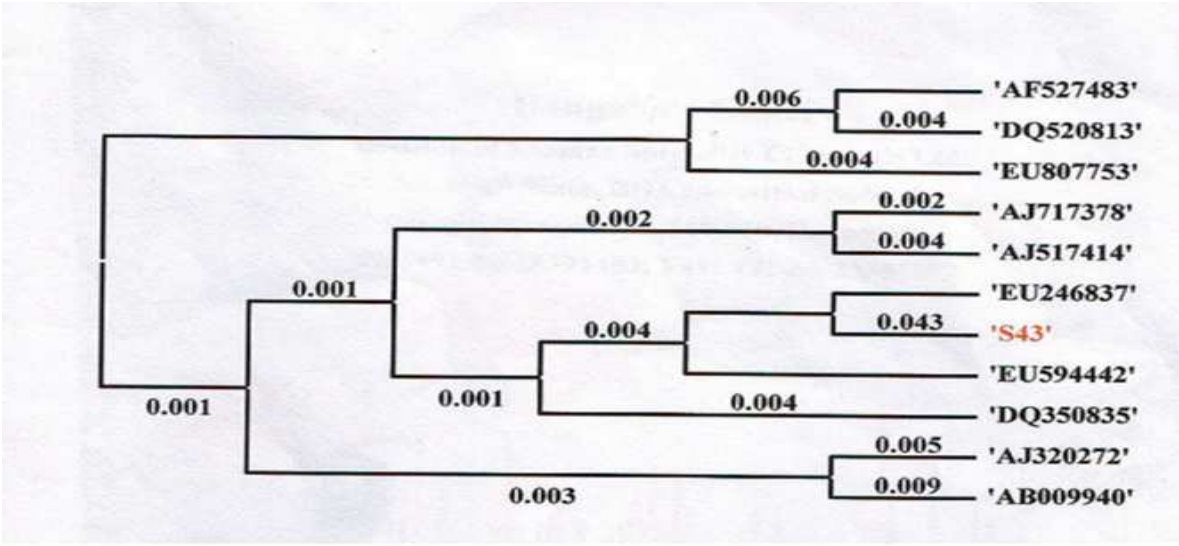



**Fig. 7: It shows phylogenetic tree in MEGA 3.1 software using neighbour joining method [28].**

**Table 2: It shows alignment view using combination of NCBI GenBank and RAPD database [30].**

| Alignment View | ID | Alignment results | Sequence description |
|---|---|---|---|
| | S43 | 0.86 | Studied sample |
| | EU594442 | 0.99 | *Staphylococcus arlettae* strain KP2 1 |
| | EU246837 | 0.98 | *Staphylococcus sp.* Cobs2Tis23 |
| | AJ717378 | 1.00 | *Staphylococcus cohnii* isolate CV38 |
| | DQ350835 | 1.00 | *Staphylococcus gallinarum* strain S1004 |
| | EU807753 | 0.95 | *Staphylococcus xylosus* strain F11 |
| | AF527483 | 0.99 | *Staphylococcus equorum* subsp. linens |
| | AJ517414 | 0.94 | *Staphylococcus nepalensis strain* CW1T |
| | DQ520813 | 0.98 | *Bacillales bacterium* NR119 |
| | AJ320272 | 0.98 | *Staphylococcus succinus strain* SB72 |
| | AB009940 | 1.00 | *Staphylococcus kloosii* |

**DISCUSSION**

*Staphylococcus has been known for its phenotypic feature of producing coagulase since a long time. However, only six species of Staphylococcus have been recognized for being coagulase – positive likewise - S. aureus, S. delphini, S. hyicus, S. intermedius, S. lutrae,S. pseudintermedius and S. schleiferi subsp. The present study has been made to investigate the coagulation factor with a novel coagulase positive strain of Staphyloccocus.*

The bacteria were characterized by individual cocci, which divided in more than one plane to form grape-like clusters. They are generally found to have diameters of 0.5 – 1.5 µm. Thus, we can conclude that the research work proved the coagulating property of this specific strain which could be further used in general clinical sector.

The Column Chromatography showed that the crude protein sample isolated from *Staphylococcus* strain consists of extracellular proteins and these extracellular proteins shows high O.D values in a spectrophotometer. Also, the molecular weight of the extracellular proteins was determined by a comparative study of SDS and Non SDS PAGE and their weight was found to be in between 16kDa and 64kDa which were compared with standard molecular marker. Only one band was observed in Non SDS-PAGE. All the isolated proteins from Staphylococcus strain were coagulase positive i.e. these proteins have a high capacity to coagulate human blood and plasma and the highest blood coagulating property was seen with 3rd fraction of the 50% ammonium sulphate precipitated protein because it coagulates human blood in just 30 seconds when compared to the control blood sample. In a nutshell, we can say that all the extracellular proteins isolated from *Staphylococcus* bacteria have a high blood coagulation property. The amino acid composition of the protein peaks was determined by reverse phase high pressure liquid chromatogram of amino acids where the peaks and elution time of the chromatogram was compared with that of standard consisting peaks of 21 amino acids. Also amino acid

quantities in each peak were determined by comparing the amino acid peaks with the standard peak of glycine. The predicted secondary structure of the protein helped to get an overview of the specific protein and its binding site. Leucine, aspartic acid and glycine were the dominant residues in the cavity. Based on nucleotide homology and phylogenetic analysis bacterial strain was identified as: *Staphylococcus sp.* (EU246837).The 16SrDNA sequence analysis confirmed the taxonomic relationship between the identified bacteria to other related bacteria to characterize the strains at species level.

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