

RESEARCH ARTICLE

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## Molecular Detection and Biological Control of Human Hair Dandruff Causing Microorganism *Staphylococcus aureus*

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### Abstract

Human hair dandruff (HHD) is a common unwanted scalp disorder that is prevalent to most human populations all over the world. This study was designed to isolate and characterize pathogens that are responsible for HHD as well as the evaluation of their biological control technique. Isolated bacteria were characterized by different biochemical tests and molecular identification methods. Here, disc diffusion methods were used to determine antibiotic and antibacterial activity against isolated bacteria. The isolated bacterial colonies were found to be Gram-positive, small, round-shaped, and purple. PCR amplification was done using 27F and 1492R primer pairs. A BlastN search of a sequenced 1465 bp region of 16S rRNA in NCBI GenBank revealed approximately 99% genome similarity with *Staphylococcus aureus*. The sequence was deposited in GenBank (Accession No. MH603394). In the antibiotic sensitivity test, Kanamycin showed the highest 31.0±0.5 mm diameter zone of inhibition (DZI) against the isolated bacteria. Moreover, as a plant-derived compound, the Methanol extract of *Allium sativum* revealed the highest, 15.0±0.5 mm DZI. The present study would give a promising direction of identification and control of this pathogen biologically.

**Keywords:** Human hair dandruff, Molecular detection, Biological control, *Staphylococcus aureus*

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(Received: December 03, 2019; accepted: February 06, 2020)

**Abbreviations:** HHD: Human hair dandruff, LB: Luria and Bertani medium, NA: Nutrient Agar, BLAST: Basic Local Alignment Search Tool, NCBI: National Centre for Biotechnology Information.

**Citation:** Md. Atik Mas-ud, Md Roushan Ali, S.M. Zia Hasan, Md Ashikul Islam, Md. Faruk Hasan, Md. Asadul Islam and Biswanath Sikdar, Molecular Detection and Biological Control of Human Hair Dandruff Causing Microorganism *Staphylococcus aureus*, *J. Pure Appl. Microbiol.*, 2020; **14**(1):147-156. <https://doi.org/10.22207/JPAM.14.1.16>

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## INTRODUCTION

Human hair dandruff (HHD) is a common disorder of the skin that affects the scalp and creates an unfavorable condition for most of the people over the world<sup>1</sup>. Dandruff is associated with irritating scalp without swelling and its pathogenesis is composed of numerous intrinsic and environmental factors<sup>2</sup>. Dandruff can contribute to several causes, including dry skin, seborrhea dermatitis, inadequate washing or scrubbing, too often shampoos, scalp psoriasis, eczema, hair-care or yeast-like fungus sensitivity. Scaling and sometimes mild itching are symptoms. Seborrheic dermatitis is a more serious form of the disease, causing skin inflammation<sup>2</sup>. The severity of dandruff varies between mild and severe, where men have higher prevalence and severity<sup>3</sup>.

Bacteria are more critical than fungi to the formation of dandruff, mostly *Staphylococcus* and *Propionibacteria*<sup>4</sup>. Xue *et al.* 2016 reported, *Staphylococcus* bacterial species are more responsible for the formation of dandruff rather than fungi<sup>5</sup>. *Staphylococcus aureus* is gram-positive and round-shaped bacteria which are a component of the firmicutes and normal body fluids, mostly seen in the nose, breathing tract and the skin<sup>6-7</sup>. Evolution of antibiotic-resistant strains of *S. aureus*, for example, methicillin-resistant *S. aureus* (MRSA) that is a global clinical problem<sup>4</sup>. In previously reported studies, staphylococci bacteria are responsible for dandruff pathogenesis. Leong *et al.*, 2019 reported a way to increase the capability of treatments against HDD caused by *Staphylococcus*<sup>8</sup>.

To address this global issue, plants are considered to be a good source of traditional medicine, as are both bioactive and new therapeutic compounds. The plants are known since ancient times and widely accepted as a crucial source of traditional medicinal compounds for specific diseases diagnosis<sup>9</sup>. There is no previously reported research where the antibacterial activity of plant extracts against human hair dandruff is discussed. We planned to treat this unfavorable scalp disorder for the first time through globally available medicinal plants.

Therefore, this study focused on the investigation of bacterial isolation from a healthy volunteer with dandruff infected hair. And to characterize the pathogen in the aspect of both

biochemical and molecular approaches and to characterize the antibacterial activities of plant extracts to control this unpleasant HHD.

## MATERIALS AND METHODS

### Hair samples collection

In present study, disease-associated hair samples were obtained from a healthy volunteer having a bad experience of this unpleasant scalp disorder who is not a member of our research group and willing to dedicate his unwanted hair scalp infected with dandruff as our research sample. Ethical clearance of the study was approved by the Director of the Institute of Biological Science, University of Rajshahi, Rajshahi-6205, Bangladesh (Approval no. IBSC.EC.5.6.18-00122).

### Isolation and culture of bacteria

The hair sample collected in a sterilized zipper bag, and then hairs were taken in a conical flask that contained 100 ml distilled water. The flasks were shaken in a rotatory shaker for 30 minutes. One milliliter of water was taken into LB liquid medium by using a sterile pipette and incubated to grow bacteria at 37°C in a shaker overnight. On the next day, after incubation, a sterile loop was used to streak the bacteria onto a solid LB agar medium. The plated bacteria were cultured and incubated overnight, 16 hours, at 37°C.

The pure culture was obtained through the streak-plate method. Finally, the plate was incubated overnight at 37°C. There were many single colonies found on the plates. To obtain the desired microorganism, sub-culturing was done, and the liquid medium was prepared for every single colony. The single colonies were taken by a loop of inoculation needle and touched on LB liquid medium on the laminar flow bench. Then it was incubated for 16-18h at 37°C temperatures. A loop of an inoculation needle was taken into the single colonies and touched on LB liquid media. It was then incubated at 37°C temperatures for 16-18 hours.

### Biochemical characterization

The morphological and biochemical characteristics of the isolated bacteria were done. Bacteriological analysis was performed using selective media<sup>10-13</sup>. After 12 to 16 hours of growth in the LB Agar plate at 37°C, the morphology of the colony, size, shape, color, and growth patterns

were recorded. Light microscopy was used to observe cell size. A series of biochemical tests namely, Potassium hydroxide, H<sub>2</sub>S production, Indole formation, Motility, Catalase, Simmon citrate, Kovac oxidase, Methyl red, MacConkey agar, Kligler Iron Agar(KIA), Triple Sugar Iron (TSI) and Urease tests were performed to characterize the isolated bacteria based on Bergey's Systematic Bacteriology Manual guidelines<sup>14</sup>.

### Molecular characterization

#### Extraction of genomic DNA

Genomic DNA was extracted from bacterial cultures by heat lysis and selective precipitation of cell debris and polysaccharides with Maxwell Blood DNA Kit (Model: AS1010) Promega, USA. The extracted DNA was re-suspended in TAE buffer (Promega, USA) and quantified using a NanoDrop Spectrophotometer (Model: ND2000, Origin: Thermo Scientific, USA), and then run 1% agarose gel electrophoresis to determine the size of the DNA.

#### PCR analysis

Amplification of the 16S rRNA gene was performed by conventional PCR technique using the universal primers: 27F forward primer 5'-TGGTAGTCCACGCCCTAAAC-3' and 1492R reverse primer 5'-GACGGGCGGTGTGTRCA-3'<sup>15-16</sup> in a 25µL reaction volume, containing nuclease free ddH<sub>2</sub>O 15µL, dNTP mix 1.0µL, forward primer 1.0µL, reverse primer 1.0µL, DNA template 1.5µL, MgCl<sub>2</sub> 2.5µL, Taq buffer B 2.5µL, and Taq polymerase 0.5µL. The PCR was done by initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 40s, annealing at 65°C for 1 min, extension at 72°C for 2 min and the final extension at 72°C for 10 min, followed by cooling at 4°C until the sample was taken out. Gel electrophoresis was used to visualize the amplified PCR product.

#### Antibiotic sensitivity test

A moderate disc diffusion method was applied to measure antibiotic susceptibility<sup>17</sup>. In this research work, to observe the sensitivity pattern against the isolated bacteria, 16 different antibiotics were selected. These isolated bacteria were grown overnight in a nutrient broth at 37°C in a shaker with 120 rpm for the antibiotic sensitivity test. Then, 1 ml (CFU 1X10<sup>6</sup>/ml) of the overnight culture of bacteria was transferred and gently spread on the nutrient agar plate and dried. Different commercially available

antibiotic disks namely, Ampicillin 10µg, Neomycin 30µg, Doxycycline 30µg, Kanamycin 30µg, Erythromycin 15µg, Tetracycline 30µg, Cefotaxime 30µg, Oxytetracycline 30µg, Azithromycin 15µg, Oxytocin 30µg, Penicillin 10µg, Nalitic acid 30µg, Amoxycillin 10µg, Vancomycin 30µg, Cefixime 5µg, and Rifampicin 5µg per disc were placed over the respective plates (1 disk per plate). And then incubated at 37°C for 16 hours.

#### Antibacterial activity of some plant extracts

Ten diverse plant varieties such as Garlic, Onion, Hog plum, Neem, Fig tree, Cassia, Pathor Kuchi, Bitter Gourd, Ginger, and Sunflower were obtained from various locations of Rajshahi University premises. Different plant parts were used as a potent source of plant extracts, described details in Table 1. They were carefully rinsed to clean off, air-dried in sunlight for the three days (8 h/ day). And then kept in the shade in an aerated place till drying up completely, and they were pulverized to a fine powder<sup>9,18</sup>. The prepared powder was drenched in Methanol solvents followed by earlier reported an experiment with slight modification (powder to solvent ratio, 1:10, w/v), and incubated for 24 hours at 25°C with shaking at 160 rpm. Then, the filtrates from the filtration through nylon membrane clothes were dried at 37°C<sup>19</sup>. Finally, the dried extracts were dissolved in Phosphate Buffered Saline (PBS) bringing the total volume of 500 ml.

The agar disc diffusion method screened the antibacterial activity of the ten plant extracts<sup>20</sup>. A suspension of the inoculum was swabbed uniformly on the solidified 20 mL LB agar. The

**Table 1.** Plants name and plant parts (Source of plant extract) used for antibacterial activity

Plant name	Scientific name	Used plant part
Garlic	<i>Allium sativum</i>	Bulb
Onion	<i>Allium cepa</i>	Bulb
Hog plum	<i>Spondias mombin</i>	Fruit
Neem	<i>Azadirachta indica</i>	Leaf
Fig tree	<i>Ficus carica</i>	Fruit
Cassia	<i>Cassia alata</i>	Leaf
Pathor Kuchi	<i>Bryophyllum pinnatum</i>	Leaf
Bitter Gourd	<i>Momordica charantia</i>	Fruit
Ginger	<i>Zingiber officinale</i>	Root
Sunflower	<i>Helianthus annuus</i>	Flower

inoculum was allowed to dry for 5 min. Aliquot of 10 $\mu$ L, 20 $\mu$ L and 30 $\mu$ L from liquids crude extract of each plant (500mg/mL) was soaked into each 6 mm Whatman paper discs on the cultured medium and kept in the bench for 1h for proper diffusion and after that incubated for 24h at 37°C. The antibacterial activity of the test specimens was evaluated by measuring DZI in millimeter (mm) with a transparent scale after incubation. If the DZI is less than 10mm, it is considered as resistance. If the DZI is greater than 10mm but less than 15mm, it is considered as intermediately resistant.

#### Statistical analysis

For the consistency of results and statistical purposes, all the above-mentioned investigations in this research were carried out three times. The results were analyzed statistically by one-way ANOVA (Analysis of Variances) as mean and standard error (Mean $\pm$ SE) using Microsoft Excel 2013 version. For the statistical analysis,  $P < 0.05$  was considered significant.

## RESULTS

### Isolation of pure culture

After repeated microbial culture of the isolated pathogen in suitable medium and conditions described earlier, pure culture was found. The isolated colony was creamy white (Fig.1A). The colonies were small to medium, smooth, convex, and mucoid in size and shape. The colony morphology was identified by microscopic and visual observation.

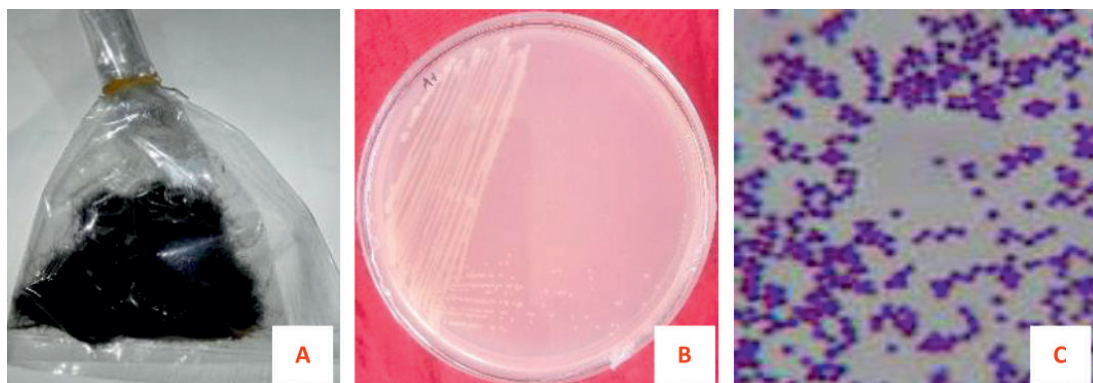
### Biochemical characterization

After isolation of the pure culture, various biochemical tests, including gram-staining (noted

below) was done to characterize the isolated pathogen precisely. Isolated bacteria confirmed as gram-positive both in gram staining reaction and KOH solubility test (Fig. 1B). In Sulfide Indole Motility (SIM) test, no gas was produced, no indole ring was found, and motility was observed in media (Fig. 1C). In the case of the Catalase test, bubbles (oxygen gas) were produced immediately after inoculating the bacteria with H<sub>2</sub>O<sub>2</sub> (hydrogen peroxide), indicated catalase positive. The Simmon's Citrate test showed dark-blue (Prussian-blue) color in the inoculating bacterial medium and the bacteria had a positive effect on the citrate medium. Isolated bacteria did not yield a purple color during the Kovac oxidase test. Therefore, it showed a negative result in the Kovac oxidase test. It was less acidic and provided yellow color following inoculation of bacteria into Methyl Red medium so that bacteria had a negative effect on methyl red. For the KIA test, it produced a yellow color on the medium following inoculation of isolated bacteria and showed positive results. For the Urease test and TSI test, negative and positive results were observed respectively by the isolated bacteria. Bacteria produced pink color rounding the colony in MacConkey agar, confirming they are lactose non-fermenting (Table 2).

### Antibiotic susceptibility assay

In antibiotic susceptibility assay, various types of standard discs of antibiotics against the isolated bacteria were used. The highest antibacterial activity was shown by Kanamycin (30 $\mu$ g/disc) with 31 $\pm$ 0.0 mm diameter zone of inhibition (DZI) followed by Oxytetracycline (30 $\mu$ g/disc) with 28 $\pm$ 0.0 mm DZI (Fig. 2A). On the contrary,



**Fig. 1.** Collection of hair samples, isolation of bacteria and gram staining of microorganism. (A) Hair sample (B) Isolated bacterial colonies and (C) Gram-positive bacteria

**Table 2.** Biochemical characteristics of the isolated bacteria

Test	Result	Optimization	Remarks
Gram staining	+ve	Small round shaped, purple color colony	Isolated bacteria were gram-positive
Potassium hydroxide	+ve	Bacterial smear becomes viscous, sticky mess	Isolated bacteria were gram-positive
H <sub>2</sub> S production	-ve	Absence of blackening along the line of inoculation	Bacteria were positive to H <sub>2</sub> S production
Indole formation	-ve	No band is formed of the medium	A negative indole test for isolated bacteria
Motility	-ve	No growth of bacteria is observed in SIM media	Non-motility was recorded for isolated bacteria
Catalase	+ve	Copious bubbles produced	Bacteria were able to produce catalase enzyme
Simmon citrate	+ve	Production color from green to Prussian blue	Isolated bacteria can utilize citrate
Kovac oxidase	-ve	No color formation within 5-10 seconds after inoculation	Isolated bacteria are negative to oxidase reagent
Methyl red	-ve	Yellow color	No red color was formed in methyl red
MacConkey agar	+ve	Pink color for colony	Lactose non fermenting
Kligler Iron Agar	+ve	Yellow color	Isolated bacteria non-ferment glucose
Triple Sugar Iron	+ve	Red color in slant and yellow color in butt	Isolated bacteria could not ferment sugars
Urease	-ve	No color change	No magenta color was formed

+ve = positive, -ve= negative



Rifampicin (5µg/disc) and Doxycycline (30µg/disc) showed the lowest; 9.4±0.0 mm and 11±0.0 mm, DZI. But Ampicillin, Oxytocin, Penicillin, and Vancomycin did not show any zone of inhibition (Fig. 3).

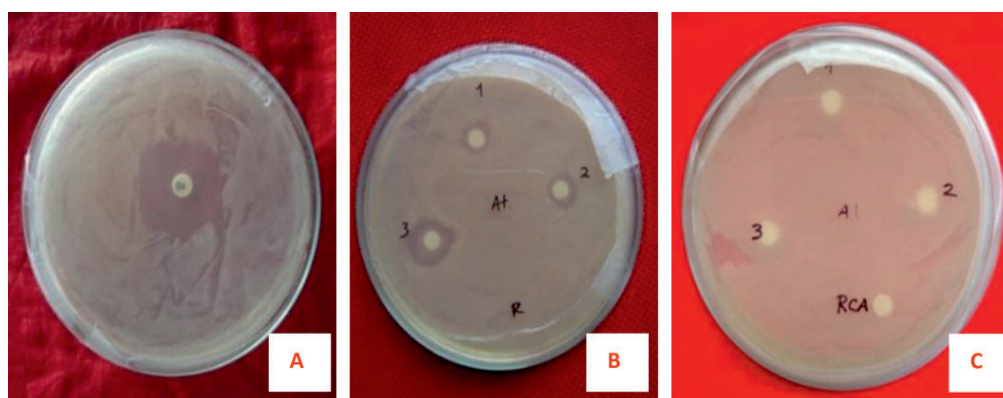
#### Antibacterial activity against isolated bacteria by some plant extracts

The antibiotics which cause antimicrobial resistance but have some adverse effects on human health. For this reason, the natural products with antimicrobial effects have been investigated to eliminate the use of synthetic antibiotics during the last decades. In recent

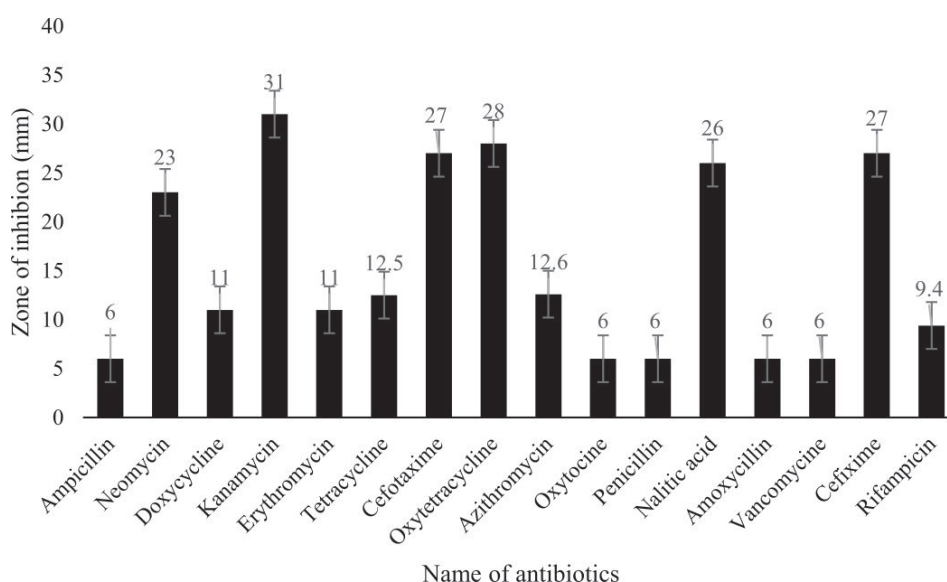
decades, the use of synthetic antibiotics was replaced by natural products with antimicrobial effects. Therefore, some medicinal plants are used to inhibit the growth of the human pathogenic microorganisms. Among the 10 medicinal plants, the Methanol extract of *Allium sativum* showed highly significant antimicrobial activity about 15.0±0.5 mm DZI (Fig. 4). This study precisely indicates that the antibacterial activity varies with plant type and solvent type.

#### Molecular characterization

The genomic DNA of isolated bacteria was PCR amplified by one pair of universal 16S



**Fig. 2.** Antimicrobial activities of drug discs against *Staphylococcus aureus*. (A) Kanamycin, (B) *Allium sativum* extract, (C) *Rhizobium* from *Cicer arietinum*.



**Fig. 3.** Graphical representation of antibiotics and their zone of inhibition

Legend: M±SE=mean and standard error, Resistant=<10 mm; Intermediate =10-15 mm; Susceptible=>15 mm

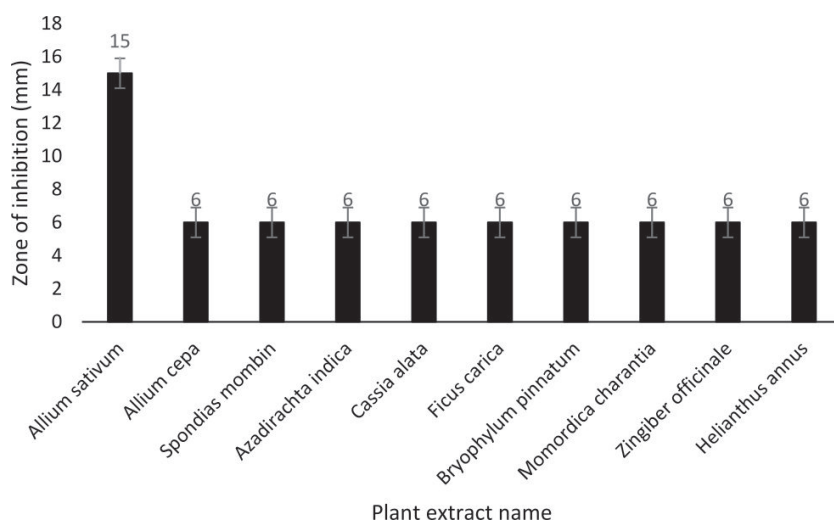
ribosomal RNA primers of the genes (27F and 1492R). The PCR amplified fragment was 1500 bp (approximately) compared to the DNA ladder of 1kb (Fig. 5).

After sequencing, the 1465 bp nucleotide sequence was performed for a BlastN search in GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The BlastN result reported approximately

99% sequence similarity with *Staphylococcus aureus* strain 8 BWI 16s rDNA gene. The obtained sequence was deposited in GenBank (Accession No. MH603394).

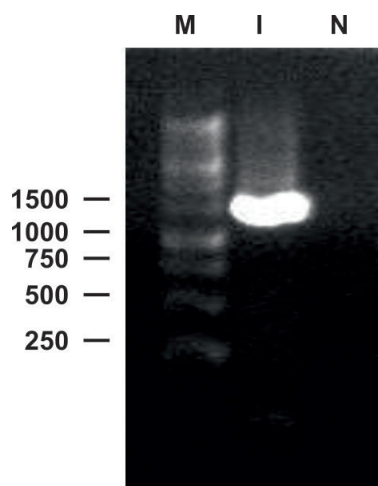
#### Phylogenetic analysis

The sequences of *Staphylococcus spp.* in FASTA format were downloaded from NCBI GeneBank. The data were matched in Multiple



**Fig. 4.** Graphical representation of plant extracts and their zone of inhibition

Legend: Mean $\pm$ SE=mean and standard error, Resistant=<10 mm; Intermediate =10-15 mm; Susceptible= $\geq$ 15 mm

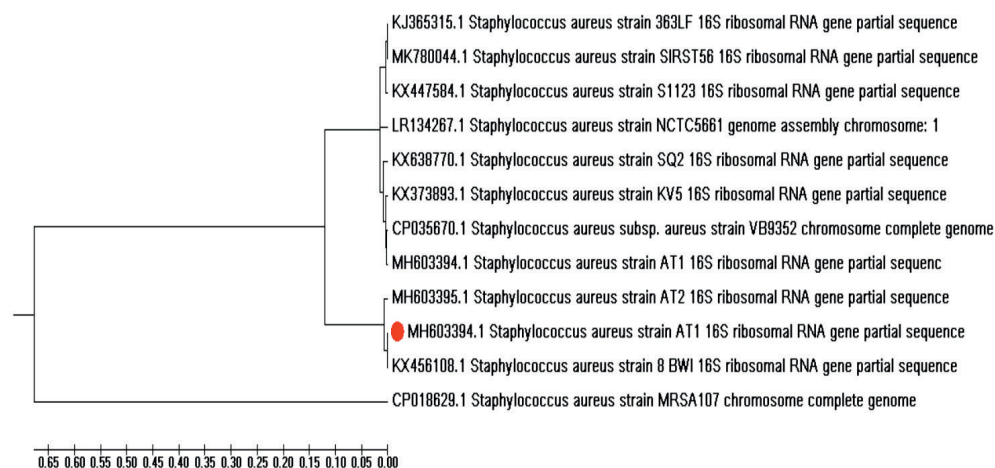


**Fig. 5 .** 16s rRNA PCR profiles of 27 F and 1492 R primers, generated from bacterial genomic DNA. M: DNA ladder, I: PCR product, N: Negative Control

Sequence Alignment (MSA) and constructed a phylogenetic tree (Fig. 6). The evolutionary tree was constructed using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) method. The tree is drawn to scale with branch lengths that are the same units as the evolutionary distances that the phylogenetic tree is used to infer. Evolutionary steps are determined using the Composite Maximum Likelihood method. Twelve nucleotide sequences were included in the study and eliminated all positions that contain gaps and missing data. In the final dataset, there were 774 positions. Molecular Evolutionary Genetics Analysis (MEGA version 7.0) software was used to analyze the evolutionary studies<sup>21-23</sup>.

#### DISCUSSION

Dandruff is a disease of the skin that mainly affects the scalp<sup>2</sup>. The actual cause of this



**Fig. 6.** Evolutionary relationships of taxa. The isolated bacteria with its accession number is indicated by red point

has some debates but is supposed to include a combination of environmental and genetic factors. According to a previous study reported by Xue *et al.* in 2016, the possibility of dandruff formation is higher due to bacteria (*Staphylococcus*) than that of fungi. The amount of sebum and water indicated the presence of bacteria<sup>5</sup>. In this study, the morphological, physiological, biochemical, antibiotic, antibacterial, antagonistic and molecular tests were performed after the isolation of bacterial strains for the bacterial identification. In gram staining, isolated bacteria from hair samples showed a purple color, indicating that it's a gram-positive bacterium. Isolated bacteria showed non-motility in SIM medium and did not produce  $H_2S$  and indole ring. Here, isolated bacteria showed a positive result to Simmons citrate and a negative result for the MacConkey agar test. Kligler Iron Agar (KIA) test may help to differentiate enteric gram-negative *bacilli* based on the fermentation of carbohydrate and production of  $H_2S$ .

In this study, isolated bacteria showed a positive result to the KIA test, which means the bacteria were glucose fermenters and non-lactose fermenters. The Catalase test is used to identify organisms producing catalase enzymes. By breakdown into water and oxygen gasses, this enzyme detoxifies hydrogen peroxide. The results of bubbles produced during the production of oxygen gas indicate the Catalase test positive. This finding indicates the similarity of the previously

reported experiment<sup>24</sup>. The Methyl red test is used to identify the stable acidic end product that confirms mixed acid-fermenting bacteria. In this test, isolated bacteria showed a negative result with a yellow color. Urease test measure the capability of a microorganism to breakdown urea by producing urease enzyme. Isolated bacteria showed a negative result in the present study. There was a similar result reported earlier<sup>25</sup>.

In the antibiotic susceptibility test, 16 antibiotics were used to perform the antibacterial activity of an antibiotic against bacterial isolates responsible for dandruff, using Kirby-Bauer antibiotics (KB test) or discuss susceptibility to diffusion antibiotics. An antibiotic sensitivity test was helpful in finding out the control measures of this disease. The zone of inhibition on a plate identified the sensitivity pattern of the isolated bacteria against the different types of antibiotics<sup>26</sup>. This result showed that isolated bacteria were very sensitive to Kanamycin, Oxytetracycline, Cefotaxime, Cefixime, Nalitic Acid, and Neomycin.

Antimicrobial activities against isolated bacterial species were evaluated *in vitro* by ten medicinal plants used as extracted material. Isolated bacteria were intermediately resistant to *Allium sativum* and the increase of that compound increased their sensitivity. These are similar to the findings of some previous studies<sup>26-27</sup>.

In the present study, we find out that the bacterial strain is responsible for the dandruff disease of human hair. And molecular



identification of bacteria creates an opportunity to identify this pathogen using specific primers. It also provides information for future research to know the facts behind this bacterial pathogenicity.

## CONCLUSION

Human hair dandruff (HDD) is a common, unpleasant scalp disorder that is one of the major concerns for the health-conscious people over the world. In our recent work, we isolated dandruff, causing pathogens from a healthy volunteer, experimented with some major biochemical and molecular characterization to control this disease using some globally available medicinal plants. In the Biochemical test, we proved that isolated bacteria as gram-positive and non-motile, glucose fermented, but lactose non-fermented. In molecular characterization, it confirms the isolated bacterial strain is *Staphylococcus aureus* that leads to identifying the associated genes or functional group for dandruff pathogenesis. In the antimicrobial test, *Staphylococcus aureus* shows the highest sensitivity to *Allium sativum* among all the plant extracts that recommend a new prospect for developing an anti-dandruff compound. Although there were some limitations as we isolated pathogens from a single volunteer, the present study would be helpful for further direction of identification and control of this pathogen with biologically active compounds.

## Application of research

Disease identification, characterization of the pathogen and management of the above-mentioned disease will increase the quality and quantity of human hair.

## ACKNOWLEDGMENTS

The authors wish to thank Professor Joarder DNA and Chromosome Research Lab, Dept. of Genetic Engineering and Biotechnology, University of Rajshahi, Bangladesh for providing financial support and other facilities during the whole research work.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## FUNDING

None.

## AUTHORS' CONTRIBUTION

MAM, MRA, SMZH, and MFH designed the experiments, developed the methodology and prepare the manuscript. MAM, MRA, MFH, and BS collected the data and carried out the analysis. MRA, MFH, MAI, and MAI assisted with data analysis and manuscript preparation. Finally, MAM, MRA, SMZH, MAI, and MFH carried out manuscript correction according to the journal's comment.

## DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

## ETHICS STATEMENT

Prior to the commencement of the study, the ethical clearance of the study was approved by the Director of the Institute of Biological Sciences (Approval no. IBSC.EC.5.6.18-00122) and ethical committees. In addition, specimens were collected and consent was obtained from patients.

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