Volume 3, Issue 2, 2025

Frontier in Medical & Health Research

ISSN: (e) 3007-1607 (p) 3007-1593

MOLECULAR EPIDEMIOLOGY OF STAPHYLOCOCCUS AUREUS ISOLATED FROM MICE. A STUDY OF ANTIBIOTIC RESISTANCE AND GENETIC DIVERSITY USING 16S-rRNA A GENE SEQUENCING

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DOI: https://doi.org/10.5281/zenodo.15363062

Keywords
Stress, Work-Life Balance, Emotional Intelligence, Ambulance personnel, Paramedics

Article History
Received on 19 March 2025
Accepted on 19 April 2025
Published on 26 April 2025

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Abstract

Staphylococcus aureus *is a clinically significant pathogen with increasing antibiotic resistance and zoonotic potential. In the current study total of bacterial strains of S. aureus strains were isolated from swab samples of mice. The isolates were then characterized on the basis of their morphology and microscopic analysis. Biochemical characterization was conducted to characterize isolates of pathogens. All strains are classified and characterized as Staphylococcus aureus by giving round, smooth, and slightly raised cream to golden yellow colonies on Mannitol salt agar. Biochemical characterization was conducted to characterize isolates of pathogens. The biochemical test results show that isolates don't react with coagulase, oxidase, methyl red test and motility test and did react with catalase test. Using 16S rRNA gene sequencing, this study further investigated the genetic diversity, antibiotic resistance trends, and molecular epidemiology of S. aureus isolates isolated from mice. Following the extraction of genomic DNA from bacterial isolates, PCR amplification and 16S rRNA gene sequencing were conducted. Phylogenetic reconstruction using MEGA X and bioinformatics analysis, such as BLAST, confirmed species identification and demonstrated evolutionary links with other isolates of S. aureus. Resistance profiles were evaluated through the use of antibiotic susceptibility testing. The findings showed substantial grouping in the phylogenetic tree and strong genetic similarity (98.37%) to known S. aureus strains, indicating potential host adaptability or environmental selection pressures. Concerns over antibiotic resistance in zoonotic strains were also raised by the observation of diverse resistance patterns. The study expands the understanding of S. aureus epidemiology and its possible consequences for public health and laboratory animal management by shedding light on the genetic diversity and antibiotic resistance of the bacteria in mice.*

INTRODUCTION

Gut microbiota refers to the wide and complex community of the microorganisms that lives an animal gastrointestinal system. The immune system, digestion, and general health of the host are all significantly impacted by these microorganisms. However, the gut microbiota may contain opportunistic bacteria like *Staphylococcus aureus*, which can cause infections, especially in hosts with

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ISSN: (e) 3007-1607 (p) 3007-1593

weakened immune systems (Sekirov *et al.*, 2010). *Staphylococcus aureus* is an opportunistic pathogen that may colonize and infect both people and animals. *S. aureus* is found in the mucous membranes and on the skin of humans and certain animals. This virus may cause a variety of infections, from minor ones that affect the skin and soft tissues to more serious ones that can be deadly, such bacteremia, osteomyelitis, and endocarditis (Papadopoulos *et al.*, 2018). *Staphylococcus aureus* is one of the gram-positive bacterial pathogens that may easily acquire antibiotic resistance determinants and employs a wide range of virulence factors to start and maintain infections. *Staphylococcus aureus* is one of the most common bacteria for invasive, possibly lethal infections (Dantes *et al.*, 2013).

Rapid changes in the gut microbiota's composition during the first two or three years of life may have an impact on risk factors for adult health (Milani *et al.*, 2017). Mice that have dysbiosis of the intestinal microbiota develop deadly diseases. Mice with aberrant gut microbiota composition have cognitive deficits (Zhan *et al.*, 2018). Mice's gut microbiota influences the development of malaria and mediates acute pancreatitis (Zhu *et al.*, 2019). There are a variety of different environmental factors that impact gut colonization, including host genetics, diet, age, lifestyle, healthcare, and antibiotic usage (Denou *et al.*, 2016). The widespread use of similar antimicrobials in human and veterinary medicine facilitates the selection of antibiotic-resistant microorganisms and their subsequent dispersion in nature. Environmental contamination, which in turn encourages AMR genes and threatens their future transmission, is a result of municipal sewage, manure, and slurry (Hendriksen *et al.*, 2019). Animals are among the most significant environmental carriers of determinants of antibiotic resistance. The ubiquitous presence of AMR in bacteria found from slaughter animals and animal-derived products in Poland is shown by the findings of AMR monitoring programs and studies on AMR in bacteria from wild animals (Wasyl *et al.*, 2018).

AMR in animal commensal intestinal flora may pose a threat to humans if viruses such as Salmonella can transfer AMR genes from this reservoir to people through contaminated animal products, intimate contact with animals, or animal husbandry facilities. Antimicrobial resistance (AMR) in animals is influenced by a variety of variables, including prevalence and transmission. The way animals behave may have an impact on the likelihood of generating antibiotic-resistant bacteria (Authority, 2018).

Despite an abundance of research on the pathophysiology of staphylococci in humans, *S. aureus* infects and causes disease in a variety of animal hosts, which has important ramifications for agriculture and public health (Peton and Le Loir, 2014). The health of animals is negatively impacted by infections, and people can get staphylococcal infections from animals. Although the incidence varies depending on the host species, 20 to 30 percent of humans have *S. aureus*. For instance, the frequency for pigs is 42%, that of sheep is 29%, and that of cows and heifers is between 14 and 35% (Nagase *et al.*, 2002). Because of its capacity to colonize a broad range of host species, *Staphylococcus aureus* is becoming recognized as a zoonotic pathogen. There is an extensive amount of research on the topic since different animal species have economic significance. It follows that the lack of study on *S. aureus* colonization and infection in wild animals is not remarkable. Red squirrels (exudative dermatitis), boars (nasal carriage), raccoons (botryomycosis), great apes (nasal carriage and sepsis), rhesus macaques (nasal carriage), and chaffinches (healthy carriage) are among the numerous wildlife species that have been found to harbor *S. aureus* (Paterson *et al.*, 2012).

Six different biotypes of *Staphylococcus aureus* have been examined in animals: non-host specific, bovine, avian-abattoir, β-hemolytic human, human, and caprine. Depending on the host organism, these isolates have different traits. These biotypes have often withstood the use of sophisticated characterization methods; clustering of isolates from different hosts using multilocus enzyme electrophoresis suggests host specificity and a restricted capacity for strain transfer between species (Peton and Le Loir, 2014). Many evidences indicate that the industrialization of livestock farming and the beginning of animal domestication during the Neolithic era (10,000–2,000 BC) produced an environment in which human-animal contact, which

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ISSN: (e) 3007-1607 (p) 3007-1593

can result in host-switching events, allowed pathogens to spread from animals to humans (Richardson *et al.,* 2018). *S. aureus* frequently has little trouble crossing species boundaries when it comes to infecting new hosts. This ability is largely due to the large number of mobile genetic elements (MGEs) in the S. aureus genome and its capacity to move them to different settings.

Infectious microorganisms several hosts are known to be infected by *Staphylococcus aureus*. Other than humans, it has been effectively isolated from a variety of companion, farm, and wild animals worldwide (Haag *et al.,* 2019). Often utilized in experimental settings, mice and rats can acquire *Staphylococcus aureus* infections or colonizations that happen "naturally" in their reproductive habitats (Raafat *et al.,* 2020). There are 45 species and 24 subspecies of Staphylococcus, which belong to the Staphylococcaceae family. Most of these species are aerobic or facultatively anaerobic (Brinkman and Leipe, 2001).

The most prevalent and virulent kind of staphylococcus is *Staphylococcus aureus*, which derives its name from the carotenoid pigment that these bacteria generate, which turns their colonies yellow when they come into contact with solid medium. Since 2005, the number of methicillin-resistant *Staphylococcus aureus* infections in people has increased (Kevorkijan *et al.,* 2019). The first recorded case of livestock-associated MRSA (LA-MRSA) in Belgium was bovine mastitis in early 1970. ST398 is the most well-known strain of LA-MRSA. Initially found in pigs, this strain has since spread to other animals and, most significantly, humans, especially those who work in occupations that require frequent contact with animals (Pantosti, 2012). Investigating *S. aureus* in many contexts, including in free-living animals, is essential to understanding its zoonotic potential and capacity for antibiotic resistance. Mice, hares, deer, foxes, mountain goats, kangaroos, bears, squirrels, shrews, bats, minks, raccoons, seals, apes, and several bird species are just a few of the wild animals that researchers have discovered and examined for *S. aureus* (Mrochen *et al.,* 2018).

The scientific understanding of the genetically determined mechanisms via which *Staphylococcus aureus* adapts to different animal hosts has only just started to take shape. For certain bacteria, host specificity is determined by how well their receptors bind to the proteins of the host they have chosen. When *S. aureus* hemolysin lyses the red blood cells, it obtains the bacterial nutrition iron from hemoglobin. Hemoglobin is released prior to degradation and attaches itself to a receptor on the surface of *S. aureus* called iron surface determinant B (IsdB). Compared to hemoglobin from other animals, human hemoglobin binds to this receptor more firmly. Since people are frequently the perfect host for *Staphylococcus aureus*, we can comprehend why bacteria thrive when exposed to human blood (Pishchany *et al.,* 2010).

Monecke *et al.,* conducted an in-depth investigation of 2855 samples of wild animal feces from Sweden, Germany, and Austria in 2016 (Pishchany *et al.,* 2010). Out of this, 155 *S. aureus* were chosen for isolation; 124 of them underwent additional research, and 29 of these were assigned to certain CCs in the manner described below: CC1 (fox, fallow deer, raven, mouflon), CC5 (hare, partridge), CC6 (fox), CC7 (fox), CC8 (fox, mouflon, marmot), CC9 (wild boar), CC12 (porpoise), CC15 (raven, elk), CC22 (raven, fox), CC25 (badger), CC30 (marmot, deer), CC49 (vole, cat), CC59 (wild boar), CC88 (crow), CC97 (eagle, wild boar, elk, roe deer), CC130 (fallow deer, hedgehog, fox, rat, hare, SARM and SASM), CC133 (swan, wild boar, roe deer, chamois), CC398 (hare, SARM), CC599 (hedgehog), CC692 (eagle, magpie, dove, owl, woodpecker, great tit), CC707 (reindeer), CC1956 (topillo), CC2767 (lynx, reindeer). Methicillin-sensitive Staphylococcus aureus, or MSSA, accounted for the majority of the bacteria discovered. The study shows that MRSA and MSSA strains obtained from wildlife are highly varied, with certain clonal lineages associated with both people and animals and others that appear to be uncommon and unique, such as CC692 (Pantosti, 2012).

28 distinct MRSA strains were discovered in German wild boar meat. In every sample, the mecA gene was found. The blaZ gene, which imparts resistance to ampicillin-penicillin, and msrA and mphC, which cause resistance to macrolides, are among the other resistance genes discovered in the isolates. A further study that tested several samples for MRSA was conducted on Austrian birds. Three methicillin-resistant *Staphylococcus aureus* infections were found

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ISSN: (e) 3007-1607 (p) 3007-1593

in 1,325 samples of wild birds (Kraushaar and Fetsch, 2014). Insects may also colonize Staphylococcus bacteria. Between the end of the 1980s and 2000, the number of *S. aureus* infections brought on by MRSA increased quickly on a global scale. Human MRSA infections may initially be categorized as either community-associated (CA) or health care-associated (HA) MRSA based on epidemiological criteria (Salgado *et al.,* 2003). Differentiation between HA-MRSA and CA-MRSA strains is made possible by their structural and functional genomic characteristics. These epidemiological criteria have been increasingly ambiguous when HA-MRSA strains were found in all persons and CA-MRSA strains were identified as the infectious agents in hospitals (Tavares *et al.,* 2013). In addition to these two varieties of MRSA, animals may retain and disperse so-called LA-MRSA strains, which have been found to cause infections in humans. Different lineages that correspond to the pathogen's separate origins can be identified thanks to the three MRSA kinds' unique genotypes and associated genotypic traits.

By isolating and analyzing Staphylococcus aureus, the study aims to investigate the patterns of antibiotic resistance observed in the gut microbiome of mice. These results might raise awareness of drug-resistant bacteria in domestic animals and provide insight into the likelihood of such problems.

MATERIALS AND METHOD

Study area and isolation of samples

Samples of the skin and nasal cavities were taken from mice in the Hazara Division of Abbottabad, Khyber Pakhtunkhwa, Pakistan. Following Standard Operating Procedures (SOPs), each sample was collected and properly labeled for diagnostic purposes.

Sample transportation and storage

Samples were collected and immediately transferred to Abbottabad University of Science and Technology's Microbiology Laboratory for bacterial identification and isolation. After being labeled, the samples were kept until further processing could be completed.

Samples process

Following collection, samples were carefully cleaned to remove surface contaminants and particle debris using distilled water or sterile phosphate-buffered saline (PBS). Using a tissue grinder or sterile mortar and pestle, the cleaned specimens were further physically homogenized to a fine particle fineness in order to ensure equal microbial dispersion. In sterile 50mL Falcon tubes, this homogenate was then suspended in a pre-measured volume of nutrient-rich liquid culture medium, such as tryptic soy broth (TSB) or Luria-Bertani (LB) broth, with exclusive emphasis on maintaining a 1:10 sample-to-media ratio for optimum growth conditions. Then, for a standard 24-hour incubation period, the inoculation tubes were put in a shaking incubator set to 37°C (±1°C) and agitated at 180–200 rpm. This promoted active aerobic microbial growth and inhibited sedimentation. This preparatory step facilitates the execution of subsequent microbiological tests, including selective culturing and antibiotic susceptibility testing, by ensuring enough biomass growth under controlled physiological conditions (David and Daum, 2010).

Isolation and Growth of Bacteria

Clinical samples were streaked onto selective medium and then cultured for 24 to 48 hours at 37°C. Examine the growth characteristics and appearance of the bacterial colonies during a 24-hour incubation period at 37°C. Several techniques, including Gram staining and biochemical characterization, were used to check the growth for the presence of the necessary microorganisms (Fernandes Queiroga Moraes *et al.,* 2021).

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Growth Media Selectively used for *staphylococcus aureus*

Table 1: Types of media used in different quantity/L

S. No	Media	Quantity/L
1.	Mannitol salt agar	111/ g
2.	Triptycase soy agar	TSA/l
3.	Blood agar	40g/l
4.	Bared parker agar	63g/l

Morphology based Characterization of Isolated *Staphylococcus aureus*

Gram staining

To perform the Gram staining, just a small amount of distilled water was applied to a transparent slide. A sterile needle was used to apply an appropriate amount of pure culture on the slide. The culture was evenly distributed throughout the surface using the needle. Using sterilized water, a drop of crystal violet was added to the slide smear. The crystal violet was swirled in and let to dry for around 30 seconds once a uniform dispersion was achieved. Following the application of crystal violet stain, the slide was carefully cleaned using sterile distilled water. The slide was properly washed with pure distilled water, and then a droplet of Lugol's iodine was applied to the smear to remove any leftover crystal violet hue. Lugol's iodine and crystal violet combine to keep the stain in place. After applying Lugol's iodine, the slide was cleaned with acetone. Acetone, a decolorizer, aids in removing excess stains from the slide. A drop of safranin was put to the slide to disguise the smear. The counterstained safranin stain gives gram-negative bacteria their distinctive color. After thoroughly cleaning the slide with water to get rid of any remaining traces of safranin, it was eroded clean. Blotting paper was used to gently remove the excess liquid from the slide. The slide was coated with a drop of mounting chemical Canada balsam in order to retain the discolored smear. To observe the slide containing the plated smear, a 100X magnification microscope was utilized (Greenwood *et al.*, 2012).

Biochemical Characterization

Biochemical assays, including catalase, indole test, urease test, citrate utilization test were carried out. Briefly described as follow:

Catalase Test

The test indicates the presence of the catalase enzyme, which causes hydrogen peroxide (H2O2) to release more oxygen. It is employed to distinguish between different bacteria that produce the catalase enzyme. The *Staphylococcus aurous* strain catalase test was carried out by gently mixing one colony with hydrogen peroxide on a sterile slide. The appearance of gas bubbles on the surface of the culture material indicated that the test was effective (Reiner, 2010).

Coagulase test

This test demonstrates the presence of coagulase, an enzyme that causes fibrinogen's peptide bonds to break and form fibrin clots. It's employed in the differentiation of coagulase-producing bacteria (Brown *et al.*, 2005). In order to perform coagulase test for *S. aureus* strains, one colony was carefully covered with either human or rabbit plasma on a sanitized slide. Human plasma was added to a bacterial culture in order to detect the presence of coagulase, which was then confirmed by clump formation or agglutination (Brown *et al.*, 2005).

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Oxidase Test

Oxidase test, technique for identifying the presence of cytochrome C oxidase, sometimes referred to as cytochrome a3, an enzyme that is present during aerobic respiration. The 1% Kovac's oxidase reagent was applied to a small piece of filter paper, which was then allowed to air dry. Using a sterile loop, a well-isolated colony of *S. aureus* strains was transferred onto filter paper from a newly cultured (18–24 hours) bacterial plate. For every colony under test, colour variations were examined. In ten to fifteen seconds, the hue changes to dark purple following an oxidase positive test. When oxidase negative organisms are present, the colour either stays the same or responds more slowly than two minutes (Shields and Cathcart, 2010).

Motility test

This test is done to find out if an organism can move using its flagella. The placement of the flagella differs depending on the type of bacterium. In order to perform the motility test for *S. aureus* strains transfer the semisolid agar into test tubes after preparing it. Apply a straight needle to a colony of a culture that has grown on nutrient agar medium for 18 to 24 hours. Once at the middle of the tube, only pierce 1/3 to ½ inch deep. Make sure the needle exits the medium in the same direction as it entered. Incubate for up to seven days at 35°–37°C to see if a diffuse growth zone has flared out from the inoculation line (Shields and Cathcart, 2011).

Methyl red (MR) test

The addition of the methyl red indicator at the end of the incubation period, changes colour, it means that the fermentation of glucose produced enough acid and that the environment was maintained in a way that allowed an old culture's pH to remain below 4.5 In order to perform the Methyl Red (MR) Test, a culture of the *S. aureus* strains was inoculated into a tube containing MR broth, which was high in glucose and peptone. Two loopful of each bacterial culture were added to the broth into test tubes, incubate it for 48–72 hours at 37°C, tagged with the organism's name. To the incubated tubes, a few drops of methyl red indicator were added after the incubation period. Every tube was inspected to check for a certain red (Tille and Bailey, 2014).

Disk Diffusion Susceptibility Testing

Mueller-Hinton agar coated with different antibacterial filter paper disks is used to cultivate facultative anaerobic and pathogenic aerobic bacteria. By figuring out how sensitive or resistant these bacteria are to various antibiotic drugs, the disk diffusion susceptibility test assists clinicians in selecting alternatives to therapy for their patients. The ability of that drug to inhibit that organism can be inferred from the proliferation surrounding the disks (Hudzicki, 2009). Bacterial suspensions were prepared using the 0.5 McFarland standard. Antibiotic disks were placed on the surface of Mueller-Hinton agar

plates after the suspension was applied. The plates were incubated at 37°C for 16–18 hours in order to determine their sensitivity to antibiotics. Next, the inhibition zones were measured in mm (Hudzicki, 2009).

Molecular characterization

DNA Extraction:

The carry out genomic DNA of tested bacterial culture was extracted by using Qiagen RTU kit. For determining the spore concentration needed for extraction, 1 mL of the culture containing 108 cfu/mL was centrifuged. The extraction tube (2.5 mL) was filled with 250 µL of proteinase K to remove any potential proteins and lysis buffer AL. After centrifuging the suspension, the supernatant was disposed of. In order to get rid of the particulates, 95% ethanol was added to the lysate. After passing the cleaned lysate through a purification micro spin column, AE buffer was used to elute the column. AW1 and AW2 were the washing buffers that were being employed. The NS1020 nano drop spectrophotometer was used to measure the extracted DNA. For subsequent downstream analysis, the isolated DNA was kept at -20 °C. 1% agarose gel electrophoresis was used to evaluate the isolated DNA's purity (Schumann and Pukall, 2013).

PCR Amplification and Sanger Sequencing

The isolated DNA was amplified with F/R primers specific to 16S. 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) and 27F 5' (AGA GTT TGA TCM TGG CTC AG) are the sequences. The PCR product was predicted to be between 1.4 and 1.6 kb.

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Exonuclease I and SAP enzymes were used for enzymatic digestion in order to sequence the PCR product. The PCR product from agarose gel electrophoresis was then run through a purification column and elution buffer. Sanger sequencing was performed on the cleaned PCR product using primers 785F 5' (GGA TTA GAT ACC CTG GTA) and 907R 5' (CCG TCA ATT CMT TTR AGT TT) (Crossley et al., 2020).

Stage	PCR Protocol	Temperature (°C)	Time (min.)
1st	Initial Denaturation	94	5.0
2nd (35 Cycles)	Denaturing	94	0.5
	Annealing	52.7	0.5
	Extension	72	2.0
3rd	Final Extension	72	5.0
4th	Hold	4	∞

Bioinformatics Analysis

Chromas and BioEdit tools were used to evaluate the sequence in order to determine the bacterial strain's evolutionary connection. The sequence was edited for low-quality and superfluous amplifications, and the peaks were adjusted. The highly matched sequences from the databank were obtained using the NCBI's basic local alignment search tool (BLASTn). The Clustal Omega bioinformatics program was used to perform multiple sequence alignment of the chosen BLASTn resulting sequences prior to constructing of the phylogenetic tree. To determine the evolutionary link between S. aureus and other bacterial species, a tree was constructed and evaluated after the MSA. The sequenced bacterial strain was used using MEGAX software to generate the phylogenetic tree for the evolutionary connection with other species. For the creation of phylogenetic trees, the Fast Minimum Evolution Method and Max Sequence Difference 0.75 were employed (Brinkman and Leipe, 2001).

Results

Samples processing

Sterile swabs were used to take samples from the mice's skin, nasal cavities, and other pertinent tissues in order to isolate Staphylococcus aureus from them. To promote S. aureus growth, the swabs were streaked onto selective medium, such as Mannitol Salt Agar (MSA), and incubated for 24 to 48 hours at 37°C.

Figure 1: Samples collection and processing

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Enrichment Culture Preparation

To facilitate microbial enrichment, the mine swab samples were homogenized and then aseptically transferred into sterile 15 mL tubes that were filled with nutritious broth medium (such as Tryptic Soy Broth or Buffered Peptone Water). The optimal nutrient availability for bacterial growth was maintained at a 1:10 (w/v) sample-to-broth ratio. The tubes were then firmly sealed and kept in a shaking incubator at 37°C for 24 hours at 150 rpm to promote aeration and the steady growth of facultative anaerobic and aerobic bacteria. By enabling low-abundance bacteria to proliferate to detectable levels and revive stressed or injured cells, this enrichment mechanism enhances the detection of these microorganisms. Standard precautions, such as employing sterile methods and negative control tubes containing just broth, were taken throughout the process to keep monitor out for any contamination (ISO 6887-2:2017; FDA BAM, 2021).

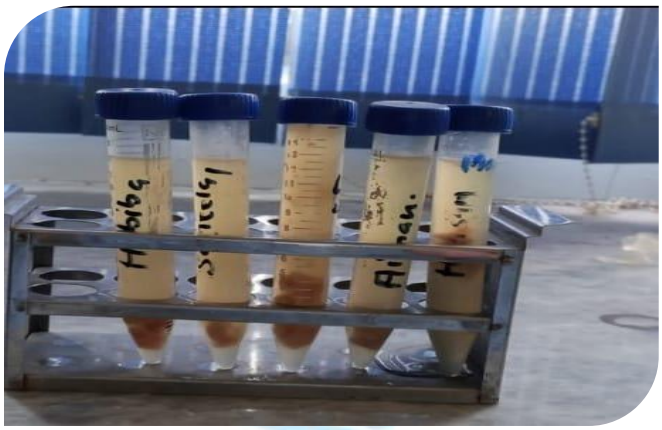
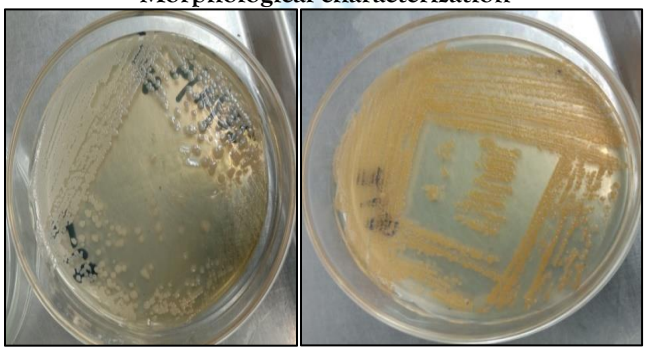


Figure 2: Enrichment of mice swab samples

Morphological characterization



Bacterial isolates were then characterized by morphology, by using Mannitol salt media. On nutrient agar all *S. aureus* strains produce cream to golden yellow colonies.

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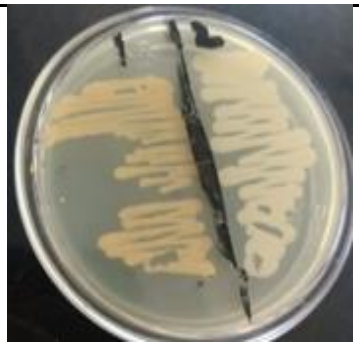

Figure 3: Morphological characterization of bacterial isolates

Gram Staining Results

Using an isolated strain of *S. aurous* cultured for a whole night, Gram staining identified the organism are Gram-positive cocci arranged in grape-like clusters. Prior to accomplishing confirmation biochemical testing, the Gram reaction and cellular organization provided first proof that these isolates were staphylococci.

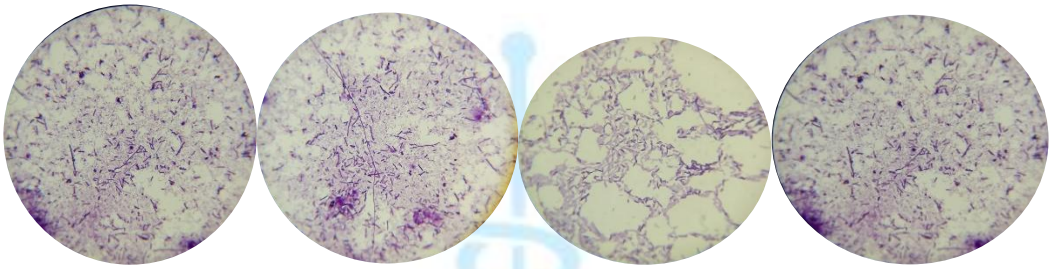

Figure 4: Gram staining results of bacterial isolates

Catalase test results

The isolated bacteria generated gas bubbles on a glass slide after being treated with a few drops of 3% H2O2, indicating that the catalase test was positive. The catalase test result showed that all the *S. aureus* bacterial strain were positive.

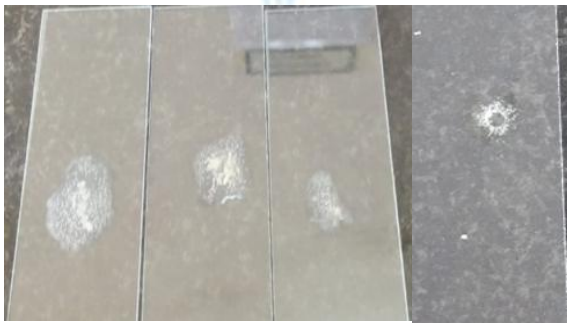

Figure 5: Catalase test results of bacterial isolate

Coagulase test

By identifying cell wall-associated clumping factor (bound coagulase), the slide coagulase test was used to quickly identify *Staphylococcus aureus*. On a glass slide, fresh colonies were suspended in saline and combined with rabbit plasma that was previously citrate-treated. Although *S. aureus* usually causes severe and instantaneous agglutination (visible clumping within 10–30 seconds), all of the four test samples exhibited no agglutination, which may indicate that bound coagulase was not present. This unfavorable result may suggest one of the following: Coagulase-negative staphylococci (CoNS) like *S. epidermidis* or *S. saprophyticus* could be the isolates; 2)

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the bacterial inoculum might have been inadequate or poorly prepared; 3) the plasma might have lost its reactivity; or 4) uncommon strains of *S. aureus* with weak clumping factor expression were found. Crucially, coagulase responses are weak or delayed in certain methicillin-resistant *S. aureus* (MRSA) strains. The more accurate tube coagulase test, which detects free coagulase and can identify *S. aureus* strains that would test negative using the slide method, is what experts recommend to validate these findings. The significance of employing many phenotypic tests for precise staphylococcal identification is shown by this disparity.

Figure 6: Coagulase test results of bacterial isolates

Oxidase test

Kovac's oxidase reagent was used for this purpose. The ability of the organism to synthesize the cytochrome c oxidase was assessed by using the oxidase test. The bacterium was positive to test whether the purple colour formed between 30-60 seconds. Our study's isolates *S. aureus* were all oxidase negative.

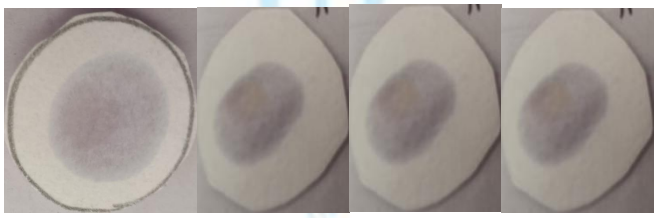

Figure 7: Oxidase test results of bacterial isolates

Motility Test Results

Semi-solid motility agar (0.3–0.4%) was used to determine the motility of *Staphylococcus aureus,* and four test samples were incubated for 24–48 hours at 37°C. Since *S. aureus* lacks flagella and other locomotive features, it is usually non-motile. None of the four samples showed any radiating development or turbidity, which is consistent with this feature and validates the anticipated lack of motility. The findings are consistent with the typical microbiological characteristics of *S. aureus*, a facultative, immobile anaerobe (Lowy,2015). The isolate's identity is further supported by this negative motility result, which also sets it apart from motile infections that show diffuse spreading in motility media, such as Proteus species or *Pseudomonas aeruginosa*.

Volume 3, Issue 2, 2025

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Figure 8: Motility test results of bacterial isolates

Methyl Red (MR) Test Results

The ability of *Staphylococcus aureus* to ferment glucose and create stable acid end products was evaluated using the Methyl Red (MR) test. After being added to MR-VP (Methyl Red-Voges Proskauer) broth, the bacterial isolates were cultured for 48 hours at 37°C. A few drops of Methyl Red indicator were added to the colony after it had been incubated. While a negative result (yellow/orange) indicates non-acidic or butanediol pathway fermentation, a positive result (red hue) would indicate mixed-acid fermentation. The test result for *S. aureus* was negative

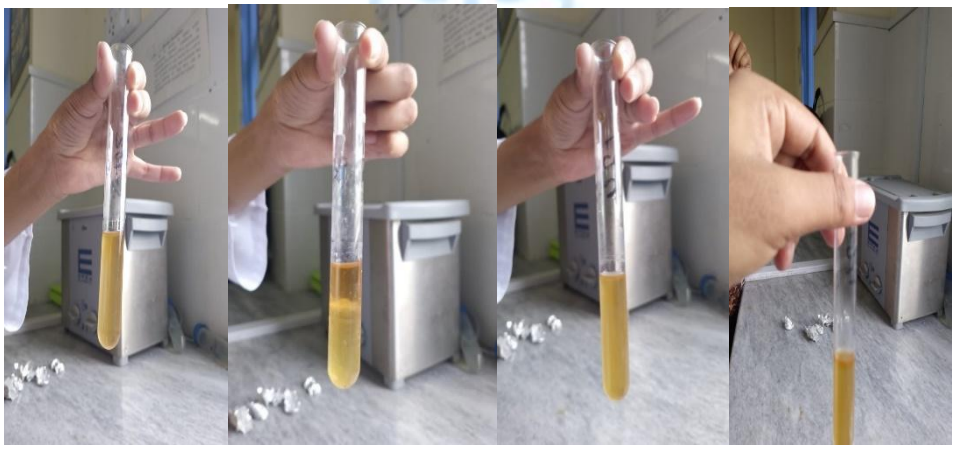

Figure 9: Methyl red test results of bacterial isolates

Antimicrobial susceptibility testing

To determine the antibiotic sensitivity pattern of isolated strains, sensitivity testing was performed. The highest antimicrobial resistance was substantial, with tetracycline (50%), erythromycin (75%).

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ISSN: (e) 3007-1607 (p) 3007-1593

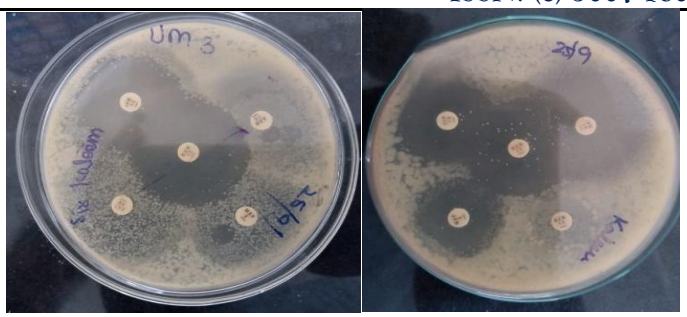

Figure 10: Antibiotic susceptibility results for all bacterial isolates

Antibiotic	Disc Concentration	CLSI Breakpoint (mm)^a	EUCAST Breakpoint (mm)^b
Penicillin	10 IU	≤28 (S), ≥29 (R)	–
Oxacillin	1 µg	≤10 (R), ≥13 (S)	≤10 (R), >10 (S)
Cefoxitin	30 µg	≤21 (R), ≥22 (S)	≤25 (R), >25 (S)
Vancomycin	30 µg	≥15 (S), ≤14 (R)	≥17 (S), <17 (R)
Erythromycin	15 µg	≤13 (R), ≥23 (S)	≤13 (R), >13 (S)
Clindamycin	2 µg	≤14 (R), ≥21 (S)	≤14 (R), >14 (S)
Gentamicin	10 µg	≤12 (R), ≥15 (S)	≤12 (R), >12 (S)
Tetracycline	30 µg	≤14 (R), ≥19 (S)	≤11 (R), >11 (S)
Trimethoprim-Sulfa	1.25/23.75 µg	≤10 (R), ≥16 (S)	≤10 (R), >10 (S)

Bacterial Ribotyping

The 16S rRNA gene sequence exhibited 98.37% similarity to the *Staphylococcus aureus* strain (Accession No. OQ626075) according to the BLASTn analysis. The evolutionary link between the isolate and other Staphylococcus species was displayed in the phylogenetic tree constructed with MEGA X software. The isolate grouped with *S. aureus* strains, according to the tree, which supported the findings of the BLAST analysis. The isolate was clearly distinguished from other Staphylococcus species by the distance-based tree, suggesting a unique phylogenetic position.

Table 2: Microbial information extracted from BLASTn results

Subject	Accession No.	OQ626075.1
	Description	*Staphylococcus aureus*
	Length (b)	1046
	Start	1
	End	1046
	Coverage	82
Score	Bit	1834
	E-value	0.0
Identities	Match/Total	1029/1046

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	Percentage (%)	98.37

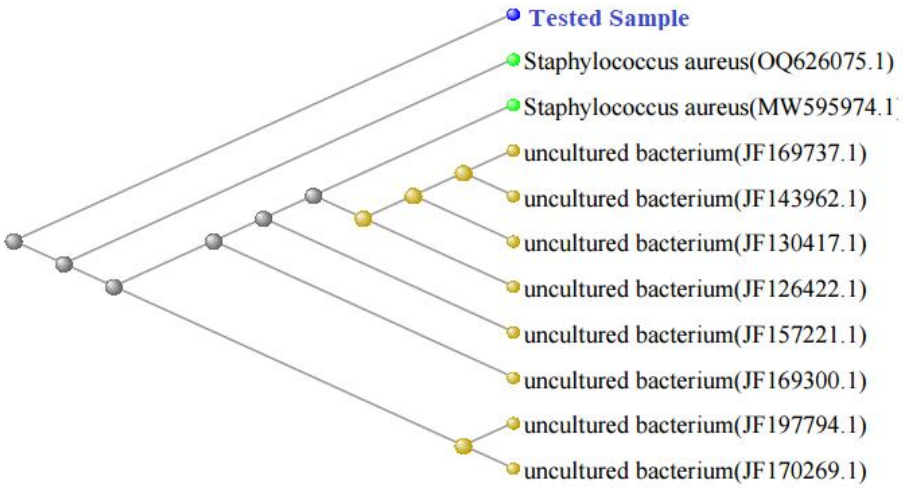
Taxonomic Hierarchy

Table 3: Taxonomic hierarchy of the identified strain

Taxon	Description
Domain	*Bacteria*
Phylum	*Bacillota*
Order	*Bacillales*
Family	*Staphylococcaceae*
Genus	*Staphylococcus*
Species	*S. aureus*

Table 4: Top 10 BLASTn Results

Scientific Name	Max Score	Total Score	Query Cover	E-value	Per. Ident (%)	Acc. Len (b)	NCBI Accession N0.
Staphylococcus aureus	1834	1834	98%	0	98.37	1075	OQ626075.1
Staphylococcus aureus	1829	1829	98%	0	98.28	1555	MW595974.1
Uncultured bacterium	1829	1829	98%	0	98.28	1372	JF197794.1
Uncultured bacterium	1829	1829	98%	0	98.28	1372	JF170269.1
Uncultured bacterium	1829	1829	98%	0	98.28	1372	JF169737.1
Uncultured bacterium	1829	1829	98%	0	98.28	1372	JF169300.1
Uncultured bacterium	1829	1829	98%	0	98.28	1372	JF157221.1
Uncultured bacterium	1829	1829	98%	0	98.28	1372	JF143962.1
Uncultured bacterium	1829	1829	98%	0	98.28	1372	JF130417.1
Uncultured bacterium	1829	1829	98%	0	98.28	1372	JF126422.1



The isolate was determined to be a strain of *Staphylococcus aureus* based on the analysis of the 16S rRNA gene sequence and the construction of a phylogenetic tree. The findings imply that the isolate and other *S. aureus* strains have a firmly evolutionary link. These results offer important new information

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ISSN: (e) 3007-1607 (p) 3007-1593

on the phylogenetic linkages and genetic diversity of Staphylococcus species.

Discussion

The study's isolation and characterization of *Staphylococcus aureus* from domestic mice's intestinal microbiota provide compelling evidence that these everyday home mice may serve as reservoirs for antibiotic-resistant strains of the bacteria. Mouse swab samples from the Abbottabad district were used in the current study to identify bacterial strains. Then, using Mannitol salt agar, the isolated bacterial strains were morphologically described. Cream to golden yellow colonies are produced on MSM by all strains of *S. aureus*. In a related work, Greenwood *et al.,* (2017) shown that *S. aureus* isolated from mouse samples has a yellow-cream hue on the surface of Mannitol salt agar.

After applying the Gram-staining method, microscopic examination demonstrated that every Staphylococcus isolate consisted of grape like clusters. A similar study was conducted by (Van Balen *et al.,* 2017) which shows after applying gram strain *S. aureus* colonies show purple grapes like clusters. The biochemical analysis of the *S. aureus* isolates was another aspect of this work. The isolates' biochemical characterization demonstrated characteristic *S. aureus* traits, such as the synthesis catalase, which are in line with accepted identification standards (Baron *et al.,* 2019). The results of this experiment showed that the *S. aureus* isolates were positive for catalase, negative for coagulase, negative for oxidase, negative for metyl red negative for motility test. Similar, study was conducted by (Price *et al.,* 2012) which shows *S. aureus* isolates were positive for catalase, negative for coagulase, negative for oxidase, negative for metyl red negative for motility test.

The high prevalence of multidrug-resistant (MDR) *S. aureus*, particularly showing resistance to beta-lactams (penicillin, methicillin) and macrolides (erythromycin), mirrors concerning trends observed in human clinical isolates (Turner *et al.,* 2019). To determine the antibiotic sensitivity pattern of isolated strains, sensitivity testing was performed. The highest antimicrobial resistance was substantial, with tetracycline (50%), erythromycin (75%). Similar study was conducted by (Abimannan et al., 2019) which shows the high rates of erythromycin (54%) and tetracycline (68%) resistance are associated with the widespread use of these antibiotics in *S. aureus* (Nairn *et al.,* 2024). The possibility of interspecies transmission or shared selection pressures in human-controlled mouse homes is raised by this comparison. The gastrointestinal tract appears to be a major colonization site that might result in prolonged bacterial shedding and environmental contamination through fecal matter, according to Acton *et al.,* (2020). Domestic mice's role in maintaining and spreading resistant infections warrants special public health attention since they commonly reside in human dwellings, food storage facilities, and agricultural settings.

In mice, intestinal colonization by *S. aureus* has a major effect on the kinetics of transmission. There is growing evidence that the gastrointestinal tract may serve as a reservoir for *S. aureus*, despite the fact that it has traditionally been believed to colonize the skin and nasal mucosa (Piewngam and Otto, 2024). Gut colonization may enhance bacterial survival and fecal-oral transmission routes, especially in unsanitary conditions. Mice may disseminate resistant strains in a range of environments, such as homes, farms, and food processing facilities, because of their high mobility and reproductive tendency (Kalan *et al.,* 2019). The findings of this investigation lend support to the hypothesis that commensal bacteria might contribute to the environmental resistome by acting as connections between natural and human clinical reservoirs (Bengtsson-Palme *et al.,* 2018). The identification of MDR *S. aureus* in domestic mice raises concerns about antimicrobial selection pressures in urban and peri-urban environments. Antibiotic residues in wastewater, agricultural runoff, or improperly disposed of medications may provide selection circumstances that favor bacteria with resistance (Bengtsson-Palme et al., 2018).

Following bioinformatics analysis and 16S rRNA gene sequencing, the bacterial isolate has been identified to be *Staphylococcus aureus*, exhibiting a high degree of similarity (98.37%) to reference strain OQ626075.1. The isolate was highly grouped with known strains of S. aureus, according to the phylogenetic tree constructed in MEGA X using the Fast Minimum Evolution approach. This finding

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ISSN: (e) 3007-1607 (p) 3007-1593

supported the BLASTn results. The isolate's clear separation from other Staphylococcus species in the tree supports its unique evolutionary position in line with its taxonomic classification. The high bootstrap values at significant nodes, whenever accessible, further support the phylogenetic inference's robustness. The discovery of closely related uncultured bacterial sequences in the BLAST results also suggests environmental or clinical variants with conserved 16S regions, highlighting the gene's utility in bacterial identification but also raising the possibility of limitations in distinguishing very similar strains.

The scientific pathway from DNA extraction to phylogenetic reconstruction proved effective in characterizing the isolate, despite the possibility that the small sequence divergences (1.63%) may be the consequence of intra-species genomic variability or PCR/sequencing artifacts. MEGA X's evolutionary model provided a reliable phylogenetic framework, while Clustal Omega's multiple sequence alignment allowed for accurate homology assessment. Even though the isolate's taxonomic hierarchy (Bacillota phylum, Staphylococcaceae family) aligns with accepted *S. aureus* classifications, the discovery of uncultured bacterial matches (like JF197794.1) emphasizes the need for additional markers (like rpoB or tuf genes) for a more precise resolution. Additional specimens may be used in subsequent investigations to assess phenotypic or geographic correlations. In the final analysis, depending on the strain's context, this approach adds to bigger microbial phylogeny databases and validates the identity, which might have implications for industrial, environmental, or medicinal applications. We found that commensal bacteria in urban wildlife often possess resistance genes that are identical to those of human illnesses, which is in line with previous study (Schröttner *et al.*, 2023). This suggests that human activities have a significant impact on the microbial ecology of synanthropic animals, like mice.

Our results emphasize the need for integrated surveillance of antibiotic resistance, which includes monitoring pest species, from a public health perspective. Given that existing One Health approaches mostly focus on livestock and companion animals; our findings suggest that commensal rodents should be considered in resistance containment efforts. According to Collignon *et al.*, (2018), these populations may be less likely to harbor resistant strains if human and veterinary medical practices such as improved cleanliness, rodent control, and sensible antibiotic usage are implemented. Furthermore, the study highlights possible shortcomings in biosecurity protocols that can let resistant bacteria to proliferate across diverse habitats due to mice's freedom of movement between human homes, livestock facilities, and food storage locations (Tălăpan *et al.*, 2023). It should be mentioned that this study has certain limitations. Despite being sufficient for preliminary characterization, the sample size may not accurately reflect the range of *S. aureus* strains seen in mouse populations. Generalizability is limited by geographic limitation to a particular place since resistance trends may vary significantly based on local antibiotic use and epidemiological factors (Klein *et al.*, 2020). Future research should employ whole-genome sequencing to investigate strain relatedness and resistance gene carriage in order to gain a better knowledge of the transmission channels among mice, humans, and other species (Stephens *et al.*, 2022). Longitudinal studies that track resistance patterns over time may help evaluate the efficacy of intervention strategies. Additionally, studies on the duration and stability of gut colonization in mice might provide insight into their role as persistent reservoirs.

Conclusion

The study shows that domestic mice have antibiotic-resistant *S. aureus* strains with resistance profiles similar to problematic human clinical isolates. The findings underscore the complex ecology of antibiotic resistance by showing how commensal animals in human settings aid in the maintenance and dissemination of resistant bacteria. To address this issue, multidisciplinary, collaborative approaches that recognize the interconnectedness of human, animal, and environmental health are required. Enhancing sanitary infrastructure, including pest species in resistance monitoring programs, and reducing antibiotic selection pressure through stewardship programs might all help mitigate this emerging public health issue.

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