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ISOLATION AND PHYLOGENETIC ANALYSIS OF *Staphylococcus aureus* STRAINS ISOLATED FROM MEAT IN TRADITIONAL MARKETS IN HA NOI

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ABSTRACT

Staphylococcus aureus is a major cause of foodborne illness worldwide, causing diarrhea and vomiting. The rising number of *S. aureus* food poisoning cases in Vietnam underscores the urgent need for effective control strategies, particularly given the bacterium's growing antibiotic resistance, complicating treatment options. This study investigates the public health risks posed by *S. aureus* in meat samples collected from traditional markets in Hanoi, Vietnam. A total of 30 bacterial isolates were obtained, of which six were identified as *S. aureus* using MALDI-TOF MS and confirmed through hemolysis testing and 16S rRNA sequencing. These isolates exhibited strong beta-hemolysis, indicating alpha-toxin production and their pathogenic potential. Notably, the antibiotic susceptibility test revealed that some isolated strains displayed varying levels of resistance to commonly used antibiotics, including aztreonam, streptomycin, amoxicillin, amikacin, and enrofloxacin. However, their uniform susceptibility to florfenicol, ceftiofur, doxycycline, and chloramphenicol suggests that these antibiotics remain effective for treating *S. aureus* infections in this region. Phylogenetic analysis revealed a high degree of genetic similarity between the isolated strains and globally distributed *S. aureus* strains associated with foodborne outbreaks in India, Korea, Japan, and Brazil, highlighting the potential for international transmission. These findings highlight the need for ongoing surveillance, improved food safety measures, and the development of updated antibiotic treatment protocols to manage the risks of *S. aureus* in the food supply.

Keywords: 16S rRNA gene, food poisoning, MALDI-TOF MS, phylogenetic tree, *Staphylococcus aureus*.

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INTRODUCTION

Staphylococcus aureus is a significant Gram-positive bacterium which is a serious threat to public health globally, especially due to its ability to cause food poisoning. Known for its characteristic grape-like cluster morphology, *S. aureus* belongs to the Staphylococcaceae family. This pathogen is commonly found on human skin and mucous membranes and can be transmitted through contaminated food, leading to widespread outbreaks of food poisoning (Lowy, 1998). Its ability to survive in a wide range of environmental conditions, including high salt concentrations, enhances its potential as a pathogen of food poisoning, allowing it to contaminate a variety of food products, particularly meats and diary (Bintsis, 2017).

The global importance of *S. aureus* is derived from its ability to produce various toxins, some of which are closely associated with its virulence. Among these toxins, heat-stable enterotoxins are particularly concerning because they are not destroyed by conventional cooking methods (Dho-Moulin & Fairbrother, 1999). These toxins cause symptoms such as nausea, vomiting, abdominal cramps and diarrhea, collectively known as Staphylococcal food poisoning (Hennekinne et al., 2012). Additionally, *S. aureus* produces a range of other virulence factors, including enzymes and cell surface proteins, which contribute to its ability to cause disease. These factors allow the bacterium to adhere to host tissues, evade the immune system, and damage host cells, further complicating the treatment of infections (Foster, 1996).

In recent years, the issue of *S. aureus*-related food poisoning has become particularly pressing in Vietnam, where food safety remains a critical concern. Kim et al. showed that there were 16 cases of food poisoning in Vietnam in the first quarter of 2024, affecting 659 patients and resulting in 3 deaths (Kim, 2024). These alarming statistics underscore the urgent need for comprehensive studies aimed to understand the prevalence, genetic diversity, and antibiotic resistance profiles of *S. aureus* strains in the Vietnamese food supply.

The increasing concern over antibiotic resistance further complicates the situation, as *S. aureus* strains resistant to commonly used antibiotics present a significant challenge to public health. Not only Methicillin-resistance *S. aureus* (MRSA), but also other *S. aureus* strains resistant to various antibiotics have become a major concern in the community, highlighting the need for ongoing surveillance and research to monitor the spread of resistant strains.

This study aims to address these important issues by isolating and characterizing *S. aureus* strains from meat samples collected from some traditional markets in Hanoi. This analysis provides valuable information for public health and the development of treatment strategies. Moreover, the result of this study could inform the creation of updated, region specific guidelines for antibiotic use, supporting broader public health efforts to reduce the impact of food poisoning in Vietnam and similar regions.

MATERIALS AND METHODS

Sampling

Thirty meat samples, including 12 beef samples, 10 chicken samples, 8 pork samples were randomly collected from various traditional markets in Cau Giay, Ha Noi, Vietnam, between June and July 2024. The samples were kept on ice and promptly transported to the Laboratory of Molecular Microbiology, Institute of Biotechnology, Vietnam Academy of Science and Technology.

Bacterial isolation

A small, minced meat sample was prepared and suspended in 10 mL of peptone water for bacterial enrichment. The mixture was then inoculated for 3 hours at 37 ^{o}C, 150 rpm. The enrichment solution of each sample was diluted with Saline solution (NaCl, 0.85%) to the appropriate concentration, and 100 µL of the dilution was spread on a Baird Parker Agar. The medium was incubated at 37 ^{o}C overnight. The obtained colonies were

streaked on Baird Parker Agar medium several times for purification. The purified colonies were stored at 4 ^{o}C and in 30% glycerol at -80 ^{o}C for further analysis.

Identification of isolates using MALDI-TOF MS

All obtained isolates were rapidly assessed using Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF MS) protein mass spectrometry (Singhal et al., 2015) with MALDI-TOF Biotyper (Bruker, Bremen, Germany). The isolates were analyzed with Biotyper Compass Explorer software (version 4.1.100) automatically. The degree of similarity between the experimental and reference strains was measured on a scale of 0 to 3.0. Results above 2.0 suggest high similarity and can be determined at the genus and species level (Clark et al., 2013).

Hemolytic activity

The hemolysis assay was employed to assess the virulence of bacterial strains by evaluating their impact on blood cells using a Blood Agar Plate containing 5% sheep blood (Puspitasari & Turista, 2019). *S. aureus* strain SA 480 exhibiting γ- hemolysis provided by the Laboratory of Molecular Microbiology, IBT, VAST was used as a reference. The blood agar plates were incubated at 37 ^{o}C for 24 hours. Colonies that produced clear zones of hemolysis were identified as hemolysin-positive.

Antibiotic susceptibility

The antibiotic susceptibility of *S. aureus* isolates was assessed using the disk diffusion method on Muller-Hinton Agar plates, as per the Clinical and Laboratory Standards Institute (CLSI) guidelines. The bacterial suspension was prepared by adjusting an overnight culture to a 0.5 McFarland standard, corresponding to approximately 1.5×10^8 CFU/ml. The standardized suspension was uniformly swabbed onto the surface of the MHA plates. Antibiotic disks were placed onto the inoculated agar surface, each containing a specific antibiotic. The plates were incubated at 37 ^{o}C for 18−24 hours. After incubation, the zones of inhibition around the disks were measured to determine the antibiotic susceptibility of the isolates. The results were interpreted according to CLSI guidelines, categorizing the isolates as susceptible (S), intermediate (I), or resistant (R) (Schreckenberger & Binnicker, 2011).

Total DNA extraction

The overnight bacterial culture was centrifuged at 12,000 rpm for 1 minute at 4 ^{o}C. The supernatant was removed and the pellets were used to extract total DNA using GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, USA) (Sola-Gines et al., 2015) following the manufacturer's instructions. The quality of extracted DNA was assessed by gel electrophoresis on a 1% agarose gel, stained with Ethidium bromide (Merck, Germany), and observed under UV light. The concentration and purity of the DNA were determined with a Nanodrop Lite (Thermo Fisher Scientific, USA).

16S rRNA amplification

The total qualified DNA samples were utilized as a template for the PCR amplification of the full-length 16S rRNA gene, using a specific pair of primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR reaction was performed in a total volume of 25 µL. The PCR thermal cycle conditions were as follows: initial denaturation at 94 ^{o}C for 5 minutes, followed by 35 cycles of denaturation at 94 ^{o}C for 1 minute, annealing at 56 ^{o}C for 45 seconds, and extension at 72 ^{o}C for 1 minute, with a final extension at 72 ^{o}C for 10 minutes. Reactions were performed in a C1000 Thermal Cycler (Bio-Rad, USA) PCR products were analyzed by electrophoresis on a 1% agarose gel stained with Ethidium Bromide (Merck, Germany) and visualized under UV light. The PCR products were subsequently purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific, USA). DNA concentration was measured with a Nanodrop Lite spectrophotometer (Thermo Fisher Scientific, USA).

16S rRNA sequencing and analysis

The bacterial 16S rRNA gene sequences were determined using the Sanger sequencing method on an ABI prism 3100 automatic DNA sequencer (Applied Biosystems, USA). Sequence data were processed and analyzed with BioEdit software (version 7.2.6.1). The resulting gene sequences were aligned and compared to 16S rRNA gene sequences of *S. aureus* strains available in GenBank (NCBI) utilizing the BLAST tool. The phylogenetic tree was constructed with MEGA X software. Phylogenetic relationships were validated with 1000 bootstrap replicates to ensure the reliability of the taxa clusters.

RESULTS

Bacterial isolation

A total of 30 colonies were isolated from the collected samples using Baird Parker Agar, a selective medium for *S. aureus*. The obtained colonies exhibited the characteristic appearance of black, shiny, convex formations measuring 1–1.5 mm in diameter, with narrow, white, entire margin, and surrounded by a clear zone - features previously indicated as characteristic of *S. aureus* (Zangerl, 2003) (Fig. 1A). All isolates were then purified and stored at 4 ^{o}C for subsequent experiments and in 30% glycerol at -80 ^{o}C for long-term preservation.

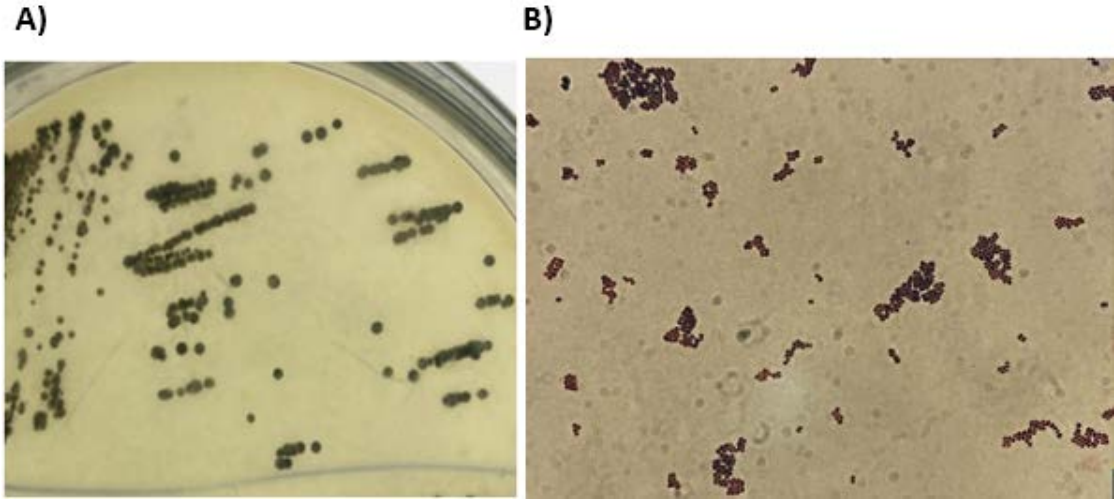

Figure 1. Morphology of bacterial colonies isolated from the samples in this study**.** A) *Staphylococcus aureus* SA 02 colony morphology on Baird Parker Agar plate and B) *Staphylococcus aureus* SA 02 cell morphology visualized using Gram staining

Identification of bacterial isolates by MALDI-TOF MS

Among the 30 isolates, six were identified as *S. aureus* by MALDI-TOF MS with score values ranging from 2.02 to 2.41 compared to the reference strain *S. aureus* (Table 1). Gram staining confirmed the presence of typical gram-positive cocci characteristic of *S. aureus* (Fig. 1B). The remaining isolates were identified as *Macrococcus* spp., *Corynebacterium* spp., *Enterococcus* spp. and *Mammaliicoccus* spp. And 10 out of the 30 isolates were not defined by MALDI-TOF MS, resulting in a 'No organism found'. All the *S. aureus* strains identified were used for further analysis.

Table 1. Representative result of identification of isolated bacteria by MALDI-TOF MS

No.	Identified strains	Score value	Reference	Note
1	No Organism Identification Possible	1.52	*Macrococcus caseolyticus CCUG 60665 CCUG*	

No.	Identified strains	Score value	Reference	Note
2	No Organism Identification Possible	1.55	*Macrococcus caseolyticus CCUG 60665 CCUG*	
3	No Organism Identification Possible	1.50	*Thauera mechernichensis Tl1 MPB*	
4	No Organism Identification Possible	1.60	*Macrococcus caseolyticus CCUG 60665 CCUG*	
5	No Organism Identification Possible	1.52	*Macrococcus caseolyticus DSM 20597T DSM*	
6	*Macrococcus caseolyticus*	1.87	*Macrococcus caseolyticus CCUG 60665 CCUG*	
7	No Organism Identification Possible	1.37	*Brevundimonas aurantiaca DSM 4731T HAM*	
8	No Organism Identification Possible	1.38	*Macrococcus caseolyticus CCUG 53809 CCUG*	
9	No Organism Identification Possible	1.40	*Paracoccus versutus B352 UFL*	
10	*Mammaliicoccus sciuri*	1.91	*Mammaliicoccus sciuri DSM 6671 DSM_dir*	
11	No Organism Identification Possible	1.63	*Macrococcus caseolyticus CCUG 28808 CCUG*	
12	*Enterococcus faecalis*	2.14	*Enterococcus faecalis DSM 20409 DSM*	
13	*Macrococcus caseolyticus*	1.89	*Macrococcus caseolyticus CCUG 60665 CCUG*	
14	*Macrococcus caseolyticus*	1.70	*Macrococcus caseolyticus CCUG 60665 CCUG*	
15	No Organism Identification Possible	1.67	*Macrococcus caseolyticus DSM 20597T DSM*	
16	*Macrococcus caseolyticus*	1.93	*Macrococcus caseolyticus CCUG 53809 CCUG*	
17	*Macrococcus caseolyticus*	1.71	*Macrococcus caseolyticus CCUG 53809 CCUG*	
18	*Macrococcus caseolyticus*	2.04	*Macrococcus caseolyticus CCUG 53809 CCUG*	
19	*No Organism Identification Possible*	1.57	*Macrococcus caseolyticus CCUG 53809 CCUG*	
20	*Enterococcus faecalis*	2.34	*Enterococcus faecalis 104575 LDW*	
21	*Staphylococcus aureus*	2.02	*Staphylococcus aureus ssp aureus DSM 20231T DSM_dir*	SA 01
22	*Staphylococcus aureus*	2.29	*Staphylococcus aureus ssp aureus DSM 20231T DSM_dir*	SA 02
23	*Corynebacterium vitaeruminis*	2.16	*Corynebacterium vitaeruminis DSM 20294T DSM*	

No.	Identified strains	Score value	Reference	Note
24	*Escherichia coli*	2.23	*Escherichia coli ATCC 25922 THL*	
25	*Staphylococcus aureus*	*2.37*	*Staphylococcus aureus ssp aureus DSM 20231T DSM_dir*	SA 03
26	*Staphylococcus aureus*	*2.41*	*Staphylococcus aureus ssp aureus DSM 20231T DSM*	SA 04
27	*Pseudomonas aeruginosa*	*2.37*	*Pseudomonas aeruginosa ATCC 27853 THL*	
28	no peaks found	*0.00*	*no peaks found*	
29	*Staphylococcus aureus*	*2.48*	*Staphylococcus aureus ssp aureus DSM 20231T DSM_dir*	SA 05
30	*Staphylococcus aureus*	*2.40*	*Staphylococcus aureus ssp aureus DSM 3463 DSM*	SA 06

Hemolytic activity

Hemolysis assay is commonly used to identify *S. aureus* due to its ability to produce hemolysin that lyses red blood cells. The hemolysis assay showed that six isolated *S. aureus* strains exhibited the ability to produce alpha-toxin, resulting in a clear, transparent zone around the colonies, indicative of beta-hemolysis (Fig. 2).

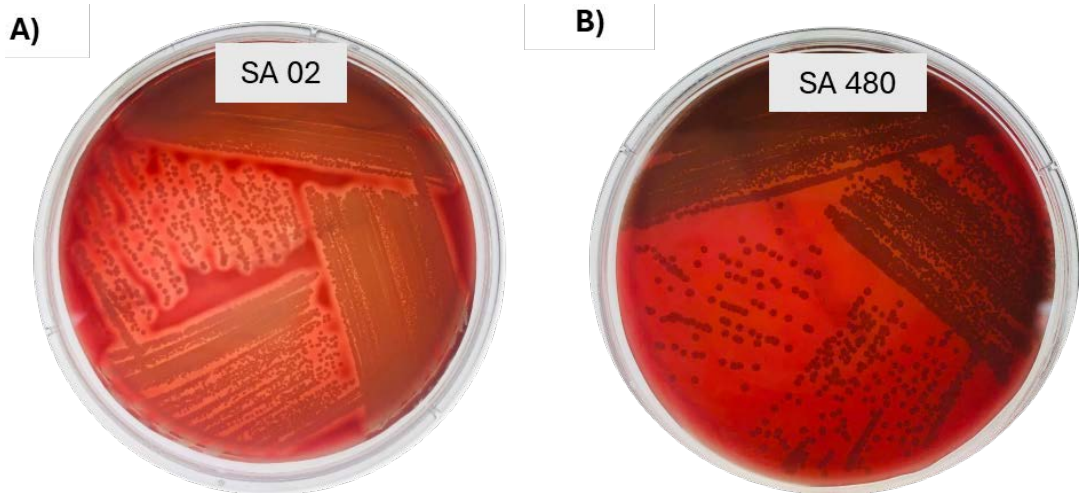

Figure 2. Hemolysis activity of *Staphylococcus aureus* strains on blood agar A) β-hemolysis of *Staphylococcus aureus* isolate SA 02 obtained in this study; B) γ- hemolysis of referenced *Staphylococcus aureus* strain, SA 480

Antibiotic susceptibility

The antibiotic susceptibility test revealed that all isolated *S. aureus* strains were susceptible to florfenicol, ceftiofur, doxycycline, and chloramphenicol, while uniformly resistant to aztreonam. Most strains were susceptible to ciprofloxacin, with one exhibiting intermediate resistance. Amikacin, streptomycin, and amoxicillin showed mixed responses, ranging from susceptibility to resistance. Enrofloxacin was generally effective, though some isolates exhibited intermediate resistance. These findings highlight the varying levels of antibiotic efficacy among the isolates (Fig. 3).

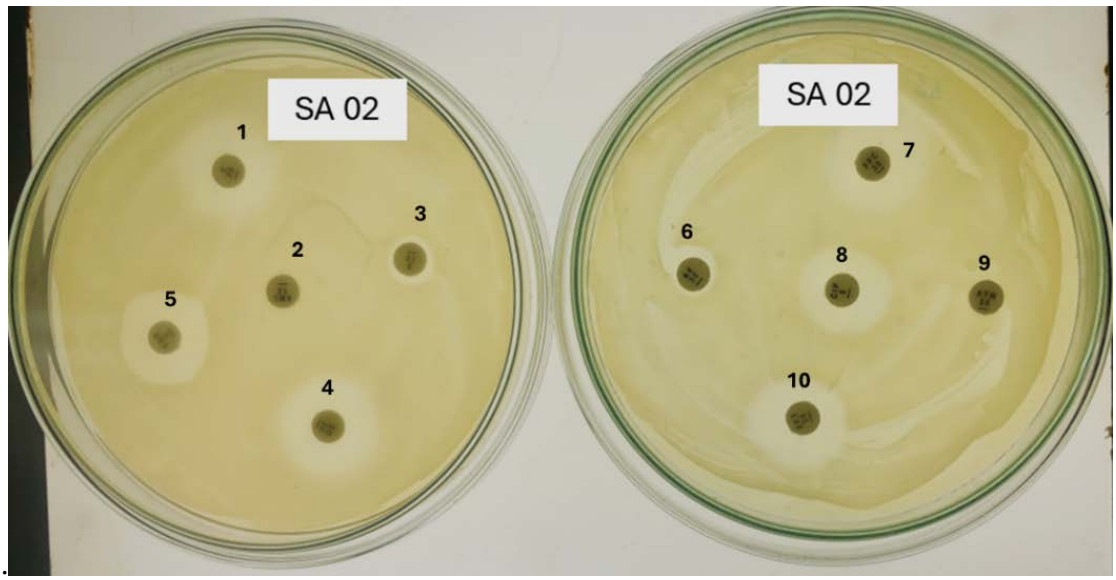

Figure 3. Representative antibiotic test result of *Staphylococcus aureus* isolates SA 02. Antibiotic abbreviation: 1- Chloramphenicol, 2 - Amoxicillin, 3 - Streptomycin, 4 - Doxycycline, 5 - Enrofloxacin, 6 - Amikacin, 7 - Florfenicol, 8 - Ciprofloxacin, 9 - Aztreonam, 10 – Ceftiofur

Table 2. Antibiotic susceptibility test result of the isolated *Staphylococcus aureus* strains. S: Susceptibility, I: Intermediate resistance, R: Resistance

Antimicrobial agents	Dose (µg)	SA01	SA02	SA03	SA04	SA05	SA06
Florfenicol	30	S	S	S	S	S	S
Ceftiofur	30	S	S	S	S	S	S
Amikacin	30	I	I	S	S	S	I
Aztreonam	30	R	R	R	R	R	R
Ciprofloxacin	5	S	S	S	S	S	I
Doxycycline	30	S	S	S	S	S	S
Streptomycin	10	S	I	S	S	I	R
Amoxicillin	10	S	R	S	-	I	R
Enrofloxacin	5	I	S	S	S	S	I
Chloramphenicol	30	S	S	S	S	S	S

DNA extraction and 16S rRNA amplification

The total DNA of the six isolated *S. aureus* strains that exhibited β-hemolysis were extracted. The quantity and quality of extracted DNA samples were confirmed by agarose gel electrophoresis and NanoDrop Lite, respectively. The concentration of the extracted DNA samples varied from 659.7 ng/µL to 1,043.2 ng/µL, with A260/280 ratios ranging between 1.80 and 1.94. These results indicated that the extracted DNA samples were of sufficient quality for subsequent analysis. Moreover, the 16S rRNA genes were successfully amplified from all the purified DNA samples, resulting in DNA bands with approximately 1,500 bp in size (Fig. 4).

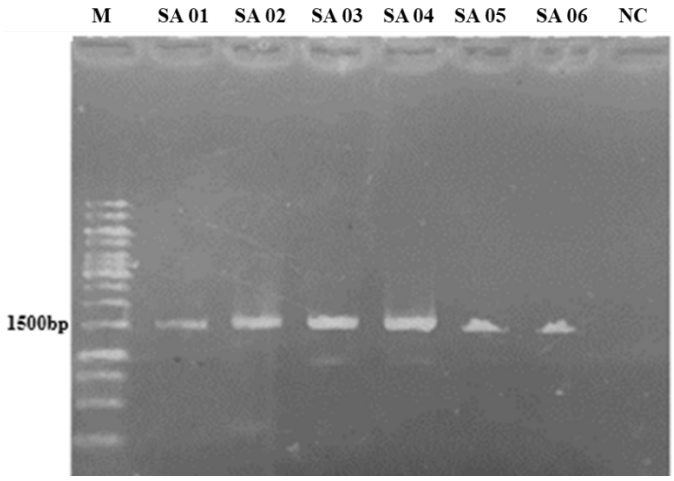

Figure 4. The 16S rRNA PCR products on 1% agarose gel M: Thermo Scientific™ GeneRuler™ 1kb DNA ladders, NC: negative control

16S rRNA sequence and phylogenetic analysis

The 16S rRNA sequence analysis showed that the six isolated *S. aureus* strains showed 99.64–100% sequence similarity with each other and 99.31–100% sequence similarity with those of five *S. aureus* strains deposited on GenBank (NCBI). These sequences were used to construct a phylogenetic tree, using *the* 16S rRNA sequence of *Salmonella enterica* strain NBRC 15187 as an outgroup and various *Staphylococcus* species other than *S. aureus* were included for comparison (Fig. 5).

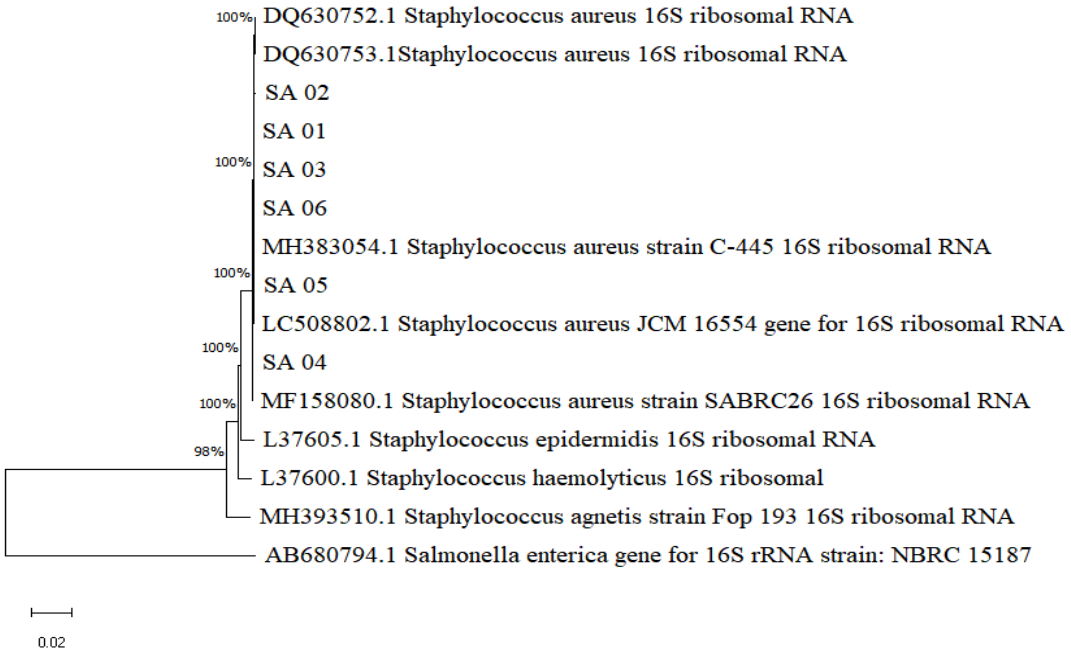

Figure 5. Phylogenetic tree based on 16S rRNA sequences of isolated *Staphylococcus aureus* strains and *Staphylococcus aureus* strain reference sequences from Genbank. *Salmonella enterica* gene for 16S rRNA strain: NBRC 15187 was used as an outgroup

As shown in the phylogenetic tree, the six *S. aureus* strains (SA 01, SA 02, SA 03, SA 04, SA 05, SA 06) from this study were clustered in the same group with the following *S. aureus* strains: *S. aureus* from the food Bhalla in India (accession number, DQ630752.1), which was isolated during a food poisoning outbreak (Vijay et al., 2007), *S. aureus* (DQ630753.1) from a clinical sample of a food poisoning outbreak in India (Vijay et al., 2007), *S. aureus* (MH383054.1) from an ice cream sample in South Korea (Samir et al., 2018), *S. aureus* JCM (LH508802.1) from a clinical sample in Japan, and *S. aureus* strain SABRC26 (MN652637) from a Minas cheese sample in Brazil, with hight bootstrap value of 100%. Additionally, these *S. aureus* strains were clearly distinguished from other *Staphylococcus* species such as *Staphylococcus epidermidis* (L37605.1), *Staphylococcus haemolyticus* (L37600.1) and *Staphylococcus agentis* strain (MH393510.1).

DISCUSSION

This study utilized a multi-technique approach to isolate, identify and characterize *S. aureus* strains from meat samples obtained at traditional markets in Cau Giay, Ha Noi, Vietnam. Initially, Baird-Parker Agar was used as a selective medium to identify colonies with distinct *S. aureus* morphologies as described previously (Krimmer et al., 1999). Further identification was carried out using MALDI-TOF MS, which identifies microorganisms by ionizing their proteins with a laser and measuring the time it takes for these ions to reach a detector. The resulting mass spectrum is then compared with a reference database for species-level identification (Patel, 2015). The result revealed that 6 out of the 30 isolates isolated were identified as *S. aureus* strains.

The hemolytic activity assay provided additional evidence of the pathogenic potential of the isolated strains, as all exhibited beta hemolysis on blood agar plates. This clear and consistent hemolytic activity is a hallmark of *S. aureus*, driven primarily by the production of alpha-toxin, a potent virulence factor that disrupts red blood cell membranes, leading to complete cell lysis (Puspitasari & Turista, 2019). This finding is concerning, as alpha-toxin is associated with severe clinical outcomes, including tissue damage and evasion of the immune system (Berube & Wardenburg, 2013).

Antibiotic susceptibility test of the isolated *S. aureus* strains revealed that they were all resistant to aztreonam, but showed mixed responses to amikacin, streptomycin, and amoxicillin. In contrast, the uniform susceptibility to florfenicol, ceftiofur, doxycycline, and chloramphenicol suggests that these antibiotics remain effective for treating *S. aureus* infections in this region. The intermediate resistance observed in some strains to ciprofloxacin and enrofloxacin may align with the global trend of increasing fluoroquiolone resistance in *S. aureus* and could indicate the early stages of resistance development (Dalhoff, 2012). These findings emphasize the importance of continuously monitoring antibiotic resistance patterns in *S. aureus* strains, particularly in regions like Vietnam where the use of antibiotics in livestock is prevalent. The variability in antibiotic resistance also highlights the potential for certain antibiotics to lose their efficacy over time, necessitating the need for updated treatment protocols and the exploration of alternative therapeutic options.

Identification of origins of bacteria using 16S rRNA sequencing confirmed the identity of the isolates, showing high sequence similarity 16S rRNA (99.31−100%) with reference *S. aureus* strains in GenBank. Phylogenetic analysis revealed that the isolated strains clustered with *S. aureus* strains from diverse geographical locations including India, Korea, Japan, and Brazil, suggesting significant phylogenetic relationships with global *S. aureus* strains. It also indicates the possibility of a common ancestor or a close link to globally occurring foodborne outbreaks, implying that transmission routes may extend beyond Asia to regions like Brazil. This phylogenetic connection underscores the importance of global collaboration in monitoring and

managing foodborne pathogens, as local outbreaks may have far-reaching implications.

Strong clustering was noticeable with strains with DQ630752.1, identified from the food, and DQ630753.1, from a clinical sample, both associated with a food poisoning outbreak in India (Vijay et al., 2007). These strains have shown heat resistance in previous studies, where heat treatment at 60 ^{o}C for 10, 20, 30, and 60 minutes resulted in two-thirds of the *S. aureus* strains surviving (Vijay et al., 2007). This characteristic is different from other bacterial causes of food poisoning like *Salmonella* spp. (Nishikawa et al., 1993) and *Vibrio* spp. (TEMMYO, 1966). This finding highlights the risk of *S. aureus* transmission through food, particularly meat, and their presence in clinical samples underscores their capability to cause human infections. This emphasizes the critical importance of effective food safety management and clinical management strategies. Moreover, the heat resistance of these strains points to the challenges in controlling *S. aureus* contamination through conventional food processing methods, suggesting the need for more robust safety protocols.

By combining different methods including culture-based method, MALDI-TOF MS, and 16S rRNA sequencing, this study achieved greater accuracy and efficiency than conventional approaches that rely on a single technique. Culture-based methods, while reliable, can be labor-intensive and prone to human error, especially dealing with visually similar colonies or isolating a single colony. MALDI-TOF MS offers rapid, species-level identification but relies on a reference database, which may have difficulties with uncommon or novel strains. 16S rRNA sequencing provides further precise genetic confirmation, further solidifying the identification. This multi-technique approach not only enhances the accuracy of pathogen identification but also provides a comprehensive understanding of the genetic diversity and antibiotic resistance profiles of the isolates, which is crucial for developing targeted intervention strategies.

In the context of global food production and distribution, the findings of this study have significant public health implications. The presence of *S. aureus* strains could be a potential source of infections, and their widespread distribution indicates a serious risk to food safety. Continuous surveillance and monitoring of *S. aureus* in the food supply chain are essential and implementing effective food safety measures is crucial.

CONCLUSION

Our study showed that *S. aureus* strains isolated from meat samples in traditional markets in Hanoi exhibited strong beta-hemolysis and high resistance to aztreonam indicating that the isolated strains are highly virulent. Phylogenetic analysis revealed that these isolates are genetically related to *S. aureus* strains from different regions, suggesting the potential for global transmission. Taken together, our findings emphasize the importance of robust food safety management.

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