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Molecular Identification of *Staphylococcus aureus* Isolated from Soy Milk Sold in Retail Shops in Enugu Metropolis, Enugu State, Nigeria Using Gel Electrophoresis and NCBI-Blast Analysis

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Abstract

Staphylococcus aureus is an important foodborne pathogen that is of major concern to public health especially when it becomes resistant to a last-line drug of choice like vancomycin. As the use of plant derived drinks such as soy milk have grown, the issue of microbial contamination and antimicrobial resistance is also of greater concern particularly in regions with little regulations. This study was done to determine the occurrence of Vancomycin-Resistant *Staphylococcus aureus* (VRSA) in soy milk retailed at shops in Thinker Corner, Enugu. Eight (8) samples of soy milk drinks were randomly purchased in retail outlets. The samples were cultured using on mannitol salt agar and subsequently identified using their morphological features, Gram stain and biochemical tests (catalase test and coagulase test). Antibiotic susceptibility test (AST) was done with vancomycin as the test antibiotic using well diffusion method on Mueller-Hinton agar after standardizing to 0.5 McFarland turbidity. The diameter of the zones of inhibition was measured in mm and interpreted using the EUCAST breakpoint guidelines. Of the eight *S. aureus* isolates tested, five (5) were resistant to vancomycin, with MIC values ranging from 60 to 30 mg/L. The result of the agarose gel electrophoresis r visualized under UV light showed strong, distinct bands in lane M2, M3, M4, which indicates successful PCR amplification of 16 S gene in these samples. The Basic Local Alignment Search Tool (BLAST) was used on the DNA sequences of the test isolates. The BLAST tree indicated similarities when the query sequence was compared to sequences found in the database. The detection of Vancomycin Resistant *Staphylococcus aureus* poses a possible health hazard and calls for routine surveillance and improved hygiene practices during the production and sales of the plant-based drinks.

Subject Areas

Applied Biology, Biotechnology

Keywords

Staphylococcus aureus, Vancomycin, Resistance, Soy Milk, Public Health

1. Introduction

Staphylococcus aureus is a widespread gram-positive bacterium that is recognised by its ability to cause diverse diseases, including foodborne diseases. It is of importance in food safety because it causes heat-stable enterotoxins, which are not inactivated by cooking or gentle pasteurization. Soy milk is a common plant drink that is gaining much attention with regard to microbial contamination, mainly in places where the manufacturing, processing, and storage procedures are uncontrolled [1]. Since *S. aureus* is able to colonize human skin, nostrils and mucous membranes, it can be contaminated during the processing of soy milk by direct Human contact, dirty equipment or dirty processing environments.

The development of antibiotic-resistant *S. aureus*, especially those resistant to vancomycin, an antibiotic commonly reserved as a last line of defense in clinical practice, is now a public health concern in control of foodborne pathogens. The danger is especially serious when such resistant strains enter the mainstream food products, such as soy milk. Vancomycin-Resistant *S. aureus* (VRSA) develops by acquiring resistance determinants, which usually include the vanA gene cluster, by horizontal gene transfer usually by Enterococcus species [2]. In addition to being resistant to glycopeptide antibiotic treatment, these strains also have expanded resistance properties that increase the danger of infections that cannot be cured in immunocompromised persons and the healthy population.

The case of soymilk contamination with VRSA carries a two-fold threat: to the food safety, on the one hand, and to the clinical antibiotics' efficiency, on the other hand. Among the reasons that cause microbial contamination of soy milk, there are inadequate pasteurization, dirty handling of raw materials, and unhygienic production or storage facilities [3] [4]. It has been found that *S. aureus* is able to develop biofilms on food-contact surfaces making regular cleaning irrelevant and allowing the survival of resistant strains in processing environments (Otto, 2023).

Although much is known about Methicillin-Resistant *S. aureus* (MRSA), especially the mecA gene that encodes penicillin-binding protein 2a (PBP2a), which mediates β-lactam resistance, there is a paucity of information on VRSA specifically in plant-based foods.

Molecular methods, especially polymerase chain reaction (PCR) have become the gold standard methods in the detection of the presence of specific antibiotic resistance genes in bacterial isolates because of their sensitivity and specificity. These protocols allow quick detection of resistance genes vanA and vanB or other determinants of vancomycin resistance providing a sensitive basis to surveillance of resistant strains in foods [5]. In addition, molecular characterization may also give information regarding the genetic mechanism of resistance and the possibility of horizontal gene transfer among foodborne pathogens [6].

Statement of the Problem

In many developing regions, including Nigeria, concerns on the safety of plant-based food products like soy milk, especially where food production and hygiene regulations are poorly enforced continue to be of concern.

Unfortunately, molecular surveillance of antibiotic-resistant bacteria in commercial plant-based beverages is almost non-existent in many parts of Nigeria. The absence of systematic screening, poor regulatory oversight, and the lack of microbiological testing standards for such products make it difficult to prevent or control outbreaks linked to resistant organisms. This knowledge gap poses a major risk to public health, particularly in urban areas such as Thinker's Corner in Enugu, where informal markets flourish and quality control is often minimal. As such the aim of this study is to identify Vancomycin-Resistant *Staphylococcus aureus* isolates obtained from commercial soy milk sold in Thinkers Corner, Enugu, Nigeria.

2. Materials and Methods

2.1. Study Area/Sampling Sites

Thinker's Corner is located in Enugu, a city in the southeastern area of Nigeria, and it is very popular. It is located at latitude 6.4693'0"N, longitude 7.5265'0"E. The study was carried out in Microbiology Laboratory of Godfrey Okoye University, Enugu, Nigeria from February to June 2025.

2.2. Sample Collection Method

Soy milk, samples were collected from different vendors namely roadside sellers, mini marts, open stalls, and mobile food carts. The variety in sampling showed where local residents typically bought their soy milk.

Eight (8) soymilk samples were collected with sterile and safe food containers to prevent any contamination from outside. Different batches of packaged soy milk were chosen by selecting sealed packages. All samples were placed in ice-filled coolers and transported to the laboratory within an hour.

2.3. Culture Media Preparation

All the media used in this study were prepared and sterilized according to the manufacturer's instructions. The media used included Nutrient agar, Mueller Hinton agar, Mannitol salt agar, Nutrient broth, peptone water.

2.4. Cultivation of Bacteria Cultures

With the aid of sterile syringes, 0.2 ml of the respective diluents of broth-cultured specimens were aseptically collected and inoculated onto the surfaces of the various solidified Mannitol salt agar in sterile petri dishes. Thereafter, it was incubated for 24 hours at 37˚C. The number of organisms on the plates with distinct growth after incubation was noted and counted [7].

2.5. Isolation of the Pure Cultures of Bacteria

With the aid of a sterile wire loop, a colony from each respective Mannitol salt agar plate was picked and streaked accordingly in a series of parallel and non-overlapping lines on the surfaces of Mannitol salt agar sterile Petri dishes for 24 - 48 hours at 35˚C [8].

2.6. Identification and Characterization of Isolates

Pure bacteria strains were identified based on their morphological and biochemical analysis.

2.7. Catalase Test

About 5 drops of 3% hydrogen peroxide were added to a test tube. With the aid of an applicator stick, small amount of the isolates was collected and placed in the test tube. The tube was placed against a dark background and observed immediately for bubbles.

2.8. Coagulase Test

Plasma is diluted with physiological saline in ratio of 1:10 in test tubes. The tube was inoculated with the isolated bacteria cultures to make a cloudy suspension; afterward, they were incubated at 37˚C for 1 - 4 hours in a hot water bath. The presence of clots denotes a positive test while the absence of clots denotes a negative test.

2.9. Preparation of McFarland Turbidity Equivalent Standard

A 1% v/v solution of sulphuric acid was prepared by adding 1 ml of concentrated sulphuric acid to 99 ml of water. Also, 1% w/v barium chloride was prepared by dissolving 0.5 g of dehydrated barium chloride in 50 ml distilled water. Then 0.5 McFarland standard equivalent was prepared by adding 0.6 ml of barium chloride solution to 99.4 ml of Sulphuric acid solution. The Mcfarland standard was determined by visual observation.

2.10. Antibiotics Susceptibility Testing (AST)

The susceptibility tests were performed following the M2A6 disc diffusion method as recommended by the Clinical and Laboratory Standards Institute [9] using Mueller-Hinton agar. The suspension was incubated for 3 hours at 37˚C to allow for the growth of test organism till the density was equivalent to the turbidity of 0.5 McFarland.

The standardized inoculums were swabbed onto Mueller-Hinton agar plate and the discs were placed on the inoculated plates and pressed firmly onto the agar plate for complete contact. The agar plates were left on the bench for 30 minutes to allow for diffusion of the antibiotics and were incubated at 37˚C for 24 h [8].

The susceptibility of each isolate to each antibiotic was shown by a clear zone of growth inhibition and this was measured using a meter rule in millimeters and the diameter of the zones of inhibition was interpreted using a standard chart [10].

2.11. DNA Extraction

Staphylococcus aureus isolates were prepared for DNA extraction using the boiling method [11]. A single loopful of *S. aureus* colonies was suspended in 1.5 mL of sterile saline and centrifuged at 12,000 × g for 5 minutes. After removing the supernatant, the bacterial pellet was resuspended in 200 µL of sterile distilled water and boiled at 100˚C for 10 minutes. The mixture was then centrifuged again at 12,000 × g for 5 minutes, and the supernatant containing the DNA was transferred to a clean microcentrifuge tube and stored at −20˚C.

2.12. Gel Electrophoresis

PCR products were analyzed using 1.5% agarose gel electrophoresis. A 100 bp DNA ladder (Thermo Fisher Scientific, USA) was used to estimate fragment sizes. The gel was stained with ethidium bromide and visualized under UV light [12].

2.13. Specie Identification

The bacteria isolates were BLASTED using National Centre for Biotechnology Information (NCBI) data to identify the bacteria species.

3. Results

3.1. Isolation and Identification of *Staphylococcus aureus*

A total of eight (8) commercial soymilk samples were inoculated on Mannitol Salt Agar (MSA) for the isolation of presumptive *Staphylococcus aureus*. Table 1 shows the results of all tests carried out.

Table 1. Morphological, Biochemical and Microscopic Characteristics of Isolates on MSA.

No of Samples	Colony Morphology (MSA)	Coagulase test	Catalase test	Gram Reaction	Presumptive Organism
Eight (8)	Yellow, shiny, smooth, convex	positive	positive	Gram-positive cocci in clusters	*Staphylococcus aureus*

Key: MSA = Mannitol Salt Agar.

The classical golden pigmentation on MSA was observed. Microscopic analysis revealed Gram-positive cocci arranged in grape-like clusters and the biochemical tests confirmed *S. aureus.* These results were suggestive of *S. aureus* and prompted further biochemical confirmation.

3.2. Phenotypic Detection of *Staphylococcus aureus*

Antimicrobial Susceptibility Testing of *Staphylococcus aureus* Isolates (See Table 2).

Table 2. Vancomycin MIC results and interpretation for *Staphylococcus aureus* isolates.

No of Samples	Vancomycin MIC (mm)	EUCAST Interpretation	Remark
SA1	60	Resistant	Vancomycin ineffective
SA2	35	Resistant	Vancomycin ineffective
SA3	30	Susceptible	Near Resistant breakpoint
SA4	30	Susceptible	Near Resistant breakpoint
SA5	33	Resistant	Vancomycin ineffective
SA6	30	Susceptible	Near Resistant breakpoint
SA7	35	Resistant	Near Resistant breakpoint
SA8	00	Resistant	Suggests vancomycin resistance

Key: VAN: Vancomycin 500 mg.

Interpretive zone diameter breakpoints guidelines in mm as advised by EUCAST:

ANTIBIOTICS VAN Sensitive Sensitivity (S) ≤ 4.

Intermediate (I) 8 - 16.

Resistant (R) ≥ 32.

According to EUCAST interpretive breakpoints (2024), the criteria for categorizing *Staphylococcus aureus* isolates based on vancomycin MIC values are:

- Susceptible (S): ≤ 2 mg/L.
- Resistant (R): > 2 mg/L.

The diameters of the inhibition zones of the isolates differed when the isolates were five were resistant to vancomycin while the three (37.5%) were of intermediate range. The results suggest that the *Staphylococcus aureus* isolates identified are antibiotic-resistant with MIC values ranging from 30 to 60 mg/L. These values fall within the EUCAST-defined susceptible range, indicating that vancomycin remains a viable treatment option for these strains [10]. However, it is notable that two isolates (SA4 and SA6) had MIC values at the upper limit of the susceptibility threshold (30 mm), suggesting a need for close surveillance, as further increases could signify reduced susceptibility or emergence of intermediate resistance (See **Figure 1**).

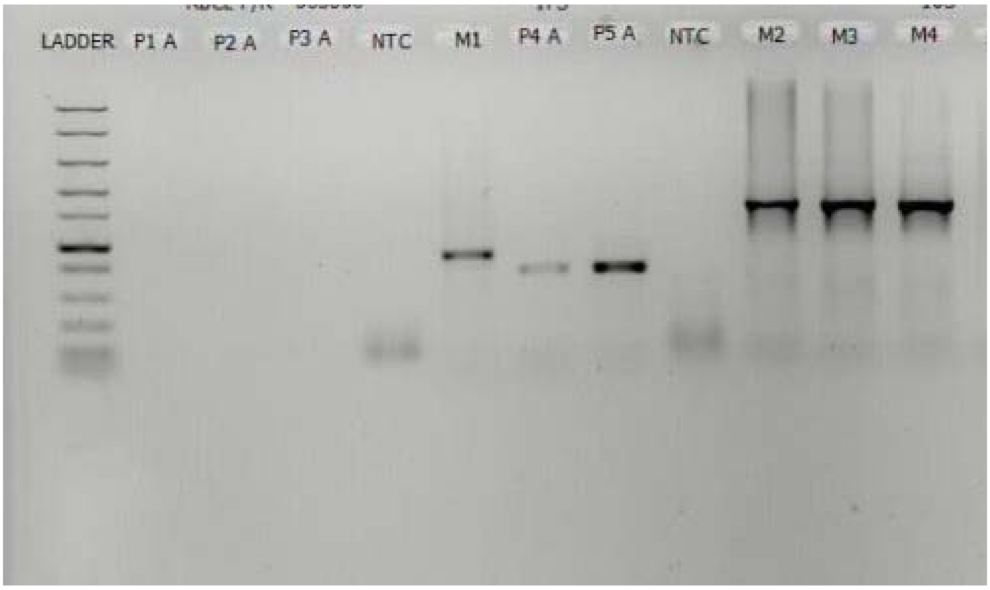

Figure 1. Gel image showing amplification of the 16 S region of samples at 1500 bp, the first lane is the 1 kb plus DNA ladder.

The results were visualized under UV light. This section uses primers; the lanes M2, M3, M4 show a strong, distinct band. This indicates successful PCR amplification of 16 S gene in these samples [12] (See **Table 3**).

Table 3. Blast table of *Staphylococcus aureus* DNA sequences.

	Accession Number	Details	Aln Length	Bit Score	e	Mismatches
M2	AY940424.1	***Staphylococcus* sp. ORG01 16 S ribosomal RNA gene, partial sequence** *Staphylococcus* sp. ORG01 16 S ribosomal RNA gene, partial sequence	681	1224	0.0	1
M3	AY940424.1	***Staphylococcus* sp. ORG01 16 S ribosomal RNA gene, partial sequence** *Staphylococcus* sp. ORG01 16 S ribosomal RNA gene, partial sequence	1501	2268	0.0	106
M4	MG517422.1	***Mammaliicoccus sciuri* strain 4D3430KR 16R ribosomal RNA gene, partial sequence MG706002.1 *Mammaliicoccus sciuri* strain APBSMLB223 16 S ribosomal RNA gene, partial sequence** *Mammaliicoccus sciuri* strain 4D3430KR 16R ribosomal RNA gene, partial sequence MG706002.1 *Mammaliicoccus sciuri* strain APBSMLB223 16 S ribosomal RNA gene, partial sequence	1515	2686	0.0	13

The blast table, produced after running a Basic Local Alignment Search Tool (BLAST) on a DNA sequence, indicates the similarity or dissimilarity of the query sequence to sequences found in a database [13].

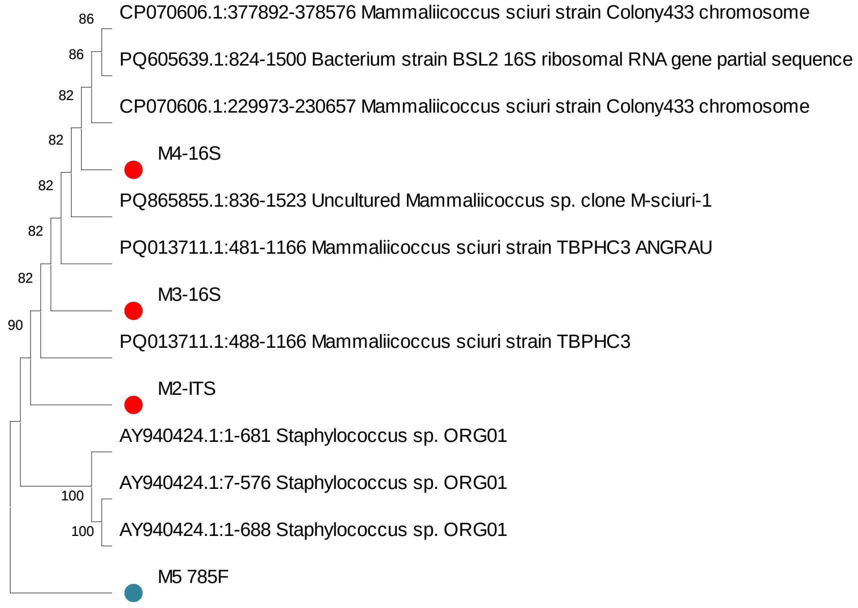

Figure 2. BLAST table or evolutionary tree.

Figure 2 shows a Phylogenetic tree also known as Phylogeny or Evolutionary tree which indicates the evolutionary relationships between organisms, showing their descent from a common ancestor.

4. Discussion

The purpose of this study was to isolate and characterize *Staphylococcus aureus* in commercially sold soy milk, as well as to define the prevalence of vancomycin-resistant strains. The phenotypic characterization of the isolates agrees with other studies [13] [14]. They reported a high prevalence of *S. aureus* in dairy based beverages in open markets in Nigeria. Similar findings were obtained in another study [15]. It was stated that the prevalence of *S. aureus* in traditional Nigerian foods was widespread and in most cases related to poor hygiene and improper food handling procedures.

Of the eight (8) *S. aureus* isolates tested, five (5) were resistant to vancomycin, which demonstrates the existence of a possible Vancomycin-Resistant *S. aureus* (VRSA) although no molecular confirmation was done [16]. The results of this study agrees with previous findings where antibiotic-resistant *S. aureus* in milk samples in Southwest Nigeria were detected [17] [18]. Another study also verified that antibiotic-resistant *S. aureus* strain is prevalent in dairy products in Nigerian markets, which is usually a result of the widespread use of antibiotics in livestock farming and poor sanitary conditions during food processing [19]. The isolation of VRSA in soymilk products is disturbing, particularly since it suggests the possibility of environmental spread of resistance genes and it places a threat on the consumers that may end up acquiring infections that are difficult to cure. The findings suggest the need for adequate surveillance in the use of antibiotics in human, animal, and environmental spheres [20] (Otto 2023). The Phylogenetic tree which indicates the evolutionary relationships between organisms, showing their descent from a common ancestor and thus confirms the isolates identified.

5. Conclusion

The study revealed that the soymilk samples tested from Thinker Corner harbored *Staphylococcus aureus* with isolates showing vancomycin resistance. The results raise the need for routine surveillance and testing of soy milk and other ready to eat food items sold in the open markets especially in the suburban and rural areas. There should be continuous public health education on hygienic practices in the production, packaging, and storage of soy milk and other foods to minimize contamination. Also of equal importance is the need for the extension of antibiotic stewardship programs to the agricultural sector so as to curb the excessive use of antibiotics in farm animal feeds as a control measure in environmental spread of resistance genes through the food chain.

Conflicts of Interest

The authors declare no conflicts of interest.

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