International Journal of Science and Research Archive

eISSN: 2582-8185

Cross Ref DOI: 10.30574/ijsra

Journal homepage: https://ijsra.net/

(RESEARCH ARTICLE)

Check for updates

Utilizing multiplex PCR as a molecular method to screen for *Staphylococcus aureus* pathogenicity and resistance

Ingelela Marian Rajini * and LK Pandey

Research Scholar, Department of Microbiology, Mansarovar Global University, Sehore, Madhya Pradesh, India.

International Journal of Science and Research Archive, 2025, 16(01), 1023-1041

Publication history: Received on 28 May 2025; revised on 05 July 2025; accepted on 08 July 2025

Article DOI: https://doi.org/10.30574/ijsra.2025.16.1.2064

Abstract

Antibiotic resistance is becoming an increasingly pressing topic as *Staphylococcus aureus* continues to be a major human pathogen that causes a broad variety of diseases. An effective strategy for monitoring and treating *S. aureus* requires molecular detection and identification of resistance and virulence genes. In order to discover important virulence and antibiotic resistance genes in *Staphylococcus aureus* quickly and simultaneously, this research sought to improve a multiplex PCR technique. Using two sets of multiplex primers targeting 10 genes (sea, seb, sec, sed, saw, femA, eta, etb, tst, and mecA), a total of two hundred *S. aureus* strains were examined. Exactness and absence of nonspecific bands were achieved by meticulous optimization of the amplification settings. Restrictive fragment length polymorphism (RFLP) analysis allowed us to verify the PCR results' authenticity by digesting each gene with a particular enzyme to produce distinctive fragment sizes. Consistent findings from both multiplex PCR and individual gene screening proved that the test was reliable.

Keywords: Toxin genes; Antibiotic; Molecular; Primers; Multiplex

1. Introduction

One of the biggest risks to human health and food safety today is foodborne illness brought on by bacterial contamination (Wu et al., 2016). Due to present processing conditions that expose meat to contaminated working surfaces, equipment, poor personal hygiene, and low-quality water, bacterial contamination of meat is inevitable (Endale and Hailey, 2013; Ruban, 2018). Numerous bacterial communities, both harmful and helpful, can be found in the human body. For this reason, in recent decades, the stresses associated with various parts of the body under various health conditions have been fully defined Normally harmless commensal bacteria, *Staphylococcus aureus* is now recognised as a significant opportunistic virus that causes a range of disorders worldwide. G. Abril et al., 2020; Momani, 2021). With an estimated 241,148 cases and 6 fatalities each year, *Staphylococcus aureus* is one of the most common causes of bacterial food poisoning in the US. Fetsch et al. (2014), Tiemersma et al. (2004), and Wang et al. (2018). *Staphylococcus aureus* is a Gram-positive coccus with a cluster-shaped organization that has been described asthe principal causative agent of a variety of clinical disorders across the world. This bacterium causes a lot of infections, both in the community and in hospitals. Unfortunately, because of the spread of microorganisms that are multi-drug resistant (MDR), therapy remains difficult. (Taylor and Unakal, 2020) *S. aureus* possesses virulent aggravating properties, produces enterotoxins, and is resistant to antibiotics, as well as having proteolytic and lipolytic activity at various temperature settings, causing food spoiling (Puah et al., 2016).

Methicillin-resistant *S. aureus* One of the most prevalent nosocomial pathogens worldwide, *S. aureus* causes pneumonia, post-operative wound infections, food poisoning, and nosocomial infections (Turner et al., 2010). *S. aureus* pathogenicity refers to the virulence factors linked to drug resistance and affinity for the formation of staphylococcal enterotoxins (Cheung et al., 2021). When *S. aureus* infects food, multiplies, and creates extracellular heat-stable

* Corresponding author: Ingelela Marian Rajini

enterotoxins, the food becomes lethal despite its look, a condition known as staphylococcal food poisoning (SFP) (Zeaki et al., 2019). Staphylococcal enterotoxins (SEs), the majority of which are type-A, are the cause of SFP symptoms, which include nausea, vomiting, and diarrhoea (CDC, 2018). *Staphylococcus aureus* isolates can be identified and classified using the accurate and efficient PCR technique. It has been employed as a quick detection technique for Methicillin-Resistant *Staphylococcus aureus* (MRSA), which amplifies a single or a small number of copies of a DNA fragment over several orders of magnitude to create thousands to millions of copies of a particular DNA sequence (Ali et al., 2014). Thus, the purpose of this work was to find the fem A gene for molecular identification of *S. aureus* and to examine the presence of harmful bacteria, particularly drug-resistant (MDR) strains, in fresh and canned meat.

Staphylococcus aureus is the most common organisms causing food-borne illnesses globally. After consuming foods that contain preformed heat-stable enterotoxins, it results in SFP. In SFP situations, contamination frequently results from lengthy or inappropriate manual handling of foods high in proteins, together with insufficient heating and storage. With multiplex PCR, a novel and efficient method for identifying multiple target genes in a single reaction, screening *S. aureus* for pathogenicity and resistance markers has never been simpler. Multiplex PCR allows the amplification of several genes in a single reaction mixture, saving time and money compared to performing multiple individual PCR assays. Many different *S. aureus* virulence factors may be targeted using this method. These include the genes that produce enterotoxins (e.g., sea, seb, sec, and sed), toxic shock syndrome toxin-1 (tst), exfoliative toxins (eta and etb), and hemolysins (hla and hlb). Infections of the skin, toxic shock syndrome, and food poisoning are among the symptoms linked to these genes. Antibiotic resistance genes may be identified using multiplex PCR. These genes include mecA, vanA, blaZ, and erm, and they give resistance to beta-lactams, vancomycin, and methicillin.

Because of its high throughput and efficacy in identifying numerous virulence and resistance determinants in a single experiment, the multiplex PCR approach finds numerous applications in clinical and epidemiological research. Through the simultaneous identification of pathogenicity and resistance genes, multiplex PCR provides a comprehensive assessment of the potential threat posed by *S. aureus* isolates. This method is particularly helpful for rapidly identifying dangerous strains that require immediate treatment because they are resistant to first-line antibiotics, such as MRSA or VRSA. The ability to detect different virulence genes gives a more nuanced picture of the pathogenic potential of the strain since some *S. aureus* isolates may contain a combination of virulence factors that boost their capacity to cause disease.

In addition to its medicinal applications, multiplex PCR is crucial for epidemiological monitoring and infection control. Healthcare professionals can use this technique to track the spread of antibiotic-resistant *S. aureus* strains in hospitals. They will be able to identify epidemics and take the appropriate action to contain them as a result. Multiplex PCR can also be used to track the prevalence of resistant and pathogenic *S. aureus* strains in food products, animals, and environmental samples. Public health initiatives can benefit from this information. However, the multiplex PCR approach has some drawbacks. The success of multiplex PCR greatly depends on the design of the primer. When amplifying their target genes, proprietors should be carefully selected to prevent reagent competition and cross-reactivity. To identify several genes in a single reaction, numerous parameters must be optimised, including primer concentration, heat cycling conditions, and detection methods. Despite these challenges, advancements like commercial multiplex kits and fluorescent probes have lately made multiplex PCR more accessible and user-friendly for routine diagnostics.

2. Methodology

2.1. Aim of research

To develop and evaluate a molecular approach using multiplex polymerase chain reaction (PCR) for the rapid detection of *Staphylococcus aureus*, focusing on the identification of key pathogenicity and antibiotic resistance genes, including those associated with methicillin resistance and toxin production.

2.2. Isolation of Bacterial Strains and Culture Conditions

This investigation made use of 220 distinct strains of *Staphylococcus aureus*. The culture collection at the Laboratory Center for Disease Control, was using appropriate maintenance medium to preserve all strains.

2.3. Source of Sample collection

In this experiment, every single food sample that was utilized to isolate *S. aureus* originated from somewhere in the Hyderabad region. A variety of meats and animal products, including paneer, pedha, butter, cheese, khawa, cream-filled pastries, ice creams, raw and cooked milk, panipuri, and more, were collected for tasting. Supermarkets, vendors, and dairies were randomly sampled for 600 food items (Table 1).

Table 1 Variety of foodstuff samples

Sl. No	Sample	Sample size
1	Raw Milk	250
2	Boiled Milk	10
3	Paneer	80
4	Peda	20
5	Butter	40
6	Cheese	25
7	Khawa	30
8	Cream filled cake	15
9	Ice-creams	10
10	Meat	60
11	Panipuri	60
Total		600

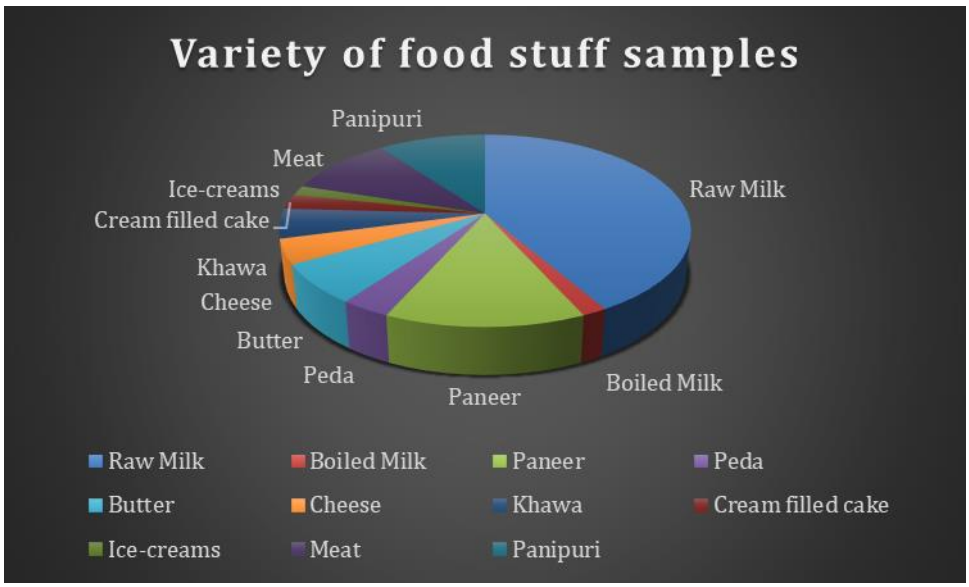

Figure 1 Variety of food stuff samples

2.4. Isolation and characterization

Isolating *S. aureus* from food samples was made possible by the selective components in Baird Parker medium and Mannitol salt agar (MSA), which only enabled bacteria that can survive high amounts of salt to thrive.

The colonies are shown digesting Mannitol on agar that contains Mannitol salt. Black jet shine is a characteristic of Baird-Parker agar, which was used to re-streak the colonies. On Blood agar large, round, smooth, golden-yellow colonies with

a clear zone of beta-hemolysis around them and on Nutrient agar round, smooth, glistening, and opaque colonies, often with a distinctive golden-yellow pigmentation were observed. Biochemical confirmation tests and microscopic examination of gram staining features, shape, and cluster were performed on these isolates.

2.5. Identification of *S. aureus*

Following established procedures, Identifying food-borne pathogens was accomplis hed via the use of morphological and biochemical investigation., with a focus on *Staphylococcus aureus*.

2.6. Morphological characterization

2.6.1. Gram's staining

We stained each bacterial isolate with Gram's solution. An optically plain, clean, grease-free glass slide was air-dried and heat-fixed with a thin layer of an 18-hour broth culture.A minute after adding the crystal violet stain (0.3%), we rinsed off any extra stain with a moderate spray of water. After 30 seconds of standing, the sample was drained off the fluid that had 0.4% gram iodine in it. To prevent the color from fading, the smear was washed with absolute ethanol (95% concentration) before being stained with the counter stain safranin (0.4% for one minute). The smear was thereafter rinsed with running water for five seconds and allowed to air dry. If the smear turned purple, the results were recorded as Gram positive, and the observations were made using an oil immersion objective. The cells' shapes and arrangements were documented.

2.6.2. Biochemical characterization

Coagulase test

The mixture was incubated at 37˚C after mixing Rabbit Plasma (Himedia) with an equivalent volume of bacterial broth culture that had been grown overnight. Clots formed within one to four hours are considered a positive result; tubes that came back negative were retested after twenty-four hours.

Catalase test

Splattering a few drops of 3% (v/v) hydrogen peroxide over a slide while mixing a loopful of culture yields positive results when gas bubbles or effervescence occur.

2.6.3. Antibiogram typing of s.aureus

All *Staphylococcus aureus* isolates discovered in food samples were tested for antibiot ic susceptibility in accordance with the guidelines laid forth by the Clinical Laboratory Standards Institute. This time around, we went with Kirby Bauer's Agar Disk Diffus ion Assay.

Inoculum preparation

Two milliliters of nutrient broth were added to colonies with comparable morphology (numbering three to five) harvested from a 24-hour culture plate. Two to four hours were thereafter spent incubating the mixture at 37˚C o. To adjust the inoculum's turbidity to 0.5 McFarland standards, sterile saline was added.

Making the mueller-hinton agar (MHA) cell culture dispersions

Muller Hinton agar, prepared as directed by the manufacturer, was poured onto 4- millimeter plates, autoclaved to sterilize, cooled, and then allowed to set on a level surface. Before being used for antibiogram typing, the plates were incubated at 37˚C for 12 hours following solidification to make sure they were sterile.

Testing plate inoculation

To ensure even inoculation of the MHA plates, a non-toxic cotton swab was immersed in the inoculum. Thoroughly spinning the swab and pressing it against the inner tube wall above the fluid level removed any extra inoculum.

Following each inoculation with the swab, the dried MHA plates were inoculated with a grass culture by rotating the plates through sixty degrees.

At last, the swab was maneuvered along the surface of the agar.

- The plates that were cultured with grass were left to dry for three to five minutes at room temperature with the lid closed.
- Using sterile forceps, the antibiotic discs were equally distributed (no closer than 24mm) across the inoculated MHA plates.
- In order to check whether there was a clear zone around the discs, the plates were inverted and incubated at 37°C for 18-24 hours after adding the discs.
- Using the zone measuring scale, the diameters of the whole zone were recorded and measured in millimeters as shown inTable (2).
- The CLSI zone interpretation criteria (M100-S15) were used to determine whether the organism was susceptible or resistant to that particular antibiotic, and hence to interpret the zone sizes.
- The standard strain utilized was *Staphylococcus aureus* ATCC 25923.

Table 2 Antibiotics chosen in accordance with CLSI standards

Name of the Antibiotics(Abr)	**Concentration(mcg/disc)**	**Classofantibiotics**
Gentamycin(GEN)	10	Aminoglycoside
CoTrimoxazole(COT)	25	Sulfonamides
Ciprofloxacin(CIP)	30	Quionolones
Tetracycline(T)	30	Tetracycline
Oxacillin(OX)	01	Penicillin
Rifampycin(RIF)	05	Rifamycin
Erythromycin(E)	15	Macrocides
Vancomycin(VA)	30	Glycopeptides
Ofloxacin(OF)	30	Fluoroquionolones
Methicillin(MET)	05	Penicillin
Cephalothin(CEP)	30	1stgenerationcephal osporin
Streptomycin(S)	10	Aminoglycoside

Antibiotic susceptibility

According to the recommendations set forth by the we assessed the antibiotic susceptibility of 300 *S. aureus* isolates using 12 different antibiotics utilizing the disc diffusion method, as recommended by the Clinical Laboratory Standard Institute (CLSI)

3. Results and discussion

3.1. Antimicrobial resistance in *Staphylococcus aureus* bacteria found in food

If a bacteria shows resistance to at least one compound from three separate families of antibiotics, it is referred to be multidrug resistant (MDR). No bacteria were found to be resistant to any of the antibiotics tested for in this investigation, as shown in Table 4 and Figure 2. Even more concerning is the fact that 3% of the microorganis ms examined showed resistance to four different medications. Only two germs, or 0.6% of the total, showed resistance to all eleven medications.

Table 3 Rate of multidrug-resistant *Staphylococcus aureus* in food-borne samples

No. of Antibiotics	% of MDR Isolates
4	3
5	02
6	08
7	4.6
8	02
9	03
10	2.3
11	0.6
12	00

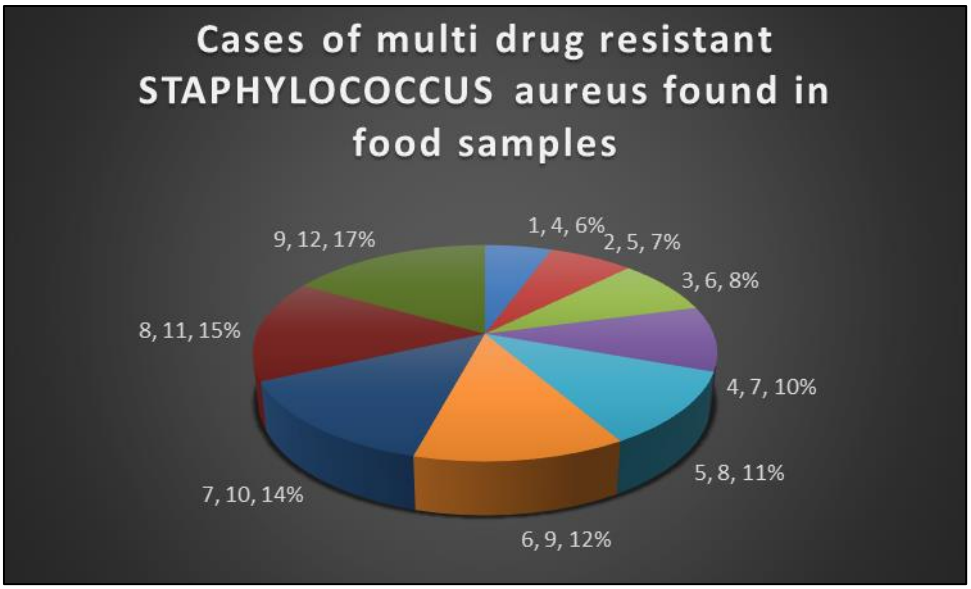

Figure 2 Cases of multidrug-resistant *Staphylococcusaureus* found in food

3.2. DNA isolation

After overnight growth in 0.5 ml of brain heart infusion broth, the complete DNA was extracted from all of the bacterial strains that were part of the study. Extraction of DNA has already been described in detail. The amount in micrograms per milliliter was determined using A260 values after DNA samples were dissolved in Tris-EDTA buffer (10 mM Tris chloride, 1 mM EDTA [pH 8.0]). Ten to one thousand nanograms of template DNA was utilized in the study.

3.2.1. Molecular typing

Purifying DNA from staphylococcal bacterium

- The DNA extraction procedure used in this work was based on that of De Baere et al. (2002) with some minor modifications.
- After 4-5 clean colonies were harvested from 24-hour overnight culture (BHA) plates, they were transferred to an Eppendorf tube with 200µL of distilled water and vortexed.
- The vortexed tubes were placed in a dry bath and left to incubate at 85˚C for 20 minutes. After that, they were frozen at -20˚C for 10 minutes. The tubes went through a 5-minute centrifugation run at 10,000 revolutions per minute.

- The liquid portion was sent to an additional sterile eppendorf tube after collection.
- Using agarose gel electrophoresis to estimate the DNA content allowed us to verify the purity.
- We used a modified version of the Gene-aid genomic kit protocol to improve DNA extraction from bacterial isolates. Instead of using enzymes to break down cell walls, we physically disrupted them. After the cells were separated, we added 200 µl of GT buffer to the supernatant and mixed it vigorously. We then incubated it at 37˚C for 5 minutes.
- Following a 10-minute incubation period at 70˚C, 200 µl of GB buffer was added and the tubes were turned over every three minutes. Following this, 200 µl of 100% ethanol was added, mixed well, and transferred to a micro column.
- The column carrying the solution was spun at 13,000 revolutions per minute for two minutes.
- After discarding the collecting tube, a fresh, sterile one was inserted.
- The mixture was centrifuged at 13,000 rpm for 30 seconds after adding 400 µl of W1buffer, and the byproduct was removed.
- The wash buffer, which had 600 µl, was then added. The mixture was centrifuged again at 13,000 rpm for 30 seconds, and the byproduct was removed. Afterwards, 400µl of washing buffer was added, the mixture was centrifuged at 13,000 rpm for 30 seconds, and the byproduct was removed. It was then centrifuged (to dry) at 13,000 rpm for three minutes with the GD column.
- After incubating the elution buffer for a while, 50µl was added to the column. After that, it was transferred to the centrifuge tube and let to stand for 35 minutes. After that, it was centrifuged at 13,000 rpm for 30 seconds.
- The remaining liquid was transferred to a new tube and put in a deep freezer at a temperature of -20˚c.
- Purifying the enterotoxin genes of *Staphylococcus aureus* requires the use of the supernatant as a template. We used the following primers and reaction conditio ns to identify enterotoxin genes (sea, seb, sec, and sed) in the stored DNA.

Purity and concentration of DNA estimation

The concentration of DNA was measured using spectrophotometry.

A total of 995 µl of distilled water was combined with 5 µl of DNA from every sample. A spectrophotometer was used to determine the optical density (O.D at 260nm /280nm). An optical density (OD) is associated with double-stranded DNA of around 50 µg/ml when scaled up to one.The following formula was used to determine the DNA concentration: DNA concentration (µg/ml) = O.D 260nm × 50 × dilution factor

DNA purity by Spectrometer DNA

DNA purity=O.D 260/O.D 280nm

Gene selection and primer design

The four genes selected for the monoplex/multiplex PCR experiment are sea, seb, sec, and sed, as shown in Table 4. By using multiplex PCR, the sea, seb, sec, and sed genes were amplified. The four staphylococcal enterotoxins—SEA, SEB, SEC, and SED—that have been the subject of the most research led to their selection as gene candidates. We reviewed the relevant literature and mined the gene bank database for primer recommendations. The selected primers were provided in lyophilized form and stored in a deep freezer until their next use.

Table 4 DNA primers designed to target the *S. Aureus* gene for polymerase chain reaction

Gene	Primer	Oligonucleotidesequence(5'-3')	Amplicon size(bp)
Sea	GSEAR-1	CCTTTGGAAACGGTTAAAACG	127
	GSEAR-2	TCTGAACCTTCCCATCAAAAAC	
Seb	GSEBR-1	TCGCATCAAACTGACAAACG	478
	GSEBR-2	GCAGGTACTCTATAAGTGCC	
Sec	GSECR-1	GAACTAGACATAAAAGCTAGG	244
	GSECR-2	CATTCTTTGTTGTAAGGTGG	
Sed	GSEDR-1	CTAGTTTGGTAATATCTCCT	317

	GSEDR-2	TAATGCTATATCTTATAGGG	
Nuc	Nuc-F:	GCGATTGATGGTGATACGGTT	270
	Nuc-R:	AGCCAAGCCTTGACGAACTAAAGC	
mecA	mecA-F	AAAACTAGGTGTTGGTGAAGATATACC	147
	mecA-R	GAAAGGATCTGTACTGGGTTAATCAG	

PCR premix composition (bioneer)

Table 5 Premix composition for Polymerase chain reaction

Components	**Amount**
1xPCRbuffer	10mMTrisHCl(pH9.0)
KCl	30mM
dNTP'smixture	250µMeach
MgCl2	1.5mM
Taq DNA polymerase	1U,stabilizer and tracking dye.

Table 6 Performance of PCR conditions for mecA gene

Initial denaturation	95˚C(5min)	
Denaturation	95˚C(1min)	
Annealing	63˚C(1min)	30 cycles
Extension	72˚C(1min)	
Final extension	72˚C(5min)	

Table 7 Performance of PCR conditions for nuc gene

Initial denaturation	94˚C(5min)	
Denaturation	94˚C(1min)	
Annealing	55˚C(40sec)	35 cycles
Extension	72˚C(1min)	
Final extension	72˚C(10min)	

Table 8 Performance of PCR conditions for Enterotoxin a, b, c and d genes

Initial denaturation	95˚C(5min)	
Denaturation	95˚C(1min)	
Annealing	52˚C(1min)	40 cycles
Extension	72˚C(1min)	
Final extension	72˚C(10min)	

3.3. Determination of PCR products by gel electrophoresis

After defrosting, vortexing, and centrifuging the tubes containing the extracted DNA and PCR pre mix components, the contents were settled. In a 25 µl total volume, the PCR mixture was mixed with 5 µl of PCR premix, 2 µl of each primer, and 4 µl of template DNA. Negative controls were added except for the template DNA, and the remaining volume was filled with deionized water. After that, it was vertexed and 4 µl of template DNA was added.

- After quickly centrifuging the PCR tubes to mix the components, they were inserted in the thermal cycler and the amplification performance settings were established.
- Agar gel electrophoresis with a 1.5% concentration was used to examine the amplified data.
- Boil 1.5 grams of agarose powder in 100 milliliters of 1 × TBE buffer, then let cool to 50˚C.
- Cool the mixture to 20˚C after adding 5 µl of bromide dye and stirring. Then, pour the agarose into the gel jar. extreme caution is required to avoid the creation of air bubbles.
- The comb was delicately removed after solidification, and the gel jar was then put into the electrophoresis tank.
- The first we l of the agarose gel was used to add 6 µl of the 100bp DNA ladder, while the other we ls were filled with 10 µl of each PCR premixed component.
- When the dye reached the end of the gel, the electrophoresis was terminated after three hours of running at 100 V.
- The gel was seen and recorded using a UV trans-illuminator.

3.3.1. Sequencing of the pcr product

The section of agarose gel containing the amplicon was marked and sliced using a sharp scalpel, and then collected in a sterile micro centrifuge tube after gel electrophores is. We called Europhins Pvt. ltd to do partial sequencing on the product, and we recorded their results.

3.3.2. Genomic profiling of toxins

Pathogenicity, epidemic detection, strain identification, and the development of prophylactic measures may all be greatly assisted by molecular techniques. For the recovery of isolates suspected of causing entero toxigenicity and Staphylococcal food poisoning, this is an essential need. The frequency of strains that generate SE and those that do not were determined using *S. aureus* toxin gene profiling tests conducted on the isolates. Isolates of food have been polymerase chain reaction typed for the enterotoxin genes (sea, seb, sec, and sed). Here we provide the results.

An antibiotic resistance gene known as mecA was amplified by polymerase chain reaction. Because the *S. aureus* bacteria is notorious for producing an exopolysaccharide barrier that makes it resistant to antibiotic treatment, and because food is a major component in the transmission of antibiotic resistance. The fact that accessory genetic elements carry the majority of the information about an organism's susceptibility to antibiotics and other harmful chemicals is common knowledge. It is still unclear if there is a relationship between *Staphylococcus aureus* enterotoxigenicity and the antibiotic resistance gene. In order to determine the geographic distribution of antibiotic-resistant gene strains, we used polymerase chain reaction to amplify the mecA gene in twenty isolates. For the 147 bp mecA gene, five out of twenty isolates were found to be positive. Figure 3 and Table 9 show the outcomes of the observations.

Table 9 Analysis of *Staphylococcus aureus* isolates from various food sources for the presence of antibiotic-resistant genes

Gene	No. of test isolates	No. of positive isolates	Incidence rate %
mecA	20	05	25%

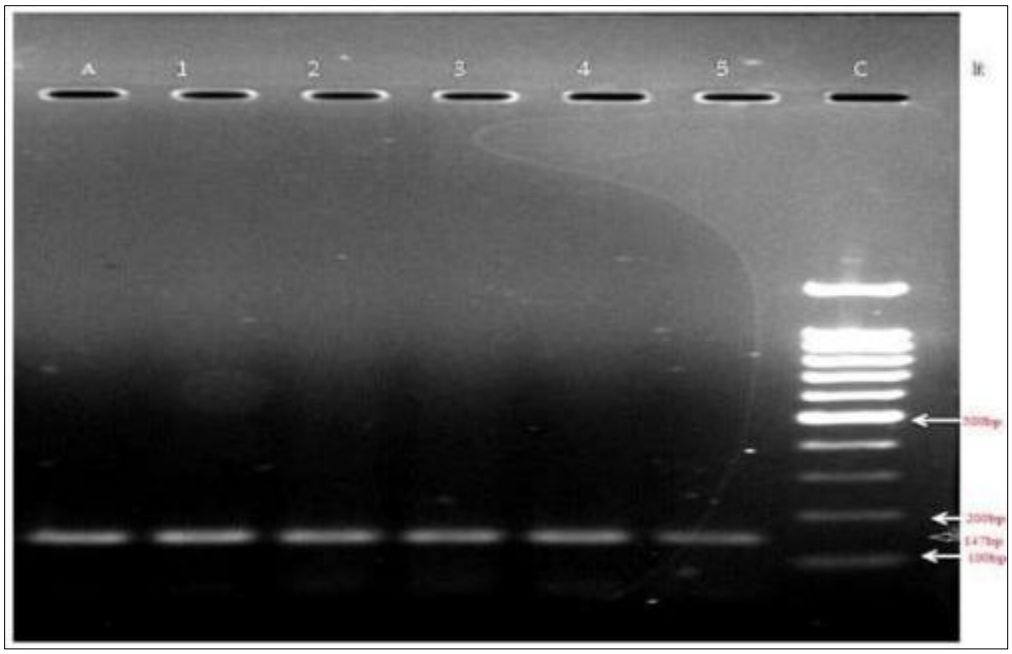

Figure 3 Lane A: ATCC 43300 strain, Lane B: 1–5 test isolates, and Lane C: 100 bp DNA marker for mecA typing

Twenty of the 300 isolates were selected for antibiotic resistance gene identification after antibiotic susceptibility testing. Twenty of the three hundred isolates tested were sent to the mecA gene identification PCR method. Five samples of milk, ten samples of white cheese, and five samples of meat were used to isolate these bacteria.

Forty percent of the meat and raw milk samples (2/5), and 10% of the isolates (1/10), showed evidence of mecA gene amplification (Table 10, Figure 3).

Table 10 *S. aureus* mecA gene frequency in various dietary samples

Sample	No. of test isolates	No. of positive isolates	Incidence rate%
Rawmilk	05	02	40
Meat	05	02	40
cheese	10	01	10

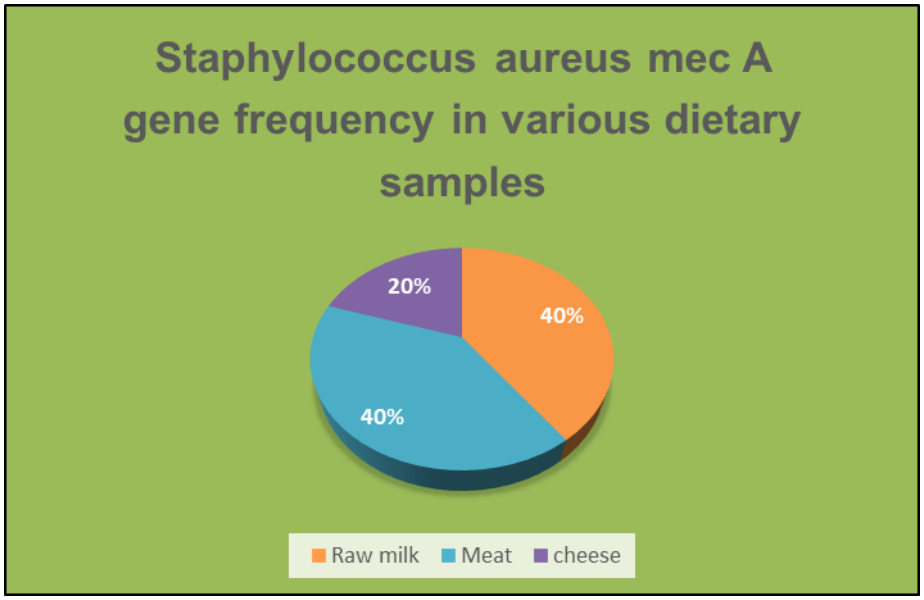

Figure 4 *S. aureus* mecA gene frequency in various dietary samples

3.3.3. Polymerase chain amplification of nuc gene

The nuc gene polymerase chain reaction is a very accurate method for establishing the presence of *Staphylococcus aureus*. The thermo nuclease enzyme is encoded by the Nuc gene, which is implicated in *S. aureus* thermal nuclease activity. Rapid diagnosis of *S. aureus* infection may be possible by amplification of the nuc gene, which encodes the thermo nuclease enzyme in *S. aureus*. This technique of detection might be quicker than existing culture methods. Due to their importance in Indian cuisine, milk and food items pose a number of health risks when contaminated. Figure 5 shows that out of 30 isolates, 4 (13.3%) showed positive amplification for the nuc gene, confirming the presence of *S. aureus* and its pathogenicity. The nuc gene amplification was performed on a randomly selected 30 isolates. Table 11.

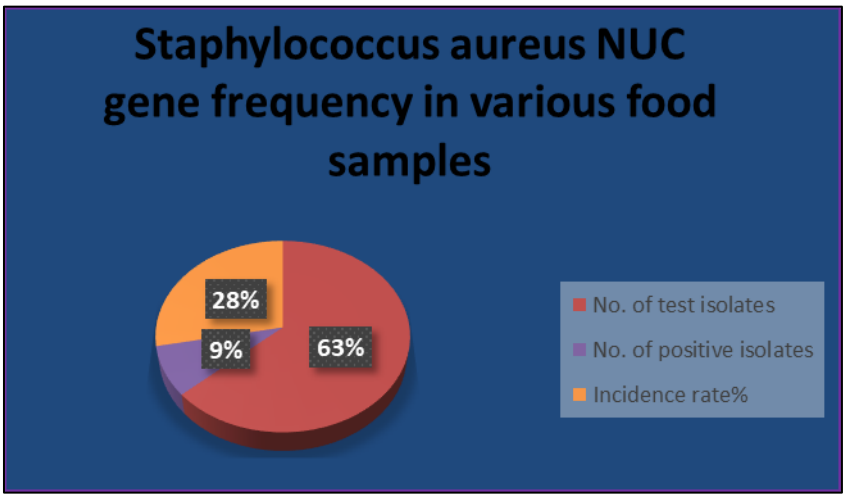

Figure 5 *Staphylococcus aureus* NUC gene frequency in various food samples

Table 11 *Staphylococcus aureus* NUC gene frequency in various food samples

No. of test isolates	No. of positive isolates	Incidence rate %
30	04	13.3

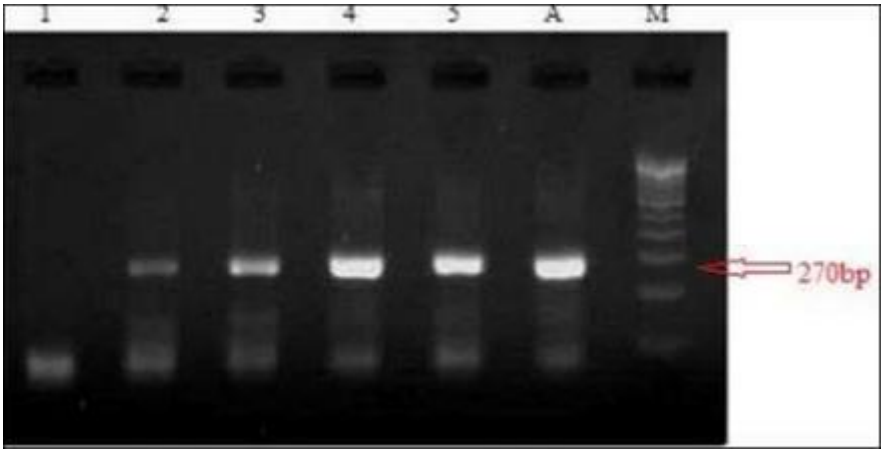

Figure 6 PCR for nucleotide typing: 100 bp DNA marker in Lane M, test isolates 1–5 in Lanes 1–5, and a positive control in Lane A

Staphylococcal enterotoxin genes (SEa, SEb, SEc, and SEd) polymerase chain reaction

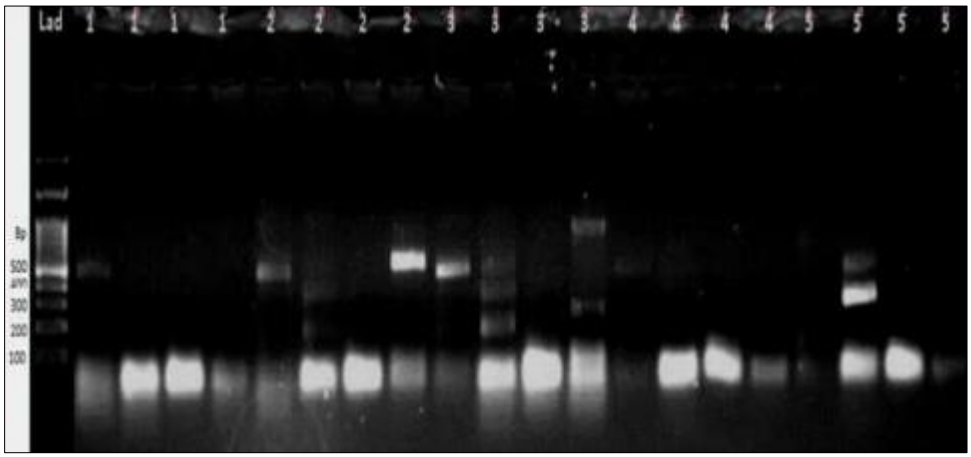

Figure 7 DNA marker (100 bp ladder) in Lane M. 1–5 Isolates for testing

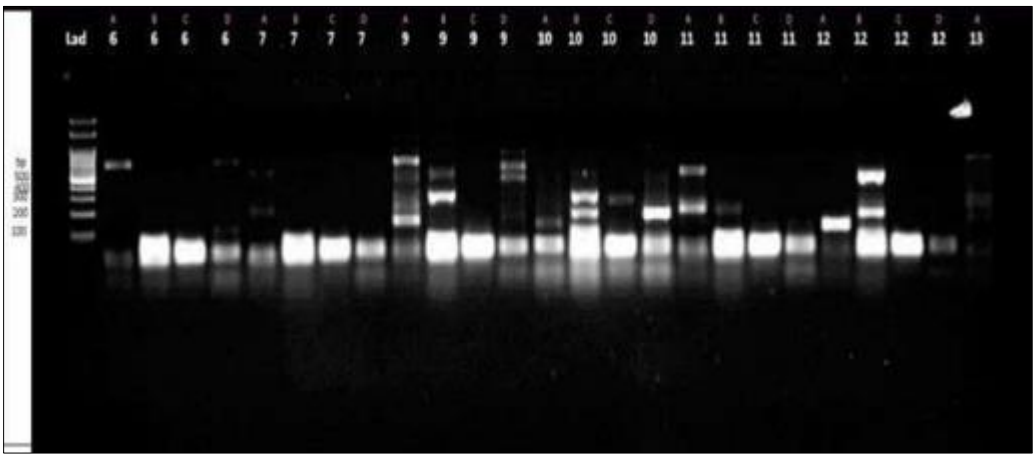

Figure 8 DNA marker (100 bp ladder) in Lane M. 6-12 Test isolates

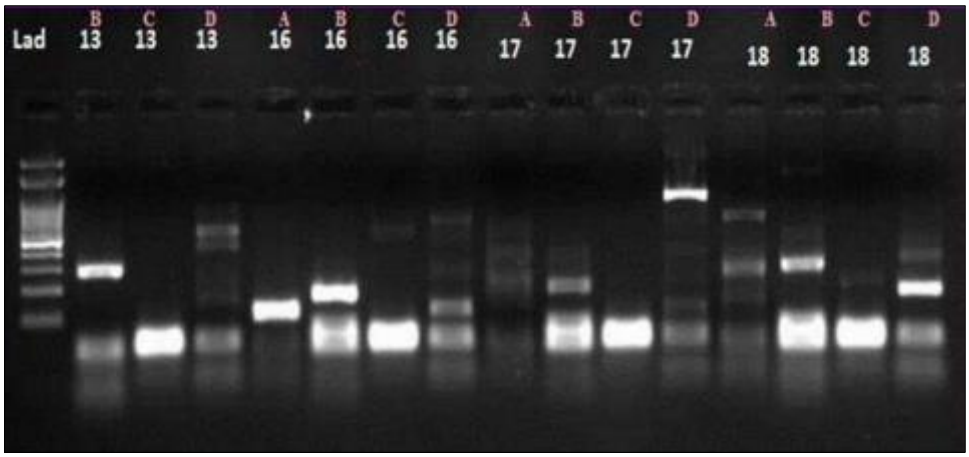

Figure 9 Lane M: - DNA Marker (100 bp Ladder) 13-18 Test isolates

Enterotoxin gene profiling by Multiplex PCR

Polymerase Chain Amplification of enterotoxin genes In order to confirm the food samples passed the first screening, Multiplex PCR amplification of enterotoxin genes was performed. The sea, seb, sec, and sed genes—which are responsible for the majority of food outbreaks globally—were amplified multiplex PCR in 20 isolates from various food sources.From the 20 isolates that were tested using multiplex PCR, five were found to be positive for the Sea gene, five for the Seb gene, two for the Sec gene, and two for the Sed gene (Table 12, Figure 10, 11).

Table 12 Prevalence of enterotoxin genes in *S. aureus* isolates from food samples

IsolateNo.	Toxin gene			
	Sea	Seb	Sec	Sed
1	-	+	-	-
2	-	-	-	-
3	-	-	-	+
4	-	-	-	-
5	-	-	+	-
6	-	-	-	-
7	-	-	-	-
8	-	+	-	-
9	+	-	-	-
10	+	-	-	-
11	-	+	-	-
12	-	-	-	-
13	+	-	-	-
14	+	-	+	-
15	-	-	-	-
16	+	+	-	-
17	-	-	-	-
18	-	-	-	-

19	-	-	-	-
20	-	+	-	+
Total-20	05	05	02	02

Figure 10 LaneL:DNAMarker(100bpLadder),+ve:positivecontrolfor Sea,Seb,Secand Sedgene, Lane1-10 Test isolates

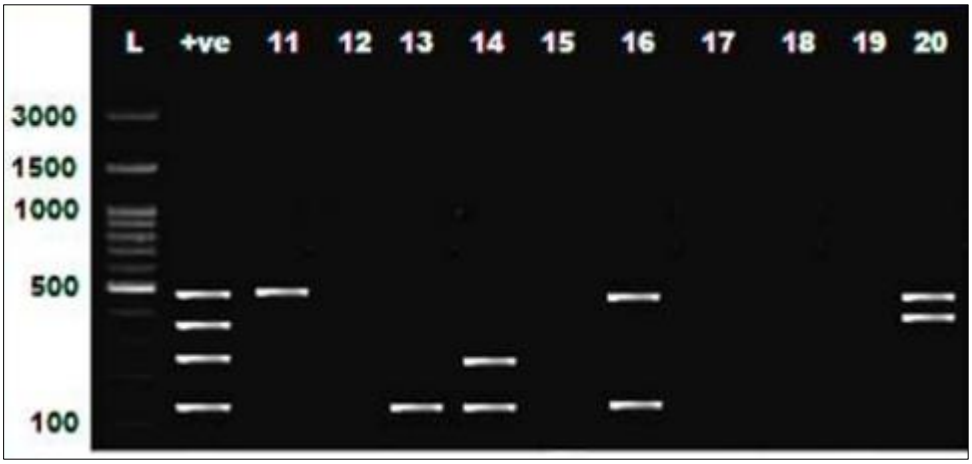

Figure 11 Lane L :- DNA Marker (100 bp Ladder), +ve: positive control for Sea,Seb,Secand Sed gene, Lane 11-20 Test isolates

3.3.4. Incidence of enterotoxin genes among S.aureus isolates from different food samples

From the Paneer samples, 150 isolates were screened. Out of them, 50 were tested for antibiotic resistance, and 4 were chosen to undergo toxin gene identification using the PCR technique. Fifty percent (2/4) of the isolates tested positive for the Seb gene, which has an amplicon size of 478 bp, and a quarter (1/4) of the isolates tested positive for the Sed gene, which has an amplicon size of 317 bp. These genes are known to encode enterotoxins. However, after testing Paneer samples for the Sea and Sec genes, not a single isolate tested positive. Table 13,14 shows that only one of the two sheep milk isolates tested positive for the Sed gene, which had an incidence rate of 50% (1/2) and an amplicon size of 371 bp. The other two isolates tested negative for all three genes.

Genomic identification of the Enterotoxins Sea, Seb, Sec, and Sed was performed on the chosen milk isolates using polymerase chain reaction (PCR). Only two out of five milk isolates tested positive for the Sea gene, which has an amplicon size of 127 bp; two out of five tested positive for the Sec gene, which has an amplicon size of 244 bp; and all five tested negative for the Seb and Sed genes as shown in Figure 11,12. In a similar vein, the highest gene was found in cow milk; it was Sea, Seb, and Sec, and it was negative for Sed. The incidence rate for all three genes was 50%.A 50%

gene incidence rate was noted for the Sea and Seb genes, while none of the meat and cheese isolates tested positive for the Sec or Sed genes.

Table 13 Incidence of toxin genes in *S. aureus* from different food samples

Sample	No.of Isolates	Toxin SEa	Toxin SEb	Toxin SEc	Toxin SEd
Paneer	04	0	02(50%)	0	01(25%)
Sheepmilk	02	0	0	0	01(50%)
Milk	05	02(40%)	0	01(20%)	0
Cowmilk	02	01(50%)	01(50%)	01(50%)	0
Cheese	05	01(20%)	01(20%)	0	0
Meat	02	01(50%)	01(50%)	0	0

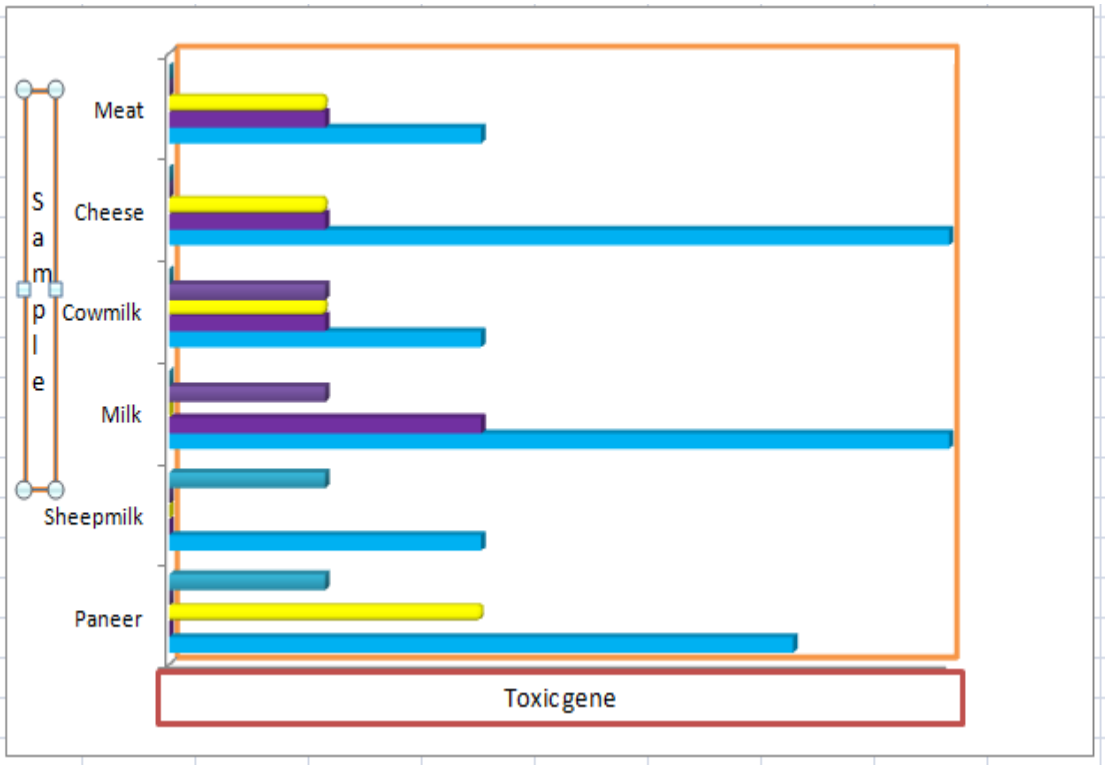

Figure 12 Incidence of toxin genes among *S. aureus* isolates from different food samples

Table 14 Incidence of toxin genes in *S. aureus* from different food samples

Toxin	No. of Positive	Prevalence%
SEa	05	25.0
SEb	05	25.0
SEc	02	10.0
SEd	02	10.0

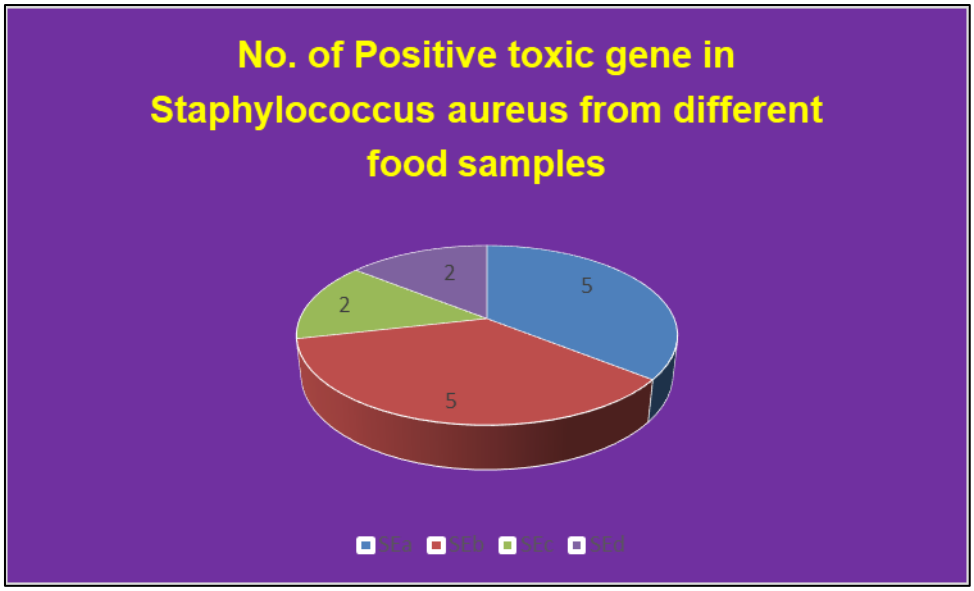

Figure 13(a) Positive toxic gene of enterotoxin genes among S.aureus isolates from different food samples

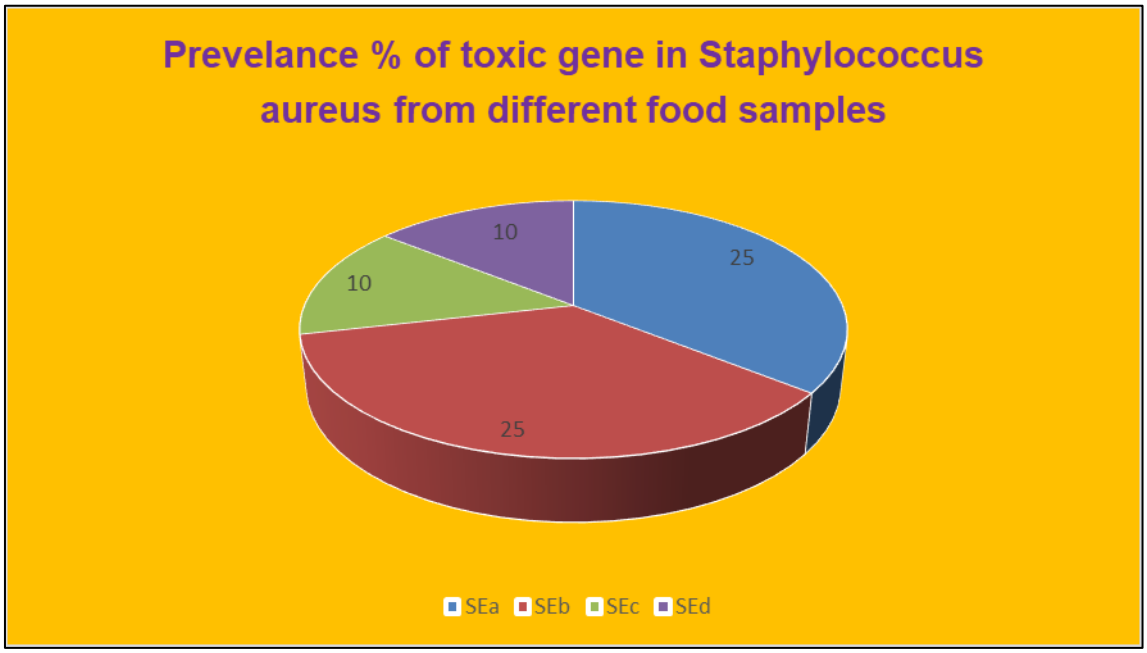

Figure 13(b) Prevalence of enterotoxin genes among S.aureus isolates from different food samples

4. Multiplex PCR conditions

Modified GeneAmp kit master mixes were utilized to generate two primer mixes. Mix 20 µM deoxynucleoside triphosphates, 5 µL 10× reaction buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl), 1.5 mM MgCl2, 20 µM sea, seb, sec, see, and femA primers, 40 µM sed primer, 25 εU AmpliTaq, and 10-1.00 nM dNTPs. Sterile water was used to reduce the mixture volume to 50 µl. Eta was 50 pmol, etb, tst, mecA, and femA were 20 pmol, and multiplex primer set B was identical to set A except for the MgCl2 concentration (2.0 mM). The reaction mixture was kept from evaporating by adding 100 µl of sterile mineral oil. The Perkin-Elmer thermocycler amplified DNA using a 5-minute denaturation at 94 °C, 35 amplification cycles (2 minutes, 2 minutes, and 1 minute at 72 °C), and a 7-minute final extension at 72 °C.

4.1. Multiplex PCR-Based Detection of Critical Staphylococcal Genes

We succeeded in amplifying the target gene sequences by fine-tuning the reaction conditions of the multiplex PCR experiment. Extreme care was made to exclude homologous regions within the enterotoxin structural genes in order to create primers that would target the coding parts of the genes. Because the annealing temperatures of each primer pair were nearly the same, there was less chance of undesired bands resulting from nonspecific amplification. In Figure 1, the amplified products can be seen following agarose gel electrophoresis. The PCR, which made use of multiplex primer sets, was carried out with DNA extracted from a common toxigenic *S. aureus* strain (the one acting as the control). The six bands in set A—sea, seb, sec, sed, saw, and femA—were reliably amplified by mixing DNAs from the same strains (Fig. 13). Additionally, as seen in Figure 1, a total of five bands were generated upon analysis of a mixture of DNAs from the corresponding strains in set B: eta, etb, tst, mecA, and femA. It was not surprising to see amplicons of the predicted sizes from many control strains. Both sets of samples failed to produce any amplicons when tested in sterile water (Fig. 1). Using genomic DNA at concentrations between 10 and 1,000 ng/reaction had no effect on the sensitivity or ability to detect all of the genes in the sample.

When using the multiplex PCR primer sets (A and B) with DNA templates from one strain of Campylobacter jejuni and two strains of Escherichia coli, no amplified products were found, further demonstrating the assay's specificity (data not shown). In addition to testing 220 *S. aureus* strains for multiplex PCR, the previously published approach was used to screen for the presence of specific toxin genes, providing further support for the multiplex PCR methodology. There was complete concordance between the two approaches. Using a total of 1,760 PCRs to test for 10 toxin genes separately was more expensive than using multiplex primer sets, which reduced the number of PCRs needed to 352. It was determined that the multiplex primer sets were highly reliable because the results from the two methods agreed. An internal control of femA was present in every sample, confirming the presence of *S. aureus* and validating the PCR conditions. Other studies have used 16S rRNA primers as an internal reference to build multiplex primers for mecA detection.

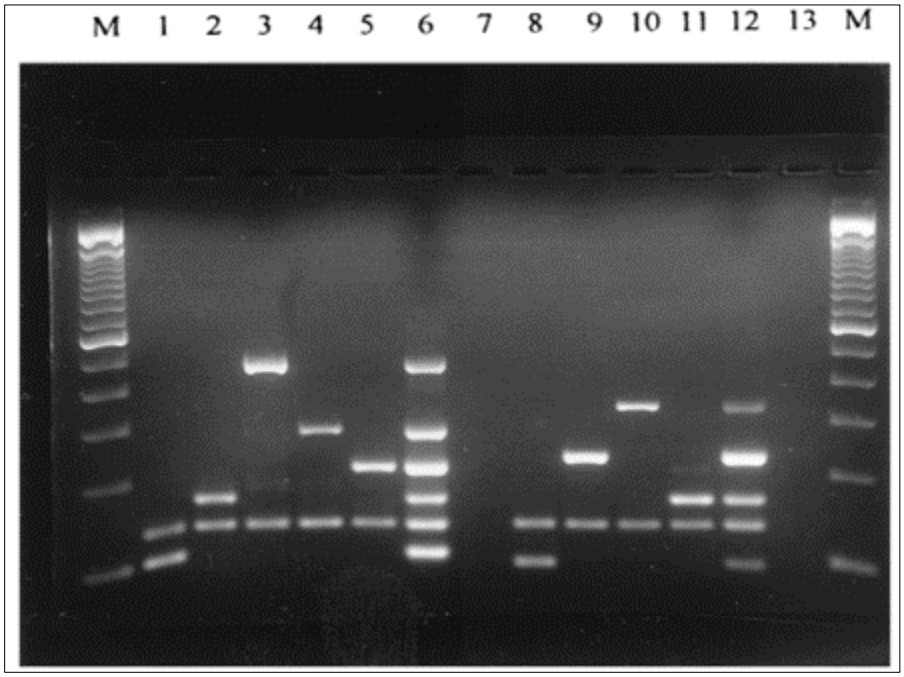

Figure 14 Agarose gel electrophoresis patterns

4.1.1. Primary validation of the amplicons

Table 14 demonstrates that the amplicon sizes generated utilising multiplex primer sets and those predicted by primer design were identical. Further confirmation and description of the sequences were made possible by the use of restriction endonucleases with cleavage sites inside the control strain amplicons. Table 14 lists the restriction enzymes that were used along with the anticipated product sizes. Enzyme fragments of the expected sizes were effectively separated in each case, according to data not shown.

Table 15 Enzymes for restriction fragment length polymorphism analysis and predicted restriction fragment sizes to validate multiplex PCR products

Gene	Amplicon size (bp)	Multiplex primer set	Restriction enzyme used	Expected sizes (bp) of restriction fragments
sea	104	A	AluI	64, 40
seb	168	A	MaeIII	121, 30, 17
sec	445	A	NdeI	302, 143
sed	280	A	MboII	172, 108
see	210	A	AccI	115, 95
femA	135	A or B	AccI	78, 57
eta	91	B	MboI	56, 35
etb	224	B	HpaII	160, 64
tst	328	B	MboI	196, 132
mecA	161	B	MaeIII	83, 78

The table above shows the expected restriction fragment sizes for several genes that were analysed using restriction fragment length polymorphism (RFLP) in order to validate multiplex PCR-amplified products. To group genes with amplicon lengths ranging from 91 bp (eta) to 445 bp (sec), multiplex primer sets A and B were utilised. Each gene was broken down by restriction enzymes, producing fragment sizes that could be uniquely described. For instance, the sea gene (104 bp) was broken down into 64 bp and 40 bp pieces using AluI, while the seb gene (168 bp) was broken down into 121 bp, 30 bp, and 17 bp pieces using MaeIII. Likewise, mecA (161 bp) was broken down by MaeIII, yielding 83 bp and 78 bp fragments, respectively. These enzyme-specific cleavage patterns establish the identification of the amplified products and validate the accuracy of multiplex PCR results.

5. Conclusion

Using a multiplex PCR technique that was refined in this work, important genes implicated in *Staphylococcus aureus* pathogenicity and antibiotic resistance were swiftly and concurrently discovered. The approach showed good specificity and reliability as all target genes amplified as expected and no non-specific products were found. Limitation fragment length polymorphism (RFLP) was used for validation in order to confirm the accuracy of the amplified products and guarantee the test's resilience. FemA made sure the results were authentic as an internal review. Since multiplex PCR requires a significantly smaller number of reactions than ordinary single-gene PCR, it is more effective for large-scale screening. Additionally, the test effectively distinguished *S. aureus* from other, non-target bacterial species, demonstrating its potential as a crucial tool for clinical and epidemiological diagnosis. By accelerating the detection and characterisation of *S. aureus*, our work highlights the necessity of integrating multiplex PCR with RFLP to enhance disease monitoring and infection control techniques. The study concluded that Staphylococcus aurous taken from different food sample showed bacteria resistance to many antibiotics. Furthermore, the presence of enteric staphylococcal genes among these isolates indicates their potential to cause food poisoning.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

References

[1] E. Kazem and J. Jarallah, "Molecular detection of *Staphylococcus aureus* enterotoxin genes," International Journal of Health Sciences, vol. 6, no. S4, pp. 6004–6019, 2022, doi: 10.53730/ijhs.v6nS4.11018.

[2] S. Alwash and R. Aburesha, "Detection of toxin-associated genes in seven spa-types of *Staphylococcus aureus* in Iraq," Indian Journal of Forensic Medicine and Toxicology, vol. 15, no. 3, pp. 4156–4164, 2021, doi: 10.37506/ijfmt.v15i3.15945.

[3] G. Y. Cheung, J. S. Bae, and M. Otto, "Pathogenicity and virulence of *Staphylococcus aureus*," Virulence, vol. 12, no. 1, pp. 547–569, 2021.

[4] F. Haghi, H. Zeighami, Z. Hajiloo, N. Torabi, and S. Derakhshan, "High frequency of enterotoxin encoding genes of *Staphylococcus aureus* isolated from food and clinical samples," Journal of Health, Population and Nutrition, vol. 40, no. 1, pp. 1–6, 2021.

[5] N. Senon, H. Al-Talib, and A. Adnan, "Detection of toxins and antibiotic resistance genes profile among methicillin-resistant *Staphylococcus aureus* (MRSA) isolates and their types of infection in a tertiary hospital in Malaysia," International Journal of Medical Research and Health Sciences, vol. 10, no. 2, pp. 151–159, 2021.

[6] B. M. Khalafallah, A. El-Tawab, A. Awad, S. Nada, and M. E. Elkhayat, "Phenotypic and genotypic characterization of Pseudomonas species isolated from frozen meat," Benha Veterinary Medical Journal, vol. 39, no. 2, pp. 47–51, 2020.

[7] S. Baniardalan, A. Mohammadzadeh, M. Pajohi-Alamoti, P. Mahmoodi, and A. Sadeghinasab, "Detection of toxic shock toxin (TST) gene in *Staphylococcus aureus* isolated from bovine milk samples," Bulgarian Journal of Veterinary Medicine, vol. 20, no. 3, pp. 236–243, 2017, doi: 10.15547/bjvm.1007.

[8] G. M. Mo. Mohammed and H. A. N. A. N. El Dahshan, "Molecular characterization of Staph. aureus and some enteric bacteria producing toxins in minced meat sold in Port-Said city markets," Assiut Veterinary Medical Journal, vol. 62, no. 151, pp. 54–63, 2016.

[9] M. A. Al-Ashmawy, K. I. Sallam, S. M. Abd-Elghany, M. Elhadidy, and T. Tamura, "Prevalence, molecular characterization, and antimicrobial susceptibility of methicillin-resistant *Staphylococcus aureus* isolated from milk and dairy products," Foodborne Pathogens and Disease, vol. 13, no. 3, pp. 156–162, 2016.

[10] R. Ali, K. Al-Achkar, A. Al-Mariri, and M. Safi, "Role of polymerase chain reaction (PCR) in the detection of antibiotic-resistant *Staphylococcus aureus*," Egyptian Journal of Medical Human Genetics, vol. 15, no. 3, pp. 293–298, 2014.

[11] A. Fetsch, M. Contzen, K. Hartelt, A. Kleiser, S. Maassen, J. Rau, et al., "*Staphylococcus aureus* food-poisoning outbreak associated with the consumption of ice-cream," International Journal of Food Microbiology, vol. 187, no. 1, pp. 1–6, 2014.

[12] E. B. Gurmu and H. Gebretinsae, "Assessment of bacteriological quality of meat contact surfaces in selected butcher shops of Mekelle city, Ethiopia," Journal of Environmental and Occupational Health, vol. 2, no. 2, pp. 61–66, 2013.

[13] C. Demir, Ö. Aslantaş, N. Duran, S. Ocak, and B. Ozer, "Investigation of toxin genes in *Staphylococcus aureus* strains isolated in Mustafa Kemal University Hospital," Turkish Journal of Medical Sciences, vol. 41, no. 2, pp. 342–352, 2011.

[14] E. R. Da Silva, L. S. do Carmo, and N. Da Silva, "Detection of the enterotoxins A, B, and C genes in *Staphylococcus aureus* from goat and bovine mastitis in Brazilian dairy herds," Veterinary Microbiology, vol. 106, no. 1–2, pp. 103–107, 2005.