RESEARCH ARTICLE

Phylogenetic Diversity of *sea* and *seb* Gene Encoding Enterotoxin in *Staphylococcus aureus* Isolated from Animal-Origin Foods

Adita Jadhav, Shubhangi Warke*

Abstract

Staphylococcus aureus is an opportunistic pathogen that causes multiple diseases in both humans and animals. It is considered a major cause of foodborne illnesses. The study was designed to investigate the prevalence and phylogenetic diversity of *sea* and *seb* gene encoding enterotoxin in *Staphylococcus aureus* isolated from animal-origin foods. A total of 150 samples were collected, including bovine milk, poultry meat, and chevon. The samples were subjected to cultural isolation and identification on Baird-Parker medium, followed by molecular detection of the thermonuclease (*nuc*) gene. The confirmed *S. aureus* isolates were further screened for Staphylococcal enterotoxin (SE) genes by singleplex PCR and studied the genetic relationship of the strain. *S. aureus* isolated on Baird-Parker medium accounted to 44% (66/150). A total of 16/50 (32%) milk samples, 18/50 (36%) poultry meat and 21/50 (42%) chevon were positive by the *nuc* gene. The frequency of SE genes in bovine milk, poultry meat and chevon was found to be 37.5% (6/16), 50% (9/18) and 28.57% (6/21), respectively. The prevalence of the enterotoxin gene *sea* and *seb* was 23.63% and 14.54 %, respectively. Phylogenetic analysis showed homology with *Homo sapiens*, which indicated the possibility of zoonotic transmission. These entero-toxigenic genes might transfer genes from animal foods to humans. Based on these findings, our data confirms the widespread proliferation of enterotoxigenic *S. aureus* strains in foods of animal origin in Nagpur and highlights major public health risks linked to the consumption of these enterotoxin gene-contaminated *S. aureus* foods, such as bovine milk, poultry meat, and chevon, emphasizing the need for surveillance and molecular characterization of these virulence genes to mitigate foodborne health risk.

Key words: Animal origin foods, , Enterotoxin gene, PCR, Sequencing , *Staphylococcus aureus*.

Ind J Vet Sci and Biotech (2026): 10.48165/ijvsbt.22.3.02

Introduction

Food poisoning poses a serious threat to consumers' health globally because of its rapid spread and high death rate. Consumption of foods contaminated by pathogenic bacteria or their toxins is responsible for food-borne illnesses. According to the WHO, 600 million foodborne disease cases occur among the human population due to the consumption of unhealthy, contaminated foods every year worldwide. Different pathogens produce different types of enterotoxins, like *E. coli,* which produces Shiga toxins and heat-labile enterotoxins; likewise, *B. subtilis* and *Salmonella* spp. also produce enterotoxins (Martinovic *et al.*, 2016). Staphylococcal enterotoxins (SEs) are produced by *S. aureus*, a non-spore-forming, non-motile, Gram positive bacteria thought to cause staphylococcal food poisoning (SFP) due to unhygienic conditions during the preparation of foods (Loir *et al.*, 2003). About 50% of the population carries the organism on their skin, hair, nasal tract, and in the throat. Food handlers are considered a potential source of contamination. Trace amounts of enterotoxins (high ng to low µg), about 10^5 CFU of CPS per gram of food, are required to develop the symptoms of SFP. The symptoms, such as severe nausea, vomiting, abdominal cramps, diarrhoea, and sometimes dehydration, usually appear within 30 min to 6 h after consumption of contaminated food (Diab *et al.*, 2021).

SEs survive in food substrates during processing because of their excellent resistance to heat, acidity, and pH alterations (Dutta *et al.*, 2020). More than 24 SEs are investigated, out of which *seb* accounts for 95% of the SFP outbreaks and is considered a classical enterotoxin (Omwenga *et al.*, 2019). *sea*

Department of Veterinary Microbiology, Nagpur Veterinary College, Maharashtra Animal & Fishery Sciences University, Nagpur-440006, Maharashtra, India

***Corresponding Author:** Dr Shubhangi Warke, Professor and Head, Department of Veterinary Microbiology, Nagpur Veterinary College, MAFSU, Nagpur-440006, Maharashtra, India. e-mail: shubhangi_warke@rediffmail.com

How to cite this article: Jadhav, A. A., & Warke, S. R. (2026). Phylogenetic Diversity of *sea* and *seb* Gene Encoding Enterotoxin in *Staphylococcus aureus* Isolated from Animal-Origin Foods. Ind J Vet Sci and Biotech, 22(3), 9-14.

Source of support: Nil

Conflict of interest: None

Submitted 14 /02/2026 **Accepted** 02 /03 / 2026 **Published** 10/05/2026

is the most frequently found enterotoxin in food, either by itself or in combination with other SEs. Due to its incredibly high resistance to proteolytic enzymes, it is also thought to be the primary cause of SFP (Argudin *et al*., 2010). *seb* causes staphylococcal food poisoning that mediates inflammation and is responsible for cytokine release. Due to its potency and stability, it may cause severe poisoning in humans under different environmental conditions. This enterotoxin may be detectable in urine, blood, respiratory tract secretions, and other body fluids (Gholamzad *et al.,* 2015). The present study aimed to determine the molecular prevalence of enterotoxins produced by *S. aureus* strains in animal food (bovine milk, poultry meat and chevon) employing conventional PCR and their phylogenetic analysis.

Materials and Methods

Collection, Processing and DNA Extraction of Samples

A total of 150 samples, including bovine milk, poultry meat and chevon, were aseptically collected and processed for the presence of *S. aureus* in the Nagpur region. The isolation was performed as per standard protocol (ISO standard 6888/1:1999 and 6888/2:1999). The samples were inoculated onto BHI broth and then incubated overnight at 37° C. One loopful of inoculum taken from BHI broth was streaked onto selective Baird Parker agar (HiMedia Pvt. Ltd., India) enriched by adding 5% egg yolk and 1% potassium tellurite (Oxide) and kept for incubation for 18-24 h at 37° C. Gram's staining was performed for the identification of the suspected colonies morphologically (Ridwana and Fayaz, 2017; Ghabbour*, et al.,* 2022).

Genomic DNA was extracted using the HiPurA® Multi-Sample DNA Kit (MB554) from overnight grown culture of *S. aureus* in BHI broth as per the manufacturer's instructions.

Molecular Detection of Thermonuclease (*nuc*) Gene of *S. aureus*

A species-specific published primer sequence of Oliveira *et al. (*2015) was employed for the amplification of the *nuc* gene (Table 1) for molecular identification of *Staphylococcus aureus*. The extracted DNA from presumptive *S. aureus* isolates was subjected to PCR targeting *nuc* gene. PCR amplification was performed in a total volume of 25 µL containing 12.5 µL master mix (2X) (Thermo Fisher Scientific), 1 µL (10 pmol/µL) each forward and reverse primer, 5 µL template DNA and 5.5 µL nuclease-free water. The PCR cycling conditions of the *nuc* gene are described in Table 2.

Detection of Enterotoxin *sea* and *seb* Gene

The published primers for the detection of enterotoxin *sea* and *seb* gene were used (Table 1). PCR amplification was performed in a total volume of 25 µL containing 12.5 µL master mix (2X) (Thermo Fisher Scientific), 1 µL (10 pmol/µL) each forward and reverse primer, 3 µL template DNA, 0.5 µL Taq polymerase and 7 µL nuclease-free water. *S. aureus* reference strain (MTCC 87) was used as a positive control and *E. coli* strain (MTCC 452) as a negative control. The cycling conditions of *sea* and *seb* were comprised of 35 cycles of denaturation, annealing and extension, which are described in Table 2. The amplification was accomplished using a thermocycler (BR Biochem Lifesciences Pvt Ltd). The PCR amplified products were resolved on 1.5% agarose gel in 1X Tris-Acetate-EDTA (TAE) buffer. Amplified PCR products were analysed using electrophoresis and visualised using the Gel documentation system (Syngene G box, UK).

Sequencing and Phylogenetic Analysis of Enterotoxin Gene *sea* and *seb*

One positive isolate of *sea* and *seb* was randomly selected and sent for sequencing to Barcode Biosciences, Bangalore, India Pvt. Ltd. The data were analyzed for identification of *S. aureus* (*sea* and *seb*) gene and compared with global enterotoxin *S. aureus* sequences in the GenBank using NCBI nBLAST and multiple sequence alignment was generated using ClustalW feature embedded in MEGA X. The phylogenetic tree was constructed using Maximum likelihood method to determine the evolutionary relationship among the sequences.

Results and Discussion

Identification of *Staphylococcus aureus*

Out of 150 samples processed, 66 (44%) samples were positive for *S. aureus* on Baird Parker agar exhibiting jet-black colonies (Table 3, Fig. 1) All the samples exhibited typical

Table 1: Oligonucleotide primer sequences used for amplification of *nuc, sea* and *seb* genes

Primer		Sequences (5'-3')	Amplicon size (bp)	Reference
nuc	Forward	5' GCG ATT GAT GGT GAT ACG GTT 3'	270	Oliveira *et al.* (2015)
	Reverse	3' AGC CAA GCC TTG ACG AAC TAA AG 5'		
sea	Forward	5' GGT TAT CAA TGT GCG GGT GG 3'	102	Abou-Khadra *et al.* (2020)
	Reverse	5' CGG CAC TTT TTT CTC TTC GG 3'		
seb	Forward	5' GTA TGG TGG TGT AAC TGA GC 3'	696	Hassanen and Shaltout (2017)
	Reverse	5' CCA AAT AGT GAC GAG TTA GG 3'		

Table 2: Cycling conditions used for amplification of *nuc, sea* and *seb* genes

Primer	Initial denaturation	Denaturation	Annealing	Extension	Final extension	No. of cycles
nuc	94^0C for 5 min	94^0C for 30 sec	60^0C for 60 sec	72^0C for 60 sec	72^0C for 10 min	35
sea, seb	94^0C for 5 min	94^0C for 30 sec	50^0C for 30 sec	72^0C for 30 sec	72^0C for 10 min	35

morphology and staining characteristic (Gram-positive cocci) of *S. aureus* (Fig. 2). The result of *S. aureus* in milk samples was 40% (20/50) on Baird Parker agar from the total examined raw milk samples. This agreed with the findings (39.09%) reported by Sudhanthiramani *et al.* (2015). However, higher incidences from 55-70% were reported by Ridwana and Fayaz (2017) and Choudhary and Kashyap (2024), while quite lower results of 10-17% were reported by Fawzy *et al*. (2017) and Patel *et al*. (2018).

Our study showed the prevalence of 44% in poultry meat, which was closer to 41% reported by Karmi (2013). Das and Mazumder (2016) and Herve and Kumar (2017) showed 48.57% and 46.61% prevalence rates of *S. aureus* by the conventional culture method in poultry meat. These results were higher compared to other studies (15%) conducted by Fawzy *et al*. (2017). Our findings revealed the presence of *S. aureus* in chevon as 48% on Baird Parker agar by culture method. This result was in agreement with that of Das and Mazumder (2016), who reported 43.33% prevalence in chevon, whereas Tefera *et al*. (2019) showed the prevalence of *S. aureus* in sheep and goat carcasses to be 33%. Lower prevalence of *S. aureus* (10% and 15.2%) in chevon was reported by Normanno *et al*. (2007) and Fawzy *et al*. (2017).

Table 3: Prevalence of *S. aureus* from bovine milk, poultry meat, and chevon

Samples	No. of samples	Positive on Baird Parker agar	Positive for the *nuc* gene
Bovine milk	50	20 (40.0%)	16 (32.0%)
Poultry meat	50	22 (44.0%)	18 (36.0%)
Chevon	50	24 (48.0%)	21 (42.0%)
Total	**150**	**66 (44.0%)**	**55 (36.66%)**

Molecular Detection of *S. aureus* by the *nuc* Gene

The prevalence of the *nuc* gene of *S. aureus* in animal food origin was recorded to be 36.66% (55/150) by PCR (Table 3, Fig 3). In the bovine milk sample isolates, 32.0% (16/50) were detected as *S. aureus* using the *nuc* gene primer set. These results correlated with the finding (37%) of Lubna *et al.* (2023), whereas Begum *et al.* (2018) observed a higher prevalence of 92.31%. A low prevalence of 16.3% in raw milk was reported by Mansour *et al.* (2017) in Giza, Egypt.

The molecular detection rate of *S. aureus* in poultry meat samples in our study was found to be 36.0% (18/50). The higher prevalence of 66.6% was reported by Shylaja *et al.* (2018) from commercial broiler chicken slaughterhouses. Abdulrahman (2020) stated that the isolation rate of *S. aureus* in poultry meat can vary from 28% to 80% in local and imported frozen chicken. The isolation rate of *S. aureus* in chevon samples in our study was found to be 42% (21/50). According to Ghabbour *et al*. (2022) the molecular prevalence of *S. aureus* in chevon was 46.0% (17/50), while a high prevalence rate of 66.6% was reported by Shylaja *et al.* (2018). Since milk, meat, and their products are so important in the Indian diet, contamination of these products can result in several health risks because of the high incidence of *Staphylococcus aureus*. As a result, care must be taken while handling, preparing, and consuming animal food and their products.

Molecular Detection of Enterotoxin *sea* and *seb* Genes

In the present study, overall 55 *S. aureus nuc* gene-positive isolates were further screened for the prevalence of enterotoxin *sea* and *seb* genes, out of which 21 isolates were found positive for the presence of enterotoxin genes. Among these, *sea* was the most frequent, carried in 13 (23.63%) isolates sharing 83.33%, 66.66% and 33.33% in bovine milk, poultry meat and chevon, respectively, followed by *seb* 8 (14.54%) including 16.66 % bovine milk, 33.33% of poultry

Fig. 1: Typical jet black colonies surrounded by a clear white halo zone showing lecithinase activity on Baird Parker Agar

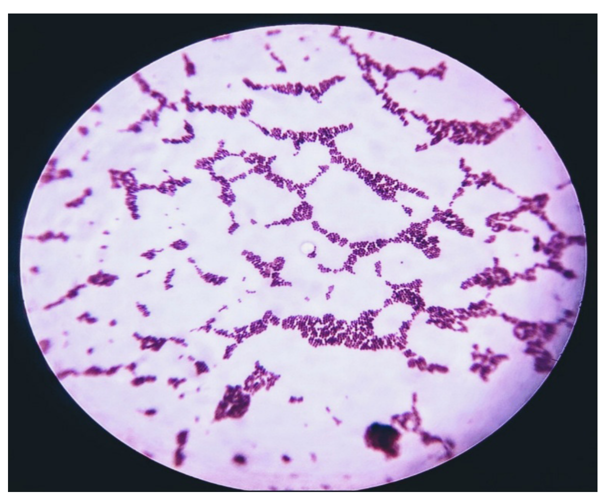

Fig. 2: Microscopic view, typical grape like clusters of *Staphylococcus aureus.* Gram's stain ×10

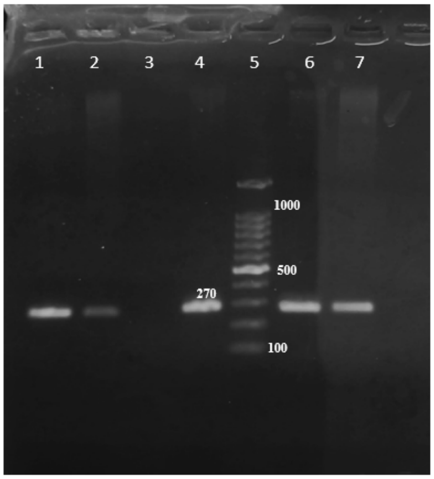

Fig. 3: Amplification of *nuc* gene at 270 bp. Lane 1: P22, Lane 2: P34, Lane 3: Negative control (*E. coli*), Lane 4: G26, Lane 5: 100 bp DNA ladder, Lane 6: Positive control, Lane 7: M10

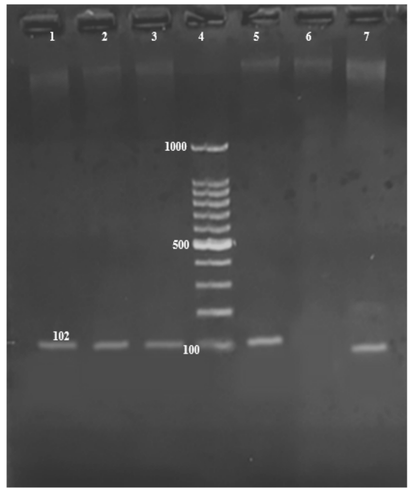

Fig. 4: Amplification of *sea* gene at 102 bp. Lane 1: M1, Lane 2: G28, Lane 3: M5, Lane 4: 100bp DNA ladder, Lane 5: Positive control, Lane 6: Negative control (*E. coli*), Lane 7: M10, Lane 8: P4

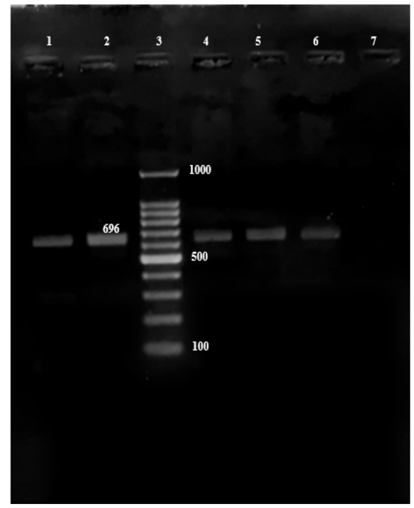

Fig. 5: Amplification of *seb* gene at 696 bp. Lane 1: G29, Lane 2: Positive control, Lane 3: 100 bp DNA ladder, Lane 4: G40, Lane 5: P31, Lane 6:P27, Lane 7: Negative control (*E. coli*)

Table 4: Prevalence of enterotoxin genes in *S. aureus* isolates from animal-origin food

Food samples	No. of isolates positive by *nuc*	No. of isolates positive for SE genes	SE	
			sea	***seb***
Bovine milk	16	6	5	1
Poultry meat	18	9	6	3
Chevon	21	6	2	4
Total	**55**	**21 (38.18%)**	**13 (23.63%)**	**8 (14.54%)**

meat and 66.66% of chevon (Table 4; Fig. 4, 5). In previous investigations, the *sea* gene was documented as being predominant SE present in 45.5% bovine milk (Forouzani-Moghaddam *et al.,* 2024). Maktabi *et al.* (2021) stated that 13% *S. aureus* isolates had *sea* gene, which is less compared to our finding. However, the *sea* gene was recorded comparatively lower in raw milk from different parts of India than the present findings, such as 53.84% by Paul *et al.* (2020) and 23.00% by Sundararaj *et al.* (2019). Out of 9 enterotoxigenic *S. aureus* isolates of poultry meat, 6 (66.66%) were positive for *sea* gene (Table 4, Fig. 4). A similar prevalence (66.66%) in poultry meat was reported by Karadal *et al.* (2024), while Hassanen and Shaltout (2017) and Sundararaj *et al.* (2019) found that *sea* was 40% in poultry meat. On the other hand, a lower prevalence rate of *sea* gene, 13.3% to 17.9%, was detected by Kitai *et al.* (2005), Abraham *et al.* (2022) and Ghanem *et al.* (2024) in poultry *S. aureus* isolates Out of 6, enterotoxigenic *S. aureus* isolate of chevon, 2 (33.33%) showed the presence of *sea* gene. A greater result of the frequency of the *sea* gene in chevon isolate was found to be 38.7% by Haghi *et al.* (2021).

The *seb* gene in bovine milk samples showed an incidence of 1 isolate out of 6 (16.66%) in our study (Table 4, Fig. 5). Nearly similar results of detection of enterotoxin, *seb* gene (17.8%) were reported by Bayrakal and Aydin (2024), while a lower prevalence of *seb* in milk from Giresun (1.9 %, 1/52) was found by Cebeci *et al.* (2024). Previous studies reported that *S. aureus* was found to be capable of producing the *seb* gene from the southern parts of India (Sundararaj *et al.,* 2019). The *seb* gene was detected in 3 out of 9 (33.34%) poultry meat isolates (Table 4), which concurred with Sundararaj *et al.* (2019), but disagreed with that of Hassanen and Shaltout (2017), with 10%, and El-Nagar *et al.* (2017), with 9.5% presence of the *seb* gene in poultry meat. In an investigation, Kitai *et al.* (2005) reported the presence of 64.1% of *S. aureus* in poultry meat collected from different supermarkets throughout Japan. In chevon samples, 4 out of 6 (66.66%) isolates were found positive for the *seb* gene that yielded 696 bp (Fig. 5). The results disagreed with Haghi *et al.* (2021), who reported that the frequency of the *seb* gene in meat sample isolates was found to be 12.9%.

Sequencing and Phylogenetic Analysis

The sequencing of the samples was performed to identify the genetic variation in the enterotoxin genes present in *S. aureus* isolates. Phylogenetic analysis showed similarities with reference and field strains (Fig. 6). *sea* gene sample G26 showed 100% homology to food poisoning outbreak samples isolated from Iraq (Accession No. JN687471 and KP402068). Sample P2 harbouring the *seb* gene showed

99.86% homology to a sample from human blood from New York (Accession N0.CP049991). A previous study revealed CC8 isolate harboured *sea* gene obtained from animal foods (Suzuki *et al.* 2014). Aziz and Mohammad-Jawad (2019) in their study reported 100% similarity of A6 and b4 isolates, which were obtained from raw milk and cheese with *seb* gene of two Indian isolates. Suzuki *et al*. (2014) found that isolate CC81 carried both *sea* and *seb* genes. Moreover, 90% of the *S. aureus* strains demonstrated *sea* and *seb* genes in an earlier study (Wang *et al.* 2018). Several studies reported the *sea* gene to be the primary cause of *S. aureus* food-borne poisoning outbreaks (Argudin *et al*. 2010; Glolamzad *et al.* 2015), which corroborated with our study.

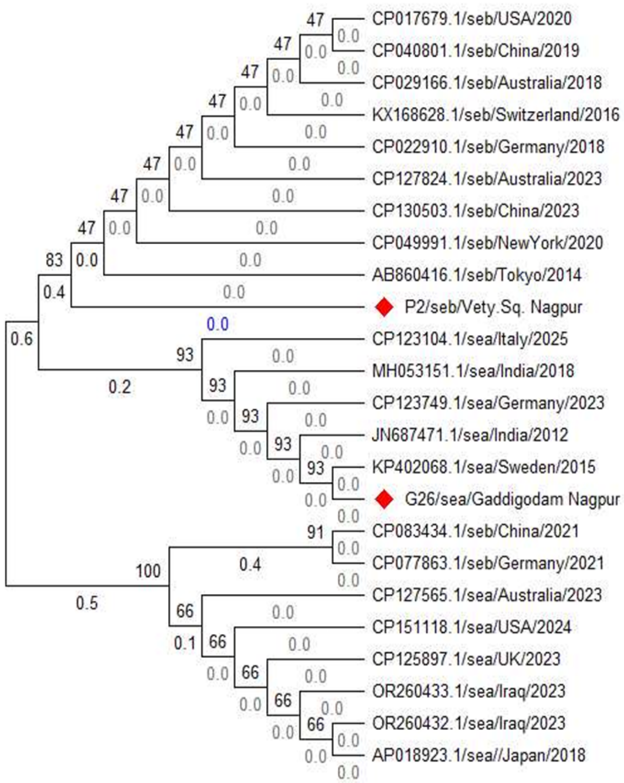

Fig. 6: Phylogenetic tree of *S. aureus* isolates based on enterotoxin (*sea* and *seb*) producing strains obtained from this study

Conclusion

Among the animal food origins, the highest enterotoxigenic *S. aureus* (18%) was found in poultry meat (9/50). The presence of enterotoxin-producing *S. aureus* in animal food origin samples represents a potential public and veterinary health risk. Therefore, it is highly advised that potentially harmful *S. aureus* be closely and continuously monitored to assess the risk to human health that comes from eating animal-origin foods.

Acknowledgement

The authors are grateful to the Associate Dean, Nagpur Veterinary College, Nagpur (MAFSU), India, for providing the necessary facilities and cooperation for this research work.

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