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RESEARCH ARTICLE

Coagulase positive Staphylococci contamination and the risk associated with the production of toxin in foods - An Exploratory Study

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Abstract: Food-borne diseases pose a threat to human health and the economy of individuals, families, and nations. *Staphylococcus aureus* is one of the most common causes of foodborne intoxication in most countries of the world. The present study was done to analyse the extent of *Staphylococci* contamination and the risk associated with the production of toxins in foods. A total of 450 Food samples were collected from retail markets, sweet shops, and households in Hyderabad. Among 143 (31.7%) cultures of *Staphylococci,* 106 (74.1%) showed coagulase enzyme production and 37 (25.9%) isolates were coagulase-negative. Only nine cultures (6.3%) showed a positive result for enterotoxin production. It is known that $>10^6$ CFU/g of *S. aureus* is likely to produce an enterotoxin, however, in the present study 17% of food samples have crossed the limit but very a small number of them were able to produce enterotoxin. For the risk assessment of *S. aureus* contamination in foods, coagulase test and toxin production of isolates have to be evaluated. The data will help set standards for the microbiological quality of foods.

Keywords: *Staphylococcus,* Enterotoxin, Coagulase, Safety

Introduction

Staphylococcus species are recognized as significant pathogens responsible for outbreaks of foodborne illnesses (Tohoyessou et al. 2020). In India, the rate of infection is still higher because of the warm and humid climate. *Staphylococcus aureus* food poisoning is an intoxication caused by the ingestion of food containing staphylococcal enterotoxins (SEs), and is one of the most common foodborne diseases in the world. *Staphylococcus aureus* produces a variety of extracellular products including the staphylococcal enterotoxins which have been implicated in human and animal diseases. The heat stability of *S. aureus* is one of the important properties of SEs in food safety (Le Loir et al. 2003). Contamination of foods by *S. aureus* may occur directly from infected food-producing animals or may result from poor hygiene during the production process or retail and storage of foods or from humans who will carry this microorganism (Doyle and Beuchat, 2007).

Coagulase-positive Staphylococci (CoPS) are opportunistic pathogens that can exist as commensals in humans, animals, and food-producing animals but have the potential to cause severe or even life-threatening diseases. They are facultative anaerobic gram-positive bacteria with a non-spore-forming spherical shape. At least nine species of CoPS have been identified, including Staphylococcus aureus, *S. hyicus, S. intermedius, S. pseudintermedius, S. lutrae, S. schleiferi subsp. coagulans, S. delphini, S. argenteus,* and *S. schweitzeri.* (Velazquez-Guadarrama et al. 2017; Gonzalez-Martín et al. 2020).

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Certain pathogenic strains of this bacterium produce heat-stable staphylococcal enterotoxins (SEs). Currently, 23 SEs have been identified, including five major classical types (SEs: SEA to SEE) and non-classical SE-like toxins (SEl: SEG to SEU). (Chajęcka-Wierzchowska et al. 2020; Saif et al. 2019). Approximately 95% of food poisoning outbreaks are caused by classical enterotoxins (Ahmed et al. 2021). Consuming food containing staphylococcal enterotoxins (SEs) can lead to the onset of severe symptoms, such as vomiting, high fever, nausea, and diarrhea, typically occurring rapidly in less than 8 hours (Jett et al. 2001).

Food Safety Standards Authority of India (FSSAI) regulations for microbiological requirements for different milk products indicate that coagulase-positive *S. aureus* should be less than 100 per gram of the milk product. Although enterotoxins are produced mainly by coagulase-positive staphylococci, some coagulase-negative staphylococci are involved in a variety of human and animal infections (Cunha et al. 2006). Taking into account that the toxigenic potential of coagulase-negative staphylococci is often neglected, the present study was done to analyze the extent of *Staphylococci* contamination and the risk associated with the production of toxins in foods.

Materials and Methods

Study area

The study was carried out in Hyderabad which is the capital of Telangana, India. As of now, it is the sixth-most populous city and the sixth-most populous urban agglomeration in India. The twin cities of Hyderabad and Secunderabad come under the ambit of a single municipal unit, the Greater Hyderabad Municipal Corporation. For administrative purposes, Greater Hyderabad Municipal Corporation has been divided into many circles with each circle being homogeneous within and different from other circles. A random sampling procedure was adopted in the study and the sample required for the study was obtained using proportionate representation according to size.

Sample collection

Food samples were collected aseptically from retail markets, sweet shops, and households of Hyderabad. Samples were transported to the laboratory in the ice box and transferred to the refrigerator until further analysis. The interval between the sampling and the analysis will be less than one hour. A total of 420 food samples including khoa (a desiccated milk product), Kulfi (ice cream), paneer (a type of milk curd cheese used in Indian cooking), Dhal (a sauce made from lentils and spices, usually served with cooked rice), nonvegetarian curry (chicken meat and mutton), pineapple fruit juice, cooked rice, vegetarian curry and sapota/sapodilla juice including 30 households hand washings were collected for the analysis.

Isolation and identification of *S. aureus*

Isolation and identification of *S. aureus* were performed according to the US FDA bacteriological analytical manual. A 25 g of the food sample was collected aseptically and added to 225ml of buffered peptone water and the mixture was homogenized for 3-5mins. A 100 µl of the inoculum was taken and inoculated on Baird Parker Agar (HIMEDIA) with egg yolk tellurite emulsion and incubated at 35^{0}C for 24-48 hr and suspected colonies (a grey-black shiny convex colony with a narrow white margin surrounded by a zone of clearing) were confirmed by doing further biochemical tests like catalase test, coagulase production, anaerobic utilization of glucose and carbohydrate (mannitol) fermentation test.

Coagulase test

The coagulase test was done by taking 0.05ml of an overnight broth culture of *Staphylococci* or 2-3 pure colonies picked from an agar plate on a clean glass tube and then by adding 0.5ml of rehydrated plasma. Coagulase plasma (from rabbit) was procured from Himedia Laboratories Pvt. ltd. Both the solutions were mixed well and incubated at 37°C in the incubator for 4h. Agglutination or clumping of cocci within 4h was considered a positive result.

Preparation of bacterial culture for enterotoxin production

Pure *S. aureus* culture was pre-enriched in Brain Heart Infusion (BHI) broth. Centrifugation of the bacterial culture was done for 5 min at a minimum of 3500g/10^{0}C. Sterile filtration of the supernatant was done and 100 µl of the filtrate per well was used in the enzyme immunoassay

Enterotoxin detection

The coagulase-negative and positive *S. aureus* strains were selected for the detection of enterotoxin. RIDASCREEN SET total sandwich enzyme immunoassay kit, manufactured in Germany was used for the combined detection of *Staphylococcus* enterotoxins (SET) A, B, C, D, and E from bacterial cultures. All reagents required for the enzyme immunoassay were there in the test kit.

Statistical analysis

A proportion test has been done to see the differences in toxin production among coagulase-positive and coagulase-negative *Staphylococci* isolates.

Results and Discussion

The incidence of *S. aureus* (percentage of contamination) in food samples sold in various localities of Hyderabad is shown in Table 1. A total of 420 food samples including 30 hand washings were

collected from retail markets, sweet shops, and households in Hyderabad. Among all the food samples analyzed, Sapota/ Sapodilla fruit juice was highly contaminated (91%) with *Staphylococcus*. Kulfi ice cream and paneer were less contaminated with *Staphylococcus* when compared to other food samples.

The results of coagulase and enterotoxin production by the *Staphylococcus* cultures are shown in Table 2. Among 143 (31.7%) cultures of *Staphylococci,* 106 (74.1%) showed coagulase enzyme production and 37 (25.9%) isolates were coagulase-negative. The number of *Staphylococcus-producing* coagulase was more than noncoagulase-producing Staphylococcus. Both coagulase-positive and negative cultures were able to produce enterotoxins. Only nine cultures (6.3%) showed positive results for enterotoxin production. It is known that $>10^6$ cfu/g of *S. aureus* is likely to produce enterotoxin, however, in the present study 17% of food samples have crossed the limit but very a small number of them were able to produce enterotoxin.

A study on the persistence and survival of *S. aureus* at different temperatures (4, 10, and 37°C) for different lengths of time (0-12 days) indicated that the *S. aureus* population varied with temperature and showed the highest population and viability at 37°C. On the 12th day differences in population were observed at lower and higher temperatures (Table 3). In the proportion test coagulase coagulase-positive isolates producing toxin was 6% (6/106) and coagulase-negative isolates producing toxin was 8% (3/37) which was not significant (P=0.620).

Table 1: Incidence of *S. aureus* (percentage of contamination) in food samples sold in various localities of Hyderabad

Food Samples	Samples positive for *S. aureus*	Mean±SD	Prevalence (%)
Rasmalai (n=60)	32	4.5±1.4	31.7
Khoa (n=60)	75	6.1±1.3	73.5
Paneer (n=60)	29	4.1±1.3	28.4
Kulfi (n=60)	22	4.1±1.3	22.0
Chiku/Sapota Juice (n=39)	36	3.0±1.1	91.8
Pineapple Juice (n=37	20	2.1±1.4	54

Table 2: Screening of coagulase and enterotoxin in Staphylococcal enterotoxin producing *S. aureus* from food sample

Type of foods	No. of *S.aureus* isolates	Coagulase +	Coagulase -	Enterotoxin (Coagulase +)	Enterotoxin (Coagulase -)
Dhal (30)	7	5	2	2	0
Khoa (60)	36	27	9	0	0
Kulfi(60)	5	4	1	0	0
Non veg(30)	12	5	7	1	1
Paneer(60)	7	6	1	0	0
Pine apple Fruit Juice (30)	2	2	0	0	0
Rasmalai(60)	22	20	2	1	0
Rice(30)	27	17	10	1	1
Sapota Fruit juice (30)	2	2	0	0	0
Veg curry(30)	16	12	4	1	1
Hand Washings (30)	7	6	1	0	0
Total (n=450)	143	106 (74.1%)	37 (25.9%)	6 (4.1%)	3* (2%)

* Out of 9 enterotoxin producing strains 3 are coagulase negative

Table 3: Population (log10cfu/g) of *S. aureus* on Rasmalai after storage at 4, 10 and 37 deg c for different length of time (0, 1,2,4,6,8,10 & 12d)

Mlik Product	Foodborne pathogen	Storage time (d)	Temperature		
			4	10	37
Rasmalai	*S. aureus*	0	2.61±0.00	2.72±0.02	2.72±0.02
		1	2.49±0.00	2.50±0.00	3.34±0.06
		2	2.45±0.01	2.53±0.04	3.32±0.02
		4	1.93±0.01	2.18±0.02	3.36±0.02
		6	1.23±0.09	2.14±0.00	2.75±0.02
		8	2.02±0.02	2.19±0.14	2.48±0.01
		10	1.77±0.04	2.79±0.02	2.92±0.03
		12	0.64±0.06	2.86±0.04	3.03±0.04

Food-borne diseases pose a threat to human health and the economy of individuals, families, and nations. In the Western hemisphere and in Europe, *Salmonella* serotype *Enteritidis* (SE) has become the predominant strain (WHO. 2011). A review of foodborne diseases in India indicated that the majority of the foodborne disease were caused due to vegetarian foods (Sudershan et al. 2011). Among the foods implicated in India, milk and milk products were predominantly involved in the foodborne disease outbreak (Sudershan et al. 2011).

Staphylococcus aureus food poisoning is an intoxication caused by the ingestion of food containing staphylococcal enterotoxins (SEs) and is one of the most common foodborne diseases in the world. The primary habitat of *S. aureus* is the nasal passage of humans and the skin and hair of warm-blooded animals (Kluytmans et al. 1997; Kuzma et al. 2003). *Staphylococcus aureus* produces a variety of extracellular products including the staphylococcal enterotoxins which have been implicated in human and animal diseases. The heat stability of *S.aureus* is one of the important properties of SEs in food safety (Le Loir et al. 2003). Contamination of foods by *S. aureus* may occur directly from infected food-producing animals or may result from poor hygiene during the production process or retail and storage of foods or from humans who will carry this microorganism (Doyle and Beuchat, 2007).

The present study showed that Staphylococcus contamination was higher in Sapodilla juice compared to other food samples indicating that they are hygienic of a low standard. Fruit juices sold on streets have been contaminated with various food and waterborne pathogens (Olorunjuwon et al. 2014; Lewis et al. 2006; Poonam, 2013; Tambekar et al. 2009; Mahale et al. 2008). The high contamination of Sapodilla fruit juice may be attributed to its neutral pH compared to other fruit juices which are of acidic pH.

In the present study, the number of coagulase-positive *Staphylococcus* cultures was more compared to coagulase-negative *Staphylococcus* (CNS). A study conducted by Cunha et al. on the detection of enterotoxin genes in coagulase-negative Staphylococci isolated from foods indicated that 22.7% were positive for CNS. Among them, four isolates were positive for enterotoxin genes (Cunha et al. 2006). A comparable study examining *S. aureus* isolates for the presence of classical enterotoxin genes revealed that none of the isolates were found to harbor any of these genes (Esemu et al. 2023). In a different study, a substantial prevalence (58.1%, 18/31) of classical staphylococcal enterotoxin genes was detected in meat samples collected in Zanjan, Iran. (Haghi et al. 2021)

In the present study, *S. aureus* population varied with temperature and showed the highest population and viability at 37°C. A study on the Influence of holding temperature on the growth and survival of *Salmonella* spp. and *Staphylococcus aureus* and the production of staphylococcal enterotoxin in egg products indicated that Staphylococcal enterotoxin A and B are detected only in the egg products held at 37 or 22 degrees C. After holding at 37 degrees C for 36 h, scrambled egg inoculated with *S. aureus* contains the highest levels of SEA and SEB (Yang et al. 2001). In terms of Staphylococcal bacterial counts, winter emerged as the season carrying the highest risk, whereas concerning enterotoxin production, the peak risk was identified in autumn, particularly during October. The susceptibility to S. aureus contamination was most pronounced in dairy products (Bianchi et al. 2022). In India, there are many studies to show the prevalence of *Staphylococcus* in food samples but studies on its enterotoxin production are scanty.

There is a need to relook at the guidelines for microbiological requirements for different milk products which indicate that coagulase-positive *S. aureus* should be less than 100 per gram of the milk product. Although enterotoxins are produced mainly by coagulase-positive staphylococci, some coagulase-negative staphylococci are involved in a variety of human and animal infections (Cunha et al. 2006).

Conclusions

While assessing the risk of foodborne disease due to *S. aureus* contamination in foods enterotoxin production needs to be examined irrespective of its coagulase enzyme production. Further molecular characterization of classical and novel genes encoding different enterotoxins is necessary to find out different types of

enterotoxins. The data will help set standards for the microbiological quality of foods.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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