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A study to check the status of enterotoxin production by *Staphylococcus aureus* in milk and milk products.

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Abstract

Staphylococcal food poisoning is of great concern for the health worldwide. It may be present in foods such as milk, milk products, meat and meat products, this condition in any type of the food material may occurs due to unhealthy practice conditions during manufacture, processing, storage and handling of food products, which are the main causes of food borne diseases. The main objective of the study is the detection of *Staphylococcus aureus* isolates obtained from food sample and also to detect the presence of coagulase test, phosphatase test and enterotoxin producing nuclease gene in the isolates.

Keywords: Enterotoxin, *Staphylococcus aureus*, food poisoning, food products, food borne diseases.

INTRODUCTION

Staphylococcus aureus is a well known pathogen of man and animals [1]. Despite the development of antimicrobial agents the staphylococcal infection still remains an important cause of morbidity and mortality [2]. The spectrum of disease produced by this organism varies from pyogenic infections to toxin mediated phenomenon. It also causes serious infections in man including deep seated abscesses, bacteraemia, endocarditis, osteomyelitis, food poisoning etc. [3,4]. The main reservoirs of infection are infected cases and carriers who spread infection in a hospital or in a community through droplets in air or through fomites. Carriage in nose plays an important role in its epidemiology and pathogenesis. *S. aureus* can readily multiply in many food items. The ability of *Staphylococci* to produce coagulase, an enzyme that clots plasma was first reported by Loch in 1903. However, there is no convincing evidence that coagulase is directly involved in pathogenicity.

S. aureus strains produce an extra cellular "Thermostable nuclease (TSN) is a rapid test (requiring 2 to 4 h) that differentiates between *S. aureus* and coagulase-negative staphylococci (CoNS), as *S. aureus* produces a nuclease that is uniquely and consistently thermostable (PDJ Sturm, *et al*., 2008). Tnase a protein with a molecular mass of 17,000 KDa and is considered as a virulence marker. The *nuc* gene is widely employed as the target gene for specific detection of *S. aureus* [5,6]. Jasper (1973) found a close correlation between thermostable nuclease and coagulase production [7].
The milk and dairy products are probably the types of foods most frequently implicated in food poisoning outbreaks [8]. A variety of exoproteins produced by *S. aureus* cause disease in the mammalian host [9]. The most notable virulence factors associated with *S. aureus* are enterotoxins [10]. Contamination of food with *S. aureus* during storage could lead to the production of enterotoxin. This intoxication resulting from the ingestion of food containing preformed heat stable enterotoxins results in acute disease known as Staphylococcal food poisoning [3].

On the other hand, *Staphylococcus aureus* is also an important agent producing infection in human; especially causing mastitis thus resulting in great economic loss and threat to food safety.The present study was carried out to evaluate the phenotypic and characters of *S. aureus*, which might shed light; on the prevalent Indian *S. aureus* clones in human infections and also in foods of animal origin, so that proper infection control measures may be undertaken.

REVIEW OF LITERATURE

2.1 General description

The genus *Staphylococcus* includes 32 species; among them *S. aureus* has been identified as one of the most important human pathogen [12]. The organism successfully adopts itself on the ectodermis of warm blooded animals. Some staphylococci are members of normal flora of the body surfaces of man and animals; others cause suppuration, a variety of pyogenic infections, food poisoning etc. *Staphylococcus aureus* causes infections in all age groups. It is responsible for sporadic infections as well as epidemics [13] and is the most common cause of infections in hospitalized patients [14].

2.2 Historical background

Von Recklinghausen (1871) first observed staphylococci in pus obtained from human pyogenic lesions. Further Robert Koch (1878) reported the presence of small spherical bacteria in pus. abscess and in infected blood of people suffering from pyaemia and named them as "*Micrococci*". Later in 1881 a Scottish surgeon Sir Alexander ogston established the functional role of cocci in abscess and other suppurative lesions. In 1882, these cocci were named as Staphylococcus. The first person to isolate pure culture of staphylococci in laboratory was Rosenhach (1884). He gave the typical characteristic features of this organism and formal description of the genus. Fie divided staphylococci

into two species namely *S. aureus* and *S. aureus* on the basis of their orange and white pigmented colonies, respectively. Third species known as *S. Citreus*, was added by Passel (1885) based on their lemon yellow pigmentation.

DNA-rRNA hybridization and comparative oligonucleotide cataloguing of 16S rRNA [15].

Habitat

The largest population of *Staphylococcus aureus* in the human body is found on the skin, mainly in the axillae, inguinal and perineal areas; and in the anterior nares [13]. Colonization by *S. aureus* may be found in 6% to24% of newborns after 3-4 days in a well baby nursery. Among healthy adults, carrier rates of 11 % to 32 %were detected in the general pharynx, conjunctiva, mouth, blood, mammary glands, faeces, body discharges, excretions and intestinal, genitourinary and respiratory tracts of their hosts [17].The occurrence of contaminating staphylococci in various food products has been studied extensively [18].

Cell morphology

Staphylococcus aureus is uniformly gram positive in young (18-24 hours) cultures, nonmotile, nonflagellated, nonspore forming bacteria. It appears spherical with an average diameter of 0.5-1.5 Mm. Thesize varies from strain to strain and influenced by the age of cultures andby the medium on which it is grown. The cells of old cultures (>48 hours)are often Gram -variable to nearly Gram-negative. *S. aureus* mainlyproduces irregular clusters of cells. Organism divides in more than one plane to form irregular clusters.

Encapsulated cells (form mucoid, glistening colonies) are surrounded by a capsule layer (>200 nm thickness) outside the cell wall [19]. Staphylococci may also have capsules that are thinner than 200 nm (microcapsules), which are not visible by light microscopy or staining by India ink method. Incidence of *S. aureus* strains exhibit in microcapsules is 80-90% [20].

2.5 Protein A (SPA)

It is a group specific antigen found in the cell wall of about 90% strains of *S. aureus* (especially Cowan l strain). Protein A is bound to the cell wall peptidoglycan and it is also shed into the medium during growth of the bacteria.

- **Cell surface adhesins**

Staphylococci have a group of cell surface proteins that act as adhesins (also known as microbial surface components recognizing adhesive matrix molecules) and hind to extracellular matrix proteins such as fibronectin, fibrinogen, laminin, elastinand collagen etc. of their host.

Slime

Is a complex extra cellular substances produced in varying amount by many Staphylococci. Crude slime is a very heterogeneous substance, usually composed of a variety of monosaccharides, including mannose, galactose, glucose, glucosamineand glucouronic acid, proteins and small peptides.

Extra- cellular proteins

The extra cellular proteins produced by Staphylococcus can act as either toxins or as enzyme activator or non-toxic enzymes. These are as follows:

Toxins

Membrane damaging toxin**:** Damage the plasma membrance of eukaryotric cells [21]

(a) Hemolytic toxin (Hemolysins):

(b) Leucocidins:

2.10 Fibrin forming and fibrinolytic enzymes

(a) Staphylocoagulase: Is a Protein that clots plasma in the absence of Ca^{**} but requires a coagulase reacting factor (CRF). CRF reacts with coagulase and resulting coagulase-CRF complex (staphylothrombin) converts fibrinogen to fibrin [22]. 98-99% of *S. aureus* strains exhibit coagulase activity. Coagulase activity may also be exhibited by the species *S. intermedius, S. hyicus, S. delphini and S. schleifer*i subsp. coagulans.

(b) Staphylokinase: Is a protein that exhibits fibrinolytic activity indirectly by binding to plasminogen, is produced by a relatively high percentage (60-95%) of human *S. aureus* strains of biotype A.

2.11 Biochemical properties

S. aureus ferments glucose, maltose, lactose, sucrose and mannitol with production of acid but no gas. Anaerobic utilization of mannitol is considered to be distinctive features of *S. aureus*. It is catalase positive, oxidase negative, methyl red and Voges-Proskauer test positive. Nitrate is reduced to nitrite and indole is not produced. *S. aureus* produces deoxyribonuclease (DNase), and heat stable nuclease (Thermostable nuclease, TNase). *S. aureus* also produce phosphatase. *S. aureus* is sensitive to novobiocin but resistant to bacitracin.

3. MATERIAL & METHODS

3.1 Collection of Sample:

A total of 460 milk and milk product samples which includes raw milk (400 samples), curd (30 samples) and pedha (30 samples) were collected from different places in and around Ghaziabad city such as milk collection centers of Co-operative milk dairies, cattle farms, individual household, milk vendors and sweet shops. The samples were collected in sterilized milk collecting tubes and polyethylene bags and transported in an icebox to laboratory of the add Post Graduate Department of Department of Life Science, Institute of Applied Medicine and research Ghaziabad.

3.2 Food Samples:

A total of 1006 food samples were collected according to the methods described by Agrawal [23]. The details of the food samples are provided below:

3.3 Milk products:

About 2 gm each of Khoa (Milk concentrate used for the preparation of sweets), Paneer (cottage cheese), Chamcham and Sweets were collected with aseptic precautions in sterile glass containers.

3.4 Laboratory procedures:

Direct Microscopic examination: Smears were prepared from all the clinical samples on clean sterile glass slides for Gram's staining. The smears were allowed to dry and then fixed by passing through flame and stained by Gram's technique. The smears were examined under oil immersion to look for Gram positive cocci in clusters.

3.5 Processing of food samples:

(a) Processing of milk products: The samples were homogenized at room temperature in 10 ml of NS in sterile beakers. The homogenization was done by electrically operated food homogenizer and then used for bacterial isolation.

(b) Processing of raw milk: Raw milk is already pasteurized, hence, it is directly used as a sample without any processing.

3.6 Bacterial culture:

All food samples were cultured according to standard procedure appropriate to the type of specimen.

Samples were streaked on 5% Sheep blood agar, nutrient agar and Baird-Parker agar medium and incubated for 24-72 hours at 37°C. The isolates were stocked in agar stabs and stored at 4^0C for further characterization.

3.7 Phenotypic tests for identification / characterization:

Staphylococci were identified as per standard methods (Mackie and McCartney, 2015).

Colony Morphology: On nutrient agar and sheep blood agar: The circular, smooth, 1-3 mm, low convex, glistening and opaque colonies, which were easily emulsifiable. butyrous in consistency golden yellow/creamy colour, surrounded by zone of β-hemolysis on blood agar were identified as the colonies of *S. aureus*. On Baird-Parker medium distinct black colored colonies were found.

Microscopic Morphology: Smears were prepared from the colonies for Gram's staining to look for the characteristic morphology of Staphylococci.

The Staphylococcal isolates were further characterized on the basis of:

Coagulase Test:

Requirements:

Rabbit plasma

Normal Saline (0.85%) or Nutrient broth

Controls:

Coagulase- positive strain (S. aureis oxford strain number 6571).

Coagulase- negative Staphylococci (S. epidermidis)

a. Slide coagulase test: [24] To detect 'bound coagulase' (Clumping factor):

Method: A drop of physiological saline solution was placed on a clean glass slide and with minimum spreading one or two colonies of culture under test was emulsified in it. A control suspension from a known coagulase-positive and negative culture was also made to confirm the reactivity of the plasma. With inoculating wire, a drop of plasma was

added at room temperature and mixed gently. The wire was flamed and the procedure was repeated for control suspensions. The appearance of coarse clumps visible to the naked eye within 10 seconds indicates positive reaction. The absence of clumping or any reaction taking more than 10 seconds to develop was considered as negative.

b. Tube Coagulase Test (Modified from Gillespie 1943): To detect 'free coagulase':

Method: A 1:10 dilution of rabbit plasma was prepared in saline (0.85% NaCl) solution. 0.5ml of the diluted plasma was placed in a small sterile tube. Few colonies of Staphylococci under test were emulsified in nutrient broth to give a dense suspension. Subsequently 0.1 ml of this suspension (about 10^9 cocci) was added to the diluted plasma tube. Similarly control test with known coagulase-positive and coagulase-negative cultures were set up. A tube of unseeded diluted plasma was also included to confirm that it does not coagulate spontaneously. The tubes were incubated at 37°C in a water-bath and examined for clot formation by tilting the tube through 90° at 1, 2 and 4 hrs and again if still negative, after standing overnight at room temperature.

Test was read positive when the plasma had been converted into a stiff gel when the tube was tilted or inverted. Test in which the plasma remained wholly liquid or showed only a flocculent or ropy precipitate or free flowing was read as negative.

DNAse Test [25]
Requirements: DNA agar (Oxoid DNAse Agar) with 0.1 toluidine blue obtained from Hi Media.

Method: Petriplates were prepared by pouring toluidine blue DNA agar. After solidification, 2 mm diameter wells (10-12 wells per plate) were made and agar plugs were removed by aspiration, 0.01 ml each heated sample (15 min. in boiling water bath) (f BHI broth culture was added into wells on prepared plates, and incubated at 35°C. Positive reaction was the formation of a bright pink halo extending at least 1 mm from the periphery of well. The results were noted after 4 hrs of incubation and observed up to 18 hrs.

Phosphatase Test:
Requirements: Phenolphthalein diphosphate agar (Hi Media), liquor ammonia solution (SG 0.88).

Method: Test strain was streaked on the phenolphthalein diphosphate agar (PPA) plates and incubated for 18-20 hours at 37°C. After incubation 01-02 ml ammonia solution was placed in the lid of petridish and culture plate was inverted above it. Bright pink colonies were considered as positive. The test was performed using the method of Agarwal [23].
Method: A heavy suspension (about 10^9 colony units / ml) was prepared in wells of a microtitre plate from an overnight culture of test organism in 0.1 ml solution of benzylpencillin (6 mg/ml in 0.1 mol/liter phosphate buffer, pH 7.3). The control tests with penicillinase negative and positive cultures were also set up. The microtitre plate was incubated for one hour at 37°C, and then two drops of freshly prepared 1% solution

of soluble starch was added to each well. In positive test, the blue colour was lost rapidly (within 10 seconds). In negative test blue colour persisted for at least 10 minutes.

RESULTS

In this study we used 1006 total number of the sample out of which 800 were from Raw Milk and 206 were milk products. During the identification we found 460 samples positive for the presence of *S. aureus*. Of the 460 isolates of *S. aureus* from milk and milk products, 357 (77.6%) were obtained from raw milk, 19 (4.1%) from chamcham (a sweet prepared from milk), 14 (3.04%) from other sweets, 42 (9.13%) from khoa (milk concentrate used in sweets) and 28 (6.08%) from paneer (cottage cheese).

Table 1:

Enzymatic Test	***Staphylococcus aureus* Detected**	***Staphylococcus aureus* Not Detected**
Catalase Test	460	-
Coagulase Test (i) Slide Coagulase (ii) Tube Coagulase	 450 460	 10 -
DNase Test	450	-
Phosphatase Test	450	-

DISCUSSION

Milk is normally sterile in the udder of the cow and buffalo provided they do not suffer from mastitis (udder infection). If they suffer from mastitis, a large number of Gram positive bacteria such as *Streptococcus* and *Staphylococcus spp*. may be present in milk when it leaves the udder.

Negligence of hygienic condition such as improper cleaning of bulk tank, dirty udder, milking equipments, milk handling technique and improper storage will increase the proportion of Gram-positive and Gram-negative bacteria in the bulk tank milk.

Food products serve as a source of nutrition as well as substrates for the growth of microorganisms. The growth of microorganisms causes food spoilage. It may result in food-borne illness. In tropical countries, raw milk and milk products are responsible for occurrences of gastro intestinal tract. It is also reported that immune-compromised individuals are prone to food-borne infection.

In the present study, out of 460 isolates of *S. aureus* from milk and milk products, 357 (77.6%) were obtained from raw milk, 19 (4.1%) from chamcham (a sweet prepared from milk), 14 (3.04%) from other sweets, 42 (9.13%) from khoa (milk concentrate used in sweets) and 28 (6.08%) from paneer (cottage cheese).The highest incidence of S. aureus

was from raw milk (77.6%), followed by khoa (9.13%), paneer (6.08 %), chamcham (4.1%) and other sweets (3.04%).

As compared to present findings higher level of incidence of *S. aureus* have been reported by Tambekar, Ekici, Sandermann, Schleifer & Lindgren [26,27,28,29,30,31] who found 17.39 %, 18.18 %, 18.80 %, 40 % and 61.70 % incidence respectively. The incidence of *S. aureus* in sweets in the present study was 3.04 % seems to be correlated with find reported by Hochkeppel while higher incidence of 20% was also reported. . The difference in the prevalence rates of *S. aureus* between milk and milk products may origin from the method of manufacture, storage and handling.

CONCLUSION

Staphylococcal food poisoning is of great concern for the health programs worldwide. It may be present in milk and milk products as a result of milk collected from the animal suffering from disease condition and excreting *S. aureus* in milk or due to unhealthy conditions during manufacture, processing, storage and handling of milk products, which are the main causes of food borne diseases. Results clearly in directed that milk and milk based products available in the market were contaminated with S. aureus, posing a high risk of food poisoning. Thus hygienic preventive measures are required to reduce the bacterial contamination, so as to increase the purity of milk and milk based products.

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