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Species Specific PCR Based Rapid Detection of *Staphylococcus aureus* from Cottage Cheese, and its Sensitivity against Antibiotics and natural products

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Abstract

Present study was aimed at isolating and identifying coagulase positive Staphylococcus aureus from cottage cheese samples produced by the traditional methods using biochemical tests as well as sequencing of amplified Sa442 DNA fragment and its control measure using antibiotics and natural products extracted in ethanol and water. Study shows that antibiotics were comparatively more effective against Staphylococcus aureus, but the purified extracts of Ajwain, Mulathi and Hime could be used as a better substitute with lesser side effects. Protective measures are also required to reduce the occurrence of Staphylococcus aureus in the milk products prepared through traditional methods.

Key words: Cottage cheese, Staphylococus aureus, antibiotics, natural products

Introduction

Staphylococcus aureus is one of the most important opportunistic pathogen among Staphylococci belonging to *Micrococaceae* family causing significant infections under appropriate conditions [Prescott, *et al*., 2002]. *S. aureus* commonly found on skin surface and in nasal passage and causes infections of the urinary tract, respiratory tract and intestinal tract (Loir, *et al*, 2003).The pathogenicity of the coagulase positive *S.aureus* as indicated by Jarvis (2001), includes manifestations such as, abscesses, boils, conjunctivitis especially in newborns, cross-infections in hospitals, septicemia, mastitis and food poisoning (of meat, milk and milk products).

About 5% of food borne staphylococcal outbreaks is caused by unidentified Staphylococcal Enterotoxins. (Letertre 2003; Omoe 2003). As little as 1.0 µg toxin in contaminated food produces symptoms of illness. This level of the toxin has been found at 8 x 10^7 cells /ml of food; this toxin cannot be denatured after boiling and storage and may be present in foods where viable cells of *S. aureus* are absent (Presscott *et al.,* 2002).

Controlling this pathogen is very difficult because it expresses certain virulence factors as it contains a thick peptidoglycan layer and teichoic acid, gives the bacterium structure, rigidity and promotes colonization of host tissue respectively, invasins like Leukocidin, cause the destruction of Leukocytes (WBC) and promote bacterial spread in tissue and cause pus formation; surface factors (Capsule, Protein A) inhibit phagocytic engulfment. Carotenoids; catalase enhances their survival in phagocytes; membrane damaging toxins (haemolysin, leukocidin, leukotoxin) lyse the eukaryotic cell membrane; exotoxins (Staphylococcal enterotoxins, Toxic Shock Syndrome toxin (TSST), and exfoloative Toxin (ET)) damage the host tissue and cause symptoms of disease. Coagulase, an enzyme that clots plasma and coats the Staphylococcal cells, prevents the cells from being phagocytosed and destroyed by macrophages. Due to these virulent determinants, it is tenacious, potentially destructive and shows increasing resistance to antimicrobial agents. *S. aureus* can be identified on the bases of various conventional, physiological or biochemical characters. The key characters for *S. aureus* are growth on BPA medium, free coagulase, clumping factor and acid production from mannitol (Murray *et al.,* 2003). These methods are not adequate enough for the characterization of bacterial species (Fox *et al.,* 1998) as they do not correspond to the actual genetic relativity of the species. In addition to this *S. aureus* can also be identified by PCR methods targeting to various gene like tst (encoding toxic shock syndrome protein), eta and etb (encoding exfoliative toxins A and B respectively), staphylococcal enterotoxin genes such as sea, sec, sei etc (encoding thermostable nuclease) and the Sa442 DNA fragment (Pinto *et al.,* 2005; Becker *et al.,* 2003). This

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sequence information is superior broadly in two ways, firstly it is reliable and precisely interpreted, and secondly it is more informative of the evolutionary relationship. This work was performed to confirm the pathogenic, coagulase positive *S. aureus* by the biochemical as well as the molecular approach by employing the sequencing of Sa442 DNA fragment. The sensitivity of *S. aureus* against antibiotics and natural products was also studied.

Materials and Methods

A standard strain of *S.aureus* (MTCC 3381) was obtained from the institute of Microbial Technology (IMTECH) Chandigarh, India. All the isolates were confirmed through biochemical tests.

Samples of cottage cheese were collected aseptically in sterilized plastic bags from different market areas of Agra city and directly transported to the laboratory under cold conditions and were analyzed within 4 hrs. The Isolation and biochemical characterization procedure was the same as employed previously by Singh and Prakash, (Singh and Prakash 2008).

Extraction of DNA. Extraction of the template DNA was done as per the method of Tsai and Olson (1991) with suitable modifications. 100 µl of 24 hrs pure culture was centrifuged at 4000 rpm for 12 minutes. The recovered pellet was suspended in 100 µl of sterilized DNase and RNase free water, heated in a boiling water bath for 10 mins and then snaps chilled in crushed ice. The obtained lysate was used as the DNA tamplate. 50 µl Master mixture was prepared at a final concentration of 1X (10 X PCR buffer), 0.2mM (2mM dNTP mix), 2mM (25mM MgCl2), 0.4 mM (10µM each primer), 1.25U Taq DNA Polymerase, 4µl template DNA and MiliQ water. Amplification was done by using primer set Sa442-1(5'-AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG-3') and Sa442-2 (5'- CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA-3'). The PCR mixture was subjected to thermal cycler (3 min at 96^0C and 40 cycles of 1s at 95^0C for denaturation step and 30 s at 55^0C for annealing-extension step). 8µl of the reaction products were resolved by electrophoresis in a 1.5% agarose gel containing 0.5µg/ml of ethidium bromide in .5X Tris-borate-EDTA buffer at 100 V for 70 minutes. A 100 bp DNA ladder (Bangalore genei) was included. The gel was visualized and photographed over the UV transilluminator (Zenith gel documentation system) and analyzed by gel doc software named UN-SCAN-IT gel 6.1.

Inhibition by antibiotics and natural products. Disk diffusion method was used to check the inhibition of the confirmed isolates of *S. aureus*, by the use of various antibiotics including, Gentamycin, Oxytetracyclin, Ampicillin, Vancomycin, Penicillin, Amoxicillin, Streptomycin, and Chloroamphenicol. Inhibition under the influence of natural products including *Trechyspermum copticum (Ajwain), Gycyrrhiza glabar (Mulethi), Chebulic myrobalan* (Hime), *Piper chaba* (Choti pepper), *Mangifera indica* (Mango seed), *Ficus religiosa* (Peepal leaves), *Syzygium cumini* (Jamun seed) and *Citrus sinensis* (Mousami) was studied by agar well diffusion method. Extracts of natural products were prepared by soaking the product in ethanol and water in a ratio of 1:4 and 1:8 respectively, for 72 hrs, in sterile conical flasks at room temperature with uniform shaking. The extracts were then filtered and concentrated by evaporating to dryness at 45^0C (Mahmood *et al.*, 2008).

Results and discussion

A total of 100 isolates were subjected to Gram's reaction. Isolates that yielded gram positive cocci in grapes like bunch, were then subjected to biochemical tests. Isolates showing catalase positive, oxidase negative, indole negative, nitrate positive, methyl red and voges-proskauer positive reactions and mannitol fermentation were confirmed as belonging to Genus *Staphylococcus* and further subjected to coagulase test for the confirmation of the species *aureus* (Fig. 1)
as coagulase test is the standard criteria for the identification of S. aureus (Burriel 1998).

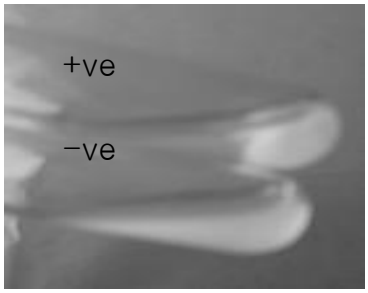
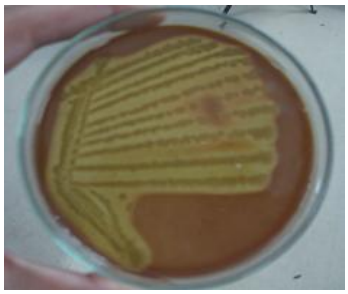
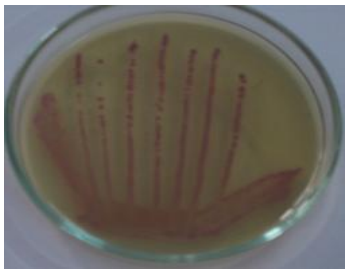


Fig 1. Coagulase test

15% isolates confirmed as *S. aureus* in cottage cheese. Confirmed isolates were further analyzed for pathogenicity by haemolysis on 5% Sheep Blood Agar (Fig. 2a) and characteristic pink coloured colonies with white halo on chromogenic medium CHROMagar for *S. aureus* (Fig. 2b).



(a)



(b)

Fig 2. Pathogenicity test (a) Haemolysis test (b) Growth on chromogenic medium

9 isolates were confirmed to be pathogenic. Pathogenicity of *S.aureus* is due to the membrane active substances i.e. cytolytic toxins, consisting of four haemolysins and a leucocidin. This genus may have alpha, beta, gamma and delta haemolysin (Presscott *et al.,* 2002). The pathogenic members of species *aureus* display beta haemolysis. Two pathogenic isolates from cottage cheese have been randomly selected for further analysis.

DNA extracted from the standard and the isolates was amplified using species specific primers. 108 bp amplified products were confirmed on Agarose gel (Fig. 3). Amplified products were submitted to the Institute of Molecular Medicine, New Delhi for sequencing. Obtained sequences were aligned through National centre for biotechnology Information (NCBI) database by using the Basic Local Alignment Tool (BLAST, 2.0 search program) and were confirmed as *S. aureus*.

Influence of antibiotics and natural products was studied on the confirmed isolates, along with standard strain.

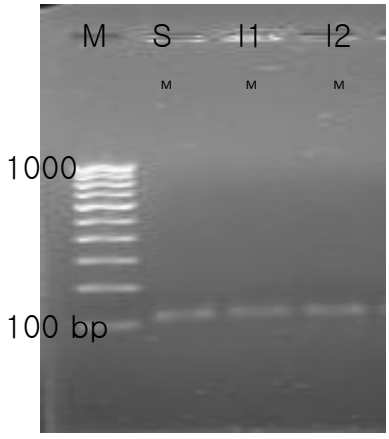
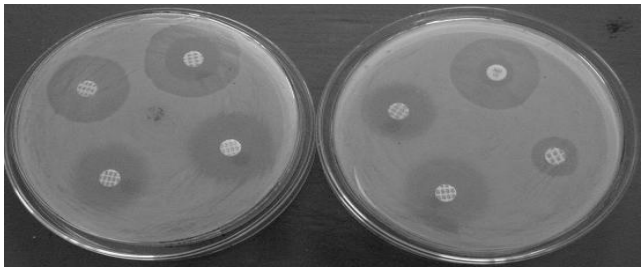
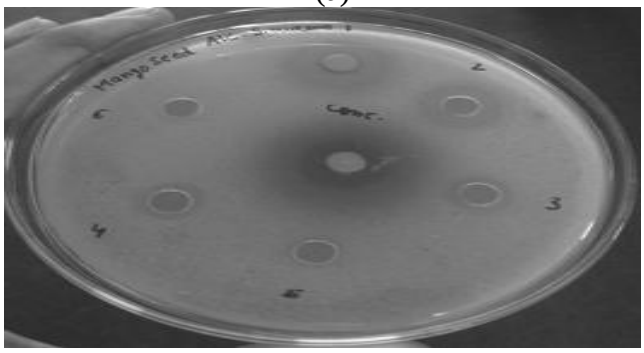


Fig 3. PCR amplified products on agarose gel. M=Marker, S=MTCC 3381, I1=Isolate 1, I2=Isolate 2

Amoxicillin and Penicillin exhibit significant zones of inhibition against standard *S. aureus* (MTCC 3381) and the isolates, while Oxytetracyclin, Chloroamphenicol and Gentamycin show comparatively smaller zones of inhibition. Though recorded to be resistant to Penicillin (Guler *et al.,* 2005) *S. aureus* yielded a zone of inhibition with Penicillin in the present study. This may be as a result of adaptation to the ever-changing environment. *S. aureus* shows a small zone of inhibition (11-19 mm) in the presence of Vancomycin. The isolates of *S. aureus* from cottage cheese exhibit a significant zone of inhibition with Streptomycin. The standard *S. aureus* (MTCC 3381) yielded a smaller inhibitory zone (Fig. 4a, Table 1).



(a)



(b)

Fig 4. (a) Plates showing zone of inhibition with different antibiotic (b) Plate showing MIC

Table 1. Zone of inhibition observed with various Antibiotics

Samples	Zone of Inhibition (in mm)							
	Gentamycin	Oxytetracyclin	Ampicillin	Vancomycin	Penicillin	Amoxicillin	Streptomycin	Chloroamphenicol
MTCC 3381	25	20	37	11	30	32	11	21
SP15d	25	20	30	15	26	27	26	27
SP10b	26	20	21	18	35	23	26	27

There are many reports available that prove antiviral, antibacterial, antifungal, antihelmintic, antimolluscal and anti-inflamatory properties of plants (Stepanovic *et al.,* 2008; Behera and Misra 2005). In the present work for determining

the MIC of the natural products against *S. aureus* the agar Agar, MHA) (Fig. 4b). The results show that all the natural products except mausami leaves yield a zone of inhibition against *S.aureus*. However, it was observed that hime was the most effective in inhibiting *S. aureus* while peepal leaves were the least effective in inhibiting both standard and the *S. aureus* isolate. Aqueous form of mulathi was also found to be effective against the standard *S. aureus* to a greater extent than the alcoholic form. Aqueous extract of ajwain was also found to be effective, but to a much lesser extent, than the alcoholic extract of ajwain which depicted a very high MIC ($2x10^{-5}$ µg/ml) as observed for the standard *S. aureus* (MTCC 3381). The *S.aureus* isolates show that ajwain extracts are not able to inhibit them effectively as the MIC required is $2x10^{5}$ µg/ml. Results showed that alcoholic extracts of natural products showed higher inhibition than aqueous extracts (Fig. 5).

This variation in the activity may be due to the better extraction of biologically active compounds (Alkaloids, flavonoides, essential oils, tennins etc.) which were enhanced in the presence of ethanol Ghosh *et al.,* 2008 Falodun et al., 2006).

Extracts of all the natural products were effective against the isolates but inhibited the isolates to a lesser extent as compared to the standard S. aureus (MTCC 3381). The genetic make up of the isolates may be different from the standard strain of S. aureus, probably the former being more virulent. From the present study it is obvious that *S. aureus* well diffusion method was employed (on Muller Hinton could be effectively treated by mulathi, hime and mango seed extracts, as these extracts were very effective in inhibiting at low concentrations (lower MIC value).

The effect of all the extracted natural products was compared with the standard antibiotics by taking ~ 800 µg/well of natural product extract and 25µg/disk of the antibiotic. It was observed that the antibiotics were more effective in inhibiting *E. coli*, *S. aureus* and *L. monocytogenes* as compared to the natural products. This may be accorded to the purity of active molecules contained in the antibiotics (Presscott *et al*., 2002). However, the present work suggests that if the active molecules are isolated from the crude extracts of the natural products they may prove to be more effective than the well known antibiotics currently in use for the control of these pathogens and can be easily incorporated in one's diet and exert no side effect as compared to antibiotics.

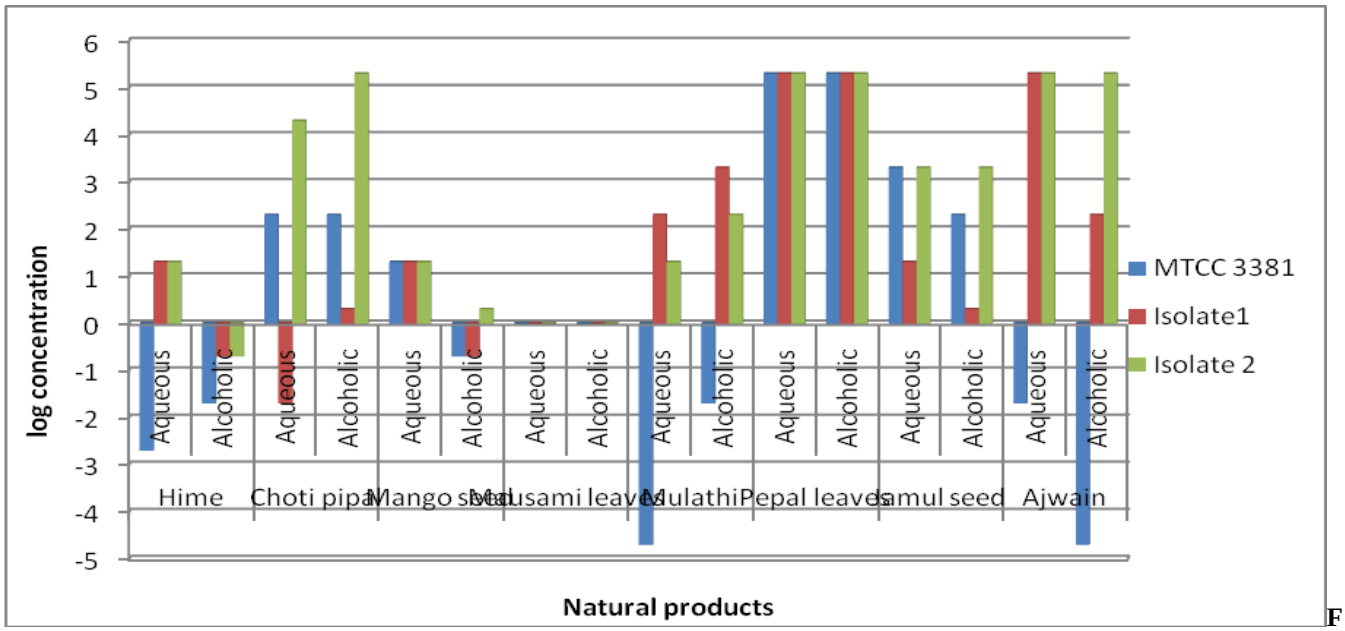


Fig 5. Minimum Inhibitory Concentration of all the natural products against S. aureus

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