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Research Article

SCREENING FOR STAPHYLOKINASE PRODUCING *STAPHYLOCOCCUS SPP.* FROM DIFFERENT ENVIRONMENTAL SAMPLES

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ABSTRACT

Staphylokinase (SAK) is a bacterial kinase produced by certain strains of *Staphylococcus spp*. It is a 15.5 kDa, consisting of 136 amino acids single chain protein, which activates plasminogen to form plasmin and digest fibrin clots. Staphylokinase (SAK) is a promising thrombolytic agent for the treatment of myocardial infarction. The present research work was carried out to screen the staphylokinase producing *Staphylococcus spp.* from different samples collected (milk, water and sewage) in and around Vellore district, Tamil Nadu, India. Out of 12 isolates, 4 organisms were producing staphylokinase effectively. The main focus of this work is to isolate and characterise potent producers of staphylokinase producing *Staphylococcus spp.* from various environmental samples involving non-pathogenic *Staphylococcus spp*. while the earlier isolation was done using pathogenic samples.

Keywords: Staphylokinase, *Staphylococcus spp.*, thrombosis, fibrinolytic.

INTRODUCTION

Thrombosis, the blockage of blood vessels with clots, can lead to acute myocardial infarction and ischemic stroke both are leading causes of death. Other than surgical interventions to remove or by pass the blockage, or the generation of collateral vessels to provide a new blood supply, the only treatment available is the administration of thrombolytic agents to dissolve the blood clot. Staphylokinase (SAK) is a 136-amino acid enzyme from *Staphylococcus spp*. (Bokarewa *et al*., 2006). It is positively regulated by the "agr" gene regulator. It activates plasminogen to form plasmin, which digest fibrin clots. This disrupts the fibrin meshwork which can often form to keep a localized infection (Steven *et al*., 2008). Staphylokinase lacks fibrin-binding and thrombin inhibitor activities, two functions which would supplement and potentially improve its thrombolytic potency (Mandi *et al*., 2009). Staphylokinase, when produced from non-pathogenic samples reduces the chances of cross contamination by any other microbe present in the sample, makes the production process safe, reduces downstream processing steps and even reduces the cost of purification, in turn making the production cheap and easy. It was also found that Staphylokinase isolated from milk and water sources, having ability to induce secretion of defensins, to complex bind them and to neutralize their bactericidal effect hence its production may therefore be responsible *in vivo* for defensin resistance during *Staphylococcus aureus* infections (Jin *et al.,* 2004*).* It has been generated as clot buster protein which shows strong promise as a 'clot-specific' thrombolytic drug (Liv *et al*., 2002).

Materials and methods

Collection of samples

Different samples of milk, water and sewage were collected from different regions of Vellore, Tamil Nadu, India (Table 1).

Table 1: Samples collected for screening of staphylokinase producing *Staphylococcus spp.*

Samples	Number of samples collected
Water	3
Sewage	2
Milk	27
Total	32

Isolation and characterization of *Staphylococcus spp.*

Blood agar

Samples were serially diluted upto 10^{-7} dilution and 0.1 ml of sample was inoculated on the blood agar plate (BAP) by using spread plate method (Fig.1).The plates were kept for 48 hours incubation at 37°C (Russell *et al*.,2006).

Mannitol salt agar

The microorganisms were checked for their ability to ferment mannitol sugar. The cultures grown on the blood agar plates were streaked on Mannitol Salt Agar (MSA). It is a selective and differential media. MSA also has nutrients appropriate for the growth of *Staphylococcus spp.* and 7.5% salt, which will enhance the growth of *Staphylococcus spp.* Plates were kept for overnight incubation at 37°C (Kateete *et al*., 2010). The bacterial cultures from the mannitol salt agar were taken and streaked on the nutrient agar plates and pure cultures were maintained.

Biochemical Characterisation

Gram's staining, Coagulase test, Catalase test and IMViC test were performed to characterize the *Staphylococcus spp.*

Screening for staphylokinase producing Staphylococcus spp.

Casein hydrolytic assay

Skimmed milk agar supplemented with 2 ml of human serum was prepared. The isolated *Staphylococcus spp.* was inoculated on the skim milk agar medium. The plates were kept for overnight incubation at 37°C (Rajamohan *et al*., 2000).

Heated plasma agar plate assay

The assay was performed to detect the thrombolytic activity of the strain. 15 ml of nutrient agar was prepared and melted at 100°C. 5 ml of heated human plasma was added (plasma to be heated at 56°C for 20 minutes). It was mixed properly. Nutrient agar medium was poured into the petriplates and kept for solidification. After solidification, the isolated strain were inoculated and kept for incubation at 37°C for 24 hours (Pulicherla *et al*., 2011).

Results and discussion

Blood agar plate

After the incubation colonies developed on the blood agar plates exhibited α, β and γ hemolysis (Fig.1). Out of 31 samples 11 showed β-hemolysis, 5 showed α-hemolysis and 4 showed γ-hemolysis (Table2).

Fig.1 : Hemolytic pattern of *Staphylococcus spp.*

Table2: Hemolytic pattern of the isolates from different samples

Sample	B-hemolytic isolates	α-hemolytic isolates	γ-hemolytic isolates
Tap water	1	1	1
Sewage water	2	0	0
Milk	9	4	3

Mannitol Salt Agar

The colonies which were able to grow on MSA, confirmed the presence of *Staphylococcus spp.* On the mannitol salt agar plates some of the *Staphylococcus spp.* showed complete fermentation of mannitol, as the color of the media was changed from orange to yellow. Some produced a pink coloured pigment (Fig.2). The golden yellow colored colonies obtained on the MSA were supposed to be *Staphylococcus aureus* whereas other color colonies were expected to be other species. On the basis of the hemolytic ability of the *Staphylococcus spp.* mannitol fermentation is tabulated (Table 3,4,5).

Fig.2 : Colonies on Mannitol Salt Agar

Table 3: Morphological characterisation of β-hemolytic isolates

Sample name	Sample source	Colony morphology	Mannitol fermentation
SPK 1	Water outside VIT	Round, slimy, yellow	+ve
SPK 2	Sewage water 1	Greyish, spreaded colonies.	+ve
SPK 3	Sewage water 2	Yellowish white, rounded	+ve
SPK 4	Milk 2	Yellow, rounded colonies	+ve
SPK 5	Milk 4	Yellow, rounded colonies	+ve
SPK 6	Milk 7	White, rounded colonies	+ve
SPK 7	Milk 8	Yellow, rounded colonies	+ve
SPK 8	Milk 9	Yellow, rounded colonies	+ve
SPK 9	Milk 12	White, rounded colonies	+ve
SPK 10	Milk 15	Yellow, rounded colonies	+ve
SPK 11	Milk 19	Yellow, rounded colonies	+ve
SPK 12	Milk 26	Greyish, slimy colonies	+ve

Table 4: Morphological characterisation of α-hemolytic isolates

Sample name	Sample source	Colony morphology	Mannitol fermentation
SSP 1	Water 1	white, rounded colonies	+ve
SSP 2	Milk 3	Slimy, greyish	Pink pigment
SSP 3	Milk 14	Slimy, greyish	Pink pigment
SSP 4	Milk 16	Slimy, white	-ve
SSP 5	Milk 20	Slimy, greyish	Pink colonies

Table 5: Morphological characterisation of γ-hemolytic isolates

Sample name	Sample source	Colony morphology	Mannitol fermentation
SAP 1	Water 2	White, raised colonies	-ve
SAP 2	Milk 1	White, raised colonies	-ve
SAP 3	Milk 5	White, raised colonies	-ve
SAP 4	Milk 23	White, raised colonies	-ve

Nutrient agar:

Fig.3 : Colonies on Nutrient Agar

Grams staining:

Out of 21 isolates, purple colored bunches of cocci are observed in 12 isolates. It indicates the presence of gram positive cocci, it may be *Staphylococcus spp.* (Fig.4).

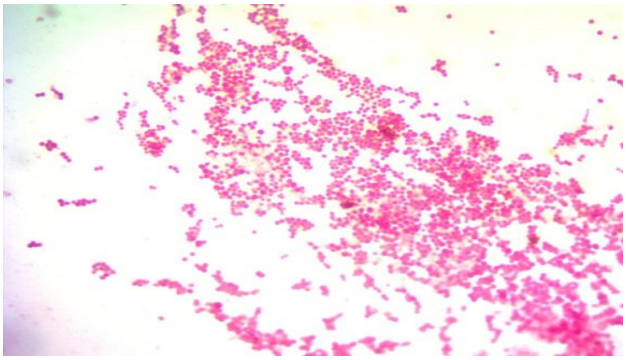
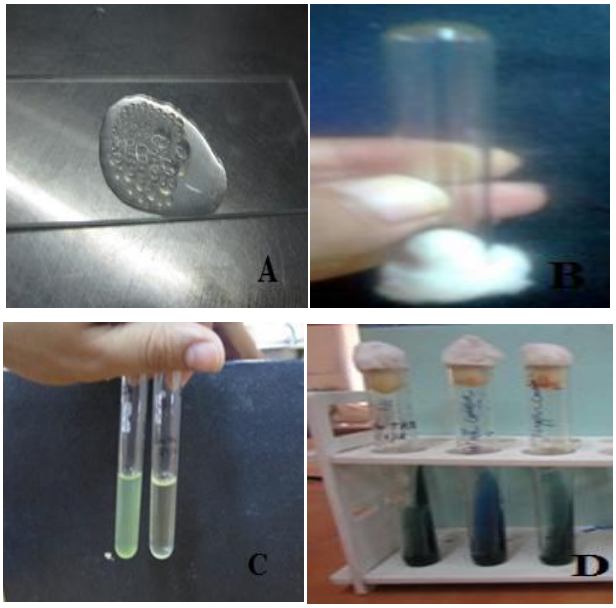


Fig.4 : Gram positive cocci

Biochemical characterisation

Biochemical tests were carried out for 12 gram positive cocci isolated from 32 different samples. 6 coagulase positive *Staphylococcus spp.* and 6 coagulase negative *Staphylococcus spp.*were obtained. All the 12 isolates showed positive results for catalase MR, VP tests and negative results for indole as well as citrate utilisation test (Table 6). Based on biochemical test the isolates were identified, it may be *Staphylococcus spp.*



A. Catalase test B. Coagulase test
C. Indole test D. Citrate utilisation test

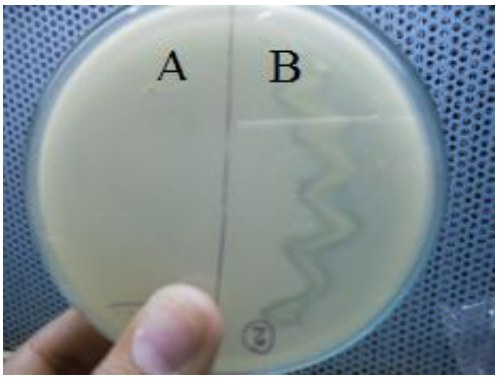
Fig.5 : Biochemical test

Table 6 : Biochemical characteristics of different isolates

Sample name	Sample source	Gram's test	Catalase test	Indole test	Citrate utilization	MR test	VP test	Coagulase test
SPK 1	Water outside VIT	+ve	+ve	-ve	-ve	+ve	+ve	+ve
SAP 1	Water 2	+ve	+ve	-ve	-ve	+ve	+ve	-ve
SPK 2	Sewage water 1	+ve	+ve	-ve	-ve	+ve	+ve	-ve
SPK 3	Sewage water 2	+ve	+ve	-ve	-ve	+ve	+ve	-ve
SPK 4	Milk 2	+ve	+ve	-ve	-ve	+ve	+ve	+ve
SSP 2	Milk 3	+ve	+ve	-ve	-ve	+ve	+ve	-ve
SPK 5	Milk 4	+ve	+ve	-ve	-ve	+ve	+ve	+ve
SPK 6	Milk 7	+ve	+ve	-ve	-ve	+ve	+ve	-ve
SPK 7	Milk 8	+ve	+ve	-ve	-ve	+ve	+ve	-ve
SPK 8	Milk 9	+ve	+ve	-ve	-ve	+ve	+ve	+ve
SPK 9	Milk 12	+ve	+ve	-ve	-ve	+ve	+ve	+ve
SPK 11	Milk 19	+ve	+ve	-ve	-ve	+ve	+ve	+ve

Screening for staphylokinase producing *Staphylococcus spp.*

Clear hollow zone was observed around the colonies. Out of the 12 isolates, 7 exhibited clear lysis around the colonies (Fig.6). It indicates the production of staphylokinase.



A: No clear zone B: Clear zone

Fig.6: Casein hydrolytic assay

Heated plasma agar plate assay**:**

Out of the 7 colonies obtained from skimmed milk agar plates, 4 showed clear fibrinolytic halos around the colonies, indicates that only 4 *Staphylococcus spp.* were producing staphylokinase.

The current research work of isolation and screening of staphylokinase producing *Staphylococcus spp.* involves the traditional methods which are simple, rapid and can be easily carried out, which help in making the production process of staphylokinase cost effective. As here environmental friendly samples are taken, which are non-pathogenic in nature, hence the chances of infection to the concerned person working with it are lowered to large extent. Moreover, the present study deals with the isolation of staphylokinase producing staphyloccus spp. from different environmental sources. This is in accordance with the earlier observation which shows that the isolates from a local wound formed due to lysogenic *Staphylococcus aureus* are used for economic production of Staphylokinase. (Pulicherla *et al., 2011*) Our work provide low cost, easier and comparatively safer method for production of staphylokinase as we used non-pathogenic environmental samples where as in all other previous research pathogenic samples were practised which increases the chances of infection and requires safer handling techniques which also leads to increase in processing cost. Staphylokinase has attracted attentions of researchers to use it as a therapeutic thrombolytic agent through special mechanism, and also cause less allergic reaction in comparison with other thrombolytic agents. (Narjes *et al., 2011*) Also it is observed that in phagocytosis assays staphylokiase proved to be a very efficient mechanism to reduce the opsonic activity of human serum IgG. The fact that SAK activates human PLG at the bacterial surface and removes IgG as well as C3b makes this protein a unique anti-opsonic molecule. (Rooijakkers *et al.*, 2011) SAK is reported to have a serine protease domain with no proteolytic activity unlike

other plasminogen activators like tissue plasminogen activator (t-PA) and urokinase. (Bhaskarjyoti *et al.,* 2009)

Conclusion:

Thrombolytic therapy has become a conventional treatment for myocardial infarction (AMI), but currently clinically prescribed thrombolytic drugs have such problems as delayed action and other side effects like bleeding, reocclusion etc. Economically the isolation, screening and production of staphylokinase are reliable. In the present study 32 different environmental samples were screened for staphylokinase producing *Staphylococcus spp.* Finally 4 *Staphylococci* isolates showed staphylokinase activity. Future prospective of the current study includes sequencing the staphylokinase producing gene, strain improvement of the *Staphylococcus spp.* and recombinant production of staphylokinase.

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