Identification of Antithrombosis, Fibrinolytic and clot lysis activity of Staphylococcus aureus in in vitro

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Abstract
Thrombotic diseases are responsible for alarming rates of mortality and morbidity worldwide. The clinical intervention to cure these disorders is carried out by the administration of thrombolytic agents. Among them staphylokinase is having relatively good clot specificity than tissue plasminogen activator. In the present study two strains of clinically isolated S. aureus were investigated for the fibrinolytic and thrombolytic properties. Antithrombotic assay results of strains JS7 and JS17 reveals that by prolonged clotting time, the formation of blood clot still occurred even with administration of high amount of the sample. These strains show the fibrinolytic activity and clot lytic activity. A zone of 6.4 mm and 6.5 mm diameter was measured in fibrin plate, which shows its fibrinolytic activity. Artificial blood clot in capillary tube was digested in both the petri plates containing JS7 and JS17.

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Key words: Staphylokinase, Thrombolysis, fibrinolysis, clot lysis, in vitro study, antithrombotic effect, staphylokinase from clinical S. aureus.

Introduction:
Acute myocardial infarction (also known as heart attack) is a leading cause of death in the world. It is commonly caused by the formation of a pathologic clot (thrombus) at a critical position that results in obstructing the blood flow to heart tissues. Fibrin is a protein that forms in the blood clots by the conversion of fibrinogen to fibrin via the proteolytic action of thrombin (1), and subsequently the formation of insoluble fibrin clots. Fibrin clot formation and fibrinolysis are normally well balanced in biological systems (2). However, when fibrin is not hydrolysed due to some disorder, thromboses can occur. Myocardial infarction is the most common of these thromboses.

The use of blood clot dissolving agents is one of the well established methods in treating patients with acute myocardial infarction (3, 4). Currently, thrombolytic/fibrinolytic agents approved under clinical investigation on patients with acute myocardial infarction include streptokinase (SK), recombinant tissue-type plasminogen activator, two-chain urokinase-type plasminogen activator, recombinant single chain u-PA and recombinant staphylokinase (5).

Staphylokinase (SAK), a 136 amino acid protein produced by lysogenic strains of S. aureus. SAK is an extracellular profibrinolytic bacterial protein and a promising thrombolytic agent that converts plasminogen to plasmin by forming an inactive 1:1 stoichiometric complex with plasminogen (6, 7).Staphylokinase mediates the lysis of platelet rich and retarded clots efficiently and shows exceptional fibrin specificity (6, 9). These properties can help minimize reocclusion and bleeding complications (10, 11, 12, 13).The present study, aims at the screening of clinically isolated strains of Staphylococcus aureus for their thrombolytic and fibrinolytic activity.

Materials and Methods:
A total of 37 Staphylococcus aureus strains were isolated from clinical samples and characterization was done by conventional methods. Among them, 28 strains of S. aureus exhibitingstaphylokinase activity based on Heated Plasma Agar assay and Casein Hydrolysis assay method were shortlisted as previously described Shagufta Naseer B, 2014 (14). Among them two strains of S. aureus have been selected for the present study, based on...
the hydrolysis of casein. These two strains labelled as JS7 and JS17, capable of producing staphylokinase. They were subjected to studies on thrombolytic and fibrinolytic activity in vitro.

In Vitro Antithrombosis Assay

Antithrombosis assay was performed according to the Jessica Trisina 2011 (15) with few modifications. The whole blood samples were taken from healthy human beings. The strains of S. aureus JS7 and JS17 were used as sample. The three different amounts of the sample ie., 50 µL, 100 µL, 200 µL were finally makeover to 250 µL by adding normal saline. To the blank, 250 µL of 20 mM potassium phosphate was added. The 250 µL of sample was mixed with 4 µL thrombin (0.92 IU) and 250 µL fresh blood on a ceramic tile. Mixture was then incubated at 37°C and coagulation of blood was observed till 1 h and the consistency of the blood clot were noted.

Fibrinolytic Properties:

Fibrin plate method was used to determine the fibrinolytic activity by plasminogen free fibrin plate method (Astrup and Mullertz, 1952; Asept A. Prihanto 2013) (16, 17). The composition of media is fibrinogen solution (2.5 ml of 1.2% (w/v) human fibrinogen in 0.1 M sodium phosphate buffer, pH 7.4), 10 U of thrombin solution, and 1% agarose. To eliminate other fibrinolytic factors, fibrin plates were heated at 80°C for 30 min. Disk of 6 mm diameter was prepared by Whatman Filter paper number 1. These discs were placed on the fibrin plate. To observe the fibrinolytic activity,75 µL of the sample (JS7 and JS17) was carefully dropped on the disc and incubated at 35°C for 24 h. Streptokinase(30,000 IU), phosphate buffer (20 mM) were used as positive control and blank. The activity of the fibrinolytic enzyme was determined by measuring the clear zone diameter.

Table1: Antithrombotic effect of JS7 and JS17 strains of S. aureus. NC: No Clotting (no clotting occurred); FC: Fully Clotted (high clotting consistency); PC: Partially Clotted (medium clotting consistency); SC: Slightly Clotted (low clotting consistency).

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<tr>
<th>Time (min)</th>
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<th>50 µL</th>
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In vitro degradation of blood clot by capillary tube method:

The samples exhibited activity to degrade blood clot. Lysis of blood clot was observed in the petri dish containing the JS7 and JS17. Clot was thoroughly lysed (complete) in the petri dish containing streptokinase and was used as positive control. There was no clot dissolution in the negative control petri dish containing distilled water.

Fibrinolytic Properties:

There was a clear zone measuring about 6.9 mm diameter and 6.4 mm diameter around the disc loaded with JS7 and JS17. Whereas in the positive control (disc having streptokinase) a zone measuring about 6.9 mm diameter was observed. There was no zone formation in the blank.

Discussion:

Thrombosis is a major life-threatening disease and effective thrombolytic agents are of clinical value for emergency treatment of such major diseases as acute myocardial infarction, cerebral infarction, or venous thromboembolism (19). SAK is a good clot buster than other chemically available chemical anticlotting agents (20, 21, 22). The thrombolytic properties of staphylokinase were indirectly investigated in the present study. The present study was performed to investigate the fibrinolytic,
antithrombotic and thrombolytic activities of two clinically isolated \textit{S. aureus} strains namely JS7 and JS17.

Antithrombotic properties of JS7 and JS17 were determined by in vitro antithrombosis assay method. The in vitro coagulation model was studied using human blood induced by thrombin which formed blood clots within 2-hour observation. Antithrombotic activity has been exhibited by the selected strains. Our results of antithrombotic effects are compatible with that of Jessica Trisina et al., 2011 (15). As there is increment in blood clotting time, the antithrombotic activity is decreasing. Thrombus formation is influenced by the presence of thrombin which promotes coagulation pathway activation (23). By prolonged clotting time, the formation of blood clot still occurred even with administration of higher amount of the sample (JS7 and JS17). The blood clot consistency was clearly observed and it has been noted that dose of sample and clot reduction (clot dissolution) is inversely proportional, that is consistency of blood clot significantly reduces in a dose-dependent manner.

The fibrinolytic activity of \textit{S. aureus} JS7 and JS17 was measured by fibrin plate method (16). Both the strains exhibited fibrinolytic activity (6.4 to 6.5 mm zone). The reports of Asep A. Prihanto 2013 (17) for fibrinolytic activity of halophilic lactic acid bacteria from fermented food shows a zone measuring of 6.8 mm in diameter. In comparison, the JS7 shows almost similar fibrinolytic activity as reported by Asep A. Prihanto(2013).

The clot lysis activity has been performed in capillary tube as per the method described by Zaichang Yang (2012). The results of JS7 and JS17 shows that it can digest the artificial blood clot and compare with Streptokinase (commercial sample).

**Conclusion:**

Over the years, thrombolytic therapies via injecting or orally administrating thrombolytic agents to lyse thrombi in blood vessels have been extensively investigated. Tissue plasminogen activator is most commonly used thrombolytic drug for thrombosis in western countries. Although these are still widely used in thrombolytic therapy today, their expensive prices and undesirable side effects, such as risk for internal haemorrhage within the intestinal tract when orally administered, prompt researchers to search for cheaper and safer sources. Researchers suggested that staphylokinase is a promising blood clot dissolving agent is having good clot specificity than that of tissue plasminogen activator.

In the present study two clinically isolated \textit{S. aureus} strains JS7 and JS17 were investigated for the fibrinolytic and thrombolytic activity. Antithrombotic activity shows that the consistency of blood clot significantly reduces as dose of sample increases. Fibrinolytic property of JS7 and JS17 strains were observed in fibrin plates. Streptokinase was used as a positive control in fibrin plate and clot lysis assay. Artificial blood clot in capillary tube was digested in the petri plates containing JS7 and JS17. JS7 shows slightly increased fibrinolytic activity and anti thrombotic activity when compared with JS17. Thus the \textit{S. aureus} strains exhibited the thrombolytic and fibrinolytic activity, due to their ability to produce staphylokinase. Further studies are needed to evaluate the Staphylokinase extracted from the isolates.

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