

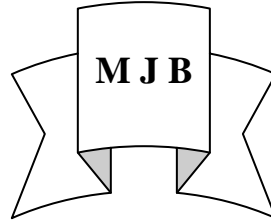
Molecular Study of Sortase Enzyme and Characterization of Some Virulence Factors in *Streptococcus pyogenes*

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Abstract

This study included 90 throat swabs collected from patients suffering from acute pharyngitis who attending to AL-Hilla General Teaching Hospital (ENT unit) during the period from October 2010 to January 2011. The age of the patients ranged from (3 to 63) years.

Only six isolates of *Streptococcus pyogenes* were obtained. It was observed that only four isolates had the fibrinolytic activity through the production of streptokinase enzyme, while two isolates failed to lyse fibrin, also the present study showed that streptokinase activity became apparent after 3 hours of incubation and clot began to decrease after 10-16 hours and disappeared completely after 24 hours of incubation.

Hyaluronidase activity was investigated, it was found that 50% (3) of GAS isolates give a positive result via the formation of a halo around the colony and this is an indicator of hyaluronidase activity.

Specific primers for PCR techniques were used for the detection of sortase enzymes (A, B and C). Sortase A was found existed in all GAS strains and this enzyme was designated as housekeeping enzyme. Sortase B and C was also detected, it was found that only two isolates were positive for both sortase B and C genes. However, only two isolates were found to have all these three enzymes whereas the others contained only the housekeeping one.

الخلاصة

من مجموع ٩٠ مسحة بلعومية قد تم الحصول عليها من مرضى تتراوح أعمارهم (٣-٦٣) سنة يعانون من التهاب البلعوم من المراجعين إلى مستشفى الحلة الجراحي (وحدة ENT) للفترة من أكتوبر ٢٠١٠ إلى آذار ٢٠١١، تم عزل ستة عزلات فقط من بكتريا المسببات القححية (*Streptococcus pyogenes*). ظهر أن أربعة عزلات من بكتريا المسببات القححية لها القابلية على تحليل الفايبرين من خلال أنتاجها لانزيم Streptokinase.

كما أظهرت بعض العزلات أن فعالية أنزيم Streptokinase تظهر واضحة بعد ثلاثة ساعات من الحضان وأن الخثرة (clot) تبدأ بالتقلص بعد ١٠-١٦ ساعة وتختفي تماما بعد ٢٤ ساعة. تم التحري عن فعالية أنزيم Hyaluronidase وقد وجد أن 50% من العزلات أعطت نتائج موجبة من خلال تكوين منطقة شفافة حول المستعمرة وهذا يعتبر مؤشر على وجود فعالية لهذا الأنزيم. تم استخدام بادئات نوعية (primers) في تقنية تفاعل البلمرة المتسلسل (PCR) للتحري عن الجينات الخاصة بأنزيمات Sortase (A, B, C) وقد أظهرت النتائج بامتلاك كافة العزلات لجين Sortase A في حين عزلتين فقط أظهرتا احتواءها لجين Sortase B و Sortase C. على أية حال فإن نتائج PCR أظهرت بأن عزلتين فقط تمتلك الجينات للأصناف الثلاثة من أنزيم Sortase.

Introduction

Group A streptococci (*Streptococcus pyogenes*) is one of the most important human pathogens causing pharyngitis. The variety of possible disease

outcomes of a GAS infection is believed to be due in part to the regulated expression of streptococcal virulence factors that are either present on the surface of the bacterium or secreted by the organism. One of the

secreted factors shown to be important for streptococcal virulence is streptokinase, encoded by the *ska* gene, *S. pyogenes* can hydrolyze fibrin clot through production of streptokinase. This enzyme play a role as mediator in causation of post streptococcal infection [1].

The another one is Hyaluronidase is an enzyme capable of degrading hyaluronic acid, a major component of the extracellular matrix of body tissues a special kind of polysaccharide found between cells as well as being the major sole component of the capsular material of certain bacteria [2]. Production of hyaluronidase by group A streptococci has been suggested to aid the organism in its spread through the connective tissue. Hence, hyaluronidase has been designated as one of the spreading factors of microbial origin. Although generally considered an extracellular product, it is known that intracellular forms of hyaluronidase which are encoded by phages integrated in the host chromosome may exist [3].

Many of Surface proteins are covalently linked by a sortase enzyme to the cell wall via a C-terminal Lipid x Triglyceride motif [4]. Sortase refers to a group of prokaryotic enzymes which form pili act as both proteases and transpeptidases [5].

Sortase is present in almost all gram-positive bacteria, anchors a range of important surface proteins to the cell wall. Sortase enzymes are cysteine transpeptidases that mediate the covalent attachment of substrate proteins to the cell walls of Gram-positive bacteria [6].

In *S. pyogenes*, sortase A is the housekeeping sortase and was shown experimentally to anchor M protein, protein F, C5a peptidase (ScpA), and protein G-related α 2-macroglobulin-binding protein (GRAB) to the cell wall [7]. In addition to sortase A, *S.*

pyogenes harbors two specialized sortases, associated with the FCT (fibronectin-binding, collagen-binding T antigen) region. Of these, one is specific for T antigen, a major component of the streptococcal pili which refer to sortase B [8], while the other one called sortase C, recognizes an altered consensus sequence, Lipid x Triglyceride and anchors a protein of unknown function [9].

Aim of Study

1. Isolate and identify of *S. pyogenes* from patient with pharyngitis.
2. Investigate on Hyaluronidase activity and Streptokinase activity in bacterial filtrate.
3. Detection of Sortase enzymes (A, B, C) in all bacterial isolates by using PCR markers.

Material and Methods

Patients

The sample were collected in Al-Hilla teaching hospital from patient suffering from pharyngitis, 90 patient samples taken, six of it is *Streptococcus pyogenes* positive.

Samples were collected from pharynx by disposable swab, *Strep pyogenes* detected by standard bacteriological methods.

Bacterial culture

The samples were cultured on blood agar base which sublimated with crystal violet 1/50000, and incubated at 37 °C over night, the identification of Gram positive bacteria were performed by clear zone of β hemolysis around the colonies due to lysis of red blood cells, and by Gram stain and standard biochemical method bacitracin positive, catalase negative, oxidase negative, according to Bergys manual for determinative bacteriology [10].

Detection of Streptokinase

Streptokinase was detected according to method mentioned by

Tillett [11].citrated human plasma was used and also CaCl₂, the clot disappeared after 24hr. of incubation [11].

Detection of Hyaluronidase:

Hyaluronidase was detected according to [13]. Hyaluronic acid was added as substrate .

DNA extraction from Gram positive bacteria

This method are made according to the genomic DNA purification kit supplemented by manufactured company (promega kit , USA).The extraction method was depended on lysis by alkali.

Detection of sortase enzyme types by PCR :-

PCR was conducted to determine the sortase types which found in isolates by targeting three genes strA ,strB and strC . Each 25µl of PCR reaction mixture for PCR contained 2.5µ l of upstream primer, 2.5 µl of downstream primer, 2.5µl of nuclease free water , 5µl of DNA extraction and 12.5 µl master mix .

Primers for sortase sequence (Srt A,B ,C) were obtained from (BioNeer company),as mentioned in table(1). Amplification was done by using PCR (cleaver, USA).

Thermal cycler conditions were as follows

The conditions of thermal cycle for each primer was investigated in table (1).

Table 1 Primers sequences and PCR condition to detect Sortase genes.

Genes	Primer sequence (5'-3')	Size of product bp	PCR condition	Reference
Srt A F	CTTAGGATCCGTCTTGCAAGCACAAATGG	500	94°C 3min 1x	paul <i>et al.</i> ,2009 [12]
Srt A R	ATGTTCTCGAGCTAGGTAGATACTTGGTTATAAGA		94°C 2min 65°C 1min 28x 72°C 1min	
			72°C 10min 1x	
Srt B F	GGTGTGGCAAAAGGCTAAGG	500	94°C 5min 1x	Timothy <i>et al.</i> ,2004 [13]
Srt B R	GCACACACTACTTCTGCCC		94°C 1min 55°C 1min 30x 72°C 1min	
			72°C 10min 1x	

Srt C F	GCATTTAAAACAGCTCAACAACAGCC		95°C 2.5min 1x	Timothy <i>et al.</i> ,2004 [13]
Srt CR	GCCTTTGTTGGTCTTGATTGG	370	94°C 30sec 55°C 1min 30x 72°C 30sec	
			72°C 7min 1x	
			94°C 30sec 55°C 30sec 30x 72°C 30sec	
			72°C 7min 1x	

The PCR amplification products were visualized by gel electrophoresis on 1% agarose gel for 40 min at 60 V., The size of the amplicons were determined by comparison to the 100 bp ladder (promega, USA).

Results

In this study, Among 90 throat swabs obtained from patients with age group ranged from (3 to 63) years, suffering from pharyngitis , who admitting to AL-Hilla General Teaching Hospital (ENT unit), for the period from October 2010 to January 2011. Only 6 (6.6 %) isolates of *Streptococcus pyogenes* (Group A streptococci) were isolated.

It was observed that only four isolates had the ability to show fibrinolytic activity through production of streptokinase enzyme,while two isolates failed to lyse fibrin ,although some studies had indicated that streptokinase was housekeeping enzyme which could be produced by all GAS isolates. In addition the present study was showed that streptokinase activity became appearent after 3 hours of incubation and clot began to decrease after 10-16 hours and disappeared completely after 24 hours of incubation.as shown in figure (1).

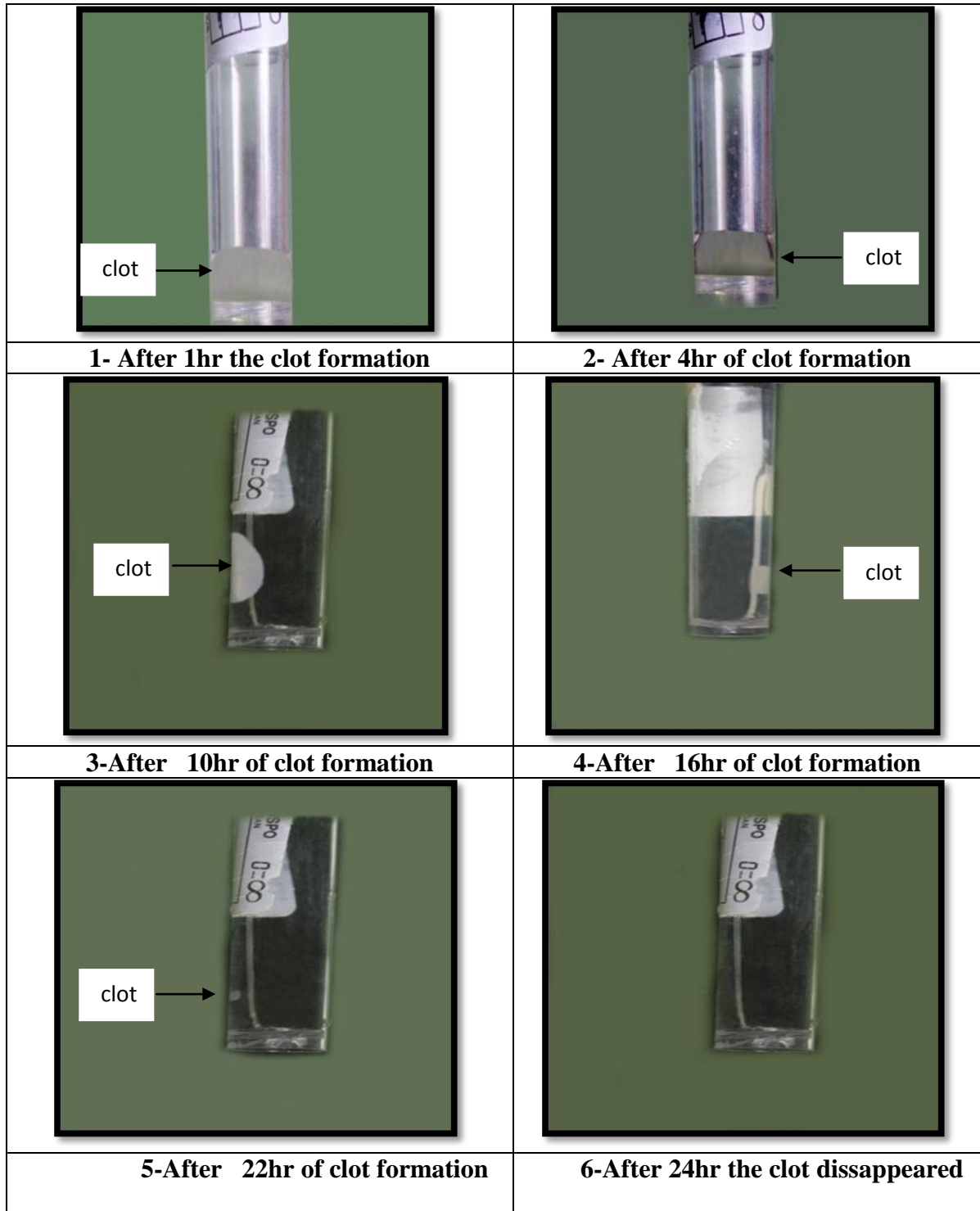


Figure1 fibrinolytic activity by production of streptokinase by *Streptococcus pyogenes*.

Hyaluronidase activity was investigated, it was found that 50% (3) of GAS isolates give positive result via formation of a halo around the

isolate and this is an indicator of hyaluronidase activity as shown in figure(2)

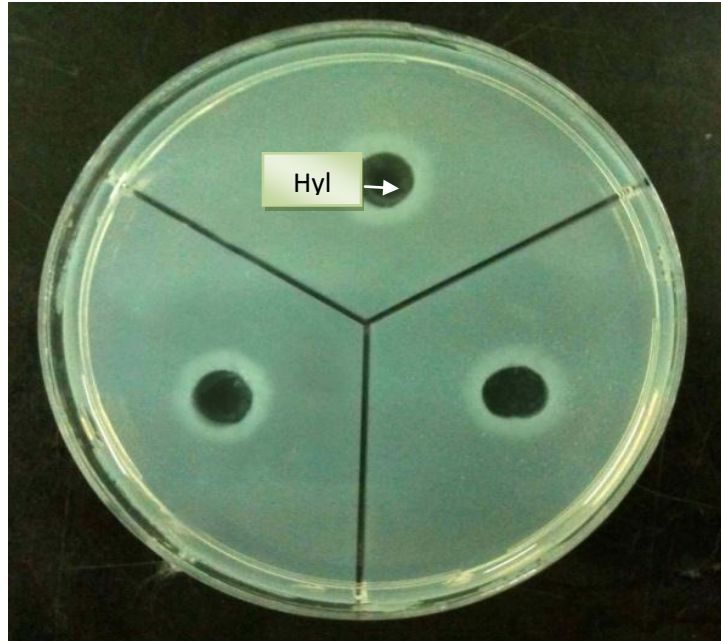


Figure 2 hyaluronidase activity in Nobel agar ,which appeared as a clear zone

Specific primers for PCR techniques are used for detection sortase enzymes . It was found that sortase A was observed in all GAS strains and this enzyme was designated as housekeeping enzyme figure (3).

whereas the others contained only the housekeeping one.

On the other hand ,sortase B was also detected and it was found that only two isolates are positive which indicates the presence of sortase B enzyme figure (4), while Srt C gene which encodes for sortase C enzyme was also present in only two isolates of *Streptococcus pyogenes* figure (5). However, only two isolates were found to have all these three enzymes

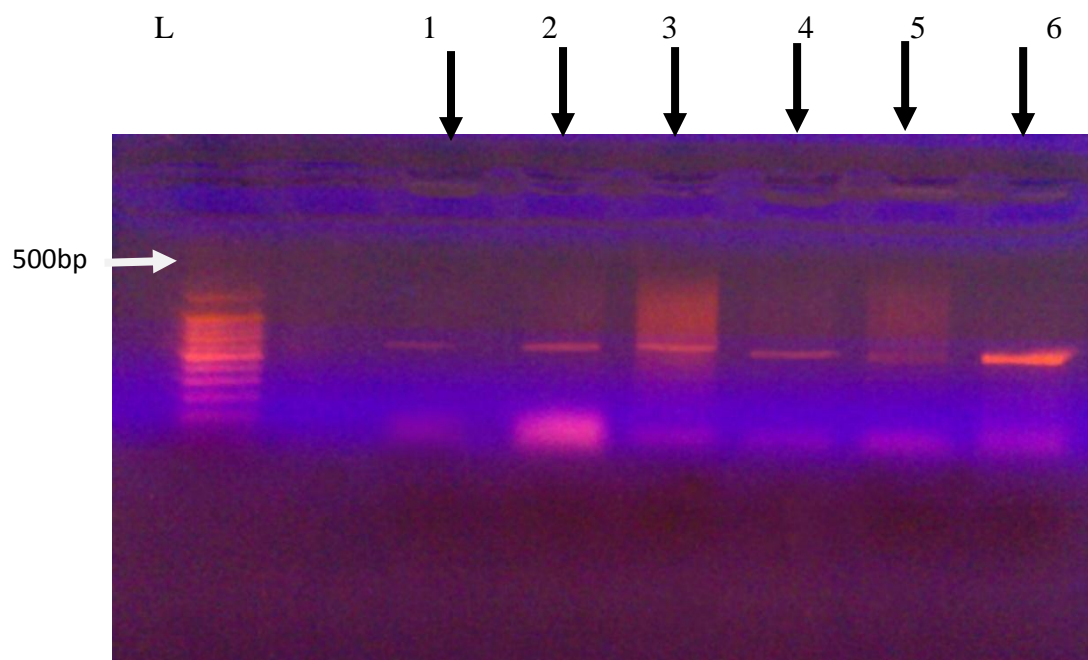


Figure 3 Gel electrophoresis of PCR product of sortase (A)
 *(1, 2, 3, 4, 5, 6) GAS isolates with positive result for sortase A

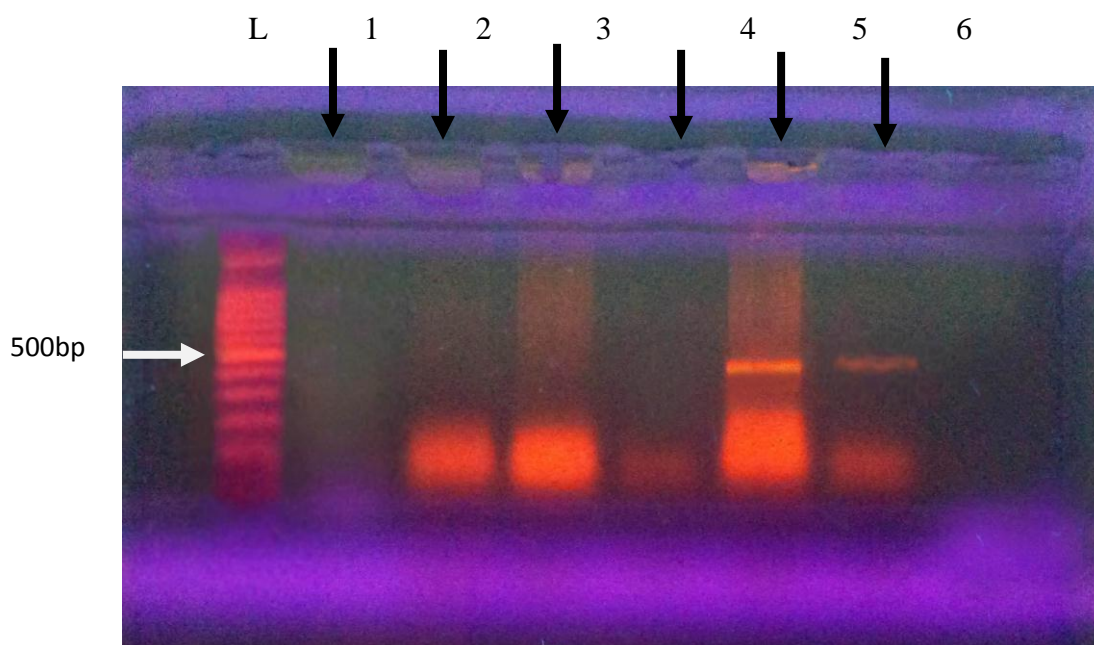


Figure 4 Gel electrophoresis of PCR product of sortase (B)
 *(1, 2, 3, 4) negative for sortase B, (5, 6) positive for sortase B.

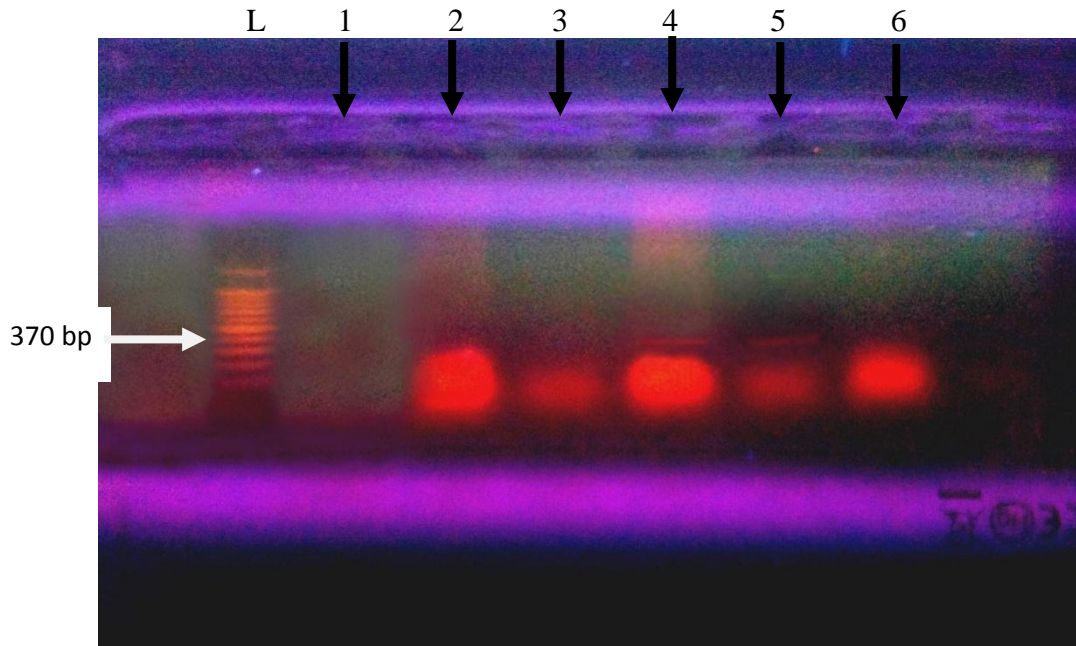


Figure 5 Gel electrophoresis of PCR product of sortase (C)

*(1, 2, 3, 6) negative for sortase C, (4,5) positive for sortase C

Discussion

Acute pharyngo – tonsilitis caused by beta – haemolytic group A *Streptococcus* is a common disease in childhood.

In this study four out six GAS isolates produce streptokinase extracellularly. Streptokinase activity became apparent after 3 hours of incubation and clot began to decrease after 10-16 hours of incubation and disappeared completely after 24 hours. This means that this enzyme was continued to synthesize extracellularly for a long time of incubation. This may be in contrast with that mentioned about streptokinase inhibition will be initiated in stationary phase of GAS growth because of synthesis of its inhibitors at this phase[14].

Moreover, it is clear that *Streptococcus pyogenes* can produce hyaluronidase extracellularly through the positive zone shown around bacteria to spread in the deep tissues. Since hyaluronidases are enzymes catalyze

the breakdown of hyaluronic acid in the body, they may increase the permeability of fluids to tissues[15].

Hyaluronidase activity is attributable to the product of the *hyla* gene and although all GAS have a *hyla* gene, only a small percentage of clinical strains (<25%) produce a detectable amount of HylA when grown in vitro[16].

Hynes, et al 2000 had pointed that the enzymatic activity against HylA had also been shown to be part of certain *Streptococcus pyogenes* bacteriophages.

Gram positive bacteria including *Strep pyogenes* assemble pili by distinct mechanism involving a transpeptidase called sortase regarding to this study, it was found that sortase A exists in all GAS isolates, this result could prove that sortase A is a housekeeping enzyme which is involved in pilus biogenesis, and also its ability to anchor most Lipid x Triglyceride motif surface protein [17].

On the other hand ,only two isolates showed positive amplification for Srt B gene which encodes sortase B this result is in correlation with the result which indicates that sortase B is not common among *Strep pyogenes* isolates [18].

In a study accomplished by Barnett .etal, 2004 indicated that Srt A is present in all strains of GAS, whereas Srt B is present in fewer than half of strains.

Moreover, Srt C which encodes for sortase C was found to be present in only two isolates of GAS. Barnett .et al , 2004 ; had indicated that Srt C was not available in all GAS strain this result came with our finding that Srt C is not present in all GAS isolates. Barnett and Scot ;2002¹⁶ observed that some strains of GAS encode only two sortases, designated SrtA and SrtB, which recognize different substrates of proteins containing an Lipid x triglycerid motif. Where as other study indicates that GAS express two distinct sortase , SrtA and SrtC.

Sortase A is the main enzyme which is responsible for anchoring proteins such as the M protein ,G related α 2 binding proteins .However our results indicates that all sortases (A , B ,C) can be encoded the same GAS isolates .

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