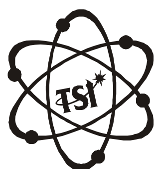
June 2010 ISSN : 0974 - 7435 Volume 4 Issue 2



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BioTechnology

An Indian Journal

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BTAIJ, 4(2), 2010 [111-118]

# Activation of plasminogen by streptokinase is a species-specific event

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*Received: 25th March, 2010 ; Accepted: 4th April, 2010*

## ABSTRACT

Streptokinase, one of the major blood-clot-dissolving agents, catalyzes the conversion of the plasma-zymogen (plasminogen) to the serine protease plasmin. Streptokinase genes from two different *S. equisimilis* strains, isolated from equine and human, were cloned and expressed in *Escherichia coli*. Cloned streptokinase genes were investigated for their expression in *E. coli* by Western blot analysis and radial caseinolysis assay. Recombinant genes of streptokinase genes were similar to their native counterparts in terms of their molecular masses and exhibited preferential plasminogen activity. PAGE and Radial caseinolysis studies demonstrated that streptokinases secreted by streptococci from different hosts were able to differentially activate only the plasminogen derived from the same host. Streptokinase-plasminogen interaction indicated that human and equine plasminogens were cleaved at the same highly conserved site and an altered form of streptokinase was produced. This work strongly supports the idea that these streptokinases must share a common plasminogen binding domain and species-specific activation of plasminogen by streptokinases which reflecting the origin of the streptococcal isolate and the role of the streptokinase in the pathogenesis of streptococcal infections. © 2010 Trade Science Inc. - INDIA

## KEYWORDS

Streptokinase;
Cloning;
Expression;
Plasminogen;
Activation;
Species.

## INTRODUCTION

Streptokinase is a group of extracellular proteins produced by a variety of beta-hemolytic streptococci. It is one of the major blood-clot-dissolving agents used in many medical treatments for the past 30 years[2,5,10,14,16,17,19,21,32,35,36]. It is used in life-threating deep-vein thrombosis, thromboembolism, thrombosed arteriovenous shunts and included in the World Health Organization Model List of Essential Medicines[38,39]. Its ability to induce reperfusion of the occluded coronary arteries and to reduce mortality has been firmly established. Two international clinical trials, GISSI-2 (trial size, 12, 490 patients)[9] and ISIS-3 (trial size, 41,299 patients)[13] resulted in conclusions that both streptokinase and tissue specific plasminogen activator, a fibrin-specific thrombolytic agent from humans, appear equally effective and safe for use in routine conditions, and no significant difference.

Most group A, C, and G streptococci isolated from human hosts secrete a plasminogen activator known as streptokinase which catalyzes the conversion of the

plasma-zymogen, plasminogen, to the serine protease plasmin. Human plasminogen and streptokinase form a 1:1 stoichiometric complex that hydrolyzes other plasminogen molecules to generate plasmin, which subsequently can degrade fibrin, the primary protein component of blood clots[2,5,10,14,16,17]. Introduced into clinical practice in the late 1950s, the intravenous infusion of streptokinase has become one of the treatments of choice in acute myocardial infarction. With regards to bacterial pathogenesis, plasmin may facilitate tissue invasion by dissolution of the fibrin barrier that forms at the site of infection, by hydrolysis of extracellular matrix proteins such as laminin or fibronectin, and by activation of latent collagenases and other zymogen forms of metalloproteinases[4-6,15,20,24,27,32].

Due to the clinical importance of this streptococcal protein, a great deal of effort has been directed toward characterizing and understanding the molecular basis of the interaction of streptokinase with plasminogen. Most previous research has focused on the streptokinase secreted by a human isolate of the group C streptococcus *S. equisimilis*. These investigations, although increasing our understanding of the streptokinase-plasminogen interaction, have also created the impression that all streptokinases belong to a family of homologous proteins, with similar biophysical and biochemical properties. This has led to the failure to fully understand the importance of the concept of species specificity, with the result that the group C streptococci *S. equi*, *S. zooepidemicus*, and *S. equisimilis*, isolated from non-human hosts, have been regarded as non-streptokinase producers simply on the basis of the inability to activate human plasminogen[1,5,7,20,24].

However, McCoy et al.[20] and Nowicki et al.[24] demonstrated that group C streptococci isolated from non-human sources secreted streptokinases which preferentially activated plasminogen obtained from the host from which the isolate had been obtained. Although these streptokinases preferred to activate only the plasminogen derived from the host, they all bound plasminogen regardless of the host source. These observations suggested that there are two major events in the activation of plasminogen by streptokinase; a primary event (binding) which is not species specific and a secondary event (activation) which is species-specific.

To achieve a better understanding of the genetic aspects of this important streptococcal product, streptokinase genes from two different strains of *S. equisimilis* (one from an *S. equisimilis* equine isolate and one from an *S. equisilimilis* human isolate) were cloned and expressed in *Escherichia coli* as (His) 6-tagged fusion proteins to study the molecular relationship between streptokinases and plasminogen from different hosts.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions

In this study the following strains of *S. equisimilis* streptococci were used: strain MZ130, isolated from an equine host; and strain H46A, ATCC 12449. Bacteria were grown at 37°C for 8 h in 500 in brain heart infusion broth (Difco) as standing cultures at 37°C. *E. coli* (XL1-Blue) strain was grown in LB medium with added selective agent [ampicillin (Ap), 50mg/l]. The pH of the cultures was monitored and maintained at 7.0 by periodic addition of 10N NaOH.

Cloning and expression of the streptokinase genes into the prokaryotic expression vector pQE-30.

Streptokinase genes from equine and human streptococcal strains were cloned into the pQE-30 vector (Qiagen) to produce recombinant streptokinases appended at the N terminus with a polyhistidine (His)6 domain. Genomic DNA from the parent strains was used as template for PCR amplification of the streptokinase genes. The forward primers used in these amplifications were 5′-cgcggatccaataattacgccaagcct-3′and 5′-cgcggatccattgctggacctgag-3′ while the reverse primers were 5′-cttatttgtttgattcgttgtacc-3′and 5′-tcccccgggttatttgtcgttagggtt-3′ for the equine and H46A streptokinase genes respectively. Bacterial cells were lysed and DNA was isolated by the procedure of Monsen et al.[22]. Genomic DNA was purified by phenol extraction and ethanol precipitation[29]. The template DNA was mixed with 5µl 10x PCR-buffer + $MgCL_2$, dNTPs, 1µl taq DNA polymerase and the reaction mixture was overlaid with 50µl of paraffin oil. Amplification of the DNA has been performed in a thermocycler-PCR-block using a program in which, the initial denaturation was at 97°C for 2min, primer annealing at 55°C for 30 sec and the elongation at 72°C for 2 min. these steps were repeated 29 times[11].

The PCR fragments were cloned in the pQE-30 (Qiagen), as *Sma*I/*Bam*HI fragment and then introduced to *E.coli* cells by transformation as described by Inoue et al.[12]. Transformed cells by cloned human streptokinase (pQE-30-HSk) and equine streptokinase (PQE-30-ESk) were grown at 30°C to mid-log phase, and expression of the streptokinase genes was induced by addition of IPTG (1mM) as described by Studier et al.[33]. Transformed cells were screened for expression of streptokinase genes by two different methods. The first method was by Western Blot analysis[34] and the second one by radial caseinolysis in plasminogen-skim milk-containing agarose as described by Saksela[28].

Miscellaneous. Purification of streptokinases was carried out as described by Peng et al.[26] while purification of equine and human plasminogen and Plasminogen activation studies with recombinant streptokinases were done as described by Caballero et al.[5].

## RESULTS

**Cloning of streptokinase genes:** The streptokinase genes from both strains were cloned into the pQE-30 vector (Qiagen) to produce recombinant streptokinases appended at the N terminus with a polyhistidine (His)6 domain. This would not only facilitate purification of the recombinant streptokinase molecules from the bacterial cytosol but also permit the study of the interaction of plasminogens with matrix-bound streptokinases.

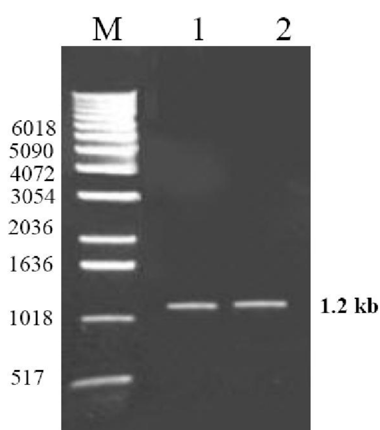


**Figure 1 : PCR amplification of streptokinase genes from *S. equisimilis* streptococci. Lane 1 and 2: are PCR products of amplified streptokinase genes of *S. equisimilis* strains H46A and MZ130, respectively; M: fragments of DNA standard (0.07-12.2 kbs, Roche, Mannheim). 3µl of each sample was analyzed on 0.7% agarose**

Genomic DNA from the parent strains was used as template for PCR amplification of streptokinase genes. Forward primers used in these amplifications were chosen to be complementary with the DNA sequences coding for the start of the mature proteins, with additional codons to facilitate directionalcloning into the *Bam*HI and *Sma*I restriction endonuclease sites of the pQE-30 vector. The reverse primers were complementary to the sequences coding for the carboxyl termini of the mature proteins. This cloning strategy resulted in a PCR product of 1251bp in case of streptokinase gene from the equine strain *S. equisimilis* MZ130 and 1242bp in case of streptokinase gene from the human strain *S. equisimilis* H46A (Figure 1).

Purified PCR fragments were digested by *Bam*HI and *Sma*I and ligated to the pQE-30 vector which was digested by the same endonucleases. Ligated streptokinase fragments with the pQE-30 vector were then introduced to *E.coli* (*Xl*-blue) cells by transformation, from which plasmid DNA was isolated and analyzed by agarose electrophoresis. Some clones of pQE-30-SK (Figure 2, lanes: 2, 3, 9 and 10) were selected and tested by restriction analysis using *Bam*HI and *Sma*I. In this analysis, all tested clones developed the right fragments of 1.2kb in both Sk genes (Figure 3).

**Expression and purification of streptokinase genes:** To investigate whether the plasmid encoding the recombinant Sk genes from both strains have been expressed, transformed cells either by human streptokinase pQE-30-HSk or by equine streptokinase pQE-30-ESk were grown at 30°C to mid-log phase. Streptokinase genes induced by IPTG and screened for their expression by Western blot analysis using antiHis6 antibody (Figure 4). Data presented in this figure illustrates that human streptokinase gene from clone pQE-30-$Az_{10}$ (Figure 4, lane: 1) and equine streptokinase from clone pQE-30-$Az_2$ (Figure 4, lane: 2) were expressed and secreted their proteins. Difference in the apparent molecular weight of both cloned genes was noticed where cloned streptokinase from the human (HSk) had an apparent molecular mass of ~47 kDa (Figure 4, lane: 1) while cloned streptokinase from the equine (ESk) had an apparent molecular mass ~49 kDa (Figure 4, lane: 2). In terms of their molecular sizes cloned streptokinases from both strains were found to be indistinguishable from their native counterparts (Fig-

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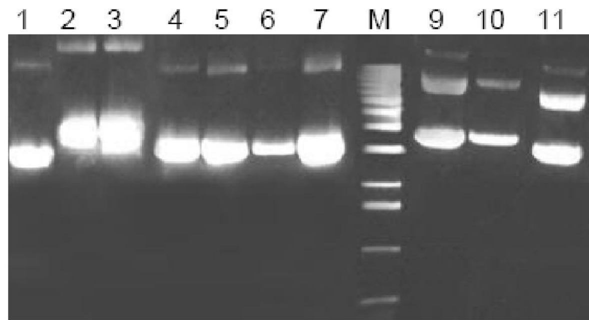


**Figure 2 : Analysis of isolated pQE-30 plasmids which containing streptokinase gene from human (lanes 9&10) and from equine (lanes: 2-7) on 0.7% agarose gel electrophoresis. In both cases, plasmid DNA of the pQE-30 without insert (lanes, 1&11) served as a control. M, fragments of DNA standard (0.07-12.2 kbs)**

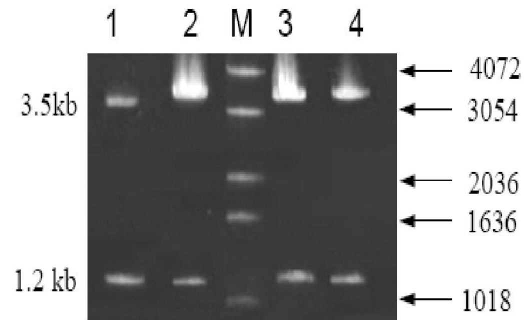


**Figure 3 : Restriction analysis of selected clones of pQE-30 which carrying Sk genes either from human (lanes: 1 & 2) or equine (lanes, 3 &4), by *Bam*HI and *Sma*I. Two fragment sizes were developed, one at 3.5 kb for the plasmid and the other at 1.2kb for the insert. Fragment sizes of the standard DNA bands written on the right**

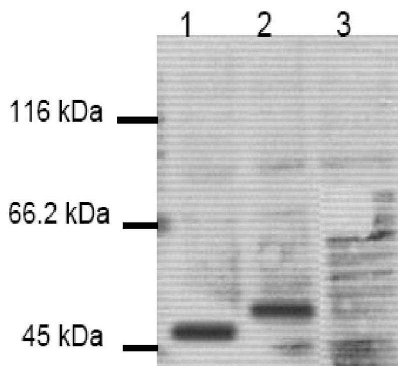


**Figure 4 : Western blot analysis of recombinant streptokinase genes. Total proteins extracted from transformed *E. coli* cells (*Xl1*-Blue), subjected to SDS-PAGE and immunodetected with anti-His6 antibody. 1: recombinant human streptokinase expressed from clone pQE-30Az$_{10}$; 2: recombinant equine streptokinase expressed from clone pQE-30Az$_2$; C: proteins of *E. coli* cells (*Xl1*-Blue) transformed with empty vector**

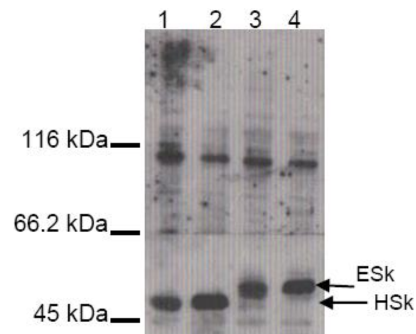


**Figure 5 : Purification of recombinant and native streptokinases. Recombinant and native proteins of streptokinase were purified from transformed *E.coli* and *S. equisimilis* cultures, respectively. Lanes, 1 and 2, are native and recombinant human streptokinases, respectively. Whereas 3 and 4, are native and recombinant equine streptokinases, respectively Proteins of streptokinases were assayed for purity by SDS-PAGE and staining by Coomassie blue, R-250**

ure 5).

**Activation of plasminogens by recombinant streptokinases:** Plasminogen activation with recombinant streptokinases was tested by the radial caseinolysis assay. In this assay, the culture supernatant containing streptokinase was applied to individual wells in an agarose gel containing skim milk and plasminogen. In case of recombinant human streptokinase produced from clone (pQE-30-Az$_{10}$), activation of human plasminogen (HPg) was noticed. This was reflected by a 2.5cm halo size (Figure 6A$_1$). In the activation of equine plasminogen (EPg) by the engineered equine streptokinase from clone pQE-30-Az$_2$ a halo size of 2.3-2.4cm was developed (Figure 6B$_1$).

Activation of HPg and EPg either by recombinant ESk or HSk, was tested by the same assay. Recombinant streptokinase HSk, however, was not able to activate plasminogen from equine (Figure 6B$_2$). The same behavior appeared upon application of recombinant ESk on human plasminogen (Figure 6A$_2$). These results indicate that the specificity of streptokinase in the interaction with the plasminogen that should be activated by streptokinase secreted by streptococci derived from the same host.

To confirm the indication developed from the radial caseinolysis assay, purified recombinant streptokinases either from human (HSk) or equine (ESk) were mixed with plasminogens derived from their hosts and subjected to SDS-PAGE analysis (Figure 7A, B). At the same time, the test was done by inverting the case, where the equine and human recombinant streptokinases were tested for their activation of human and equine plasmi-

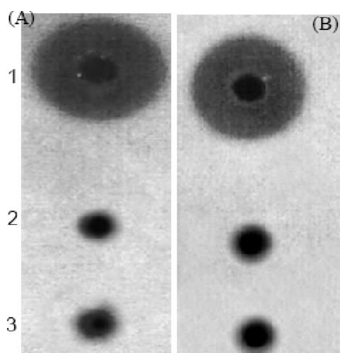


**Figure 6 : Activation of plasminogen by recombinant streptokinase using radial caseinolysis assay. $A_1$: activity of recombinant HSk, clone pQE-30Az$_{10}$ on HPg; $B_1$, activity of ESk, clone pQE-30Az$_2$ on EPg; $A_2$, activity of recombinant ESk on HPg; $B_2$, activity of HSk on EPg; $A_3$ & $B_3$, control well which contained supernatants from *E.coli* cells (*Xl1*-Blue) transformed with empty vector**

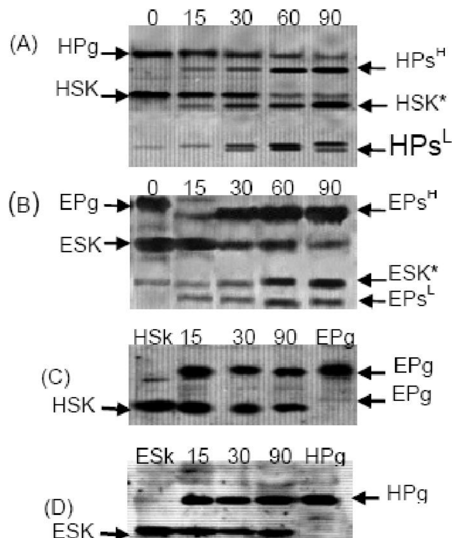


**Figure 7 : SDS-PAGE of plasminogen activation by recombinant streptokinases. (A): PAGE analysis of the interaction of HSk with HPg; (B): ESk with EPg; (C): HSk with EPg; (D): ESk with EPg over time, in minutes, as indicated above each panel**

nogens, respectively (Figure 7C, D). This test was done by mixing equal samples from purified recombinant streptokinases with plasminogens. Samples were incubated at 30°C and the reaction was terminated at different intervals (from 15-90 min). PAGE analysis showed that binding and interaction of plasminogen by streptokinase should be activated by streptokinase of streptococci derived from the same host. Human plasminogen was activated only by HSk and equine plasminogen was activated only by ESk. Activation of plasminogens started 10-15 min after incubation with the purified recombinant streptokinases (Figure 7A, B). Incubation of HSk with HPg resulted in the in the generation of several breakdown products. One of these products was a protein band at ~41-kDa which corresponded to processed or mature streptokinase (HSk*). However, PAGE analysis of equine plasminogen activation by (ESk) developed another protein band at ~27kDa which corresponded a cleaved product of equine streptokinase (ESK*).

Activation analyses of EPg by HSk and HPg by ESk using PAGE procedure developed consistent results to that developed from the radial caseinolysis assay. In these analyses, EPg was not activated by HSk and vice versa. As a control, purified HSk, ESk, HPg and EPg were applied separately in this step to detect any binding activity between the recombinant streptokinases to different plasminogens (Figure 7C, D). The activation profiles obtained from the radial caseinolysis assay and PAGE analyses confirm that the activation of plasminogen by streptokinase is specie-specific event.

## DISCUSSION

Streptokinase is considered as one of the major blood-clot- dissolving agents which produced by a variety of beta-hemolytic streptococci[19] and by many bacterial expression hosts including the pathogenic *S. equismilis* H46A, the natural streptokinase producer[37]. Most of sequenced streptokinases were originally isolated from streptococci which had infected human hosts[5,10,14,16,17,19,25,.32,35,36]. These streptokinases have been shown to be remarkably similar to one another, both functionally and structurally, with greater than 85% homology at the amino acid level. In addition, these streptokinases have the same number of amino acid residues, namely, 414. In contrast, the streptokinase secreted by *S. equisimilis* isolated from an equine host was 380 amino acids in length and exhibited at the amino acid level only 25.4% identity with streptokinase secreted by a streptococcus (*S. equisimilis* H46A) isolated from a human host which has 414 amino acids in length. The second streptokinase studied, a streptokinase secreted by an *S. equisimilis* porcine isolate was 374 amino acids in length and had only 35.3% identity with the streptokinase from the *S. equisimilis* human isolate H46A[5].

As a preliminary step in a comprehensive analysis,

two different streptokinase genes, (HSk) from an *S. equisilimilis* human isolate (H46A) and (ESk) from an *S. equisimilis* equine isolate (MZ130), were cloned and expressed in *Escherichia coli* as (His)6-tagged fusion proteins in order to study the interaction of these proteins with different mammalian plasminogens. Both of (HSk) and (ESk) streptokinase genes was cloned into the expression vector pQE-30. This vector permits the expression of cloned genes containing the initial ATG codon of the signal sequence and is expressed with an appended (His) 6 tail to facilitate purification. Genomic DNA from both the H46A and MZ130 streptococcal strains was amplified by PCR using nucleotide primers complementary to the 5′ and 3′ termini of the genome-fragment representing the immature protein. Cloned HSk and ESk genes were investigated for their expression in *E.coli* by western analysis. According to the protein bands produced in this analysis, a difference in their molecular mass was noticed. SDS-PAGE analysis indicated molecular masses of ~49 and ~47 kDa for ESk and HSk, respectively. Comparison between the cloned proteins of HSk and ESk and their native counterparts by PAGE protocol indicated that they have the same apparent molecular sizes.

Recombinant streptokinases expressed in *E. coli* showed the same substrate specificity as that of the streptococcal donor strain[10,15,16]. The identity of recombinant streptokinases of this study (HSk and ESk) has been demonstrated through the activation of human and equine plasminogens (HPg and EPg) by PAGE analysis and radial caseinolysis assay. In both techniques, however, HSk was only able to active HPg into human plasmin and EPg can be activated only by ESk and not by HSk. These results confirmed the substrate specificity of streptokinase protein where plasminogens can only activated by the streptokinase derived from the same streptococcal donor strain. In the activation of equine plasminogen (EPg) by the recombinant protein of ESk, PAGE developed a ~27kDa protein band. This result was in agreement with the N-terminal amino acid sequencing analysis of Caballero et al.[5] for the breakdown products resulting from the streptokinase-plasminogen interaction. It was indicated that a ~27-kDa protein band had the N-terminal amino acid sequence AGKPI which represented a cleaved product of ESk. From the PAGE analysis in this work and the sequencing data of Caballero et al.[5], it was apparently that the equine streptokinase had been cleaved between $Lys^{147}$ and $Ala^{148}$ to yield that altered product (ESk*) of the 27.173kDa.

At the same time, activation of human plasminogen occurred within 5-10 min after incubation with HSk, resulting in the generation of several breakdown products. A ~ 41-kDa protein corresponded to altered streptokinase (HSk*) was developed upon PAGE analysis. N-terminal amino acid sequencing of Caballero et al.[5] for these breakdown products indicated that this altered streptokinase had the N-terminal sequence SKPFA and it had been cleaved between $Lys^{59}$ and $Ser^{60}$, which resulted in the generation of altered human streptokinase (HSk*) of a 40.998-kDa. The interaction of recombinant streptokinases with their corresponding host plasminogens resulted in the generation of plasmin heavy and light chains. The human plasmin light chain ($HPs^{L}$) developed at ~25 kDa had the N-terminal amino acid sequence VVGGC while the equine plasmin light chain had N-terminal sequence of IVGGC and a size of ~25 kDa[5]. These observations indicated that cleavage of the plasminogen molecules occurred at the same cleavage site ($Arg^{561}$-$Val^{562}$) in human and ($Arg^{561}$-$Ile^{562}$) in equine plasminogens, a site which is highly conserved in a number of mammalian plasminogens[8,18,19,30]. It should be noted that Val and Ile are both hydrophobic amino acids with an aliphatic side chain bearing no net charge and are thus considered to be similar by Myers and Miller's method[23,31] of protein comparison.

An initial binding event is followed by a conformational change imposed by the streptokinase molecule on the plasminogen moiety. This is according to the "activator complex model" of plasminogen activation by streptokinase[20,24]. This event uncovers an active site in the plasminogen which leads to a series of catalytic and autocatalytic events that result in the generation of a streptokinase-plasmin complex with the cleavage of streptokinase to form an altered streptokinase still associated with the complex. Recombinant streptokinases in this study behaved in a manner very similar to that observed in the fluid-phase studies[3], where the recombinant HSk was cleaved at the same trypsin-sensitive peptide bond.

The observation that streptokinases secreted by

streptococci from different hosts were able to differentially activate only the plasminogen derived from the same host suggests that these streptokinases must share a common plasminogen binding domain. Logically, primary structural differences in the regions involved in activation among streptokinases from different species should be reflected in differences in secondary structure and finally in three-dimensional conformation; otherwise, activation would be nonspecific. As the streptokinase from the equine isolate is unable to activate human plasminogen and the streptokinase from the human isolate can not activate equine plasminogen, one would expect the primary structural differences between the equine and human streptokinases. This observation support the view that streptokinase belongs to a family of plasminogen activators whose members display greater diversity in primary structure than previously suspected. This, in fact, is the case, as shown by the alignment studies and homology indexes[5]. Also, confirm that activation of plasminogen by streptokinase is a species-specific event reflecting the origin of the streptococcal isolate and implies a role for streptokinase in the pathogenesis of streptococcal infections.

## REFERENCES


[1] M.Arditi, S.T.Shulman, T.Davis, R.Yoger; Rev. Infect.Dis., **11**, 34-45 **(1989)**.

[2] P.W.Armstrong; Nat.Rev.Cardiol., **7(2)**, 67-9 **(2010)**.

[3] W.J.Brockway, F.J.Castellino; Biochemistry **10**, 2063-2070 **(1974)**.

[4] M.D.P.Boyle, R.Lottenberg; Haemost., **77**, 1-10 **(1997)**.

[5] A.R.Caballero, R.Lottenberg, K.H.Johnston; Infect.Immun., **67(12)**, 6478-6486 **(1999)**.

[6] M.Cuchacovich, Héctor Gatica, Paula Vial, Jorge Yovanovich, Salvatore V.Pizzo, Mario Gonzalez-Gronow; Clin.Diagn.Lab.Immunol., **9**, 1253-1259 **(2002)**.

[7] R.H.Diebel, W.H.Steeley; 'Streptococcacea', In: R.E.Buchanan, N.E.Gibbons (Ed.,); Bergey's Manual of Determinative Bacteriology, Williams and Wilkins, Baltimore, Md., 490-517 **(1974)**.

[8] M.Forsgren, B.Raden, M.Israelsson, K.Larsson, L.O.Heden; FEBS Lett., **213**, 254-260 **(1987)**.

[9] Gruppo Italiano Per Lo Studio Della Soprawivenza Nell'Infarto Miocardico; Lancet, **336**, 65-71 **(1990)**.

[10] T.T.Huang, H.Malke, J.J.Ferretti; Mol.Microbiol., **3**, 197-205 **(1989)**.

[11] M.A.Innis, D.H.Gelfand, J.J.Sninsky, T.J.White; 'PCR Protocols', A Guide to Methods and Applications, Academic Press, Inc., San Diego, **(1990)**.

[12] H.Inoue, H.Nojima, H.Okayama; Gene, **96(1)**, 23-28 **(1990)**.

[13] ISIS-3 Collaborative Group; Lancet, **339**, 753-770 **(1992)**.

[14] S.Lizano, K.H.Johnston; Infect.Immun., **73(7)**, 4451-3 **(2005)**.

[15] R.Lottenberg, C.C.Broder, M.D.Boyle, S.J.Kain, B.L.Schroeder, R.Curtiss 3rd; J.Bacteriol., **174**, 5204-5210 **(1992)**.

[16] H.Malke, J.J.Ferretti; 'Streptokinase', Cloning, Expression, and Excretion by Escherichia Coli.Proc. Natl.Acad.Sci.USA, **81**, 3561 **(1984)**.

[17] H.Malke, B.Roe, J.J.Ferretti; Gene, **34**, 357-362 **(1985)**.

[18] T.Marti, J.Schaller, E.E.Rickli; Eur.J.Biochem., **149**, 279-285 **(1985)**.

[19] M.Martin; Streptokinase in Chronic Arterial Diseases (CRC, Boca Raton, FL), **(1982)**.

[20] H.E.McCoy, C.C.Broder, R.Lottenberg; J.Infect. Dis., **164**, 515-521 **(1991)**.

[21] Minami, Masaaki, Kamimura, Takuya, Isaka, Masanori, Tatsuno, Ichiro, Michio Ohta, Hasegawa, Tadao; Antimicrob.Agents Chemother., **54**, 98-102 **(2010)**.

[22] J.J.Monsen, S.E.Holm, L.G.Burman; FEMS Microbiol.Lett., **16**, 19-24 **(1983)**.

[23] E.W.Myers, W.Miller; Comput.Appl.Biosci., **4**, 11-17 **(1988)**.

[24] S.T.Nowicki, D.Minning-Wenz, K.H.Johnston, R.Lottenberg; Thromb.Haemost., **72**, 595-603 **(1994)**.

[25] H.Ohkuni, Y.Todome, H.Suzuki, M.Mizuse, N.Kotanl, K.Horiuchi, N.Shikama, A.Tsugita, K.H.Johnston; Infect.Immun., **60**, 278-283 **(1992)**.

[26] Peng, Sheng-Bin, Li Wang, John Moomaw, B.Robert Peery, Pei-Ming Sun, B.Robert Johnson, Jin Lu, Patti Treadway, L.Paul Skatrud, Q.May Wang; J.Bacteriol., **183(2)**, 621-627 **(2001)**.

[27] K.N.N.Reddy; Enzyme, **40**, 79-89 **(1988)**.

[28] O.Saksela; Anal.Biochem., **111**, 276-282 **(1981)**.

[29] J.Sambrook, E.F.Fritsch, T.Maniatis; 'Molecular Cloning: A Laboratory Manual', 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.,

**(1989)**.

[30] J.Schaller, C.Straub, U.Kampfer, E.E.Rickli; Protein Seq.Data Anal., **4**, 69-74 **(1991)**.

[31] E.W.Myers, W.Miller; Comput.Appl.Biosci., **4**, 11-17 **(1988)**.

[32] K.Steiner, H.Malek; Infect.Immun., **70**, 3627-3636 **(2002)**.

[33] F.W.Studier, A.H.Rosenburg, J.J.Dunn, J.W.Dubendorff; Methods Enzymol., **185**, 60-89 **(1990)**.

[34] H.Towbin, T.Staehelin, J.Gordon; Proc.Nati.Acad. Sci.USA, **76**, 4350-4354 **(1979)**.

[35] F.Walter, M.Siegel, H.Malke; Nucleic Acids Res., **17**, 1261 **(1989a)**.

[36] F.Walter, M.Siegel, H.Malke; Nucleic Acids Res., **17**, 1262 **(1989b)**.

[37] Wong, Sui-Lam, Ruiqiong Ye, Shyroze Nathoo; J.Appl.Environ.Microbiol., **60(2)**, 517-523 **(1994)**.

[38] World Health Organization; 'WHO Model Formulary Antithrombotic Drugs and Myocardial Infarction', http://whqlipdoc.who.int/publications/2004/92414, **(2004)**.

[39] World Health Organization; 'The WHO Mode List of Essential Medicines', 14th Edn., March 2005, http://whqlipdoc.who.int/hq/2005/a87017_eng.pdf, (10th February 2007), **(2007)**.