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Exploring the *in vitro* thrombolytic potential of streptokinase-producing β-hemolytic *Streptococci* isolated from bovine milk

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The aim of this study was to isolate and characterize streptokinase-producing β-hemolytic *Streptococcus* sp. from bovine milk. A total of 50 milk samples were collected randomly from different breeds of cow and goat (Vellore, Tamil Nadu, India). The samples were characterized and screened for streptokinase-producing isolates using microbial and biochemical analysis. About 97 colonies were isolated from milk samples showing hemolytic patterns of α (19.6%), β (24.7%) and γ (55.6 %). Out of 20β-hemolytic isolates, only 6 colonies (VB2, VB3, VB8, VB14, VB16, and VB17) were identified as β-hemolytic *Streptococci* as potent producers of streptokinase. VB2 and VB14 showed the greatest streptokinase activities of 265 U mL^{-1} and 225 U mL^{-1}, respectively. Based on biochemical and molecular characterization, the potent isolates VB2 and VB14 were identified and confirmed as *S. equinus* and *S. agalactiae*, respectively. The identified strains were named *Streptococcus equinus* VIT_VB2 (GenBank accession no. JX406835) and *Streptococcus agalactiae* VITVS5 (GenBank accession No. KF186620) The strains isolated from bovine milk provide a variance in the fibrinolytic activity on blood clots. The current study has demonstrated that the isolation of streptokinase producers from bovine milk, and the production of streptokinase from novel strain, enhanced the fibrinolytic activity. This study is the first to report that *Streptococcus equinus* produces streptokinase.

Key Words: clot buster; *Streptococcus equinus* VIT_VB2; *Streptococcus agalactiae* VITVS5; streptokinase; thrombolytics; thrombosis

Introduction

Thrombolytic disorders have emerged to be an important cause of human mortality worldwide (AHA, 2009; Allender et al., 2008). A blood clot (thrombus) in blood vessels can cause vascular blockage (thrombosis) leading to life-threatening consequences. A healthy homeostatic system suppresses the development of such blood clots in normal circulation; however, it reacts extensively during vascular injury to prevent blood loss (Macfarlane, 1977). The failure of the system to produce clot lysins such as tissue plasminogen activator (t-PA) and urokinase leads to strokes, pulmonary embolisms, deep vein thrombosis and acute myocardial pathologies. Therefore, thrombolytic therapy has become a conventional treatment for myocardial infarction (AMI), but currently clinically prescribed thrombolytic drugs have problems such as delayed action and other side effects like bleeding, re-occlusion, etc. (Subathra et al., 2012, 2013). Clinical interventions to cure these disorders are carried out by the external administration of thrombolytic agents (Collen, 1996). Streptokinase (SK), a fibrinolytic agent secreted from *Streptococcus* sp., is a potential alternative plasminogen activator (Collen et al., 1992). The medical significance of the structure and the mechanism of the action of SK has increased. Streptokinase forms a 1:1 stoichiometric complex with plasmin, which activates plasminogen to plasmin thereby contributing to clot lysis by its proteolytic action on fibrin, a major constituent of a thrombus (Jackson and Tang, 1982). The increasing potential of a streptokinase application prompted us to screen for novel streptokinase-producing organisms. Also the exponential increase of the application of streptokinase in various fields during the last few decades demands an increase in both qualitative improvement and quantitative enhancement. It is now the leading fibrinolytic agent, and is included in the World Health

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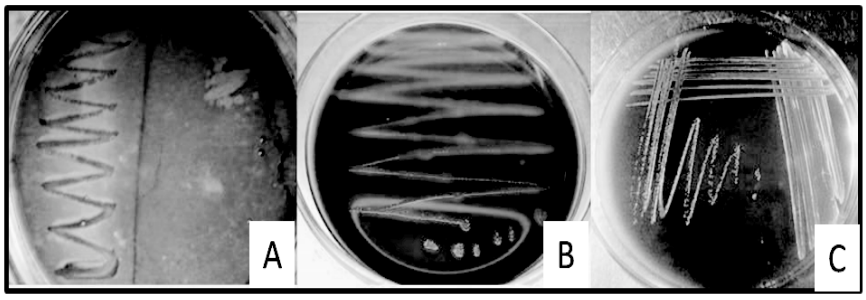

Fig. 1. Blood agar plates showing hemolysis (A) α-hemolysis (B) β-hemolysis (C) γ-hemolysis.

Table 1. Distribution of hemolytic isolates from milk samples.

Source of culture	No. of samples	No. of isolates	No. of α-hemolytic	No. of β-hemolytic	No. of γ-hemolytic
Cow Milk	37	86	16	20	50
Goat Milk	9	9	3	4	2
Packet Milk	4	2	—	—	2
Total	50	97	19	24	54

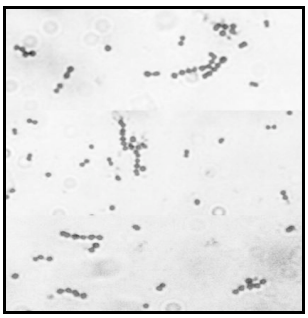

Fig. 2. 100× microscopic view of Gram positive cocci in chains.

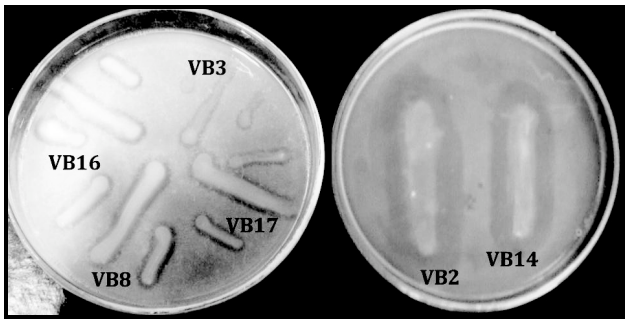

Fig. 3. Streptokinase activity of the potent isolates VB2, VB3, VB8, VB14, VB16 and VB17 on casein plasminogen overlay medium.

Organization Model List of Essential Medicines, and used clinically as an intravenous thrombolytic agent for the treatment of acute myocardial infarction. It is well established that the fibrinolytic activity of streptokinase originates in its ability to activate plasminogen (Abdelghani et al., 2005; Tough, 2005; Vaishnavi et al., 2011). The results of the present study signify the thrombolytic potential of streptokinase-producing β-hemolytic *Streptococci* from cow and goat milk samples. The study focuses on screening the possible amount of milk samples from different breeds of cows and goats for the isolation of potential β-hemolytic *Streptococci* producing streptokinase.

Materials and Methods

Isolation and identification of β-hemolytic Streptococcus strains. Forty-one cow milk samples were collected from milking cows (breeds—friesian and red sindhi), which includes four samples from commercially available packed milk and nine milk samples from goats (breeds—surti and deccania) from different locations in Vellore (Tamil Nadu, India). Twenty mL of milk were collected in a sterile container and transferred to the laboratory with minimum delay. One mL of a thoroughly mixed sample (gravity cream was removed as it enhances the growth of other contaminating microbes) was emulsified in a test tube containing 10 mL of a freshly prepared sterile 1% sodium carbonate solution, then incubated at 37°C for 24 h. This inhibits the growth of gram negative organisms (Dundar et al., 2003). Incubated milk samples were then serially diluted up to 10^{-6}. 0.1 mL of a dilution of 10^{-4} and 10^{-5} of each sample was spread onto 5% beef (cow) blood agar plates containing 0.0016% (w/v) sodium azide and cultured at 37°C for 24–48 h. The colonies with a clear zone of hydrolysis resembling β-hemolysis, with morphological characters such as small, circular, moist, convex and translucent, were selected. Each of these colonies were transferred to blood agar plates and Pike streptococcal agar plates supplemented with 5% beef blood, 0.001% (v/v) *Streptococcus* supplement and 0.0016% (w/v) sodium azide. The purity of the selected isolate was assured and cultured in 5 mL of Pike streptococcal broth broth (Pike, 1944). The strains that indicated β-hemolysis on beef blood agar and gram positive cocci in chains were considered for screening.

Screening for streptokinase-producing β-hemolytic Streptococci. The isolated bacterial colonies were routinely detected by an overlay of casein and human plasminogen in soft agar with slight modification (Sahni et al., 2007). The isolated colonies were streaked on a plate containing

Table 2. Morphological characterization and SK activity of 20 selected β-hemolytic strains.

Isolate No.	Colony morphology	Gram staining	Catalase activity	Casein-plasminogen overlay SK activity
VB1	Tiny, elevated, round, smooth, white	+ve short cocci in chains	-ve	Minimum activity
VB2	Pinpoint colonies, smooth, translucent	+ve short cocci in chains	-ve	Maximum activity
VB3	Pinpoint colonies, smooth, translucent	+ve long cocci in chains	-ve	Maximum activity
VB4	Large, irregular, white, flat	+ve long cocci in chains	-ve	Minimum activity
VB5	Large, round, mucoid, yellow	+ve rods	+ve	No activity
VB6	Pinpoint colonies, smooth, white	+ve short cocci in chains	-ve	No activity
VB7	Pinpoint colonies, undulated, yellow	+ve cocci in clusters	+ve	No activity
VB8	Pinpoint colonies, smooth, white	+ve short cocci in chains	-ve	Maximum activity
VB9	Large, round, mucoid, yellow	+ve cocci in clusters	+ve	No activity
VB10	Pinpoint colonies, smooth, white	+ve short cocci in chains	-ve	No activity
VB11	Pinpoint colonies, smooth, round, opaque	-ve cocci in clusters	+ve	Minimum activity
VB12	Large, irregular, white, flat	+ve long cocci in chains	-ve	Minimum activity
VB13	Pinpoint colonies, smooth, translucent	+ve long cocci in chains	-ve	No activity
VB14	Pinpoint colonies, smooth, translucent	+ve short cocci in chains	-ve	Maximum activity
VB15	Pinpoint colonies, smooth, opaque	+ve short cocci in chains	+ve	No activity
VB16	Pinpoint colonies, smooth, translucent	+ve long cocci in chains	-ve	Maximum activity
VB17	Pinpoint colonies, smooth, translucent	+ve short cocci in chains	-ve	Maximum activity
VB18	Tiny, elevated, round, smooth, white	+ve long cocci in chains	-ve	No activity
VB19	Pinpoint colonies, smooth, translucent	+ve short cocci in chains	-ve	Minimum activity
VB20	Pinpoint colonies, smooth, translucent	+ve short cocci in chains	-ve	Minimum activity

20 mL of soft agarose mixture containing 0.8% agarose, 10% skimmed milk, 200 μL of human plasma, 150 mM NaCl and 50 mM Tris-HCl (pH 8.0). The plates were incubated at 37°C for 12–16 h. Streptokinase activity was indicated by the appearance of clear zone around the colonies (Ozegowski et al., 1983; Sahni et al., 2007). Further identification was then carried out using the strains that resulted in maximum zone of clearance.

Morphological and biochemical characterization. The morphological characteristics were observed for isolated β-hemolytic strains. The potent producers of streptokinase were selected for biochemical characterization. The tests were conducted in reference with Cowan and Steel's Manual for the identification of bacteria (Holt et al., 1993).

Production of streptokinase (SK). The potent culture colonies (VB2, VB3, VB8, VB14, VB16 and VB17) producing streptokinase were inoculated in 100 mL of the production medium (g/100 mL: Glucose - 0.5 g, Yeast Extract - 0.5 g, KH_2PO_4 - 0.25 g, $MgSO_4 \cdot 7H_2O$ 0.04 g, $NaHCO_3$ - 0.1 g, $CH_3COONa \cdot 3H_2O$ - 0.1 g, $FeSO_4 \cdot 7H_2O$ - 0.002 g, $MnCl_2 \cdot 4H_2O$ - 0.002 g, pH 7.5) and incubated at 37°C for 24 h (Baewald et al., 1975). After incubation, the individual cultures were centrifuged at 10,000 *g* for 30 min. The cell free supernatants were filtered through a 0.45 μm cellulose acetate filter and the filtrates were considered as crude enzyme.

Enzyme activity—Casein digestion method. The 6 potent strains (VB2, VB3, VB8, VB14, VB16, and VB17) were cultured in Todd Hewitt broth at 37°C for 18–24 h. The culture supernatant was collected by centrifuging at 4°C, 8000 rpm for 10 min. The culture supernatant was used as a crude enzyme source to determine streptokinase activity using casein digestion method, which is based on the determination of the liberated tyrosine from casein after plasminogen activation. The values obtained are converted to 1 μM of tyrosine released per minute (Mounter and Shipley, 1957).

Determination of total protein content. The culture supernatant of the 6 potent strains (VB2, VB3, VB8, VB14, VB16, and VB17) were used as crude enzyme source for the estimation of protein content by the Lowry method. The quantity of protein was determined by the concentration of standard BSA versus absorbance at 550 nm in a UV spectrophotometer (Lowry et al., 1951).

In vitro blood clot lysis assay. The crude enzyme extracted from the strains (VB2, VB3, VB8, VB14, VB16, and VB17) were examined for thrombolytic activity by *in vitro* blood clot lysis activity. Streptokinase (50 μL of 1000 IU/mL) and water were used as positive and negative (non-thrombolytic) control (Holmstrom, 1965; Prasad et al., 2006). The percentage of clot lysis was calculated as given below.

Clot weight
= weight of clot-containing tube – weight of tube alone.

Percentage(%) of clot lysis = (weight of the lysis clot/ weight of clot before lysis) × 100.

Molecular characterization. The potent producers of streptokinase, VB2 and VB14, were characterized at the molecular level for species identification. The total genomic DNA was isolated using phenol chloroform method (Stainsby and Nicholls, 1932). The 16SrDNA gene was amplified using the following primers FC27 (5′ to 3′AGAGTTTGATCCTGGCTCAG) and RC1492 (5′ to 3′ACGGCTACCTTGTTACGACTT). The reaction mixture was carried out in a 25 μl final volume containing 50 ng of DNA, 10 mM dNTPs 2.5 μl of 10 × PCR buffer with 1.5 mM $MgCl_2$, 50 pmol of each primer, 200 μg bovine serum albumin (nuclease free) and 0.2 U *Taq* DNA polymerase in an cycler. The thermal cycling was as follows: initial denaturation at 80°C for 5 min, followed by 30 cycles—denaturation for 30 sec 94°C, annealing for 30 sec at 55°C, elongation for 60 sec at 72°C, with a final elongation at 72°C for 7 min (Petersen and Scheie, 2000). The PCR products were analyzed by agarose gel electrophoresis. The 16SrDNA sequences were integrated into

Table 3. Biochemical characterization of the potent streptokinase producers.

S. No.	Biochemical tests	VB2	VB3	VB8	VB14	VB16	VB17
1	Oxidase test	–	–	–	–	–	–
2	Motility	–	–	–	–	–	–
3	CAMP test	–	–	–	+	–	–
4	Voges proskauer test	–	+	+	+	–	–
5	Pyrrolidonylarylamidase (PYR)	+	+	–	+	+	–
6	ONPG	–	–	–	–	–	–
	Growth at						
7	10°C	–	–	–	–	–	–
8	45°C	+	+	+	–	–	–
	Growth in medium containing						
9	Methylene blue (0.1% in milk)	+	+	+	+	+	+
10	Sodium chloride (6.5%)	+	+	–	+	–	–
11	Bile esculin	+	+	+	+	–	–
	Sensitive to						
12	Bacitracin	–	–	+	–	–	+
13	Optochin	–	–	–	–	–	–
14	Vancomycin	+	+	–	+	+	–
	Hydrolysis of						
15	Aesculin	+	+	–	+	+	–
16	Arginine	+	+	+	–	–	+
17	Starch	–	–	–	–	–	–
	Fermentation of						
18	Glucose	+	+	–	+	+	–
19	Ribose	–	–	–	–	–	–
20	Arabinose	–	+	–	–	–	–
21	Mannitol	+	+	+	–	+	–
22	Sorbitol	–	–	–	–	–	–
23	Adonitol	–	–	–	–	–	–
24	Sucrose	+	+	+	–	–	+
25	Lactose	+	+	+	–	+	–
26	Trehalose	–	–	–	+	–	–
27	Raffinose	–	–	–	+	–	–
28	Starch	–	–	+	–	+	–
29	Glycerol	–	–	+	–	–	–
30	Gelatin	–	–	–	–	–	–
	Production of extracellular polysaccharide						
31	5% Sucrose agar	–	–	–	–	–	–
32	Reduction of TTC (2,3,5-triphenyltetrazolium chloride)	–	–	–	+	–	–

the database with an automatic alignment tool. A phylogenetic tree was generated by performing distance matrix analysis using the neighbourhood joining method (Altschul et al., 1990). Database search and comparisons were carried out with the BLAST search using the National Center for Biotechnology Information (NCBI) database (Saitou and Nei, 1987).

Results

Isolation and screening of streptokinase-producing β-hemolytic Streptococci

Streptococcus has become a major focus of streptokinase production in the pharmaceutical industry, as well as in the medical field worldwide. From 37 cow milk samples, out of 86 strains isolated, 16 isolates were α-hemolytic, 20 were β-hemolytic and 50 were γ-hemolytic. Similarly, 9 colonies isolated from 9 goat milk samples were 3α-hemolytic, 4β-hemolytic and 2γ-hemolytic strains. Among the 4 commercialized milk samples, only 2γ-hemolytic strains were isolated (Fig. 1) (Table 1).

Colonies showing similar morphology were grouped as a single colony. 20β-hemolytic strains were selected for morphological characterization and streptokinase activity (VB1 to VB19 from cow milk samples and VB20 from goat milk samples). The morphological and cultural characteristics of β-hemolytic isolates VB2, VB3, VB8, VB14, VB16 and VB17 showed pinpoint, smooth margin, trans-

Table 4. Streptokinase activity.

Isolates	Volume (mL)	Protein content (mg mL^{-1})	Total protein (mg·mL)	Relative activity (U mL^{-1})	Total activity (U·mL)	Specific activity (U mg^{-1})
VB2	50	0.33 ± 0.08	16.5	265 ± 3.4	13250	813
VB3	50	0.30 ± 0.056	15.0	196 ± 2.8	9800	644
VB8	50	0.30 ± 0.07	15.0	218 ± 4.02	10900	735
VB14	50	0.30 ± 0.092	15.0	225 ± 2.6	11250	749
VB16	50	0.31 ± 0.073	15.5	216 ± 3.65	10800	698
VB17	50	0.29 ± 0.068	14.5	207 ± 2.99	10350	721

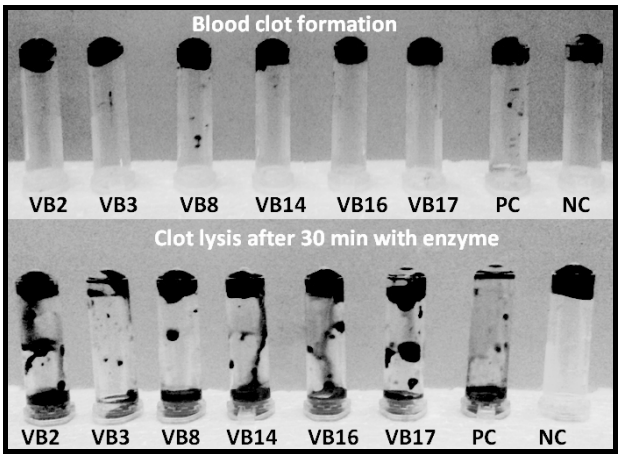

Fig. 4. Blood clot lysis activity of crude enzyme from VB2, VB3, VB8, VB14, VB16 and VB17′. PC - positive control-streptokinase, NC - negative control-distilled water.

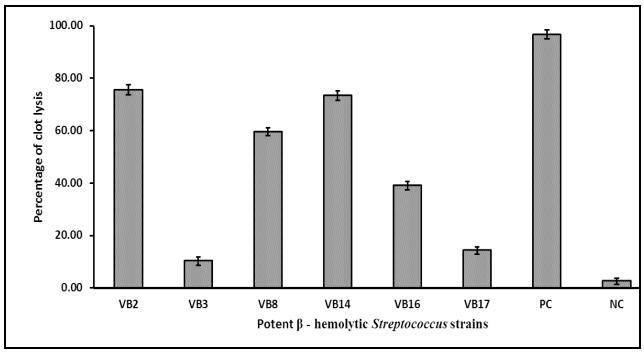

Fig. 5. Percentage of blood clot lysis activity of crude enzyme from VB2, VB3, VB8, VB14, VB16 and VB17. PC - positive control-streptokinase, NC - negative control-distilled water.

lucent or white, gram positive cocci in short and long chains (Fig. 2) with catalase negative indicating maximum streptokinase activity by showing a zone of hydrolysis with the casein plasminogen overlay method (Fig. 3). VB1, VB4, VB10, VB11, VB19 and VB20 showed minimum SK activity (Table 2). The casein plasminogen overlay method is a reliable and quick method for the detection of streptokinase by zone of clearance in order to monitor enzymatic activity (Subathra Devi et al., 2012). VB2, VB3, VB8, VB14, VB16 and VB17 were biochemically characterized and determined as the isolates belong to the genus *Streptococcus* (Table 3).

The total activity of crude SK extracted from VB2 and VB14 was found to be 13250 U and 11250 U, respectively. The selected strain showed a high activity compared to other isolates. The isolate VB3 showed a minimum SK activity of 9800 U and the total protein content was found to be around 15 mg for all the isolates (Table 4). The blood clot lysis activity was observed visually after 3 h and 6 h of incubation for crude SK extracted from VB2, VB3, VB8, VB14, VB16 and VB17. The enzyme volume of 50 μL was able to liquefy the clot completely by 6 h of incubation at room temperature (Fig. 4). The percentage of clot lysis was quantified along with positive control using 1000 U standard streptokinase (10,000 U, Sigma Aldrich) and negative control as distilled water. The crude SK extracted from VB2 showed 78.5% of clot lysis when compared to the positive control. VB14 showed 75.2% of clot lysis activity. The remaining isolates VB8, VB16, VB17, and VB3 showed 60%, 40%, 12.7%, and 10% of blood clot lysis, respectively (Fig. 5).

Molecular characterization

The molecular characterization was carried out using 16S rRNA sequencing. The PCR amplification of the genomic DNA with *Streptococcus* forward and reverse primers resulted in a partial sequence of 873 bp and 950 bp amplicon for the isolates VB2 and VB14. The sequenced strains VB2 and VB14 were confirmed and named as *Streptococcus equinus* VIT_VB2 and *Streptococcus agalactiae* VITVS5, respectively. The BLAST search result of the partial 16S rRNA gene sequences of *S. equinus* VIT_VB2 showed a 98% similarity to *Streptococcus equinus*, and *S. agalactiae* VITVS5 showed a 98% similarity to *Streptococcus agalactiae*. The sequences were submitted to Genbank with accession numbers JX406835 and KF186620, respectively. The phylogenetic tree was constructed based on Evolview software with bootstrap values ≥50%. (Figs. 6 and 7). The current study shows that *Streptococcus equinus* and *Streptococcus agalactiae* isolated from bovine milk samples are capable of producing streptokinase with a good activity equal to other group A, C and G *Streptococcus*.

Discussion

In the present study, we have investigated the biochemical and molecular characterization of isolated strains from milk samples to screen for potent producers of streptokinase. The morphological and biochemical characteristics were similar to those previously reported on *Streptococci*

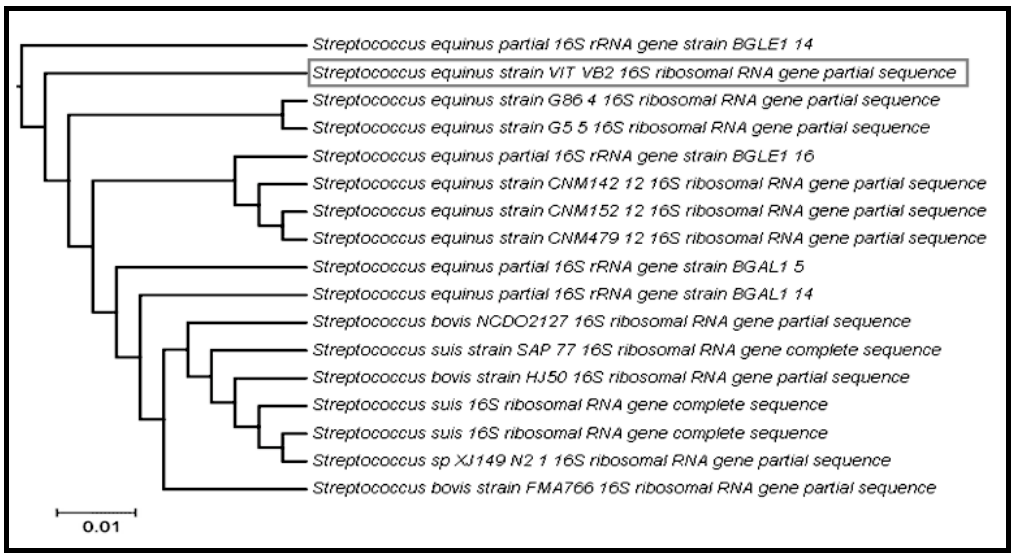

Fig. 6. The neighbor-joining method cladogram showing a phylogenetic relationship between *Streptococcus equinus* VIT_VB2 and other related *Streptococcus equinus*.

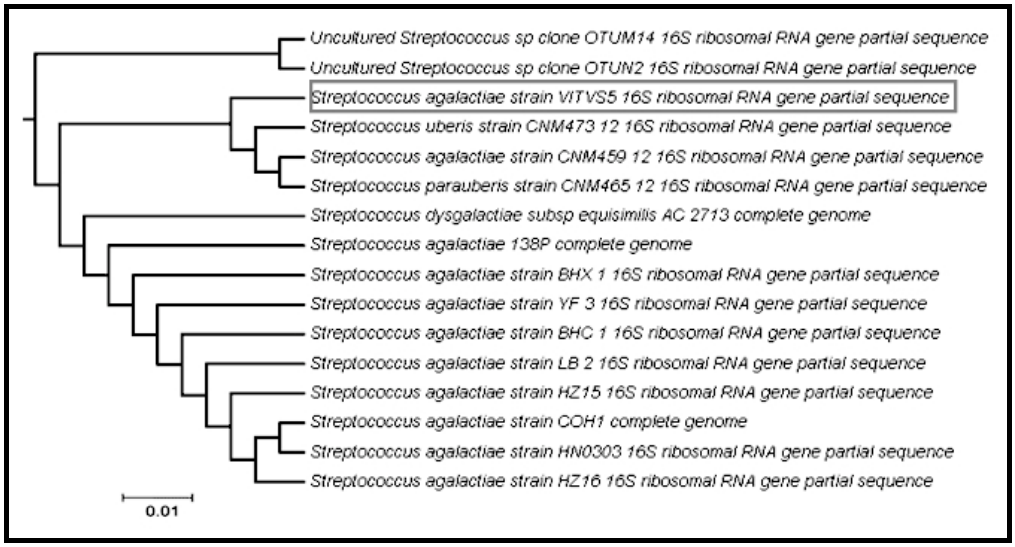

Fig. 7. The neighbor-joining method cladogram showing a phylogenetic relationship between *Streptococcus agalactiae* VITVS5 and other related *Streptococcus agalactiae*.

with a few variations. The six potent producers of streptokinase were identified as *Streptococci* by gram staining (gram positive cocci in chains) and β-hemolysis on blood agar with pinpoint isolated colonies. Previous reports described the biochemical features of *S. equinus* by its tolerance of bile and 6.5% NaCl as well as the hydrolysis of aesculin, acidification of sucrose and lactose characteristics, which identifies the isolate VB2 to be *S. equinus* (Bridge and Sneath, 1983; Facklam and Washington, 1991). While the characterization of *S. agalactiae*, as reported by Van den Heever and Erasmus (1980), namely the acidification of raffinose and trehalose, tolerance of bile and of 6.5% NaCl with vancomycin resistance, aesculin negative and positive CAMP test against *Staphylococcus aureus* identifies the isolate VB14 to be *S. agalactiae*. *S. equinus* (Group D) is an important intestinal bacteria frequently isolated from human and animal specimens as well as from food and vegetables (Bridge and Sneath, 1983). The most common important cause of mastitis in bovines is attributed to *S. agalactiae* (Group B) throughout the world (Keefe, 1997). Previous reports have mentioned the genetic classification of the group D *Streptococci*, among which *S. equinus* belongs to DNA group 1 (Farrow et al., 1984). In parallel with our investigations, the phylogenetic analysis of 16S rDNA sequences reinforces the separation of *S. equinus* from other streptococcal species (Bentley et al., 1991). The Lancefield group B corresponds to a single streptococcal species which is *S. agalactiae* (Daignault et al., 2003). The phylogenetic relationship of *S. equinus* and *S. agalactiae* was found to be similar with their respective strains with significant bootstrap values of 97 for *S. equinus* with *S. equinus* G864, and 99 for *S. agalactiae* with *S. agalactiae* CNM459. Streptokinase produced from different strains of *Streptococci* possesses an intrinsic species specificity for their target plasminogen molecules that parallels the host range

of the microorganisms (Madhuri et al., 2011). Based on the literature cited, two novel plasminogen activators have been described. They were derived from the bovine mastitis-inducing pathogens *Streptococcus uberis* and *Streptococcus dysagalactiae* (Leigh, 1994; Leigh et al., 1998). The streptokinase from *Streptococcus equisimilis* (Lancefield group C) activates human plasminogen, whereas the streptokinase of a Lancefield group E *Streptococcus* activated porcine plasminogen (Ellis and Armstrong, 1971). The total activity of the streptokinase from *S. equinus* was found to be 13250 U, and that from *S. agalactiae* 11250 U, which was relatively higher than the other isolates VB3, VB8, VB16 and VB17. Recently, the activity of purified streptokinase from *S. equinus* VIT_VB2 has been reported by Vaishnavi and Subathra Devi (2014). The increasing potential of SK application prompted us to screen for streptokinase-producing *Streptococcus* from bovine milk. The application of SK in various fields in the last few decades demands an increase in both qualitative improvement and quantitative enhancement. Quantitative enhancement requires strain improvement and medium optimization for the overproduction of the enzyme, as the quantities produced by wild strains are usually too low. The reason may be the changes in the environmental factors such as humidity, temperature and inducers. This study updates the non enterococcal group D *Streptococcus equinus* and group B *Streptococcus agalactiae* and their new variances in the production of streptokinase. The results have revealed that group B and non enterococcal group D *Streptococcus* are also the producers of streptokinase, which can be easily isolated from cow milk and screened for streptokinase by simple microbiological techniques. The study is further continued with strain improvement for enhanced SK productivity. It is believed that the isolates *S. equinus* VIT_VB2 and *S. agalactiae* VIT VS5 can be exploited commercially for large-scale industrial production of SK which can join with other SK producing *Streptococcus* like *S. pyogenes*, *S. equisimilis*, and *S. dysagalatiae*. Future studies should include sequencing of the other isolates and the expression of the SK gene in a suitable recombinant host.

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References

Abdelghani, T. T. A., Kunamneni, A., and Ellaiah, P. (2005) Isolation and mutagenesis of streptokinase producing bacteria. *Amer. J. Immunol.*, **1**, 125–129.

AHA (American Heart Association) (2009) Dallas Heart Disease and Stroke Statics-2009.

Allender, S., Scarborough, P., Peto, V., and Rayner, M. (2008) European Cardiovascular Disease Statistics 2008.

Altschul, S. F., Gish, W., Miller, M., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.*, **215**, 403–410.

Baewald, G., Mayer, G., Heikel, R., Volzke, K. D., Roehlig, R. et al. (1975) Fermentative production of *Streptococcus* metabolites, especially streptokinase. German Patent DD 111096.

Bentley, R. W., Leigh, J. A., and Collins, M. D. (1991) Intrageneric structure of *Streptococcus* based on comparative analysis of small-subunit rRNA sequences. *Int. J. Syst. Bacteriol.*, **41**, 487–494.

Bridge, P. D. and Sneath, P. H. A. (1983) Numerical taxonomy of *Streptococcus*. *J. Gen. Microbiol.*, **129**, 565–597.

Collen, D. (1996) Fibrin-selective thrombolytic therapy for acute myocardial infarction. *Circulation*, **93**, 857–865.

Collen, D., Decock, F., Vanlinthout, I., Declerck, P. J., Lijnen, H. R. et al. (1992) Comparative thrombolytic and immunogenic properties of staphylokinase and streptokinase. *Fibrinolysis*, **6**, 232–242.

Daignault, D., Guévremont, E., Guillemette, J. M., Messier, S., Gottschalk, M. et al. (2003) Serotypes of *Streptococcus agalactiae* cultured from dairy milk samples in Québec. *Can. Vet. J.*, **44**, 217–220.

Dundar, Y., Hill, R., Dickson, R., and Walley, T. (2003) Comparative efficacy of thrombolytics in acute myocardial infarction: A systematic review. *QJM*, **96**, 103–113.

Ellis, R. P. and Armstrong, C. H. (1971) Production of capsules, streptokinase, and streptodornase by *Streptococcus* group E. *Amer. J. Vet. Res.*, **32**, 349–356.

Facklam, R. R. and Washington, J. A. (1991) *Streptococcus* and related catalase-negative Gram-positive cocci. *In* Manual of Clinical Microbiology, 5th ed., ed. by Balows, A., Hausler, W. J., Herrmann, K. L., Isenberg, H. D., and Shadomy, H. J., American Society for Microbiology, Washington, D.C., pp. 238–257.

Farrow, J. A. E., Kruze, J., Phillips, B. A., Bramley, A. J., and Collins, M. D. (1984) Taxonomic studies on *Streptococcus bovis* and *Streptococcus equinus*: description of *Streptococcus alactolyticus* sp. nov. and *Streptococcus saccharolyticus* sp. nov. *Syst. Appl. Microbiol.*, **5**, 467–482.

Holmstrom, B. (1965) Streptokinase assay on large agar diffusion plates. *Acta Med. Scand.*, **19**, 1949–1954.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., and Williams, S. T. (1993) Group 17. Gram-positive cocci. *In* Bergey's Manual of Determinative Bacteriology, 9th ed., Williams & Wilkins, Baltimore, MD, pp. 527–533.

Jackson, K. W. and Tang, J. (1982) Complete amino acid sequence of streptokinase and its homology with serine proteases. *Biochemistry*, **21**, 6620–6625.

Keefe, G. P. (1997) *Streptococcus agalactiae* mastitis: a review. *Can. Vet. J.*, **38**, 429–437.

Leigh, J. A. (1994) Purification of a plasminogen activator from *Streptococcus uberis*. *FEMS Microbiol. Lett.*, **118**, 153–158.

Leigh, J. A., Hodgkinson, S. M., and Lincoln, R. A. (1998) The interaction of *Streptococcus dysgalactiae* with plasmin and plasminogen. *Vet. Microbiol.*, **61**, 121–135.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

Macfarlane, R. D. (1977) Hemostasis: Introduction. *Br. Med. Bull.*, **33**, 183–194.

Madhuri, D. H., Madhuri, M., Neha, A. S., Mohanasrinivasan, V., and Subathra Devi, C. (2011) Studies on screening, strain development, production and optimization of streptokinase from β-hemolytic *Streptococci*. *World J. Sci. Technol.*, **1**, 7–11.

Mounter, L. A. and Shipley, B. A. (1957) The inhibition of plasmin by toxic phosphorus compounds. *J. Biol. Chem.*, **226**, 855–861.

Ozegowski, J. H., Gerlach, D., and Kohler, W. (1983) Influence of physical parameters on the production of streptococcal extracellular proteins in cultures with stabilized pH: 2. Temperature dependence of extracellular protein production. Zentralbl Bakteriol. *Microbiol. Hyg.*, **254**, 361–369.

Petersen, F. C. and Scheie, A. A. (2000) Genetic transformation in *Streptococcus mutans* requires a peptide secretion-like apparatus. *Oral Microbiol. Immunol.*, **15**, 329–334.

Pike, R. M. (1944) An enrichment broth for isolating hemolytic *Streptococci* from throat swabs. *Proc. Soc. Exp. Biol. Med.*, **57**, 186–187.

Prasad, S., Kashyap, R. S., Deopuj, J. Y., Purohit, J. H., Taori, G. M. et al. (2006) Development of an in vitro model to study clot lysis activity of thrombolytic drugs. *Thromb. J.*, **4**, 14–16.

Sahni, G., Kumar, R., Roy, C., Rajagopal, K., Nihalani, D. et al. (2007) Clot specific streptokinase proteins processing altered plasminogen activation characteristics and a process for their preparation. US Patent No. 7,163,817, B2.

Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*., **4**, 406–425.

Stainsby, W. J. and Nicholls, E. E. (1932) Technic for isolation of *Streptococci*. *J. Lab. Clin. Med*., **17**, 530–538.

Subathra Devi, C., Sinha, D., Sharma, V., and Mohanasrinivasan, V. (2012) Screening for staphylokinase producing *Staphylococcus* spp. from different environmental samples. *Asian J. Pharm. Clin. Res*., **5**, 125–128.

Subathra Devi, C., Mohanasrinivasan, V., Vaishnavi, B., Selvarajan, E., and Jemimah Naine, S. (2013) Optimization studies for enhanced production of streptokinase by *Streptococcus equisimilis* UVM6. *J. Pure App. Microbiol*., **7**, 2337–2341.

Tough, J. (2005) Thrombolytic therapy in acute myocardial infarction. *Nurs. Stand*., **19**, 55–64.

Vaishnavi, B., Mohanasrinivasan, V., and Subathra Devi, C. (2011) Streptokinase: A novel clot buster. *J. Pharm. Res*., **4**, 3784–3788.

Vaishnavi, B. and Subathra Devi, C. (2014) In vitro thrombolytic activity of purified streptokinase extracted from *Streptococcus equinus* VIT_VB2 isolated from bovine milk. *J. Thromb. Thrombolysis*, doi:10.1007/s11239-014-1093-2.

Van den Heever, L. W. and Erasmus, M. (1980) Group B *Streptococcus*—comparison of *Streptococcus agalactiae* isolated from humans and cows in the Republic of South Africa. *J. S. Afr. Vet. Assoc*., **51**, 93–100.