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Research Article

CHARACTERIZATION AND PURIFICATION OF STREPTOLYSIN O TOXIN BY AMMONIUM SULPHATE FRACTIONATION METHOD

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ABSTRACT

It is a Gram-positive, non-motile, non-spore forming coccus that occurs in chains or in pairs of cells. Investigatin of streptococci species from throat sample by blood agar plate method. Morphological and biochemical characterization of the throat swab culture was identifying the streptococci. Streptolysion O isolated from the group A beta haemolytic *Streptococci* has the ability to lysis the RBC's which was confirmed by haemolytic assay. It was confirmed such as Bacitracin disc diffusion test, Catalase test and coagulase test. Streptolysin O toxin purification employed by ammonium sulphate fractionation method. After that total protein estimation followed by Lowry's method.

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INTRODUCTION

Group A *Streptococcus* (institution A strep) is a bacterium that could reason many exceptional infections, consisting of strep throat, scarlet fever, impetigo, and others. The bacteria stay within the nose and throat. One of the ways you can get ill is if you breathe in those droplets or if you touch something that has the droplets on it and then touch your mouth, nostril, or eyes. [Shulman *et al*., 2012]. It is a Gram-positive, non-motile, non-spore forming coccus that occurs in chains or in pairs of cells. Individual cells are round-to-ovoid cocci, 0.6-1.0 micrometer in diameter. When the bacteria are introduced or transmitted to vulnerable tissues, a variety of types of suppurative infections can occur [Graziella et al., 2001].

Virulence elements of organization A streptococci include: (1) M protein, fibronectin-binding protein (Protein F) and lipoteichoic acid for adherence; (2) hyaluronic acid tablet as an immunological cover and to inhibit phagocytosis; M-protein to inhibit phagocytosis (three) invasins which includes streptokinase, streptodornase (DNase B), hyaluronidase, and streptolysins; (4) exotoxins, consisting of pyrogenic (erythrogenic) toxin which reasons the rash of scarlet fever and systemic toxic surprise syndrome [Gabriele Sierig *et al*., 2003].

Beta -hemolysis is related to entire lysis of purple cells surrounding the colony, whereas alpha-hemolysis is a partial or "green" hemolysis associated with reduction of purple mobile hemoglobin. Non hemolytic colonies had been termed gamma-hemolytic. The mobile wall is composed of repeating gadgets of N-acetyl glucosamine and N-acetyl muramic acid, the standard peptidoglycan. Traditionally, the definitive identification of streptococci has rested on the serologic reactivity of "mobile wall" polysaccharide antigens as initially defined through Rebecca Lancefield. More than 20 serologic groups have been identified and designated by letters (eg, A, B, C) [Azar et al., 2016]. Of the non–group A streptococci, group B is the most important human pathogen. Streptolysin O is toxic to a wide variety of cell types, including myocardium, and is highly immunogenic. The determination of the antibody responses to this protein (antistreptolysin O titer) is often useful in the serodiagnosis.

MATERIALS AND METHODS

Isolation of bacteria

The inoculums of streptococcus was taken from the throat infection in human, as these bacteria feed on the blood cells it was usually available on wounded throat. *Streptococcus* used in this study is listed in the table -1. GAS isolate from a patient with severe throat infection that are able to produce streptolysin O (SLO). Group A *Streptococcus* were grown in Brain Heart Infusion agar with 5% blood.

Characterization of bacteria

Primary screening of throat infection causing agents were done by gram staining test, which conforms the presence of gram positive bacteria. Streptococcus was confirmed by the biochemical tests, hemolysis reaction on blood agar, agglutination test, and bacitracin disc test and Gram staining, catalase, coagulase, Oxidase etc.,

Bacitracin susceptibility test

The Bacitracin disk is sensitivity test used to differentiate the beta-hemolytic streptococcus. An overnight culture grown on 5% blood agar incubated 35^0C. Select a beta- hemolytic colony and heavily inoculate a quadrant of a 5% blood agar plate. Drop a bacitracin disk in the heaviest zone of

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inoculation. Tap disk lightly to ensure that it adheres to the agar and incubate plate overnight in 35^0C.

Hemolysis activity

Blood is used because of the convenience in testing throat swabs for beta –hemolytic streptococci. Sheep blood does not support the growth of *Haemophilus haemolyticus* which appears similar to streptococci on agar containing rabbit, horse or human blood ß-hemolytic streptococci. Streak culture for isolation on Brain Heart infusion agar plate with 5% blood and incubate plate at 35^0C in 24 hrs [Tapsall. J. W.1987].

Fractional precipitation by ammonium sulfate

Saturated Ammonium sulphate (GR Himedia) solution of pH 7.0 was added to the diluted plasma slowly with constant stirring [Dieter Gerlach *et al*., 1993]. The mixture was kept overnight to complete precipitation. Next day, the precipitated protein was filtered through chain cloth bags and the precipitates were washed again with Ammonium sulphate solution. The mixture was again kept overnight at room temperature. Next day, the precipitated mixture was filtered through chain cloth. The precipitated was collected, dissolved in D.H_2O and dialyzed using cellophane bags. The dialyzed protein, 0.85% (w/v) NaCl was added to make it isotonic condition. The purified protein was estimated for total protein, albumin and globulin.

Streptococcal antigen preparation

Streptococcus pyogens was used to prepare the test antigen. Cells were grown in flasks containing 100 ml of Brain Heart Infusion broth in overnight and cells were harvested by centrifugation. Cells were washed three times with distilled water and the heat killed by placing the suspension in a water bath at 65^0C for 30 minutes. Cell wall proteins were removed enzymatically; first used 0.1 percent pepsin solution prepared from pepsin powder (Himedia) and pepsinization allowed continuing for two hours at room temperature. Finally suspension titrated against positive ASO serum [Mark Reglinsk *et* al., 2016]. The antigen remains stable for many months under ordinary refrigeration and frequent removal to room temperature during use [John J Redys *et al*., 1972].

Total estimation of protein by Lowry's method

BSA was weighed accurately 100mg of BSA and dissolved in distilled water and make up to 100 mL in a standard flask.1mL of the solution contains 1 mg of protein. Pipette out 0.1, 0.2, 0.3, 0.4, 0.5 mL of the working standard into a series of test tubes. Pipett out different volume of the sample extract in separate test tubes. Make up the volume to 1mL in all the test tubes. A tube with 1mL of water serves as the blank. Added 5mL of reagent C to each tube including the blank and mix well and allow standing for few minutes. Then added 0.5mL of reagent D, mix well and incubated at room temperature in the RT for 10 min. Blue color is developed. The procedure was performed the same for test's samples respectively. The readings were taken at 660nm. The amount of protein in the sample was calculated by plotting the standard graph. The samples were analyzed for the molecular weight determination using SDS-PAGE and Gel documentation.

SDS-PAGE analysis

The gel was prepared between two glass plates. The glass plates were cleaned first with a detergent and then thoroughly with distilled water. The two glass plates were placed together, with the 3 spacer between them (along the side edges and along the bottom edge) and the whole assembly was fixed tightly with clamps. The two side edges and the bottom edge were sealed with 2% agar and the assembly was kept vertically on a flat surface. The running gel was prepared and mixed solutions without TEMED in a 100 mL conical flask and deserted. TEMED was added and mixed and poured immediately between the glass plates, upto about 2.5 cm below the notch. After the completion of run, the power supply was disconnected and the gel assemble was removed from the electrophoresis chamber. Then the side spacers and the notched glass plates were removed with the help of spatula. Finally the gel stained with Coomassie brilliant blue staining.

RESULTS AND DISCUSSION

In present investigation of isolation of streptococci from sore throat of different severities, 3 samples were positive in which the isolates were identified as group A *Streptococci* on the basis of phenotypic identification criteria. Which was further identified using gram's stain, catalase test, oxidase test [Lowbury *et al*., 1964].

Morphological and biochemical test results

Table 1- Gram staining results

S. No	Isolates	Morphology	Gram stain	Catalase	Coagulase
1	P1	Rod	-	-	-
2	P2	Cocci	+	-	-
3	P3	Rod	-	-	-
4	P4	Rod	-	-	-
5	P5	Cocci	+	-	-
6	P6	Rod	-	-	-
7	P7	Rod	-	-	-
8	P8	Rod	-	-	-
9	P9	Cocci	+	-	-
10	P10	Rod	-	-	-
11	P11	Rod	-	-	-

(+ Positive) (- Negative)

Morphological, physiological and biochemical analysis characterization of the throat swab cultures were carried out to identify the streptococci species. Catalase test was negative because of absence of bubble formation. Coagulase test result indicates the selected isolates fail to produce coagulase positive reaction. Thus the cultures were differentiated from *staphylococcus*. Gram positive cocci were observed in under oil immersion lens. Which were isolated from throat swab culture in blood agar. Zone of inhibition was found in bacitracin disc diffusion test and considered as positive result. The catalase test was negative as there was no bubble formation. Thus the culture was differentiated from staphylococcus. The catalase test gives the majority of differentiations very efficiently. Sometime observe false positive results will result if any red blood cells are transferred. Weak positive results should be repeated on a blood free medium.

A beta hemolytic reaction (Joseph E Alouf *et al*., 1988) resulted as complete clearing around the colony (Fig: 2). an alpha hemolytic reaction is observed as greening around the colony and gamma haemolysis is interpreted as no change in

the media surrounding the colony. The hemolytic reaction on blood agar is complex and subject to many variables.

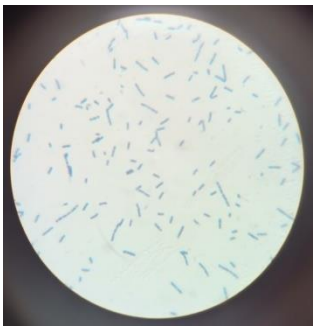


Fig: 1- Microscopic observation **Fig: 2** Hemolysis in blood agar plate

Haemolysin was present in Brain Heart Infusion Agar medium supplemented with 5% blood. After centrifugation the supernatant was collected. Supernatant have a protein O molecules. Group A *Streptococci* to produce hemolytic properties on blood agar [Evelyn M. Molloy *et al*., 2014].

Fig: 3, 4 bacitracin disc diffusion test

Fig: 5 -Brain Heart Infusion Broth Fig: 6 Dialysis

Isolated pure culture inoculums into Brain Heart Infusion broth and incubated 37^0C for 24 hours. After completion of incubation the culture medium was centrifuged at 3000 rpm for 20 minutes. The supernatant was collected which is contain erythrogenic toxin. The Purification of the erythrogenic toxin is the supernatant to reduce the volume to an amount erythrogenic protein concentrated by this way of centrifugation. This material was precipitated with saturated ammonium sulphate solution. After overnight incubation the supernatant loaded in cellophane bag for dialysis. Finally, the purified protein molecules obtained from cellophane bag. [vipin and Nandha 2015].

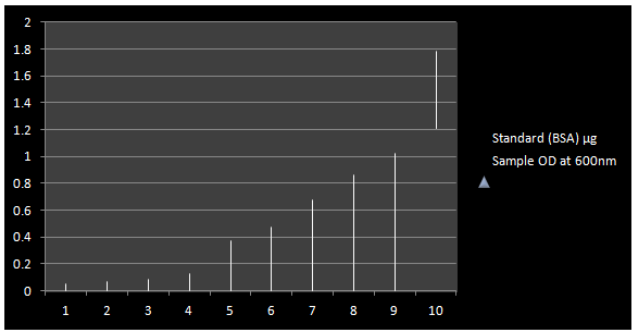
After dialysis the samples was estimated in total protein concentration by Lowry's method. Thus it is concentration of total protein 169 µg/ mL. (Table-2) previous study report agreed with total protein concentration estimation and quantity [John J Redys *et al*., 1972].

Protein estimation by Lowry's method

Calculation

Total protein (µg / mL) = 160µg / 1.210 x 1.783 = 169µg / mL

Estimation of protein for the sample was done by Lowry's method. Optical Density was observed at 600 nm and protein molecules concentration was found to be 169 µg / mL.



Graph: 1 Estimation of protein from sample

SDS- PAGE

Sample purity was analysed by SDS PAGE lane 1 was loaded with marker. Lane 2 and lane 3 shows the band for Streptolysin O which was separated from dialysis bag. Dialysed SLO molecular weight as 65kDa.SDS PAGE of purified erythrogenic toxin from *streptococcus pyogens* showed band with molecular weight in the range of 66 kDa. There is no difference in the molecular weight of the isolated *streptococcus pyogens* toxin. Serologically active erythogenic toxin was found in the identity of the toxin band of SDS PAGE. The purity of samples was monitored and the molecular weight was estimated by SDS- poly acrylamide gel electrophoresis with a 12.5% running gel by the method. SLO had a molecular weight of 75000 according to SDS –PAGE [Dieter Gerlach *et al*., 1993].

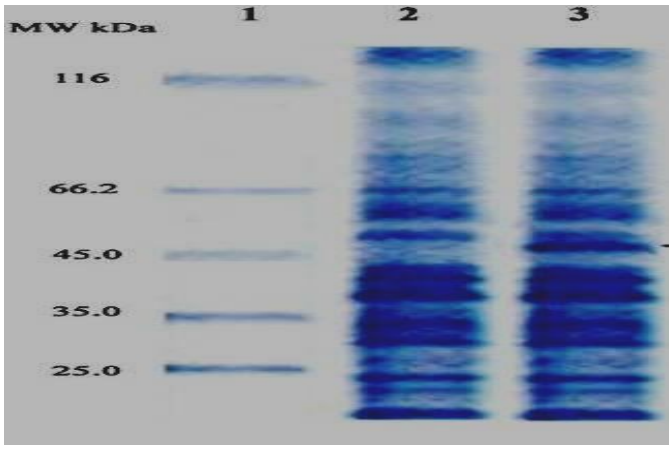


Fig: 7 purity analysis of streptolysin O toxin by SDS-PAGE

CONCLUSION

The work reports that the streptococci present in the throat swab were isolated by blood agar plate method. Morphological and biochemical characterization of the throat swab culture was identifying the streptococci. Streptolysion O isolated from the group A beta haemolytic *Streptococci* has the ability to lysis the RBC's which was confirmed by haemolytic assay. It was confirmed such as Bacitracin disc diffusion test, Catalase test and coagulase test. Streptolysin O

toxin purification employed by ammonium sulphate fractionation method. After that total protein estimation followed by Lowry's method. Total protein was found to be 169µg/mL. It was confirmed through SDS PAGE that the molecular weight of purified toxin is 65kDa.

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