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# Partially Purification M Protein from Streptococcus Pyogens and Molecular Study of Some Virulence Factors

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**Abstract**
The current study was conducted to isolation one type of surface protein of *Streptococcus pyogens* this protein separated using partially purification method. Purifiction process include precipitation of crude M protein one peak of this protein was appeared, the best precipitate rate was 30% . PCR assay were performed to identify the presence of some genes related to pathogenicity in bacteria responsible from Tonsillitis .*Streptococcus pyogens* represented the largest causative agent of inflammation of the tonsil so the study revealed the presence of certain gene in some isolate of bacteria, including the gene *SpeB* that gave large proportions in the study isolates 12 out of 37 at a rate of (32.4%).
**Keywords** : *S.pyogens* , Tonsillitis , M protein ,PCR ,*SpeB*

## Introduction

M proteins are cell-surface attached proteins that are composed of two polypeptide chains that forms an alpha-helical, coiled-coil configuration .They have an LPXTG motif at their carboxyl terminal part. This protein Confers *S. pyogenes* with the property to resist killing in human blood. The M proteins occur in pairs, twisted around each other in a coiled coil structure (Fischetti *et al*, .1990). The major virulence factor reported in GAS is the M protein encoded by emm gene. The M protein confers resistance to phagocytosis by polymorph nuclear leukocytes (Oehmcke *et al*, .2004). M-protein belongs to a large pathogenicity island that was probably acquired before the *S. pyogenes* speciation. Its potential instability could have practical implications for species identification in the clinical laboratory (Pahlman *et al*., 2008).SpeB gene is a chromosomally located on every GAS strains and has cysteine protease activity (James *et al*, .2002) which enables evasion of innate immunity via degradation of complement factors (Honda-Ogawa *et al*, .2013). It carries laminin- binding activity of the bacteria, it is not only secreted molecule but also occurs unexpectedly tightly bound to the bacterial surface (Hytonen *et al*,.2001).SpeB is the predominant extracellular protein in streptococcal culture supernatant and secreted as a 42-kDa zymogen and auto catalyzed into an active 28-kDa cysteine protease It cleaves or degrades host extracellular matrix, immunoglobulins, complement components, and even GAS surface adhesions (M protein and protein F1), C5a peptidase, and several secreted proteins (Chen *et al*,.2003).and tumor necrosis factor-β, with weak T helper 2 cytokines responses (Cunningham, 2008).

## Material and Method

### Detection presence of M protein

M protein was detected in GAS by using the indirect bacterial test as follows:
Serial dilution $10^{-1}$ to $10^{-8}$ was conducted for testing bacteria which grown for 18 hours. 0.1 ml of each dilution was pipetted onto plates with blood- brain heart infusion media; spread with a glass spreader and incubated at 37 ºC for 18 hours, then we calculated the number of living cells.
After that, 0.1 ml of each dilution was transferred to sterile plan tubes (duplicate), then 0.4 ml of blood of healthy persons which considered the source of phagocyte cells. One of the tubes was incubated at shaker incubator for 20 RPM/min and the other was incubated at 37 ºC for 3 hours. 0.1 ml of fixed and mobile treatment was pipetted onto plates with blood- brain heart infusion media using sterile spreader and incubation at 37ºC for 18 hours. The numbers of living cells were calculated and the test is positive when the growth in symmetric treatment (Lancefield and Perlmann, 1952).

### Partially purification M protein from *Streptococcus pyogens*

Crude M protein was extracted by limited pepsin digestion according to the method of (Manjula and Fischetti, 1980).

**Determination of protein Concentration :** The protein concentration was determined according to Bradford (1976) method.

 

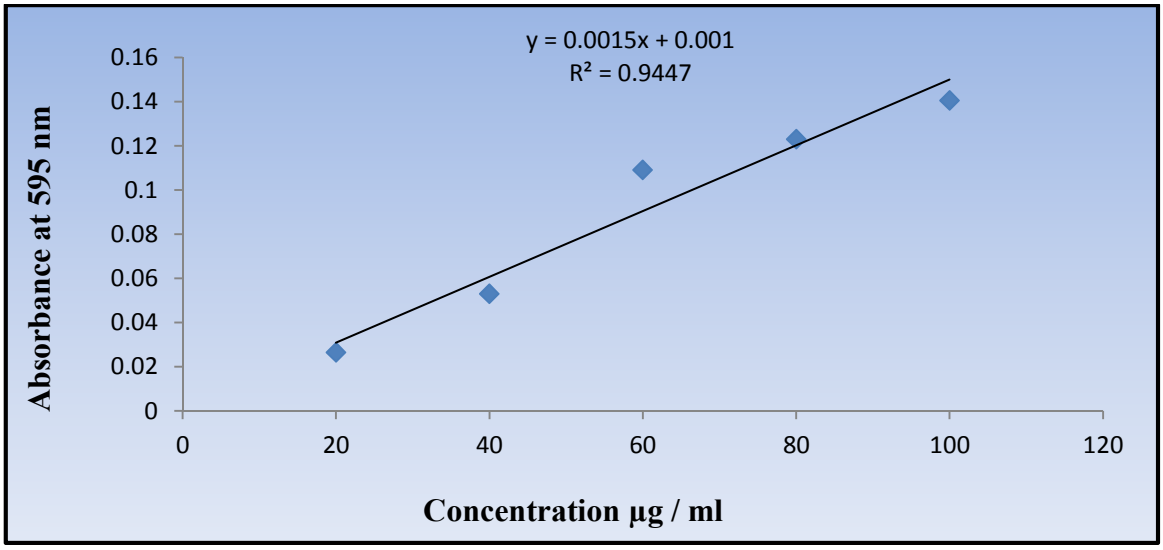


**Figure (1):** Standard curve of protein concentration.

**Ammonium sulphate precipitation**

The ammonium sulfate was added in different saturation ratio (30%,40% 50% 60%)to reach the optimum ratio of ammonium sulfate by adding gradually the amount of salt to each 25 ml protein solution in ice bath and magnetic stirrer for 1hour ,the procedure include centrifugation the solution at 3500xg for 10 minutes. The supernatant was dropped while the precipitate was dissolved in minimum volume of phosphate buffer (0.01 PH, 8).

**Gel Filtration Chromatography:** Separation and purification protein was caring out using sphedaxG-200gel method.

**Preparation of sephadex G-200 gel**

The preparation of gel was achieved as recommended by supplied company, since by take 3gram from gel sephadex G-200 was suspended in 105ml D.W and put in water bath at 90 °C for 5hours then wash twice with phosphate buffer prepared in (3-7-3), after that the gel was suspended in amount of the same buffers, then the gel was degassed by using vacuum pump, the gel was packaged gently in glass Colum with dimension (1.5x16.5). The Colum was equilibrated using same buffers which used in gel.

**Molecular Assay**

**DNA Extraction**

A single colony of *Streptococcus pyogens,* which had been incubated overnight, transfer to sterile Brain heart infusion Broth and incubated at 37 ◦C for 24 hour. The DNA extracted using the genomic DNA purification kit (Geneied) according to manufacturing instructions.

**PCR assay**

A PCR (Polymerase Chain Reaction) is performed in order to make a large number of copies of a gene. Otherwise, the quantity of DNA is insufficient and cannot be used for other methods of testing. A PCR is performed on an automated cycler, which heats and cools the tubes with the reaction mixture in a very short time. PCR is performed in three major steps denaturation ,annealing and extension.

**SpeB gene**

One gene primer for *Streptococcus pyogens* virulence factor was used for genetic detection of these bacteria, this gene is *SpeB.* Sequence and size product of this primer found in table 1.

**Table (1): Primer used in PCR**

| Primer name | Product size (pb) | Sequences | References |
|---|---|---|---|
| SpeB F<br>SpeB R | 423 | 5'GTAGCAACACATCCTGTAGCTGCA'3<br>5'AGGTGCAC GAAGCG CAG AAG ATAT'3 | (Naglaa *et al*,.2014) |

**The reaction mixture**

Amplification of DNA was carried out in a final volume of 20µl containing master mix (Bioneer) , SpeB primer and the bacterial extracted DNA with a volume pointed in table 2.

 

**Table (2): Protocols of PCR reaction mixture volumes**

| NO | Content s of reaction mixtures | Volume |
|---|---|---|
| 1 | Master mix | 5 µl |
| 2 | Forward primer | 2.5 µl |
| 3 | Reveres primer | 2.5 µl |
| 4 | DNA template | 5 µl |
| 5 | Nuclease free water | 5 µl |

**Thermal reaction Cycling Conditions**

Reaction processes for each gene in thermal cycle was differing in type of gene .Specificity and reference recommendation as follow:

**Table (3)** Thermal cycling conditions.

| steps | Temperature | Time | NO. of cycles |
|---|---|---|---|
| 1- Initial denaturation | 94 °C | 3min | 1 |
| 2- Denaturation | 94 °C | 7s | 3 |
| 3- Annealing | 55 °C | 10s | 25 |
| 4- Elongation | 72 °C | 5s | 2 |
| 5- Final elongation | 72 °C | 3min | 2 |
| 6-Hold | 4 °C | α | 1 |

**Results**

**Purification of M protein**

**Ammonium sulphate precipitation**

The crude extract of M protein was precipitated by ammonium sulphate precipitation method . Results of this study showed that 30% of the crude extract M protein is best ratio for precipitate from *Streptococcus pyogens.*

**Gel filtration chromatography for M protein**

When the ammonium sulphate precipitated, M protein solution was passed through sephadex G-200 Colum that equilibrated with phosphate buffer (0.01 M, pH 8), the fraction was collected and measured at 280 nm absorbency, one peak of protein was appeared, and one peak is M protein show in figure (2)

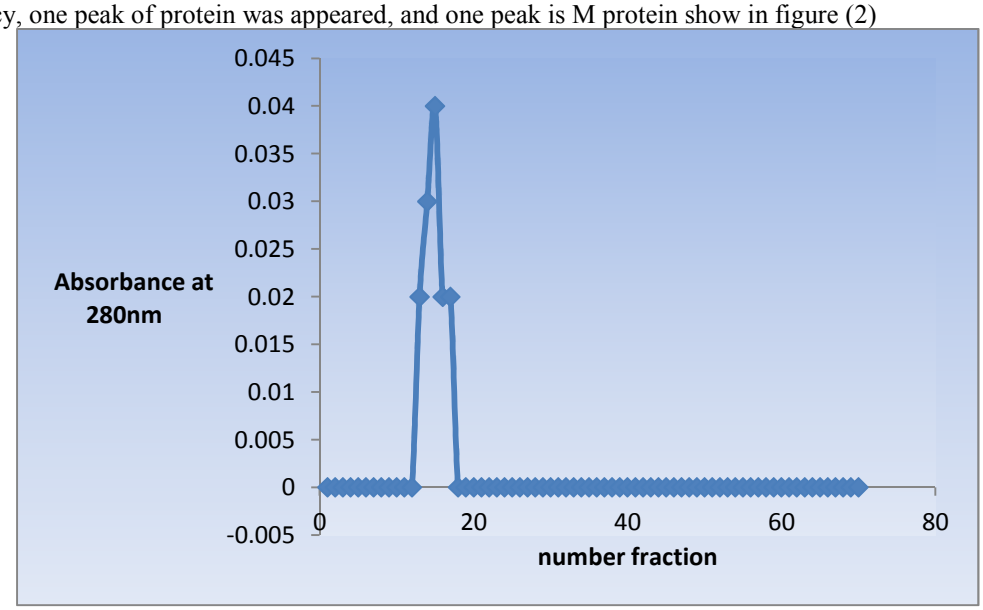


**Figure (2)** Gel filtration chromatography using G-200 Colum (1.5x16.5) for purification M protein from *Streptococcus pyogens* with 0.01M and PH 8 phosphate buffer, flow rate 36ml/60min and fraction volume 3ml.

**Molecular study**

Because of *Streptococcus pyogens* appear to be predominant bacterial in tonsillitis samples .These isolates were applied in genetic study.DNA was extracted for all *S.pyogens* isolates and the DNA concentrated was measured using Nano drop apparatus. DNA samples that show positivity for DNA concentration were used for searching for SpeB**.** Molecular study reveals12 positive isolates from 37 samples (32.4%).

 

***SpeB gene***

The *SpeB* gene considered virulence factor gene for *Streptococcus pyogen*s and used for diagnosis *Streptococcus pyogens* and molecular weight for423.Positive results for amplification this gene that gave large proportions in the study isolates 12 out of 37 at a rate of (32%) figure (3)

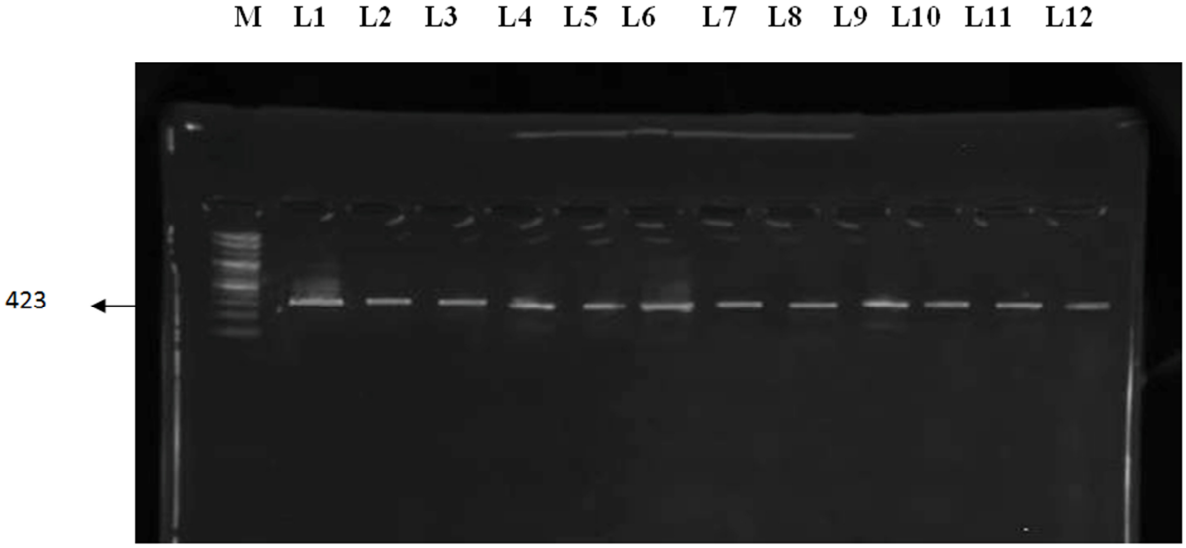


**Figure (3)** 2% Agarose Gel Electrophoresis of423bp PCR products of SpeB gene, lane M represent 2000bp molecular marker, lane 1, 2, 3, 4, 5,6, 7,8,9,10,11,12 represent sample no. of positive results .

**Discussion**

**Purification of M protein**

In this study was showed 30% the best ratio to precipitate because it gave highly phagocytosis. These results agreed with (Edwin *et al*, .1977). The ammonium sulphate was used in enzyme or protein precipitation because of its highly solubility and cheap cost compared with the other salts - unaffected in PH and enzyme equilibrating the charges found in protein surface and disruption of the water layer surrounding it that leads to its precipitation. Gel filtration chromatography by using sephadex G-200 Colum was the next step of M protein purification after the step of ammonium sulphate precipitation. The results showed one peak of protein**,** one peak mean that only found M protein this result is agreements with (Dahee *et al*,.2002).

Sephadex G-200 has many advantages included fast run, high recovery separation, simple preparation and its stability for long time permits reusing of the gel many times in protein separation (Thomas, 2011).

**Molecular study**

*SpeB* gene is virulence factor used for diagnosis of *Streptococcus pyogens* results found 12 isolates out of 37 at rate (32.4%) positive result and molecular weight 423pb this study is correlated with (Santos *et al*,.2003) who found *SpeB* isolates is (25%) in contrast with (Dmitrieva *et al*,.2000) who found *SpeB* isolates (69%) .

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