British Journal of Medicine & Medical Research
13(6): 1-10, 2016, Article no.BJMMR.22800
ISSN: 2231-0614, NLM ID: 101570965

SCIENCEDOMAIN *international*
www.sciencedomain.org

The Inductive Effect of Purified Streptococcal M-protein on Immunity against *Streptococcus pyogenes* in Rabbit Model

Jawad K. Tarrad Al-Khafaji[1*]

[1]Department of Microbiology, College of Medicine, Babylon University, Iraq.

Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

Article Information

DOI: 10.9734/BJMMR/2016/22800
Editor(s):
(1) Renu Gupta, Department of Microbiology, Institute of Human Behaviour and Allied Sciences, New Delhi, India.
(2) Arun Chauhan, Department of Immunology and Microbiology, School of Medicine and Health, University of North Dakota, USA.
(3) Philippe E. Spiess, Department of Genitourinary Oncology, Moffitt Cancer Center, USA and Department of Urology and Department of Oncologic Sciences (Joint Appointment), College of Medicine, University of South Florida, Tampa, FL, USA.
Reviewers:
(1) Charbell Miguel Haddad Kury, Medical School of the Municipality of Campos dos Goytcazes, Brazil.
(2) Anonymous, Nagoya City University, Japan.
(3) Maen Mahfouz, Al-Zafer Hospital, Najran, Saudi Arabia.
Complete Peer review History: http://sciencedomain.org/review-history/13081

Original Research Article

Received 27th October 2015
Accepted 18th December 2015
Published 26th January 2016

ABSTRACT

Aims: The study aimed to know the effects of the purified M-protein on immune system to produce protection against *Streptococcus pyogenes* in rabbits.
Study Design: Case-control study.
Place and Duration of Study: In this study, collection samples and bacterial identification were carried out in two hospitals; Child Protection Hospital and Central Child Hospital in Baghdad city, and experimental work was done in Department of Medical Microbiology, College of Medicine-Babylon university, Iraq. The study was done during the period between January to July 2014.
Methodology: A total of 260 samples were collected from tonsillitis and pharyngitis cases. Three main parts involved in this study: the first part is bacterial diagnosis based on relied diagnostic procedures. The second part is detection of serogroup of GAS and antistreptolysin O (ASO) antibodies by using latex agglutination test, and the third part is experimental study conducted on the protective immune response against the group A streptococci using rabbit model. M-protein was purified by using Ion exchange chromatography. The rabbit models were immunized with purified M protein according to standard method. The immune response generated against the M protein was

**Corresponding author: E-mail: mbmc.kadhum70@gmail.com;*

checked in an rabbit population.
Results: From a total of 260 samples of tonsillitis and pharyngitis cases among children, only 8 (3.07%) isolates were identified as *Streptococcus pyogenes*. High amount of M-protein was detected in two isolates by indirect bacterial test. The concentration of purified M-protein ranged from 20-24.68 µg/ml. The purified M protein has important role in an induction of the immune response in experimental model. It leads to increased phagocytosis, stimulation of T-cell, and high level of antibody in serum of an immunized rabbits.
Conclusion: The purified streptococcal M protein has strong antigenicity, and it has important role in an induction the strong protective immune response in experimental rabbit model. It may be used in future studies as vaccine against streptococcal infection among humans.

Keywords: Streptococcus pyogenes; M-protein antigen; immune response.

ABBREVIATIONS

a.k.a	*also known as*
ASO	*antistreptolysin O*
CFU	*colony-forming unit*
GAS	*group A streptococci*
HA	*hyaluronic acid*
IBT	*indirect bactericidal test*
STSS	*streptococcal toxic shock syndrome*

1. INTRODUCTION

Streptococci are considered one of the predominant flora which colonize the respiratory tract of human [1]. One of these, *Streptococcus pyogenes* is an obligate human pathogen. Infections caused by group A streptococcus (GAS) represent a public health problem especially among children in both developing and developed countries. It is a cause of major human morbidity and mortality worldwide [2]. Group A streptococci (GAS) cause 18 million cases of severe diseases resulting in 517,000 deaths each year. GAS is responsible for a number of diseases ranging from common clinical illnesses such as pharyngitis, impetigo, cellulites and scarlet fever to severe invasive infections such as puerperal sepsis, streptococcal toxic shock syndrome (STSS) and post infectious sequelae such as rheumatic fever and acute glomerulonephritis [3].

Upper respiratory infection due to *Streptococcus pyogenes* remains one of the most common infections occurring in childhood. All ages are susceptible to spread of the organism under crowded conditions, such as those at schools and military facilities. School-age children (5-15 years) are considered as the major reservoirs of group A beta-hemolytic Streptococci [3,4]. GAS is further subdivided into different serotypes based on highly variable N terminal sequences of the cell surface M protein as described by Dr. Rebecca Lancefield. M protein is a filamentous molecule that is expressed on the GAS surface as a coiled-coil dimer. Currently, there are over 150 serotypes of M-protein (a.k.a: Emm protein that encoded by *emm* gene) identified in GAS with prevalent strains varying over time and geography [5].

GAS produces several surface-associated and secreted components that have been implicated in internalization. M protein and hyaluronic acid (HA) capsule are two major virulence factors of GAS that inhibit phagocytosis and enhance virulence in animal models [6]. M-protein is responsible for adherence to epithelial cells of upper respiratory tract of human and it inhibits phagocytosis by cleavage of C3b and escape from opsonization. M-protein also has important role in the pathogenesis of rheumatic fever [7].

M-protein is limited almost to group-A streptococci, but it also found in group C and G. This substance is essential factor in pathogenicity of *St. pyogenes* (if absent result in avirulent). Virulent strains produce matted colonies (granular) because that produces much M-protein, whereas avirulent strains produce glossy colonies. Therefore the name “M protein” stems from the mucoid morphology of encapsulated cells [4,6].

The immunity to M proteins appears to elicit not just protective immunity, but the potential for

autoimmunity as well, and these cross-reactive antibodies have been implicated in the post-infectious sequelae of GAS infection. Thus, immunity to native M protein, both naturally acquired and vaccine induced, looms as a double-edged sword [8].

There is no licensed vaccine available against GAS so far, but some studies under trial vaccine based on M protein used to harness the protective immunity of M protein non-react and without incurring the risks of molecular mimicry and autoimmune diseases, thus considered as a single-edged sword [9].

The present study was aimed to evaluate the effects of the purified M-protein of *Streptococcus pyogenes* on immune system to produce protective immunity against streptococcal infection in rabbits model.

2. MATERIALS AND METHODS

The present study involved three parts; bacteriological part, serological part and experimental part.

2.1 Part I: Bacteriological Study

2.1.1 Collection of clinical materials

A total of 260 samples were collected by swabbing from clinical cases of tonsillitis and pharyngitis in children patients (5-15 years old) who were admitted to Child Protection Hospital and Central Child Hospital in Baghdad City, Iraq, during the period between January to July 2014.

2.1.2 Cultivation of clinical materials

The clinical materials that taken from tonsillitis and pharyngitis cases were inoculated on blood agar plates (Bioanalyse, Turkey) and Azide agar plates (Hi-Media, India). The plates were incubated at 37℃ for 18 to 24 hours under 5-10% CO_2, then the primary diagnosis was done.

2.1.3 Identification of *S. pyogenes*

2.1.3.1 Colonial morphology and microscopic examination

A pure colony was taken from each positive culture. Its identification was based on the morphological properties include colony size, hemolysis around colony, color, shape, translucency, edge, and elevation of texture and then investigated by Gram stain to observe shape and arrangement of bacterial cells [10].

2.1.3.2 Biochemical tests

The identification of each isolate of *Streptococcus pyogenes* was based on conventional biochemical tests that recommended by Forbes et al. [10].

The Vitek II System (Biomerieux-France) and API 20 Strep test (Biomerieux, France) were used to confirm the biochemical tests. The assays had been performed according to the manufacturer's instructions. Recently, these systems were used for identification and detection of *Streptococcus pyogenes* in rapidly and more accurate identification.

2.2 Part II: Serological Study

2.2.1 Lancefield reaction test

The Lancefield streptococcal latex test kit was used for the qualitative detection and identification of the serogroup of isolated Streptococci by slide agglutination procedure according to protocol of manufactured company (Abon, UK).

2.2.2 Antistreptolysin O (ASO) test

Five milliliter of blood samples were collected from children patients in evacuated tubes (without anticoagulant) and were centrifuged at 4000 rpm for 5 minutes. Detection and determination of specific streptococcal antibody titer in sera of the patients were achieved by tube agglutination procedure according to directions of ASO test kit (Spenreact, Spain).

2.3 Part III: Experimental Model

This part was done in laboratory of microbiology department of Medical College, Babylon University, Iraq.

2.3.1 Indirect Bactericidal Test (IBT)

The presence of M protein was detected in each isolate of GAS by using IBT 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 (Difco, USA); spread with a glass spreader and incubated at

37℃ 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 (in duplicate), then 0.4 ml of blood of healthy persons which considered the source of phagocyte cells. One of the tubes was incubated at 37℃ in shaker incubator with 20 rpm and the other was incubated at 37℃ in non-shaker incubator for 3 hours. From each tube in both incubation, 0.1 ml was pipetted onto plates with blood-brain heart infusion media using sterile spreader and incubation at 37℃ for 18 hours. The bactericidal activity was determined by measuring Colony-Forming Units (CFU) [11,12].

2.3.2 Extraction and purification of M-protein

M-protein was extracted by limited pepsin digestion method [13]. The crude M protein was purified from *Streptococcus pyogenes* using ion exchange chromatography (DEAE-cellulose). The ion-exchange column was prepared according to modified method that described in [14]. The results were interpreted and proved by chemist Dr. Moaid O. AL-Gazali (Biochemical Research Center, Babylon Medicine College, Iraq).

2.3.3 Determination of M protein

The final concentration of purified M-protein was determined by Lowry method [15].

2.3.4 Immunization of rabbits

Fifteen White New Zealand male rabbits (originated from Babylon Medical house for Lab. animals) were divided into five groups, each group consists of 3 male rabbits that were left for one week for adaptation in an animal house. Each rabbit was 6 to 8 month ages and 1.3 –2.4 kg of body weight. The injection area was a sterilized with 70% alcohol, and the M protein was injected intramuscularly (skin test) by a needle with 23 gauges. The injection partition table was divided into 5 injections for 5 weeks, one injection in every week.

The first group of animals was injected with 100 µl of crude M protein. The second group was injected with 100 µl of precipitated M protein by 30% ammonium sulphate. The third group was injected with 100 µl of washing M protein step of purification by ion-exchange chromatography. The fourth group was injected with 100 µl of elution M protein step of purification, and the fifth group (control group) was injected with 100 µl of buffer phosphate (pH8) [16,17].

2.3.5 Estimation of immune response by phagocytosis and antibodies

After five weeks from immunization of rabbits, a 5 ml of blood was collected slowly from the rabbits by needle with 23 gauges that injected into the lateral thoracic region toward the area of the maximal heart beat. The blood collection was performed under general anesthesia with 98% diethyl ether. The fresh blood samples were placed in sterile two tubes, the first tube was treated with anticoagulant (EDTA); and this tube was used for estimation of phagocytosis by procedure that described by Domingue and Pierce [16]. Second tube was not treated with anticoagulant. This tube was used for estimation of specific antibody level in serum of rabbit by tube agglutination (ASO test).

2.3.6 Detection of immune response of T-cell

The immune response of T-cells in an immunized rabbits was determined after 4 and 24 hours from immunization with purified M-protein by modified procedure described in [17]. In briefly, In order to study the effect of M protein on T cell proliferation, peripheral blood mononuclear cells were stimulated with various concentrations of M protein by using serial dilutions of it for 4 and 24 hr. The M protein-induced responses with cells from a single animal were demonstrated in three separate experiments.

The main steps in present study can be summarized in schematic presentation (Fig. 1).

2.3 7 Ethical considerations

The author declares that written informed consent was obtained from the patient for publication of this research article.

The permission was obtained from animal house office (in Babylon medical college) as ethical justification for using Rabbits in this study.

3. RESULTS AND DISCUSSION

3.1 Bacteriological Study

3.1.1 Identification of *Streptococcus pyogenes*

A total of 260 samples taken from clinical cases of tonsillitis and pharyngitis in children patients, only 8 (3.07%) isolates were identified as *Streptococcus pyogenes*, while 252(96.92%) isolates were represented other bacterial

causative agents in this population of patients as shown in Fig. 2. All eight isolates belong to serogroup A streptococci were confirmed by latex slide agglutination test (Lancefield reaction).

3.2 Serological Study

3.2.1 Detection of anti-streptolysin O antibodies

The present study documented that 8 samples diagnostic with Vitek-II system, API 20 strep and Lancefield reaction test but when applying ASO test only 5(62.5%) samples out of 8 give high titer of ASO more than 200 U, the reminder 3(37.5%) samples gives negative results less than 200U, as shown in Table 1. These results were documented in previous study by Danchin et al. [18] while Ma et al. [19] who found more than 90% positive rate of ASO antibodies against SLO antigen in sera of GAS-infected patients.

Antibodies against SLO are generated by the humoral immunity and can be quantified as Anti-streptolysin O antigen, they appear in serum from one week to one month after the onset of streptococcal infection [6]. The ASOT test used as an indicator of recent infection [20]. The negative results of ASO test in some cases that involved in present study may be suggest either colonization of *St. pyogenes* in upper respiratory tract of tested patients or the patients were suffering from infection for long or past infection.

Table 1. The percentage of positive and negative ASO test results

Antistreptolysin O test	No. of isolates	%
Positive	5	62.5
Negative	3	37.5
Total	8	100

3.2.2 Detection of M protein in *S. pyogenes* isolates

The present study demonstrated that 2 out of 8 isolates of *Streptococcus pyogenes* have M protein in high content by IBT. The bactericidal activity was determined by calculating the number of Colony-Forming Units (CFU) which incubated at shaker and non-shaker incubator. The difference between them after incubation indicates low M-protein amount, while the symmetric between them indicate high M protein amount in *Streptococcus pyogenes* isolates. The two out of eight isolates that contain high M - content given local name: first isolate FJM-1 and second isolate FJM-2 as local isolates within our study.

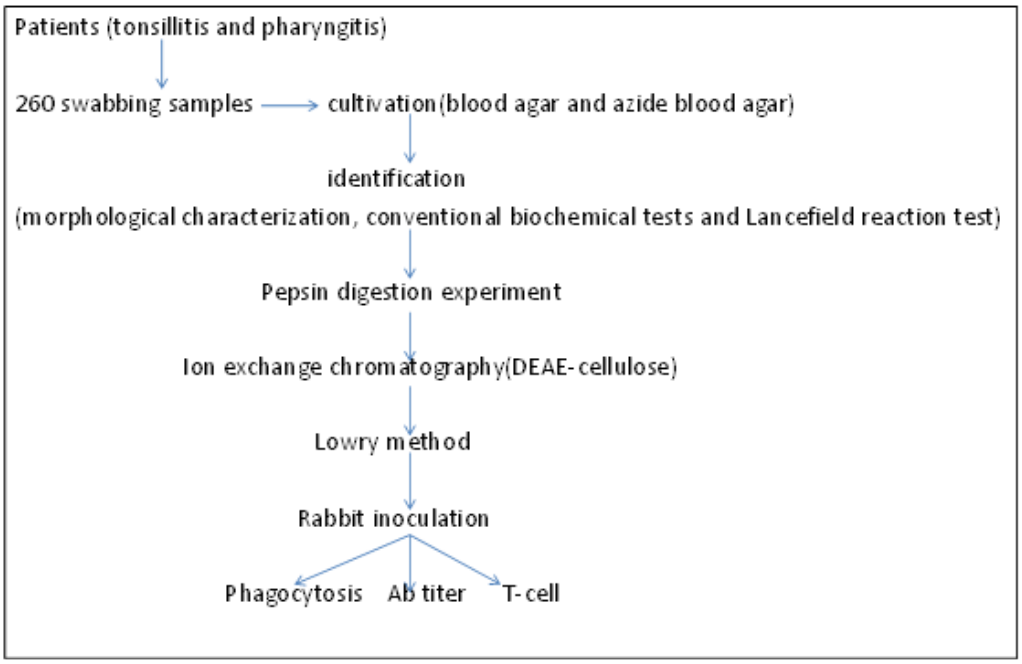

Fig. 1. Schematic presentation for main steps in present study

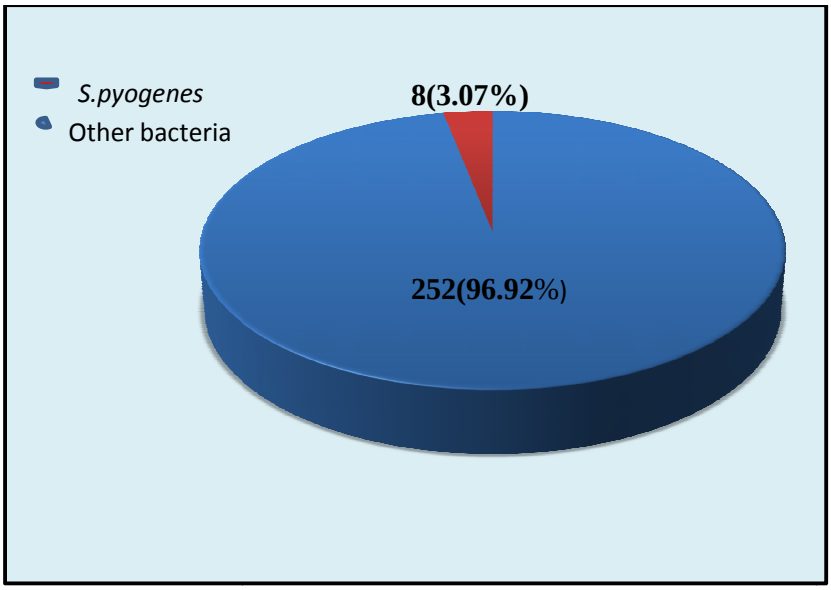

Fig. 2. Percentage of *S. pyogenes* isolates and other bacterial causes among children patients with tonsillitis and pharyngitis

3.2.3 Extraction and purification of M protein

The M-protein was extracted and purified from local isolates that above mentioned: Isolate FJM-1 and isolate FJM-2 because they detected with high content of M-protein, while other isolates with very low content, therefore 6 isolates were excluded. It is possible that M proteins are degraded in this study.

The crude M protein outcome from ammonium sulphate step was passed through DEAE–cellulose ion exchange chromatography column that already equilibrated with phosphate buffer (0.1 M, PH 7.0) and then the absorbance was read at wave 280 nm for wash fraction (unbounded proteins have positive charge). The elution step for bounded protein (that carries a negative charge) is done by using NaCl gradient (0.1 M–1 M).

The results showed one peak of protein in elusion step as in Fig. 2, While the wash step as in Fig. 3 showed appearance of two peaks of protein when it is eluted by gradient NaCl. These results indicate the M protein was more purified in elution step than in wash step [14,21].

DEAE-cellulose chromatography has many advantages, including high resolution power, high capacity, easy handling, good separation, ability of reactivation for using many times besides the simplicity of separation principle which depending on charge differences [22,23].

The concentrations of M protein were determined by procedure of Lowry method. The purified M protein concentration was ranged from 20-24.68 µg/ml. as in Table 2.

3.2.4 Determination of anti-M protein titer

The efficiency of antisera obtained from the rabbits (when injected by wash, elution, crude, and precepitiated by ammonium phosphste) were investigation by tube agglutination test towards group A Streptococcus. The result revealed strong agglutination, since clear visible clumping was formed within one minute of reaction.

For the determination of anti-M protein titer in immunized rabbits, the agglutination test was done and the results obtained from rabbits usually has different levels of titration of group A streptococci antibody which gave the following values: agglutination titer for crude protein was 1/80 while an agglutination titer in ammonium sulfate precipitation was 1/320. The agglutination titer for wash step using DEAE-Cellulose was 1/640, and for Elution step using the same column was 1/1280 while the control (buffer phosphate) was given negative results.

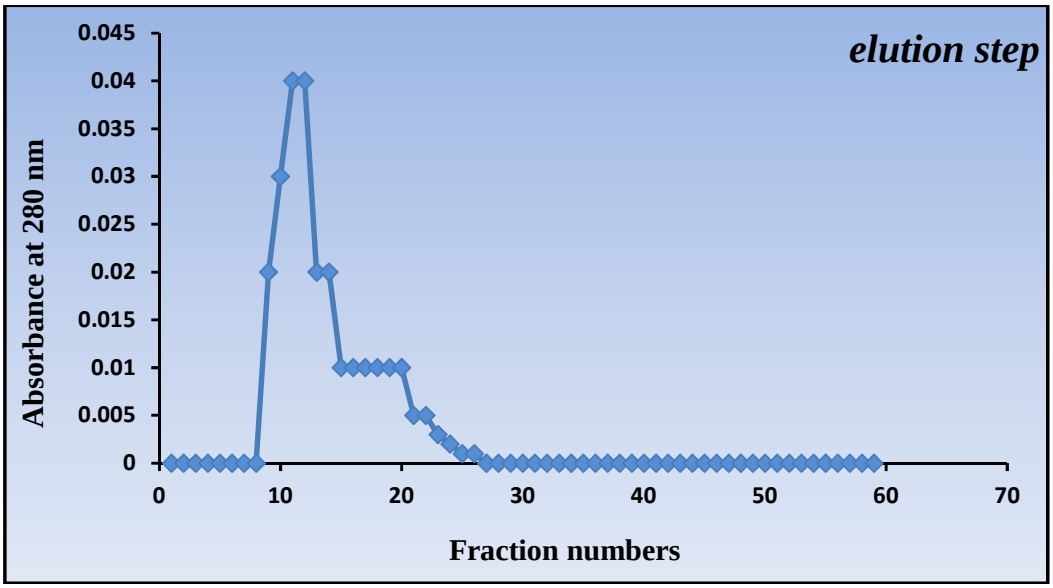

Fig. 3. Ion exchange chromatography using DEAE- cellulose column (28×1.6 cm), for purification the M protein from *Streptococcus pyogenes* equilibrated with 0.1 M and pH 8 phosphate buffer, flow rate 30 ml /hr and fraction volume 3 ml

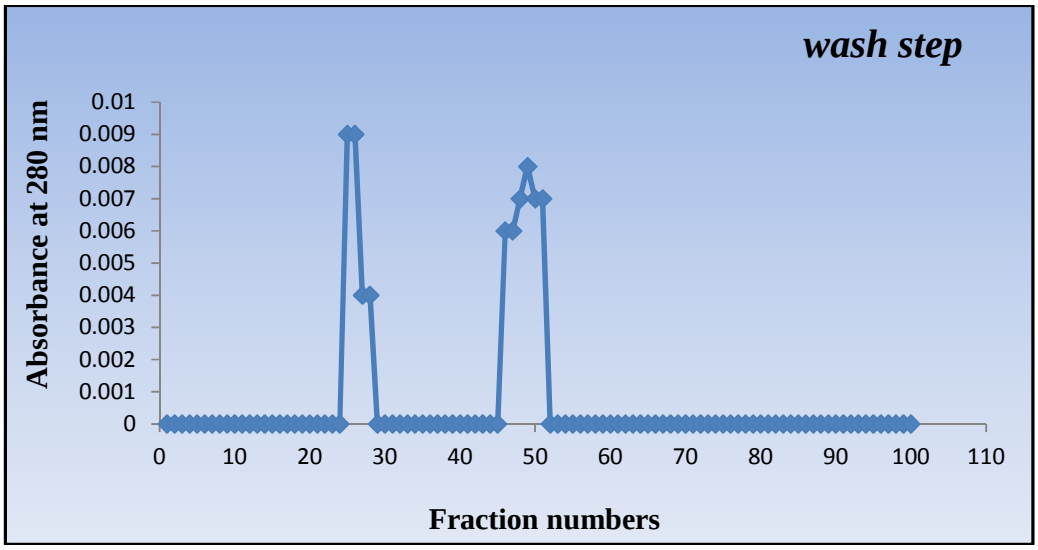

Fig. 4. Ion exchange chromatography using DEAE- cellulose column (28×1.6 cm), for purification the M protein from *Streptococcus pyogenes* equilibrate with NaCl gradient (0.1-1M), flow rate 30 ml /hr and fraction volume 3ml

From the above results we note that M protein which Elution from DEAE-Cellulose give high stimulation of immune response in rabbits.

M protein has the ability to stimulate the immune system to produce antibodies quality privacy immune (Specific Anti-M protein Antibodies) [24].

3.2.5 Phagocytosis activity tests

M protein concentration was estimated in each protein purification steps and in accordance with the method of Lowry, it was highly concentrated (807.15 µg/ml) in the crude extraction. This shows that it is not pure and contains other proteins. Then followed by

the precipitation of ammonium sulfate in a way (245.68 µg/ml), but it was low concentration (ranged from 20-24.68 µg/ml.) of protein in purification steps by Ion exchange chromatography. This refers to the purity of M protein (See Table 2).

In our study, the rabbits were injected intramuscularly with different solutions of protein purification steps. We note that the percentage of the immune response may patch when the injection of purified M-protein in elution step better than anyone else unlike other purification steps.

The phagocytosis activity test shows increased the phagocytic cells number when injected with elution protein concentration through purification steps. This indicate the purify of M protein which obtained from *Streptococcus pyogenes* and refers to specific immune response. The activity test gave highly phagocytosis, these results agreed with result of Abid et al. [5]. The virulence factor protein M is high-capacity antigens (Supper antigens) which gives the ability to resist phagocytosis process by macrophages [8].

3.2.6 Detection of immune response for T-cell

In the present study, the rabbit skin test was used to investigate the effect of M protein on T cell activation, we note that the immune response has led to the emergence of inflammation, and inflammation diameters have increased directly proportional to the degree of purity of protein after 24 hours, see Table-3. These refer to the effect of M protein of *S. pyogenes* in sensitivity of laboratory animals (rabbits) and stimulation of immune response by T-cell activation. The some studies show that a highly purified soluble form of M protein from *S. pyogenes*, which lacks the membrane spanning region, is a potent inducer of T cell proliferation and release of Th1 type cytokines [17]. B cell and T cell appear to a play a role in propagating immunity , although the exact mechanisms of cell-mediated immunity against GAS are still unclear [25].

The current study in an immunized rasbbits has demonstrated that type-specific immunity against M protein *In vivo*, and we tried to study the role of M protein in providing protective immunity against GAS in rabbit model to prepare or use in vaccine development in future for control and prevent GAS infections.

Table 2. Activity of phagocytic cell in rabbit after immunization with M protein of *Streptococcus pyogenes*

No. of animal group	Steps of purification	Protein concentration (µg/ml)	Phagocytic percentage (%)
1	Crude extracted protein (total proteins)	807.15	52
2	30% ammonium sulphate precipitation (precipitated protein)	245.68	71
3	Ion exchange chromatography DEAE-cellulose (Wash step)	24.58	83
4	Ion exchange chromatography DEAE-cellulose (elution step)	20.0	89
5	Control group	0.0	0.0

Table 3. Skin test after 4 and 24 hours for rabbits which injected with M antigen of *S. pyogenes*

No. of animal group	Purification steps	Skin test diameter of inflammatory zone (cm)	
		4 hours	24 hours
1	Crude protein	Negative	2.6
2	30% preciptation ammonium sulphate	Negative	2.8
3	Wash step	Negative	3
4	Elution step	Negative	3.1
5	Control group	Negative	Negative

4. CONCLUSION

Thus from the results expressed above, we are concluded the purified streptococcal M protein has activity in stimulation of immune response, increased phagocytosis, stimulation of T cells and antibody level in serum of treated animal with M protein which indicate it has strong antigenic that may be used in protective immunity or it may used in vaccine development against streptococcal infection in future.

ACKNOWLEDGEMENTS

I am extremely thankful to Firas A. AL-Taei, for helping and providing the needed facilities, which are essential for successful completion of the present work. I am also deeply indebted to Dr. Moaid O. AL-Gazali and to staff and patients of Child Protection Hospital and Central Child Hospital in Baghdad City, Iraq for their kind assistance.

COMPETING INTERESTS

Author has declared that no competing interests exist.

REFERENCES

1. Schleiss MR. Streptococcal infections, Group A sreptococci. Pead. Infect. Immun. 2009;124:557-563.
2. Cho K, Caparon M. tRNA modification by GidA/MnmE is necessary for *Streptococcus pyogenes* virulence. Infect. Immun. 2008;76:3176-3186.
3. AL-Kareem F, Abbas A, Hussein M. Comparative study of antibody response to *Streptococcus pyogenes* between school children carriers and patients with tonsillitis. Iraqi J Sc. 2014;55(2A):403-410.
4. Kotloff KL, Dale JB. Progress in group A streptococcal vaccine development. Pediatr Infect Dis J. 2004;23:765–767.
5. Abid AJ, Jabur MA, Nabat Z. Partially purification of M protein from *Streptococcus pyogenes* and molecular study of some virulence factors. Adv. Life Sc. Technol. 2015,28:46-50.
6. Cunningham, M. Pathogenesis of group A streptococcal infection. Clin. Microbiol. 2000;13:470-511.
7. Oehmck S, Shannon O, Mörgelin M, Herwald H. Streptococcal M proteins and their role as virulence determinants. Clinica Chemica Acta. 2010;411:1172-1180.
8. Madoff LC. Immunity to group A Streptococcal M protein: Forging a single-edged sword. CID. 2005;41:1123-1124.
9. McNeil S, Halpern S, Langley J, et al. Safety and immunogenicity of 26-valent group A *streptococcus* vaccine in healthy adult volunteers. Clin Infect Dis. 2005;41: 1114–1122.
10. Forbes B, Sahm D, Weissfeld A. Baily and Scott Diagnostic Microbiology, Twelve edition, Elsevier Company; 2007.
11. Lancefield R, Perlmann G. Preparation and properties of type-specific M protein isolation from group A type I streptococci. J. Exp. Med. 1952;96:71-82.
12. Johnson DJ, Kaplan E, Sramek J, et al. Laboratory diagnosis of group A streptococcal infections, World Health Organization, Geneva, Switzerland; 1996.
13. Manjula BN, Fischetti VA. Studies on group A streptococcal-M-proteins. Purification of type M5 proteins and comparison of its amino terminal sequences with two immunological unrelated M-protein molecules. J. Immunol. 1980;124(1):261-276.
14. Dahee M, Yousif S, Mustafa M. Protein-M extraction from locally isolated *Streptococcus pyogenes*. Iraqi J. Sc. 2002; 43(2):26-42.
15. Lowry O, Rosebrough N, Farr A, Randall R. Protien measurement with Folin phenol reagent. J. Biol. Chem. 1951;139:265-275.
16. Domingue GR, Pierce WA. Effect of partially purified streptococcal M protein on the *in vitro* phagocytosis of *Streptococcus pyogenes.* J. Bacteriol. 1965;89(3):583-588.
17. Pahlman L, Olin AL, Darenberg J, Morgelin M, Kotb M, Herwald, Norrby-Teglundm A. Soluble M1 protein of *Streptococcus pyogenes* triggers potent T cell activation. Cell. Microbiol. 2008;10:404-414.
18. Danchin M, Carlin J, Devenish W, Nolan T, Cerapetis J. New normal ranges of antistreptolysin O antideoxyribonuclease B titer for Australia children. J Pead. Child. Health. 2005;41(11):583-586.
19. Ma C, Liu Z, Li W, Qian X, Zhang S, Gao X, Jiang S, Wei L. FbaA- and M protein-

based multi-epitope vaccine elicits strong protective immune responses against group A streptococcus in mouse model. Microbes Infect. 2014;16(5):409-18.
20. Shet A, Kaplan E. Addressing the burden of group A streptococcal disease in India. Indian J Pediatr. 2004;71:41-48.
21. Rosenberg IM. Protein analysis and purification. Second edition. XXI, Berlin, Germany; 2005.
22. Goyal M, Basak A. Purification of human erythrocytes catalase by ion exchange chromatography. J. Enzym. Res. 2011; 2(1):11-17.
23. AL-Kubaissy K. Biochemistry, third edition, Al-Balkaa University, Dar Waeel publisher, Jordan; 2011.
24. Gupta VK, Sekhar S, Dhanda V, Toor D, Kumar R, Chakraborti A. Immune response against M protein-conserved region peptide from group A streptococci in India population. J. Microbiol. Immunol. Infect. 2014;20:1-7.
25. McMillan DJ, Dr`eze PA, Vu T, et al. Updated model of group A *Streptococcus* M proteins based on a comprehensive worldwide study. Clin. Microbiol and Infection. 2013;1(5):E222–E229.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/13081