World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
Published by Atom and Cell Publishers
Available online at: http://www.wjpsonline.org/
Original Article

Immunogenetic and Bacteriological Study of Acute Otitis Media and Tonsillitis among Children Patients

Abbas Zeki Mehdi*, Hammadi A. Al-Hilali*, Mohammed Sh. Jebur**

*College of Medicine, Al-Qadisiya University, Iraq
**Institute of Medical Technology, Middle Technical University, Iraq

Received: 06-04-2015 / Revised: 25-05-2015 / Accepted: 10-06-2015

ABSTRACT

A total of 200 specimens (105 males and 95 females) were included (100) swabs of otitis media and (100) swabs of tonsillitis. These swabs were obtained from patients consulted to different hospitals of Baghdad city during the period from 6 February 2014 to 18 May 2014. There were 27(27%) positive isolates for *Strep. pyogenes*, which obtained from patients with age ranged from less than 5.5 years, who suffered from tonsillitis and ear infection. The phenotypic characterizations of twenty seven isolates (27%) of *Strep. pyogenes*, which were obtained, showed characteristics colony morphology when cultured on different agar plates, microscopic appearance and biochemical tests. Also these results of isolation and identification were confirmed by API 20 Strep test and VITEK 2 card 2GN (Biomeriux company, France). Results of molecular detection of Streptokinase as a virulence factor of *Strep. pyogenes*, which is responsible for disrupt host cell structures, showed that 20(74.1%) *Strep. pyogenes* isolates of tonsillitis infection and otitis media were positive except 7(25.9%) isolates were negative on 1.5% agarose gel. with highly significant differences ($P<0.01$). The size was 1322 bp of the DNA ladder (100-1500bp). While PAM gene SF1 detection from Strep. pyogenes isolates were amplified by PCR using specific forward and reverse primers were revealed that 20 *Strep. pyogenes* isolates of tonsillitis infection were negative 18(90%) except 2(10%) isolates were positive. Seven isolates from otitis media cases presented 6(85.7%) isolates had negative results except 1(14.3%) showed positive result on 1.5% agarose gel as a result of PCR reaction, the size of the 1322bp of the DNA ladder (100-1500bp).

Key words: Tonsillitis, otitis media, Strep. pyogenes.

INTRODUCTION

Acute otitis media and tonsillitis diseases are one of a major public health concern both in developed and developing countries. *Strep. Pyogenes* is one of the most prevalent human pathogens, which causes a wide array of infections and the most frequent of them are acute tonsillitis and acute otitis media, so it responsible for a variety of infectious diseases and immunological complications [1]. More than 30 million cases of Streptococcal tonsillitis occur each year in the USA. Worldwide, *Strep. pyogenes* causes over 18 million cases of severe diseases resulting in over a half million annual deaths [2].

Strep. pyogenes is Gram-positive, Non-motile, and non-spore forming spherical cocci with 0.5 to 1.0 μm, which can form long chains in liquid medium, although they occur in short chains or pairs in clinical specimen, *Strep. pyogenes* cells is covered with a capsule of hyaluronic acid, a high molecular weight [3]. Pathogenesis of *Strep. pyogenes* infections is multi factorial, as suggested by the number and wide array of virulence determinants possessed by cell-surface molecules have been reported as possible adhesions due to their ability to bind specific components of the host tissues [4].

The Streptokinase gene which is responsible for is produced by different strains of hemolytic Streptococci and due to its non-human origin, is immunogenic and can evoke the immune system; hence, frequent administrations of Ska can result in production of neutralizing antibodies, which in turn reduces the efficacy of therapy, and eventually may lead to extensive allergic reactions [5]. Applied the PCR technique was useful for detecting some virulence factor of *Strep. pyogenes* from tonsillitis and otitis media infections. PAM which mean peptidylglycine alpha-amidating monooxygenase is a protein-coding gene, which go annotations related

***Corresponding Author Address:** Prof Dr. Mohammed Sh. Jebur, Institute of Medical Tech. Email: d_mohamed_1959@yahoo.com*

to this gene include copper ion binding and calcium ion binding [6], while SF1 gene elucidate specific primers used for splicing factor 1 that known as zinc finger protein. So it has been shown to interact with Transcription elongation regulator [7].

Some human yields, such as fibronectin and plasminogen there exists a multitude of separate Streptococcal structures adept of mediating these interfaces. Many host tissue and plasma proteins are specifically documented by *Strep. pyogenes* through structurally discrete binding domains which comprise a major amount of surface fibrils that are collectively known as M and M-like proteins. Included among the M-protein-bound host proteins are devices of the complement cascade. The product of the emm gene of M serotype 53 streptococci binds both human plasminogen and plasmin with high attraction. The plasminogen binding site of PAM has been restricted to a 13-amino-acid repeated domain [8].

The present study was designed to improve the immunogenetic and bacteriologic cross reaction of *Strep. pyogenes* isolates from acute otitis media (AOM) and tonsillitis as a causative agent through some virulence factor from patients in Baghdad city. Patients presenting to five hospitals in Baghdad city, during the period of 6 February 2014 to 18 May.

MATERIALS AND METHODS

Patients: Two hundred patients (105 males and 95 females) with age less than 5.5 years, who suffered from tonsillitis and ear infection, that presenting to five hospitals in Baghdad city during the period from 6 February 2014 to 18 May 2014.

Specimen's collection: Tonsillitis and otitis media specimens were collected using cotton swabs. These swabs were directly inoculated onto the plates of transport medium, then incubated overnight at 37˚C [9]. The history for each case was recorded in a questionnaire.

Identification of Streptococcus pyogenes

Morphological Examination: Culturing of throat and ear specimens: Each swab was immediately taken by transport medium and then inoculated on blood ager plates and 5% sheep blood agar plates and incubated in a (5-10%) CO_2 to prove the facultative anaerobic-enriched atmosphere for 24 hours at 37°C. [9]. Also different media were used for laboratory isolation and identification.

Colony Morphology and Microscopic examinations: A single colony was taken from each primary positive culture and its identification depended on the morphology properties (colony, size, shape, color and natural of pigments, translucency, edge, elevation and texture). Suspected colonies were selected and investigated by gram stain to observe as specific color reaction, shape aggregation and specific intracellular compounds after staining the bacteria by gram stains, tests were done to reach the final diagnosis [10].

Biochemical Tests: Different biochemical tests were achieved for the suspected colonies, which include appeared as beta hemolysis, lactose non-fermenter and negative reaction for catalase, indole and oxidase [10]. Biochemical tests were performed to support results using API 20 Strep test, which is accurate technique for Confirm identification of *Strep. pyogenes* isolates [11]. Also VITEK 2 was used for confirmation of identification of suspected isolates of *Strep. pyogenes* by used card 2GN (biomeriux company, France).

Molecular study: Detection of Streptokinase, Plasminogen (PAM) genes by PCR Assay: Genomic DNA was extracted from *Strep. pyogenes* isolates using ZR Fungal/Bacterial DNA Mini PrepTM kit Following manufacture instructions. The sequences of primer sets were used in PCR to amplify species were shown in Table (1).

Amplification of Ska and PAM genes of *Strep. pyogenes* were studied according to protocol of [12]. Amplified products were electrophoresed on 1.5% agarose for 1hr at 75 V/cm.

Statistical analysis: Statistical programs performed by SPSS (V-15). Descriptive statistics were included tables creation (number, percentage, mean and standard error) and Graphical presentation such as figures [13].

Table (1): Oligonucleotide primers sequence used for PCR amplification of specific gene for *Strep. pyogenes*.

Genes	Sequence (5' to 3')	Size (bp)	References
Ska	**F-** GGGATCCATGAAAAATTACTTATCT	1322	(Molaee *et al*. 2013)
	R- ACTCGAGTTATTTGTCTTTAGGGT		
PAM	**F-**GAGTTGA/GAACGACTTAAAAA/GCGAGAGACATG	1322	(Molaee *et al*. 2013)
	SF1- R GTGCTTGACCTTTACCTGGAACAGATT		
	SF3- R GCTGTTTGAGCAGCTCTACC		
	SF4-A CT CTAGGTTCAGCTAAGCGTGAGTTG		
	SF4-L GAAATCCAAACAAGCACTACCTACTG		

RESULTS AND DISCUSSION

Isolation and Identification

Macroscopic and Microscopic characterizations: From total of Two hundred samples were obtained from tonsillitis and otitis media infections patients presenting to five hospitals in Baghdad city. The phenotypic characterization of twenty seven isolates (27%) of *Strep. pyogenes*, which were obtained showed characteristics colony morphology when cultured on blood agar plates, so its appear as white to grey colonies(1-2) mm in diameter [14]. While macroscopic features of *Strep. pyogenes* isolates reported G +ve cocci chains or pairs non-motile bacterium and non-spore forming cells [15].

Biochemical tests: All 27 *Strep. pyogenes* isolates were positive for oxidase, catalase, Simmon's citrate utilization, indole negative and negative MR-VP tests. It did not ferment carbohydrates, but many isolates oxidize glucose. All these results were confirmed by Mini API 20 STREP system and VITEK 2 system *Strep. pyogenes* [16].

Frequency of *Strep. pyogenes* isolates: Results of culture growth from tonsillitis throat swabs by bacteriological methods showed that the most commonly isolated bacterium was *Strep. pyogenes*, which forms 20(20%), 9(9%) *Staphylococcus aureus*, 11(11%) *Pseudomonas aeruginosa*, 5(5%), Candida and only 1(1%) isolate of *H. influenza*. Also these results were agreement with Gonzalez *et al*., (2000) [17] who found that the isolation of *Strep. pyogenes* were (30.5%) and (69.5%) for other bacteria in Las Palmas City (USA), while Abdullah, (2003)[18], reported that the isolation of *Strep. pyogenes* were (46.8%) in Baghdad and Kofa cities (Iraq).(Table-2)

Table (2): Frequency of Culture growth (Tonsillitis)

Culture growth (Tonsillitis)	N	%	Chi-Square (P-value)
No growth	**54**	**54%**	**P=0.00 HS (P<0.01)**
Strep. pyogenes	**20**	**20%**	
Staphylococcus aureus	**9**	**9%**	
Pseudomonas aeruginosa	**11**	**11%**	
H. influenza	**1**	**1%**	
Candida	**5**	**5%**	
Total	**100**	**100%**	

Culture growth from otitis media, ear swabs were observed that there was highly significant differences (p>0.01), and there was no growth 54(54%) were seen in samples. Bacterial isolates distribution of otitis media cases was presented 7(7%) *Strep. pyogens*, 24(24%) *Staphylococcus aureus*, (4%) *Pseudomonas aeruginosa*, 9(9%) Candida and only 3(3%) *H. influenza*. (Table-3) Grevers *et al*., (2012) [19] reported that (100) children with severe acute otitis media, (21%) isolates were *Strep. pneumoniae*, (10%) *Strep. pyogenes*, (13%) *Moraxella catarrhalis* and 1% *H. influenzae*, and these findings harmony with present study [20].

Table (3): Frequency of Culture growth (Otitis media)

Culture growth (Ear infection)	N	%	Chi-Square (P-value)
No growth	**47**	**47%**	**P=0.00 HS (P<0.01)**
Strep. Pyogenes	**7**	**7%**	
Staphylococcus aureus	**24**	**24%**	
Pseudomonas aeruginosa	**4**	**4%**	
Proteus sp	**6**	**6%**	
H. influenza	**3**	**3%**	
Candida	**9**	**9%**	
Total	**100**	**100%**	

Genotypic detection of isolates

Detection of Streptokinase gene by PCR technique: PCR technique is a primer mediated enzymatic amplification of specifically cloned or genomic DNA sequences [1]. In present study polymerase chain reaction (PCR) technique was used to further confirming the diagnosis of *Strep. pyogenes.* The DNA of twenty seven isolates was extracted and purified by using ZR Fungal/Bacterial DNA Mini Prep kit. The results were detected by electrophoresis on 1.5% agarose gel and exposed to UV light in which the DNA appeared as compact bands. (Figure-1)

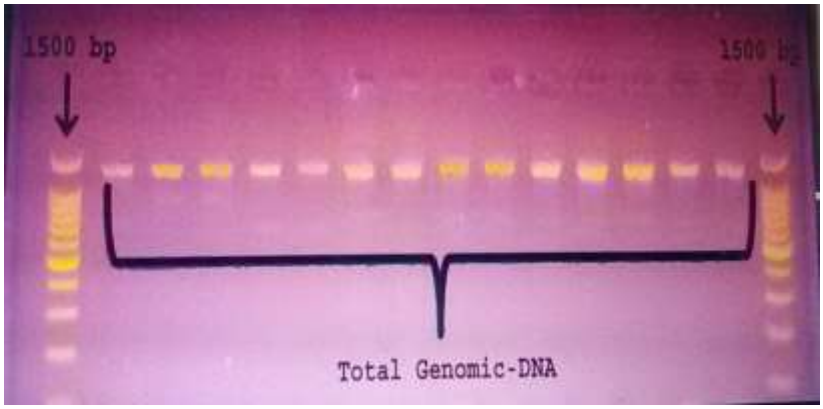

Figure (1): Total genomic DNA extracted from isolates using 1.5% agarose gel electrophoresis.

The results of Streptokinase gene was amplified by PCR using Ska gene specific forward and reverse primers. The results presented in (Figure-2) showed that 20(74.1%) *Strep. pyogenes* isolates of tonsillitis infection and otitis media were positive except 7(25.9%) isolates were negative on 1.5% agarose gel. The size was 1322 bp of the DNA ladder (100-1500bp). These results were approve with Gangwar *et al*., (2010) [21] who revealed that Streptokinase of *Strep. pyogenes* by PCR product was 1353 bp size. Molaee *et al*., (2013) [12] were showed that streptokinase gene was the predominant extracted gene in *Strep. pyogenes* isolates of acute cases patients, so that these results were with agreement to study results.

Elevation percent of positive detection Ska gene by (PCR) in tonsillitis were 15(75.0%) and otitis media 5(71.4%), while a negative (25.0%) and (28.6%) respectively with non-significant differences (P<0.05) between kind of infections and Ska gene distribution. (Table-4) McArthur *et al*., (2008) [22] were indicated that variant streptokinase proteins secreted by *Strep. pyogenes* isolates display differing plasminogen activation characteristics and might play distinct roles in disease pathogenesis.

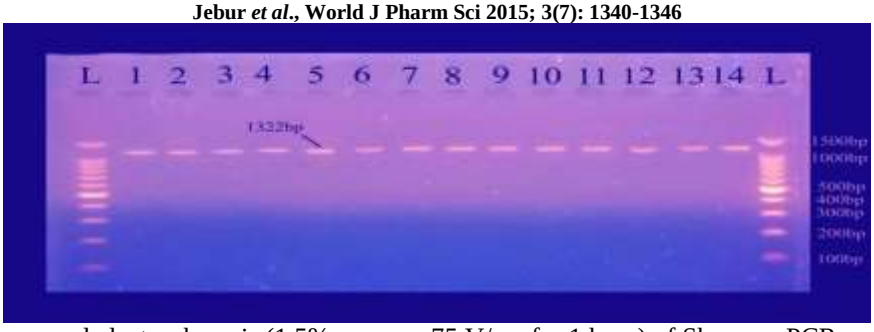

Figure (2): Agarose gel electrophoresis (1.5% agarose, 75 V/cm for 1 hour) of Ska gene PCR products (1322 bp amplicon) in *Strep. pyogenes* isolates. Lane L: (DNA ladder) 100-1500bp molecular marker, lanes: 1, 2, 3, 5, 6,7, 8, 9, 10, 11, 12, 13, 14 isolates were positive results.

Table (4): Distribution of Streptokinase through kind of infection

Ska gene (PCR)		Kind of infection		Total	Chi-Square (P-value)
		Tonsillitis	Otitis media		
Positive	N	15	5	20	P= 0.853 NS (P>0.05)
	%	75.0%	71.4%	74.1%	
Negative	N	5	2	7	
	%	25.0%	28.6%	25.9%	
Total	N	20	7	27	
	%	100.0%	100.0%	100.0%	
Positive / Negative Ratio		3.0	2.5	2.857	

Al-Khalifawi, (2011) [23] reported that from 72 *Strep. pyogenes* isolates of tonsillitis patients, 43 (59.7%) were detected streptokinase gene by PCR, and these results nearly to present study. While Mohammed and Al-Jebori, (2012) [24] was showed 6(6.6%) of *Strep. pyogenes* isolates positive gene detection through its ability for production of streptokinase virulence factor from tonsillitis cases.

PAM gene detection by PCR technique: PAM (peptidylglycine alpha-amidating monooxygenase), which is a protein-coding gene. That go annotations related to gene include copper ion binding and calcium ion binding [6]. While SF1, is a specific primers used for splicing factor 1 that known as zinc finger protein. SF1 gene has been shown to interact with transcription elongation regulator [7]. Results of current study of PAM gene SF1 detection from *Strep. pyogenes* were amplified by PCR using specific forward and reverse primers were presented in Figure-3 showed that 20 *Strep. pyogenes* isolates of tonsillitis infection were negative 18(90%) except 2(10%) isolates (L2 and L5) were positive. While 7 *Strep. pyogenes* isolates of otitis media infection, there were 6(85.7%) isolates had negative results except 1(14.3%) (L5) showed positive result (clear band) on 1.5% agarose gel as a result of PCR reaction, the size of the 1322 bp of the DNA ladder (100-1500bp).

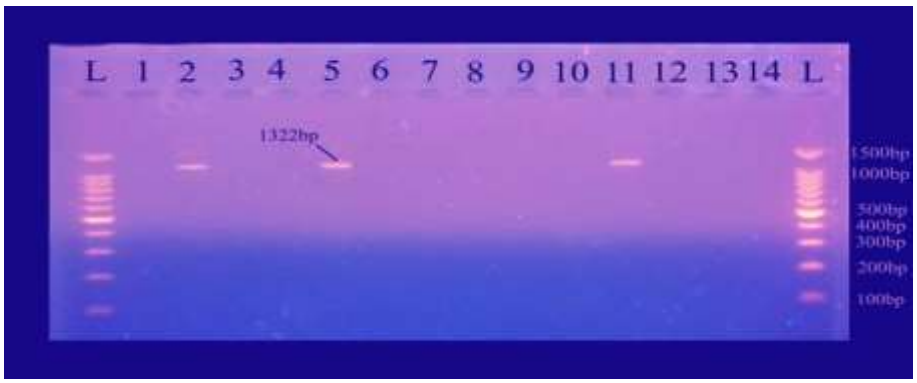

Figure (3): Agarose gel electrophoresis (1.5% agarose, 75 V/cm for 1 hour) of PAM gene and SF1 for tonsillitis and otitis media PCR products (1322 bp amplicon) in *Strep. pyogenes* isolates. Lane L : (DNA ladder) 100-1500bp molecular marker, (lanes: L2, L5 and L11) isolates are positive results.

There were no-significant differences (P>0.05) of PAM gene SF1(PCR) with raised of negative result tonsillitis 18(90.0%), otitis media 6(85.7%), positive 2(10.0%) and 1(14.3%) respectively. (Table-5)

Table 5: The relationship between type of infection and PAM gene SF1

PAM gene SF1 (PCR)		type of infection		Total	Chi-Square (P-value)
		Tonsillitis	Ear infection		
Positive	N	2	1	3	P= 0.753 NS (P>0.05)
	%	10.0%	14.3%	11.1%	
Negative	N	18	6	24	
	%	90.0%	85.7%	88.9%	
Total	N	20	7	27	
	%	100.0%	100.0%	100.0%	
Positive / Negative Ratio		0.111	0.1666	0.125	

Wezenet and Kronvall, (2005) [25] were reported that PAM gene (SF-1) was 26 (11.9%) for *Strep. pyogenes* isolates, through that these findings confirmed by results of Sakata, (2014) [26] who reported that PAM gene SF1 was the most prevalent type of *Strep. pyogenes* isolated from children with invasive infections between 2003 and 2012. According to the molecular distributions results of streptokinase and PAM genes among *Strep. pyogenes* isolates obtained in present study as a virulence factors in acute otitis media and tonsillitis documented the same origin or source of causative agent. Also results indicate that these virulence factors of *Strep. pyogene* confers an important selective advantage for survival of the bacterium in infected throat and middle ear.

Acknowledgements: To College of Medicine / Department of Microbiology / Al-Qadisiya University and College of Health and Medical Technology laboratories staff.

REFERENCES

1. Liu D, Susan H, Edwin S, *et al*. Rapid identification of *Strep. pyogenes* with PCR primers from a Putative transcriptional regulator gene. J. Medicine 2005, 156: 564-567.
2. Carapetis JR, Steer AC, Mulholland EK, Weber M. The global burden of group a Streptococcal disease. Lancet Infect. Dis. 2005, 5(11): 685-694.
3. Jawetz Melnick, and Adelberg's . Medical microbiology 24th: 2014, Pp. 978.
4. Nobbs AH, Lamont RJ, Jenkinson HF. Streptococcus adherence and colonization. Microbiology and molecular biology reviews MMBR 2009, 73: 407-450.
5. Banerjee A, Chisti C, Banerjee UC. Streptokinase-a clinically useful thrombolytic agent. Bio technol. Adv. 2004, 22: 287–307.
6. Morris K, Cao F, Onagi H, Altamore T, Gamble A. and Easton C. Prohormone-substrate peptide sequence recognition by peptidylglycine α-amidating monooxygenase and its reflection in increased glycolate inhibitor potency. Bioorganic & Medicinal Chemistry Letters 2012, 22 (23):7015-7018.
7. Goldstrohm AC, Albrecht TR, Suñé C, Bedford MT. and Garcia-Blanco MA. The transcription elongation factor CA150 interacts with RNA polymerase II and the pre-mRNA splicing factor SF1. Mol. Cell. Biol. 2001, 21 (22): 7617-7628.
8. Berge, A. and Sjobring U. PAM, a novel plasminogen-binding protein from Strep. pyogenes. J. Biol. Chem. 1993, 268: 25417-25424.
9. Mims C, Goering RV, Dockrell HM, Wakelin D, Zuckerman M, Chiodini PL, Roitt IM. Mims Medical Microbiology 4thed., Elsevier Mosby, USA, 2008.
10. Salma AM, Hossain MA, Musa AK, Shamsuzzaman AK. *et al*. Aerobic and anaerobic bacterial causes of puerperal sepsis at Mymensingh medical college hospital. Mymensingh Med J. 2010, 19(1):10-15
11. O'Hara CM, Miller JM. Evaluation of the Vitek 2 ID-GNB assay for identification of members of the family Enterobacteriaceae and other nonenteric gram-negative bacilli and comparison with the Vitek GNI card. J. Clin. Microbiol. 2003, 41: 2096-2101.
12. Molaee N, Hamid A, Mosayebi G. Expression of Recombinant Streptokinase from *Strep. pyogenes* and its Reaction with Infected Human and Murine Sera Iran J. Basic Med Sci 2013, (16): 985-989.
13. McCall RB. Fundamental Statistics for Psychology Harcourt brace. Jovanvich, Inc. 3rd ed.: 1980, Pp:102-114.
14. Bisno AL. and Stevens DL. *Strep. pyogenes* (including Streptococcal Toxic Shock Syndrome and Necrotizing Fasciitis). (***http://www.nos.nl/nieuws/artikelen/2004/10/31/balkenendeoverziekbed.html***)
15. Kayser M. Selective cytokine production by epithelial cells following exposure to *Escherichia coli*. Infec. Immun. 2005, 6: 602-609.
16. Lin Ching-nga. Characterization of a novel streptococcus species associated with marine environment. University of Hong Kong. 2011, http://hub.hku.hk/bib/B46699521
17. Gonzalez LZ, Lupiola P, Tejedor MT. Carriers of BHS from groups A, B, and C among school children in Las Palmas. Enferm. Infect. Microbial. Clin 2000, (18): 271-274.
18. Abdullah M.A. Group A Streptococcal Tonsillar infection among primary school children in samara. MSc. Thesis College of Medicine University of Tikrit.2003.
19. Grevers G, Wiedemann S, Jan-Christof B, *et al*. Identification and characterization of the bacterial etiology of clinically problematic acute otitis media after tympanocentesis or spontaneous otorrhea in German children. BMC Infectious Diseases 2012, 12:312.

20. MaanAbd AS, IshraqAbd A, NhidAbd AS. and Mohammed SM. Prevalence of Streptococcus pyogenes among children. MSc thesis. College of Science. University of Babylon- Iraq. 2014.
21. Gangwar S, Pramesh CL, Anant R. Amplification and cloning of Streptokinase gene in pTargeT mammalian expression vector Biotechnology International 2010, (3): 33-49.
22. McArthur JD, McKay FC, Ramachandran V. et al. Allelic variants of streptokinase from Strep. pyogenes display functional differences in plasminogen activation. FASEB J. 2008, 22: 3146–3153.
23. Al-Khalifawi M, Alaa A. and Al-Zobaei A. Molecular study of beta-haemolytic streptococci in patients with Tonsillitis in Ramadi City. Egypt. Acad. J. biolog. Sci. 2011, 3(1): 33-39.
24. Mohammed SA. and Al-Jebori RR. Molecular study of sortase enzyme and characterization of some virulence factors in Strep. pyogenes. Medical Journal of Babylon. 2012, 9:1.
25. Wezenet T. and Göran K. M Protein Gene (emm Type) Analysis of Group A Beta-Hemolytic Streptococci from Ethiopia Reveals Unique Patterns. J. Clin. Microbiol, 2005, 43 (9): 4369-4376.
26. Sakata H. Susceptibility and emm type of Streptococcus pyogenes isolated from children with severe infection. J Infect Chemother, 2013, 19:1042–1046.