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Real Time RT RCR Detection of *m*RNA encoding Enterotoxin B *Staphylococcus aureus* in Synovial fluid of Patients with Rheumatoid arthritis

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Staphylococcal enterotoxins (superantigens) in Rheumatoid arthritis patients are considered. Nevertheless, there is unclear where is the origin of these superantigens in the SF of RA patients. This study aimed to assess mRNA encoding Staphylococcal enterotoxin B gene in Synovial fluid of the patients with RA. This experimental study, eighty Synovial fluids of patients with RA was assayed. The mRNA extraction carried out. Based on Staphylococcal enterotoxin B gene specific primers for RT Real Time PCR designed. Then, cDNA was synthesized and amplification by SYBR®Green Real Time RCR was down. The S. *aureus* strain ATCC 14458 as positive control was used. The results analyzed descriptively. The results indicated, using specific primers and probe for enterotoxin B could amplify the related cDNA gene. SYBR®Green Real Time RT RCR method has been outline that 38 cases (47.5%) were showed. In fact, the reverse transcriptase convert *m*RNA to cDNA for partially encoding staphylococcal enterotoxin B (superantigen) gene. In comparison positive and negative control, the results descriptively analyzed.The results showed, Real Time RT RCR were able to detect and characterized the mRNA producing enterotoxin B gene in Synovial fluid of 47.5% of the RA patients. Despite the finding may be discussed the direct role of enterotoxin B as a classic superantigen in the pathophysiology of RA disease. It may demonstrate the causative of disease and facilitate the rapid detection. However, more research is needed this finding could change the perspective approach treatment of the RA diseases.

Keywords: Real time RT RCR, Staphylococcal Enterotoxin B, Synovial fluid, rheumatoid arthritis.

Rheumatoid arthritis (RA) is a chronic inflammatory disease, which has been remained unknown exact etiology and its pathophysiology[1]. The RA is one of the most important autoimmune diseases that 0.5% - 1% populations of the world are affected. The prevalence of RA disease in Iran is about 0.33%[2, 3]. The disease can lead to decrease the daily activities and quality of life[4]. However, different environmental and genetic factors may have been involved in as causative of the RA diseases[5]. In the terms of genetic, the people with MHC-type HLA-DR4 have more prone to the disease and the male patients after 8 years with RA

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has been shown HLA-B27[6]. Among environmental factors, the bacterial and viral agents, nutrition[7], and hormonal[8] have mentioned. Recently, the results researches revealed that superantigens (SAgs) have also implicated in chronic inflammatory diseases such as psoriasis and rheumatoid arthritis[9-11]. *Staphylococcus aureus* (*S. aureus*) SAgs as inflammatory agents attached to MHC II and T cell receptor and lead to release of the large amounts of cytokines including IL-1 and TNF-± which consequent inflammation and damages of tissues occurred[7]. Thus, SAgs are predisposing factors for RA disease[12]. In addition, the superantigenic toxins may play a role in causes of diseases such as Atopic dermatitis, psoriasis, and chronic sinusitis[13]. Few researchers have focused to detect the Enterotoxin C and B genes from clinical isolates of S. *aureus*[14, 15]. The staphylococcal enterotoxin P, detection, and characterization have reported[16]. Furthermore, recognition of Staphylococcal enterotoxin A (superantigens A) in Synovial fluid of RA children have been discussed[12]. Because of, rapid and accurate detection of causative agents of RA disease can guide to appropriate treatment and decrease the risk of permanent disabilities. So far, several methods for detection of Enteroxins producing gene in body fluid including uniplex PCR[17], Multiplex PCR[18], and Sandwich ELISA[19] have reported. Due to published results of Staphylococcal, enterotoxins act as superantigen and causes of inflammation disease. The precise, accurate and rapid identification of superantigens and evaluate of in Synovial fluid has been remained unresolved. However, an exciting and important outline for patients with chronic rheumatoid arthritis is determine and clarified related etiologic agents. Especially, once the body fluids of patients had no identifiable organisms or diagnostic determinants. Despite, several studies have reported gene encoding of SAgs, but the origin was unclear. It is also not clear whether these gene encoding of SAgs are expresse in site of involvement or not? In order to answer the question this study has designed. Thus, the aim of this research was to develop the Real time RT RCR method to detection of mRNA producing enterotoxin B gene in Synovial fluid of RA patients, while the lack of isolation of *S. aureus* bacterium.

MATERIALS AND METHODS

This experimental study had conducted in Baqiyatallah University of Medical Sciences microbiological department laboratory Tehran Iran. The study protocol approved by the Ethical Committee of the Baqiyatallah University of Medical Sciences, Teheran, IR Iran, on November 29, 2011, with Code No: 24, Paragraph 28. During three years (January 2012- March 2015). Totally, 80 Synovial fluids (SF) samples from patients with RA who had no significant of infectious disease, based on American college of Rheumatology (ACR) criteria, provided by expert rheumatologist. All patients were from different cities of Iran that referred to Baqiyatallah Hospital rheumatology clinic and they have medical records. Because of repeated visits the clinic they agreed to fill consent form and donation synovial fluid sample were enrolled in this study. The samples stored at -80°C until used. The *S. aureus* 14458 ATCC strain used as positive control.

Real time RT RCR method

Primer and probe Design

Primers pairs and probes for cDNA synthesis was designed and Real time PCR reaction were performed. The primer was based on the reference sequence of *S. aureus* ATCC 14458, enterotoxin B gene, evaluate by Primer3 software and GenScript Real-time PCR (TaqMan) Primer Design (Table 1) and synthesized by Pishgam Company Iran.

Set up of mRNA Extraction from Reference Bacteria

As a positive control, *S. aureus* ATCC 14458 strain producing enterotoxin B has inoculated to LB broth and incubated in 37 ºC for 18 hours. After that, based on mRNA extraction Ribex kit (Gene All Company), the bacterial mRNA has extracted. Briefly, 0.25 ml of bacterial suspension was add to 0.75 ml of Ribex solution and were pipette for lysis. The homogenates lysate were incubated for 5 minutes in room temperature and then they were centrifuged in 12000× g for 10 minutes in 4°C and Colorless supernatant were transferred to a new micro tube. 0.2 ml of Chloroform was added to 0.75 ml of lysate supernatant solution to a new micro tube and they were gently mixed for 15 seconds and were incubated for 2 minutes at room temperature. The

solution was centrifuged in 12000× g for 15 minutes in 4 °C, and then the aqueous phase was transferred to a new micro tube. 0.25 ml of isopropyl alcohol and 0.125 of 0.8 M sodium citrate were added to aqueous phase separately and ten times by upside-down were slowly mixed. The samples have placed in room temperature for 10 minutes and then they were centrifuged in 4 °C and Supernatant was discarded. 1 ml of 75% ethanol was added for washing the mass mRNA and then the solution were centrifuged in 7000×g for 5 minutes in 4 °C and the Supernatant was carefully discarded and mass mRNA was air dried for 5 minutes. The resultant mRNA dissolved in DEPC water and was placed for 15 minutes in 56°C. The DNase enzyme were added to above solution in order to remove the DNA contamination. Finally, the amounts of mRNA measured by Nano drop and subjected to cDNA synthesis. The resultants were stored in -70ºC.

mRNA Extraction from Synovial fluids Samples

According to the above protocol, the 80 synovial fluids samples stored in -80ºC were subjected to mRNA extraction and cDNA synthesis separately.

cDNA Synthesis

Based on the manufacture's instruction the cDNA was synthesized as briefly: respectively, 2 and 5 *p*mol from 100 *p*mol stoke solution of specific Forward and Reveres primers and added to 2 ¼l of purified mRNA. In purpose of denaturation of RNA, the solution incubated for 5 min at 65°C, and cooled on ice immediately. After that, 10µl Master Mix RT (Reverse transcriptase) manufactured by Gen all, (Hyrer script RT master mix cat No. 601-710) was added to the tube, and were slowly over a brief centrifuges. Solution was incubated for 1 h at 55° C, and finally reaction stopped with incubation in 95° C water bath for 5 minutes and cooled on ice. 2 units of RNaseH was added to synthesized cDNA and were incubated for 20 min at 37 ° C. in each case, due to quality control of the results agarose gel electrophoresis was performed.

Real time PCR reaction

Real time PCR reaction was performed using SYBR®Green day. 10 ¼l Master Mix Real Q plu Z master mix (Amplicon; Cat No. A323499), 1¼l of each Real Time primer pairs and probe by concentration of 5 *p*mol, 5¼l of synthesized cDNA and 4¼l water with DEPC quality added to micro-tubes and the final volume reached 20¼l. Three steps Amplification program was as follows in Corrbet RG 6000 instrument. The initial temperature was 95°C for 15 minutes and then 40 cycles of denaturation at 95°C for 15 seconds, Annealing for 30 seconds at 56°C the final stage in 72°C for 30 seconds. The digital output were monitor and saved.

RESULTS

Patient's analysis

In this study, 80 rheumatoid arthritis patients were included. The demographic analysis revealed that the mean age of patients participating was 52± 13 years old, with 49 (61%) women and 31 (39%) men. Mean duration of chronic rheumatoid arthritis was 5.2 years. They were all under treatment with steroid, azathioprine, methotrexate, and or biologic drugs.

Bacterial *m*RNA Extraction and cDNA

The results of *m*RNA extracted and cDNA synthesized from positive control sample of *S. aureus* strain ATCC 14458 that producing enterotoxin B has shown in Figure 1.

The results of optimized Tm of real time PT PCR reactions were determined e" 77.2°C for positive control (No. 27 bright purple color). The results of real time RT PCR a number of synovial fluids samples of patients with RA is shown in Figure 2. In the right table, the real time RT PCR synovial fluid samples with TM above 77.2°C indicated the presence of mRNA encoding enterotoxin B of S*taphylococcus aureus*. Out of 80 samples of synovial fluid of patients suffering from RA, 38 cases (47.5%) of synovial fluid sample have shown enterotoxin B *m*RNA by converting to cDNA. The results of positive reaction (cDNA gene amplification) by Real Time PCR methods were shown in Figure 3.

The results of one run Real Time RT PCR including 20 samples can be seen in Figure 3, after the fifteen cycles of Real Time RT PCR amplification reaction and depending of initial gene copy number and reaction conditions the maximum out coming were on 30 to 40 cycles. The horizontal lines with threshold near zero indicate the absence of the target gene in the samples. While, the ascending sigmoid curve line showed the presence of the

Table 1. Characteristics of Primers and Probes used in this study

Name	Sequence	Strand	Modification
	The cDNA synthesis primers Set: Amplicon Size = 165		
Query- L	TGTATGTATGGTGGTGTAAC	Forward	
Query- R	ATAGTGACGAGTTAGGTA	Reveres	
	Real- Time PCR Primer Set : Amplicon Size = 70		
Query-L1	GCAGAGAGTCAACCAGATCCT	forward	
Query-R1	TTTCCATCAAACCAGTGAATTT	reverse	
Query-P1	TCGCTTTGTGCAACTCATCTGGTTT	reverse	5'Fam - 3'Tamra

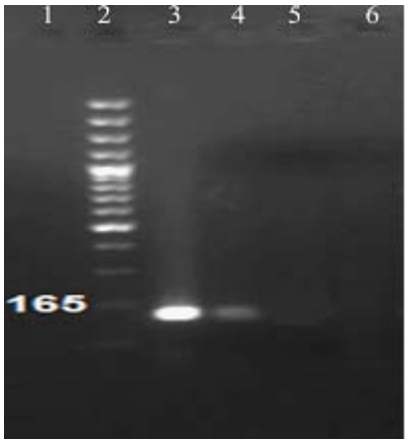

Fig. 1. Gel electrophoresis of Real time RT PCR product from cDNA synthesized based on *S. aureus* mRNA optimization. Lines 1, 5 and 6 are negative control. Line 2 DNA marker 100 bp. Lines 3 and 4 is positive control.The sequencing of the Real Time PCR products confirms the accuracy of the results

target gene and amplifications. The finding results confirmed by electrophoresis.

DISCUSSION

However, the role of the Staphylococcus and Streptococcus toxins in pathogenesis of infectious diseases has discussed[20]. The pathophysiology of bacterial exotoxins impact remains unresolved. Several studies have pointed out the role of staphylococci producing toxins in pathogenesis of acute form of the inflammatory diseases. For example, the role of these toxins in Kawasaki and atopic dermatitis has discussed[21]. The researches results have showed that bacterial SAgs may have a role in chronic sinusitis and nasal polyposis[22, 23]. A research result has pointed out to pathogenicity of staphylococcal enterotoxins (SAgs) in the airway inflammation[24].

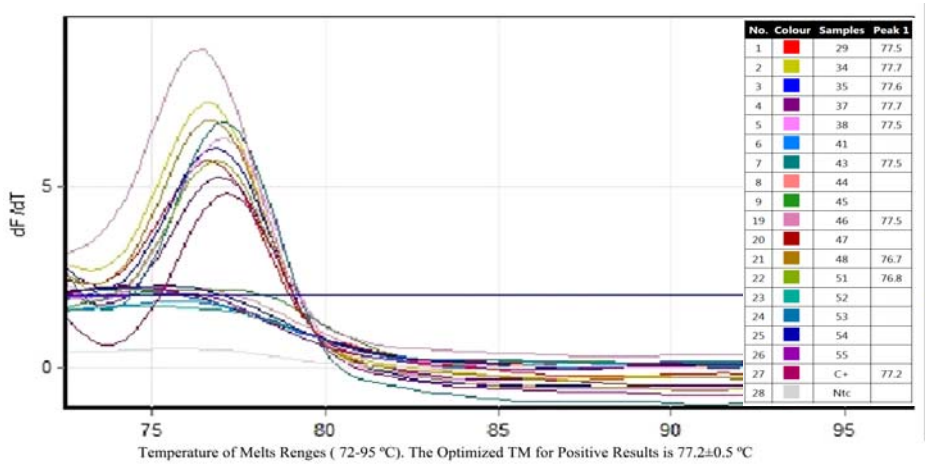

Fig. 2. The results of optimized reaction for TM Real Time RT PCR peak assay for Staphylococcal Superantigen (Enterotoxin B) detection in the synovial fluids of patents with RA have shown

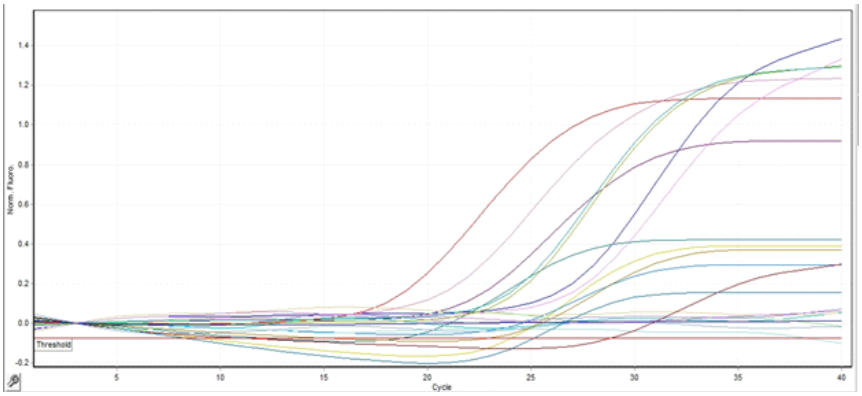

Fig. 3. An example of device-normalized optimization of the Real Time RT PCR reaction for Staphylococcal SAgs (Enterotoxin B) detection in the synovial fluids of patents with RA are shown

Staphylococci producing enterotoxins have isolate from patients with psoriasis and atopic erythroderma and compare the results with healthy individuals[25]. In addition, isolating staphylococcal producing enterotoxin B in clinical samples was reported[15]. Enterotoxins production from Coagulase Negative Staphylococci (CoNS) isolated from Cerebrospinal fluid were explored[26]. Furthermore, Enzyme-linked immunosorbent assay (ELISA) was used to detect staphylococcal SAgs in the synovial fluids of seronegative rheumatoid arthritis patients and immunoblot confirming has reported[27]. However, detection of staphylococcal enterotoxin A, B, C, D and E from synovial fluid of patients with rheumatoid arthritis by PCR were reported. Of course, not only the staphylococcal SAgs in SF of patients with RA have investigated but also other superantigens, such as Mycoplasmas in SF of RA patients by frequency of 53.4% were reported[28, 29]. Detection of exotoxins producing bacteria in clinical samples is not surprising. However, there is controversial and noteworthy, why in non- acute disease and in absence of bacteria, different toxins being recognized. Where is the resources of toxins production?.

The previous researches findings have erased several important questions. Why in absence of the bacteria? The enterotoxins (SAgs) and its encoding genes were detectable. For response this question, the Real Time RT PCR methods have set up. The 80 synovial fluids samples of RA patients have tested. Our study showed the mRNA encoding staphylococcal enterotoxin B in the SF of patients. This finding may explain the causality serogenative rheumatoid arthritis of some patients[30.]

Because of, these exotoxins have ability to activate the immunological reactions with resultant cytokines storm.

On the other hand, despite negative culture of Staphylococcal species, the presence of mRNA encoding staphylococcal enterotoxin B (superantigen B) was existed. The results of current study suggest one-step closer to find out the origin of staphylococcal enterotoxins in the SF of RA patients. Because of, the results of this study described two points that are more important. First, staphylococcal enterotoxin B DNA has expression in local of inflammation. Secondly, this finding induced two important hypotheses.

a) The possibility of the normal form of staphylococcal producing enterotoxin converted to fastidious and or non- cultivable form of the bacteria.

b) Other hypothesis is that the possibilities of plasmids carrying genes encoding staphylococcal enterotoxins and penetrate into the synovial tissue cell and expression.

The approval of both hypothesis required further investigation with evidence immune-electron microscopy.

However, based on the result of this study out of 80 samples of synovial fluid of patients suffering from RA, 38 (47.5%) of them have shown

enterotoxin B *m*RNA existence separately. This finding was differing from the Nourbakhsh study results (47.5% vs 18%). These differences may be reflecting the more reliability of molecular method RT Real Time PCR used in this study. In addition, there was no correlation between detected the staphylococcal SAgs with positive cultures of *S. aureus* from synovial fluids of patients with RA[12]. Despite, the current research conducted in order to assay the synovial fluids samples of non-septic rheumatoid arthritis patients. Several researchers had investigated the etiologic agents of septic arthritis in children. They showed that, in 23% of suspected patients the 16sDNA of bacterial genomic from synovial fluid was detected[31]. Clinical aspects have demonstrated many differences between rheumatoid arthritis and septic arthritis. This study investigates the SF patients with RA while a few researchers have reports the finding results from SF septic arthritis patients. Therefore, no available similarity is there between the results of this study with other reported studies. Because of, the diseases were different.

Staphylococcal SAgs may play an important role in pathophysiology of inflammatory disease such as RA. While, is not possible isolated bacteria from the synovial fluid of patients. Because of, bacteria named non-cultivable or Viable-But-Non culture able-State (VBNC) reported[32]. Thus, it is necessary designing rapid and simple technique to identify the superantigenic exotoxins in the synovial fluid or blood of patients. Further study is requiring for determined the roles of the other bacterial SAgs such as *Prevotella copri*[33] in SF of RA patients.

In conclusion, this study result suggests possible role of Staphylococcal SAgs in the pathophysiology of RA as an autoimmune disease. Due to this reason, rapid identification and treatment of the disease caused by these organisms may prevent the autoimmune system activation of the patients. This finding may provide specific and appropriate cure for some patients with RA. Bacteriological assay of synovial fluid of patients with RA is laborious and time consuming. However, set up the fast and easy method to detect exact etiology of RA patients may provide specific treatment. Hence, there is a relative lack of available information. Further studies with larger sample size is needed this areas of RA patients.

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REFERENCES

1. Eftekharian M. Risk factors for rheumatoid arthritis: Level of education, tonsillectomy and appendectomy . *Marmara Medical Journal*, 2013; **26**:30-33.
2. Davatchi F et al. The prevalence of musculoskeletal complaints in a rural area in Iran. *Clin Rheumatol*, 2009; **28**: 1267-1274.
3. Jamshidi AR, Tehrani BA, Roknsharifi S, Akhlaghi M, Salimzadeh A, Davatchi F. Estimating the prevalence and disease characteristics of rheumatoid arthritis in Tehran: A WHO -ILAR COPCORD Study (from Iran COPCORD study, Urban Study stage 1). *Med J Islam Repub Iran* 2014; **28**:93.
4. Lee J, Kim SH, Moon SH, Lee EH. Measurement properties of rheumatoid arthritis-specific quality-of-life questionnaires: systematic review of the literature. *Qual Life Res*, 2014; **23**:2779-91.
5. Reckner Olsson A, Skogh T, Wingren G. Comorbidity and life style,reproductive factors, and environmental exposures associated with rheumatoid arthritis. *Ann Rheum Dis*, 2001; **60**: 934–9. doi:10.1136/ard.60.10.934.
6. Kallbeg H, Padyukov L, Plenge RM. Gene–gene and gene–environment interactions involving HLA-DRB1, PTPN22, and smoking in two subsets of rheumatoid arthritis. *Am J Hum Genet*, 2007; **80**:867–75. doi: 10.1086/516736.
7. Balandraud N, Meynard, JB, Auger I. Epstein-Barr virus load in the peripheral blood of patients with rheumatoid arthritis: Accurate quantification using real-time polymerase chain reaction. *Arthritis Rheum*, 2003; **48**:1223–8. doi: 10.1002/art.10933.
8. Heikkilä R, Aho K, Heliövaara M. Serum androgen-anabolichormones and the risk of rheumatoid arthritis. *Ann Rheum Dis*,1996; **57**: 281–5. doi: 10.1136/ard.57.5.281.
9. Li J, Yang J, Lu YW, Wu S, Wang MR, Zhu JM.

Possible Role of Staphylococcal Enterotoxin B in the Pathogenesis of Autoimmune Diseases. *Viral Immunol,* 2015; **28**(7):354-9. doi: 10.1089/vim.2015.0017.

10. Ataee RA, Ataee MH, Alishiri GH, Esmaeili D. Staphylococcal enterotoxin C in synovial fluid of patients with rheumatoid arthritis. *Iran Red Crescent Med J,* 2014; **16**:e16075.
11. Torres BA, Kominsky S, Perrin GQ, Hobeika AC, Johnson HM. Superantigens: the good, the bad, and the ugly. Exp Biol Med (Maywood), 2001; **226**:164-76.
12. Noorbakhsh S. Identification of bacterial antigen& superantigens in synovial fluid of patients with arthritis .*Tehran university medical jornal*, 2012; **70**(1): 41- 48.
13. Yarwood JM,Leuny DY,Schlivert PM. Evidence for involvement of bacterial superantigens in psoriasis,aptopic dermatitis and Kawasaki syndrome. *FEMS microbial let*, 2000; **192**(1): 1-7.
14. Ataee RA, Kamali M, Karami A, and Ghorbani M. Standardization of molecular diagnostic of the EntC S. aureus isolated from human infection and determine its sequence. *Iranian journal of military medicine .Autumn,* 2012; **14**(3): 226-235.
15. Ataee RA , Kamali A, Morteza Izadi, Aref Aghania, Ataee MH.. Molecular screening of Staphylococcal entrotoxin B in clinical isolated .*Cell J.Fall* . **13**(3):87 92.
16. Ataee RA ,Hedaiati M, Khanshan R, Ataee MH. *Molecular screening of Staphylococcal entrotoxin P encoding gene from clinical isolates .Jundishapur jornal of microbiology,* 2012*;* **6**(5):e 6365.
17. Johnson WM, Tyler SD, Ewan EP, Ashton FE, Pollard DR, Rozee KR. Detection of genes for entrotoxins , exfloiative toxins and toxic shock syndrome toxin 1 in Staphylococcus aureus by PCR.Jornal of microb . *J Clin Microbiol*. 1991; **29**(3): 426–430.
18. Mehrota M ,Wang G,Wendy M. Multiplex PCR for detection gene Staphylococcus aureus entrotoxins ,exfoliative toxin, TSST1 and methicillin resistance.*Jornal of clinical microbiology* ,2000; (38)3: 1032- 1035.
19. Kuang H, Wang W, Xu L, Ma W, Liu L, Wang L, Xu C. Monocolonal antibody base sandwich ELISA for the detection of SEA. *Environmental research and public helth*, 2013;.**10**(4): 1598-1608.
20. Floret D .Clinical aspects of streptococcal and staphylococcal toxinic diseases .*Arch Pediatr,* 2001; 8(Suppl 4):762s-78s.
21. Yarwood JM, Leung DY, Schlievert PM. Evidence for the involvement of bacterial superantigens in psoriasis, atopicdermatitis, and Kawasaki syndrome. *FEMS Microbiol Lett,*2000; **192**(1):1-7.
22. Tripathi A, Kern R, Conley DB, Seiberling K, Klemens JC, HarrisKE, et al. Staphylococcal exotoxins and nasal polyposis: analysis systemic and local responses. *Am J Rhinol,* 2005; **19**(4):327-33.
23. Tripathi A, Conley DB, Grammer LC, Ditto AM, Lowery MM,Seiberling KA, et al. Immunoglobulin E to staphylococcal and streptococcal toxins in patients with chronic sinusitis/nasal polyposis. *Laryngoscope,* 2004; **114**(10):1822-6.
24. Bachert C, Gevaert P, van Cauwenberge P. Staphylococcus aureus superantigens and airway disease. *Curr Allergy Asthma Rep*,2002; **2**(3):252-8.
25. Tomi NS, Kränke B, Aberer E. Staphylococcal toxins in patients with psoriasis, atopic dermatitis, and erythroderma, and in healthy control subjects. *J Am Acad Dermatol,* 2005; **53**(1):67-72.
26. Ataee RA, Mehrabi-Tavana A, Izadi M, Hosseini SMJ, Ataee MH. Bacterial Meningitis: a new risk factor. JRMS. 2011; **16**(2): 207-210.
27. Ataee RA, Alishiri GH, Mohoseni- Moghadam Z, Latifi AM and Ataee MH. Enzyme-linked Immunosorbent Assay for Detection Staphylococcal Enterotoxins in Synovial fluid of heumatoid Arthritis patients. Journal of Pureand Applied Microbiology 2013; **7**(2): 1113-1119.
28. Golmohammadi R, Ataee RA, Alishiri GH, Mirnejad R, Mehrabi Tavana A, et al. Design of PCR-based method for detection of a geneencoding Mycoplasma arthritidis mitogen superantigen in synovial fluid of rheumatoid arthritis patients. Iranian J Microbiol. 2014; **6**: 415 – 420.
29. Ataee RA, Golmohammadi R, Alishiri GH, Mirnejad R, Esmaeili D, Jonaidi-Jafari N. Simultaneous Detection of Mycoplasma pneumoniae, Mycoplasma hominis and Mycoplasma arthritidis in Synovial Fluid of Patients with Rheumatoid Arthritis by Multiplex PCR. Archives of Iranian Medicine 2015; **18**(6): 345- 350.).
30. Ataee RA, Alishiri GH, and Ataee MH. Laboratory characteristics of a prospective of patients with rheumatoid arthritis: new biomarkers for diagnosis. *Journal of Health Policy and Sustainable Health* 2015; **2**: 153-159

31. Rosey AL, Abachin E, Quesnes G, Cadilhac C, Pejin Z, Glorion C,et al. Development of a broad-range 16S rDNA real-time PCR for the diagnosis of septic arthritis in children. *J Microbiol Methods*, 2007; **68**(1):88-93.
32. Fakruddin M, Mannan KS, Andrews S. Viable but nonculturable bacteria: food safety and public health perspective. *ISRN Microbiol*, 2013; e703813. doi: 10.1155/2013/703813.
33. Salinas M , Madero G (2015) Prevotella copri and the microbial pathogenesis of rheumatoid arthritis *Reumatol Clin*, 2015; **11**(2):61–63