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Study of Three Pair Primers PCR to Detect SEC Gene in Synovial Fluid of Rheumatoid Arthritis Patients and Comparison with Elisa

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More than 20 types of *S. aureus* enterotoxins have been identified so far. Detection each of them is very important in clinical samples. Different detection primers pair have been designed and used. The aim of this study was to assay the efficacy of three pair primers to detect staphylococcal enterotoxin type C in synovial fluid of RF patients and compare the results with Elisa. In this study, seventy synovial fluids of RF patients were assayed. Three pair primers were used. The primers were amplified 102, 206 and 1223bp fragment respectively. The PCR products were sequenced and compared multiple alignments with reference gene. In addition, the Elisa plate was designed for detection of the SEC. The obtained data was subjected to descriptive analysis. The results showed that the three pair primers were amplified by different frequencies. The results of amplified of amplicons 1223, 206 and 102bp were 9(18%), 34(68%) and 17(44%) positive for ent C gene respectively. The results of Elisa revealed that 22 cases (44%) were positive for staphylococcal enterotoxin C. The amplicon 206bp were the most abundant probability of the amplification product. The inter gene amplicons amplification pair primers were shown more specify. The results indicated that the staphylococcal enterotoxin C gene existed in synovial fluids of RF patients. Therefore candidate specific primers to amplify 206bp fragments of ent C and Elisa method are reliable. This finding may help to detect the etiology of rheumatoid arthritis.

Key words: Staphylococcal enterotoxin C, PCR, Elisa, Rheumatoid arthritis.

S. aureus, the most common bacteria resistant to antibiotics is able to secrete various types of exoproteins with superantigenic properties[1]. Probable causes of sudden unexpected death and other serious disease in adults are reported in literature[2-3]. In addition, the superantigens may also reduce responsiveness to corticosteroids resulting in more severe asthma[4], and may play a role in the induction of autoimmune diseases[5]. Additionally, the roles of the superantigens in non-gastrointestinal diseases such as airway disease are identified to be crucial[6-7]. Thus, detection and diagnosis of staphylococcal superantigens in clinical and environmental samples have taken priority[8], Especially in culture negative samples is critical. Various methods based on Polymerase chain reaction (PCR) for the detection of Staphylococcal enterotoxin genes have been used[9-11]. In all of these investigations enterotoxin genes have been studied in bacteria

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isolated from samples. However, few researches are available with negative the bacteriology report where Staphylococcal enterotoxins have been detected. In a study, the *S. aureus* clinical isolates was subjected to staphylococcal enterotoxin C gene determined by PCR[12]. Prevalence and expression of the staphylococcal superantigen genes between methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) was investigated and showed that 65% isolates of *S. aureus* strains from patients harbored superantigen genes[13]. It is reported that 70.0% of TSST-1 producers were also enterotoxigenic with staphylococcal enterotoxin C (SEC) were positive[14]. The result of other investigation revealed that 53% children with atopic dermatitis (AD) were colonized with toxigenic strains of *S. aureus* producing staphylococcal enterotoxin C, A, TSS-1, and B types which had higher disease severity compared with the nontoxigenic and *S. aureus*-negative groups[15]. Recently, enterotoxigenic potential of coagulase- negative *S. aureus* has been shown (3;16). The result of our previous study revealed that 20.46% of coagulase negative *S. aureus* strains isolated from CSF meningitis were able to produce superantigenic enterotoxin[17]. Staphylococcal enterotoxin C is a common supantigen whose expression and secretion is aynchronized with the staphylococcal TSST-1. In order to determine the exact frequency of *S. aureus* producing enterotoxin C in clinical samples, several pair primers were designed and applied for the detection of *S. aureus* enterotoxin C has been reported. However, it is not clear what type of primers is the best and have higher sensitivity and specificity. The aim of this study was to compare three pair primers and select the most sensitive and specific attributes for the detection of staphylococcal enterotoxin C in synovial fluid (SF) of rheumatoid arthritis (RA) patients and compare the results with Elisa test.

Materials and Methods

Bacterial strain

In this study, a strain of *Staphylococcus aureus* (*S. aureus*) which was previously confirmed to harbor entertoxin C gene was used as the standard strain[18].

Primers used

In this study three pair primers were applied. The first primer pair amplified a 102bp that was selected from previous research[19]; the second and third primers amplified 206bp and 1223-bp fragment respectively were designed in this study. Using online Genscript software the primers pair were designed on the basis of the reference sequence (*S. aureus* MW2 enterotoxin C gene, accession number AB084256.1) and were analyzed by primer 3 software. In addition, multiple alignments were carried out by DNASIS MAX trial version.

Sampling

During 22 months, from September 2010 to July 2012, a total of 70 samples of synovial fluid were collected by a rheumatologist from patients with rheumatoid arthritis were provided in the study. In aseptic conditions, the samples were immediately divided into two parts; bacteriological culture was performed from samples in Castaneda's medium. The other part was subjected to ELISA and also PCR. The remaining samples of SF were kept at – 20 °C to next stages of the research.

Bacterial DNA extraction

DNA extraction of standard bacteria was performed by modified salting out method[20] from 24- hours culture suspension of bacteria. Then, the extracted DNA was evaluated by Nano- Drop (Thermo Scientific Nano- Drop 2000 Spectophotometer USA) and conditions to perform PCR were optimized.

DNA amplification

The PCR was performed and optimized conditions for amplifying the three pair primers were carried out separately. The optimized condition for the first primers pair which amplified a 1223 bp in a total volume of 25 µl and included: 1 µl (10ng/µl) of template DNA, 1.5*U* Taq DNA Polymerase, 0.12*mM*dNTP Mix, 0.4 *p*mol of each Primer and 2*mM*MgCL2. DNA amplification was performed in a thermal cycler (Eppendorf AG 22331 Germany) using the following conditions: initial denaturation for 4 min at 94 °C followed by 35 cycles of denaturation (94 °C for 30s), annealing 60 °C 35S and extension 72 °C for 40s. Final extension step 72 °C for 5 min was performed.

The optimized condition for the second primers pair which amplified a 206 bp fragments was carried out in a total volume of 25 µl and included:1 µl (15 ng/µl) of template DNA, 1.4*U*/0.7 µl Taq DNA Polymerase, (0.3 µl) 0.12*mM*dNTP Mix, 10 *p*mol/µl of each Primer and 2*mM*MgCL2/µl. DNA amplification was performed in a thermal cycler

(Eppendorf AG 22331 Germany) using the following conditions: initial denaturation for 4 min at 95 °C followed by 40 cycles of denaturation (94 °C for 40s), annealing 60 °C 35s and extension 72 °C for 40s. Final extension step 72 °C for 5 min was performed. Each case as positive controls, PCR containing template DNA extracted from the standardized strain were used.

The optimized condition for the amplification of the third primers pair which amplified a 102 bp fragment in a total volume of 25 µl and included, 0.3 µl (15 ng/µl) of template DNA, (1 µl)1.5*U* Taq DNA Polymerase, (0.3µl). 12*m M*dNTP Mix, 0.4 *p* mol of each Primer and (0.7 µl) 1.4 *mM*MgCL2. DNA amplification was performed in a thermal cycler (Eppendorf AG 22331 Germany) using the following conditions: initial denaturation for 3 min at 95 °C, followed by 40 cycles of denaturation (94 °C for 40s), annealing 50.5 °C 30S and extension 72 °C for 40s. Final extension step 72 °C for 5 min was performed after complete the cycles.

Visualization of amplified DNA

A 5 µl aliquot of the PCR product was analyzed on 0.8 to 1.5% TBE agarose (Maxpure agarose, Lot No. K452410. Spain). The electrophoresis was carried out in Horizontal gel tanks at 100V for 45minute or until the desired resolution was obtained. Then, agarose slab gel was stained with flooding in ethidium bromide solution (0.5 mg/µl) for 10 min and after that washing was carried out. Gel were viewed by UV transillumination (Biorad, Universal Hood ™™, USA) and photographed by using a 35-mm camera.

DNA extraction from SF

DNA extraction was performed from SF according to manufacturer's guidelines (CinnaPure DNA; Cat. No. PR881612; CinnaGen Co; Iran). Extracted DNA was examined by Nano- Drop and one micro liter of it was used as template to perform PCR.

PCR product was subjected to electrophorese by 1.5% TBE agarose gel. It was then placed in the ethidium bromide solution for 10 minutes. After that gel documentation was studied by ultraviolet light. Because additional bands were observed in the gel, the PCR product was electrophoresed again by 1.5% low melting temperature of agarose gel. Then, the equal bands were isolated from the gel and DNA clean up was performed by kit (Gel DNA Recovery Kit, GF-GP-050, Cinnagen, Iran). After ensuring no additional bands were in the gel, finally it was sent to Cinnagen Company to determine the sequence.

ELISA plate design

For this purpose, anti-staphylococcal enterotoxin C antibody (Rb PAb enterotoxin C Ab 15899 500 lot 722163; from Abcam Germany) was used. The antibody was monospecific polyclonal. Bovine Serum albumin (Lot 057K0737, Sigma Life Science), Horse Radish peroxidase conjugated Ab (Anti Rabbit IgG, Code No: AP7181, Lot 102, Razi Biotech, Iran), Substrate (urea peroxide) and Cromogen (tetramethylbenzidine) and stopper (sulfuric acid) were purchased from Merck, Germany.

Primary antibody (*S. aureus* enterotoxin C antibodies) was coated onto the plates. The working dilution was 1 ng/µl. Each one of 96 wells micro plate ELISA was used.

In each plate, the rows A, B, C, D and E were coated with anti-enterotoxin type C antibody. Rows F and G were considered as negative controls. Row H was selected as a positive control. In negative control rows, the antibody was not coated. For positive control row, a specific antibody of a known toxin was used. The plate was prepared by the above mentioned method was kept at 37 °C overnight so as to evaporate the remaining liquid. Then all wells were treated with 3% BSA solution as free site blocker. This was done in order to saturate free binding sites. After that, plates were sealed in aluminum foil pocket and stored in a refrigerator at 4 °C until use.

After preparation of ELISA plates all samples were assayed as follows: 50 µl of SF (diluted with sterile PBS to 50%) was loaded in each well and the plates were incubated at 37 °C for one hour. After incubation period, the remaining samples were then evacuated from the plates and they were washed three times by washing buffer for 45 seconds. After that, 50 microliters of working diluted secondary antibody was added to each well. Then it was incubated for 50 minutes at 37 °C and then the sink drain was diluted three times and washed. In the final stage, 50 µl of substrate solution (urea peroxide) and then 50 µl chromogene solution (tetramethylbenzidine) was added. After 15 minutes when the color was observed, the plates were subjected to 100 µl of stopper (sulfuric acid

2*M*) which was immediately poured into each well. Then, the opacity each well of ELISA plates at 450 *nm* were measured using ELISA Reader (TECAN Austia GMbH, Modwl: Sunrise, Serial No: 501000095). The cut off value was calculated by using the mean OD of negative controls and a constant factor 0.15 was added to them and the test cut-off value were obtained.

RESULTS

The Routine bacteriological culture of 70 SF samples revealed no bacterial growth. The result of measurement of DNA extracted from the reference strains used in this study was 117ng/µl. The Primer design results are shown in Table 1. Each of these pair's primers could amplify specific fragments. Figure 2 represent the results of PCR product electrophoresis and suggested that, part A for fragment of 102 bp. Part B for fragment 206 bp and Part C showed the fragment of 1223 bp respectively.

The sequencing results of PCR products approved the optimization condition of the PCR method and confirmed the *S. aureus* enterotoxin C. The study results of 70 SF samples by each of the primers according to the above optimization conditions separately have shown some similar bands and also several exceeded bands (Figure 2). Gene sequencing of PCR product of SF sample confirmed the presence of *S. aureus* enterotoxin C gene. Figure 2 shows an example of the results of electrophoresis of PCR products. The intended DNA bands were subjected to extraction from the gel and purification. The PCR was then repeated and sequencing was carried out. The results confirmed the gene sequence.

The results of Elisa method showed that

Table 1. Three pair primers characteristics used in this study. In the first primer pairs the restriction enzymes Xho and BamHI sequences were added

Number	Primers	Nucleotide sequence	Product size
1	forward (Xho)	5'- TAGCCTCGAGGGAATGTTGGATGAAGGAG -3'	1223 bp
	reverse (BamHI)	5'- CTAGGGATCCTATGGACACAATGATACTGG -3'	
2	forward	5'- GAAACACAATTTCTTTTGAAGTGC -3'	206 bp
	reverse	5'- AAACTTATCGCCTGGTGCAG -3'	
3	forward	5'- TGTATGTATGGAGGTGTAAC -3'	106 bp
	reverse	5'- AATTGTGTTTCTTTTATTTTCATAA -3'	

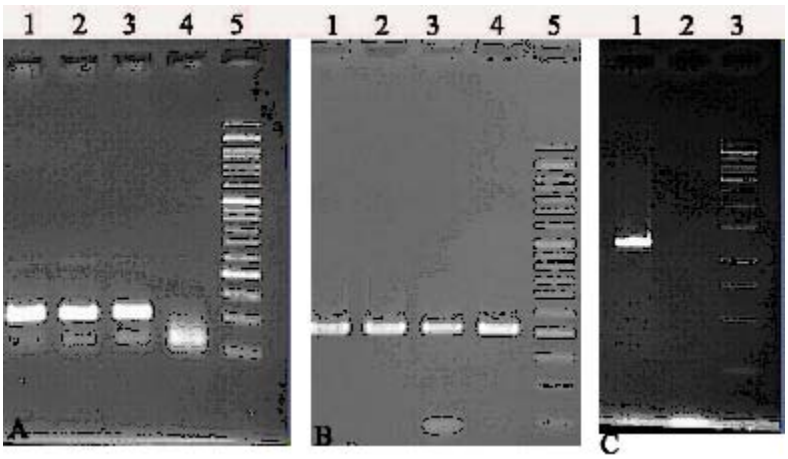

Fig. 1. Optimum conditions for the amplification of three primers of Staphylococcus enterotoxin C genes with reference template bacterial genome were shown. In part A, lines 1 to 3 show 102 bp amplified fragment band as a positive control, line 4 is negative control and lane 5 represents 50 bp DNA ladder. In part B, lines 1 to 4 show the 206 bp amplified fragment band as a positive control and lane 5 represents 50 bp DNA ladder. In part C, lines 1 show 1223 bp amplified fragment band as a positive control, line 2 is negative control and lane 3 represents 1Kb DNA ladder

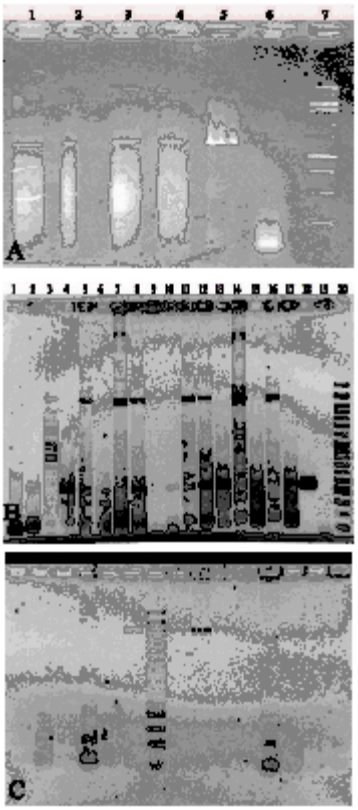

Fig. 2. Examples of electrophoresis and the PCR product of each primer with DNA extracted from synovial fluid samples are shown separately. In part A, PCR product with primers of the 1223 bp amplified fragment (lines 1 to 4) with a positive control (lane 5), negative control (lane 6) and 1Kb DNA ladder (lane 7).is shown.

In part B, the results of the PCR product with primer 206 bp amplified fragment and DNA template extraction of the synovial fluid from patients with rheumatoid arthritis is shown. The PCR results were negative for SF sample in lines 1, 3, 9 and 16. Lines 2, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15 and 17 are examples of the positive PCR results. In fact, this primer could be amplified 206 bp fragment in samples of synovial fluid. Line 18 is positive control, line 19 is negative control and Lines 20 is 50 bp DNA ladder. As it was shown in part B, the lined 2, 4, 13 and 17 only a single band amplification is Intended, whereas in other samples extra bands in range 1100bp fragments can be observed. Part C shows the results of electrophoresis PCR product with amplified 102 bp fragment. As it was shown the lines 1, 3, 5, 6, 9, 10, 11, 13 and 14 are negative results. Lines 2, 4, 12 and 15 show fragment of 102 bp as positive cases. Line 7 is 50bp DNA ladder and Line 8 is a negative control. Furthermore, as it has seen in part B, in addition the 206 bp fragments amplified, the amplification of 1100 bp fragments in Lines 3, 5, 7, 8, 11, 12, 14 and 16 is shown. Also, in part C the result of electrophoresis of PCR product revealed the non-specific band of 1000 bp fragments in the line 6 and 9.

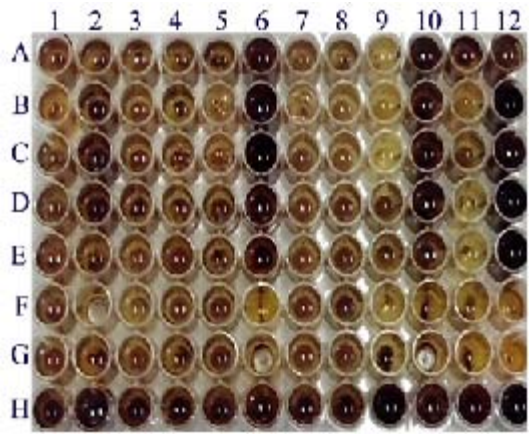

Fig. 3. The results of an Elisa test plate for the detection of *S. aureus* enterotoxin C form the SF of patients with rheumatoid arthritis is shown.

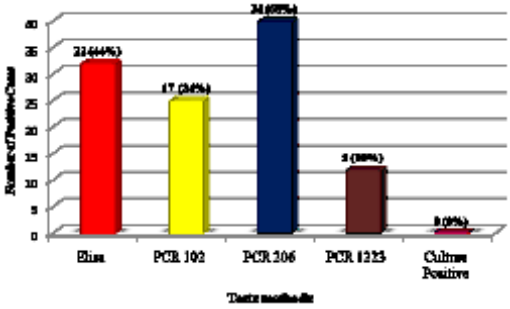

Fig. 4. Comparison of Elisa results with the PCR performed by different primers

44% (22 cases) of SF of rheumatoid arthritis patients' samples contained staphylococcal enterotoxin C. As shown in figure 4, results of PCR with primers amplified of amplicons 1223bp, 206 and 102bp were 9 (18%), 34 (68%) and 17 (44%) positive for ent C gene respectively were positive for staphylococcal enterotoxin C gene.

Figure 3 is shown an example of an Elisa plate. The plate rows A, B, C, D and E were loaded with anti-Staphylococcal enterotoxin C antibody. The deep brown color of wells indicated the *S. aureus* enterotoxin C. Also, in positive control row (row H) brown color can be observed. Positive controls wells in rows 9 and 12 contain 10 ng/µl and the other positive row wells contained 5 ng/µl standard toxins as positive control.

The comparison of Elisa results with the results of the PCR with different primers amplification and bacterial culture are shown in Figure 4. As it was shown no bacterial growth

occurred in Castaneda even after 4 weeks of incubation.

DISCUSSION

In our previous study, by using the commercial Elisa kit the presence of common Staphylococcus enterotoxins (superantigens) in the SF of rheumatoid arthritis patient's refinement and the confirmatory Western blotting tests were reported[21]. The results of that study raised several important questions. For example, why, none of the samples of SF had bacteria, while Staphylococcal enterotoxins were detected? From where, these toxins entered SF? Where are the original sources of the enterotoxins? Whether, the gene coding for enterotoxin is detectable or not? The inflammatory role of super- antigens has been reported by several investigations[22; 23]. Various biomarkers for the differential diagnosis of rheumatoid arthritis and other inflammatory diseases are available[24], however, there is no research that has suggested the precise etiology of rheumatoid arthritis and the direct role of super-antigens in SF has not been shown. In addition, the presences of super-antigens in SF have not been reported. However, a recent report claimed that rheumatoid arthritis is a systemic autoimmune disease with uncertain etiology[25]. There are abundant reports which showed the role of superantigens in triggering inflammatory disease[26-28]. In addition, the results of one study revealed that the level of antibodies against peptide mimicking urease is significantly higher in sera from patients with rheumatoid arthritis in comparison with the volunteer blood donor sera[29]. In another study, the etiopathogenesis of the autoimmune disease of RA were investigated and association between periodontopathic bacteria (*Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella melaninogenica*, and *Tannerella forsythia*) and the etiology of RA have been shown[30], as these bacteria have also been identified in the SF of RA patients. While in this study no bacteria had been isolated from SF of the patients.

However, in order to answer these questions, the present study was designed to detect the common Staphylococcus enterotoxin C (super- antigen C). In addition to Elisa method, the PCR method for the detection of Staphylococcus enterotoxin C with three different primers pairs were designed and set up. Thus, each of the SF samples was assayed with PCR separately with the three primers pairs and also the Elisa. Then, the results were compared. The first primer was amplified a 102 bp fragment. These primers alone in 34% (17 cases) of SF samples of 102 bp fragments were shown. However, the primer was designed to amplify a 206 bp fragment were amplified in 68% (34 cases) of samples to track. Similarly, the third primer was designed so that the upstream and downstream which contain full length enterotoxin C gene is amplified. It amplifies a fragment of 1223 bp. These primers were also able to show that 18% of the samples were amplified with 1223 bp fragment. However, the ELISA test showed the presence of enterotoxin C in 44% of SF samples. Comparison of both cross tab two tests gave considerable results. For example, in the two samples of synovial fluids the PCR amplified the1223 bp fragment. While, the Elisa results was negative for these samples. The reasons of this phenomenon are not clear. Perhaps it's because no gene was expressed or the amount of the expression was less than sensitivity of the Elisa test. Comparing the results of Elisa with PCR amplified 206 bp fragments indicated similarity, so that in all the cases that were positive by Elisa also gave positive results by PCR. Only 11 cases were positive by PCR amplified the 206 bp fragment but the results of Elisa tests were negative for enterotoxin C. Furthermore, comparison of the result of PCR amplification 102 bp fragments with Elisa revealed that in 14 cases of synovial fluids, the Elisa showed the positive results. While, primers amplified 102 bp fragments PCR has not been able to show the gene that has enterotoxin C. whereas, only in six samples PCR amplified the 102 bp fragments but the Elisa test was not been able to demonstrate the presence of *S. aureus* enterotoxin C. Comparison of two PCR methods that amplified the 102 and 206 bp fragments respectively was not 100 % compliant. Thus, in six samples of SF, the PCR amplified the 102 bp fragments but the results of PCR for amplification of 206 bp fragments for these samples were negative. The reason for this finding is not clear. It may be related to genomic extraction procedure. Because of, in this study we had used to two

different gene extraction kits. Similarly, in 20 samples of SF PCR was positive for amplified 206 bp fragments. Whereas PCR results with primers amplified 102 bp fragments were negative. This discrepancy could be related to the different region of enterotoxin gene selected for amplification. An interesting finding was that, only seven cases (10%) of SF samples of RA patients were confirmed to have *S. aureus* enterotoxin C by means of PCR amplicons 102, 206 bp that were also matched by the Elisa test.

CONCLUSION

Interesting finding of this study was the detect Staphylococcal ent C genes in SF of patients and also Staphylococcal enterotoxin C which were confirmed by sequencing and Elisa test. But we are unable to explain why it is that the no organisms were isolated, the staphylococcal ent C gene have detectable. Whether, The amplified gene has been hidden in SF cells? It may needed more future research could provide an appropriate response to this question. However, this study is for the first time existence gene encoding *S. aureus* enterotoxins C in the SF of patients are reported. These findings suggest that Staphylococcal enterotoxin C in the SF of patients as a new biomarker will support investigation on diagnosis of other staphylococcal enterotoxins (superantigens) for the clarification of the etiology of rheumatoid arthritis. This can present a novel approach in future for the diagnosis and designing appropriate prevention and treatment methods of rheumatoid arthritis.

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