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# Enzyme-linked Immunosorbent Assay for Detection of Staphylococcal Enterotoxins in Synovial Fluid of Rheumatoid Arthritis Pateints

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**Experimental evidence suggests that staphylococcal superantigenic enterotoxins are involved in arthritis. The aim of this study was to detect staphylococcal enterotoxins in synovial fluid of seronegative rheumatoid arthritis patients. A total fifty synovial fluid samples from patients with seronegative rheumatoid arthritis were analysed. A commercial sandwich-enzyme immunoassay for the detection of *Staphylococcus aureus* enterotoxins SET A, B, C, D and E were used. The samples were separately processed by Amicon Ultrafiltration system. The results were confirmed by western blotting test. The results indicated that, more than 60 percent of the synovial fluid smaples of the patients with rheumatoid arthritis have at least one of the staphylococcal enterotoxins. Based on our study, the most abundant enterotoxin seen in the synovial fluid of patients with rheumatoid arthritis were enterotoxin A, C, E, B and D respectively in order of presence. However, bacteriological culture was negative for *Staphylococcus aureus* isolation. The results showed the existence of Staphylococcal enterotoxins in the synovial fluid of seronegative rheumatoid arthritis patients. While none of the samples, bacterial growth was observed. This finding could provide a new test method to diagnosis of rheumatoid arthritis and was designed based on the specific treatment of disease. In addition, these findings suggest that, detection of Staphylococcal enterotoxins in synovial fluid as a new biomarker can help identify causes and select specific traetment options.**

**Key words:** ELISA, *Staphylococcus aureus*, Enterotoxins, Western-blotting, Rheumatoid Arthritis, Seronegative.

Rheumatoid arthritis (RA) is a chronic inflammatory arthropathy that can affect most joints. Specific bacterial pathogens such as: Gram positive cocci, Mycobacteria, Proteus, *Escherichia coli* and Mycoplasma are suspected to have a role in Rheumatoid Arthritis[1]. However in many cases, the bacteriological cultures of blood or synovial fluid samples were all negative. Additional complicate is that, a subset of patients with RA is seronegative. The term seronegative polyarthritis encompasses a spectrum of inflammatory joint disorders characterized by persistent absence of serum rheumatoid factors.

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Staphylococcal pathogenesis is caused by several virulence factors including enterotoxins such as SEA, SEB, SEC, SED, SEE and toxic shock syndrome toxin-1 [2]. These exotoxins are members of the superantigen (SAg) family that induces massive secretion of inflammatory cytokines[3].

Moreover, staphylococcal enterotoxins as main classic superantigens have been considered potential causes of inflammatory diesease such as rheumatic fever, arthritis, Kawasaki syndrome, atopic dermatitis, and guttate psoriasis, because of their potent immune system-altering capacity (4). In addition, staphylococcus aureus is one of the dominant pathogens that induce septic arthritis and RA[5]. Rheumatoid arthritis (RA) as a major global public health problem is an autoimmune inflammatory disease that mainly affects synovial joints[6]; however, it¢s exact etiology remains unknown. Few experimental studies have indicated the possible role of superantigens in rheumatoid arthritis and related diseases[7]. For example, intravenous inoculation of a toxic shock syndrome toxin-1 (TSST-1) could induce arthritis in mice[8]. This and other similar investigations suggest that superantigens may have the role in experimental arthritis models[9;10]. Some researchers have suggested that the sera from patients with rheumatic diseases contained elevated antibodies to staphylococcal enterotoxins A and B (SEA and SEB). However, the elevation of antibodies to SEA and SEB in sera from patients with rheumatic diseases was less specific and have not provided clear understanding of the pathogenetic mechanisms[11-12]. The most widely accepted cause of rheumatoid arthritis is an infection with a micro-organism in a genetically susceptible host[13]. A 10 year retrospective study on septic arthritis identified that the predominant causative pathogens were methicillin-susceptible *Staphylococcus aureus* (44.6%) and Streptococci (14.2%) respectively[14]. However, differentiation of severe joint damage between septic and nonseptic arthritis were controversial[15].

*Staphylococcus aureus* superantigen exotoxins stimulate production of inflammatory cytokines from peripheral blood mononuclear cells[16]. However, the mechanisem contribution of superantigenic exotoxins production to the outcome of staphylococcus-related diseases is not clearly understood. This is of particular importance, because the effects of toxins could potentially be neutralized with specific antobodies[17]. In recent years, only few clinical studies have actually demonstrated the presence of staphylococcal enterotoxins in the serum of patients[18]. The results of some investigations revealed that the amount of staphylococcal superantigens may not be sufficient to induce a serum antibody response[19-20]. Hence, direct detection superantigens in synovial fluids is crucial. The aim of this investigation was to apply the sensitive capture enzyme-linked immunosorbent assay (ELISA) to detect the staphylococcal enterotoxins (superantigens) in the synovial fluids of seronegative rheumatoid arthritis patients and confirm the results by immonoblatting test.

## MATERIALS AND METHODS

### Subjects and Sample collection

From August 2010 to November 2011 a total of 50 patients (35 males and 15 females, with an average age of 50 years old) diagnosed with rheumatoid arthritis were selected for this study. The institutional ethics committee approved the protocols involved in this study. Informed consent was taken from the patients. Aspiration of the synovial fluid (SF) was performed by expert rheumatologist. Approximately 3 to 5 ml of SF was aspirated from each patient and then aliquated the into the stile microtube subsequently. The aliquated samples were stored at -20°C until they were used.

### Negative Control Samples

In this study, healthy human synovial fluid as negative control was needed. During the research, three cadavers at the time being brain death were available. They had no history of arthritis or other disease and his organs were donated. From each 3 ml of synovial fluid was aspirated and used as control.

### Sample Preparation Methods for ELISA

The suspected of Staphylococcal enterotoxins from synovial fluids was extracted as below. Equal volumes of synovial fluids were added to strile Phosphate-buffered saline (0.05 M phosphate in 0.15 M NaCl containing 0.05% $NaN_3$; pH 7.5) and mixed them by vortexing. Then, the solutions were subjected to ultrafiltration by centrifuge (3000×g for 10 min) solution over 50 Kda filter device (Amicon- Ultra -15; Ultracel-

50KDa). The filtrates were then used for the SEs assays.

**Enzyme-linked Immunosorbent Assay**

For this purpose, we used the RIDASCREEN SET A, B, C, D, E (Art. No. R4101, R-Biopharm AG, Darmstadt, Germany) which is an enzyme immunoassay for the detection of *Staphylococcus aureus* enterotoxins A, B, C, D and E in fluid and solid foods as well as in bacterial cultures. All reagents required for the enzyme immunoassay are contained in the test kit. The test kit is sufficient for 12 determinations. A microtiter plate spectrophotometer was required for determination. The procedure of Enzyme-linked Immunosorbent Assay (ELISA) was performed based on kit direction. In this study a microtiter plate spectrophotometer (TECAN Austia GMbH, Modwl: Sunrise, Serial No: 501000095) were used.

**SDS- PAGE**

The native and extracted and also estimated molecular weight of the susceptive enterotoxins was determined using SDS-PAGE (SDS-poly acrylamide gel electrophoresis was carried out at 120 mv for 60 min). The slob gel was stained using colloidal Coomassie brilliant blue G-250. In each case a paralel slob gel was prepared for western- blotting test.

**Western-blot for confirmation of Staphylococcal Enterotoxins A and B**

In this method, SEA and SEB antibody (Abcam, Ab 15897 500 lot 796734 Ab 15898, 500; lot 693317 an) was used. The immunoblotting method was performed described briefly (21) as follows: 20 µl prepared samples of each synivial fluids were separated by SDSPAGE (two casset of PAGE were prepared one for SEA and another for SEB) and then electrophoretically transferred in a semidry transfer apparatus (Trans-Blot SD Electrophoretic Transfer Cell cat. no. 170-3940) to the PVDF membrane. The transferred proteins were bound to the surface of the membrane, which provided access for reaction with immunodetection reagents. All remaining binding sites were blocked by immersing the membrane in a solution that contained 3% bovine serum albumin (BSA) for an hour. The membrane was washed three times with phosphate buffer saline (PBS): 0.23 g $NaH_2PO_4$, 1.15 g $Na_2HPO_4$, 9.00 g NaCl, to which $H_2O$ was added to reach 1000 ml and adjusted to pH=7.2. After washing, the membrane was immersed in a primary antibody solution (1 to 15,000 diluted in BSA 0.5%) overnight. The membrane was then washed three times and the antibody-antigen complex was identified with horseradish peroxidase (HRPO) coupled to the secondary anti-IgG antibody (e.g. rabbit IgG1 was diluted up to 10,000 fold in 0.5% BSA). Chromogenic substrates (BM Blue POD substrate precipiting Ref 11 442 066 001, Roch, Co Germany) were then used to visualize the specific bindings.

## RESULTS

The mean age of patients who participated in this study were 50 years old. All of them suffered from arthritis of the knee joint. The results of bacteriological assessement revealed that bacterial was not recovered from synovial fluid samples from rheumatoid arthritis. All the synovial fluids were subjected to enzyme-linked immunosorbent assay (ELISA) test plate. Figure 1 is shows one of the ELISA tested plate. This figure illustrates the results of 12 synovial fluid samples tested for five common enterotoxins. Yellow color indicates the positive results. In the A row, the yellow color of each well indicated the persence of the staphylococcal enterotoxin A. The yellow colors in row B, C, D and E were marked for staphylococcal enterotoxins B, C, D and E respectivly. The wells of G and F rows were negative control and the wells in H row is positive controls for five staphylococcal enterotoxins.

As seen in figure 1, the intensity of the yellow color indicated the concentrations of the

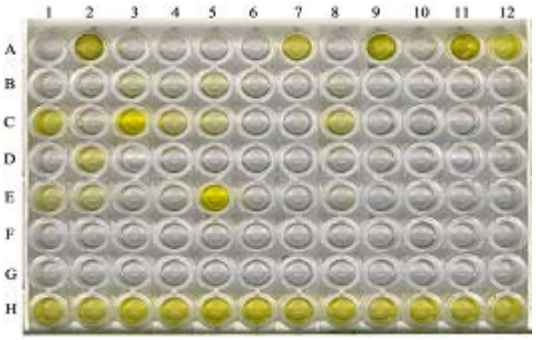


**Fig. 1.** The result of ELISA tested plate with 12 samples of synovial fluid is shown. The rows of A to E show the presence of enterotoxin A to E respectively. the rows F and G are negative controls and the H rows is positive controls included in the plate

toxin. Based on the results of this study, the most abundance enterotoxin were seen in the synovial fluid of patients with rheumatoid arthritis were enterotoxin A and C respectively. After that, enterotoxin E, B and D can be named.

The results of the spectrophotometer scanned plates in 450 nm showed the average OD of the positive control row wells was 2.1 ± 0.051. Based on the kit detection the Cut-off value of the test was defined as OD = 0.24

The intensity of yellow color generated in positive control row wells was due to the presence of approximately 10 ng mixture of five enterotoxin in each wells. However, based on the amount of positive control, the concentration of each enterotoxin was estimated between 0.5 to 3

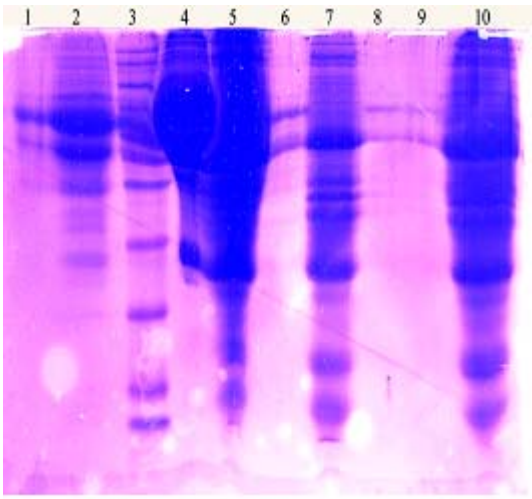


**Fig. 2.** The reesults of SDS- PAGE of the synovial fluid's samples from patient with rheumatoid arthritis is shown. Columns 5, 10, & 7 were loaded with 30, 20 and 10ml of the synovial fluid samples respectively. Column 2 was laoded by another sample. Columns 1, 4, 6, 8 and 9 is overflow of the adjacent columns. Column 3 is the molecular weight marker

ng in each wells that contained the sample. An intersting finding of this study was that, more than one enterotoxin was found in few synovial fluid samples. However, *Staphylococcus aureus* was not isolated from the samples.

In this study each sample was separately subjected to the SDS- PAGE. As shown in the figure 2, the results of electrophoresis of untreated the synovial fluids irrespective of their nature indicated that there are several bands over the

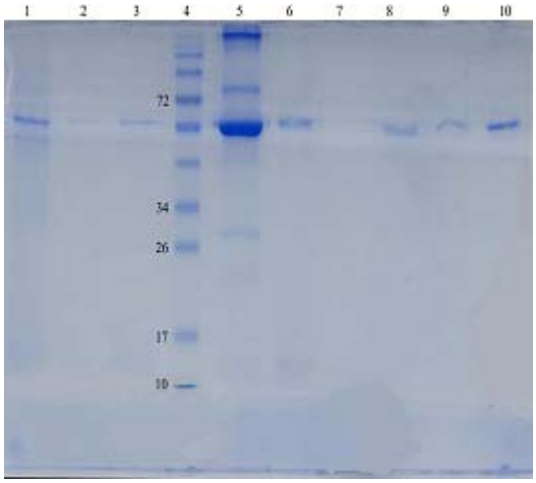


**Fig. 3.** Results of electrophoresis samples of the synovial fluid of patients with rheumatoid arthritis that was diluted with phosphate buffer in ratio 1:1 and was passed through the Amicon ultrafilter of 50 kDa by centrifuged at 4000 rpm for 5 min. As shown in columns 1, 2, 3, 5, 6, 8, 9 and 10 a 56 kDa protein band were observed. In addition that, in columns 1 and 6, a weak band at 12 kDa area is visible. In column 5 in the 28 kDa area a protein band was seen

region of 50 KDa. Using Amicon ultrafiltration system in individual samples removed most bands of proteins over 50 kDa. In this case, the passed proteins from ultrafilteration were those with the molecular weight below 100 kDa (Figure 3).

In our study as the confirmatory test, western blot was carried out and the results shows that, the existence of proteins which reacted with antibodies specific to enterotoxin A and B.

As shown in Figure 3, Amicom ultracel ultrafiltration (centrifugal solution by over 50kda filter device) product electophoresis has made 56KDa band and lower band. By using the Rb PAb Staphylococcus Enterotoxins A and B (15897 500 lot 796734 and 15898 500 lot 693317, Abcam), the presence of SEA and SEB in synovial fluids was confirmed by the Western-blot.

## DISCUSSION

Rheumatoid arthritis is a chronic inflammatory arthropathy of unknown causes that can affect most joints. However, It is well defined that superantigens include bacterial products, stimulate T cells to proliferate nonspecifically

through interaction with class II major histocompatibility complex products on antigen-presenting cells and then with variable regions on the beta chain of the T cell receptor complex. These proteins should be considered as the potential causes of illnesses such as arthritis because of their potent immune system-altering capacity[22].

In recent years, the results of many investigations indicated that the *Staphylococcus aureus* produce a large number of virulence factors, notable among these are the superantigenic enterotoxins (enterotoxin A to E) and toxic shock syndrome toxin-1 (TSST-1) that are involved in the pathogenesis of arthritis (23;24). There are no reasons to believe as to why the administration of antibodies did not affect the development of arthritis, suggesting inefficacy of such a procedure in neutralization of enterotoxin mediated disease manifestations. The results of some studies suggest that increased responses to SEB relate to increased colonisation and hence exposure to superantigen (SAG) producing Staphylococcus in atopic dermatitis, and that the inflammation of atopic dermatitis is not caused by an inability to make antibody to SAG[25]. Moreover, in addition to a sharp increase in the incidence of septic arthritis, some researches reveal that more than half of arthritis patients suffered from sterile inflammatory arthritis such as rheumatoid arthritis[26]. However, septic or nonseptic arthritis in adults is a global challenging diagnosis, however, prompt differentiation of etiology is crucial to minimize morbidity and mortality[27].

In this study, we focused on the detection of Staphylococcal superantigenic enterotoxins in the synovial fluid of patients with rheumatoid arthritis spondylitis artheropathy. Because of this reason, in these patients, no organisms were found in synovial fluid. Our aim was to show whether in the synovial fluid of patients the *Staphylococcus aureus* enterotoxins are found or not. For this reason, we supposed that it is possible that these superantigen compounds can be absorbed in small amounts from other body sites into the bloodstream and tropism to other place of the body such as synovial fluid by unknown mechanisms. For this purpose, ELISA kit (r- Biopharm Co Germany) was used. The results indicated that more than 60 percent of the synovial fluid of patients contained at least one of staphylococcal enterotoxin and could be detected by this kit. We must consider that already 20 Staphylococcus aureus enterotoxin or enterotoxin-like protein with superantigenic property have been identified. It is likely that there are other Staphylococcal enterotoxins that are out of the detection range of ELISA kits.

According to manufacturer's instructions, the ELISA kit is recommended for the detection of common Staphylococcus aureus enterotoxin in samples of liquid cultures and food extracts only. This is the first time this kit has been used as medical diagnosis tool for enterotoxin detection in pathological samples (synovial fluid of patients) with minor modification.

There are some investigations that demonstrated that the RIDASCREEN kit (r-Biopharm GmbH, Darmstadt, Germany) is a convenient, rapid, and reliable tool for the detection and identification of SEs in foods[28]. In addition, another study has developed a highly sensitive capture enzyme-linked immunosorbent assay that detects SEB in body fluids at very low levels. In that assay, the peak levels of SEB in serum and renal clearance can be measured in mice and the results suggested that this test is a potentially useful tool for the study of the pharmacokinetics of SEB and the effects of potential therapeutic reagents on serum SEB levels[29].

In an nother experimental study, severe arthritis in DBA/1J mice was produced by the subcutaneous injection of Staphylococcal enterotoxin B (SEB), This evidence suggests that this experimental arthritis model may provide a means to examine the role of superantigens and the efficacy of pharmacological agents for the treatment of rheumatoid arthritis[30].

## CONCLUSION

Two important findings of this study are the presence of Staphylococcus enterotoxins in the synovial fluid of patients as a new biomarker in the etiology of seronegative rheumatoid arthritis that can be a novel approach in future for the diagnosis and designing an appropriate treatment of rheumatoid arthritis.

Second, the development of confirmatory western blotting test for the diagnosis. Of course, this is not the first time that the ELISA test is used

to detect enterotoxin. However, it is the first time that this test was used to as a clinical diagnostic tool and the results were confirmed with western blotting.

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