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# Staphylococcal Enterotoxin A Detection from Rheumatoid Arthritis Patients' Blood and Synovial Fluid

Ramezan Ali Ataee[1], Mahboobeh Sadat Kahani[2], Gholam Hossein Alishiri[3], and Zyenab Ahamadi[4]

[1] Ph.D. of Medical Microbiology, Professor, Department of Virology and Bacteriology, and Clinical Development Medical Center of Baqiyatallah Hospital, Faculty of Medicine, Baqiyatallah University of Medical Sciences, Tehran, Iran
[2] M.Sc. of Microbiology, Researcher, Department of Virology and Bacteriology, Faculty of Medicine, Baqiyatallah University of Medical Sciences, Tehran, Iran
[3] M.D. of Rheumatology, Clinical Development Medical Center of Baqiyatallah Hospital and Department of Rheumatology, Faculty of Medicine, Baqiyatallah University of Medical Sciences, Tehran, Iran
[4] Ph.D. of Biology, Assistant Professor, Research Center of Biology, Baqiyatallah University of Medical Sciences, Tehran, Iran

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**Abstract**

**Introduction:** Direct detection of microbial super antigens in synovial fluid of patients with rheumatoid arthritis may be able to guide to the design of cost-effective therapies. The purpose of this study was to assess the existence of *Staphylococcal* enterotoxin A (superantigen A) in the synovial fluid of patients with RA by the PCR and ELISA methods.

**Methods:** This experimental study was conducted on the synovial fluid of 103 RA patients from Baqiyatallah University of Medical Sciences' Rheumatology Clinic in Tehran, Iran in 2011-2014. Bacterial cultures, polymerase chain reaction with specific primer pairs and enzyme-linked immunosorbent assay (ELISA) methods were used. The PCR products were subjected to sequence as a confirmatory molecular method results. The data were descriptively analyzed by SPSS Version 19.

**Results:** The bacteriological study result indicated that, in four cases (3.8%) of the patients, bacterial strains were isolated. The result of PCR molecular method for staphylococcal enterotoxin A gene showed that, 42 of the patients (40.7%) tested positive for the *ent* A gene. The results of ELISA were positive for staphylococcal enterotoxin A (superantigen A) in 51 cases (49.51%) of the patients' synovial fluids. The results indicated that the possibility of detecting superantigen A in the SF of RA patients, but the origin of the enterotoxin A gene remained unknown.

**Conclusions:** The findings of this study may be able to alter the actual theory on the pathogenesis, diagnosis, and treatment of RA patients. In addition, the results have shown the probability of an endogenous origin for the involved superantigen A in RA patients' synovial fluids.

**Keywords:** rheumatoid arthritis, staphylococcal enterotoxin A, ELISA, PCR, synovial fluid

## 1. Introduction

Staphylococcal enterotoxins (SEs) (superantigens) have been considered as one of the most common causative agents of bacterial food-borne disease outbreaks worldwide (1), and intensive research has been performed on both people's (2) and animal's staphylococcal toxigenic isolates (3). Consequently, it is well established that SEs are the major cause of food poisoning (4). In addition all SEs possess superantigenic activity and induce the inflammatory cytokines storm and inflammatory disease (5, 6). In this regard, the non-gastrointestinal roles of these toxins, such as atopic dermatitis (AD), have been demonstrated (7). The results of one study indicated that the staphylococcal and

**Corresponding author:**
Dr. Gholam Hossein Alishiri, Clinical Development Medical Center of Baqiyatallah Hospital and Department of Rheumatology, Faculty of Medicine, Baqiyatallah University of Medical Sciences, Tehran, Iran.
Tel: +98 21 82483427, Fax: +98 21 88620843, Email: Ghahlishiri@gmail.com
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group A streptococcal superantigens possess the ability to cause the toxic shock syndrome starting from a colonized mucosal surface (8). Therefore, the inflammatory disease caused by *Staphylococcus aureus (S. aureus)* superantigens also has been taken into consideration. Several reports have suggested that superantigens may act as the triggering factor in the pathogenesis of rheumatoid arthritis (RA) and other inflammatory diseases (9-11). These findings provide adequate knowledge for the development of serological diagnostics, immune prophylaxis, individual prognosis tools, and therapies against the invasive *S. aureus*, based on extracellular toxins (12). Therefore, achieving rapid, sensitive, and cost-effective diagnostic tools for the detection of staphylococcal superantigens in the body fluids of patients has become a health priority. In this regard, enzyme-linked immunosorbent assays (ELISA) for the detection and quantization of antibodies against staphylococcal enterotoxins A, B, C, and toxic-shock syndrome toxin-1, which are expected to be significant tools for the study of superantigens in several diseases, were developed (13). In addition, recent research represented the sensitive and specific colorimetric ELISAs for *S. aureus* enterotoxins A and B detection in urine and buffer samples (14). The results of several studies are felt to be the most important for toxins (superantigens) detection instead of organisms detection, because the organisms were not isolated from pathological body fluids, while the superantigens existed or perhaps were produced by non-pathogenic organisms, such as coagulase negative staphylococci (15,16). In this respect, the integration of ELISA and PCR methods may also enhance the accuracy and credibility of the test and could be useful in revealing the origin of the etiological agents. In this regards, our hypothesis was to show whether or not the most common classical superantigens existed in the synovial fluid (SF) of the patients with RA. Thus, the purpose of this study was to assess the existence of SEA (superantigen) in the SF of patients with RA by PCR and ELISA methods.

## 2. Material and Methods

### *2.1. Research Design and Bacterial Reference Strain*

This experimental study was conducted on the synovial fluid of 103 RA patients from Baqiyatallah University of Medical Sciences Rheumatology Clinic, Tehran, Iran, during 2011-2014. Bacteriological, polymerase chain reaction (PCR) with specific primer pairs and enzyme-linked immunosorbent assay (ELISA) methods were used separately. The PCR products were sequencing. Non- parametric statistical methods and descriptive analyses by SPSS Version 19 were used. In our previous study, a clinically-isolated *S. aureus* strain contained the *ent* A gene (producing enterotoxin A had characterized as Gen Bank reference (ATCC 25923) used as positive control (17).

### *2.2. Synovial Fluid and Blood Samples Collection*

Over a period of three years, synovial fluid (SF) samples were taken from 103 RA patients who were referred to our laboratory by rheumatologists. Inclusion and exclusion criteria of the RA patients were based on the American College of Rheumatologists' (ACR's) 2010 criteria (18). Sampling was conducted based on the Standard Protocol for sampling with constant monitoring of perfect aseptic conditions. The procedure indicated that, after disinfection of the sampling area, 5-10 ml of SF were aspirated by an expert rheumatologist. Immediately afterwards, 3-5 ml of the SF were inoculated onto Castaneda medium (Baharafshan Co., Tehran, Iran, Lot no: 9 nkB) and then incubated at 37 °C for 48 hr. The remaining SF samples were subjected aseptically to DNA extraction and stored at -80 C of freezer. All patients provided informed consent orally to the rheumatologist. Then, they agreed to be referred to the clinical laboratory to provide blood samples. The protocol of this study was approved by the Ethics Committee of the Baqiyatallah University of Medical Sciences, Tehran, Iran, on November 29, 2011, with Code No: 24, Paragraph 28.

### *2.3. Bacteriological procedure*

After inoculation of the Castaneda medium and incubation at 37°C for 48 hr, 1 ml of the medium was aspirated and subcultured on a blood agar plate (Blood Agar Base, CONDA SA, Madrid, Spain, Lot 812171, Cat No: 1108). After incubation at 37°C for 24 hr, the isolated bacterial strains were assessed by using the bacterial standard protocol (19).

### *2.4. Bacterial DNA Extraction and DNA Extraction from Synovial Fluid*

The reference bacterial was inoculated into the LB broth medium (Lot VM1322685, Merck, Darmstadt, Germany), after 24 hr of incubation at 37°C. The bacterial suspension was centrifuged, and the sediments were obtained. DNA extraction was performed by using the salting-out method (20) with minor modifications. Then, the PCR protocol was set up. Free DNA tubes containing 500 µl of the patient's SF and 500 µl of DNA-free distilled water (DW) (mixed in aseptic conditions) were centrifuged for 1 min at 3000 g at 4 °C. Afterwards, the resulting supernatants were transferred to DNA-free sterile tubes, and the genomes were extracted with the CinnaPure-DNA Extraction Kit

(Cat. NO. PR881612, Cinnagen Co., Tehran, Iran). According to the manufacturer's instructions, 200 µl of diluted SF were added to the micro tube, and then 400 µl of Lyses Buffer were added, and the mixture was spun for 20 s. Afterwards, 300 µl of the precipitation solution was added to the micro tubes and spun again for 5 s. During this stage, all of the contents of the micro tubes were transferred to a new column and centrifuged for 1 min at 13000 g at 5°C. The column was transferred to the new micro tube and washed by 400 µl Washing Buffer 1, and centrifuged for 1 min at 12000 g at 5°C. The resulting mixture was transferred to a clean micro tube and washed by 400 µl of Washing Buffer 2, and centrifuged for 1 min at 12000 g at 5°C. After evaporation and changing the column, 30 µl of Elution Buffer were added and centrifuged for 5 min at 12000 g at 5 C. The quality and quantity of the DNA were measured using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

*2.5. Primer*

Based on previously published primers pair (21), with the following sequences: forward 5-TGTATGTATGGAGGTGTAAC-3 and reverse 5-ATTAACCGAAGGTTCTGT-3. These primers pair amplified a 270bp fragment. However, for increased reliability and to prevent the occurrence of errors, the primers pair was analyzed on Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). In addition, multiple alignments were conducted by the DNASIS® MAX trial version software (Hitachi Solution America Ltd., San Bruno, CA, USA) for gene reference.

*2.6. Polymerase Chain Reaction and Enzyme-linked Immunosorbent Assay*

A set of specific primer pairs, which amplified a 270 bp fragment, were used for the diagnosis of enterotoxin A gene in SF and blood samples of the RA patients by Polymerase Chain Reaction. The 25 µl mastermix reaction contained 1 µl DNA template, 0.3 U of Taq DNA polymerase, 2.5 µl of 10X PCR buffer, 0.16 mM of each dNTPs, 2 mM MgCl2 (all reagents were from Cinnagen Co, Tehran, Iran), 10 pmol (1 µl) of each primer pair (synthesized by Cinnagen Co., Teheran, Iran) and double-distilled water. The amplification reactions were conducted in a thermal cycler (Bio-Rad C1000, Bio-Rad Laboratories Inc., Hercules, CA, USA). PCR cycles were: initial denaturation at 95 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 61 °C for 40 s and extension at 72 °C for 40 s, followed by a final extension at 72 °C for 5 min. The amplified PCR products were electrophoresed in a 1.5% agarose gel for 45 min and then stained with ethidium bromide (0.05 mg/ml) for 20 min. The gels were photographed under ultraviolet light using the Universal Hood II Gel Doc (Bio-Rad Laboratories Inc., Hercules, CA, USA). Also, molecular size markers (50 and 100 bp) were included in each sample for analysis. All of the samples of SF genomes were assayed after PCR optimization. In order to validate the enterotoxin detection in the patients' SF samples, the indirect ELISA was performed. The SF sample was centrifuged for 10 min at 5000 g at room temperature and the supernatant was subjected to ELISA, as follows: Based on a previous report (17), 50 µl of SF with the 50 µl of DW as diluents were mixed. Then, 50 µl of sample were added to the defined wells of ELISA plates that were coated previously with a polyclonal non-specific antibody against staphylococcal enterotoxin A (Rb. PAb. Staphylococcus enterotoxins A 15897 500, lot 796734, Abcam). After one hour of incubation and being washed three times, 50 µl of the conjugate antibody (horseradish peroxidase = HRPO) coupled to the secondary anti-IgG antibody were diluted up to 10,000-fold in 0.5%, after which BSA was added. Then, the enzyme substrate was added along with a final 100 µl of the stop solution. After 10 minutes from this process, the color changed. After this color variation indicator, the wells absorption was measured by the ELISA reader device at 450-nm wavelength and then the cut-off value was calculated for each plate.

**3. Results**

*3.1. The Result of the Bacteriological Assay*

From August 2011 to August 2014, synovial fluid samples from 103 RA patients were assessed. During this study and after sequential sub-cultures, only four (3.8%) bacterial species were isolated. Based on the results of the biochemical diagnostic tests, in all four cases, *S. aureus* was detected. The *ent* A gene was not PCR amplified in the isolated *S. aureus* strains.

*3.2. The result of DNA extraction*

The quality and quantity of the standard strain and patients sample genomes were examined by both gel electrophoresis (the gel concentration was 0.8%, in 100 V for 45 min) and Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) measurement. Both results were comparable (Figure 1).

| # | Sample ID | User name | Date and Time | Nucleic Acid Conc. | Unit | A260 | A280 | 260/280 | 260/230 | Sample Type | Factor |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | Dear-User | 2013/06/30 08:40:53 ق.ظ | 1147.1 | ng/µl | 22.942 | 12.459 | 1.84 | 2.87 | DNA | 50.00 |

Method Information

| Parameter | Value |
|---|---|
| Workbook File | C:\Documents and Settings\Dear-User\Desktop\2013_06_30_Nucleic Acid.twbk |
| Application | Nucleic Acid |
| Data range | 220nm 350nm |
| Analysis wavelength | 260 nm |

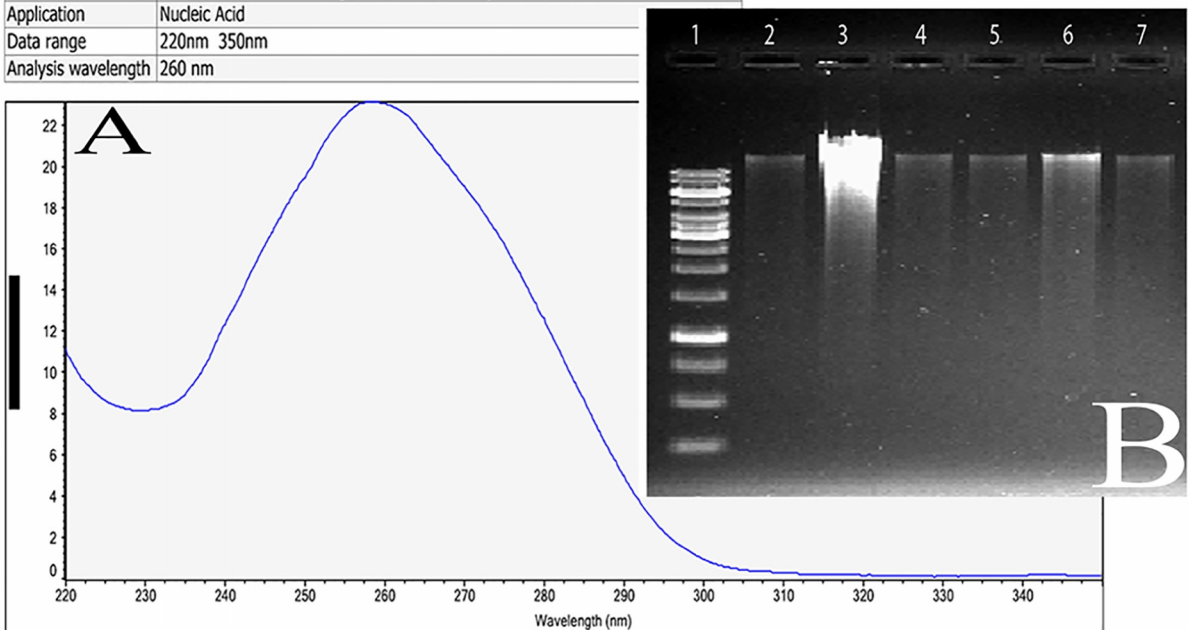


**Figure 1.** Left: the result of Nanodrop DNA scan; Right: electrophoresis result of DNA extraction from SF samples. Line 1 is the 100bp molecular marker; Lines 2 to 6 represent DNA that has been extracted from different patients' synovial fluids.

*3.3 The PCR Results of the Rheumatoid Arthritis Patients' SF*

After examination based on the standard strain genome and Master Mix components, PCR was set up and applied as a positive control for each test (Figure 2). The results of PCR performed with amplifier primer of a 270bp fragment revealed that 40.7% of the samples have amplified a 270bp fragment as it was a part of ent A gene. Because of sequencing of the PCR product confirmed the results. Figure 2 exemplifies the results of PCR product electrophoresis with amplifier primer of a 270bp fragment for a number of samples.

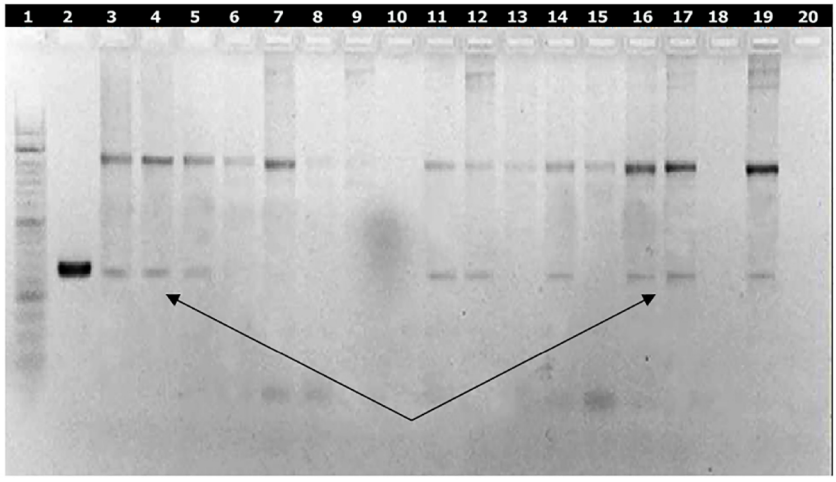


**Figure 2.** Example of the result PCR amplicon 270 bp fragment from rheumatoid arthritis patients' synovial fluid: Line 1 is a 50 bp MW (molecular weight) Marker, line 2 is the positive control, lines 3, 4, 5, 11, 12, 14, 16, 17, and 19 show the 270bp fragment, and lines 6, 7, 8, 9, 13, 15, 18, and 20 are the negative samples.

*3.4. The Result of ELISA*

The results of the ELISA method indicated that 49.51% of RA patients' SF samples were positive for SEA (Figure 3). Comparative study results of PCR, ELISA, and bacterial culture for staphylococcal enterotoxin A assay of 103 rheumatoid arthritis patients' synovial fluid were 40.7, 49.51, and 3.8%, respectively, and the assays of 103 blood samples were 37, 45, and 2.9%, respectively.

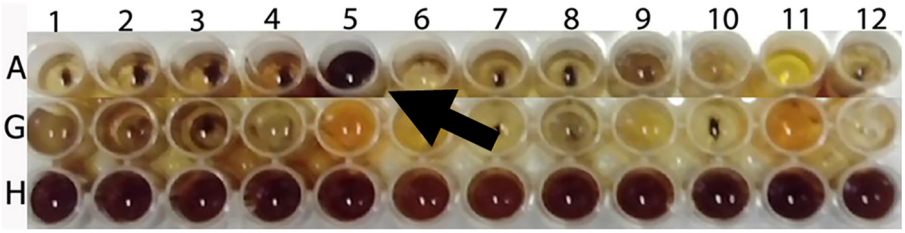


**Figure 3.** Example results of the ELISA plate for staphylococcal enterotoxin A: This figure represents an assembly of rows for the purpose. The vertical A row represents patient's number of synovial fluid samples (Well 5 shows a positive result). The G and H rows are negative and positive control, respectively.

**4. Discussion**

*Staphylococcus aureus* strains are the most common causative agents of septic arthritis, and it has been demonstrated that superantigens produced by *S. aureus* represent major arthritogenic factors (22) in septic RA cases. Clarifying the causative agents of non-septic RA is crucial for successful treatment and reduction in the costs for the patient. However, staphylococcal superantigen A (enterotoxin A) is being increasingly recognized for its possible roles in many human diseases other than non-gastrointestinal disorders, such as atopic dermatitis, Kawasaki syndrome, nasal polyposis, and certain autoimmune disorders, especially RA (23), which makes it mandatory to perform assays for the detection of the enterotoxin A.

Our previous report was focused on the ELISA method to investigate the common staphylococcal enterotoxins (classical superantigens) in the SF of RA patients (24), the results of which raised the critical question concerning the source of these toxins in non-septic RA. Therefore, in the present study, a total of 103 SF and synchronically blood samples of patients with RA were assayed for the presence of *S. aureus* enterotoxin A by using bacterial culturing, ELISA, and PCR methods. The result of bacterial culturing showed that four isolated Staphylococcal strains were non introducing enterotoxin A was isolated from SF samples, while in 2.9% of blood cases, bacteria were isolated. The result of the ELISA and PCR assessments of 103 SF samples indicated in 51 cases (49.51%) the presence of enterotoxin A and in 42 cases (40.70%) the presence of enterotoxin A gene. In fact, these findings indicated that the accuracy and credibility of the ELISA method was higher than that of the PCR method (49.51% vs. 40.70%). It is noteworthy that in only 31 cases (30.09%) of all samples, the PCR and ELISA results were essentially the same. In nine cases (8.73%), the PCR results showed the presence of enterotoxin A gene, whereas the ELISA results for the existence of enterotoxin A were negative. The reason may be related the lack of gene expression or inadequate production of enterotoxin, which was under the ELISA sensitivity threshold. We found 19 cases (18.44%) that were positive for ELISA, whereas negative results were obtained for PCR, but the PCR reactions were repeated by a different Genomic DNA Extraction method. The output results showed that differences between PCR and ELISA still persisted, and perhaps they were due to the similarities between enterotoxin A and other enterotoxins (cross reaction), such as enterotoxins E and P.

The interpretation of these results is very difficult. First, *S. aureus* produces more than 20 types of enterotoxins with great similarities between them. Second, the different structural features may have similar mechanisms of action. Meanwhile, this study's aim was only focused on the detection of staphylococcal enterotoxin A in synovial fluid of patients with RA. However, the results of this study indicated that the PCR molecular method and ELISA could detect enterotoxin A in the SF of patients with RA. The results have elicited two important questions. The first question concerns why the toxin was found in the patient's SF even though the bacterial growth in the SF cultures was negative. Further research is needed to provide answers to this question. The second important question concerned the role of staphylococcal enterotoxins as superantigens in inflammatory disease and rheumatoid arthritis. However, no direct attempt to detect these enterotoxins in patient's SF and/or blood has been reported to date. Perhaps one reason is the lack of proper and valid methods for testing the measured superantigen. Another important

reason may be the fact that the SF aspiration is very difficult. We believe that the main reason concerns inaccurate reporting. For example, the lack of evidence for the role of staphylococcal enterotoxins in rheumatoid arthritis was reported (25). However, in recently-reported data, the majority of S. aureus strains colonized in the skin of atopic dermatitis patients carried genes encoding SEA and demonstrated the importance of SEA as an immunoinflammatory triggering factor of atopic dermatitis (26). However, this study was designed to determine the existence of staphylococcal enterotoxin A (superantigen A), by ELISA and by PCR amplification simultaneously in RA patients' SF and blood, and the PCR product sequencing confirmed the results. There were several important limitations in this study, including the limit on the collection of SF samples from the patients with confirmed cases of the disease. Another important limitation was that, despite the fact that 270 bp PCR amplicon (part of *ent* A gene) was amplified in this study, sequencing confirmed that the partially enterotoxin A gene belonged, but further research is needed to perform for the full length gene determined.

**5. Conclusions**

The findings of this study indicated that, despite the absence of enterotoxin A producing staphylococcal bacterium in the synovial fluid and blood of the RA patients, the existence of SEA and also the encoded *ent* A gene were detectable by PCR methods. This finding may alter the actual theory on the pathogenesis of RA disease. Therefore, the hypothesis that different staphylococcal strains are present in the human body must be emphasized. Therefore, they probably release and incorporate the encoded enterotoxins gene elements that are far from the site of colonization. However, due to the fact that there are no similar studies, this hypothesis should be compared and analyzed. Therefore, more research with a larger sample size is recommended.

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**Conflict of Interest:**

There is no conflict of interest to be declared.

**Authors' contributions:**

All authors contributed to this project and article equally. All authors read and approved the final manuscript.

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