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ORIGINAL ARTICLE

Design of PCR-based method for detection of a gene-encoding *Mycoplasma arthritidis* mitogen superantigen in synovial fluid of rheumatoid arthritis patients

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ABSTRACT

Background and Objectives: *Mycoplasma arthritidis* mitogen (MAM) superantigen has been shown to induce chronic arthritis, which resembles human rheumatoid arthritis (RA) in a rodent model. However, its role as a causative agent in human RA is not well understood yet. The aim of this study was to investigate the presence of MAM superantigen gene in the synovial fluid (SF) of RA patients.

Materials and Methods: The MAM superantigen gene a reference was synthesized based on GenBank Data base (Gene ID: 6418105). Specific primer pairs were designed and PCR amplification was performed for MAM superantigen gene detection. A total of 133 SF samples of RA patients were assayed. The PCR products were subjected to sequencing and were descriptively analyzed.

Results: The results of the PCR product sequencing showed the method has objective applicability and accuracy. The sensitivity of the PCR reaction for the reference DNA template was 1*n*g/ml. The PCR results assay of the 133 SF samples raveled that, 9.7% and 22.5% of them were positive for the MAM superantigen gene and *Mycoplasma pneumoniae (M. pneumoniae)*, respectively.

Conclusion: In this study, two *Mycoplasma* genomes were detected with increased frequency in RA SF patients' samples. This finding appears to be a promising instrument in the etiological diagnostic of RA patients and could also lead to improved treatment selection. Further research on the other *Mycoplasma* species present in the SF of RA patients is essential.

Keywords: Rheumatoid arthritis, *Mycoplasma arthritidis* mitogen, Synovial fluid, Superantigen, PCR

INTRODUCTION

Mycoplasma arthritidis (*M. arthritidis*), as an animal pathogen is unique among *Mycoplasma* species harboring the lysogenized bacteriophage MAV1, whose contribution to virulence, and production of the potent *Mycoplasma arthritidis* mitogen (MAM), that confers increased toxicity, lethality

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and arthritogenicity in experimental animals (1,2). However, several reports mention that this bacterium was isolated from the synovial membranes, synovial fluid (SF), middle ear, eye, abscessed bone, abscessed ovary, oropharynx of wild and captive rats and also from joint fluid of non-human primates, including rhesus and wild boars (3-5). Reports on the isolation or molecular detection of this bacterium from human infections are unavailable. Based on the evidence, it has been postulated that superantigens might play a role in the autoimmune diseases and it was shown that the MAM superantigen can both trigger and exacerbate mouse autoimmune arthritis (6). Current interest in MAM superantigen relates to its ability to modulate the immune system *in vivo* that may lead to the development of autoimmune diseases, such as rheumatoid arthritis (RA), respiratory, reproductive and inflammatory joint diseases in rodents (7-9). MAM has the potential for triggering autoimmune disease in mice (10). Other studies indicate that MAM causes acute polyarthritis in rats and chronic proliferative arthritis in mice (11). In addition, macrophage activation by *M. arthritidis* could play a significant role in the inflammatory response and induces toxicity, arthritis, and dermal necrosis in mice (12).

The evidences indicating that *M. arthritidis* may cause experimentally induced acute and chronic arthritis in animals has led several investigators to look for a similar potential in humans. Direct involvement of toll-like receptor (TLR2 and TLR4) in MAM binding and presentation to T cells was investigated and revealed that coexpression of TLR2 or TLR4 with the human leukocyte antigen-D related (HLA-DR) significantly increases MAM binding. The subsequent T cell activation may play an important role in the outcome of diseases induced by *M. arthritidis* (13,14). A recent study demonstrated the reactivity of antibodies to MAM in the sera of RA patients (15). Furthermore, the results of another study proved that MAM has the capacity to induce proinflammatory cytokine transcription in monocytes via major histocompatibility complex (MHC) class II molecules. This phenomenon has been shown to activate one pathway of autoimmune diseases arthritis in rodents, which closely resembles human RA (16). However, cultivation and isolation of *M. arthritidis* from clinical samples is more costly and time consuming. In addition, the role of *M. arthritidis* in human arthritis remains unsolved.

The aim of this study was to design PCR-based molecular methods for the detection of gene-encoded MAM superantigen in SF samples of patients with RA.

MATERIALS AND METHODS

Standardization protocol. In this study, the complete sequences of MAM superantigen gene (Gene ID: 6418105) belongs to *M. arthritidis* strain 158L3-1 with GenBank reference NC_011025.1 was synthesized with as positive control in vector pGEM-B1 (Bioneer Corp, Daejeon, South Korea). The vector pGEM-B1 containing MAM superantigen gene (717bp) was transferred into *E. coli* DH5α by the electroporation method (17). Ampicillin (100 μg/ml) resistant transformants were selected and subjected to purification. This recombinant gene was used as *M. arthritidis* PCR positive control standard. In addition, a *Mycoplasma pneumoniae (M. pneumoniae)* strain supplied by the Mycoplasma Reference Laboratory of the Razi Vaccine and Serum Research Institute, Karaj, Iran, as a Mycoplasma test control.

DNA Extraction. Two methods of DNA extraction were performed. The first method was DNA extraction from recombinant bacterial cells while the other method was the DNA extraction from samples of SF of RA patients.

DNA Extraction from Bacteria. One ml of an overnight culture of *E. coli* DH5α containing a high-copy of pGEM-B1, which endorsed the MAM superantigen gene, was centrifuged (5000×g, for 5 min at 4 °C). Then, the bacterial pellet was suspended in 500 μl of free DNA distilled water and the cell lysis was carried out by boiling for 10 min. An amount of 500 ul of phenol was added and the mixture was gently vortexed for 1 min. Afterwards, the tube was centrifuged (10000 × g, for 5 min at 4 °C). Carefully, the upper phase was decanted into a fresh tube. This step was repeated twice. Double volume of the harvested supernatant, 96% cold ethanol and one-tenth volume of 3*M* sodium acetate were added to the impression. The tube was inverted several times until the content was well mixed. Then, the tube was centrifuged (14000 × g, for 5 min at 4 °C). The supernatant was discarded and the pellet was carefully rinsed with 1 ml of 70% ethanol. The tube was centrifuged for 2 min at room temperature and the ethanol was removed by aspiration. The pellet of

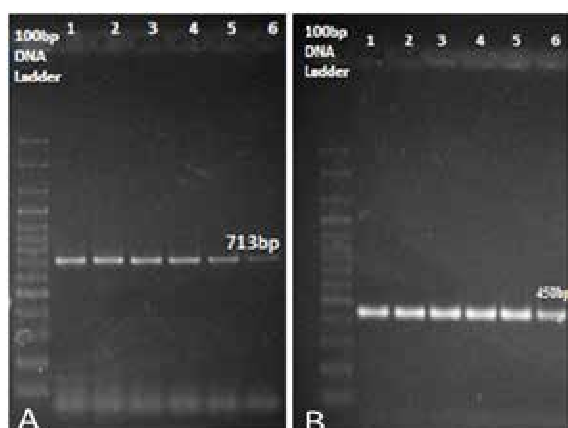

Fig. 1.
A: The results of PCR product electrophoresis for *Mycoplasma* genus
B: *M. pneumoniae* detection.

nucleic acid was dried in the air for 10 min. Then, the DNA pellet was dissolved in 50 mM of TE buffer (pH 8.0) and the purity of DNA extracted was evaluated with the Nanodrop spectrophotometer.

DNA extraction from synovial fluid. A total of 133 SF samples were subjected to DNA extraction separately by the above mentioned method and also with the CinnaPure DNA Kit for the isolation of DNA (CinnaGen Co., Teheran, Iran).

Primer Design. A single primer pair was designed to amplify the MAM superantigen using online GeneScript software (GeneScript, Piscataway, The USA) based on the reference sequence (MAM superantigen with Gene ID: 6418105 of *M. arthritidis* strain 158L3-1 with GenBank reference NC_011025.1) and were analyzed using Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, Massachusetts, The USA). In addition, multiple alignments were carried out using DNASIS MAX trial version (Hitachi Solutions America Ltd., San Bruno, California, The USA). This primer pair enabled amplification of a 203bp fragment.

In addition, for the detection of *M. pneumoniae*, based on Gene ID sequences reference: 15152184, a single primer pair was designed. Furthermore, to detect *Mycoplasma* genus, the primers pair was selected from a previous study (18). The sequences of this primer were F 5'-ACTCCTACGGGAGGCAGCAGT-3' and R 5'-TGCACCATCTGTCACTCTGTTAACCTC-3'. All primer pairs were synthesized by Bioneer Corp., Daejeon, The Republic of South Korea.

Polymerase chain reaction. The PCR method used for the detection of MAM superantigen gene was based on the specific primer pairs that amplified a 203bp fragment. The amplification reaction was carried in 25 µl reaction mixture containing 1 µl DNA template, 0.3 U of Taq DNA polymerase, 2.5 µl of 10X PCR buffer, 0.16 mM of each dNTPs and 2 mM $MgCl_2$, 0.1 *p*mol of the primer pair (CinnaGen Co., Teheran, Iran) and double-distilled water, to a final volume of 25 µl. The cycling program consisted of an initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 sec, primer annealing temperature each gradients (54-64°C) for 40 sec and extension at 72°C for 40 sec, followed by a final extension at 72°C for 5 min using Bio-Rad C1000 thermal cycler (Bio-Rad Laboratories Inc., Hercules, California, The USA).

The amplified PCR products were electrophoresed in a 1.5% agarose gel and then stained with ethidium bromide. The gels were photographed under ultraviolet light using Gel Documentation (Bio-Rad Laboratories Inc., Hercules, California, USA).

RESULTS

The results of primers pair design for the PCR detection of the MAM superantigen gene and *M. pneumoniae* P1 gene were F1 5'- GAGGCAAATAACGTCGAACA-3', R1 5'-ATTGCAACTTCACCATCACG-3', F2 5'-AAAGGAAGCTGACTCCGACA-3' and R2 5'-TGGCCTTGCGCTACTAAGTT-3', respectively. Agarose gel electrophoresis of DNA extracted by this method showed a favorable and sharp band.

The results of optimization PCR set up with specific primers for the detection of *Mycoplasma* genus and *M. pneumoniae* are shown in Fig. 1. The selected gradient temperature revealed that the best annealing temperature was 58 and 56°C, respectively. The result of the PCR sensitivity determination for *M. pneumoniae* was 4.92 *n*g/ml.

The results of NCBI Blasting of PCR product sequencing for *Mycoplasma* genus and also *M. pneumoniae* confirmed the ATCC: 29342 *M. pneumoniae* strain.

The result of optimization PCR set up with specific primers for the detection of MAM superantigen gene is presented in Fig. 2. In this case, applied

gradient temperature revealed that the best annealing temperature was 56°C. The result of the sensitivity determination for detection of MAM superantigen gene by the PCR method was 1 *n*g/ml.

The DNA sequencing of the 203bp amplicon fragment of MAM superantigen gene, as PCR product, was carried out, and the results of multiple alignment of the reference gene (MAM superantigen with Gene ID: 6418105 of *M. arthritidis* strain 158L3-1, with GenBank reference NC_011025.*S.*) with the outcome sequenced PCR product (a 203bp fragment amplicons) obtained in this study are shown in Fig. 3.

In order to prevent contamination, each of the samples was separately analyzed. The results indicate that the PCR method was optimized for MAM superantigen gene detection. All the 133 SF samples of RA patients were studied. However, the results of detection of *M. pneumoniae* and MAM superantigen genes in the SF samples suggested that a substantial number of samples evidenced these genes (Fig. 4). Therefore, the genes of *M. pneumoniae* and MAM superantigen were present in 30 (22.5%) and 13 (9.7%) cases, respectively.

DISCUSSION

Based on the results of recent studies, the existence of different *Mycoplasma* species other than MAM superantigen has been demonstrated in mononuclear leukocytes (ML) from the blood and SF of patients with RA (19). It has been postulated that *Mycoplasma* or their superantigens might play a critical role in human RA (20). In this regard, the MAM superantigen, as a potent stimulator of the immune system, has been widely considered (15). Therefore, the detection of MAM superantigen in the blood and

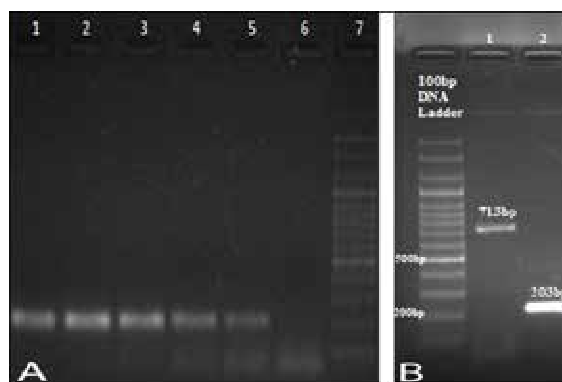

Fig. 2.
A: Temperature gradient of PCR detection *Mycoplasma arthritidis* mitogen (MAM) superantigen gene.
B: optimization of condition detection for *M. pneumoniae* (713) and MAM superantigen gene (203).

SF of patients with RA can help to design accurate diagnostic methods and effective treatments for this disease. However, isolation and characterization of *Mycoplasma* from blood and SF of RA patients are associated with several technical issues. In order to overcome some of these difficulties, this study was designed to detect the MAM superantigen in SF of RA patients. Because of the lack of bacterial standard strain and necessity of a gold standard, we synthesized the MAM superantigen gene according to the GenBank database. Therefore, the MAM superantigen sequence (713bp fragment) was obtained as a reference sequence from GenBank. Then, bioinformatics analysis and gene synthesis were carried out and the PCR protocol was performed. All 133 SF samples of RA patients were assayed. The PCR product was subjected to sequence determination and, by the match of the results with the reference gene, 100% homology was demonstrated. In comparison, the results of Petrov AV reported 15.9% detection

Fig. 3. The purified PCR product (203bp fragment) was sequenced and the alignment with reference *Mycoplasma arthritidis* mitogen (MAM) gene is shown. The first row sequence is related to the PCR product obtained from this research while the second row is the reference MAM gene.

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                                             370        380        390        400        410        420
PCR Product This Study   Sequence   ---------- -------AAA AAATATCGCT ATTTTAGATG AAATAATGGG CAAAAGCAGA
Reference MAM gene       Sequence   AACGTCGAAC AAATCAAAAG AAATATCGCT ATTTTAGATG AAATAATGG- CAAAAGCAGA

                                             430        440        450        460        470        480
PCR Product This Study   Sequence   TAACGATTTA TCTTACTTTA TATCTCAGAA TAAGAATTTT CAAGAGTTAT GGGATAAAGC
Reference MAM gene       Sequence   TAACGATTTA TCTTACTTTA TATCTCAGAA TAAGAATTTT CAAGAGTTAT GGGATAAAGC

                                             490        500        510        520        530        540
PCR Product This Study   Sequence   TGTCAAACTA ACTAAAGAAA TGAAAATAAA ACTTAAAGGC CAAAAACTAG ATCTTCGTGA
Reference MAM gene       Sequence   TGTCAAACTA ACTAAAGAAA TGAAAATAAA ACTTAAAGGC CAAAAACTAG ATCTTCGTGA

                                             550        560        570        580        590        600
PCR Product This Study   Sequence   TGGTGAAGTT GCAATAAACT TAGT------ -TCACTTCAC TTTAGTTAAC TTCCCCAGGA
Reference MAM gene       Sequence   TGGTGAAGTT GCAATAAACA AAGTAAGAGA ATTATTTGGC AGCGACAAAA ATGTAAAAGA
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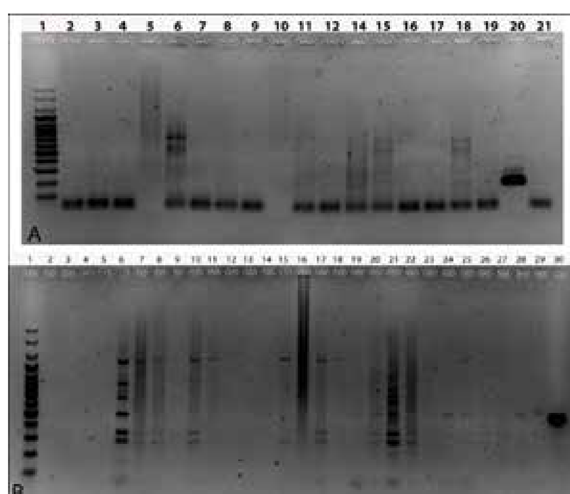

Fig. 4.
A. the result of PCR product electrophoresis of several samples of SF from patients with rheumatoid arthritis (RA) that shown the presence of MAM superantigen gene (203 bp). Lanes6, 14 and 18 showed the existence of the genes. Lane 20 is the positive control, whereas lane 21 is the negative control.
B. the same samples SF of patients with RA were evaluated for the presence of *M. pneumoniae* gene (450bp) are shown. Lanes 6, 15, 19, 20, 21, 24, 25, 28 and 29 showed the existence of the *M. pneumoniae* genes. Lane 30 is the positive control.

of *M. arthritidis* in SF (19), while the results of the PCR method used in this study were 9.7% positive for the MAM superantigen gene. These differences may reflect the absence of MAM superantigen gene in reported *M. arthritidis* bacteria or cross reactivity in Petrov's research. However, despite these results, its pathogenic role in rodents has been discussed. The research of Sawitzke et al. indicated the elevation of antibodies to MAM in RA patients' sera. They suggest that MAM or a MAM-like molecule might be associated with RA disease (21). Another study suggested that the binding of MAM to HLA-DR leads to a conformational change in MAM structure, which allows its interaction with TLR2 and TLR4 and makes feasible the recognition by T cells (13).

In addition, there is a report that showed the MAM overproduction and knockout mutants of *M. arthritidis* were mitogenic in mice, compared to the wild-type strain (1). Therefore, the existence of this bacterium or their MAM superantigen gene in body fluids may better explain the pathogenic role in human inflammatory diseases. However, the main finding of this research indicated the presence of MAM superantigen gene in SF of RA patients by this PCR method. Also, its sequencing and alignment with the reference gene, as a confirmatory method, indicate the accuracy of the procedure. In order to confirm the validity of this study and to prevent the false positive results of MAM superantigen gene in SF, *Mycoplasma* genus primers pairs were used. Furthermore, in this study, the *M. pneumoniae* ATCC 29342 strain was used as a test control. In addition, all SF samples were assayed for *M. pneumoniae* specific primers pair's detection.

In conclusion, this study revealed the design of a PCR method for the detection of the MAM superantigen, and also determined the sensitivity and accuracy of the test. The main finding of this study was that 9.7% of patients' SF samples with RA were found to contain the MAM superantigen, while 22.5% had *M. pneumoniae* as a superantigen. In addition, the results indicated that further investigations on other *Mycoplasma* species are essential. In fact, the results showed that in total 32.25% of SF samples of RA patients were positive for superantigens. Therefore, further studies on larger cohorts are necessary in order to accurately diagnose RA in suspected cases and for the application of this method in medical diagnostic laboratories.

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