Original Article

Simultaneous Detection of *Mycoplasma pneumoniae*, *Mycoplasma hominis* and *Mycoplasma arthritidis* in Synovial Fluid of Patients with Rheumatoid Arthritis by Multiplex PCR

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Abstract

Background: It has been recognized that infectious agents, such as different bacteria and viruses, may play a role in the developing of rheumatoid arthritis (RA). Recently, the mycoplasma species has been implicated in the pathogenesis of RA.

Aim: The aim of this study was to design a multiplex PCR for rapid and simultaneous detection of *Mycoplasma pneumoniae*, *Mycoplasma hominis,* and *Mycoplasma arthritidis* in the synovial fluid of patients with rheumatoid arthritis (RA).

Methods: A total of 131 synovial fluid (SF) samples from patients with RA were assayed. *Mycoplasma pneumoniae* (ATCC: 29342), *M. hominis* (native strain), and the synthetic complete genome of *M. arthritidis* mitogen (MAM) superantigen were used as controls. All SF samples were subjected to DNA extraction separately and multiplex PCR was performed. The PCR products were confirmed by sequencing.

Results: The designed multiplex PCR was able to detect *M. pneumoniae*, *M. hominis,* and *M. arthritidis* in the SF of patients with RA with a frequency of 30 (22.9%), 23 (17.5%) and 13 (9.9%), respectively.

Conclusion: In this study, the overall detection of the Mycoplasma species in RA patients was 53.4%; thus, we recommend the application of multiplex PCR assays when searching for a specific anti mycoplasma treatment for RA patients.

Keywords: Multiplex PCR, *Mycoplasma arthritidis, Mycoplasma hominis, Mycoplasma pneumoniae*, Rheumatoid arthritis, synovial fluid

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by erosive synovitis, persistent synovitis, systemic inflammation, and detection of autoantibodies.[1] Annually, a global prevalence of 0.5%–1% in adults and an incidence of 5–50/100,000 new cases of RA are reported. The disease is most typically encountered in women and the elderly, which can lead to joint and cartilage damage, disability, and a decrease in quality of life.[2]

Although the exact etiology of RA is unknown, various infectious agents loaded with superantigens, including bacteria, fungi and viruses, have been regarded as the cause of this disorder.[3] Bacterial pathogens, such as *Coxiella burnetii*,[4] *Enterobacteriaceae* spp.[5,6] oral anaerobic bacteria[7], Staphylococcus spp.,[8,9] Streptococcus spp., *Niesseria* spp., *Haemophilus* spp.,[10] and *Mycoplasma* spp.[11] have been reported as causes of RA.

The *Mycoplasma* spp. belong to the Mollicutes family (cell wall deficiency bacteria).[12] Cultivation and isolation of mycoplasmas in clinical specimens are associated with many difficulties and have not been set-up in most medical laboratories. However, it has been reported that mycoplasma superantigens stimulate the immune system to develop autoimmune diseases such as RA.[13,14] A relationship has been reported between RA and Mycoplasma members, including *M. pneumoniae*, *M. fermentans*, *M. hominis*, *Ureaplasma urealiticum* and *M. arthritidis* separately,[11] yet such relationships are not supported by strong evidence, and also, their prevalence remains unclear.[10] Therefore, the detection of mycoplasmas in the synovial fluid (SF) of RA patients has not been sufficiently investigated. Furthermore, exact detection of these causative agents is a critical step forward in the treatment plan for RA disease.

The results of recent investigations indicate that culturing mycoplasma is difficult and time-consuming and is less sensitive than molecular methods. Several molecular detection studies based on polymerase chain reaction (PCR),[15,16] such as the multiplex PCR, are performed for detection of human[17,18] and animal mycoplasmas.[19] Also, multiplex PCR is rapid and enables the simultaneous detection of multiple bacteria in clinical samples.[20] Therefore, the aim of this study was designing a multiplex PCR method for rapid and simultaneous detection of *M. pneumoniae*, *M. hominis,* and *M. arthritidis* in the SF of patients with RA. At present, this is the first study in this field performed in Iran.

Materials and Methods

Bacterial strains

Mycoplasma pneumoniae (ATCC: 29342) obtained from the

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Razi Vaccine and Serum Research Institute, Alborz, Iran, *M. hominis* (native strain) obtained from the department of bacteriology, Tarbiat Modares University, Teheran, Iran, and the complete genome of *M. arthritidis* mitogen (MAM) superantigen (GeneID: 6418105 of *M. arthritidis* strain 158L3-1 with Gen Bank reference NC_011025.1) from the Bioneer Company, Daejeon, Republic of Korea, were synthetized and then, set up the PCR method[21] was used as controls.

Clinical specimens

Totally, 131 SF samples from patients with RA who had no significant infectious diseases or fever, based on the American College of Rheumatology (ACR) criteria, were collected by rheumatology specialists during three years (2011–2013). The samples were transferred to the laboratory in sterile conditions. The approval of the Ethics Committee of the Baqiyatallah University of Medical Sciences, Tehran, Iran, was obtained before sampling.

Bacterial DNA extraction

To perform DNA extraction, 0.5 mL of *M. hominis* and *M. pneumoniae* suspension were centrifuged at 12000 rpm, separately. The DNA was extracted from sediments by the hybrid method of boiling and phenol-chloroform. [22]

The extraction of DNA from synovial fluid samples

A quantity of 100 µL of synovial fluid was diluted in 200 µL sterile distilled water and DNA extraction was performed by the hybrid method of boiling and phenol-chloroform.[23] The amounts of DNA for both bacterial and synovial fluid samples were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA).

Primers

The characteristics of the selected or designed primers are listed in Table 1.

Uniplex PCR

The uniplex PCR technique was performed separately on each strain, as follows: the 25 µL reaction mixture contained 2 µL of template DNA, 0.5 µL (10 pmol) of each primer and 12.5 µL of Master Mix (Ampliqon, Odense, Denmark), composed of 1.5 mM $MgCl_2$, 0.2% Tween 20, 0.4 mM dNTPs, 0.05 units/µL Ampliqon Taq DNA Polymerase, inert red dye and 9.5µL of distilled-deionized water (DDW). Wide ranges for optimal cycling conditions were tested. The uniplex PCR final setup was as follows: predenaturation at 95°C for five minutes, denaturation at 95°C for 35 seconds, annealing at 58°C for 40 seconds, extension at 72°C for 45 seconds and final extension at 72°C for five minutes.[24]

Multiplex PCR

The 25 µL reaction mixture of multiplex PCR for reference bacteria were setup and contained 2 µL of template DNA of each bacterial sample (6 µL for all three bacterial samples), 0.25 µL (10 pmol) of each primer (2 µL for all four primer pairs: GPO1, MGSO, RNAH1, RNAH2, MPP1F, MPP1R, and MAMF, MAMR), 12.5 µL Master Mix (Ampliqon, Odense, Denmark), and 4.5 µL of DDW.

The 25 µL reaction mixture of multiplex PCR for SF sample setup contained 2 µL template DNA which was extracted from the SF samples (variable concentrations of 13.5-155 ng/µL), 0.25 µL (10 pmol) of each primer (2 µL for all four primer pairs), 12.5 µL Master Mix, and 8.5 µL of DDW.

The final setup of the multiplex PCR protocol was as follows: predenaturation at 95°C for five minutes, denaturation at 95°C for 35 seconds, annealing at 60°C for 1 minute, extension at 72°C for 1 minute, and final extension at 72°C for five minutes.[27]

PCR product d analysis

A solution of 1.5% agarose gel was prepared, using 1X tris-borate-EDTA (TBE) buffer. For staining, a 7 µL volume of 1X GelRed Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA, The USA) was used. A volume of 5 µL of PCR product was electrophoresed in TBE buffer at 90V for 40 minutes.[28] The bands were verified under UV light using a gel documentation system (Bio-Rad Laboratories, Hercules, CA, The USA).

Sensitivity

Due to the limitations of this study, the sensitivity tests for separate microorganisms were performed separately for each uniplex PCR. The DNA content was measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). The PCR reaction was performed on the DNA solution with dilutions ranging between 10^{-1} and 10^{-5} at 58°C annealing temperature. Finally, the sensitivity of each technique was calculated.[29]

Table 1. Characteristics of primers used in this study.

For detection of	Primer name	Primer sequence	Product length	Target gene	Reference
Mycoplasma Genus	GPO1	Forward primer: 5'-ACTCCTACGGGAGGCAGCAGT-3'	704–713 bp	16sRNA	Van Kuppeveld F et al. 1993[25]
	MGSO	Reverse primer: 5'-TGCACCATCTGTCACTCTGTTAACCTC-3'			
M. hominis	RNAH1	Forward primer: 5'-CAATGGCTAATGCCGGATAC	335 bp	16srRNA	Blanchard A. et al 1993[26]
	RNAH2	Reverse primer: 5'-GGTACCGTCAGTCTGCAAT-3'			
M. pneumoniae	MPP1F	Forward primer: 5'-AAAGGAAGCTGACTCCGACA-3'	450 bp	P1	In this study
	MPP1R	Reverse primer: 5'-TGGCCTTGCGCTACTAAGTT-3'			
M. arthritidis	MAMF	Forward Primer: 5'-GAGGCAAATAACGTCGAACA-3'	203 bp	MAM	In this study
	MAMR	Reverse Primer: 5'-ATTGCAACTTCACCATCACG-3'			

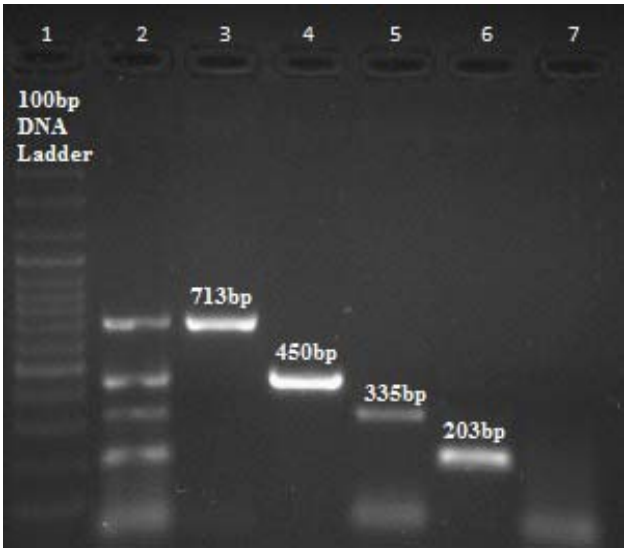

Figure 1. The PCR electrophoresis results of Multiplex and Uniplex PCR of *M. pneumoniae*, *M. hominis*, and *M. arthritidis* mitogen (MAM) gene products (with species and genera primers).

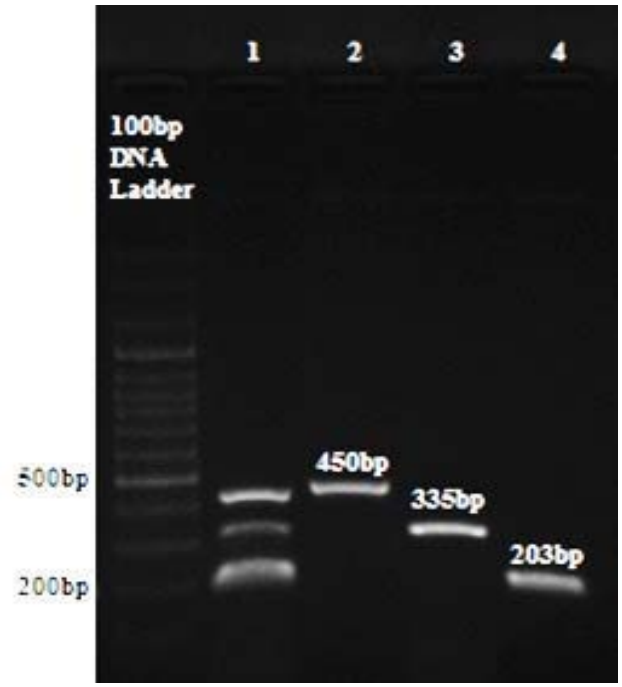

Figure 2. PCR electrophoresis results of Multiplex and Uniplex PCR of *M. pneumoniae*, *M. hominis* and *M. arthritidis* MAM gene products (with species primers): Line 1 - results of multiplex PCR of *Mycoplasma pneumoniae* (450 bp), *M. hominis* (335 bp) and *M. arthritidis* mitogen gene (203 bp) with species primers; Line 2 - uniplex PCR of *M. pneumoniae* (450 bp) with species specific primer; Line 3 - uniplex PCR of *M. hominis* (335 bp) with species specific primers; Line 4 - uniplex PCR of *M. arthritidis* mitogen gene with specific primers.

Specificity

The specificities of the primers were evaluated using the NCBI primer blast tool and *in silico* PCR amplification. Furthermore, the PCR reactions were performed with the genome set of bacteria other than those associated with this study (*M. orale*, *M. arginini*, *M. gallisepticum*, *M. genitalium*, *Ureaplasma urealyticum*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli*, *Shigella sonnei*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, and *Chlamydia trachomatis)*, and the results were confirmed as negative.

Sequence analysis

The PCR products were analyzed by sequencing (Macrogen Corp., Rockville MD, The USA). The sequencing data were aligned with *M. pneumoniae*, *M. hominis*, and *M. arthritidis* target genes in the NCBI database to assess homology.

Results

Patients analysis

Demographic analysis revealed that the mean age of patients participating in this study was 52 years, with 79 (60.3%) women and 52 (39.7%) men.

Sensitivity

To determine the sensitivity of the PCR for detection of Mycoplasma genus, serial dilutions of DNA extracted from *M. pneumoniae* was used as a template. As a result, it was found that this PCR reaction was able to detect at a rate of 0.0492 ng/μL of DNA in the total volume of 25 μL (about 5.58×10^4 copies of the genome).

The sensitivities of PCR for detection of the three species for this study were as follows: 0.00492 ng/μL of DNA in a total volume of 25 μL (5.58×10^3 copies of the genome) for the *M. pneumoniae* species, 0.4032 ng/μL of DNA in a total volume of 25 μL (4.98×10^3 copies of the genome) for *M. hominis*, and 1×10^3 ng/μL of DNA in a total volume of 25 μL (1.13×10^3 copies of the genome) for *M. arthritidis*.

Multiplex PCR

Figure 1 depicts the results of multiplex and uniplex PCR set up for the detection of *M. pneumoniae*, *M. hominis* and *M. arthritidis* mitogen (MAM) superantigen gene, with species and genera primers at 60°C annealing temperature.

Line 1 - DNA Ladder; Line 2 - multiplex PCR of the Mycoplasma genus (704–713 bp), *M. pneumoniae* (450 bp), *M. hominis* (335 bp), and *M. arthritidis* MAM gene (203 bp), which were amplified with species and genus primers; Line 3 - uniplex PCR of the Mycoplasma genus (704–713 bp) with genus primers; Line 4 - uniplex PCR of *M. pneumoniae* (450 bp) with species specific primer; Line 5 - uniplex PCR of *M. hominis* (335 bp) with species specific primers; Line 6 - uniplex PCR of *M. arthritidis* mitogen (MAM) gene with specific primers; Line 7 - negative control with genus and species primers without DNA templates.

Figure 2 shows the results of multiplex and uniplex PCR set up for the detection of *M. pneumoniae*, *M. hominis* and *M. arthritidis* mitogen superantigen gene only with the species primers at 60°C annealing temperature.

In this study, a total of 131 SF samples from 131 patients with RA, 79 (60.3%) women and 52 (39.7%) men, were assayed. The results of this study showed that this molecular diagnosis method is able to detect *M. pneumoniae*, *M. hominis*, and *M. arthritidis* in the SF of patients with RA. *Mycoplasma pneumoniae*, *M. hominis*, and *M. arthritidis* were confirmed in 30 (22.9%), 23 (17.5%) and 13 (9.9%) of 131 samples, respectively. Also, Mycoplasma

Table 2. Mixed *Mycoplasma* Species Detected by the PCR of 131 Synovial Fluid Samples From Rheumatoid Arthritis Patients.

	M. pneumonia	*M. hominis*	*M. arthritidis*
Number (Percentage)	30 (22.9%)	23 (17.5%)	13 (9.9%)
Mixed species of *Mycoplasma* that were detected			**Number**
M. pneumoniae + M. hominis + M. arthritidis			1
M. pneumoniae + M. hominis			4
M. pneumoniae + M. arthritidis			3
M. hominis + M. arthritidis			1

genus was detected in 70 (53.4%) samples (Table 2).

One of the most important and interesting findings of this research was that at least two species of Mycoplasma were detected simultaneously in the SF samples, as follows: *M. pneumoniae*, *M. hominis*, and *M. arthritidis* in one case; *M. pneumoniae* and *M. hominis* in four cases; *M. pneumoniae* and *M. arthritidis* in three cases; *M. hominis* and *M. arthritidis* in one case (Table 2).

Discussion

Although, there is currently no strong evidence, the role of *Mycoplasma* spp. has been considered in various diseases and it is thought to be a causative factor in RA.[30] The culture and isolation of Mycoplasmas is costly and time-consuming at present. Therefore, most of the research has focused on serological and molecular PCR-based diagnostic methods, which are preferred due to rapidity of detection and because they eliminate the need for bacterial cultures.[31] Several reports have been reported on the benefits of multiplex PCR in being able to simultaneously detect multiple bacteria in clinical samples.[32] The advantages of the multiplex PCR method have made it the first choice for this study. As far as we are aware, no studies have been done in the field of multiplex PCR method for the detection of Mycoplasmas in SF samples. This study aimed to develop a multiplex PCR method applicable for the detection of *M. pneumoniae*, *M. hominis*, and *M. arthritidis* in SF of RA patients. Thus, 131 SF samples were assayed. The results of this study indicate that the presented method can detect *M. pneumoniae*, *M. hominis*, and *M. arthritidis* in the SF of RA patients.

However, similar researches have not been conducted up to now, and only limited reports are available on bacterial cultures from the serum and SF of patients with RA.[33,34] Also, it should be noted that this study has been carried out for the first time in Iran.

Based on the results of this study, the multiplex PCR molecular method was able to detect *M. pneumoniae* in 30/131 (22.9%) samples SF of patients with RA. In the study of Johnson, *et al.* on SF samples with nested PCR, *M. pneumoniae* was found in 19/24 (79%) of RA patients, 6/6 (100%) of non-rheumatoid inflammatory arthritis, and in 8/10 (80%) of osteoarthritis cases.[35] The comparison of the results of our study with those of Johnson *et al.* revealed significant differences, which may be due to the methods used, the number of investigated cases, and the geographical region where the studies were performed. However, the most important finding of these two studies is that *M. pneumoniae* was reported by both, therefore suggesting that RA may play a significant causative role.

In the study of Haier, *et al.* on the serum of patients with RA, *M. pneumoniae* was found in 5/28 (17.85%) of samples.[36] Their results are in accordance with the present study (the difference is in the reported amounts), with the difference being that serum was used by Haier, *et al*.[36]

The findings of Hakkarainen, *et al.* via the immunosorbent assay and immunoblotting methods on 1259 patients with serological confirmation of *M. pneumoniae* showed that 11 (0.8%) patients were diagnosed with arthritis.[11] Although their report differs from the present study in terms of methods and the rates reported, both researches suggest a possible role of *M. pneumoniae* in RA disease.

Based on the results of this study, both the uniplex and multiplex PCR molecular methods were able to detect *M. hominis* in 23 (17.5%) of the 131 samples of SF from patients with RA.

The results of the study by Haier, *et al.* on the blood of patients with RA revealed the existence of *M. hominis* in six out of 28 (21.4%) cases. As stated earlier, although in accordance with our findings, the major difference rests in the fact that their study was performed on serum samples.

According to the report of Hakkarainen, *et al.* and our present research, it can be concluded that the survey of these organisms is important in the management of RA patients. In addition, the results of our study showed that multiplex PCR molecular methods detected *M. arthritidis* in 13/131 (9.9%) of the SF of patients with RA. Also, the study of Petrov, *et al.* assessed blood and SF samples of 218 RA patients and revealed the existence of *M. arthritidis* in 20.5% of blood samples and 15.9% of SF samples.[37] A comparison of their results with ours showed great similarities. However, according to the results of both studies, a survey of the role of this organism in RA seems important. Shah hosseini M, *et al.* studied the serum of 100 anti-CCP positive and 100 anti-CCP negative samples of patients with RA by the PCR method, revealing the existence of Mycoplasma genus in 12 of 100 (12%) and 4 of 100 (4%) of the anti-CCP positive and anti-CCP negative samples, respectively.[32] The differences between the study of Shah hosseini M, *et al.* and our research are most probably related to the origin of the samples. In conclusion, the results of this study proved that the uniplex and multiplex PCR molecular methods are compatible with the specific detection of *M. pneumoniae*, *M. hominis* and *M. arthritidis* in SF samples of patients with RA, facilitating the recommendation of the specific treatment for these patients.

The clinical implication of this study is that it suggests a possible role for Mycoplasmas in the pathophysiology of RA as an autoimmune disease. Due to this, in addition to use of the primers for the mycoplasma genus, three additional pair primers were used for the detection of three species of mycoplasmas: *Mycoplasma pneumoniae*, *Mycoplasma hominis*, and *Mycoplasma arthritidis*. Early identification and treatment of the disease caused by these organisms may prevent the autoimmune system activation of the patients. Thus, the results of this study may provide

specific and appropriate cure for some patients with RA. Given the importance of this topic, there have been attempts to design an accurate and rapid method to identify members of the *Mycoplasma* genus. At present, routine bacteriological cultures of synovial fluid of patients with RA have reported negative results for *Mycoplasma*. Therefore, the laboratories are not able to grow these bacteria with the conventional bacteriological methods. The detection of mycoplasmas is thus expensive and time consuming. However, application of fast and easy ways to detect *Mycoplasma* species in SF of patients with RA may provide specific treatment to the patient. Hence, further studies with larger sample size are needed and it is recommended that because there is a relative lack of available information in these areas, the interaction should be enhanced between clinicians and laboratories specialists for molecular detection of *Mycoplasma* species in SF of RA patients.

Authors' Contribution

Ramezan Ali Ataee developed the original idea and the protocol; Gholam Hossein Alishiri selected the SF samples, Ramezan Ali Ataee, and Reza Golmohammadi designed the experimental protocols, abstracted the data, and prepared the manuscript. Madi Tate provided technical assistance.

Conflict of interest

The authors have no conflicts of interest.

Financial Disclosure

None declared.

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