



Real-time PCR of *Mycoplasma pneumoniae* in Synovial Fluid of Rheumatoid Arthritis and Osteoarthritis Patients

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Abstract

Objective: The present study aims to explicate the role of *Mycoplasma pneumoniae* (*M. pneumoniae*) in RA pathogenesis by detecting its species-specific DNA in patients' synovial fluid.

Methods: The 100 patients with RA and 100 patients with knee Osteoarthritis (OA) were recruited. Knee synovial fluid of participants was analyzed for *M. pneumoniae* by real-time PCR.

Results: *M. pneumoniae* was only detected in two percent of RA patients' and 1% of OA patient's synovial fluid (p=1.000).

Conclusion: Our result shows a weak possibility of the role of *M. pneumoniae* in RA pathogenesis.

Keywords: *Mycoplasma pneumoniae*; Osteoarthritis; Real-time PCR; Rheumatoid arthritis

Introduction

Rheumatoid Arthritis (RA) is the most common autoimmune arthritis, that many predestined and acquired factors are involved in the pathogenesis of it, some are or caused by random events; either one is necessary but not sufficient for the development of RA [1]. It seems that primary stages of the disease are probably caused by repeated activation of innate immunity, by some stimulators such as bacterial products [2]. Different mechanisms are known for an infection to participate in the disease development, such as direct synovial invasion, stimulation of innate immunity resulted from pattern-recognition receptors or through the inducement of an autoreactive adaptive immune response by molecular mimicry [1]. Subtypes of mycoplasma specimen have been suspected to be stimulators of RA development. The possible mechanisms that contribute to mycoplasma are direct synovial infection and super antigenicity, which can activate the immune response Mycoplasma species [3,4], including *M. fermentans* [5,6] and *M. arthritidis* [3] have been studied vastly; but there are few researches about *Mycoplasma pneumoniae* (*M. pneumoniae*). There is some evidence that among patients with positive *M. pneumoniae* antibody, only around 0.9% have developed arthritis [7,8]. In other cases, patients suffered from hypogammaglobulinemia had been found to have *M. pneumoniae* in their joints [9,10]. In some studies, RA patients, compared with controls, showed an elevated prevalence of anti-*mycoplasma pneumoniae* IgG antibodies [11], but this could represent an epiphenomenon or a non-specific B-cell activation. Despite these efforts, researchers have not been completely successful to answer one important question "Are there any relationship between existence of *M. pneumoniae* in the joint and development of RA?"

Methods and Materials

The following study includes every consecutive RA and OA patients above 18 years old who referred to Rheumatic Diseases Research Center (RDRC) of Mashhad University of Medical Sciences from April 2015 to June 2016. Two diseases were diagnosed according to the related American College of Rheumatology (ACR) criteria [12]. Exclusion criteria were as follows: 1) Patients with suspected septic arthritis, 2) Crystal observation in synovial fluid, 3) Antibiotics consumption in the recent two weeks, 4) Patients whose required information was not available.

One hundred RA patients and 100 OA patients were considered as the case and control group respectively. Synovial fluid samples (2 cc to 5 cc) from the swollen knee joints using a sterile needle aspiration in a sterile tube with no additive materials were sampled and stored at -20°C. The laboratory operator was blinded to clinical diagnosis. Demographic data and drug history of antibiotics were obtained. The study was approved by the ethics committee of Mashhad University

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of Medical Sciences. Statistical analyses were performed with SPSS version 17.0.

Laboratory analyses

PCR for *Mycoplasma pneumoniae* detection: Hyaluronidases was used to remove the viscosity of synovial fluid, then after washing with PBS, DNA extraction was performed by using a Genetbio commercial kit (South Korea) regarding the manufacturer's instructions. Real-time PCR test was carried out using GeneProof *Mycoplasma pneumoniae* PCR Kit (Czech Republic) regarding the manufacturer instructions. This kit is designed for *Mycoplasma pneumoniae* detection based on the amplification of a specific conservative DNA sequence coding CARDS toxin and then measuring the amplification product concentration in the course of the PCR process by means of fluorescence marked probe. *M. pneumoniae* presence is indicated by FAM fluorophore fluorescence growth. An Internal Standard (IS) is included in the reaction mix, controlling the possible inhibition of the PCR reaction or the efficiency of the DNA isolation process. PCR amplification was done by using Rotor-Gene 6000 thermal cycler (QIAGEN, Germany). A 30 µl of Master Mix was added to 10 µl of the isolated DNA and underwent PCR cycling conditions; 37°C for 2 minutes followed by 45 cycles of 95°C for 5 seconds, 60°C for 40 second, and 72°C for 20 seconds. After the amplification reaction, the data were analyzed using Rotor-Gene 6000 software.

Results

There was no statistical difference between age and disease duration of two groups, but OA patients were older than RA ones ($p < 0.001$). Real-time PCR method detected *Mycoplasma pneumoniae* in 2% of RA and 1% of OA patient's synovial fluid with no statistical difference ($p = 1.000$). Table 1 contains complete information of three patients who were positive for *Mycoplasma pneumoniae*. As 77% of RA and 72% of OA patients were female, all *Mycoplasma* positive patients were female.

Discussion

According to our results, *M. pneumoniae* DNA was detected in two RA and one OA patient's synovial fluid using real-time PCR method with no significant difference. The number of studies on the prevalence of *M. pneumoniae* in synovial fluid of RA patients was relatively low. We discuss here the most important ones. Johnson et al. [13] assessed the distribution of *M. pneumoniae* in synovial fluid of 25 RA patients and 17 controls with other inflammatory or OA disease or trauma. Their results revealed that *M. pneumoniae* DNA sequence was present in 79% of RA, 80% of OA, and 100% of inflammatory arthritis patients, but none of the traumatic injury cases were positive [13]. This polarity with our study may stem from the method used to detect *M. pneumoniae*; by them [13] (sequencing the amplicon after PCR with universal mycoplasma primers), and us (species-specific primers in PCR to detect *M. pneumoniae*). Another study investigated the presence of *M. pneumoniae* [14], *M. hominis* and *M. arthritidis* simultaneously in synovial fluid of RA patients using Multiplex PCR method. They assessed 131 synovial fluid samples of patients with no significant infectious diseases or fever. Their results indicated that 22.9% of SF sample were positive for *M. pneumoniae* which was not in concordance with our results. Considerable differences between those and our study include: multiplex PCR method was performed by them to detect several species simultaneously, whereas we focused only on *M. pneumoniae* species using real-time PCR. In another

study [15], immunoblot method carried out on synovial fluid and sera of arthritis patients to find out mycoplasma. Furthermore, an ultrasensitive PCR method was developed to investigate mycoplasma DNA in synovial fluid. They revealed that sera of 55% of RA, 88% of JRA, 90% of OA and 86% of other inflammatory arthropathy patients were positive for *M. hominis* antibody, and 15% of RA, 90% of JRA, 50% of OA, and 43% of other inflammatory arthropathies samples were positive for *M. fermentans*. However, they failed to detect any evidence of mycoplasma DNA in synovial fluid of all patients by PCR-based tests. Beyond the synovial fluid, there are some studies about the presence of *M. pneumoniae* in the blood samples of RA patients. Haier et al. [11] claimed that the *Mycoplasma spp.* sequence was extracted from 15 out of 28 RA blood samples (53.6%), whereas in three out of 32 healthy controls (9.4%) it was extracted. Using specific primers to detect species-specific DNA, five of 28 RA (18%) and none of healthy controls were positive for *M. pneumoniae*. In another study significantly higher level of *M. pneumoniae* antibody (IgG) in RA serum was reported in comparison with healthy controls [16]. It should be mentioned that this study was conducted by antibody detection instead of PCR. Another study used PCR method to study the serum of 100 anti-CCP positive, 100 anti-CCP negative and 91 RA patients sample (whose disease was confirmed clinically), demonstrating the presence of mycoplasma genus in 12%, 4% and 11% of the samples, respectively [17]. One of the most important differences between our study and previous mentioned studies is that we assessed SF but they worked on blood samples. As a result, although we cannot deny the possible relationship between *M. pneumoniae* blood infection and RA, our results is against the presence of joint inflammation by *M. pneumoniae*. Although we could not detect *M. pneumoniae* DNA in most of our samples, we cannot conclude the patients' previous exposure to the germ. Negative results of *M. pneumoniae* could be due to their disappearance caused by the immune system. But, humeral response produces specific serum antibodies, which is easily detectable, and would exist longer.

Possible role of *M. pneumoniae* chronic infection in RA development or future injuries to articular tissue is weakened by our results.

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