



The Detection of *Mycoplasma* spp. DNA in Synovial Fluid of Patients with Inflammatory Arthritis Using PCR-RFLP

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ABSTRACT

Aim: Certain *Mycoplasma* species, the smallest and simplest free-living bacteria which lack a rigid cell wall, are considered as important pathogenic organisms in human and recognized to have a role in rheumatoid arthritis. The aim of this study was to use molecular methods to detect *Mycoplasma* spp. in synovial fluid of patients with reactive arthritis in comparison with patients suffering from non-inflammatory arthritis as a control group.

Materials & Methods: Synovial fluid samples were collected from 99 patients with arthritis, all of which fulfilled the standard criteria of American College of Rheumatology for the diagnosis of inflammatory arthritis (59 patients) or non-inflammatory arthritis (40 patients). The DNA of all synovial fluid samples was extracted, and PCR was performed with a specific set of general primers for 16S rRNA of *Mycoplasma* genus. The PCR products were confirmed via restriction enzyme digestion using *Bam*HI and sequencing.

Finding: A total of 11 out of 99 (11.1%) samples of patients with reactive arthritis revealed a 270bp amplification band. Digesting the PCR product of 16S rRNA by *Bam*HI confirmed the PCR assay. The sequencing also confirmed the amplified products.

Conclusion: The pathophysiology of inflammatory arthritis could be attributed, at least in part, to the persistence of bacterial DNA in the joint of patients with reactive arthritis.

Keywords: *Mycoplasma* spp., RFLP, PCR, Synovial fluid

CITATION LINKS

[1] Current imaging strategies in rheumatoid arthritis. [2] Ultrastructural Changes during the life cycle of *Mycoplasma salivarium* in oral ... [3] Systematic detection of *Mycoplasmas* by culture and polymerase... [4] *Mycoplasma pneumoniae*: Clinical features and management. [5] Vaccines for *Mycoplasma* diseases in animals and man. [6] *Mycoplasma*-induced arthritis in animals... [7] Identification of *Mycoplasma fermentans* in ... [8] Detection of *Mycoplasma* infections in... [9] Multiplex PCR testing detection of higher-than-expected rates of cervical *Mycoplasma*, *Ureaplasma*, and *Trichomonas*... [10] The American Rheumatism Association 1987 revised... [11] Comparison of multiplex PCR assay with culture for detection of genital *Mycoplasmas*. [12] Identification of three novel *Mycoplasma* ... [13] Molecular biology and pathogenicity of *Mycoplasmas*. [14] *Mycoplasma pneumoniae*: A potentially severe infection. [15] Characteristics of the *Mycoplasmas* as a group. [16] *Mycoplasma pneumoniae*: Current Knowledge on Nucleic Acid Amplification Techniques and Serological Diagnostics. [17] Laboratory diagnosis of *Mycoplasma pneumoniae* infection. [18] Unexpectedly high frequency... [19] Polymerase chain reaction is superior to serology for the diagnosis of acute *Mycoplasma pneumoniae* ... [20] Comparison of two PCR-based assays for detecting *Mycoplasma genitalium*... [21] Polymerase chain reaction ... [22] Molecular diagnosis of ... [23] Mix-ups and mycoplasma: The enemies within. Leuk Res. [24] RFLP analysis of a PCR-amplified ... [25] Detection and identification of avian ... [26] Relationship between ... [27] Simultaneous detection of *Mycoplasma* ... [28] Molecular diagnosis of *Mycoplasma pneumoniae* in synovial fluid of rheumatoid arthritis patients. [29] Detection of *Mycoplasma salivarium* and *Mycoplasma fermentans* in synovial fluids of temporomandibular joints of patients with disorders in the joints. FEMS Immunol Med Microbiol. [30] Increased risk of rheumatoid arthritis among patients with *Mycoplasma pneumoniae*: A nationwide population-based cohort study in Taiwan. [31] Intra-articularly localized bacterial DNA containing CpG motifs induces arthritis. [32] The features of arthritis induced by CpG motifs in bacterial DNA. Arthritis Rheum. [33] CpG motifs in bacterial DNA activate leukocytes through the pH-dependent generation of reactive oxygen species.

Introduction

Rheumatoid arthritis (RA) is defined as a chronic inflammatory disease characterized by synovitis, systemic inflammation, and the detection of autoantibodies [1]. It has been widely discussed that like other autoimmune diseases, it could be caused by an infectious agent, virus, or slow growing bacteria. Among which, *Mycoplasma* spp. is the smallest and simplest form of freely replicating bacteria with 16 species, including *M. pneumoniae*, *M. salivarium*, *M. buccale*, *M. orale*, *M. faucium*, *M. lipophilum*, and *M. fermentans*, which are usually found in the oropharynx as commensal bacteria [2].

The reports showed that *M. pneumoniae* is associated with a wide range of extrapulmonary complications, from mild to severe, looking like a very good candidate [3-4].

It was demonstrated that *Mycoplasmas* could be isolated from the arthritic joints of many animals [5-6], while *M. fermentans* could be isolated from joints of patients with rheumatoid arthritis and other seronegative patients with inflammatory arthritis and cellular infiltrates [7].

Mycoplasma spp. commonly found in the oral cavity are a part of symbiotic gut flora and considered to have a low pathogenic potential. However, when they penetrate into the blood vessels and reach major organs, certain species could cause acute and chronic illnesses [8].

In the 1960s and the early 1970s, the possibility of isolating *Mycoplasma* from synovial specimens of patients with various rheumatoid disorder including RA, has been largely discussed, but very few teams reported such isolation, and these results were sometimes challenged [3]. Recently, researchers have focused on serological and molecular PCR-based methods as diagnostic tools to avoid bacterial cultures

methods [9].

Objectives: The aim of this study was to use molecular methods to detect *Mycoplasma* spp. and to confirm the results by Restriction Fragment Length Polymorphism (RFLP) method and sequencing.

Material and Methods

Patients and specimens: In this case/control study, after obtaining a written informed consent from the participants, synovial fluid samples were collected from 99 patients with arthritis according to ACR (American College of Rheumatology) [10]. Among which 59 and 40 cases satisfied the standard criteria for the diagnosis of inflammatory and non-inflammatory arthritis, respectively.

The synovial fluid samples were obtained by sterile aspiration from the knees of patients when it was needed, as a part of the clinical treatment procedure routine. The samples were centrifuged at $500 \times g$ for 30 min at 4°C to remove the cells. The supernatants were removed and then stored at -70°C for further assays.

DNA extraction: A total of 500 μL of the synovial fluid samples were centrifuged at 14 000 g for 60 min after thawing. Tris-EDTA buffer (Merck, Germany) was used for re-suspending the pellet and incubated at 56°C for 1 hr by 1% sodium dodecyl sulphate (SDS) and 100 mg/mL proteinase K (Sigma, USA). Nucleic acids were extracted by phenol-chloroform-isoamyl alcohol (25:24:1), followed by chloroform-isoamyl alcohol (Sigma, USA), according to Stellrecht et al. (2004) [11]. The aqueous phase was precipitated by adding 2 volumes of 95% ethanol and 0.1 M sodium acetate and centrifuged at 14 000 g for 30 min. DNA was recovered, and the pellet was re-suspended in 100 μL distilled water. Then 10 μL of each DNA sample was used in PCR assay [3].

PCR assay: Amplification was performed using specific primers for 16SrRNA region between GPO3F and MGS0 in 25 µL volumes. The general *Mycoplasma* primers were able to amplify a 270 bp fragment of 16S rRNA gene for the detection of *Mycoplasma* genus. Used primers in this study were as follows: GPO3F (5' TGGGGAG-CAAACAGGATTAGATACC 3') and MGS0 (5' TGCACCATCTGTCACTCTGTTAACCTC 3') [12]. PCR was performed in a reaction mixture with a total volume of 25 µL, containing 2.5 µL 10x Taq polymerase buffer, 0.3 µL dNTPs (10 mM), 1 U Taq DNA polymerase, 0.6 µL MgCl₂ (50 mM), and 0.3 M of each primer. Amplification consisted of a 3 min thermal delay step at 95°C, followed by 30 cycles comprising of a 20 sec denaturation step at 94°C, a 30 sec step at 64 °C for annealing, and a 40 sec step at 72°C for extending. After amplification, 1% agarose gel electrophoresis and red safe staining were used to visualize the PCR products. *M. pneumoniae* ATCC 29342 was used as control strain.

RFLP with *BamH1*: The PCR product confirmation was performed using *BamH1* endonuclease. The nucleotide sequence of *Mycoplasma* genus representatives, deposited in GenBank under the accession numbers of CP017332.1, was used as a reference strain for determining the cleavage site with *BamH1* enzyme. Gene Runner (version 6) and Primer3(version 0.4.0) software were used for evaluating the cleavage site with *BamH1* (G/GATCC), producing 145bp and 125bp bands.

Then genomic DNA was digested with 10 units of restriction endonucleases *BamH1* (Fermentas Inc., Glen Burnie, MD) according to the manufacturer's instructions. Incubation time was 2-4 hrs at 37°C.

Electrophoresis: The PCR products and digested genomic DNA were separated on a 2% agarose gel electrophoresis and

stained with red safe in TBE (Tris/Borate/EDTA) buffer.

Sequencing: To confirm the obtained findings from the electrophoresis, sequencing was done for the PCR products and the fragments of the RFLP by Macrogen Inc. (Seoul, Korea).

Findings

PCR assay for *Mycoplasma* detection: A 270bp amplification band was obtained for 11(11.1%) out of 99 arthritis samples, which means that 11.1% of the patients with arthritis were positive for the presence of *Mycoplasma* DNA. All of the patients in whom *Mycoplasma* DNA was detected belonged to the reactive arthritis group. Thus, 18.6% (11 of 59) of patients with inflammatory arthritis harbored *Mycoplasma* spp. DNA. No *Mycoplasma* spp. DNA was detected in synovial fluid samples of patients with non-inflammatory arthritis.

In silico analysis of amplified fragments and Restriction Fragment Length Polymorphism Analysis: In silico analysis of the 270bp product, it was showed that *BamH1* restriction endonuclease, with 1 restriction site, was able to produce 2 bands with 145bp and 125bp sizes, producing a good discrimination. In agreement with theoretical data, the digestion of 16S rRNA gene PCR products with *BamH1* also showed a uniform pattern with 2 specific bands (145 and 125bp) in each analyzed PCR product. A representative of PCR products before and after the digestion with *BamH1* and the resultant restriction products are shown in Figure 1.

Sequencing: The results of sequencing confirmed that *Mycoplasma* spp. DNA target region was successfully amplified using the PCRs. The sequencing data of the created fragments by restriction enzyme also confirmed that RFLP was correctly

performed.

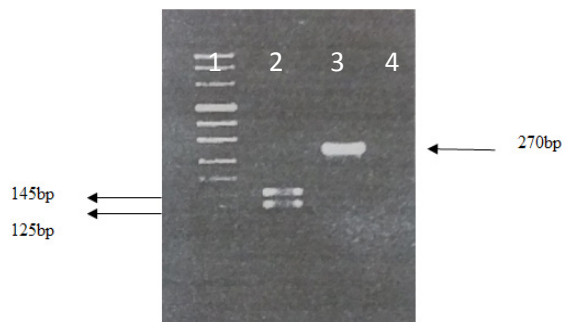


Figure 1) A representative of PCR products and created fragments after digestion with *Bam*H1. Lane 1. 100bp DNA ladder. Lane 2. PCR product after digestion with *Bam*H1. Lane 3. 270bp PCR product of *Mycoplasma* 16SrRNA. Lane 4. Negative control.

Discussion

Mycoplasma spp. usually exhibits a rather strict host and tissue specificity, probably reflecting their nutritionally exacting nature and obligate parasitic mode of life. However, there are numerous examples of the presence of *Mycoplasma* in hosts and tissues which are different from their normal habitats. The complex network of the interactions between *Mycoplasma* and host immune system involves *Mycoplasma*-induced specific and nonspecific immune reactions which may play an important role in the development of lesions and exacerbation of *Mycoplasma*-induced diseases [13]. Consequently, many chronic inflammatory or non-inflammatory infections have been proposed to be induced or triggered by *Mycoplasma* spp. [14].

Detection of *Mycoplasma* spp. by culture methods is a gold standard assay, but positive results could be obtained after several days or even several weeks: therefore, it is not appropriate for acute cases of diseases [15-16].

Serology as another assay for diagnosing the bacteria has been developed due to its

extensive accessibility for separate detection of immunoglobulin classes. It is recommended that both serological assay and direct *Mycoplasma* identification should be done [17]. Therefore, due to a significant degree of variability and non-specificity, routine serological methods for the identification of *Mycoplasma* infections are limited [18]. PCR assay is defined as a reliable test for the detection of *Mycoplasma* spp. such as *M. pneumonia* [19], *M. genitalium* [20], *M. hominis* [21], and *Ureaplasma urealyticum*. Moreover, Deguchi et al. (1995) [20] suggested that PCR is significantly more sensitive than culture methods for the detection of *M. genitalium*. In another study, it was shown that PCR assay is reliable for detecting *M. pneumoniae* in respiratory specimens [22].

Molecular methods are widely used for the detection of *Mycoplasma* because culture procedures are cost and time consuming [23]. Most of the research has been focused on the serological and molecular PCR-based diagnostic methods due to the rapidity in detection and eliminating the need for bacterial cultures [9].

In this study, 11 out of 99 (11.1%) arthritis samples contained *Mycoplasma* DNA; all of which were confirmed by RFLP method and sequencing. The cleavage patterns produced by the electrophoresis of *Bam*H1 digesting PCR products of *Mycoplasma* DNAs, proved the accuracy of the PCR performed for this study, and supported the validity of the results. In Scheidegger et al. (2009)'s study, PCR-RFLP of the 16S rRNA gene was introduced as a useful tool for accurate and rapid detection of *Enterococcus* [24]. In another project conducted for identifying avian *Mycoplasmas* by PCR-RFLP, it was shown that the results obtained by this method could be in agreement with those obtained by conventional assay [25].

No *Mycoplasma* DNA was detected in samples of patients with non-inflammatory ar-

thritis. This could be confirmed by Ramirez's study, suggesting that *M. pneumoniae* could be considered as a cofactor in rheumatoid arthritis [26].

In a study by Johnson et al. (2000), *M. fermentans* was found in 31 (91.17%) out of 34 samples of patients with reactive arthritis [7]. In another study by Ataee et al. (2015), a total of 131 synovial fluid samples of 131 patients with reactive arthritis were analyzed, and *Mycoplasma* genus was detected in 53.4% (70) of the evaluated samples [27]. Golmohammadi et al. (2014) confirmed *M. pneumoniae* species and *Mycoplasma* genus in 30 (22.9%) and 70 (53.4%) samples by molecular methods, respectively [28]. In a study by Watanabe, 16 synovial fluid samples from 13 female patients were screened for *Mycoplasma* spp. using a commercial kit, and *Mycoplasma* was observed in 8 (50%) out of 16 samples under study [29]. Haier et al. (1999) also evaluated *Mycoplasma* infections in the blood of 28 patients with rheumatoid arthritis. They were able to detect *M. fermentans* (8 cases), *M. pneumoniae* (5 cases), *M. hominis* (6 cases), and *M. penetrans* (1 case) [8]. In a cohort study done in Taiwan, the relationship between *M. pneumoniae* and the high risk of developing rheumatoid arthritis, especially in people less than 2 years old, was reported [30].

A major attractive hypothesis, which has been set forward, suggests that bacterial DNA could act as an immunostimulator which may directly be responsible for synovial inflammation. The bacterial DNA, which differs from eukaryotes DNA by the presence of more non-methylated CpG motifs, stimulates monocytes/macrophages and is sufficient to trigger arthritis in mice model [31-33].

Conclusion

In conclusion, inflammatory arthritis is probably associated with the particular

ability of *Mycoplasma* spp. to hide in certain cells and/or to induce a specific immune tolerance through molecular mimicry and/or to promote immunostimulatory effect probably due to the persistence of bacterial DNA in joints.

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