Detection of mycobacteria in joint samples from patients with arthritis using a genus-specific polymerase chain reaction and sequence analysis

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Abstract

Objective. Mycobacteria have been implicated in the pathogenesis of various forms of arthritis. The aim of this study was to examine the diagnostic potential of molecular biological techniques as well as to investigate the pathogenetic role of mycobacteria in chronic arthritis.

Patients and methods. DNA, extracted from synovial fluid and synovial tissue samples from patients with mycobacterial septic arthritis (n = 2), seronegative spondyloarthropathies (SpA) (n = 18), undifferentiated arthritis (UA) (n = 21) and rheumatoid arthritis (RA) (n = 40), was analysed using a mycobacterial genus-specific polymerase chain reaction (PCR) applied to amplify mycobacterial DNA. Subsequently, automated sequencing was performed for speciation. Samples from patients with either non-mycobacterial septic arthritis, osteoarthritis (OA), crystal arthritis or joint trauma served as negative controls (n = 19).

Results. Mycobacterium tuberculosis complex and Mycobacterium marinum were detected in the two patients with mycobacterial septic arthritis. The other species identified were Mycobacterium hodleri (in one RA patient), Mycobacterium smegmatis (in one OA patient and two RA patients) and Mycobacterium austroafricanum (in one crystal arthritis patient). All other samples were negative.

Conclusions. The results suggest that the mycobacterial genus-specific PCR applied on DNA extracts isolated directly from joint samples may be employed as an additional diagnostic tool in the case of clinical suspicion of a mycobacterial infection. No evidence was obtained for a pathogenetic role of mycobacteria in SpA, UA or RA.

Key words: Arthritis, Seronegative spondyloarthropathies, Undifferentiated arthritis, Rheumatoid arthritis, 16S rRNA gene PCR, Sequence analysis, Diagnosis, Mycobacterium tuberculosis, Mycobacterium marinum.

The arthritogenicity of mycobacteria is illustrated by their capacity to induce septic arthritis. Although Mycobacterium tuberculosis is the most extensively documented example, several non-tuberculous mycobacteria are also increasingly being recognized, including Mycobacterium avium, Mycobacterium fortuitum, Mycobacterium chelonae, Mycobacterium kansasii, Mycobacterium terrae and Mycobacterium marinum [1, 2].

The methods to identify mycobacterial species from clinical samples usually require cultured mycobacteria [3]. The diagnosis of a mycobacterial infection can be hampered by the slow growth of mycobacteria or difficulties in culturing. Although more rapid methods for the identification of cultured mycobacteria have been developed, alternatives to ensure a specific detection of mycobacteria are provided by nucleic acid amplification techniques [3]. The principal advantages of polymerase chain reaction (PCR)-based DNA amplification are that it can be applied directly on clinical specimens and provides a rapid diagnosis [3].

As molecular microbiological techniques are evolving, they are also increasingly being utilized in studies addressing the aetiology and pathogenesis of various idiopathic chronic arthritides [4–14]. Infectious agents may be involved in the aetiology of chronic arthritis, either as an inciting trigger or as a persistent antigenic drive in synovial inflammation. Recently, there has been...
renewed interest in the role of the intestinal bowel flora in the pathogenesis of seronegative spondyloarthropathies (SpA), undifferentiated arthritis (UA) and rheumatoid arthritis (RA). The close relationship between the gut and the joints is suggested by many clinical observations, e.g. reactive arthritis (ReA) after gastrointestinal infections and arthritis associated with chronic inflammatory bowel disease [15]. Results from animal models showed an important role for normal enteric bacteria, bacterial products and host genetic susceptibility in the pathogenesis of experimentally induced arthritis [16–18]. Furthermore, several groups have shown evidence of macroscopic and/or microscopic gut inflammation, which were unrelated to clinical intestinal symptoms, in subsets of patients with SpA and UA [15]. A recent study also presented evidence for ultrastructural lesions along the gut wall in 50% of the patients with RA [19]. Damaged intestinal epithelium in these patients could result in a defective defence against components of the microbial flora [20]. Consequently, dissemination into the joint may take place, resulting in local deposition of bacterial antigens and triggering of an inflammatory response, as has been argued, for example, for Yersinia enterocolitica-related arthritides [21–23]. As mycobacterial species are members of the normal bowel flora, it is conceivable that they might cause arthritis by similar mechanisms [24].

The aim of this study was to detect the local presence of mycobacterial species in the inflammatory joints of patients with mycobacterial septic arthritis, SpA, UA or RA. For this purpose, a mycobacterial genus-specific PCR, based on the 16S rRNA gene, was applied on DNA extracts isolated directly from synovial fluid (SF) and/or synovial tissue (ST) samples derived from inflamed joints. The sequences of the amplified mycobacterial DNA were subsequently analysed. Patients with non-mycobacterial septic arthritis, osteoarthritis (OA), gout or joint trauma served as negative controls.

Patients and methods

Patients

One hundred patients with an arthritis of at least one joint were included in the study. These 100 patients fulfilled the criteria for either septic arthritis, acid fast positive or culture proven (n = 5); SpA, including anklyosing spondylitis (n = 2), ReA (n = 7), enteropathic arthritis (n = 2) and psoriatic arthritis (n = 7) [25]; UA, based on the exclusion of other rheumatic diseases (n = 21); RA (n = 40) [26]; OA (n = 7) [27]; crystal arthropathy (n = 8) [28]; or post-traumatic joint effusion (n = 1). The disease duration, as measured from the first clinical signs of arthritis, was documented. The patients gave informed consent and were followed for at least 1 yr to allow confirmation of the accuracy of the diagnosis. The study protocol was approved by the medical ethics committee of Leiden University Medical Centre.

Specimen collection

SF and/or ST samples were collected from knee joints using the Parker–Pearson biopsy procedure with several modifications [13]. Prior to the biopsy procedure, a skin biopsy was removed which included the hair follicles to avoid contamination of joint samples by skin flora. This was followed by the sampling of SF and the application of an intra-articular anaesthetic (lidocaine 0.5%) via the site of the removed skin. The Parker–Pearson needle was also inserted via the skin biopsy site and 6–8 randomly taken ST biopsy samples were collected from the inflamed joint. From all joints other than the knee, SF was obtained during routine aspiration.

All samples were stained, using a standard acid fast staining technique, and cultured for mycobacteria on Lowenstein-Jensen medium, with or without pyruvate or glycine [3]. In the positive mycobacterial culture, speciation was performed by a separate PCR using the insertion element IS6110, in a region coding for a M. tuberculosis-specific insertion, as the genetic target [3]. All samples were also Gram stained and cultured using standard techniques for common bacteria.

After embedding formalin-fixed ST samples in paraffin, sections were cut, and the tissue stained with haematoxylin and eosin for histological analysis.

DNA extraction

The samples (500 μl SF and/or 6–8 biopsy specimens) were transferred into digestion buffer [500 mM Tris (pH 9), 20 mM EDTA, 10 mM NaCl, 1% SDS, 0.5 mg/ml proteinase K] and incubated for 18 h at 60 °C to release total DNA. The digested material was extracted with Tris–EDTA-buffered phenol (pH 7.4), ethanol precipitated and the DNA pellet was resuspended in 25–100 μl of sterile water, depending on the viscosity of the DNA extract.

Mycobacterial PCR

For the PCR, primers based on the mycobacterial 16S rRNA gene were used which correspond to residues 8–27 and 590–571 of the Escherichia coli 16S rRNA sequence: 5′-AGAGTTTTGATCCTGGYTCAG-3′ and 5′-TTTTCAGGAAAACGCAGCAA-3′, respectively. DNA amplification was carried out in 25 μl reaction volumes. Each reaction contained 5 pmol of each primer, 0.25 U of SuperTh polymerase (HT Biotechnology, Cambridge, UK), 55 ng TaqStart (Clontech, Palo Alto, USA), 200 μM of each dNTP, 50 mM Tris–HCl (pH 9), 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100 and 0.01% gelatin. A 35 μl overlay of sterile mineral oil was added, followed by 5 μl DNA extract. A touch-down PCR was used [29]: 3 min at 95 °C followed by two cycles of 20 s at 95 °C, 1 min at 66 °C and 1 min at 72 °C; then two cycles identical to the previous two cycles, but with an annealing temperature of 64 °C. The annealing temperature was subsequently decreased by 2 °C every two cycles, until an annealing temperature of 56 °C was reached. At this temperature, 40 additional cycles were run and the programme ended with 7 min at 72 °C. All PCRs were carried out in an OmniGene Thermal Cycler.
Detection of mycobacteria in joint samples by PCR

(IBM Ltd, Teddington, Middlesex, UK). Primers were synthesized by Perkin-Elmer Cetus (Gouda, The Netherlands).

**PCR product detection**

The PCR amplicons were checked by electrophoresis of 20 μl of the reaction mixture in an ethidium bromide-stained 1.5% agarose gel. A 1 kilobase (kb) DNA ladder (Gibco BRL, Life Technologies B.V., Breda, The Netherlands) was used as a DNA size standard. The DNA was visualized on a UV transilluminator and photographed. Positive bands were excised and eluted O/N in 100 μl H2O. Depending on the presence of inhibitory factors in individual clinical samples, the detection limit of the PCR was 1–5 copies of the 16S gene, corresponding to 20–100 copies/ml SF (data not shown). Re-amplification of the eluted amplicons after 1:1000 dilution was performed (25 cycles at 56 °C under the same conditions) with the same primers and amplicons were purified using a Qiagen PCR purification kit (Diagen, Hilden, Germany).

**Sequence analysis**

The PCR amplicons were sequenced using fluorescently-labelled dideoxynucleotides in the Applied Biosystems cycle sequencing system (Applied Biosystems/Perkin Elmer). Searches for sequence similarity were carried out using the FastA program on EMBL data banks [30].

**Prevention of contamination and controls**

During the collection of SF and biopsy specimens, and during the transfer of SF and ST into digestion buffer in a biosafety hood, control vials with digestion buffer were simultaneously opened and closed with sample collection vials (collection controls). DNA extraction and the preparation of the PCR mixtures were performed in separate, specially dedicated positive-pressure laboratories (>10 Pa) with a lock-gate in a biosafety hood, equipped with UV germicidal lamps. Dedicated pipettes with disposable filtertips, disposable gloves and labcoats, and non-reusable waste containers were used in both rooms. PCR spiking and product detection, and sequence analysis, were performed in a different laboratory.

Negative controls, to check for cross-contamination of samples, were included after every second clinical sample (extraction controls). These negative controls consisted of digestion buffer without clinical material, and were handled in the same way as the clinical samples. Positive as well as negative controls were included in each experiment. Positive controls consisted of reaction mixtures, spiked with 0.2 pg of chromosomal DNA of *Mycobacterium paratuberculosis* in H2O. In addition, each clinical DNA extract was also spiked to detect the presence of PCR inhibiting factors. To test the PCR reaction mixture itself for contamination of components, a negative control consisting of reaction mixture without DNA template was also included in each experiment (reaction mix control).

As gel extraction control, excision of a gel slice not containing a PCR band was performed. This gel slice was extracted and reamplified to monitor contamination due to environmental mycobacteria present during electrophoresis.

Sample processing and analysis were performed by investigators who were unaware of the clinical features of the patients.

**Results**

**Patients**

The clinical features and demographic characteristics of the various patient groups are shown in Table 1. One of the two patients with mycobacterial septic arthritis visited the out-patient clinic for analysis of pain and swelling of the right knee joint which had persisted for 2 yr. Direct acid-fast staining of the SF collected from the knee was negative, but mycobacterial culture, using Lowenstein-Jensen agar, was positive. The mycobacterial PCR, here using the IS6110 as genetic target, performed on the cultured isolate, revealed the presence of *M. tuberculosis* complex. Histopathological examination of the sampled ST showed a white central caseating granuloma. Separate biopsy specimens were used for PCR analysis directly on the clinical samples. The second patient with mycobacterial septic arthritis visited the out-patient clinic because of persisting swelling of the right hand and wrist. He mentioned that he owned a tropical fish tank. This patient had been treated with several courses of oral antibiotics, namely floxapen and augmentin. Tissue examination of an early biopsy specimen revealed granulomatous inflammation and long acid-fast particles. Culture of the affected tissue was negative. Treatment with doxycycline, and later ciproxin, was prescribed in view of a possible *M. marinum* infection. As the tenosynovitis persisted, a radical excision of the inflamed tissue was performed, and samples were used for PCR analysis in this study. Histopathological examination of the specimen revealed a granulomatous lesion with fibrinoid necrosis. Mycobacterial stain and culture of the tissue were now negative.

*Staphylococcus aureus* (n = 1), *Neisseria gonorrhoeae* (n = 1) and *Streptococcus pyogenes* (n = 1) were cultured from the SF of a knee and elbow joint, respectively, from two patients, and from the SF and ST of a knee joint from a third patient with septic arthritis. Bacterial agents involved in the cases of ReA determined using serological assays for *Yersinia, Chlamydia* and streptococci, and culture of the faeces and urethra, were *Salmonella typhimurium* (n = 2), *Campylobacter jejuni* (n = 1), *Chlamydia trachomatis* (n = 1) and *Streptococcus group A* (n = 2). In one patient with Reiter’s syndrome, the bacterial agent could not be determined.

Except for the samples from the two patients with mycobacterial septic arthritis, none of the SF and/or ST samples from the other patients were either acid-fast positive or mycobacterial culture positive. Pathology reports of ST examination did not reveal any granulomatous lesions.
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Table 1. Clinical and demographic data of patients studied for the intra-articular presence of mycobacterial DNA using a mycobacterial genus-specific PCR and sequence analysis

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>No. of patients</th>
<th>PCR positive</th>
<th>Mycobacterial species (% sequence identity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycobacterial septic arthritis</td>
<td>2</td>
<td>2</td>
<td>Synovial fluid: M. tuberculosis (100%)</td>
</tr>
<tr>
<td>(n = 2)</td>
<td></td>
<td></td>
<td>Synovial tissue: M. marinum (100%)</td>
</tr>
<tr>
<td>Seronegative spondyloarthropathy</td>
<td>18</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Undifferentiated arthritis</td>
<td>21</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>40</td>
<td>3</td>
<td>Synovial fluid: M. hodleri (97%)</td>
</tr>
<tr>
<td>(n = 21)</td>
<td></td>
<td></td>
<td>Synovial tissue: M. smegmatis (98%)</td>
</tr>
<tr>
<td>Non-mycobacterial septic arthritis</td>
<td>3</td>
<td>0*</td>
<td></td>
</tr>
<tr>
<td>Osteoarthritis</td>
<td>7</td>
<td>1</td>
<td>Synovial fluid: M. smegmatis (94%)</td>
</tr>
<tr>
<td>Crystal arthropathy</td>
<td>8</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Joint trauma</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>7</td>
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</table>

na, not available.

*In two patients with septic arthritis, synovial tissue was not available.

**PCR analysis**

In the positive control group, a DNA product of ~582 bp was amplified using a mycobacterial genus-specific PCR from DNA extracts of both SF and ST samples from the first patient. On sequencing this DNA product, 100% identity was found with both *M. tuberculosis* complex and *Mycobacterium bovis* (this fragment of the 16S gene contains no difference between these two species). Analysing the inflamed tissue samples from the second patient also showed a positive PCR result. Sequence analysis revealed 100% homology to that of *M. marinum*. Synovial fluid was not available from this patient.

Mycobacterial DNA was detected in SF and/or ST from three of the 40 patients with RA, but in none of the samples from the 18 patients with SpA or the 21 patients with UA. Furthermore, mycobacterial DNA was detected in the SF, but not ST, from one of seven OA patients and in the ST, but not SF, from one out of the eight patients with crystal arthropathy. After sequencing and comparative data analysis of the mycobacterial 16S gene amplicons from the SF and/or ST samples, the following percentages of sequence similarity with known mycobacterial species were found: *M. hodleri* (97%) in one patient with RA; *M. smegmatis* was detected in the joint samples from one patient with OA, and two other patients with RA: 94% and twice 98%, respectively; and *M. austroafricanum* (98%) in the joint samples from a patient with a crystal arthropathy. No specific clinical features were observed in the patients in whom mycobacterial DNA was detected.

In the collection, extraction and reaction mix controls, no mycobacterial DNA product was present, indicating the effect of the stringent measures to prevent cross-contamination. During PCR analysis of the clinical samples, no amplicons of the spike DNA (*M. paratuberculosis*) were found, showing that no laboratory cross-contamination had occurred.

**Discussion**

In this study, mycobacterial DNA was amplified from SF and ST samples of two patients with mycobacterial septic arthritis. This was accomplished using a genus-specific PCR for mycobacteria on DNA extracts isolated directly from the joint samples, followed by sequence analysis of the PCR products. Using the same approach, mycobacterial DNA was identified in the joint samples of three patients with RA, one patient with a crystal-induced arthropathy and one patient with OA.

A similar PCR assay based on DNA coding for 16S rRNA for the detection and identification of mycobacteria in clinical samples has been described previously [31, 32]. The 16S rRNA gene is a suitable target for amplifying mycobacterial nucleic acids at the genus level, and differentiating at the species level with a set

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**Table 2.** Results of mycobacterial DNA detection by means of a genus-specific PCR in synovial fluid and/or tissue from patients with various forms of arthritis

<table>
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</tr>
</tbody>
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na, not available.

*In two patients with septic arthritis, synovial tissue was not available.*

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The data represent the median (range).

*Other arthritides: osteoarthritis (n = 7); crystal arthropathies (n = 8); joint trauma (n = 1); non-mycobacterial septic arthritis (n = 3).
of species-specific probes or by sequence analysis [33, 34]. The possible applications of a specific mycobacterial PCR in rheumatological practice have been proposed previously [32, 35]. One case report describes species diagnosis using the analysis of the mycobacterial 16S rRNA gene on a cultured isolate derived from excised s.c. nodules [32]. In another case report, M. avium complex was identified in DNA extracts isolated directly from both skin and ST [35]. Three weeks later, culture confirmed the presence of M. avium. The potential of molecular microbiological methods as a diagnostic tool in culture-negative tuberculous tenosynovitis and arthritis was also demonstrated in a patient who had just been treated for pulmonary tuberculosis [36].

The results from the present study further support the potential of this molecular microbiological tool in the diagnosis of mycobacterial septic arthritides. However, further investigation of larger numbers of patients is clearly warranted. The detection of nucleic acid sequences from mycobacteria in the joint samples from five of the 98 patients in the study group with other arthritides, for example, indicates the importance of only applying this technique when a high degree of suspicion of a mycobacterial infection exists. The discordant results between the culture and the mycobacterial genus-specific 16S rRNA-based PCR results (negative culture, positive PCR) in the second patient with mycobacterial arthritis may be explained by a non-uniform distribution of microorganisms under paucibacillary conditions, or by the use of antimicrobial treatment prior to the radical excision of the diseased tissue.

The detection of mycobacteria in the joint samples of only three of the 40 patients with RA, and in none of the patients with SpA and UA, does not favour a pathogenetic role for mycobacteria, or mycobacterial DNA, in the inflamed joints of patients with chronic arthritis. Previous work has also failed to provide such evidence [4, 5]. We cannot, however, exclude the possibility that mycobacteria are involved in the pathogenesis of arthritis in the subset of three RA patients. Nevertheless, the detection of mycobacterial DNA in the joint samples from two patients with OA and crystal arthropathy, respectively, warrants a note of caution in the interpretation of these findings.

Three mycobacterial species, namely M. smegmatis, M. austroafricanum and M. hodleri, were detected in the SF and/or ST samples from three patients with RA, one patient with a crystal arthropathy and one patient with OA. Only M. smegmatis, an environmental saprophyte, has been associated with human disease [37]. Mycobacterium austroafricanum and M. hodleri have been characterized biochemically and genetically [38, 39]. These two strains were isolated from fluoranthene-contaminated water and from soil, respectively. After sequencing and comparative data analysis of the mycobacterial 16S amplicons from the SF and/or ST samples, the similarities found with known mycobacterial species were rarely 100%. This could also mean the presence of an as yet unidentified mycobacterial species. The detection of disease-non-specific mycobacterial DNA in joint samples could mean one of the following. Firstly, it is possible that the detection of the mycobacterial species reflects the presence of irrelevant bacterial DNA in, for example, phagocytes entering the inflammatory joints. This would accord with the data from a previous study, applying a universal PCR with broad-range 16S rRNA gene primers, in which we demonstrated that DNA products from several bacterial species can be present in the inflamed joints of patients with ReA and UA [13]. Secondly, the detection of the mycobacterial sequences could be explained by the occurrence of contamination. However, this is highly unlikely because of the extensive precautions taken during tissue sampling and analysis, and the absence of any mycobacterial DNA in the negative collection and laboratory controls.

Although this study does not substantiate a significant local role for mycobacteria in the pathogenesis of SpA, UA and RA, its place in the pathogenesis of these arthritic processes should not yet be completely dismissed. It is still possible that a mycobacterial infection occurs outside the joints, inducing a cross-reactive immune reaction affecting the joints. Studies on the cellular and humoral immune responses in these diseases will have to clarify this issue. Alternatively, a mycobacterial infection in the synovium, or at an alternative site in the body, might be present at a very early stage and could disappear after triggering the immune system. Furthermore, perhaps not whole bacteria or bacterial nucleic acids, but only mycobacterial cell wall fragments that are transported from, for example, the bowel, are deposited locally in the joint, resulting in inflammation. Such a mechanism has also been postulated for the pathogenesis of Yersinia-induced ReA [21–23]. Antigen detection, performed on SF and ST samples, may confirm or contest this hypothesis.

In conclusion, this study supports the further exploration of the diagnostic potential of a genus-specific PCR for mycobacteria on DNA extracts isolated directly from joint samples with subsequent sequence analysis. The results do not provide evidence for a local role of mycobacteria in the joints of patients with RA, SpA or UA.

Acknowledgement

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