BACTERIAL PATHOGENICITY

Detection of *Ureaplasma urealyticum* in urine of patients with systemic lupus erythematosus and healthy individuals by culture and polymerase chain reaction

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A method was developed to detect *Ureaplasma urealyticum* in urine by the polymerase chain reaction (PCR). A 457-bp fragment of the urease gene of *U. urealyticum* was amplified by PCR. Before PCR, components disturbing the amplification had to be reduced. This was possible by diluting the urine 1 in 10 with distilled water and by the extraction of the *U. urealyticum* DNA. Urine specimens from 41 patients with systemic lupus erythematosus (SLE) and 21 healthy individuals were treated by the dilution method and investigated by PCR for *U. urealyticum* DNA. The results were compared with those obtained by culture and the detection rates of PCR and culture were found to be identical. Also there was no difference in the detection rates of *U. urealyticum* from urine of SLE patients and healthy individuals; 10 (24.4%) of the 41 urine specimens from SLE patients and five (23.8%) of the 21 urine specimens from healthy individuals gave positive results for *U. urealyticum*. The results of this study do not indicate a decisive role for *U. urealyticum* in SLE.

Introduction

Ureaplasma urealyticum was described by Shepard *et al.* [1] who isolated it in 1954 from the genitourinary tract of men with non-gonococcal urethritis. It belongs to the family Mycoplasmataceae and differs from *Mycoplasma* species by its ability to hydrolyse urea. Robertson and Stemke [2] identified 14 serotypes of *U. urealyticum*. These serotypes can be divided into two biotypes based on protein patterns obtained by gel electrophoresis, percentage DNA-DNA hybridisation, restriction fragment length polymorphism, sensitivity to Mn²⁺ and enzyme profiles [3].

U. urealyticum has been associated with several diseases, such as urethritis, prostatitis, vaginitis, infertility, certain complications during pregnancy and pneumonia in neonates [4–7]. In a study of patients suffering from systemic lupus erythematosus (SLE), a multisystemic autoimmune disease of unknown aetiology, Ginsburg *et al.* [8] suggested a link between SLE and chronic colonisation of the female genitourinary tract with *U. urealyticum*.

The aim of the present study was to develop a method for the detection of *U. urealyticum* by polymerase chain reaction (PCR) and to perform further investigations about the possible role of *U. urealyticum* in SLE.

Materials and methods

Specimens

Early stream urine samples were taken from 41 female patients with SLE and 21 healthy individuals (17 female, four male). These urine specimens were examined for *U. urealyticum* by culture and PCR. All patients satisfied at least four American College of Rheumatology (formerly, American Rheumatism Association) criteria for SLE [9]. The mean duration of the disease was 96 months. The mean age of the patients was 44 years (range 16–60 years). The mean age of the healthy individuals was 33 years (range 22–64 years).

Micro-organisms and media

The following strains representing the 14 serotypes of *U. urealyticum* (kindly provided by Dr J. Robertson, University of Alberta, Canada) were used as controls for the sensitivity and specificity of the PCR: strain 7 (serotype 1), strain 23 (serotype 2), strain 27 (serotype

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3), strain 58 (serotype 4), strain 354 (NIH; serotype 5), strain Pi (serotype 6), strain Co (serotype 7), strain T960 (CX 8) (serotype 8), strain Vancouver (serotype 9), strain Western (serotype 10), strain K2 (serotype 11), strain U24 (serotype 12), strain U38 (serotype 13) and strain U26 (serotype 14).

U. urealyticum strains were cultivated in bromothymol blue broth according to Robertson [10] and in urea-plasma differential basal (A7) agar medium according to Shepard and Lunceford [11]. These media were also used for the cultural investigation of the urine specimens from the SLE patients and healthy individuals. PPLO broth (Difco Laboratories Detroit, Michigan, USA) containing PPLO serum fraction (Difco) 1% v/v and penicillin (Hoechst, Frankfurt, Germany) 100 000 IU was used as transport medium.

Mycoplasma bovis PG45 – a member of the family Mycoplasmataceae – and *Proteus vulgaris* (field strain; Institut für Mikrobiologie und Tierseuchen, Tierärztliche Hochschule, Hannover, Germany), a micro-organism with an urease gene, served as negative controls in the PCR.

Culture method

One ml of the urine sample was diluted immediately upon receipt with 2 ml transport medium. The remaining urine was kept in a separate vial. In the laboratory, 0.2 ml each of the diluted and undiluted urine were transferred to 1.8 ml of bromothymol blue broth and 10 µl of each were plated on to A7 agar medium. A further 1 ml of the diluted and 1 ml of the undiluted urine samples were centrifuged for 10 min at 14 000 g at 4°C. A portion (700 µl) of each supernate was decanted and the sediments were resuspended in the remaining 300 µl. From these suspensions, 0.2 ml were transferred to 1.8 ml of bromothymol blue broth and 10 µl were plated on to A7 agar medium. Liquid media were incubated in an atmosphere of CO₂ 5% at 37°C and examined twice daily for colour change without turbidity. Immediately after colour change, 0.1 ml was plated on to A7 agar. Agar media were incubated in an anaerobic atmosphere (Gas generating kit for anaerobic bacteria; Oxoid, Wesel, Germany) at 37°C and examined after incubation for 48 h and then daily for the characteristic small dark brown colonies. Urine samples were considered as positive by culture only after the detection of ureaplasma colonies, as colour change of the liquid medium, not combined with turbidity – occasionally used as an indication for growth of ureaplasma – can also originate from a small number of other urease-producing micro-organisms.

Determination of the number of colony-forming units (cfu)

The numbers of cfu were determined according to the method of Albers and Fletcher [12] with slight

modifications. Four-fold dilutions of the broth cultures of *U. urealyticum* were produced by charging the wells of sterile microtitration plates with 150 µl of bromothymol blue broth and serially transferring 50 µl volumes. By this procedure dilutions of 1 in 4, 1 in 16 up to 1 in 65 536 were obtained. Ten µl of each dilution were plated on to A7 agar immediately after production and incubated for 6 days as indicated above. Dilutions yielding 10–100 colonies of *U. urealyticum* were counted. The number of cfu/ml of the original *Ureaplasma* culture was determined as a mean of three investigations according to the formula: cfu/ml = cfu × 100 × dilution.

Development of a protocol for the detection of *U. urealyticum* in urine by PCR

Urine samples of healthy individuals which were culture negative for *U. urealyticum* were inoculated with different numbers of cfu of *U. urealyticum*. These samples were used for the development of a method suitable for the detection of *U. urealyticum* in urine by PCR. To reduce the amount of the components disturbing PCR, two approaches were tried before PCR: dilution of the urine specimens, and extraction of the ureaplasma DNA.

Dilution of urine specimens. The urine specimens were diluted with distilled water before PCR. Dilutions containing 10–90% urine and undiluted urine were used as templates in the PCR.

DNA extraction. Two methods were used for the extraction of the ureaplasma DNA. The first was a modification of the method described by Schleif and Wensingk [13]: 100 µl of liquid culture containing 10⁵ cfu of *U. urealyticum* were mixed with 900 µl urine and subjected to microwave irradiation (Philips, Space Cube 50) for 20–30 s at 650 W. After centrifugation at 12 000 g and 4°C for 30 min, the supernate was removed and mixed with 50 µl of a reaction buffer containing 0.1 M Tris, pH 7.8, 0.05 M EDTA, SDS 5% w/v and proteinase K (Boehringer, Mannheim, Germany) 500 µg and then incubated for 1 h at 56°C. After centrifugation at 12 000 g and 4°C for 15 min the DNA was separated by adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) to the supernate and shaking for 15 min at room temperature. After centrifugation for 10 min at 3000 g the DNA was precipitated from the aqueous phase with 2 volumes of absolute ethanol containing glycogen 1 µl and 3 M sodium acetate, pH 5.2, 40 µl and incubated overnight at –20°C. After centrifugation for 30 min at 12 000 g and 0°C, the ethanol was decanted and the DNA was dried and resuspended in 10 µl of distilled water. This solution was used for PCR. In the second method DNA was extracted by the Qiaex gel extraction kit (Diagen, Hilden, Germany). The extraction procedure was performed according to the manufacturer's instructions. Briefly, a suspension of

urine 90 μ l and liquid culture of *U. urealyticum* 10 μ l containing 10^4 cfu was added to a mixture of QX1 buffer (3 M NaI, 4 M NaClO₄, 10 mM Tris-HCl, pH 7.0, 10 mM Na₂S₂O₃) 300 μ l and Qiaex silica gel 10 μ l. After incubation for 10 min at 50°C the mixture was centrifuged for 30 s at 12 000 *g*. The pellet was resuspended in 500 μ l of QX2 buffer (8 M NaClO₄, 10 mM Tris-HCl, pH 7.0) and again centrifuged. After repeating this step the pellet was resuspended in QX3 buffer (ethanol 70% v/v, 100 mM NaCl, 10 mM Tris-HCl, pH 7.5) and centrifuged again. Following air drying, the pellet was resuspended in 10 μ l of TE buffer, incubated for 5 min at 50°C and centrifuged. The supernate was used for amplification.

PCR

The two oligonucleotides Uu1 (5'-GATGGTAAGT-TAGTTGCTGAC-3') and Uu2 (5'-ACGACGTCCA-TAAGCAACT-3'), published by Blanchard [14], flanking a 457-bp fragment of the urease gene of *U. urealyticum*, were used as primers. The PCR was performed on an automated DNA thermal cycler (Perkin Elmer Cetus, Überlingen, Germany) with *Taq*-DNA-polymerase (Stratagene, Heidelberg, Germany). The following served as templates: (i) broth cultures of *U. urealyticum*; (ii) undiluted urine and diluted urine (containing 10–90% urine) inoculated with different numbers of cfu of *U. urealyticum*; and (iii) *U. urealyticum* DNA extracted from urine experimentally infected with *U. urealyticum*. *M. bovis* and *P. vulgaris* were used as negative controls. Amplification was done in a reaction mixture containing 10 μ l of the sample to be analysed, 10 mM Tris-HCl, pH 8.8; 50 mM KCl, gelatine 0.01%, 1.5 mM MgCl₂ 125 μ M each of dATP, dCTP, dTTP and dGTP, 10 pmol of each primer and 2.5 U of *Taq*-polymerase per 50 μ l. Samples were denatured at 92°C for 1 min. Primers were annealed at 60°C for 90 s and extended at 72°C for 2 min. A total of 50 cycles was performed.

Analysis of amplification products

PCR amplification products were analysed on a vertical polyacrylamide 8% gel by applying 10 μ l of product mixed with 2 μ l of sample buffer containing bromophenol blue 4 mg, 0.5 M EDTA, 100 μ l pH 8.0, glycerol 565 μ l and TBE-buffer (consisting of Tris 5.4 g, borate 2.75 g, 0.5 M EDTA 1.0 ml, pH 8.0, in 100 ml of distilled water) 400 μ l. TBE-buffer diluted 1 in 5 with distilled water was used as running buffer. Gel electrophoresis was carried out for 45 min at 15 W. Bands were visualised by UV fluorescence after soaking the gel for 30 min in a solution containing ethidium bromide 0.01 μ g, glycerol 2 ml and 400 ml of the diluted TBE-buffer or by silver staining according to Blum *et al.* [15]. To ensure the specificity, amplification products showing the 457-bp fragment were cleaved with *Mbo*I (20 U/20 μ l; Pharmacia, Freiburg, Germany) for 3 h at 37°C in a solution

containing Tris-buffer (6 mM Tris-HCl, 50 mM NaCl, 6 mM MgCl₂) 5 μ l and distilled water 23 μ l. After terminating the reaction by applying 4 μ l of sample buffer the sample was subjected to gel electrophoresis. A 1-kb DNA ladder (Gibco BRL, Eggenstein, Germany) was used as molecular size marker.

Protocol used for the detection of *U. urealyticum* in urine of SLE patients and healthy individuals

Urine specimens were investigated by the culture method as indicated above. The PCR used for the clinical evaluation was performed on (i) urine specimens from SLE patients and healthy individuals diluted 1 in 10 with distilled water and (ii) on urine specimens from SLE patients and healthy individuals pre-incubated in liquid medium (0.2 ml of urine in 1.8 ml of bromothymol blue broth). Amplification was performed as indicated above. The amplification products were visualised by silver staining according to Blum *et al.* [15].

Results

Suitability of the methods investigated for the detection of U. urealyticum in urine artificially inoculated with U. urealyticum by PCR

The detection of *U. urealyticum* in urine by PCR depended on the preparation of the urine for PCR. While detection was impossible with untreated urine, it was successful with diluted urine as well as with the extracted *U. urealyticum* DNA.

With the dilution method the best results were obtained with a 1 in 10 dilution of the urine (10% urine). Amplification did not occur in samples containing >40% urine and occurred only sporadically in samples containing 20–30% urine. After 1 in 10 dilution of the urine, 1 cfu of *U. urealyticum* – corresponding to 10^3 cfu of *U. urealyticum*/ml of urine – could be detected in the 10- μ l sample used as a template in the reaction mixture (Fig. 1).

The detection limit of the PCR performed on DNA of *U. urealyticum* extracted by the Qiaex kit corresponded to 10^2 cfu of *U. urealyticum* in the 10- μ l sample, i.e., to 10^5 cfu of *U. urealyticum*/ml of urine. Fragments of *U. urealyticum* DNA extracted by the method according to Schleif and Wensingk [13] could not be amplified in the PCR performed.

The 457-bp fragment of the *U. urealyticum* urease gene amplified by PCR could be cleaved with *Mbo*I into four fragments of 53, 98, 139 and 166 bp, corresponding to the sizes stated (Fig. 2) [14]. DNA from all serotypes of *U. urealyticum* but not from *M. bovis* or *P. vulgaris* was amplified.

Fig. 1. Detection limit of *U. urealyticum* in samples containing 10% urine by PCR. Lane 1, molecular size standard; 2–6, 8, *U. urealyticum*-free urine samples, diluted 1 in 10 with distilled water and inoculated with 10^4 (2), 10^3 (3) 10^2 (4), 10 (5), and 1 (6, 8) cfu of *U. urealyticum*; 7, 9, *U. urealyticum*-free controls. Samples were separated on a polyacrylamide gel and stained with ethidium bromide (lanes 1–7) or silver (lanes 8 and 9). A 457-bp fragment could be seen after staining with ethidium bromide in samples inoculated with 10^4 –10 cfu of *U. urealyticum* (lane 5) and after silver staining in samples containing 1 cfu of *U. urealyticum* (8). An amplification product could not be observed by ethidium bromide (lane 7) or by silver staining (9) in the negative controls.

Fig. 2. Characterisation of the 457-bp DNA fragment of the urease gene of *U. urealyticum* amplified by PCR. Lane 1, molecular size standard; 2, 457-bp amplification product of *U. urealyticum*; 3, 457-bp fragment cleaved with *Mbo*I into four fragments. Samples were electrophoresed on a polyacrylamide gel and stained by ethidium bromide.

Clinical results

U. urealyticum could be cultivated from the urine of eight (19.5%) of the 41 SLE patients investigated and from the urine of four (19.0%) of the 21 control individuals (Table 1); 10^3 – 10^5 cfu *U. urealyticum*/ml urine were isolated from SLE patients and 10^2 – 10^4 cfu of *U. urealyticum*/ml of urine from healthy individuals.

PCR was performed on urine from the SLE patients and the healthy individuals diluted 1 in 10 with distilled water and on urine pre-incubated in bromothymol blue broth. Without pre-incubation, *U. urealyticum* could be detected in urine specimens of seven (17.1%) of the 41 SLE patients investigated and in three (14.3%) urine specimens of the 21 healthy individuals. After enrichment in bromothymol blue broth, *U. urealyticum* was demonstrated in two more urine specimens, one from an SLE patient and one from a healthy individual (Table 1).

SLE patients revealed eight (19.5%) urine specimens

Table 1. Detection of *U. urealyticum* in urine samples from patients with SLE and from healthy individuals by culture and PCR

Source	Number of individuals positive for <i>U. urealyticum</i> detected by				Total (%)
	Culture and PCR*	Culture only	PCR* only without pre-incubation	PCR* only after pre-incubation	
SLE patients (n = 41)	6	2	1	1	10 (24.4)
Healthy individuals (n = 21)	3	1	0	1	5 (23.8)

*Amplification products were visualised by silver staining

positive for *U. urealyticum* by culture and eight (19.5%) urine specimens positive for *U. urealyticum* by PCR. In healthy individuals four (19.0%) urine specimens were positive for *U. urealyticum* by culture and four (19.0%) by PCR; i.e., identical numbers of *U. urealyticum*-positive urine specimens were detected by culture and by PCR. However, it has to be considered that three urine specimens (two from SLE patients and one from a healthy individual) were *U. urealyticum* positive only by culture and three urine specimens (two from SLE patients and one from a healthy individual) only by PCR. In total, *U. urealyticum* was demonstrated in urine specimens of 10 (24.4%) of the 41 SLE patients investigated and in five (23.8%) urine samples of the 21 healthy individuals (Table 1).

Discussion

In the present investigation, a method was developed to detect *U. urealyticum* in urine by PCR and was compared with culture for sensitivity. This method has been used to detect *U. urealyticum* in urine of SLE patients and of healthy individuals.

As *U. urealyticum* could not be detected by PCR in untreated urine specimens inoculated with *U. urealyticum* – probably because of PCR-inhibiting substances – a method had to be developed to reduce the concentration of these components. Two different approaches were pursued: firstly, dilution of the urine and secondly, extraction of the *U. urealyticum* DNA; both approaches appeared to be suitable. However, of the two DNA extracting methods investigated, only the extraction by the Qiaex kit was successful, whereas the method of Schleif and Wensingk [13] did not yield positive results. This may have been due to the high concentration of salts accumulating in the DNA-containing aqueous phase. As the dilution method was more sensitive (detection limit 10^3 cfu/ml of urine), and also much simpler and cheaper than the extraction of DNA (detection limit 10^5 cfu/ml of urine), it was applied to the detection of *U. urealyticum* in the urine specimens of patients with SLE and healthy individuals by PCR.

With regard to sensitivity, no difference was observed between the culture method and the PCR. However, since in about one-third of the urine specimens *U. urealyticum* could be detected only by culture, and in one-third only by PCR, the detection rate was increased considerably by using both methods.

The detection rates of *U. urealyticum* in urine of patients with SLE and healthy individuals were almost identical. These results are in contradiction to the results reported by Ginsburg *et al.* [8] who found a significant difference in the isolation rate of *U. urealyticum* from patients with SLE (63%) and control

individuals (4.5%) for which they used patients with chronic fatigue syndrome. The age and the socio-demographic factors of both groups were the same. However, it is questionable as to whether patients with chronic fatigue syndrome are suitable control individuals. It is well known that the colonisation of the genitourinary tract with *U. urealyticum* depends not only on age and sociodemographic factors, but also on sexual activity [4, 16, 17]. The low colonisation rate of the patients with chronic fatigue syndrome could also be the consequence of a lower level of sexual activity by these individuals.

According to the present investigations, which were performed on SLE patients and healthy control individuals of about the same age, *U. urealyticum* does not play a decisive role in SLE. However, since mycoplasmas are apparently involved in several chronic inflammatory disorders [18–25], further investigations not restricted to the detection in urine may be necessary to definitely rule out any relationship between infection with ureaplasmas and SLE.

Nevertheless, the detection of *U. urealyticum* remains important and necessary because of its involvement in several urogenital diseases. As the cultivation of *U. urealyticum* is laborious, time consuming, and requires specific expertise, the PCR may be a suitable alternative for the detection of *U. urealyticum* in urine.

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