MOLECULAR DIAGNOSIS AND TYPING

Use of PCR to detect mycoplasma DNA in respiratory tract specimens from adult HIV-positive patients

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The polymerase chain reaction (PCR) was evaluated retrospectively for its ability to detect *Ureaplasma urealyticum* and *Mycoplasma* spp. in respiratory tract specimens obtained from adult patients with AIDS. Mycoplasma DNA was detected in specimens from 12 of 84 patients. Of the 107 specimens tested, 13 and seven positive PCR results were obtained with the genus- and species-specific oligonucleotide primers used, respectively, in two different steps. With the latter, one sample was positive for *U. urealyticum* plus *M. hominis*, another for *M. fermentans* plus *M. salivarium*, and five others were positive for *M. salivarium*. The unexpected detection of *U. urealyticum* DNA in respiratory secretions from an adult AIDS patient suggested that this urogenital mycoplasma could have a role in determining or exacerbating respiratory tract infections in the HIV-positive population, but that its low rate of isolation could be related to the frequent failure of methods used currently to detect mycoplasmas.

Introduction

Ureaplasma urealyticum is a main cause of non-chlamydial, non-gonococcal urethritis and acute prostatitis, and can cause chorioamnionitis with pre-term delivery in pregnancy [1]. It also appears to be involved in respiratory disease of very low birth weight infants, and recent evidence indicates that *U. urealyticum*, alone or in addition to *Mycoplasma hominis*, is able to cause meningitis, particularly in the first few days of life in premature infants with respiratory disease or hydrocephalus [2]. *U. urealyticum* has also been isolated from immunosuppressed or hypogammaglobulinaemic patients with septic arthritis, but is isolated infrequently from respiratory specimens of children outside the neonatal period [3]. Moreover, evidence that *U. urealyticum* is involved in infections in AIDS patients is lacking.

Routine laboratory diagnosis is based mainly on serology and, to a lesser extent, on time-consuming cultivation techniques [4]. Considering the lack of

antibody production by immunosuppressed patients, the polymerase chain reaction (PCR) may represent a rapid, sensitive and specific tool to detect mycoplasmas in various biological fluids. This study investigated the use of PCR to detect DNA of *U. urealyticum* and *Mycoplasma* spp. in respiratory secretions from adult HIV-infected patients to assess the frequency of isolation and the clinical settings in which mycoplasmas were identified.

Materials and methods

Specimen collection

In total, 130 respiratory tract specimens, obtained by bronchoalveolar lavage (BAL) or from induced sputum (IS) or nasopharyngeal aspirates (NPA), were collected and frozen between March 1991 and April 1996 from 84 HIV-positive patients (54 males, 30 females; age range 24 SD 7.1 years; CD4⁺ cell counts 52 SD 2.1/mm³) attending the Section of Infectious Diseases of the Department of Clinical and Experimental Medicine, University of Ferrara, and the Department of Infectious and Tropical Diseases of University 'La Sapienza' of Rome. The patients presented with radiographic abnormalities and mild respiratory symptomatology in the absence of concomitant pulmonary

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opportunistic pathogens, including *Pneumocystis carinii*, *Toxoplasma gondii* and mycobacteria. In addition, 15 HIV-negative patients, chosen randomly from people who demanded HIV testing spontaneously in our institutions, were included in the study. This control group consisted of patients with negative serological tests for mycoplasmas, but from whom four BAL or IS specimens were positive for *Mycoplasma* spp. by routine culture examination at the time of sampling.

Sample processing

BAL was performed according to Hartman *et al.* [5]. For IS and NPA specimens, the method of Hague *et al.* [6] was used. Such specimens (*c.* 5–10 ml) were liquefied with Sputasol™ (Unipath) and then vortex mixed with glass beads for 3 min. An equal volume of phosphate-buffered saline (PBS, pH 7.2) was added and samples were centrifuged at 2000 *g* for 15 min. The pellet was resuspended in 1 ml of PBS and stored until required for PCR evaluation. Sample lysis was by the method of Kellog and Kwok [7]. Briefly, 100 μ l of each sample were centrifuged at 10 000 *g* for 15 min at 4°C, the pellets resuspended in washing buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂), and then recentrifuged as above. Pellets were lysed in 25 μ l of

solution A (10 mM Tris-HCl, pH 8.3, 100 mM KCl, 2.5 mM MgCl₂) and 25 μ l of solution B (10 mM Tris-HCl, pH 8.3, 2.5 mM MgCl₂, Tween 20 1% v/v, Triton X-100 1% v/v) containing proteinase K 120 μ g/ml. After incubation for 1 h at 60°C, samples were heated at 95°C for 10 min in a water-bath and then cooled to room temperature.

PCR conditions

Standard precautions (keeping autoclaved reagents in small, single use volumes, UV irradiation treatment for surface laboratory benches, filter-tips, adding DNA last) were used to avoid the risk of contamination by DNA or PCR product carryover [8].

Initially, 2- μ l portions of undiluted and 10-fold dilutions of each sample were amplified with human globin-specific primers (GLO5/GLO6, 989 bp; Table 2) to test whether lysis had occurred [9]. Plasmid pBR322 DNA (Promega, Madison, WI, USA) was added to each sample, and PCR was performed with primers pBR1/pBR2 (719 bp; Table 2) to identify any inhibition of amplification caused by the presence of inhibitors in the sample [10]. Mycoplasmas have been detected previously with genus-specific primers

Table 1. Sequences of primers used to amplify DNA from samples

Designation	Sequence 5'–3'	Reference
GLO5	TGGTAGCTGGATTGTAGCTG	[9]
GLO6	ATTTTCCCACCCTTAGGCTG	[9]
PBR1	CATCTCGGGCAGCGTTGGGT	[10]
PBR2	ACAAGCTGTGACCGTCTCCG	[10]
PBR6	AGCGCAGCGAGTCAGTGAGC	[10]
MGSO	TGCACCATCTGTCACTCTGTAAACCTC	[11]
GPO1	ACTCCTACGGGAGGCAGCAGTA	[11]
UNI ⁻	TAATCCTGTTTGCTCCCCAC	[10]
RNA5	AGAGTTTGATCCTGGCTCAGGA	[10]
PNEU-GEN	CCTGCAAGGGTTCGTTATTT	[10]
HOM ⁺	TGAAAGGCGCTGTAAGGCGC	[11]
SAL	GCTGCGTCAACAGTTCTCTG	[10]
PIR	GTCCGTTTGGACCGCTATAG	[10]
ORA5	GGAGCGTTTCGTCGCTAAG	[10]
FER	AAGAAGCGTTTCTTCGCTGG	[10]
PEN2	CAATCCTGATACATCCCGTA	Unpublished data
UU3	GATGGTAAGTTAGTTGCTGAC	[12]
UU4	ACGACGTCCATAAGCAACT	[12]
UU5	CAATCTGCTCGTGAAGTATTAC	[13]

Table 2. Targets of primers used in PCR amplifications

Target	Primer 1 (sense)	Primer 2 (antisense)	Amplicon length (bp)
β -Globin gene	GLO5	GLO6	989
Plasmid pBR322 DNA	PBR1	PBR2	719
Plasmid pBR322 DNA	PBR1	PBR6	974
Mycoplasmas	GPO1	MGSO	717
<i>M. pneumoniae</i> + <i>M. genitalium</i>	PNEU-GEN	MGSO	851
<i>M. fermentans</i>	FER	UNI ⁻	579
<i>M. pirum</i>	PIR	UNI ⁻	579
<i>M. salivarium</i>	RNA5	SAL	839
<i>M. hominis</i>	HOM ⁺	UNI ⁻	589
<i>M. penetrans</i>	RNA5	PEN2	850
<i>M. orale</i>	ORA	UNI ⁻	583
<i>U. urealyticum</i>	U3	U4	456
<i>U. urealyticum</i>	U5	U4	429

(MGSO/GPO1, 717 bp; Table 2), followed by amplification of positive samples with species-specific primers for *M. pneumoniae*, *M. genitalium*, *M. hominis*, *M. salivarium*, *M. orale*, *M. pirum*, *M. fermentans*, *M. penetrans* and *U. urealyticum* (Table 2). Mycoplasma genus- and species-specific oligonucleotide primers (Unité de Chimie Organique, Institut Pasteur, Paris, France) were deduced mostly from either the conserved or variable 16S RNA regions; *U. urealyticum* primers UU3, UU4 and UU5 were obtained from the urease gene sequence (Tables 1 and 2) [10–13]. *M. penetrans* primer PEN2 was chosen from the V5 variable region of the 16S rRNA gene after multiple alignment of several human mycoplasmas. The specificity of this primer was tested and it was found not to cross-react with human mycoplasmas other than *M. penetrans* (data not shown). PCR mixtures (50 µl) contained 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 25 µM tetramethylammonium chloride (Aldrich, Milwaukee, WI, USA), 40 pmol of each primer, 0.2 mM dNTPs and 1 U of *Taq* DNA polymerase (Promega) and were overlaid with two drops of mineral oil. DNA sample (2 µl) was added through the oil. PCR conditions comprised initial denaturation for 15 min at 95°C, followed by 30 cycles of 30 s at 95°C, 90 s at 58°C, and 90 s (increased 1 s/cycle) at 72°C, with final extension period at 72°C for 10 min [10].

Analysis of amplified DNA

Portions (20 µl) of amplified products were electrophoresed in agarose 0.8% w/v gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8), together with positive and negative controls, and DNA bands were visualised under UV illumination after ethidium bromide staining.

Results and discussion

Sensitivity of the PCR assay

M. pneumoniae strain FH was grown and harvested in late exponential phase and DNA was extracted and purified as described previously [10]. Serial 10-fold DNA dilutions were used for amplification with the genus-specific primers MGSO/GPO1. Visible PCR products (717 bp) could be detected on stained agarose gels at a dilution representing ≤ 10 fg, which corresponded to *c.* 10 mycoplasmas/ml. This procedure was followed for all the mycoplasmas mentioned above and the results indicated that 5–15 micro-organisms could be detected, depending on the species tested.

Detection of mycoplasmas in respiratory secretions

Of the total specimens amplified, 23 failed to yield a PCR product from the pBR322 control DNA at two dilutions. These specimens were not used in the analysis for mycoplasma DNA by PCR, which was thus performed on the remaining 107 specimens. Of these, 13 were positive for mycoplasma DNA, either with genus-specific (*n* = 13) or with species-specific (*n* = 7) primers in 12 and six AIDS patients, respectively (Table 3). One specimen contained both *U. urealyticum* and *M. hominis* DNA after amplification with UU3/UU4 primers (456-bp DNA band) followed by another amplification with UU4/UU5 primers (429-bp DNA band). Another specimen contained *M. salivarium* DNA plus *M. fermentans* DNA, while five other samples were also PCR-positive for *M. salivarium* DNA. All samples from HIV-negative patients which were positive by culture for *Mycoplasma* were also positive for *M. salivarium* DNA by PCR.

Table 3. Detection of mycoplasma DNA by PCR in respiratory secretions of HIV-infected patients

Patient no.	Clinical data	DNA detection (positive samples/ total samples)		Mycoplasma DNA amplification							
		genus	species	sal	ora	hom	pneu/gen	pen	ferm	pir	Uu
1	AIDS, past PCP, atypical bronchovascular markings	1/3	1/3	+	–	–	–	–	+	–	–
2	AIDS, past PCP, endocarditis,	1/3	1/3	+	–	+	–	–	–	–	–
3	AIDS	1/2	0/2	–	–	–	–	–	–	–	–
4	Advanced AIDS, past PCP, bronchovascular markings, lung infiltrates	2/3	2/3	+(2)	–	–	–	–	–	–	–
5	AIDS, past TBC, bronchovascular infiltrates	1/1	1/1	+	–	–	–	–	–	+	–
6	AIDS, CMV retinitis, disseminated TBC	1/1	0/1	–	–	–	–	–	–	–	–
7	AIDS, past TBC, cough, interstitial infiltrates	1/1	1/1	–	–	+	–	–	–	–	+
8	AIDS, KS	1/1	0/1	–	–	–	–	–	–	–	–
9	ARC, pneumonia	1/1	0/1	–	–	–	–	–	–	–	–
10	ARC, hepatitis	1/1	0/1	–	–	–	–	–	–	–	...
11	AIDS, bronchovascular infiltrates	1/1	1/1	+	–	+	–	–	–	–	–
12	AIDS, cerebral lymphoma, CMV retinitis, lung nodular infiltrates	1/1	0/1	–	–	–	–	–	–	–	...

sal, *M. salivarium*; ora, *M. orale*; hom, *M. hominis*; pneu/gen, *M. pneumoniae* and *M. genitalium*; pen, *M. penetrans*; ferm, *M. fermentans*; pir, *M. pirum*; Uu, *U. urealyticum*; PCP, *Pneumocystis carinii* pneumonia; TBC, *Mycobacterium tuberculosis* infection.

BAL samples from HIV-infected patients are contaminated frequently by upper respiratory tract and oral flora, including mycoplasmas, for which the frequency of isolation is higher than that from HIV-negative patients [14]. The present study raises the question of the possible role of *U. urealyticum* in triggering pulmonary disease in HIV-infected patients. To date, this organism has been implicated, in addition to urogenital tract infections, in causing bronchospasm and chronic lung diseases in infants and young children [15]. In the present report, *U. urealyticum* DNA was detected in lung secretions from an adult patient with AIDS who presented with cough and chest signs of bronchovascular markings in the absence of severe respiratory disease. The low rate of isolation may not be representative of the frequency with which *U. urealyticum* is involved in the causation of lung injury, and the possibility that this mycoplasma could be involved as a pathogen in respiratory tract infections or self-limiting interstitial pneumonia cannot be excluded. Furthermore, it is unclear whether it is found alone or in combination. *M. hominis* is habitually associated with genitourinary tract infections, pelvic inflammatory disease, and post-abortion fever. The unusual association of *U. urealyticum* with *M. hominis* observed in the present study could contribute to, cause, or worsen lung disease in the HIV-infected population.

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