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Mycoplasmas in the urine of HIV-1 infected men

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SUMMARY

The aim of this study was determine the prevalence of *Mycoplasma hominis, M. genitalium*, *M. fermentans*, *M. pirum*, *M. penetrans* and *Ureaplasma urealyticum* in HIV-infected patients. Culture and PCR were used to detect six species of *Mycoplasma* in first-void urine of HIV-1 infected men. A total of 497 HIV/AIDS patients (age range 5–75 years, mean 37 years) were screened in the study. All presented positive for at least one kind of mycoplasma, especially *U. urealyticum* and *M. hominis*. Six mycoplasmas were significant in the homosexual contact and heterosexual contact groups. The distribution of *M. hominis*, *M. penetrans*, and *M. pirum* were significantly different in this four-transmission category. $CD4^+$ cell count levels were lower in the AIDS-associated *Mycoplasma*-positive group than in the *Mycoplasma*-negative group ($P < 0{\cdot}01$). This study indicates that *U. urealyticum*, *M. hominis* and *M. fermentans* are prevalent in HIV-1-infected male patients. This may be an indication of whether mycoplasmas are co-factors in the progression of HIV disease.

Key words: Culture, HIV, mycoplasmas, PCR, prevalence.

INTRODUCTION

Infection with human immunodeficiency virus type 1 (HIV-1) is characterized by complex pathological alterations in which the immunological system presents a significant decrease in T-helper cell-dependent cellular immune response. Clinical signs and symptoms of AIDS appear after an asymptomatic period ranging from months to years [1, 2]. The pathogenic mechanisms underlying HIV infection are multifactorial, multiphasic, and extremely complex [3]. Persistent HIV replication, chronic and aberrant immune activation and cytokine secretion are major components of the pathogenic process. It may be that there are various factors and/or co-factors to be considered. Some of the widely known factors affecting the period of latency are genetic susceptibility, patient's age, viral strain diversity, route of contamination and the characteristics of the inoculums [4]. Activators of the immune system may accelerate the degradation of the immune system and the progression of the disease [5].

Studies have suggested the possibility that certain *Mycoplasma* species may act as co-factors accelerating AIDS progression. Mycoplasmas are microorganisms that evolved regressively (by genome reduction) from Gram-positive bacterial ancestors with a low DNA content of guanine plus cytosine [6].

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Many of their properties, such as small genome size, small number of rRNA operons and tRNA genes, lack of a cell wall, fastidious growth and limited metabolic activities, are seen as the result of this evolution [7]. The class Mollicutes comprises about 150 distinct species including more than 92 *Mycoplasma* species found in humans, animals, insects and plants. Of these, 16 species have been isolated from human tissues or fluids, of which 15 constitute the normal human mollicute flora. While *Mycoplasma pneumoniae* has been definitively distinguished as a human pathogen, other species have been isolated in significant quantities from patients and healthy individuals [8, 9]. The role of these mycoplasmas in human diseases has intrigued researchers.

The presence of *Mycoplasma fermentans*, *M. pirum* and *M. penetrans* in patients with AIDS, and their association with the progression of HIV infection is drawing attention to the possible mechanisms by which mycoplasmas may influence the pathogenesis of HIV-1. It has been suggested that these organisms contribute to the pathogenesis of AIDS [4, 8, 9]. *In vitro* they enhance the cytopathic effect of HIV, and are powerful immunomodulators: they kill undifferentiated myelomonocytic cell lines; induce cytokine secretion by several cell types of the monocyte-macrophage lineage in both murine and human immune systems; and they activate B and T lymphocytes. They have also been shown to produce superantigens [10]. Some species of this group (*M. penetrans, M. fermentans, M. pirum*) have been isolated from AIDS patients, but the role of mycoplasmas in humans infected by HIV has not yet been established.

Some reports are available on the prevalence of *M. hominis, Ureaplasma urealyticum* and four other HIV-associated mycoplasmas (*M. genitalium*, *M. fermentans*, *M. pirum*, *M. penetrans*) in the family Mycoplasmataceae in HIV/AIDS populations in other countries, but data on HIV-associated mycoplasmas in HIV-infected individuals in China are entirely lacking. Therefore, we designed this study to determine the prevalence of these six species in HIV/AIDS patients in Jiangsu Province, China.

MATERIALS AND METHODS

Patients and sample preparation

In March 2009, 497 HIV-1-infected men at the Jiangsu Centers for Diseases Prevention and Control were recruited for this study. Written or verbal informed consent was obtained from each individual or their legal representatives before study enrolment. The HIV infection was confirmed by Western Blot test at Jiangsu Centers for Diseases Prevention and Control.

For each subject, 3 ml of blood was drawn into a 5-ml collection tube containing 5% EDTA. At the same time, the first-void urine (FVU) specimens (15–30 ml) were collected in sterile containers and then transferred to a sterile plastic centrifuge tube. All the specimens were transported to the laboratory within 4 h. The FVU specimens were centrifuged at 3500 ***g*** for 15 min. The pellet was then suspended in 1 ml 0·9% sodium chloride solution and equated to two parts, one was immediately used to culture *U. urealyticum* and *M. hominis*, the other was stored at −70 °C for subsequent PCR detection.

CD4$^+$ T cell counts

Absolute CD4$^+$ T cell counts were determined in EDTA-treated peripheral blood samples using an Ortho Cytoron Absolute flow cytometer (Ortho Diagnostic Systems Inc., USA) and anti-CD4-FITC monoclonal antibodies (Ortho Diagnostic System Inc.), according to the manufacturer's instructions. Three-colour flow cytometry was also performed for supplemental CD4$^+$ T lymphocyte subset identification. After staining of whole blood with monoclonal antibodies, samples were lysed with FACS lysing solution (Becton Dickinson, USA). Ten thousand lymphocytes were analysed with the FACSCalibur (Becton Dickinson) system. The absolute counts of the above-mentioned CD4$^+$ T cells were obtained using the TRUCount system (Becton Dickinson).

Culture of *M. hominis* and *U. urealyticum*

Detection of *M. hominis* and *U. urealyticum* was performed by culture according to the manufacturer's instructions (Identifying Culture Medium, Liming Co., China).

Detection of *M. genitalium, M. fermentans, M. pirum*, and *M. penetrans* by PCR DNA extraction

The saline suspensions described above were used for DNA extraction. Briefly, 0·5 ml of the suspension was centrifuged at 15 000 ***g*** for 15 min at 4 °C. The pellet was washed three times with PBS at pH 7·3 and then

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Table 1. *Primers used in this study*

	Primers (5′→3′)	Product sizes
Nested primer	Primer 1: 5′-GAGTTTGATCCTGGCTCACG-3′ Primer 2: 5′-ATTACCGCGGCTGCTGGCAG-3′	535 bp
M. genitalium	Primer 3: 5′-GCCATATCAGCTAGTTGGT-3′ Primer 4: 5′-CTCCAGCCATTGCCTGCTA-3′	281 bp
M. penetrans	Primer 5: 5′-CATGCAAGTCGGACGAAGCA-3′ Primer 6: 5′-AGCATTTCCTCTTCTTACAG-3′	410 bp
M. pirum	Primer 7: 5′-ATACATGCAAGTCGATCGGA-3′ Primer 8: 5′-ACCCTCATCCTATAGCGGTC-3′	180 bp
M. fermentans (IS sequence)	RW004: 5′-GGACTATTGTCTAAACAATTTCCC-3′ RW005: 5′-GGTTATTCGATTTCTAAATCGCCT-3′	206 bp

centrifuged again as described above. The sediment obtained was re-suspended in 100 μl TE buffer [10 mM Tris–HCl (pH 8·0), and 1 mM EDTA], and then lysed by the addition of 1% sodium dodecyl sulfate (SDS). The mixture was then incubated with 40 μl proteinase K (Boehringer, Germany) at 55 °C for 4 h. The samples were subsequently heated for 10 min at 95 °C to inactivate the proteinase K. The DNA extracted was quantified by the use of a nucleic acid fixed-quantity machine at 260 wavelengths. The samples were then frozen and stored at −20 °C. For PCR, 5 μl of the mixture was used.

PCR assays of *M. pirum*, *M. genitalium*, and *M. penetrans*

A nested PCR was designed for the amplication of *M. pirum*, *M. genitalium*, and *M. penetrans*. In order to improve the sensitivity and the specificity, the primers were designed against the conserved region of 16S rRNA. A two-step procedure was used, first to amplify the general genus and then to amplify the specific species. The primers are listed in Table 1.

PCR amplification was performed at a final volume of 50 μl. PCR buffer (10 mM Tris–HCl (pH 8·3), 50 mM KCl, 0·1% Triton X-100) containing 1·5 mM $MgCl_2$, 200 μM of each dNTP (Shanghai Shenergy Biocolor, China), 0·5 μM of each primer (Invitrogen Life Technologies, China), and 1 U *Taq* polymerase (Shanghai Shenergy Biocolor). Five microlitres of each sample were added to a final volume of 50 μl per reaction tube. The samples were heated at 95 °C for 2 min. This was followed by 35 cycles of denaturation at 93 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min and a final cycle of 5 min at 72 °C. Three microlitres of the first PCR reaction product were used for the second-step PCR. The second-step PCR conditions were the same as for the first step.

PCR assays of *M. fermentans*

The primers were designed against the insert sequence (IS) of *M. fermentans* (Table 1). The reaction mixture was the same as the nested PCR described above, except that the $MgCl_2$ concentration was 2·0 μM. The samples were heated at 95 °C for 2·5 min, followed by 45 cycles of denaturation at 94 °C for 35 s, annealing at 55 °C for 45 s, extension at 72 °C for 50 s and a final cycle of 5 min at 72 °C. The products were incubated at 4 °C until electrophoresis.

Detection of DNA products

Ten microlitres of the second-step PCR product and 15 μl of the 16S rRNA PCR product were electrophoresed in a 2·0% agarose gel for 45–60 min at 75 V in TAE buffer (pH 8·0). The gel was stained with ethidium bromide and the products were visualized with a UV transilluminator and photographed (UVP GDS-8000). Controls consisted of known mycoplasmas and the DNA markers were provided by Tiangen Company (China).

Rigorous precautions were taken to avoid contamination of the PCR mixture, including reagent aliquoting, the use of positive displacement pipettes, the isolation of PCR reagents and products, and the physical separation of the DNA extraction site and *Mycoplasma* culture.

Every step of the experiments included both negative and positive controls. Type strains of *U. urealyticum* (ATCC 27618), *M. penetrans* (ATCC 55252),

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Table 2. Mycoplasma *prevalence in urine by age*

Age (yr)	No.	Uu	Mh	Mg	Mf	Mpe	Mpi
<20	10	3 (30·0)	4 (40·0)	0	0	0	3 (30·0)
20–34	193	46 (23·8)	54 (28·0)	40 (20·7)	15 (7·8)	1 (0·5)	23 (11·9)
35–49	220	66 (30·0)	64 (29·1)	83 (37·7)	12 (5·5)	6 (2·7)	38 (17·3)
⩾50	74	20 (27·0)	23 (31·1)	22 (29·7)	6 (8·1)	1 (1·4)	17 (23·0)

Uu, *Ureaplasma urealyticum*; Mh, *M. hominis*; Mg, *M. genitalium*; Mf, *M. fermentans*; Mpe, *M. penetrans*; Mpi, *M. pirum*.
$\chi^2_{Mg} = 19{\cdot}923$, $P = 0{\cdot}000$.

Table 3. Mycoplasma *prevalence in urine by mode of transmission* (%)

Transmission category	No.	Uu	Mh	Mg	Mf	Mpe	Mpi
Homosexual contact	144	41 (28·5)	49 (34·0)	34 (23·6)	11 (7·6)	7 (4·9)	19 (13·2)
Heterosexual contact	207	53 (25·6)	55 (26·6)	70 (33·8)	12 (5·8)	0	30 (14·5)
Injecting drug use	53	12 (22·6)	17 (32·1)	12 (22·6)	5 (9·4)	1 (1·9)	5 (9·4)
Others	93	29 (31·2)	24 (25·8)	29 (31·2)	5 (5·4)	0	27 (29·0)

For abbreviations see Table 2.
$\chi^2_{Mh} = 9{\cdot}754$, $P = 0{\cdot}021$; $\chi^2_{Mpe} = 14{\cdot}546$, $P = 0{\cdot}002$; $\chi^2_{Mpi} = 14{\cdot}397$, $P = 0{\cdot}002$.

M. hominis (ATCC 23114), *M. fermentans* (incog.), *M. genitalium* G37 (ATCC 33530), and *M. pirum* (ATCC 25960), were kindly provided by Statens Seruminstitut (Denmark).

Statistical analysis

Statistical analysis was performed using SAS 9.0 software (SAS Institute Inc., USA). The prevalence of the mycoplasmas is presented as a percentage. Categorization variables were analySed using χ^2 test. Continuous variables were analysed by the *U* test. All reported *P* values were two-tailed, and a *P* value $<0{\cdot}05$ was considered significant.

RESULTS

Patient population

In this study, 497 HIV/AIDS patients were screened in. The age range of the patients was 5–75 years (mean age 37 years). The results obtained by culture technique and by PCR using generic primers are as follows. Other coincident sexually transmitted diseases or opportunistic infections during *Mycoplasma* infection were not diagnosed in the study patients, and there was no vaccine administration during this study period.

Table 4. *$CD4^+$ cell count in relation to the AIDS-associated mycoplasmas*

	AIDS-associated mycoplasmas	
$CD4^+$ cell (/μl)	Positive detection ($n = 222$)	Negative detection ($n = 275$)
P_{50}	333·5	393
$P_{2·5}$–$P_{97·5}$	18·7–1068·5	29·9–976·6

Prevalence of mycoplasmas in the study population

Of the 497 samples analysed, all presented positive for at least one kind of mycoplasma, with especially high infection rates for *U. urealyticum* (27·2%), *M. hominis* (29·2%) and *M. genitalium* (29·2%). The infection rates for *M. fermentans* and *M. pirum* were 6·6% and 16·3%, respectively. Eight homosexual HIV-1-infected patients were positive for *M. penetrans*.

Prevalence of mycoplasmas by age

Urine samples of males aged between 5 and 75 years were evaluated in this study. Table 2 shows the distribution of detection of mycoplasmas in different age groups. The only significant difference was in the *M. genitalium*-infected group.

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Prevalence of mycoplasmas by HIV transmission category

As shown in Table 3, six mycoplasmas were significantly different in homosexual and heterosexual contact groups. The distribution of *M. hominis*, *M. penetrans*, and *M. pirum* were significantly different in this four-transmission category.

Relationship between the detection of AIDS-associated mycoplasmas and CD4$^+$ cell count

Table 4 shows the mean values of CD4$^+$ cell counts in relation to the detection of these four mycoplasmas. The level of CD4$^+$ cells in the AIDS-associated *Mycoplasma*-positive group was lower than in the negative group ($P < 0{\cdot}01$).

DISCUSSION

This study is an extension of our previous study. The existence of six mycoplasmas in males with HIV infection was observed in our study. Therefore, it is important to clarify the role of these mycoplasmas in HIV-infected patients. However, the prevalence of mycoplasmas in HIV-infected male patients reported in the literature is different according to the studied population and the qualitative or quantitative evaluation of the specimens [11, 12]. In the present study, prevalence of *M. hominis* was higher than *U. urealyticum* (29·2 *vs.* 27·2%) but of no statistical significance. This finding differs from our previous study. No difference in *Mycoplasma* prevalence was observed in the different age groups, with the exception of the *M. genitalium*-infected group (Table 2).

Compared to our previous study [13], the prevalence of these six mycoplasmas was higher than that in STD clinic attendees and healthy subjects. This may be further indication that mycoplasmas are co-factors in HIV disease progression. Immunodeficiency associated with HIV could be a factor of predisposition for *Mycoplasma* infection [14]. One of the objectives of this study was to re-examine the presence of these six mycoplasmas in the urine of male patients; we confirmed the existence of these pathogens and, in particular, we found a higher infection rate of *Mycoplasma genitalium* than da Costa *et al.* [14], while another study conducted by Soni *et al.* [15] showed a similar infection rate of mycoplasmas as our study. Although we did not find any valuable results like age tendency or different modes of transmission, discussion remains necessary before the role of these mycoplasmas in the progression of HIV infection becomes clear. In humans, the association of mycoplasmas with HIV was supported by its significantly more frequent presence in patients with HIV infection [5]. However, whether these mycoplasmas act as co-factors in HIV progression cannot be concluded from this cross-sectional study; further studies need to be designed.

Concerning CD4$^+$ cell changes between the AIDS-associated *Mycoplasma*-positive and -negative groups, *Mycoplasma* infection was clearly associated with a decrease in CD4$^+$ cell count. As the CD4$^+$ cell is the target cell of HIV, this result may suggest that *Mycoplasma* infection is related to immune status as expected.

We acknowledge that our study has certain limitations, such as its cross-sectional design and the lack of a control group of *Mycoplasma*-infected patients not infected with HIV. However, our study is the only study to analyse the association between HIV and *Mycoplasma* infection. In summary, more molecular and epidemiological studies with a larger number of samples are needed to identify the relationship between HIV and *Mycoplasma* infection.

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DECLARATION OF INTEREST

None.

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