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Epidemiology of *Mycoplasma* acquisition in male HIV-1 infected patients: a multistage cross-sectional survey in Jiangsu, China

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SUMMARY

Mycoplasma infections are most frequently associated with disease in the urogenital or respiratory tracts and, in most cases, mycoplasmas infect the host persistently. In HIV-infected individuals the prevalence and role of genital mycoplasmas has not been well studied. To investigate the six species of *Mycoplasma* and the risk factors for infection in Jiangsu province, first-void urine and venous blood samples were collected and epidemiological questionnaires were administered after informed consent. A total of 1541 HIV/AIDS patients were recruited in this study. The overall infection rates of six *Mycoplasma* species were: *Ureaplasma urealyticum* (26·7%), *Mycoplasma hominis* (25·3%), *M. fermentans* (5·1%), *M. genitalium* (20·1%), *M. penetrans* (1·6%) and *M. pirum* (15·4%). The *Mycoplasma* infection rate in the unmarried group was lower than that of the married, divorced and widowed groups [adjusted odds ratio (aOR) 1·432, 95% confidence interval (CI) 1·077–1·904, $P < 0·05$]. The patients who refused highly active antiretroviral therapy (HAART) had a much higher risk of *Mucoplasma* infection (aOR 1·357, 95% CI 1·097–1·679, $P < 0·05$). Otherwise, a high $CD4^+$ T cell count was a protective factor against *Mycoplasma* infection (aOR 0·576, 95% CI 0·460–0·719, $P < 0·05$). Further research will be required to confirm a causal relationship and to identify risk factors for *Mycoplasma* infection in HIV/AIDS populations.

Key words: HIV, mycoplasmas, 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. Human immunodeficiency virus (HIV) is recognized as the aetiological agent of acquired immune-deficiency syndrome (AIDS). However, the progression of AIDS is highly variable in different individuals, and several factors, such as viral strains or host factors, have been attributed as the possible cause of such variations [1]. Infectious agents, including various viruses, parasites and bacteria, are considered to be co-factors in the progression of AIDS [2–5]. Sexually transmitted infections (STIs) are thought

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to increase the infectiousness of HIV-1-seropositive individuals by recruiting HIV-1-infected lymphocytes and macrophages to genital mucosal sites and by disrupting epithelial integrity [6].

Mycoplasma is a bacterial group with a morphologically small shape, possessing a reduced small genome. Some species of *Mycoplasma* cause STI associated with reproductive tract syndromes in both men and women. *Mycoplasma* infections are most frequently associated with diseases of the urogenital or respiratory tracts and, in most cases, mycoplasmas infect the host persistently [7]. This facultative-pathogenic cell wall-less bacterium is found as a commensal in the urogenital tract of sexually active people [8]. A great deal of effort has been devoted to understanding the role of AIDS-associated mycoplasmas in recent years [9]. At least 16 species of mycoplasmas from humans and their major colonization sites, including oropharynx, upper respiratory tract, and genitourinary tract, have been isolated and considered to be of human origin [10]. As a conditional pathogenic organism, it associates with various diseases, including pneumonia, arthritis, meningitis and chronic urogenital tract disease [8]. Although the target cells of mycoplasmas have yet to be defined, significant associations exist between mycoplasmas and sexually transmitted diseases, especially *Mycoplasma genitalium* infection and male non-gonococcal urethritis (NGU) [11].

Several *Mycoplasma* species have been detected in the human respiratory and urogenital tracts [12]. The species most frequently isolated from the urogenital tract of sexually active adults are *M. hominis* and *Ureaplasma urealyticum* [13], although they have been considered responsible for genital diseases, infertility and obstetric complications [14, 15], not all colonized patients show signs and symptoms of disease. *M. genitalium* is an emerging cause of male and female urogenital inflammation as it has been implicated in male urethritis [11] and can persist for extended periods of time at urogenital sites including the endocervix [16–18]. The presence of *M. fermentans* in AIDS patients was reported in 1989 [19]. *M. fermentans* and other two mycoplasmas (*M. penetrans* and *M. pirum*) were proposed as co-factors of HIV for transmission and progression of virulence [1, 20]. However, mechanistic evidence for establishment and persistence of infection remains scarce. Importantly, the host's innate and adaptive immune responses are largely unknown and serve as impediments to our understanding of the pathogenesis of mycoplasmas.

For many pathogenic mycoplasmas, a first step in causing disease is adherence to host cells, often by adhesions associated with a polarized organelle [21] in species such as *M. genitalium, M. penetrans* and *M. pirum*. For the last 20 years studies have been interested in the characterization of pathogenic factors of mycoplasmas. Consistent with the ability to survive long-term in urogenital tissues [17, 22], and similar to other sexually acquired urogenital pathogens, it is hypothesized that mycoplasmas have evolved specific mechanisms to evade the host's immune system [18, 23] and have been associated with chronic symptoms. The potential of *M. fermentans* to interact with the immune system has been intensively investigated and the molecular basis of *M. fermentans* as an immunomodulatory agent has been reviewed [24, 25]. Previous researches have implicated that lipoproteins are mitogenic [26] and stimulate transcription of the HIV genome in HIV-infected cells *in vitro* via Toll-like receptors, suggesting that *M. penetrans* may play a role in expediting the progression of AIDS [1].

Although prevalence of AIDS-associated mycoplasmas, e.g. *M. fermentans, M. genitalium, M. penetrans* and *M. pirum*, has been discussed on numerous occasions, there is still a paucity of good epidemiological data on their frequency and potentially pathogenic roles. Whether this association is causal or coincidental needs further evaluation [27].

In HIV-infected individuals the prevalence and role of genital mycoplasmas has not been well studied. The potential for increased HIV transmission in the presence of other STIs has been observed in several studies [28–30], but less is known about the influence of *Mycoplasma* infections in HIV/AIDS patients. *M. genitalium* has been shown to increase HIV replication in peripheral blood mononuclear cells *in vitro* [31]. Considering the clinical associations of mycoplasmas with urogenital inflammation, it remains imperative to investigate the basic mechanisms of mucosal infection and disease induced by this organism [32]. Therefore, the objectives of this study were to determine the population prevalence of six *Mycoplasma* species and their risk factors associated with infection.

MATERIALS AND METHODS

Cross-sectional surveys design and procedure

The study consisted of four consecutive cross-sectional surveys of HIV-1-infected males between 2009 and 2011 at 8-month intervals. Patients were eligible for

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enrolment in the study if they were aged >18 years. The study was conducted in Jiangsu province; 13 cities of Jiangsu province were selected for this study and part details of the baseline study have been described previously [33].

This cross-sectional study focused on the relationship between HIV-1 infection and persistent infection of mycoplasmas. Comparisons were made between subgroups of patients according to demographic characteristics.

Patient recruitment and samples preparation

Between 2009 and 2011 male HIV/AIDS patients from Jiangsu province enrolled in a population-based cross-sectional study to assess the association between *Mycoplasma* infections and HIV-1 acquisition. A total of 1541 men were recruited from the Jiangsu Provincial Center for Disease Prevention and Control. Part details of the first survey have been published previously [33]. After informed consent was obtained, the patients were asked to provide blood and first-void urine samples and were interviewed by outreach workers, using a structured questionnaire consisting of questions related to demographic characteristics. Demographic information, behavioural and STI data were collected at each visit. First void of urine was collected for testing of mycoplasmas. Immediately after collection urine was placed in 1 ml Amplicor transport medium, with 1 ml diluents added and stored at −5 °C, the samples were frozen at −20 °C within 48 h of collection. Five millilitres of venous blood was collected in anticoagulant tubes for $CD4^+$ T cell counts.

Detection of *U. urealyticum, M. hominis, M. fermentans, M. genitalium, M. penetrans*, and *M. pirum* by polymerase chain reaction (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 centrifuged again as described. The sediment obtained was resuspended in 100 μl TE buffer (10 mM Tris hydrochloride, 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.

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

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

The 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, China), 0·5 μM of each primer (Invitrogen Life Technologies, China), and 1 U *Taq* polymerase (Shanghai shenergy). Five microlitres of each sample were added, to a final volume of 50 μl per reaction tube. The samples were heated at 94 °C for 2 min. This was followed by 35 cycles of denaturation at 94 °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 products were used for the second-step PCR. The second-step PCR conditions were the same as for the first step.

PCR assays for U. urealyticum, M. hominis *and* M. fermentans

The primers were designed against the insert sequence (IS) of *M. fermentans* (Table 1). The reaction mixture was the same as for the nested PCR described above, except that the concentration of $MgCl_2$ was 2·0 μM. The samples were heated at 94 °C for 2·5 min, followed by 35 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 gene 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

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Table 1. *Primers used in this study*

	Primers (5′→3′)	Product size
U. urealyticum	Primer 1: 5'-CAG ATA CAT TAA ACG AAG CAG G-3' Primer 2: 5'-GTG GTG ACA TAC CAT TAA C-3'	225 bp
M. hominis	Primer 1: 5'-CAA TGG CTA ATG CCG GAT ACG C-3' Primer 2: 5'-GGT ACC GTA AGT CTG CAA T-3'	334 bp
Genus-specific 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

(UVP GDS-8000). Controls consisted of known mycoplasmas and the DNA marker was 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.

Every step of the experiments included both negative and positive controls. Type strains of *U. urealyticum* (ATCC 27 618), *M. hominis* (ATCC23114), *M. fermentans* (*incognitus*), *M. genitalium* (G37), *M. penetrans* (ATCC55252), and *M. pirum* (ATCC25960), were kindly provided by SSI (Denmark).

$CD4^+$ T cell counts

Absolute $CD4^+$ T cell counts were determined in EDTA-treated peripheral blood samples by using an Ortho Cytoron Absolute flow cytometer (Ortho Diagnostic Systems Inc., USA) and anti-CD4-FITC monoclonal antibodies (Ortho Diagnostic Systems 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). A total of 10 000 lymphocytes were analysed on FACSCalibur (Becton Dickinson) equipment. The absolute counts of the above-mentioned $CD4^+$ T cells were obtained using the TRUCount system (Becton Dickinson).

Statistical considerations

Statistical analysis was performed using SAS v. 9.0 software (SAS Institute Inc., USA). The crude prevalence rates of these mycoplasmas were calculated for the participating male HIV-1 patients in this study. Multiple logistic regression analyses were used to adjust the estimates of the temporal changes of the prevalence of mycoplasmas. The results are presented as adjusted odds ratios (aORs) with 95% confidence intervals (CIs). Categorized variables were analysed using χ^2 test. All reported P values were two-tailed, and a P value <0·05 was considered significant.

Ethical approval

Verbal or written consent were obtained from all participants. The ethical protocol was approved by the Jiangsu Provincial Center for Disease Prevention and Control Ethics committee.

RESULTS

Demographic and behavioural characteristics of HIV/AIDS patients

Of the 1541 eligible male patients who participated in this study, 851 (55·2%) were at HIV infection status and 690 (44·8%) had progressed to AIDS. The mean and the median ages were 39·20 (S.D. = 11·28) years and 39·14 (range 18–70) years, respectively. More than half of the patients were aged <40 years, and 54·0% of participants had an education level of <9 years. More than half (51·1%) of the participants

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Table 2. *Characteristics of the respondents in the repeated cross-sectional study, 2009–2011*

Characteristics	No.	%
Education		
⩽9 years	832	54·0
10–12 years	442	28·7
>12 years	267	17·3
Marriage status		
Unmarried	469	30·4
Married/divorced/widowed	1072	69·6
HIV infection mode		
Homosexual	623	40·4
Heterosexual	668	43·3
Other	250	16·2
History of STIs (except HIV)		
Yes	271	17·6
No	1270	82·4
History of HAART		
Yes	700	45·4
No	841	54·6
Disease progression		
HIV	851	55·2
AIDS	690	44·8

STI, Sexually transmitted infection; HAART, highly active antiretroviral *therapy*.

had a CD4$^+$ T cell count <350/μl, and 45·4% of the patients were receiving highly active antiretroviral therapy (HAART). Of all the recruited patients, 40·4% and 43·3% became infected with HIV via homosexual or heterosexual interactions, respectively, while 17·6% had other STIs except HIV and mycoplasmas. Other details of demographic and behavioural characteristics are given in Table 2.

Overall prevalence of mycoplasmas in the cross-sectional study, 2009–2011

The overall infection rates of the six *Mycoplasma* species were: *U. urealyticum* (26·7%), *M. hominis* (25·3%), *M. fermentans* (5·1%), *M. genitalium* (20·1%), *M. penetrans* (1·6%) and *M. pirum* (15·4%). The infection rates of *Mycoplasma* classified by CD4$^+$ T cell count in this cross-sectional study are presented in Table 3. The group with CD4$^+$ T cell counts ⩽350 had a higher infection rate of *M. genitalium* ($\chi^2 = 17{\cdot}750$, $P < 0{\cdot}001$) and *M. pirum* ($\chi^2 = 16{\cdot}173$, $P < 0{\cdot}001$).

The risk factors of AIDS-associated *Mycoplasma* infections in patients

Three *Mycoplasma* species, i.e. *M. fermentans, M. penetrans* and *M. pirum*, have been named AIDS-associated mycoplasmas since 1994 [20]. For the high infection rate of *M. genitalium* in male HIV/AIDS patients, we added this to the above three species to calculate the risk factors of AIDS-associated *Mycoplasma* infections between them. Table 4 analyses the relationship between AIDS-associated *Mycoplasma* infections and some sociodemographic data. *Mycoplasma* infection rates in the unmarried group was lower than that in the married, divorced and widowed groups (aOR 1·432, 95% CI 1·077–1·904, $P < 0{\cdot}05$). The patients who refused HAART had a higher risk of being infected with these mycoplasmal species (aOR 1·357, 95% CI 1·097–1·679, $P < 0{\cdot}05$). The infection rate of AIDS-associated mycoplasmas was significantly high in the group with CD4$^+$ T cell count ⩽350 (aOR 0·576, 95% CI 0·460–0·719, $P < 0{\cdot}05$).

Table 3. Mycoplasma *infection rates by CD4$^+$ T cells in the cross-sectional study, 2009–2011*

	CD4$^+$ ⩽350		CD4$^+$>350			
	N	(%)	*n*	(%)	χ^2	*P*
U. urealyticum	202	(26·1)	210	(27·4)	0·359	0·549
M. hominis	197	(25·4)	193	(25·2)	0·010	0·920
M. genitalium	202	(24·0)	107	(15·3)	17·750	<0·001*
M. fermentans	42	(5·0)	36	(5·2)	0·024	0·876
M. penetrans	14	(1·7)	10	(1·4)	0·130	0·719
M. pirum	158	(18·7)	79	(11·3)	16·173	<0·001*

* $P < 0.05$.

DISCUSSION

Mycoplasma infections are not currently a part of standard STI screening programmes in China and in the absence of rapid diagnostic tests, mycoplasmas would be treated syndromically. This is the first study to longitudinally assess *Mycoplasma* infections in male HIV/AIDS patients in Jiangsu, China. The overall infection rates of six *Mycoplasma* species were: *U. urealyticum* (26·7%), *M. hominis* (25·3%), *M. fermentans* (5·1%), *M. genitalium* (20·1%), *M. penetrans* (1·6%) and *M. pirum* (15·4%), *U. urealyticum* co-infection with *M. hominis* is common in this study. The co-infection of *U. urealyticum* and *M. hominis* was higher than in gynaecological outpatients referred for occasional or routine gynaecological examinations [12]. The infection rate of *M. genitalium* in the current study was higher than in a STI clinic female population [34] and in

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Table 4. *Logistic regression analysis of relationship between AIDS-associated* Mycoplasma *infections and sociodemographic data*

Covariates	Infection rate	Crude OR (95% CI)	Adjusted OR (95% CI)
Age group, years			
⩽29	155 (34·9)	–	–
30–39	165 (32·6)	0·902 (0·689–1·181)	0·774 (0·570–1·050)
40–49	195 (36·9)	1·089 (0·837–1·416)	0·880 (0·629–1·231)
⩾50	121 (33·5)	0·940 (0·701–1·260)	0·782 (0·537–1·139)
Education			
⩽9 years	349 (34·4)	–	–
10–12 years	179 (34·4)	0·997 (0·798–1·246)	1·076 (0·853–1·359)
>12 years	108 (35·4)	1·045 (0·799–1·366)	1·199 (0·892–1·614)
Marriage status			
Unmarried	178 (31·4)	–	–
Married/divorced/widowed	458 (36·0)	1·228 (0·994–1·517)	1·432 (1·077–1·904)*
HIV infection mode			
Homosexual	237 (33·2)	–	–
Heterosexual	271 (33·4)	1·010 (0·816–1·250)	0·990 (0·787–1·246)
Other	128 (40·6)	1·378 (1·048–1·811)	1·328 (0·985–1·791)
History of STIs (except HIV)			
Yes	112 (34·1)	–	–
No	524 (34·7)	1·023 (0·795–1·315)	0·928 (0·713–1·208)
History of HAART			
Yes	278 (31·3)	–	–
No	358 (37·6)	1·322 (1·090–1·604)	1·357 (1·097–1·679)*
Disease progression			
HIV	355 (35·7)	–	–
AIDS	281 (33·2)	0·895 (0·738–1·086)	0·920 (0·749–1·130)
$CD4^+$ group			
⩽350	350 (41·5)	–	–
>350	193 (27·7)	0·538 (0·434–0·668)	0·576 (0·460–0·719)*

OR, Odds ratio; CI, confidence interval; STI, Sexually transmitted infection; HAART, highly active antiretroviral therapy.
* $P < 0·05$.

HIV-positive women [27]. As HIV shedding is associated with high *M. genitalium* organism burden, *M. genitalium* infection may facilitate HIV transmission [35]. However, *M. fermentans* and *M. penetrans* were described at lower rates in the present study compared to other studies [36].

Immunodeficiency associated with HIV could be a factor predisposing to *Mycoplasma* infections [37]. Of the 1541 participants, more than half of the patients were aged <40 years, and 55·1% of the participants had an education level of <9 years. The logistic regression in this study analysed the relationship between AIDS-associated *Mycoplasma* infections and some sociodemographic data. *Mycoplasma* infection rates in the unmarried group were lower than in the married, divorced and widowed groups. Some of the patients had homosexual practices, perhaps indicating a risk factor for the spread of infection [38, 39]. Patients who refused HAART had a much higher risk of mycoplasmal infection. Further, a high $CD4^+$ T cell count was a protective factor against *Mycoplasma* infection. Studying the result that revealed the relationship between $CD4^+$ T cell counts and *Mycoplasma* infections, indicated that HIV/AIDS patients with low $CD4^+$ T cell counts were more easily infected with *M. genitalium* and *M. pirum*. Thus regular treatment with HAART and stable $CD4^+$ T cell counts >350 seem vital to these populations. Further research on the prevalence and natural history of mycoplasmas is required, but given its high prevalence in these populations, inclusion of *M. genitalium* as part of routine syndromic management may be worth considering. This would, however, require revision to current guidelines.

The present study showed an increased susceptibility to *Mycoplasma* infections in male HIV/AIDS patients. The limitations of our main study include its cross-sectional design. We will focus on prospective

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and cohort designs to assess genital *Mycoplasma* infections and how they work in HIV progression in future studies. Further research will be required to confirm a causal relationship and to identify risk factors for *Mycoplasma* infections in HIV/AIDS populations; and to assess the clinical significance on HIV transmission, impact on HIV viral load and its use in the clinical practice setting. If findings from this research are confirmed, *Mycoplasma* screening and treatment in these patients may be warranted.

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DECLARATION OF INTEREST

None.

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