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BRIEF REPORT

Absence of *Pneumocystis jirovecii* Colonization in Human Immunodeficiency Virus-Infected Individuals With and Without Airway Obstruction and With Undetectable Viral Load

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Pneumocystis jirovecii colonization has been associated with non-acquired immune deficiency syndrome (AIDS) pulmonary comorbidity. We used spirometry to measure pulmonary function and analyzed oral wash specimens by quantitative polymerase chain reaction (PCR), targeting the large mitochondrial ribosomal subunit. For sensitivity control, a blinded subsample was subjected to touch-down PCRs, targeting both large and small ribosomal subunits and the major surface glycoprotein. *Pneumocystis jirovecii* deoxyribonucleic acid (DNA) was detected in 1 of 156 (95% confidence interval, .1%–3.5%) virologically suppressed human immunodeficiency virus (HIV)-infected individuals confirmed by all PCR methods. Thus, prevalence of *P jirovecii* colonization was low and unlikely to be a major cause of pulmonary comorbidity in this group of well treated HIV-infected individuals.

Keywords. airway obstruction; HIV-1; Pneumocystis; colonization.

With the widespread use of combination antiretroviral therapy (cART), the incidence of *Pneumocystis* pneumonia (PCP) has decreased [1], albeit PCP remains a common acquired immune deficiency syndrome (AIDS)-defining opportunistic infection [2]. Moreover, colonization with *P jirovecii* has been linked to non-AIDS pulmonary comorbidity in human immunodeficiency virus (HIV)-infected individuals in the cART era [3].

Chronic obstructive pulmonary disease (COPD) is more prevalent in individuals infected with HIV compared with uninfected individuals [4], but the underlying mechanisms are unclear. In the pre-ART era, PCP was recognized as a risk factor for subsequent airway obstruction [5]. Furthermore, evidence from the early cART era suggested that *P jirovecii* colonization may be an independent risk factor for HIV-associated COPD [6]. However, studies of *P jirovecii* colonization were unable to determine causality.

The prevalence of *P jirovecii* colonization has been investigated in several HIV-infected and uninfected populations. In the early cART era, the prevalence of colonization varied from 10% to 70% depending on the population studied [3]. However, most studies included HIV-infected individuals with profound immunodeficiency, respiratory symptoms, or AIDS [3].

In this study, we determined the prevalence of *P jirovecii* colonization in a cohort of cART-treated HIV-infected individuals with and without airway obstruction using 2 independent polymerase chain reaction (PCR) methods and spirometry. We hypothesized that *P jirovecii* colonization would be detectable even in well treated individuals and a higher prevalence would be found in those with COPD.

METHODS

Patients

This study was approved by the Committee on Health Research Ethics of the Capital Region of Denmark (protocol no. H-8-2014-004) and performed in accordance with the Helsinki Declaration. Oral and written informed consent was obtained from all patients before participation. The study was registered at clinicaltrials.gov (NCT02382822) as part of the ongoing Copenhagen Comorbidity Study in HIV infection. We initially planned to study colonization longitudinally, but, due to the results, only a cross-sectional was performed. Inclusion period was from May to October 2015. All participants were recruited from the University of Copenhagen outpatient clinics at Rigshospitalet and Hvidovre Hospital.

Data Collection

Information about HIV-related variables including CD4 T-cell count, HIV viral load, previous PCP, intravenous drug use, and coinfection with hepatitis C virus were retrieved from patient records. All participants completed a questionnaire

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that included information on tobacco exposure and use of inhaled medication.

Spirometry

Spirometry was performed using an EasyOne ultrasonic spirometer (ndd Medizintechnik, Zürich, Switzerland) in accordance with American Thoracic Society (ATS)/European Respiratory Society (ERS) guidelines [7]. For bronchodilation (in patients with a ratio between forced expiratory volume in 1 second and forced vital capacity [FEV_1/FVC] <0.7), 400 µg of salbutamol was given by inhalation (Ventoline Diskus; Glaxo Smith Kline, Middlesex, United Kingdom). Airway obstruction was defined according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria for COPD and defined as a post-bronchodilator FEV_1/FVC <70% [8]. Chronic obstructive pulmonary disease was also assessed by the lower limit of normal (LLN) (ie, fifth percentile) of the FEV_1/FVC ratio according to ATS/ERS recommendations using Global Lung Function Initiative prediction equations [9].

Quantitative Real-Time Polymerase Chain Reaction (PCR) and Touch-Down PCRs

Oral wash was collected after gargling 10 mL saline (9 mg/mL) for 1 minute. Samples were either analyzed on the same day or stored at 5°C overnight before analysis. First, 500 µL sample was digested with 21 µL proteinase K (Roche, Basel, Switzerland) at 56°C for 30 minutes followed by PCR. For the quantitative PCR (qPCR), sensitivity was based on the use of 0.5 mL oral wash sample that was vortexed to secure equal distribution of cells but not further processed before proteinase K treatment. Using an in-house protocol and the BD MAX Platform (Becton-Dickinson, Durham, NC), *P jirovecii* deoxyribonucleic acid (DNA) was determined by qPCR targeting the large subunit of mitochondrial (mtLSU) ribosomal ribonucleic acid (rRNA) (Supplementary Material 1). Blinded for previous results, one quarter of the samples (N = 39) were also tested for *P jirovecii* DNA in a reference laboratory (Department of Microbiology and Infection Control, Statens Serum Institut) using touch-down PCRs targeting mtLSU rRNA, mitochondrial small subunit rRNA, and the major surface glycoprotein (MSG) (see Supplementary Material 1). Both PCR tests were done on the clinical sample material that had been stored at −20°C for additional testing, thus DNA extraction was repeated for the repeat test.

Data Analysis

Comparison of individuals with airway obstruction versus those without airway obstruction were done using 2-sample Student's *t* tests or Mann-Whitney *U* test for continuous data, as appropriate. Fisher's exact test was used for categorical data. The 95% binomial confidence intervals (CIs) for colonization prevalence were calculated based on the Wilson method using the "binom" package in R. The statistical software package R (V.3.2.0) was used [10].

RESULTS

One hundred fifty-eight participants were recruited, including 44 with airway obstruction and 114 without airway obstruction. Two participants were not receiving cART and omitted from further data analysis (Table 1). Both were negative for *P jirovecii* DNA. Furthermore, 2 participants had detectable HIV viral replication (222 and 223 copies/mL) corresponding to a viremic bleep and recently initiated cART, respectively, and both had fully suppressed viral replication upon follow up. All remaining participants had undetectable HIV viral load. HIV-infected individuals with airway obstruction were older ($P < .001$) and had higher cumulative smoking ($P < .001$), current smoking ($P < .001$), and inhaled corticosteroid use ($P = .03$). The groups were similar with regard to sex distribution, ethnicity, current $CD4^+$ T-cell count, CD4 nadir, and cART use. Most of the participants with airway obstruction had either mild or moderate airway obstruction, and only 5 were classified with severe airway obstruction (FEV_1 30%–49% of predicted) (Table 2). Consequently, 42% of those with airway obstruction, according to the GOLD fixed ratio criteria, were identified as obstructed using the LLN criteria. Participants with airway obstruction also had lower FVC compared with individuals without airway obstruction (FVC 4.0 vs 4.5, $P < .01$) (Table 1).

qPCR on oral wash was carried out for every participant. No tests showed inhibition. *P jirovecii* DNA was detected in only 1 of the 156 participants (0.6%; binomial 95% CI, .1–3.5). This individual had no respiratory complaints (ie, dyspnoea, wheezing,

Table 1. Clinical Characteristics of Study Participants[a]

Clinical Features	HIV-Infected With Airway Obstruction	HIV-Infected Without Airway Obstruction	*P* Value
N	44	112	
Age, y (SD)	58.9 (9.28)	52.2 (12.0)	<.001
Male, n (%)	37 (84.1)	102 (91.1)	.25
Ethnicity (white), n (%)	43 (97.7)	108 (96.4)	1.0
Duration of HIV infection, y (SD)	19.8 (8.1)	14.2 (8.5)	<.001
Prior clinical AIDS	9 (20.5)	19 (17.0)	.47
Current CD4, cells/µL (SD)	746 (303)	720 (264)	.58
CD4 nadir, cells/µL (IQR)	177 (94–336)	260 (120–383)	.20
Undetectable viral load, n (%)	44 (100)	110 (98.2)	1.0
HCV coinfection, n (%)	4 (9.0)	4 (3.5)	.22
Current IDU, n (%)	0 (0)	1 (0.9)	1.0
Current smoking, n (%)	20 (48.8)	21 (19.3)	<.001
Cumulative pack years, y (IQR)[b]	24.0 (12.5–35)	4.0 (0–17.0)	<.001
Inhaled corticosteroid use, n (%)	5 (11.4)	3 (2.7)	.03
Previous PCP, n (%)	5 (11.4)	6 (5.3)	.7

Abbreviations: AIDS, acquired immune deficiency syndrome; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IDU, intravenous drug use; IQR, interquartile range; PCP, *Pneumocystis* pneumonia; SD, standard deviation.

[a] Characteristics were summarized using mean (SD) or median (IQR or range). *P* values were calculated using Student's *t* tests or Mann-Whitney *U* tests, as appropriate, or Fisher's exact test.

[b] Cumulative pack years were calculated for entire cohort.

Table 2. Pulmonary Function of Study Participants[a]

Pulmonary Function and Colonization	HIV-Infected With Airway Obstruction	HIV-Infected Without Airway Obstruction	*P* Value
N	44	112	
FEV_1, l (SD)	2.6 (0.8)	3.5 (0.7)	<.001
FEV_1, percent predicted (SD)	72.9 (19.5)	91.9 (11.6)	<.001
FVC, l (SD)	4.0 (1.1)	4.5 (0.8)	<.01
FVC, per cent predicted (SD)	88.4 (19.8)	92.7 (10.9)	.174
FEV_1/FVC ratio (SD)	63.2 (7.5)	78.2 (6.3)	<.001
GOLD Stage			
$FEV_1 \geq 80\%$ predicted	15 (34.1)	98 (87.5)	<.001
FEV_1 50%–79% predicted	23 (52.3)	14 (12.5)	
FEV_1 30%–49% predicted	4 (9.0)	0 (0)	
FEV_1 <30 predicted	2 (4.5)	0 (0)	
Pneumocystis jirovecii colonization, n (%)	0 (0)	1 (0.9)	1

Abbreviations: FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; SD, standard deviation.

The FEV_1 percent predicted was calculated using Global Lung Function Initiative prediction equations.

or coughing) nor any other clinical or immunologic evidence consistent with PCP. For confirmation of qPCR results, frozen samples from patients with and without airway obstruction (16 and 23 random samples, respectively) were subsequently assessed by conventional PCR blinded to previous results. In this analysis, the same individual was identified as being colonized, and all others were negative for *P jirovecii* DNA.

Seventy-three participants had 1 or more potential risk factors for colonization including airway obstruction (28%), current smoking (26%), previous PCP (7%), inhaled corticosteroid use (5%), or current CD4 count <200 cells/μL (2%).

DISCUSSION

Despite the presence of potential risk factors for colonization, the prevalence of *P jirovecii* colonization was surprisingly low in this study population of virologically suppressed HIV-infected individuals and not related to airway obstruction.

Contrary to its relationship with PCP, the association between immune function and *P jirovecii* colonization is unclear. A previous study in 30 HIV-uninfected individuals was not able to detect *P jirovecii*, but it detected the fungus in 8 of 50 uninfected individuals with COPD [11]. One study found that low CD4 count was associated with colonization [12], whereas CD4 was not a risk factor in another study [13]. We tested HIV-infected individuals on cART with undetectable viral replication and high CD4 counts, which may explain the low prevalence in our study compared with previous studies [3].

Other possible explanations for the low prevalence in this study may include geographic area. Thus, one study found city of residence to be a risk factor for colonization [13]. In addition, seasonal variation may explain differences in colonization prevalence, because PCP has been associated with higher mean temperatures [14]. Although we assessed colonization from a restricted geographical area between May and October, it seems unlikely that these factors alone explain the low prevalence.

Another explanation for the low prevalence may include the methods applied. Other studies assessed *P jirovecii* colonization using autopsies, bronchoalveolar lavage (BAL) fluid, induced sputum, or oral wash specimens [3]. Furthermore, several different methods have been used including conventional PCR, real-time, or nested PCR. Oral wash had 88% sensitivity and 85% specificity for PCP using qPCR targeting the MSG and direct microscopy on BAL or induced sputum as the gold standard [15]. A nested PCR may yield a better sensitivity, but, on the other hand, it may not take into account false-positive results and may amplify a potential contamination. Quantitative PCR and nested PCR have been compared for diagnosis of PCP showing similar sensitivity (94% vs 94%) and higher specificity for qPCR (94% vs 81%) [16]. However, diagnostic performances for PCP may not be applicable for colonization. To minimize the risk of underestimating the prevalence due to methodological errors, we used both qPCR and conventional PCR. Importantly, identical results were obtained using both of these methods. Because the low prevalence of *P jirovicii* positives was unexpected, a subset of the specimens was re-examined in a reference laboratory where 3 independent targets were detected as part of an accredited analysis. We initially re-examined one quarter of the samples. Because these results were identical to the qPCR results, we found it unnecessary to re-examine all samples.

Pneumocystis jirovecii colonization has been associated with various non-AIDS pulmonary conditions, ie, COPD and interstitial lung disease, although causal relationships have not been established [3]. Several studies have suggested a higher incidence of COPD in HIV-infected individuals than in uninfected individuals [4], but the underlying mechanism(s) have not been established. We initially planned a longitudinal study aimed at studying a causal relationship between *P jirovecii* colonization and development of airway obstruction. Based on the low prevalence of colonization, we were not able to carry out such a study.

The present study has strengths and limitations. To our knowledge, we have conducted the largest population study of *P jirovecii* colonization in individuals infected with HIV, and 2 different methods were used to detect colonization. Due to ethical considerations, only oral washings, and not samples from the lower airways, were sampled. The lack of lower respiratory tract sample may have compromised the ability to detect *P jirovecii*. However, previous studies found high detection rate in oral washes of HIV-infected patients [6]. Finally, the study was restricted to a limited geographical area, and it may not be representative of other parts of the world.

CONCLUSIONS

In conclusion, the prevalence of *P jirovecii* colonization was low and may indicate declining prevalence of *P jirovecii* colonization in HIV-infected individuals on cART. Based on the data at hand, *P jirovecii* colonization does not appear to be a major contributor to the development of COPD in well treated, HIV-infected individuals. However, longitudinal studies in populations with a higher prevalence of *P jirovecii* colonization and the use of lower respiratory tract specimens would be needed to draw conclusions about a causal relationship between *P jirovecii* colonization and pulmonary disease.

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Author's contribution. All authors have seen and approved the manuscript and contributed significantly to the work. A. R., T. B., J. K., and S. D. N. designed the study. A. R. and D. M. K. collected the data. J. S. J., A. O. K., and J. K. set up the polymerase chain reactions. A. R. was responsible for the statistical analysis. A. R., T. B., J. G., J. V., J. S. J., J. K., and S. D. N. interpreted the data. A. R. and S. D. N. drafted the manuscript. All authors have critically revised and approved the final version.

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Supplementary Data

Supplementary material is available online at Open Forum Infectious Diseases online (http://OpenForumInfectiousDiseases.oxfordjournals.org/).

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