

Pneumocystis colonisation is common among hospitalised HIV infected patients with non-*Pneumocystis* pneumonia

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ABSTRACT

Background: When *Pneumocystis* DNA is recovered from respiratory specimens of patients without *Pneumocystis* pneumonia (PCP), patients are said to be colonised with *Pneumocystis*, although the significance of this state is unknown. Understanding risk factors for and outcomes of colonisation may provide insights into the life cycle and transmission dynamics of *Pneumocystis jirovecii*.

Methods: We performed a cross sectional study of the prevalence and clinical predictors of *Pneumocystis* colonisation in 172 HIV infected, PCP negative inpatients undergoing diagnostic evaluation of 183 episodes of pneumonia at either the Medical Center of Louisiana at New Orleans between 2003 and 2005 or San Francisco General Hospital between 2000 and 2005. DNA was extracted from sputum and bronchoalveolar lavage specimens and amplified using a nested PCR assay at the mitochondrial large subunit (18S) ribosomal RNA locus. Colonisation was deemed present if *Pneumocystis* DNA was identified by both gel electrophoresis and direct DNA sequencing.

Results: 68% (117/172) of all patients were colonised with *Pneumocystis*. No strong associations with colonisation were identified for any demographic factors. Among clinical factors, having a CD4+ T cell count ≤ 50 cells/ μ l (unadjusted OR 2.4, 95% CI 1.09 to 5.48; $p = 0.031$) and using PCP prophylaxis (unadjusted OR 0.55, 95% CI 0.29 to 1.07; $p = 0.077$) were associated with *Pneumocystis* colonisation, although the latter association may have been due to chance. After adjustment for CD4+ T cell count, use of PCP prophylaxis was associated with a decreased odds of colonisation (adjusted OR 0.45, 95% CI 0.21 to 0.98; $p = 0.045$). 11 patients who were colonised were subsequently re-admitted for evaluation of a second episode of pneumonia; three were found to be colonised again, but none had PCP.

Conclusions: The majority of hospitalised HIV infected patients with non-PCP pneumonia are colonised with *Pneumocystis*. Failure to use co-trimoxazole prophylaxis and severe immunosuppression are associated with an increase in the odds of colonisation. *Pneumocystis* colonisation among hospitalised patients does not commonly lead to PCP.

The fungus *Pneumocystis jirovecii* (formerly *P. carinii*) frequently causes pneumonia and death in HIV infected patients worldwide.^{1,2} Because *Pneumocystis* pneumonia (PCP) prophylaxis and treatment are neither universally available nor universally effective, understanding the progression from preclinical

infection to overt pneumonia could improve transmission control and prevention strategies. The lack of a reliable in vitro method of culturing *Pneumocystis* long hindered research in these areas until indirect methods such as nucleic acid amplification tests were developed and used to provide evidence of infection.³

The discovery of *Pneumocystis* DNA in the respiratory specimens of individuals without clinical PCP and without demonstrable *Pneumocystis* organisms on microscopy led researchers to define a state called *Pneumocystis* colonisation.^{3,4} Most epidemiological studies of this state have used a PCR assay targeting the mitochondrial large subunit (18S) ribosomal RNA (mtLSU rRNA). This gene has multiple copies and a highly conserved nucleotide sequence, features which theoretically give the PCR assay high sensitivity and specificity, and make it an ideal procedure for studies of colonisation.⁵ Although detecting *Pneumocystis* DNA does not prove the viability of the organism, it does imply recent infection, an assumption which has fostered insights into how *Pneumocystis* may be acquired and transmitted.⁶ Colonisation nevertheless remains a state of uncertain clinical significance.

Studies applying the mtLSU rRNA assay suggest that colonisation with *Pneumocystis* DNA is prevalent in a variety of respiratory specimens and populations. Among individuals without clinical PCP, *Pneumocystis* DNA is present in 20% of oral washes from healthy adults in the general population,⁷ 44% of bronchoalveolar lavage fluid samples from non-HIV-infected immunocompromised adults⁸ and 46% of lung autopsy specimens from HIV infected hosts.⁹ Chronic lung disease,¹⁰ corticosteroid use⁸ and HIV infection⁹ are all associated with an increased risk of *Pneumocystis* colonisation.

Because *Pneumocystis* has the greatest impact on those with HIV, and remains a leading cause of opportunistic infection and death even in the era of combination antiretroviral therapy, characterising risk factors more fully in this population is important.² While one small study showed lower CD4+ T cell counts to be a risk factor for colonisation,¹¹ this hypothesis was rejected in a subsequent large study.⁹ The same study also found no association with other HIV related factors, including a history of PCP and the use of *Pneumocystis* chemoprophylaxis.⁹

The outcomes of colonisation, which may be even more important, are much less well described. Studies of mice¹² and humans⁶ suggest that carriers

of *Pneumocystis* may transmit it to other hosts. Limited information suggests that *Pneumocystis* colonisation can lead to pneumonia.^{11–13}

We designed a cross sectional study to determine the risk factors for and outcomes of *Pneumocystis* colonisation using respiratory specimens from HIV infected hosts admitted to two large public hospitals with pneumonia. We hypothesised that lower CD4+ T cell counts would predict a higher likelihood of colonisation, and that colonisation would predict a higher likelihood of subsequent PCP in a prospective cohort of patients at one of the hospitals. Some of the results of this study have been previously reported in abstract form.^{14–15}

MATERIALS AND METHODS

Participants, design and setting

The sample population for this study was consecutive HIV infected adults suspected of PCP and admitted to the inpatient wards of one of two acute care public hospitals: San Francisco General Hospital, San Francisco, California, USA, between 2000 and 2005, or the Medical Center of Louisiana, New Orleans, New Orleans, Louisiana, USA, between 2003 and 2005. Enrolment followed the diagnostic procedure for HIV associated pneumonia that is standard at each hospital. In San Francisco, the pulmonary consult team screens all requests for diagnostic testing for PCP. Patients with clinically appropriate presentations undergo sputum induction, followed by bronchoscopy with bronchoalveolar lavage (BAL) if the sputum stains are negative for *Pneumocystis* and alternative pathogens.¹⁶ In New Orleans, the diagnostic strategy is to offer BAL to all patients within the first 48 h of admission at the discretion of the primary healthcare team. The study's inclusion criteria required that the subjects be referred for PCP testing, and be proven to be PCP negative by microscopic examination of stained induced sputum or BAL fluid in accredited on-site clinical laboratories. Institutional review boards at the University of California, San Francisco, Louisiana State University and the Centers for Disease Control and Prevention approved the study protocol, and all subjects provided written informed consent.

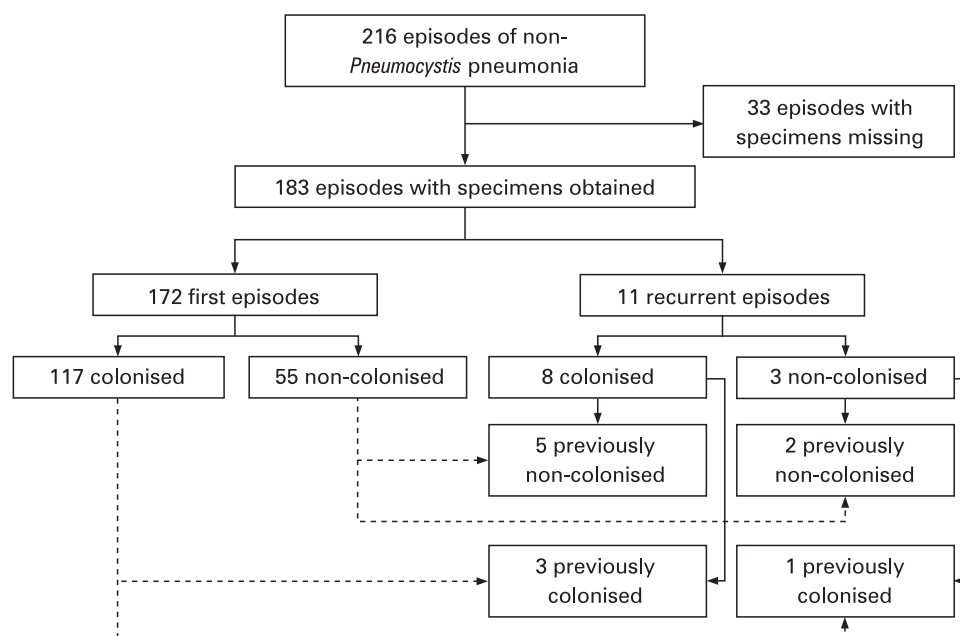
Data collection

Demographic information (age, gender, race and ethnicity) and data on clinical history at admission (previous episodes of PCP, serum CD4+ T cell count within 6 months, plasma HIV RNA level within 6 months and type of PCP prophylaxis medication, if any) were abstracted from medical records using standardised forms. At San Francisco General Hospital, patients undergoing evaluation for PCP are listed in a registry, which was monitored to identify participants subsequently admitted for evaluation for PCP. Similarly, patients hospitalised for evaluation for HIV associated pneumonia at the Medical Center of Louisiana at New Orleans are included in a clinical database, from which the variables used in this study (except for data on empiric PCP therapy prior to BAL, which were unavailable) were abstracted. Final aetiological diagnoses were adjudicated based on microbiological and radiographic results and clinician judgment. A diagnosis of PCP required microscopic identification of *Pneumocystis* organisms on sputum or BAL. Vital status 6 weeks after discharge was determined for all patients enrolled in San Francisco by reviewing electronic medical records for deaths occurring prior to that date and patient contacts (eg, outpatient clinic visits) occurring after that date.

Specimen processing, DNA extraction and DNA amplification

Pellets remaining after microbiological processing of induced sputum and BAL specimens were stored in ethanol at -80°C , and later thawed for DNA extraction using a commercially available DNA extraction kit (Promega Corporation, Madison, Wisconsin, USA). Nested PCR with positive and negative internal controls was performed using first round primers pAZ102-E and pAZ102-H, as has been described previously.¹⁷ Second round primers were pAZ102-X¹⁸ and M292R (5'-TAT CCA ACA ACT TTT ATT TC-3'). Several procedures were consistently applied to avoid DNA carryover, including unidirectional workflow, daily decontamination of the processing area with 70% ethanol and ultraviolet light, and testing of positive and negative controls with every round of amplification. Products of PCR were run on 1.0% ethidium bromide gels with standard DNA ladders, and the assays were considered

Figure 1 Patient flow diagram



positive for *Pneumocystis* DNA if a 252 base pair band was visualised and subsequently confirmed with sequencing. If *Pneumocystis* DNA was isolated from any sputum or BAL specimen obtained during the hospitalisation, the patient was defined as colonised for that episode.

DNA sequencing

After purification using a commercially available kit (Millipore, Billerica, Massachusetts, USA), DNA sequencing was performed using the dye terminator chemistry method (Applied Biosystems, Foster City, California, USA).

Statistical analysis

STATA V.9.0 (STATA Corporation, College Park, Texas, USA) was used for statistical analysis. Assuming a two-sided alpha of 0.05 and power of 0.8, we used pilot data to estimate that 180 episodes of non-PCP pneumonia would be required to evaluate our primary hypothesis, that CD4 counts <200 cells/μl would be associated with a 25% or higher increase in *Pneumocystis* colonisation prevalence compared with CD4 counts ≥200 cells/μl.

Table 1 Baseline characteristic of 172 HIV infected patients without *Pneumocystis* pneumonia

Characteristic	No (%) [*]
City	
New Orleans	111 (65)
San Francisco	61 (35)
Age (y)	
<35	27 (16)
35–44	75 (45)
45–54	57 (34)
≥55	8 (5)
Sex	
Female	48 (28)
Male	123 (72)
Ethnicity	
African-American	123 (72)
Caucasian	33 (19)
Other	16 (9)
CD4+ T cell count (cells/μl)	
> 200	36 (25)
101–200	32 (22)
51–100	23 (16)
≤ 50	55 (38)
HIV RNA (copies/ml)	
<75	9 (8)
≥75–<20 000	20 (17)
≥20 000–<100 000	32 (28)
≥100 000	54 (47)
Prior PCP	53 (31)
PCP prophylaxis on admit	
Co-trimoxazole	51 (30)
Other	20 (12)
Aetiology of pneumonia	
Bacterial	104 (60)
Opportunistic†	22 (13)
Non-infectious‡	18 (10)
Undiagnosed	28 (16)

^{*}Up to 57 observations may be missing (as for HIV RNA), but percentages are calculated as a proportion of actual observations. Percentages may not total 100% due to rounding. †Includes fungal, mycobacterial and viral pneumonias. ‡Includes Kaposi's sarcoma, lymphoma and other non-infectious pulmonary processes, such as chronic obstructive pulmonary disease exacerbations and pulmonary oedema. PCP, *Pneumocystis* pneumonia.

Demographic and clinical variables were examined according to *Pneumocystis* colonisation status; for those with multiple episodes, only data from the first episode were analysed. Sensitivity analyses were performed to compare individuals with and without colonisation data. Individuals missing predictor or outcome variables were excluded from multivariate analyses. Associations were evaluated with reference to a type I error ratio (p) less than 0.05, and assessed using a χ^2 or Fisher's exact test for categorical variables, or Student's t test or the Mann-Whitney rank sum test for continuous variables. In addition, unadjusted odds ratios (OR) with 95% confidence intervals (CI) were calculated using logistic regression. Multivariate analyses were performed using stepwise forward and stepwise backward logistic regression, including predictors with face validity and empirical association at $p < 0.2$. The final model was evaluated for influential observations, and goodness of fit of the model was assessed using the Hosmer-Lemeshow statistic. Two-way interactions between CD4+ T cell count and both PCP history and use of PCP prophylaxis were explored, with significance evaluated using the Wald test.

RESULTS

Episodes

Overall, specimens from 183 of 216 episodes of non-PCP pneumonia were available for testing, 63 from San Francisco (34%) and 120 from New Orleans (66%) (fig 1).

A sensitivity analysis showed that missing colonisation status was correlated with older age (42 vs 46; $p = 0.006$), but not with other predictors. Sixty-eight per cent (125/183) of episodes involved patients whose respiratory specimens were positive for *Pneumocystis* by DNA amplification at the mtLSU rRNA locus. Under the conservative assumption that all missing PCR results were negative for *Pneumocystis*, the prevalence of colonisation would have been 58% (125/216). Among those with multiple specimens (eg, sputum induction and BAL) obtained during the same hospitalisation, 11 patients were colonised on one specimen but not on another (five on sputum but not on BAL, six on BAL but not on sputum). Of the 172 participants, 11 (two in San Francisco, nine in New Orleans) were re-admitted and underwent evaluation for a second episode of pneumonia.

Subjects

Subjects were predominantly middle-aged (35–54 years), male and African-American (table 1).

A high proportion had advanced AIDS, with median CD4+ T cell count of 90.5 cells/μl (interquartile range (IQR) 17–197), and median plasma HIV RNA 5 log copies/ml (IQR 4.2–5.4). Thirty-one per cent (53/172) had previously had PCP, but only 46% (50/109) of those with CD4+ T cell counts less than 200 cells/μl reported taking PCP prophylaxis. Out of all patients, 30% were taking co-trimoxazole, 9% dapsone and 2% other prophylaxis regimens. Among San Francisco patients, 48% received empiric PCP therapy prior to sputum induction or BAL (median time between treatment and diagnostic specimen collection, 2 days). Final diagnoses included bacterial pneumonia (60%); other opportunistic pneumonias (13%), including fungal (6%), mycobacterial (5%) and viral (2%) pneumonias; and non-infectious pulmonary processes (10%), including pulmonary KS (3%), lymphoma (1%) and other non-infectious conditions (6%). In 16% of patients, no specific final diagnosis was made.

Table 2 Factors associated with *Pneumocystis* colonisation in 172 HIV infected patients without *Pneumocystis* pneumonia.

Characteristic	No colonised (%)	OR (95% CI)*	p Value
City			0.86
New Orleans	75 (68)	Referent	
San Francisco	42 (69)	1.06 (0.54–2.08)	
Age (y)			0.84
<35	19 (70)	Referent	
35–44	49 (65)	0.79 (0.31–2.06)	
45–54	41 (72)	1.08 (0.39–2.96)	
≥55	5 (63)	0.70 (0.13–3.66)	
Sex			0.60
Female	34 (71)	Referent	
Male	82 (67)	0.82 (0.40–1.70)	
Ethnicity			0.42
African-American	81 (66)	Referent	
Caucasian	23 (70)	1.19 (0.52–2.74)	
Other	13 (81)	2.25 (0.61–8.32)	
CD4+ T cell count (cells/μl)			0.12
>200	25 (69)	Referent	
101–200	19 (59)	0.64 (0.24–1.75)	
51–100	15 (65)	0.83 (0.27–2.51)	
≤50	45 (82)	1.98 (0.74–5.31)	
HIV RNA (copies/ml)			0.24
<75	7 (78)	Referent	
≥75 and <20 000	11 (55)	0.35 (0.06–2.12)	
≥20 000 and <100 000	26 (81)	1.24 (0.20–7.53)	
≥100 000	39 (72)	0.74 (0.14–3.99)	
Prior PCP			0.16
No	77 (65)	Referent	
Yes	40 (75)	1.68 (0.81–3.48)	
PCP prophylaxis on admit			0.077
No	72 (73)	Referent	
Yes	43 (61)	0.55 (0.29–1.07)	
PCP prophylaxis category			0.059
None	72 (73)	Referent	
Co-trimoxazole	28 (55)	0.44 (0.22–0.89)	
Other	15 (75)	1.08 (0.36–3.28)	
Aetiology of pneumonia			0.79
Bacterial	72 (69)	Referent	
Opportunistic†	13 (59)	0.64 (0.25–1.65)	
Non-infectious‡	12 (67)	0.89 (0.31–2.58)	
Undiagnosed	20 (71)	1.11 (0.44–2.79)	

*Up to 57 observations may be missing (as for HIV RNA), but OR are calculated using actual observations only. †Includes mycobacterial, fungal and viral pneumonias. ‡Includes Kaposi's sarcoma, lymphoma and other non-infectious pulmonary processes, such as chronic obstructive pulmonary disease exacerbations and pulmonary oedema. PCP, *Pneumocystis* pneumonia.

Predictors of *Pneumocystis* colonisation

Similar proportions of subjects were colonised in both cities. In unadjusted analysis, there was no strong evidence that any of the demographic or clinical variables listed in table 2 were associated with *Pneumocystis* colonisation.

Those with CD4+ T cell counts ≤50 were more likely than those with CD4+ T cell counts >50 to be colonised (OR 2.4, 95% CI 1.09 to 5.48; $p = 0.031$). As shown in table 2, odds of colonisation increased progressively with each progressive decrease in CD4+ T cell category, although the global test of association ($p = 0.12$) and the test for linear contrast ($p = 0.15$) suggested that this may have been due to chance. Those with a previous history of PCP were more likely than those without such a history to be colonised (75% vs 65%), but the 95% CI for the OR included the possibility of no difference (OR 1.68, 95% CI 0.81 to 3.48; $p = 0.16$). The use of PCP prophylaxis was associated with a lower proportion colonised with *Pneumocystis* (61% vs 73%), but the 95% CI for the OR for this association

also included no difference (OR 0.55, 95% CI 0.29 to 1.07; $p = 0.077$). Among those taking the most effective form of prophylaxis, co-trimoxazole, colonisation was less common than among those not taking any prophylaxis (55% vs 73%, OR 0.44, 95% CI 0.22 to 0.89; $p = 0.023$). Among patients in San Francisco, the proportion colonised was similar among those who received empiric PCP treatment prior to sputum induction or BAL to the proportion among those who did not (64% vs 73%, OR 0.68, 95% CI 0.23 to 2.00; $p = 0.48$). Final diagnosis was not associated with colonisation status.

In the multivariate regression model presented in table 3, the use of PCP prophylaxis was strongly associated with a decreased odds of *Pneumocystis* colonisation (adjusted OR 0.45, 95% CI 0.21 to 0.98; $p = 0.045$). This model included PCP prophylaxis and ordinal categories of CD4+ T cell count, on the basis of both empirical and face validity. Having a history of PCP did not alter the effects of these variables nor did it improve the overall model, so it was excluded. The final model proved robust across

Table 3 Adjusted predictors of *Pneumocystis* colonisation in 144 patients without *Pneumocystis* pneumonia

Characteristic	OR (95% CI)	p Value
CD4+ T cell count (cells/ μ l)		0.068
>200	Referent	
101–200	0.69 (0.25–1.92)	
51–100	1.04 (0.33–3.28)	
≤ 50	2.45 (0.88–6.86)	
PCP prophylaxis on admit		0.045
No	Referent	
Yes	0.45 (0.21–0.98)	

PCP, *Pneumocystis* pneumonia.

strata ($p = 0.99$, by the Hosmer–Lemeshow statistic), and the exclusion of influential points did not significantly change the model. Two-way interactions between each of the combinations of the predictors PCP history, use of PCP prophylaxis and CD4+ T cell count were explored, but none was observed.

Outcomes of *Pneumocystis* colonisation on subsequent hospitalisation

Eleven patients were readmitted and evaluated a second time for non-PCP pneumonia, but there was no discernible pattern to *Pneumocystis* colonisation status on readmission. Of these 11, four (36%) were readmitted in the same calendar year as the first admission. None had PCP. Three of the 11 were persistently colonised with *Pneumocystis*. Of these three, two had the same genotype as on the previous hospitalisation; one acquired a mixed infection (two new genotypes in addition to the previous one). Five patients who were not colonised on the first admission became colonised on the second admission. Three of these five were colonised with a single genotype; two developed mixed infections. One patient colonised on the first admission was not colonised on the second admission, and two patients who were not colonised remained not colonised. Of the 61 patients enrolled at San Francisco General Hospital, 58 (95%) were alive at 6 weeks. Of the three who died, none had PCP, as confirmed by a negative microscopic examination of BAL and by the presence of an alternate diagnosis as the cause of death.

DISCUSSION

In this study, the majority (68%) of hospitalised HIV infected patients with pneumonia but without clinically evident PCP were colonised with *Pneumocystis jirovecii*. After adjustment for relevant covariates, use of prophylactic medication against PCP was associated with a dramatic reduction in the odds of colonisation, while there was a trend towards an increased odds of colonisation in those with low CD4+ T cell counts. Finally, *Pneumocystis* colonisation did not appear to be a risk factor for subsequent PCP within 6 weeks.

The proportion of patients colonised with *Pneumocystis* in this study was higher than reported in other studies involving several different patient populations. Among adults in these studies, the proportion of patients who were colonised with *Pneumocystis* ranged from 0% to 20% in immunocompetent patients with and without underlying pulmonary disease, from 35% to 60% in immunosuppressed, non-HIV-infected patients and from 10% to 46% in HIV infected patients.^{7 9 11 19–21} Because previous studies included patients with similarly low CD4+ T cell counts, the higher prevalence of colonisation reported here likely reflects the low proportion using PCP prophylaxis.

The unadjusted finding that the odds of colonisation increases dramatically among those with CD4+ T cell counts ≤ 50 relative to

those with CD4+ T cell counts >50 supports the hypothesis that immunosuppression predisposes patients to colonisation. This threshold for an increased odds of colonisation is lower than expected given the abundant evidence that the risk of PCP increases below a CD4+ T cell count of 200 cells/ μ l.²² The low threshold identified here may be an artefact of the narrow spectrum and right skewed distribution of CD4+ T cell counts in a population referred for testing for PCP. In contrast with a threshold model of the relationship between immunosuppression and *Pneumocystis* colonisation, we would hypothesise a proportional relationship as a more biologically plausible model of this host–pathogen interaction. Although possibly due to chance, the increasing odds of colonisation seen with decreasing CD4+ T cell counts in this study are consistent with such a hypothesis. Moreover, this trend is consistent with a previous study that also showed this relationship to be stepwise and inversely proportional.¹¹

Limited statistical power rather than confounding likely explains the lack of an adjusted association between host immune status and colonisation, as adjustment for PCP prophylaxis use actually strengthened the association of colonisation with the lowest strata of CD4+ T cell counts. Measurement error inherent to chart abstraction likely contributed, as HIV RNA and CD4+ T cell counts were frequently obtained weeks to months before hospital admission. Use of antiretroviral therapy, another unmeasured potential confounder, may have also biased these results in an uncertain direction.

Use of *Pneumocystis* prophylaxis was associated with a decreased prevalence of colonisation, especially among those taking co-trimoxazole. Many studies have shown that the use of PCP prophylaxis reduces the risk of PCP.^{23 24} Thus the finding that prophylaxis is also associated with a decreased odds of *Pneumocystis* colonisation is consistent with a biological model in which colonisation leads to PCP. This finding is further strengthened by the evidence that the most effective form of PCP prophylaxis, co-trimoxazole, is associated with lower odds of colonisation than dapsone and other forms of prophylaxis. Although higher doses of co-trimoxazole, in the form of empiric treatment for PCP, did not contribute to the multivariate model of colonisation, the time period between initiation of empiric treatment and diagnostic testing may have been too short for *Pneumocystis* DNA to be cleared from the body.

It is possible that patients acquire *Pneumocystis* while in hospital, as suggested by the observation that several patients had within-episode discordance in colonisation status between respiratory specimens. Studies collecting serial specimens during hospitalisation and correlating genotype with location within the hospital would help explore this possibility.^{25 26} Longitudinal studies to determine the frequency and duration of *Pneumocystis* colonisation among outpatients with a wider range of CD4+ T cell counts would also help further explain the timing and mechanism of transmission.

The low cumulative incidence of recurrent pneumonia among the cohort from San Francisco prevents strong conclusions about the natural history of colonisation as a risk factor for subsequent PCP, but this study does imply that such outcomes are rare. While non-human studies suggest that progression from *Pneumocystis* colonisation to infection is common,²⁷ the effectiveness of prophylaxis against PCP likely prevents these findings from being replicated in humans who adhere to recommended therapies for HIV. Although this study did not collect specific data on medication use after discharge, most patients were likely prescribed prophylaxis and many likely also started antiretroviral therapy after discharge. Among those who were re-admitted, the frequency of changes in colonisation

status and mtLSU rRNA genotype between episodes suggests that colonisation may be a highly dynamic state. Furthermore, the high prevalence of colonisation with both first (68%) and recurrent (73%) episodes of pneumonia raises the possibility that *Pneumocystis* colonisation could be a nearly universal experience over time in a population with advanced HIV.

This study builds on previous work in several ways. Firstly, this study examined hospitalised patients, a group for whom there are limited data about *Pneumocystis* colonisation. Secondly, the method of sampling all patients referred for diagnostic testing for opportunistic respiratory infections is a new approach for this field and makes the study's conclusions applicable to the population most at risk for PCP. Thirdly, the study's large sample size allowed identification of novel risk factors for colonisation that previous studies may have lacked adequate power to detect.⁹ Fourthly, this study used highly accurate measures to define the study outcome, *Pneumocystis* colonisation. All patients underwent the reference standard test, microbiological staining of induced sputum or bronchoalveolar lavage specimens, to exclude PCP. The presence of *Pneumocystis* DNA was confirmed by sequencing all positive specimens, which made the outcome measurement not only highly sensitive but also highly specific. Finally, this study adds longitudinal data on outcomes in HIV infected patients colonised with *Pneumocystis*.

There are several limitations to the conclusions that can be drawn from this study. DNA assays cannot distinguish between the presence of living and dead organisms, and some positive mtLSU rRNA assays may have arisen from non-viable remnants of remote infection or from inhalation of non-viable *Pneumocystis* DNA from the environment. Nevertheless, the association of PCP prophylaxis, especially co-trimoxazole, with decreased odds of detecting *Pneumocystis* DNA, is consistent with a biological effect of prophylaxis in modifying the presence of presumably viable organisms. A second limitation is that colonised patients were not systematically followed after hospital discharge to determine whether they developed PCP, and the conclusions of this study about colonisation among patients who were re-admitted are limited by the small sample size. However, as diagnostic testing for PCP in these hospitals requires approval of the pulmonary services, which record all referrals in registries, it is unlikely that patients re-admitted with PCP were missed. Furthermore, as both hospitals in the study serve primarily indigent populations for whom they are the primary local providers of HIV care, it is unlikely that many patients sought care elsewhere for recurrent pneumonia.

In conclusion, *Pneumocystis* colonisation affects the majority of patients with advanced HIV who are hospitalised with non-*Pneumocystis* pneumonia. While much evidence suggests that *Pneumocystis* is transmitted from person-to-person,³ the high prevalence of colonisation in HIV infected patients on hospital admission suggests that respiratory isolation may not be a viable strategy for preventing disease transmission. In contrast, this study does support the use of co-trimoxazole prophylaxis against PCP in accordance with well established clinical guidelines for patients with AIDS. Finally, this study offers further evidence in favour of the hypothesis that advanced immunosuppression increases the odds of *Pneumocystis* colonisation.

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