A. BACKGROUND AND SIGNIFICANCE

Human immunodeficiency virus (HIV) has infected more than 40 million people, an overwhelming majority of whom live in middle- or low-income countries. In recent years, scientists have shown that people can be sequentially infected with different strains of HIV, a phenomenon termed 'superinfection'. Though fewer than 20 such cases have been described[3, 7], individuals recently infected with HIV may be at greater risk for superinfection than those with chronic, asymptomatic infection[8]. Additionally, detecting HIV superinfection during acute infection is more likely because the probability of one strain becoming predominant in the circulating virus population rises as the duration of infection increases. Ascertaining the frequency of superinfection is important because there is anecdotal evidence that individuals with HIV superinfection progress more rapidly to AIDS[9].

Superinfection studies have largely focused on volunteers in the United States[9]. Assessing HIV superinfection in resource-constrained settings where the HIV pandemic is concentrated is essential to understand whether superinfection is more common in such environments with higher HIV incidence and viral diversity. It is very difficult to maintain cohorts of patients in resource limited conditions. In addition to the challenges of maintaining cohorts of patients willing to participate in research, technologically sophisticated DNA sequencing machines and highly skilled laboratory technicians are needed to detect HIV superinfection. Consequently, few research centers outside of North America and Western Europe have the infrastructure to study HIV superinfection.

The research laboratories of Dr. Esper Kallas, at the University of São Paulo and the Federal University of São Paulo, Brazil comprise one of the sites with the expertise and clinical ties necessary to study HIV superinfection. In the last five years, Dr. Kallas has established a cohort of 200 people newly infected with HIV in Sao Paulo, Brazil. In this proposal, we will collaborate with Dr. Kallas to quantify HIV superinfection in this cohort. The primary objectives are to (1) assess the frequency of HIV superinfection in newly infected persons living in Sao Paulo and (2) enroll those individuals infected with multiple strains of HIV in a long-term study to evaluate the implications of superinfection on disease progression. Secondary objectives include strengthening research ties between UW-Madison and Brazilian scientists and developing generalized protocols for assessing HIV superinfection that can be applied to resource-constrained settings.
B. PRELIMINARY DATA

The O'Connor laboratory has extensive expertise with viral nucleic acid sequencing. Dr. O'Connor designed PCR primers and nucleic acid sequencing protocols that were used to describe one of the first known cases of HIV superinfection[1]. Additionally, he runs the Genetics Service unit of the Wisconsin National Primate Research Center and his group has developed amplification and sequencing strategies for a variety of simian immunodeficiency viruses (SIVs)[10, 11]. Recently, his group successfully PCR amplified and sequenced viral nef genes from a cohort of HIV+ patients receiving care at the University of Wisconsin Hospitals and Clinics.

The Kallas clinical site has been working since 2002 to follow a cohort or recently HIV-infected subjects in Sao Paulo, Brazil[6]. The project was piloted to identify, recruit and enroll recently infected HIV positive individuals into a prospective study of HIV infection. Subjects testing HIV-positive at one of five clinical trial sites in the municipal area of São Paulo were also tested using the STARHS criteria (sensitive EIA reactive, less sensitive EIA non-reactive) to identify recently acquired HIV infection[5]. As of November 10, 2007, 210 people with recently acquired HIV infection have been successfully enrolled into the cohort for follow up. Participants in the cohort are predominantly male (91%), the majority (81%) of whom self-identify as men who have sex with men (MSM). They are recruited from sites in the central, north and southern areas of São Paulo, distributed through different ethnicities. In an important difference from US based cohorts, almost all Brazilian subjects only start antiretroviral therapy when their CD4 T cell counts decline to 250 cells/µl, according to the Brazilian Guidelines. This approach provides a better opportunity to study the early natural course of HIV-1 infection without the interference of antiretroviral drugs. For example, researchers working with this cohort recently made the unexpected observation that HSV-2-co-infection leads to higher CD4+ T cell counts[2], while syphilis seropositivity may accelerate progression to immunodeficiency (data not shown).

C. RESEARCH PLAN

i. Methodology. HIV superinfection can be detected by retrospective nucleic acid sequencing of individual viruses cloned from blood plasma. During a typical HIV infection, the sequences of HIV clones from a single individual will form a single phylogenetic cluster with a readily identifiable common ancestor. In suspected superinfections, phylogenetic analyses will reveal two or more distinct sequence clusters (Figure 1).
For this project, we will work with Dr. Kallas to develop methods for sequencing and analyzing individual clones of the viral env gene from the 200 newly infected patients in his cohort. The env gene shows the greatest diversity between HIV strains[4]. Therefore, the probability of two independent virus sources having similar or identical env nucleic acid sequences is low. We will examine existing databases of Brazilian HIV sequences and design PCR and sequencing primers that will robustly detect env from volunteers in Dr. Kallas's cohort.

Two additional analyses will be performed on individuals where superinfection is suspected on the basis of env sequencing. A second HIV gene (pol) will be sequenced to confirm the presence of two independent viral strains and to examine strain-specific differences in antiretroviral drug susceptibility. Additionally, we will sequence the env gene at additional timepoints in order to detect the precise timing of superinfection.

**ii. Coordination of work.** All sample processing and viral sequencing from newly infected patients will be performed under Dr. Kallas's supervision in São Paulo in accordance with Brazilian regulations governing research studies involving their nationals. Together, Drs. Kallas and O'Connor will develop protocols for amplifying and sequencing env genes from Brazilian strains of HIV. Sequencing results will be shared electronically and analyzed jointly by the O'Connor and Kallas laboratories.

The O'Connor and Kallas laboratories have been collaborating for nearly two years and the communications infrastructure needed for this project is already in place. Our laboratories have held joint lab meetings via Skype videoconferencing for more than a year. For this project, we will increase the frequency of videoconferencing to at least once per month. In addition to scheduled joint lab meetings, ad

![Figure 1. Identification of HIV superinfection by env sequencing.](image1.jpg)

Circles indicate three individuals (A, B, and C) suspected of being HIV superinfected intermingled with sequences from other acutely HIV infected persons and laboratory HIV strains. Note that the independent origins of the superinfecting viruses can be easily visualized using this type of phylogenetic analysis. Reprinted from Smith et al., 2004.
hoc videoconferencing (already available to scientists at both sites) allows collaborating staff to interact continuously and in real-time at minimal cost.

The majority of funds requested for this project will support periodic trips for Dr. O'Connor and his research staff to the study site in order to jointly analyze data and provide on-site nucleic acid sequencing support and training. In addition, funds are requested for Dr. Kallas to visit Madison at least once during the two year period of support to study other technological advances that could improve study of superinfected individuals and to meet with other UW-Madison staff interested in HIV and global health.

D. SUMMARY

The O'Connor and Kallas laboratories are uniquely qualified to determine the frequency of HIV superinfection in the context of incipient HIV infection. Successful completion of this project could provide an explanation for why certain individuals develop AIDS rapidly and will establish the foundation for additional collaborative studies marrying advanced virology and immunology research at UW-Madison with clinical research sites in resource-constrained settings.

E. LITERATURE CITED