2013

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Recommended Citation

Heipertz, Richard A.; Sanders-Buell, Eric; Kijak, Gustavo; Howell, Shana; Lazzaro, Michelle; Jagodzinski, Linda L.; Eggleston, John; Peel, Sheila; Malia, Jennifer; Armstrong, Adam; Michael, Nelson L.; Kim, Jerome H.; O'Connell, Robert J.; Scott, Paul T.; Brett-Major, David; and Tovanabutra, Sodsai, "Molecular Epidemiology of Early and Acute HIV Type 1 Infections in the United States Navy and Marine Corps, 2005–2010" (2013). *Journal Articles: Epidemiology*. 99.

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Molecular Epidemiology of Early and Acute HIV Type 1 Infections in the United States Navy and Marine Corps, 2005–2010

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Abstract

The U.S. military represents a unique population within the human immunodeficiency virus 1 (HIV-1) pandemic. The last comprehensive study of HIV-1 in members of the U.S. Navy and Marine Corps (Sea Services) was completed in 2000, before large-scale combat operations were taking place. Here, we present molecular characterization of HIV-1 from 40 Sea Services personnel who were identified during their seroconversion window and initially classified as HIV-1 negative during screening. Protease/reverse transcriptase (*pro/rt*) and envelope (*env*) sequences were obtained from each member of the cohort. Phylogenetic analyses were carried out on these regions to determine relatedness within the cohort and calculate the most recent common ancestor for the related sequences. We identified 39 individuals infected with subtype B and one infected with CRF01_AE. Comparison of the pairwise genetic distance of Sea Service sequences and reference sequences in the *env* and *pro/rt* regions showed that five samples were part of molecular clusters, a group of two and a group of three, confirmed by single genome amplification. Real-time molecular monitoring of new HIV-1 acquisitions in the Sea Services may have a role in facilitating public health interventions at sites where related HIV-1 infections are identified.

Introduction

UNAIDS RECOGNIZES MILITARY MEMBERS as a group at risk of human immunodeficiency virus 1 (HIV-1) infection¹ and the U.S. military portion of the HIV-1 pandemic is unique for several crucial reasons. Screening for anti-HIV-1 antibodies has been employed on all applicants for military service since 1985 and on active and reserve component members of the armed services since 1986.² In 2004, the Department of Defense instituted mandatory testing for all service members every 2 years. Testing is also conducted when clinically indicated and as related to predeployment and postdeployment processing.^{2,3} In addition, U.S. military personnel constitute a globally deployed risk group who are subject to unique HIV-1 risk environments and the stress of 11

years of continuous large-scale combat and support operations in support of the Global War on Terrorism and Overseas Contingency Operations, most prominently in Afghanistan and Iraq.

Several epidemiological and molecular epidemiological studies of HIV-1 have evaluated incident infections in the U.S. Army and Air Force with the most recent cohort ending in 2007.^{4–7} Studies have also been undertaken investigating infections in the U.S. Navy and Marine Corps,^{8–10} but the most recent of these considered data through May 2000, before the beginning of large-scale combat operations. Recent annual rates of infection per 1,000 HIV-1 tests range from 0.13 to 0.35 depending upon service and component (whether active or reserve force) in the Sea Services.^{2,3} Since the tempo of deployments and geographic breadth of missions undertaken by

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the Navy and Marine Corps in the post-9/11 era have significantly increased, important questions remain for epidemiology and prevention.

This current study aims to molecularly characterize acute and very early incident HIV-1 infections in the U.S. Navy and Marine Corps with a first known positive HIV-1 screening test between January 1, 2005 and August 3, 2010.³ The overarching public health investigation and its epidemiology have been described elsewhere.³

Materials and Methods

Participants and HIV-1 testing

The overall study was comprised of 496 participants who are enlisted U.S. Sailors and Marines with a first known positive HIV-1 screening test between January 1, 2005 and August 3, 2010.³ From January 2005 through April 2009, screening utilized the second generation enzyme-linked immunosorbent assay (ELISA) confirmed through Western blot.³ All 496 participants had their last-negative HIV-1 test rescreened using the third-generation ELISA confirmed through Western blot and nucleic acid testing (NAT) using the Abbott RealTime HIV-1 Test. The subset described in this publication is comprised of 54 participants whose last negative HIV-1 test occurred during their seroconversion window where anti-HIV-1 antibody testing was nonreactive but retrospective look back testing of repository samples detected HIV-1 nucleic acid. The seroconversion window lasts from approximately 5–19 days postinfection.¹¹ Therefore, these 54 participants were initially misclassified as negative in their HIV-1 screening. Of the 54 samples drawn during this seroconversion window of the 496 in this cohort, 12 samples were not available because volumes were entirely consumed during HIV-1 diagnostic look back testing. Sample identification numbers given to each individual were coded to protect their personal information.

Viral RNA extraction

A volume of 0.5 ml of plasma was extracted per sample. For samples with viral load (VL) below 10,000 copies/ml, 1 ml of plasma was extracted. The plasma was centrifuged for 1 h at 4°C at 20,000×g. One hundred and forty microliters of plasma was left behind following the spin. The viral pellet was resuspended and extracted with the QIAamp viral RNA mini kit (Qiagen, USA) per the manufacturer's instructions.

Complementary DNA (cDNA) synthesis

Viral RNA was reverse transcribed using the SuperScript III RT kit (Invitrogen, USA) following the manufacturer's instructions. Of oligo(dT) 50 μM was utilized as the primer along with kit contents diethylpyrocarbonate (DEPC) H₂O and 10 mM deoxynucleotide triphosphates (dNTP). Seven microliters of RNA was added to the mix and incubated for 5 min at 65°C. A mix of 10×RT buffer, DTT, MgCl₂, RNase OUT, and SuperScript III RT enzyme was added to the RNA/primer mix for a total reaction volume of 20 μl. The reaction proceeded for 3 h at 50°C, followed by 85°C for 5 min. In some cases, an internal primer (HXB2 5983–5956) was used to generate the *pro/rt* cDNA fragment when oligo(dT)-generated cDNA was not conducive to yielding a positive *pro/rt* polymerase chain reaction (PCR). The cDNA obtained was used as a template for PCR at near end-point dilution. For cDNA amplification, the primers used in this study are shown in Table 1 with the PCR conditions described below.

Pro/rt PCR

The PCR for the *pro/rt* region (HXB2 2094–3866) utilized the AmpliTaq Gold PCR kit (Applied Biosystems, USA) per the manufacturer's instructions. Briefly, MgCl₂, 10×PCR buffer, dNTP (1.25 mM), forward and reverse primers (20 μM for each), and AmpliTaq Gold enzyme were made in a master mix and dilutions of cDNA were added with water to a final volume of 50 μl. The nested PCR strategy utilized first round PCR primers at HXB2 positions 1808–1834 and 3892–3870 and second round PCR primers at HXB2 positions 2071–2093 and 3886–3864.

Env PCR

The PCR for the *env* region (HXB2 5984–9063) utilized the Platinum Taq kit (Invitrogen, USA) per the manufacturer's instructions. Briefly, 10×buffer, MgSO₄, dNTP (1.25 mM), forward and reverse primers (20 μM for each), and Platinum Taq enzyme were made in a master mix and dilutions of cDNA were added with water to a final volume of 20 μl. The seminested PCR strategy utilized the same forward primer in both first and second round PCR reactions and was positioned at HXB2 5958–5983. The first round reverse primer was positioned at HXB2 9520–9497 and the second round reverse primer at HXB2 9089–9064. For samples where a molecular link was observed, SGA was carried out using the same PCR protocol and the PCR products from a cut-off of 30% positive wells were used.¹²

TABLE 1. LIST OF PRIMER SEQUENCES USED FOR POLYMERASE CHAIN REACTION

Region (HXB2 number)	Primer/probe	Sequence (5'–3')
<i>pro/rt</i> (2094–3866)	Outer forward	GGGCTTCATTAGAAGAAATGATGACAG
	Outer reverse	CTATTTRGCTGCCCCATCTACATA
	Inner forward	GARAGRCAGGCTAATTTTTTAGG
	Inner reverse	GCTGCCCCATCTACATAGAAAGT
<i>env</i> (5984–9063)	Outer+inner forward	TAGGCATTTCCTATGGCAGGAAGAAG
	Outer reverse	GCAGCTGCTTATATGCAGGATCTGAGGG
	Inner reverse	TCCAGTCCCCCTTTTCTTTTAAAAA
Full genome (789–9,496)	Outer forward	AAATCTCTAGCAGTGGCGCCCCGAACAG
	Outer reverse	GCACTCAAGGCAAGCTTTATTGAGGCTT
	Inner forward	TGACTAGCGGAGGCTAGAAGGAGAGA
	Inner reverse	GCAGCTGCTTATATGCAGGATCTGAGGG

FIG. 1. Consort figure sample selection criteria. Out of the total cohort of 496 individuals with incident HIV-1 infections, 54 were misclassified as HIV-1 negative in their last negative screening. These samples were anti-HIV-1 antibody negative but nucleic acid positive leading to the misclassification of these samples.

Full-genome PCR

The PCR for full-genome sequence generation (HXB2 789–9496) utilized the Advantage GC Genomic LA kit (Clontech, USA) per the manufacturer's instructions. Briefly, 2×GC-Melt LA Buffer, dNTP (10 mM), forward and reverse primers (20 μM for each), and GC Genomic LA polymerase mix were made in a master mix and dilutions of cDNA were added with water to a final volume of 25 μL. The nested PCR strategy utilized first round PCR primers at HXB2 623–649 and 9632–9605 and second round PCR primers at HXB2 763–788 and 9524–9497.

Sequencing

PCR products generated by the most dilute cDNA for *pro/rt*, *env*, or full-genome sequencing were purified using the PureLink Genomic DNA Purification kit (Invitrogen, USA) per the manufacturer's instructions. For PCR reactions

yielding secondary or nonspecific bands, the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, USA) was utilized for gel extraction and purification of PCR products. Sequencing reactions of PCR products were performed using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, USA) along with 5×Sequencing Buffer, and water to a final volume of 16 μL. Sequencing reactions were purified with a PCR 96 Well Kit (Princeton Separations, USA) and loaded on an Applied Biosystems 3730 DNA Analyzer.

Sequence alignment

Sequences generated from this cohort were manually edited using Sequencer 5.0 (Gene Codes Corporation, USA) and aligned using Gene Cutter (HIVDB).¹³ The initial HIV-1 genotype was assigned for each sequence in this cohort using the HIV-1 Genotyping Tool at the National Center for Biotechnology Information (NCBI).¹⁴ Appropriate reference sequences that covered the entire region of *pro/rt*, *env*, or full-genome were downloaded from the HIVDB and aligned. The whole alignment covering each region and consisting of sequences from this cohort plus references was manually edited using MacGDE 2.4.

Phylogenetic analysis

Reference sequences were comprised of only subtype B for the *pro/rt* and *env* regions and subtype B and CRF01_AE for the full-genome alignments. A single reference was kept per individual (earliest time point). Reference sequences with excessive "N" base calls and/or containing large insertions or deletions were removed from the analysis. Alignments for *pro/rt*, *env*, and full-genome regions were phylogenetically analyzed using Molecular Evolutionary Genetics Analysis (MEGA) 5.0.¹⁵ In all regions, subtype B references belonging to Thai B, termed B', were excluded from analysis since sequences from this cohort did not belong to B'. In the *pro/rt* region, 144 nucleotide positions were removed from analysis due to their association with drug resistance that may confound the analysis. This comprehensive list of drug resistance sites was taken from the Stanford University HIV Drug Resistance Database. Maximum likelihood (ML) analysis was utilized to phylogenetically categorize the *pro/rt* and *env* regions. The ML method used the General Time Reversible (GTR) substitution model and gaps were treated as partial deletion with a cutoff of 50% of site coverage. Neighbor-joining analysis with the Kimura two-parameter model of substitution¹⁶ was utilized to determine the subtype of the full-genome sequences. Gaps were also treated as partial deletion with a cutoff of 50% of site coverage. Phylogenetic trees generated from the analyses were visualized using FigTree 1.3.1.

Pairwise genetic distance analysis

Calculations for pairwise genetic distance of *env* and *pro/rt* regions were performed in MEGA 5.0.¹⁵ The Kimura two-parameter substitution model¹⁶ was utilized with partial deletion (50%) of gaps. The distance matrix data were analyzed using JMP Genomics Software 4.0 (SAS Institute Inc., USA).

Inferring most recent common ancestor (MRCA) using Bayesian Markov chain Monte Carlo (MCMC) relaxed molecular clock

To determine the time to the MRCA, we used a Bayesian MCMC-based approach. BEAST version 1.6.2 was used for

FIG. 2. Maximum likelihood (ML) phylogenetic trees generated from *pro/rt* and *env* sequencing. ML phylogenetic tree of the *pro/rt* region (HXB2 2094–3866) from 1,296 subtype B reference sequences and 39 sequences from this cohort are shown (A). Five hundred and three subtype B references and 38 sequences from this cohort were analyzed by ML and covered the *env* region (HXB2 5984–9063) (B). The five samples shown to be part of a cluster were further sequenced to generate 10 *env* SGA sequences per sample. Red *, cohort sequences; green *, cohort sequences part of the dual cluster; blue *, cohort sequences part of the tri cluster; black box, indicates dual quasiespecies.

all calculations.¹⁷ SGA sequences generated from the same individual were grouped into a single taxon to allow for calculations. Analyses were carried out using the GTR substitution model and a relaxed uncorrelated exponential clock^{17,18} with exponential growth prior. The MRCA calculations reported were based on an ESS of greater than 300 via Tracer version 1.5. Trees generated from the BEAST runs were visualized after 10% burn-in with TreeAnnotator

version 1.6.2. A graph of the MRCA with 95% confidence intervals was generated using Prism 5.0 (GraphPad Software, USA).

GenBank submission

The sequences from this study have been submitted to GenBank under the numbers JX863919–JX864047.

FIG. 3. Neighbor-joining tree generated from full-genome sequencing. An alignment was generated covering all gene-coding regions of the HIV-1 genome (HXB2 789–9496). A total of 299 subtype B reference sequences was compared alongside the five linked samples from this cohort. Additionally, 88 CRF01_AE references and the single CRF01_AE sequences from this cohort are represented. Red *, CRF01_AE cohort sequence; green *, cohort sequences part of the dual cluster; blue *, cohort sequences part of the tri cluster.

Results

Participants

The Sea Services study identified 496 HIV seroconversions³ and look back testing of the last seronegative sample in each of these cases showed that 54 were positive by NAT. Twelve of 54 samples were not available because volumes were entirely consumed during HIV-1 diagnostic look back testing. Additionally, two samples were not amplifiable either in protease/reverse transcriptase (*pro/rt*) or envelope (*env*) and one sample was amplifiable only in *pro/rt* (Fig. 1). The collection dates ranged from April 5, 2002 to December 21, 2009. The mean VL for these samples was 6.8 with a range of 3.7 to 7.8 log copies per ml.

HIV-1 subtype designation and cluster identification

Based on sequenced regions of *pro/rt* and *env*, 39 of the 40 samples were subtype B while one was CRF01_AE. None of the subtype B sequences clustered with Thai B, so-called B'. We further examined the genetic diversity of the subtype B sequences with other subtype B reference sequences obtained from the LANL HIVDB.¹³ Given that sequences in our cohort were not classified as B', B' sequences were excluded from our analysis.

Thirty-nine sequences from our cohort (red *) and 2,296 *pro/rt* subtype B references were analyzed by ML (Fig. 2A). Prior to tree construction, 144 nucleotide positions (48 amino

acids) corresponding to potential drug resistance were considered potentially confounding and were removed from analysis. Most of the sequences of interest were dispersed throughout the subtype B reference sequences. However, there was a group of two samples (green *) [306159, 306512] and a group of three samples (blue *) [306344, 306376, 306517] that formed tight monophyletic clusters.

A similar analysis approach was performed on the *env* sequences. Thirty-eight sequences from our cohort and 538 reference sequences were analyzed in *env* by the ML method, confirming the subtypes identified in *pro/rt* (Fig. 2B). Figure 2B also shows an even distribution of our cohort samples (red*) throughout the reference sequences. We have employed SGA, a powerful technique that allows the fine analysis of sequence differences. It has been particularly useful in the analysis of transmitted founder viruses and in the evaluation of breakthrough infections in vaccine studies.^{19,20} SGA of *env* was performed on the five molecularly linked samples. The generation of multiple sequences from the same patient for *env* SGA further strengthens the link within each cluster and also reveals that 306159 and 306344 have two predominant quasiespecies (green or blue * surrounded by a black box) that were also confirmed by visual inspection and HIVDB Highlighter Plot (data not shown).¹³

Furthermore, we obtained full-genome sequences (HXB2 789–9496) of the five samples from these two linked clusters as well as the single CRF01_AE sequence. Two hundred and ninety-nine subtype B and 88 CRF01_AE reference sequences

FIG. 4. Pairwise genetic distance calculations for the *env* region. The pairwise genetic distance for the *env* region was calculated for reference and cohort sequences using the Kimura two-parameter model. For each figure section, the mean pairwise genetic distance is annotated in the center of each graph. (A) A comparison of references and sequences from this cohort; (B) cohort sequences compared to references identified prior to the year 2000; (C) cohort sequences compared to references identified from 2000 to 2004; (D) cohort sequences compared to references identified from 2005 to the present; (E) cohort sequence comparison without any references.

from the HIVDB were included in the neighbor-joining phylogenetic analysis. In concordance with our previous data, the clusters were readily apparent over the full genome, and the CRF01_AE sequence clustered with other CRF01_AE reference sequences identified from Southeast Asia (Fig. 3).

Genetic distance calculations of molecularly linked samples

We further characterized the relationship of the two molecularly linked groups through comparison of sequence

diversity of the two groups to the reference sequences, stratified by the following years: pre-2000, 2000–2004, and 2005–present. Reference sequences that did not have a date were removed from this analysis.

Initially, we considered the *env* region since it is more diverse than *pro/rt*, thereby providing a greater likelihood to observe differences between our linked samples and others from the cohort. For the three sequences suspected to be molecularly linked and having a single quasispecie, we generated a consensus sequence of the 10 *env* SGA obtained per individual and used each consensus for analysis of the genetic distance. For the two sequences with dual quasispecies, a separate consensus was generated for each quasispecie such that each of these dually infected individuals would be represented by two consensus sequences in the analysis. We chose to use consensus *env* sequences in the analysis because we did not want to overemphasize the signal observed if each of the SGA sequences was used separately. The overall comparison of Sea Service samples plus reference sequences showed a mean genetic distance of 0.142 (Fig. 4A). Interestingly, when sequences from this cohort were compared to the references characterized as pre-2000, 2000–2004, and 2005–present, a significant increase in genetic distance was observed from 0.136 to 0.149 to 0.152 (p -values<0.0001), respectively (Fig. 4B–D). Additionally, the comparison of cohort sequences to each other, without references, yielded a mean genetic distance of 0.152 (Fig. 4E). A comparison of the genetic distances of the consensus sequences from each member of the cluster is shown in Table 2 (top portion). The genetic distance of sequences in the two molecular clusters was less than 0.025 and well outside of the bell curve distribution observed in all comparisons, providing further evidence of the molecular link between these groups of sequences.

After removal of nucleotide positions associated with drug resistance, comparison of cohort and reference sequences in the *pro/rt* region showed a mean genetic distance of 0.066 (Fig. 5A). In parallel with *env* data, cohort samples from pre-2000, 2000–2004, and 2005–present showed a significant increase in genetic distance from 0.059 to 0.066 to 0.069 (p -values<0.0001), respectively (Fig. 5B–D). Through comparison between these cohort sequences, the same genetic distance of 0.069 was observed for reference sequences over the same time period (Fig. 5E). A comparison of the genetic distances of the consensus sequences from each member of the cluster is shown in Table 2 (bottom portion). The genetic distances of sequences in the two molecular clusters were less than 0.01 and fall outside the bell curve distribution.

Dating of linked molecular clusters

We used the Bayesian MCMC approach to calculate time to the MRCA of each linked sample and the overall time to MRCA for each cluster. All 10 *env* SGA sequences generated per individual were used in calculations and grouped as a single entity. The draw dates for each sample were used in the MRCA calculation. Additionally, the last negative test dates (4–18 months, see Table 3) were used as a guide to verify the MRCA calculations produced in the analysis.

With the dual cluster, 306159 and 306512, there was a large period of time between the two samples (Fig. 6A and Table 3). Sample 306512 had a mean MRCA calculation of 0.58 months

TABLE 2. PAIRWISE GENETIC DISTANCE OF THE LINKED GROUPS

	306159_b	306512	306344_b	306376	306517
<i>Env</i>					
306159_a	0.008	0.024			
306159_b		0.024			
306344_a			0.008	0.009	0.018
306344_b				0.010	0.017
306376					0.014
<i>Pro/rt</i>					
306159		0.006			
306344				0.003	0.009
306376					0.008

The two linked groups and the pairwise genetic distance of the consensus sequence of each sample within the group are shown. The top portion of the table shows *env* distances and the bottom portion shows *pro/rt* distances.

(95% upper–lower of 2.0–0.02) while that for 306159 was 53.5 months (95% upper–lower of 56.83–52.05) prior to the 306512 collection date (Fig. 6B). The MRCA of this dual cluster was calculated to be 56.1 months (95% upper–lower of 65.41–52.06), in agreement with the last known negative HIV-1 test for each individual (Table 3).

The cluster of three sequences, 306344, 306376, and 306517, had a shorter time period between samples, including two test dates within 3 months (Fig. 6C and Table 3). Sequence 306517 had a mean MRCA of 0.47 (95% upper–lower of 1.26–0.04), 306376 of 30.38 (95% upper–lower of 30.94–30.03), and 306344 of 34.32 months (95% upper–lower of 36.34–33.16) prior to the 306517 collection date (Fig. 6D). Overall, this cluster had an MRCA date of 34.41 months (95% upper–lower of 36.49–33.18), consistent with the last known negative HIV-1 test (Table 3).

Discussion

Using samples from enlisted U.S. Navy and Marine Corps personnel whose first known positive HIV-1 screening test occurred between January 1, 2005 and August 3, 2010, we characterized sequences from 40 of 54 cohort members with samples drawn in the seroconversion window. Non-B subtypes, including recombinant viruses, have been identified previously in U.S. military cohorts.^{7–10} In this study, all but one sequence in this cohort was subtype B, suggesting that 11 years of continuous large-scale combat and support operations has not significantly altered the HIV-1 subtypes previously reported to be circulating in Sea Services personnel.^{8–10}

The sequencing of two regions of the HIV-1 genome increased the likelihood of correct subtype designation. While linkage analysis did not change in this study from the use of two regions, others have used a similar strategy of sequencing multiple HIV-1 genes and have found this to be an effective tool to characterize transmission groups.^{21–25} The use of ML phylogenetic analysis was one method used to identify transmission clusters, but we also employed the calculation of the pairwise genetic distance over the *pro/rt* and *env* regions.²² Comparing the genetic distance of our linked samples to all subtype B reference sequences spanning the same regions, we observed that the genetic distance of our linked samples was below the mean distance of all reference

FIG. 5. Pairwise genetic distance calculations for the *pro/rt* region. The pairwise genetic distance for the *pro/rt* region was calculated for reference sequences and cohort sequences using the Kimura two-parameter model. For each figure section, the mean pairwise genetic distance is annotated in the center of each graph. (A) A comparison of references and cohort sequences; (B) cohort compared to references identified prior to the year 2000; (C) cohort sequences compared to references identified from 2000 to 2004; (D) cohort sequences compared to references identified from 2005 to the present; (E) cohort sequence comparison without any references.

sequences compared. Our group of two (306159 and 306512) and three (306344, 306376, and 306517) linked samples was separated by years between diagnoses. Samples 306159 and 306512 are separated by more than 4 years while 306344 and 306376 are separated by 3 months and 306376 and 306517 by

more than 2 years. These data are consistent with the findings of Redd *et al.*,²⁶ who reported that transmitted viruses more closely resemble donor archival sequences than the diverse quasiespecies observed in the donor prior to transmission. This further supports the idea that unique features of transmitted

TABLE 3. DATES OF IMPORTANCE FOR THE LINKED SAMPLES

Spin number	Last negative test date	First positive test date	Calculated HIV-1 acquisition date
306159	24-Feb-2004	14-Apr-2005	08-Mar-2005
306512	15-Apr-2009	20-Aug-2009	03-Aug-2009
306344	18-May-2006	09-Mar-2007	12-Feb-2007
306376	14-Dec-2005	04-Jun-2007	30-May-2007
306517	22-Aug-2009	21-Dec-2009	07-Dec-2009

Important dates associated with the five samples demonstrated to be molecularly linked. The last negative HIV-1 test date for each individual is given along with the first positive test date, which is the collection date of the sequences described here. The approximate acquisition date based on Bayesian MCMC analysis is also shown.

founder viruses are required to overcome the transmission bottleneck.^{27,28}

Even in the setting of short transmission periods, distinguishing between common or linked sources is problematic.^{29,30} To determine direction of transmission through these methods

would require multiple samples taken before and after infection for each individual in the transmission cluster.²⁹ This is not feasible in a force-wide screening program. A convenience survey of a subgroup of this cohort revealed several self-identified risk factors including multiple and varied sexual exposures during the risk window,³¹ further complicating any such determination. However, these methods combined with conventional epidemiologic approaches could provide strong transmission information relevant to public health.

Bayesian MCMC calculation for the time to the MRCA of each sample agrees with the initial date of diagnosis regarding which individual was diagnosed before another in each cluster. It is not known if others are involved in each network/cluster, either from the larger group of samples from this cohort that were not analyzed³ or part of the civilian population that interacts with this cohort. In addition, two samples, 306196 and 306501, whose genetic distance calculations to each other were relatively low, 0.074 for *env* and 0.035 for *pro/rt*, were characterized as unlinked because the genetic distance between the two sequences in *env* and *pro/rt* fall within the bell curve distribution of all reference sequences for each

FIG. 6. Relaxed-clock use for cluster analysis. The most recent common ancestor (MRCA) was calculated for each of the linked clusters using a Bayesian Markov chain Monte Carlo (MCMC)-based approach on the *env* region. The date of sample collection was used in the calculation of the MRCA. The dual cluster (306159, 306512) MCMC-based consensus tree is calibrated to the time prior to the 306512 collection date (A). A graphic representation, including the upper and lower 95% confidence interval for MRCA of each sample and the cluster as a whole, is shown (B). The tri cluster (306344, 306376, 306512) MCMC-based consensus tree is calibrated to the time prior to the 306517 collection date (C). A graphic representation, including the upper and lower 95% confidence interval for MRCA of each sample and the cluster as a whole, is shown (D).

region, as shown in Figs. 4A and 5A. A bridging sample was not identified in this subgroup of seroconversion, early-incident infections within this cohort.

A second observation made during this ancestor analysis was that the mean genetic distance of sequences from this cohort compared to reference sequences increased over the time period evaluated: pre-2000, 2000–2004, and 2005–present (Figs. 4B–D and 5B–D, respectively). Previous studies reported that HIV-1 genetic diversity increases by approximately 1% per year^{32,33} due to its high mutation rate.³⁴ Therefore, it is not unexpected that HIV-1 genetic diversity within subtype B increases over time. In fact, others have made similar observations with HIV-1 subtype B and C through comparison of the *gag* and *env* genes.³⁵ Taken together with previous observations regarding HIV-1 diversity increase over time, acquired subtype B HIV-1 in this cohort continues to be part of the larger HIV-1 epidemic rather than comprising an isolated network of transmitted viruses (compare Figs. 4D and E and 5D and E).

Among these 40 analyzed sequences, five (12.5%) were linked. While patients identified in the seroconversion window may differ from the overall cohort for a number of reasons including risk behavior prompted self-referral for testing, extrapolating to the entire cohort of Sailors and Marines in the parent public health investigation, as many as 62 individuals may have acquired linked HIV-1 viruses. While this would represent a minority of new HIV-1 infections in this cohort, minimizing the number of individuals who are part of transmission clusters is essential. More analysis is needed to incorporate chronic infections in such analyses, particularly ones identified in similar time frames and at the same locations as seroconverting infections. This would allow more robust mapping and characterization of transmission networks to include identification of persistent, common transmission nodes.

Previous studies have shown that HIV-1 transmission outbreaks often occur over a limited time frame^{21,30,36} and early identification of transmission groups would aid in the design of specific public health interventions to prevent future spread. A previous publication highlighted the use and potential positive impact of molecular surveillance on control of HIV-1 spread within targeted populations.³⁷ While care must be taken to protect personal confidentiality, real-time identification of transmission networks within the U.S. military may enhance public health interventions against HIV-1 transmission in the force and is an area of need for further study. Real-time molecular surveillance of HIV-1 acquisition in the U.S. military should be evaluated for its potential public health impact.

Acknowledgments

The authors would like to thank Dr. Angie Eick and the Department of Defense Serum Repository for reconciling and pulling the archived serum samples. We would also like to thank retired Navy Captain Neal Naito when at the Bureau of Medicine and Surgery who assisted in obtaining financial sourcing for this effort and Dr. Shilpa Hakre and Mr. Mark Milazzo of the U.S. Military HIV Research Program, Department of Epidemiology and Threat Assessment who assisted with force testing data management and interpretation. Finally, we would like to thank Taylor Cervenka, Holly Hack,

Janelle Kuroiwa, Annemarie O'Sullivan, and Laurin Stevenson for their technical support.

This project was funded in part by the U.S. Navy Bureau of Medicine and Surgery and the Military Infectious Diseases Research Program, project MIDRP-H0140100TPPOC. These studies were supported by a cooperative agreement (W81XWH-07-2-0067) between the Henry M. Jackson Foundation for the Advancement of Military Medicine and the Department of Defense.

The purpose of this research is to inform public health decisions regarding force health protection of U.S. military service members. The results obtained from this study will in no way be used in a criminal setting for any of the individuals described. This public health activity was reviewed by the Walter Reed Army Institute of Research (WRAIR) Institutional Review Board and termed RV314. The views expressed in this article are those of the authors and do not reflect the official policy or position of the Departments of the Army, Navy, or Defense, the Uniformed Services University, or any agency of the U.S. Government. Mention of trade names, commercial products, or organizations does not imply endorsement by the U.S. government.

Author Disclosure Statement

No competing financial interests exist.

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