Here we present genomic and in vitro analyses of temporally separated episodes of herpes simplex virus type 1 (HSV-1) shedding by an HSV-1–seropositive and human immunodeficiency virus (HIV)/HSV-2–seronegative individual who has frequent recurrences of genital HSV-1. Using oligonucleotide enrichment, we compared viral genomes from uncultured swab specimens collected on different days and from distinct genital sites. We found that viral genomes from 7 swab specimens and 3 cultured specimens collected over a 4-month period from the same individual were 98.5% identical. We observed a >2-fold difference in the number of minority variants between swab specimens from lesions, swab specimens from nonlesion sites, and cultured specimens. This virus appeared distinct in its phylogenetic relationship to other strains, and it contained novel coding variations in 21 viral proteins. This included a truncation in the UL11 tegument protein, which is involved in viral egress and spread. Normal immune responses were identified, suggesting that unique viral genomic features may contribute to the recurrent genital infection that this participant experiences.

**Keywords.** clinical; high-throughput sequencing; genital; oligonucleotide enrichment; comparative genomics.

Herpes simplex virus type 1 (HSV-1) is a neurotropic pathogen that is transmitted via close contact of mucosal surfaces. This large, double-stranded DNA virus is geographically widespread, with the highest seroprevalence (up to 90%) in low-income countries [1]. HSV-1 typically causes painful oral and/or genital lesions, and it can also cause infectious keratitis, eczema herpeticum, or, in rare cases, encephalitis [2]. The virus can transit via nerve endings from the skin or mucosa into neuronal nuclei, such as the trigeminal ganglia and dorsal root ganglia. By establishing latency in these neurons, HSV can evade the immune system and remain in the host throughout life [3, 4]. Upon reactivation in neurons, viral progeny transit back to the epithelium, where they may replicate further and transmit to new hosts [3]. Viral genome shedding can be detected in skin swabs by polymerase chain reaction (PCR) analysis. Although viral lesions generally have a higher genome copy number than nonlesion sites, both symptomatic and asymptomatic shedding can transmit virus to new hosts [5, 6].

There are 2 serotypes of HSV: historically, type 1 was the more prevalent cause of oral lesions, and type 2 was the more prevalent cause of genital lesions [3]. However, the proportion of first-episode genital HSV-1 cases has risen in recent years in high-income countries [1, 7]. Unlike genital HSV-2, which is characterized by frequent outbreaks, genital HSV-1 recurrences tend to be infrequent, even in the first year of infection [8, 9]. Recent studies have characterized the range of human immune control of HSV-1 and HSV-2 in the oral and genital mucosa [10–13]. These studies also demonstrated that specific HSV-1 proteins such as U39 (large ribonucleotide reductase subunit) and U48 (VP16 transactivator) are recognized by human CD8+ T cells [11, 13]. Owing to the similarity in pathogenesis of HSV-1 and HSV-2, it is likely that genital HSV-1 infection would cause comparable immune responses and mucocutaneous T-cell infiltration as genital HSV-2 [14].

A small percentage of individuals with genital HSV-1 infection have a high rate of recurrence beyond the first year of infection. It is unknown whether HSV-1 in these individuals differs genetically from other HSV-1 isolates that are successfully controlled by the host immune system, or if the ongoing recurrences represent an innate failure of the immune system. Prior approaches studying HSV-1 genetic variation in clinical cases such as these have relied on PCR analysis of single genes or on viral samples that have been expanded in tissue culture [15, 16]. Recent advancements in next-generation sequencing and targeted oligonucleotide enrichment now enable capture of whole viral genomes directly from mucosal surfaces, without expansion in culture [17–21]. This approach has been applied
to viruses such as human immunodeficiency virus (HIV) and human cytomegalovirus to reveal viral adaptation or evolution within a host and to deduce its potential impacts on pathogenesis and disease [18, 22, 23].

Here, we present the first genome sequences of uncultured mucosal swab specimens of HSV-1, which were collected from a study participant who has an unusually high recurrence rate of genital HSV-1. We used oligonucleotide enrichment to capture and sequence uncultured viral genomes directly from symptomatic lesions and nonlesion sites, and standard library preparation methods to sequence viral genomes from diagnostic lesion material expanded in culture. With these data, our goal was to establish the level of viral diversity present within a single host over a time period involving several rounds of latency, reactivation, and shedding. We were particularly interested in capturing viral genomes from nonlesion sites, as this has proven difficult prior to oligonucleotide enrichment. We hypothesize that the unique genetic signatures of this virus may correlate with the clinical characteristics of genital HSV-1 disease that this participant experiences.

**METHODS**

**Participant and Sample Collection**

The participant had positive results of a type-specific HSV immunoblot assay [24]. Swab specimens were collected every weekday for 4 weeks by using a spatial grid of the female genital tract, as previously described [25]. An aliquot of each swab specimen was used for column-based DNA isolation and quantitative real-time PCR (qPCR) analysis to detect HSV-1 genomes [25, 26]. The participant also had symptomatic genital HSV-1 recurrences before and after the swabbing study; specimens from associated lesions were collected for HSV-1 culture. This individual's virus is referred to hereafter as "v.29." The University of Washington Human Subjects Division approved the study, and the participant provided informed consent.

**Cell Culture and Virus Expansion**

Virus stocks were propagated and titered on African green monkey kidney cells (Vero cells [ATCC CCL-81]; Supplementary Materials). Cultured virus was obtained from samples collected over 4 months: v.29_day(-88)_culture1 was collected in March, v.29_day1_culture2 was collected in June, and v.29_day32_culture3 was collected in July (Figure 1). HSV-1 strain McKrae was provided by Lynda Morrison [27], and strain KOS63 was provided by Richard Dix [15]. Viral nucleocapsid DNA was isolated from v.29 cultures after 5 passages, using published protocols [28].

**Sample Library Prep, Illumina Sequencing, and Viral Assembly**

HSV-positive swab specimens were processed using phenol:chloroform DNA extraction (Supplementary Materials).

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**Figure 1.** Cultures and a 30-day survey of spatially distinct viral shedding in a participant with frequent recurrence of genital herpes simplex virus type 1 (HSV-1). A, Genital HSV-1 shedding detected over 1 month in a single study participant is depicted on spatial grids (expanded view, center) of the female genital tract. HSV-1 genome copy number was detected by real-time quantitative polymerase chain reaction (qPCR) analysis and color-coded as log_{10} copies/mL detected in each genital location (see color-coded legend on right). The time line depicts days on which lesions (L) were observed, asymptomatic shedding (A) was present, and no swab specimens were collected (eg, on weekends; white boxes). Lesion sites are outlined in a thick black line on the genital grids. B, Expanded view of individual genital lesions from 3 consecutive days, where the extracted regions illustrate the specific day and site of each swab used for viral genome sequencing (Figure 4). C, Tiled phase image of v.29 plaque size and morphology 72 hours after infection of a Vero cell monolayer. The v.29 culture is from the third round of passaging of v.29_day(-88)_culture1. This image highlights the heterogeneity present in the plaque phenotypes of this participant’s virus, similar to what has been observed for other HSV-1 isolates [30]. The inset is an expanded view of the entire well. The scale bar represents 25 μm.
Sample DNA was sheared into approximately 500-bp fragments, processed using a KAPA LTP Library Preparation Kit, and enriched for viral DNA by using Roche/Nimblegen custom oligonucleotide baits designed and validated by the Bloom lab (Dhummakupt et al, unpublished). Enriched libraries were sequenced on an Illumina MiSeq, using version 3 chemistry and 300-bp paired-end reads. Viral genomes were assembled using the VirGA work flow as previously described [29]. Supplementary Table 1 contains GenBank identifiers and sequencing statistics for all v.29 genomes.

Consensus Genome Comparison and Phylogenetic Analysis
Trimmed versions of viral genomes were used for consensus genome comparison [30]. MAFFT, version 7.313, was used for pair-wise global nucleotide alignments; ClustalW2, version 2.1, was used for pair-wise amino acid alignments; and custom Python scripts were used to calculate protein coding and DNA differences between samples (Supplementary Materials). Phylogenetic networks were constructed with SplitsTree4, version 4.13.1. Supplementary Table 2 has details of previously sequenced isolates. Additional files related to comparative genomics analyses are available at: https://scholarsphere.psu.edu/collections/bcc08hg256.

Intrastrain Minority Variant (MV) Detection and Validation
Each consensus genome was analyzed for MV loci, using VarScan, version 2.2.11; SnfEff; SnpSift; and the Integrative Genomics Viewer (Supplementary Materials). Parameters used to differentiate true MVs from technical artifacts were as follows [17]: minimum variant allele frequency of ≥0.02 (2%), base call quality of ≥20, position read depth of ≥100, and ≥5 independent reads supporting the minor allele. Polymorphisms with directional strand bias of ≥90% were excluded.

T-Cell Responses
CD8+ T-cell responses were studied by an interferon-γ enzyme-linked immunospot assay, using peripheral blood mononuclear cells and 3 pools of 117 HSV-1 peptides (Supplementary Materials). Single peptides were identified through deconvolution, as previously described, with a peptide concentration of 1 μg/mL [11]. Interferon-γ expression by CD4+ T cells was determined using UV-inactivated HSV-infected Vero cells [31]. Net values were obtained by normalizing experimental findings to values for the negative control wells.

Western Blotting
Vero cells were lysed in radio immunoprecipitation assay buffer, and soluble protein supernatants were electrophoresed and blotted (Supplementary Materials).

RESULTS
Genital Shedding of HSV-1 Varies in Space and Time
During a study of spatial localization of HSV-2 shedding patterns, we enrolled a single participant with a clinically severe phenotype of genital HSV-1 infection. This 32-year-old HSV-1–seropositive, HIV/HSV-2–seronegative woman (participant 29) acquired genital HSV-1 five years earlier and had experienced 4 recurrences in the past year.

Swab specimens were collected from 22 sites throughout the genital tract of the participant during every weekday over a 30-day period (Figure 1). HSV-1 shedding was detected by qPCR on 15 of 20 days (75%), with a lesion observed on 8 (40%). On day 1 of the anatomic shedding survey, there was a genital lesion at site 16, which persisted until day 5 (Figure 1A). A separate lesion developed at a physically distant location (site 5) on days 8–10. On other days, there was either asymptomatic shedding or no virus detected (Figure 1A). On days when a lesion was present, the median number of sites with HSV-1 detected was 20, and the median quantity of virus detected was 7.2 log10 genome copies/mL. On days with only asymptomatic shedding, the median number of HSV-positive sites was 4, and the median quantity of virus was 2.3 log10 copies/mL. These data were similar to those found previously for HSV-2, where shedding was detected throughout the female genital tract in the presence of a lesion [25].

Genomic Comparison of v.29 to Other Known HSV-1 Isolates
We hypothesized that the severe phenotype of genital HSV-1 infection in this participant might be related to unique genetic features of this virus (referred to as v.29). Using HSV-positive DNA from 7 mucosal swab specimens collected from anatomically-separate areas during days 1–3 (Figure 1B), we performed oligonucleotide enrichment and high-throughput sequencing, followed by de novo genome assembly, of this virus (Supplementary Table 1). These swab specimens were chosen because they had relatively high viral loads (≥5.0 log10 HSV-1 genome copies/mL) and included lesions as well as nonlesion sites (Figure 1B). Using the viral consensus genome assembled from each swab specimen, we graphed a network of genetic relatedness between v.29 and 47 other published HSV-1 genomes. We found that v.29 formed its own branch in this network, separate from viral isolates of known geographic clades (Figure 2).

We also performed amino acid alignments of every HSV-1 protein to compare the coding sequences of v.29 to those of other known HSV-1 strains. This revealed 21 viral proteins containing a total of 27 unique amino acid differences that have not been previously observed in any HSV-1 strain (Supplemental Table 3). These novel coding variations likely contributed to the distinct location of v.29 in the phylogenetic network (Figure 2). We also identified a truncation of the last 10 amino acids of UL11 (Figure 3A and 3B), a viral tegument protein involved in secondary envelopment, viral egress, and cell-to-cell spread [32]. This UL11 truncation has been observed only once before, in an HSV-1 isolate from a German patient with eczema herpeticum, whose viral genome clustered near v.29 in the phylogenetic network (Figure 2) [33]. To test whether this truncation...
had an effect on UL11 protein size or expression in v.29, we used Western blot analysis. We identified a shift in the observed molecular weight of the UL11 protein in v.29 and a loss in overall expression of UL11 relative to strains of HSV-1 with an intact UL11 (Figure 3C).

Comparison of HSV-1 Genomes From Genital Swab Specimens Versus Cultured Specimens, at the Consensus Level
To determine whether genital HSV-1 genomes vary over space and time in an infected individual, we compared the v.29 genomes derived from 7 swab specimens collected over 3 days to each other and to those of 3 culture specimens of v.29 collected over 4 months (Figure 1B and 1C). Viral cultures were analyzed using standard techniques for the isolation of viral nucleocapsid DNA and subsequent viral genome sequencing [28, 29]. Comparison of the consensus-level viral genomes from each swab specimen and culture to one another revealed an average pair-wise DNA identity of 98.5% (Figure 4). Comparing among these, genomes from the day 1 lesion swab specimen and cultured specimen were 98.4% identical, those from the day 2 swab specimens were 98.5% identical, and those from both day 3 swab specimens were 98.6% identical. We observed 1 single-nucleotide variant (SNV) at the consensus level, which occurred in the v.29_day32_culture3 sample (Figure 4). This SNV was located in a noncoding section of the genes LAT and RL2 in the repeat region, and no micro-RNAs were impacted. There were 27 unique insertions or deletions (in/dels) observed in the alignment of these 10 viral genomes, and many of these in/dels manifested as fluctuations in length of homopolymers and tandem repeats. The overall level of viral nucleotide identity between swab specimens and related viral cultures was comparable to that observed between subclones of a single HSV-1 strain [29].

Different Minority Variants Detected in Anatomically- and Spatially-Separated Swabs and Cultures
Minority variants (MVs) within a viral population can provide insights on viral fitness, evolution, and intra-host and inter-host bottlenecks [17, 23, 34]. In contrast to SNVs, which occur at the consensus level and represent the major allele in the population at a particular nucleotide position, MVs exist below the consensus level as alternative minor...
alleles (with a frequency ≥2% and <50%). We examined the MVs in each v.29 sample to determine whether their frequencies or genomic locations differed between spatially or temporally separated swab specimens or cultured specimens. Although only a portion of MVs occurred inside of genes, the codon-specific annotation of these “genic” variants allowed for straightforward comparison across samples. We found that MVs in the 7 swab specimens and 3 cultured specimens were distributed across the genome, with many in the viral genes UL13 (7 MV), UL36 (66 MV), RL1 (15 MV), and RL2 (6 MV) (Figure 5A). After normalization of these data for gene length, UL11, UL36, and RL1 contained the most MVs (data not shown). We found that the largest number of MVs were present in the uncultured lesion swab specimens isolated on days 1 and 2 (302 total). Nonlesion swab specimens had 29–83 MVs present per sample, while cultured specimens had 46–72 MVs each (Figure 5A). Surprisingly, the cultured specimen collected on day 1 had only 67 MVs, in contrast to the matching day 1 uncultured lesion swab specimen (271 MVs). We also examined the penetrance or frequency of each minor allele and found that most had a frequency of ≤10% (Figure 5B). Higher-frequency MVs (>10%) occurred in the cultured virus samples, in genes UL13 and UL14, and in all genomes at sites of homopolymer or tandem repeats (eg, in UL36 and RL1). Taken together, these data suggested that there was substantial variation present in the viral populations of lesions, compared with nonlesion sites, and that variation decreased during viral introduction to culture.

Analysis of HSV-1–Specific Immune Responses

Another explanation for the high rate of genital HSV-1 shedding and recurrence in this participant could be an insufficient immune response. To test this, we analyzed CD8+ T-cell responses to HSV-1 peptides in a standard assay used in prior HSV studies [11, 31]. We found that this individual had CD8+ T-cell responses to peptide

![Figure 3.](https://academic.oup.com/jid/article-abstract/218/4/595/5039658)
pools containing HSV-1 epitopes (Figure 6A). Following deconvolution, specific epitopes mapping to known HLA-A*0101-restricted CD8+ T-cell epitopes in HSV-1 protein VP11/12 (encoded by *UL46*) and VP16 (encoded by *UL48*) were identified (Figure 6B) [10, 13]. The participant was verified as positive for HLA-A*0101.

The HSV-1–specific CD4+ T-cell interferon-γ response was also intact (Figure 6A). This individual also tested seropositive for HSV-1 by Western blot (data not shown) [24]. These results suggested that a defective cellular immune response was not the cause of the frequent recurrence of genital HSV-1 in this individual.

DISCUSSION

This is the first study to examine anatomically separated genital HSV-1 shedding patterns for a single participant over a multiday period (Figure 1). We present data from an individual with frequent genital HSV-1 lesions and a high rate of viral shedding, even several years into her infection. Recurrences of genital HSV-1 after the first year occur in only 5% of infected individuals [25]. We found that v.29 had unique phylogenetic signatures and did not cluster with known HSV-1 genomes (Figure 2). v.29 also had a unique truncation in the UL11 viral tegument protein (Figure 3). We observed conservation in viral genomes—at the consensus level—between swab specimens collected over 3 days and cultured specimens collected over 4 months (Figure 4). However, at the level of MVs, we observed a >2-fold decrease in the average number of MVs—and a 6-fold decrease in genic MVs—in nonlesion swab specimens and cultures relative to the lesion specimens (Figure 5). The available data indicate that this participant has a typical CD4+ and CD8+ T-cell response (Figure 6), although it is possible that a quantitative
or qualitative difference in these responses is casually associated with her clinical phenotype. These data suggest that viral genomic factors may be impacting the relatively severe clinical phenotype that this participant experiences. Alternatively, she may not have localized HSV-1–specific T-cells to the genital region either acutely, during recurrences, or chronically, as tissue-resident memory cells that locally persist after resolution of episodic infection [12, 35, 36].

Among the unique amino acid variants observed in v.29, we identified a truncation in the viral tegument protein UL11, which is involved in secondary envelopment, viral egress, and cell-to-cell spread [32]. This truncation, due to the presence of an early stop codon, is associated with decreased expression of the UL11 protein in v.29 (Figure 3C). UL11 has homologues in all known herpesviruses and interacts directly with the HSV-1 proteins glycoprotein E, UL21, and UL16 [32, 37]. This truncation affected the UL11 C-terminus near its known site of interaction with glycoprotein E, which functions in both cell-to-cell spread and in binding to the Fc domain of HSV-specific immunoglobulin antibodies [38]. It is unclear what advantage a truncated UL11 would provide, but perhaps it could lead to a hyperactive glycoprotein E interaction with the Fc domain of immunoglobulin G, resulting in improved immune evasion. The effects of UL11 and other unique amino acid variants in v.29 warrant exploration in future work.

Prior studies of clinical HSV infection have entirely used cultured virus to examine genetic variation, typically via restriction fragment–length polymorphism assays [33, 39–41]. The data from this study suggest that, at the consensus level, anatomically-separated swab specimens of genital HSV-1 are relatively stable over 3 days (Figure 4). The high level of DNA identity between v.29 genomes from swab specimens is comparable to that of HSV-2 genomes from longitudinal samples of a single person [42, 43], while it is less divergent than HSV genomes recovered from different individuals [29, 30, 43].

Deep sequencing of viral genomes can detect variants with frequencies below the detection limit of Sanger sequencing (ie, approximately 20%). Analyses of RNA viruses such as HIV...
and hepatitis C virus (HCV) often include MVs at frequencies of ≤2% [22, 44]. We used a conservative cutoff of ≥2% to detect, with high confidence, MVs in the v.29 genomes [17, 23]. We observed more MV diversity in the viral populations of lesions from this participant than from any other sample type analyzed (ie, nonlesion swab specimens and cultured specimens). One of the lesions (Supplementary Table 1) analyzed (ie, nonlesion swab specimens and cultured specimens from this participant) had high coverage, so we controlled for depth of coverage by randomly downsampling these data to match the average coverage of the other samples. Following downsampling, we still detected more MVs in this lesion swab specimen than in all other sample types analyzed (data not shown). This raises the intriguing possibility that viral diversity could be linked to the level of immune surveillance associated with a viral lesion [25]. In line with potential evidence of immune selection, we also identified 7 MVs in VP11/12 (UL146) and VP16 (UL48), which appeared to be under T-cell surveillance in this participant (Figures 5A and 6B). When the v.29 lesions were cultured, there was an overall loss of MV diversity, suggesting a bottleneck in the viral population at the initial point of culture (Figure 5A). However, these cultured isolates also contained MVs that were not detected in uncultured swab specimens of v.29, suggesting genetic drift or adaptation following culture (Figure 5). These “culture-specific” MVs (eg, in UL13 and UL14) resemble those documented in other herpesviruses during culture adaptation and loss of gene functions while passing in vitro [29, 45–47].

We studied an individual who acquired HSV-1 5 years prior, and, therefore, any viral adaptation to the genital niche or her immune response may have already occurred [8, 48]. Although it is plausible that daily swabbing of the genital mucosa could affect the observed genital shedding patterns, the participant’s prior 5 years of repeated recurrences suggests otherwise. We also cannot rule out that the viral genomes and shedding patterns observed here could represent spatial cross-contamination, rather than simultaneous reactivation of virus from multiple ganglia. However, in previous work exploring anatomic shedding patterns of HSV-2, several controls were used to address this possibility [25], and it was concluded that simultaneous reactivation, rather than contamination, accounted for the observed HSV-2 shedding patterns. In this study, we found minor genetic variants between swab specimens collected on the same day at different genital sites, providing further support for simultaneous shedding, rather than cross-contamination.

One of the strengths of this study was the implementation of an oligonucleotide enrichment strategy that enabled successful sequencing and assembly of the first uncultured genital HSV-1 genomes. We observed that the differences in HSV-1 genome copy number and the ratio of viral to host DNA impacted the overall yield and viral genome recovery of each library. The coverage of all v.29 genomes met or exceeded that of prior genomic studies of cultured HSV-1 [29, 30, 33]. However, the high G+C-content, repetitive elements, and potential recombination breakpoints in the large structural repeats (Figure 4A) could affect the reliability of these regions in each viral genome assembly and subsequent alignments [30, 49].

Obtaining a genomic snapshot of spatially- and temporally-separated uncultured HSV-1 swab specimens to study viral adaptation within a host is now achievable. This intensive study of a person with a phenotypically severe genital HSV-1 infection suggests that viral diversity exists over a short time frame within a person, albeit
on the level of minority variants, rather than consensus-level coding changes. These findings also suggest that viral genetic variation may be a key factor contributing to clinical phenotype. Future studies can now focus on detecting how transmission between individuals affects the frequency of these minority variants, and how viral populations adapt during the initial phase of infection, when an individual’s innate and adaptive immune response are first developing. Determining the genetic relationship of virus isolated from oral versus genital niches is also of interest, given that oral-genital transmission is likely a key component of current trends in HSV epidemiology [1, 9].

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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