CHAPTER TWO

A holistic perspective on herpes simplex virus (HSV) ecology and evolution

Molly M. Rathbun and Moriah L. Szpara*

Department of Biochemistry and Molecular Biology, Department of Biology, Center for Infectious Disease Dynamics, and Huck Institutes of the Life Sciences, Pennsylvania State University, University Park, PA, United States

*Corresponding author: e-mail address: moriah@psu.edu

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Abstract

Herpes simplex viruses (HSV) cause chronic infection in humans that are characterized by periodic episodes of mucosal shedding and ulcerative disease. HSV causes millions of infections world-wide, with lifelong bouts of viral reactivation from latency in neuronal ganglia. Infected individuals experience different levels of disease severity and frequency of reactivation. There are two distantly related HSV species, with HSV-1 infections historically found most often in the oral niche and HSV-2 infections in the genital niche. Over the last two decades, HSV-1 has emerged as the leading cause of first-episode genital herpes in multiple countries. While HSV-1 has the highest level of genetic diversity among human alpha-herpesviruses, it is not yet known how quickly the HSV-1 viral population in a human host adapts over time, or if there are population bottlenecks associated with viral reactivation and/or transmission. It is also unknown how the ecological environments in which HSV infections occur influence their evolutionary trajectory, or that of co-occurring viruses and microbes. In this review, we explore how HSV accrues genetic diversity within each new infection, and yet maintains its ability to successfully infect most of the human population. A holistic examination of the ecological context of natural human infections can expand our awareness of how HSV adapts as it moves within and between human hosts, and reveal the complexity of these lifelong human-virus interactions. These insights may in turn suggest new areas of exploration for other chronic pathogens that successfully evolve and persist among their hosts.

1. Introduction

Virus genetic diversity is vast and presents itself in many ways. For instance, RNA viruses lacking polymerase proof-reading capability can mutate quickly via base misincorporation. The accrual of polymerase error can cause genetic drift in the genotypes circulating among populations of infected hosts (e.g., influenza virus). Under the right conditions, viruses can spread rapidly among many hosts, and mutations can expand within the population along the way (e.g., severe acute respiratory syndrome coronavirus 2). Other viruses evolve rapidly within individuals, creating an explosion of within-host diversity to compete with the host immune system (e.g., human immunodeficiency virus). In contrast to these RNA viruses, DNA viruses with polymerase proof-reading capability do not accrue misincorporation errors as quickly, and yet these viruses infect billions of human hosts around the world and show a unique genetic signature in each randomly sampled infection (e.g., herpes simplex virus 1). In this review, we probe the question of how herpes simplex virus 1 (HSV-1) has become so genetically diverse around the world and so prolific at spreading in the human population, and what ecological factors make this possible. This

virus, also known as *Human alphaherpesvirus 1*, is part of the *Simplexvirus* genus, along with the distinct species herpes simplex virus 2 (HSV-2). The factors that contribute to the overall ecology of both HSV species are many. At the smallest scale (e.g., of cellular infections), these factors include the molecular life cycle of the virus, its ability to generate genetic diversity, and its cellular tropism. At the level of individual hosts, these ecological factors include host-pathogen immune interactions, viral capacity to induce reactivation and shedding, within-host flux in the viral population, and interactions among coincident microbial species. At the scale of human populations, virus ecology includes host behaviors that enable transmission, the prevalence of naïve vs infected hosts, and the efficiency of viral spread between hosts. Understanding these ecological factors in a holistic sense will improve our understanding of how HSV adapts as it moves within and between human hosts, help reveal the complexity of these lifelong human-virus interactions, and expand our awareness of how viruses successfully evolve and persist among their hosts.

To make sense of the wide array of virus life cycles, human and animal viruses are often categorized as either acute or chronic. This generalization was historically useful, as it helped researchers generate predictions for how quickly a new virus might evolve or how the infection might progress (e.g., potential disease manifestations and severity). In general, RNA viruses generate new mutations quickly—e.g., "mutant swarms"—and are often associated with acute infections. In contrast, DNA viruses tend to develop mutations more slowly and are often capable of establishing chronic or long-lasting infections. Early measurements of HSV-1 and other herpesviruses in laboratory settings indeed indicated very low rates of polymerase error. However, over the past two decades, new research on the evolution of large, complex DNA viruses has challenged this paradigm. Lab-based experimental evolution of HSV-1 populations has demonstrated the accumulation of minor variants and changes in population diversity that lead to relatively rapid consensus-level changes (Kuny et al., 2020). Studies of multiple herpesvirus species in clinical settings have shown significant diversity between randomly sampled infections (e.g., between-host diversity), as well as varied levels of within-host diversity (Akhtar et al., 2019; Depledge et al., 2014; Johnston et al., 2017a; Lassalle et al., 2020; Renzette et al., 2011, 2013; Shipley et al., 2018, 2019). Laboratory studies suggest that population bottlenecks and expansions significantly impact the observed prevalence of mutations in HSV-1 populations, and the ability of accumulated variants to be carried over from one generation to the next. (Jaramillo et al., 2013;

Kuny et al., 2020; Sasadeusz et al., 1997). Population bottlenecks and expansions could in turn lead to an observed frequency of genetic differences that is much higher on the global scale than that observed during a single viral replication cycle. Moreover, the rate of fluctuations in insertions and deletions is rarely mentioned, despite prior data showing that repetitive elements in the HSV genome differ in length between isolates. The variety of mutation mechanisms and population dynamics cataloged through studies of HSV-1 infections, in the context of a high seroprevalence in the population, may begin to explain how even rare mutation events occur frequently enough to impact the evolutionary trajectory of this life-long human pathogen. To attain a better understanding of HSV-1 and other DNA virus mutation rates, future studies will need to encompass virus population dynamics and higher-level ecological factors. Here we review HSV-1 from both the molecular and clinical perspectives, and we propose a holistic ecological paradigm for future studies of HSV-1 evolution.

2. Mechanisms for generating viral diversity at the molecular level

2.1 Polymerase error and mutation frequency

Herpesviruses have large, dsDNA genomes, and encode their own polymerases with proof-reading capability (Sanjuán and Domingo-Calap, 2016; Sanjuan et al., 2010). This fact sets up the expectation that these viruses would have a low mutation rate, or number of base misincorporations per replication cycle, compared to non-proofreading RNA viruses. Early measurements of mutation rate in specific HSV-1 genes tended to be on the order of 1×10^{-8} mutations per base per infectious cycle (Drake and Hwang, 2005). However, later examination via sequential plaque-to-plaque transfers of HSV-1 estimated the number of mutations at 3.6×10^{-4} per base per plaque transfer (Jaramillo et al., 2013). These data were used to suggest that the higher observed mutation frequency in the HSV-1 population could be due in part to bottlenecks and the effects of Muller's ratchet—the hypothesis that small asexual populations will accumulate deleterious mutations over time (Jaramillo et al., 2013). Additionally, continued characterization of the high G+C content among herpesviruses, which is highest among human herpesviruses in the HSV-1 genome, has provided evidence for non-standard DNA structures such as G-quadruplexes (Artusi et al., 2015; Biswas et al., 2016; Guiblet et al., 2021). These structures can challenge polymerase processivity, increasing the local error rate and ergo local

mutation frequency (Guiblet et al., 2021). HSV-1 mutation rates are likely influenced by polymerase activity, DNA repair mechanisms, and genome characteristics such as G + C content and quadruplexes, as has been reviewed extensively elsewhere (Kuny and Szpara, 2020; Renner and Szpara, 2018; Wilkinson and Weller, 2003).

2.2 Standing variation in the viral population

Whole genome sequencing of HSV-1 samples from around the globe indicate an appreciable 3–4% difference in DNA identity—across the ~152 kb genome—between any two randomly selected infections (Bowen et al., 2019; Szpara et al., 2014). Such genome-wide genetic diversity, in combination with the in vitro mutation rate studies mentioned above, strongly suggest that HSV-1 infections generate some degree of genetic diversity, or standing variation in the viral population, within each infected host. Standing variation within individual human infections has been detected in clinical specimens for multiple herpesvirus species (Lassalle et al., 2020; Minaya et al., 2017; Renzette et al., 2014; Shipley et al., 2018, 2019; Weiss et al., 2018). It is likely that natural HSV-1 infections also carry genetic diversity in the form of insertions, deletions, and fluctuations in tandem repeat length, although these features are less well-studied due to technical limitations on their validation by deep sequencing technologies (Deback et al., 2009, 2010; Merkel and Gemmell, 2008; Szpara et al., 2014).

2.3 Within-host vs between-host variation

While viral polymerase error has been measured experimentally, and within-host variants have been detected in clinical studies of HSV-1 in humans, it is still not clear how these data relate to between-host differences observed around the world. At the level of within-host variation, HSV-1 nucleotide diversity is reported to be less than 1.5×10^{-3}, with an average of $\sim 3 \times 10^{-4}$, as detected from clinical isolates (Bowen et al., 2019; Cudini et al., 2019). Thus, the question remains as to whether this level of standing variation is sufficient to account for the 3–4% difference in viral genetic identity between infections of independent hosts. These comparisons between hosts are typically made at the level of the viral consensus genome, which represents the most common nucleotide detected at each genomic position during deep sequencing of the viral population (see Fig. 1). It is not yet known how many HSV-1 infections exist with above-average levels of within-host diversity. A recent study of the beta-herpesvirus human

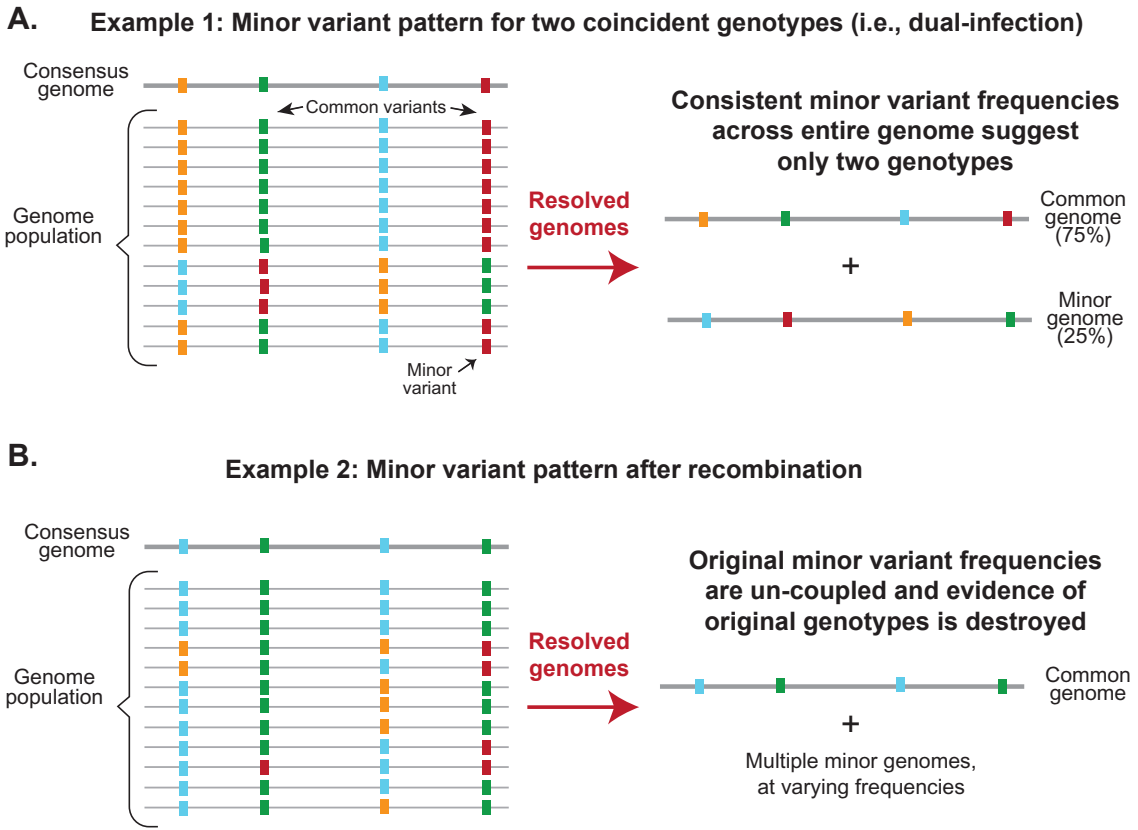

Fig. 1 See figure legend on opposite page.

cytomegalovirus used viral haplotype reconstruction to reveal that patient samples with higher than average within-host diversity did not have unusually high mutation rates, but rather reflected superinfection of the human host with distinct virus strains (Cudini et al., 2019). Superinfections or dual-strain infections occur when an individual acquires two genetically distinct infections of the same species, either coincidentally or through subsequent exposures. While not many examples have been documented, this has been shown to occur for both HSV-1 and HSV-2 infections (Heller et al., 1982; Johnston et al., 2017b). When applying deep sequencing technologies, as in Cudini et al. (2019), superinfections can be reflected in the number of minor variants detected and their frequencies. Observing many minor variants that are scattered across the viral genome at similar frequencies would suggest superinfection, while non-correlated frequencies may instead reflect mutation accumulated through replication errors (Fig. 1). Similar studies of clinical specimens collected from HSV-1 cerebral spinal fluid also suggest that some individuals carry above-average levels of within-host viral genetic diversity, although this may differ between samples and niches within the same person (Lassalle et al., 2020; Shipley et al., 2018, 2019).

2.4 Viral recombination within human hosts

Experimental infections and phylogenetic analyses both suggest that HSV-1 genomes are able to undergo extensive recombination (Lee et al., 2015; Loncoman et al., 2017; Norberg et al., 2004; Sakaoka et al., 1994). Such

Fig. 1 Minor variant patterns in deep sequencing data can reflect different genomic states of HSV-1 infections. The consensus genome, shown as a thick gray line at the top of each genome population, represents the most common allele or variant that has been identified at each nucleotide locus. Within-host or within-sample genetic diversity in the viral population can be quantified by examining the "minor variants" at each nucleotide locus (depicted as colored boxes on linear genomes). Because all deep-sequencing approaches to date involve fragmentation of the viral genome and computational re-assembly after sequencing, the co-linearity of these allelic variants has been inferred from allele frequency distributions or verified using secondary methods (Houldcroft et al., 2017; Renner and Szpara, 2018). (A) In some cases, minor variant frequencies are nearly uniform across the entire ~152 kb genome, which is suggestive of the presence of a second, low frequency genome, especially if any variants are physically linked on the same sequencing reads. (B) Experimental infections and phylogenetic analyses have demonstrated that HSV-1 genomes undergo rampant, genome-wide recombination (Lee et al., 2015; Loncoman et al., 2017). This molecular mechanism would allow for constant "shuffling" of variants between infecting genomes, and it could result in a minor variant pattern with numerous loci having a wide range in variant frequencies.

recombination events have the potential to drastically increase the rate of within-host shifts in minor variant frequency, by shuffling variants between viral genomes. This adds nuance to the explanation of minor variant patterns above, since genetically distinct strains within a superinfection may undergo recombination in a way that de-couples variant frequencies (Fig. 1) (Lassalle et al., 2020). HSV-1 circulates within the human population at roughly 60% seroprevalence (Looker et al., 2015), and infected individuals carry the virus with them for their entire lives. The high prevalence of a lifelong infection transmitted through close contact results in new opportunities for virus transmission with every new partner. Under these conditions, even rare events, such as low mutation rates and sporadic recombination, could be compensated for by a high prevalence rate of a lifelong chronic infection. It is unclear how often HSV-1 dual-infections occur. However, recombination between HSV-1 genomes circulating world-wide may contribute to the high level of genetic diversity observed in an otherwise-stable viral genome. It will be interesting to compare the within-host viral genetic diversity of individuals who are multiple years into their infection relative to that observed in primary infections. If individuals who have been infected for multiple years have had more time to be exposed to multiple HSV-1 genomes from different sources (e.g., partners) their within-host viral genetic diversity may reflect this in a trend toward the minor variant pattern shown in Fig. 1B. Comparing primary and established infections using whole-genome deep-sequencing approaches may help to shed light on the development of within-host HSV-1 diversity, and the generation of new consensus genotypes.

3. Clinical sampling and experimental design considerations

3.1 Challenges for direct human sampling

Collecting samples of any virus within its natural ecological context often entails complications well beyond those involved in laboratory-based experimental infections. For herpesviruses that infect wild animals, access to those natural populations might be limited, permits to work with the host species may be required, and the ideal sample types may or may not be feasible to collect. These factors are just as important to consider when sampling HSV-1 from natural human infections. While seroprevalence indicates that HSV-1 is widespread—e.g., 50% of adults in the Americas

(Looker et al., 2015)—not all infected individuals experience symptomatic outbreaks. Because HSV infections are lifelong and incurable, the primary clinical goal for infected individuals is to manage symptoms, and to reduce the likelihood of transmission to uninfected partners (e.g., through antiviral suppression therapy, and/or avoidance of contact during symptomatic outbreaks) (Johnston and Corey, 2015). There is a social stigma associated with genital herpes, and to a lesser extent with orolabial herpes, which can present a barrier to identification and clinical study enrollment of infected individuals. However, even for individuals willing to contribute samples for clinical studies, there is the added challenge that HSV reactivation occurs spontaneously and unpredictably in each individual. There is no way to predict the timing of viral shedding (i.e., viral release from the skin), nor to intentionally trigger a reactivation to allow precise sample collection on a clinically-controlled timeline (Johnston and Corey, 2015). Thus most studies on the natural ecology of HSV shedding have utilized repeated sample collections, spanning days or weeks, in order to document the rate and frequency of viral shedding and lesions (Johnston et al., 2014; Ramchandani et al., 2016; Schiffer et al., 2011; van Velzen et al., 2013).

3.2 Enriching for HSV DNA in direct-from-patient samples

With recent advances in the sensitivity and affordability of deep sequencing technologies, an increasing number of studies are examining within-host diversity for multiple human herpesviruses (Casto et al., 2020; Cudini et al., 2019; Depledge et al., 2014; Johnston et al., 2017a; Kaymaz et al., 2020; Lassalle et al., 2020; Renzette et al., 2014; Shipley et al., 2018, 2019). Several recent studies have successfully applied in-solution oligonucleotide enrichment to retrieve HSV-1 DNA from sparse clinical samples, with sufficient quantity for the assembly of complete viral genomes, as well as the deep sequencing of the viral population within the host (Lassalle et al., 2020; Shipley et al., 2018, 2019, 2020). Deep sequence coverage of the viral population allows for the detection of standing variation, or minor variants, that are otherwise not captured in a consensus-level genome assembly (see Fig. 1). This approach can be utilized to detect viral variants that arise over time, whether in sequential clinical samples from a single individual (Minaya et al., 2017; Shipley et al., 2018, 2019), or in lab-based experimental evolution studies (Jaramillo et al., 2013; Kuny et al., 2020). Studies applying a similar approach will continue to be important to capture HSV-1 genetic diversity and associate it with distinct aspects of clinical infection.

3.3 Analyzing deep sequencing data for all types of genetic variation

Many deep-sequencing studies of HSV-1 are limited to analyzing single-nucleotide differences at the consensus level, or to single-nucleotide minor variants in the viral population (e.g., Fig. 1). While useful, these metrics only capture one aspect of genetic diversity. The focus on single-nucleotide loci excludes a whole swath of diversity mediated by insertions and deletions, tandem repeat length fluctuations, and larger structural variants. Length differences arise at these loci at both the consensus-level and the minor-variant level. Other recent reviews have covered the potential for new long-read sequencing technologies to improve the resolution of analyses of these genome features (Houldcroft et al., 2017; Kuny and Szpara, 2020; Renner and Szpara, 2018). Here, we seek to emphasize the importance of these features in the ecological context.

The HSV-1 genome is packed with hundreds of homopolymer tracts and other types of tandem repeats and microsatellites. At several genomic sites, these repeats are so long that their full extent can only be precisely measured using long-read sequencing technologies (e.g., Oxford Nanopore or Pacific Biosystems), or estimated via restriction-fragment length polymorphism (RFLP) approaches. However, recent improvements in statistical methods combined with short-read sequencing (e.g., Illumina) offer an opportunity to characterize shorter microsatellites as they exist in HSV-1 genomes (Loiseau et al., 2020; Mousavi et al., 2019). Earlier studies using gene-targeted sequencing, RFLPs, and microsatellite-typing suggest that there is a significant quantity of variability between HSV-1 genomes at these repetitive sites (Deback et al., 2009, 2010; Umene and Kawana, 2003; Umene et al., 1984). Thus, we anticipate that these differences can also be tracked in clinical isolates, to understand how viral genetic changes accumulate over the course of natural infections in vivo. Short homopolymer frameshifts can already be tracked with short-read sequence data, since these fit entirely within the length of a single sequence read. The accessibility of detecting homopolymer length changes has already led to the detection of a frameshift in HSV-1 UL13 that arises during sequential infections in cell culture (Kuny et al., 2020; Szpara et al., 2010). Analysis of HSV-1 genomes collected from human transmission pairs in two prior studies indicate that insertions and deletions can accumulate rapidly to create genomic diversity both within and between hosts (Pandey et al., 2017; Shipley et al., 2019). Even HSV-1 infections sampled multiple times over a period as short as 3 days, i.e., within a single shedding episode, have revealed viral genomes

with short insertions and deletions (Shipley et al., 2018, 2019). The ability of these repetitive elements to fluctuate in length over very short time periods warrants exploration in future studies of HSV-1 evolution within and between hosts.

4. Integrating the whole body: Chronic infection, shedding patterns, and tissue compartmentalization

HSV-1 causes life-long infection by establishing a reservoir of latent, episomal viral genomes within the soma of neurons in the sensory and sympathetic ganglia. Periodically these genomes spontaneously reactivate and undergo replication. While the latent reservoir remains intact, new viral progeny are transported in a retrograde direction along neurites, back to peripheral epithelial cells and mucosal membranes. This compartmentalization allows the virus to evade full eradication by the immune system during latency in neurons, and to achieve full productive replication and shedding at epithelia, on multiple occasions over a human host's lifetime.

4.1 Distinct phases of the viral life cycle underlie each "shedding episode"

Reactivated virus that is actively replicating at the mucosal surface produces new progeny virus that can either spread to cells in close juxtaposition or be transmitted to a new host upon contact. The release of progeny virus from epithelial cells is termed "viral shedding." Samples of newly shed viral progeny can be collected by swabbing the skin surface. These samples contain a milieu of human DNA, other skin-associated microbes, and the target HSV DNA. While HSV genomes can be detected and quantified in this mixture by quantitative PCR, the complexity and rarity of the target genomes relative to human DNA have necessitated the use of oligonucleotide bait-based enrichment in order to fully characterize these samples (Shipley et al., 2020). Viral shedding has a spatial aspect, involving both asymptomatic and symptomatic sites on the body. Viral shedding also has a temporal aspect, termed "episodes." Shedding episodes have been functionally defined as consecutive days with detectable viral genomes at the skin surface, allowing for up to 1 day of negative or missing data amidst a continuous sequence (Johnston et al., 2014; Tronstein et al., 2011). These episodes can include shedding from symptomatic lesions, as well as days where shedding appears to be asymptomatic, i.e., without active lesions or other symptoms. Quantification of HSV genomes in clinical studies suggests that viral

shedding is more frequent and more intense from symptomatic lesions than from non-lesion sites (Tronstein et al., 2011; Wald et al., 1995). However, cytotoxic T lymphocytes (CD8+ T-cells) have been detected in epithelial peripheral tissues during both asymptomatic and symptomatic HSV-2 shedding, and this response is anticipated to occur during HSV-1 infection as well (Johnston et al., 2014). Current areas of research in this field focus on the molecular mechanisms that trigger viral entry into latency in neurons, vs productive replication in epithelial cells, as well as the mechanisms that govern reactivation from latency (Roizman and Whitley, 2013; Thompson and Sawtell, 1997). Anecdotal evidence suggests that individual humans differ in the stressors that precede outbreaks of viral shedding, suggesting that there is individual variation in the triggers of reactivation (Douglas and Couch, 1970; Dréno et al., 2012; Jenkins and Baum, 1995).

4.2 Viral compartmentalization within the human body

While there are many unanswered questions regarding HSV-1 latency and reactivation, we can identify different stages between neurons and epithelial cells where changes in the quantity of co-occurring viral genomes could impact within-host genetic diversity—e.g., population bottlenecks and expansions. These population dynamics influence the within-host genetic diversity of other herpesviruses, such as human cytomegalovirus. Populations of cytomegalovirus compared between urine and plasma samples from individual patients have revealed rapid genetic divergence between virus populations from each compartment (Hage et al., 2017; Renzette et al., 2013). These findings are consistent with population bottlenecks and expansions as the virus moves between compartments, in addition to certain genome positions being under positive selection (Renzette et al., 2013). Since HSV undergoes compartmentalization by its requirement for movement between the skin and the nervous system, we anticipate that similar population dynamics may occur between epithelial and neuronal cells.

4.3 Bottlenecks and population expansions via latency and reactivation

Initially, within-host genomic diversity is established from the original transmission event that launches infection of a new host. This initial infection seeds a reservoir of latent episomal HSV-1 genomes in neuronal nuclei. Studies using in vivo mouse models of viral latency have detected a range of 10–1000 genomes per infected neuron, and revealed that this varies

between neurons of the same host, and is influenced by viral strain and input dose (Sawtell, 1997; Sawtell et al., 1998). The frequency of reactivation differs by viral strain, and it appears that only a subset of genomes or neurons reactivate at any given time (Kobiler et al., 2010; Sawtell and Thompson, 2004; Strelow et al., 1994; Wilson and Mohr, 2012). Thus, each reactivation may start with a population bottleneck, animating only a subset of the HSV-1 genetic diversity (e.g., minor variants) from the latent pool of standing variation (Fig. 2). The latent population of genomes may exist from the initial infection, or it could arise from minor variants that accumulate over cycles of reactivation within the host, and/or be influenced by additional co-infecting or subsequently infecting viral genomes (e.g., a superinfection scenario). Each reactivation event involves a small viral population expansion within neurons, and a larger expansion of the viral population during productive replication within epithelial cells. These expansion events may shift the frequency of any minor variants that existed in the initial reactivated genomes.

Viral replication in epithelial cells, and surveillance by local immune cells, provide further opportunities to accrue replication-associated mutations in the viral population during viral shedding. In this way, the viral progeny shed after reactivation can vary in minor variant composition, or total genetic diversity, while retaining a largely consistent consensus genotype. Statistical models of HSV-2 shedding from the mucosa suggest that the extent and duration of viral shedding can be predicted from the density of cytotoxic T-cells at the localized area of infection (Schiffer et al., 2010). Moreover, these models suggest that the entire process from viral reactivation in neurons, to detectable shedding of virus at the mucosal surface, could occur at a constant subclinical "slow viral drip" (Schiffer and Corey, 2013). It is possible that this "slow drip" only becomes detectable by quantitative PCR when the quantity of viral shedding reaches a certain threshold (Schiffer and Corey, 2013). These data suggest that if population bottlenecks and expansions impact HSV-1 within-host viral population diversity, then minor variant frequencies could also be in constant flux between neuronal and epithelial cells.

4.4 Population dynamics of viral movement between neurons and epithelial cells

We propose three models to describe how within-host HSV-1 genetic diversity might fluctuate over the course of reactivation within neurons, to active replication, and shedding in epithelial cells (Fig. 2). All three

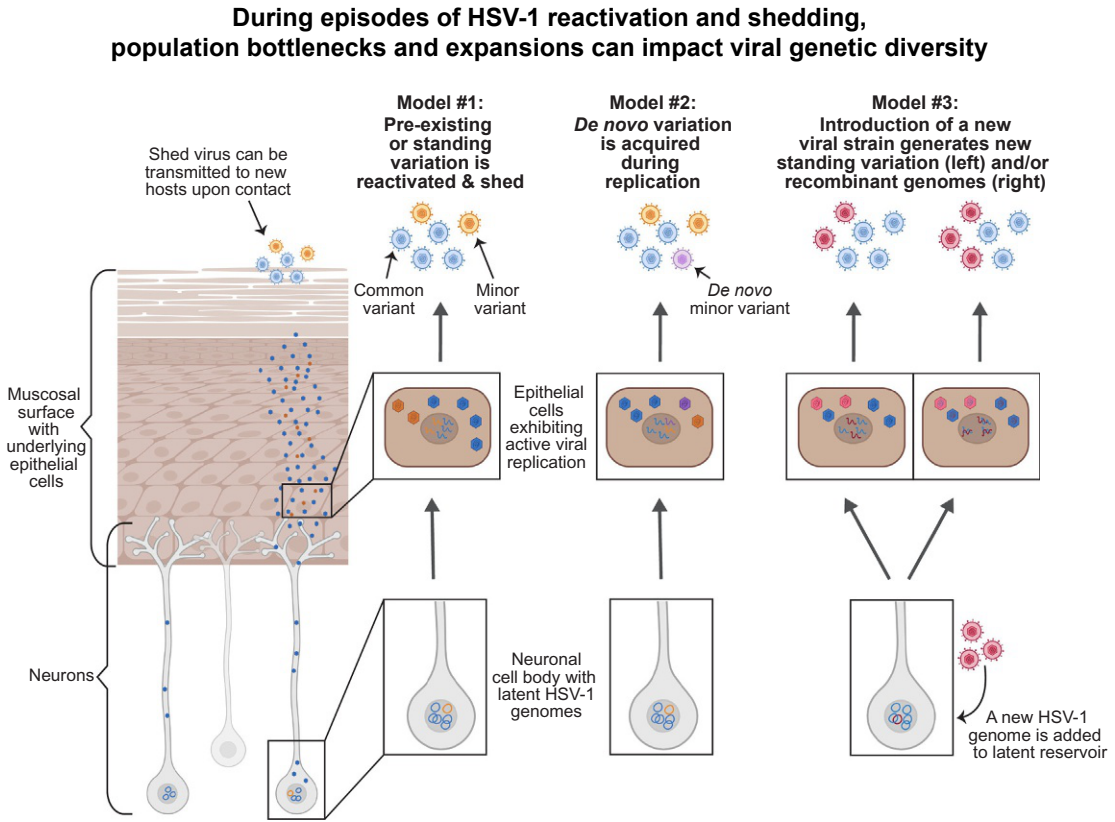

Fig. 2 See figure legend on opposite page.

models are possible, although each might occur at different frequencies, or be more likely under certain conditions. In one model, only a subset of pre-existing standing variation, which is present in the latent reservoir of episomal viral genomes, reactivates (Fig. 2, Model #1). During expansion of the reactivated viral population, the frequencies of minor variants among these genomes shift such that shed virus carries a different composition of genetic diversity than that in the initial infection, and possibly different from previous shedding episodes (Shipley et al., 2018). Another possibility is that standing variation does not exist at the outset, but instead accumulates over time. This would occur if minor variants arose mostly from polymerase error during replication of reactivated genomes within neurons, and/or during active replication in epithelial cells (Fig. 2, Model #2). This model of generating diversity also allows for fluctuation of variant frequencies between shedding episodes. The accumulation of minor variants during a period of population expansion after a bottleneck event is most similar to what is described by Muller's ratchet (see Section 1). If Muller's ratchet is in effect, it could explain the low number and frequencies of minor variants seen across clinical HSV-1 deep-sequencing studies thus far (Greninger et al., 2018; Pandey and Szpara, 2019). However, these studies were not designed to capture extremely low frequency variants (e.g., $<2\%$) and/or deleterious variants that did not persist to the point of viral shedding. In the third model, genetic diversity appears within an HSV-1 infection only after a superinfection, or dual-infection, from a second coincident or subsequently-infecting strains (Fig. 2, Model #3). If two HSV-1 strains can even transiently co-exist within one host, they may undergo recombination to produce a genetically

Fig. 2 Models for the impact of population bottlenecks and expansions on viral genetic diversity, during episodes of HSV-1 reactivation and shedding. During each reactivation event, only a subset of viral genomes begin replication and are transported to epithelial cells. This suggests that a population bottleneck occurs at the start of each reactivation event, with a subsequent population expansion during active viral replication in epithelial cells. We propose three models for how these events may influence genetic diversity in the viral population shed to new hosts. In Model #1, pre-existing genetic variation that was seeded in the ganglia at the time of initial infection undergoes reactivation and may shift in frequency during this bottleneck event. In Model #2, de novo genetic diversity arises during the population expansion within epithelial cells. In Model #3, a superinfection event has occurred, providing either shifting ratios of two HSV-1 genomes, or recombination generating a new HSV-1 genome. We propose that each model could potentially occur across different shedding episodes and between different hosts, creating the genetic fodder that fuels worldwide HSV-1 genome diversity. *Image created with BioRender.com.*

unique third strain. To better understand this scenario, studies are warranted to investigate whether exposure to a new HSV-1 strain could induce reactivation of the original strain, simply by initiating a new reactivation event within the ganglia. The degree to which each of these models may contribute to the generation and fluctuation of HSV-1 within-host diversity can be further explored via clinical and viral genomic studies that examine patient infections longitudinally over multiple shedding episodes. It is possible that all three of these models are active during HSV-1 infection, through the continuous ebb and flow of viral reactivation and shedding across the host's lifetime.

4.5 Transmission between human hosts

The potential for fluctuation of within-host genetic diversity also brings into question the role of transmission bottlenecks in the observed global HSV-1 genetic diversity between hosts. Previous analyses of HSV-1 transmission using paired samples examined either a subset of genomic markers via comparison of RFLPs, or most recently, deep sequencing of whole viral genomes and within-host diversity (Pandey et al., 2017; Shipley et al., 2019; van der Wiel et al., 1985). So far, these studies suggest that transmission results in near complete conservation of the consensus-level HSV-1 genotype between familial partners, with the exception of insertions or deletions at homopolymers and other repetitive sites. Each of the cases examined thus far involved transmission between genetically-related family members, and as such it is unclear to what extent inherited immune genotypes may have reduced the selective pressure for the incoming viral population to acquire new host-specific adaptations (Casanova, 2015; Casto et al., 2020). A recent deep sequencing study involving a rare case of viremic HSV-1 with maternal-neonate transmission pair revealed considerable within-host genetic diversity that was mostly shared between hosts, with a smaller amount of de novo variation (Shipley et al., 2019). Each examination of HSV-1 transmission to date has provided new insights on these individual scenarios, and this is likely to be the case in future work as well.

There are two main variables to consider that may impact the composition of the transmitted viral population and the impact of genetic bottleneck(s) during transmission. The first is the amount of shedding from the source partner in a transmission pair, and the second is the route and frequency of physical contact(s) that may lead to transmission. As mentioned previously, shedding can occur at either asymptomatic sites or symptomatic

lesions, and the frequency and intensity of viral shedding can vary between individuals. We have discussed how the viral genome population composition might change under different circumstances following reactivation (Fig. 2). During transmission, the quantity and composition of genomes in newly shed virus will influence the consensus level viral genotype and underlying minor variants that enter the recipient partner (Fig. 1). A more diverse population may be transmitted when high quantities of virus are shed (e.g., a looser bottleneck), whereas the diversity may be limited during low-level viral shedding (e.g., a tighter bottleneck). This is akin to the tight transmission bottleneck (i.e., very few virions) that has been proposed to influence the highly diverse populations of influenza virus, as well as the looser bottleneck (i.e., hundreds of virions) that has been proposed to occur during in utero transmission of human cytomegalovirus (Leonard et al., 2017; McCrone and Lauring, 2018; Renzette et al., 2011).

Each case of HSV-1 transmission is further complicated by the different potential route(s) of transmission. HSV-1 is transmitted upon contact with virus shed at mucosal and epithelial skin surfaces, often during events such as kissing, sexual intercourse, or oral sex. Depending on the nature of these events, the number of virions transmitted to a new host may be quite variable. Repeated opportunities for transmission, such as between long-term sexual partners, may also have implications for the extent of shared viral genetic diversity between hosts. Repeated contacts between partners may involve more than one body niche on each partner, creating complex opportunities for repeated exposures and/or transmission events. Further studies are warranted to understand how genetic bottlenecks under these various transmission circumstances impact the population(s) of viral genetic variants that establish infection in each new host. This in turn will impact the consensus level genotype detected in each transmission partner and influence the long-term assessment of between-host diversity.

5. Extending beyond individual hosts, strains, and species

5.1 Epidemiologic shift of HSV-1 into the genital niche

HSV can establish infection in multiple niches throughout the human body. Historically, HSV-1 was most commonly observed in the oral niche, while HSV-2 was more common in the genital niche (Roizman et al., 2007). In rare cases, such as in immunocompromised patients or neonates, both viral species can become disseminated across the derma, or be viremic throughout

the body (Berrington et al., 2009; Johnston et al., 2008). Recently, the epidemiology of HSV species has shifted such that HSV-1 is now the leading cause of new primary genital infections in many regions of the world (James et al., 2020; Looker et al., 2015; Xu et al., 2006). This shift has impacted infection incidence mostly in the Americas and Europe, and less so in Africa and Southeast Asia. These observations raise new questions about the reasons for this epidemiologic shift, and what consequences it will have on the future evolutionary trajectory of these viruses and their human hosts. The geographic aspects of this pattern of HSV-1 incidence in different body niches are likely not due to any viral genetic components. Early on, viral comparative genomics studies of randomly sampled HSV-1 infections around the globe suggested that the geographic relatedness of viral genomes reflected human migratory patterns (Kolb et al., 2013; Szpara et al., 2014). However, more recent studies have demonstrated that these historical patterns do not reflect the spread of modern HSV-1 genetic diversity (Bowen et al., 2019; Lassalle et al., 2020). At present there seems to be no predisposition for HSV-1 genomes in one locale to have evolved a capacity for crossing into the genital niche. Rather, the shift appears driven by a combination of host behavioral factors.

5.2 Changes in human behavior impact viral opportunities for niche expansion

Historically HSV-1 infection was often acquired during childhood via nonsexual routes, such as a kiss or other contact with a family member's saliva (e.g., via a pacifier or bottle) (Rhoads et al., 2007; Young et al., 1988). However, recent studies have detected a statistically significant decrease in HSV-1 seroprevalence among male and female children ages 14–19 in the U.S.A. (Bradley et al., 2014). These data indicate that younger generations are less likely to acquire HSV-1 via nonsexual routes during childhood, and consequently do not develop a protective antibody response to this pathogen before reaching sexual maturity. Thus, while the percentage of adolescents who have had sex prior to age 13 has decreased over the past decade, those who are sexually active are more susceptible to acquiring genital HSV-1 or -2 (Kann et al., 2018). The trend toward increasing genital HSV-1 infections may also be influenced by shifts in sexual behaviors. In the same study from Bradley et al., 2014, the seroprevalence of HSV-1 in study participants between ages 14–19 was higher for those who reported being sexually active, and for those with three or more sexual partners. Additional studies of sexual behaviors among the same age group confirm

an increase in frequency of oral sex between opposite-sex partners, which increases opportunities for HSV-1 to transmit between the oral and genital niche (Copen et al., 2012). Together, these findings suggest that the changing epidemiology of HSV-1 seroprevalence may be driven in large part by changes in human behavioral patterns. It remains to be seen how this shift in epidemiology will impact HSV-1 genetic diversity and evolution. Changes in early sexual behaviors, number of lifetime partners, and overall HSV prevalence may lead to increased opportunities for superinfection, and/or recombination, and resulting shifts in viral genetic diversity. These recombination events would not be limited to HSV-1 alone, as co-infection with subsequent recombination between genital HSV-1 and -2 genomes has already been reported (Casto et al., 2019). Only continued genomic surveillance of natural HSV-1 infections, using approaches such as those described here, will reveal if these changes occur. Such studies will also be important to inform the development of vaccines capable of targeting the full range of HSV-1 genotypes in present-day circulation.

5.3 Multiple microbes co-exist and interact in these human niches

Along with their behavioral practices, humans infected by HSV are also carrying a vast microbiome and virome that varies between anatomical niches and over time. An increasing number of studies suggest that these other members of the host ecosystem may play important roles in the acquisition, shedding, and transmission of HSV. While HSV can occupy several body sites depending on the circumstances, the most common sites of infection are the oral and genital niches. Since HSV is widespread and causes life-long infection, the challenge lies not in finding HSV alongside other microbes, but rather in identifying causal links between coincident microbes and specific events such as HSV reactivation and shedding.

5.4 Virus-virus interactions and their impacts on genetic diversity

Much of the evidence for virus-microbial interactions between HSV and other species has been described in the genital niche. For instance, an initial viral infection may affect the human host's susceptibility to subsequent infections, through its impacts on the local ecology of the genital niche. A primary example has been the observation that individuals with genital HSV-2 infection experience an elevated risk of also contracting human immunodeficiency virus (HIV), which is particularly problematic in areas

that experience high rates of both infections (e.g., Sub-Saharan Africa) (Corey et al., 2004; Glynn et al., 2009). This overlap in epidemiology is in part due to HSV-shedding and lesions that lead to an increase in the amount of circulating helper T-cells (CD4+ T-cells) in peripheral tissues—which are the target cells for HIV infection (Corey et al., 2004). Once acquired by an HSV-2 positive individual, HIV infection then increases the frequency of reactivation and intensity of HSV-2 shedding, due to the reduced function of the host immune system (Augenbraun, 1995; Corey, 2007). With the prevalence of genital HSV-1 on the rise, one may predict that a similar interaction with HIV will be observed in time due to its similar pathogenesis to HSV-2. It remains to be studied how instances of HSV co-infection with HIV impact the development of within-host genetic diversity for HSV. However, if a compromised immune system creates an opportunity for higher rates of HSV reactivation and viral replication, then this might also lead to an increased number of minor variants in the viral population, and faster rates of genetic drift or the development of antiviral resistance. This could create challenges in treating HIV positive patients with co-infecting HSV, as immunocompromised patients commonly experience antiviral resistance and can present with high levels of HSV within-host genetic diversity (Casto et al., 2020; Horsburgh et al., 1998). Specifically, HSV antiviral resistance mutations have been documented to occur at ~4–7% prevalence in those who are immunocompromised due to HIV infection (Levin et al., 2004). These effects may not be restricted to one anatomical niche. A study of men seropositive for HSV-1 and -2 indicated that co-infecting HIV led to more frequent viral shedding, and is a particular risk factor for oral HSV-2 shedding (Kim et al., 2006). Genomic studies of either or both anatomical niches are warranted to assess the impact(s) of co-infection with HIV on the diversity and evolution of minor variants in HSV shed during these co-morbid infections.

5.5 Virus-bacteria interactions and their impacts on genetic diversity

When replicating in the host mucosal and epithelial layer, HSV encounters an environment regulated by host cells and by various microbial species. The composition of these species can fluctuate over time, which can lead to an imbalance between species resulting in clinical conditions, such as seen in bacterial vaginosis. This condition can be asymptomatic or symptomatic, and occurs when lactobacilli species decline, and anaerobic species increase

(e.g., *Gardnerella*). Similar to the case with human immunodeficiency virus, patients with bacterial vaginosis are at an increased risk for acquiring HSV-2 (Esber et al., 2015). Bacterial vaginosis causes a disruption of typical vaginal conditions, such as increased pH and irritation of the mucosa. It is a highly prevalent condition that occurs in ~29% of women ages 14–49 in the United States (Koumans et al., 2007), and ~33% of women age 25–49 in Sub-Saharan Africa (Torrone et al., 2018). The presence of bacterial vaginosis-associated flora can also impact established HSV-2 infections, leading to an increase in viral shedding (Nardis et al., 2013). However, changes to the genital flora during bacterial vaginosis are not restricted to women. A recent study used high-throughput sequencing to characterize the microbial composition of sexual partners with and without HSV-2. In female participants with an HSV-2 positive partner, the investigators identified an increase in vaginal microbiome alpha diversity, i.e., a greater imbalance between species, that specifically impacted *Gardnerella and Lactobacillus* species (Mehta et al., 2020). Likewise, male participants showed greater penile species richness, or abundance of microbes, if their partner was HSV-2 positive (Mehta et al., 2020). These data suggest that the microbial flora of sexual partners may also influence the potential for acquiring, shedding, or transmitting HSV-2 (Mehta et al., 2020). While these observations have focused on HSV-2, the effect of bacterial vaginosis on genital HSV-1 infections may be quite similar. If so, it would be interesting to compare the HSV-1 within-host genetic diversity, shedding rates, and microbial composition of hosts with a history of bacterial vaginosis. Going forward, it may also be important to know how often HSV-1 superinfections can be detected in hosts with increased risk of acquiring infection due to a pre-existing condition. These data may inform public health recommendations on the management of coincident infections.

5.6 Signs of positive selection in viral genomes

In considering the total ecology of HSV-1, from viral replication and latency to individual-host shedding and partner microbiomes, it becomes more clear how the factors contributing to viral evolution can vary between hosts and over time. This variability may then result in the discordance between early estimates of the HSV-1 mutation rate via polymerase error, and the observed world-wide strain diversity (Drake and Hwang, 2005; Szpara et al., 2014). Often, HSV-1 studies involving genome sequencing of new strains provide

a phylogenetic analysis of how those genomes compare to others around the globe (Bowen et al., 2019; Lassalle et al., 2020; Shipley et al., 2018). While these analyses have been useful to confirm the genetic uniqueness between randomly sampled infections, they do little to decipher the details of strain diversity, or to inform us on which localized areas of the genome may drive functional differences between strains. In the first comparative genomic study of HSV-1, investigators made a conservative estimate of at least 54 amino acid residues within 13 genes being under positive selection (Szpara et al., 2014). As viral genome sequencing studies continue, more sites of positive selection will presumably be recognized. There are also specific amino acid positions within the HSV-1 genome that co-segregate with other amino acids at nearby sites, creating distinct genotypes (Bowen et al., 2019; Szpara et al., 2014). In a recent examination of 10 HSV-1 samples collected in Finnish individuals, investigators noted two subgroups within the sequenced viral genomes (Bowen et al., 2019). These subgroups were particularly evident at co-segregating amino acid sites, which in certain genes created two major genotypes. These data and matched experimental studies with these viral isolates indicated the potential for functional phenotypic variability between strains (Bowen et al., 2019). As more clinical HSV-1 deep-sequencing data becomes available, it will be interesting to explore whether within-host minor variants also occur at these segregating sites. The mutation rate at these sites may also be influenced by overlapping or nearby genomic features such as non-B DNA structures (e.g., G-quadruplexes), or intrinsically disordered regions (Guiblet et al., 2021; Mozzi et al., 2020). However, it is likely that these linked loci within genotypes do not accrue from polymerase error-based mutation alone. Rather, these co-varying sites can be shuffled between genomes via recombination within a single host. A recent examination of linkage disequilibrium among HSV-1 genome sequences from the cerebral spinal fluid of infected individuals identified very few linked genes (Lassalle et al., 2020). If recombination is indeed occurring freely and frequently genome-wide, it would allow for extensive flexibility and shuffling of genotypes, or segregating sites (Bowden et al., 2004). In this way, the pre-existing variation of HSV-1 genomes worldwide provides a variety of sequence diversity that can produce novel genotypes, especially if the frequency of HSV-1 dual-infections is currently underestimated. Expanding efforts to catalog global diversity, including a thorough comparison of unique variants vs those that circulate at a high frequency in the global population, will be critical to piece together the evolutionary trajectory of contemporary HSV-1 infections.

5.7 Potential for balancing selection to impact viral diversity

The vast genetic diversity of HSV-1 across the globe should not be a complete surprise, as this virus has co-evolved over at least seven million years with its human hosts, who in turn vary in the efficacy and robustness of their immune responses (Wertheim et al., 2014, 2021). It would also be worthwhile to explore whether co-varying viral loci (or segregating sites) correlate with the variability observed in human immunity (e.g., MHC types and HLA alleles) (Hosken et al., 2006, p. 8). Immune-related genes in humans are known to harbor great allelic diversity across the world (Robinson et al., 2016). This diversity is important for maintaining a human population capable of surviving various pathogen infections, and thus selective pressures encourage maintenance of this diversity in a process known as balancing selection (Charlesworth, 2006). Given the *co*-evolutionary history and potential for antigenic polymorphisms in the HSV-1 genome, it would be useful to test co-varying viral loci for evidence of both positive and balancing selection by their human hosts. For instance, viral glycoproteins on the outer envelope of virions are common antigenic targets of the host-immune system, which leads to these genes being under frequent selective pressure. The glycoproteins are among the most variable viral proteins of both HSV-1 and HSV-2, with the highest gene diversity across many strains (Akhtar et al., 2019; Bowen et al., 2019; Johnston et al., 2017a; Szpara et al., 2014). If certain HSV glycoprotein genotypes or segregating sites do have an evolutionary correlation with differences in the human immune response, this may have implications for viral genetic contributions to virulence, particularly during transmission to genetically distant hosts. It is possible that severe manifestations of HSV-1 disease reflect a mismatch between viral virulence and host immune control (or lack thereof). Comparative genomics analyses between clinical isolates of human cytomegalovirus identified hotspots of linkage disequilibrium, or "hyper-variable regions," that were primarily located within viral glycoproteins (Lassalle et al., 2016). These findings propose a mechanism for host-specific adaptability in an otherwise stable herpesvirus species.

6. Summary and perspectives

Considering the total ecology of HSV-1 allows us to observe that its evolution likely occurs on both a short- and long-term time frame, as has been proposed for human cytomegalovirus (Lassalle et al., 2016). During a single shedding episode, genes under strong selective pressures (such as

glycoproteins) can undergo rapid antigenic shift by minor variant fluctuations and/or recombination. During a life-long HSV-1 infection, short term exposure to a new virus strain could produce a transient instance of dual-infection. This would allow for recombination between independent viral genomes (and genotypes) and create a long-term and large-scale reshuffling of viral variant loci (segregating sites). This can lead to de-coupling of within-host minor variants, which may be transmitted to new partners, thus leading to new standing variation of HSV genomes. These patterns of viral variation may then occur within the next host, while still maintaining the overall conservation of genomic sites that enable optimal infection of many hosts. The rate at which these instances of variation arise within one host and are transmitted to another host may also be impacted by diversity in the efficacy of each host immune system. Similarly, viral shedding patterns can be influenced by co-infecting microbes, or by differences in host behavior (e.g., frequency of interaction with sexual partners). In this way, a holistic perspective on HSV-1 evolution reveals a complex viral system that is easily capable of achieving very high prevalence within the human population, while maintaining standing variation both within-host and between-hosts. This perspective may be applicable to other large DNA viruses, as well as other chronically infecting viruses. A focus on the natural ecology of infection may help to improve our understanding of how complex and chronic viruses balance host-pathogen competition with transmissibility. The benefits of exploring such concepts for HSV may also extend to the public health sector, where they can be used to inform prophylactic intervention protocols for patients who are at high risk of acquiring multiple HSV infections (e.g., immunocompromised individuals). Contemporaneously, investigations that further our ability to link viral genotypes to virulence, particularly for genes identified as under positive selection in clinical studies, will continue to be of high priority.

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