The Effect of Antiretrovirals on HIV-1 Evolution in the US

Introduction

The human immunodeficiency virus (HIV) is a retrovirus that attacks the human immune system and as of 2019, has infected 1.2 million individuals in the United States [1]. HIV was first identified by Luc Montagnier and his group in 1983, but evolutionary analyses have revealed a zoonotic origin dating back to the 1800s [2]. Viral transmission first likely occurred through human exposure to the bodily fluids of simians infected with the simian immunodeficiency virus (SIV) whilst hunting primates as a food source [3]. Two SIV strains, *SIVcpz* from chimpanzees and *SIVsm* from sooty mangabeys, with a host-range wide enough to cross the species barrier and infect humans, are credited to have given rise to HIV-1 and HIV-2 respectively [4]. HIV-1 due to its highly infectious nature, has caused a global pandemic, whilst the less virulent HIV-2 remains largely endemic to West Africa [1,4]. The focus of this project will be the HIV-1 type due to its prevalence in the United States.

The disease progression of HIV-1 infectees can be divided into three stages: (1) acute HIV infection, clinical latency, and AIDS [5,6]. In the acute stage, those newly infected experience flu-like symptoms only due to a robust immune response to the virus [5,6]. In the next stage, the virus becomes established but continues to replicate at a low rate [5,6]. As such, infectees may have viral loads below the detection limit, thus remaining asymptomatic and non-contagious [5,6]. This stage can be prolonged through the appropriate use of antiretrovirals (ARVs). However, if unchecked, the viral load of a HIV-1 infectee will increase exponentially throughout the years [5,6]. A high HIV-1 viral load leads to a severely impaired immune system unable to respond to opportunistic infections [5,6]. This clinical outcome is known as acquired immunodeficiency syndrome (AIDS), the final most severe stage of HIV-1 infection [5,6]. AIDS symptoms involve pneumonia, persistent diarrhoea, rapid weight loss and neurological disorders [5,6].

Due to the highly infectious nature of HIV-1, AIDS quickly spread throughout the US. The government and pharmaceutical companies in response, poured vast amounts of research funding into developing a cure for it. As a result, the HIV-1 replication cycle is well-characterised, and its mechanisms exploited extensively as drug targets. The cycle starts when an *Env* glycoprotein on the HIV-1 envelope binds to a receptor on the CD4 outer membrane and triggers a series of conformational changes allowing the viral envelope to fuse with the host cell membrane [7]. This fusion enables the deposition of viral RNA and the proteins (e.g., reverse transcriptase and integrase) necessary to hijack the host replication machinery into the cell [7]. Reverse transcriptase converts HIV-1 RNA into DNA, which is then transported into the host nucleus [7]. Once imported, HIV-1 DNA is integrated into the host DNA by the integrase enzyme, thus enabling it to be transcribed and translated by the replication machinery of the immune cell [7]. New HIV-1 proteins and RNA are transported near the cell surface and assembled into immature viruses that will be pushed out of the cell as virions [7]. Once extracellular, virions are attacked by proteases triggering their maturation into viruses ready to infect other CD4 T-cells [7]. Each CD4 T-cell can produce hundreds of virions, and this burden eventually causes apoptosis of the cell [8]. As such, CD4 T-cell depletion is characteristic of HIV-1 infectees that have progressed to AIDS [8].

Initial management of HIV-1 in North America, merely involved prevention and symptomatic treatment of the disease [9, 10]. However, by 1987 the first ARV drug azidoythymidine (AZT),

also known as Zidovudine®, was approved by the FDA [9, 10]. AZT worked to prevent viral replication by inhibiting HIV-1 reverse transcriptase function [9, 10]. The first "wave" of antivirals was administered as monotherapies [9, 10]. However, most patients treated with this method soon developed drug resistance, and viral rebound followed [11]. This rapid viral response can be attributed to the hypermutability of HIV, caused by its error-prone reverse transcription process [12, 13]. This process has been estimated to cause up to 10-point mutations per genome per round of replication [12, 13]. A high error rate increases the chances of generating a virion with a mutation that confers drug immunity [12, 13]. For example, HIV-1 positive participants in an early clinical trial developed integrase inhibitor resistance mutations as soon as 24 weeks after the administration of Dolutegravir monotherapy [11].

To prevent drug resistance, researchers strongly advised clinicians to implement the use of dual therapy (i.e., the concurrent use of two HIV-1 ARVs) [9, 10]. This multidrug attack strengthens and accelerates viral suppression and with lower replication rates, the chances of developing a drug resistance mutation (DRM) significantly lowers for a HIV-1 patient. In the late 90s, a treatment regimen comprising of three or more ARV drugs, known as highly active ARV therapy (HAART), was implemented [9, 10]. Since then, this regimen has been standardised around the world and continues to be the gold standard treatment strategy. A HIV-1 patient responsive to HAART may potentially reduce their viral load to levels below detection limits and lower their chances of sexually transmitting the disease [5, 6]. A recent clinical trial involving antibody infusions of 3BNC117 and 10-1074 bNAbs, which target the HIV-1 glycoprotein, also demonstrated significant suppression of plasma viraemia in HIV-1 positive participants [14]. Some of the benefits of antibody infusions include lower costs for the patient and fewer treatment-associated symptoms [14]. However, despite the vast improvements in the quality of life and the life expectancy of HIV-1 patients, there remains the possibility of rebound due to persisting viral reservoirs.

A HIV-1 reservoir is a cluster of immune cells that are infected by transcriptionally silent HIV-1 [15, 16]. As such, these infected cells do not express viral antigens enabling them to escape immune surveillance [15, 16]. If a HIV-1 patient develops drug-resistance or stops taking medication, these reservoirs can re-activate and replenish the viral load of the patient with potentially multidrug resistant HIV-1 copies. These viral copies can then be transmitted to other people and create "transmission clusters" in the population [17]. In fact, the increased use of ARTs has been accompanied by the steady accumulation of DRMs in sampled individuals [18, 19]. It serves as a reminder that while beneficial, ARVs can also act as strong selective forces for more virulent strains. Moreover, HIV-1 treatments are expensive and lifelong; this makes treatment inaccessible for low socioeconomic groups. Low-cost HIV-1 therapy is essential to prevent the development of multidrug resistant strains in HAART-experienced patients due to treatment discontinuation.

Almost 25 years after its introduction, HAART continues to be effective against HIV-1. However, due to the hypermutable nature of the virus, several studies have already underscored the importance in ongoing surveillance of HIV-1 diversity [20-24]. Our study aims to investigate the evolution of HIV-1 in the US across time: from the introduction of ARVs to present day. This study is unique as the US-wide evolution of HIV-1 in respect to ARVs has not yet been explored. (1) It is hypothesised that the combined use of ARV drugs has successfully impeded most significant changes in the HIV-1 genome, thus reflecting the continuous annual decrease in the

number of new HIV infections and deaths. (2) It is also hypothesised that there are individuals within the US population infected with HIV-1 that have accumulated multiple genetic mutations.

Methodology

The genetic sequences used in the analyses were collected from the NCBI Virus data repository. The database was filtered for the following: HIV-1 (taxid: 11676) sequences, sequence lengths 8000-10000 nucleotides, collected from North America: USA, and originating from human (taxid: 9605) hosts. The sequences collected were grouped into seven "periods", according to the collection dates of the sequenced samples. This grouping was based on the timelines created by Cheney et al. (2021), which detailed the Food and Drug Administration (FDA) approval dates of HIV-1 ARV drugs, and Arts et al. (2012), which marked the approximate dates ARV treatment methods were implemented. The database was filtered for USA collected samples only, as analyses were based on major drug approval dates of the FDA (a US institution). It should be noted, that the NCBI Virus repository had a severe lack of full HIV-1 genomes sequenced prior to 1996. As such, database filtering was relaxed to include sequences that were ~83% complete to increase data collection within reasonable limits.

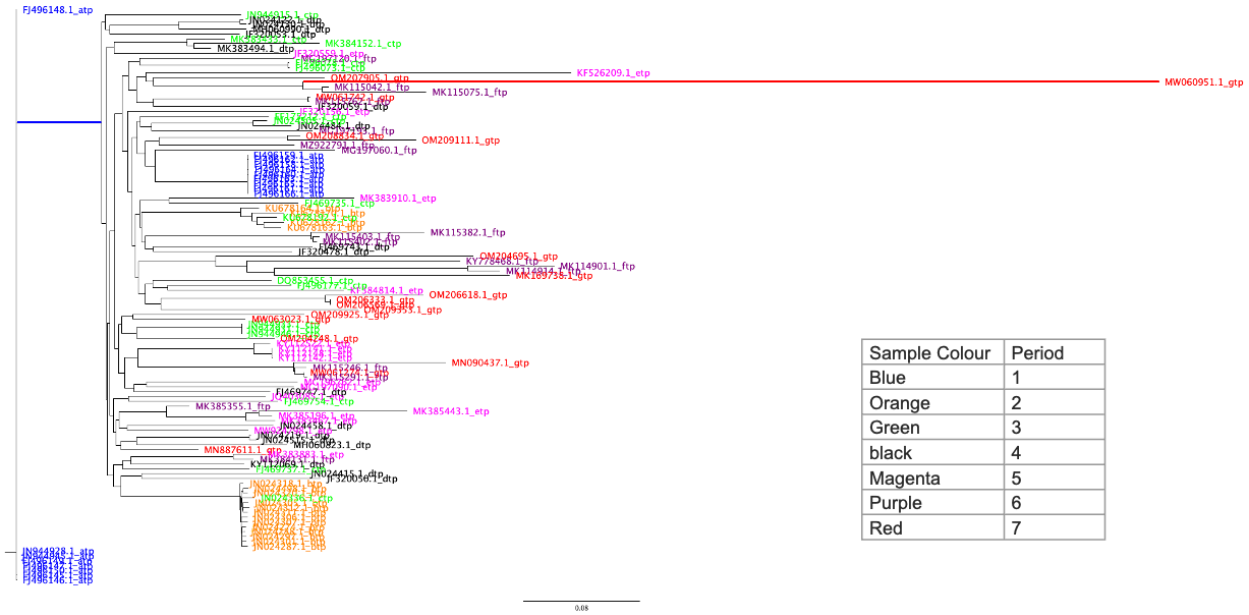
The first period (1987) refers to the FDA approval date of Zidovudine®, the first HIV-1 mono-therapeutic drug. The second period (1992) represents the introduction of dual-therapy methods, although monotherapy was still used. The third period (1997) marks the FDA approval date of Combivir®, the first HIV-1 combination therapy. The fourth period (2003) denotes the FDA approval date of Enfuvirtide®, the first fusion inhibitor drug. The fifth period (2007) refers to the FDA approval dates of Maraviroc® and Raltegravir®, the first CCR5 antagonist and Integrase Strand Transfer Inhibitor (ISTI) drug, respectively. The sixth period marks FDA approval of Truvada® for PrEP[2], the first combination drug retailed to significantly reduce the risk of HIV infection. The final period denotes the FDA approval date of Ibalizumab®, the first monoclonal antibody treatment that effectively targets HIV-1 epitopes. Due to difficulties in retrieving the exact date of FDA approvals, the beginning of each period was set to 1 January (e.g., the first period spans from 01/01/1987 - 12/031/1991). See the time interval for each period is detailed in Appendix 1.

A set of genetic sequences for each period was retrieved as a FASTA file. All data analyses were performed using the Anaconda Software Distribution [29]. Each file was randomly sampled without replacement *n* times using the bbmap package [25], so that downstream statistical analyses would be balanced. Each file was sampled twice, first for a set of 17 sequences and then a set of 78 sequences. Note that the first period definitively contained 17 sequences and as such, could not be down sampled for 78 sequences. Each file representing a "drug/treatment" period was subsampled so that downstream analyses are balanced. Moreover, balancing is essential when generating phylogenetic trees, so that later time periods with better HIV-1 sampling are not overrepresented.

The set of FASTA files containing 17 sequences were concatenated and aligned using the mafft package [26]. The set of FASTA files containing 78 sequences were also concatenated and aligned using the mafft package [26]. A phylogenetic tree derived from a maximum likelihood model was

generated for each aligned file using the RAxML package [27]. The Newick tree outputs were visualised using FigTree (v1.4.4) [28].

Results/Discussion



Sample Colour	Period
Blue	1
Orange	2
Green	3
black	4
Magenta	5
Purple	6
Red	7

Figure 1: Phylogeny of HIV-1 samples collected in the US and analysed under a maximum likelihood model

A phylogenetic tree was generated from 119 sequences obtained from 1987 (the FDA approval year of the first ARV drug) to present day (see Figure 1). These HIV-1 sequences are colour-coded according to their respective period (e.g., sequences in blue belong to the first time period when monotherapy was first implemented). This colouration was implemented to easily visualise the gradual evolution of HIV-1 across time in the US. There are two groups of sequences (blue) located towards the root of the tree. These are samples from the earliest time period, thus explaining their close relation to the most recent common ancestor (TMRCA). However, there is a distinct "break-away" group from the first time period that is more closely related to the rest of the sequences. This "break-away" group and the rest of the sequences are distinguished from the previous two (blue) groups by a distinct branch (blue line).

This branch (blue line) represents a strong bottleneck caused by Zidovudine®. This reverse transcriptase inhibitor works to prevent the conversion of HIV-1 RNA to DNA, thus halting viral replication. Whilst highly effective, HIV-1 quickly evolved resistance mutations against this drug as it was initially implemented as a monotherapy. The "breakaway" group represents a group of individuals carrying HIV-1s with Zidovudine® resistant mutations. These DRMs are what enabled the "fitter" viral copies to persist past the bottleneck and continue replicating to give rise to the next generation of HIV-1s. This phenomenon explains why the "breakaway" group is so genetically similar to the rest of the sequences from later time periods.

However, the rest of the sequences from samples taken after the first time period are remarkably similar to each other. This can be explained by the implementation of dual therapy in 1992 which

impeded drug-induced natural selection of fitter HIV-1 copies. A combination of two ARVs quickly lowers viral replication rates and thus reduces the chances of generating a DRM. The efficacy of combination therapy as shown in Figure 1, would have been further cemented by the standardisation of HAART in 1996. Despite the efficacy of combination therapy, the gradual (albeit much slower) evolution of HIV-1 can still be observed in the phylogenetic tree. The HIV-1 sequences are growing more genetically diverse to TMRCA (increasingly situated farther right on the tree) as time progresses. Nevertheless, the lack of a staircase-like tree topology, characteristic of a virus under strong, continuous positive selection (Norstrom et al., 2012) showcases the success of HAART and supports the first hypothesis.

On the right-hand side of Figure 1, there is a sequence (MW060951.1_gtp) from the most recent period that is distinguished by a long branch (red line) from the rest of the tree. The long branch indicates that this sequence descends from HIV-1 copies that have accumulated multiple mutations over time, leading to its significant divergence from TMRCA. The apparent conservation of these mutations over time, strongly indicates that these mutations confer some type of fitness towards the virus, such as drug resistance or increased virulence. However, such definitive conclusions cannot be made from a phylogenetic tree generated from whole HIV-1 sequences. Future analyses studying specific proteins, such as the envelope glycoprotein in HIV-1 samples from infected individuals taking ARVs targeting that protein, may better reveal the presence of a drug-mediated directional selection.

Still, it is possible that this long branching could be due to sequencing issues or poor sequence alignment. As such, phylogenetic analysis was repeated with a larger subsample of the HIV-1 sequences collected (Figure 2). This resampling without replacement was conducted to increase sample size and test the validity of the phylogenetic relationships initially observed.

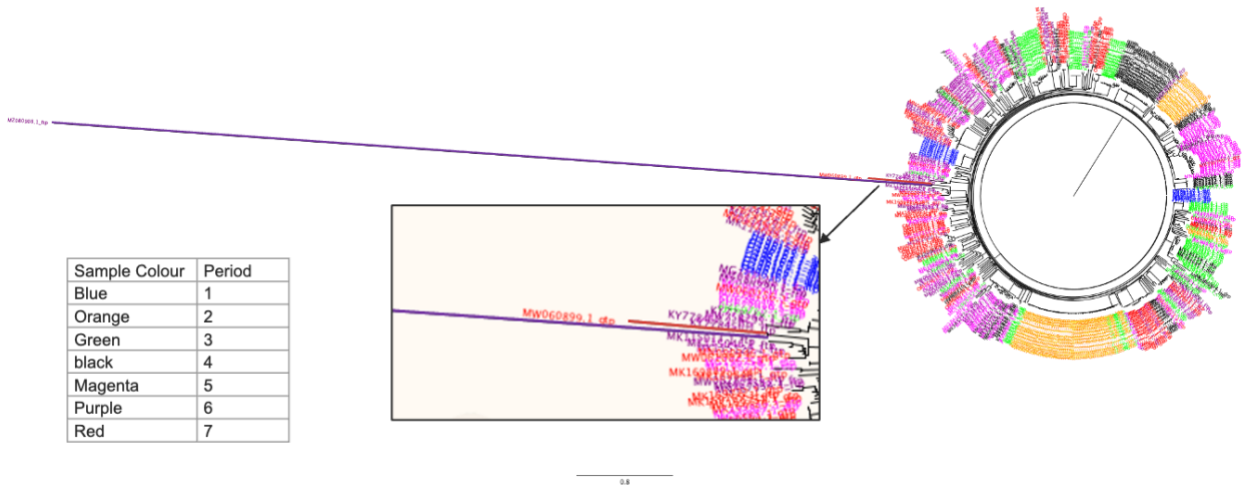


Figure 2: Circular phylogeny of HIV samples collected in the US and analysed under a maximum likelihood model

Figure 2 depicts a circular phylogeny of 485 HIV-1 samples collected in the US. Note that a circular form of the phylogenetic tree was chosen for easier viewing. A rectangular version of the

tree above can be viewed in Supplementary 1. Similar phylogenetic patterns were observed in Figure 2. There are two groups of sequences (blue) from the first time period separated by a distinct branch: one group closer to TMRCA and another "break-away" group closer to the rest of the sequences. As such, the bottleneck (created by Zidovudine® treatments) observed from the first phylogenetic tree was also present in Figure 2. The group of sequences closest to TMRCA is notably closer to the centre of the circular phylogeny.

Whilst it appears that HIV-1 has accumulated few genetic changes over time due to the short branch lengths, it is likely that any evolutionary patterns that can be gleaned from the phylogenetic tree above is obscured by the extremely long branch (purple line) distinguishing the MZ080908.1_ftp sample. This long branching effect demonstrates the limitations of phylogeny. Nevertheless, there are two samples from recent time periods that appear to have notably diverged from TMRCA. The MZ080908.1_ftp sequence (node farthest left) comes from a sample taken during the time period beginning 2012. This indicates that highly divergent HIV-1 strains have been around almost a decade ago. Still, the distinct placement of this node could be accounted for by sequencing errors or a sequence misalignment.

However, a closer look at the circular phylogeny reveals a much shorter but still distinct branch (red line) showcasing the MW060899.1_gtp sequence from the most recent time period; see black box in Figure 2. The divergence of this sequence indicates that an individual in the US population is infected with a HIV-1 strain carrying multiple mutations that have been accumulated through time. As the branch length distinguishing the MW060899.1_gtp node (red line) is not as remarkably long as the previous branch (purple line). it is unlikely that the differences observed in this HIV-1 sequence is caused by sequencing errors or a misalignment. It is far more likely that the MW060899.1_gtp sequence is a clear example of a HIV-1 strain that has survived ARV therapies as it carries several mutations conferring multidrug resistance or increased virulence that has been conserved through time.

Conclusion

This study aimed to investigate the effect of ARV therapies on HIV-1 evolution in the US, across time. In support of earlier studies, it was found that monotherapies such as Zidovudine® were insufficient to sustain viral suppression in HIV-1 patients, as the hypermutable HIV-1 strains quickly evolved DRMs. However, a treatment regimen combining several ARV drugs, targets multiple parts of the HIV-1 replication cycle and viral replication is rapidly suppressed. As such, treatment responsive HIV-1 patients have a significantly lower likelihood of developing DRMs. This is reflected in the phylogenetic analyses above, wherein HIV-1 evolution is greatly impeded after the standardisation of HAART.

Despite ongoing HAART efficacy, several research groups utilising genomics have found increasing prevalence of DRMs in HIV-1 strains from samples recently collected from certain cities of South Africa, Brazil, New Zealand, China, and the US [20-24]. The phylogenetic analyses of this study corroborate their findings, as a handful of HIV-1 sequences from samples collected across the US, were also shown to have accumulated multiple mutations (potentially DRMs) despite widespread HAART use in the country. In conclusion, the results in this study underscore the importance of ongoing HIV-1 drug development and monitoring the transfer of HIV-1 DRMs.

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Appendix 1

Period	Period Significance	From	To	Number of Sequences Retrieved
1	1987 (Monotherapy implementation)	01/01/1987	12/31/1991	17
2	1992 (Dual-therapy implementation, mono therapy still used)	01/01/1992	12/31/1995	78
3	1996 (First combination pill is FDA approved, HAART implementation)	01/01/1996	12/31/2002	208
4	2003 (First fusion inhibitor is FDA approved)	01/01/2003	12/31/2006	822
5	2007 (First ISTI and CCR5 antagonist is FDA approved)	01/01/2007	12/31/2011	803
6	2012 (Truvada is FDA approved)	01/01/2012	12/31/2017	2595
7	2018 (First monoclonal Ab approved)	01/01/2018	12/31/2022	5124

* This grouping was based on the timelines created by Cheney et al. (2021), which detailed the Food and Drug Administration (FDA) approval dates of HIV-1 ARV drugs, and Arts et al. (2012), which marked the approximate dates ARV treatment methods were implemented.