REVIEW

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Adaptation of advanced clinical virology assays from HIV-1 to SARS-CoV-2

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Purpose of review
In response to the HIV–AIDS pandemic, great strides have been made in developing molecular methods that accurately quantify nucleic acid products of HIV-1 at different stages of viral replication and to assess HIV-1 sequence diversity and its effect on susceptibility to small molecule inhibitors and neutralizing antibodies. Here, we review how knowledge gained from these approaches, including viral RNA quantification and sequence analyses, have been rapidly applied to study SARS-CoV-2 and the COVID-19 pandemic.

Recent findings
Recent studies have shown detection of SARS-CoV-2 RNA in blood of infected individuals by reverse transcriptase PCR (RT-PCR); and, as in HIV-1 infection, there is growing evidence that the level of viral RNA in plasma may be related to COVID disease severity. Unlike HIV-1, SARS-CoV-2 sequences are highly conserved limiting SARS-CoV-2 sequencing applications to investigating interpatient genetic diversity for phylogenetic analysis. Sensitive sequencing technologies, originally developed for HIV-1, will be needed to investigate intrapatient SARS-CoV-2 genetic variation in response to antiviral therapeutics and vaccines.

Summary
Methods used for HIV-1 have been rapidly applied to SARS-CoV-2/COVID-19 to understand pathogenesis and prognosis. Further application of such methods should improve precision of therapy and outcome.

Keywords
COVID-19, COVID-19 prognosis, SARS-CoV-2 diagnostics, SARS-CoV-2 genetics, SARS-CoV-2 viremia

INTRODUCTION

In response to the COVID-19 pandemic, researchers across the globe have collectively shifted focus to the common goal of understanding the newly emerged SARS-CoV-2. HIV-1 researchers have accumulated nearly 40 years of experience studying an epidemic caused by a newly emerged viral pathogen, uniquely positioning them to quickly respond to a new viral pandemic. Many methods and technologies used during the HIV-1 epidemic can and have been applied to understanding the SARS-CoV-2 pandemic. In the following review, we discuss how molecular technologies developed for HIV-1 have been applied successfully and swiftly to the study of SARS-CoV-2.

SENSITIVE METHODS FOR MEASURING VIRAL RNA

Early in the AIDS epidemic disease was diagnosed and monitored clinically and by lymphocyte counts. Once HIV-1 was identified as the causative agent of AIDS [1,2], tests were developed to diagnose infection serologically and quantify HIV-1 RNA in blood [3–5]. The importance of quantifying HIV-1 RNA in plasma (also referred to as viral load) for prognosis was then demonstrated [6,7] and it soon became routine practice to measure viral load for disease staging and assessing response to treatment (Table 1). The importance of viral load as a marker of treatment failure and emergence of drug resistance from a highly diverse viral population became clear with the discovery of more potent inhibitors of viral replication. Indeed, the importance of viral load in disease monitoring became so widely accepted that

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KEY POINTS

- An important breakthrough in HIV-1 science was the development of sensitive RT-PCR assays of plasma HIV-1 RNA to estimate prognosis, understand pathogenesis and identify effective treatments.
- Improved HIV-1 RNA assays with single copy sensitivity have provided insights in HIV-1 persistence on antiretroviral therapy and can help guide efforts to eliminate HIV-1 reservoirs.
- Until recently, detection of SARS-CoV-2 RNA by RT-PCR assays in nasopharyngeal and oropharyngeal samples have been the sole method for diagnosis of COVID-19.
- Similar to HIV-1, quantification of SARS-CoV-2 RNA in plasma has been associated with disease severity and more sensitive assay may strengthen the association and provide further insights in disease pathogenesis and persistence.
- Despite the limited genetic diversity of SARS-CoV-2 compared with HIV-1, the development of vaccines and therapeutics for COVID-19 will likely create the need for more sensitive sequencing methods to detect the emergence of minor populations of escape variants and to determine the linkage of mutations detected.

the WHO recommended implementation of viral load testing for monitoring individuals on antiretroviral therapy (ART) in resource-limited settings in 2010 [8]. As viral load monitoring has become more important automated platforms have increased the ease and throughput of testing. Roche, Abbott, Hologic, and Cepheid offer FDA-cleared nucleic acid amplification-based HIV-1 RNA quantification tests on automated platforms that are widely used across the world (Table 1).

Table 1. Nucleic acid amplification tests for HIV-1 and SARS-COV-2

Automated NAAT	HIV	Quantitative Diagnosis confirmation Treatment and drug resistance monitoring
	SARS-CoV-2	Qualitative/Semiquantitative Respiratory samples only Diagnosis (EUA)
Manual NAAT	HIV	Increased sensitivity (single copy) Nonplasma sample types Cure, treatment, and drug resistance trials
	SARS-CoV-2	Quantitative Nonrespiratory sample types Increased sensitivity

NAAT, nucleic acid amplification tests.

Persistent HIV-1 viremia on antiretroviral therapy

Monitoring HIV-1 RNA levels below the limit of quantitation of commercial assays has become an important endpoint for many studies aimed at HIV cure. This is because, although ART results in logarithmic decreases in viral load, assays capable of detecting single copies of HIV-1 RNA have shown that the majority of those on ART have detectable HIV-1 RNA in the range of 1–3 cps/ml [9–17]. Studies of decay dynamics of virus in plasma have given important insights into disease progression and treatment, including the existence of a long-lived viral reservoir [12,13,15]. In addition, clearance of low-level persistent viremia may be a marker for HIV cure so great focus has been given to detection of viral RNA below 20 cps/ml. Some iteration of manual reverse transcriptase PCR (RT-PCR) assays have been used for most studies of persistent low-level viremia (Table 1) [9,14,16]. Since manual single copy assays are inherently low-throughput and technically difficult there have been recent efforts to adapt high-throughput commercial platforms for detection of low-level persistent viremia [18▪,19]. Incorporating high throughput single copy assays will facilitate large studies aimed at clearing persistent viremia and achieving functional cure.

Molecular testing for SARS-CoV-2 RNA

One of the largest and most critical efforts at the start of the COVID-19 pandemic was the search for a reliable, quick, and broadly available test for SARS-CoV-2 infection. Since the beginning of the HIV epidemic in the 1980s the field of molecular diagnostics has made tremendous strides, enabling the release of a molecular test for SARS-CoV-2 RNA very quickly after the sequence of the viral genome was made available [20▪▪,21,22▪,23]. In addition to significant technical advances in molecular diagnostics, the low genetic diversity of SARS-CoV-2 as compared with HIV-1 and other RNA viruses has also contributed to the quick release and implementation of molecular assays to detect SARS-CoV-2 RNA [24▪▪]. Similar to HIV-1 the first SARS-CoV-2 molecular diagnostic tests developed were largely manual, but diagnostic companies quickly followed with their own high-throughput versions with varying amounts of automation, including Roche, Abbott, Hologic, and Cepheid (Table 1). Importantly, these tests are designed to test respiratory specimens and are qualitative with some simply returning a positive or negative result. Others return a cycle threshold value, allowing approximation of levels of viral RNA in the sample. As the pandemic

evolves and data accumulates the importance of quantitative tests for understanding infection dynamics is becoming clear, particularly as it relates to disseminated infection.

Plasma RNAemia in SARS-CoV-2 infection

Little focus has been given to the potential presence of virus in the blood (viremia) in most respiratory infections since primary target cells and general symptoms point to respiratory containment of infection. However, analyses of SARS-CoV-2 RNA in the nasopharynx have not shown consistent associations with symptomatic disease, including the severity of symptomatic disease [25–30]. While the lack of consistent association is partly attributable to inconsistencies in sample collection [31], variable viral replication kinetics in the upper respiratory tract over the infection course represent another possible explanation for the lack of consistent association between diagnostic test cycle threshold value and disease severity [32]. In addition, sampling of only the nasopharynx may miss contributions to disease caused by viral replication in the lower respiratory track or dissemination of virus through blood to extrapulmonary sites. Reports of both expanded receptor expression beyond the primary target cells in the lung and presence of virus in peripheral tissues such as the gastrointestinal (GI) tract, endothelium and central nervous system (CNS) may point to disseminated viral infection as an important contributor to severe disease [33–41]. Indeed, an unusually wide range of symptoms can accompany SARS-CoV-2 infection, including thromboses, loss of smell, vomiting, and diarrhea [37,42–44]. Unlike HIV-1, current data do not suggest that SARS-CoV-2 replicates to a significant degree in peripheral blood cells; however, normal blood flow could provide a means for virus dissemination to extrapulmonary sites. Aside from a possible role in disease pathogenesis, blood–borne viral RNA could also be an important indicator of lung tissue breakdown leading to release of intact virions, viral proteins and nucleic acids, or infected cells into the bloodstream. Along these lines, a few groups have found that the detection of SARS-CoV-2 RNA in plasma (SARS-CoV-2 RNAemia) is associated with severe disease [45▪,46▪,47,48▪–50▪], although to inconsistent degrees. The inconsistency in proportion of patients with plasma RNA and the amount of plasma RNA is likely due to differences in definitions of disease severity and in the RT-PCR methods used. SARS-CoV-2 RNA has been detected in plasma from variable proportions of patients with COVID-19, ranging from 35% of hospitalized patients [46▪] to 88% of critically ill patients [49▪], with clear trends in each study toward severe disease in those with RNAemia. Our group has found a similar trend using a highly sensitive N-targeting RT-PCR assay, with ~90% of ICU patients having RNAemia and ~60% of hospitalized, non-ICU patients showing RNAemia (unpublished data). Longitudinal analysis from patients across the spectrum of disease severity, including those with progressive disease during the observation period, is ongoing and is expected to provide additional insight into the usefulness of SARS-CoV-2 plasma RNAemia as a prognostic marker. In addition, it is possible that application of single copy assays to blood samples similar to those discussed above for HIV-1 will increase the frequency of SARS-CoV-2 RNA detection in less severe disease (Table 1). These studies are also ongoing. Important centrifugation and antibody pull-down studies to determine whether viral nucleic acid detected in plasma corresponds to viral particles and/or infected cells are ongoing as well; and future studies should address whether any virions present are infectious, and if infected cells are present, if they are producing infectious virus by assessing virus growth in cell culture in a Biosafety Level 3 facility. These studies will help define the clinical significance of SARS-CoV-2 RNAemia by addressing whether detection of viral RNA in plasma signals uncontrolled infection and a risk for complications requiring earlier intervention. Further studies focused on whether therapies that prevent or reduce RNAemia could improve outcome will be important and could accelerate development of effective therapies for COVID-19, similar to what occurred with ART of HIV-1 infection [51].

HIV-1 SEQUENCING TECHNOLOGIES FOR SARS-COV-2

Applying sequencing analysis to HIV-1 has provided tremendous insight into its global spread, transmission patterns, pathogenesis, immune escape, diagnostics, and viral response to treatments including small molecules and neutralizing antibodies and by identifying mutations associated with viral drug resistance to ART. Although the initial epidemiology of HIV-1 and AIDS was based on serological studies, sequencing technologies provided rapid and new insights [52–54], revealing large diversity between HIV-1 isolates, and it is now understood that HIV-1 has an extraordinarily high genetic variability with a mutation rate of 4×10^{-5} nucleotide per site per replication cycle [55–57] resulting in rapid adaptation to selective pressures. Because of the high mutation rates and highly divergent lineages, no vaccine exists to prevent the transmission of HIV-1.

HIV-1 sequencing technology for HIV-1 drug resistance

In addition to applications for improving primer design for more accurate virus quantification, a major application of HIV-1 sequencing is to detect mutations in the virus that are associated with drug resistance. By identifying mutations in HIV-1 that may have been selected for in persons receiving ART or examining the effectiveness of ART regimens in individuals with viruses containing suspected drug-resistance mutations, sequencing technology was used as a tool for establishing the most effective ART regimens [58].

To this aim, first-generation Sanger-based sequencing technologies provided a foundation for the commercialization of clinical drug resistance assays, which have allowed clinicians to tailor treatment to an individual patient's mutational pattern. Although Sanger-technology was dependable for accurately identifying mutations associated with drug resistance in the majority (>20%) of viruses circulating in plasma from persons living with HIV-1, elucidating minor intraindividual variation required sequencing cDNA templates diluted to single copies, known as single-genome sequencing (SGS) [59–61] (Table 2). Although SGS increased the sensitivity for minor variants over Sanger technology, it was limited by the number of sequencing reactions required for each patient. Next-generation sequencing (NGS) increased the throughput capacity and accuracy of sequencing by allowing for the simultaneous sequencing of thousands of copies of DNA in a single run. Multiplex amplified samples with unique index sequences greatly reduced the complexity of SGS allowing multiple amplified cDNA products in the same sequencing run. Subsequently the incorporation of cDNA barcoding into NGS increased the sensitivity for minor variant detection [62,63] even more than SGS [i.e., ultrasensitive SGS (uSGS) [64]] and was recently used to uncover rare mutations linked on the same HIV-1 genome [65].

SARS-CoV-2 diversity and sequencing

Since the first sequence of SARS-CoV-2 was generated [22▪], more than 95 000 SARS-CoV-2 sequences have been deposited in the global initiative on sharing all influenza data (GISAID). Unlike HIV-1, which has high-level heterogeneity even within an infected individual with an estimated substitution rate of $0.8–1.7 \times 10^{-3}$ per site per year [66–68], SARS-CoV-2 sequences have a high-sequence identity with a substitution rate estimated to be $8.4 \times 10^{-4}–1.1 \times 10^{-3}$ per site per year at the population level [69–72]. This conservation has largely been attributed to the exoribonuclease (ExoN) activity of the nonstructural protein 14 (nsp14) as it was demonstrated that wild-type SARS-CoV has a mutation rate of 9.0×10^{-7} substitutions per nucleotide per replication cycle [73]. However, a mutant form with an inactivating substitutions in nsp14 reduced the fidelity to 1.2×10^{-5} substitutions per nucleotide per replication cycle, strikingly similar to HIV-1 and other RNA viruses [73–75]. Significantly, it was shown that a common SARS-CoV-2 mutation 14408C>T that causes a P323L substitution in the RNA dependent RNA polymerase (nsp12) may also

Table 2. Sequencing platforms used for the detection of HIV drug resistance

Sequencing generation	Technology	Manufacturer	Applications to HIV DR testing
First	Sanger	ThermoFisher (Applied Biosystems)	Population-based sequencing for mutations >20% viral population Single-genome sequencing by limiting dilutions of cDNA to detect linkage
Second 'Next'	Pyrosequencing	Roche – discontinued	High-throughput multiplexing Sensitive sequencing for mutations >5% viral population uSGS by cDNA PrimerID tagging for minor variants (1–5%) [61,62]
	Ion Torrent	ThermoFisher (Life Technologies)	
	SBS	Illumina	
Third	SMRT sequencing	PacBio	Long HIV-1 reads Sensitive sequencing for mutations >5% viral population (looped amplification)
	Nanopore sequencing	Oxford Nanopore Technologies	

DR, drug resistance; SBS, sequencing-by-synthesis; SGS, single-genome sequencing; uSGS, ultrasensitive single-genome sequencing.

Table 3. SARS-CoV-2 phylogenetic categorization systems Nextstrain, global initiative on sharing all influenza data and PANGOLIN

Clades		Lineages[a]	
Nextstrain (79)	**GISAID (78)**	**PANGOLIN (80)**	**GISAID Clade marker variants relative to WIV04-reference**
19B	S	A	C8782, T28144C
19A	L	B	C241, C3037, A23403, C8782, G11083, G25563, G26144, T28144, G28882
	V		G11083T, G26144T NSP6-L37F + NS3-G251V
20A	G	B.1	C241T, C3037T, A23403G includes S-D614G
20C	GH		C241T, C3037T, A23403G, G25563T includes S-D614G + NS3-Q57H
20B	GR	B.1.1	C241T, C3037T, A23403G, G28882A includes S-D614G + N-G204R

[a]Pangolin lineages are also based upon phylogenetic evidence, but because they also factor in active local geographical outbreaks, they are too numerous for this table. For a more detailed information go to cov-lineages.org.

increase the mutation rate, suggesting that mutations in regions outside of the ExoN could also alter SARS-CoV-2 fidelity [76].

In contrast to the complex HIV-1 classification systems, the conservation of SARS-CoV-2 required that slight differences in sequences were used to develop the three major nomenclature classification systems [77▪▪] (Table 3). The first two classification systems, GISAID and Nextstrain, are focused on investigating large scale diversity patterns of SARS-CoV-2 for understanding of patterns and determinants of the global spread by identifying clades that persist for at least several months and have significant geographic spread [78,79]. The PANGOLIN lineage system is the third classification system and was developed to have short-term epidemiological significance by utilizing a more dynamic system for tracking active virus lineages [77▪▪,78,80].

Sequencing SARS-CoV-2 for clinical research

As with HIV-1, SARS-CoV-2 sequences have facilitated the investigation of transmission dynamics to guide public health action and to elucidate sequence conservation for the development of robust molecular diagnostic methods. For these reasons, SARS-CoV-2 whole genome sequencing (WGS) will continue to be essential in tracking the pandemic. In addition, as case studies have recently documented reinfections of SARS-CoV-2, the limited amount of sequence diversity in SARS-CoV-2 in any given gene region necessitates WGS for differentiating the first and second episodes by showing that each of the viruses belonged to a different lineages rather than a few mutations having been a consequence of RT-PCR or sequencing error [81,82]. Although the sequence conservation in SARS-CoV-2 genomes has limited the application of sequencing technologies relative to HIV-1, selective pressures from both host immune as well as novel vaccine and therapeutic interventions are likely to drive novel mutations in SARS-CoV-2 in the future. The SARS-CoV-2 envelope glycoprotein or Spike mediates viral entry into host cells and is thus currently the major target for vaccine and monoclonal neutralizing antibody development. As such, Spike gene sequencing will be a key focus for identifying immune escape variants. Several studies have shown that patients infected with SARS-CoV-2 strains having Gly614 variant in Spike had on average lower RT-PCR cycle threshold values relative to individuals infected with Asp614 [83▪,84] and it was also shown that the D614G substitution increases the infectivity *in vitro* [83▪,85]. However, there has been no conclusive evidence that any of the emerging SARS-CoV-2 substitutions have resulted in a change in transmissibility or the disease severity of COVID-19 [24▪▪]. There is also a growing body of evidence to suggest that deletions as well as mutations may be important for SARS-CoV-2 infection. For example, a deletion in Spike at the S1/S2 cleavage site that causes a premature stop codon after S1 was observed and it was speculated that this deletion may cause an increase in free S1 as a mechanism to mitigate the host response to SARS-CoV-2 infection and cause less severe disease [86]. Such deletions may also exist outside of Spike; in fact, a group recently showed that a 382-nucleotide deletion in SARS-CoV-2 ORF7b and ORF8 eliminates ORF8 transcription and, because there is a robust antibody response to ORF8, they suggest that it may be the result of immune-driven selection [69].

It is possible that more sensitive sequencing approaches are needed, such as SGS or uSGS, to completely understand the intrapatient variation and consequences of SARS-CoV-2 mutations. In addition, as the selection of mutations through immunotherapy or vaccination will often come at the expense of fitness, genetic variants will likely exist at different frequencies depending on the

levels of the factor suppressing viral replication within an infected host. HIV-1 research has shown that when investigating intrapatient variation and selection it is crucial to obtain a sufficient sequence read depth to overcome the error rate of the specific sequencing platform used and to ensure that a sufficient number of templates have been sampled.

CONCLUSION

Application of the advances made through HIV-1 science to SARS-CoV-2 have provided rapid, major insights into virus quantification and sequencing in support of efforts to diagnose, treat and prevent COVID-19. Measuring HIV-1 RNA using RT-PCR-based assays greatly accelerated the development of effective therapies for HIV-1 infection. Similarly, RT-PCR-based HIV-1 for SARS-CoV-2 RNA has been routinely used for diagnosis of COVID-19 and is being extended to quantify viral dissemination and assess disease severity. Although SARS-CoV-2 isolate sequences are homogeneous and sequencing applications have been limited to WGS, it is likely that more sensitive sequencing technologies, including SGS and uSGS, will be needed to detect mutations that emerge with selective pressures from vaccines, small molecule inhibitors, and immune-based therapies.

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Conflicts of interest

J.W.M. is a consultant to Gilead Sciences and holds shares or share options in Co-Crystal Pharma, Inc., Abound Bio, Inc. and Infectious Disease Connect. K.D.M. and J.L.J. declare no conflicts of interest.

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