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REVIEW

Agnostic Sequencing for Detection of Viral Pathogens

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SUMMARY The advent of next-generation sequencing (NGS) technologies has expanded our ability to detect and analyze microbial genomes and has yielded novel molecular approaches for infectious disease diagnostics. While several targeted multiplex PCR and NGS-based assays have been widely used in public health settings in recent years, these targeted approaches are limited in that they still rely on *a priori* knowledge of a pathogen's genome, and an untargeted or unknown pathogen will not be detected. Recent public health crises have emphasized the need to prepare for a wide and rapid deployment of an agnostic diagnostic assay at the start of an outbreak to ensure an effective response to emerging viral pathogens. Metagenomic techniques can nonspecifically sequence all detectable nucleic acids in a sample and therefore do not rely on prior knowledge of a pathogen's genome. While this technology has been reviewed for bacterial diagnostics and adopted in research settings for the detection and characterization of viruses, viral metagenomics has yet to be widely deployed as a diagnostic tool in clinical laboratories. In this review, we highlight recent improvements to the performance of metagenomic viral sequencing, the current applications of metagenomic sequencing in clinical laboratories, as well as the challenges that impede the widespread adoption of this technology.

KEYWORDS viral diagnostics, metagenomic, respiratory viruses, SARS-CoV-2, molecular epidemiology, diagnostic, next-generation sequencing

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INTRODUCTION

The role of clinical and public health microbiology laboratories is to aid in the diagnosis and treatment of microbial pathogens, including bacterial, viral, fungal, and parasitic infections (1), to contribute to surveillance and monitoring of infectious agents, and to play an integral role in the response to emerging infectious diseases. Over the past few decades, these laboratories have become more reliant on highly sensitive molecular diagnostic assays to aid in the identification, characterization, and surveillance of endemic and emerging infectious diseases. The 2003 severe acute respiratory syndrome (SARS) epidemic was one of the first instances where molecular methods, including reverse transcription-PCR (RT-PCR) and amplicon sequencing, were widely implemented for disease control and rapid characterization of an emerging pathogen (2, 3).

The advent of next-generation sequencing (NGS) technologies has led to the slow but gradual implementation of sequencing into clinical and public health settings. The 2009 H1N1 influenza pandemic was the first public health crisis to occur during the NGS era. Multiplexed molecular assays and genome sequencing were utilized to characterize the virus and perform public health surveillance during the pandemic (4–6). The 2014–2016 Ebola epidemic posed unique challenges in that molecular testing and sequencing efforts had to be deployed remotely in Western Africa to contain the outbreak. That crisis saw the deployment of rapid molecular tests and portable next-generation sequencing for viral detection and genome characterization that required minimal hands-on time and laboratory training (7), as well as complex phylogenomic analysis to identify outbreak clusters (8).

More recently, the words "PCR" and "rapid antigen test" have become household vocabulary due to the 2019 coronavirus disease (COVID-19) pandemic, caused by SARS coronavirus 2 (SARS-CoV-2). The COVID-19 pandemic has brought molecular diagnostic technologies to the forefront of the fight against emerging infectious diseases. Within weeks of the emergence of SARS-CoV-2, RT-PCR-based diagnostic tests were developed and disseminated worldwide (9–11). Genomic surveillance for emerging variants was also aided by tiled-amplicon sequencing (12, 13) and tools such as NextStrain (14) and GISAID (15) to track the genetic relationship between sequenced samples in real time. However, despite recent advancements in molecular diagnostic and sequencing techniques for emerging pathogens, most techniques are targeted in what they detect, relying on *a priori* knowledge of a pathogen's genome. This makes these methods unsuitable for the detection of new or emerging pathogens or for detection of multiple agents in patients with coinfections.

The COVID-19 pandemic has emphasized the need for the development, translation, and deployment of pathogen-agnostic molecular diagnostic techniques for use in clinical and public health laboratories. Metagenomic next-generation sequencing (mNGS)-based approaches are distinct in that they nonspecifically detect the nucleic acid composition of a biospecimen and can enable early detection of emerging, novel, or rare diseases. Recent research has showcased the utility and feasibility of mNGS in clinical settings for the diagnosis of bacterial infections of unknown etiology (16–19); this application of mNGS has been reviewed extensively (20–22). This narrative review will focus on the advancements in pathogen-agnostic sequencing and sequence data analysis for viral infections, the current role of mNGS in viral diagnostics and surveillance, as well as the challenges that have hindered the translation of pathogen-agnostic sequencing to clinical reference laboratories. We end with an outlook for the future of pathogen-agnostic viral testing.

MOLECULAR DIAGNOSIS OF VIRAL INFECTIONS

History of Viral Diagnostics

Viral pathogens harness host-derived proteins to replicate. Viral particles may be enveloped or nonenveloped and contain a single- or double-stranded DNA or RNA genome. Due to the heterogeneity in viral structure and genome composition, a multitude of techniques have been developed over the past several decades to effectively

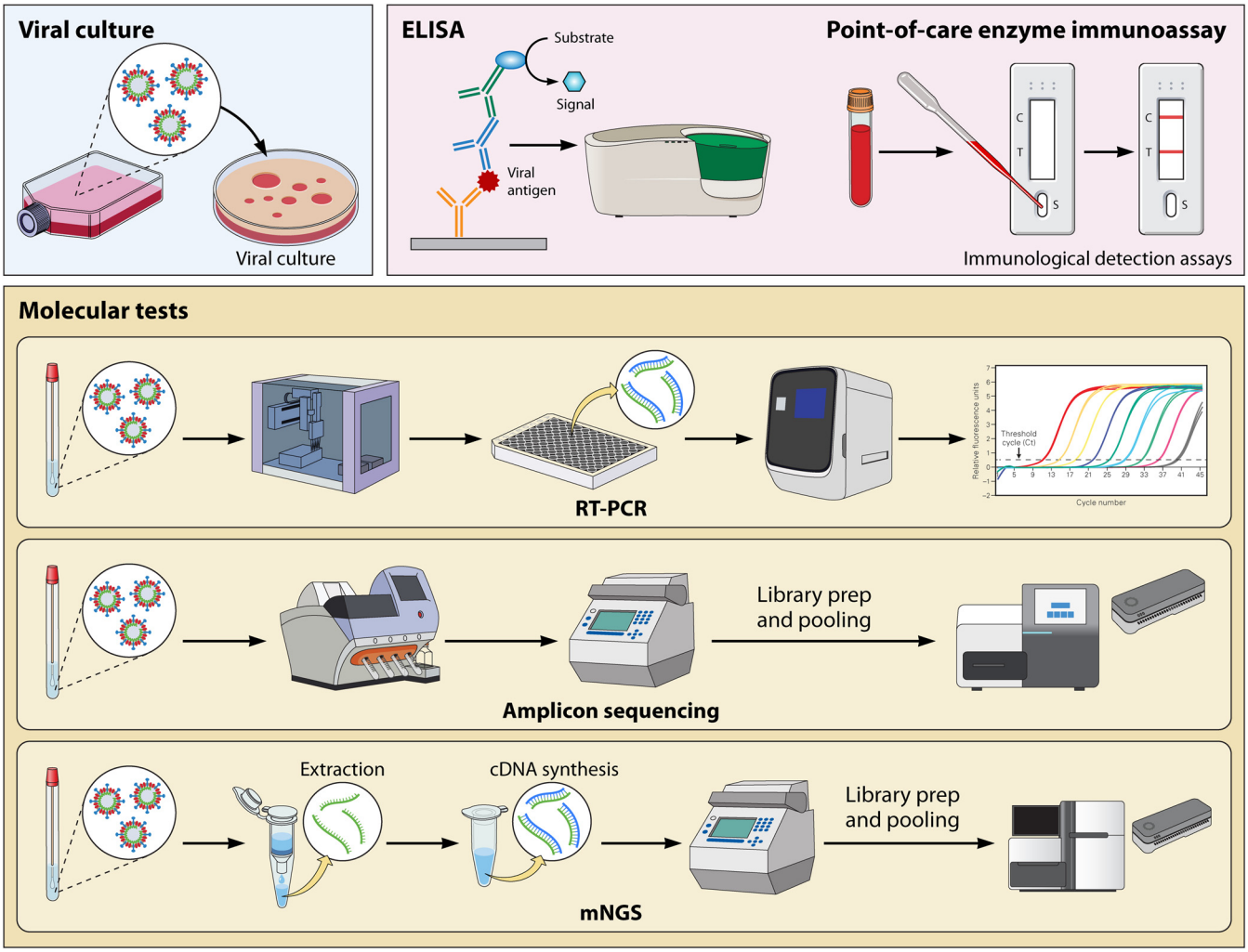


FIG 1 Summary of nonmolecular and traditional methods for molecular diagnosis and characterization of viral pathogens (top panels), along with more detailed workflows for molecular diagnostic techniques (bottom panels).

diagnose viral infections. Recently, many of these approaches have relied on molecular biology techniques to detect viral nucleic acids directly from patient samples. Traditionally, direct viral detection has been accomplished through cell culturing methods (23). Despite viral culture showing high sensitivity for some viruses, this method has a roughly 3- to 5-day lead time from sample collection to diagnosis and relies on robust sample storage to ensure the integrity of the viral particles (23), rendering the sensitivity of this method extremely variable for different viruses (24, 25). This complexity, in addition to high costs, are limitations of culturing techniques for virus detection. Immunological methods, including enzyme-linked immunosorbent assays, have been developed and extensively adopted for diagnosis of viral infections (26, 27). The 2009 H1N1 influenza pandemic and the COVID-19 pandemic led to widespread deployment of rapid antigen point-of-care tests (enzyme immunoassays) as a frontline tool for viral diagnosis; however, these tests have been shown to suffer from variable sensitivity depending on the brand of test, and sensitivity can decline as a virus' genome mutates (28, 29). Improvements in molecular techniques for viral diagnosis have overcome limitations in test performance and assay turnaround time associated with viral culture and certain immunological methods. This review will briefly summarize the role of molecular biology techniques, such as nucleic acid amplification tests (NAATs), that have been adopted for diagnosis and surveillance of viral infections and then focus in more detail on NGS and mNGS techniques (Fig. 1).

Nucleic Acid Amplification Tests for Viral Diagnostics

The development of PCR by Mullis (30) led to widespread research into the applications of nucleic acid amplification in diagnostic medicine. Several related techniques, including RT-PCR, in which RNA is reverse transcribed into cDNA prior to PCR amplification, multiplex PCR (mPCR), which includes multiple primer sets in the PCR that target two or more genomic regions of a single pathogen or multiple pathogens, and real-time PCR, a quantitative or semiquantitative method for nucleic acid detection in PCR, have all been developed and adapted to be used in clinical laboratories for viral diagnostics (31). More recently, droplet digital PCR has been utilized for viral detection due to its high sensitivity and specificity, as well as its ability to provide absolute quantification of viral nucleic acid in a patient sample. However, automation and cost reduction are required for the widespread implementation of this method (32). Loop-mediated isothermal amplification has been adopted for the diagnosis of viral infections to overcome the shortcomings of PCR, such as the presence of PCR inhibitors in clinical specimens (33). Transcription-mediated amplification is an additional NAAT that has been used for viral diagnostics (34). However, due to its low cost, reliability, and adaptability, PCR has been the gold standard for viral diagnostics over the past 2 decades.

Several platforms have been developed in recent years to meet the demands for NAATs in clinical laboratories. These large-scale multiplex NAATs have been at the forefront of COVID-19 diagnostics worldwide due to their low cost and reliability. Several prominent platforms for mPCR diagnostic testing have emerged in recent years. Laboratory-developed PCR tests (LDTs) (e.g., the U.S. CDC SARS-CoV-2 assay) and commercial platforms, including but not limited to Cepheid GeneXpert (Cepheid, CA, USA), Panther Fusion and Aptima assays (Hologic, WI, USA), Abbott m3200 (Abbott, IL, USA), BioFire respiratory panel (BioFire Diagnostics, UT, USA), and the Roche Cobas platform (Roche Diagnostics, IN, USA), have provided public health and clinical laboratories with expanded capacity for routine testing and the ability to respond effectively to emerging public health threats. Many of these and other platforms received emergency use authorization from the U.S. Food and Drug Administration (FDA) and other regulatory agencies around the world during the COVID-19 pandemic. While assay throughput, accuracy, turnaround time, and cost vary substantially across the above-mentioned platforms, clinical laboratories often use a multitude of commercial platforms and LDTs to meet testing needs (11). The expanded testing capacity available to diagnostic laboratories has been especially helpful in the public health response to COVID-19. Additionally, point-of-care NAATs, such as the Cepheid Xpert Xpress and Abbott ID NOW have been developed to provide clinical laboratories with rapid and actionable diagnostic results.

mPCR assays have been extensively validated against each other for multiple respiratory viral pathogens (35–39). Overall, the diagnostic performance of commercial and laboratory-developed mPCR panels is very high and considered the gold standard for viral detection. However, differences in positive and negative percent agreement are common when comparing commercial and LDT mPCR assays (37–42). Similarly, analytical limits of detection are variable across PCR platforms, with the Roche Cobas platform having a very low limit of detection for SARS-CoV-2 (35), which has led this platform to become the primary diagnostic comparator for validation of alternative commercial mPCR platforms (43). There is also varying concordance in diagnostic performance levels between LDTs, depending on the methodology and chosen primer target, with some countries' tests performing better than others (44). Overall, point-of-care tests appear to exhibit lower diagnostic sensitivity than LDTs or larger commercial platforms (35, 43, 45), although the Cepheid Xpert Xpress point-of-care test had comparable performance to an LDT for SARS-CoV-2 in one study (46).

Other considerations when evaluating the performance of viral diagnostic assays include sampling time and matrix (defined as the collection device and associated media used to collect and store a clinical sample), as well as the selection of primers within the assay. One study found that, among samples tested with the same assay kit, there was a difference in SARS-CoV-2 diagnostic performance depending on the PCR

primer-probe sets that were chosen (47). Additionally, sample matrices can have a large impact on an assay's diagnostic performance (48). Finally, consideration must be given to the timing and anatomical site of sample collection, as a substantial number of positive cases may be missed if sample collection does not occur at a time or site where sensitivity is optimal (49). Although PCR-based diagnostic assays have been at the forefront of viral diagnostics over the past 2 decades, this technique is limited in its ability to reveal genomic characteristics about a given pathogen that may be useful for infection treatment and public health surveillance.

Next-Generation Sequencing-Based Diagnostic and Surveillance Strategies

The widespread adoption of next-generation sequencing platforms to biomedical research has opened the door for the translation of these technologies to clinical and public health laboratories. Not only is detection of viral pathogens possible with NGS, but sequencing data can also allow users to characterize a pathogen's genome. The genomic information contained within NGS data provides useful information to clinical and public health laboratories, including the ability to call variants and trace outbreaks through phylogenetic analyses. Mutations in a pathogen's genome can also be identified through NGS data that may impact a virus's pathogenicity, infectiousness, detection (via changes in primer regions), and response to therapeutics and vaccines. Methods to sequence viral genomes in a research setting have existed for the past several decades since the invention of Sanger sequencing (50) and the early application of whole viral genome sequencing. However, until recently, this technology has been largely limited to research activities and sporadic cases where NGS has been used to diagnose pathogens of unknown etiologic origin that could not be diagnosed using alternative methods (51). Widespread translation of viral sequencing to the clinical laboratory has only occurred in recent years and has been largely driven by the reduction in the cost of sequencing reagents and platforms, improvements in library preparation kit ease of use, and the ubiquity of liquid-handling robots in diagnostic laboratories to drive sample preparation, making these techniques more feasible for use in clinical and public health settings.

Unlike bacterial pathogen identification, where the highly conserved 16S rRNA gene can be used to amplify and detect bacterial pathogens directly from clinical specimens, a universally conserved gene does not exist across all viruses, which is in part due to the vast diversity of viral genomes. Therefore, methods to effectively sequence viral genomes have historically relied on designing virus-specific primers to amplify and sequence each virus' genome. This labor and cost-intensive process has precluded whole-genome sequencing of viruses in clinical laboratories beyond several case reports to diagnose diseases of unknown etiologic origin that were RT-PCR negative for commonly targeted pathogens (52–55) or for outbreak investigations (56). Prior to the COVID-19 pandemic, routine sequencing for viral pathogens in the clinical and public health laboratory was largely limited to influenza virus surveillance (57) or multiplex PCR sequencing (i.e., amplicon sequencing) using virus-specific primers to amplify and sequence genomic fragments from known viral agents (58–60). This method of amplicon sequencing was used for characterization of viral genomes and outbreak surveillance during the 2014–2016 Ebola epidemic (8, 61), as well as for the 2015 Zika virus epidemic (12). The need to effectively characterize SARS-CoV-2 genomes to identify variants of concern with mutations that may drive transmission or increase pathogenicity led to the widespread use and optimization of SARS-CoV-2 amplicon sequencing in clinical and public health laboratories. This was performed using both clinical (62–64) and wastewater samples (65) for viral surveillance. However, while targeted sequencing methods are a highly scalable and cost-effective method for detection and characterization of viral pathogens, targeted sequencing methods are limited in that they rely on *a priori* knowledge of a pathogen's genome; untargeted agents or unknown pathogens will be missed. Therefore, pathogen-agnostic sequencing approaches are preferred for the detection and characterization of rare or emerging pathogens, as well as for effective pandemic prevention by public health.

NEXT-GENERATION SEQUENCING IN THE CLINICAL AND PUBLIC HEALTH LABORATORY

NGS Platforms: Advantages and Disadvantages

The introduction of 454 pyrosequencing (454 Life Sciences) in 2005 was the first commercially available, massively parallel (i.e., next-generation) sequencing platform and opened novel avenues of research with its increased simplicity and lower costs (66, 67). In 2007, Illumina launched their own NGS platform that has now been widely adopted in clinical settings for both research and clinical service. Ion Torrent, like Illumina (Thermo Fisher Scientific), enables massively paralleled short read sequencing that has been used for whole-genome sequencing for SARS-CoV-2 (68). Both 454 pyrosequencing, Illumina, and Ion Torrent are sequencing-by-synthesis platforms, meaning that they record signals produced by nucleotide incorporation using a polymerase enzyme. The Illumina platform can also be used for either single-read sequencing or paired-end sequencing in which both ends of the DNA fragment are sequenced. Unlike Illumina sequencing, which relies on clonal bridge amplification and sequences clusters of fragments as a single "read," or Ion Torrent, which sequences read clusters associated with individual beads coated with amplified fragments, third-generation sequencing platforms are characterized by single-molecule sequencing. More recently, Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT) have launched their own third-generation sequencing platforms. PacBio sequencing functions by sequencing adaptor-ligated circular DNA loops by recording light pulses as a polymerase adds nucleotides to the sequenced DNA fragment. Nanopore sequencing records changes in electrical potential that are produced as a DNA fragment flows through a protein nanopore, where each nucleotide produces a different electrical potential. One key difference between early and third-generation sequencing platforms is the ability of third-generation sequencing platforms to sequence long reads, versus short reads, that are produced on the Illumina, Ion Torrent, and pyrosequencing platforms. While PacBio has a read length limit in the range of 10^4 bp, ONT sequencing can produce reads up to 10^6 bp in length (69). The ONT MinION device is uniquely suited to clinical settings as it is user-friendly, highly portable, and less costly compared to the much larger Illumina, Ion Torrent, and PacBio sequencing devices. The comparably higher error rates of ONT sequencing have, until recently, precluded their translation to clinical laboratories (70); however, improvements in ONT chemistry and post-sequencing base-calling have reduced these error rates (71). Traditionally, Illumina has given the high accuracy necessary for public health surveillance efforts; however, recently, PacBio HiFi sequencing has produced comparable accuracy (72). Despite these differences, each of these platforms has been utilized for viral diagnostics and surveillance. Detailed comparisons of these platforms have been explored elsewhere (21, 73–75).

Targeted versus Nontargeted Sequencing

There are two key approaches to sequencing a virus' genome, targeted and untargeted (i.e., pathogen-agnostic). While the initial sample collection, processing, and nucleic acid extraction can be similar for these two approaches, untargeted sequencing may require more sample volume, or a pretreatment step to reduce host nucleic acid. Additionally, the library preparation procedures and data analysis workflows can differ substantially (Fig. 2). Regardless of whether a targeted or untargeted approach is used, one key consideration when designing or implementing a sequencing-based diagnostic assay for viral detection is the nature of the virus' genome. Viral genomes can be single or double stranded and contain DNA or RNA. While DNA viral genomes can be directly amplified or sequenced from a biological sample, RNA genomes generally must be reverse transcribed to cDNA prior to amplification or DNA sequencing.

Pathogen-agnostic sequencing holds several key advantages over targeted sequencing approaches for viral diagnostics and genome characterization. Untargeted sequencing approaches do not rely on prior knowledge of a pathogen's genome and can detect multiple pathogens within a sample. This is especially valuable when sequencing an emerging

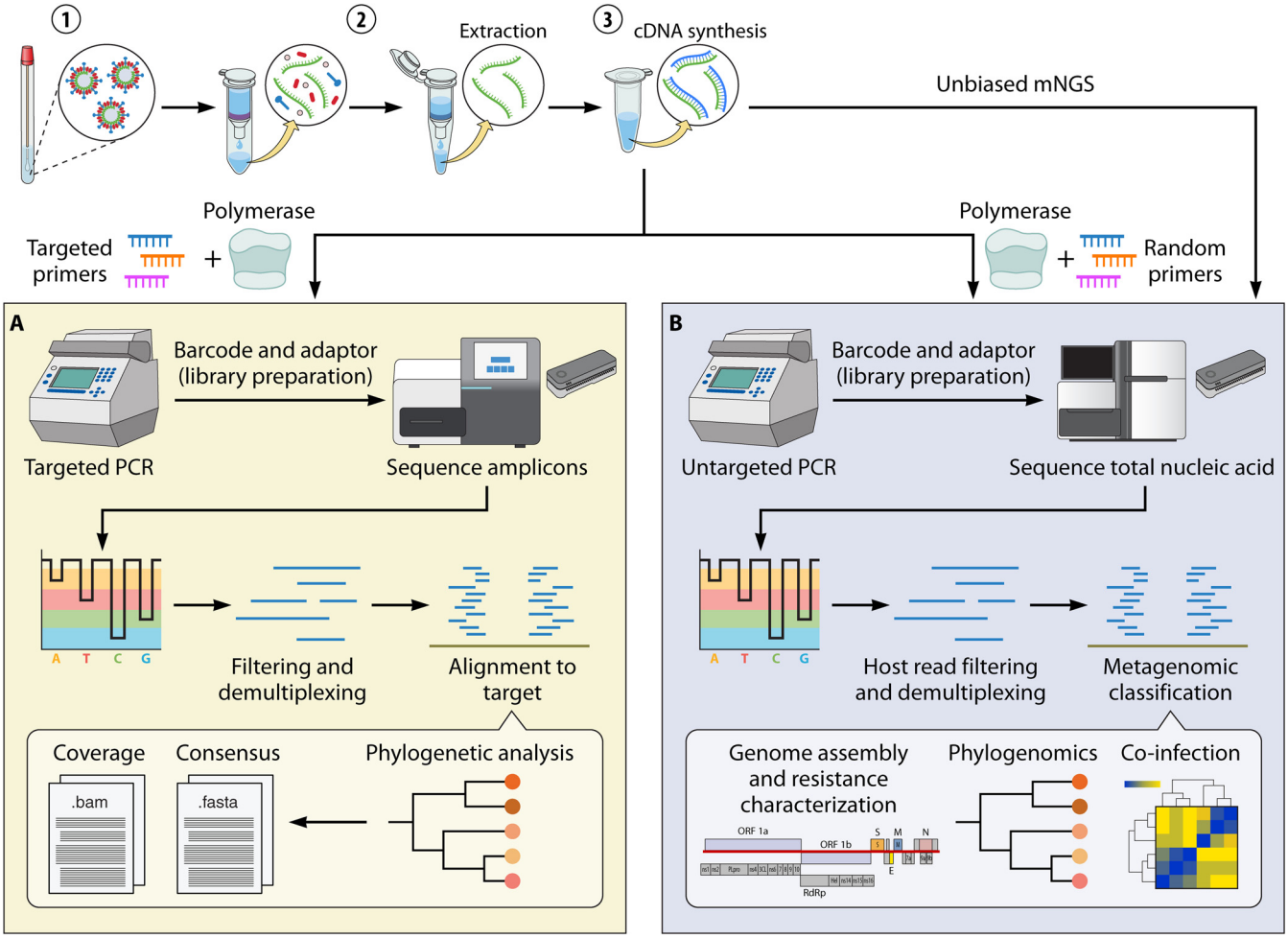


FIG 2 Sample preparation, library preparation, sequencing, and data analysis workflows for both targeted (i.e., amplicon NGS) (A) and untargeted (i.e., mNGS) (B) for detection of viral pathogens. The basic workflow for mNGS and amplicon sequencing is often similar until the cDNA synthesis and PCR step, where amplified mNGS approaches are characterized by random amplification techniques rather than targeted multiplex PCR. mNGS approaches may also be unbiased, as cDNA is directly sequenced without amplification to avoid amplification bias. Unbiased mNGS is difficult for samples with low abundance of viral nucleic acid or high abundance of host nucleic acid. mNGS often requires deeper sequencing for detection of viral pathogens. Detection of coinfections and unknown pathogens is possible with mNGS but not amplicon sequencing.

pathogen (where validated tests have not been developed or deployed), rare pathogens, clinical specimens from infections of unknown etiologic origins, or coinfections. Pathogen-agnostic approaches allow for both diagnosis of infection and characterization of a virus' genome (76–78). Clinically actionable information from viral genomes can include information about drug resistance and mutations that may confer changes to transmissibility or pathogenicity. Targeted sequencing on the other hand can allow clinical laboratories to perform massively parallel sequencing for outbreak investigation and public health surveillance for a single viral agent (61, 63). Cost should also be considered when implementing untargeted sequencing approaches. Due to the potential for there to be low pathogen abundance in a clinical sample, deeper untargeted sequencing is often required to detect viral pathogens within a patient sample. In contrast, targeted PCR amplification prior to targeted sequencing allows for lower-depth sequencing and permits extensive sample multiplexing, thereby reducing sequencing costs (64). Decreased diagnostic performance of mNGS for viral pathogens in samples with low viral loads is also a significant concern (76, 78–80). Poor diagnostic sensitivity may, in part, be due to the high relative abundance of host nucleic acid occupying most of the sequencing output. However, there is reason to be optimistic for the future of pathogen-agnostic untargeted sequencing due to advancements in sample preparation, library preparation, host nucleic acid depletion, and sequencing technology.

NGS in the Clinical and Public Health Laboratory: Success Stories

Although the applications of NGS technologies in clinical and public health laboratory settings have been limited until recently, there have been several recent success stories. Following the 2009 H1N1 influenza pandemic, there was an imperative to implement sequencing-based influenza surveillance systems to track changes in influenza subtypes and to detect clades which may compromise vaccine or antiviral treatment effectiveness. Early influenza surveillance sequencing efforts were largely driven by Sanger sequencing (81, 82) or deep sequencing of clinical samples to recover influenza genomes (83). However, due to the presence of resistant variants across many influenza genes, Sanger sequencing does not have the scaling capacity to effectively track resistance and emerging variants across the entire genome or over multiple gene targets (84, 85). NGS-based protocols can overcome some of the limitations of Sanger sequencing. In recent years, several groups have worked to optimize and implement NGS-based amplicon sequencing approaches for influenza surveillance (61, 86, 87) and identification of novel RT-PCR target sites within the influenza genome (88). More recently, mNGS has also been shown to be highly effective at detection and characterization of influenza virus genomes (78, 79).

The 2014–2016 Ebola virus epidemic and recent Zika virus outbreaks have presented novel challenges to molecular epidemiology of viral pathogens. While NGS platforms for viral surveillance had performed well in clinical laboratories of high-income countries with established molecular testing infrastructure, these public health crises offered the new challenge of integrating molecular surveillance into regions that did not already have extensive molecular diagnostic laboratory infrastructure. To effectively respond to outbreaks in these regions, sequencing workflows needed to be adapted to produce sequence data and analyze viral sequences in real time in regions with limited resources. RNA sequencing had been effectively used for phylogenetic linkage of Zika infection clusters (53). However, Quick and colleagues were able to effectively develop a real-time amplicon sequencing protocol that could be remotely deployed to difficult to access regions using the portable MinION sequencing device (ONT) (61). This method was effectively utilized for molecular epidemiology of both the Zika and Ebola outbreaks (8, 12, 61).

The COVID-19 pandemic led to the rapid deployment of NGS-based surveillance and diagnostics worldwide. While RT-PCR has led the way for SARS-CoV-2 diagnosis in clinical laboratories (9), targeted NGS has been a widely used and optimized tool for phylogenetically tracking mutations in the SARS-CoV-2 genome and detecting emerging variants of concern (62–64). Recently, wastewater surveillance has played a major role in SARS-CoV-2 surveillance as widespread clinical testing declines and we approach a state of endemic COVID-19 in the population (65). Never have rapid advancements in molecular surveillance and diagnostic technologies been developed and translated at a rate we have witnessed since March 2020.

Recently, responses to the 2022 Monkeypox (MPOX) outbreak (89) have incorporated many of the same diagnostic testing technologies developed during the COVID-19 pandemic. Currently, the gold standard for MPOX diagnosis is either MPOX PCR alone or orthopoxvirus PCR coupled with downstream MPOX PCR or sequencing to confirm diagnosis (90, 91). Several PCR assays have been developed to distinguish MPOX clades (92); however, as mentioned in the section above, one limitation of PCR is that its ability to detect individual mutations in a virus' genome is limited without designing an entirely new assay. There have been several success stories of NGS for diagnosis and surveillance of MPOX. The first case of MPOX in Brazil was diagnosed with a modified, randomly primed metagenomic sequencing technique termed SMART-9N (93). Additionally, phylogenetic analysis of NGS data from MPOX patients provided evidence to suggest sexual transmission of MPOX in Italy (94), and evidence showing transmission between humans and dogs was reported (95). Phylogenetic analysis of MPOX sequence data has also suggested the virus has been circulating outside endemic regions for quite some time (96). Sequence data provided by these studies

has allowed public health officials to effectively track its progression and to enact a robust response to this outbreak.

Although there have been numerous examples of success stories of NGS implementation in the public health laboratory, many of the applications of NGS have been limited to targeted approaches. Pathogen-agnostic approaches (mNGS) remain largely confined to research applications or to specialized clinical diagnostic services at a select group of Clinical Laboratory Improvement Amendments (CLIA)-certified laboratories to identify the cause of infections of unknown etiology (97). Widespread implementation in clinical service has been limited. However, there are advantages to using mNGS for routine diagnostics over targeted approaches, including the ability to detect coinfections, rare pathogens that circulate at low prevalence in the population, or emerging pathogens where gold standard diagnostic assays have not been developed. However, there are multiple disadvantages or barriers. Cost considerations, laboratory logistics, diagnostic performance, and access to user-friendly and robust bioinformatics pipelines have prevented mNGS from being readily translated from research settings to clinical and public health service laboratories. Several key recent advancements have pushed this technology toward more widespread adoption. These key advancements in mNGS include sample preparation, library preparation, host depletion, and data analysis and are discussed in the following section.

VIRAL METAGENOMIC SEQUENCING AND ANALYSIS TECHNIQUES

Advances in Sample Processing and Library Preparation

The basic workflow for conducting an mNGS experiment includes sample collection, nucleic acid extraction (RNA, DNA, or whole nucleic acid, depending on the target of interest), reverse transcription of RNA to cDNA (for RNA viruses), followed by nucleic acid amplification, sample-specific barcode adaptor ligation, and library quantification and pooling. While an untargeted amplification step is not always necessary for metagenomic sequencing of bacterial pathogens, this step is often required to effectively sequence viruses due to their small genomes and the comparatively low concentration of viral nucleic acid in clinical samples. Host nucleic acid depletion or viral enrichment protocols are frequently utilized to increase diagnostic sensitivity and can be introduced into the workflow (Fig. 3). A summary of recent advancements in these fields is provided in this section.

While protocols to extract nucleic acids from clinical specimens have existed and been extensively validated for many years, the COVID-19 pandemic led to a rapid expansion of workflows to perform massively parallel extractions on clinical samples. This has been largely driven by an expansion of workflows and platforms that utilize a walk-away extraction strategy that can be achieved with programmed protocols on liquid-handling robots (98, 99).

While methods for random amplification of DNA from clinical samples have been explored extensively, many of the recent advancements in viral mNGS have surrounded the development and optimization of techniques to target RNA viruses. RNA viruses are of particular importance, as these viruses have been responsible for the majority of recent pandemics and public health crises (e.g., SARS, influenza, respiratory syncytial virus, Ebola, Zika, dengue, SARS-CoV-2). Additionally, there is evidence that DNA viruses may be detected with RNA sequencing (100), as all viruses have an RNA stage. The low biomass of viral RNA in clinical specimens, along with the need for DNA as input to many sequencing platforms, requires RNA extracts to be reverse transcribed and undergo nonspecific PCR amplification prior to sequencing. Several studies investigating the feasibility and utility of mNGS for viral detection and characterization utilized protocols that harnessed random hexamers to randomly prime, reverse transcribe, and amplify cDNA (101, 102). While these approaches have achieved good performance and demonstrated the feasibility of mNGS for viral detection, several recent studies have suggested that sequence-independent single-primer amplification (SISPA) (103) may offer improved diagnostic performance over random hexamers for

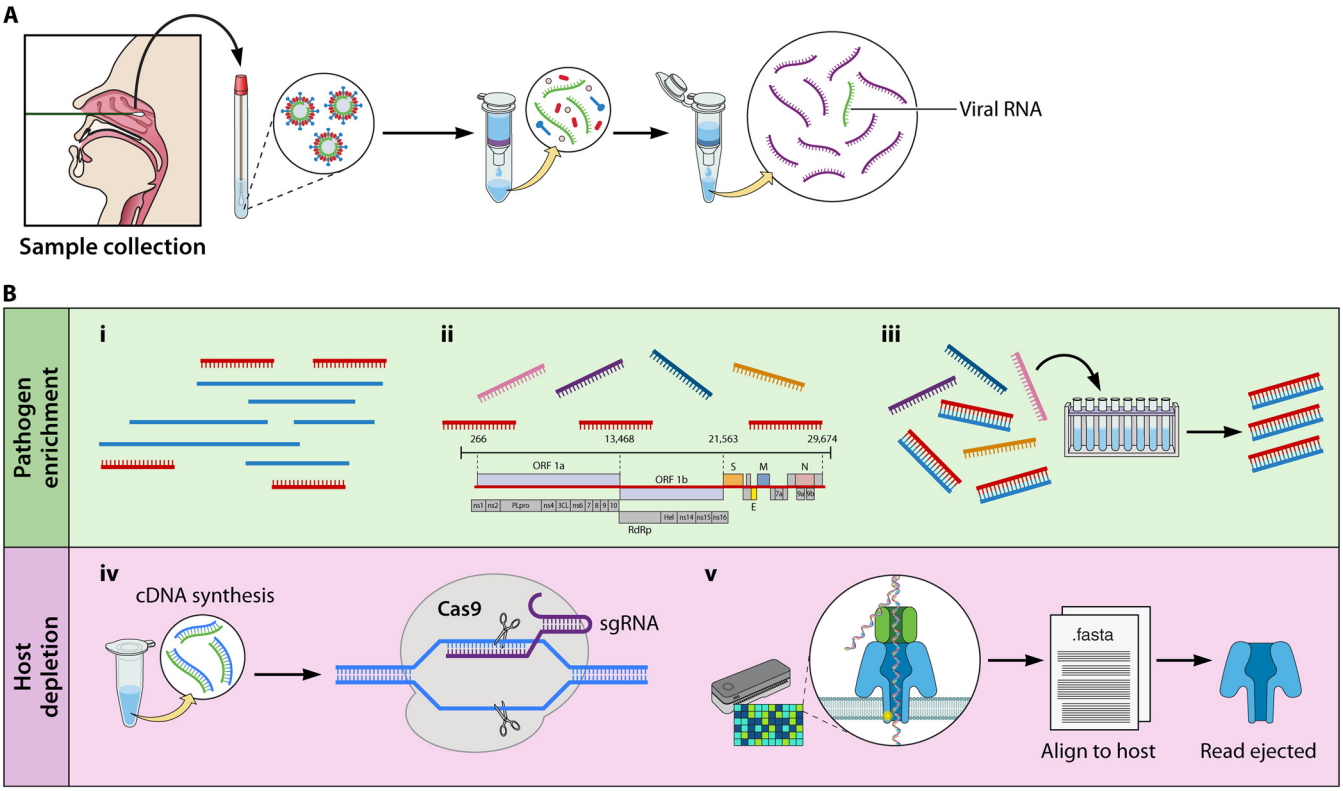


FIG 3 (A) Model of initial upper respiratory specimen collection and processing workflow detailing the nucleic acid composition of many clinical samples. Viral nucleic acid often represents a very small proportion of the total nucleic acid within a sample. (B) Schematics of approaches for pathogen enrichment (i to iii) and host depletion (iv and v) that have been utilized in mNGS workflows. Pathogen-enriched primers-probes are highlighted in red. Pathogen enrichment approaches include the following: (i) not-so-random primers that do not bind to off-target nucleic acid; (ii) metagenomic sequencing with spiked primer enrichment (MSSPE), in which primers for targeted pathogens are spiked at higher concentrations; and (iii) capture probe enrichment, where hybridized fragments are enriched following a magnetic bead pulldown. (iv) The host depletion approaches are illustrated. Depletion of abundant sequences by hybridization (DASH) (iv), and versus ONT's adaptive sampling (v).

respiratory syncytial virus (104) and SARS-CoV-2 (105). SISPA is a technique that utilizes random nonamers tagged to a known linker oligonucleotide sequence (primer A) to randomly prime and reverse transcribe RNA to cDNA in a two-step process. A second primer complementary to the linker sequence of the first primer (primer B) is then added to the mix and used to PCR amplify the cDNA product. The feasibility and performance of this technique for detection and characterization of RNA viruses from clinical samples has been demonstrated for several viruses, including Ebola, Chikungunya (77), avian RNA viruses (106), dengue (80), influenza (78, 79), and SARS-CoV-2 (76), among others (76, 78). A related technique, SMART-9N, performs random priming and one-step cDNA synthesis to reduce workflow time (93, 107). Rapid SMART-9N also couples random PCR amplification to Nanopore library preparation to further reduce workflow time (107, 108). A modified version of this approach was used to detect the first case of MPOX in Brazil (93). However, SISPA and SMART-9N can suffer from low sensitivity compared to RT-PCR when viral abundance is low (68, 70).

Not-so-random primers (109) have been utilized to minimize the reverse transcription of host and bacterial transcripts while preserving the random reverse transcription of RNA viruses. While several variations of this method have been developed (110–113), in general, they use a pool of pseudorandom hexamers that systematically exclude hexamers that would bind to human rRNA. Not-so-random primers enrich for viral pathogens by preventing the amplification of off-target DNA fragments, with greater than 90% reduction of host rRNA (114). Although not-so-random primers have shown promise, one drawback to this method is the possibility that certain viral targets will be missed using the reduced primer pool. More extensive validation of these methods is necessary on a phylogenetically diverse panel of viruses.

Several other host depletion or pathogen enrichment approaches have been developed to improve the diagnostic performance of mNGS. One enrichment approach is metagenomic sequencing with spiked primer enrichment (MSSPE), a method that spikes primers targeting a specific virus or panel of viruses in addition to random primers to detect untargeted viruses (115). This method increases genome coverage by 47% and produced comparable sensitivity to PCR for detection of targeted pathogens. Capture probe enrichment is a method that uses taxon-specific probes to pull down targeted viral genome fragments from metagenomic libraries prior to NGS. Several techniques have been developed and tested using this method, including SeqCap probes (Roche Diagnostics, Mannheim, Germany) and VirCapSeq-VERT (116). These methods have shown increased performance and up to 10,000-fold enrichment (116) of targeted viruses compared to traditional metagenomic sequencing methods, but notably this approach does not reliably detect viruses excluded from the target panel (117). As well, lengthy hybridization times for capture probe enrichment may preclude the translation of these techniques to clinical laboratories due to the delay in receiving clinically actionable results. While VirCapSeq-VERT was shown to have improved sensitivity over mNGS, this approach can lead to lower coverage for genome termini compared to mNGS (118).

In contrast to enrichment approaches, which attempt to increase the abundance of target reads in the sequencing output, host depletion approaches attempt to reduce the abundance of host reads, which are often in high abundance in patient samples. Host depletion approaches may enable the improvement in assay sensitivity without requiring a target list of pathogens for target enrichment. Several commercial kits have been developed to deplete host nucleic acid. However, these kits are not used extensively, as the cost per sample is often quite high. Recently, several promising host depletion approaches have emerged and are currently being tested. One of the more promising techniques is depletion of abundant sequences by hybridization (DASH), a Cas9-based approach that utilizes a pool of single guide RNAs (sgRNAs) that are designed to target human rRNA (119). When combined with the Cas9 nuclease, this sgRNA-Cas9 complex cleaves host rRNA into small fragments that are removed from the sequencing library through a magnetic bead cleanup. Jumpcode Genomics (San Diego, CA, USA) has developed and commercialized the first CRISPRclean kit based on this approach and has shown that mNGS combined with Cas9-based rRNA depletion using their kits improves pathogen genome coverage and yields diagnostic performance comparable to PCR (120). One final approach that can be applied to the Nanopore sequencing platform is adaptive sampling (Oxford Nanopore Technologies, Oxford, United Kingdom), a method to select for and physically eject nucleic acid molecules from the protein nanopores in real time following alignment of the newly sequenced read against a reference database (e.g., human rRNA). However, this method is currently only optimal for the depletion of larger DNA fragments, since the computation required to align new reads to the reference is considerably slower than the Nanopore sequencing speed (121). Further research and clinical validation of these enrichment and depletion approaches is necessary to assess their diagnostic utility. Tradeoffs between diagnostic performance and end-to-end assay completion time will likely also guide the decision of whether to include enrichment or depletion approaches in a clinical workflow.

Finally, recent advancements in sequencing technology have driven viral diagnostic mNGS toward clinical translation. Sequencer throughput has improved across all technologies, enabling the improved detection of low-abundance sequences by simply sequencing larger numbers of reads. Sequencing technology has become more compact and affordable, including the release of the Illumina iSeq and ONT MinION, the latter of which costs less than $1,000 and is slightly larger than a chocolate bar. The reduction in sequence error rate for the Nanopore sequencing platform is a major advance. Improved accuracy, in addition to portability and ease of use, will allow for the expansion of the Nanopore technology into clinical and public health laboratories. The release of locked-down sequencing devices (e.g., Illumina MiSeqDx and ONT

GridION Q) will facilitate regulatory approval of mNGS for diagnostic purposes. Overall, these advances in wet lab techniques for pathogen-agnostic viral sequencing will improve the translatability and diagnostic potential of mNGS, which will ensure that pathogen-agnostic assays are available to tackle the next pandemic.

Advances in Data Analysis

In parallel to advancements in laboratory protocols, important strides in bioinformatic analysis of mNGS data have been made, which will further encourage mNGS technology transfer. Bioinformatic analysis of metagenomic data has traditionally been computationally challenging, requiring extensive computing resources and a highly trained bioinformatics workforce. Tools to taxonomically classify metagenomic sequencing reads, such as Kraken2 (122) and Centrifuge (123), while powerful, are difficult for users without a computational background to run. In 2014, Naccache and colleagues described SURPI, a clinically oriented rapid pathogen identification pipeline that was cloud compatible (124). Since then, the SURPI pipeline has been utilized in clinical settings to provide actionable diagnoses (18, 51, 125) and has been upgraded to SURPI+ to enable the software to be used for locked-down mNGS assays (97). More recently, Chan Zuckerberg ID (formerly IDSeq) was released as an open-source, cloud-based platform for pathogen identification and global pathogen surveillance (126). This method has since been used to detect coinfections among confirmed SARS-CoV-2 cases (127). BugSeq is another recently deployed cloud-based metagenomic classification pipeline with support for long-read sequencing platforms that provides classification based on read alignment (128), as well as contigs (129). This pipeline has been validated for clinical samples for detection of SARS-CoV-2 (76) and novel viruses (129) present in metagenomic data.

Along with analytic advances, there has been emphasis on the curation and validation of reference databases for clinical metagenomics; however, much of this focus has been on bacterial metagenomics, omitting some critical issues for viral detection. Recently, the FDA has released the FDA-ARGOS database (130), which aims to provide high-quality genomes for diagnostic assays. Notably, FDA-ARGOS omits many respiratory viruses (e.g., influenza viruses A and B and respiratory syncytial virus, as of October 2022) and should therefore not be used for viral detection in its current state. Others (122, 123) have built databases based on NCBI RefSeq, the curated and higher-quality section of GenBank, or NCBI nt, which includes greater diversity in albeit lower-quality sequences from public repositories. Progress toward creating translatable and clinically actionable bioinformatics pipelines and comprehensive and high-quality genome reference databases will drive the development and validation of accurate, agnostic, and deployable mNGS assays for detection of viral pathogens.

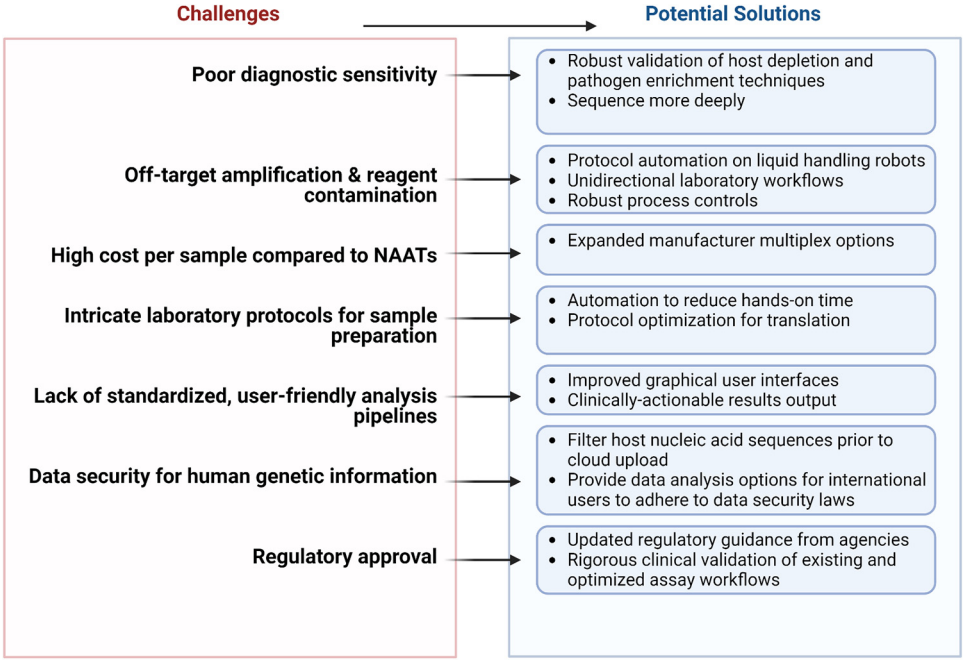
VIRAL METAGENOMICS: CHALLENGES TO WIDESPREAD ADOPTION

Concerns Regarding Test Performance

While recent advancements in wet lab technique and sequencing technology have increased the focus on the unique benefits of mNGS for viral diagnostics, several studies have highlighted a disparity in diagnostic performance between mNGS and gold standard PCR assays (76, 78). As outlined in the recent advancements section above, many related but distinct sample preparation approaches have been developed, but emerging evidence suggests that these approaches are not all equal in terms of their diagnostic performance (104, 105, 131). There are a diverse range of extraction techniques, both in-house within clinical laboratories as well as among purchased kits and analysis platforms from commercial suppliers. Moreover, priming and amplification methods differ. Finally, the choice of sequencing platform can influence the output quantity (i.e., number of reads per sample), as well as the quality (i.e., read length and error rate). Downstream analysis pipelines may perform differently depending on which sequencing platform is used. There is a gap in the literature of studies systematically evaluating diagnostic performance across these diverse methodologies (Table 1).

Diagnostic sensitivity for weakly positive specimens remains somewhat low, which

TABLE 1 Challenges to translation of agnostic sequencing for routine viral diagnosis and potential solutions to each challenge



may in part be due to the abundance of off-target host nucleic acid in clinical samples; in one review, there was a median relative abundance of host sequences of 91% across a range of sample matrices (132). While pathogen enrichment and host depletion approaches show promise in improving diagnostic performance of agnostic sequencing, robust validation studies of these methods in clinical settings are lacking. Indeed, a recent systematic review of the accuracy of mNGS for diagnosis of infectious diseases highlighted comparatively low sensitivity for mNGS compared to PCR and variable sensitivity across sample matrices (132). The sensitivity of different diagnostic techniques can vary by matrix type (48, 133, 134), perhaps due to a combination of variable viral loads in different collection sites and tissues, as well as a difference in abundance of host nucleic acids among specimen types. Sampling matrix remains a key consideration when designing validation studies and drafting intended use statements for diagnostic assays.

Not only should sampling matrix be considered when evaluating the diagnostic performance of a molecular assay, but also the time point of sample collection should be considered. Incubation time, the period between infection and symptom onset, can vary substantially between viral pathogens (135). These pathogen-specific characteristics can impact the expected viral load in a specimen at the time of sampling and can subsequently influence the performance of a diagnostic assay. Indeed, the timing of sample collection from symptom onset was shown to influence the diagnostic sensitivity for SARS-CoV-2 detection (136). Therefore, consideration must be given to the expected viral load in tissues based on patient exposure history. As mNGS technology improves, this information will help to define the potential assay utility. mNGS may also lead to a better understanding of the pathogen-specific spectrum of clinical disease, as mNGS data can also enable public health laboratories to characterize a pathogen's genome. This may enable more effective diagnostic and treatment approaches across an array of pathogens.

Sensitivity is an obvious consideration when evaluating mNGS assays, but specificity in assay performance is also critical due to the need to detect small amounts of viral nucleic acid from clinical specimens. Robust process controls are necessary to ensure positive results are not due to reagent or carryover contamination that can be

amplified during PCR or introduced during other steps in the wet lab workflow. Barcode cross talk, the misclassification of sequence reads to different indices due to sequencing error, or ligation errors during barcode attachment, can also influence the results of an mNGS assay. Adjustment for this error must be made, taking into consideration the error rate of the sequencing platform being used to inform the adjustment threshold and results (137). A related and very challenging issue is determining a threshold for target sequence read abundance within a sample to define sample positivity. High sensitivity and data throughput produced by NGS platforms means the decision surrounding the choice of sequence read abundance threshold to yield a positive diagnosis needs to be defined carefully and be informed by both the specifications of the sequencing platform as well as the method of library preparation. Robust controls, including negative (blank) controls to account for contamination during wet lab processes, positive controls containing known pathogens, and spike-in process controls to ensure nontarget analytes do not impact sensitivity, will aid in the validation of mNGS assays. Further guidance from regulatory agencies in this regard will guide research groups in designing validation studies for new mNGS assays.

Cost Considerations and Laboratory Workflow

Perhaps two of the largest considerations with which clinical laboratories are faced when choosing to integrate mNGS into their service workflow are cost, time to result, and ease of use. While the cost of sequencing has been rapidly declining, metagenomic sequencing remains costly (between $130 and $685 per sample, depending on the approach used) (132) compared to lower-cost PCR or amplicon sequencing approaches (~$12 per sample for reagent costs [62]). The issue of cost is especially important for laboratories considering implementing mNGS as a first-line diagnostic tool, as the decision to conduct these costly tests may need to be justified to insurance providers. The high cost is partially due to the depth of mNGS sequencing required to detect low-abundance viruses compared to targeted NGS approaches. Even when samples contain pathogens at high viral loads, users of commercially produced library preparation kits are limited in their ability to multiplex many samples in parallel by the number of sample indices provided by the sequencing platform manufacturer, although some sequencing platforms provide more multiplexing options than others. Enhanced dialog between clinical users and commercial manufacturers of extraction and library preparation kits and sequencing platforms is necessary to bridge these significant cost differentials before this technology can be translated.

Much of the work that has led to advancements in this field has been performed by specialized clinical research laboratories that employ highly qualified personnel to execute mNGS workflows. Currently published protocols for agnostic sequencing of viruses are labor-intensive and lengthy, making them unsuitable for use in high-volume clinical laboratories where staff are often overworked and have varied degrees of molecular biology expertise. This workflow is further complicated if host depletion or pathogen enrichment methodologies are integrated into the protocol. Additionally, mNGS protocols necessitate strong process controls and unidirectional workflows to reduce the risk of contamination. Clinical laboratories, perpetually lacking in resources, will find mNGS workflow implementation challenging. Automating the workflow reduces the hands-on time and risk of contamination and improves the clinical usefulness of the results as automation can accelerate testing time frames. Partial or complete end-to-end assay automation on liquid-handling robots or miniature microfluidic devices may be required to handle sample testing volume and quality control, as well as reduce per sample costs, but this introduces another barrier to routine pathogen-agnostic sequencing implementation in clinical service laboratories. Additionally, while many clinical laboratories now have staff trained to execute molecular diagnostic assays, many of these staff have little to no experience handling NGS data. User-friendly computing interfaces for data upload and reporting are necessary to improve the translatability of mNGS into settings where bioinformatics expertise is limited. Further challenges to translating bioinformatic workflows for mNGS data are described in the section below. Funding, automation and

sequencing infrastructure, and personnel training and support for clinical and public health laboratories stemming from the COVID-19 pandemic may be leveraged to make the integration of automated mNGS workflows into clinical laboratories feasible.

Data Analysis and Privacy

Advancements in bioinformatics have improved translatability; however, advances in several other areas are still required to integrate mNGS workflows into clinical laboratories. While there are some bioinformatics pipelines that provide clinically interpretable results reporting, many recently published viral metagenomic classification pipelines (138–143), while promising in terms of their analysis performance, do not contain a graphical user interface, nor were they designed with clinical users' needs in mind. Pipeline performance evaluation prior to selection is also needed, as pipelines vary in quality and function (144). Not only should pipeline performance be considered, but also the output format of testing results can also determine the clinical utility and application of the assay. For example, mNGS diagnostic results can be reported in a qualitative (e.g., presence or absence), semiquantitative (e.g., relative abundance), or quantitative manner. Certain viruses, such as Epstein-Barr virus or cytomegalovirus in the post-transplant setting, may require accurate quantitation to guide clinical management, such as antiviral therapy and immunosuppression; until further methods enabling accurate quantification from mNGS are available, these applications will continue to require alternative assays. Implementation of data analysis workflows that are characterized by expert back-end management with user-oriented front-end interfaces will improve the user experience and reduce the delay of actionable results from mNGS. The size and complexity of mNGS data often preclude analysis of these data on standard computers; cloud computing enables cost-effective and often better-maintained computational infrastructure. Not only does cloud computing enable scalable computing, hardware acceleration on graphical processing units and dedicated servers can enable faster, more cost-efficient data analysis.

Privacy and data security concerns have slowed or prevented regulatory approval and clinical integration of mNGS. These concerns are two-pronged. First, due to the considerable abundance of host nucleic acid in clinical samples, there is a need to either remove these reads from metagenomic data sets in advance of data upload for analysis or filter and discard these host reads immediately after uploading data to a secure analysis server. Second, the location and relative security of analysis servers remain a concern. Many regulatory agencies require data to be hosted on a server within the borders of their jurisdiction for data security purposes. Choosing a bioinformatics pipeline and a location to host data storage and analysis requires careful consideration. Data storage for metagenomic assays will be another challenge, and how long, in what format, and where to store data are currently unresolved questions. Clinical laboratories should store final assay reports to comply with regulation, and many will also choose to store raw or intermediary files for more granular analysis of results, bioinformatic validation, and research use. Barriers to and recommendations for clinical deployment of analysis workflows have been reviewed in more depth elsewhere (145).

Regulatory Approval

Regulatory approval is another challenge presented to clinical laboratories looking to adopt pathogen-agnostic workflows for their diagnostic and public health services. Approval has been hindered by a lack of recent FDA guidance (146). Several recent studies have been published that validated mNGS for viral pathogen detection in clinical specimens (97, 147, 148). While there are numerous resources available for designing these validation studies for diagnostic assays and devices (149–151), the unique agnostic nature of mNGS presents several challenges to obtaining regulatory approval over standard diagnostic approaches. As these assays can, in theory, detect any known organism from a clinical specimen, validation of the diagnostic performance for every viral pathogen would be an impossible task. One potential solution is to perform vigorous clinical validation of the assay based solely on a panel of viral pathogens that

occurs at higher incidence in the population. Validation of positive diagnostic results by mNGS on nonvalidated, rare pathogens would be followed by a second, confirmatory RT-PCR test. Extensive wet lab recommendations for the implementation of mNGS in clinical settings have recently been published and may further aid in the design of validation studies (152). While we believe these validation approaches may be sufficient for regulatory approval, there is no consensus regarding what constitutes a gold standard for an agnostic mNGS viral assay. We believe that an mNGS assay gold standard would be a highly sensitive and specific diagnostic assay that could confirm or refute the findings of mNGS results, based on the presence or absence of pathogen nucleic acid in a sample (i.e., a molecular diagnostic test). Regulatory guidance on gold standards for mNGS assay validation will guide this process.

While the rapid evolution of sequencing platforms and chemistries have yielded enormous improvements in sequencing accuracy and throughput, these advancements ironically make validation testing and regulatory approval of a "final" or locked-down version of these assays hard to do. Overall, updated guidance from regulatory agencies regarding the pathway to approval for agnostic sequencing assays for diagnosis of viral infections is integral to the future implementation of these technologies. While several research groups have obtained approval for clinical use of mNGS for bacterial pathogens in a select set of circumstances (97), widespread regulatory approval for routine viral diagnosis is lacking. Adequately addressing the barriers outlined in this review, particularly regarding patient data security, will help push research groups and commercial ventures toward the successful approval of pathogen-agnostic sequencing assays for viral infection diagnosis.

PATHOGEN-AGNOSTIC DIAGNOSTICS: FUTURE OUTLOOK

Recent public health emergencies have emphasized the need to respond rapidly to new and emerging pathogens. Current multiplex RT-PCR and targeted NGS approaches for viral diagnosis and characterization of viral genomes are limited in that they rely on prior knowledge of a pathogen's genome. This limitation precludes the ability of public health and clinical laboratories to both effectively respond to emerging pathogens in which a validated molecular diagnostic test does not already exist or provide a clinically actionable diagnosis of a rare viral pathogen that is not included on existing viral diagnostic panels. Metagenomic sequencing for viral diagnosis can overcome the limitations of targeted approaches, potentially providing time-sensitive diagnostic results and enabling a robust response to public health crises. Rapid decreases in the cost of whole-genome sequencing using NGS technologies over recent years (153) have led to the expansion and increased translation of NGS into clinical settings. This review has highlighted many success stories of the implementation of NGS for viral diagnostics and molecular epidemiology for viral outbreaks. Metagenomic approaches have been utilized for characterization and detection of influenza (78), SARS-CoV-2 (76), Ebola, hepatitis C, and Chikungunya virus (77), as well as for diagnosis of astrovirus encephalitis, which was not captured on standard viral diagnostic panels (154). Several commercial platforms have been developed for viral detection by mNGS, including Galileo ONE (Arc Bio, Scotts Valley, CA, USA) for wet lab sample preparation, as well as DRAGEN and IDbyDNA Explify (Illumina, San Diego, CA, USA) for bioinformatic analysis of pathogens in mNGS sequence data. However, these platforms are currently limited to research-use-only applications. The implementation of mNGS in clinical settings has so far been limited to a select group of CLIA-certified laboratories, and no commercially produced, clinically validated, and regulatory approved assay for agnostic detection of viral pathogens currently exists.

Recent advancements in sequencing technology, including third-generation and portable sequencing platforms, improvements in sequencing throughput and error rate, and reductions in time to result and cost, have all worked to overcome limitations to the translation of mNGS methods to clinical and public health settings. Additionally, advancements in sample preparation and pathogen enrichment and host nucleic acid depletion have driven improved diagnostic sensitivity of mNGS workflows. Finally, the

development of several user-friendly and robust bioinformatics pipelines for metagenomic classification of mNGS data may provide clinical laboratories with timely and medically actionable results that would significantly boost the uptake of mNGS into service laboratories.

Several barriers remain to obtaining regulatory approval and supporting widespread adoption of agnostic sequencing for viral diagnostics. Relatively low diagnostic sensitivity on specimens with low viral load compared to RT-PCR and amplicon sequencing (76, 78, 80) remains an issue precluding regulatory approval of this technology for routine diagnostics. While advancements in sample preparation and host depletion or pathogen enrichment may help increase diagnostic performance, these methods have been somewhat limited to research settings and have not undergone vigorous clinical validation. Cost and workflow considerations also limit the adoption of mNGS-based diagnostics as current library preparation methods remain expensive, lack validated liquid-handling robot automation protocols, are prone to contamination, and are technically challenging. This complicates their use by laboratory technicians in service laboratories. NGS data analysis has historically been plagued by non-user-friendly programs and challenges to translating sequencing results into clinically interpretable and actionable information. While advancements have been made on this front, standardized and secure analysis pipelines and reference databases are needed for the translation of mNGS results. Enhanced regulatory guidance and subsequent approval of mNGS assays for diagnostic uses comprise perhaps the most important challenge to overcome for mNGS technology translation.

We remain optimistic that mNGS will play an integral role in future infectious disease diagnostics and public health surveillance. We anticipate that in the next few years, several clinically validated assays will be subject to regulatory approval. Although RT-PCR will likely remain the gold standard diagnostic technology for viral infections for quite some time, reductions in sequencing cost and workflow length will likely lead mNGS to gradually replace some existing diagnostic assays. We believe that as sequencing costs continue to decline and laboratory workflows become more streamlined, the per-sample cost of mNGS will draw closer to that of NAATs, such that the transition of mNGS to first-line diagnostics will become more cost-effective. Fully automated laboratory workflows (going from raw sample to result) using liquid-handling robots or miniaturized microfluidics will be developed and validated to reduce the chance of nucleic acid contamination, reduce end-to-end assay completion time, and eliminate skill barriers to mNGS execution. One such example where mNGS use may become more widespread is for diagnosis of central nervous system (CNS) infections. Diagnosis of CNS infections can be a significant financial and resource burden on hospitals, as many tests may be necessary for diagnosis of these infections. mNGS may speed up the time to pathogen detection and reduce the time needed for patients to occupy intensive care unit beds, as well as the cost of conducting many diagnostic assays. Another application where we believe mNGS will undergo rapid expansion is for public health surveillance and molecular epidemiology of viral outbreaks, as these applications are already well under way. One universal pathogen-agnostic assay to surveil all endemic viral pathogens is a far more cost-effective and comprehensive surveillance approach. Although it is challenging to predict how innovation in viral diagnostics will evolve over the next 10 years, we expect metagenomic sequencing to become as cost-competitive as NAATs for the quantity and quality of actionable information generated, and mNGS may replace NAATs as a primary tool for viral detection.

In this review, we have attempted to convey the recent technical advancements of metagenomic sequencing for diagnosis of viral infections. While these advancements give cause for optimism for the technological translation of mNGS into clinical and public health settings, several key technical, analytical, and regulatory challenges remain. Nevertheless, recent success stories of NGS and mNGS in these settings, coupled with the urgent need for nations to establish more robust pandemic preparedness strategies,

are pushing many to collaborate to overcome these obstacles in the pursuit of a universal viral diagnostic assay.

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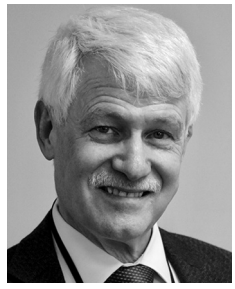

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