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Perspectives on Molecular Diagnostic Testing for the COVID-19 Pandemic in Delaware

Erin L. Crowgey;[1] Mary M. Lee;[2] Brett Sansbury;[3] and Eric B. Kmiec[4]

1. Nemours Alfred I duPont Hospital for Children

2. Nemours Alfred I duPont Hospital for Children

3. ChristianaCare Christiana Hospital

4. ChristianaCare Christiana Hospital

Abstract

The United States has quickly transitioned into one of the epicenters for the coronavirus pandemic. Limitations for rapid testing for the virus responsible for the pandemic, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is the single most important barrier for early detection and prevention of future outbreaks. Combining innovative molecular biology techniques, such as clustered regularly interspaced short palindromic repeats (CRISPR)/Cas nuclease systems and next generation sequencing (NGS) may prove to be an effective solution to establish a high-throughput diagnostic and genomic surveillance workflow for COVID-19 in the State of Delaware. Integrating key expertise across the medical institutions in Delaware, including ChristianaCare and Nemours/Alfred I. duPont Hospital for Children, is one potential solution for overcoming current barriers and driving a successful implementation of these techniques.

Introduction

A public health crisis

As of June 15, 2020, there were over 7.8 million confirmed cases of COVID-19, across 213 countries with over 430,000 confirmed deaths. The virus responsible for causing COVID-19 is severe acute respiratory syndrome coronavirus 2, referred to as SARS-CoV-2. In February of 2020, the United States Secretary of Health and Human Services (HHS) determined that there was a public health emergency related to COVID-19 and authorized the Emergency Use Authorization (EUA) for diagnostic tests for COVID-19. Unfortunately, several of the approved tests have had poor specificity or sensitivity,[1] availability of supplies / reagents is limited,[2] and pipelines for implementing high-throughput workflows for these assays has been challenging. The impact of insufficient diagnostic capacity has been devastating for the healthcare industry and economic infrastructure and the United States has unfortunately transitioned into one of the epicenters for COVID-19.

Over 2 million cases and approximately 115,000 deaths have been reported in the United States, with a case mortality rate of 6%.[3] Of concern, over 40 million Americans have filed for unemployment during this pandemic, highlighting the significant financial crisis unfolding. Currently, individual states are trying to grapple with measures needed to permit safe reopening and to lessen the stay at home orders to help prevent further economic hardship and the growing unease of the general population. It remains to be seen how effective these public health safety measures will be to avoid a second wave of viral infections, as predicted following the relaxation

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of social distancing guidance. In the State of Delaware – which has a population of over 900,000 – there have been over 10,000 cases with more than 500 deaths as of June 22, 2020. The majority of cases have been reported in Sussex and New Castle County, followed by Kent County.

Overview of current diagnostic techniques

Currently, there are a number of rapid real time quantitative polymerase chain reaction (RT-qPCR) assays available to enable a rapid diagnosis; however, these assays detect the presence of viral particles and are unable to track transmission patterns or virus evolution. Moreover, reagents for the various RT-PCR assays and the assay kits are in high demand, creating a supply chain limitation. The inability to conduct rapid and wide-spread testing for COVID-19 is a critical barrier for early detection in order to appropriately quarantine and implement contact tracing as public health measures to prevent further transmission and future outbreaks. Over the last decade there have been several advancements in molecular biology techniques that facilitate rapid diagnostics and genomic analysis of viruses, including SARS-CoV-2.

Next generation sequencing (NGS) is a molecular biology technique that enables scalable, massively parallel DNA / cDNA sequencing. The ability to sequence cDNA molecules and the entire genome of a small virus enables the precise identification and order of nucleotides.[4] NGS enables this process using a method that simultaneously sequences millions of fragments of cDNA at once, which is a significant improvement compared to the gold standard of Sanger sequencing.[4] Recently, a team of scientists developed an NGS pipeline for studying COVID-19,[5] now termed the ARTIC pipeline. This technique coupled with advanced translational bioinformatics enables the precise analysis of genomic differences of COVID-19 strains, which enables scientists to study transmission patterns and viral evolution.[6] Furthermore, this technique ensures that the diagnostic test primers are sensitive (region of the genome are not mutating) and can be used to monitor the environmental warning system via metagenomics, which is being shown to be a useful indicator of community transmission.[7]

In addition to novel sequencing-based testing, breakthroughs in gene editing technology, specifically clustered regularly interspaced short palindromic repeats (CRISPR)/Cas nuclease systems, have been adapted for numerous assays.[8] CRISPR/Cas has been shown to be effective in almost every animal species tested and its molecular activities go far beyond site-specific DNA cleavage in the activation of the DNA damage response pathway in mammalian cells.[8–12] CRISPR's unique ability to target RNA and DNA sequences, in a precise fashion, has given rise to an important and exciting application of the fundamental technology. CRISPR/Cas can now be used to determine if someone is infected with a novel coronavirus[13]; the basis of this diagnostic application lies in the alignment of the central piece of the CRISPR with the target site on the genome. Interesting applications of CRISPR/Cas for viral detection of SARS-CoV-2 have begun to emerge including the trans reporter (DETECTR) platform.[13,14] Currently, research and development studies have begun to determine if CRISPR is a more reliable and sensitive alternative to the traditional RT-qPCR assay for detection of SARS-CoV-2.

Antibody screening is useful in limited clinical situations and is being used as a public health screening tool to assess prior COVID-19 infection.[15] Antibody tests for SARS-CoV-2 include many that are not yet approved by the Food and Drug Administration (FDA) and have variable sensitivity and specificity.[16] Some cross-react with other types of Coronavirus, not just SARS-CoV-2. With COVID-19 infections, the antibody response is delayed and may not be produced until 2-3 weeks after the onset of symptoms, thus an elevated IgM antibody is not a reliable

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indicator of acute infection and must be confirmed with a RT-PCR test. A detectable IgG antibody is consistent with prior COVID-19 infection,[15] although it may detect other coronaviruses and may not be protective against COVID-19. Antibody testing may be useful in cases that may have been potentially missed, for screening of plasma donors, or for diagnosis in patient with multi-system inflammatory syndrome associated with COVID-19 (MIS-C).

An effective response to a pandemic such as COVID-19 will need to leverage all of these techniques to have an efficient and robust outcome. The RT-qPCR assay is fast and inexpensive but does not provide enough data for studying transmission patterns or identify viral variants to assess response to targeted treatments or host interactions (see Table 1). The NGS approach enables a robust analysis of the viral genome to enable analysis, such as genomic surveillance and viral variant analysis, but is slower and more costly than RT-qPCR and there are still several hurdles for scaling this assay for high-throughput efforts – although companies like Illumina are actively working on these barriers. The CRISPR technique is also a fast and inexpensive assay but the limit of detection is not well established, and it doesn't provide the ability to analyze the viral genome.

Table 1. Characteristics of each of the diagnostic tests currently active in Covid 19 detection.

Technique	Diagnosis acute infection (< 7 days)	Detect previous exposure / not currently shedding virus	Fast Turnaround (hours)	Genomic Surveillance and Transmission Patterns	Costs
qPCR	Yes	No	Yes	No	$$
CRISPR	Yes	No	Yes	No	$
NGS	Yes	No	No	Yes	$$$
Antibody	No	Yes	Yes	No	$$

Screening for COVID-19 in the state of Delaware: acute diagnosis and long term surveillance

An analysis of the current state of molecular testing and diagnostics reveals both good intentions and unwanted consequences. While a rush to produce rapid testing assays is a meritorious response to a pandemic, it is now becoming clear that many of the early screening methodologies have suboptimal test parameters. If we are to respond in a more efficacious fashion to future public health challenges of a viral pandemic, a more comprehensive and systematic approach is needed.

Currently the most common forms of sample collection for COVID-19, regardless of the down-stream diagnostic assay, involves a nasopharyngeal or oropharyngeal swab (see Figure 1). These methods have several shortcomings including potential cross-contamination with other viral material, operator dependent variability in quality of the sample, and pediatric subjects may not cooperate well.[17] Improving alternative collection methods and nucleic acid extraction techniques will enhance the ability to provide high-throughput rapid diagnostic pipelines for the COVID-19 pandemic. The sample collection method and sample processing should be considered an essential aspect of developing any diagnostic assay, as this is a major bottle-neck in the workflow.

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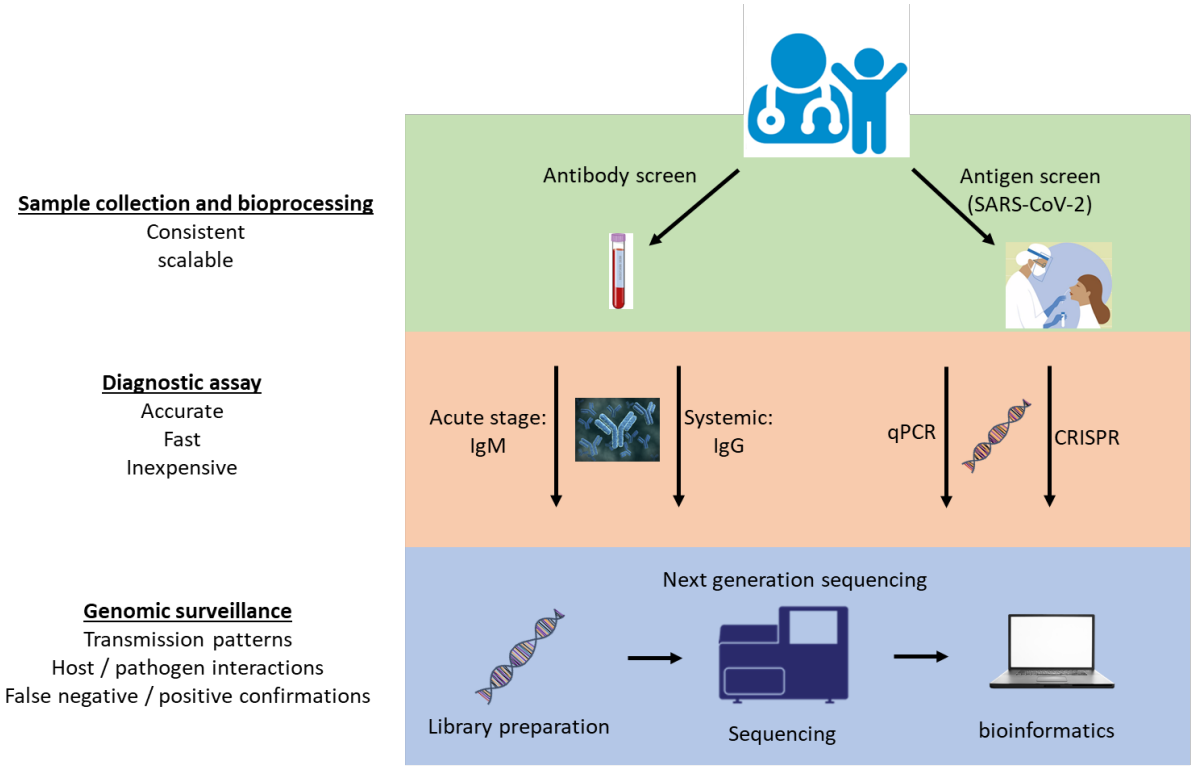
Figure 1. Overview of a comprehensive diagnostic and screening workflow for COVID-19. Workflow and processing activities from collection through detection is presented highlighting the three phases of diagnostic analyses.



Regardless of the acute diagnostic test for the antigen, CRISPR or RT-qPCR, a robust workflow for COVID-19 long term should include NGS as a follow up for a positive diagnostic test or a suspected false negative. NGS is proving to be essential for understanding transmission patterns and potential host / viral interactions.[6] As mentioned previously, these analyses cannot be conducted using the RT-qPCR or CRISPR technique. It is therefore possible that combining the potential for point-of-care diagnosis using the CRISPR technique outlined above with NGS could lead to a more comprehensive diagnostic and screening system for the current pandemic and could serve as a foundation for subsequent public health challenges.

The combined skill sets of the ChristianaCare Gene Editing Institute (Director Dr. Kmiec) and Nemours Alfred I duPont Research Laboratories, including the Computational Medicine team (Director Dr. Crowgey) and the Nemours Biobank (Director Dr. Corao), enable an opportunity to carefully and precisely evaluate the capacity of enhancing viral nucleic acid preparation and combinatorial testing in an unbiased and unimpeded fashion. We are doing just that by optimizing novel viral RNA collection and extraction methods that are agnostic to downstream approaches. It is important to note that collection methods for COVID-19 samples involves the collection of a diverse metagenome, which is a community of bacteria, phages, viruses, and host cells, regardless if it is a nasopharyngeal or oropharyngeal swab or saliva sample. This is a key consideration when considering limit of detection for downstream assays.

Currently, the gold standard for extracting high-quality RNA from a nasopharyngeal swab involves a manual preparation using a vacuum manifold. This process does not scale well as it requires laboratory technicians working multiple shifts to extract large numbers of samples and

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lacks automation. Additionally, there are limitations for automating high-throughput methods for extracting viral RNA specific for COVID-19 from samples, as these methods do not yield enough viral RNA for appropriate limit of detection for downstream assays, regardless of the method of collection. The teams at ChristianaCare and Nemours / Alfred I. duPont Hospital for Children are teaming up to conquer these issues using advanced robotics, to offer a scalable and robust workflow for implementing a high-throughput workflow for the state of Delaware.

The Gene Editing Institute at ChristianaCare is uniquely positioned in the state of Delaware to lead the efforts in adapting and implementing a CRISPR diagnostic test for COVID-19. These applications have begun to emerge across the country, including the specific high-sensitivity enzymatic reporter unlocking (SHERLOCK)[18] and trans reporter (DETECTR) platforms. It is important to leverage translational research efforts to determine if CRISPR technology is a more reliable and sensitive alternative to traditional RT-qPCR assay for detection of SARS-Cov2 virus. The Delaware ACCEL program (PI Binder-Macleod, grant U54-GM104941) has efficiently positioned research funding for COVID-19 in Delaware to help the scientists at Nemours and ChristianaCare drive these new types of molecular biology approaches.

CRISPR-based technologies, classic SHERLOCK, STOP (One-step SHERLOCK), and DETECTR must be validated for sensitivity and lower limits of detection of SARS-CoV-2 (see Figure 2) using synthetic RNA templates, in order to model the reaction safely. Scientists do not fully understand the lower limits of detection in patient derived samples using either SHERLOCK or DETECTR. Thus, it is important to evaluate each of these technologies to determine which provides the most sensitive and accurate outcome, which assay is the easiest to use at point-of-care, and which can instill the highest degree of confidence in the validity of the test. The key to any point of care acute testing process is to ensure that it is not only reproducible, but robust. Key elements of the CRISPR-based approaches must involve a full assessment of scalability and applications to enable high throughput NGS population surveillance for statewide screening.

Figure 2. Overview of CRISPR-based Technologies for COVID-19 Detection. A comparison is shown of reaction steps, reaction components, end readout, limit of detection and assay duration between three current CRISPR-based COVID-19 detection technologies Classic SHERLOCK, DETECTR, and STOP (One-step SHERLOCK).

CRISPR-based Detection	Reaction Steps	Isothermal Amplification	CRISPR Detection	Readout (RT, 2 min)	Limit of Detection (copies/µl)	Duration (minutes)
Classic SHERLOCK	2	RT-RPA (42°C, 25 min)	IVT + Cas13a (37°C, 30 min)	Lateral Flow	10-100	~60
DETECTR	2	RT-LAMP (62°C, 20 min)	Cas12a (37°C, 10 min)	Lateral Flow	70-300	~30
STOP (One-step SHERLOCK)	1	RT-LAMP + Cas12b (60°C, 60 min)		Lateral Flow	100-300	~70

The rapid diagnostic tests, such as the CRISPR technique described above, are only focused on the detection of the virus and do not yield any information on the actual sequence of the viral genome (see Table 1). As seen with other viruses, slight modifications of a viral genome can make vaccinations more or less effective, can be linked to disease outcome / severity, and can be used to trace transmission patterns. A team of scientists have developed a PCR-based NGS assay

that enables the rapid sequencing of the COVID-19 viral genome.[5,6] This technique was originally established for the Illumina MiSeq platform, which is one of the lowest throughput sequencing platforms (in terms of number of reads / samples sequenced) offered by Illumina. A need to scale this platform to larger sequencing instruments, such as the NextSeq or NovaSeq are still needed, and the research teams at Nemours are in a position to help drive these efforts in the state of Delaware. Collectively these efforts will enable Delaware to generate viral genomic data that can and help drive national collaborative research efforts.

NGS techniques are essential in a robust response to the COVID-19 pandemic, as acute testing methodologies provide a snapshot of the penetration of any viral or bacterial infection, none are conclusive enough to predict outcomes or establish surveillance. One way to address this issue is to implement NGS protocols to analyze for COVID-19 specific variants through linkage to transmission patterns, outcomes and scalability. Such a procedure is based on the collection of positive test samples – in this case, by the Nemours Immunology diagnostic lab – followed by a PCR NGS library preparation and sequencing using a high-throughput Illumina platform, such as the NextSeq. Of interest, this type of sequencing platform can also be leveraged to sequence the coding regions of the infects host, called whole exome sequencing, which has the potential of providing even more clues regarding pathogen-host interactions.

Bioinformatics, a multi-disciplinary field focused on creating and implementing computer algorithms for analyzing complex biological data, is proving to be essential aspect to this workflow. A variety of software programs capable of analyzing COVID-19 genomic data should then be employed; for example, the database https://nextstrain.org, an open-source project that enables the comparisons of COVID-19 for genomic surveillance and outbreak response. But, importantly, it will be crucial to follow the transmission of the viral load through the population by utilizing the Nemours Biobank system to continue to screen healthcare workers and community members. This approach will also allow us to determine the presence of Delaware specific variants within the virus and link variants to transmission patterns that have been previously reported. Taken together, our efforts will contribute to the national efforts to understand how such strain mutations correlate with disease outcome and responsiveness to therapy.

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